Phenotypic and genotypic analysis of *bla*$_{\text{CTX-M}}$ encoding plasmids isolated from bovine *E. coli* samples in the United Kingdom

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Declaration of Authorship

I, **Christine J. Boinett** hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

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Date:

ABSTRACT

The purpose of this study was to characterize *bla*$_{CTX-M}$ plasmids originating from bovine *Escherichia coli* and investigate their contribution to bacterial host fitness.

In this study 52 bovine *Escherichia coli* strains collected between March and October 2007 encoding *bla*$_{CTX-M,}$ an extended spectrum β-lactamase (ESBL) gene conferring resistance to 3rd generation cephalosporins, were characterized. The majority of strains belonged to *E. coli* commensal phylogroups A and B1 expressing a multi-drug resistance (MDR) phenotype and harboured multiple plasmids of which 90% were transferred by conjugation. Transconjugants or transformants were made successfully from all 52 strains when selecting for resistance to cefotaxime. All plasmids were shown by PCR and sequence analysis to harbour *bla*$_{CTX-M}$ and nearly 80 % encoded multiple resistances. Plasmid sequence analysis of four plasmids encoding *bla*$_{CTX-M-14b}$ (IncI1-X1), $_{-15}$ (IncFII-FIA-FIB) and $_{-32}$ (IncX1 and IncB), identified genes necessary for stable plasmid maintenance and spread.

Five representative plasmids encoding *bla*$_{CTX-M-1}$, $_{-15}$, $_{-14b}$ and $_{-32}$ were assayed for their fitness impact upon the host. Efficiencies of β-lactam hydrolysis using whole cell extracts were determined in the same *E. coli* BL21 host strain with the most efficient encoded by *bla*$_{CTX-M-14b}$ and *bla*$_{TEM-1}$ ESBL genes and least efficient encoded *bla*$_{CTX-M-15}$ only. A 160 kb plasmid encoding 13 resistance genes was grown in the presence of 380 different metabolites and differences in metabolite utilisation between this and the plasmid-free BL21 strain determined. The plasmid-harbouring strain utilized less phosphor-sulphur compounds, suggesting the metabolic cost incurred by acquiring the plasmid may have implications of cellular utilization of alternative phosphate sources. There were no differences in growth was observed in nutrient rich media. The contribution of active efflux to resistance was investigated using L-phenylalanyl-L-

arginyl-b-naphthylamide (PAβN) in combination with ampicillin, cefotaxime or ceftazidime. Minimum inhibitory concentration (MIC) values were found to decrease ≥ 2 fold in the presence of the efflux pump inhibitor (EPI), in some cases becoming completely susceptible to ampicillin. This indicates that the possible use of EPIs in combination with previously failed antimicrobial drugs to potentially restore efficacy of treatment.

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Contributions of others

I carried out all the work in this thesis, with the exception of the following:

- The isolates presented in this study were screened for ESBL production by by colleagues at the AHVLA (Chapter 3).
- Plasmid DNA extractions for sequencing were carried out by Dr. Manal AbuOun , Mathew Stokes and Hannah Preedy.
- Plasmid sequencing was carried out by the AHVLA Central Sequencing Unit headed by Dr. Richard Ellis (Chapter 5).

Abbreviations

°C	Degrees Celsius
aac	Aminoglycoside acetyltransferase
aad	Adenylacetyltransferase
ATP	Adenosine triphosphate
ATPase	ATP hydrolase
bla	Beta-lactamase
C	Chloramphenicol
cat	Chloramphenicol acetyltransferase
Caz	Ceftazidime
cml	Chloramphenicol transporter
Ctx	Cefotaxime
bla_{CTX}	Cefotaximase β-lactamase (ESBL)
dfr	Dyhydrofolate reductase resistance gene
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamenetetra acetic acid
ESBL	Extended spectrum β-lactam
HGT	Horizontal gene transfer
HRP	Horseradish peroxidase
Inc	Incompatibility
Int	Integron gene
IS*Ecp1*	Insertion element *cp1*
kb	Kilobase
M	Molarity (mol/L)
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
µg	Microgram
µl	Microlitre
ml	Mililitre
MLS	Macrolide Lincosamide Streptogramin
µM	Micromolar
NaPOi	Sodium Phosphate dibasic
ng	Nanogram
nmol	Nanomol
dNTP	2'-Deoxynucleotide-5'-triphosphate
bla_{OXA}	Oxacillinase β-lactamase
PCR	Polymerase chain reaction
PMF	Proton motive force
Rep	Replicon
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Rate per minute
sul	Sulphonamide resistance gene
str	Streptomycin resistance gene
tet	Tetracycline resistance gene
bla_{TEM}	Temorina β-lactamase (ESBL)
TMB	Trimethylbezedene
U	Units/ml
dUTP	2'-Deoxyuridine

1 INTRODUCTION

1.1 Antibiotic Resistance: An Introductory Overview

Antimicrobials are defined as substances with the ability to inhibit bacterial growth (bacteriostatic) or kill bacteria (bactericidal). Antimicrobials broadly refer to anti-infective agents against bacteria, from microbial (termed antibiotics), synthetic, semi-synthetic, plant and animal origin (Courvalin 2006). Antimicrobials target several mechanisms in bacteria, nucleotide synthesis (RNA, DNA), protein synthesis, cell wall synthesis, metabolic pathways and cell membrane disruption.

Resistance to antimicrobial compounds can be mediated by intrinsic and acquired mechanisms. The former mechanisms referrers to bacteria of a particular species that are innately resistant to particular compounds, without prior exposure to the compound. Acquired resistance is where a bacterial population, initially susceptible to a given antimicrobial, become resistant, proliferate, and spread in a population upon selective pressure to the given antimicrobial. This clonal expansion of the resistant strain is known as vertical transmission of resistance. Another method of resistance is the transfer of resistance determinants by mobile genetic elements such as plasmids, transposons and bacteriophages. Collectively this method of transmission is known as horizontal gene transfer (HGT).

Resistance can be achieved by several mechanisms including acquisition of genes that encode enzymes that inactivate the antimicrobial e.g. β-lactamases that cleave the amide bond of the β-lactam ring, efflux systems that enable the extrusion of the compound, acquisition of genes that encode proteins with altered drug resistant variants (e.g. *sul* genes that confer resistance to sulphonamides), or protect the target (*tetO tetM* ribosomal protection proteins that confer resistance to tetracycline). Alternatively

bacteria can acquire mutations in target molecules that render them less able to bind efficiently to the antimicrobial (*gyrA* mutations that confer resistance to fluoroquinolones), or may incur mutations in regulatory regions that could result in the overexpression of target proteins (e.g. in trimethoprim resistance), or resulting in down regulation of porin genes that would limit access of the antimicrobial into the cell (OmpF in *E. coli*) (see reviews; Normak et al., 2002).

Acquired resistance poses a great threat to treatment of bacterial infections. The acquisition and spread of resistance has partially been linked to the transfer of resistance genes by HGT. Plasmids have been implicated in much of this spread, readily transferring by conjugation (Caratolli, 2009). The persistence of resistance to antimicrobials despite the cessation of prescription e.g. sulphonamides, supports the hypothesis that some bacteria may act as potential reservoirs of resistance. An example would be the *Enterobacteriacieae* and other gut microbes (Levy 1982; Osterblad, Leistevuo et al. 1995; Sunde and Sorum 1999). Previous studies have shown *E. coli* to be one of the most significant contributors to persistence of antimicrobial resistance in healthy humans, with a large number of resistance genes encoded on plasmids (Osterblad, Hakanen et al. 2000). *E. coli*, a commonly found commensal, can harbour multiple plasmids that may encode some resistances, including plasmids that harbour $bla_{\text{CTX-M}}$, conferring resistance to third generation cephalosporins. This class of β-lactam antimicrobials were commonly prescribed to treat a wide variety of infections. However due to the increased incidences of resistance, mediated by extended spectrum β-lactamases, such as CTX-M, treatment failure was quickly observed. This class of antimicrobial target membrane anchored enzymes involved in cell wall synthesis, preventing peptidoglycan crosslinking thus weakening the cell wall. The relative ease of accessing these enzymes (localised on the outer cell membrane), low production costs and high specificity, makes this an ideal anti-infective target. Therefore investing in

prolonging the efficacy of this drug is of great interest to public health. Understanding the mechanisms that govern resistance to these antimicrobial is the first step. This thesis aims to investigate in detail the genetic platform to which these ESBL enzymes are encoded on and the effect to the host.

1.2 Pathogenic *E. coli*

E. coli is one of the most abundant Gram-negative facultative anaerobes inhabiting the gastrointestinal (GI) tract, and its potential to act as a reservoir for resistance genes makes this an interesting organism to study.

E. coli strains are largely non-pathogenic, however if the host becomes immunocompromised or suffers damage to the GI lining, then even these non-pathogenic strains of *E. coli* can mount an infection (Kaper et al., 2004). However, the acquisition of virulence factors which, like antibiotic resistance genes may be carried on a variety of MGEs, gives rise to *E. coli* pathotypes with the ability to cause disease in the host, leading to complications such as; sepsis, meningitis, urinary tract infections, diarrhoeal diseases, mastitis, enterotoxaemia, homolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) (Erskine et al., 2002; Lanz et al., 2003; Nataro and Kaper, 1998). These differ from their non-pathogenic counterparts, because of their ability to express virulence factors that aid colonisation of host surfaces and ensure survival within the host.

Enteropathotypes of *E. coli* are classified into at least seven different classes based on their virulence mechanisms which include; (i) enterotoxigenic *E. coli* (ETEC), (ii) enteropathogenic *E. coli* (EPEC), (iii) enterohaemorrhagic *E. coli* (EHEC), (iv) enteroinvasive *E. coli* (EIEC), (v) enteroaggregative *E. coli* (EAEC), (vi) diffuse adherent *E. coli* (DAEC) and (vii) necrotoxigenic *E. coli* (NTEC) (Nataro and Kaper, 1998). In addition to these intestinal pathogens, other clinically relevant *E. coli*

subspecies include; uropathogenic *E. coli* (UPEC), which causes urinary tract infections; and increasing cases of extraintestinal pathotypes of *E. coli*, found to be the causative agent in some cases of meningitis and sepsis, are collectively known as extraintestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2000).

E. coli encode virulence factors not usually found in commensals, that enable pathogenic *E. coli* to overcome host defences e.g. enabling the bacterium to colonize a variety of different host cell surfaces (adhesins), to evade host immunity and survive within serum (bacterial capsule, endotoxins and exotoxins), to divide within and escape from macrophages, to cause damage to the host tissue, often mediated by toxins. These capabilities are achieved by possession and expression of multiple virulence genes carried by the bacterium of which many are also be found located on plasmids such as heat labile toxin genes in enterotoxigenic *E. coli* and fimbrial adhesins on many differing *E. coli* (ETEC, EHEC etc). These include the presence of fimbriae, toxins, siderophores (iron capture), intimate adherence and acid tolerance. These features are essential in ensuring survival of the bacterium in the host (Castanie-Cornet et al., 1999; Nataro and Kaper, 1998; Richard and Foster, 2003).

The different pathotypes of *E. coli* can be classified by their 'O' antigens (lipooligosaccharides), 'H' (flagella) and 'K' (capsular) antigens. A combination of the O and H antigens defines a serotype (F. Kauffman 1946, Nataro and Kaper, 1998). To date there are over 100 different K groups, 180 O groups and over 60 H groups described, giving rise to approximately 700 different antigenic types (Robins-Browne and Hartland, 2002). Although still used today, serotyping methods are now supplemented by other molecular methods to distinguish between strains in addition to more traditional biochemical tests, Gram staining and colony morphology observation, summarized in table 1 below.

Table 1-1: Properties used to identify *E. coli*

Test/observation	Result
Colony morphology	Colonies appear round, smooth and shiny
Gram stain	Gram-negative rods
Catalase positive	Yes
Oxidase positive	No
Indole production	Yes
Nitrate reduction	Yes
Methyl red	Yes
Glucose, lactose, sucrose, maltose and lactose fermentation	Yes
Raffinose fermenter	No
Hemolysis	Some yes, β-hemolysis
Coagulase production	Yes

1.2.1 Molecular subtyping of *E. coli* strains

Molecular typing/grouping methods to distinguish different *E. coli* species have provided a useful tool for monitoring *E. coli* strains that may be responsible for systematic outbreaks. Described below is a brief summary of some of the tools that may be used, some of which were employed in identification of the epidemic international *E. coli* clone ST131 O25:H24

1.2.1.1 Pulse field gel electrophoresis

Pulse gel electrophoresis (PFGE) is a molecular subtyping tool used to distinguish strains of *E. coli* based on their restriction digestion patterns. DNA fragment patterns are interpreted based on the PFGE pattern. The principle of this typing relies on random genetic changes that result in differential restriction pattern. Strains can be categorised based on their relatedness; 1) indistinguishable (part of an outbreak): which have an identical number and size of bands, 2) closely related (probably part of an outbreak): a difference of 2-3 DNA fragments, 3) possibly related (possibly part of an outbreak): a

difference of 4-6 bands and 4) unrelated (not part of the outbreak): a difference of ≥ 7 (Tenover et al., 1998). PFGE has been employed in previous studies to type the ST131 clones, however this method did not cluster these strains as part of an outbreak under the Tenover criteria, and often did not meet the 'possibly related' criteria.

1.2.1.2 MLST

Multi-locus sequence typing was developed in later studies and showed one *E. coli* known, now known commonly as sequence type (ST) 131, to belong to the same clone. This method is based on sequencing short nucleotide sequences (400-500bp) of 5-7 housekeeping genes that have diversified resulting in polymorphisms within the genes to give sequence types. The method relies on the low mutation rates of the housekeeping genes, making it a suitable method for monitoring strain epidemiology (Enright et al., 1999). MLST transformed how molecular epidemiology data was analysed, whereas with PFGE, single nucleotide polymorphisms could results in changes to the digestion pattern and consequently migration. This would result in almost no information as to the clonality. Using housekeeping genes that are strongly conserved for typing in MLST enables information on the population biology and evolutionary history to be assimilated.

1.2.1.3 Other typing methods

As described above MLST offers a robust typing system when studying strain epidemiology. However due to the time consuming protocol and expensive, this method is rarely used for high throughput screening. Other methods such as random amplified polymorphic DNA (RAPD), Enterobacterial repetitive intragenic consensus sequences (ERIC) PCR and ribotyping provide information on strain relationships.

Other more broad identification tools include phage typing; based on the lytic patterns denoting resistance or sensitivity to bacteriophage infections and *E. coli* Phylogrouping. Phage typing is limited to *E. coli* O157:H7 and its susceptibility to 16 different bacteriophages, to which 88 different bacteriophages are used in combination to distinguish between *E. coli* strains (Ahmed et al., 1987; Woodward et al., 2002). Phylogrouping is a method used to type strains based on electrophoretic mobility (Multi-locus enzyme electrophoresis – MLEE) or more recently the PCR amplification of variable genes in different phylogroups of *E. coli*, of which there are four A, B1, B2 and D. Later whole genome sequencing techniques found the non-pathogenic/commensal group B1 to be further subdivided into phylogroup E and B1, a non-O157 EHEC pathogenic group and a commensal group respectively (Sims and Kim, 2011).

1.3 E. coli structure

E. coli is a Gram-negative rod, typically measuring 2-6 μm, which inhabits both human and veterinary gastrointestinal tracts as part of the gut flora characteristic to the members of the *Enterobacteriaceae* family (Nataro and Kaper, 1998).

The Gram-negative cell wall consists of an inner and outer membrane (Fig. 1-1), with multiple protein constituents. Adjacent to the inner membrane is the rigid peptidoglycan layer consisting of alternating residues of β-(1,4) linked N-acetylglucosamine and N-acetylmuramic acid, liked by a peptide chain consisting of 3-5 amino acids (to be described in greater detail in section 1.9). Undecaprenyl phosphate sugars are essential in peptidoglycan synthesis they act as glucosyl carriers enabling the transfer of hydrophilic motifs across the cytoplasmic membrane (Bouhss et al., 2008). Membrane derived oligosaccharides (MDO); a class of β-glucans containing β-1-2 and β –1-6 linked glucose subunits with substitutions of membrane derived moieties, in addition to

the peptidoglycan layer forms an extensive porous mesh-like polymer matrix (Lequette et al., 2008; van Golde, 1973). Between the peptidoglycan layer and the outer membrane lies the periplasmic space containing soluble proteins e.g. enzymes. The peptidoglycan layer is anchored to the outer membrane by lipoproteins. Embedded on the outer membrane are the outer membrane proteins also involved in transport e.g. porins and components of the bacterial secretion systems. Forming a monolayer covering the outer membrane are lipopolyssaccharides, which contribute to cellular integrity and provide protection from chemical attack. (Holtje, 1998; Robins-Browne and Hartland, 2002).

The cell wall and membrane encases the cytoplasm, containing proteins, enzymes and the DNA. The genetic components of *E. coli* are discussed in the section that follows.

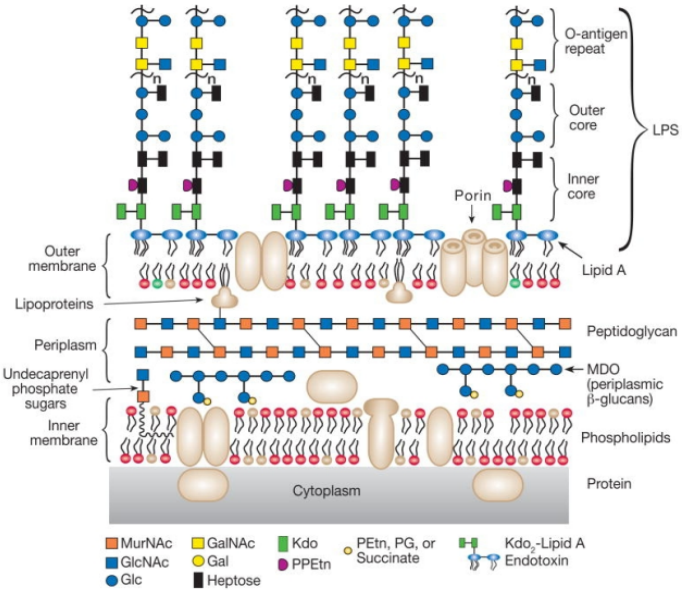


Figure 1-1: Schematic representation of the structure of a gram-negative cell wall. In red are the phosphate heads of the phospholipids; yellow are the phosphatidylethanolamine phospholipids, yellow phospholipids; phosphatidylglycerol; green are Kdo; 3-deoxy-D-*manno*-octulosonic acid, black heptose consisting of L-glycero-D-manno-heptose, 'n' is a variable number of O-antigen repeats, Pink/purple are PPEtn; pyrophosphoethanolamine. Picture taken from Essentials of glycobiology (2nd Edition) 2008.

1.4 Genetics of *E. coli*

The *E. coli* genome is made up of a single, circular double stranded DNA molecule about $4x10^6$ base pairs (bp) in length, localised within the cytoplasm in a region termed the nucleoid. In the era of whole genomic sequencing the size of *E. coli* genomes can be variable due to core and accessory genes. In addition to the genophore (prokaryotic genome), *E. coli* can harbour plasmids; circular double stranded DNA molecules capable of autonomously replicating within the bacterial cell. These can vary in number (estimated to be up to 11 plasmids) and size (1kb to 200kb). These extrachromosomal DNA molecules have the capacity to encode a multitude of genes including antimicrobial and heavy metal resistance, antibiotic production, bacteriocins, toxins and various virulence factors. Of particular relevance to this thesis is the frequent but non-exclusive carriage of antibiotic resistance genes on plasmids.

1.5 Horizontal gene transfer

Horizontal gene transfer (HGT) is the acquisition of genetic material from an exogenous source, within or outside a phylogenetic group or even between different organisms. HGT is a major driving force behind evolution and adaptation in bacteria especially in the development of antimicrobial resistance. HGT can be mediated by three primary mechanisms: a) conjugation, mediated by plasmids or conjugative transposons; b) transduction, mediated by bacteriophages; and c) transformation of cell-free DNA. These three mechanisms enable the transfer of DNA *inter*cellularly and *intra*celluarly increasing the genetic diversity, collectively known as mobile genetic elements (MGE). MGEs are DNA molecules that are capable of movement either by transposition or homologous recombination. Other MGEs include insertion sequences (IS) or insertion elements (ISE), transposons, integrons and cassettes (Carattoli, 2003). Of all the

mechanisms, plasmid conjugation is currently regarded by many authors as the most common mechanism of transfer among prokaryotes (Bennett, 2008; Carattoli, 2001; Frost et al., 2005; Liebert et al., 1999; Mahillon et al., 1999; Sota, 2008). Irrespective of whether plasmids mediate most HGT, of importance to this thesis is not only the carriage but also the transmission of β-lactam resistances by plasmids.

1.5.1 Transduction and Transformation

The transfer of DNA between bacterial cells can occur by either of these mechanisms. Natural bacterial transformation can occur in natural environments, the contribution to HGT cannot be quantified, because the competency of the bacteria cannot be determined in the environment, however studies using naturally competent bacteria have demonstrated the ability of transformation to contribute to bacterial evolution (Rizzi et al., 2008).

Transduction is mediated by bacteriophages and is generally limited to phages that accommodate large DNA segments 50-100kb genomes. Bacteriophages, which can lie dormant in the host bacterial chromosome as prophages, may switch to a virulent replication state and result in maturation of the virus particle, and packing of its own DNA (specialized transduction) or accidentally packaging host DNA (generalized transduction). The lysis of the cell and infection of another recipient cell, could result in recombination (Frost et al, 2005).

1.5.2 Bacterial Plasmids

Plasmids are extranuclear genetic elements capable of autonomous replication. Plasmids commonly exist as double stranded covalently closed circular molecules, but increasingly, linear plasmids have been found in some bacterial species (Hinnebusch

and Tilly, 1993). Plasmids can also be integrated into the bacterial chromosome; known as integrated conjugative elements (ICEs), increasing the diversity of the genome from laterally acquired DNA from distantly related sources (Andam et al., 2011; Burrus et al., 2004). Plasmids encode a number of genes that control their replication and maintenance, in addition to genes that may confer some advantage to the host e.g. antimicrobial resistance, metabolic function or virulence genes. Bacterial plasmids can be self-transmissible by conjugation, often encoding these proteins necessary for conjugative transfer on the plasmids itself. Mobilizable plasmids do not encode these genes and consequently cannot transfer by conjugation unless with other larger conjugative plasmids or integrative conjugative elements (ICE) by a process known as mobilization. Other plasmids, like the cryptic plasmids, do not encode any genes that confer a known phenotype (Frost et al., 2005; Francia et al., 2004; Sota, 2008). With the advent of plasmid DNA sequencing, recent studies have reported the presence of extensive plasmid transfer genes that make up the large structures necessary for conjugative transfer, resistance and virulence. Woodford and colleagues reported three ESBL plasmids found in the ST131:O25 international *E. coli* clone (Woodford et al., 2009).

1.5.3 Transposons

Transposons are part of the MGE family of transposable elements that move by two main mechanisms, conservative transposition and by replicative transposition. The conservative transposition mechanism simplistically is a "cut and paste" mechanism between donor and recipient DNA. Replicative transposition, involves a co-integrate intermediate between donor and recipient, after recognition of target DNA by recombination. The exchange of DNA is catalysed by a transposase (*tnpA*) and the cointegrate resolved by a resolvase (*tnpR*) (Derbyshire et al., 1986). There are two types

of transposons commonly found in Gram-negative bacteria, composite and complex. Composite transposons are characterised by the presence of insertion sequences (IS) either side of the intervening gene. Complex transposons are more diverse in nature and have been identified as the most prevalent class associated with antimicrobial resistance genes. Complex transposons transposase by replicative transposition. They are characterised by flanking 38bp inverted repeats (IR), *tnpA* and *tnpR* genes and a *res* site, at which resolvase acts. The Tn*3* family of complex transposons consist of a family of transposons including the most widely studied member of this group Tn*21* (Grinsted et al., 1990; Liebert et al., 1999) (Fig. 1-2). These transposons can encode a multitude of resistance genes, but can also carry other mobile genetic elements independent of the transposon; including integrons, gene cassettes (open reading frames), metabolic operons and ISEs (Hall and Collis, 1995; Liebert et al., 1999). Tn*21* transposons have been found in association with β-lactamase genes; CTX-M-9 and CTX-M-2 and CTX-M-15, thought to have influenced the spread of these genes (Novais et al., 2006, Sandergen et al., 2011; Valverde et al., 2006).

1.5.4 Conjugative transposons

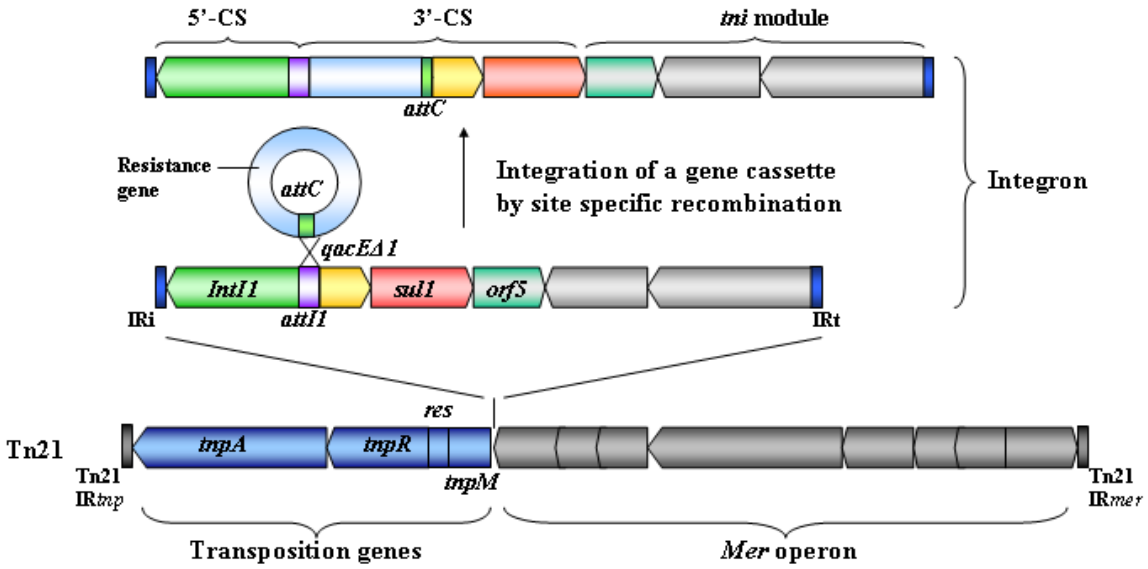
Conjugative transposons are non-replicative self-transmissible integrative elements derived from bacteriophages, with the ability to trigger the transfer of other non-self transmissible elements and plasmids. Transfer of genetic material occurs via a covalently closed intermediate, integrating into the chromosome/DNA without duplicating the target site at the point of integration. Conjugative transposons cannot replicate independently and thus require a further recombination event once transfer to the recipient cell has taken place to ensure its maintenance. To date, no conjugative transposons have been found in *E. coli* (Hochhut and Waldor, 1999; Scott and Churchward, 1995; Waters, 1999).

1.5.5 Integrons

Integrons are gene capture systems that employ site-specific recombination to integrate gene cassettes at specific insertion sites (*attI*) in addition to a secondary recombination site (*attC*) within the integron. Integration is mediated by a site-specific recombinase (*intI),* which encodes an integrase (Carattoli, 2001; Fluit and Schmitz, 2004; Recchia and Hall, 1995).

Multiple gene cassettes can be integrated into one integron with expression driven from two integron specific promoters, Pc and P2 upstream of the gene cassettes (Hall and Collis, 1995;). The promoters are located on the 5' conserved region of the *intI* gene and the *attI*. The secondary *attC* site, located at the 3' end of the gene cassette mobilizes the gene, which integrates the gene cassette to an integron via the *attI* site (Fig. 1-2) (Jove et al., 2010; Mazel, 2006; O'Brien, 2002).

There are four classes (1-4) of integrons, defined by the sequence identity of the integrase enzymes. Of the four, class 1 and 2 are the most prevalent among pathogenic bacteria (Carattoli, 2001). Integrons themselves are not mobile but aid the capture of genes, however they are sometimes found in association with part of the *tni* module, thought to encode transposition genes because of its similarity to *tni* genes of Tn*5053 and* Tn*402* (Fig. 1-2). These genes (*tni*) are thought to be part of the larger transposition module of related transposons such as Tn*5053*, but the loss of some of the *tni* genes classifies most clinical class 1 integrons as defective transposons (Betteridge et al., 2011; Brown et al., 1996; Kholodii et al., 1995; O'Brien, 2002,).



Adapted from Carattoli, 2001 and Liebert et al., 1999

Figure 1-2: Schematic diagram of a transposon, Tn*21*. The flanking inverted repeat (IR) sequences are represented by the blue line, a *mer* operon (genes encoding mercury resistance and its regulation) are in grey. The *tnp* region, which encodes the transposase gene (*tnpA*), the resolvase gene (*tnpR*) the putative transposon regulator (*tnpM*) and a resolution site (*res*) are in the blue-arrowed boxes. Also shown is a classic integron flanked with IR sequences (grey lines). The integron harbours a 5' conserved segment (CS); which includes the integrase gene (*intI1*), an *attI1* insertion site. Gene cassettes within the integron include the quaternary ammonium compound disinfectant resistance gene (qacEΔ1) and sulphonamide resistance gene (*sul1*), *orf5* (unknown function) and the transposition (*tni*) module shaded in grey. Gene acquisition by recombination via the (*attC*) site is outlined (Carattoli, 2001; Liebert et al., 1999). Depicted by block arrows is the direction the genes are transcribed.

1.5.6 Insertion elements

Insertion elements (ISs) are major contributors to genetic plasticity in bacterial genomes. They are commonly associated with transposons and together cooperate in the mobilization of genes they encompass. IS elements have been found amongst bacterial chromosomes, pathogenicity islands and plasmids, found to assemble an array of genes of different functions including virulence, catabolic utilization and antimicrobial resistance (commonly associated with class II transposons like Tn*3*). Insertion elements are typically 0.5-2 kb in size, that encode a transposase (TnpA or InsAB fusion protein)

for transposition, recognising the terminal inverted repeats. These elements can however mobilise independently of the transposon. Their mobilization is catalysed by a DDE transposase characterized by the amino acid triad (aspartate-aspartate-glutamate) and can be mobilized by either the cut and paste mechanisms (described in section 1.5.2) or the rolling circle mechanism, which results in the circularisation of the IS elements bringing the IRs together, which contain the -35 or -10 box forming the promoter. A third mechanism involves a conintegrate intermediate (Shapiro intermediate) between the donor and recipient. Once resolved, two copies of the IS element exist, one in the donor and the other in the recipient (Bennet et al., 2004; Mahillon et al., 1999).

1.6 Plasmid transfer and maintenance

1.6.1 Conjugation in Gram-negative bacteria

Bacterial conjugation is by far the most studied of the DNA translocation systems (Waters, 1999). Conjugation is fundamental in HGT, not only limited to genes that confer antimicrobial resistance but also includes genes that enable the bacterium to occupy a new niche e.g. hydrocarbon degradation, production of nitrogen fixing nodules and /or to exert virulence (Lamb et al., 1982; Lawley, 2004).

Plasmid mediated bacterial conjugation is dependent on successful transfer of plasmids to the recipient. For this to occur, close contact between the donor cell and recipient must be achieved. The success of conjugative transfer relies on three essential proteins, (i) the transferosome: a multi-protein complex that forms the conjugative pilus via a type IV secretion system (T4SS), also known as the membrane pair formation (Mpf) complex, (ii) the relaxasome; a DNA/protein complex that is formed by the binding of transfer proteins to the origin of transfer (*oriT*), introducing single stranded nicks in the DNA and finally (iii) the coupling protein (CP); a cytoplasmic membrane protein that

links the two components together enabling transfer of DNA to the recipient (Cascales and Christie, 2004; Gilmour M. W., 2000; Lawley, 2004; Waters, 1999).

Initial contact of the donor and recipient cells can occur in two ways; random collision or be mediated by the conjugative pilus. The pilus mediates initial attachment recognising a carbohydrate receptor on the surface of the recipient cell and thus stabilizing the mating pair (Fig. 1-3). This triggers the rapid initiation of DNA transfer independent of prior transcription and translation events (Gilmour M. W., 2000; Lawley, 2004; Waters, 1999). The relaxosome, mediated by a relaxase enzyme, cleaves the negatively supercoiled plasmid DNA at the *oriT* junction; an AT rich sequence comprised of tandem repeats that contains a *nic* site (the site at which DNA cleavage occurs). This results in the covalent attachment of the tyrosine residue from the relaxase enzyme to the 5' end of the nicked DNA (Pansegrau et al., 1990). Strand replacement synthesis occurs at the 3' end of the nicked DNA, catalyzed by DNA polymerase III via a rolling circle mechanism (Lawley, 2004; Pansegrau et al., 1990). Once transferred, both the donor nicked DNA and the newly synthesised single stranded DNA molecule in the recipient is re-circularized by the relaxase complex and conjugational transfer is halted (Becker and Meyer, 2000). The 3' end then acts as a primer for synthesis of the replacement strand in the recipient (Becker and Meyer, 2000).

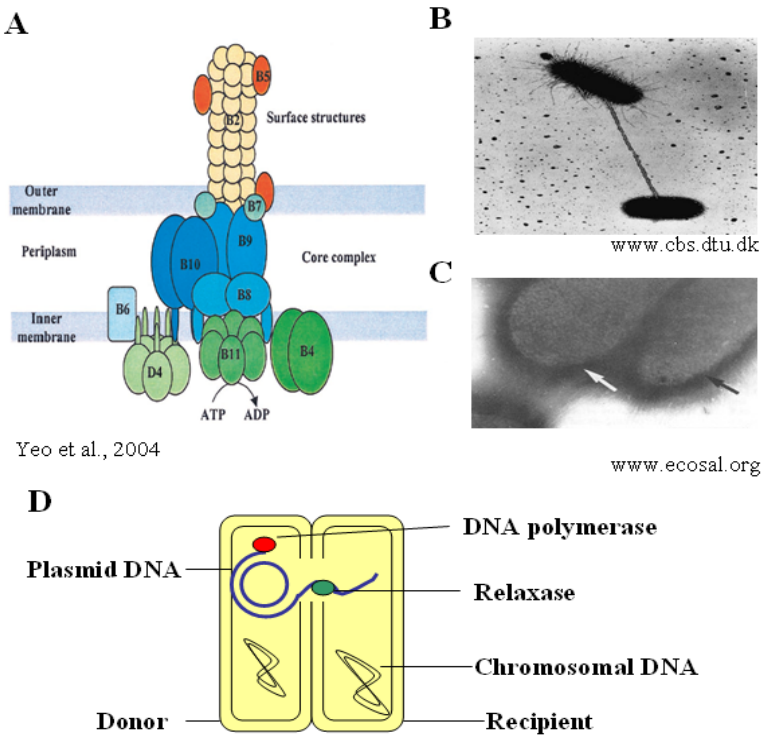


Figure 1-3: Bacterial conjugation in Gram-negative bacteria. (A): schematic diagram of the components of the type IV secretion system in *A. tumefaciens*. In green is the ATPase that provides energy for the translocation of molecules across to the recipient. (B): mating bridge formation between cells. (C): Mating aggregates; donor strains have an abundance of pili and flagella (black arrows) and recipients are 'bald' indicated by the white arrow. (D): schematic representation of rolling circle replication of plasmid DNA and translocation of genetic material.

1.6.2 Pilus structure

The T4SS is a complex of 13 core proteins that span the cell envelope facilitating the transfer of DNA or effector proteins through a pore to the recipient and even take up DNA during natural transformation (Chen and Dubnau, 2004; Hofreuuter et al., 2001; Lawley et al. 2003). The T4SS system is made up of core proteins; TraA (pilin), L, E, K, B, V, C and G and auxiliary proteins; TraF, G, H, N, U, W and TrbC. These proteins make up the pilus structure and proteins necessary for mating pair stabilization. Additional proteins, TraD (coupling protein) and TraI (relaxase), M and Y respectively, are necessary for binding to DNA and subsequent transfer.

Two types of pilus have been described in the conjugative systems: the F-like pili and the P-like pili. These pili differ in their structure, the F-like pili being long and flexible and the P-like pili short and rigid (Lawley et al. 2003; Schroder and Lanka, 2005).

1.6.3 Plasmid establishment in the recipient cell

The incoming plasmid DNA may experience a hostile environment upon entry to the new host. In order to exist symbiotically with the host, the plasmid must initially overcome any host defences for example activation of bacterial SOS systems that result in plasmid destruction or cell death, which can be triggered by the transient single stranded plasmid DNA molecule upon entry into the recipient (Baharoglu et al., 2010).

Upon entry into the recipient cell, plasmid encoded *ssb* and *psiB* proteins are expressed. SSB is a DNA binding protein that binds to single stranded DNA during DNA replication amongst other metabolic processes. Previous studies showed that *ssb* was able to depress genes necessary for bacterial conjugation in bacteria that encoded a *drd* mutation; a gene required for the suppression of conjugation. PsiB is a protein that is involved in inhibition of the SOS response in *E. coli*, possibly by inhibiting the activation of the RecA protein; a protein involved in the activation of the *E. coli* SOS response (Althorpe et al., 1999). Some plasmids in addition to *ssb* and *psiB* possess an anti-restriction system encoded by the *ardA* gene. This gene when expressed protects the incoming plasmid from digestion by type I restriction enzymes, which recognise and digest unmethylated 'exogenous' DNA, allowing the unmodified plasmid DNA to evade restriction (Althorpe et al., 1999). All three genes (*ardA, ssb* and *psiB*) are transferred early during plasmid translocation to alleviate the hostile environment in the recipient cell.

1.6.4 Plasmid maintenance

Once the plasmid has established a replication system in the recipient cells, ensuring its maintenance in the host is vital. Bacterial plasmids employ several mechanisms to ensure this, which include, postsegregational killing, partitioning systems and site-specific recombination systems (Funnell, 2005).

1.6.4.1 Postsegregational killing (PSK)

This mechanism refers to the maintenance of a genetic element in a host, known as genetic addiction. It relies on the action of a toxin and antitoxin encoded on the plasmid, to which the activity of the toxin is inhibited by the antitoxin. The antitoxin can counteract the toxin in three ways, (i) by inhibiting expression of the toxin (ii) by inhibiting the toxin itself or (iii) or by protecting the cellular target. An imbalance or loss of the toxin/antitoxin system (due to plasmid loss) results in the rapid decomposition of the antitoxin due to its proteolytic activity, leaving the toxin to exert its action of the cellular target that subsequently leads to programmed cell death (Jaffe et al., 1985).

There are three types of genetic addiction systems (or PSK) which include, a proteic system, a restriction modification system and an anti-sense RNA regulated system. Below is a brief overview of the above mechanisms involved in genetic addiction (Gerdes, 2000).

1.6.4.1.1 Proteic system

The proteic system is the classical toxin/antitoxin system where the antitoxin serves as the antidote to the toxin by binding and inhibiting its activity (Critchlow et al., 1997). An example of this system is the CcdB/CcdA: toxin/antitoxin protein set. The CcdB toxin binds to the DNA gyrase, specifically the GyrA subunit, forming a CcdB-GyrA complex which results in the formation of double stranded DNA breaks mediated by

DNA gyrase (Critchlow et al., 1997; Gerdes, 2000). Although the exact method of eventual cell death is unknown, it has been proposed that the CcdB-GyrA-DNA complex forms a lesion at the replication fork that blocks DNA replication much like the quinolones that block DNA replication. Blocking polymerase access inevitably affects downstream processes that are thought to lead to cell death (Critchlow et al., 1997; Jaffe et al., 1985).

1.6.4.1.2 The restriction modification system

The toxin in this case is a restriction enzyme that recognises and cleaves specific DNA sequences (see REBASE for extensive study of these proteins; http://rebase.neb.com). The introduction of these breaks in the DNA in turn induces the bacterial SOS response that is signalled by DNA damage, consequently leading to cell death. The antitoxin negates the effect of the toxin by binding to the sequence specific regions and methylating the DNA, which prevents cleavage of the DNA. Loss of the gene results in dilution of the antitoxin and subsequent restriction enzyme mediated cleavage of chromosomal DNA and cell death. Although both the toxin and antitoxin are diluted at the same rate, the restriction enzyme need only cleave one site in the chromosome to induce cell death in contrast to the methyltransferase that requires methylation of all available sites to preserve cellular integrity (Kobayashi, 2001).

1.6.4.1.3 Antisense-RNA regulated addiction systems

The antisense-RNA regulated system relies on repression of toxin gene expression by its own antisense-RNA, encoded on the same locus. The antitoxin is an unstable antisense RNA molecule that binds to the leader region of the toxin gene and inhibits translation of the toxin gene (Blomberg et al., 1990). The RNA-RNA duplex is then cleaved by a ribonuclease III (RNAseIII) enzyme and prevents translation of the toxin protein. Loss of the gene results in the rapid decay of the RNA by the RNAse, and expression of the toxin is no longer repressed (Gerdes et al., 2000).

1.6.4.2 Partitioning systems

Plasmid partitioning systems are positioning systems, but also confer some incompatibility. This differs from the classical replication mediated incompatibility system (see later), although it is loosely based on the same concept. That is to say, plasmids with the same partitioning system cannot coexist in the same cell. The Par proteins, ParA and ParB are encoded by the *par* gene. The ParB protein bind to a *cis* acting centromere-like partition site, multiple ParB proteins are recruited and bind to the DNA forming a nucleosome complex called the segrosome. This results in plasmid pairing of the newly replicated plasmid, and subsequently the recruitment of ParA, an ATPase that stimulates the separation of the plasmid pairings. By adhering to these strict subcellular localizations, plasmids ensure faithful segregation into daughter cells during cell division (Funnell, 2005).

1.7 Plasmid incompatibility

The success of genetic transfer between cells lies on the ability of the recipient cell to accept the donor plasmids. Plasmids are classified by incompatibility (Inc) grouping (Novick, 1987). This is defined as the inability for two plasmids to coexist in a cell in the absence of selection. That is to say, that if by conjugation (or transformation) a plasmid were to be introduced into a strain carrying an unknown Inc group and was eliminated in the progeny, the incoming plasmid would be termed incompatible, and assigned to the same Inc group (Carattoli, 2003; Datta and Hedges, 1971). This method of classification allows the identification and grouping of plasmids used to study the epidemiology of plasmids known to harbour antimicrobial resistance genes (Anderson et al., 1977).

Plasmid incompatibility stems from the need for plasmids to control their copy number within a host. Replication control is inversely proportional to the copy number i.e. a high copy number results in a low replication rate, therefore preventing plasmid loss due to low copy numbers in the cell. The alternative is the loss of replication control termed 'runaway replication', resulting in high copy numbers that eventually lead to cell death after about four generations (Brantl, 2004).

Prior to the development of molecular Inc typing methods, plasmid grouping was based on the elimination of an incoming unknown plasmid from the recipient containing a plasmid of a known group (Datta and Hedges, 1971). Plasmids were then assigned an incompatibility group denoted by a letter in the alphabet. Using phenotypic methods such as plasmid elimination to group the plasmids proved difficult when trying to identify non-conjugative plasmids or plasmids that did not transfer across due to entry exclusion (surface exclusion), which arise from donor plasmid inhibiting an incoming plasmid, preventing redundant transfer of the same or related plasmid (Perumal and Minkley, 1984).

In 1988, Couturier et al. developed a scheme to classify plasmids based on genetic similarities in their replication regions (replicons) using DNA hybridization techniques. This technique further propelled plasmid classification but could not discriminate between closely related plasmids leading to cross-hybridization reactions (Carattoli, 2009; Couturier et al., 1988).

In recent years the development of a PCR based replicon typing (PBRT) method for typing plasmids occurring in *Enterobacteriaceae* has enabled the rapid identification of plasmid groups (Carattoli et al., 2005). To date 26 Inc groups have been identified in *Enterobacteriaceae* by the plasmid section of the National Collection of Type Cultures (NCTC; London, United Kingdom). The PBRT technique uses 18 different primer pairs; 5 multiplex and 3 simplex reactions recognizing HI1, HI2, I1-γ, X, L/M, N, FIA,

FIB, FIC, W, Y, P, A/C, T, K, B/O, FrepB (which detects 20 of the 26 FII, FIII, FIV, FV and FVI variants) and $FIIA_s$. However with every technique, limitations are expected and although the PBRT offers a high throughput screening of plasmid groups, identification of new and divergent variants require sequencing of the plasmids (Carattoli, 2009).

1.8 Plasmid multi-locus sequence typing (MLST)

Plasmid MLST is another typing scheme that is based on the amplification of several housekeeping genes and assigning plasmid types based on sequence variation, as in traditional MLST. To date plasmid typing schemes exist for IncI1, HI1, HI2, F and N plasmids. Sequences of alleles are input into the pMLST database (www.pubmlst.org) and sequence types assigned (Garcia-Fernandez et al., 2008; Garcia-Fernandez et al., 2011; Villa et al., 2010).

1.9 Plasmid sequencing

Plasmid sequencing has proved to be an essential tool in understanding the structure of plasmids. Due to the ease of genetic exchange, typing systems may not account for the variability of the plasmid as a result of HGT. For example a plasmid may be typed as an IncI1 plasmid, but have a large amount of sequence from another plasmid group or may be a co-integrate of two plasmids. Plasmid sequencing is one way to obtain this information however, some challenges come with it. Firstly, extraction of large low copy plasmids, without genomic DNA contamination, and at high enough concentrations for sequencing to be performed has proved challenging. Nevertheless DNA extraction kits for bacterial artificial chromosomes have proved useful, but plasmids larger than 250 kb become more of a challenge to obtain using commercially available kits. Another challenge is the assembly. Assembly of whole genomic

sequencing (WGS) data uses a reference to assemble the sequencing reads into a scaffold. Due to the variability of the plasmid, aligning the contigs to a reference for assembly is not a viable option, instead de novo assembly methods are required. In addition, the presence of numerous repeat sequences as a result of IS elements or iterons (which control plasmid replication), are unable to be resolved by assembly programs. This will leave gaps in the sequence and primer walking is currently the only option to close gaps in the sequence (Frost et al., 2005). Despite the technical difficulties, a lot of information about the plasmids genetic content can be obtained.

1.9.1 Antimicrobial chemotherapy and resistance

Antimicrobials are defined as substances with the ability to inhibit bacterial growth (bacteriostatic) or even kill bacteria (referred to as bactericidal agents). This is a general term used to describe substances from microbial (termed antibiotics), synthetic, semi-synthetic, plant and animal origin (Courvalin, 2006).

As with most chemotherapeutic agents, antimicrobials target various structures and metabolic pathways, resulting in arrested bacterial growth (bacteriostatic) or cell death (bactericidal). These include substances that inhibit bacterial cell wall synthesis, protein synthesis, folic acid biosynthesis, DNA transcription and replication, all processes vital in ensuring growth and survival of the bacterium (Courvalin, 2006). See Table 1-2 for a list of antimicrobials, their targets and mechanisms of resistance.

Resistance can be mediated by six chief mechanisms; (i) altering the drug targets (e.g. fluoroquinolone resistance), (ii) mutations resulting in the over-expression of enzymes (e.g. resistance to sulphonamides and trimethoprim) and (iii) acquisition of genes that encode enzymes that alter the drug (e.g. aminoglycosides, MLS antibiotics, tetracyclines and β-lactams), (iv) altered membrane permeability, (v) increased efflux

and (vi) expression of alternative proteins with reduced affinity for the drug (e.g. resistance to sulphonamides and trimethoprim) (Alekshun and Levy 2007).

In addition, resistance to antimicrobials may be attributed to physiological changes including biofilm formation. *Pseudomonas aeruginosa* is an opportunistic bacterial pathogen that forms biofilms in lung infections of cystic fibrosis patients. These infections are notoriously difficult to treat because of the difficulty of the antimicrobial compound to penetrate the exopolysaccharide matrix (Drenkard et al., 2002). Another recent study by Llobert et al. 2011 found that *Klebsiella pneumoniae* strains exposed to polymixin, upregulated the expression of the capsule operon, causing an increase in capsule production. Polymixin is a cyclic lipopeptide that disrupts the LPS by displacing divalent cations that stabilise it. Resistance is thought to be mediated by decreased penetration of this compound through to the cell membrane (Llobert et al., 2011).

Table 1-2: Antimicrobial drugs and modes of inactivation.

Target mechanism	Target	Antimicrobial class	Mechanism of action	Mode of inactivation	Reference
DNA	Toposiomerase IV/DNA gyrase	Flouroquinolones	Bind to topoisomerase-DNA complex, resulting in accumulation of double stranded breaks in DNA.	Mutations in *gyrA* and *parC* . Qnr protection proteins; low-level resistance. Efflux (*norA* in *S. aureus)*.	Chen et al., 2003 36
		Coumarins	Competitive inhibitors of ATP for DNA gyrase and Topoisomerase IV.	Mutations in target GyrB subunit of DNA gyrase.	Gellart et al., 1976 Hardy et al., 2003
	RNA polymerase	Rifamycins	Binds beta subunit of RNAP thereby preventing trancription initiation.	Mutations in target RNAP *(rpo*B).	Kohanski et al., 2010
	DNA integrity	Nitrofurans	Double stranded DNA breaks mediated by free radicals.	Nitrofuran reductase; structural and functional changes. Mediated by *nsf*A and *nsf*B.	Whiteway et al., 1998
	Folic acid synthesis	Trimethoprim	Competitive inhibitors of DHFR thus preventing folic acid synthesis.	Mutations resulting in overproduction of DHFR. Mutations to decrease affinity of the drug. Expression of alternative DHFR enzymes.	Huovinen et al., 1995
		Sulfonamide	Competitive inhibitors of DHPS thus preventing folic acid synthesis.	Mutations resulting in overproduction of DHPS. Expression of alternative DHPS enzymes.	Huovinen et al., 1995
Protein synthesis	50S ribosomal subunit	Macrolides	Prevents peptide elongation.	Esterification and phosphorylation. MLS resistance efflux (*mef*A) and *erm* methyltransferase.	Leclercq et al., 1991
		Lincosamides	Bind to the 23S rRNA blocking both the 'A' and 'P' site and preventing peptide synthesis.	Acetylation. MLS resistance efflux (*mefA*) and *erm* methyltransferase..	Leclercq et al., 1991
		Streptogramins	Streptogramin A binds the A site and prevent elongation of protein synthesis. Conformational changes allow Streptogramin B to bind and prevent peptide elongation and release of incomplete peptides.	Streptogramin A; Acetylation and Streptogramin B; hydrolysis. MLS resistance efflux (*mefA*) and *erm* methyltransferase.	Leclercq et al., 1991
		Chloramphenicols	Binds to the 'A' site of the ribosome, thus preventing binding of tRNA.	Acetylation and efflux.	Schawrz et al., 2004
	30S ribosomal subunits	Tetracylcins	Binds to the 'A' site of the ribosome, thus preventing binding of tRNA and growing peptide.	Reduction of drug, ribosomal protection proteins. Efflux mediated by PMF.	Chopra et al., 2001
		Aminoglycosides	Reversibly bind to 16S rRNA resulting in incorporation of incorrect amino acids; misread.	Acetylation, phosphorylation, nucleotidylation. Altered target.	Kotra et al., 2000

Abbreviations: ATP; adenosine triphosphate, MRSA; methicillin resistant *S. aureus*, DHFR; Dihydrofolate reductase, DHPS; Dihydropteroate synthase, MLS; Macrolide lincosamide, streptogramin. LPS; lipopolysaccharide. Ala; Alanine, Lac; Lactate, Ser; serine. RNA; ribonucleic acid, rRNA; ribosomal RNA, tRNA; transfer RNA.

Table 1-2: ***continued.***

Target mechanism	Target	Antimicrobial class	Mechanism of action	Mode of inactivation	Reference
Cell wall	Peptidoglycan synthesis	Beta-lactams	Inhibiting transpeptidases therefore inhibiting crosslinking of peptidoglycan.	Hydrolysis of beta-lactam ring by beta-lactamases. Altered PBP. Porins (OmpF); reduced drug uptake. Target site duplication (MRSA).	Mascaretti, 2003
		Glycopeptides	Bind to D-ala-D-ala terminus of nascent peptidoglycan chain and inhibit addition of new amino acid subunits.	Alter chemical composition of target i.e. D-ala-D-lac (-D-ser) pentapeptide instead. Drug trapping; thicker cell wall (*S. aureus*).	Reynolds, 1989
		Beta lactam inhibitors	Co-administered with beta-lactams. Reversible/Irreversible inhibitor of beta-lactamases.	Over-expression of target beta-lactamase. Point mutations resulting in structural/functional changes.	Bush, 1988
Cell membrane	Phospholipids	Polymixins (Lipopeptide)	Bind to LPS and eliminate disulphide bridges by displacing metal cations (Mg+ and Ca+). Physiochemical disruption of membrane.	Efflux and target alteration.	Zavascki et al., 2007
		Bacitracins (cyclic polypeptide)	Inhibits regeneration of isoprenyl phosphates (by inhibiting isoprenyl phosphatase), a lipid carrier involved in biosynthesis of the cell wall.	Drug efflux (BcrABC).	Stone et al., 1971

Abbreviations: ATP; adenosine triphosphate, MRSA; methicillin resistant *S. aureus*, DHFR; Dihydrofolate reductase, DHPS; Dihydropteroate synthase, MLS; Macrolide lincosamide, streptogramin. LPS; lipopolysaccharide. Ala; Alanine, Lac; Lactate, Ser; serine. RNA; ribonucleic acid, rRNA; ribosomal RNA, tRNA; transfer RNA.

1.9.2 Disruption of DNA Synthesis

There are two main classes of antimicrobials that target nucleic acid synthesis: the quinolones and coumarins that target nucleic acid synthesis; and rifamycins that target RNA synthesis. Quinolones target two tetrameric enzymes; DNA gyrase, made up of two GyrA and GyrB subunits or the topoisomerase IV subunits made up of two ParC and ParE subunits. DNA gyrase catalyses the negative supercoiling of DNA in an energy dependant reaction to relieve torsional stress and maintain DNA topology during replication and transcription. Topoisomerase, a homologue of DNA gyrase, decatenates interlinked daughter chromosomes after replication. Quinolones bind enzyme-bound DNA complexes and arrests the DNA bound enzymes where single stranded breaks have been made by the enzyme. Although the mechanisms of cell death is unknown, it has been suggested that cell death occurs as a result of activation of the SOS response due to the single stranded breaks. Mutations in the quinolone resistance determining region (QRDR) of these enzymes (GyrA and ParC) alter the structure thereby preventing binding of quinolones and thus confer reduced susceptibility to these compounds (Drlica et al., 2008, Hopkins et al., 2005).

Coumarins are competitive inhibitors of ATP, binding to the ATP sites in the GyrB and ParE subunits of DNA gyrase and topoisomerase respectively, and inhibiting ATPase activity of these enzymes. Mutations that confer resistance come at the cost of losing enzymatic activity because of the close proximity of the ATP binding site and the coumarin binding site (which overlap). Consequently any changes to the tertiary structure to inhibit binding of the compound will also result in loss of ATP binding (Collin et al., 2011).

Rifamycin inhibits transcription by binding to the β-subunit of RNA polymerase (RNAP), made up of 4 subunits (α, β, β' and σ), forming a stable RNAP-drug complex.

Missense mutations in the β-subunit of RNAP, encoded by *rpoB* confer resistance to rifamycins due to structural changes (Kohanski et al., 2010).

1.9.3 Disruption of Proteins synthesis

These antimicrobials target bacterial ribosomes, which are made up of two ribonucleoprotein subunits, 30S and 50S. Together, these form the 70S bacterial ribosome, which bind to the mRNA molecule to form the initiation complex, resulting in the addition of aminoacyl-tRNA subunits to nascent polypeptide. Antimicrobials that inhibit the 50S subunit include; macrolides-lincosamides-streptogramins (collectively MLS), chloramphenicol and oxazolidones. Antimicrobials that target 30S ribosomal subunit include tetracycline and aminoglycosides. 50S ribosomal inhibitors prevent translation initiation (oxazolidones) or peptide elongation. 30S ribosomal inhibitor bind to the A site of the ribosome, preventing binding of the aminoacyl-tRNA subunits.

Resistance to these compounds involves the modification of the drug by; acetylation, phosphorylation and nucleotidylation resulting in chemically modified compounds unable to bind to the ribosomes. In addition, ribosomal protection proteins have been found to bind to ribosomes in proximal sites to elongation and dislodge the compounds from the binding site (Connell et al., 2003).

1.9.4 Alterations to metabolism and expression of alternative drug targets

Folic acid synthesis is the target for sulphonamides and trimethoprims, which act on two steps in the synthesis of tetrahydrofolic acid; a derivative of folic acid used in the production of thymine. Thymine is an essential precursor in the production of nucleic acid synthesis in bacteria, as bacteria cannot utilise exogenous sources of folic acid and must produce this compound. Dihydropteroic acid, a derivative of folic acid is produced from dihydropteroate diphosphate and p-aminobenzoic acid (PABA), catalysed by dihydropteroate synthase (DHPS). Tetrahydrofolic acid is produced from dihydrofolic acid. Dihydrofolic acid is produced from dihydropteroic acid catalysed by dihydrofolic

reductase (DHFR). Sulphonamides and trimethoprims inhibit DHPS and DHFR respectively. Resistance to these compounds is mediated by the production of drug resistant enzymes encoded by *sul* and *dhfr* genes, conferring resistance to sulphonamides and trimethorpims respectively. In addition, mutations in the promoter regions of *dhfr* have been found to increase the amount of DHFR produced. Trimethoprims act as competitive inhibitors for DHFR. By increasing the enzyme concentration, the number of enzyme active sites increase and the effect of the inhibitor is reduced as the enzyme active sites are no longer saturated with the antimicrobial compound, subsequently leading to reduced susceptibility (Huovinen et al., 2001).

In addition to overexpression of the target, resistance to sulphonamides can be mediated by the presence of naturally drug insensitive variants of the DHPS enzyme, commonly borne on plasmids (Huovinen et al., 2001).

1.9.5 Decreased intracellular drug concentration or increased efflux

Decreased antibiotic influx in an important mechanism of resistance in Gram-negative bacteria. The hydrophobic lipid bilayer and porins of Gram-negative bacteria act like a selective barrier enabling the selective diffusion or transport of molecules into the cell. Hydrophobic molecules such as macrolides easily diffuse through the lipid bilayer and small hydrophilic molecules are transported via porins. Resistance mediated by altered LPS has been reported in polymixin resistant *E. coli*, *K. pneumoniae* and *Salmonella* strains, resulting in the reduced binding of antimicrobial peptides such as polymixins. These strains were found to have higher levels of 4-aminoarabinose and phosphoethanolamine effectively reducing the charge in the LPS layer (Delcour, 2009, Llobert et al., 2011). Other mechanism of resistance mediated by decreased influx is the decreased expression of porins. In *E. coli* the main channels are OmpF, OmpE and PhoE, made up of a trimer of 16-strand β-barrels making a pore. Changes to Omp expression can be triggered by exposure to antimicrobials. The multiple-antibiotic-

resistance (Mar) phenotype has been shown to be a result of exposure to antimicrobial treatment, resulting in insensitivity to drugs, mediated by expression of global regulators such as *micF,* a small RNA that control the expression of *ompF*. The Mar phenotype has also been responsible for the increased expression of the efflux proteins including the *acrAB* locus (Alekshun and Levy, 1997). Other efflux mechanisms thought to contribute to resistance include ErmAB-TolC (*E. coli*), MexAB-OprM (*P. aeroguinosa*) and VexAB (*Vibrio spp.)* (Li and Nikaido, 2009).

1.9.6 Alterations to the cell membrane

Antimicrobials such as polymixin B, colistin (polymixin E) and daptomycin target the cell membrane. These are cationic polypeptides that target the anionic phospholipids in the cell membrane and are thought to destabilise the LPS by displacing the divalent cations. This leads to increased permeability and leakage, eventually leading to cell death. Resistance to these compounds is thought to be mediated by alterations to structure and level of LPS production, outer membrane proteins, Mg^{2+} and Ca^{2+} content, and expression of the capsular polysaccharide (Falagas et al., 2005).

1.9.7 Disruption of cell wall synthesis

The bacterial cell wall is reinforced with a layer of covalently cross-linked glycan matrix known as the peptidoglycan layer (murein). The peptidoglycan layer is made up of alternating sugar units of N-acetyl muramic acid (NAM) and N-acetylglucosamine linked via a β-(1,4) glycosidic bond. These carbohydrate moieties are crosslinked by a peptide chain consisting of 3 to 5 amino acids namely, D-alanine, D-glutamine, mesodiaminopimelic acid (Gram-negative) or L-lysine (Gram-positive), and two terminal D-alanine residues. The crosslinking of the glycan chains are catalysed by transpeptidase enzymes, which first cleaves the terminal D-alanine in the precursor linking peptide, then utilizes a serine residue in the active site that acylates the serine residue forming an acyl-enzyme intermediate. The subsequent de-acylation step results

in regeneration of the enzyme and formation of the peptide bond, crosslinking the peptide to the peptidoglyan layer (Fig. 1-4) (Mascaretti, 2003; Wilke et al., 2005).

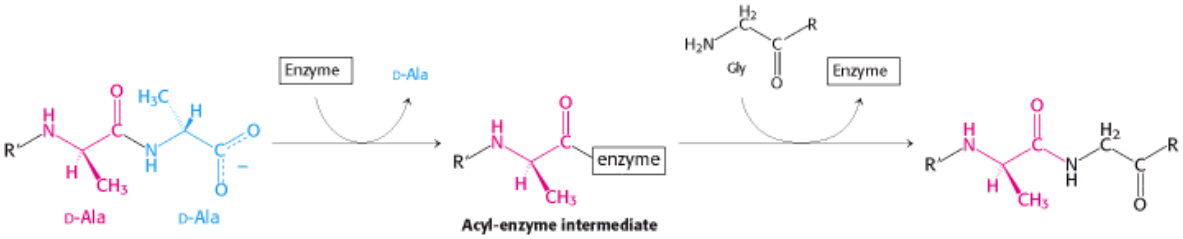


Figure 1-4: Transpeptidation reaction resulting in the crosslinking of the peptidoglycan layer. The molecule on the left depicts the D-ala-D-ala terminal residues, the substrates of PBP (transpeptidase) in the peptidoglycan crosslinking reaction, depicted as the incoming enzyme. The 'R' group representes D-alanine-D-glutamine-mesodiaminopimelic acid polypeptide. The transpeptidase enzyme cleaves the terminal D-ala residue forming an acyl-enzyme intermediate (middle complex). The enzyme catalyses a further de-acylation step with an adjacent peptide chain, resulting in the formation of a peptide bond and regeneration of the enzyme. From Biochemistry (5th Edition) W.H Freeman and Co.

Vancomycin and β-lactams both target the transpeptidase enzyme involved in peptidoglycan synthesis. β-Lactams inhibit transpeptidation (crosslinking) of the peptidoglycan layer by binding to the active site and inhibiting the transpeptidases (also known as penicillin-binding proteins or PBP) that catalyse this reaction. Vancomycin covalently binds to the D-ala-D-ala subunits of transpeptidation and prevents access to the active site by steric hindrance. Resistance to these compounds is mediated by the expression of altered PBP with lower affinity for the compounds or production of enzymes that hydrolyse the active compounds.

1.10 Beta-lactams

Beta-lactam antibiotics are a broad class of antibiotics that include penicillin and its derivatives: cephalosporins, monobactams, carbapenems, and β-lactamase inhibitors.

Their chemical structure includes a four membered cyclic amide known as the β-lactam ring. β-Lactam inhibitors are usually co-administered with β-lactam antibiotics and function as competitive inhibitors for β-lactamases that bind irreversibly to the enzyme active site and forming an acyl-enzyme complex consequently inactivating the enzyme (Drawz et al., 2010). β-Lactams target membrane anchored bacterial enzymes known as transpeptidases, which are involved in synthesis of the peptidoglycan layer. The relative ease of accessing these enzymes (localised on the outer cell membrane), low production costs and its specificity (no functional or structural homologues in humans) makes this an ideal chemotherapeutic. Therefore investing in prolonging its efficacy is of great interest to healthcare workers and patients alike (Mascaretti, 2003; Wilke et al., 2005).

Beta lactams mimic the D-ala-D-ala substrate, enabling efficient binding to the transpeptidase enzymes or PBP. The β-lactam antibiotics bind irreversibly to the PBPs forming a covalent penicilloyl-enzyme complex unable to catalyse transpeptidation reactions. β-Lactams bind to the enzymes active site, where a serine residue present in the active site, forms an ester bond with the carbonyl carbon of the β-lactam ring, resulting in the formation of a stable penicilloyl-enzyme complex (Fig. 1-3). As a consequence the acyl group cannot be transferred to the amino group of the nascent polymer. As the transpeptidase enzymes lay inactive, the constitutive autolysis and recycling of the peptidoglycan layer results in weakening of the cell wall polymer. The fragile cell wall coupled to the high osmotic internal pressure results in lysis of the bacterial cell (Mascaretti, 2003; Drawz et al., 2010).

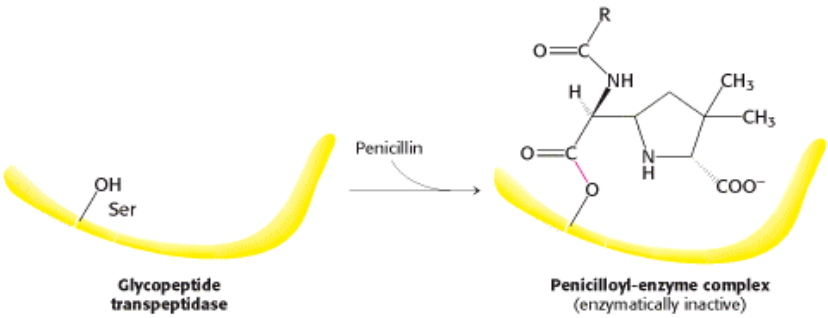


Figure 1-5: Action of penicillin antimicrobial and the target (transpeptidase) molecule. It is proposed that an enzyme (yellow) mediated lysine molecule abstracts a proton from the active site serine molecule (OH-Ser), enabling a nucleophilic reaction to occur between the now electron rich moiety and the carbonyl group (red line), consequently forming an irreversible penicilloyl-enzyme complex (depicted in the picture on the right). From Biochemistry (5th Edition) W.H Freeman and Co.

1.10.1 Resistance to β-lactams

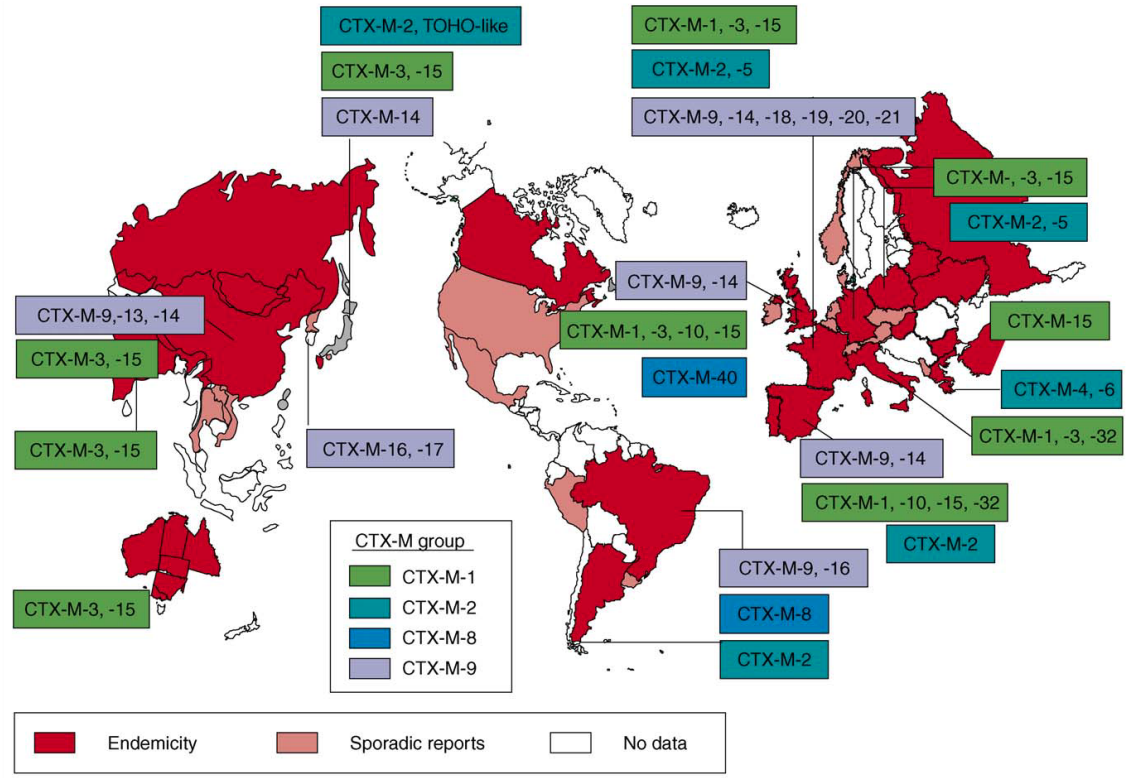
There are three mechanisms by which bacteria acquire resistance to β-lactams; (i) altering the permeability, (ii) altering the target molecule (PBPs) and (iii) inactivation by enzymes (Wilke et al., 2005).

Mutations in the active site of the target molecule (PBPs) render the β-lactam inactive, as it is less able to bind to and inhibit transpeptidation (Wilke et al., 2005). In methicillin resistant *S. aureus* the *mecA* gene encodes a PBP2a transpeptidase with a lower affinity for methicillin (Lowy et al., 2003).

Resistance to β-lactams can also be conferred by the production of β-lactamases. These enzymes are secreted into the periplasmic space where they hydrolyze the amide bond within the β-lactam ring rendering it inactive and are unable to bind to the enzyme active sites due to steric hindrance. There are over 470 β-lactamases known to date classed into four different groups (A to D) based on amino sequence similarity; known as the ambler classification system (Bradford, 2001; Paterson, 2006; Ambler, 1980). The class B enzymes are metallo-β-lactamases, harbouring a zinc ion coordinated to the enzyme. The remainder (A, C and D) are all serine β-lactamases. The class A enzymes

are the most frequently observed in clinical isolates, commonly associated with transferable plasmids. The first plasmid mediated β-lactamase gene described in *E. coli* was TEM-1 in the 1960's. This gene was originally isolated in Greece, but was later found to have spread to other parts of the world and in different species of bacteria. The cause was attributed to its location within mobile genetic elements. New β-lactam antimicrobials were developed in an effort to resist inactivation by β-lactamases with a broader spectrum of activity targeting an array of β-lactamase enzymes. However resistance quickly developed which saw the emergence of extended spectrum β-lactamases (ESBLs). ESBLs have an increased spectrum of activity against oxyimino-cephalosporins and monobactams but not cephamycins or carbapenems. To date there are over 200 ESBLs described in a range of *Enterobacteriaceae* (Patterson and Bonomo, 2005).

Surveillance studies of the late 1980's saw the emergence of isolates conferring higher MICs against cefotaxime and ceftazidime (Bonnet, 2004). This non-TEM, non-SHV ESBL was designated CTX-M-1, thought to be a derivative of chromosomal β-lactamases from *Kluvyera* spp. The early 1990s saw a rapid rise in E. coli strains with ESBL phenotypes of presumptive CTX-M production and by the mid 2000's it was reported in countries all over the world (Fig 1-6). Nucleotide sequencing of these enzymes (circa 1996) revealed variants of these enzymes to exist, grouped according to their amino acid sequence (Bonnet, 2004; Livermore and Woodford, 2006).



Picture taken from Rafael Canton and Teresa M Coque (2006). Current opinion in microbiology.

Figure 1-6: Global distribution of CTX-M β-lactamases. CTX-M enzymes are a heterogeneous group of enzymes found to have spread globally. Predominant enzymes include the CTX-M-1, -2, -8 and -9 variants. The rapid spread of CTX-M producing isolates has been attributed to the association of these enzymes with mobile genetic elements such as plasmids and insertion elements.

1.10.2 CTX-M enzymes

Cefotaximases (*bla*$_{\text{CTX-M}}$) are Ambler class A β-lactamases that hydrolyze extended spectrum cephalosporins and monobactams, but are inactive against cephamycins and carbapenems. In the recent years they have been the most prevalent β-lactamases found in animal isolates (Ewers et al., 2012). CTX β-lactamases hydrolyze cefotaxime, but also provide a high level resistance to penicillins, narrow-spectrum cephalosporins and third generation cephalosporins.

CTX-M enzymes can be classed into five different classes based on their amino acid sequence identity, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (see Fig 1-7). To date over 50 allelic variants of CTX-M genes have been identified, but the

CTX-M-14 (CTX-M group 9) and -15 (CTX-M group 1) variants are by far the most clinically (and veterinary) relevant (Canton et al., 2012). The prevalence of CTX-M enzymes has been attributed to their effective cleavage of cefotaxime in addition to other narrow, broad and extended spectrum β-lactams. However, the activity between the different variants of CTX-M is heterogeneous (Rossolini et al., 2008, Patterson, 2006).

CTX-M enzymes are folded into two domains, the α-helical domain and five β-strands surrounding the α-helical domain. The active site is located between the two domains, with the B3 β-strand forming part of the active site. The bottom of the enzyme is denoted the Ω Loop. Mutations in the B3 and Ω loop regions results in increased flexibility and accommodation of compounds with bulkier side chains of the extended spectrum β-lactams, such as cefotaxime, in comparison to the broad-spectrum compounds such as ampicillin. Mutations in the B3 strand at position 240 from aspartate to a neutral glycine is thought to improve flexibility and increase access of the substrate to the active site. Mutations in the Ω-loop region (Pro167Ser) are thought to increase flexibility further enabling hydrolysis of larger side chains like ceftazidime. Although these CTX-M enzymes harbour the same mutations, their kinetic activity is variable, due to additional mutational variants that may enhance/decrease hydrolysis of certain substrates or differential expression of the variants (Chen et al., 2005).

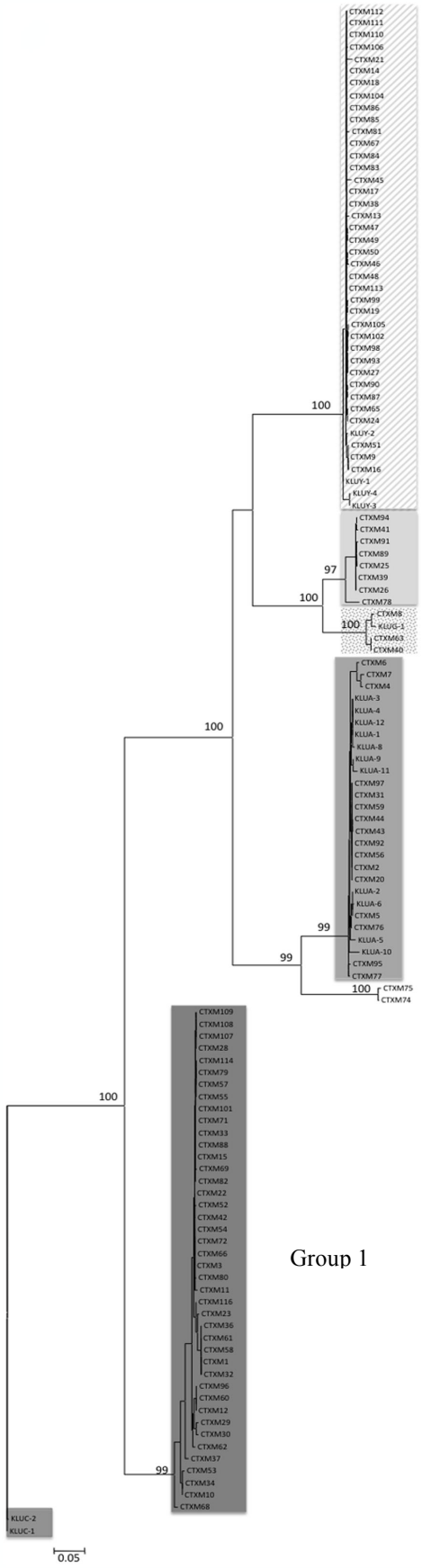


Figure 1-7: Phylogenetic tree of the five CTX-M clusters identified by amino acid sequence variation. The most prevalent enzymes found in clinical and veterinary samples today are the CTX-M-14 and CTX-M-15 enzymes belonging to CTX-M group 9 and 1 respectively. Picture taken from Canton et al., 2012 (Frontiers in Microbiology).

1.11 Mobile genetic elements and *bla*$_{CTX-M}$ genes

CTX-M enzymes are thought to have originated from *Kluyvera* species harbouring *klu* enzymes. They are thought to have been mobilised from the *Kluyvera* chromosome by insertion elements, namely IS*Ecp1*. IS*Ecp1* has also now been associated with mobilising CTX-M group 1 and 9 variants (Toleman and Walsh, 2011). IS*Ecp1* elements are found upstream of the genes they mobilise and transpose the adjacent gene. In addition IS*Ecp1* encodes -35 and -10 promoter sequences and is thought to drive expression of downstream genes. This quality is thought to be exploited when genetic transfer occurs from one bacterial species to another, to drive expression in the new species (Poirel et al., 2003).

CTX-M group 2 and 9 enzymes have been associated with insertion sequence common region 1 (IS*CR1*) upstream of the CTX-M gene. This insertion element mobilises genes via a rolling circle mechanism enabling transposition of large segments of DNA. IS*CR1* elements recognise their own terminal sequences, resulting in transposition to more genetically diverse regions and contributing further to their dissemination (Canton et al., 2012; Toleman and Walsh, 2011). However attempts to monitor this transfer in vitro has been unsuccessful, but the potential for transfer of large sections of DNA may become a future concern in the dissemination of resistance genes.

The spread of CTX-M variants has been highly attributed to their presence on plasmids (Carattoli, 2009). CTX-M variants have been associated with plasmids of particular incompatibility groups. For example the CTX-M-15 enzyme has been commonly found in association with the IncFII plasmid group. This particular plasmid has been very successful in the spread of this variant due to its presence on the conjugative (self-transmissible) IncFII plasmid, and is commonly found in an *E. coli* clone ST131 that appears to have co-evolved with the plasmids, further aiding the spread of this CTX-M variant (and plasmid) (Canton et al., 2012; Coque et al., 2008; Woodford et al., 2011).

This is usually a large, low copy plasmid, but has evolved multiple mechanisms to ensure maintenance in the host, including multiple addition systems. This plasmid has been found to have undergone multiple recombination events resulting in the acquisition of multiple replication origins in addition to multiple drug resistance genes including; other β-lactamases such as bla_{TEM} and bla_{OXA} and genes that confer resistance to aminoglycosides, chloramphenicols, sulphonamides, trimethoprims and tetracyclines (Woodford et al., 2009). However, IncF plasmids are narrow host range plasmids limited to the *Enterobacteriaceae* family. Other broad host range plasmids associated with CTX-M enzymes include the IncN, IncL/M and IncI1, commonly found in *Salmonella* spp. and *E. coli* commonly isolated in food producing animals, thought to be reservoirs of resistance (Carattoli, 2011).

1.12 Multi-drug resistance

Altering membrane permeability is more commonly found in Gram-negative bacteria, making them more resistant to a larger repertoire of antibiotics. Multidrug transporters have been found to be responsible for conferring resistance or reducing susceptibility to antifungals, antiprotozoal and antimicrobials (Lomovskaya et al., 2007). Multidrug transporters in bacteria are classified into five families based on sequence similarity. These families are; the major facilitator (MFS), resistance-nodulation-cell division (RND), small multidrug resistance (SMR), multidrug and toxic compound extrusion (MATE), and ATP-binding cassette (ABC) families (Piddock et al., 2006). Many putative and proven drug transporters of all five families exist in the *E. coli* genome, and a large fraction of them are multidrug transporters. The RND efflux system plays a major role in drug resistance in Gram-negative bacteria by allowing the direct extrusion of a wide spectrum of lipophilic and amphiphilic substrates, including several classes of

antimicrobials, antiseptic compounds, dyes and detergents into the extracellular space (Nikaido, 1996).

In Gram-negative bacteria, transport of β-lactams into the periplasm occurs via outer membrane porins (Nikaido et al., 1983). The combination of reduced drug uptake (via porins) increased efflux and inactivation by periplasmic β-lactamase enzymes can effectively contribute to reduced susceptibility to these compounds (Wilke et al., 2005). With such broad specificity offered by MDR efflux pumps, reduced susceptibility and in some cases increased resistance is inevitable in Gram-negative bacteria (Nikaido and Normak, 1987; Nagano and Nikaido, 2009; Sawai et al., 1988). One of the most studied efflux systems is the AcrAB-TolC tripartite efflux protein in *E. coli* (Nikaido and Takatsuka, 2009). AcrB is the inner membrane protein, the AcrA protein is located in the periplasmic space and the TolC protein forms the outer membrane protein channel (Fig 1-8). Efflux is driven by an electrochemical potential gradient of H^+. The substrate binds to the AcrB protein, where it is translocated through the TolC pore to the extracellular space bypassing the cell membrane and periplasmic space. Substrates for the AcrAB-TolC system include a number of antimicrobial classes including β-lactams (Yu et al., 2003).

Clinically relevant Gram-negative organisms such as *P. aeruginosa*, *S. enterica, C. jejuni*, *K. pneumoniae*, *H. pylori* have all been described as having homologues of the AcrAB-TolC efflux systems or other known RND transporters that contribute to intrinsic resistance, challenging efficacy of current therapies (Piddock, 2006). The broad spectrum of antimicrobials that may be extruded by efflux transporters have made efflux proteins an attractive drug target. Efflux pump inhibitors (EPIs) target the substrate-binding region of the transporter (Lomovskaya et al., 2001). This however limits the type of the antimicrobial compounds inhibited, as the EPI either acts as a competitive inhibitor, preventing binding of substrates or cooperatively acts to prevent

disassociation of the antimicrobial substrates resulting in blocking of the channel. Consequently the rates of efflux inhibition depend on the type of EPI used (Lomovskaya et al., 2001; 6, Lomovskaya et al., 2007).

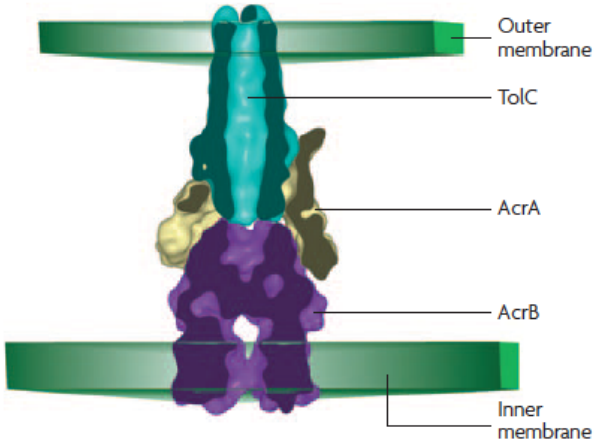


Figure 1-8: Simplified ICM model of the tripartite AcrAB-TolC efflux transporter, part of the RND (resistance nodulation division) transporters. The substrate is guided to the efflux transporter by the AcrB inner membrane protein, and then it is translocated through the TolC transmembrane protein to the extracellular space by a H^+ antiport system. Taken from Lomovskaya *et al*. 2007. Nature.

1.13 The fitness cost of antibiotic resistance

Resistance is thought to come at a cost, namely bacterial fitness, which is strictly defined as the capability of a genotype to survive and reproduce (Andersson and Hughes, 2010). The acquisition of resistance, through chromosomal mutation or HGT is thought to reduce bacterial fitness, but this cost can be alleviated through counteracting this cost by increasing fitness e.g. compensatory mutations or survival in a limiting condition such as an antibiotic environment where the bacterium encodes the resistance gene, resulting in survival and out-competition of strains that lack these traits. The relative fitness of a bacterial species can me be measured in different ways including *in vitro* and *in vivo*. *In vitro* fitness measurements can be detected by phenotypic markers, such as the expression of resistant variants including *rpsL* and *rpoB* mutations as a result of exposure to streptomycin and rifampicin respectively. *In vivo* measurements include competition assays with mutant and wild-type strains, and detection of the

ratio's of the two strains after several days of infection and bioluminescent tags may be used to monitor the infection (Andersson and Hughes, 2010). Bacterial growth experiments have also been used to measure fitness, either in pairwise competition assays or measuring exponential growth rates of the two strains, these techniques have been used to measure fitness costs of plasmid acquisition to host strains (Humphrey et al., 2012, Lim et a., 2010, Platt et al., 2011).

1.14 Antimicrobial resistance and its application to livestock

Resistance to antimicrobials can be observed in an array of bacterial species, pathogenic and non-pathogenic, in human and animal populations. Monitoring resistance is therefore of great interest to researchers and clinicians alike. In doing so, resistance patterns can be identified for prevalence studies, in addition to detecting emerging resistance genes and providing suitable treatment courses (McEwen, 2006).

Bacteria resistant to currently used antimicrobials can easily spread amongst livestock. Establishing a surveillance system allows the monitoring of common zoonotic pathogens like *Enterobacteriaceae* e.g. *Salmonella*, *Campylobacter* and in some cases *Yersinia* for the carriage of resistance phenotypes. Monitoring commensal bacteria such as *E. coli*, *Enterococcus faecium* and *Enterococcus faecalis* is crucial when studying resistance acquisition, as these bacteria can colonize the gastrointestinal tract of a range of animal species and even serve as a reservoir for these genes (McEwen, 2006). This may lead to contamination of food for human consumption. The array of bacterial species that can be found in the gut dramatically increases the probability of resistance gene acquisition (through various methods described earlier) both intra and interspecies. And now with the ever-increasing movement of animals across borders, the problem of resistance is not only a domestic but also an international one (O'Brien, 1997).

1.15 Surveillance of antimicrobial resistance

Monitoring and surveillance systems track and record emerging resistance patterns provide early detection of outbreaks, improving treatment managements and guide policy for current and future prescription practices (www.who.int). Surveillance methods involve sampling, identifying the bacterial species, antimicrobial susceptibility profiling, data collection and analysis. The data is collated and presented through networks like the European Antimicrobial Resistance Surveillance Network (EARS-Net) and European Surveillance of Antimicrobial Consumption (ESAC). These data are collected in various laboratories in their respective countries, producing yearly reports on resistance and antimicrobial usage patterns. Therefore it is crucial to maintain the same sampling techniques and lab practices to compare the data. The set up of councils such as the British Society of Antimicrobial Chemotherapy (BSAC) and Clinical and Laboratory Standards Institute (CLSI) ensure laboratory interpretations for laboratory tests such as minimum inhibitory concentration (MIC) determination and antimicrobial disc diffusion assays are comparable between labs, but no high throughput routine method of assaying the genetic determinants of resistance is used. In recent years, the development of PCR-based techniques and miniature microarray technology enabled high throughput screening of strains, providing clinically relevant information e.g. genes encoding virulence and antibiotic resistance clinically relevant information including genes encoding virulence and antibiotic resistance (Anjum et al., 2007; Anjum et al., 2011).

1.16 CTX-M encoding plasmids in the UK

The most frequently detected CTX-M enzyme in the UK in human *E. coli* isolates is CTX-M-15 and -3, however in animals, CTX-M-14 has been found to be the most prevalent (Livermore and Hawkey, 2005, Randall et al., 2011, Stokes et al., 2012). The

detection of this enzyme was first reported in 2004 (Teale et al., 2005). These genes are commonly associated with multi-drug resistant plasmids, capable of transferring by conjugation, which may aid the persistence and dissemination of these resistance genes (Randall et al., 2011). In recent reports however, an epidemic plasmid, pCT, encoding the CTX-M-14 varaint in *E. coli*, was not found associated with any other resistance genes suggesting the drive for persistence could be driven solely by the presence of β-lactam compounds (Cotell et al., 2011). Sequence analysis of this plasmid revealed multiple addition and conjugative systems that may explain the apparent spread of this plasmid worldwide (Cotell et al., 2011). This further expresses the need to study the horizontal spread of MDR plasmids as they may impact the clinical strategies used to combat disease.

1.17 Aims

The increasing isolation of *bla*$_{\text{CTX-M}}$ encoding plasmids from zoonotic pathogens and commensal *E. coli* strains suggests the genetic platform in which these enzymes exists are a significant contributing factor to the dissemination and persistence of CTX-M enzymes.

The aim of this study was to characterize *bla*$_{\text{CTX-M}}$ encoding plasmids and investigate those aspects of these plasmids that may contribute to their global spread. The prevalence of particular CTX-M variants suggests that some variants of these enzymes may favor particular plasmids, which in turn contribute to their dissemination.

The working testable hypothesis is that, in the absence of selection, *bla*$_{\text{CTX-M}}$ variants harbored within a plasmid solely define the prevalence of that *bla*$_{\text{CTX-M}}$ variant observed globally. However, it is recognized that the plasmid itself may play a contributing role and alternatively it could be a combination of the genetic platform to which *bla*$_{\text{CTX-M}}$ is found that stabilizes the gene and the plasmid harboring the gene within a population. The stability or success of the gene; defined by the ability to spread amongst a population, would depend on the plasmid it is located on, but in the presence of selection the genes including CTX-M enzymes would provide an advantage. This thesis aims to explore the contribution, if any, of plasmids harboring CTX-M enzymes to overall fitness in the presence and absence of selection and whether they are linked to particular plasmid incompatibility groups or the CTX-M variant. In addition the genetic structure of these plasmids will be explored through plasmid sequencing to identify potential genetic traits that may enable the plasmid to persist within a population.

The specific aims are as follows:

- To characterize field isolates from bovine *E. coli* species, collected from regions across the United Kingdom, conferring the ESBL phenotype.
- To characterize the plasmids possessing $bla_{\text{CTX-M}}$ genes to identify their transferability, drug resistance phenotype and genotype and any linkage to plasmids isolated from other regions.
- To identify the genetic structure of plasmids encoding the CTX-M variants.
- To investigate the cost of acquiring the plasmid to host fitness.

2 MATERIALS AND METHODS

2.1 Bacteriological techniques

2.1.1 Initial screening of bacterial isolates

Fifty-two *E. coli* isolates were selected from samples collected from farm visits received from regional laboratories in England and Wales in 2007. All 52 isolates had already been screened by colleagues at the AHVLA (bacteriology) for resistance to cefotaxime by plating onto Chromagar™ ECC supplemented with 2 and 8 mg/L of cefotaxime (ctx) (Randall, Kirchner et al. 2009). Chromagar ECC™ is a chromogenic media that enables identification of *E. coli* (blue colonies) and other coliforms (mauve/red colonies). Each positive colony (blue in colour) was selected and plated onto Chromagar ECC™, and analysed further by real time PCR to detect $bla_{\text{CTX-M}}$ genes.

2.1.2 Bacterial Cultivation

All bacterial strains were stored at -80 °C in Luria Bertani (LB) Broth containing 25 % glycerol (v/v). Strains were routinely grown from frozen stocks on LB agar plates (or Broth) at 37 °C for 16-18 hours. Liquid cultures were incubated aerobically shaking at 150- 200 rpm at 37 °C for 16-18 hours.

2.1.3 Bacterial enumeration

Colony forming units (CFUs) per ml were determined by the Miles and Misra method used for surface variable counts (Miles & Misra, 1938). Ten-fold serial dilutions of a bacterial suspension in 0.1 M phosphate buffered saline (PBS) at pH 7.2, was used to spot three 20 µl droplets onto dry LB agar plates and allowed to soak into the agar. The

plates were incubated at 37 ˚C for 16-18 hours and colonies were counted at the appropriate dilution factor.

2.1.4 *E. coli* identification

All the isolates were plated onto Brilliant Green Agar (BGA) to confirm the isolates were not contaminated during storage. BGA is a selective media used to select for lactose and/or sucrose fermenters including *Salmonella* and *E. coli*. Brilliant Green included in the medium inhibits growth of Gram-positive bacteria and most Gram-negative bacteria. The agar also includes a phenol red indictor, which discolours in the presence of lactose/sucrose fermentation. *E. coli* ferments lactose and sucrose producing yellow/green colonies, non-fermenters, including *Salmonella*, produces red/pink colonies surrounded by bright red medium.

Isolates were recovered from frozen stocks as outlined above. Overnight colonies were streaked onto BGA plates to single colonies and incubated for 16-18 hours at 37°C. Included, were a positive and negative control: *S. Nottingham* (NCTC 7832) and *E. coli* (NCTC 10418) respectively.

2.1.5 Preparation of competent cells

Competent cells were prepared by three different methods, two chemically competent methods (Sambrook et al.1989) using Calcium chloride ($CaCl_2$) and Rubidium Chloride ($RbCl_2$), and an electrical competent procedure (Sheng et al., 1995). These cells were used in the transformation of $bla_{CTX\text{-}M}$ plasmids for subsequent phenotypic studies and are detailed below.

2.1.5.1 $CaCl_2$ Chemically competent cells

A fresh overnight culture (LB broth) was diluted 1:150 for growth in pre-warmed LB broth. Cells were grown at 37 ˚C with shaking at 250 rpm to an OD_{600} of 0.4 and

thereafter immediately placed on ice for 20 minutes. Cells were harvested by spinning the culture for 10 minutes at 1250 g (4 ˚C) and re-suspended in 12 ml ice cold 0.1 M $CaCl_2$, then incubated on ice for 30 minutes. The cells were pelleted by centrifuging at 1250 g at 4 ˚C for 10 minutes. After decanting the supernatant, the cells were re-suspended in ice cold 0.1 M $CaCl_2$ containing 15 % glycerol (v/v) and aliquoted into pre-chilled microcentrifuge tubes to be stored at -80 ˚C in 100-200 µl aliquots.

2.1.5.2 $RbCl_2$ Chemically competent cells

To prepare $RbCl_2$ competent cells, a fresh overnight culture was diluted 1:100 with fresh pre-warmed LB broth supplemented with the following: 5 mM glucose, 10 mM $MgSO_4$, 10mM $MgCl_2$, and incubated in a shaking incubator (250 rpm) at 37 ˚C to an OD_{600} of 0.4. The culture was immediately placed on ice for 20 minutes, and then centrifuged for 10 minutes at 1250 g at 4 ˚C. The cells were re-suspended with 50 ml ice cold 0.1 M $CaCl_2$ and pelleted again as above. Cells were re-suspended in 5ml transformation buffer (100 mM $CaCl_2$, 50 mM $MgCl_2$, 50 mM $MnCl_2$, 50 mM $RbCl_2$) and incubated on ice for 1 hour. The chemically competent cells were stored in 100 - 200 µl aliquots at -80 ˚C in 10 % glycerol (v/v).

2.1.5.3 Electrically competent cells

Electrically competent cells were prepared as described previously by Sheng et al. 1995, with a modification to the centrifuging speeds and an additional 20 minute incubation step on ice following growth. An overnight culture was diluted 1:1000 in LB broth to an OD_{600} of 0.4. Cells were placed on ice for 20 minutes harvested by centrifuging at 2500 g for 10 minutes at 4 ˚C. Cells were re-suspended in 10 % glycerol (v/v), equal to the original culture volume and spun at 2500 g for 10 minutes (4 ˚C) to pellet cells. This washing and pelleting step was then repeated again using 10 % glycerol (v/v), equal in volume to the original culture. Once decanted, the cells were re-suspended in 60 ml of

10 % glycerol (v/v) and pelleted once more. The cells were re-suspended in 2 ml of 10 % glycerol (v/v) per litre of initial culture volume and stored in 100 µl aliquots at -80 ˚C.

2.1.5.4 Competent cell lines

Table 2-1: Listed in the table below are the competent cell lines used in this study.

Competent Cell	Supplier	Competency type	Genotype
E. coli DH10B™	Invitrogen	Chemical (Cat. No. 18297-010) and electrocompetent (Cat. No. 18297-015)	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G λ⁻
E. coli BL21	Novagen	Chemical (Cat. No 69449-3)	F⁻ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm lon*
E. coli BL21KAMR	Prof. Nikaido UC Berkley	Made Chemical and electrocompetent	BL21 *_acrAB _mdtABC _*(*srl-recA*)*306*::Tn*10*(Tcr) (Kim et al. 2010)
E. coli K12 (20R764)	AHVLA	Made chemical and electrocompetent	F⁻ *lac*$^+$ *rif*$^+$

2.2 Molecular Methods

2.2.1 Standard PCR

Polymerase chain reaction (PCR) was performed in a GeneAmp® PCR system (9600) cycler machine (Applied biosystems).

Thermostable DNA *Taq* polymerse (Promega; Cat. No. M186B or Qiagen; Cat. No. 201203) was used in all standard PCR reactions according to the manufacturer's instructions, unless otherwise stated. Unless stated the following PCR reaction mixtures and conditions were used; 5 µl 10 x reaction buffer, 1 U of *Taq* polymerase, 10 µM of each primer, 10 mM of each deoxynucleoside triphosphate (dNTP), 1-2 µl of and made up to 50 µl with nuclease free water. PCR cycling conditions were as follows; 5 minutes at 94 ˚C, 30 cycles of 94 ˚C for 1 minute, 60 ˚C for 30 seconds, 72 ˚C for 1 seconds and

a final extension at 72 ˚C for 5 minutes. Primers used in this study can be found in appendix B.

2.2.2 Gel electrophoresis

DNA was run on a 0.8 – 2 % agarose gel. The gels were stained in 0.8 µg/ml ethidium bromide and visualized under ultraviolet light using the AlphaImager™ system (Alpha Innotech).

2.2.3 Colony PCR

A single colony was re-suspended directly into the PCR mixture. This crude lysate method was employed for screening for $bla_{\text{CTX-M}}$ PCR, however was not used in the instance of multiplex PCR's due to the low sensitivity of the reaction that may result in non-specific products.

2.2.4 DNA extraction

Different DNA extraction methods were employed. Below are details of the methods used.

2.2.4.1 Cell lysates

From a fresh overnight culture, 3 to 5 five single colonies were picked and re-suspended in 100 µl of nuclease free water. This was heated to 95 ˚C for 15 minutes, and then chilled on ice for a further 1 minute before 3-5 µl was used in subsequent PCR reactions.

2.2.4.2 Alkaline lysis

Bacteria from a fresh overnight culture (plate or broth) were pelleted and re-suspended in a tris base buffer, with a detergent (SDS or Sarcosyl) and adjusted to pH 8.0. The suspension was incubated at temperatures ranging from 54- 60 ˚C as per indicated by the subsequent method i.e. PFGE, DNA microarray genotyping or Plasmid DNA extraction.

2.2.4.3 Plasmid DNA extraction

Two methods were used to extract plasmids; a) phenol chloroform extraction for plasmid content analysis by gel electrophoresis, and b) plasmid midi prep extraction (QIAGEN Cat. 12143); for subsequent transformation, PCR and sequencing procedures.

2.2.4.3.1 Phenol Chloroform extraction

Plasmid DNA was extracted from 1 ml of a fresh overnight culture grown in LB broth. Cells were pelleted by centrifuging at maximum speed for 5 minutes and supernatant discarded. The pellet was re-suspended in 200 µl alkaline lysing solution (3 % SDS, 50 mM trizma base (Sigma Cat. 93349), and 30 mM NaOH) and incubated at 55 ˚C for 30 minutes. 200 µl of phenol:chloroform:isoamyl alcohol mix 25:24:1 (Sigma Cat. P3803) was added and the suspension was mixed by inverting five to six times. This was centrifuged at maximum speed for 10-15 minutes to separate the top aqueous phase (containing the nucleic acids) and the bottom organic phase (contains mainly proteins and chloroform). The aqueous phase was carefully aspirated and stored at -20 ˚C.

2.2.4.3.2 Plasmid Midi prep

Plasmid DNA was prepared as per manufacturers instructions; unless otherwise stated, using the Qiagen plasmid midi kit (Cat. No. 12142) or the HiSpeed plasmid midi kit (Cat. No. 12662). A higher initial culture volume was used and all subsequent buffer volumes were adjusted accordingly. Briefly, 50 ml of an overnight culture was pelleted

and re-suspended in 8 ml buffer P1. 8ml of buffer P2 and pre-chilled buffer P3 were added separately and mixed by gently inverting 4-6 times between buffers. The mixture was centrifuged at maximum speed for 30 minutes (4 ˚C), decanted, and then spun again at maximum speed for 15 minutes (4 ˚C). The supernatant was applied to a column, previously equilibrated with 8 ml buffer QBT and allowed to empty by gravity flow. The column was washed twice with 20 ml buffer QC and eluted with buffer QF. DNA was concentrated by one of the methods described below.

2.2.5 Concentrating plasmid DNA

2.2.5.1 Ethanol precipitation

The eluate from the plasmid extraction was precipitated either by adding 0.7 volumes of room temperature 100 % isopropanol then incubated at room temperature for 15 minutes, or 2.2 volumes 100 % ethanol and placed at -80 ˚C for 30-60 minutes. The mixture was centrifuged at 15,000 g for 60 minutes (4 ˚C). The isopropanol/ethanol mixture was carefully decanted and the DNA pellet was washed with 0.4 volumes of the original sample volume with 70 % ethanol (v/v). This was incubated at -80 ˚C for 30 minutes and then centrifuged at 15,000 g for 60 minutes (4 ˚C). The pellet was re-suspended in an appropriate volume with nuclease free water.

2.2.5.2 Sodium acetate precipitation

Sodium acetate was added to a final concentration of 0.3 M. To that, 2.2 volumes of 100 % ethanol was added and placed at -80 ˚C for 30-60 minutes. The mixture was washed, pelleted and re-suspended as above.

2.2.6 Bacterial plasmid transformation

Two methods were employed to introduce plasmids to host plasmids; chemical transformation and electroporation. The latter of the two methods was employed when

transformation was unsuccessful, which was the case for some of the large plasmids (>90 kb).

2.2.6.1 Chemical transformation

Approximately 100-500 ng of DNA (1-2 µl) was mixed with 30 µl of *E. coli* chemically competent cells prepared as previously described (section 2.1.5.1) and incubated on ice for 30 minutes. Cells were heat shocked at 42 ˚C for 30 seconds and placed on ice for 2 minutes. 250 µl of room temperature S.O.C media (super optimal broth with catabolite repression media) was added and incubated for 1 hour at 37 ˚C. This was plated onto dried LB agar plates supplemented with 3 mg/L cefotaxime for selection of strains harbouring $bla_{\text{CTX-M}}$ encoding plasmids. Strains were confirmed to harbour $bla_{\text{CTX-M}}$ by colony PCR.

2.2.6.2 Bacterial electroporation

100 - 500ng DNA was mixed with 30-50 µl of competent cells and incubated on ice for 10 minutes. The mixture was transferred to a 0.1 cm cuvette (BioRad Cat. No 165-2089). Electroporation parameters were as follows; 1.25 kV (12.5 kV/cm), 25 µF and 100Ω. The cells were recovered in 500 µl of S.O.C media for 1 hour and plated on selective media LB agar plates as above. Strains harbouring $bla_{\text{CTX-M}}$ encoding plasmids were screened by PCR.

2.3 Bacterial conjugation

Plasmid transferability for all the strains was determined by *in vitro* conjugation, using a rifampicin resistant *E. coli* K12 (20R764) strain as the recipient. Isolates were plated on LB agar and incubated for 16-18 hours at 37 °C and three representative colonies were used to inoculate LB broths. These were incubated for 16-18 hours in a shaking incubator at 37 °C.

Optical density readings (OD_{600}) of the overnight liquid cultures were taken and appropriate volumes were calculated to result in a 1:10 ratio of donor strains to recipient strain (*E. coli* K12 - 20R764) in a final volume of 3 ml. 200 µl of the resulting donor/recipient mixture was dispensed onto a 0.2 µm membrane filter (Millipore GTTP02500) and incubated at 37 °C for 1 hour. The same was repeated on the donor only and recipient only cultures as positive and negative controls for the selective plates (see below). The reaction was stopped by placing the filter into a 15 ml falcon tube containing 5 ml of ice cold 0.1 M PBS (pH 7.2) and placed on ice. The filters were washed by gentle vortexing and cells pelleted by centrifuging at 3200 g for 10 minutes, then re-suspended in 1 ml 0.1 M PBS (pH 7.2). The cells were then further diluted in 0.1 M PBS (pH 7.2) and spread onto a selective LB media containing 1 mg/L cefotaxime and 150 mg/L rifampicin. These were incubated for 16-18 hours at 37 °C.

As controls, the recipient cultures were spread onto selective LB media containing 1 mg/L cefotaxime and 150 mg/L rifampicin, and recipients were spread onto LB media containing150 mg/L rifampicin, to quantify any spontaneous mutants.

The recipient overnight cultures were also plated on LB plates (minus selection) for enumeration of initial viability counts using the Miles & Misra method described above (section 2.1.3).

Three transconjugants were picked and plated to single colonies on selective plates and incubated for 16-18 hours at 37 °C. The presence of $bla_{\text{CTX-M}}$ was analysed by PCR to confirm successful transfer of $bla_{\text{CTX-M}}$ plasmids to the recipient strain. Plasmid transfer rates (frequency of transfer) were determined as the number of transconjugants (CFU/ml) per initial recipient (CFU/ml) (Fernandez-Astorga, Muela et al. 1992).

2.4 Molecular subtyping methods

2.4.1 *E. coli* phylogrouping PCR

E. coli phylogenetic characteristics were determined by PCR using a multiplex PCR reaction devised by Clermont et al. 2000. Three primers pairs; ChuA.1/2, YjaA.1/2 and TscpE4C2.1/2 (see appendix B for primer sequences) were used in a triplex reaction with 2 µl 10 x reaction buffer, 2.5 U of *Taq* polymerase, 20 µM of each primer, 2 µM of each deoxynucleoside triphosphate (dNTP), 3 µl of crude lysates (recovered in selective media form frozen stocks) and made up to 20 µl with nuclease free water. PCR cycling conditions were as follows; 5 minutes at 94 ˚C, 30 cycles of 94 ˚C for 30 seconds, 55 ˚C for 30 seconds, 72 ˚C for 30 seconds and a final extension at 72 ˚C for 7 minutes. Fragments were run on a 2 % gel and visualised under UV light subsequent to ethidium bromide staining. Phylogroups were assigned according to the dichotomous key approach described by Clermont and colleagues (Clermont et al., 2000). Controls included *E. coli* K12 20R764 (phylogroup A) and an *E. coli* ST131 strain (phylogroup B2; AHVLA, Dr. G. Wu).

2.4.2 Pulse field gel electrophoresis

Pulse field gel electrophoresis (PFGE) was used to further subtype the *E. coli* strains used in this study. Isolates were subjected to genomic DNA *XbaI* restriction digest according to the PulseNet protocol for subtyping *E. coli* 0157 (Centre of Disease Control, Atlanta) (CDC 2008). In brief, the isolates were suspended in a buffer containing 1 M tris and 0.5 M EDTA at pH 8.0. The cell suspension was adjusted to OD_{610} 1.3-1.4. Agarose plugs were prepared containing a 1:1 (v/v ratio) mixture of the bacterial suspension and melted 1 % SeaKem Gold agarose containing 1 % SDS and 0.5 mg/ml Proteinase K.

The plugs were lysed in 5 ml lysis buffer containing: 50 mM Tris, 50 mM EDTA pH 8.0, 1 % sarcosyl and Proteinase K to a final concentration of 0.1 mg/ml. The plugs were incubated at 54°C (150 rpm) for 2 h. After cell lysis, plugs were washed with preheated (50°C) 10 ml HPLC (high performance liquid chromatography) water twice, followed by four washes with 10 ml preheated (50°C) TE buffer (10 mM Tris, 1 mM EDTA pH 8.0). Each wash was incubated for 10 minutes in a shaking incubator (150 rpm).

Plugs were digested with 50 U (per sample) of *XbaI* for 2 hours at 37 ˚C, and reaction stopped by adding an equal volume (200 µl) of 0.5 x Tris-Borate EDTA buffer (TBE). The samples were run on a 1 % agarose gel in 0.5 x TBE buffer, a 0.5 x TBE running buffer (2.2 L) containing 1 ml thiourea (8 mg/ml). Electrophoresis was performed with a CHEF DR-II apparatus (Bio-Rad) with the following settings; an initial switch time of 2.2 seconds with final switch time of 54.2 seconds, 6 V, 120˚ included angle for 18 hours at 14 ˚C. The gels were stained with 0.8 µg/ml ethidium bromide and visualized under ultraviolet light using the AlphaImager™ system (Alpha Innotech).

Salmonella Ser. Braenderup H9812 standards were used to estimate the band sizes (Hunter, Vauterin et al. 2005) and gel images were analysed using the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Macro-restriction profiles were used to construct a Dice coefficient-based similarity dendogram using the BioNumerics program, Applied Maths, Sint-Martens-Latem, Belgium (Carrico, Pinto et al. 2005; Hunter, Vauterin et al. 2005; Karlowsky, Kasloff et al. 2007).

2.4.3 Antimicrobial resistance array

All 52 isolates were analysed using an oligonucleotide array tube, used to detect antimicrobial resistant genes in Gram-negative bacteria. The array platform (ArrayTube™ AMR05) is mounted at the bottom of a 1.5 ml microcentrifuge tube, with 68 different probes printed in triplicate. These include 56 different oligonucleotide

probes for genes conferring resistance to aminoglycosides, β-lactams, chloramphenicols, macrolides, tetracyclines, quinolones, sulphonamides and trimethorpims, in addition to two integrase genes associated with class 1 and 2 integrons. Certain gene probes e.g. *bla*$_{\text{CTX-M}}$, are represented by more than one probe to account for allelic variants (Anjum et al., 2011).

2.4.3.1 Crude lysis

The method was carried out as previously described (Anjum et al., 2011). Briefly, samples were plated onto nutrient agar plates and grown overnight at 37˚C. A 10 µl loopfull of bacteria was re-suspended in 400 µl of bacterial lysis buffer (0.1 M Tris-HCl pH 8.0, 0.05 % Tween 20, 240 mg/L proteinase K). Bacteria were lysed by incubating the suspension at 60˚C for 2 hours in a lysis buffer, followed by incubating the sample at 95 ˚C for 15 minutes. The lysates were centrifuged at 15000 g for 5 minutes and 6.55 µl (1-2 µg) of the supernatant (crude extract) was used in subsequent linear multiplex DNA amplification reactions.

2.4.3.2 DNA labelling

The crude extract (6.55 µl) containing genomic DNA was added to a mix containing: 1 µl dNTPs (1 mM dA/C/GTP and 0.65 mM dTTP; GE Healthcare), 0.1 µl Therminator DNA polymerase (2000 units/ml, NEB), 1 µl Therminator 10 x reaction buffer (NEB), 0.35 µl Biotin-16-dUTP (1 mM, Roche), 1 µl primer mix (Indentibac) to a final volume of 10 µl for linear multiplex amplification. The amplification cycling conditions were as follows; 96 ˚C for 5 minutes, followed by 40 cycles of; 62 ˚C for 20 seconds, 72 ˚C for 40 seconds and 96 ˚C for 60 seconds.

2.4.3.3 Hybridisation

The hybridisation step was carried out as per the manufacturer's instructions (www.identibac.com). Briefly; the array tube was initially preconditioned using 500 µl of water for 5 minutes at room temperature on an agitating thermomixer set at 550 rpm. This was followed by another 500 µl wash with a hybridisation buffer containing 1 M NaPOi, 20 % SDS, 0.5 M EDTA, 20 x SSC (0.3 M sodium chloride and 0.03 M sodium citrate) made up in water. 10 µl of the biotin labelled amplicon was added to 90 µl of the hybridisation buffer, which was heated for 1 minute at 95˚C and placed on ice for a further minute. The mixture was added to the array tube and incubated at 55˚C for one hour at 550 rpm.

The array tube was washed with a low stringent wash containing 2 x SSC and 0.01 % Triton and placed in the thermomixer for 5 minutes at 40 ˚C (550 rpm). The tube was washed a further two times with buffers containing 2 x SSC and 0.2 x SSC incubated at 40˚C and 30˚C respectively (550 rpm). 100 µl of freshly prepared 2 % blocking solution; 0.02 g of milk powder dissolved in 1 ml of 6 x SSPE (0.9 M saline, 0.06 M sodium phosphate and 0.06 M EDTA)/0.005 % triton buffer was added to the array tube and incubated for 15 minutes at 30 ˚C (550 rpm).

2.4.3.4 Staining, detection and analysis

Horseradish peroxidase conjugated to streptavidin was used to stain the hybridised biotin-labelled DNA. Staining is mediated by an enzyme catalysed reaction producing a coloured precipitate upon addition of the substrate Trimethylbenzene (TMB). 100 µl of freshly prepared poly-HRP streptavidin was prepared by diluting a 1.5 mg/ml stock of Streptavidin in a 1: 50 v/v ratio with 6 x SSPE/0.005 % Triton buffer to obtain a final concentration of 0.2 µg/ml. This was incubated at 30 ˚C at 550 rpm for 15 minutes. The array tube was washed as before in 2 x SSC/0.01 % Triton, 2 x SSC and 0.2 x SSC incubated at 30 ˚C and 20 ˚C for the latter two washes at 550 rpm.

To detect the bound DNA, 100µl of the peroxidise substrate TMB Seramun grün, (Diagnostica GmbH) was added to the chip, incubated for 10 minutes at room temperature. The images were captured and visualised using the ArrayMate™ (www.identibac.com). Figure 2-1 shows an example of the image generated from the array tube. Signal intensity values were measured using the Iconoclust imaging software (www.identibac.com). Data was normalised against the signal intensity value of the *ihfA* positive control probe and the median values (signal substance) for each triplicate gene probe was used in subsequent analysis.

From previously published data (Batchelor et al., 2008), normalised intensity values for the corresponding gene probes of value ≥ 0.4, were considered present whilst those < 0.2 were considered absent. Values between 0.4 and 0.2 were considered as ambiguous. The purpose of normalization was to limit the sources of systematic variation that may affect signal intensity values e.g. non-specific binding to the probes.

Normalised data was analysed using Genespring GX v7.3.1 (Agilent technologies) to cluster the results using the Pearson correlation coefficient.

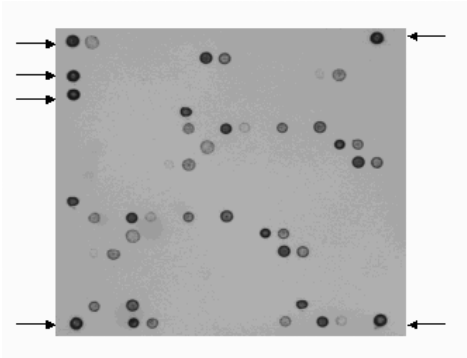


Figure 2-1: An example of the image generated using the ArrayMate™ imaging software. The cells were lysed and DNA amplified by linear multiplex PCR labelled with biotin. The amplicons were hybridized to the arrays and the signal intensity measured using the Iconoclust imaging software. There are 6 biotinylated spots (indicated by arrows) on the AMR05 chip, which serve as marker spots for array chip validation, 4 control genes: *ihfA, gapA, dnaE* and *hemL*. The *ihfA* gene probe was used to normalise signal intensity values, 56 antimicrobial resistance genes and 2 integrase associated genes.

2.4.4 Validation of array data by PCR

PCR was used to validate the array results that conflicted with phenotypic data (see section 2.2 above). 5 µl of template DNA was added to a PCR reaction mixture containing 10 x PCR buffer (Promega M186B) containing 2 mM or 3 mM $MgCl_2$ depending on primer pair used to improve stringency, 2.5 Units of *Taq* polymerase (Promega M186B; 2500 Units), 10 mM of each dNTP, 20 µM of the forward and reverse primer and adjusted to a 50 µl reaction volume with nuclease free water.

PCR cycling conditions for the validation PCR were as follows: 94 °C for 3 minutes, followed by 30 cycles of 95 °C for 30 seconds, annealing for 1 minute (annealing temperatures between 50 °C and 64 °C were used for the different PCR primers corresponding each antimicrobial resistance genes present on the probes), 72 °C for 1 minute; and a final extension at 72 °C for 5 minutes. Fragments were run on a 1 % agarose gel, stained in 0.8 µg/ml ethidium bromide and visualized under ultraviolet light using the AlphaImager™ system (Alpha Innotech).

2.4.5 PCR subtyping of *bla*$_{\text{CTX-M}}$

The strains were confirmed to harbour *bla*$_{\text{CTX-M}}$ by multiplex PCR, devised by Woodford et al., 2005 to identify the alleles as previously described. For the sequencing PCRs *bla*$_{\text{CTX-M}}$ group 9 (Sabate, Navarro et al. 2002) and group 1 specific primers (Carattoli, Garcia-Fernandez et al. 2008) or *bla*$_{\text{CTX-M}}$ universal primers (Batchelor et al., 2005) were used to amplify the genes. A 30 cycle program (for *bla*$_{\text{CTX-M}}$ group 1 and 9 primers) or 35 cycle (*bla*$_{\text{CTX-M}}$ universal primers) with a 60°C annealing temperature was used to amplify amplicons with sizes: 849 bp (group 1), 836 bp (group 9) and 585 bp (universal). The fragments were visualised on an agarose gel stained with ethidium bromide, extracted and purified using the Qiagen PCR purification kit (Cat. No. 28104)

as per the manufacturers instructions. Gene products were sequenced using the ABI PRISM sequence analyser (3700) and analysed using DNAStar LaserGene v 9 EditSeq.

2.4.6 Genetic environment surrounding *bla*$_{CTX-M}$ genes

Commonly associated with *bla*$_{CTX-M}$ genes is the insertion element, IS*Ecp1*. Primers described by (Poirel, Gniadkowski et al. 2002) were used to amplify the IS*Ecp1* element upstream of *bla*$_{CTX-M}$ group 1 encoding strains. Reverse primers described in (Sabate, Navarro et al. 2002) were used in conjunction with forward primers described by (Poirel, Gniadkowski et al. 2002) to amplify the IS*Ecp1* element upstream of *bla*$_{CTX-M}$ group 9 genes (see appendix for a list of primer sequences). Briefly the PCR was performed as follows: 8 µl of crude lysates or 3 µl of purified DNA was used in a 50 µl reaction consisting of 5 µl 10 x reaction buffer (containing 15mM $MgCl_2$), 10 mM of each dNTP, 10 µM of each primer and 1 U of *Taq* polymerase. PCR conditions were as follows; 94 °C for 5 minutes, 30 cycles of 94 °C for 25 seconds, 52 °C for 40 seconds, 72 °C for 70 seconds and a final extension at 72 °C for 6 minutes. Fragments were run on a 1 % agarose gel, stained in 0.8 µg/ml ethidium bromide and visualized under ultraviolet light using the AlphaImager™ system (Alpha Innotech).

2.4.7 Plasmid content analysis

Plasmid DNA was isolated by alkaline lysis and phenol chloroform extraction as described above (Kado and Liu 1981). Plasmid DNA was run on a 0.8 % agarose gel in TBE buffer at 21°C (maintained using a BioRad cooling module) for 6 hours at 150 V. Plasmid DNA was visualised by staining with 0.8 µg/ml ethidium bromide and visualised under UV light.

Plasmid sizes were estimated using *E. coli* 39R 861 (Threlfall, Rowe et al. 1986) and a DNA supercoiled ladder (2-16Kb, Sigma) as markers. Approximate plasmid sizes were calculated using the Rochelle method (Rochelle et al., 1986), which uses a multiple

linear regression of the molecular weight (kb $\log_{10}$), against the relative mobility (mm $\log_{10}$) and the reciprocal square root of the relative mobility (mm). Statistical analysis was carried out using the ANOVA (analysis of variance) tool in Excel, which separates the sum of squares (ss) into their components. The resulting table gives the regression coefficients, which were used to estimate the plasmid sizes using the quadratic equation below:

$$\mathbf{Log_{10}\ M_r = a + b_1\ (log_{10}\ r) + b_2\ (1/r^{1/2})}$$

Where M_r is the estimated molecular weight (kb), 'r' is the distance migrated (mm). '*a*', 'b_1' and 'b_2' are the regression coefficients obtained from running the ANOVA statistical tool.

2.4.8 S1 PFGE to size plasmids

S1 nuclease PFGE is a method used to size plasmid DNA by linearizing the circular covalently closed macromolecules by introducing 'nicks' in the DNA. This method was used to screen transconjugants for strains harbouring single plasmids encoding $bla_{\text{CTX-M}}$. Transconjugants that harboured more than a single plasmid were transformed into DH10B™ (Invitrogen) and/or *E. coli* K12 20R764 competent cells (prepared as described in section 2.2.6) and S1 PFGE repeated to confirm the presence of a single plasmid.

The method of S1 nuclease digestion followed by PFGE was performed as described previously (Barton et al., 1995; Dierikx et al., 2010). Whole genomic DNA extraction was performed essentially as described by the standard PFGE protocol (PulseNet; section 2.4.2). Any modifications to the protocol are described. Bacterial suspensions were adjusted to OD_{610} 1.6-1.7. Subsequent to bacterial lysis and washes, the plugs were equilibrated in 200 µl S1 buffer (0.5M sodium acetate (pH4.5), 2.8M NaCl, 45mM $ZnSO_4$) for 15 minutes at room temperature. S1 nuclease (Promega Cat. No. M5761-E576B) was diluted with fresh S1 buffer to a final volume of 100 µl, and this enzyme-buffer mix was added to the plug already equilibrated in 200 µl of buffer to a final

volume of 300 µl and a final enzyme concentration of 8 U/plug. This reaction was incubated at 37 °C for 45 minutes exactly, when the reaction was stopped by adding 100 µl of 1 x TBE buffer. Electrophoresis was conducted in a CHEF DR-II system using the following conditions: initial and final switch times; 1 and 25 seconds respectively, voltage 6 V, included angle 120 ° for 17 hours at 14 °C. Plasmids were sized using a low range PFGE marker (NEB Cat. No. N0350S).

2.4.9 Plasmid incompatibility group typing

Plasmid incompatibility (Inc) groups were determined of all isolates by a PCR-based replicon typing (PBRT) method described by Carattoli *et al*., 2005. This method was also applied to plasmids transferred by conjugation. Plasmids that were unable to conjugate, or transferred more than one plasmid were transformed into DH10B™ and/or *E. coli* K12 20R764 chemically competent cells (Invitrogen/ this study) according to the manufacturers' specifications; and subsequently typed by PBRT. The method employed 3 simplex and 5 multiplex PCR reactions recognising FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA, the major incompatibility groups among *Enterobacteriaceae* (Carattoli et al., 2005). Briefly, 2 µl of DNA extract (plasmid or whole genome) or 5 µl of crude lysates were added to a PCR reaction containing 2.5 µl 10 x reaction buffer (with 15 mM $MgCl_2$), 10mM of each dNTP, 50 µM of each primer pair (see appendix for a list of the primers), 0.5 U of *Taq* polymerase and made up to 25 µl with nuclease free water. All PCR amplifications were performed under the following conditions; 94 °C for 5 minutes, 30 cycles of 94 °C for 1 minute, annealing at 60 °C (52 °C for F simplex PCR) for 30 seconds, 72 °C for 1 minute and a final extension step at 72 °C for 5 minutes. Products were run on a 2 % agarose gel and visualised under UV light. Positive controls used in this study were a gift from Dr. Alessandra Carattoli (ISS Rome, IT).

2.4.10 pMLST of IncI1 group

Plasmids belonging to the IncI1 incompatibility group were further typed by multi-locus sequence typing (pMLST) methods described by (Garcia-Fernandez, Chiaretto et al. 2008). Six plasmid genes involved in plasmid maintenance and replication were identified as markers for typing plasmids by pMLST; *pilI* (254 bp), *sogS* (254 bp), *ardA* (342 bp), *repI1* (104 bp) and *trbA-pndC* (812 bp). Crude lysates from field strains known to harbour an IncI1 plasmid were typed by this method. Briefly; 2 µl of DNA extract (plasmid or whole genome) or 5 µl of crude lysates were added to a PCR reaction containing 5 µl 10 x reaction buffer (with 15 mM $MgCl_2$), 10mM of each dNTP, 50 µM of each primer pair (see appendix B for a list of the primers), 0.5 U of *Taq* polymerase and made up to 50 µl with nuclease free water. PCRs were performed as follows; 1 cycle at 94 °C for 5 minutes, 30 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 1 minute and a final extension at 72 °C for 5 minutes. Amplicons were purified using the QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen Cat. No. 28104). Using the online pMLST database (http://pubmlst.org/plasmid) alleles were assigned to sequence types (ST) based on the sequencing data (Jolley, Chan et al. 2004).

2.4.11 Next Generation plasmid sequencing and annotation

Plasmid DNA was extracted as previously described using a Qiagen large construct kit that uses a larger initial culture volume (500 ml) (Qiagen Cat. No. 12462). Plasmid DNA (5 µg DNA) was sent to the AHVLA Central Sequencing Unit for 454 Next Generation Sequencing using the GS FLX Titanium series, (Roche). The reads were assembled into contigs using the Newbler assembler software.

All contigs generated from the 454 sequencing data were joined into a pseudomolecule with a linker sequence (NNNNNCACACACTTAATTAATTAAGTGTGTGNNNNN)

that puts stop codons in all six reading frames (J. C Venter institute). The contigs were annotated using RAST (Rapid Annotation using subsystems Technology), which predicted the open reading frames (ORFs) in addition to using the SEED framework to provide further functional annotation (www.rastdb.com). The sequences were annotated further using the BLAST non-redundant database from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against other published plasmid sequences and the annotation results compared to that generated from RAST.

2.5 Phenotypic methodology

2.5.1 Antimicrobial resistance phenotype

Antimicrobial resistance phenotypes for all 52 isolates was determined using the disc diffusion assay (Davis and Stout 1971). Briefly, *E. coli* cultures were plated onto Iso-sensitest agar (Oxoid) and incubated overnight at 37 ˚C in the presence of the following antimicrobials: amikacin (AK, 30 μg), amoxycillin/clavulanic acid (AMC, 30 μg), ampicillin (AMP, 10 μg), ampramycin (APR, 15 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), ciprofloxacin (CIP, 1 μg), chloramphenecol (C, 10 μg), furazolidone (FR, 15 μg), gentamacin (CN, 10 μg), nalidixic Acid (NA, 30 μg), neomycin (N, 10 μg), streptomycin (S, 25 μg), sulphamethoxazole/trimethoprim (SXT, 25 μg), compound sulphonamides (S3, 300 μg), tetracycline (TE, 10 μg). Zone sizes were interpreted using a ProtoZONE (Don Whitley Scientific) automated reader and resistance phenotypes assigned using breakpoints set by the British society of Antimicrobial Chemotherapy (BSAC) (Andrews 2001).

2.5.2 Susceptibility testing

Minimum inhibitory concentrations (MIC's) of three β-lactams; ampicillin (amp), cefotaxime (ctx) and ceftazidime (caz), were determined using the agar dilution method

(Andrews 2001). Briefly, serial double dilutions of the selected β-lactams were prepared in distilled water. The range of antimicrobial dilutions was determined using the BSAC MIC breakpoints for *Enterobacteriaciae* as a guideline. The ranges used were as follows; amp: 1 - 1024 mg/L (BSAC breakpoint ≥16), caz: 0.5 - 512 mg/L (BSAC breakpoint ≥2), ctx 0.5 - 512 mg/L (BSAC breakpoint ≥2). Antimicrobials were reconstituted in distilled water, filter sterilised using 0.2 μM filters then serially diluted to make a series of eleven double dilution stocks used to determine MIC. Control plates did not contain any antimicrobial agents. Agar dilution plates were then prepared by mixing 10 ml antimicrobial stocks (double strength) with 10 ml of molten double strength Iso-senitest agar.

The inoculum was prepared from an overnight culture (approximately 10^9 CFU/ml) grown for 16-18 hours in a 37 °C shaking incubator (200 rpm) and adjusted to 10^4 CFU/ml by diluting with 0.1 M PBS (phosphate buffered saline) pH 7.2. A standard 10^4 CFU (colony forming units) per spot was delivered using a multipoint inoculator. A control plate (with no antibiotic) and *E. coli* control strain NCTC 10418 was included in every MIC determination. All the plates were incubated for 18 hours at 37 °C and the MIC interpreted according to BSAC guidelines.

2.5.3 Bacterial growth kinetics

Bacterial growth kinetics was determined using FLUOstar OPTIMA plate reader (BMG LABTECH). Liquid cultures grown in 3 ml LB broth were re-suspended at 0.1 % (v/v) in minimal media (1 x MOPS; 1 M 3-N-morpholinopropanesulfonic acid, 0.132 mM K_2HPO_4, 0.4 % glucose) or LB broth and incubated in the FLUOstar OPTIMA at 37 °C for 24 h. In some instances the media was supplemented with antimicrobials to measure growth kinetics in the presence of ctx, caz or amp. Control media (no bacteria) was also present in triplicate as were three technical repeats for each strain. Absorbance was measured every 15 minutes at OD_{600}. At least two biological repeats were performed for

each strain. Bacterial viability counts expressed as CFU/ml were taken at timed intervals (0, 2.5, 5 and 24 h) as previously described (see section 2.1.3). The area under the curve was calculated and a two-tailed T-test carried out to assess statistical significance.

2.5.4 Plasmid system enzyme kinetics

2.5.4.1 Cell lysis by osmotic shock

E. coli BL21 competent cells were transformed with $bla_{CTX\text{-}M}$ harbouring plasmids as described previously. Overnight liquid cultures grown at 37 °C were harvested by centrifuging at 10,000 x g for 30 minutes at 4 °C. Cells were washed once in an equal volume of sodium phosphate buffer (0.01M $NaPO_4$ pH 7) then pelleted at 10,000 x g for 30 minutes at 4 °C (Hedberg et al., 1995).

Cells were lysed by osmotic shock (Berg JO, 1981) by re-suspending the cells in 1/10 original volume of 0.02 M TrisHCl buffer (pH 8.0) containing 20 % (w/v) sucrose and incubated for 10 minutes at room temperature. Cells were then subjected to a freeze thaw cycle to improve recovery of protein by incubating at -80 °C and thawed in a 37 °C water bath, this process was repeated once more. Cells were centrifuged at 10,000 x g for 30 minutes at 4 °C and the supernatant fraction was collected and stored at -20 °C.

2.5.4.2 Measuring protein concentration

The total protein content [T.prot] was measured using the BCA protein assay kit (Thermoscientific), using BSA as a standard.

2.5.4.3 Determining the extinction coefficient of hydrolysed nitrocefin at 492nm

The molar extinction coefficient (absorption coefficient) measures the amount of light absorbed by a substance at a given wavelength. Previously reported molar extinction coefficients of nitrocefin at various experimental conditions included 20 500 $M^{-1}cm^{-1}$ at 492 nm (Heras, 2008; 370) and 15 000 $M^{-1}cm^{-1}$ at 482 nm (Perez-Llarena 1997; 179).

The aforementioned molar extinction coefficients were determined at conditions that differ from this study. For this study the molar extinction coefficient was determined from fully hydrolysed nitrocefin in a final volume of 200 µl. Hydrolysis of nitrocefin (100 µM) was monitored at 492 nm over 10 minutes using Beckman Coulter's PARADIGM™ detection platform. Absorbance was monitored using a flatbottom microplate (IWAKI®; 3860-096), using a path length defined by the volume of liquid in the well. The pathlength (0.53 cm) was determined mathematically using the following formula:

Equation 1 $$V = \pi r^2 h$$

r; *radius (cm),* ***h;*** *pathlength,* ***V;*** *sample volume(cm^3)*

The change in absorbance per minute was used to calculate the extinction coefficient of fully hydrolysed nitrocefin using the Beer Lambert Law (equation 2). The Beer-Lambert law states that the absorbance is proportional to the concentration of a given substance dissolved in a given solution at a given wavelength:

Equation 2 $$A_\lambda = \varepsilon c l$$

A_λ; absorbance, ***ε;*** *molar extinction coefficient,* ***c;*** *molar concentration of substance,* ***l;*** *length of light path.*

The molar extinction coefficient for nitrocefin was calculated to be 20 469 $M^{-1}cm^{-1}$ under these conditions at 492nm, 25 °C ± 0.5 °C, 50 mM sodium phosphate buffer pH 7.0.

2.5.4.4 Determining β-lactamase activity

β-Lactamase activity of the supernatant fraction was assayed spectrophotmetrically (**λ**= 492 nm) in the presence of 100 μM nitrocefin (Calbiochem Cat. No. 484400) in 50 mM $NaPO_4$ buffer (pH 7.0) (Hedberg et al., 1995). The extracts were diluted appropriately to a measurable rate, typically 90 μl of protein extract was added to 10 μl of 100 μM nitrocefin in 50 mM sodium phosphate buffer (pH 7.0) to a final volume of 100 μl (Hedberg 1995; 39). All determinations were carried out in triplicate over a period of 10 minutes (25°C) using a Beckman Coulter's PARADIGM™ detection platform.

The β-lactamase activity of the crude extracts were measured as units of activity, where one Unit of β-lactamase is the amount of product formed per min in 1 ml of reaction at 25 °C ± 0.5 °C, 50 mM sodium phosphate buffer pH 7.0 (Bisswanger, 2004).

The reduction of nitrocefin was monitored over 10 minutes and changes in absorbance at 492nm were plotted against time (in minutes). The gradient of the linear portion of the curve (OD/min) was used to calculate the enzyme activity per minute (or Units min^{-1}). The Beer-Lambert equation (equation 2) was used to calculate the Units of enzyme per minute using the determined molar absorption coefficient of nitrocefin, ε_{492}=20 469 $M^{-1}cm^{-1}$ (section 2.5.5.3), a path length of 0.27 cm; corresponding to the volume of liquid in the well was used and the OD/min.

2.5.4.5 Determining the total enzyme concentration; $[E]_T$

The amount of enzyme (mg) produced in one millilitre was calculated by dividing the total enzyme activity (μmol min^{-1}) by the specific activity (μmol min^{-1} mg^{-1}) (Campbell and Farrell, 2006). The total enzyme activity and the total specific enzyme activity were

expressed as a measure in a 1ml reaction therefore the amount of enzyme could be expressed as mg ml^{-1}. Molecular weights were estimated from amino acid sequences using the ExPASy PI/Mw computation tool, Swiss-Prot (http://web.expasy.org). The enzyme concentration (mol L^{-1}) was calculated by dividing the concentration of enzyme (mg ml^{-1}) by the molecular weight (g mol^{-1}).

2.5.4.6 Kinetic parameters of β-lactamase enzymes

Crude lysates were used to assay for enzyme activity (Hedberg et al., 1995; Ocallaghan et al., 1972). Each well contained a total volume of 100 µl and the change in optical density at λ = 492 nm was measured at timed intervals for up to 15 minutes with a plate photometer (Paradigm Beckman Coultier). All determinations were carried out in triplicate.

The reaction was started by addition of 90µl of 1U of β-lactamase (crude lysate) with a multichannel pipette. The initial rates of hydrolysis (v_0) were determined over a range of nitrocefin concentrations (10 µM to 400 µM) in 50 mM $NaPO_4$ buffer (pH 7.0, 25 ± 2 °C) (see Fig. 2-).

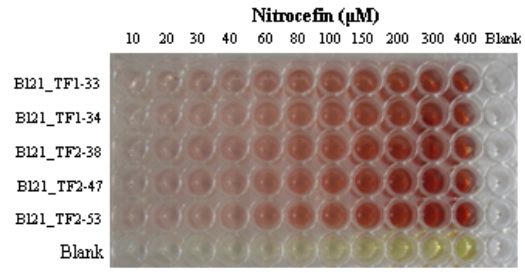


Figure 2-2: Layout of plate used to determine kinetic parameters of β-lactamase in the presence of nitrocefin. The β-lactamase activity was monitored following the reduction of nitrocefin at varying concentrations (10 – 400µM) measured at λ = 492nm in a 100 µl volumetric reaction. The reaction was measured at timed intervals for up to 15 minutes with a plate photometer. BL21_TF denotes the crude extracts of BL21 bacteria harbouring the CTX-M producing plasmids.

The initial velocity was determined by plotting absorbance readings (A_{492}) determined by monitoring the reduction of nitrocefin (see above) against time (s). The gradient of the linear portion of the curve gave the rate in abs min^{-1} (see Fig. 6-4). The mean rate, from three technical replicates, (abs min^{-1}) at each concentration of substrate (10μM to 400μM) was used to calculate initial velocity (v_0) using the Beer-Lambert Law [Equation 2] and molar absorption coefficient (20 469 $M^{-1}cm^{-1}$) obtained from fully hydrolysed nitrocefin.

Initial velocity values were plotted against substrate concentration and a Lineweaver-Burk double reciprocal plot was used to estimate K_M and V_{MAX} values. K_M and V_{MAX} values were calculated for the plasmid free *E. coli* BL21 host strain to obtain residual enzyme efficiencies, which were subtracted from the K_M and V_{MAX} values of the strains harbouring $bla_{CTX\text{-}M}$ plasmids. The enzyme turnover or catalytic constant (K_{CAT}) was calculated as a measure of the efficiency of the enzyme by dividing the V_{MAX} by the total enzyme concentration $[E]_T$.

2.5.5 Biolog™ Phenotypic microarray

The phenotypic microarray (Biolog, Inc.) assay was used to assess phenotypic effects of plasmid acquisition. Single colonies grown on LB agar plates were re-suspended in inoculating fluid (IF-0A; Biolog) containing a redox dye, supplemented with 20 mM sodium succinate, 2 mM ferric citrate and 2.5 mg/ml thiamine. The turbidity was adjusted to 85 % using a turbidity standard (Cat. No. 3431). The Biolog™ plates were inoculated with 100 μl/ well of the inoculum for each strain. Bacterial growth was monitored by a colourimetric assay monitoring the reduction of a tetrazolium dye (colourless) to formazan (purple) by the cellular respiration process. Plates were incubated at 37°C for 96 hours and growth or cellular respiration was measured every 15 minutes using the OmniLog® reader by capturing a digital image, where the image pixilation was used to measuring the intensity of formazan (purple) dye present and

convert it to arbitrary units. Kinetic data (time versus OmniLog® signal) was analysed with the OmniLog-PM software and area under the curve (AUC) was calculated to assess substrate utilisation for each strain using the OmniLog® software. Wells without substrate (colourless) were used as negative controls for each plate.

The AUC of the test wells was divided by the AUC negative controls wells (A1) to give a fold change relative to the negative control of each strain. The fold change from the plasmid free strains was subtracted from the strains harbouring the plasmid to give relative changes in metabolism between the two strains. The results were reported as differences in fold change relative to the plasmid free strain.

3 RESULTS CHAPTER 3: Characterisation of *E. coli* field isolates conferring resistance to 3rd generation cephalosporin's

3.1 Introduction

The rapid rise in strains conferring resistance to new classes of antimicrobials has increased the need for monitoring and surveillance of antimicrobial resistance. Acquiring and managing information on the emergence and trends of resistance in bacteria of human and animal origin is vital in improving our understanding of resistance with respect to the use of antimicrobials in humans and animals. By monitoring the persistence and spread of resistance, intervention and policies regarding the prudent use of antimicrobials in treatment strategies may be applied to circumvent the further spread of resistance (McEwen S. A. 2006).

In this chapter, the aim is to characterise field isolates from the United Kingdom, known to be ESBL producers, by determining the clonality of the *E. coli* strains harbouring $bla_{\text{CTX-M}}$ genes, identifying the associated antimicrobial resistance genes and mobile genetic elements that may contribute to the prevalence of these ESBL producing *E. coli* strains in cattle and through the food chain.

3.2 Results

3.2.1 Study isolates

The isolates in this study were part of an enhanced surveillance programme conducted in England and Wales in 2007. The programme was introduced to monitor veterinary bacteria for the presence of ESBLs, as part of a larger surveillance strategy to detect, assess and respond to new and re-emerging animal related threats.

Prior to the studies to be reported in this thesis the ESBL field isolates had already been collected and characterised as containing *bla*$_{CTX-M}$. The following section details the steps involved. Samples were collected over regular time periods, sampled from individual animals (floor faeces) or environmental samples (unidentified faeces, slurry, water/feed troughs, rodent/bird droppings, collecting yard and driveways. Samples were tested against a panel of cephalosporins including; cefotaxime, ceftazidime cefoxitin, cefepime, ceftiofur, cefoperazone, cephalexin and imipenem, only the floor faeces (from Cattle) were found to confer resistance to at least on of these compounds. Susceptibilities were determined for these isolates and interpreted using the BSAC guidelines (www.bsac.org.uk). Isolates found to be resistant to one or more of these compounds at the AHVLA reference laboratories were selected for further analysis as part of the enhanced surveillance programme. The samples were subsequently screened for ESBL production at the Animal Health and Veterinary Laboratories Agency (AHVLA). This work was performed by colleagues at the AHVLA whom I am grateful to acknowledge here.

Fifty-two *E. coli* isolates from the enhanced surveillance project, mainly from cattle faeces, with presumptive ESBL phenotypes were selected for this study. They represent a cross-section of isolates made from 3 specific farm visits in Pembrokeshire (*n = 9*), Dorset (*n = 7*) and Shropshire (*n = 22*), and from routine submissions made to the AHVLA regional laboratory network in Hertfordshire (*n = 6*) and Bedfordshire (*n = 8*) (see Table 3-1 for details).

3.2.2 Confirmatory identification of *E. coli*

The isolates were plated onto brilliant green agar (BGA), containing phenol red indicator. The colonies appeared yellow/green in colour indicating the presence of lactose and sucrose fermentation, indicative of the *E. coli* species.

Table 3-1: Details of isolates used in the study including; origin, sampling and host species.

Isolate	Origin species	Animal group	Sample	Location
I1-21	Bovine	Adult cows	Field faeces	Farm visit, Pembrokeshire
I1-22	Bovine	Adult cows	Field faeces	Farm visit, Pembrokeshire
I1-23	Bovine	Adult cows	Field faeces	Farm visit, Pembrokeshire
I1-24	Bovine	Adult cows	Field faeces	Farm visit, Pembrokeshire
I1-25	Bovine	Adult cows	Field faeces	Farm visit, Pembrokeshire
I1-26	Bovine	Adult cows	Field faeces	Farm visit, Pembrokeshire
I1-27	Bovine	Adult cows	Field faeces	Farm visit, Pembrokeshire
I1-28	Bovine	Adult cows	Field faeces	Farm visit, Pembrokeshire
I1-29	Bovine	Calf	Floor faeces	Farm visit, Pembrokeshire
I1-30	Bovine	Calf	Faecal sample	RVC labs, Hertfordshire
I1-31	Bovine	Calf	Faecal sample	RVC labs, Hertfordshire
I1-32	Bovine	Calf	Faecal sample	RVC labs, Hertfordshire
I1-33	Bovine	Calf	Faecal sample	RVC labs, Hertfordshire
I1-34	Bovine	Calf	Faecal sample	RVC labs, Hertfordshire
I1-35	Bovine	Calf	Faecal sample	RVC labs, Hertfordshire
I1-36	Bovine	Calf	Floor faeces	Regional labs, Bedfordshire
I1-37	Bovine	Calf	Floor faeces	Regional labs, Bedfordshire
I1-38	Bovine	Calf	Floor faeces	Regional labs, Bedfordshire
I1-39	Bovine	n/a	Floor faeces	Regional labs, Bedfordshire
I1-40	Bovine	Calf	Floor faeces	Regional labs, Bedfordshire
I1-41	Bovine	Calf	Floor faeces	Regional labs, Bedfordshire
I1-42	Bovine	n/a	Floor faeces	Regional labs, Bedfordshire
I1-43	Bovine	Calf	Floor faeces	Regional labs, Bedfordshire

Table 3-1: ***continued.***

Isolate	Origin species	Animal group	Sample	Location
I2-37	Bovine	Milking cows	Floor faeces	Farm visit, Dorset
I2-38	Bovine	Milking cows	Floor faeces	Farm visit, Dorset
I2-39	Bovine	Milking cows	Floor faeces	Farm visit, Dorset
I2-40	Bovine	Milking cows	Floor faeces	Farm visit, Dorset
I2-42	Bovine	Milking cows	Floor faeces	Farm visit, Dorset
I2-43	Bovine	Collecting yard	Environmental swab	Farm visit, Dorset
I2-45	Bovine	Driveway	Environmental swab	Farm visit, Dorset
I2-46	Bovine	Weaned calves	Floor faeces	Farm visit, Shropshire
I2-47	Bovine	Weaned calves	Floor faeces	Farm visit, Shropshire
I2-48	Bovine	Weaned calves	Floor faeces	Farm visit, Shropshire
I2-49	Bovine	Weaned calves	Floor faeces	Farm visit, Shropshire
I2-50	Bovine	Weaned calves	Floor faeces	Farm visit, Shropshire
I2-51	Bovine	Weaned calves	Floor faeces	Farm visit, Shropshire
I2-52	Bovine	Weaned calves	Floor faeces	Farm visit, Shropshire
I2-53	Bovine	Weaned calves	Floor faeces	Farm visit, Shropshire
I2-54	Bovine	Weaned calves	Floor faeces	Farm visit, Shropshire
I2-55	Bovine	Weaned calves	Floor faeces	Farm visit, Shropshire
I2-56	Bovine	Weaned calves	Floor faeces	Farm visit, Shropshire
I2-57	Bovine	Weaned calves	Floor faeces	Farm visit, Shropshire
I2-58	Porcine	Pig	Floor faeces	Farm visit, Shropshire
I2-59	Bovine	Milking cows	Floor faeces	Farm visit, Shropshire
I2-60	Bovine	Milking cows	Floor faeces	Farm visit, Shropshire
I2-61	Bovine	Milking cows	Floor faeces	Farm visit, Shropshire
I2-62	Bovine	Milking cows	Floor faeces	Farm visit, Shropshire
I2-63	Bovine	Milking cows	Floor faeces	Farm visit, Shropshire
I2-64	Bovine	Milking cows	Floor faeces	Farm visit, Shropshire
I2-65	Bovine	Milking cows	Floor faeces	Farm visit, Shropshire
I2-66	Bovine	Milking cows	Floor faeces	Farm visit, Shropshire
I2-67	Bovine	Milking cows	Floor faeces	Farm visit, Shropshire

3.2.3 *E. coli* phylogrouping

The phylogenetic background of each isolate was determined by a triplex PCR assay devised by Clermont et al., 2000. This classification method is based on sequence specific genes or DNA fragments thought to be specific to a phylogenetic group and uses a subtractive library from two *E. coli* strains to assign phylogenetic groups based on amplification of two genes; *chuA*, a gene required for heame transport in enteroheamorhagic O157:H7 *E. coli* (Torres and Payne 1997), *yjaA;* a gene involved in the cellular response to hydrogen peroxide and acid stress (Lee, Hiibel et al. 2010) and TSPE4.C2, a DNA fragment of unknown function identified from the subtractive library (Bonacorsi, Clermont et al. 2000).

Based on the PCR amplicons identified, *E. coli* phylogenetic groups were assigned to the panel of 52 isolates. They belonged to all four phylogenetic groups with the most abundant phylogenetic group being group B1, with 46 % (*n = 24*) of isolates; 33 % (*n = 17*) of strains belonged to group A, 11 % (*n = 6*) belonged to group D and finally 10 % (*n = 5*) of the isolates belonged to group B2.

Figure 3-1 below describes the abundance of these groups relative to the region of isolation. Isolates from the Shropshire farm (*n = 22*) were largely represented by phylogroup B1 (*n = 18*) and the remaining four isolates from phylogroup A. Six of isolates from the Pembrokeshire farm (*n = 9*) belonged to phylogroup A, two from phylogroup B1 and one isolate belonging to phylogroup D. Isolates from the Dorset farm (*n = 7*) demonstrate the largest diversity of the three farms, with three of the four phylogroups represented; phylogroup D (*n = 2*), phylogroup B2 (*n = 3*) and lastly phylogroup A (*n = 2*). Isolates from the Hertfordshire region belonged to phylogroups A (*n = 3*), B1 (*n = 2*) and B2 (*n = 1*). All four phylogroups were represented in isolates from the Bedfordshire region with a quarter of the isolates (*n = 2*) belonging to group A

and B1, and three isolates belonging to phylogroup D and a single isolate from phylogroup B2.

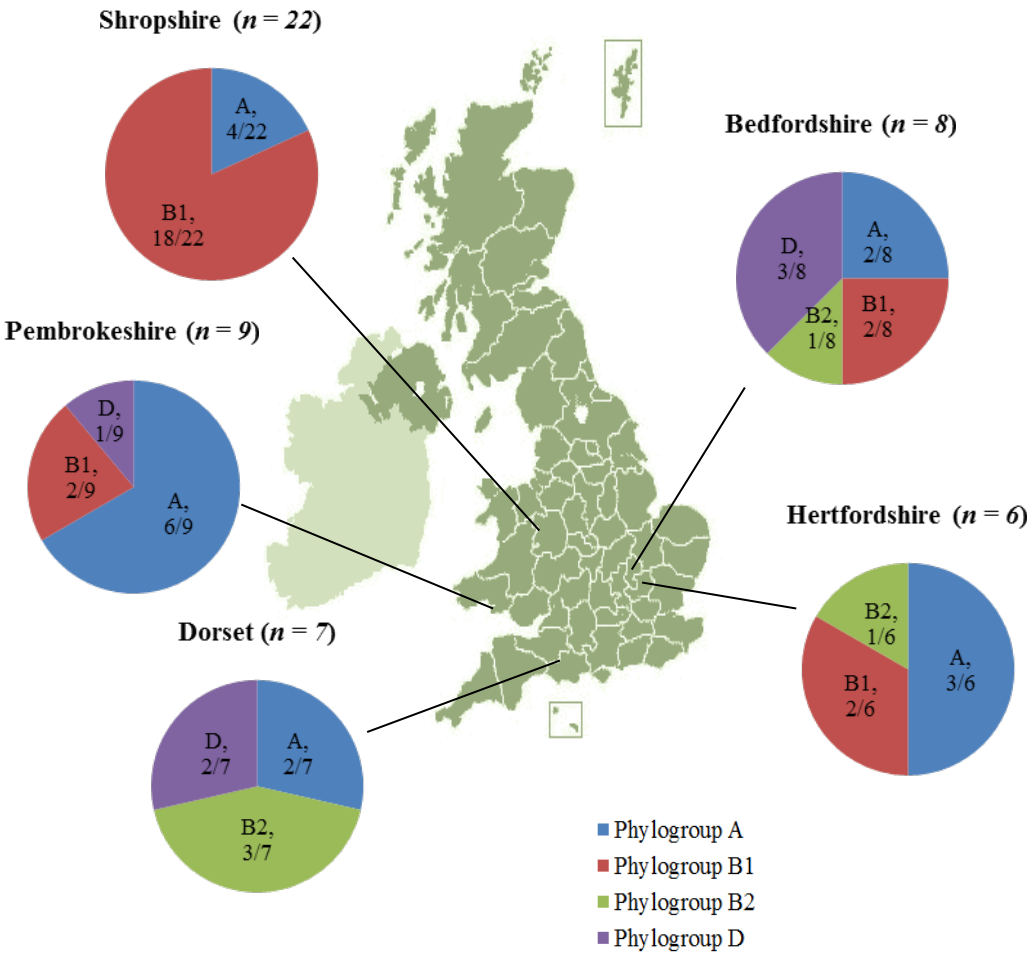


Figure 3-1: Map showing the regions to which the study isolates (n = 52) originated from and their respective phylogroups. The method of phylogenetic analysis was carried out as described by Clermont et al., 2000 using a triplex PCR to group the *E. coli* isolates into four main groups; A (blue), B1 (red), B2 (green) and D (purple).

3.2.4 Molecular subtyping of *E. coli* isolates

All 52 isolates used in this study were analysed by pulse field gel electrophoresis (PFGE), a molecular method used to cluster food borne disease pathogens for identification of common source for outbreaks. The isolates used in this study were subjected to *XbaI* PFGE analysis. Strain relatedness was calculated using the Dice coefficient (BioNumerics). Strains with the threshold linkage value at ≥ 81 %, were assigned the same type or subtype (denoted as numbers). Two strains were said to be of the same 'subtype' if they have identical banding patterns or of the same 'type' with up to six banding differences (Carrico, Pinto et al. 2005). Strain clusters were assigned letters (A-E), as shown in Fig. 3-2. Table 3-2 also shows the PFGE groups assigned using the Dice coefficient threshold.

The highest similarity (≥ 81 % similarity threshold) belonged to isolates from the Shropshire farm (designated '^{S}'), with 59 % (13/22) of the isolates assigned to this cluster (cluster E). Four other isolates from this region showed 60 % similarity to cluster 'E' isolates. Clustering of the remaining isolates from this region (*n = 5*) demonstrated ≤ 40 % similarity to the other isolates from this farm. Two of these isolates (I2-46 and I2-49) demonstrated approximately 85 % similarity to each other but with only 40 % similarity to cluster E isolates. Another isolate; I2-47 demonstrated a 35 % similarity to cluster 'E' Shropshire isolates, but with a higher similarity percentage (60 %) to I1-35, an isolate from the Hertfordshire region. Isolate I2-50 appeared to have undergone incomplete digestion, therefore clustering of this isolate could not be determined.

Of the seven strains isolated from the Dorset farm (designated '^{D}'); five were found to have 50 % similarity. The remaining two isolates, I2-40 and I2-37 were 40 % and 30 % similar to other isolates from this farm respectively (cluster D).

Isolates form the Pembrokeshire farm (designated 'P') were more variable with only two sets of isolates, I1-24 and I1-27 (cluster B) and I1-28 and I1-29 (cluster D), clustering together with 90 % and 95 % similarity respectively, suggesting a clonal origin. The remaining isolates from this region were found to have between 35 % and 40 % similarity to each other.

Isolates from the Bedfordshire regional laboratories (designated 'B') were also variable with only two isolates clustered within the dice coefficient threshold (≥ 81 %), I1-42 and I1-43 (cluster A). These demonstrated a 95 % similarity suggesting possible clonality. Two isolates, I1-38 and I1-40 (cluster B) demonstrated 80 % similarity. These stains fall outside the Dice coefficient criteria but can be designated as 'possibly related' strains (Tenover, Arbeit et al. 1995). The isolates from this region, when compared to each other, were found to have 35-40 % similarity with each other.

Only two strains of the seven strains from the Hertfordshire regional laboratories (designated 'H') fulfilled the Dice coefficient criteria, I1-31 and I1-32 with 95 % similarity, suggesting possible clonality. The remainder of the strains belong to clusters B, C and D with only 30 – 50 % similarity with strains from this region.

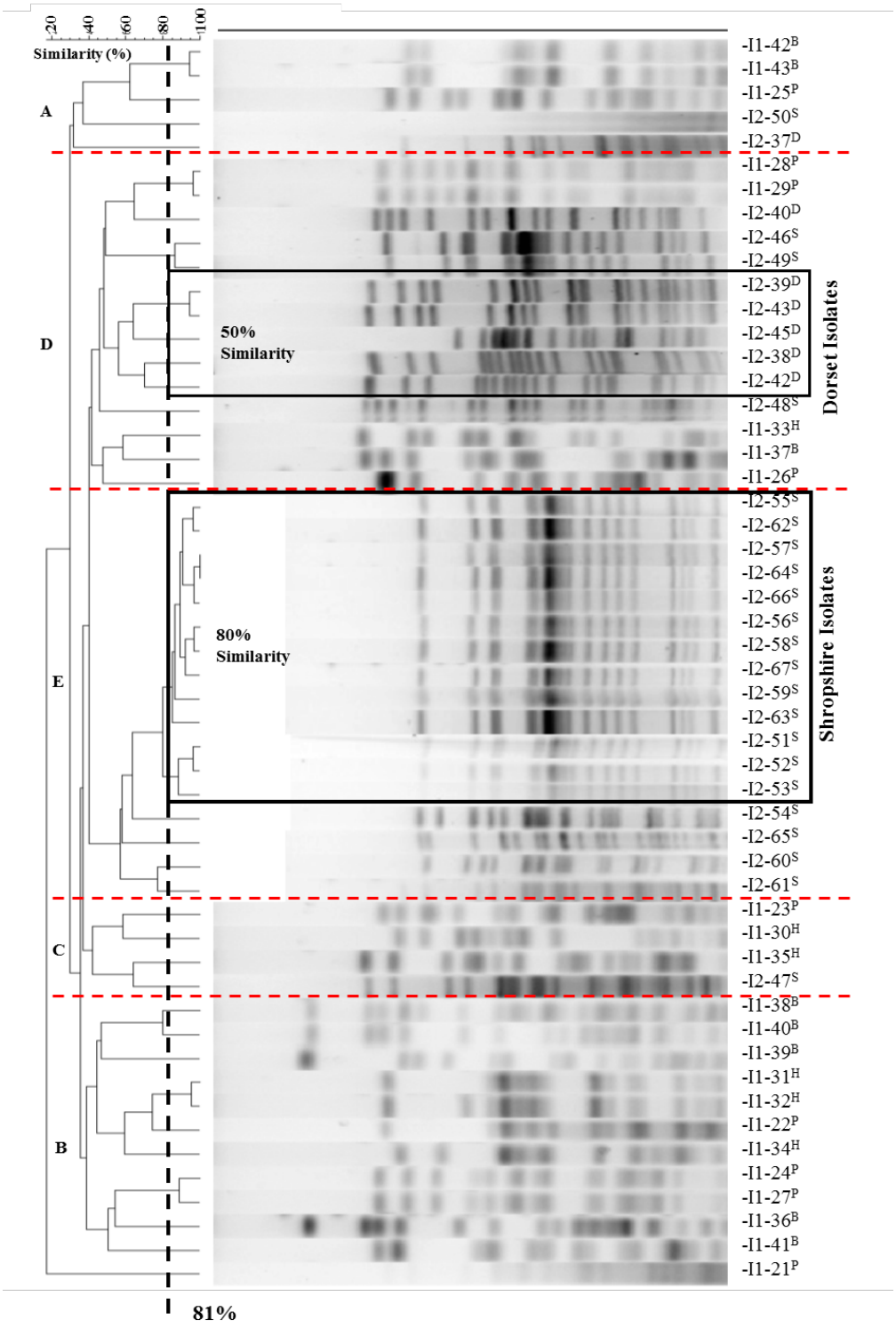


Figure 3-2: PFGE macrorestriction profiles. Isolates were digested with *XbaI* and analysed by agarose gel electrophoresis. The dendogram shows sequence similarity from 20 % to 100 %. Superscripts adjacent to the isolate name denote farm locations; 'P'; Pembrokeshire farm, 'H'; Hertfordshire regional labs, 'B'; Bedfordshire regional labs, 'D'; Dorset farm, 'S'; Shropshire farm. DNA relatedness was calculated using the Dice coefficient (BioNumerics). The dotted vertical line represents ≥ 81 % similarity threshold. Separated by the red dotted line, are Clusters A to E. The largest clusters of genetically related isolates are indicated by the box (solid line), from the Shropshire (80 % similarity) and Dorset (50 % similarity) farms.

3.2.5 Antimicrobial susceptibility

Antimicrobial resistance phenotypes for all 52 isolates were determined by the disk diffusion assay. Susceptibility testing showed all isolates to be resistant to β-lactams, ampicillin (amp), cefotaxime (ctx) and ceftazidime (caz). Antimicrobial resistance phenotypic patterns varied greatly within isolates from the same farm; however isolates found to be epidemiologically indistinguishable (identical number of bands) by PFGE displayed the same phenotypic makers in most cases. This was true for isolates; I1-28 and I1-29 (Pembrokeshire), I1-38 and I1-40, I1-42 and I1-43, from the Bedfordshire region and the E1 clusters of the Shropshire farm isolates, suggesting the clonal expansion of these strains within these regions (see table 3-2).

Two isolates from the Hertfordshire region; I1-31 and I1-32, which were found to have 95 % similarity, were found to have the same phenotypic markers indicative of their close genetic relation

70 % (n = 15) of the isolates from the Shropshire farm had the same phenotypic markers of resistance characterised by a Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip profile (see table 3-2). These isolates were found to have ≥ 81 % similarity from their macrorestriction profile (Fig. 3-2), suggesting a possible clonal amplification of a strain harbouring these strains, however these strains were not identical.

All but one isolate from the Dorset farm (cluster D) were found to harbour the same phenotypic markers (amp, caz and ctx), however these strains were found to be related with ≤ 50 % similarity, suggesting that they may harbour the same mobile genetic element. Isolates from different regions; I1-21/26/24 (Pembrokeshire) and I1-30 (Hertfordshire) and I1-28/29 (Pembrokeshire) and I1-33 (Hertfordshire), demonstrated similar phenotypic markers of resistance, but these strains were unrelated geographically, with the two regions lying in the West and East of the United Kingdom.

Table 3-2: Detailed results of *E. coli* phylogrouping, PFGE grouping and antimicrobial susceptibility assay on the isolates used in this study.

Isolate	Phylogenetic group	PFGE type	Associated antibiotic resistance markers
Pembrokeshire Farm			
I1-21	B1	*nd*	Te, Amp, Caz, S, Ctx
I1-22	A	B5	Na, Te, Amp, Caz, Sxt, S3, Ctx, Cip
I1-23	D	C1	Na, Te, N, Amp, Caz, Sxt, C, S, S3, Ctx
I1-24	A	B7	Te, Amp, Caz, S, Ctx
I1-25	B1	A2	Amp, Caz, S, Ctx
I1-26	A	D10	Te, Amp, Caz, S, Ctx
I1-27	A	B7	Na, Te, Amp, Caz, Sxt, S, S3, Ctx, Cip
I1-28	A	D1	Amp, Caz, Ctx
I1-29	A	D1	Amp, Caz, Ctx
Hertfordshire regional labs			
I1-30	B2	C2	Te, Amp, Caz, S, Ctx
I1-31	A	B3	Na, Te, Amp, Caz, C, S, S3, Ctx, Cip
I1-32	A	B4	Na, Te, Amp, Caz, C, S, S3, Ctx, Cip
I1-33	B1	D8	Amp, Caz, Ctx
I1-34	A	B6	Na, Te, Amp, Fr, Caz, Stx, C, Cn, S, S3, Ctx, Cip
I1-35	B1	C3	Te, N, Amp, Caz, S, S3, Ctx
Bedfordshire regional labs			
I1-36	B1	B8	Te, Amp, Caz, Stx, S, S3, Ctx
I1-37	B1	D9	Te, N, Amp, Caz, Stx, C, AmC, S, S3, Ctx
I1-38	B2	B1	Te, Amp, Caz, Stx, S, S3, Ctx
I1-39	D	B2	Te, N, Amp, Caz, Sxt, S, S3, Ctx
I1-40	D	B1	Te, Amp, Caz, Sxt, S, S3, Ctx
I1-41	D	B9	Te, N, Amp, Caz, Sxt, C, S, S3, Ctx,
I1-42	A	A1	Na, Te, Amp, Caz, Sxt, C, AmC, S, S3, Ctx, Cip
I1-43	A	A1	Na, Te, Amp, Caz, Sxt, C, AmC, S, S3, Ctx, Cip

Abbreviations: Am; Amoxycillin/clavulanic acid, Amp; Ampicillin, Ctx; Cefotaxime, Caz; Ceftazidime, Cip; Ciprofloxacin, C; Chloramphenicol, Fr; Furizolidone, N; neomycin, Na; Nalidixic acid, Te; Tetracyclin, S; Streptomycin, Sxt; sulphamethoxazole, S3; compound sulphonamide. nd*; not determined. Phylogenetic groups include A, B1, B2 and D. PFGE clustering is indicated by a letter (A to E) followed by a number (1-10).*

Table 3-2: ***continued*****:**

Isolate	Phylogenetic group	PFGE type	Associated antibiotic resistance markers
		Dorset farm isolates	
I2-37	A	A4	Amp, Caz, Cn, Ctx,
I2-38	D	D6	Amp, Caz, Ctx,
I2-39	B2	D4	Amp, Caz, Ctx
I2-40	B2	D2	Amp, Caz, Ctx
I2-42	D	D6	Amp, Caz, Ctx
I2-43	B2	D4	Amp, Caz, Ctx
I2-45	A	D5	Amp, Caz, Ctx
		Shropshire farm isolates	
I2-46	B1	D3	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-47	A	C4	Te, N, Amp, Caz, Sxt, C, AmC, S, S3, Ctx
I2-48	B1	D7	Amp, Caz, Ctx
I2-49	B1	D3	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-50	A	*nd*	Te, N, Amp, Caz, Sxt, C, AmC, S, S3, Ctx
I2-51	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-52	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-53	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-54	B1	E2	Amp, Caz, Sxt, C, S3, Ctx
I2-55	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-56	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-57	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-58	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-59	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-60	A	E4	Te, N, Amp, Caz, Sxt, C, AmC, S, S3, Ctx
I2-61	A	E4	Te, N, Amp, Caz, Sxt, C, AmC, S, S3, Ctx
I2-62	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-63	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-64	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-65	B1	E3	Amp, Caz, Ctx
I2-66	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip
I2-67	B1	E1	Na, Te, N, Amp, Caz, Sxt, C, S3, Ctx, Cip

Abbreviations: Am; Amoxycillin/clavulanic acid, Amp; Ampicillin, Ctx; Cefotaxime, Caz; Ceftazidime, Cip; Ciprofloxacin, C; Chloramphenicol, Fr; Furizolidone, N; neomycin, Na; Nalidixic acid, Te; Tetracyclin, S; Streptomycin, Sxt; sulphamethoxazole, S3; compound sulphonamide. nd*; not determined. Phylogenetic groups include A, B1, B2 and D. PFGE clustering is indicated by a letter (A to E) followed by a number (1-10).*

3.2.6 Resistance gene profiles; characterisation of β-lactam encoding genes

All 52 isolates were analysed using an oligonucleotide array tube, used to detect antimicrobial resistant genes in Gram-negative bacteria. The array consists of 68 antimicrobial resistance genes. DNA microarray data confirmed the presence of one or more ESBL genes to be present in all the isolates, conferring resistance to β-lactams (*bla*$_{\text{CTX-M}}$ groups 1, 2, 8, 9 and 26); which reflected the susceptibility profiles observed in the disc diffusion assay (see Fig. 3-3 below).

The Dorset farm isolates, previously found to harbour the same antimicrobial resistance markers (see table 3-2), were found to harbour *bla*$_{\text{CTX-M}}$ group 1 genes conferring resistance to β-lactams.

Sixteen out of twenty-two isolates from the Shropshire region were found to encode *bla*$_{\text{CTX-M}}$ group 1 genes, with 13 of these isolates belonging to the E1 PFGE cluster. The remaining six isolates from this farm harboured *bla*$_{\text{CTX-M}}$ group 9 genes, four of which also harboured an additional β-lactamase gene, *bla*$_{\text{OXA-1}}$.

Pembrokeshire isolates were all found to harbour *bla*$_{\text{CTX-M}}$ group 1 genes, with all but one isolate harbouring additional β-lactamase genes; *bla*$_{\text{TEM-1}}$ or *bla*$_{\text{OXA-1}}$. Isolates from the Hertfordshire regional labs, also harboured *bla*$_{\text{CTX-M}}$ group 1 genes in addition to *bla*$_{\text{TEM-1}}$ or *bla*$_{\text{OXA-1}}$ in all but one of the isolates.

Four of the eight isolates from the Bedfordshire regional labs harboured *bla*$_{\text{CTX-M}}$ group 9 genes, three with additional *bla*$_{\text{TEM-1}}$ genes (I1-36, I1-40 and I1-41) and I1-39 having both *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{OXA-1}}$ genes. Only one isolate harboured a *bla*$_{\text{CTX-M}}$ group 1 gene with additional *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{OXA-1}}$ genes. The remaining three isolates from this region; I1-38, I1-42 and I1-43, all harboured both *bla*$_{\text{CTX-M}}$ group 1 and group 9 genes in addition to a *bla*$_{\text{TEM-1}}$ gene.

3.2.7 Resistance gene profiles; characterisation of non-β-lactam encoding genes

Non-β-lactam genes included on the array platform confer resistance to aminoglycosides (*aac, aad* and *strA/B)*, chloramphenicol/florfenicol (*cat* and *floR*), sulphonamides (*sul*), tetracycline (*tet*) and trimethoprims (*dhfr*). 75 % of the isolates were found to encode at least two or more non-β-lactam resistance genes. Interestingly none of the isolates from the Dorset farm harboured any non-β-lactam resistance genes (see Fig. 3-3 below).

Often isolates found to have differing PFGE macrorestriciton profiles of ≤ 50 %, classified as unrelated, were found to have the same antimicrobial resistance genes. This was observed in isolates from the Shropshire region, namely D3 and E1 clusters (see table 3-3) suggesting non-clonal relationship. This was also observed in isolates I2-47, I2-50, I2-60 and I2-61, which all had differing macrorestriction profiles with similarities between 35 – 75 % (clusters C4 and E4) but with the same antimicrobial genes (see table 3-3).

Interestingly, isolates from two different regions; Hertfordshire (I1-30) and Pembrokeshire (I1-21 and I1-24), also had the same antimicrobial genes: $bla_{\text{CTX-M-1}}$, *strB*, $bla_{\text{TEM-1}}$, *tetA*. These strains were isolated from the East (Hertfordshire) and West (Pembrokeshire) of the UK, with only 35 % similarity between them. Under the genetic relatedness scheme described by Tenover et al., 1995 these strains would be described as unrelated, further suggesting the transfer of these resistance markers by mechanisms other than clonal spread.

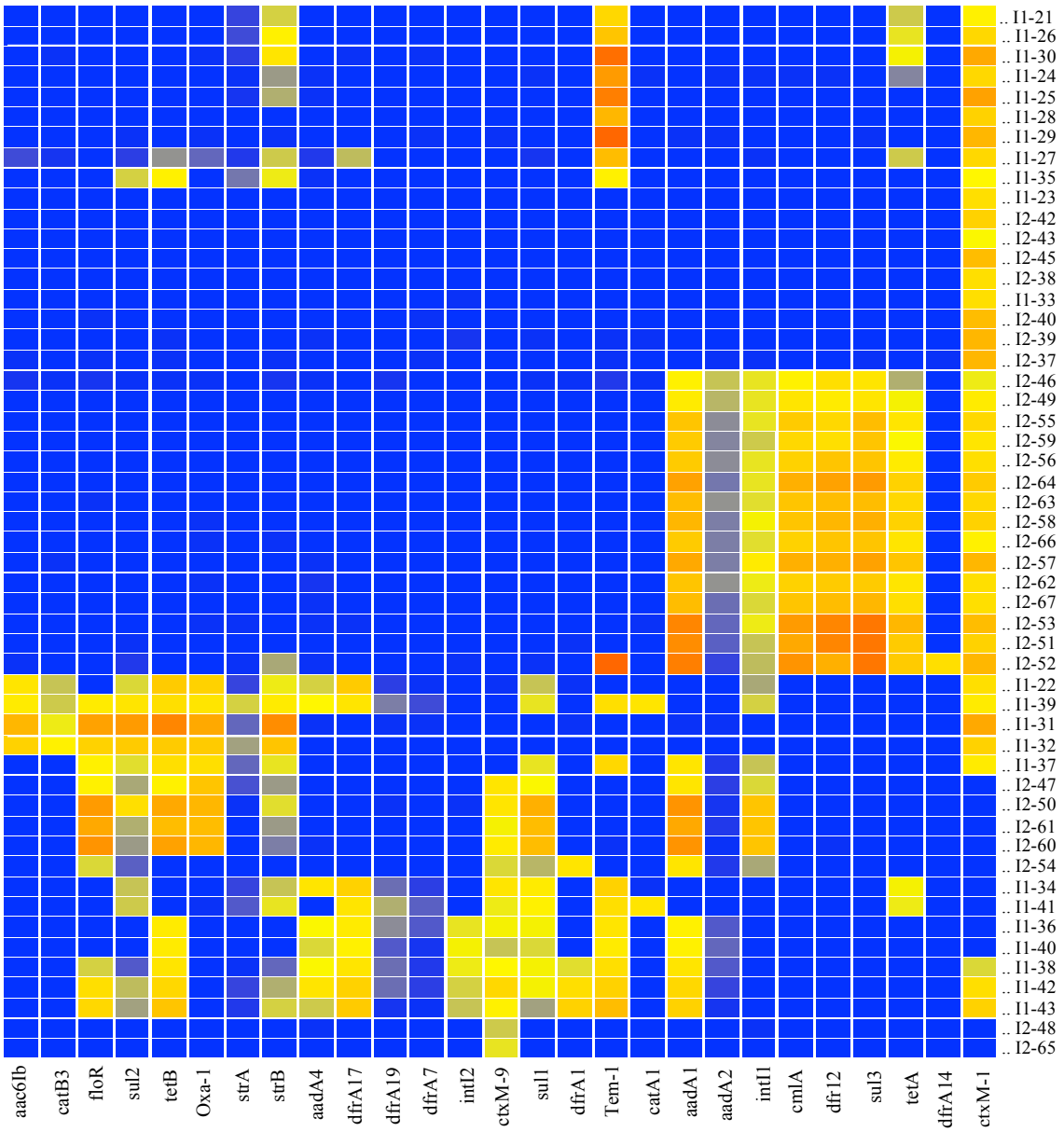


Figure 3-3: Antimicrobial resistant genes identified using miniaturized DNA microarray technology. Data was analysed with GeneSpring GX v7.3.1(Agilent technologies) and clustered using Pearson correlation coefficient. Red/yellow denotes genes present, blue denotes absent genes. All the isolates were found to encode either CTX-M group 1 (*bla*CTX-M-1*, -15* and *-32*) or CTX-M group 9 (*bla*CTX-M-14 and -14b) variants, with three isolates encoding both *bla*CTX-M-14 and *-15* genes. Majority of the isolates also encoded other antimicrobial resistance determinants commonly found in multiple drug resistant bacteria. ***Abbreviations*****:** *aac6lb*, *aadA1*, *aadA2*, *aadA4*, *strA* and *strB* confer resistance to aminoglycosides. *catA1*, *catB3* and *cmlA* confer resistance to chloramphenicols. *floR* confers resistance to chloramphenicol/florfenicol. *ctxM1*, *ctxM9*, *tem1* and *oxa1* confer resistance to β-lactams. *dfrA7*, *dfrA14*, *dfrA17* and *dfrA19* confer resistance to trimethoprims. *sul1*, *sul2* and *sul3* confer resistance to sulphonamides. *tetA* and *tetB* confer resistance to tetracylcines. *Int1* and *Int2* are integrons (mobile genetic elements) commonly associated with antimicrobial resistance genes.

3.3 Phenotypic and genotypic correlation between antimicrobial resistance markers and phenotype represented using the disc diffusion assay.

The antimicrobial susceptibility phenotype (determined by the disc diffusion assay) and genotype (determined by the array) were compared for all the isolates (see table 3-3). The phenotypic markers of resistance were grouped into the general class the antimicrobials fall into (aminoglycosides, β-lactams, chloramphenicols/florfenicols, sulphonamides, tetracyclines, trimethoprims and quinolones). The genotypic markers were also grouped into the antimicrobial class that the genes conferred resistance to: β-lactams (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{OXA-1}}$), aminoglycosides (*aac, aad* and *strA/B*), chloramphenicols/florfenicols (*cat* and *floR*), sulphonamides (*sul*), tetracyclines (*tet*), trimethoprims (*dhfr*) and quinolones (*qnr*).

Genotypic and phenotypic correlation with each antimicrobial group from the DNA array and susceptibility profiles of the field isolates was assessed using kappa statistics. Sulphonamides, trimethoprims and β-lactams scored kappa coefficients of 1, denoting 'perfect' correlation. Aminoglycosides, chloramphenicols/florfenicols and tetracyclines showed very good correlation (≥ 0.8). Quinolones scored 'poor' correlation. This was investigated further (see below).

Genes that were identified using the array but were absent for the phenotypic marker were amplified by PCR to confirm the presence of these genes. This is because some alleles were unable to be resolved by the array, possibly due to polymorphic differences in the genes, but the presence or absence could be confirmed by PCR. The confirmed genotypic profiles were used to construct table 3-3.

Table 3-3: Genotypic and phenotypic correlation of donor isolates

Antimicrobial class	Phenotype	Genotype	Kappa
β-lactams	52	52	1
Choramphenicol/Florfenicols	26	29	0.885
Sulphonamides	32	35	0.875
Trimethoprims	28	30	0.922
Aminoglycosides	37	39	0.902
Tetracylines	35	37	0.910
Quinolones*	23	0	0

The kappa coefficient was used to assess the correlation between phenotypic and genotypic data prior to validation of the DNA array genes by PCR. Scores of 1; denote complete agreement and 0 indicates very poor correlation. Scores < 0.2 were interpreted as poor, 0.21 to 0.4; fair, 0.41 to 0.6; moderate, 0.61 to 0.8; good and 0.81 to 1; very good correlation.

3.3.1 Quinolone resistance

The twenty-three isolates conferring resistance to quinolones (nalidixic and ciprofloxacin) did not harbour any plasmid mediated *qnr* resistance markers included on the DNA array (*qnrA*, *-B* and *-S*). These isolates were screened for mutations that give rise to quinolone resistance in the DNA gyrase (*gyrA*) and topoisomerase (*parC*) genes of the quinolone resistance determining region (QRDR) (Fig. 3-4 and 3-5). Single point mutations in the QRDR region gives rise to quinolone resistant strains, however this difference between wild type and mutant strains cannot be resolved by the array, as it cannot identify single base pair changes reliably. In cases where there was no correlation between the quinolone resistance susceptibility and array data, nucleotide sequencing was employed. All isolates except one, I1-27, were found to encode mutations within QRDR region of *gyrA* and *parC*. This strain was found to harbour the *aac(6)lb* aminoglycoside resistance gene that confers low level cross-resistance to fluoroquinolones in addition to its acetyl-transferase activity (Robicsek, Strahilevitz et al. 2006).

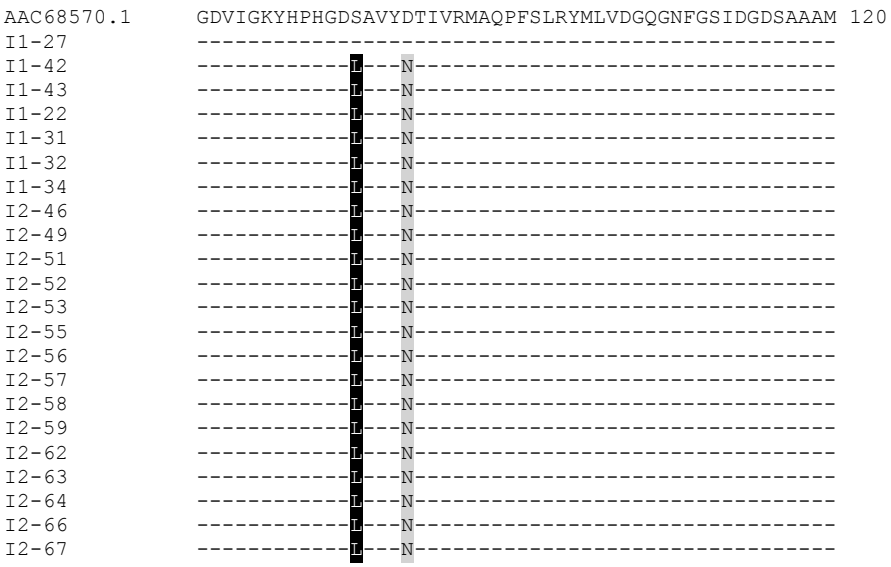


Figure 3-4: Sequence alignment of the GyrA gene that confers resistance to quinolones.

Quinolone resistance determining regions were amplified by PCR and sequenced using the primers described in Rodriguez-Martinez et al., 2006. The QRDR substitution S83L (shaded black) confers resistance to quinolones. D87N (shaded grey) of the QRDR region, confers low level resistance to fluoroquinolones (Chen and Lo 2003; Kim, Park et al. 2009). This amino substitution does not alter the steriochemical structure greatly and is therefore unlikely to confer resistance to quinolones on its own. The GyrA reference sequence was obtained from the NCBI BLAST database; accession number AAC68570.1.

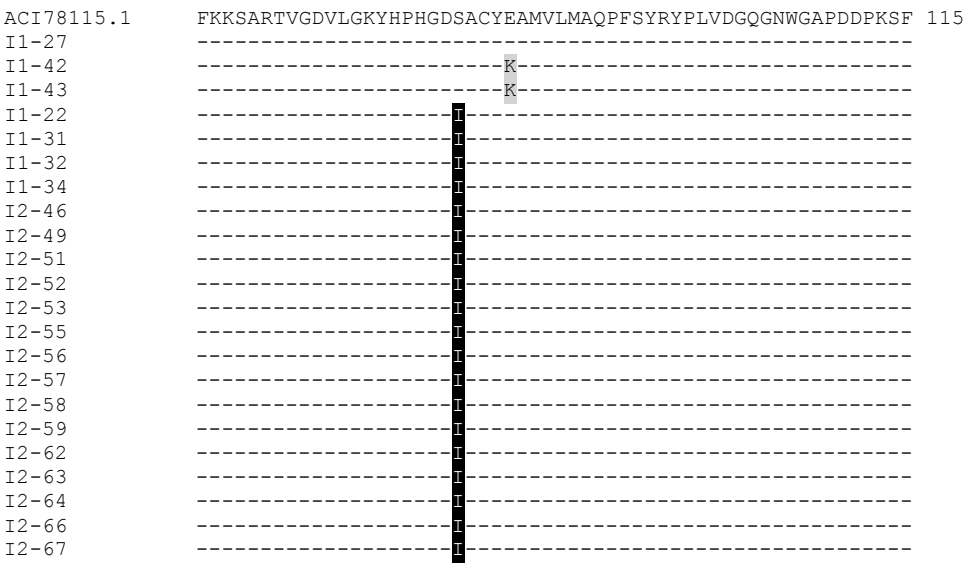


Figure 3-5: Sequence alignment of the ParC gene that confers resistance to quinolones.

Quinolone resistance determining regions were amplified by PCR and sequenced using the primers described in Rodriguez-Martinez et al., 2006.

The S80I substitution (shaded black) is an accessory QRDR substitution that gives rise to quinolone resistance. The E84K substitution (shaded grey) results in a positively charged amino acid from an otherwise negative charge (wild type), thought to decrease affinity to ciprofloxacin. The ParC reference sequence was obtained from the NCBI BLAST database; accession number ACI78115.1.

3.3.2 Plasmid content analysis

Plasmid profiles were determined using gel electrophoresis (an example can be found in Fig. 3-6 overleaf). Plasmid sizes were calculated according to the method described by Rochelle *et al*.

The results showed that all the isolates harboured at least one large plasmid (60-150 kb) (see table 3-4), with 65 % (34/52) of the strains harbouring two or more plasmids. The large plasmids are characteristic of multidrug resistant (MDR) plasmids, and further sequencing of selected plasmids was performed to gain insight into the mobile elements present on these plasmids (see Chapter 5).

Plasmid size calculation:

$$\mathbf{Log_{10}\, M_r = a + b_1\, (log_{10}\, r) + b_2\, (1/r^{1/2})}$$

Where M_r is the estimated molecular weight, r is the distance migrated (mm) and a, b_1 and b_2 are the regression coefficients obtained from running the ANOVA statistical tool.

3.3.3 Plasmid typing

Plasmid incompatibility groups were determined by a PCR-based replicon typing method (PBRT) (Carattoli, Bertini et al. 2005). About half the isolates (n = 25) were found to harbour multiple replicons (two or more) with IncF plasmids (FrepB, IncFIA, IncFIB) being present in 54 % of the isolates; in addition to IncN, IncB/O, IncI1, IncK and IncP plasmids (see table 3-4). Fourteen isolates from the Shropshire farm (n = 22) were untypeable (14/22) using the PBRT scheme. Isolates from the Dorset farm, that had indicated a single plasmid carriage correlated these findings by harbouring a single incompatibility group, IncN.

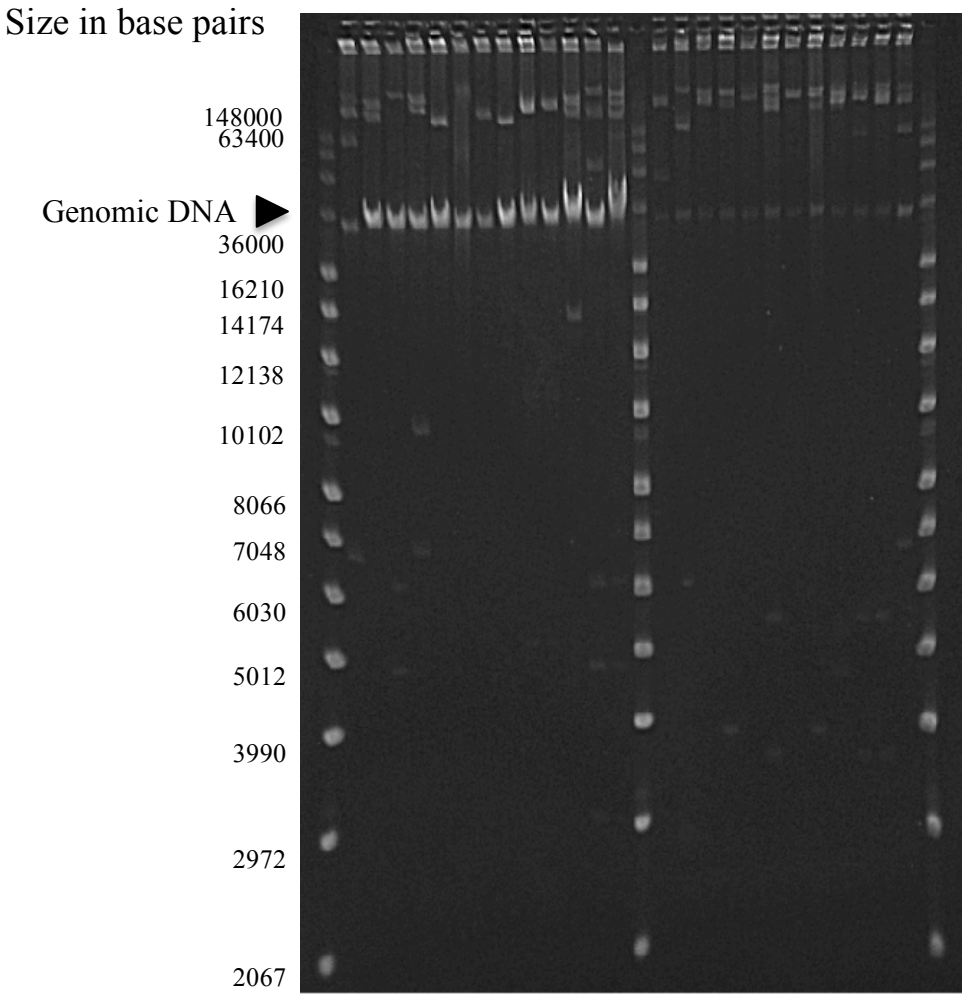


Figure 3-6: Representative gel picture of the plasmid profile used to estimate plasmid sizes of field isolates. A supercoiled ladder and plasmids from *E. coli* were used to estimate plasmid sizes.

Table 3-4: Results of the plasmid profile analysis and PCR based replicon typing of the project isolates.

Region isolated	Isolate	No. of plasmids	Estimated plasmid Size (kb)	PBRT
Pembrokeshire Farm	I1-21	2	80, 50	N, Y
	I1-22	3	150, 6, 4.5	F, FIA, FIB
	I1-23	4	140, 60, 10, 7	B/O, F, FIB, P
	I1-24	1	50	F, N
	I1-25	1	100	F, I1
	I1-26	1	55	N
	I1-27	1	50	F, FIA, FIB, N
	I1-28	2	100, 5.5	F
	I1-29	2	100, 5.5	F
Hertfordshire regional labs	I1-30	3	100, 50, 14	FIA, FIB, N
	I1-31	6	150, 50, 30, 6, 4.5, 3	F, FIA, FIB, N
	I1-32	6	150, 100, 50, 6, 4.5, 3	F, FIA, FIB, I1, N
	I1-33	2	80, 30	F, K
	I1-34	3	150, 50, 6	F, FIB, K, N
	I1-35	2	150, 80	F, FIA, FIB
Bedfordshire regional labs	I1-36	3	140, 80, 4	F
	I1-37	3	150, 100, 80	F, FIA, FIB, I1
	I1-38	5	150, 80, 58, 5.5, 3.8	F, FIA, FIB, I1, K
	I1-39	2	100, 80	B/O, F, FIB, P, Y
	I1-40	3	140, 80, 4	F, FIB
	I1-41	7	150, 140, 100, 80, 58, 5.5, 4.5	F, FIA, FIB, P
	I1-42	5	100, 80, 50, 5.5, 3.8	F, FIA, FIB
	I1-43	5	140, 100, 80, 5.5, 3.8	F, FIA, FIB

Table 3-4: ***continued.***

Region	Isolate	No. of plasmids	Estimated plasmid Size (kb)	PBRT
Dorset Farm	I2-37	2	50, 4.5	N
	I2-38	1	60	N
	I2-39	1	60	N
	I2-40	1	60	N
	I2-42	1	60	N
	I2-43	1	60	N
	I2-45	1	60	N
Shropshire Farm	I2-46	1	70	-
	I2-47	6	120, 80, 60, 7, 5.5, 4.5	F, FIA, FIB, I1
	I2-48	2	100, 80	F, FIA, FIB
	I2-49	2	100, 60	-
	I2-50	5	100, 80, 7, 5.5, 4.5	F, FIA, FIB, I1
	I2-51	2	100, 60	-
	I2-52	2	100, 60	B/O
	I2-53	1	100	-
	I2-54	1	70	F
	I2-55	1	60	-
	I2-56	1	60	-
	I2-57	2	100, 60	-
	I2-58	2	100, 60	-
	I2-59	1	60	-
	I2-60	2	100, 80	F, FIA, FIB
	I2-61	5	100, 80, 7, 5.5, 4.5	F, FIA, FIB, I1
	I2-62	2	100, 60	-
	I2-63	2	100, 60	-
	I2-64	2	100, 60	-
	I2-65	1	80	F, FIB, I1
	I2-66	1	60	-
	I2-67	1	60	-

3.3.4 Sequence analysis of *bla*$_{CTX-M}$ variants and genetic environment surrounding *bla*$_{CTX-M}$

DNA microarray analysis detects five groups of CTX β-lactamases; groups 1, 2, 8, 9 and 26. With over 80 allelic variants of CTX-M enzymes sequencing these genes was necessary to identify the variants represented within the isolates. *bla*$_{CTX-M}$ genes were amplified by PCR and sequenced. DNA sequences were compared against the BLAST non-redundant database from NCBI (www.ncbi.nlm.nih.gov/) and *bla*$_{CTX-M}$ variants subsequently assigned. As identified by the DNA array results, only two variants of the CTX-M enzymes were present; groups 1 and 9 (see table 3-5). The CTX-M group 1 enzymes included variants: CTX-M-1, -15 and -32. CTX-M group 9 enzymes included CTX-M-14 and a closely related variant CTX-M-14b, differing by only one amino acid substitution (A231V) (Navarro, Mesa et al. 2007).

All the isolates from the Pembrokeshire farm and Hertfordshire regional labs were found to encode *bla*$_{CTX-M-15}$ variants. Half of the isolates from the Bedfordshire regional labs harboured *bla*$_{CTX-M-14}$, a single isolate encoded *bla*$_{CTX-M-15}$ and three isolates harboured both a *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$ variants. All the Dorset isolates harboured *bla*$_{CTX-M-1}$ variants. Unlike the other farm isolated strains (Dorset and Pembrokeshire), isolates from the Shropshire farm were found to harbour four types of CTX-M enzymes including: *bla*$_{CTX-M-1}$ ($n = 1$), $_{-14}$ ($n = 1$), $_{-14b}$ ($n = 5$) and $_{-32}$ ($n = 15$). Interestingly, all the isolates in the E1 PFGE cluster and two from the D3 cluster encoded *bla*$_{CTX-M-32}$.

3.3.5 Analysis of the additional mobile genetic elements harboured within the isolates

3.3.5.1 IS*Ecp1*

The genetic environment surrounding the β-lactamase gene was analysed by PCR and 67 % of the strains (n=35) were found to harbour the IS*Ecp1* insertion sequence element upstream of the β-lactamase gene (see table 3-5). Three of the isolates encoding both $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ genes were found to harbour the IS*Ecp1* element upstream of both β-lactamase genes. These elements may be on separate plasmids, as these strains were found to harbour multiple plasmids.

3.3.5.2 Integrons

Integron associated genes *IntI1* and *IntI2*, part of the mobile genetic element (MGE) family, were also present in 52 % (n=27) of the isolates of which 20 of these were in isolates from region 5 (see table 3-5). *IntI1* has been previously associated with sulphonamide resistance gene; *sul1*. This common association of *sul* genes with integrons is thought to contribute to the spread and persistence of sulphonamide resistance, despite restricted use of this drug class (Perreten and Boerlin 2003).

Table 3-5: Mobile genetic elements identified in the field isolates and associated resistance genes identified by micro-array analysis.

Region isolated	Strain	CTX-M type	ISE*cp1*	Integrons	Associated antimicrobial genes identified by microarray analysis
Pembrokeshire Farm	I1-21	CTX-M-15	+	-	*bla*CTX-M-1, *strB, bla*TEM-1, *tetA*
	I1-22	CTX-M-15	+	*intI1*	*aac(6')lb, aadA4, catB3, bla*CTX-M-1, *dfrA17, bla*OXA-1, *strA, strB, sul1, sul2, tetB*
	I1-23	CTX-M-15	+	-	*catA1, bla*CTX-M-1, *dfrA7, dfrA17, strA, strB, sul2, bla*TEM-1, *tetA*
	I1-24	CTX-M-15	+	-	*bla*CTX-M-1, *strB, bla*TEM-1, *tetA*
	I1-25	CTX-M-15	+	-	*blaC*TX-M-1, *strB, bla*TEM-1
	I1-26	CTX-M-15	+	-	*bla*CTX-M-1, *strB, bla*TEM-1, *tetB*
	I1-27	CTX-M-15	+	-	*bla*CTX-M-1, *dfrA17, strB, bla*TEM-1, *tetA, tetB*
	I1-28	CTX-M-15	+	-	*bla*CTX-M-1, *bla*TEM-1
	I1-29	CTX-M-15	+	-	*bla*CTX-M-1, *bla*TEM-1
Hertfordshire regional labs	I1-30	CTX-M-15	+	-	*bla*CTX-M-1, *strB, bla*TEM-1, *tetA*
	I1-31	CTX-M-15	+	-	*aac(6')lb, bla*CTX-M-1, *floR, bla*OXA-1, *strB, sul2, tetB*
	I1-32	CTX-M-15	+	-	*aac(6')lb, catB3, bla*CTX-M-1, *floR, bla*OXA-1, s*trA, strB, sul2, tetB*
	I1-33	CTX-M-15	+	-	*bla*CTX-M-1
	I1-34	CTX-M-15	+	-	*aadA4, bla*CTX-M-9, *dfrA17, strB, sul1, sul2, bla*TEM-1, *tetA*
	I1-35	CTX-M-15	+	-	*bla*CTX-M-1, *strB, sul2, bla*TEM-1, *tetB*
Bedfordshire regional labs	I1-36	CTX-M-14	+	*intI2*	*aadA1, aadA4, bla*CTX-M-9, *dfrA7, dfrA19, sul1, bla*TEM-1, *tetB*
	I1-37	CTX-M-15	+	*intI1*	*aadA1, bla*CTX-M-1, *floR, bla*OXA-1, *strB, sul1, sul2, blaT*EM-1, *tetB*
	I1-38	CTX-M-14 and 15	+ +	-	*aadA1, aadA4, bla*CTX-M-1, *bla*CTX-M-9, *dfrA1, dfrA17, sul1, sul2, bla*TEM-1, tetB
	I1-39	CTX-M-14	+	*intI1*	*aac(6')lb, aadA4, blaCTX-M-1, dfrA17, dfrA19, bla*OXA-1, *sul1, sul2, strA, strB, bla*TEM-1
	I1-40	CTX-M-14	+	*intI2*	*aadA1, aadA4, bla*CTX-M-9, *dfrA17, sul1, bla*TEM-1, *tetB*
	I1-41	CTX-M-14	+	-	*catA1, bla*CTX-M-9, *dfrA17, dfrA19, strB, sul1, sul2, bla*TEM-1, *tetA*
	I1-42	CTX-M-14 and 15	+ +	*intI2*	*aadA1, aadA4, bla*CTX-M-1, *bla*CTX-M-9, *dfrA1, dfrA17, floR, strB, sul1, sul2, bla*TEM-1, *tetB*
	I1-43	CTX-M-14 and 15	+ +	*intI2*	*aadA1, aadA4, bla*CTX-M-1, *bla*CTX-M-9, *dfrA1, dfrA17, floR, strB, sul1, sul2, bla*TEM-1, *tetB*

Table 3-5: continued

Region	Strain	CTX-M type	ISE*cp1*	Integrons	Associated antimicrobial genes identified by microarray analysis
Dorset Farm	I2-37	CTX-M-1	+	-	*bla*CTX-M-1
	I2-38	CTX-M-1	+	-	*bla*CTX-M-1
	I2-39	CTX-M-1	+	-	*bla*CTX-M-1
	I2-40	CTX-M-1	+	-	*bla*CTX-M-1
	I2-42	CTX-M-1	+	-	*bla*CTX-M-1
	I2-43	CTX-M-1	+	-	*bla*CTX-M-1
	I2-45	CTX-M-1	+	-	*bla*CTX-M-1
Shropshire Farm	I2-46	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-47	CTX-M-14b	-	*intI1*	*aadA1, bla*CTX-M-9, *floR, bla*OXA-1, *strB, sul1, sul2, tetB*
	I2-48	CTX-M-14	+	-	*bla*CTX-M-9
	I2-49	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-50	CTX-M-14b	+	*intI1*	*aadA1, bla*CTX-M-9, *floR, bla*OXA-1, *strB, sul1, sul2, tetB*
	I2-51	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-52	CTX-M-32	-	*intI1*	*aadA1, cmlA1, bla*CTX-M-1, *dfrA12, dfrA14, strB, sul3, bla*TEM-1, *tetA*
	I2-53	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-54	CTX-M-1	-	*intI1*	*aadA1, bla*CTX-M-9, *dfrA1, floR, sul1*
	I2-55	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-56	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-57	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-58	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-59	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-60	CTX-M-14b	+	*intI1*	*aadA1, bla*CTX-M-9, *floR, bla*OXA-1, *strB, sul1, sul2, tetB*
	I2-61	CTX-M-14b	+	*intI1*	*aadA1, bla*CTX-M-9, *floR, bla*OXA-1, *strB, sul1, sul2, tetB*
	I2-62	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-63	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-64	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-65	CTX-M-14b	+	-	*bla*CTX-M-9
	I2-66	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*
	I2-67	CTX-M-32	-	*intI1*	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfrA12, sul3, tetA*

3.3.6 Analysis of β-lactam susceptibility

The MICs were determined for all the strains against three β-lactams; ampicillin, cefotaxime and ceftazidime. The susceptibility profiles were compared for the strains producing the five CTX-M variants; CTX-M-1, -14, -14b, -15 and -32. In addition susceptibility profiles were compared for the three strains producing both the CTX-M-14 and -15 enzymes. Median MIC values were taken from each CTX-M variant producing strain and results presented in the graph below (see Fig. 3-7).

The results indicate that all the CTX-M variants were equally resistant to ampicillin (MIC ≥ 1014 mg/L).

CTX-M-14 producing isolates were found to have the lowest MIC values against cefotaxime (MIC 128 mg/L). CTX-M-1, -14b, -15 and -32 were shown to have higher cefotaxime MIC values at 256 mg/L, however the highest cefotaxime MIC values (512 mg/L) were observed with the three strains harbouring both $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ variants.

CTX-M group 1 variants; CTX-M-15 and -32 were found to have the highest MIC values for ceftazidime at 512 mg/L and 256 mg/L respectively. However CTX-M-1 producing strains, a member of the same CTX-M group had lower MIC values at 64 mg/L. Strains harbouring CTX-M-14 and strains harbouring both CTX-M-14 and -15 variants also gave MIC median values at 64 mg/L.

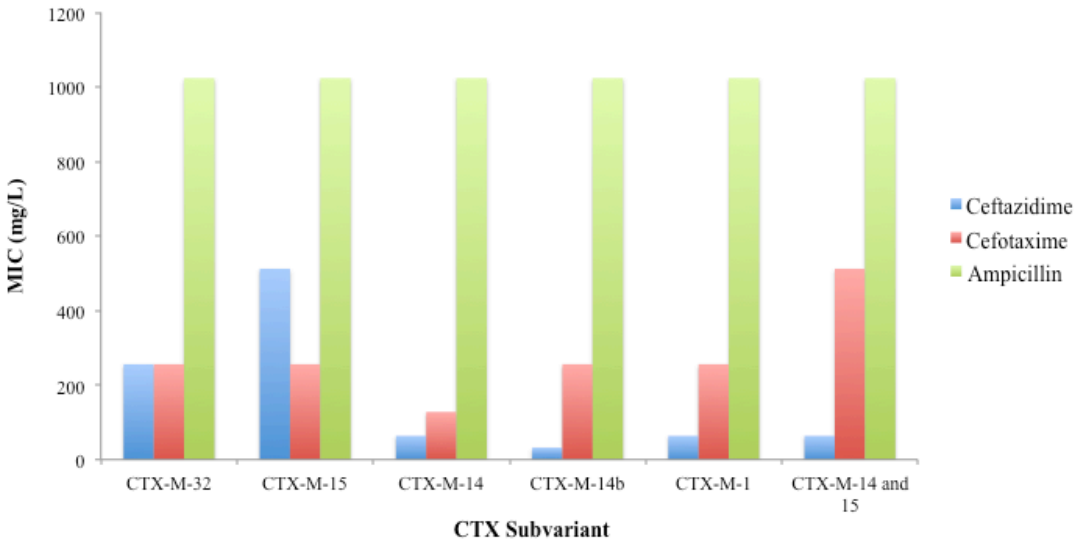


Figure 3-7: Detailed analysis of β-lactam susceptibility and CTX-M producing strains. MIC values were interpreted using the BSAC plate dilution method.

3.4 Discussion

The isolates presented in this study were part of a collection from the enhanced surveillance study from two farms and submissions from 3 regional laboratories. Phylogenetic analysis of these isolates demonstrated the representation of all four major groups; A, B1, B2 and D (Herzer, Inouye et al. 1990). These isolates were not collected as a result of an outbreak and therefore it was not surprising that a majority (79 %) of the strains belonged to the commensal phylogenetic group A and B1. The B1 phylogenetic group is unique because it is further divided into two subgroups; B1c and B1e. B1c falls into the commensal phylogroup A subtype and the subgroup B1e falls into the phylogroup E, a cluster of non-O157 EHEC, EAEC and ETEC pathogenic strains (Sims et al., 2011).

Only 21 % of the isolates were found to belong to the pathogenic *E. coli* phylogroups D and B2, which typically harbour EPEC, UPEC, APEC and ExPEC strains (Sims et al., 2011). These pathogenic strains can successfully colonize the gastrointestinal tract in as early as two week old calves (China et al., 1998). These pathogenic strains of *E. coli* are non-pathogenic to adult cattle; however EHEC (China et al., 1998; Mainil et al., 1987) and ETEC (Acres et al., 1977; Nagy and Fekete, 1999) strains have been identified as one of the major causes of neonatal calf diarrhoea, which can lead to high calf morbidity and mortality rates (Svensson et al., 2003).

Through consumption of contaminated feed, water or other environmental sources, persistence of *E. coli* 0157:H7 in healthy adult cattle suggests a potential reservoir for these species (Besser et al., 2001; Brown et al., 2001; Carlson et al., 2009). Brown et al., 2001 found that persistent colonisation of particular *E. coli* strains led to the increased efficiency in attachment to the endothelial cells (Carlson et al., 2009). This coupled to persistent shedding of the *E. coli* strains may lead to clonal expansion of a particular

subtype of *E. coli*, pathogenic or commensal (Carlson et al., 2009; Robinson et al., 2009), which may be a reason for the abundance of some clones from the Shropshire and Dorset farms.

Antimicrobial susceptibility profiles of these isolates revealed the presence of multiple antimicrobial resistance genes, both in the commensal and pathogenic strains. The successful dissemination of these *E. coli* strains within the farm by clonal expansion, as suggested by the PFGE profiles, consequently increases the incidence of spread of antimicrobial resistance through vertical transmission, this was observed in the strains isolated from the Shropshire and Dorset farms (Petersen, Christensen et al. 2006; Carlson, Nightingale et al. 2009; Johnson, Menard et al. 2009; Johnson, Johnston et al. 2010; Woodford, Turton et al. 2011).

Vertical transmission (clonal expansion) of strains harbouring antimicrobial resistance genes is viewed as an efficient vehicle of dissemination because of the already established niche, the ability to transfer (vertically) any associated mobile genetic element to daughter cells and thirdly the ability to act as a donor in the transfer of these mobile genetic elements through horizontal gene transfer (Carlson, Nightingale et al. 2009; Woodford, Turton et al. 2011).

Resistance to quinolones can be mediated by four chief mechanisms; 1) chromosomal mutations that occur in the quinolone resistance determining region (QRDR), 2) reduction in accumulation by active efflux, 3) acquired resistance collectively known as plasmid mediated quinolone resistance (PMQR) and 4) decreased uptake or increased efflux of quinolones, thought to be associated with the multiple-antimicrobial resistance (MAR) phenotype or other efflux systems such as AcrAB efflux pump or the plasmid-

mediated quinolones efflux pumps, OqxAB and Qep. Expression of MarA, encoded within the *mar* operon, controls expression of over 60 chromosomal genes including genes that are involved in decreased influx (down-regulation of OmpF) or increased efflux (Hopkins, Davies et al. 2005, Kern et al., 2000, Nordman et al., 2008). The *qnr* genes confer the PMQR phenotype, although these resistance genes are though to confer a low-level resistance phenotype (Rodriguez-Martinez et al., 2003). Qnr proteins is a 218 amino acid protein of the pentapeptide family that typically interacts with other proteins, binding to DNA gyrase and preventing the action of quinolones (Tran and Jacoby 2002). For the isolates examined in this study, none of the twenty-three isolates conferring resistance to quinolones (nalidixic and ciprofloxacin) were found to harbour the *qnr* gene from DNA microarray analysis (*qnrA*, *B* and *S*).

Sequence analysis of the QRDR of the 23 isolates determined these isolates (*n = 22*) to harbour single point mutations in the target genes; *gyrA* and *parC*. The gene products, DNA gyrase and topoisomerase IV respectively, are targets for quinolones. DNA gyrase and topoisomerase IV are thought to be homologues of each other, with high amino acid sequence similarity in the QRDR region at the N-terminus (Peng and Marians 1993). Mutations in the QRDR region prevent binding of the quinolones to these enzymes, thus conferring resistance (Ouabdesselam, Hooper et al. 1995; Vila, Ruiz et al. 1996).

A single isolate (I1-27) did not harbour any QRDR mutations, despite conferring resistance to quinolones. This strain was found to harbour an aminoglycoside resistance gene; *aac(6')-lb-cr* by PCR analysis. This encodes an acetyl transferase enzyme that alters the drug by acetylation, thereby resulting in reduced binding to the ribosomes. However, Robicsek *et al*. described the ability for AAC(6')-lb-cr to confer low level resistance to floroquinolones by acetylating the amino group in the piperzinyl group of

ciprofloxacin and is a plasmid mediated quinolone resistance (PMQR) trait (Robicsek, Strahilevitz et al. 2006).

Antimicrobial resistance genes located on plasmids can be transferred efficiently from one host to another by horizontal gene transfer (HGT) and is thought to be a major contributor to the persistent dissemination of antimicrobial genes (Grohmann, Muth et al. 2003; Fluit 2005; Andam, Fournier et al. 2011). Multidrug resistance phenotypes have been associated with complex mobile genetic elements that enable their efficient transmission (Liebert, Hall et al. 1999). It is widely accepted that the rapid dissemination and consequently the prevalence of CTX-M β-lactamases is attributed to their association with mobile platforms including plasmids, integrons, and insertion (IS) elements (Canton and Coque 2006; Novais, Canton et al. 2006; Valverde, Canton et al. 2006; Novais, Canton et al. 2007; Poirel, Naas et al. 2008; Vinue, Lantero et al. 2008; Woodford, Carattoli et al. 2009).

Twenty-seven (52 %) of the isolates were found to harbour integrase genes (*int1 and int2*) found within class 1 and class 2 integrons respectively. Often located on plasmids, these gene capture systems incorporate open reading frames (gene cassettes) within them, facilitating expression of these genes (Hall and Collis 1998). Multiple antimicrobial resistance (AMR) genes can be found in tandem within integrons, making them vital contributors to multidrug resistance phenotypes (Hall and Collis 1998). Fifteen of the 27 *int1* harbouring strains were found to have four AMR genes (*dfrA12, aadA2, sul3* and *cmlA1*) recently identified in a class 1 integron (Sunde, Solheim et al. 2008). This association of the *sul3* and *cmlA* genes with class 1 integrons is thought to contribute to its rapid spread in the former case (Perreten and Boerlin 2003) and persistence of resistance to chloramphenicol's in the latter case despite the cessation of use in food producing animals in 1988 (Gilmore 1986; de Jong, Thomas et al. 2012).

Previous studies have found *bla*$_{CTX-M}$ genes associated with complex *sul1* harbouring class 1 integrons (Sabate, Navarro et al. 2002; Novais, Canton et al. 2006; Riano, Moreno et al. 2006; Vinue, Lantero et al. 2008). The genetic platform surrounding these *bla*$_{CTX-M}$ genes is thought to be a major factor in the dissemination of *bla*$_{CTX-M}$ genes (Canton and Coque 2006). 63 % of the isolates were found to harbour the IS element IS*Ecp1*uspstream of *bla*$_{CTX-M}$, including the strains harbouring two variants of *bla*$_{CTX-M}$. IS*Ecp1*-*bla*$_{CTX-M}$ genes have been associated with transposons and this is thought to be a means of gene acquisition by transposable events including conjugative transposition and by site specific homologous recombination, enabling *inter* and *intra*-cellular transfer of genetic material (Liebert, Hall et al. 1999; Poirel, Decousser et al. 2003; Canton and Coque 2006).

The association of IS elements, integrons and transposons with plasmids is thought to be a major contributor to the evolution and spread of plasmids and the genes they carry. The strains in this study were found to harbour IS*Ecp1* elements upstream of *bla*$_{CTX-M}$ genes, in addition to integrons, capable of capturing antimicrobial gene cassettes and plasmids.

Plasmids are commonly found to harbour multiple drug resistance genes (Watanabe 1963). These are typically large plasmids (60kb to 400kb) conferring resistance to multiple drug classes (Boyd, Tyler et al. 2004; Garcia, Navarro et al. 2007; Welch, Fricke et al. 2007; Woodford, Carattoli et al. 2009). All the isolates in thus study were found to harbour at least one plasmid and showed a MDR phenotype. Plasmids have previously been found to harbour CTX-M β-lactamase genes, which are have been attributed with the rapid dissemination of these genes. CTX-M-9, -14, -15 and 32 are such β-lactamases thought to be associated with epidemic plasmids, which include narrow host range plasmids IncF, IncI, IncB/O and IncHI2 and broad host range

plasmids IncK, IncN and IncP (Cottell et al., 2011, Boyd, Tyler et al. 2004, Lavollay, Mamlouk et al. 2006; Novais, Canton et al. 2006, Valverde et al., 2009). The isolates included in this study were found to harbour multiple replicon types including both narrow (IncF, IncI, IncB/O) and broad host range (IncN and IncP) plasmids mentioned above, with the potential to transfer genetic material across different bacterial species.

E. coli 0157:H7 strains which are pathogenic for humans have been found in mature cattle that show no obvious symptoms (Whipp, Rasmussen et al. 1994; Armstrong, Hollingsworth et al. 1996) are thought to be reservoirs of these pathogenic organisms (Armstrong, Hollingsworth et al. 1996). Similarly, in the panel of isolates chosen 32 % of the strains harbouring a multidrug resistance phenotype were found to belong to phylogroup A and another 46 % belonging to phylogroup B1; both groups have been associated with pathogenic *E. coli*. Non-pathogenic *E. coli* strains of bovine origin are thought to be able to colonize the human gut, if only temporarily, thereby increasing the chance of horizontal gene transfer resistance determinants (Marshall, Petrowski et al. 1990; Oppegaard, Steinum et al. 2001). The ability for these non-pathogenic and pathogenic strains to harbour multiple drug resistance, adapt to new hosts and stabilise in the absence of selection are all factors that contribute to the persistence of resistance (Salyers and Amabile-Cuevas 1997; Blake, Hillman et al. 2003), make them potentially difficult to treat when human infection occurs.

These results suggest the possible association of these resistance determinants on mobile platforms such as plasmids, thus enabling efficient horizontal transfer of resistance to other strains, in addition to vertical transfer by clonal expansion. Furthermore, the detection of these MDR phenotypes in both pathogenic and non-pathogenic *E. coli* species, suggests the ability of *E. coli* to act as a reservoir of

resistance genes. In addition the presence of CTX-M-15 producing isolates, a variant commonly associated with human infections was observed in these isolates. This may have implications in the transfer of CTX-M-15 harbouring strains between humans and animals in close contact to these animals further aiding the dissemination of these ESBL producing strains.

4 RESULTS CHAPTER 4: Molecular characterisation of *bla*$_{CTX-M}$ encoding plasmids

4.1 Introduction

Work from the previous chapter has shown that the *E. coli* field isolates chosen for this study harbour a multidrug resistance phenotype, containing in most cases more than one antimicrobial resistance gene. In addition, these isolates were found to harbour multiple plasmids up to 150 kb in size.

In this chapter the characteristics of *bla*$_{CTX-M}$ plasmids identified in the first chapter will be investigated. The plasmids were transferred to the same *E. coli* K12 host by conjugation or transformation in cases where conjugation was unsuccessful or in instances where multiple plasmids transferred across. Plasmid sizes were estimated using S1 nuclease PFGE and resistance profiles of the subsequent transconjugant or transformant was investigated using antimicrobial disc diffusion assays and miniature DNA microarray analysis.

4.2 Results

4.2.1 Identifying IncI1 plasmid phylogenetic relationship by plasmid multi-locus sequence typing

In the previous chapter, the isolates were found to be a mixture of all four phylogenetic groups including commensal *E. coli* strains harbouring multiple plasmids. The field isolates were typed by the PCR based replicon typing (PBRT) method. Isolates that harboured IncI1 plasmids (see table 3.4) were typed by plasmid MLST (pMLST) to

determine the possible extent of evolution of the IncI1 plasmid harboured within the field isolates, which at the time this work was done only the IncI1 and IncH plasmid typing system were available (http://pubmlst.org/plasmid).

Seven of the ten field isolates harbouring IncI1 plasmids were picked for pMLST studies. These include; I1-25 (Pembrokeshire), I1-37, I1-42, I1-43 (Bedfordshire), I2-47, I2-50 and I2-61 (Shropshire). Briefly, the pMLST method involved PCR amplifying and sequencing genes required for replication and maintenance, which include: *pill*, a gene within the *pil* locus involved in pilus biogenesis; *sogS*, a primase involved in discontinuous plasmid DNA replication; *ardA*, encodes a type-I restriction enzyme, *repI1*, the RNAI antisense regulating system in the IncI1 replication system and the intragenic *trbA- pndC* region involved in maintenance and plasmid transfer respectively (Garcia, Navarro et al. 2007). Amplicons were compared with known allelic variants and sequence types (ST) assigned using the pMLST database (http://pubmlst.org/plasmid) (see table 4.1).

The I1-25 field isolate harbouring both $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-15}}$ β-lactamases was assigned to ST 24, with additional resistance markers conferring resistance to sulphonamides. I1-37, assigned to ST 48, harboured three β-lactamase genes; $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-1}}$ but with no additional resistance markers apart from those conferring resistance to β-lactams; ampicillin (amp), ceftazidime (caz) and cefotaxime (ctx). I1-42 and I1-43 harboured two CTX-M enzymes; $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ in addition to $bla_{\text{TEM-1}}$ were assigned to ST 31 of clonal complex (cc) 31; denoting the number of nucleotide differences between alleles and groups of isolates genetically related clusters differing by one or two loci respectively. I2-47, I2-50 and I2-61 isolates from the same farm were assigned to ST 38 of the cc-3 clonal complex. These strains harboured only the $bla_{\text{CTX-M-15}}$ β-lactamase gene which conferred resistance to ampicillin, ceftazidime and cefotaxime.

Table 4-1: pMLST results of IncI1 harbouring of *E. coli* field isolates.

Region	Isolate	Phylogroup	Resistance markers	β-lactamase	pMLST
Pembrokeshire farm	I1-25	B1	Amp, Caz, S, Ctx	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$	ST 24
Bedfordshire regional labs	I1-37	B1	Amp, Caz, Ctx	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bl_{a\text{OXA-1}}$	ST 48
Bedfordshire regional labs	I1-42	A	Amp, Caz, Ctx	$bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$	ST 31 CC-31
Bedfordshire regional labs	I1-43	A	Amp, Caz, Ctx	$bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$	ST 31 CC-31
Shropshire farm	I2-47	A	Amp, Caz, Ctx	$bla_{\text{CTX-M-14b}}$	ST 38 CC-3
Shropshire farm	I2-50	A	Amp, Caz, Ctx	$bla_{\text{CTX-M-14b}}$	ST 38 CC-3
Shropshire farm	I2-61	A	Amp, Caz, Ctx	$bla_{\text{CTX-M-14b}}$	ST 38 CC-3

Abbreviations: *E.* coli phylogroups were designated using the method described by Clermont *et al.,* 2000 (see chapter 3, 3.2.3). pMLST (plasmid multi-locus sequence tying). Amp; ampicillin, Caz; ceftazidime, Ctx; cefotaxime. Sequence types (ST) assigned using http://pubmlst.org/plasmid database. Genetically related plasmids are given as clonal complex (cc).

4.2.2 Plasmid characterisation

Conjugation experiments were performed on all 52 isolates to determine if the CTX-M producing isolates harboured these and other antimicrobial resistance genes on plasmids and to what extent these were transferable. Plasmid transferability for all the strains was determined by *in vitro* conjugation experiments using a rifampicin (rif) resistant *E. coli* K12 (20R764) strain as a recipient. Transconjugants were selected on the basis of rifampicin and cefotaxime resistance. Of 52 donors, transfer was achieved with 49 and in these the presence of $bla_{\text{CTX-M}}$ genes confirmed by PCR amplification. Based on the initial donor and transconjugants counts, frequency of transfer rates were determined (see chapter 2; 2.2.6 for methodology).

All 49 plasmids were able to transfer $bla_{\text{CTX-M}}$ harbouring plasmids by conjugation. The frequency of transfer rates; measured as the number of transconjugants (cfu/ml) per

initial recipient (cfu/ml) ranged between 1.02 x 10^{-2} (i.e. approximately 1 transconjugant per 100 initial recipients) to 9.23x10^{-9} (approximately 1 transconjugant per 100 million initial recipients), see table 4-4 for the individual transfer rates.

The plasmid content for each of the 49 resulting transconjugants was determined by the alkaline lysis method of Kado and Liu (1981) for estimating plasmid sizes (section 2.3.1). The alkaline lysis method extracts plasmids predominantly in their covalently closed form, however, estimating plasmid sizes by this method is limited due to the different conformations of the plasmid that migrate at different speeds within the electrophoresis gel i.e. nicked open circular, covalently closed DNA, linear, supercoiled and denatured supercoiled DNA. By this method 11 of the 49 transconjugants were identified as having multiple plasmids. These plasmids were later transformed into *E. coli* K12 (20R764), selected with cefotaxime and rifampicin. Transformants were obtained for all 11 of transconjugants that harboured multiple plasmids. In addition the three plasmids that could not conjugate were subjected to transformation into *E. coli* K12 and transformants selected in the presence of cefotaxime and rifampicin. All the resulting transconjugants (*n = 38*) and transformants (*n = 14*), representing $bla_{\text{CTX-M}}$ harbouring plasmids from the isolates were resized using S1 nuclease PFGE (method described in chapter 2; 2.3.8) (Dierikx, van Essen-Zandbergen et al. 2010). S1 nuclease linearizes the plasmid DNA by introducing 'nicks' in the DNA, thus enabling a more accurate method of sizing the plasmid using a linear DNA marker. A standard curve of the linear DNA marker sizes (kb) was plotted against the distance migrated (mm) and plasmid sizes were approximated from the resulting quadratic equation of the polynomial curve. Plasmids ranged in size from 28 kb (IncN) to 175 kb (IncF). The plasmids were grouped based on their Inc type and the average sizes of plasmids belonging to a particular Inc group was calculated (see Fig. 4-1).

Having ensured that each ESBL plasmid was in the same host background without other plasmids, it was now possible to type them by PBRT (PBRT) (Carattoli, Bertini et al. 2005). Plasmid transfer rates were grouped according to their Inc group; IncF (including FIA and FIB) IncN, IncI, IncB/O, 'untypeable' (untypeable by this method) and co-transfer plasmids (see Fig. 4-1).

The non-conjugable plasmids (I1-39TF, I2-55TF and I2-66TF) from strains I1-39, I2-55 and I2-66 were transformed into *E. coli* K12 (20R764). Two of the three non-conjugable plasmids (I2-55TF and I2-66TF) could not be typed using the PBRT method and the third plasmid (I1-39TF) was found to harbour an IncF replicon (see table 4-4 for detailed results).

IncF plasmids were found to have the highest transfer rates averaging at $8.00x10^{-4}$ with an average plasmid size of 80 kb. IncN (45 kb) and IncI1 (86 kb) averaged at frequency of transfer rates of $1.14x10^{-4}$ and $9.71x10^{-5}$, approximately eight times less efficient in transfer than plasmids of the IncF group. For plasmids from the Shropshire farm 14 of the 22 isolates were untypeable by the PBRT method. Seven of the transconjugants from this region were found to have more than one plasmid and thus grouped under the co-transfer plasmids with a mean transfer rate of $2.83x10^{-8}$. The remaining seven with plasmid sizes averaging 61 kb, were found to have mean transfer rates of $4.48x10^{-6}$. Only one transconjugant was identified as harbouring the IncB/O (151 kb) group with the lowest transfer rates ($4.62x10^{-8}$).

Thirteen transconjugants from different regions were found to harbour more than one plasmid suggesting that mobilisation of the ESBL plasmid may have been dependent upon characteristics of the plasmid itself. These varied in size, usually with one smaller plasmid and were not limited to particular Inc types (see table 4-3 for details). To calculate the average transfer rate of co-transferred plasmids, the sizes were arbitrarily separated into large (ranging from 86-175 kb) and small (ranging from 28-45 kb) and mean sizes determined to be 118 and 36 kb respectively. The transfer rate for co-transferred plasmids was 2.83×10^{-8}, approximately 30, 000 times less efficient than the IncF plasmids.

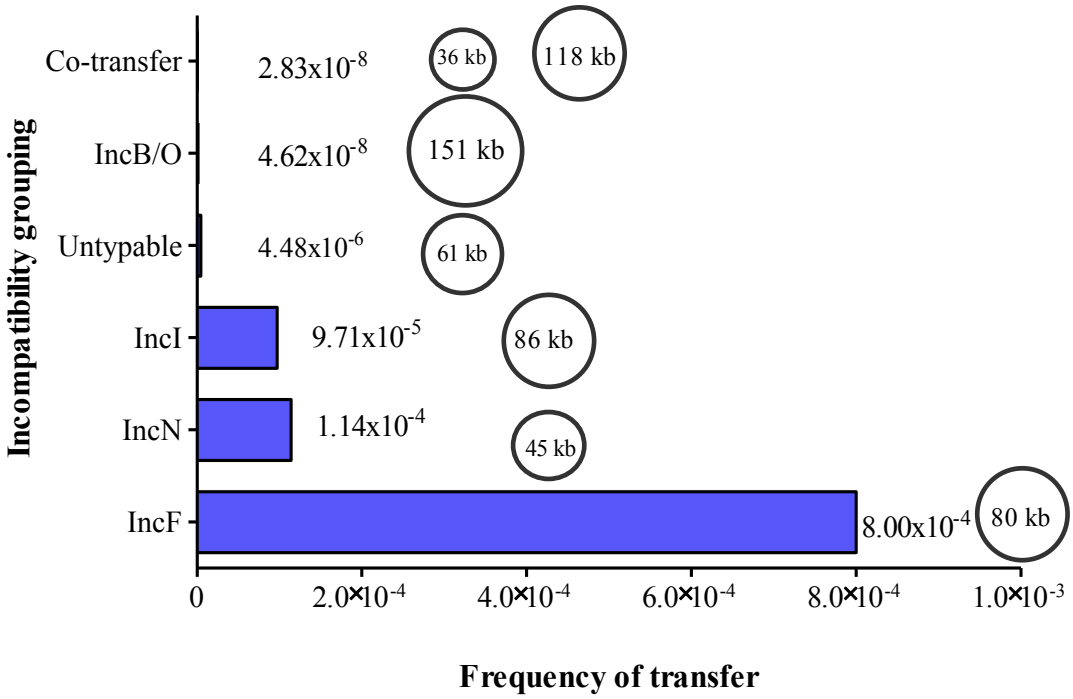


Figure 4-1: Graphical representation of plasmid transferability rates and their respective plasmid incompatibility groups and their average plasmid sizes. Frequency of transfer rates were calculated as the number of transconjugants (cfu/ml) per initial donor (cfu/ml). Plasmids were typed by PBRT. Plasmid sizes were approximated using S1 nuclease PFGE and the mean values calculated for all the plasmids belonging to the particular Inc group. IncF (*n =13*) plasmids were found to be the highest frequency of transfer rates, followed by conjugative plasmids IncN (*n =12*) and IncI1 (*n =3*). Fourteen plasmids from the Shropshire region were untypeable by the PBRT method. Thirteen transconjugants were found to harbour more than one plasmid, these were grouped as co-transferred plasmids (see table 4-2 for details). Three plasmids were unable to conjugate and were not included in this data analysis.

4.2.3 Transferable phenotypic antimicrobial resistance markers

To determine the overall co-transfer of resistance markers with the ESBL producing plasmids, resistance profiles of the field isolates were compared between the transconjugants/transformants harbouring $bla_{CTX\text{-}M}$ encoding plasmids (selected with cefotaxime) and the field isolates. For this the antimicrobial resistance profiles for all 49 transconjugants and the three transformants that were unable to conjugate, were determined using the disc diffusion assay against a panel of 16 antimicrobials (see methodology section 2.4.1).

The percentage of co-transferred resistance markers was calculated as the number of antimicrobial class markers (i.e. β-lactams, aminoglycosides, chloramphenicols, trimethoprims, sulphonamides, quinolones and nitrofurans) present in each transconjugant/transformant (recipient) divided by the number present in their donor; 72 % of the markers were found to co-transfer with the ESBL phenotypic markers (amp, ctx and caz), irrespective of plasmid Inc group (Fig. 4-2).

All the isolates were found to have markers of β-lactam resistance, conferring resistance to ampicillin and cefotaxime (table 4-2). Interestingly only 35 % (*n = 18*) of the transconjugants were found to be no longer resistant to ceftazidime. Resistance to the aminoglycosides (amikacin, apramycin, gentamacin, neomycin and streptomycin) was the most abundant resistance phenotype, present in 75 % (*n = 39*) of the field isolates but of these 62 % of these markers (*n = 24*) were present in the resulting transconjugant/transformant derivatives. Interestingly resistance to this class of drugs was limited to markers conferring resistance to streptomycin only (*n = 10*), neomycin only (*n = 13*) and gentamicin and streptomycin (*n* = 1).

Thirty-two field strains conferred co-resistance to sulfamethoxazole/trimethoprim (Sxt^R) and compound sulphonamide ($S3^S$), a marker for resistance to both sulphonamides and trimethoprims; 66 % (*n = 21*) of these resistance makers were transferred to the resulting transconjugant/transformants.

Tetracycline resistance was the third most common resistance maker in the field isolates, with 73 % (*n = 38*) of the strains conferring resistance to tetracycline (Tet^R); 53 % of plasmids transferred this resistance phenotype (*n = 20*), associated with the IncN, IncF and untypeable plasmid groups.

Chloramphenicol resistance (C^R) markers were present in 54 % (*n = 28*) of the isolates. Only half (*n = 14*) of these markers of resistance were present in the transconjugant/transformant derivatives.

Twenty-three donor field strains (44 %) were found to harbour markers that confer resistance to quinolones. Interestingly, four strains were able to transfer resistance to quinolones; nalidixic acid and ciprofloxacin (see table 4-4). This was unusual because resistance to quinolones were previously found to be a result of chromosomal mutations (chapter 3), however these were transferred into an *E. coli* K12 background and no known plasmid mediated quinolone resistance genes were identified by the DNA microarray analysis (see section 4.2.4).

Only one field isolate was resistant to the nitrofuran; furazolidone (Fr). This resistance marker was transferred to the transconjugant (I1-34TC); which incidentally had multiple plasmids. When the ESBL was transformed into *E. coli* K12 (I1-34TF), this

resistance phenotype marker was not transferred. Collectively these data suggest the two markers, ESBL and Fur were not on the same replicon.

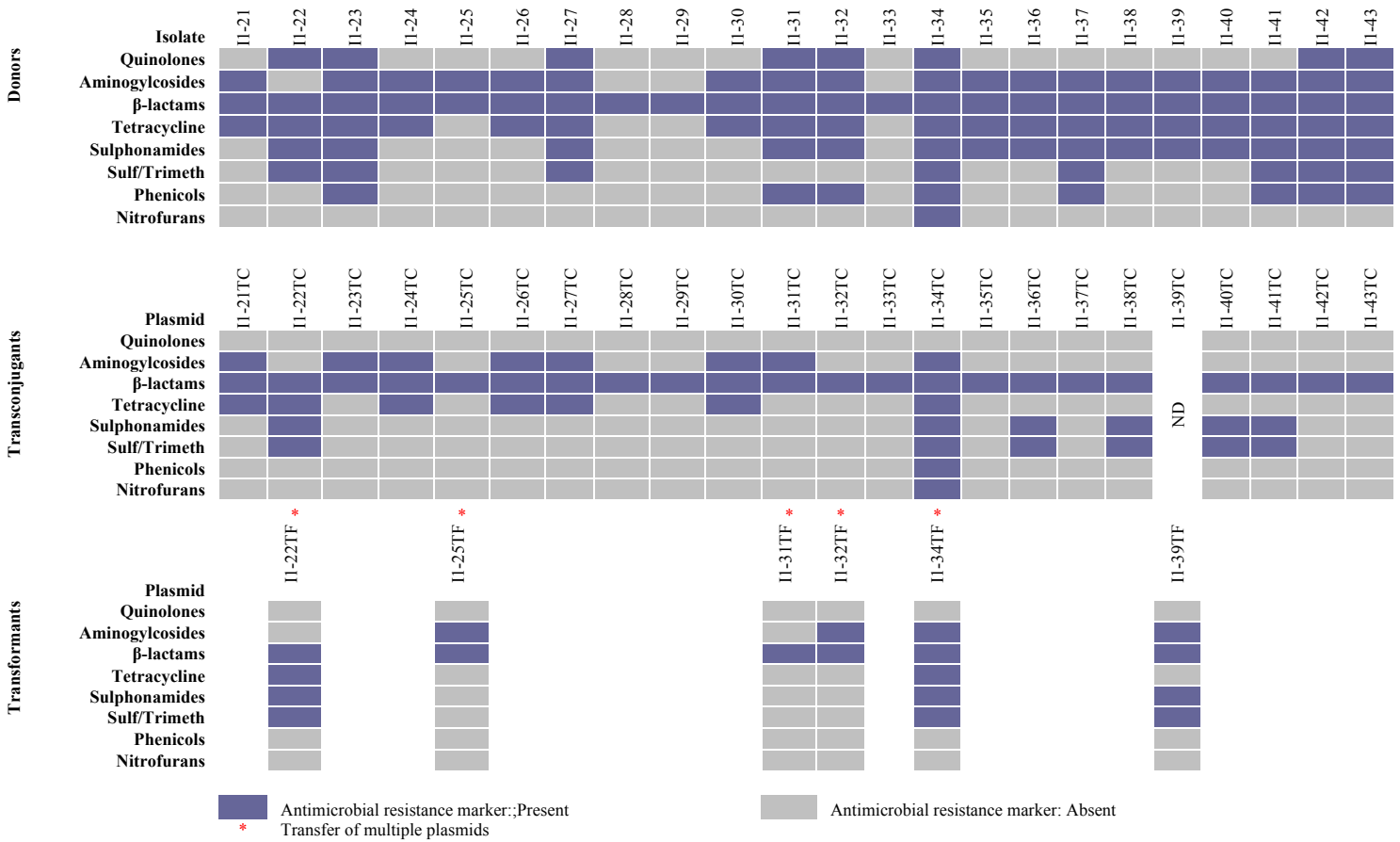


Figure 4-2: Schematic representation of transferable antimicrobial resistance markers determined by disc diffusion. Represented are phenotypic profiles for the donor isolates, transconjugants (TC) and transformants (TF) of plasmids that transferred multiple plasmids during conjugation. Abbreviations: Sulf/Trimeth: Sulfamethoxazole/trimethoprim. ND: not done. 72 % of the resistance markers were found to be transferable.

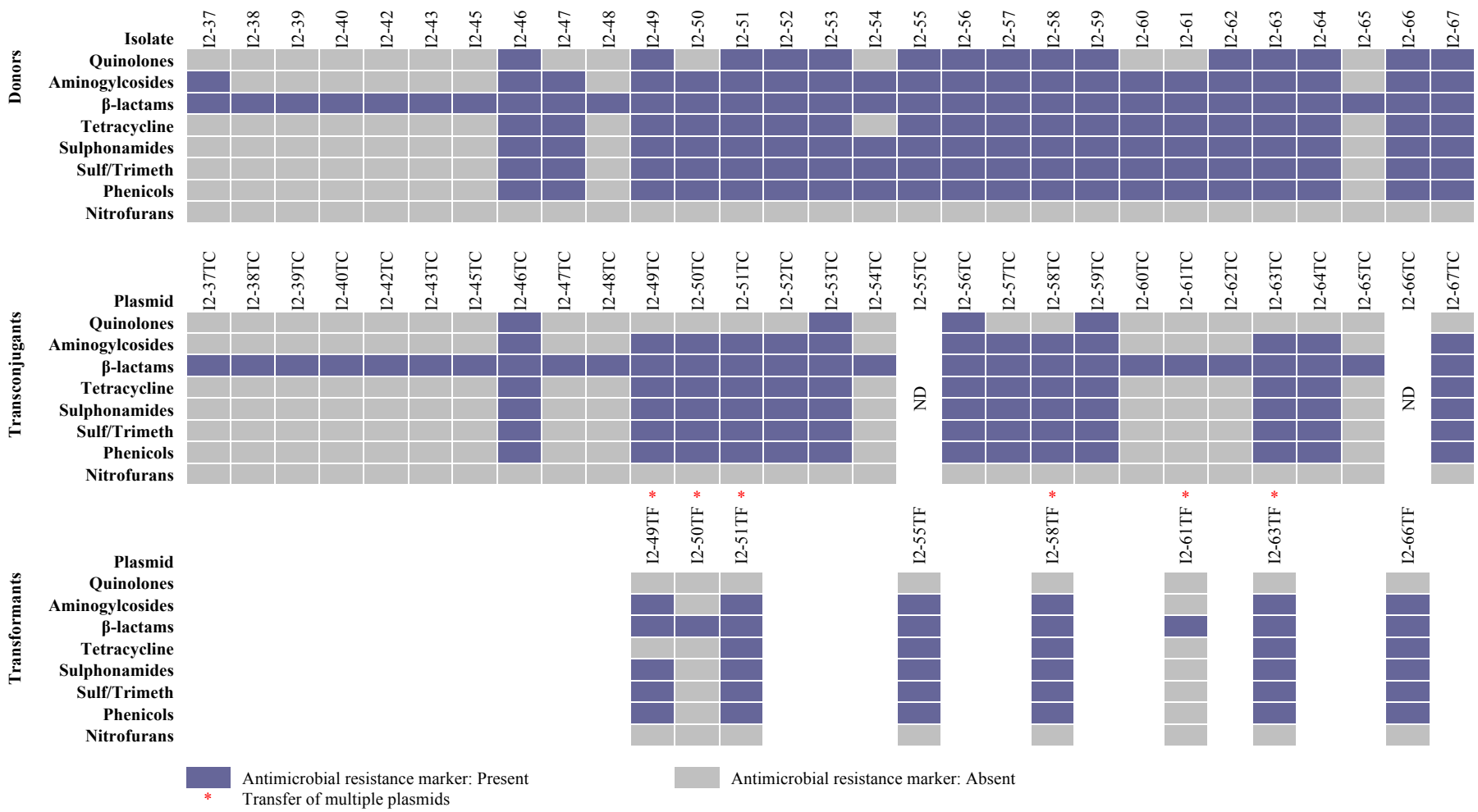


Figure 4-2: ***continued.***

4.2.4 Transferable genes of resistance

In order to define the genetic basis of the resistances that were in the donor field isolates and those associated with the ESBL, DNA microarrays were carried out (see Fig. 4-4). All the plasmid-harbouring derivatives were found to harbour *bla*$_{CTX-M}$ group 1 or 9 genes. Seventeen (32 %) of the CTX-M producing plasmids had no additional resistance markers aside from *bla*$_{CTX-M}$, in agreement with the phenotypic data. Additional genes that confer resistance to β-lactams; *bla*$_{TEM-1}$ and *bla*$_{OXA-1}$ were present in 34 % (*n = 18*) and 6 % (*n = 3*) of the transferred plasmids respectively.

Fifty-eight percent of *bla*$_{CTX-M}$ plasmids transferred had additional multidrug resistance gene profiles and this was to be anticipated given the data arising from the phenotypic assays described above. Other antimicrobial resistance genes include markers that associated with aminoglycoside resistance; *aadA2* (27 %), *strB* (13 %), *aadA4* (12 %), *strA* (8 %) and *aac(6)'lb* (8 %). Frequently detected sulphonamide and trimethoprim resistance genes included: *sul3* (27 %), *sul1* (13 %), *sul2* (2%) and *dfrA12* (23 %), *dfrA17* (13 %) and *dfrA14* (2 %) respectively. Genes associated with resistance to tetracyclines included; *tetA* (35 %) and *tetB* (4 %). Markers for chloramphenicol/florfenicol resistance included *cmlA1* (27 %) and *catA1* (2 %).

Only sixteen (45 %) of the plasmids were found to harbour the integrase associated gene (*int1*), 7 % less than the donor field isolates. A majority (*n = 14*) were harboured within the plasmids from a single farm (Shropshire region). Interestingly, no *int2* integrase associated genes were observed in *bla*$_{CTX-M}$ encoding plasmids.

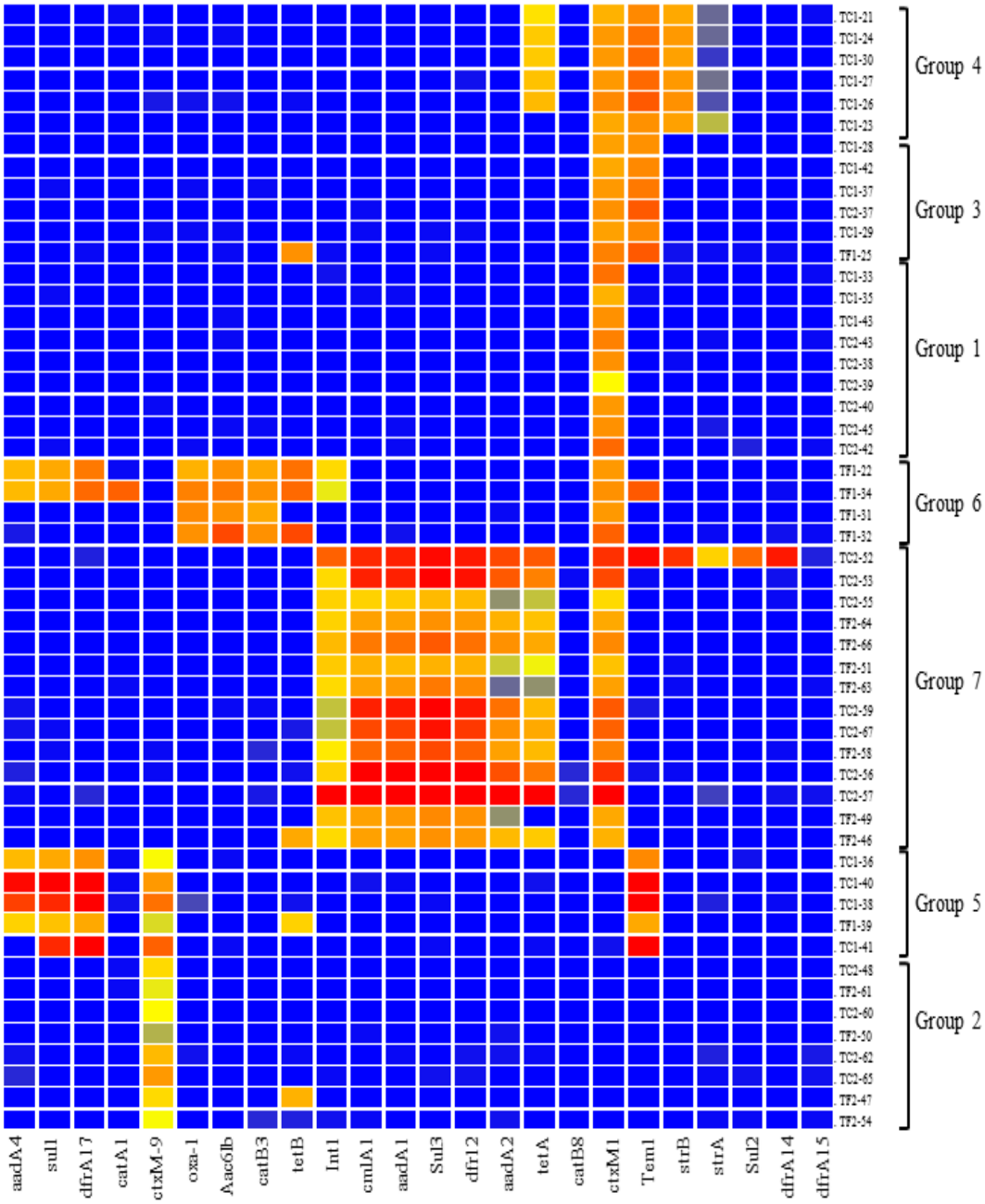


Figure 4-3: Antimicrobial resistant genes identified in the transferred plasmids using miniaturized DNA microarray technology. Data was analysed with GeneSpring GX v7.3.1(Agilent technologies) and clustered using Pearson correlation coefficient. Red/yellow denotes genes present, blue denotes absent genes. The plasmids were grouped into 7 groups based on Pearson coefficient clustering. All the plasmid harboured either $bla_{\text{CTX-M}}$ group 1 or 9 genes.

***Abbreviations*:** *aac6lb*, *aadA1*, *aadA2*, *aadA4*, *strA* and *strB* confer resistance to aminoglycosides. *catA1*, *catB3* and *cmlA* confer resistance to chloramphenicols. *floR* confers resistance to chloramphenicol/florfenicol. *ctxM1*, *ctxM9*, *tem1* and *oxa1* confer resistance to β-lactams. *dfrA7*, *dfrA14*, *dfrA17* and *dfrA19* confer resistance to trimethoprims. *sul1*, *sul2* and *sul3* confer resistance to sulphonamides. *tetA* and *tetB* confer resistance to tetracylcines. *Int1* and *Int2* are integrons (mobile genetic elements) commonly associated with antimicrobial resistance genes.

4.2.5 Gene clustering

Seven major gene clusters were identified in *bla*$_{\text{CTX-M}}$ encoding plasmids, clustered using the Pearson correlation coefficient (see Fig. 4-4).

Group 1: This group included nine plasmids (IncI1, F and N) harbouring the *bla*$_{\text{CTX-M-1}}$ gene only and included plasmids from donor isolates from three different regions, Bedfordshire, Hertfordshire and Dorset. Interestingly, only the Dorset field isolates clustered together when analysing the donor strains (see chapter 3).

Group 2: Eight plasmids (IncI1, F and untypeable) clustered together harbouring only *bla*$_{\text{CTX-M-9}}$ gene only and were from donor field isolates from the Shropshire farm. These plasmids were previously clustered together in the donor field strains although additional resistance genes were present but clearly not associated with the *bla*$_{\text{CTX-M-9}}$ gene.

Group 3: Six plasmids (IncF and N) were clustered together in this group harbouring *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{TEM-1}}$. The plasmids in this cluster were primarily from the Pembrokeshire field isolates and one plasmid was from the Dorset farm. Interestingly the Pembrokeshire field isolates from this group were clustered with other donor field isolates from this farm, but after transformation/conjugation, now clustered in group 4, encoding *strB* and *tetA* genes in addition to the ESBL genes transferred by plasmids.

Group 4: This group included six plasmids (IncF and N) harbouring the ESBL gene, *bla*$_{\text{CTX-M-1}}$ in addition to *bla*$_{\text{TEM-1}}$, *strA*, *strB* and *tetA*. These plasmids were from strains isolated from Pembrokeshire (*n = 6*) and Hertfordshire (*n = 1*).

Group 5: Five plasmids (all IncF), all from donor field strains isolated in the Bedfordshire region, harboured *bla*$_{\text{CTX-M-9}}$, *bla*$_{\text{TEM-1}}$, *aadA4*, *dfrA17* and *sul1* multi-drug resistance genes. These plasmids also clustered together when the genes for the donor field isolates were analysed.

Group 6: Four plasmids (IncFIA-FIB and N) were clustered in this group; three plasmids from the Pembrokeshire (*n = 1*) and Hertfordshire (*n = 3*) field isolates. Genes included, $bla_{\text{CTX-M-1}}$ and $bla_{\text{OXA-1}}$ in addition to the aminoglycoside- quinolone cross-resistance gene *aac(6)'lb*. I1-34TF and I1-22TF harboured additional resistance markers *aadA4*, *dfrA17*, *sul1*, *tetB* and *intI*. The I1-34TF plasmid encoded three additional resistance genes, *catA1* and $bla_{\text{TEM-1}}$. These plasmids also clustered together in the DNA array analysis for the field isolates, but upon transfer lost all lost *strA*, *strB* and *sul2* genes between them.

Group 7: The largest cluster of plasmids (*n = 14*) was derived from the Shropshire farm isolates. These plasmids, one IncB/O and the remaining unytypeable, encoded several multi-resistance genes including; *aadA1*, *aadA2*, *cmlA1*, $bla_{\text{CTX-M-1}}$, *dfr12*, *sul3*, *tetA* and *int1*. The I2-52TC plasmid harboured four additional resistance genes including, *dfrA14*, *strB*, *sul2*, and $bla_{\text{TEM-1}}$. All plasmids found to cluster together from this farm in the transconjugants/transformants, were also grouped together in the analysis of genes of the donor field isolates, found with the same resistance genes, suggesting the same plasmid may be responsible for the transfer of resistance genes in this farm.

4.2.6 Genotypic and phenotypic correlation

Genotypic and phenotypic correlation with each antimicrobial group from the DNA array and susceptibility profiles of the transconjugants/transformants was assessed using kappa statistics. Sulphonamides, trimethoprims and β-lactams scored kappa coefficients of 1, denoting 'perfect' correlation. Aminoglycosides, chloramphenicols/florfenicols and tetracyclines showed very good correlation (≥ 0.8). Quinolones scored 'poor' correlation because of resistance due to mutations in topoisomerase genes rather than

qnr genes, which were present on the array; however four plasmids (all transconjugants) conferred resistance to quinolones.

Genes that were identified using the array but were absent for the phenotypic marker were PCR amplified to confirm the presence of these genes on the plasmids. This is because some alleles were unable to be resolved by the array, but presence/absence could be confirmed by PCR. The confirmed genotypic profiles were used to construct table 4-4.

Table 4-2: Genotypic and phenotypic correlation between transferred plasmids

Antimicrobial class	Phenotype	Genotype	Kappa
β-lactams	52	52	1
Choramphenicol/florfenicols	15	18	0.867
Sulphonamides	21	21	1
Trimethoprims	21	21	1
Aminoglycosides	24	28	0.885
Tetracylines	20	22	0.92
Quinolones*	4	0	0

The kappa coefficient was used to assess the correlation between phenotypic and genotypic data prior to validation of the DNA array genes by PCR. Scores of 1; denote complete agreement and 0 indicates very poor correlation. Scores < 0.2 were interpreted as poor, 0.21 to 0.4; fair, 0.41 to 0.6; moderate, 0.61 to 0.8; good and 0.81 to 1; very good correlation.

4.2.7 Co-transfer plasmids

Eleven plasmids were co-transferred during the original conjugations with selection for the transfer of the ESBL phenotype. Multiple plasmids present in these transconjugants were identified and sizes estimated by the alkaline lysis method described by Kado and Liu (1981). All 11 plasmids were transformed and the antimicrobial gene profiles of the resulting $bla_{\text{CTX-M}}$ plasmids assessed (see table 4-3).

Eight plasmids that harboured quinolone resistance markers (Cip^R and Na^R) became susceptible to these antimicrobials once transformed into their *E. coli* hosts (K12 or

DH10). This suggests, the resistance determinant may have been encoded on the other plasmids co-transferred with the transformed *bla*$_{CTX-M}$ plasmid. These could include allelic variants of the *qnr* genes not included on the AMR05 DNA array chip e.g. *qnrC* and *qnrD*.

Three donor strains (I2-47, I2-46 and I2-64) transferred two plasmids, a single 'large' (> 60kb) and a smaller plasmid (< 60kb). I2-47TC harboured an 86 kb IncI1 plasmid and a 35 kb plasmid untypeable by the PBRT method. DNA array analysis of the antimicrobial genes of the transconjugants (harbouring both plasmids) and the transformant (harbouring the 35 kb plasmid) revealed both plasmids to encode only *bla*$_{CTX-M}$ group 9 genes and no other resistance genes. Two transconjugants; I2-46TC and I2-64TC, each harboured two plasmids; 111 kb, 45 kb and 90 kb, 40kb respectively, all untypeable by the PBRT scheme. These plasmids harboured the same resistance genes, however their phenotypic profiles showed I2-46TC to confer resistance to nalidixic acid (Na) and ciprofloxacin (Cip). *bla*$_{CTX-M}$ genes were found to be encoded on the smaller 45 kb (I2-46TC) and 40kb (I2-64TC) plasmids respectively.

I1-40TC was found to harbour two large plasmids; 156 kb and 122 kb, both belonging to the IncF group. The plasmids were subjected to S1 nuclease digest and run on a PFGE and bands corresponding to the two plasmids extracted. PCR amplification of *bla*$_{CTX-M}$ group 9 genes suggested that both plasmids harboured this gene. The transformant and transconjugant were found to have the same susceptibility profile and belonged to the same Inc group (IncF), suggesting these may be the same plasmid migrating at different speeds, as an artefact of agarose gel separation. This would explain the existence of these similar plasmids, of the same Inc group, in the same cell, which by definition would be unable to co-exist in the same cell.

Two transconjugants, I2-62TC and I2-52TC were found to harbour a 58 kb IncF and a 151 kb IncB/O plasmid respectively. The larger IncB/O plasmid (I2-52TC) harboured a multi-resistance gene profile determined by DNA microarray analysis, harbouring an integrase associated gene *intI* and genes conferring resistance to aminoglycosides (mediated by *aadA1*, *aadA2* and *strB*), chloramphenicols (*cmlA1*), trimethoprims (*dfrA12* and *dfrA14*), tetracycline (*tetA*), sulphonamides (*sul2* and *sul3*) and two β-lactam genes ($bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-1}}$) conferring resistance to β-lactams determined by antimicrobial susceptibility assays (disc diffusion). Plasmid extraction was carried out on the original parent field isolate (donor; I2-52) and transformed into *E. coli* DH10. Plasmid characterisation of the resulting transformant (I2-52TF) derivative revealed a 40 kb plasmid untypeable by the PBRT scheme. This plasmid was found to have fewer resistance genes, however still conferring resistance to the same group of antimicrobials as the transconjugant derivative. This smaller plasmid still harboured the integrase associated gene *intI* and interestingly had the same resistance gene profile as the plasmids from this region also harbouring untypeable 40-50 kb plasmids (see table 4-3).

The IncF harbouring I2-62TC plasmid was also extracted (from the original donor; I2-62) and transformed into *E. coli* DH10. The resulting transformed plasmid (I2-62TF) was found to be a 40 kb plasmid, 12 kb smaller than the transconjugant I2-62TC. This 40 kb plasmid was untypeable by the PBRT scheme, similar to other plasmids from this farm (see table 4-4). The IncF plasmid (I2-62TC; 58 kb) harboured only the $bla_{\text{CTX-M-9}}$ variant of the CTX-M β-lactamase. The smaller 40 kb plasmid (I2-62TF) was found to harbour several resistance genes conferring resistance to aminoglycosides (*aadA1*, *aadA2*), chloramphenicols (*cmlA1*), trimethoprims (*dfrA12*), tetracycline (*tetA*), sulphonamides (*sul3*) and β-lactams ($bla_{\text{CTX-M-1}}$), a profile identical to the other plasmids also from this region encoding a CTX-M group 1 variant (see table 4-4).

Table 4-3: Summary of co-transfer plasmids identified.

Plasmid	Recipient *E. coli* strain	Estimated plasmid size (kb)	Associated antimicrobial resistance markers	Antimicrobial resistance genes	*Int*	PBRT
I1-22TC[b]	*E. coli* K12	150, 6, 4.5[c]	Amp, Caz, Ctx, Cip, Na, S3, Sxt, Te	ND	ND	F, FIA, FIB
I1-22TF	*E. coli* DH10	171	Amp, Caz, Ctx, Sxt, S, S3, Te	*aac6lb, aadA4, bla*CTX-M-1, *dfrA17, bla*OXA-1, *sul1, tetB*	*int1*	F, FIA, FIB
I1-31TC[b]	*E. coli* K12	150, 6[c]	Amp, Ctx, S3, Sxt	ND	ND	F, FIB, N
I1-31TF	*E. coli* DH10	28	Amp, Caz, Ctx	*aac6lb, bla*CTX-M-1, *bla*OXA-1		N
I1-32TC[b]	*E. coli* K12	150, 100, 4.5[c]	Amp, Caz, Ctx, Na	ND	ND	F, FIB
I1-32TF	*E. coli* DH10	151	Amp, Caz, Ctx, S	*aac6lb, bla*CTX-M-1, *bla*OXA-1		F, FIA, FIB
I1-34TC[b]	*E. coli* K12	50, 6[c]	Amp, Caz, Ctx, Cip, C, Cn, Fr, Na, S, S3, Stx, Te	ND	ND	F, FIA, FIB, N
I1-34TF	*E. coli* DH10	175	Amp, Caz, Ctx, C, Cn, S, S3, Stx, Te	*aac6lb, aadA4, catA1, bla*CTX-M-1, *dfrA17, bla*OXA-1, *sul1, bla*TEM-1, *tetB*	*int1*	F, FIA, FIB
I1-40TC[a]	*E. coli* K12	**156, 122**	Amp, Ctx, S3, Sxt	*aadA4, bla*CTX-M-9, *dfrA17, sul1, bla*TEM-1		F
I1-40TF	*E. coli* DH10	ND	ND	*aadA4, bla*CTX-M-9, *dfrA17, sul1, bla*TEM-1		F
I2-46TC[a]	*E. coli* K12	111, **45**	Amp, Caz, Ctx, Cip, C, Na, N, S3, Stx, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	-
I2-47TC[a]	*E. coli* K12	86, **35**	Amp, Ctx	*bla*CTX-M-9		I1
I2-47TF	*E. coli* K12	35	ND	*bla*CTX-M-9		-
I2-49TC[b]	*E. coli* K12	100, 60[c]	Amp, Ctx, Cip, C, Na, N, S3, Sxt, Te	ND	ND	-
I2-49TF	*E. coli* K12	40	Amp, Caz, Ctx, C, N, S3, Sxt	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3*	*int1*	-
I2-50TC[b]	*E. coli* K12	100, 80, 60, 7, 5.5, 4.5[c]	Amp, Ctx, C, N, Sxt, Te	ND	ND	F, FIB, I1
I2-50TF	*E. coli* K12	40	Amp, Caz, Ctx	*bla*CTX-M-9		-
I2-51TC[b]	*E. coli* K12	100, 60[c]	Amp, Caz, Ctx, Cip, C, Na, N, S3, Sxt, Te	ND	ND	-
I2-51TF	*E. coli* K12	40	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	-
I2-58TC[b]	*E. coli* K12	100, 60[c]	Amp, Caz, Ctx, Cip, C, Na, N, S3, Sxt, Te	ND	ND	-
I2-58TF	*E. coli* K12	40	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	-
I2-61TC[b]	*E. coli* K12	100[c]	Amp, Ctx	*bla*CTX-M-9		I1
I2-61TF	*E. coli* K12	40	Amp, Ctx	*bla*CTX-M-9		-
I2-62TC*	*E. coli* K12	58	Amp, Ctx	*bla*CTX-M-9	ND	F
I2-62TF*	*E. coli* DH10	40	ND	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	-
I2-63TC[b]	*E. coli* K12	100, 60[c]	Amp, Caz, Ctx, Cip, C, Na, N, S3, Sxt,Te	ND	ND	-
I2-63TF	*E. coli* K12	40	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	-
I2-64TC[a]	*E. coli* K12	92, **40**	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	-
I2-52-TC*	*E. coli* K12	151	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, dfrA14, strB, sul2, sul3, bla*TEM-1, *tetA*	*int1*	B/O
I2-52TF*	*E. coli* DH10	40	ND	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12,, sul2, tetA*	*int1*	-

*; These plasmids were found to transfer two different plasmids by the two methods employed conjugation and transformation. Both plasmids were typed by PBRT, sized by S1 PFGE and resistance gene profiles determined by miniaturized DNA micro-array analysis. [a]; These plasmids transferred as multiple plasmids to the recipient *E. coli* K12 strains. The plasmids were extracted from these transconjugants and transformed into *E. coli* K12 and used in subsequent plasmid characterization studies. [b]; Transconjugants found to harbour multiple plasmids by plasmid profile were transformed into *E. coli* K12 and resulting transformants were used in subsequent disc diffusion assays, DNA array analysis and plasmid sizing by PFGE. [c]; These plasmids were sized by the alkaline lysis method. Abbreviations:Amp; Ampicillin, Ctx; Cefotaxime, Caz; Ceftazidime, Cip; Ciprofloxacin, C; Chloramphenicol, N; neomycin, Na; Nalidixic acid, Te; Tetracycline, S; Streptomycin, Sxt; sulfamethoxazole, S3; compound sulphonamide, *aac6lb*, *aadA1*, *aadA2*, *aadA4*, *strA* and *strB* confer resistance to aminoglycosides. *catA1* and *cmlA* confer resistance to chloramphenicols. *ctxM1*, *ctxM9*, *tem1* and *oxa1* confer resistance to β-lactams. *dfr12*, *dfrA14* and *dfrA17* confer resistance to trimethoprims. s*ul1*, *sul2* and *sul3* confer resistance to sulphonamides. *tetA* and *tetB* confer resistance to tetracylcines. *Int1* and *Int2* are integrons (mobile genetic elements) commonly associated with antimicrobial resistance genes. *ND: not done*.

4.2.8 Changes to β-lactam susceptibility upon plasmid transfer

In order to investigate if the susceptibility to β-lactams varied between the donors and transconjugant/transformant derivatives, MICs were determined against ampicillin, cefotaxime and ceftazidime. The susceptibility profiles were compared for the plasmids producing $bla_{\text{CTX-M-1}}$ variants; CTX-M-1, CTX-M-15, CTX-M-32 and $bla_{\text{CTX-M-9}}$ variants; CTX-M-14 and CTX-M-14b. Median MIC values were taken from each CTX-M variant producing transconjugants/transformants and comparisons between the CTX-M variants and the parent strains were made. The results can be found in the bar chart below (Fig. 4-5).

The results indicate that all the CTX-M variants were equally resistant to ampicillin (MIC ≥ 1024 mg/L). High MIC values were also observed in the donor isolates (see table 4-4 for details).

CTX-M group 1 variants (CTX-M-32 and CTX-M-15) had the highest median MIC values against ceftazidime (MIC ≥ 32 mg/L and ≥ 64 mg/L respectively); eight and four-fold lower than the donor field strains (MIC ≥256 mg/L) respectively (Fig. 4-5). CTX-M-14 producing plasmids followed with median MIC values of 32 mg/L, the same as in the donor strains. CTX-M-1 and CTX-M-14b producing plasmids had the lowest median MIC values with ceftazidime; ≥12 mg/L and ≥8 mg/L respectively, these were 5 (64 mg/L) and 4 (32 mg/L) times lower than the donor strains.

Susceptibility to cefotaxime dropped only 2-fold in the transconjugants/transformants encoding the CTX-M-32 and -15 (MIC ≥ 128 mg/L) variants compared to the donor strains (MIC ≥ 256 mg/L). The remaining MIC values for variants; CTX-M-1 (MIC ≥ 32 mg/L), CTX-M-14 (MIC ≥ 64 mg/L) and CTX-M-14b (MIC ≥ 64 mg/L) decreased four-fold from their field isolate donors.

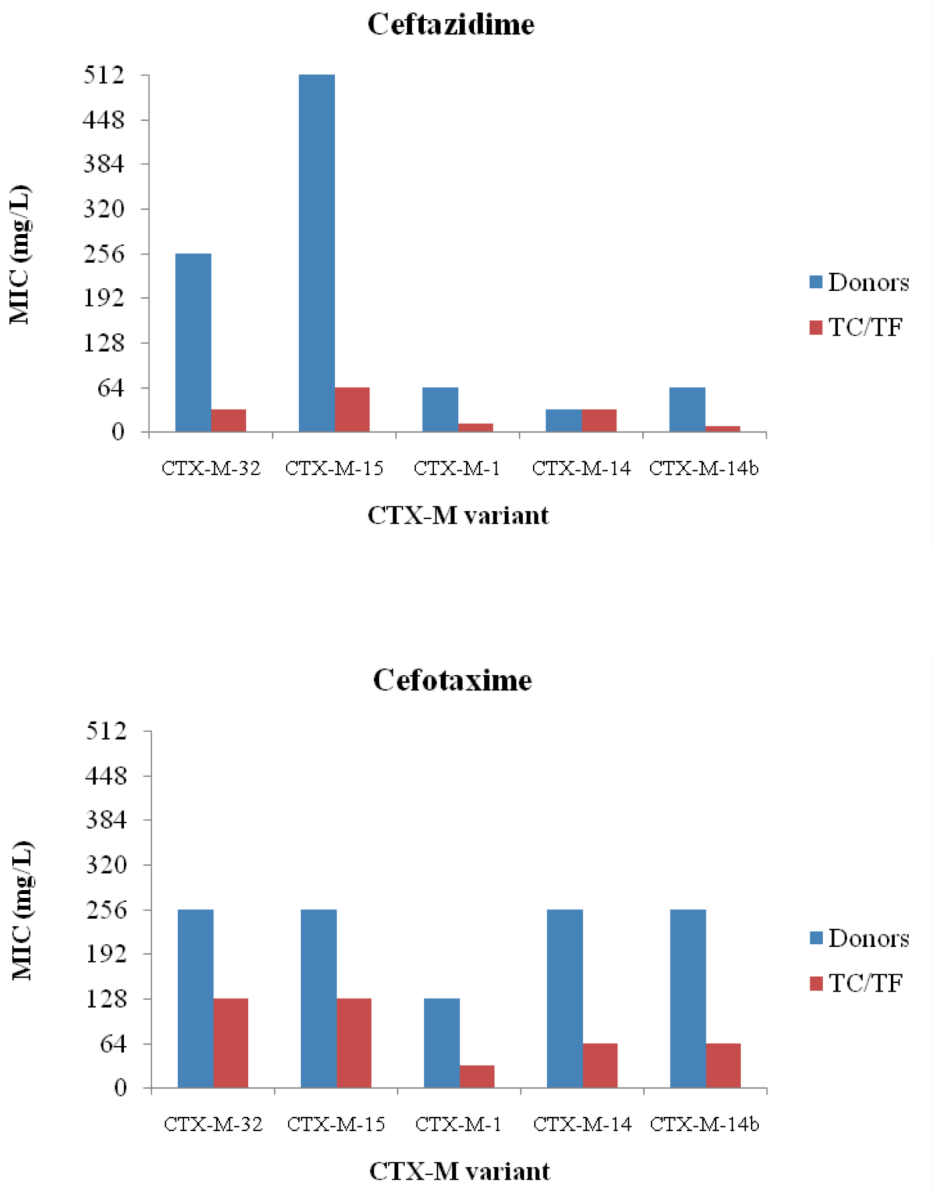


Figure 4-4: Analysis of β-lactam susceptibility testing on CTX-M producing transformants (TF) and transconjugants (TF) in comparison to donor strains. MIC values were interpreted using the BSAC plate dilution method. Results show all the CTX-M variants (donor strains and plasmid derivatives) were resistant to ampicillin (MIC ≥ 1024 mg/L) (data not shown). MIC values dropped 2-8 fold in the presence of ceftazidime (top) and cefotaxime (bottom) between the donor field isolates and transconjugant (TC) and transformants (TF).

Table 4-4: Summary of *bla*$_{CTX-M}$ harbouring plasmids isolated from bovine *E. coli* in the UK in 2007. *(See legend on pg. 149)*

Region isolated	Plasmid	Frequency of transfer	PBRT	Plasmid size (kb)	Associated antibiotic resistance markers	Antimicrobial resistance genes	Integron	MIC (mg/L) Caz	Ctx	Amp
Hertfordshire	I1-33TC	2.35×10^{-5}	F	60	Amp, Caz, Ctx	*bla*CTX-M-1		64	128	> 1024
Hertfordshire	I1-35TC	1.07×10^{-4}	F	60	Amp, Caz, Ctx	*bla*CTX-M-1		64	128	> 1024
Dorset	I2-38TC	1.43×10^{-5}	N	45	Amp, Ctx	*bla*CTX-M-1		32	32	> 1024
Dorset	I2-39TC	5.25×10^{-6}	N	45	Amp, Ctx	*bla*CTX-M-1		8	32	> 1024
Dorset	I2-40TC	1.15×10^{-5}	N	45	Amp, Ctx	*bla*CTX-M-1		8	64	> 1024
Dorset	I2-42TC	3.86×10^{-5}	N	45	Amp, Ctx	*bla*CTX-M-1		16	32	1024
Dorset	I2-43TC	2.10×10^{-5}	N	45	Amp, Ctx	*bla*CTX-M-1		8	32	> 1024
Dorset	I2-45TC	6.00×10^{-6}	N	45	Amp, Ctx	*bla*CTX-M-1		8	32	> 1024
Bedfordshire	I1-43TC	2.18×10^{-6}	I1	86	Amp, Caz, Ctx	*bla*CTX-M-1		32	64	> 1024
Shropshire	I2-48TC	1.10×10^{-7}	F	70	Amp, Ctx	*bla*CTX-M-9		32	16	1024
Shropshire	I2-65TC	1.93×10^{-5}	-	35	Amp, Ctx	*bla*CTX-M-9		8	64	> 1024
Shropshire	I2-60TC	1.19×10^{-5}	-	35	Amp, Ctx	*bla*CTX-M-9		8	128	> 1024
Shropshire	I2-61TF	8.57×10^{-9}	-	35	Amp, Ctx	*bla*CTX-M-9		8	64	> 1024
Shropshire	I2-50TF	1.00×10^{-8}	-	40	Amp, Caz, Ctx	*bla*CTX-M-9		8	128	> 1024
Shropshire	I2-47TC	6.60×10^{-8}	I1	86, **35**	Amp, Ctx	*bla*CTX-M-9		8	64	> 1024
	I2-47TF	-	-	35	ND	*bla*CTX-M-9		ND	ND	ND
Shropshire	I2-54TC	1.50×10^{-6}	F	75	Amp, Ctx	*bla*CTX-M-9		16	64	1024
Shropshire	I2-62TC	6.67×10^{-7}	F	58	Amp, Ctx	*bla*CTX-M-9		8	32	1024
	I2-62TF	-	-	40	ND	*aadA1*, *aadA2*, *cmlA1*, *bla*CTX-M-1, *dfr12*, *sul3*, *tetA*	*int1*	ND	ND	ND
Dorset	I2-37TC	1.25×10^{-3}	N	45	Amp, Ctx	*bla*CTX-M-1, *bla*TEM-1		32	64	> 1024
Pembrokeshire	I1-28TC	9.88×10^{-6}	F	60	Amp, Caz, Ctx	*bla*CTX-M-1, *bla*TEM-1		64	128	> 1024
Pembrokeshire	I1-29TC	5.37×10^{-6}	F	60	Amp, Caz, Ctx	*bla*CTX-M-1, *bla*TEM-1		64	128	> 1024
Bedfordshire	I1-37TC	2.85×10^{-4}	I1	86	Amp, Caz, Ctx	*bla*CTX-M-1, *bla*TEM-1		32	64	> 1024
Bedfordshire	I1-42TC	4.00×10^{-6}	F, I1	86	Amp, Caz, Ctx	*bla*CTX-M-1, *bla*TEM-1		32	64	> 1024
Pembrokeshire	I1-25TF	2.10×10^{-5}	F	70	Amp, Caz, Ctx, S	*bla*CTX-M-1, *bla*TEM-1		32	128	> 1024
Pembrokeshire	I1-23TC	1.02×10^{-2}	F	54	Amp, Caz, Ctx, S	*bla*CTX-M-1, *strA*, *strB*, *bla*TEM-1		64	128	> 1024
Pembrokeshire	I1-26TC	3.88×10^{-6}	N	45	Amp, Caz, Ctx, S, Te	*bla*CTX-M-1, *strB*, *bla*TEM-1, *tetA*		32	128	> 1024
Hertfordshire	I1-30TC	4.25×10^{-6}	N	45	Amp, Caz, Ctx, S, Te	*bla*CTX-M-1, *strB*, *bla*TEM-1, *tetA*		64	128	> 1024
Pembrokeshire	I1-24TC	1.33×10^{-5}	N	45	Amp, Caz, Ctx, S, Te	*bla*CTX-M-1, *strA*, *strB*, *bla*TEM-1, *tetA*		256	128	> 1024
Pembrokeshire	I1-21TC	1.71×10^{-6}	N	45	Amp, Caz, Ctx, S, Te	*bla*CTX-M-1, *strA*, *strB*, *bla*TEM-1, *tetA*		64	128	> 1024
Pembrokeshire	I1-27TC	4.29×10^{-8}	N	45	Amp, Caz, Ctx, S, Te	*bla*CTX-M-1, *strA*, *strB*, *bla*TEM-1, *tetA*		128	128	> 1024

Table 4-4 *continued*.

Region isolated	Strain	Frequency of transfer	PBRT	Plasmid size (kb)	Associated antibiotic resistance markers	Antimicrobial resistance genes	Integron	MIC (mg/L) Caz	Ctx	Amp
Bedfordshire	I1-41TC	3.20×10^{-5}	F	86	Amp, Ctx, Sxt, S3	*bla*CTX-M-9, *dfrA17, sul1, bla*TEM-1		32	64	> 1024
Bedfordshire	I1-36TC	1.60×10^{-7}	F	122	Amp, Ctx, Sxt, S3	*aadA4, bla*CTX-M-9, *dfrA17, sul1, bla*TEM-1		16	64	> 1024
Bedfordshire	I1-38TC	1.09×10^{-7}	F	122	Amp, Ctx, Sxt, S3	*aadA4, bla*CTX-M-9, *dfrA17, sul1, bla*TEM-1		64	128	> 1024
Bedfordshire	I1-40TC	1.58×10^{-6}	F	**156, 122**	Amp, Ctx, Sxt, S3	*aadA4, bla*CTX-M-9, *dfrA17, sul1, bla*TEM-1		16	128	> 1024
	I1-40TF	-	F		ND	*aadA4, bla*CTX-M-9, *dfrA17, sul1,* blaTEM-1		ND	ND	ND
Bedfordshire	I1-39TF	≥0	F	86	Amp, Caz, Ctx, S, Sxt, S3	*aadA4, bla*CTX-M-9, *dfrA17, sul1, bla*TEM-1		16	64	> 1024
Hertfordshire	I1-32TF	7.02×10^{-8}	FIA-FIB	151	Amp, Caz, Ctx, S	*aac6lb, bla*CTX-M-1, *bla*OXA-1		64	32	> 1024
Hertfordshire	I1-31TF	1.20×10^{-7}	N	28	Amp, Caz, Ctx	*aac6lb, bla*CTX-M-1, *bla*OXA-1		16	32	1024
Pembrokeshire	I1-22TF	1.00×10^{-8}	FIA-FIB	171	Amp, Caz, Ctx, S, S3, Sxt, Te	*aac6lb, aadA4, bla*CTX-M-1, *dfrA17, bla*OXA-1, *sul1, tetB*	*int1*	64	128	> 1024
Hertfordshire	I1-34TF	4.00×10^{-9}	FIA-FIB	175	Amp, Caz, Ctx, C, Cn, S, S3, Sxt, Te	*aac6lb, aadA4, catA1, bla*CTX-M-1, *dfrA17, bla*OXA-1, *sul1, bla*TEM-1, *tetB*	*int1*	>512	256	> 1024
Shropshire	I2-49TF	1.71×10^{-8}	-	40	Amp, Caz, Ctx, C, N, S3, Sxt	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3*	*int1*	32	32	> 1024
Shropshire	I2-46TC	6.67×10^{-9}	-	111, **45**	Amp, Caz, Ctx, Cip, C, Na, N, S3, Stx, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	32	128	1024
Shropshire	I2-56TC	4.50×10^{-8}	-	51	Amp, Caz, Ctx, Cip, C, Na, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	32	128	> 1024
Shropshire	I2-59TC	4.80×10^{-8}	-	40	Amp, Caz, Ctx, Cip, C, Na, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	64	64	> 1024
Shropshire	I2-53TC	1.50×10^{-8}	-	40	Amp, Caz, Ctx, Cip, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	32	64	> 1024
Shropshire	I2-66TF	≥0	-	40	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	32	128	> 1024
Shropshire	I2-55TF	≥0	-	51	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	32	64	> 1024
Shropshire	I2-67TC	5.14×10^{-9}	-	125	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	32	64	> 1024
Shropshire	I2-51TF	7.50×10^{-8}	-	40	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	64	128	> 1024
Shropshire	I2-57TC	1.20×10^{-8}	-	100	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	64	128	> 1024
Shropshire	I2-58TF	3.60×10^{-8}	-	40	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	64	128	> 1024
Shropshire	I2-63TF	9.23×10^{-9}	-	40	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	128	128	> 1024
Shropshire	I2-64TC	4.36×10^{-8}	-	92, **40**	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, sul3, tetA*	*int1*	64	128	> 1024
Shropshire	I2-52TC	4.62×10^{-8}	B/O	151	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12, dfrA14, strB, sul2, sul3, bla*TEM-1, *tetA*	*int1*	32	128	> 1024
	I2-52TF	-	-	40	ND	*aadA1, aadA2, cmlA1, bla*CTX-M-1, *dfr12,, sul3, tetA*	*int1*	ND	ND	ND

See legend below.

Table 4-4: Summary of *bla*$_{\text{CTX-M}}$ harbouring plasmids isolated from bovine *E. coli* in the UK in 2007.

The table shows transconjugant (TC) and transformant (TF) derivatives of the field isolates and the frequency of transfer rates of the TCs and plasmid sizes of the resulting CTX-M harbouring plasmids. Transconjugants that harboured multiple plasmids were screened for *bla*$_{\text{CTX-M}}$ and these highlighted in bold. The plasmids were typed by PBRT, untypeable plasmids by this method were denoted by a dash (-). Antimicrobial resistance markers were determined by disc diffusion (phenotype) and DNA microarray analysis (genotype).
Abbreviations:Amp; Ampicillin, Ctx; Cefotaxime, Caz; Ceftazidime, Cip; Ciprofloxacin, C; Chloramphenicol, N; neomycin, Na; Nalidixic acid, Te; Tetracycline, S; Streptomycin, Sxt; sulfamethoxazole, S3; compound sulphonamide, *aac6lb*, *aadA1*, *aadA2*, *aadA4*, *strA* and *strB* confer resistance to aminoglycosides. *catA1* and *cmlA* confer resistance to chloramphenicols. *bla*CTX-M-1, *bla*CTX-M-9, *bla*TEM-1 and *bla*OXA-1 confer resistance to β-lactams. *dfr12*, *dfrA14* and *dfrA17* confer resistance to trimethoprims. s*ul1*, *sul2* and *sul3* confer resistance to sulphonamides. *tetA* and *tetB* confer resistance to tetracylcines. *Int1* and *Int2* are integrons (mobile genetic elements) commonly associated with antimicrobial resistance genes. *ND: not done*.

4.3 Discussion

The donor field isolates were found to confer a multi-drug resistance phenotype and harboured plasmids, a not unexpected result given the significance of plasmid mediated resistance in *E. coli* as outlined in the introductory chapter to this thesis. The plasmids were typed by PBRT and in some isolates found to harbour multiple replicon types, as a result of multiple plasmids. Ten field strains were found to harbour the IncI1 replicon. Seven of these plasmids were typed by plasmid multi-locus sequence typing (Garcia-Fernandez et al., 2008). These plasmids were found to belong to groups ST24, ST31 (CC-31) and ST38 (CC-3). ST24 has been previously identified in an *E. coli* isolate in the Netherlands in 2004 (pST-24). This plasmid however was not recorded as harbouring any β-lactamases genes (http://pubmlst.org).

The clonal complex (CC) 3 ST38 plasmid type has previously been identified in a plasmid from the UK isolated from a human *E. coli* strain harbouring a $bla_{\text{CMY-2}}$ AmpC-like β-lactamase, but no CTX-M enzymes were identified. CMY-2 encoding plasmids were first reported in 1990 and have since spread globally in multiple *Enterobacteriaceae* species (Bauernfeind et al., 1990; Bauernfeind et al., 1996; Navarro et al., 2001). These plasmids have been associated with $bla_{\text{TEM-1}}$ β-lactamases in addition to other multidrug resistance phenotypes and mobile genetic elements such as IS*Ecp1* upstream of the $bla_{\text{CMY-2}}$ (Navarro et al., 2001). The ST38 plasmid was also found to harbour an IS*Ecp1* upstream of the $bla_{\text{CTX-M-15}}$ gene, which was present in an IncF plasmid. This isolate also harboured a plasmid bearing an IncI1 gene. The association of the β-lactamase genes with a mobile genetic element in the IncF plasmid, would enable mobilisation of adjacent genes (Poirel et al., 2003) and thus serve as an ideal platform for genetic recombination events that could result in the acquisition of

bla$_{CTX-M}$ genes to another plasmid in close proximity such as the ST38 IncI1 bearing plasmid that was identified.

The ST31 CC 31 plasmid (found in strains I1-42 and I1-43), has previously been identified in *S. sonnei* and *E. coli* strains (http://pubmlst.org/plasmid). These *bla*$_{CTX-M-15}$ harbouring plasmids have been found in different geographical regions including Belgium, Germany, France, the Netherlands, UK and USA. These strains have been isolated from several species including horses, humans and cattle. Interestingly, one ST31 plasmid previously identified (Folster et al., 2010) harboured a 90 kb plasmid encoding *bla*$_{CTX-M-15}$ and *bla*$_{TEM}$ β-lactamases. This plasmid also displayed a multi-drug resistance phenotype conferring resistance to ampicillin, sulfamethoxazole, nalidixic acid, streptomycin and tetracycline (Folster, Pecic et al. 2010). The two ST31 plasmids identified in this study also harboured these β-lactamase genes and observed a similar antimicrobial resistance profile. These pMLST results demonstrate the widespread nature of these plasmids, not least in their isolation from multiple *Enterobacteriaceae* species and from multiple hosts.

Plasmid typing of the field isolates found 73 % of the isolates harboured known conjugative plasmids including narrow host range plasmids; IncB/O, IncF (IncFIB and IncFIA), IncI1 and broad host range plasmid IncN. These plasmid Inc types are amongst those most commonly found to be associated with large multi-drug resistance plasmids (Boyd et al., 2004; Couturier et al., 1998; Lavollay et al., 2006, Waters, 1999). Conjugation frequency studies were employed to determine the extent horizontal gene transfer play in the dissemination of *bla*$_{CTX-M}$ harbouring plasmids identified in this study.

IncF plasmids were found to have the highest transfer rates followed by IncN, IncI1 and the least efficient IncB/O. The conjugation experiments highlighted the transfer rate of

particular Inc groups irrespective of size, as these varied in each group; with IncF plasmids ranging from 54 to 175 kb, IncI1 plasmids from 86 kb and the IncB/O plasmid at approximately 151 kb. No plasmids smaller than 28 kb were shown using the S1 nuclease method, unlike the plasmid profile gel (Fig. 3-6) where plasmids as small as 3.8 kb were detected. This could be a limitation of the PFGE gel, which in the time period (16hrs) could have excluded the smaller plasmids from the gel. This experiment was performed at 37 °C, an optimal temperature of IncF and IncF like plasmids. At this temperature, DNA transfer is thought to occur at an approximate rate of 45 kb per minute (Lawley et al., 2004). IncF and IncI1 plasmids have been also been shown to harbour adhesins at the tip of the pilus, thereby mediating efficient donor-recipient contact through higher incidence of cell-cell contact (Lawley et al., 2004). The IncB/O plasmid frequency transfer rates were low, although these plasmids have the same pilus structure as the IncI1 plasmids (Waters, 1999). This suggests that the plasmid itself may limit the transfer by conjugation. Plasmids such as the IncH plasmids are known to transfer at higher rates at lower temperatures (22 - 30°C) (Lawley et al., 2004). In the first instance, this experiment could be repeated at varying temperatures to determine if the frequency of transfer is affected.

Three IncF plasmids (I1-22TF, I1-32TF and I1-34TF) bearing *bla*$_{\text{CTX-M-15}}$ (CTX-M group 1), harboured FIA-FIB replicons. These multi-replicon plasmids have previously been associated with *bla*$_{\text{CTX-M-15}}$ genes (Carattoli et al., 2009). The FIA-FIB is commonly found in enteric bacteria and it is thought that in such instances where multiple replicons are available, one replicon remains highly conserved, leaving the other able to mutate and possibly alter the compatibility group (Carattoli et al., 2008).

All the plasmids included in this study were found to harbour at least one gene conferring resistance to β-lactams. Seventeen plasmids (33 %) were found to only harbour genes that conferred resistance to β-lactams mainly from bla_{CTX-M} group 1 and 9. Sequence of bla_{CTX-M} genes present in the donor strains, indicated that these were most likely to be the CTX-M-1, -15 (group 1) and CTX-M-14 and 14b (group 9) variants that had been transferred in the recipients.

The remainder of the plasmids (56 %; *n = 35*) all harboured a multi-drug resistance (MDR) phenotype. This was reflected from the transfer of multiple genes between donor and recipient strains, as identified from DNA microarray analysis. It must be noted that the transfer of the plasmids from possible clonal donor strains (e.g. isolates from the Shropshire Farm, see Fig.3-2) may skew the frequency of these resistance genes observed and impact the resistance reporting of this data. Nineteen of these MDR plasmids encoded additional bla_{TEM-1} genes, and eleven of these were IncF plasmids. Previous studies have found bla_{TEM-1} genes to co-exist with $bla_{CTX-M-15}$ variants due to the association of bla_{TEM-1} with Tn*3* transposons. It is hypothesised that IncF plasmids can acquire CTX-M β-lactamases via an integration mediated Tn*3*::bla_{TEM} transposition event with the IS*Ecp1* containing $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ variants (Boyd et al., 2004; Carattoli, 2009; Marcade et al., 2009).

Also commonly found in association with $bla_{CTX-M-15}$ genes are bla_{OXA-1} and *aac'(6)lb* genes, which are thought to have originated from a gene cassette (Boyd et al., 2004). This is a common feature of IncF plasmids (Carattoli, 2009), as was demonstrated in the results with three plasmids (I1-22TF, I1-32TF and I1-34TF), which harboured these resistance genes. Interestingly, an IncN plasmid (I1-31TF) was grouped with these plasmids found to harbour the bla_{OXA-1} and *aac'(6)lb* resistance genes (see table 4-4).

Seven plasmids, from the same farm (Dorset) harboured IncN plasmids encoding the $bla_{CTX-M-1}$ variant. Previous studies have demonstrated the successful transfer of a

bla$_{\text{CTX-M-1}}$ encoding IncN plasmid between farm workers and pigs on an unrelated farm, and like the IncN plasmids observed in this study, the majority had no additional resistance genes (Moodley et al., 2009).

Two plasmids were found to harbour the IncI1 incompatibility group encoding *bla*$_{\text{CTX-M-14b}}$, and *bla*$_{\text{CTX-M-15}}$ variants. Although not largely represented in this panel of strains, IncI1 plasmids are widely distributed amongst *Enterobacteriaceae* (Carattoli, 2009). Smet et al., 2010 sequenced a 92 kb IncI1 plasmid encoding only *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM-1}}$ resistance genes, showing similar gene profiles to the three IncI1 plasmids identified in this study which also harbour the same CTX-M variants and an additional TEM-1 β-lactamase in one of the plasmids (I1-37TC) (Smet et al., 2010). Their sequenced plasmid was also found to have an extensive number of genes required for conjugation, in addition to a gene cluster encoding the type-IV pili, required for adhesion and invasion of Shigatoxigenic *E. coli* (Smet et al., 2010). The association of resistance determinants with this effective conjugative platform and virulence factors are factor for the successful dissemination of this plasmid family (Carattoli, 2009).

Plasmids harbouring *bla*$_{\text{CTX-M-32}}$ genes from the Shropshire region were found to confer a distinct resistance profile, conferring resistance to multiple antimicrobials including aminoglycosides, β-lactams, chloramphenicols, sulphonamides, trimethoprims and tetracyclines. These plasmids were identified as possessing the *intI* integrase marker, associated with class 1 integrons (Hall and Collins, 1998). Resistance determinants (*aadA1*, *aadA2*, *cmlA1*, *dfr12*, *sul3*) in these plasmids have been commonly found amongst class 1 integrons (Mazel, 2006; Sunde et al., 2008).

Class 1 integrons (InS21, In35, In116 and In60) have been found to harbour *bla*$_{\text{CTX-M}}$ genes within them; these include *bla*$_{\text{CTX-M-1, -2, -3, -9}}$ (Arduino et al., 2002; Di Conza et al., 2005; Eckert et al., 2006; Novais et al., 2006; Power et al., 2005; Riano et al., 2006;

Sabate et al., 2002; Vinue et al., 2008), however no *bla*$_{\text{CTX-M-32}}$ genes have been described amongst these.

Previous studies analysing the genetic environment surrounding the *bla*$_{\text{CTX-M-32}}$ variant have found these genes to be associated more commonly with the insertion elements IS*1*, IS*5* and IS*Ecp1* (Cartelle et al., 2004; Farnandez et al., 2007). The plasmids in this study did not harbour the IS*Ecp1* element upstream of the *bla*$_{\text{CTX-M-32}}$ gene. However Fernandez and colleagues have found a partially truncated IS*Ecp1*element, with insertions of IS*1* and IS*5* elements upstream of the *bla*$_{\text{CTX-M-32}}$ gene (Farnandez et al., 2007). The primers used to detect the IS*Ecp1* in this study were located at the 3' end of the IS*Ecp1* element (Poirel et al., 2002) and the 3' end of *bla*$_{\text{CTX-M}}$ (Carttoli et al., 2008). If the IS*1* and IS*5* insertions were present, the resulting amplicon would be ~ 3 kb instead of the estimated 1 kb fragment. The resulting large PCR amplicon may not have been amplifiable using the conditions used in this study and explain the negative results obtained during IS*Ecp1* screening.

The *bla*$_{\text{CTX-M-32}}$ plasmids harbouring these uniform resistance genes from the Shropshire farm were untypeable by the PBRT scheme. This may be due to genetic changes in the replicon region making them unyteable by the PBRT scheme. Previous studies have observed a 35 kb 'untypeable' plasmid harbouring an IncX-like backbone (pJIE143), with conjugative machinery that enables the transfer of this plasmid by conjugation (Partridge et al., 2011). This type of genetic changes may explain the characteristics of the similarly sized plasmids observed in the Shropshire region. The same group identified another 'untypeable' plasmid belonging to the IncN family of plasmids. Unlike the pJIE143 plasmid, pJIE137 harboured class 1 integrons (*intI*) in addition to *aadA2*, *dfrA12* and *sul1* integron associated gene cassettes (Partridge et al., 2012), which were also observed in the plasmids of the Shropshire region. Plasmid pJIE137 also harboured genes encoding conjugation machinery similar to the Tra proteins of

R46 IncN plasmid (Partridge et al., 2012). The pJIE137 plasmid from this study was also found to harbour an IS*CR1* element; a complex IS*91*-like insertion element commonly associated with class 1 integrons (Toleman et al., 2006). This unique mobile genetic element may offer a secondary horizontal gene transfer mechanism to classical conjugation, by way of a rolling-circle mechanism of self-transposition (Toleman et al., 2006). This element has also been associated with other $bla_{\text{CTX-M}}$ variants including $bla_{\text{CTX-M-2, -9, -14}}$ (Bae et al., 2007; Toleman et al., 2006).

Characteristics found in the pJIE137 and pJIE143 plasmids demonstrate the importance of understanding the genetic environment of $bla_{\text{CTX-M}}$ genes. Although IS*CR1* elements have not been found in conjunction with $bla_{\text{CTX-M-32}}$ genes, further work on the untypeable plasmids from the Shropshire farm will be required to ascertain the exact nature of transfer of these plasmids by conjugation or the unique rolling-circle mechanism of the IS*CR1* element. Determining the plasmid backbone will also further our understanding of how the plasmid had spread across the farm.

Eleven transconjugants (21 %) transferred multiple plasmids during conjugation. Upon transformation 26 % of their resistance determinants were lost, most frequently quinolone resistance markers (Na and Cip). The loss in quinolone resistance is most likely due to the chromosomal mutations found in the QRDR, although this does need verification.

Furthermore, due to the limitations of plasmid sizing of supercoiled plasmids, estimating the sizes did not take into account migration of the different conformations of the plasmid. Transconjugants that presented multiple plasmids of varying sizes may have been a result of the different forms of plasmid migrating at different rates on the plasmid profile gel. These plasmids often harboured the same plasmid Inc groups, and by definition, plasmids with the same Inc group cannot co-exist in the same cell (Datta

and Hedges, 1971). It is likely that the multiple plasmids in these instances were merely different forms of the plasmid. DNA micro-array and S1- nuclease PFGE analysis on the transconjugants would have to be performed to confirm this postulation.

Two transconjugants (I2-46TC and I2-64TC), were found to have co-transferred untypeable plasmids. These plasmids would need to be sequenced to identify the replicon types or any possible recombination events that may have taken place *in vitro* to give rise to the larger plasmids observed in the transconjugant derivatives.

As shown in this chapter, all resistance to ampicillin and cefotaxime was transferred, although only 67 % of ceftazidime resistance was transferred. Susceptibility to these three antimicrobials was determined in recipients' after transfer and in the donor strains. MIC levels for all the donor strains and plasmid derivatives (transconjugants or transformants) to ampicillin were ≥ 1024 mg/L. Very high levels of resistance to aminopenicillins (ampicillin and amoxicillin) have previously been observed with CTX-M enzymes (Bonnet, 2004). However, the MIC values for the plasmid derivatives were in general lower than the donor field strains for ceftazidime and cefotaxime. Genotypic results showed 100 % transfer of $bla_{\text{CTX-M}}$ genes and 95 % and 57 % transfer of additional β-lactamase genes; TEM-1 and OXA-1 respectively. Therefore this reduction in MIC levels despite the transfer of the CTX-M enzymes suggests other elements present in the donor strains account for the differences including possibly chromosomally located resistance genes and multidrug resistance efflux systems.

At least 2 fold lower ceftazidime MIC values were seen with all the CTX-M variants compared to cefotaxime MIC values. CTX-M enzymes are generally more active against cefotaxime than ceftazidime (Barthelemy et al., 1992). This is because CTX-M enzymes have more flexibility within the β3 strand and Ω-loop due to amino acid substitutions in this region, enabling access of larger side chains such as the

cephalosporins. In addition amino acid substitutions including; Asn-104, Ser-237, Asp-240 and Arg-276 in the CTX-M enzymes are thought to increase substrate specificity enabling the efficient hydrolysis of oxyimino-cephalosporinas including ceftazidime and cefotaxime, unlike the earlier ESBL enzymes TEM and SHV which poorly hydrolyse these compounds (Bonnet, 2004).

Differences between the group 1 enzymes, CTX-M-1, -15 and -32 was observed, with CTX-M-1 having the lowest MIC against cefotaxime (median MIC 32 mg/L) and the second lowest against ceftazidime (median MIC 12 mg/L). CTX-M-1 is described as the least efficient *bla*$_{\text{CTX-M}}$ group1 enzyme in comparison to CTX-M-15 and -32. This is because it lacks the D240G mutation in the omega loop; a mutation thought to increase the flexibility of the β-strand thus rendering the active site more accessible to bulkier side chains such as that of ceftazidime (Novais, Canton et al. 2008; Rossolini, D'Andrea et al. 2008; Novais, Comas et al. 2010).

CTX-M-15 and -32 had the same median MIC values against cefotaxime (median MIC 128 mg/L); however the median MIC value for ceftazidime was two-fold less for CTX-M-32 in comparison to CTX-M-15. Previous studies have demonstrated CTX-M-32 enzymes as the most efficient inactivator of oxyimino-cephalosporins with respect to the five variants (CTX-M-1, -14, -14b, -15 and -32) represented in this data set, thought to be because of its ability to efficiently inactivate cefotaxime and ceftazidime (Novais, Comas et al. 2010). However, the MIC data shows that the CTX-M-32 enzyme is similar, if not less efficient than CTX-M-15, owing to the lower MIC values of CTX-M-32 against ceftazidime (Novais, Comas et al. 2010). The presence of other β-lactamase genes on *bla*$_{\text{CTX-M-15}}$ encoding strains may account for their higher levels of resistance to ceftazidime. Although ceftazidime is a poor substrate for the TEM-1 enzyme; previous studies have shown that mutations in the omega loop, results in increased hydrolytic activity towards ceftazidime (Petrosino and Palzkill 1996).

CTX-M-14 and 14b producing strains, both CTX-M group 9 variants, had lower cefotaxime and ceftazidime MIC values than their group 1 counterparts (CTX-M-1, -15 and -32). This decrease in MIC between the different group 9 and 1 enzymes has previously been observed (Bonnet, 2004). These two enzyme variants differ in substrate specificity due to the active site substitution D240G. CTX-M group 1 enzymes (CTX-M-15 and -32) encode a glycine residue at position 240 (Ambler numbering), an uncharged residue thought to negate any steric hindrance in binding of the cephalosporins. The charged aspartate residue present in the group 9 enzymes (CTX-M-14 and14b) may restrict binding of the bulkier side chains of cephalosporins, especially ceftazidime (Bonnet, 2004). This difference in substrate accommodation may explain the lower MIC values obtained for these group 9 enzymes.

Four fold differences were observed in CTX-M-14 and -14 b ceftazidime MIC values. CTX-M-14b enzymes are thought to have evolved from the CTX-M-9 variant, with only one base pair difference (G**C**A→G**T**A) resulting in an A231V substitution (numbering of Ambler et al., 1980). Whilst, CTX-M-14 harbours two base pair substitutions with respect to the CTX-M-9 variant (G**CA**→G**TG**), coding for the same amino acid as the CTX-M-14b enzyme, A231V (Sabate, Tarrago et al. 2000; Poirel, Naas et al. 2001; Navarro, Mesa et al. 2007). The CTX-M-14 enzymes encode two additional silent base pair mutations, not present in CTX-M-14b and CTX-M-9. CTX-M-14 and -14b enzymes have the same tertiary structure and therefore there should be no difference in their substrate specificity (Chen et al., 2005), although four-fold differences in ceftazidime MIC values were observed in this study. Interestingly all the CTX-M-14 enzymes were encoded within IncF plasmids and the CTX-M-14b enzymes were encoded within the ‘untypeable’ plasmids. These IncF plasmids harboured additional

TEM-1 enzymes, which as previously postulated for CTX-M-15, may have resulted in increased specificity to the ceftazidime substrate (Petrosino and Palzkill, 1996).

The differences in MIC values between the different CTX-M enzymes may give some insight to the reason for the prevalence observed of CTX-M 15 enzymes in recent years in clinical *E. coli* samples in Europe (Livermore et al., 2007). That is to say, that CTX-M-15 enzymes should, in theory be the most abundant of the β-lactamase variants due to its relative tolerance to β-lactam agents, making it a more efficient enzyme and a possible criterion for positive selection under Darwinian theory of evolution (Gillespie 2004). The relative efficiency of the other CTX-M variants in this study in susceptibility to cefotaxime and ceftazidime suggests other factors may influence drug susceptibility in the plasmid system, including the presence of other, albeit less, efficient B-lactamases, whose activity has an additive effect.

In the next chapter, further analysis of the role of CTX-M variants and plasmids in the relative fitness of the strain will be investigated.

5 RESULTS CHAPTER 5: Nucleotide sequencing of $bla_{CTX\text{-}M\text{-}14b}$, $_{-15}$ and $_{-32}$ encoding multidrug resistance plasmids from *E. coli*

5.1 Introduction

The rapid spread of antimicrobial resistance determinants has been attributed to the ease of transfer of plasmid encoding these genes (Salyers et al., 1997). As discussed in chapter one, transfer is mediated by a complex conjugation system usually encoded on the plasmid by a cluster of co-ordinately regulated genes, the so-called *tra* genes, along with other genes for pilus assembly (*pil*) and plasmid establishment in the host cell (*ssb* and *psiB*). Rates of transfer vary between plasmids as we have seen in previous chapters (see section 4.2.2). Also, transfer is limited by the competence of the recipient bacterium to receive the plasmid, as also discussed in chapter one, such factors as surface exclusion and incompatibility play a role in limiting plasmid transfer. Once transferred to a new host bacterium, the plasmid usually becomes established and maintained more or less irreversibly. To achieve this state of 'successful maintenance' in a new host background, the plasmid encodes genes that counter mechanisms that would result in random plasmid loss during cell division. These maintenance processes, that are thought to impart a fitness cost to the host, include plasmid partitioning systems, that ensure plasmid segregation during replication and cell division, site specific recombinases that resolve plasmid multimers during replication and recombination and post-segregational killing systems (PSK), also known as addiction systems that ensure survival of only bacteria which retain the plasmid (Gerdes, 2000 in; Slater et al., 2008). In addition, for those plasmids encoding a selectable trait such as antibiotic resistance, the presence of selection will provide a selective advantage over their plasmid free strains. Often encoded on plasmids, are genes conferring resistance to multiple drug

classes. These genes can be found adjacent to each other owed to by the arrangement of gene cassettes within an integron (Recchia and Hall, 1995). Other contributors to genetic diversity include the association of resistance genes with insertion elements (ISEs), Integrons and ISEs have been found to drive expression of genes they encompass and play an important role in host adaptability (Hall and Collins, 1995; Poirel et al., 2003). Collectively these elements provide a source of genetic diversity. By providing a selective advantage, metabolic or physical, HGT enables the organism to adapt to a new ecological niche (Andam et al., 2011).

In this chapter, the aim is to sequence a number of ESBL plasmids to gain a complete understanding of the biology of the encoding plasmids. The sequence data will enable interrogation of plasmid transfer and maintenance systems, define the MDR regions within its genetic context, discover other factors associated with the plasmids that the studies in chapters 3 and 4 have not revealed and perhaps gain insights into plasmid evolution.

5.2 Results and Discussion

5.3 Plasmid sequence analysis

Four plasmids were selected for sequencing harbouring the *bla*$_{\text{CTX-M-32, -15}}$ and $_{-14}$ variants from the transformant or transconjugant derivatives of the field isolates collected from various regions in the UK, characterised in the previous chapter. Two plasmids harboured narrow host range conjugative replicons IncI1 (pI2-47TC), IncB/O (pI2-52TC) and a multireplicon plasmid harboured IncF-FIA-FIB (pI1-34TF), identified using the PCR based replicon typing (PBRT) scheme. The fourth plasmid (pI2-53TF) was one of the 17 plasmids from the Shropshire farm that could not be typed by this scheme and was representative of these untypeable plasmids (see Table 5.1). Sequencing of this plasmid would help determine its replication control mechanism and possibly ascertain its conjugative or mobilisation system. These four plasmids harbour the *bla*$_{\text{CTX-M-32, -15,}}$ and $_{-14}$ variants, which have previously been reported in isolates from both human and animal origin (Boyd et al., 2004; Cartelle et al., 2004; Cottell et al., 2011; Ma et al., 2002; Smet et al., 2010; Woodford et al., 2009). Sequence analysis of these plasmids originating from bovine *E. coli* strains presenting *bla*$_{\text{CTX-M}}$ variants commonly found in human as well as animal origins, will enhance our understanding as to the mechanisms that enable efficient dissemination and persistence in different species. The plasmid contigs arising from sequence data were concatenated and compared with other fully sequenced plasmids for any similarities in gene arrangements within the concatenated plasmid sequence, which may give insight into the possible mechanisms of plasmid evolution.

Table 5-1: Summary of *bla*$_{CTX-M}$ plasmids selected for sequencing.

Plasmid	PBRT	Plasmid size (kb)	Associated resistance profile	Antimicrobial resistance genes	*Int*
pI1-34TF	F, FIA, FIB	175	Amp, Caz, Ctx, C, Cn, S, S3, Sxt, Te	*aac6lb, aadA4, catA1, bla*$_{CTX-M-15}$, *dfrA17, bla*$_{OXA-1}$, *sul1, bla*$_{TEM-1}$, *tetB*	*intI1*
pI2-47TC	I1	86, **35**	Amp, Ctx	*bla*$_{CTX-M-14b}$	
pI2-52TC	B/O	151	Amp, Caz, Ctx, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*$_{CTX-M-32}$, *dfr12, dfrA14, strB, sul2, sul3, bla*$_{TEM-1}$, *tetA*	*intI1*
pI2-53TF	-	40	Amp, Caz, Ctx, Cip, C, N, S3, Sxt, Te	*aadA1, aadA2, cmlA1, bla*$_{CTX-M-32}$, *dfr12, sul3, tetA*	*intI1*

The four plasmids were selected from the available 52 strains analysed in the previous chapter based on their *bla*$_{CTX-M}$ variant, plasmid Inc group and antimicrobial resistance gene profiles (phenotype/genotype) and sequenced including: pI1-34TF, pI2-47TC (the **35** kb plasmid in bold), pI2-52TC and pI2-53TF. Shown are the Inc groups (determined by PBRT), plasmid sizes (determined by S1-nculease PFGE), phenotypic and genotypic resistance genes determined by disc diffusion and miniaturized DNA microarray (see chapter 4). *Abbreviations:* Amp; Ampicillin, Ctx; Cefotaxime, Caz; Ceftazidime, Cip; Ciprofloxacin, C; Chloramphenicol, N; neomycin, Na; Nalidixic acid, Te; Tetracycline, S; Streptomycin, Sxt; sulfamethoxazole, S3; compound sulphonamide, *aac6lb*, *aadA1*, *aadA2*, *aadA4*, *strA* and *strB* confer resistance to aminoglycosides. The *catA1* and *cmlA* confer resistance to chloramphenicols. The *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-9}$, *bla*$_{TEM-1}$ and *bla*$_{OXA-1}$ genes confer resistance to β-lactams. The *dfr12*, *dfrA14* and *dfrA17* genes confer resistance to trimethoprims. Sulphonamide resistance genes include; s*ul1*, *sul2* and *sul3*. Tetracycline resistance genes included; *tetA* and *tetB*. The integron class 1 associated integrase gene is denoted as *intI1* and is commonly associated with multidrug resistance gene cassettes. *ND: not done*.

5.3.1 Analysis of pI1-34TF

This plasmid was identified as a ~175 kb IncFIA-FIB fusion replicon plasmid, isolated from the I1-34 field stain. Nucleotide sequence analysis (see chapter 2, section 2.4.11) revealed this plasmid to harbour a *bla*$_{CTX-M-15}$ ESBL variant. This plasmid was selected for sequence analysis due to the multiple drug resistance genes identified by DNA microarray analysis in the transformant pI1-34TF. This plasmid was found to harbour resistance genes to six classes of drugs including, aminoglycosides, β-lactams, chloramphenicols, sulphonamides, tetracyclines and trimethoprims, with a total of 10 antimicrobial resistance genes (see table 5-1).

5.3.1.1 Plasmid scaffold

Plasmid pI1-34TF was found to have an overall 57 % nucleotide identity to pIP1206; a multidrug resistance *E. coli* IncFII-FIA-FIB plasmid isolated from bovine faeces (GenBank AM886293.1) (Perichon et al., 2008) (see Fig. 5-1A). This plasmid

harboured 217 predicted open reading frames (ORFs, see table 5-2). Regions of homology included the *tra* and *trb* conjugative systems, antimicrobial resistance genes conferring resistance to chloramphenicol (*catA1*), tetracycline (*tetB*) and quinolones (*qepA*) (Lawley et al., 2003). Plasmid pI1-34TF was found to encode the *repE* gene of the FIA replicon, *repA* of FIB and the regulators of FII replicon (*repA1-A4* and *A6*). Plasmid pIP1206 however did not encode a $bla_{\text{CTX-M}}$ gene.

The $bla_{\text{CTX-M-15}}$ gene and surrounding regions of pI1-34TF was found to be similar to two plasmids, pEK499 (GenBank EU935739) (Woodfoord et al., 2009) (Fig. 5-1B) and pEC-L8 (GenBank GU371928) (Smet et al., 2010) (Fig. 5-1C), with 39 % (66 kb) and 37 % (62 kb) overall nucleotide identity respectively to pI1-34TF encoding mainly the transfer genes (*tra*), resistance genes (*aac(6)lb*, $bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-15,}}$ $bla_{\text{TEM-1}}$) and post-segregational killing (PSK) systems (*ccdA/ccdB, hok/mok*). Both of these plasmids harboured fusion replicons IncFII-FIA and were previously found to have 75 % homology to each other (Smet et al., 2010).

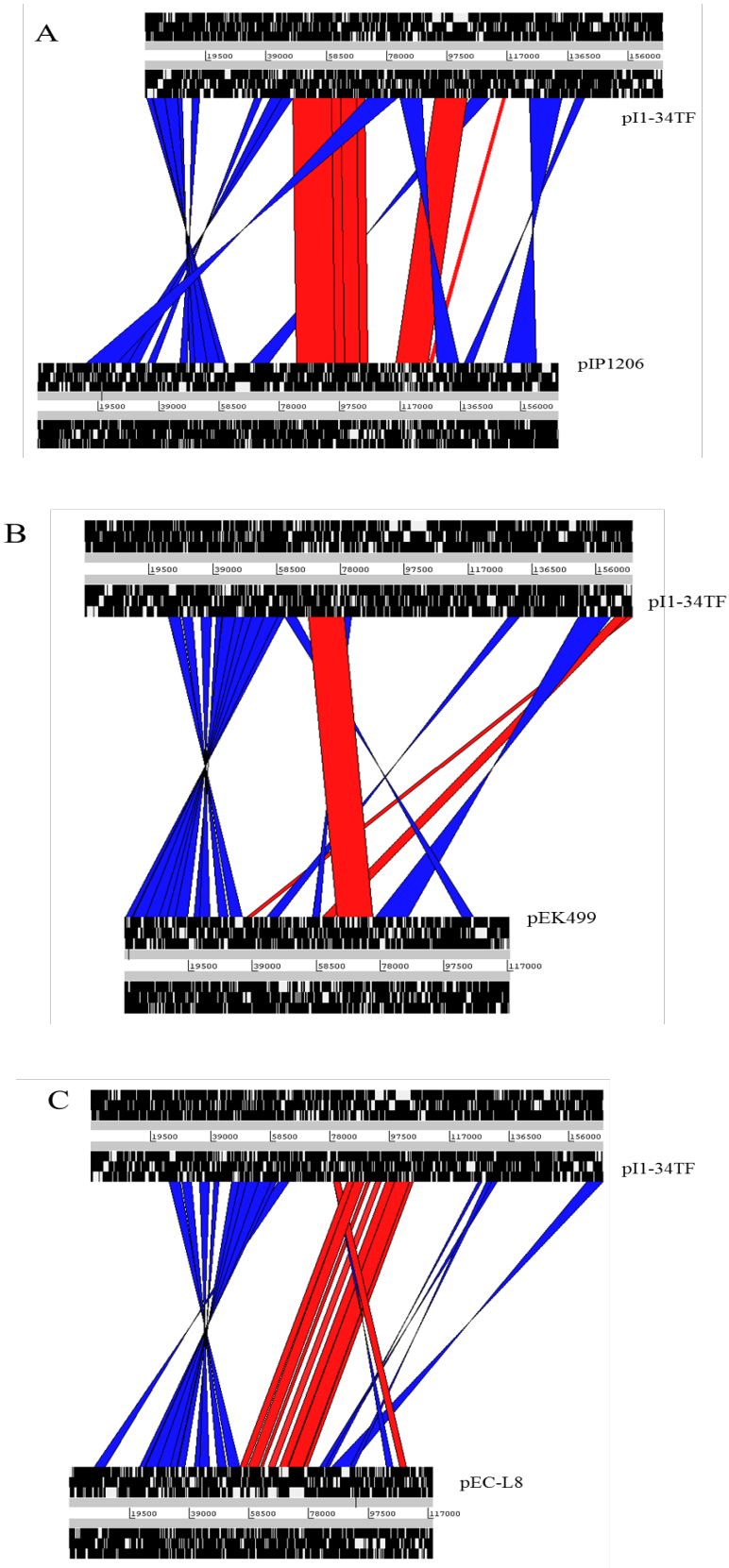


Figure 5-1: Sequence alignments of pI1-34TF with (A) pIP1206 and (B) pEK499 using WebACT (www.webact.org) with a cut-off of 1000 bp. (A) Plasmid pI1-34TF was found to have 57 % nucleotide identity to pIP1206, an IncFIA-FIB-FII plasmid. pIP206 did not encode any $bla_{\text{CTX-M}}$ genes. (B) Plasmid pI1-34TF was found to have 39 % nucleotide identity to IncFII-FIA $bla_{\text{CTX-M-15}}$ plasmid pEK499. (C) Plasmid pI1-34TF was found to have 37 % nucleotide identity to IncFII-FIA $bla_{\text{CTX-M-15}}$ pEC-L8 plasmid.

Table 5-2: Predicted open reading frames identified in pI1-34TF.

Contig	Function	Start	Stop	Strand
1	Aerobactin siderophore receptor IutA @ TonB-dependent siderophore receptor	2672	471	-
1	L-lysine 6-monooxygenase [NADPH] (EC 1.14.13.59), aerobactin biosynthesis protein IucD	4031	2754	-
1	Citrate:6-N-acetyl-6-N-hydroxy-L-lysine ligase, alpha subunit (EC 6.3.2.27), aerobactin biosynthe	5770	4028	-
1	N6-hydroxylysine O-acetyltransferase (EC 2.3.1.102), aerobactin biosynthesis protein IucB	6717	5770	-
1	Citrate:6-N-acetyl-6-N-hydroxy-L-lysine ligase, alpha subunit (EC 6.3.2.27), aerobactin biosynthe	8442	6718	-
1	hypothetical protein	8578	9771	+
1	hypothetical protein	9835	9978	+
1	Enolase (EC 4.2.1.11)	11306	10872	-
1	Manganese ABC transporter, inner membrane permease protein SitD	12328	11774	-
1	Manganese ABC transporter, inner membrane permease protein SitD	12630	12310	-
1	Manganese ABC transporter, inner membrane permease protein SitC	13484	12627	-
1	Manganese ABC transporter, ATP-binding protein SitB	14308	13481	-
1	Manganese ABC transporter, periplasmic-binding protein SitA	15222	14308	-
2	COG2963: Transposase and inactivated derivatives	99	455	+
2	unknown in ISEc8	452	802	+
2	COG3436: Transposase and inactivated derivatives	833	1054	+
2	COG3436: Transposase and inactivated derivatives	1162	1347	+
2	Arginine pathway regulatory protein ArgR, repressor of arg regulon	1890	1411	-
2	Arginine/ornithine antiporter ArcD	3374	1971	-
2	Ornithine carbamoyltransferase (EC 2.1.3.3)	4426	3422	-
2	Carbamate kinase (EC 2.7.2.2)	5422	4511	-
2	Arginine deiminase (EC 3.5.3.6)	6653	5433	-
2	hypothetical protein	7459	7313	-
2	Hemolysin	7494	7739	+
2	FIG00244926: hypothetical protein	7742	7861	+
2	RepA1	8419	7967	-
2	Replication regulatory protein repA2	8979	8722	-
2	hypothetical protein	9159	9281	+
2	SrnB	9412	9263	-
2	hypothetical protein	9838	9656	-
3	hypothetical protein	2061	2381	+
3	FIG00641101: hypothetical protein	2563	2420	-
3	IncF plasmid conjugative transfer protein TraR	2738	2517	-
3	IncF plasmid conjugative transfer pilus assembly protein TraV	3388	2873	-
3	IncF plasmid conjugative transfer protein TrbG	3636	3385	-
3	IncF plasmid conjugative transfer protein TrbD	3845	3648	-
3	IncF plasmid conjugative transfer protein TraP	4422	3832	-
3	IncF plasmid conjugative transfer pilus assembly protein TraB	5839	4412	-
3	IncF plasmid conjugative transfer pilus assembly protein TraK	6567	5839	-
3	IncF plasmid conjugative transfer pilus assembly protein TraE	7120	6554	-
3	IncF plasmid conjugative transfer pilus assembly protein TraL	7453	7142	-
3	IncF plasmid conjugative transfer pilin protein TraA	7833	7468	-
3	IncF plasmid conjugative transfer regulator TraY	8261	7866	-
3	IncF plasmid conjugative transfer regulator TraJ	9049	8360	-
3	IncF plasmid conjugative transfer mating signal transduction protein TraM	9619	9236	-
3	X polypeptide	9949	10542	+
3	putative ORF	10702	10568	-
3	unnamed protein product	11767	10838	-
3	FIG00638017: hypothetical protein	12064	11777	-
3	hypothetical protein	12128	12364	+
3	hypothetical protein	12420	12698	+
3	hypothetical protein	12860	12705	-
3	F leading maintenance	13143	12985	-
3	hypothetical protein	14094	13243	-
3	Transposase	14375	14091	-
3	Protein sok	14669	14857	+
3	PsiA protein	15588	14869	-
3	PsiB protein	16019	15585	-
3	Probable chromosome partitioning protein parB	16208	16074	-
3	Putative cytoplasmic protein	16532	16299	-
3	Single-stranded DNA-binding protein	17052	16600	-
3	Retron-type reverse transcriptase	19148	17247	-
3	hypothetical protein	19890	20093	+
3	hypothetical protein	20424	20272	-
3	FIG00643413: hypothetical protein	20654	20442	-
3	Plasmid pO157 DNA, complete sequence	20916	20680	-
3	FIG00638607: hypothetical protein	21245	20889	-
3	hypothetical plasmid protein	22654	21293	-
3	YdaB	22936	22706	-
3	FIG00639443: hypothetical protein	23168	23028	-
3	hypothetical protein	23283	23155	-
3	hypothetical protein	23482	23763	+
3	hypothetical protein	24159	23968	-
3	Orf52 protein	24578	24156	-

Continued on the following page.

Table 5-2: *continued.*

Contig	Function	Start	Stop	Strand
3	Putative antirestriction protein	25050	24625	-
3	hypothetical protein	25206	25346	+
3	FIG00638373: hypothetical protein	26234	25464	-
3	YcgB	26713	26279	-
3	putative cytoplasmic protein	26948	26727	-
3	Adenine-specific methyltransferase (EC 2.1.1.72)	27632	26949	-
3	FIG00637984: hypothetical protein	27740	27865	+
3	Plasmid PO157 DNA, complete sequence	28943	28017	-
3	Chromosome (plasmid) partitioning protein ParB	30628	29657	-
3	Chromosome (plasmid) partitioning protein ParA	31785	30628	-
3	FIG00639997: hypothetical protein	31750	31902	+
3	Replication initiation protein RepE	33137	32382	-
3	FIG00638134: hypothetical protein	33665	33546	-
3	Resolvase	34665	33859	-
3	CcdB toxin protein	34971	34666	-
3	CcdA protein (antitoxin to CcdB)	35191	34973	-
3	hypothetical protein	35633	35436	-
3	Virulence-associated protein vagC	35751	35981	+
3	VagD	35978	36394	+
3	FIG131328: Predicted ATP-dependent endonuclease of the OLD family	36469	38034	+
3	FIG116849: hypothetical protein	38019	39041	+
3	hypothetical protein	39261	39097	-
4	aminoglycoside-(3)-N-acetyltransferase	36	896	+
4	hypothetical protein	2124	1933	-
4	hypothetical protein	2130	2375	+
4	hypothetical protein	2977	2861	-
5	Transposon modulator protein	349	149	-
5	Integron integrase IntI1	1715	702	-
5	Dihydrofolate reductase (EC 1.5.1.3)	1873	2346	+
5	Streptomycin 3"-O-adenylyltransferase (EC 2.7.7.47)	2477	3265	+
5	Ethidium bromide-methyl viologen resistance protein EmrE	3471	3818	+
5	Dihydropteroate synthase (EC 2.5.1.15)	3812	4651	+
5	similar to puromycin N-acetyltransferase	4779	4982	+
5	Chromate transport protein ChrA	5138	6343	+
5	Transcriptional regulator, PadR family	6354	6659	+
5	FIG00643008: hypothetical protein	6798	6685	-
5	Transposase	6886	7650	+
5	Repressor protein MphR(A)	8619	8143	-
5	major facilitator superfamily MFS_1	9965	8727	-
5	Macrolide 2'-phosphotransferase I	10219	9962	-
5	unnamed protein product	10253	10888	+
6	Beta-lactamase (EC 3.5.2.6)-$bla_{\text{OXA-1}}$	1457	582	-
7	orf; Unknown function (Insertion Sequence Associated)	1518	1171	-
7	Insertion Sequence Associated	1919	1515	-
7	IncF plasmid conjugative transfer pilus assembly protein TraC	1918	2280	+
7	IncF plasmid conjugative transfer protein TrbI	2277	2663	+
7	IncF plasmid conjugative transfer pilus assembly protein TraW	2660	3292	+
7	IncF plasmid conjugative transfer pilus assembly protein TraU	3289	4281	+
7	IncF plasmid conjugative transfer protein TrbC	4290	4928	+
7	IncF plasmid conjugative transfer protein TraN	4925	6733	+
7	IncF plasmid conjugative transfer protein TrbE	6760	7017	+
7	IncF plasmid conjugative transfer pilus assembly protein TraF	7010	7753	+
7	IncF plasmid conjugative transfer protein TrbA	7767	8108	+
7	hypothetical protein	8424	8089	-
7	IncF plasmid conjugative transfer protein TraQ	8505	8789	+
7	IncF plasmid conjugative transfer protein TrbB	8776	9330	+
7	IncF plasmid conjugative transfer protein TrbJ	9320	9634	+
7	IncF plasmid conjugative transfer protein TrbF	9588	9980	+
7	IncF plasmid conjugative transfer pilus assembly protein TraH	9967	11340	+
7	IncF plasmid conjugative transfer protein TraG	11337	14162	+
7	IncF plasmid conjugative transfer surface exclusion protein TraS	14159	14668	+
7	IncF plasmid conjugative transfer surface exclusion protein TraT	14682	15413	+
7	hypothetical protein	15623	15501	-
7	IncF plasmid conjugative transfer protein TraD	15665	15805	+
7	IncF plasmid conjugative transfer protein TraD	15826	17862	+
7	IncF plasmid conjugative transfer DNA-nicking and unwinding protein TraI	17862	22982	+
7	IncF plasmid conjugative transfer pilin acetylase TraX	23002	23748	+
7	IncF plasmid conjugative transfer fertility inhibition protein FinO	23752	24363	+
7	FIG00640595: hypothetical protein	24497	24709	+
7	putative nuclease	24894	25016	+
7	hypothetical protein	25177	25010	-
7	Insertion Sequence Associated	25184	25831	+
7	orf, conserved hypothetical protein	25831	26178	+
7	Transposase	26198	27769	+

Continued on the following page.

Table 5-2: *continued.*

Contig	Function	Start	Stop	Strand
8	FIG00640301: hypothetical protein	97	216	+
8	insertion sequence IS100, ATP-binding protein	1039	260	-
8	Transposase	2061	1039	-
8	Transposase	2833	2075	-
8	Transposase	3092	2835	-
8	hypothetical protein	3434	3141	-
9	transposase TnpA	755	51	-
10	Transposase	436	41	-
11	Right origin-binding protein	670	254	-
11	transposon tn10 tetc protein	758	1351	+
11	Tetracycline efflux protein TetA	2687	1464	-
11	Transcriptional regulator, ArsR family	3897	3352	-
11	IS1 ORF2	5062	4685	-
11	Insertion element protein	5382	5107	-
11	Chloramphenicol acetyltransferase (EC 2.3.1.28)	5661	6320	+
11	YbjA protein	6898	6521	-
11	TnpA transposase	9931	6965	-
11	resolvase	10308	9934	-
12	Beta-lactamase (EC 3.5.2.6)-$bla_{\text{TEM-1}}$	181	1041	+
12	InsA protein (Fragment)	1318	1136	-
12	hypothetical protein	2628	1318	-
12	Programmed cell death toxin PemK	3245	2913	-
12	Programmed cell death antitoxin PemI	3504	3247	-
12	microcin M activity protein McmM	4250	3597	-
12	hypothetical protein	4369	4247	-
12	hypothetical protein	4488	4348	-
12	RepA1	6046	5189	-
12	Replication regulatory protein repA2 (Protein copB)	6605	6348	-
12	COG3547: Transposase and inactivated derivatives	7010	8032	+
12	hypothetical protein	8504	8857	+
12	COG1112: Superfamily I DNA and RNA helicases and helicase subunits	9770	9270	-
13	Transposase	23	268	+
13	stable plasmid inheritance protein	270	446	+
13	Error-prone, lesion bypass DNA polymerase V (UmuC)	837	448	-
13	Transposase	1451	1095	-
13	hypothetical protein	1897	1673	-
13	hypothetical protein	2034	2204	+
13	RepFIB replication protein A	2197	3174	+
13	FIG00243898: hypothetical protein	3369	3250	-
13	RepFIB associated resolvase	4199	3459	-
13	hypothetical protein	4356	4475	+
13	hypothetical protein	4559	4699	+
13	FIG00642198: hypothetical protein	5033	6043	+
13	FIG00643263: hypothetical protein	6259	6372	+
13	FIG00641477: hypothetical protein	7493	7377	-
13	Transposase insC for insertion element IS2A/D/F/H/I/K	8121	7756	-
13	Colicin I receptor precursor	8487	10373	+
13	FIG00640645: hypothetical protein	10380	11171	+
14	hypothetical protein	257	376	+
14	TnpA transposase	90	2411	+
14	Glucose-1-phosphatase (EC 3.1.3.10)	3331	2588	-
14	hypothetical protein	3475	3353	-
14	hypothetical protein	3938	3453	-
14	Putative thioredoxin precursor	4448	3963	-
14	ABC transporter, ATP-binding subunit precursor	5130	4435	-
14	membrane protein	6265	5135	-
14	Similar to ABC transporter: eg YBJZ_ECOLI hypothetical ABC transporter	7538	6255	-
14	Putative membrane protein	8920	7541	-
14	Periplasmic protein p19 involved in high-affinity Fe2+ transport	9551	9024	-
14	Putative high-affinity iron permease	11532	9592	-
14	FIG00640820: hypothetical protein	11593	11751	+
14	Na(+)-translocating NADH-quinone reductase subunit C (EC 1.6.5.-)	12586	11825	-
14	Membrane protein, suppressor for copper-sensitivity ScsD	13329	12823	-
14	Secreted protein, suppressor for copper-sensitivity ScsC	13477	13319	-
14	hypothetical protein	13758	13627	-
15	Tryptophan synthase beta chain like (EC 4.2.1.20)	2321	2593	+
15	Beta-lactamase (EC 3.5.2.6)-$bla_{\text{CTX-M-15}}$	3515	2640	-
15	hypothetical protein	3790	3668	-
15	transposase InsC3 for insertion sequence ISEc9	5033	3771	-

5.3.1.2 Plasmid transfer, establishment and maintenance

Plasmid pI1-34TF was found to harbour three replication systems of the IncFIA, FIB and FII group. Multiple replicons have been identified previously on plasmids harbouring the $bla_{CTX\text{-}M\text{-}15}$ gene isolated from human samples in America, Australia, Europe and India (Carattoli, 2009). Multireplicon plasmids are thought to be advantageous due to the ability of the plasmid to drive replication from any of the available replication origins. If a plasmid with the same replicon were to be introduced into the same host, provided the resident plasmid is driving expression from one of its alternative replicons, the incoming plasmid will be able to replicate, because in this instance they would be compatible (Villa et al., 2010). Furthermore, the replicon that is not involved in replication initiation can undergo mutational changes resulting in divergent replicons that will enable other IncF plasmids to be acquired, thus breaking the incompatibility barrier.

Plasmid pI1-34TF was found to encode *tra* and *trb* conjugative genes, which code for the F-pilus, which accounted for at least 16 % of the genes identified. Plasmid maintenance systems identified on this plasmid included four toxin-antitoxin genes (*ccdB/ccdA*, *hok/mok*, *pemK/pemI* and *vagD/vagC*) and partitioning systems (*sopA/sopB*). These PSK systems were also identified in pEK499 carrying a total of five killing systems and plasmid pIP1206 carrying three of the four toxin-antitoxin genes identified on this plasmid (Perichon et al., 2008; Woodford et al., 2009). These systems are thought to ensure plasmid inheritance in the absence of antimicrobial selection.

5.3.1.3 Mobile genetic elements

Eighteen percent of the genes identified from the draft plasmid genome were associated with horizontal gene transfer including transposases and resolvases, which include genes associated with transposons Tn*21*, and Tn*3* and IS elements: IS*1*, IS*2*, IS*26*, IS*6100*, and IS*Ecp1B*. Also identified were class 1 integron associated genes *tnpM*

(transposase) and *intI1* (integrase). The integron associated genes were found in one contig encoding a Tn*21* modulator protein (*tnpM*), and two transposase genes (*tnpA*); one of which was associated with IS*6100*, an integrase gene (*intI1*), a putative chromate transporter (*chrA*) and four resistance genes (*dfrA7, aadA4, sulI, mphA*). This region showed 99 % similarity to a homologous region in pEK499 (Woodford et al., 2009), but unlike pEK499, which was flanked by an IS*26* transposase, the $bla_{\text{CTX-M-15}}$ gene of pI1-34TF harboured an IS*Ecp1*- $bla_{\text{CTX-M-15}}$-*orf477Δ* transposon unit similar to a $bla_{\text{CTX-M-15}}$ harbouring IncFII plasmid pEK516 (GenBank EU935738) (Woodford et al., 2009) (see Fig. 5-2).

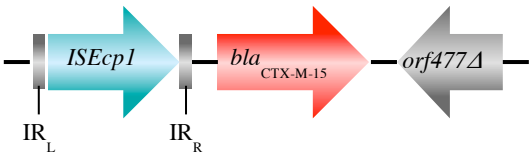


Figure 5-2: A schematic diagram of the genetic organisation surrounding the blaCTX-M-15 gene. This region was found to be similar (100 % homology) to an IncFII plasmid pEK516 (GenBank EU935738). The arrows indicate the orientation of each of the genes identified, which encoded the IS*Ecp1* transposase upstream of the $bla_{\text{CTX-M-15}}$ gene and downstream was the hypothetical protein orf477Δ (*amb*).

5.3.1.1 Drug resistance

Ten antimicrobial resistance genes were identified by sequencing conferring resistance to aminoglycosides (*aac(6')Ib* and *aadA4*), chloramphenicol (*catA1*), trimethoprims (*dfrA17*), sulphonamides (*sul1*), a macrolide inactivation cluster (*mphR-mrx-mphA*) and ESBL genes $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$ and $bla_{\text{CTX-M-15}}$. Genes conferring resistance to tetracycline included *tetR* (repressor), *tetC*, *tetD* and *tetA(B)* (efflux pumps). The nanoarray results identified this plasmid as encoding the *tetB* gene, however, the sequencing results identified the *tetA(B)* gene. The *tetA(B)* gene encoded on this plasmid was found to have 100 % nucleotide identity to *tetA(B)* of pIP1206 (GenBank CAP07771), a gene highly similar (99 % amino acid alignment) to *tetB* of *Haemophilus*

parasuis (GenBank YP_195816.1), but only 44 % similar (amino acid alignment) to *E. coli tetA* (GenBank ACQ42041) (Perichon et al., 2008) (see Fig. 5-3). These results suggest that the array is capable of detecting this variant of *tet* gene. Both proteins belong to the major facilitator superfamily involved in tetracycline efflux in an electrochemical proton gradient depended process (Kimura et al., 1998; Saraceni-Richards and Levy, 2000; Speer et al., 1992).

Other genes conferring resistance to various compounds included resistance to chromates (*chrA*), quaternary ammonium compounds (*qacE*) and copper (*scsS/scsD*).

Also identified were ATP-binding cassette (ABC) proteins, permeases and major facilitator (MFS) transporters predicted to be involved in iron/manganese transport (*sitABCD*). The role of these transporters in virulence and plasmid maintenance have not been confirmed, although the plasmid mediated iron transporters are thought to contribute to the virulence of pathogenic bacteria harbouring these plasmids, by enabling iron capture in the low iron environment, such as in the humans and animal hosts (Janakiraman and Slauch, 2000; Szczepanowski, et al., 2005).

```
tetAB_CAP07771        ---------------------------MNSSTKIALVITLLDAMGIGLIMPVLPTLLREF 33
tetB_YP_195816.1      MNCEFKLIYC----ILKIRKVKIEKSEMNSSTKIALVITLFDAMGIGLIMPVLPTLLREF 56
tetA_ACQ42041         MSTNLSVIKNPRVQSDQRRLVRRPDVKPNRPLIVILSTVALDAVGIGLIMPVLPGLLRDL 60
                                                * .  : *  . :**:********** ***::

tetAB_CAP07771        IASEDIANHFGVLLALYALMQVIFAPWLGKMSDRFGRRPVLLLSLIGASLDYLLLAFSSA 93
tetB_YP_195816.1      IASEDIANHFGVLLALYALMQVIFAPWLGKMSDRFGRRPVLLLSLIGASLDYLLLAFSSA 116
tetA_ACQ42041         VHSNDVTAHYGILLALYALMQFACAPVLGALSDRFGRRPVLLVSLAGAAVDYAIMATAPF 120
                      : *:*:: *:*:*********.  ** ** :***********:** **::** ::* :.

tetAB_CAP07771        LWMLYLGRLLSGITGATGAVAASVIADTTSASQRVKWFGWLGASFGLGLIAGPIIGGFAG 153
tetB_YP_195816.1      LWMLYLGRLLSGITGATGAVAASVIADTTSASQRVKWFGWLGASFGLGLIAGPIIGGFAG 176
tetA_ACQ42041         LWVLYIGRIVAGITGATGAVAGAYIADITDGDERARHFGFMSACFGFGMVAGPVLGGLMG 180
                      **:**:**:::**********.: *** *...:*.: **::.*.**:*::***::**: *

tetAB_CAP07771        EISPHSPFFIAALLNIVAFLVVMFWFRETKNTRDNTDTEVGVETQSNSVYITLFKTMPIL 213
tetB_YP_195816.1      EISPHSPFFIAALLNIVTFLVVMFWFRETKNTRDNTDTEVGVETQSNSVYITLFKTMPIL 236
tetA_ACQ42041         GFSPHAPFFAAAALNGLNFLTGCFLLPESHKGERRPLRREALNPLASFRWARGMTVVAAL 240
                       :***:*** ** ** : **.  * : *::: . ..  . .::. :.  :   :..:. *

tetAB_CAP07771        LIIYFSAQLIGQIPATVWVLFTENRFGWNSMMVGFSLAGLGLLHSVFQAFVAGRIATKWG 273
tetB_YP_195816.1      LIIYFSAQLIGQIPTTVWVLFTENRFGWNSMMVGFSLAGLGLLHSVFQAFVAGRIATKWG 296
tetA_ACQ42041         MAVFFIMQLVGQVPAALWVIFGEDRFHWDATTIGISLAAFGILHSLAQAMITGPVAARLG 300
                      : ::*  **:**:*:::**:* *:** *::  :*:***.:*:***: **:::* :*:: *

tetAB_CAP07771        EKTAVLLGFIADSSAFAFLAFISEGWLVFPVLILLAGGGIALPALQGVMSIQTKSHQQGA 333
tetB_YP_195816.1      EKTAVLLGFIADSSAFAFLAFISEGWLVFPVLILLAGGGIALPALQGVMSIQTKSHQQGA 356
tetA_ACQ42041         ERRALMLGMIADGTGYILLAFATRGWMAFPIMVLLASGGIGMPALQAMLSRQVDEERQGQ 360
                      *: *::**:***.:.: :*** :.**:.**:::***.***.:****.::* *....:**

tetAB_CAP07771        LQGLLVSLTNATGVIGPLLFAVIYNHSLPIWDGWIWIIGLAFYCIIILLSMTFMLTPQAQ 393
tetB_YP_195816.1      LQGLLVSLTNATGVIGPLLFAVIYNHSLPIWDGWIWIIGLAFYCIIILLSMTFMLTPQAQ 416
tetA_ACQ42041         LQGSLAALTSLTSIVGPLLFTAIYAASITTWNGWAWIAGAALY----LLCLPALRRGLWS 416
                      *** *.:**. *.::*****:.**  *:. *:** ** * *:*    **.:. :     .

tetAB_CAP07771        GSKQETSA 401
tetB_YP_195816.1      GSKQETSA 424
tetA_ACQ42041         GAGQRADR 424
                      *: *.:.
```

Figure 5-3: Amino acid alignment of TetA(B) of pI1-34TF, which has 100 % nucleotide identity to TetA(B) of pIP1206 (GenBank CAP07771). This protein was found to have 99 % homology to TetB of *Haemophilus parasuis* (GenBank YP_195816.1) but only 44 % homologous to *E. coli* TetA (GenBank ACQ42041). Alignments were performed using the web based ClustalW algorithm (ww.ebi.ac.uk). Key: "*"; identical, ":" (colon) and "." (dot/full stop) are conserved and semiconserved substitutions respectively.

5.3.1.2 Hypothesis of plasmid evolution

Plasmid pI1-34TF was found to harbour three replicons FIA, FIB and FII. The plasmid scaffold was a mosaic of different plasmids, in that only about 60 % of the plasmid was homologous to pIP1206; previously identified as harbouring the IncFIA-FIB-FII multiple replicon and only about 45-50 % similarity to pEK499 and pEC-L8 harbouring the *bla*$_{\text{CTX-M-15}}$ gene on an IncFII-FIA fusion plasmid. The ability of the plasmid to switch replication to a different replicon (IncFIA, FIB or FII) could enable the acquisition of an additional plasmid harbouring an IncF replication system to replicate in the same cell. The coexistence with other IncF-like plasmids may enable recombination between other incoming (but not limited to) IncF plasmids resulting in the variability observed between this plasmid and other IncF mutlireplicon plasmids.

Furthermore, the parent strain was found to harbour multiple plasmids leading to the possibility of recombination events occurring between the pI1-34TF plasmid and other plasmids harboured within the strain, further expanding the genetic pool available to contribute to genetic plasticity of the plasmid. The mosaic nature of pI1-34TF plasmid scaffold is most likely the result of multiple recombination or transposition events that have occurred mediated by regions of homology (site specific or homologous recombination).

5.3.2 Analysis of pI2-47TC

Replicon typing identified this plasmid as belonging to the IncI1 group and it was found to be ~ 86 kb in size. This plasmid co-transferred along with a smaller ~35 kb plasmid (pI2-47TF) found to be untypeable using the PBRT scheme (table 5-1). DNA was extracted from the transconjugant derivative of this plasmid (pI2-47TC) and introduced into *E. coli* DH10B by transformation. This plasmid was selected for sequencing because of the nature of the co-transfer of the two plasmids. The resulting sequenced plasmid pI2-47TC was found to be approximately 127 kb in size. Sequence analysis of this plasmid and the possible hypothesis of the evolution of this plasmid are discussed below.

5.3.2.1 Plasmid scaffold

The sequence pI2-47TC was found to have 59 % similarity (75 kb) to a 93 kb IncI1 plasmid; pEK204 (GenBank EU935740) isolated in *E. coli* encoding the $bla_{\text{CTX-M-3}}$ variant (Woodford et al., 2009) (see Fig. 5-4A). This plasmid harboured 175 predicted ORFs (see table 5-3). Regions of similarity included; extensive conjugative transfer genes *tra* (~ 25 kb), *trb* (~ 5 kb) and *pil* (~ 11 kb) loci; plasmid maintenance proteins encoded by *psiB* and *ssb*; and the IncI1 *repZ* replicase gene. The *repZ* gene and type IV pili are characteristics of IncI1 plasmids (Woodford et al., 2009).

In addition to the IncI1-like conjugative systems identified, pI2-47TC was found to encode genes showing 99-100% identity to *taxABC* and *pilX* genes and a π replication protein (*pir*). These are conjugative systems characteristic of IncX plasmids (Chu et al., 2008; Nunez et al., 1997; Norman et al., 2008; Partridge et al., 2011). Plasmid pI2-47TC was found to have 25 % (31 kb) overall similarity to a 34.3 kb IncX1 plasmid pJIE143 (GenBank JN194214), isolated in *E. coli* encoding the $bla_{\text{CTX-M-15}}$ variant (Partridge et al., 2011) (see Fig. 5-4B).

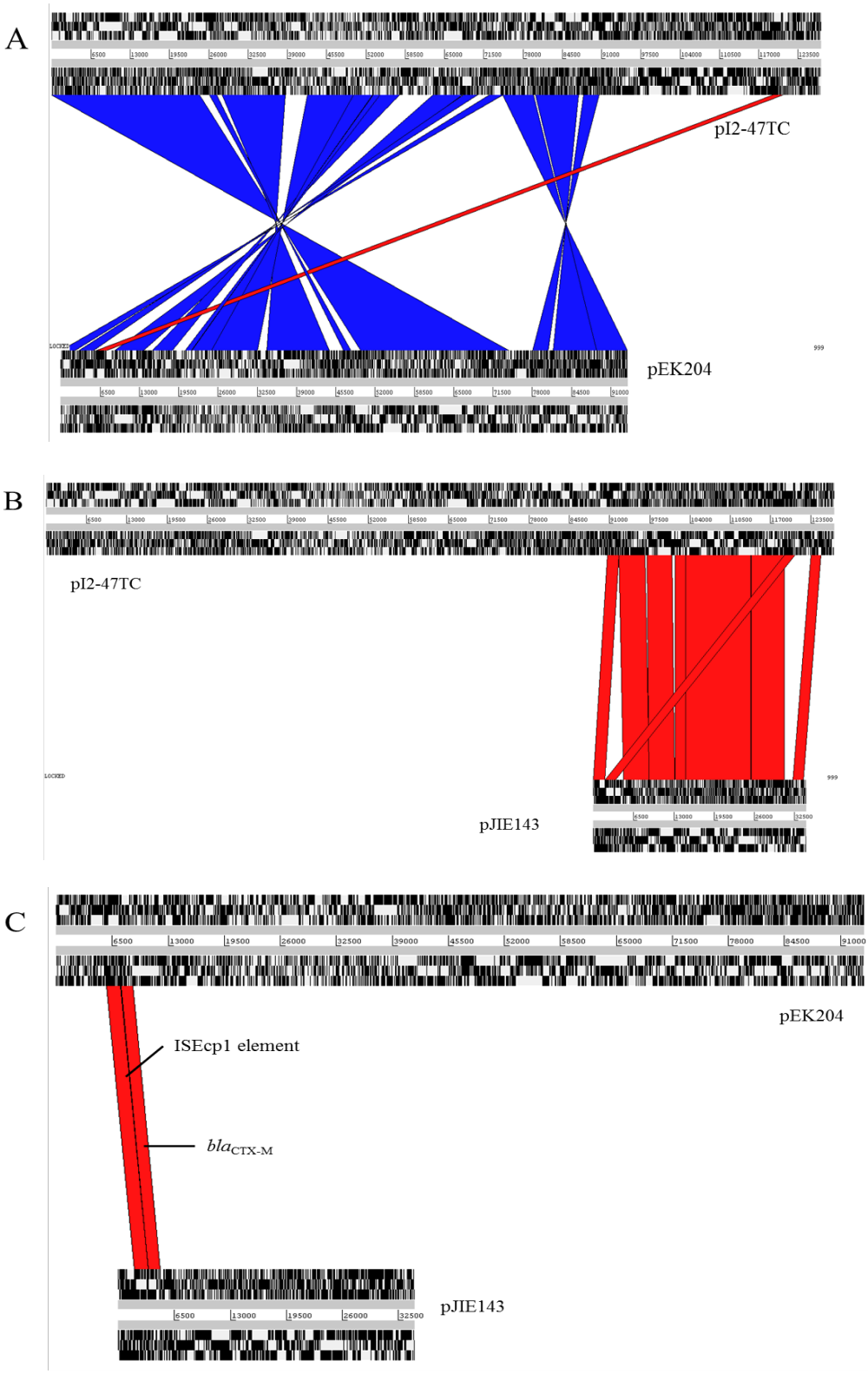


Figure 5-4: Sequence alignments of (A) pI2-47TC with pEK204 and (B) pJIE143 using WebACT (www.webact.org) with a cut-off of 1000 bp. A) Plasmid pEK204 (GenBank EU935740) was found to have 59 % similarity to pI2-47TC. **B)** Plasmid pJIE143, an IncX1 plasmid was found to have 25 % similarity to pI2-47TC. **C)** Homology of a ~ 3kb region encoding the IS*Ecp1* mobile element (100 % similarity) and bla_{CTX-M} gene (99 %) was observed between the pJIE143 ($bla_{CTX-M-15}$) and pEK204 $bla_{CTX-M-3}$, which only differ by one amino acid base change (D240G). The IS*Ecp1* region homology was shared with pI2-47TC but this plasmid was found to harbour a $bla_{CTX-M-14b}$ gene.

Table 5-3: Predicted open reading frames identified in pI2-47TC.

Contig	Function	Start	Stop	Strand
1	Shufflon-specific DNA recombinase	33	1220	+
1	IncI1 plasmid conjugative transfer protein TraE	1371	2195	+
1	IncI1 plasmid conjugative transfer protein TraF	2281	3483	+
1	IncI1 plasmid conjugative transfer protein TraG	3543	4127	+
1	hypothetical protein	4290	4141	-
1	IncI1 plasmid conjugative transfer protein TraH	4522	4980	+
1	IncI1 plasmid conjugative transfer protein TraI	4977	5795	+
1	IncI1 plasmid conjugative transfer protein TraJ, related to pilus biogenesis/retracton protein	5792	6940	+
1	IncI1 plasmid conjugative transfer protein TraK	6937	7227	+
1	Plasmid conjugative transfer endonuclease	7242	7793	+
1	IncI1 plasmid conjugative transfer DNA primase	7883	11650	+
1	IncI1 plasmid conjugative transfer protein TraL	11668	12015	+
1	IncI1 plasmid conjugative transfer protein TraM	12012	12704	+
1	IncI1 plasmid conjugative transfer protein TraN	12715	13698	+
1	IncI1 plasmid conjugative transfer protein TraO	13701	14990	+
1	IncI1 plasmid conjugative transfer protein TraP	14990	15694	+
1	IncI1 plasmid conjugative transfer protein TraQ	15694	16221	+
1	IncI1 plasmid conjugative transfer protein TraR	16272	16676	+
1	IncI1 plasmid conjugative transfer protein TraS	16740	16928	+
1	IncI1 plasmid conjugative transfer protein TraT	16912	17712	+
1	IncI1 plasmid conjugative transfer protein TraU	17709	20846	+
1	IncI1 plasmid conjugative transfer protein TraV	20846	21460	+
1	IncI1 plasmid conjugative transfer protein TraW	21427	22629	+
1	IncI1 plasmid conjugative transfer protein TraX	22658	23242	+
1	IncI1 plasmid conjugative transfer integral membrane protein TraY	23270	25507	+
1	surface exclusion protein	25581	26231	+
1	hypothetical protein	26512	26303	-
1	hypothetical protein	26747	26875	+
1	FIG00641806: hypothetical protein	26903	27079	+
1	hypothetical protein	27695	27567	-
1	FIG01047462: hypothetical protein	27738	27989	+
1	post-segregation killing protein	28216	28061	-
1	TrbA	28505	29713	+
1	TrbB protein	29732	30802	+
1	FIG00640314: hypothetical protein	30795	33086	+
1	involved in conjugative DNA transfer	35822	33123	-
1	Nickel ABC transporter, periplasmic nickel-binding protein NikA (TC 3.A.1.5.3)	36165	35833	-
1	hypothetical protein	36293	36409	+
1	hypothetical protein	36399	36734	+
1	YdiA	37668	36820	-
1	hypothetical protein	37874	37710	-
1	hypothetical protein	37900	38013	+
1	hypothetical protein	38017	38214	+
1	FIG00642528: hypothetical protein	38248	38499	+
1	FIG00639209: hypothetical protein	38727	38530	-
1	Transposase	39454	38750	-
1	hypothetical protein	39684	39568	-
1	Mobile element protein	39686	41221	+
1	Mobile element protein	41238	41993	+
1	YDFB protein	42639	42373	-
1	FIG01046738: hypothetical protein	43167	42733	-
1	hypothetical protein	43166	43360	+
1	hypothetical protein	43353	43475	+
1	hypothetical protein	43912	43769	-
1	Antirestriction protein ArdA	44575	43896	-
1	hypothetical protein	45454	44858	-
1	PsiA protein	46170	45451	-
1	PsiB protein	46604	46167	-
1	FIG01047988: hypothetical protein	48614	46656	-
1	UPF0401 protein yubL	48906	48673	-
1	Single-stranded DNA-binding protein	49491	48964	-
1	hypothetical protein	50189	49875	-
1	FIG00638431: hypothetical protein	50451	50260	-
1	FIG01047188: hypothetical protein	50870	50448	-
1	Putative antirestriction protein	51342	50917	-
1	hypothetical protein	51637	51308	-
1	unknown; orf44	51752	51591	-
1	FIG00638373: hypothetical protein	52531	51755	-
1	YcgB	53011	52577	-
1	putative cytoplasmic protein	53246	53025	-
1	Adenine-specific methyltransferase (EC 2.1.1.72)	53930	53247	-
1	FIG00637984: hypothetical protein	54037	54162	+
1	FIG01048508: hypothetical protein	55216	54314	-
1	hypothetical protein	55258	55371	+

Continued on the following page.

Table 5-3: *continued.*

Contig	Function	Start	Stop	Strand
1	hypothetical protein	55473	55882	+
1	Error-prone repair protein UmuD	55879	56316	+
1	Error-prone, lesion bypass DNA polymerase V (UmuC)	56316	57590	+
1	stable plasmid inheritance protein	58008	57592	-
1	Putative stability/partitioning protein encoded within prophage CP-933T-stbB	59023	58001	-
1	ybiA	59702	59394	-
1	FIG01048970: hypothetical protein	60466	59789	-
1	Resolvase	61392	60613	-
1	FIG00642857: hypothetical protein	61807	61394	-
1	Virulence-associated protein vagC	62381	62611	+
1	VagD	62608	63024	+
1	Beta-lactamase (EC 3.5.2.6)	64082	63222	-
1	Resolvase/integrase Bin	64822	64265	-
1	transposase	64986	67991	+
1	hypothetical protein	69218	69367	+
1	FIG00644632: hypothetical protein	69684	70286	+
1	FIG00643565: hypothetical protein	70303	70836	+
1	hypothetical protein	71054	71203	+
1	stability (stb) locus of IncFII plasmid NR1; similar to SwissProt accession number P11907	71575	71297	-
1	FIG01069112: hypothetical protein	71956	71681	-
1	hypothetical protein	72234	71956	-
1	hypothetical protein	72676	72792	+
1	replication initiation protein	74224	73148	-
1	hypothetical protein	74155	74475	+
1	hypothetical protein	75333	75620	+
1	hypothetical protein	75934	76056	+
1	IncI1 plasmid conjugative transfer NusG-type transcription antiterminator TraB	76062	76595	+
1	hypothetical protein	76745	76629	-
1	IncI1 plasmid conjugative transfer protein TraC	76794	77531	+
1	hypothetical protein	77704	78255	+
1	IncI1 plasmid conjugative transfer protein PilI	78417	78671	+
1	hypothetical protein	79040	78777	-
1	IncI1 plasmid conjugative transfer protein PilK	79431	79724	+
1	IncI1 plasmid conjugative transfer protein PilL	80053	81120	+
1	IncI1 plasmid conjugative transfer protein PilM	81120	81557	+
1	IncI1 plasmid conjugative transfer lipoprotein PilN	81571	83253	+
1	IncI1 plasmid pilus assembly protein PilO	83246	84541	+
1	IncI1 plasmid pilus assembly protein PilP	84528	84980	+
1	IncI1 plasmid conjugative transfer ATPase PilQ	84991	86544	+
1	IncI1 plasmid conjugative transfer inner membrane protein PilR	86557	87642	+
1	IncI1 plasmid conjugative transfer prepilin PilS	87659	88273	+
1	IncI1 plasmid conjugative transfer putative membrane protein PilT	88589	88843	+
1	Type-IV sectretion leader peptidase/N-methyltransferase	88828	89484	+
1	hypothetical protein	435	560	+
2	hypothetical protein	920	804	-
2	PI protein (Replication initiation protein)	1009	1881	+
2	hypothetical protein	1936	2298	+
2	hypothetical protein	2433	2960	+
2	hypothetical protein	3304	3444	+
2	FIG01069805: hypothetical protein	3795	3544	-
2	FIG01069787: hypothetical protein	4133	3831	-
2	Chromosome (plasmid) partitioning protein ParA	4886	4227	-
2	Resolvase	5857	4991	-
2	hypothetical protein	6349	6155	-
2	hypothetical protein	7365	6709	-
2	DNA-binding protein H-NS	7933	7469	-
2	Haemolysin expression modulating protein	8155	7949	-
2	DNA topoisomerase III (EC 5.99.1.2)	10305	8152	-
2	hypothetical protein	10894	10310	-
2	Exodeoxyribonuclease V beta chain (EC 3.1.11.5)	11395	10991	-
2	hypothetical protein	11905	11411	-
2	hypothetical protein	12210	11902	-
2	hypothetical protein	12567	12313	-
2	IncN plasmid KikA protein	12860	12573	-
2	hypothetical protein	13281	12862	-
2	Type IV secretion system protein VirD4	15155	13293	-
2	ATPase provides energy for both assembly of type IV secretion complex and secretion of T-DNA c	16170	15142	-
2	Inner membrane protein forms channel for type IV secretion of T-DNA complex (VirB10)	17290	16172	-
2	Forms the bulk of type IV secretion complex that spans outer membrane and periplasm (VirB9)	18188	17283	-
2	Inner membrane protein forms channel for type IV secretion of T-DNA complex (VirB8)	18874	18188	-
2	hypothetical protein	18995	18867	-
2	hypothetical protein	19383	19213	-
2	Inner membrane protein of type IV secretion of T-DNA complex, VirB6	20483	19389	-
2	Minor pilin of type IV secretion complex (VirB5)	21211	20495	-

Continued on the following page.

Table 5-3: *continued.*

Contig	Function	Start	Stop	Strand
2	ATPase provides energy for both assembly of type IV secretion complex and secretion of T-DNA c	23970	21223	-
2	FIG01068513: hypothetical protein	24315	23989	-
2	Bores hole in peptidoglycan layer allowing type IV secretion complex assembly to occur (VirB1)	24948	24376	-
2	hypothetical protein	25127	24951	-
2	IncQ plasmid conjugative transfer protein TraG	25520	25305	-
2	ORF2	25810	25517	-
2	IncQ plasmid conjugative transfer DNA nicking endonuclease TraR (pTi VirD2 homolog)	27116	25887	-
2	ORF8	27623	27120	-
2	hypothetical protein	27846	27971	+
2	COG4226: Uncharacterized protein encoded in hypervariable junctions of pilus gene clusters	28331	27984	-
2	HicA	28576	28328	-
2	Mobile element protein	28860	30122	+
2	hypothetical protein	30103	30225	+
2	hypothetical protein	30392	30270	-
2	Beta-lactamase (EC 3.5.2.6)	30396	31247	+
2	S-methylmethionine permease	31897	31649	-
2	hypothetical protein	32046	31909	-
2	hypothetical protein	32472	32122	-
2	hypothetical protein	32557	32432	-
2	hypothetical protein	32781	32563	-
2	hypothetical protein	283	56	-
3	FIG00643296: hypothetical protein	521	270	-
3	FIG00643553: hypothetical protein	1060	518	-
3	COG2214: DnaJ-class molecular chaperone	1680	1165	-
3	IncI1 plasmid conjugative transfer pilus-tip adhesin protein PilV	88	336	+
4	hypothetical protein	17	223	+
5	hypothetical protein	191	18	-

5.3.2.2 Plasmid transfer, establishment and maintenance

Like the IncI1 pEK204 plasmid (Woodford et al., 2009), pI2-47TC encoded genes associated with IncI1 plasmids including the type IV pilus (*pil* prepilin genes); thin pili required for liquid mating and the RepZ replicase gene (Kim and Komano, 1997; Woodford et al., 2009). In addition to the F-like pili encoded by the *pil* genes (part of the type IV secretion system), this plasmid also encoded the *tra* genes that make up the rigid pili of IncI1 plasmids (Komano et al., 1990). The conjugal transfer associated proteins; *exc* (surface exclusion protein), *traE-Y* (F-pilus), *trbABC* (putative transfer proteins), *sogS/sogL* (DNA primase and regulatory protein), and *nikAB* (relaxase and regulatory protein) were found make up 22 % of the total number of predicted genes in pI2-47TC.

Interestingly this plasmid also encoded Pilx/Tax conjugation proteins previously identified in IncX plasmids (Norman et al., 2008; Nunez et al., 1997; Partridge et al., 2011). Plasmid pJIE143 was found to (GenBank JN194214) have 25 % similarity to pI2-47TC, and found to encode a π-like replication protein encoded by *pir*. Also identified, were the seven oriV-γ iterons, which provide binding sites for the π protein (Germino and Bastia, 1983). This protein has been studied extensively in the archetypal IncX2 plasmid R6K and is known to be involved in the replication and copy number control of this plasmid (Filutowicz et al., 1986; Nunez et al., 1997; Partridge et al., 2011).

The plasmid encoded two toxin-antitoxin genes: *pndA/pndC* and *hicA/hicB*, identified in pEK204 and pJIE143 respectively (Partridge et al., 2011; Woodford et al., 2009). Other plasmid establishment and maintenance systems included the Psi, SsB; involved in conjugation and HHA; a histone-like protein found on IncX1 pJIE143 (Partridge et al., 2011).

5.3.2.3 Mobile genetic elements

A Tn*3* resolvase (*tnpR*), transposase (*tnpA*) and a 38bp inverted repeat were identified adjacent to the $bla_{\text{TEM-1}}$ gene, all genes essential for Tn*3* transposition (Kostriken et al., 1981).

The IS*Ecp1* element was identified 42 base pairs upstream of $bla_{\text{CTX-M-14b}}$. The IS*Ecp1* element is commonly associated with $bla_{\text{CTX-M}}$ genes and has been implicated in driving expression of downstream genes in addition to mobilising $bla_{\text{CTX-M}}$ (Poirel et al., 2003; Poirel et al., 2005).

5.3.2.4 Drug resistance

This plasmid was found by sequencing to harbour two resistance genes namely, $bla_{\text{CTX-M-14b}}$ and $bla_{\text{TEM-1.}}$ The $bla_{\text{TEM-1}}$ gene was not identified using the array (table 5-1). This anomaly was not acknowledged because the phenotype conferring resistance to ampicillin, ceftazidime and cefotaxime, denoted the presence of at least one ESBL to which $bla_{\text{CTX-M}}$ was attributed to give rise to the resulting phenotype. This reveals a greater accuracy of plasmid sequencing in detecting additional genes that may not be detected during phenotypic and high-throughput screening methods.

Although the $bla_{\text{CTX-M-14b}}$ gene was not identified in either pEK204 (IncI1, $bla_{\text{CTX-M-3}}$) or pJIE143 (IncX1, $bla_{\text{CTX-M-15}}$), the genetic environment surrounding $bla_{\text{CTX-M}}$ genes was similar to that of pI2-47TC. All three plasmids harboured the IS*Ecp1*element upstream $bla_{\text{CTX-M}}$ (99 % similarity); encoding the IR_L (left inverted repeat), IS*Ecp1* associated transposase (*tnpA*) and the IR_R (right inverted repeat) (Partridge et al., 2011; Woodford et al., 2009).

The $bla_{\text{CTX-M-14b}}$ gene and associated *ISEcp1* element was found to be 100 % similar to the genetic platform of plasmid pCT (GenBank FN868832); an epidemic IncK $bla_{\text{CTX-M-14b}}$ plasmid from *E. coli*, which was isolated from a calf on a dairy farm in the UK (Cottell et al., 2011) (Fig. 5-5A). Interestingly, in pCT the region encoding the IS*Ecp1*

element and associated transposase had 100 % nucleotide identity to pEK204 and pJIE143 and was also inverted as in pI2-47TC (Fig. 5-5B). Analysis of this region suggests possible acquisition of the *bla*$_{\text{CTX-M-14b}}$ gene via an IS*Ecp1* associated transposition event recognising the 14 bp inverted repeat sequences flanking these genes (Poirel et al., 2003; Poirel et al., 2005). This could explain the presence of the *bla*$_{\text{CTX-M-14b}}$ variant encoded by pI2-47TC within a scaffold similar to pEK204 and pJIE143, encoding *bla*$_{\text{CTX-M-3}}$ and *bla*$_{\text{CTX-M-15}}$variants, respectively.

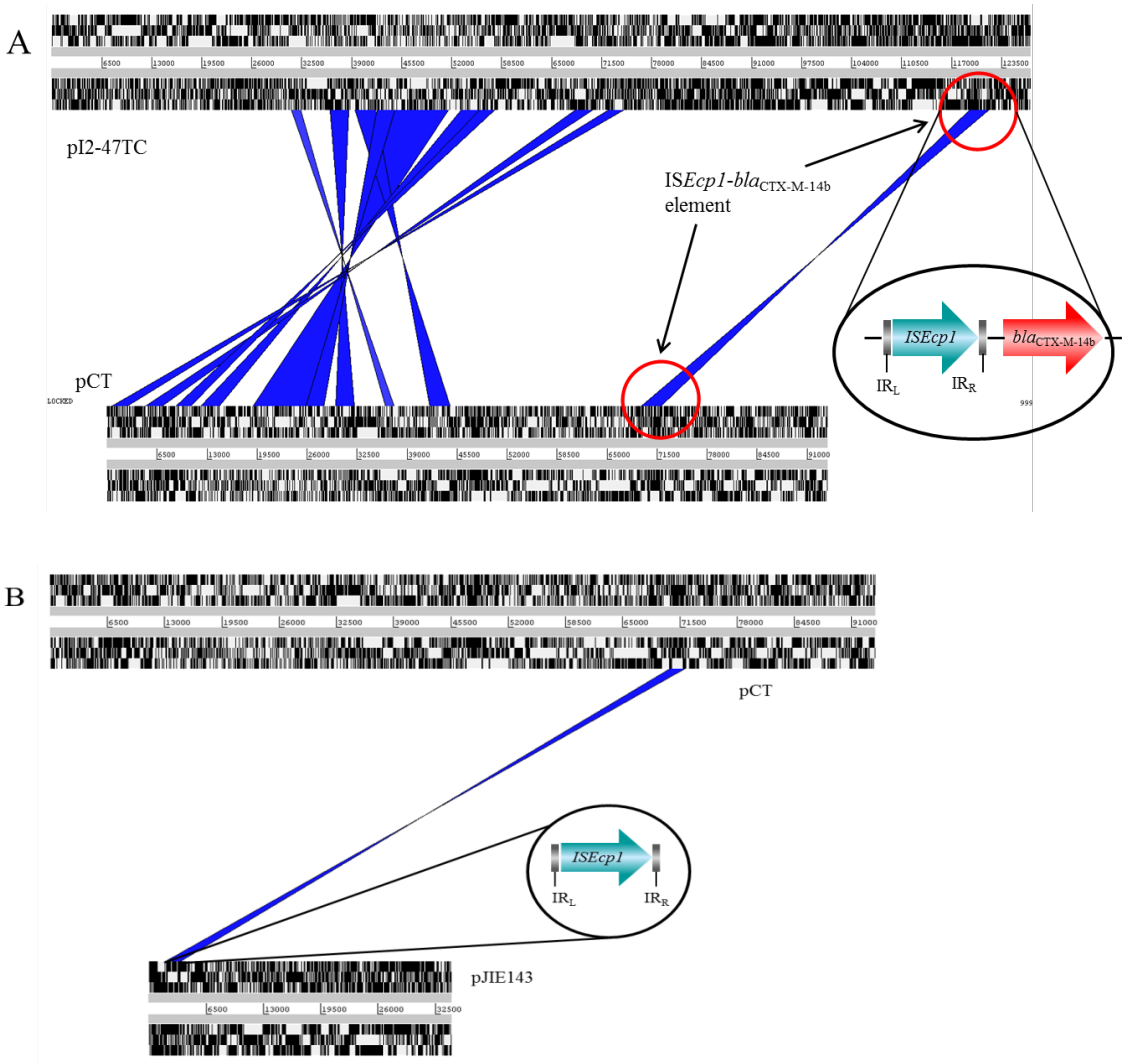


Figure 5-5: Genetic platform surrounding the *bla*$_{\text{CTX-M-14b}}$ gene. The genetic environment surrounding the *bla*$_{\text{CTX-M-14b}}$ gene was found to have 99 % nucleotide identity to pCT a *bla*$_{\text{CTX-M-14b}}$ harbouring IncK plasmid. The IS*Ecp1* element was also identified in the pJIE143 (*bla*$_{\text{CTX-M-15}}$) plasmid. The presence of both elements could result in possible recombination events leading to the mobilisation of *bla*$_{\text{CTX-M}}$ downstream.

5.3.2.5 Hypothesis of plasmid co-integration

The *bla*$_{\text{CTX-M-14b}}$ gene was identified on a single contig, which also harboured the *pil*X, *tax* and *pir* genes with 92 % nucleotide identity to pJIE143. This suggests that the IncX1 pJIE143-like scaffold harbours the *ISEcp1-bla*$_{CTX\text{-M-14}}$ region and the IncX conjugative elements, but also has association with an IncI1-like plasmid scaffold, encoding IncI1 conjugative proteins.

The identification of two replication control systems; IncX1 and IncI1, could be the result of a fusion of the two replicons (IncX1 and IncI1). This phenomenon has been observed previously in IncFII-FIA plasmids (Perichon et al., 2008; Szczepanowski et al., 2005; Woodford et al., 2009). Another possibility is the recombination of the two plasmids *in vivo* during conjugation, leading to the formation of a con-integrate resulting in a larger sequenced plasmid, a phenomenon that has been described previously in IncFII-HI2 co-integrate plasmid (Bradley et al., 1986). Evidence that supports this theory includes results from the initial plasmid sizing of the transconjugant by S1 nuclease PFGE (linear DNA), which identified two distinct plasmids approximately 86 kb and 35 kb in size (Fig. 5-6B). The resulting plasmid sequenced (pI2-47TC) was found to be ~ 130 kb, which is approximately the sum of both these plasmids. In addition, when IncI1-pEK204 and IncX-pJIE143 were merged into a pseudomolecule and compared with the pI2-47TC pseudomolecule, the alignments showed two distinct plasmid scaffolds of the IncI1 and IncX1 plasmids within the co-integrate (Fig. 5-6A)

These plasmids could to be sequenced individually (pre-integration) to determine any regions of homology that may have facilitated the co-integration events between an IncX1 and IncI1 plasmid, by homologous recombination to form pI2-47TC.

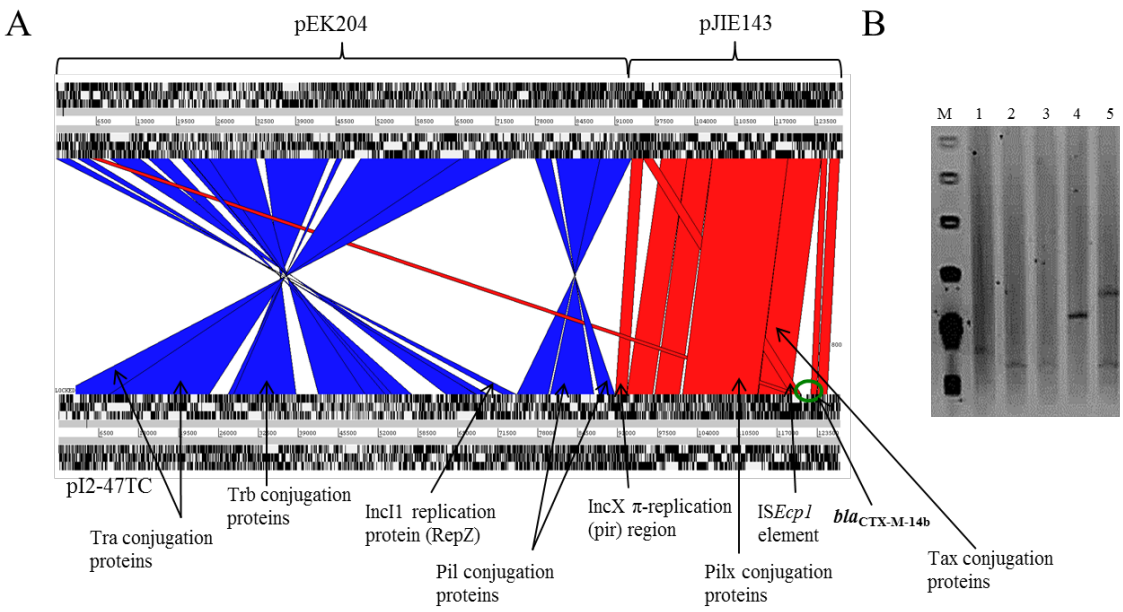


Figure 5-6: Hypothesis of pI1-47TC co-integration of a smaller mobilizable plasmid and larger conjugative plasmid. A) Sequence alignment of pI2-47TC pseudomolecule with pEK204 and pJIE143 pseudomolecule depicts the hypothesis of proposed plasmid co-integration. Plasmid pI2-47TC was found to harbour both IncI1 and IncX1 replication systems, most similar to IncI1 pEK204 ($bla_{CTX\text{-}M\text{-}3}$) and IncX1 pJIE143 ($bla_{CTX\text{-}M\text{-}15}$). **B)** S1 nuclease-PFGE gel image showing the two ~35 kb and ~86 kb plasmids in the transconjugant I2-47TC (lane 5) in addition to other plasmids untypeable (by PBRT) plasmids of similar size to the smaller 35 kb plasmid isolated from the same farm (lane 1-4).

5.3.3 Analysis of pI2-52TC

Plasmid pI2-52TC was isolated from a strain from the same farm as pI2-47TC and pI2-53TF. This plasmid was transferable by in vitro conjugation and was identified as belonging to the IncB/O group by PBRT. S1 nuclease PFGE estimated the size of this plasmid at 151 kb. The resistance gene profile of plasmid pI2-52TC differed from other plasmids of this region with profiles similar to pI2-53TF encoding: *aadA1, aadA2, cmlA1,* $bla_{CTX\text{-}M\text{-}32}$*, dfr12, sul3* and *tetA*. However, plasmid pI2-52TC was found to harbour four additional resistance genes: *dfrA14*, *sul2*, *strB* and $bla_{TEM\text{-}1}$. To investigate whether this donor field isolate also harboured a plasmid with a similar resistance gene profile and a plasmid of similar size to other plasmids (untypeable 30-40 kb plasmids) isolated from the same farm. DNA extracted from the field strain (I2-52, see chapter 3), and transformed into *E. coli* DH10B and plasmid sizing and DNA array experiments

performed (see chapter 2 for details). The resulting plasmid sizes and resistance gene profile of the transformant was found to be similar to other untypeable ~30 – 40 kb plasmids from this farm e.g. pI2-53TF (see table 5-1). The isolation of two plasmids encoding bla_{CTX-M} group 1 genes from the donor strain by two different methods (conjugation and transformation) gave two plasmids; pI2-52TC (~150 kb) and pI2-52TF (~40 kb). These plasmids were found to have similar resistance gene profiles.. To investigate the relationship of the larger conjugative plasmid encoding similar resistance genes and other untypeable plasmids of this farm, the genetic composition of the ~150 kb pI2-52TC plasmid was analysed, and discussed later is the sequence analysis of the smaller 40 kb plasmid pI2-53TF.

5.3.3.1 Plasmid Scaffold

This plasmid was found to have 67 % similarity to a 110 kb IncB plasmid (pR3521: GenBank GU256641) from *E. coli* isolated from a hospitalised patient in Greece (Papagiannitsis et al., 2011) (Fig. 5-7A). However, pR3521 did not encode a bla_{CTX-M} gene. The genetic organisation surrounding bla_{CTX-M} was found to be similar to a $bla_{CTX-M-32}$ *E. coli* strain ECO39 (GenBank EU921825) (Fig. 5-9) (Lee et al., 2009). ECO39 plasmid was found to encode a Pir (initiator RepB) replication protein, toxin-antitoxin system (StbED), plasmid partition protein (ParA) and resolvase (TnpR) similar to an IncX1 plasmid (pOU1114, GenBank DQ115387) isolated from *E. coli* (Chu et al., 2008) (Fig. 5-8). This plasmid harboured 186 predicted ORFs (see table 5-4).

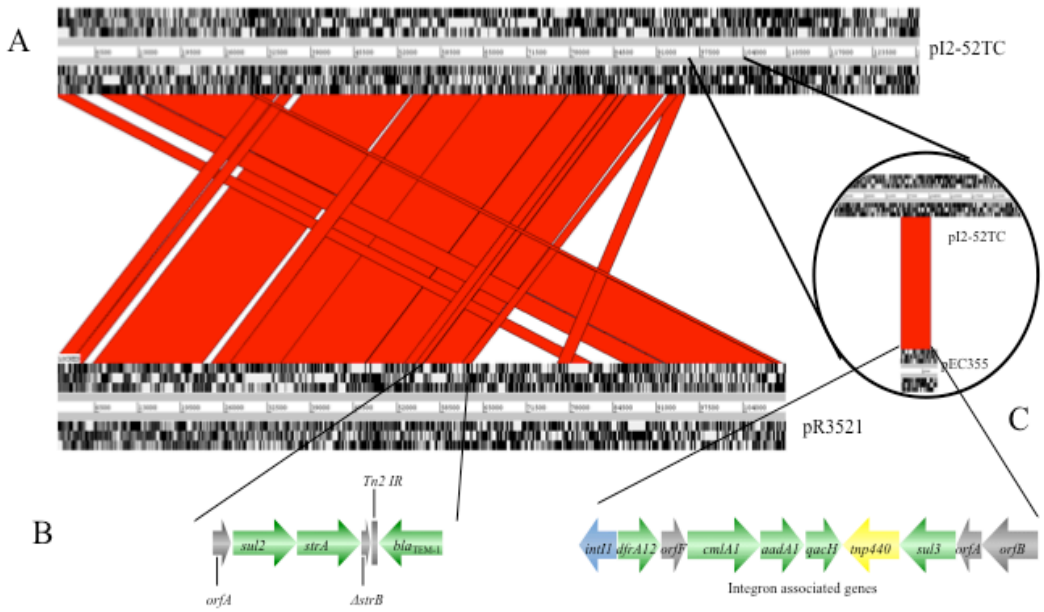


Figure 5-7: Plasmid scaffold of pI2-52TC. A) Plasmid pI2-52TC was found to have 67 % similarity (89 kb) to an IncB plasmid isolated from *E. coli* (pR3521: GU256641). **B)** The similarity regions harboured the *tra* and *pil* conjugative transfer genes and antimicrobial resistance genes $bla_{\text{TEM-1}}$, *strA* and *sul2*. **C)** The integron 1 associated multi-resistance gene region was similar in genetic organisation to *E. coli* plasmid pEC355 (partial sequence) (FM244708), a region spanning 7 % of the 130 kb sequence encoding *dfrA12-orfF-cmlA1-aadA1-qac-sul3* resistance genes. WebACT (www.webact.org) was used to make the alignments (1000 bp cut-off) and determine regions of similarity.

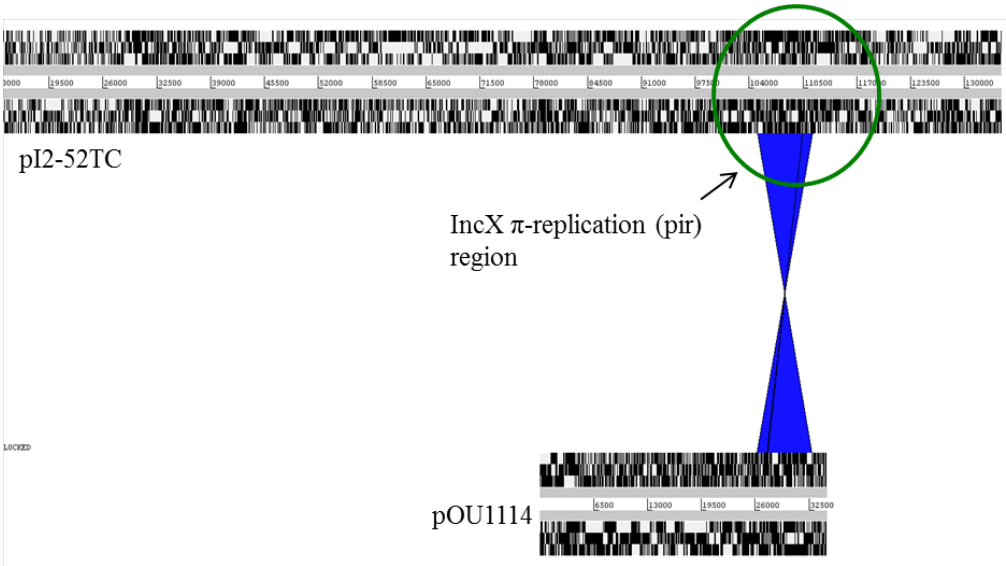


Figure 5-8: Plasmid pI2-52TC IncX replication region. Plasmid pI2-52TC encoded a π replication protein (*pir*) previously identified in IncX plasmids including the archetypal R6K plasmid. This region of similarity (95-98 %) to pOU1114 also harboured toxin-antitoxin (*stbE/D*), partition protein (*parA*) and resolvase (*tnpR*) genes. WebACT (www.webact.org) was used to make the alignments (1000 bp cut-off) and determine regions of similarity.

Table 5-4: Predicted open reading frames identified in pI2-52TC.

Contig	Function	Start	Stop	Strand
1	transposase	198	2747	+
1	streptomycin phosphotransferase	2740	3513	+
1	Insertion Sequence Associated	5361	3790	-
1	orf, conserved hypothetical protein	5728	5381	-
1	Insertion Sequence Associated	6405	5728	-
1	putative transposase	6722	6405	-
1	hypothetical protein	6858	6983	+
1	FIG00638738: hypothetical protein	7476	7081	-
1	involved in conjugative DNA transfer	10291	7580	-
1	Nickel ABC transporter, periplasmic nickel-binding protein NikA (TC 3.A.1.5.3)	10635	10303	-
1	hypothetical protein	10762	10878	+
1	hypothetical protein	10868	11203	+
1	YdiA	12137	11289	-
1	hypothetical protein	12343	12179	-
1	hypothetical protein	12369	12482	+
1	hypothetical protein	12542	12682	+
1	FIG00642528: hypothetical protein	12716	12967	+
1	FIG00639209: hypothetical protein	13204	12998	-
1	Transposase	14141	13263	-
1	CcgAII protein	14602	14138	-
1	hypothetical protein	14639	14511	-
1	hypothetical protein	14590	14703	+
1	YDFB protein	14970	14704	-
1	FIG01046738: hypothetical protein	15498	15064	-
1	hypothetical protein	15710	15495	-
1	hypothetical protein	15736	15936	+
1	hypothetical protein	15981	16115	+
1	Antirestriction protein ArdA	17121	16231	-
1	hypothetical protein	17789	17193	-
1	PsiA protein	18505	17786	-
1	PsiB protein	18936	18502	-
1	FIG00638906: hypothetical protein	20955	18991	-
1	Putative cytoplasmic protein	21241	21008	-
1	Single-stranded DNA-binding protein	21826	21299	-
1	YchA	22458	22210	-
1	FIG00638431: hypothetical protein	22787	22596	-
1	FIG01047188: hypothetical protein	23206	22784	-
1	Putative antirestriction protein	23678	23253	-
1	hypothetical protein	23835	23975	+
1	hypothetical protein	24094	23927	-
1	hypothetical protein	24864	24094	-
1	hypothetical protein	25343	24909	-
1	putative cytoplasmic protein	25578	25357	-
1	Adenine-specific methyltransferase (EC 2.1.1.72)	26262	25579	-
1	hypothetical protein	26369	26494	+
1	FIG01048508: hypothetical protein	27549	26647	-
1	FIG00640646: hypothetical protein	27806	28216	+
1	Error-prone repair protein UmuD	28213	28650	+
1	Error-prone, lesion bypass DNA polymerase V (UmuC)	28650	29921	+
1	Chromosome (plasmid) partitioning protein ParA	30210	30743	+
1	hypothetical protein	30740	31042	+
1	hypothetical protein	31544	31380	-
1	FIG00641828: hypothetical protein	32899	31553	-
1	hypothetical protein	32977	33096	+
1	FIG00644632: hypothetical protein	33253	33855	+
1	FIG00643565: hypothetical protein	33872	34387	+
1	FIG00732908: hypothetical protein	34384	34653	+
1	stability (stb) locus of IncFII plasmid NR1; similar to SwissProt accession number P11907	35125	34844	-
1	FIG01069112: hypothetical protein	35506	35231	-
1	hypothetical protein	35790	35506	-
1	hypothetical protein	36345	36211	-
1	RepA1	37567	36695	-
1	hypothetical protein	37792	37604	-
1	IncI1 plasmid conjugative transfer NusG-type transcription antiterminator TraB	38270	38911	+
1	IncI1 plasmid conjugative transfer protein TraC	39052	39714	+
1	hypothetical protein	39981	40601	+
1	Inner membrane protein YqiK	40628	42322	+
1	hypothetical protein	42403	42645	+
1	hypothetical protein	43072	42941	-
1	hypothetical protein	43129	43245	+
1	IncI1 plasmid conjugative transfer protein PilL	43323	44393	+
1	IncI1 plasmid conjugative transfer protein PilM	44397	44834	+
1	IncI1 plasmid conjugative transfer lipoprotein PilN	44866	46485	+
1	IncI1 plasmid pilus assembly protein PilO	46506	47801	+

Continued on the following page.

Table 5-4: *continued.*

Contig	Function	Start	Stop	Strand
1	IncI1 plasmid pilus assembly protein PilP	47791	48249	+
1	IncI1 plasmid conjugative transfer ATPase PilQ	48353	49861	+
1	IncI1 plasmid conjugative transfer inner membrane protein PilR	49863	50957	+
1	IncI1 plasmid conjugative transfer prepilin PilS	51019	51555	+
1	IncI1 plasmid conjugative transfer putative membrane protein PilT	51600	52085	+
1	Type-IV sectretion leader peptidase/N-methyltransferase	52101	52727	+
1	IncI1 plasmid conjugative transfer pilus-tip adhesin protein PilV	52745	54106	+
1	Cob(I)alamin adenosyltransferase (EC 2.5.1.17)	54351	54145	-
1	hypothetical protein	54340	54492	+
1	IncI1 plasmid conjugative transfer protein TraE	54579	55400	+
1	IncI1 plasmid conjugative transfer protein TraF	55502	56704	+
1	hypothetical protein	56813	56694	-
1	IncI1 plasmid conjugative transfer protein TraH	56808	57266	+
1	IncI1 plasmid conjugative transfer protein TraI	57263	58099	+
1	IncI1 plasmid conjugative transfer protein TraJ, related to pilus biogenesis/retracton protein	58083	59231	+
1	IncI1 plasmid conjugative transfer protein TraK	59228	59518	+
1	IncI1 plasmid conjugative transfer DNA primase	59582	63643	+
1	IncI1 plasmid conjugative transfer protein TraL	63660	64010	+
1	IncI1 plasmid conjugative transfer protein TraM	64022	64717	+
1	IncI1 plasmid conjugative transfer protein TraN	64728	65702	+
1	IncI1 plasmid conjugative transfer protein TraO	65706	67043	+
1	IncI1 plasmid conjugative transfer protein TraP	67040	67753	+
1	IncI1 plasmid conjugative transfer protein TraQ	67750	68280	+
1	IncI1 plasmid conjugative transfer protein TraR	68327	68725	+
1	IncI1 plasmid conjugative transfer protein TraS	68782	69033	+
1	IncI1 plasmid conjugative transfer protein TraT	69053	69766	+
1	FIG00644353: hypothetical protein	69995	69759	-
1	IncI1 plasmid conjugative transfer protein TraU	70061	73105	+
1	IncI1 plasmid conjugative transfer protein TraV	73105	73725	+
1	IncI1 plasmid conjugative transfer protein TraW	73683	74888	+
1	IncI1 plasmid conjugative transfer protein TraX	74885	75454	+
1	IncI1 plasmid conjugative transfer integral membrane protein TraY	75529	77688	+
1	surface exclusion protein	77776	78422	+
1	hypothetical protein	78528	78373	-
1	COG0568: DNA-directed RNA polymerase, sigma subunit (sigma70/sigma32)	78696	79595	+
1	COG0568: DNA-directed RNA polymerase, sigma subunit (sigma70/sigma32)	79774	80286	+
1	Protein pndA	80576	80424	-
1	hypothetical protein	80859	80740	-
1	FIG00642036: hypothetical protein	80828	81148	+
1	hypothetical protein	81236	81427	+
1	FIG00641173: hypothetical protein	81424	81807	+
1	FIG00643543: hypothetical protein	81811	82026	+
1	FIG00641946: hypothetical protein	82904	82224	-
1	putative nuclease	83007	83474	+
1	FIG00641806: hypothetical protein	83624	83800	+
1	hypothetical protein	84105	83863	-
1	hypothetical protein	84180	84365	+
1	hypothetical protein	84362	84490	+
1	hypothetical protein	84841	84695	-
1	TrbA	85179	86477	+
1	TrbB protein	86474	87598	+
1	FIG00640314: hypothetical protein	87579	89948	+
1	hypothetical protein	89995	90288	+
1	replication initiation protein	90360	90557	+
1	Dihydropteroate synthase (EC 2.5.1.15)	90745	91560	+
1	Aminoglycoside 3'-phosphotransferase (EC 2.7.1.95)	91621	92163	+
1	Dihydrofolate reductase (EC 1.5.1.3)	92169	92642	+
1	Aminoglycoside 3'-phosphotransferase (EC 2.7.1.95)	92747	92992	+
1	Beta-lactamase (EC 3.5.2.6)	94108	93248	-
1	Resolvase/integrase Bin	94848	94291	-
2	Streptomycin 3"-O-adenylyltransferase (EC 2.7.7.47) @ Spectinomycin 9-O-adenylyltransferase	436	654	+
2	Dihydrofolate reductase (EC 1.5.1.3)	651	1148	+
2	FIG01049527: hypothetical protein	1522	1397	-
2	Streptomycin 3"-O-adenylyltransferase (EC 2.7.7.47) @ Spectinomycin 9-O-adenylyltransferase	1568	2347	+
2	Chloramphenicol resistance protein	2609	3868	+
2	Streptomycin 3"-O-adenylyltransferase (EC 2.7.7.47) @ Spectinomycin 9-O-adenylyltransferase	3961	4752	+
2	Ethidium bromide-methyl viologen resistance protein EmrE	4922	5254	+
2	Transposase	6109	5501	-
2	Dihydropteroate synthase (EC 2.5.1.15)	7225	6368	-
2	hypothetical protein	7938	7693	-
2	short-chain dehydrogenase/reductase SDR	8839	7976	-
2	macrolide-efflux determinant	9239	8985	-
3	Resolvase	1088	444	-
3	Chromosome partitioning protein ParA	1469	2131	+

Continued on the following page.

Table 5-4: *continued.*

Contig	Function	Start	Stop	Strand
3	unknown	2229	2510	+
3	hypothetical protein	2883	3017	+
3	DNA distortion protein 3	3864	3508	-
3	FIG01047678: hypothetical protein	4123	4001	-
3	FIG01047678: hypothetical protein	4447	4322	-
3	PI protein (Replication initiation protein)	5221	4487	-
3	hypothetical protein	5262	5492	+
3	FIG01046213: hypothetical protein	5640	5771	+
3	hypothetical protein	6141	6296	+
3	RelB/StbD replicon stabilization protein (antitoxin to RelE/StbE)	6557	6808	+
3	RelE/StbE replicon stabilization toxin	6798	7079	+
3	hypothetical protein	7125	7337	+
4	Mobile element protein	285	19	-
4	DNA-cytosine methyltransferase (EC 2.1.1.37)	385	1818	+
4	FIG01046921: hypothetical protein	2445	1852	-
4	IS5 transposase	3782	2802	-
4	Lipid A acylation protein PagP, palmitoyltransferase	3781	3915	+
4	Beta-lactamase (EC 3.5.2.6)	4083	4958	+
4	Tryptophan synthase beta chain like (EC 4.2.1.20)	5202	5005	-
4	FIG01046921: hypothetical protein	5950	5261	-
5	Relaxase /helicase	24	266	+
5	Transcriptional regulator, TetR family	975	298	-
5	Tetracycline efflux protein TetA	979	2253	+
5	Permease of the drug/metabolite transporter (DMT) superfamily	3169	2285	-
5	Amidases related to nicotinamidase	3699	3307	-
6	Integron integrase IntI1	33	512	+
6	Tn21 protein of unknown function Urf2	667	1065	+
6	Resolvase for Tn21	1191	1751	+
7	TnpA transposase	16	1752	+
8	Uncharacterized outer membrane usher protein yqiG precursor	17	1462	+
8	hypothetical protein	1462	1674	+
9	hypothetical protein	207	10	-
9	hypothetical protein	721	212	-
9	hypothetical protein	1220	909	-
9	hypothetical protein	1388	1558	+
11	aminoglycoside 3'-phosphotransferase	87	902	+
12	transposase	65	769	+
13	IS1 protein InsB	98	475	+

5.3.3.2 Plasmid transfer, establishment and maintenance

Like the pR3521 IncB plasmid, pI2-52TC encoded the type IV thin conjugative pili required for liquid mating (*pilI*, *pilL- pilV*) (Komano et al., 1990). Additional proteins involved in conjugation included 22 *tra* genes, *trbABC* and *sogS/L* to which SogS acts as a regulator for the SogL DNA primase that is involved in priming DNA during the synthesis of the transferred DNA strand during conjugation (Komano et al., 2000).

Other genes necessary for plasmid establishment in the recipient include *ssb*, *psiB*, *nik* (Althrope et al., 1999; Furaya and Komano, 1991; Shereda et al., 2007). Also encoded were surface exclusion genes *excA* (an analogue to *exc* of IncI)*, traT and traS,* which function to reduce redundant transfer of same or closely related plasmid between cells (Furuya and Komano, 1994; Perumal et al., 1984). The plasmid encoded two types of post segregation killing systems *stbE/stbD* and *pndC/pndA*.

5.3.3.3 Mobile genetic elements

This plasmid harboured transposes associated with Tn*172*, Tn*2*-like, Tn*3* and Tn*21*. Also identified were IS*1* and IS*5* mediated transposases and a truncated IS*Ecp1* adjacent to the β-lactamase gene. In addition a class 1 integron associated integrase gene (*intI1*) was identified, closely associated with antimicrobial resistance genes *dfrA12-aadA2-cmlA1-aadA1-qac-sul3*. This genetic organisation was similar to plasmid pEC355 (partial sequence) (GenBank FM244708) (Fig. 5-6C), but similar genetic organisations have been previously identified in *E. coli* and *Salmonella* strains (Sunde et al., 2008).

5.3.3.4 Drug resistance

The two β-lactamase genes identified were $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-32}}$. The $bla_{\text{TEM-1}}$ gene was adjacent to a Tn*3* associated transposase. Tn*3* is frequently associated with

mobilisation of the *bla*$_{TEM-1}$ gene (Partridge and Hall, 2005). The *bla*$_{CTX-M-32}$ gene was not found in any of the plasmids similar to pI2-52TC i.e. pOU1114 and pR3521. This gene was identified adjacent to an IS5-associated transposase and a truncated IS*Ecp1* element similar to a *bla*$_{CTX-M-32}$ encoding region of an *E. coli* strain, ECO39 (GenBank EU921825) (Fig 5-9) (Lee et al., 2009). This structure is commonly found among *bla*$_{CTX-M-32}$ harbouring plasmids (Cartelle et al., 2004; Vinue et al., 2009).

Other drug resistance genes identified included aminoglycoside resistance genes *aadA1*, *aadA2*, *aph*, *strB*, chloramphenicol resistance genes *cmlA*, trimethoprim resistance genes *dfrA12, dfrA14,* sulphonamide resistance genes *sul1*, *sul3*, *tetA*, a quaternary ammonium compound resistance gene *qac* and a macrolide drug efflux gene *mefE*.

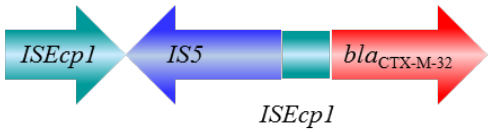


Figure 5-9: A schematic diagram of the genetic organisation surrounding the *bla*$_{CTX-M-32}$ gene. This IS*Ecp1*- *bla*$_{CTX-M-32}$ region was found to be similar (100 % homology) to a *bla*$_{CTX-M-32}$ harbouring plasmid ECO39 (EU921825) from an *E. coli* strain. The arrows indicate the orientation of each of the genes identified. The IS*Ecp1*-like element was disrupted by another insertion element IS*5* upstream of the *bla*$_{CTX-M-32}$ gene.

5.3.3.5 Virulence associated genes

Additional genes were identified that may confer an advantage and include the putative adhesion genes *yqiJ* and *yqiJ*, thought to be crucial in colonization of avian pathogenic *E. coli* (APEC) during infection (Antao et al., 2009). The plasmid also encoded *umuDC* and *impABC* UV-protection genes. The proteins mediate repair of error-prone DNA, thereby contributing to cell survival upon exposure to mutagens (Runyen-Janecky et al., 1999). This may provide a selective advantage especially in environments where UV is the primary sterilising technique e.g. sterilising equipment and environment in hospitals.

5.3.3.6 Hypothesis of plasmid formation

Plasmid pI2-52TC was found to have 67 % nucleotide identity with an IncB plasmid (p3521) encoding the Tra, Trb and Pil conjugative mechanisms. However the RepA replication protein encoded by pI2-52TC only had 45 % nucleotide identity to the repA gene of p3521 but was 99 % similar to the RepA gene of an IncZ plasmid pIE545 (GenBank M93064), which is not included in the PBRT scheme (Praszkier et al., 1989). Praszkier and colleagues found the IncZ and IncB plasmid to be incompatible, thus indicating that these plasmids share the same replication mechanism using small counter transcript RNA molecules. The RNAI and RNAII sequence of IncB plasmids control the copy number of these plasmids by forming a stem-loop structure (Praszkier et al., 1989). This region was found to be 97 % similar (nucleotide identity) to RNAI-RNAII and promoter regions of the IncB plasmid pMU720 (GenBank M28718) (Fig. 5-10). Alignment with these typical IncB plasmids, classified by their replication control mechanisms, strongly suggests the presence of an IncB plasmid scaffold in pI2-52TC. A second Pir mediated replication system was identified in pI2-52TC that is a protein previously identified amongst plasmids of the IncX family (Chu et al., 2008; Norman et al., 2008; Partridge et al., 2010). Three (*sul2, strB* and $bla_{\text{TEM-1}}$) of the four additional resistance genes markers that were not identified in other plasmids of this farm were also identified in the IncB p3521 plasmid (see Fig 5-7B). The remainder of the resistance genes were found associated with an IntI1 integrase gene, characteristic of a class I integron. Thus the acquisition of the multiple antimicrobial resistance genes harboured within a class I integron to an IncB plasmid scaffold via an integrase mediated recombination event is proposed.

The acquisition of the IncX replication region (*pir* and *oriV* sites) may have occurred via another transposition event resulting in the coexistence of two replicons.

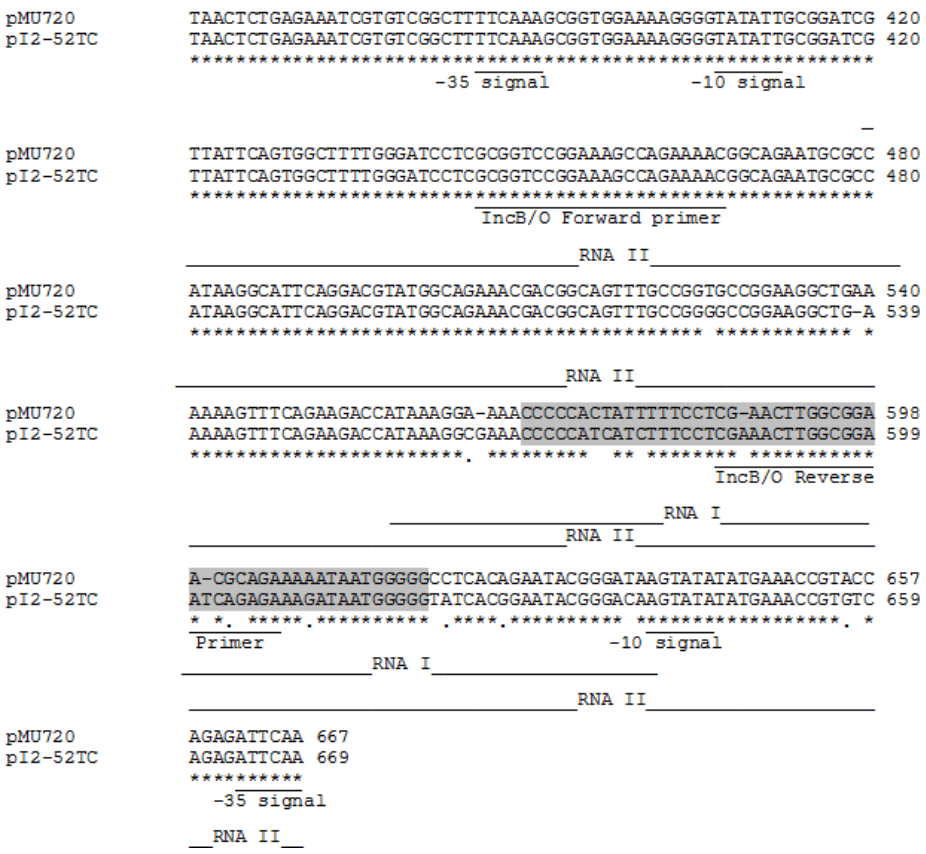


Figure 5-10: Sequence alignment of the RNA I and RNA II region of the IncB minireplicon (pMU720: GeneBank M28718) and pI2-52TC using ClustalW (www.ebi.ac.uk). The RNA II (complement) is located at position 420-790, the RNA I; 569 -631, stem loop (shaded grey). The putative transcription initiator sites RNA II and RNA I at located at positions 420 and 423, and 629 and 622 respectively. The symbols: "*"; identical, ":" (colon) and "." (dot/ fill stop) are conserved and semiconserved substitutions respectively. Adapted from Praszkeir et al., 1989.

5.3.4 Analysis of pI2-53TF

Two plasmids from the field isolate (I2-52) were both found to encode the *bla*$_{\text{CTX-M-32}}$ gene on a 40 kb transformant (pI2-52TF) and a 171 kb transconjugant (pI2-52TC) (see section 5.3.3 above). Analysis of the genetic composition pI2-53TF was thought to elucidate possible linkage of this ~ 40 kb plasmid with the 150 kb IncB pI2-52TC plasmid (see above section), which harbours the same resistance genes as pI2-53TF (see table 5-1).

5.3.4.1 Plasmid Scaffold

Plasmid pI2-53TF, found to be untypeable by the PBRT scheme, was found to have 52 % similarity to a 140 kb *Salmonella enterica* subsp. enterica serovar Choleraesuis pSC138 plasmid (GenBank AY509004) (Chiu et al., 2005; Chiu et al., 2011) (Fig 5-11A). Plasmid pI2-53TF was also was found to be 47 % similar to a 35 kb *Salmonella enterica* subsp. enterica serovar Dublin plasmid pOU1114 (GenBank DQ115387) (Chu et al., 2008) (Fig. 5-11B). However, neither of these plasmids (pSC138 or pOU1114) was found to possess the $bla_{\text{CTX-M-32}}$ gene, unlike pI2-53TF. The region harbouring the $bla_{\text{CTX-M-32}}$ gene was found to be 100 % similar to an *E. coli* strain EC039 (GenBank EU921825) also identified in the IncB-IncX1 pI2-52TC plasmid from this farm. This plasmid harboured 56 predicted ORFs (see Table 5-5).

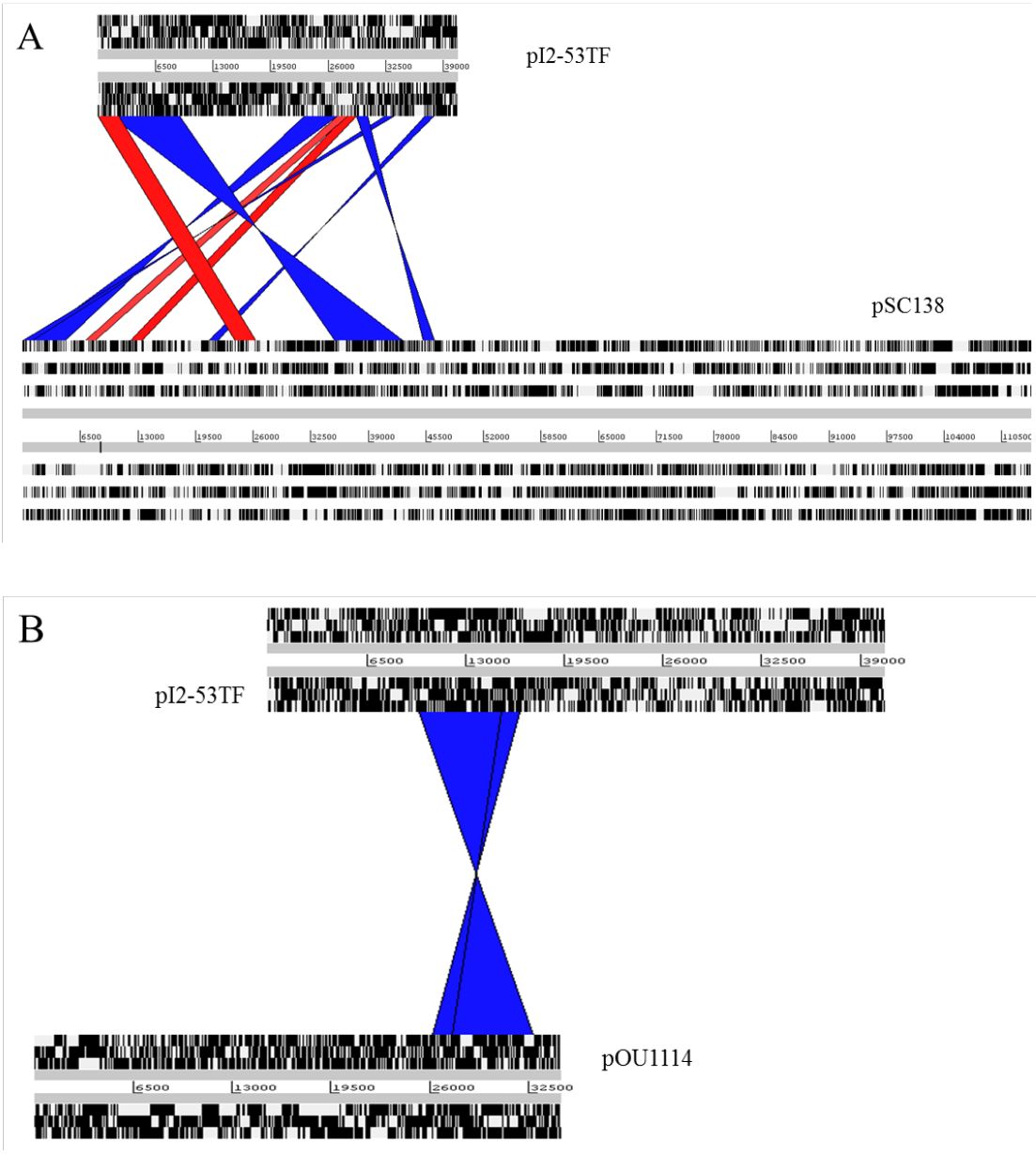


Figure 5-11: Plasmid pI2-53TF scaffold. Plasmid pI2-53TF was compared to the scaffold of two plasmids; pSC138 and pOU111 found to have 52 % and 47 % similarity respectively. Sequence comparisons were performed using WebACT (www.webact.org) sequence comparison tool, using a cut-off of 1000 bp. **A**) IncI1-like plasmid pSC138 (AY509004) was found to have 46 % (18.5 kb) nucleotide identity to pI2-53TF. **B)** IncX1 35 kb plasmid pOU1114 (DQ115387) was found to have 17 % (6.6 kb) nucleotide identity to pI2-53TF.

Table 5-5: Predicted open reading frames identified in pI2-53TF.

Contig	Function	Start	Stop	Strand
1	Dihydrofolate reductase (EC 1.5.1.3)	651	1148	+
1	FIG01049527: hypothetical protein	1522	1397	-
1	Streptomycin 3"-O-adenylyltransferase (EC 2.7.7.47) @ Spectinomycin 9-O-adenylyltransferase	1568	2347	+
1	Chloramphenicol resistance protein	2609	3868	+
1	Streptomycin 3"-O-adenylyltransferase (EC 2.7.7.47) @ Spectinomycin 9-O-adenylyltransferase	3961	4752	+
1	Ethidium bromide-methyl viologen resistance protein EmrE	4922	5254	+
1	Transposase	6109	5501	-
1	hypothetical protein	6156	6431	+
1	Dihydropteroate synthase (EC 2.5.1.15)	7225	6434	-
1	hypothetical protein	7564	7316	-
1	hypothetical protein	7939	7694	-
1	short-chain dehydrogenase/reductase SDR	8840	7977	-
1	short chain dehydrogenase	8969	8853	-
1	macrolide-efflux determinant	9240	8986	-
2	Resolvase	1306	662	-
2	Chromosome partitioning protein ParA	1687	2349	+
2	unknown	2447	2728	+
2	hypothetical protein	2753	3235	+
2	hypothetical protein	3268	3729	+
2	DNA distortion protein 3	4082	3726	-
2	FIG01047678: hypothetical protein	4482	4219	-
2	FIG01047678: hypothetical protein	4665	4540	-
2	PI protein (Replication initiation protein)	5439	4705	-
2	hypothetical protein	5480	5710	+
2	FIG01046213: hypothetical protein	5858	5989	+
2	hypothetical protein	6359	6514	+
2	RelB/StbD replicon stabilization protein (antitoxin to RelE/StbE)	6775	7026	+
2	RelE/StbE replicon stabilization toxin	7016	7297	+
3	Mobile element protein	285	19	-
3	DNA-cytosine methyltransferase (EC 2.1.1.37)	385	1818	+
3	FIG01046921: hypothetical protein	2445	1852	-
3	hypothetical protein	2744	2598	-
3	IS5 transposase	3782	2802	-
3	Lipid A acylation protein PagP, palmitoyltransferase	3781	3915	+
3	Beta-lactamase (EC 3.5.2.6)	4083	4958	+
3	Tryptophan synthase beta chain like (EC 4.2.1.20)	5274	5005	-
3	FIG01046921: hypothetical protein	5950	5261	-
3	transposase	6500	6306	-
4	Relaxase /helicase	24	266	+
4	Transcriptional regulator, TetR family	975	298	-
4	Tetracycline efflux protein TetA	1054	2253	+
4	Permease of the drug/metabolite transporter (DMT) superfamily	3169	2285	-
4	Amidases related to nicotinamidase	3699	3307	-
5	Resolvase for Tn21	2269	1709	-
5	Tn21 protein of unknown function Urf2	2745	2395	-
5	Integron integrase IntI1	3427	2948	-
6	hypothetical protein	207	10	-
6	hypothetical protein	721	212	-
6	hypothetical protein	1220	909	-
6	IncN plasmid KikA protein	1570	1256	-
7	TnpA transposase	17	1753	+
8	hypothetical protein	75	770	+
9	hypothetical protein	141	902	+
10	transposase	248	54	-
11	macrolide-efflux determinant	220	11	-

5.3.4.2 Plasmid transfer, establishment and maintenance

A π replication protein (encoded by *pir*) was identified, similar to pOU1114 (GenBank DQ115387), an IncX1 plasmid from *E. coli*, originally identified in the archetypal IncX2 plasmid R6K (Chu et al., 2008; Filutowicz et al., 1998).

Plasmid pI2-53TF was found to be conjugative with a frequency of transfer rate of $1.50x10^{-8}$. No conjugative systems typical of conjugative plasmids including IncX1 plasmids (Pilx and Tax proteins) or the type IV conjugative pilus systems were identified (Chu et al., 2008; Norman et al., 2008; Nunez et al., 1997). A putative auxiliary conjugative protein TaxD was identified; however this gene had very weak homology; 36 % and 43 % to genes identified in pE001 (GenBank JF776874) and pOU1114 IncX plasmids (see Fig. 5-12) (Bielak et al., 2011; Chu et al., 2008). In addition, a putative IncN associated conjugative transfer protein KikA, lethal to *Klebsiella oxytoca* was identified (Holcik et al., 1996). As these plasmids were able to transfer by conjugation (see chapter 4), closure of this plasmid would enable the conjugative system to be elucidated.

Plasmid partition gene *parA* and post-segregational killing genes *stbE/stbD* were identified, involved in plasmid maintenance ensuring stable inheritance of the plasmid (Funnel, 2005).

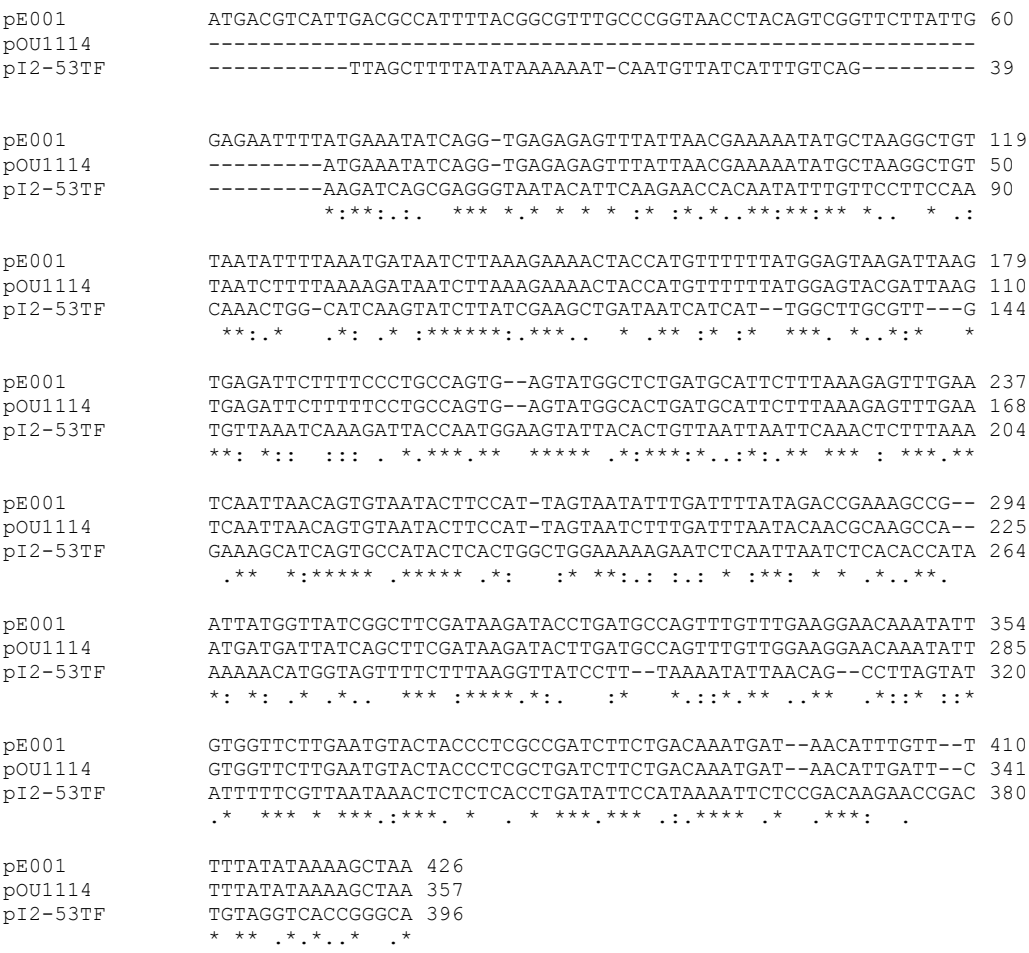


Figure 5-12: Base pair alignment of TaxD putative auxiliary conjugative protein found identified in pE001 (JF776874) and pOU1114 IncX plasmids. This gene only shared 36 % and 43 % homology to these genes respectively. The TaxD sequence of pOU1114 was 69 bp shorter than the taxD gene of pE001, another IncX plasmid. It is not known whether the differences in the sequences will impacts on the function of this protein. Key: "*"; identical, ":" (colon) and "." (dot/full stop) are conserved and semiconserved substitutions respectively.

5.3.4.3 Mobile genetic elements

A class 1 integron associated integrase gene (*intI1*) was identified in the same contig as several resistance determinants in similar arrangement to a *sul3* harbouring class 1 integron carried on an *E. coli* plasmid pEC355 (GenBank FM244708) (see Fig. 5-13A). This contig harboured integrase (*intI*), transposase (*tnp*), a putative oxidoreductase (*orfB*) and resistance genes in the following arrangement: *intiI-dfrA12-orfF-cmlA1-aadA1-qacH-tnp440-sul3-orfA-orfB* (Fig. 5-13). A similar integron structure was also

observed in a type II designated integron with the structure *intI-dfrA12-gcuF-cmlA1-aadA1-qacH-tnp440-sul3*, carried on an IncI1 plasmid pRYC301 (HQ875012) isolated from *E. coli* (Curiao et al., 2011). The *gcuF* gene of unknown function had a 100 % base pair similarity to *orfF* (unknown function) of pEC355 (see Fig. 5-14).

The *sul3* gene was adjacent to the IS*440* associated transposase. This *sul3-IS440* platform may be involved in acquisition of the class I integron via an integrase mediated transposition event from a plasmid harbouring the multidrug resistance gene cassette, resulting in the acquisition of this integron onto an IncX plasmid backbone (Curiao et al., 2011).

Tn*21* associated resolvase (*tnpR*) and transposase (*tnpA*) was encoded on pI2-53TF. Tn*21* has previously been identified in *sul3* containing integrons of *E. coli* and *Salmonella* strains (Curiao et al., 2011; Sunde et al., 2008). Also identified were IS*1*, IS*5*, IS*26* and Tn*1721* associated transposases. The abundance of these MGEs increases the probability of both intra- and inter-molecular movement that may not require homology to a DNA sequence, although preference for particular base pair sequences have been reported (Craig, 1997).

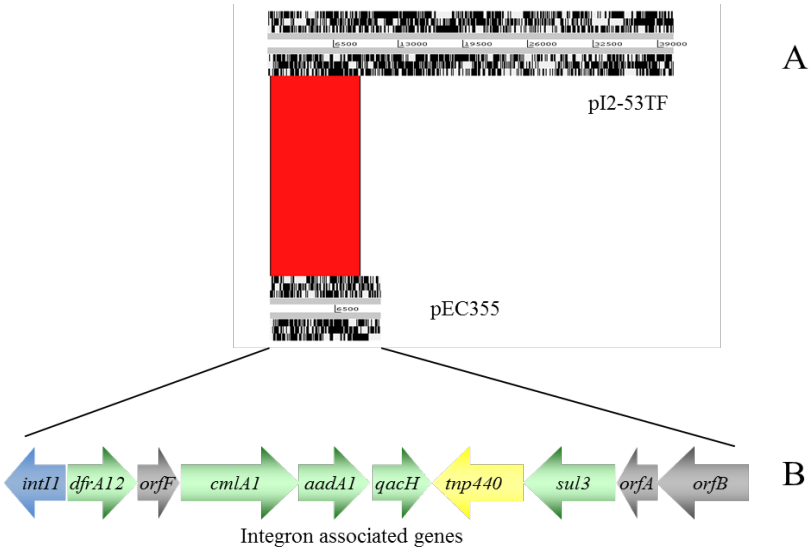


Figure 5-13: Integron associated genes identified in a single contig of pI2-53TF. This genetic organisation of a *sul3* containing integron was been observed in pEC355 (FM244708) in a class 1 integron. **A)** The alignment of the pEC355 integron with pI2-53TF using WebACT (www.webact.org) using a 1000 bp cut-off. Schematic diagram B shows the five resistance genes (green boxes) identified in one contig on pI2-53TF, these were found in the same genetic organisation as in pEC355.

```
gcuF            ATGTTTATTCAAACGGCATTTAGCTTTTCAGGCGTTATTCAGTGCCTGTTTTGCCTTTTT 60
orfF            ATGTTTATTCAAACGGCATTTAGCTTTTCAGGCGTTATTCAGTGCCTGTTTTGCCTTTTT 60
                ************************************************************

gcuF            TCCGGGCTTCGCCTGCATGGGCTGCGCAGGTTTTCAGTCTTTTTGGCCTCTAGCCCTTGC 120
orfF            TCCGGGCTTCGCCTGCATGGGCTGCGCAGGTTTTCAGTCTTTTTGGCCTCTAGCCCTTGC 120
                ************************************************************

gcuF            GTAGCAAGCGCAAGCAGCTATCGTTTTTGCAGTGCTGTGCCGCCTCGGTGGCGCAGCGTT 180
orfF            GTAGCAAGCGCAAGCAGCTATCGTTTTTGCAGTGCTGTGCCGCCTCGGTGGCGCAGCGTT 180
                ************************************************************

gcuF            TTTTCACGGTTAGCGCCCGTCGCCAAATTCAAGTTATCCGTTTTGGCTTCTGGTTCTAAC 240
orfF            TTTTCACGGTTAGCGCCCGTCGCCAAATTCAAGTTATCCGTTTTGGCTTCTGGTTCTAAC 240
                ************************************************************

gcuF            ATTTCGGTCAAGCCGACCCGCATTCTGCGGTCGGCTTACCTCGCCCGTTAG 291
orfF            ATTTCGGTCAAGCCGACCCGCATTCTGCGGTCGGCTTACCTCGCCCGTTAG 291
                ***************************************************
```

Figure 5-14: Base pair alignment of integron associated gene *gcuF* (pRYC301, HQ875012) and orfF (pEC355, FM244708) encoding proteins of unknown functions. Alignments were performed using the ClustalW2 program (www.ebi.ac.uk), these genes were found to have 100 % similarity. Key: "*"; identical nucleotide base.

5.3.4.4 Drug resistance

Plasmid pI2-53TF encodes multiple drug resistance region similar to the class I integron associated region of a partial sequence plasmid pEC355 (GenBank FM244708). Resistance genes included, *aadA1*, *aadA2* (aminoglycoside), *cmlA1* (chloramphenicol transporter), *dfrA12* (trimethoprim), *qacH* (quaternary ammonium resistance), *sul3* (sulphonamide). Additional resistance genes identified included *aphA1* (aminoglycoside), a macrolide efflux determinant *mefB*, tetracycline resistant determinants *tetA* (efflux) and *tetR* (repressor), adjacent to a *pecM* gene; a member of the drug metabolite superfamily thought to export antioxidant compounds protecting the pathogen from host defences (Jack et al., 2001).

The region harbouring the $bla_{\text{CTX-M-32}}$ gene was found to be similar to an *E. coli* strain ECO39 (GenBank EU921825) (Fig. 5-15), with an IS*Ecp1* like element disrupted by another insertion element IS*5*, upstream of the $bla_{\text{CTX-M-32}}$ gene (Cartelle et al., 2004; Fernandez et al., 2007; Lee et al., 2009).

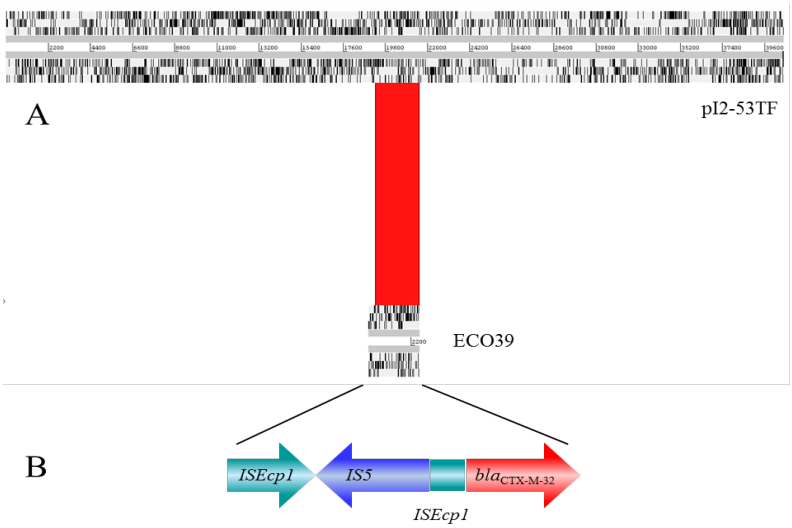


Figure 5-15: Genetic environment of $bla_{\text{CTX-M-32}}$ in pI2-53TF. A) Alignment of pI2-53TF with ECO39 (EU921825); a blaCTX-M-32 harbouring E. coli strain encoding an ISEcp1 disrupted by an IS5 insertion element upstream of the blaCTX-M-32 gene. B) Schematic diagram of the genetic organisation of this region, with arrows indicating the orientation of each of the genes identified. WebACT (www.webact.org) was used to make the alignments (1000 bp cut-off) and calculate similarity.

5.3.4.5 Hypothesis of plasmid formation

Plasmid pI2-53TF was found to harbour elements of the IncX replication machinery, however this region only covered approximately 17 % of the plasmid genophore. The plasmid was found to harbour class I integron associated genes including the *sul3* gene previously found on IncI1 plasmids. Interestingly these integron and the genes cassettes encompassed were previously identified on another plasmid from the same farm (pI2-52TC, IncB). The difference in the genetic background of these two plasmids suggests the acquisition of these genes cassettes was mediated by an integrase dependent transposition mechanism into the IncX plasmid backbone. Sequence comparison of the smaller 40 kb plasmid pI2-53TF to the 150 kb pI2-52TC revealed a 93 % sequence identity between the two plasmids. This suggests for the latter plasmid, the acquisition of IncX1 $bla_{\text{CTX-M-15}}$ onto an IncB plasmid backbone already encoding the bla_{TEM}, *strA* and *sul2* genes, genes not identified in pI2-53TF, resulting in the plasmid cointegrate pI2-52TC harbouring IncB-IncX1 replicons (see Fig. 5-16).

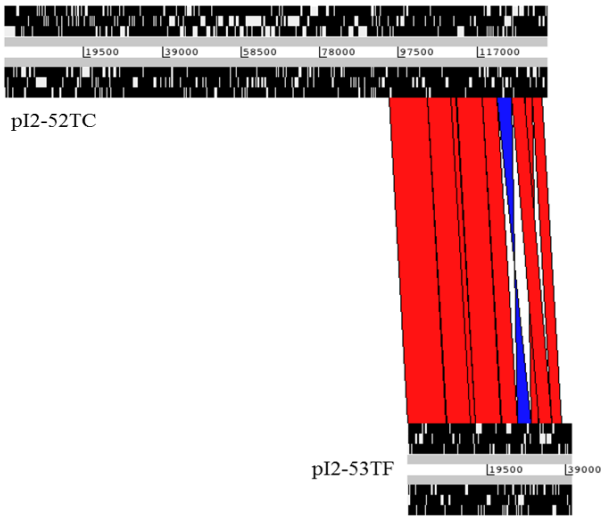


Figure 5-16: Sequence comparison of two $bla_{\text{CTX-M-32}}$ plasmid isolated from *E. coli* strain from the same farm. The smaller IncX1 plasmid (40 kb) pI2-53TF was found to have 93 % sequence identity to the larger IncB-IncX1 (~ 150 kb) plasmid pI2-52TC, suggesting a possible cointegration of an Inc1 and IncB plasmid forming the larger pI2-52TC. WebACT (www.webact.org) was used to make the alignments (1000 bp cut-off) and calculate similarity.

IncX plasmids have previously only been associated with $bla_{CTX\text{-}M\text{-}15}$ and $_{-14}$ plasmids. However, like these plasmids, pI2-53TF encodes an IS*Ecp1* element upstream of the $bla_{CTX\text{-}M\text{-}32}$ gene. Plasmids harbouring the $bla_{CTX\text{-}M\text{-}32}$ gene have been identified previously in IncN harbouring plasmids (Novais et al., 2007), but so far have not been identified in plasmids of the IncX group. This implicates the IS*Ecp1* element, a known mobilising agent for adjacent $bla_{CTX\text{-}M}$ genes, to be involved in the acquisition of the $bla_{CTX\text{-}M\text{-}32}$ gene onto an IncX plasmid backbone by transposition or homologous recombination, or by a combination of both mechanisms, thus mobilizing IS*Ecp1*-$bla_{CTX\text{-}M\text{-}32}$ as a transposed unit (Poirel et al., 2005; Zong et al., 2010).

What is not clear about the pI2-53TF plasmid is the mechanism of conjugation. This plasmid was found to have conjugation frequencies of $1.50x10^{-8}$, which fell below the interquartile range when compared with the frequency of transfer rates of all the plasmids regardless of size and Inc type included in this study (mean = $2.28x10^{-4}$, standard deviation = $1.42x10^{-3}$, lower quartile = $1.66x10^{-8}$, upper quartile = $1.16x10^{-5}$). However, no classical conjugative systems (*pil, tax, tra* or *trb*) were identified on this plasmid nor were any mobilisation proteins. That is not say that these systems were absent, but they might be encoded within the sequence not covered by the contigs and would require further investigation by gap closure. Although showing only 43 % homology, the identification of *taxD* gene, a putative auxiliary conjugative protein found on IncX plasmids (Bielak et al., 2011), may indicate the presence of a Tax mediated mobilising system. Another alternative could be that this plasmid may not have been conjugative at all, but could have been a result of spontaneous mutants of the donor strains conferring resistance to rifampicin. To eliminate this possibility a PFGE could be carried out on this and the donor strain.

5.4 Conclusion

Sequence analysis of these plasmids identified multiple systems encoded within the plasmids to ensure efficient transfer including plasmid partitioning systems, addiction systems and extensive conjugative proteins in IncB, IncI1 and IncFIA-FIB-FII plasmids. In the case of the IncB plasmid (pI2-52TC), a second conjugative system, belonging to the IncX plasmid group (*tax* and *pilx*) was identified in addition to its replication control (*pir* and *oriV*). Co-integration of a large conjugative plasmid and a smaller mobilisation plasmid has previously been observed in *Enterobacteriaciae* (Olsen et al., 2004). The distinct plasmid systems identified in pI2-52 suggest possible co-integration of the IncX scaffold and the IncB plasmids. This is different from the IncFIA-FIB-FII (pI1-34TF) fusion plasmid, which form the plasmid scaffold and the abundant transposase genes identified appears to be the result of multiple independent transposition and recombination events resulting in a mosaic structure. Common to all the plasmids were plasmid maintenance systems to ensure stable inheritance in the absence of selection. In some cases, genes thought to contribute to virulence were also identified. All these mechanisms; plasmid transfer (conjugation and mobilization), maintenance (plasmid, replication, multimer resolution and post-segregational killing systems), resistance and virulence determinants and even the ability to acquire multiple replicons; may provide a selective advantage to the host or the plasmid itself and cumulatively contribute to the general success of a plasmid with respect to spread and persistence.

It is thought that plasmids impose a metabolic burden on the host, potentially because of the mechanisms in place to ensure plasmid maintenance, which could result in differential growth rates between the plasmid free and plasmid containing bacteria (Bennett, 2008; Dahlberg & Chao, 2003; De Gelder et al., 2007; Sengupta et al., 2011; Turner et al., 1998). In the next chapter, the impact of plasmid acquisition upon the host

will be investigated. Differential growth patterns between plasmid harbouring and plasmid free strains will be compared. In addition other the plasmid traits that may confer advantage will be investigated including; resistance genes in the context of the plasmid environment and the contribution of efflux systems to resistance.

The plasmids described here display mosaic structures that do not resemble any one plasmid in the GenBank database. This may well indicate promiscuous re-assortment as a common phenomenon in plasmids that may aid the rapid dissemination of antimicrobial resistance genes and contribute to the persistence of resistance genes in commensal *E. coli* strains, thereby increasing reservoirs of multiple drug resistance genes.

6 RESULTS CHAPTER 6: Analysing plasmid impact on host fitness

6.1 Introduction

The acquisition of resistance determinants by horizontal gene transfer is thought to impart a fitness cost to the host in the absence of selection (Dahlberg and Chao, 2003). The cost is thought to be attributed to maintaining the plasmid in the host during replication and partitioning with every generation (Ebersbach and Gerdes, 2005). In addition, plasmid encoded determinants are thought to be contributors to the decreased fitness observed due to the expression of these determinants, which may inadvertently affect bacterial growth or other basic cellular functions (Dahlberg and Chao, 2003; Turner et al., 1998).

The fitness impact on the host is thought to be alleviated by the strict control of plasmid copy number (Brantl, 2004). In addition, fitness trade-offs have been observed between the plasmid and host, whereby the rate of conjugation was shown to increase but at the expense of host fitness (Turner et al., 1998).

In the absence of selection, plasmid maintenance systems ensure the stable inheritance of the plasmids and in the presence of selection, the plasmid harbouring genes confer an advantage e.g. plasmid encoding antimicrobial resistance genes will be positively selected in the presence of the said antimicrobial. Sometimes the success of the plasmid harbouring strain is due to its efficient clonal dissemination, for example in the case of the ST131 clones harbouring a CTX-M-15 β-lactamase (Peirano and Pitout, 2010). In some instances however, the prevalence of particular plasmids has been attributed to the plasmids themselves, loosely termed epidemic plasmids (Livermore et al., 2007). The rapid rise in CTX-M producing plasmids cannot be solely explained by the genetic platform these genes are harboured within i.e. the plasmids, as this enzyme confers resistance to the same class of antimicrobials as its ancestral predecessors: TEM and

SHV ESBLs, often encoded on similar plasmid backbones (Livermore et al., 2007; Rossolini et al., 2008). Previous studies have demonstrated the fitness advantage conferred between variants of the *bla*$_{TEM}$ enzymes; TEM-1, TEM-10 and TEM-12 in the presence of selection. The differences in the relative growth and susceptibility to ESBLs, was thought to be due to the efficiency of β-lactam hydrolysis (Mroczkowska et al., 2008). Mroczckowska and colleagues proposed that the relative abundance of TEM-1 producing strains was due to higher catalytic efficiencies of this variant over TEM-10 and TEM-12 in the presence of ampicillin. As TEM-1 is the most common *bla*$_{TEM}$ allele, it was proposed that the global β-lactam based antibiotic consumption selected for this allele, resulting in higher frequencies of this allele amongst clinical populations of Gram-negative bacteria (Mroczkowska et al., 2008).

Novais *et al*., later proposed that the evolutionary trajectory of *bla*$_{CTX-M}$ diversification and consequently abundance of CTX-M variants was driven by the higher efficiency of cephalosporin inactivation by this enzyme, more frequently used in treatment (Novais et al., 2010). Here, Novais *et al*. reported the increase in minimum inhibitory concentration (MIC) values as a measure of fitness based on two assumptions; a) only organisms able to survive high concentrations of the antimicrobial will survive and b) where there is a concentration gradient established, such as in the human body, only those populations able to withstand higher concentrations of the drug will be selected (Baquero and Blazquez, 1997; Novais et al., 2010).

In chapter 4, the different CTX-M variants: CTX-M-1, CTX-M-14, CTX-M-14b, CTX-M-15 and CTX-M-32, displayed varying MIC values against ceftazidime. To investigate the possible fitness advantage conferred by these CTX-M variants, enzyme kinetic studies were employed to determine the efficiency of β-lactam hydrolysis with respect to the plasmid system encoding *bla*$_{CTX-M}$ variants and any additional β-lactamases encoded. Five representative plasmids from the total isolate set (*n = 52*)

were transformed into *E. coli* BL21 strains, two of which were sequenced (reported in chapter 5): pI1-34TF, a ~160 kb CTX-M-15 plasmid; and pI2-53TF, a ~44 kb CTX-M-32 plasmid. In addition, three plasmids only found to encode one resistance gene were studied: pI1-33TF, CTX-M-15; and pI2-38TF, CTX-M-1 and finally, pI2-47TF; a ~44 kb derivative of I2-47. This field strain (I2-47) harboured two $bla_{\text{CTX-M-14b}}$ encoding plasmids, one a transconjugant derivative approximately ~130 kb in size (pI2-47TC) and a second ~44 kb plasmid (pI2-47TF) transferred by transformation. The larger ~130 kb plasmid was sequenced and revealed a possible cointegrate plasmid consisting of an IncI1 and IncX1 scaffold (refer to chapter 5). To ensure single plasmids were transferred to the BL21 host, the plasmid DNA extracted from the donor field strain was used to transform the BL21 cells. Upon screening only the ~44 kb plasmid was transferred. This ~44 kb plasmid was used in subsequent phenotypic studies.

Discussed in this chapter is the impact of plasmid acquisition to the host with respect to fitness. The above plasmids were assayed for difference in growth rates between the plasmid-free strain and strains harbouring the CTX-M plasmids in the presence and absence of selection, as a measure of fitness. In addition the impact of plasmid acquisition to host growth in limited carbon sources was tested using the Biolog™ phenotypic microarray. The 160 kb large plasmid pI1-34TF, which was found to harbour >200 predicted open reading frames from sequencing was transformed into *E. coli* BL21 and the stability of the plasmid upon acquisition into a host was determined by maintenance of this strain on solid media by subculturing for 30 days. Growth differences between the plasmid-free and plasmid-harbouring strains were determined in 380 different metabolites using the phenotypic microarray.

This plasmid was also found to encode multiple membrane proteins including ATP-binding cassette (ABC) proteins, permeases and major facilitator (MFS) transporters. Both ABC and MFS families of transporters have been known to contribute to drug

resistance by indiscriminate or specific efflux of antimicrobial drugs (Mahamoud et al., 2007; Piddock, 2006). The effect of these and other plasmid encoded drug transporters, to drug resistance was investigated by monitoring changes in MIC to β-lactams in the presence of the efflux pump inhibitor phenyl-arginine-β-napthylamide (PAβN).

This chapter addresses the question as to what features of the plasmid ensure the success of the plasmid with respect to persistence and abundance within the Gram-negative bacterial population observed in the clinical and veterinary setting, or if in fact the plasmid system as a whole entity including the genetic platform that the genes are encoded on may be a reason for the success of $bla_{CTX\text{-}M}$ harbouring plasmids, as suggested by Livermore et al., 2007.

6.2 Results

6.2.1 Growth kinetics

The impact of plasmid acquisition on growth rates to the host was determined by monitoring the optical density (OD_{600}) over a 24-hour period in defined minimal media (MM) with glucose as the sole carbon and energy source (see chapter 2, section 2.5.3). The initial assay measured the differences in growth rate between the plasmid harbouring strains and the plasmid free *E. coli* BL21 host strain. The differences in growth were given as a measure of the area under the curve (AUC) from 6 replicates. The AUC was used to measure the total growth of the plasmid harbouring strains over the 24-hour period (presumed stationary phase). This measurement was taken to account for the total growth change in the 24-hour period. The limitation of this method being, the initial growth rates, whilst in exponential phase may not be obvious after AUC after 24 hours is taken.

The AUC followed similar profiles as the apparent rates of growth and thus AUC values were used instead of rate constants to take into account total growth for the duration of the experiment.

A non-paired student t-test was used to compare growth kinetics.

6.2.1.1 Growth kinetics in the absence of selection

The plasmid pI1-34TF harboured in BL21, was found to have significantly higher ($p < 0.001$) total growth (measured by the AUC) than the plasmid free BL21 host strain in LB-broth (Fig. 6.1A). Host strains (BL21) harbouring plasmids pI2-38TF and pI2-47TF were found to have significantly lower ($p < 0.001$) growth compared to the plasmid free BL21 strains and no difference in growth was observed with BL21 strains harbouring pI1-33TF and pI2-53TF.

When grown in defined minimal media (MM) no significant difference ($p < 0.001$) in growth was observed between the plasmid harbouring and plasmid free strains (Fig. 6-1B). Here the growth of the strains show a biphasic pattern, indicating growth on minimal media may be limited approximately after 8 hours, probably due to exhaustion of the carbon source.

To determine if plasmid introduction to ΔBL21KAMR; an AcrAB-MdtABC deficient mutant (RND deficient), had any effect on growth, measurements were taken in both LB-broth and MM. No significant differences ($p < 0.001$) in growth between the plasmid free and plasmid harbouring strains were seen in either growth medium (Fig. 6-1C and D).

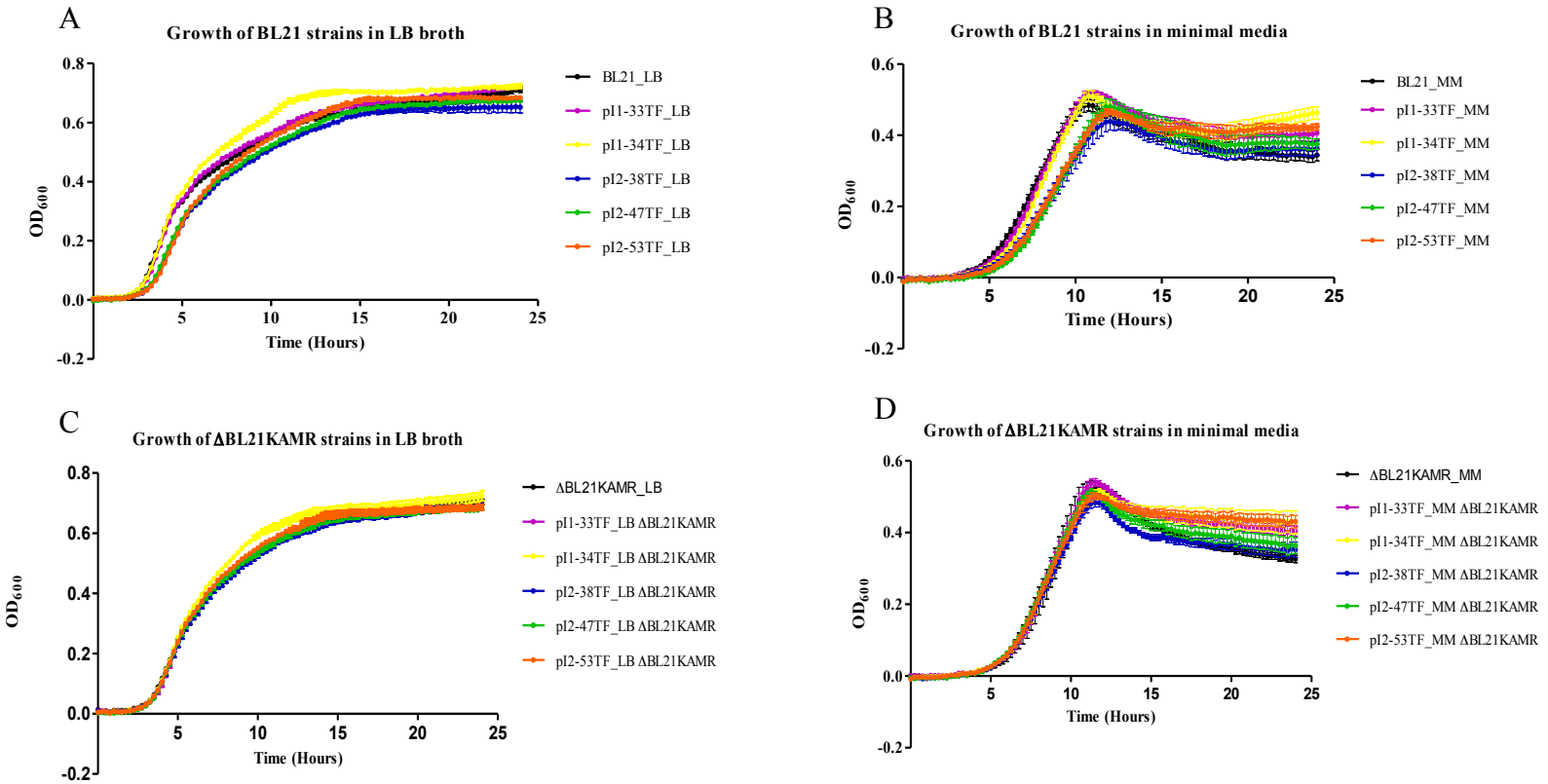


Figure 6-1: Growth of plasmid in host strains in the absence of selection in LB-broth and minimal media supplemented with 0.1 % glucose. (A) and (B) Growth kinetics of CTX-M producing plasmids harboured in BL21 in LB-broth and minimal media respectively. (C) and (D) Growth kinetics of CTX-M producing plasmids harboured in ΔBL21KAMR in LB-broth and minimal media respectively. Two biological replicates and three technical replicates were averaged to give the mean OD600 in the 24 hour period used to monitor the growth of the strains, measured in 15 minute intervals.

6.2.1.2 Growth kinetics is the presence of selection

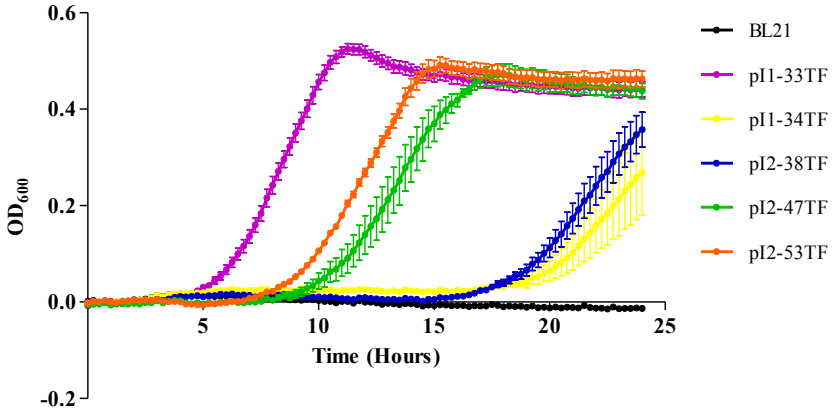
Defined media was used in determining the growth kinetics in the presence of selection. Cefotaxime and ceftazidime were chosen due to differences in MICs observed in previous experiments (see section chapter 4).

The plasmids, harboured within BL21 background were grown in the presence of cefotaxime (1 mg/L) (Fig. 6-2A). Significantly ($p < 0.001$) faster growth was observed in strains harbouring pI1-33TF (CTX-M-15), pI2-47TF (CTX-M-14b) and pI2-53TF (CTX-M-32) plasmids compared to the plasmid free strain. When compared to the untreated strains (no cefotaxime) in MM, no significant differences ($p < 0.001$) in growth were observed in strains harbouring these plasmids. Strains harbouring pI1-34TF (CTX-M-15) and pI1-38TF (CTX-M-1) had significantly lower growth rates than the untreated strains harbouring these plasmids. These results show differences in the two IncF plasmids producing the CTX-M-15 enzyme upon exposure to the antibiotic cefotaxime, despite these enzymes being regarded as the more efficient inactivators in MIC experiments in chapter 4 (section 4.2.8) that may suggest intrinsic differences in these plasmids independent of the CTX-M variant produced.

Interestingly, pI1-34TF in the RND deficient background (ΔBL21KAMR) treated with cefotaxime grew better than the same plasmid harbored in the BL21 host, having significantly higher growth rates ($p < 0.001$) than the plasmid free strain (Fig. 6-2B). Strains harboring pI1-33TF, pI2-47TF and pI2-53TF grew at similar rates as when in the BL21 host. The strain harbouring pI2-38TF had no significant difference in growth compared to the plasmid free strain. These results suggests, that even in the absence of the AcrAB efflux system, strains harbouring the CTX-M plasmids are able to survive exposure to cefotaxime, possibly due to efficient drug inactivation by the enzymes or by other compensatory efflux systems or reduced porin expression in the host background.

Transcriptional studies and possibly the use of transposon directed insertion-site sequencing (TraDIS) could be employed to determine the genes upregulated and or essential for resistance to cefotaxime.

A



B

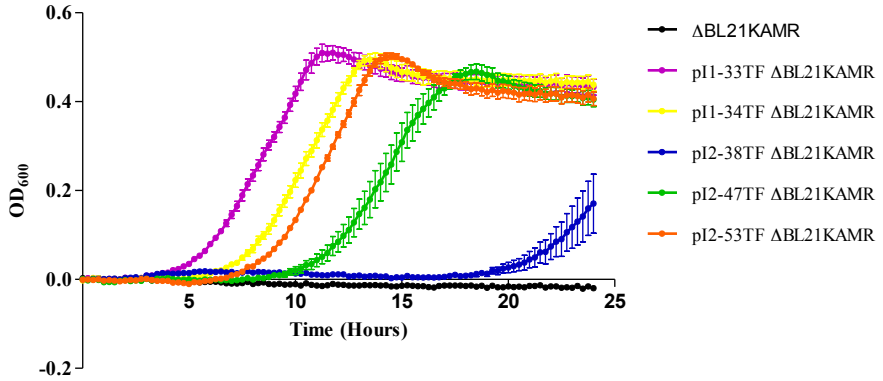


Figure 6-2: Growth kinetics of CTX-M producing plasmids in the presence of cefotaxime (1 mg/L). (A) Depicts growth of plasmids harboured within E. coli BL21 strains and (B) is the growth of these plasmids in the ΔBL21KAMR RND deficient background. Two biological replicates and three technical replicates were averaged to give the mean OD600 in the 24 hour period used to monitor the growth of the strains, measured in 15 minute intervals.

Growth of the strains in the presence of 0.5 mg/L of ceftazidime was monitored (Fig. 6-3A and B). The MIC for the host strains, BL21 and ΔBL21KAMR was < 0.5 mg/L. Strains were grown in minimal media supplemented with 0.5 mg/L ceftazidime to inhibit growth of the plasmid free strains. However, both the plasmid free control strains grew in the presence of ceftazidime, with no significant ($p < 0.001$) difference in growth compared to the plasmid bearing strains (see Fig. 6-3A and B).

Moulin and colleagues found differences in growth of *E. coli* strains in different carbon sources in the presence of ceftazidime (Malouin et al., 1991). To determine if the growth of the control strains was due to the use of glucose (0.1 %) as a supplement in the defined media, the plasmid free strains and CTX-M-15 producing plasmid pI1-33TF, in the BL21 and ΔBL21KAMR background, were streaked on minimal agar plates supplemented with 1% (w/v) sodium succinate and ferric citrate (AbuOun 2009) and 10 % (w/v) glutamate (Malouin et al., 1991). The plates were supplemented with 0.5, 1 and 2 mg/L ceftazidime (above the MIC of the plasmid free host strains). Control plates contained LB-agar supplemented with 0.5, 1 and 2 mg//L ceftazidime. Both the plasmid free and plasmid harboring strains grew in the presence of all concentrations of ceftazidime in LB-agar. No growth was observed in the other two carbon sources (in minimal media) in all three concentrations of ceftazidime. Growth of the plasmid harboring strain (pI1-33TF; CTX-M-15) was only observed at the initial streaking point. The change in carbon source appears to recover efficacy of the drug and even limit the growth of the CTX-M plasmid. Future work will employ MIC experiments to investigate the effect of the different carbon sources on the efficacy of ceftazidime with different CTX-M producing plasmids to determine if the MICs observed using Iso-sensitest agar (which contains glucose) has an effect on the efficacy of the drug.

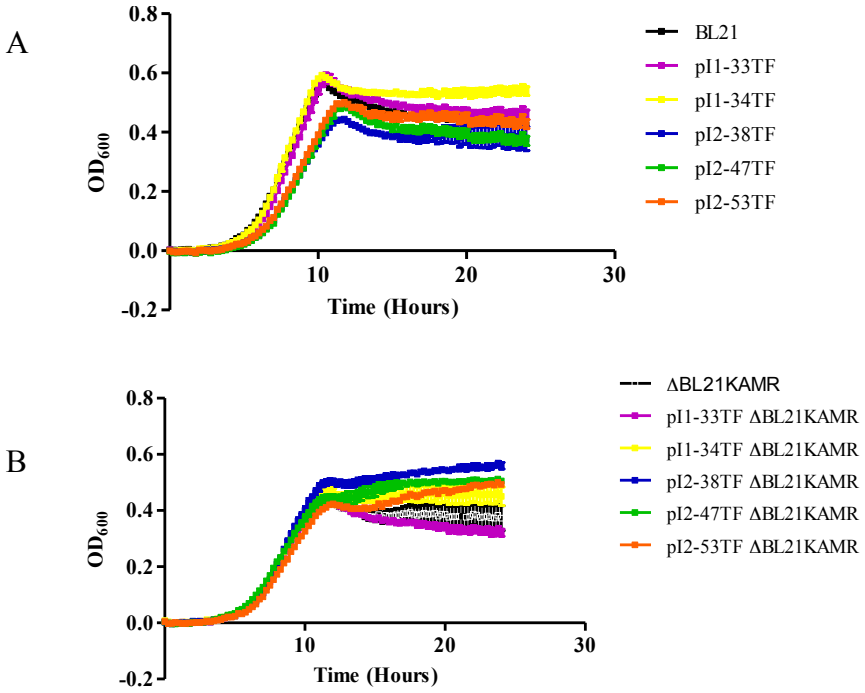


Figure 6-3: Growth kinetics of CTX-M producing plasmids in the presence of ceftazidime (0.5 mg/L) in BL21 (A) and BL21KAMR (B). Growth of both of the host strains in concentrations of ceftazidime above their pre-determined MICs deemed these results as inaccurate, requiring optimisation in different growth medium. Two biological replicates and three technical replicates were averaged to give the mean OD600 in the 24 hour period used to monitor the growth of the strains, measured in 15 minute intervals.

6.2.2 Plasmid stability in ageing colonies

To investigate the fitness cost of plasmid acquisition to the host strain, a single plasmid (pI1-34TF; CTX-M-15) was selected for analysis because of the severe growth retardation when grown in the presence of cefotaxime in the BL21 host strain, the surprising recovery once introduced into the RND deficient strain (Fig. 6-1) and the large size (150 kb) with over 200 predicted ORFs identified by the RAST prediction annotation tool (rast.nmpdr.org) (see chapter 5).

6.2.2.1 Plasmid stability in *E. coli* BL21

Plasmid pI1-34TF was introduced into *E. coli* BL21 by transformation. The plasmid bearing and plasmid free (control) strains were maintained on solid LB-agar

supplemented with 0.2 % glucose and 3 mM $CaCl_2$ for 30 cycles. Fresh BL21 host strain and the plasmid harbouring (pI1-34TF) BL21strain, were streaked on the plate to a single colony, this was defined as cycle 0. A cycle was defined as growth of a single colony after an overnight incubation, which is approximately 28 generations, totalling approximately 900 generations in 30 cycles (Andersson and Hughes, 1996). A single colony from the overnight plate was picked and streaked onto a fresh plate giving cycle 1 strains. This was repeated a further 29 times to obtain 30 cycles. Picking a single colony is thought to provide an intense genetic drift due to lineages being generated through forceful bottleneck selection (Andersson and Hughes, 1996). The single colony picked for re-streaking was grown overnight in liquid media and frozen at -80 °C for subsequent phenotypic experiments.

6.2.2.2 Growth kinetics between the plasmid free and plasmid bearing strain at cycle 0, 15 and 30

To determine if plasmid acquisition and maintenance affects growth of the host over the 30-cycle period, the plasmid free and plasmid (pI1-34TF) harbouring BL21 was monitored over a 24-hour period in minimal media supplemented with 0.2 % glucose (Fig. 6-4). No significant difference ($p < 0.001$) in growth between the cycles 0, 15 and 30 (approximately 28, 420 and 840 generations respectively) was observed between the plasmid free and the plasmid bearing strain. However, what is interesting to note is the growth rate between the plasmid bearing strains increased with time (i.e. growth at cycle 30 > cycle 15 > cycle 0), the reverse of BL21, suggesting some form of adaptation of the plasmid bearing strains in accommodating the plasmid.

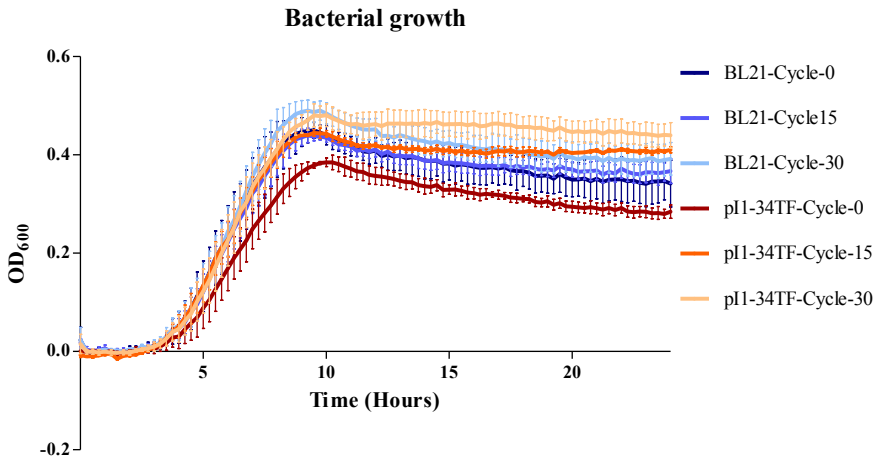


Figure 6-4: Graph showing the growth kinetics (measured at OD_{600}) of the plasmid (BL21) and plasmid bearing (pI1-34TF) strain maintained on solid media for 0 cycles (28 generations), 15 (420 generations) and 30 (840 generations) cycles. The strains were grown in defined minimal media containing glucose. Two biological replicates, each with three technical replicates were measured.

6.2.3 Assessing the impact of plasmid acquisition and maintenance to host metabolism

The plasmid harbouring strains and the control (plasmid free BL21) was assessed for differences in growth in 380 different metabolite sources using the Biolog™ phenotypic microarray. Metabolites tested included phosphorous and peptide nitrogen sources. The peptide nitrogen sources were selected because of the significant differences in growth observed between an *E. coli* 0157 harbouring a 92 kb F-like MDR plasmid; pO157 and the cured strains (Lim et al., 2010). The phosphorous compounds were selected because phosphorous is an important compound in numerous signal transduction pathways, which may affect downstream genes or pathways that may be involved in expression of resistance mechanisms (Wanner, 1996).

The three cycles 0, 15 and 30, from the 30 cycle sustained growth on solid media were selected for phenotypic analysis. The experiment was run for 96 hours, with differences in growth represented at 26 (Fig. 6-5), 48 (Fig. 6-6) and 96 (Fig. 6-7) hours.

At 26 hours (Fig. 6-5), for cycle 0 (28 generations) bacteria, the plasmid bearing strain only utilised (1-2 fold) 4 of the 380 (1 %) metabolites tested. All were dipeptide compounds. In the cycle 15 passage strains (420 generations), the plasmid bearing strain grew better in phosphorous compounds (1-2 fold). The cycle 30 strains (840 generations) utilised less phosphorous compounds than the plasmid free strain (up to 5 fold). Interestingly, the plasmid harbouring strain in this older generation (30 cycles) utilised dipeptide compounds better than the cycle 0 and 15 passage.

At 48 hours (Fig. 6-6), no difference was observed between BL21 and the plasmid bearing strain of cycle 0. However with the cycle 15 passage, the plasmid bearing strain did not utilise D-Manose-1-phospate as well as the plasmid free strains (3 fold less) also at the cycle 15 passage, but utilised the dipeptide compounds more (1-2 fold) than BL21-cycle 15 passaged strain. The cycle 30 passaged plasmid bearing strain utilised less phosphorous compounds (up to 4 fold less) but was able to utilize the dipeptide compounds 1-2 fold better than the plasmid free in the than BL21-cycle 30 passaged strain.

At 96 hours (Fig. 6-7), the cycle 0 and 15 passaged strains utilized the similar amounts of compounds as the plasmid free strain, a reduction in respiration in only 0.3 % and 1.6 % and an increase in 1.1 % and 2.4 % of the metabolites tested respectively. However the older generation (cycle 30) plasmid bearing strain utilized the phosphorous-based compounds up to 3 fold less than the plasmid free (cycle 30) passages strain. However, this cycle 30 passaged strain utilized the dipeptide compounds four times better than the plasmid free cycle 30 passaged strain. The differences in compound utilization at the 96 hour time point relative to the plasmid free BL21, may be skewed because other better utilized compounds may be provided by the break-down of dead cells, resulting in the reduced number of compounds affecting the plasmid harbouring strains. These results suggest that as the passages and therefore the

age of the strains increase, the plasmid harbouring strain is less able to utilise the same compounds as its plasmid free counterpart, but this phenotype is not reflected in the growth of the strains (Fig. 6-4). Further work could look at the growth by measuring changes in OD_{600} of the strains in compounds, which show variability in metabolite usage between the cycles.

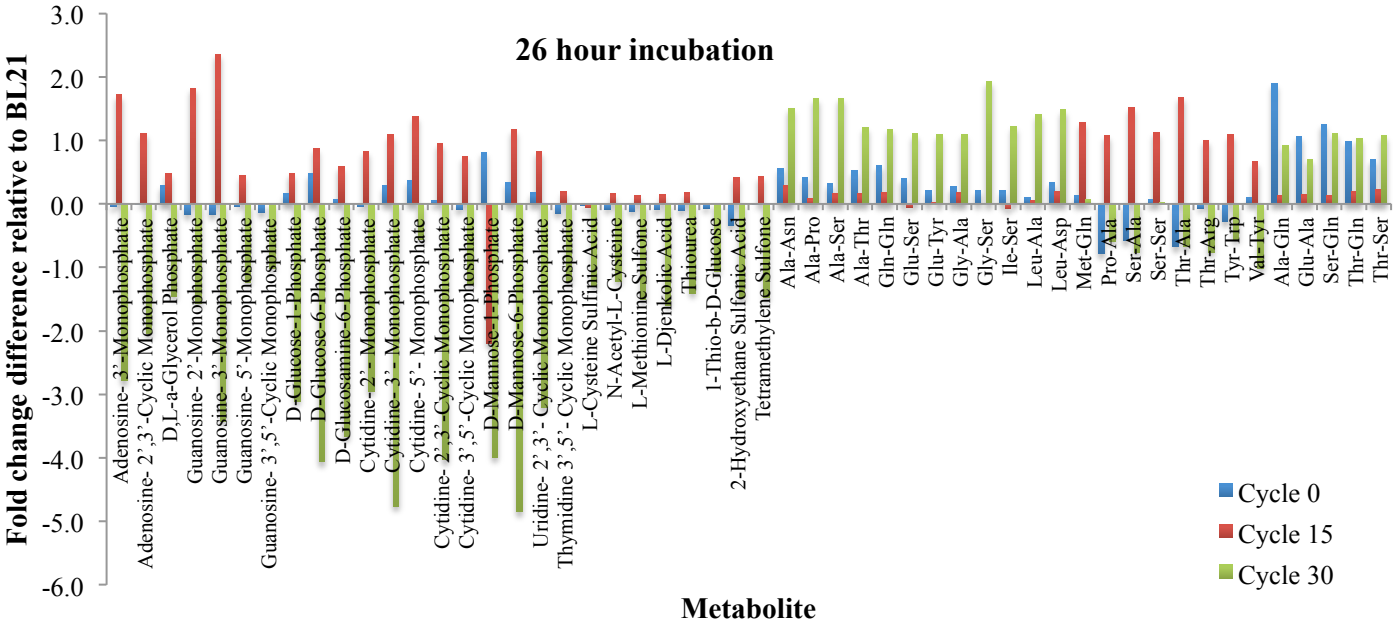


Figure 6-5: Graph showing fold change differences in metabolite utilization relative to the plasmid free BL21 strain, using fold change cut-offs of ≥ 1 or ≤ -1 for increased and decrease in growth respectively relative to BL21 after 26 hours of incubation. The area under the curve of the kinetic graphs were taken at each time interval for two biological replicates and the fold change relative to each of their respective negative controls; present on each of the 96 well phenotypic plates, was calculated. The differences in the fold change were calculated to compare differences in metabolite utilization between the plasmid harbouring and host (plasmid free) strains and Fold changes of the plasmid bearing strain (pI1-34TF) are shown in blue (cycle 0), red (cycle 15) and green (cycle 30) relative to the plasmid free host strains at the corresponding cycles.

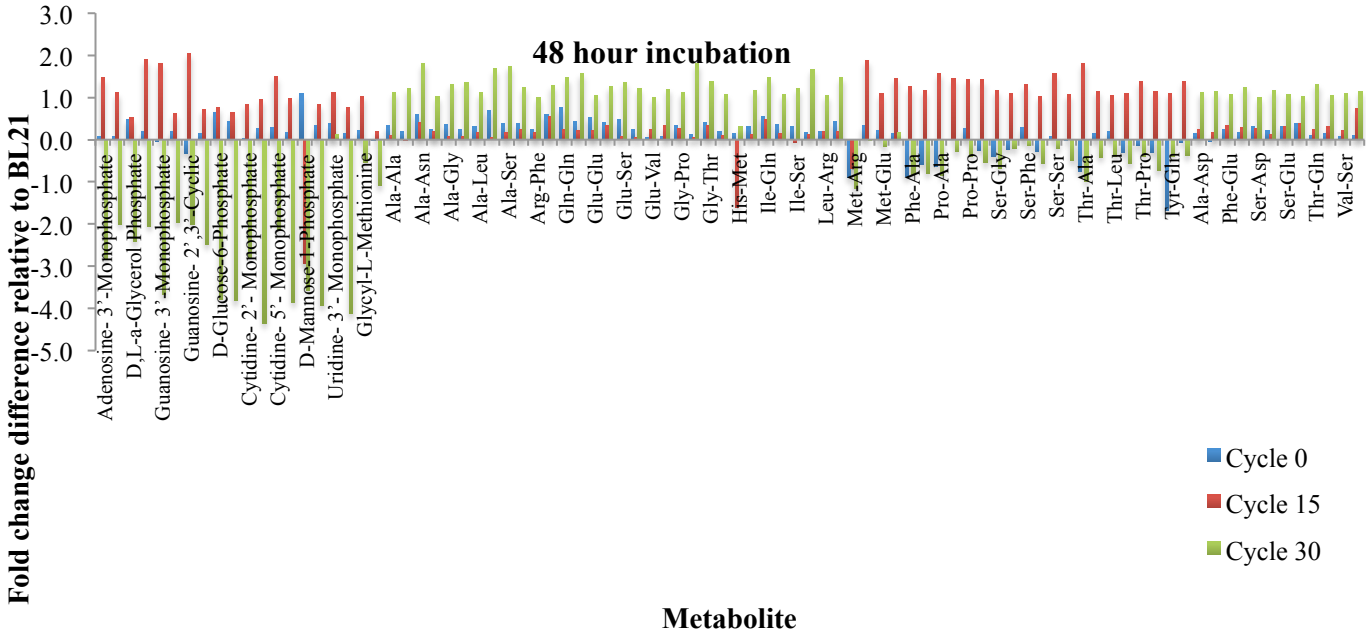


Figure Error! No text of specified style in document.**-1: Graph showing fold change differences in growth relative to the plasmid free BL21 strain, using fold change cut-offs of ≥ 1 or ≤ -1 for increased and decrease in growth respectively relative to BL21 after 48 hours of incubation.** The area under the curve of the kinetic graphs were taken at each time interval for two biological replicates and the fold change relative to each of their respective negative controls; present on each of the 96 well phenotypic plates, was calculated. The differences in the fold change were calculated to compare differences in metabolite utilization between the plasmid harbouring and host (plasmid free) strains and Fold changes of the plasmid bearing strain (pI1-34TF) are shown in blue (cycle 0), red (cycle 15) and green (cycle 30) relative to the plasmid free host strains at the corresponding cycles.

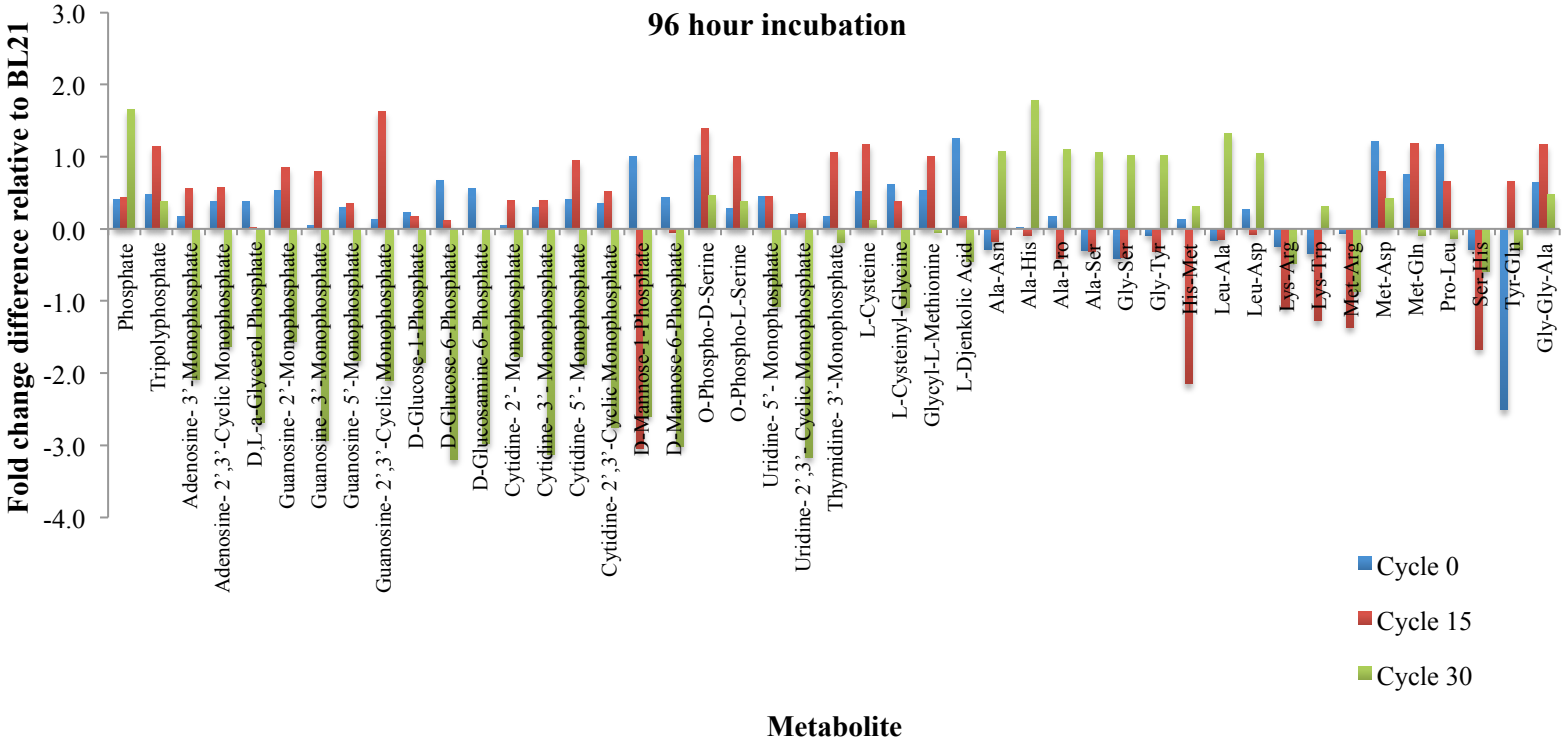


Figure Error! No text of specified style in document.**-2: Graph showing fold change differences in growth relative to the plasmid free BL21 strain, using fold change cut-offs of ≥ 1 or ≤ -1 for increased and decrease in growth respectively relative to BL21 after 96 hours of incubation.** The area under the curve of the kinetic graphs were taken at each time interval for two biological replicates and the fold change relative to each of their respective negative controls; present on each of the 96 well phenotypic plates, was calculated. The differences in the fold change were calculated to compare differences in metabolite utilization between the plasmid harbouring and host (plasmid free) strains and Fold changes of the plasmid bearing strain (pI1-34TF) are shown in blue (cycle 0), red (cycle 15) and green (cycle 30) relative to the plasmid free host strains at the corresponding cycles.

6.2.4 Enzyme kinetics studies

Kinetics measurements of purified CTX-M enzymes have previously been reported (Barthelemy et al., 1993; Bradford et al., 1998; Cartelle et al., 2004; Ishii et al., 1995; Kimura et al., 2004; Ma et al., 2002). To investigate the efficiency of the CTX-M producing plasmids, the plasmids were transformed into an *E. coli* BL21 strain. Crude cell extracts were used to determine the contribution of plasmid encoded β-lactamase genes i.e. CTX-M or additional TEM and OXA enzymes (see table 6-1).

Characteristically, purified CTX-M-32 enzymes are the most efficient variant at hydrolysing ceftazidime between all the variants tested, followed by CTX-M-15, CTX-M-1 and finally CTX-M-14(b) (Bonnet, 2004; Novais et al., 2010). Using crude lysates for the kinetic measurements would in theory take into account other plasmid characteristics which may affect the efficiency of β-lactam hydrolysis including additional β-lactamases and plasmid copy number, and as a consequence the relative expression levels of the β-lactamase enzyme. This measurement could therefore reflect the efficiency of the plasmid as an entity is deactivating β-lactamases. The plasmid free host strain was used as a control to measure any residual β-lactamase activity produced by the host.

Table 6-1: Plasmids selected to determine the impact of plasmids to host fitness. The resistance gene profiles of the plasmids are also given.

Plasmid	Plasmid Size (kb)	Antimicrobial resistance genes
pI1-33TF	70	*bla*$_{\text{CTX-M-15}}$
pI1-34TF	150	*aac6lb, aadA4, catA1,* *bla*$_{\text{CTX-M-15}}$*, dfrA17,* *bla*$_{\text{OXA-1}}$*, sul1,* *bla*$_{\text{TEM-1}}$
pI2-38TF	44	*bla*$_{\text{CTX-M-1}}$
pI2-47TF	44	*bla*$_{\text{CTX-M-14b}}$, *bla*$_{\text{TEM-1}}$
pI2-53TF	44	*aadA1, aadA2, cmlA1,* *bla*$_{\text{CTX-M-32}}$*, dfrA12, sul3, tetA*

Gene targets: *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{OXA}}$; ESBLs, *aadA* and *aac*; aminoglycosides, *cmlA1* and *catA1*; Chloramphenicols, *dfrA12* and *dfrA17*; trimethoprims, *sul1* and *sul3*; sulphonamides, *tetA*; tetracyclines.

Steady state kinetics of the five plasmids was determined by monitoring the colour change of nitrocefin. Nitrocefin (Fig. 6-8) is a chromogenic cephalosporin with a highly reactive β-lactam ring, readily hydrolyzed by all known β-lactamases produced by both Gram-negative and Gram-positive bacteria. It undergoes a distinctive colour change from yellow (λ=390 pH7.0) to red (λ=486nm pH 7.0) upon hydrolysis of the β-lactam ring, consequently producing a colour change. This is thought to be a result of cleavage of the amide bond within the β-lactam ring in an already highly conjugated system (emitting the yellow colour) leading to further conjugation, thus producing the red colour, which can be measured spectrophotometrically (O'Callaghan, 1972; 39). The colour change is monitored in the 480-500 nm range (Herdberg, 1995; 39, Reifferscheid, 2006; 46).

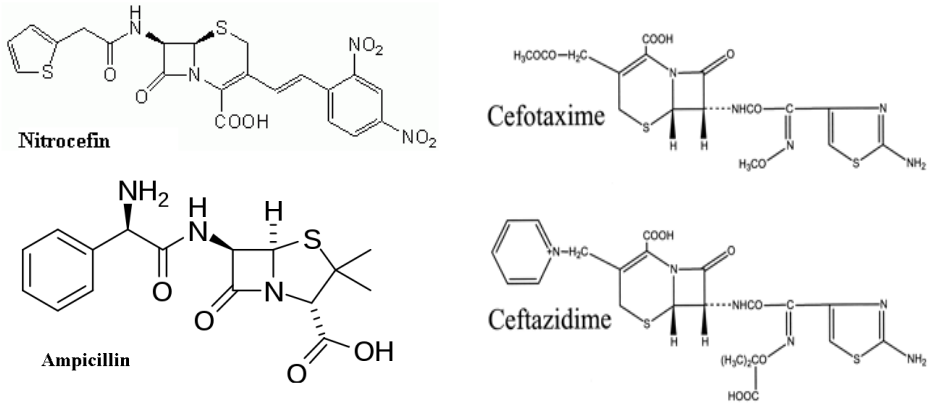


Figure 6-8: Chemical structures of broad spectrum (ampicillin), extended spectrum (cefotaxime and ceftazidime) and the colorimetric substrate used to measure β-lactam hydrolysis, nitrocefin.

6.2.4.1 Determining the protein concentration

The protein content was measured using the BCA protein assay kit (Thermoscientific), using BSA as a standard (see table 6-2). BSA absorbance values corrected against the blank and known BSA protein concentrations (μg/ml) were plotted to give a standard curve (Fig. 6-9). The total protein [T.Prot] was calculated from the standard curve (polynomial; order 3) (table 6-3). Four of the plasmid harbouring strains had similar protein concentrations (approximately 200 μg/ml) compared to the plasmid free host (BL21). One plasmid pI2-53TF/BL21 had a slightly lower protein concentration at 178.85 μg/ml.

Table 6-2: Absorbance at λ=562 nm of BSA standards determine using the BCA assay.

		Absorbance			
Dilution	**Concentration (μg/ml)**	**Replicate 1**	**Replicate 2**	**Mean ABS**	**Corrected**
A	2000	1.856	2.161	2.0085	1.861
B	1500	1.899	2.02	1.9595	1.812
C	1000	1.283	1.318	1.3005	1.153
D	750	0.952	0.879	0.9155	0.768
E	500	0.734	0.813	0.7735	0.626
F	250	0.434	0.349	0.3915	0.244
G	125	0.303	0.32	0.3115	0.164
H	25	0.173	0.177	0.175	0.0275
I (Blank)	0	0.153	0.142	0.1475	0

The bovine serum albumin (BSA) protein standards were prepared according to the manufacturer's instructions using an albumin standard (BSA). Protein measurements were made at 562 nm. The mean readings were subtracted from the blank (I) readings t give the 'corrected' readings, used to plot a graph (standard curve of absorbance at 562nm versus protein concentration (μg/ml).(below; Fig. 6-2).

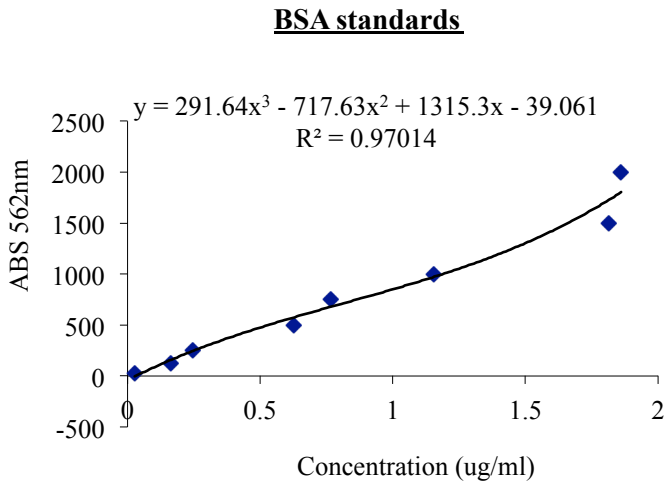


Figure 6-9: BSA standard curve used to estimate total protein concentration of crude cell extracts [T.Prot]. The total protein [T.Prot] was calculated from the standard curve (polynomial; order 3).

Table 6-3: Protein concentration of crude lysates

Sample	Replicate 1	Replicate 2	Mean ABS	Concentration (µg/ml)
pI1-33TF	0.199	0.208	0.2035	201.34
pI1-34TF	0.202	0.205	0.2035	201.34
pI2-38TF	0.211	0.21	0.2105	208.73
pI2-47TF	0.205	0.201	0.203	200.81
pI2-53TF	0.182	0.183	0.1825	178.85
BL21	0.199	0.192	0.1955	192.83

Equal volumes of the crude lysates were mixed with the bicinchonic acid (BCA; Pierce) to measure the absorbance, according to the manufacturer's instructions. Protein concentrations [T Prot] were estimated from the standard curve generated from the BSA standards (Fig. 6-1).

6.2.4.2 Enzyme activity

The enzyme activity is the amount of substrate converted per unit of time under the given conditions. The β-lactamase activity (enzyme activity) of the crude extracts were measured as units of activity, where one Unit of β-lactamase is the amount of enzyme required to form 1 µmol of product per min in 1 ml of reaction. See chapter 2; section 2.5.5.4 for details.

Figure 6-11 shows the graph used to estimate the β-lactamase activity given as Units of enzyme per minute (Units min^{-1}). Two plasmids, pI2-38TF and pI2-47TF reached

saturation before measurement could begin (see Fig. 6-10). These two extracts were diluted 1 in 10 and measurements repeated. The β-lactamase activities are displayed in table 6-4. From this data, plasmid pI2-38TF (CTX-M-1) and pI2-47TF (CTX-M-14b) had the highest β-lactamase activity, followed by pI1-34TF (CTX-M-15), 16 times lower activity than pI2-38TF, pI2-53TF (CTX-M-32) with 20 times less activity than pI2-38TF (the highest activity of the plasmids tested) and finally pI1-33TF with 35 times less activity compared to pI2-38TF. The host strain (with no plasmids) was also measured to obtain residual β-lactamase activity, found to have 540 times lower activity than pI2-38TF and 154 times lower than the least active of the plasmids; pI1-33TF.

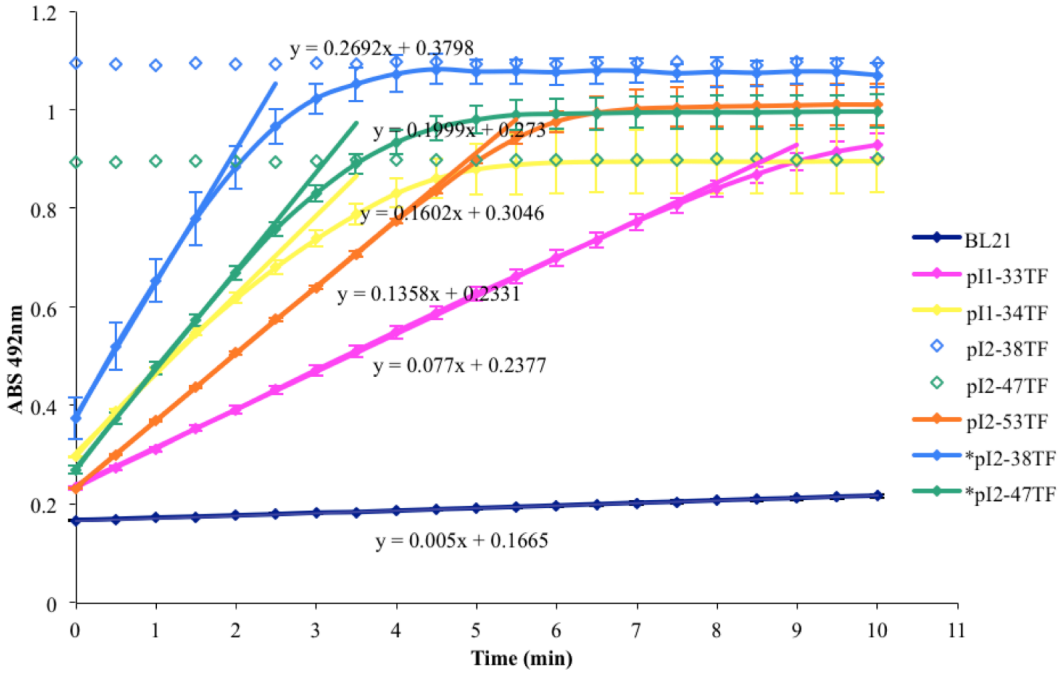


Figure 6-10: Measuring β-lactamase activity from crude extracts in the presence of 0.1 mM nitrocefin. Absorbance values were plotted against time (minutes). Rates (OD/min) were determined by the gradient of the linear portion of the non-linear regression. The rates (OD/min) were used to calculate the enzyme activity (Bisswanger 2004). *; pI2-38TF and pI2-47TF extracts were diluted 1in 10 to allow the measurements of initial rates.

Table 6-4: Calculating total enzyme activity in Units (µmol min^{-1}).

Plasmid	OD/min	Product converted (M) in 100ul	Product converted (M) in 1ml	Product converted (µM) in 1ml	Enzyme activity (Units)[a]
BL21[b]	0.005	$4.57x10^{-7}$	$4.57x10^{-6}$	0.46	$4.57x10^{-4}$
pI1-33TF	0.077	$7.03x10^{-6}$	$7.03x10^{-5}$	7.03	$7.03x10^{-3}$
pI1-34TF	0.1602	$1.46x10^{-5}$	$1.46x10^{-4}$	14.63	$1.46x10^{-2}$
*pI2-38TF	2.692	$2.46x10^{-4}$	$2.46x10^{-3}$	245.89	$2.46x10^{-1}$
*pI2-47TF	1.999	$1.83x10^{-4}$	$1.83x10^{-3}$	182.59	$1.83x10^{-1}$
pI2-53TF	0.1358	$1.24x10^{-5}$	$1.24x10^{-4}$	12.40	$1.24x10^{-2}$

*pI2-38TF and pI2-47TF extracts were diluted 1in 10 to allow the measurements of initial rates. The dilution factor was accounted for in calculating the units of enzyme. [a]Enzyme units are the amount of product in µmoles formed in one minute in 1ml of a reaction with units (µmol min^{-1}).

[b]The total enzyme activity for the plasmid free *E. coli* BL21, to which all test plasmids were transformed in.

6.2.4.3 Calculating the specific enzyme activity

The specific activity (µmol min^{-1} mg^{-1} or Units mg^{-1}) (table 6-5) was calculated by dividing the enzyme activity (µmol min^{-1}) by the amount of protein (mg). In the crude lysates a large percentage of the total protein [T.Prot] may not be the protein of interest. The purer the protein sample, the higher the proportion of [T.Prot] attributed to the enzyme. In a crude extract, the Units of activity remain the same as in the purified state of the same sample, but the protein content would decrease because of other protein contaminants, and consequently the specific activity attributed to the enzyme would be less. The specific enzyme activity of the BL21 host was subtracted from the specific activity of the CTX-M producing plasmids to discount the residual β-lactamase activity produced by the host strain.

The CTX-M-1 and CTX-M-14b producing plasmids, pI2-38TF and pI2-47TF had the highest specific β-lactamase activities. Interestingly the CTX-M-32 producing plasmid, pI2-53 was found to have a higher specific activity compared to pI1-34TF; the CTX-M-15 producing plasmid that had a higher enzyme activity, suggesting the CTX-M-32 plasmid has a higher activity despite having a lower protein concentration. The lowest activity was observed with the CTX-M-15 producing plasmid, pI1-33TF.

Table 6-5: Calculating specific enzyme activity.

Plasmid	[T. Prot] (µg)	[T. Prot] (mg)	Enzyme activity (Units/ml)[a]	Total specific activity (µmol $min^{-1}mg^{-1}$)	Specific activity (µmol $min^{-1}mg^{-1}$)
BL21	7713	7.71	$4.57x10^{-4}$	$5.92x10^{-5}$	-
pI1-33TF	8054	8.05	$7.03x10^{-3}$	$8.73x10^{-4}$	$8.14x10^{-4}$
pI1-34TF	8054	8.05	$1.46x10^{-2}$	$1.82x10^{-3}$	$1.76x10^{-3}$
pI2-38TF	8349	8.35	$2.46x10^{-1}$	$2.95x10^{-2}$	$2.94x10^{-2}$
pI2-47TF	8032	8.03	$1.83x10^{-1}$	$2.27x10^{-2}$	$2.27x10^{-2}$
pI2-53TF	7154	7.15	$1.24x10^{-2}$	$1.73x10^{-3}$	$1.67x10^{-3}$

a; Enzyme units are the amount of product in µmoles formed in one minute in 1ml of a reaction with units (µmol min^{-1}).
b; The total enzyme activity for the plasmid free *E. coli* BL21, into which all test plasmids were transformed. The specific enzyme activity for the test plasmids was subtracted from the specific enzyme activity of plasmid free *E. coli* BL21 (residual β-lactamase activity).

6.2.4.4 Determining the total enzyme concentration; $[E]_T$

The enzyme concentration (mol L^{-1}) was calculated as described in section 2.5.5.5 (chapter 2). The concentration of β-lactamase in CTX-M producing plasmids was similar averaging at $2.6x10^{-10}$ (see table 6-6), indicating that all the plasmids produce the same amount of enzyme.

Table 6-6: Calculating $[E]_T$.

Plasmid	[E] (mg ml^{-1})	[E] (g L^{-1})	M_W (Da)	$[E]_T$ (mol L^{-1})
pI1-33TF	8.64	$8.64x10^{-6}$	31143.60	$2.77x10^{-10}$
pI1-34TF	8.32	$8.32x10^{-6}$	31143.60	$2.67x10^{-10}$
pI2-38TF	8.37	$8.37x10^{-6}$	31245.69	$2.68x10^{-10}$
pI2-47TF	8.05	$8.05x10^{-6}$	30979.38	$2.60x10^{-10}$
pI2-53TF	7.41	$7.41x10^{-6}$	31187.66	$2.37x10^{-10}$

The total enzyme concentration $[E]_T$ (mol L^{-1}) was calculated by dividing the total enzyme activity (µmol min^{-1}), determine in section 6.1.5 of this chapter by the specific activity (µmol min^{-1} mg^{-1}), determined in section 6.1.6. $[E]_T$ is the concentration of β-lactamase enzyme in a 1 ml reaction.

6.2.4.5 Kinetic parameters

The kinetic parameters of the crude extracts from CTX-M producing plasmids showed pI2-53TF (CTX-M-32) to have the lowest K_M with 10.09 μM, followed by pI1-33TF (CTX-M-15); 14.46 μM, pI2-47TF (CTX-M14b); 20.05 μM and finally pI2-38TF (CTX-M-1) and pI1-34TF (CTX-M-15) with K_M values of 38.67 μM and 38.81 μM respectively (Fig. 6-11 and table 6-7). An enzyme with a low K_M achieves maximal catalytic efficiency at low substrate concentration and is considered to have a higher affinity for the substrate. Therefore by this definition, the CTX-32 producing plasmid has the highest affinity for nitrocefin and the enzyme to have the lowest affinity is the CTX-M-15 producing plasmid, pI1-34TF. This plasmid produces the same CTX-M variant as pI1-33TF, but these plasmids may sit on very different genetic platforms, which may explain the variation observed.

Catalytic efficiencies (k_{CAT}/K_M **)** of the five test plasmids showed the CTX-M-14b producing plasmid, pI2-47TF to be the most efficient system with the highest k_{CAT}/K_M value, 211.63 $\mu M^{-1}s^{-1}$ (table 6-7). This was followed by CTX-M-32 pI2-53TF (103.09 $\mu M^{-1}s^{-1}$), then CTX-M-1 pI2-38TF (97.45 $\mu M^{-1}s^{-1)}$ then finally the CTX-M-15 producing plasmids pI1-34TF (37.02 $\mu M^{-1}s^{-1}$) and pI1-33TF (28.59 $\mu M^{-1}s^{-1}$). Plasmids pI2-47TF, pI2-53TF and pI2-38TF were estimated to be approximately 44 kb in size by S1-nuclease PFGE. Given the relative sizes; 44 kb verses the 70-160 kb CTX-M-15 plasmids, the two-fold drop of the enzyme efficiency between the 44 kb plasmids and the two larger IncF CTX-M-15 plasmids suggests a possible link with copy number; which is tightly regulated for IncF plasmids, and the enzyme efficiency. The lower enzyme efficiencies observed with the IncF CTX-M-15 plasmids might be due to lower plasmid copies of the CTX-M producing plasmid leading to lower gene expression and consequently lower enzymatic activities of these enzymes.

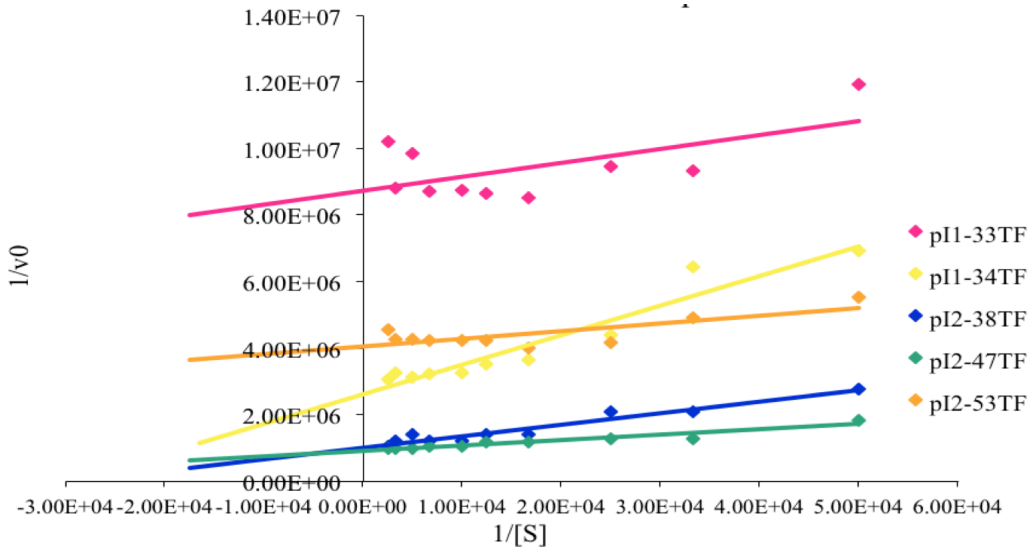


Figure 6-11: Double reciprocal (Lineweaver-Burke) plot used to determine K_M and V_{MAX} values using the intercept; X and Y intercepts respectively using the formula function in Microsoft excel.

Table 6-7: Kinetic parameters of crude extracts from transformed *E. coli* BL21 strains harbouring *bla*$_{CTX-M}$ variants.

Genotype	Plasmid	K_M (μM)	V_{MAX} (μM s^{-1})	$[E]_T$ (μM)	K_{CAT} (s^{-1})	K_{CAT}/K_M ($\mu M^{-1}/s^{-1}$)
bla$_{CTX-M-15}$	pI1-33TF	14.46	0.11	$2.77x10^{-4}$	413.51	28.59
bla$_{CTX-M-15}$	pI1-34TF	38.81	0.38	$2.67x10^{-4}$	1436.86	37.02
bla$_{CTX-M-1}$	pI2-38TF	38.67	1.01	$2.68x10^{-4}$	3768.07	97.45
bla$_{CTX-M-14b}$	pI2-47TF	20.05	1.10	$2.60x10^{-4}$	4242.59	211.63
bla$_{CTX-M-32}$	pI2-53TF	10.09	0.25	$2.37x10^{-4}$	1040.29	103.09

The enzyme turnover or catalytic constant (K_{CAT}) was calculated as a measure of the efficiency of the enzyme by dividing the V_{MAX} by the total enzyme concentration [E]T. Catalytic efficiencies are given as K_{CAT}/K_M.

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6.2.5 Susceptibility of plasmid harboring *bla*$_{CTX-M}$ plasmid in a BL21 host

PAβN was used to investigate the clinical relevance of using the EPIs in successfully inhibiting extrusion of β-lactams; which are transported via the AcrAB-TolC efflux system, the substrate for this EPI.

The concentration at which PAβN (100 μg/ml) was used, was shown to effectively inhibit efflux in a dose dependent manner (Coldham et al., 2010). To eliminate toxicity related cell death, which may be associated with prolonged exposure to the inhibitor during susceptibility assays, a range of concentrations were used. The cells were incubated overnight in the presence of PAβN at 25, 50 and 100 μg/ml in the presence of ampicillin, cefotaxime or ceftazidime.

For three of the plasmids, the decrease in MIC in the presence of increasing concentrations of PAβN was indirectly correlated. Plasmids, pI2-38TF and pI2-47TF and pI2-53TF showed up to a 2000 fold decrease in MIC in the presence of increasing concentrations of PAβN, making these strains susceptible to ampicillin (Fig. 6.12A). The CTX-M-15 producing plasmids pI1-33TF and pI1-34TF did not demonstrate a decrease in MIC in the presence of the EPI, maintaining MIC values of > 1024 mg/L (Fig. 6.12A).

MICs against the cephalosporins cefotaxime (Fig. 6.12B) and ceftazidime (Fig. 6.12C) demonstrated a similar pattern with the three plasmids pI2-38TF, pI2-47TF and pI2-53TF having over a 2000 fold decrease in MIC in the presence of 100 μg/ml PAβN. CTX-M-15 producing plasmids, pI1-33TF and pI1-34TF were found to only have two-fold differences, in some cases resulting in an increase in MIC. Interestingly the MIC increased two fold for both plasmids in the presence of 25 μg/ml PAβN, however the MIC reduced two-fold in the presence of higher concentrations (50 and 100 μg/ml).

This suggests these two plasmids at low EPI concentrations have a decreased susceptibility to the EPI or may induce the expression of a different efflux system.

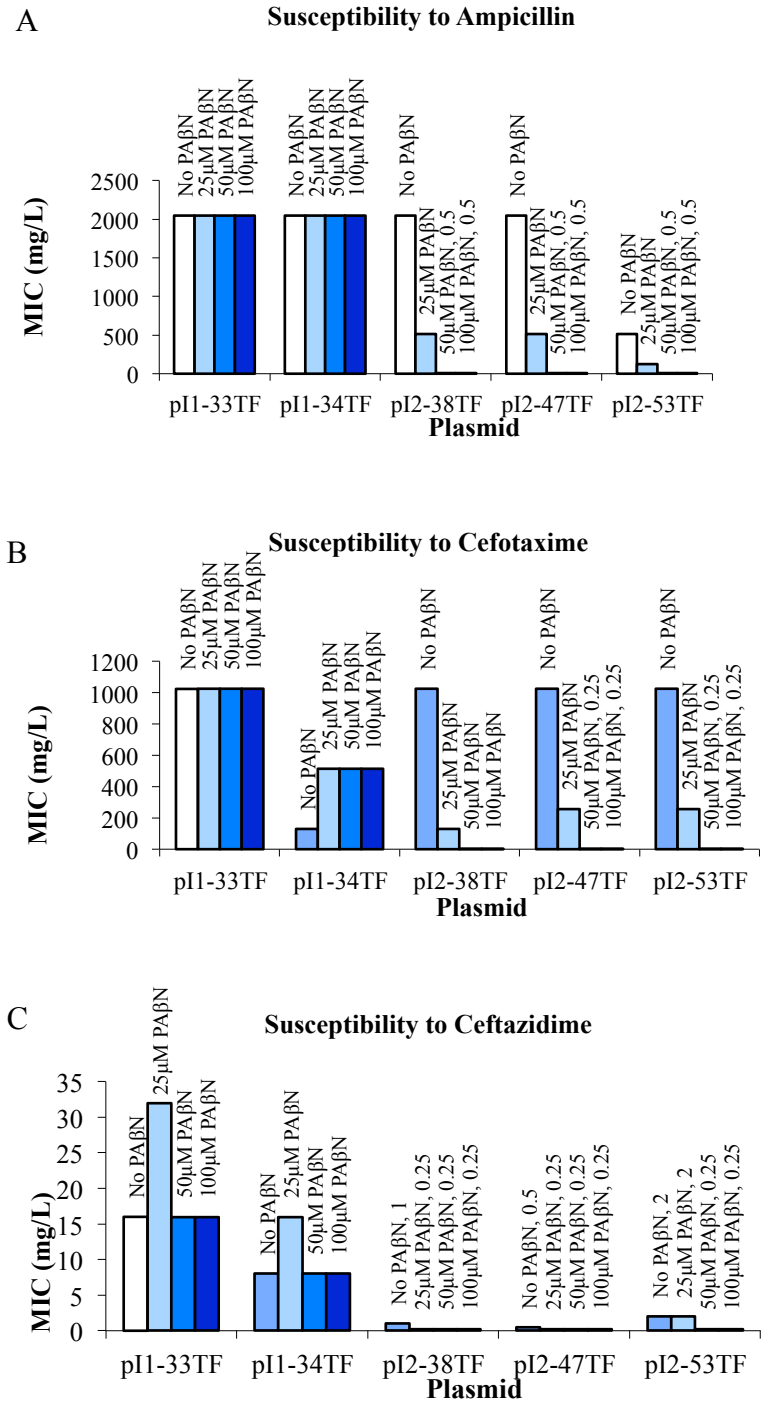

Figure 6-12: Susceptibility to ampicillin, cefotaxime and ceftazidime in the presence of PAβN at 25, 50 and 100μM. The white bars represent strain grown in the antibiotic but untreated with PAβN, the blue bars, in increasing blue intensity (light to dark blue) denote increasing concentrations of PAβN; 25 (light blue), 50 (medium blue) and 100μM (dark blue). A range of concentrations lower than that used to inhibit efflux (100 μg/ml) in the H33343 accumulation experiment were chosen to limit toxicity, however the bacterial cells were able grow in the presence of the highest concentration of PAβN used.

6.3 Discussion

The effect of plasmid acquisition and maintenance for 840 bacterial generations, to host fitness was investigated. Differential growth patterns in defined media were measured in the presence of phosphorous, sulphur and peptide nitrogen sources.

Plasmid pI1-34TF was selected for analysis of plasmid maintenance and its impact on host fitness using the Biolog phenotypic microarray. The size of the pI1-34TF (150 kb) made this an ideal candidate, because the size may impose a greater fitness cost to the host. Daily single colonies were picked, artificially forcing a genetic bottleneck of a random single cell, a process thought to limit mutations that may outcompete each other in a heterogeneous population, thereby decreasing the likelihood of bias estimates of the mutation rate (Andersson et al., 1996; Sniegowski et al., 2010; Stevens et al., 2011).

After passaging on plates for 30 cycles, the "aged" plasmid harboring strain was found to utilize phosphorous compounds less than the BL21 strain but interestingly metabolized some dipeptide nitrogen sources better. This reduction of phosphorous utilization compounds may be a result of the stress conditions of growing the plasmid on solid media for prolonged periods. In periods of stress, the RpoS; a stress inducible RNA polymerase (RNAP) σ factor or σ^S is produced. To confirm this hypothesis, RT-PCR could be performed on selected genes of the RpoS regulon to assess the contribution to the phenotype observed. When complexed with RNAP, the holoenzyme induces expression of stress response genes including genes that control phosphate (P_i) transport and metabolism (Ruiz and Silhavy, 2003). The induction of RpoS during carbon and P_i limitation would result in activation of the *pst* regulon, that encodes proteins involved in P_i transport. Ruiz and colleagues found that in limited P_i *rpoS* translation and stability increase, resulting in increase P_i transport and metabolism. In the present study the abundance of P_i compounds may result in the opposite function,

that would decrease the translation of *rpoS* and consequently the expression of the Pst transport system, resulting in decreased utilization of phosphorous observed.

To investigate the role of possible reduced growth rates as a result of acquiring the plasmid, direct competition experiments with the plasmid free and plasmid-harboring strains were performed. The results did not show any significant difference in growth between the two strains, suggesting no little or not cost to growth rate.

Plasmid acquisition and maintenance is thought to impart a fitness cost due to the expression of genes encoded within it (Andersson and Hughes, 2010). During selection the plasmid confers some advantage (e.g. antibiotic resistance) enabling it to be retained in the host. It can be assumed due to its fitness cost that the plasmid should be lost, but rarely is this observed (Andersson and Hughes, 2010). In this chapter the *in vitro* effects of plasmid acquisition and maintenance with respect to β-lactamase enzyme kinetics, growth kinetics and metabolite usage in defined carbon sources were investigated.

The CTX-M-14 producing plasmid was found to be the most enzymatically efficient plasmid system followed by the CTX-M group 1 plasmids, then CTX-M-32, CTX-M-1 and the CTX-M-15 plasmids in descending order. Previously published studies report CTX-M group 1 enzymes to have higher enzymatic efficiencies than the group 9 enzymes (Bonnet, 2004).

In the results presented above the efficiency of the plasmid system was investigated, which includes the CTX-M enzymes in addition to other β-lactamases that may be encoded within the plasmid, as in pI1-34TF and pI2-47TF, which encoded TEM-1 and OXA-1 enzymes.

Evolutionary trajectories of the CTX-M group 1 enzymes published by Novais et al. put forward the hypothesis that the CTX-M-3 enzyme was likely to be the evolutionary predecessor of the group 1 enzymes (Novais et al., 2010). They then proposed the diversification of this group was a result of exposure to cefotaxime and ceftazidime. As a consequence higher MICs are observed for both antibiotics in later variants. CTX-M-1, -15 and -32, are thought to be derivatives of CTX-M-3 variant and are thought to be the more efficient inactivators of ceftazidime when compared to the group 9 enzymes (CTX-M-14 and -14b) because of a D240G amino acid substitution observed in CTX-M-15 and -32 variants, thought to render the bulkier ceftazidime side chain more accessible to β-lactamase (Bonnet, 2004; Chen et al., 2005; Novais et al., 2010).

In the kinetic measurements, nitrocefin, a narrow spectrum cephalosporin was used to measure the efficiency of β-lactam inactivation. In this instance the lowest efficiency was observed in plasmids producing CTX-M-15 (pI1-33TF and pI1-34TF). This was unexpected. These enzymes had higher affinities (low K_M) but were found to be the least efficient compared to the other plasmids. Plasmid pI2-53TF producing CTX-M-32 was found to the second most efficient enzyme after CTX-M-14b, with higher MIC values against cefotaxime. Previous studies have found CTX-M-14 enzymes to hydrolyze cefotaxime more efficiently than ceftazidime. The Ser237Ala amino acid substitution in CTX-M-14 variants is thought to contribute to the increased efficiency observed between cefotaxime and ceftazidime. The decreased steric hindrance and the formation of a hydrogen bond with the carboxylate functional group of cefotaxime is thought to bring the β–lactam ring into position for acylation (Ma et al., 2002). This difference between the CTX-M-14 (group 9) enzyme and the remaining group 1 enzymes (CTX-M-1, 15 and 32) that lack this mutation may account for the higher tolerance to cefotaxime.

Subtle differences between the two CTX-M-15 producing plasmids were observed in in the enzyme kinetic measurements and the MIC. Lower susceptibility to ampicillin and ceftazidime was observed in pI1-33TF encoding just the CTX-M-15 β–lactamase (from DNA microarray data) compared to pI1-34TF encoding TEM-1 and OXA-1 in addition to CTX-M-15. Although TEM-1 and OXA-1 are poor substrates of ceftazidime and cefotaxime (Aubert et al., 2001), they are efficient inactivators of aminopenicillins and in the case of TEM-1 reported to be a more efficient than CTX-M at hydrolyzing aminopenicillins (Bonnet, 2004). This additional β-lactamse activity may have resulted in higher efficiency values for pI1-34TF compared to pI1-33TF, despite both plasmids encoding the same CTX-M variant.

Previous studies have reported lower β-lactamase activities upon acquisition of an SHV encoding plasmid from *K. pneumoniae* to *E. coli* by conjugation, thought to be a result of a lower plasmid copy number in the new host (Xiang et al., 1997). Another later study also associated an increase in plasmidic CMY-2 expression and subsequent MIC values to increased plasmid copy number as a result of a point mutation in the IncI1 plasmid replication control RNA antisense molecule (Kurpiel et al., 2012). This suggests that plasmid copy number may influence the overall kinetics of β-lactam hydrolysis as part of the plasmid maintenance mechanisms the plasmid copy number is tightly controlled, especially in large IncF plasmids such as pI1-34TF (~160 kb) and pI1-33TF in this study. As a result the amount of enzyme expressed may be lower in comparison to a smaller high copy plasmid harbouring the same resistance gene. As a consequence of lower expression, the amount of enzyme catalysing the hydrolysis of the β-lactam may be affected leading to lower efficiencies observed (Valenzuela et al., 1996). By using the method of crude lysis to measure the kinetic parameters of the β-lactamases expressed by the plasmid, variables such as plasmid copy number, β-lactam expression and multiple β-lactamases encoded on the same plasmid, which may all

influence the overall efficiency of β-lactam inactivation, and should be taken into account. The low efficiencies for the two CTX-M-15 enzymes possibly reflects this variability; an efficient enzyme in an inefficient plasmid system (large, low copy plasmid). To confirm this, real time PCR analysis and transcriptomics (RNAseq) could be performed to assess the levels of CTX-M expression should be performed to confirm whether the size and therefore the copy number of the plasmid influences β-lactamase expression. This experiment should also be repeated with a larger sample set with strains harbouring varying sizes of plasmids, to determine if the β-lactamase efficiency is affected by plasmid size. It must be mentioned here the limitations of this assay is also the possible contamination of the lysate with inhibitory molecules or degrading enzymes that may alter kinetic data. An improvement of this assay would be to include protease inhibitors during the cell lysis stage.

Other effectors of multidrug resistance include the active efflux of antimicrobial compounds. Plasmid sequence analysis identified multiple transport and membrane associated proteins were encoded in pI1-34TF and pI2-47TC. Based on this observation, the ability to extrude antimicrobials by diminishing host efflux by using EPIs was monitored by chages in MIC to amp, caz and ctx. In the presence of PAβN, complete susceptibility of some of these ESBL plasmid-harbouring strains to ampicillin was achieved.

In conclusion, the effect of plasmid acquisition on the host depends on the variant of CTX-M encoded in addition to the plasmid genetic platform on which it is encoded (Bennett, 2008). For example, plasmid copy number, which may have effectors that result in increased tolerance to the drug by way of decreased efflux, or increased/decreased survival in carbon limiting sources. In addition, the plasmid

appears to have no effect or slightly increased fitness in non-aged colonies in the absence of selection in phosphate and dipeptide sources. Previous studies observed an increased in fitness upon plasmid acquisition, which may have implications in persistence of MDR plasmids (Enne et al., 2004; Michon et al., 2011). The aged colonies appeared to have a reduced fitness in the absence of selection, however these were grown on selected metabolites and do not necessarily reflect 'natural' environments i.e. a host strain that has evolved to accommodate multiple plasmids without a fitness burden or the ecosystem these strain may be surviving (the gastro-intestinal tract) may have other metabolites abundances/shortages not tested in the phenotypic microarray. Using a BL21 lab adapted strain as a host, may however add some limitations to the interpretation of this data, as this strain has been passaged over a prolonged period and may have accumulated detrimental mutations that could affect the reduced metabolite usage. This experiment could be improved by using a field strain, naive to this plasmid and compare differences in metabolite usage upon acquiring the plasmid.

These results indicate the contribution of a multitude of factors: plasmid regulation; copy number and gene expression, encoded resistance genes and MDR efflux, are involved in persistence of antimicrobial resistance plasmids without impacting on host fitness unless grown under nutrient limiting conditions.

7 GENERAL DISCUSSION

The increased isolation of CTX-M producing *E. coli* is of great concern to human and veterinary health (Li et al., 2007; Livermore et al., 2007; Rossolini et al., 2008). *E. coli* is one of the most common species isolated from clinical and veterinary samples, and dissemination of CTX-M producing strains is not limited to either setting, which impacts greatly on the epidemiology of the ESBL producing strains by increased incidences of *E. coli* harbouring the ESBL phenotype (Bradford, 2001; Brinas et al., 2003; Bonnet, 2004; Li et al., 2007).

The diverse nature of $bla_{\text{CTX-M}}$ encoding strains collected from both commensal and pathogenic *E. coli* species in the United Kingdom was demonstrated by PFGE analysis (see chapter 3), with all of the strains harbouring a MDR phenotype, however it must be said that these strains were initially screened for ESBL production and therefore may only be representative sample of ESBL producing strains and not the complete *E. coli* population that may have not harboured the ESBL containing plasmids. An interesting approach would have been to isolate *E. coli* strains from a single sampling (time or location) and subsequently screened for the resistance phenotype, accounting for the percentage of strains conferring the ESBL phenotype.

All the isolates conferred a MDR phenotype and interestingly were found to belong to the avirulent commensal *E. coli* strains (A and B1 phylogroups) and virulent D and B2 phylogroups (Chattaway et al., 2011; Sims and Kim, 2011). These results emphasise the potential of cattle to act as reservoirs of multi-drug resistant pathogenic *E. coli* strains, which may be easily passed to humans through the food chain. The presence of these MDR strains in food producing animals and domestic animals have previously been implicated in spread of *E. coli* clones between humans and animals (Johnson et al., 2009). The global spread of a MDR *E. coli* clone ST131 O25:H4, is one such successful

clone (Coque et al., 2008; Nicholas-Chanoine et al., 2008). This UPEC strain is commonly associated with IncFII, IncFIA-FIB and IncI1 plasmids producing CTX-M-15 (Woodford et al., 2009) and CTX-M-14 enzymes (Periano and Pitout, 2010). These two types of plasmids are known to be transmissible by conjugation. The association of *bla*CTX-M genes on conjugative plasmids is thought to have contributed to the dissemination of these plasmids (Naseer and Sundsfjord, 2011). Plasmid MLST studies on the IncI1 plasmids from the present study demonstrate the widespread nature of these plasmids, with some of the IncI1 plasmids reported in Belgium, France, Germany, The Netherlands, UK and the USA. Interestingly, these IncI1 STs have been isolated from *E. coli* and *S. sonnei* species from human sources indicating the potential of transfer of these plasmids between different bacterial species and between human and animals.

The isolates in this study were found to harbour at least one plasmid encoding a minimum of one ESBL gene. These were limited to $bla_{\text{CTX-M}}$ group 1 and $bla_{\text{CTX-M}}$ group 9 variants, harboured amongst conjugative plasmids with IncF (IncFIA, FIB, FII), IncI1, IncN, IncB/O and IncX1 groups which were identified by replicon typing and plasmid sequencing for the IncX1 plasmid; although the latter has more recently been incorporated as part of a new subtyping system for the IncX plasmids (Johnson et al., 2012). The plasmids represent known conjugative narrow (IncB/O, IncF, IncFIB and IncI) and broad (IncN) spectrum plasmids and are amongst those commonly found to be associated with large plasmids encoding multiple-antimicrobial resistance genes (Couturier et al., 1988; Boyd et al., 2004; Lavollay et al., 2006; Waters, 1999).

These plasmids were found to be transmissible by conjugation in most cases after one hour incubation, resulting in the transfer of the MDR phenotype in plasmid sizes ranging from 28 to 100 kb. This transfer appeared to be irrespective of size with some of the 45 kb IncN plasmids having slower transfer rates than a 171 kb IncF plasmid (see Table 4-4). In the presence of selection, plasmids conferring a selective advantage i.e.

antimicrobial resistance will be maintained in the host. However in the absence of selection the plasmids were still maintained in the host. This was observed with the introduction of a 160 kb IncFIA-FIB-FII plasmid into an *E. coli* BL21 host. *E. coli* genomes vary in size, ranging from 4.6 Mb (*E. coli* K12) to 5.4 Mb (EHEC 0157:H7). It is estimated that *E. coli* genomes could potentially encode 4500 genes giving rise to approximately 4300 known polypeptides (http://ecocyc.org/ECOLI), therefore the effect of plasmid gene expression, in this case approximately 200 genes, may not impact greatly on the host. Sequencing (DNA and RNA-seq) of both the plasmids and the chromosome may give insight as to the origins of the phenotypes observed in the Biolog and growth kinetics assays.

Plasmid sequencing revealed multiple plasmid maintenance systems were present to ensure stable inheritance of the plasmid in the absence of selection, which included multimer resolving systems (*parA*) and multiple post segregation killing systems *(hok/mok*, *ccdB/ccdA, pemK/pemI*), with one IncFIA-FIB-FII plasmid encoding at least four of these addiction systems, a feature commonly found in IncF plasmids encoding $bla_{\text{CTX-M}}$ (Mnif et al., 2010). These maintenance systems encoded within the plasmid may enable the 160 kb plasmid to be maintained over the 840 generations despite not having the compensatory *rpoB* mutations or observable phenotypes

The establishment and maintenance of these plasmids in a new host that may be of a different bacterial genus would need to express the genes essential for plasmid replication and maintenance. In addition to plasmid based promoter sequences, all the plasmids identified in this study harboured IS*Ecp1* elements upstream of the CTX-M genes, a feature commonly associated with $bla_{\text{CTX-M}}$ genes (Poirel et al., 2003; Miriagou et al., 2006). IS*Ecp1* elements are part of the MGE superfamily that can mobilize the intervening gene (Mahillon et al., 1999; Poirel et al., 2003; Canton and Coque, 2006;

Miriagou et al., 2006; Novais et al., 2010). IS*Ecp1* elements not only contribute to their dissemination but have also been linked to driving expression of the genes downstream; a quality thought to be exploited when genetic transfer occurs between bacteria of differing species origin, where expression in the recipient species is low due to a weak promoter (Poirel et al., 2003), thereby increasing the potential of the plasmid to occupy a new niche.

Sequence analysis of plasmids provided additional evidence of their mosaic nature, especially in the IncFIA-FIB-FII triple replicon plasmid. The presence of three replicons provided evidence of fusion of these three subgroups of IncF plasmid types, and comparisons of this plasmid with previously sequenced plasmids gave further evidence for genetic exchange, with similarities between 37-57 % observed with other IncF plasmids from human origin (see Fig. 5-1) (Perichon et al., 2008; Woodford et al., 2009; Smet et al., 2010). The presence of multi-replicon plasmids may be advantageous if the host were to acquire another plasmid of the same Inc group. The replication could occur at another replication region enabling the plasmids to co-exist and as seen in the IncF plasmid reported in this thesis, HGT may occur, suggesting the ability to accommodate multiple plasmids of the same Inc group may result in variability enabling the exchange of genetic material from a larger pool of plasmids.

The presence of mobile genetic elements such as, insertion elements; IS*Ecp1*, IS*26*, IS*1*, integrons; *intI1*, *intI2* and complex transposons Tn*21* and Tn*3*, facilitate the capture of genetic material aiding the evolution of these plasmids and their dissemination. Previous studies have shown the common association of $bla_{\text{CTX-M}}$ variants with class 1 and 2 integrons (Carattoli, 2001). These integrons are often found within transposons, which capture genes that confer resistance, namely to aminoglycosides (*aadA*), chloramphenicols (*cmlA*), sulphonamides (*sul1*) and trimethoprims (*dfrA*). The Tn*21* family of transposons have previously been associated with $bla_{\text{CTX-M}}$ genes, and are

thought to influence the spread of genes they encompass within integrons (Liebert et al., 1999; Novais et al., 2006; Valverde et al., 2006). The identification of the Tn*21* transposon associated transposases and resolvases on all of the sequenced plasmids, pI2-53TF, pI2-47TC, pI2-52TC and pI1-34TF belonging to the IncX1, IncI1-X1, IncB/O and IncFIA-FIB-FII plasmid groups, indicates that this Tn*21* element contributes to their multidrug phenotype and the high probability of its role in the spread of MDR.

Plasmid sequencing of pI2-53TF revealed a) a backbone to be similar to IncI1 plasmid, b) acquired resistance gene cassettes commonly associated with a class I integrons, c) an IncX1 replication protein (*pir*) and d) the $bla_{\text{CTX-M-32}}$ gene associated with the IS*Ecp1* element (see Fig. 5-11). This plasmid was found to be approximately 35 kb in size by S1 nuclease PFGE and able to conjugate at frequencies of 1.50×10^{-8}. In 2011, Partridge and colleagues published the first $bla_{\text{CTX-M-15}}$ harbouring IncX1 plasmid pJIE143, which is 35 kb in size (Partridge et al., 2011). To date, this is the first CTX-M-32 producing IncX1 plasmid identified. Due to the similar size and replication system as pJIE143, it could have been assumed that mutations in the CTX-M-15 genes resulting in the CTX-M-32 variant giving rise to pI1-53TF. The mutation may have been brought about from intense antibiotic selection pressure, as suggested by Novais et al., 2010. However, sequence comparison of these two plasmids revealed the only the CTX-M region encoding $bla_{\text{CTX-M}}$ gene and the IS*Ecp1* element upstream to show 98% similarity (see Fig 7-1). The 2% difference corresponds to the 11 base pair difference between the two CTX-M variants. Therefore it can be proposed that the $bla_{\text{CTX-M-32}}$ gene on an IncX1 background was acquired via an IS*Ecp1* mediated transposition event by other plasmids such as IncN, commonly associated with this CTX-M variant (Diestra et al., 2009) or via a Asp240-Gly substitution of a CTX-M-1 variant (Cartelle et al., 2004).

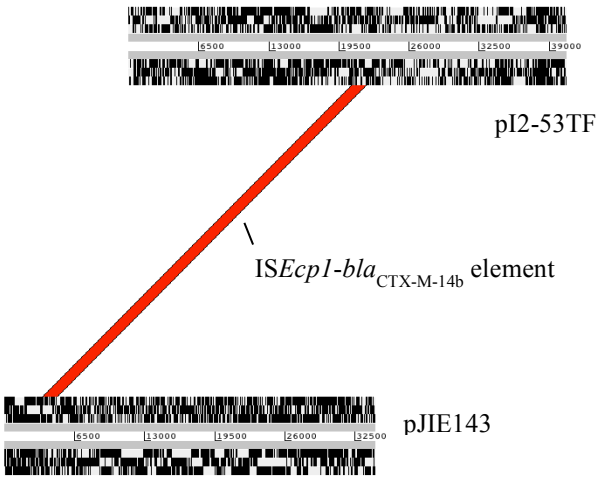

Figure 7-1: Sequence alignment of pI2-53TF an IncX1 plasmid harbouring the *bla*$_{CTX-M-32}$ variant and pJIE143 another IncX1 plasmid harbouring the *bla*$_{CTX-M-1}$ variant.

IncX1 plasmids have also been associated with *bla*$_{CTX-M-1}$ and *bla*$_{TEM-1}$ as a conintegrate with an IncHI1 plasmid (Dolejska et al., 2011; Literak et al., 2010). Another IncX1 plasmid from *S. enterica* Dublin was also described as a cointegrate with an IncF plasmid (Chu et al., 2008). Like the two plasmids sequenced in this study; pI2-53TF (IncX1) and pI2-47TF (IncX1-IncI1proposed cointegrate), these IncX1 plasmids have the potential to transfer by conjugation independently (Partridge et al., 2011; Ong et al., 2009) or as a cointegrate (Chu et al., 2008, Dolejska et al., 2011) thereby maximising the chance of persistence of in a population.

Before a recently published typing system for the IncX plasmids (Johnson et al., 2012) that further subdivided the IncX group into four different subtypes, the initial typing system by PBRT only identified plasmids of the IncX2 subgroup (Carattoli et al., 2005; Johnson et al., 2012). When sized by plasmid profiling techniques previously, this 35 kb band migrated at the same rate as the genomic DNA contaminant, this coupled with the previous undetected IncX1 plasmids by PBRT suggests that there may be a gross underestimation of IncX1 plasmids during screening of strains harbouring *bla*$_{CTX-M}$ genes with the ability to independently mobilize in Enterobacteriaceae.

Consistent within all the sequenced plasmids in this study was the genetic variability between them and with other previously sequenced plasmids. It has been suggested that in order for a plasmid to be successful and persist in a population, it must retain the most efficient inheritance systems, low fitness burden and a high transfer rate (Sorensen et al., 2005). The adaptation of the plasmid by genetic exchange resulting in gene loss or gain can be advantageous in some cases to the host (Enne et al., 2005). One such advantage may be the acquisition of a more efficient CTX-M enzyme by mutation or lateral gene transfer; mediated by MGEs. The rapid rise in CTX-M enzymes in the bacterial population that quickly replaced TEM ESBLs may have been the result of a selective advantage offered by the more efficient enzyme; CTX-M at hydrolysing 3rd generation cephalosporins as their use in the clinical and veterinary setting began to increase (Bonnet, 2004; Livermore et al., 2007). However TEM-1 enzymes are still prevalent within the plasmid population and was present in nearly half the plasmids tested, all of which were also harbouring CTX-M variants (see chapter 4). However, noted is the selection bias these isolates present due to initial screening on chromagar media supplemented with 2 mg/L cefotaxime, which would consequently not select for isolates encoding TEM only, and therefore there may be an under-representation of TEM producing isolates in the isolates. The question arises that why, if a more efficient inactivator of the extended spectrum cephalosporins were present, would other β-lactamase enzymes such as TEM-1 and OXA-1 persist? The results of the plasmid enzyme kinetics revealed the most efficient systems to be plasmids producing CTX-M-14 and CTX-M-1 enzymes, with CTX-M-15 producing plasmids being the least efficient. Previous enzyme kinetics studies of purified CTX-M β-lactamases using ceftazidime and cefotaxime substrates, report CTX-M-14 enzymes to have higher efficiencies at hydrolysing ceftazidime than the CTX-M-15 and CTX-M-1 variants (Barthelemy et al., 1992 1122; Dutour et al., 2002; Poirel et al., 2002 50). Although

those studies have reported differences in efficiencies (K_{CAT}/K_M) between the group 9 (CTX-M-14) and group 1 (CTX-M-1 and -15) enzymes to be less than 1 fold. In this study, using nitrocefin as the substrate, a 6-fold difference was observed with the cell extracts of *E. coli* K12 harbouring plasmids encoding these derivatives (see Table 6-8). Enzyme efficiencies may be affected as a result of plasmid copy number, which when studied in plasmids used in recombinant gene expression, show a maintenance burden proportional to the plasmid size, thought to be a result of high replication and gene expression (Camps, 2010). By maintaining low copy numbers for the larger IncF plasmids (producing CTX-M-15), the burden of maintaining these plasmids may decrease but at the cost of gene expression and the opposite may be said for the other smaller ~44kb plasmid harbouring the CTX-M-1, -14b and -32 variants. This may in turn affect the efficiency of nitrocefin hydrolysis and result in the efficiencies observed.

Differences were also observed when the plasmid harbouring strains were grown in the presence of cefotaxime. The largest CTX-M-15 producing plasmid (160 kb); pI1-34TF (in a BL21 host strain), experienced an extended lag phase prior to growth for the first 20 hours, which in the absence of host efflux mechanisms (AcrAB and MdtABC) resulted in the recovery of growth to similar levels to another CTX-M-15 producing plasmid (pI1-33TF) after this time, suggesting the presence of the plasmid had some effect of host permeability (Fig. 6-2). This was reflected when MICs against cefotaxime, ceftazidime and ampicillin in BL21 strains harbouring these CTX-M-15 plasmids were unaffected upon treatment with the EPI, PAβN (Fig. 6-21). These results suggest that these two plasmids may not rely heavily on host efflux for decreased susceptibilities to these compounds as may be the case for the other CTX-M producing plasmids, but use the plasmid mediated β-lactamase enzyme to efficiently cleave the antimicrobial to a lower inhibitory concentration, enabling the bacteria to survive to a point when the drug concentration is negligible or has degraded. The half-life of cefotaxime is

approximately 1 hour (Patel et al., 1995). The combined effect of degraded cefotaxime by natural or enzymatic processes may be a contributing factor to the survival of bacteria harbouring pI1-34TF-like plasmids.

A proposed hypotheses for this stark difference in enzyme kinetics and the growth of bacteria harbouring these plasmids is the genetic platform in which the β-lactamase enzyme is encoded, includes: 1) the type of β-lactamase encoded; which will determine the efficiency of inactivation, 2) the copy number of the plasmid the enzymes are encoded on; which will subsequently determine the amount of genes expressed and subsequently proteins synthesised. This may in turn may affect the efficiency of the hydrolytic reaction and 3) any additional β-lactamases encoded on the plasmid such as TEM-1, which has been shown to have increased hydrolytic activity towards ceftazidime (Petrosino and Palzkill, 1996); or OXA-1, which confers resistance to β-lactam inhibitors (Zhou et al., 1994).

The dissemination and persistence of a plasmid in a population would be the contributing factor to its success, but is dependant on a complex interplay of multiple factors including the growth of the bacterium in the absence of selection, which would depend on plasmid encoded maintenance systems (partitioning, multimer resolution and post-segregation killing systems); and in the presence of selection the expression of antimicrobial resistance genes, adjacent to IS*Ecp1* elements that may function as promoters resulting in high level expression. In addition to these factors, other attributes that enable coexistence of the plasmid in the host such as the ability to transfer by conjugation, which may create an advantage by promoting cell-to-cell interaction, thereby increasing the chances of biofilm formation (Sorensen et al., 2005), compensatory mutations to alleviate fitness costs, copy number control mechanisms, and permeability alterations; which may serve as an advantage in the presence of antimicrobial selection and the constant genetic exchange that ensures the existence of a

well established plasmid harbouring all these features to exist and spread globally. One such example of plasmid-host harmony is the success of the UPEC O25:H4-ST131 *E. coli* strain harbouring a multidrug resistance IncF conjugative plasmid. With the ability for genetic exchange to readily occur, the genetic shift provided by this exchange could be the determining feature of a successful plasmid, resulting in the balance between selective advantage and fitness costs. This would suggest that the novel IncX1 plasmid identified in this study, harbouring the CTX-M-32 variant with the ability to conjugate, harbour several antimicrobial resistance genes and being relatively small in size, may be able to achieve an ideal symbiotic relationship with the host bacterium. Future studies could involve monitoring the spread this plasmid from its original isolate to a population naïve to this plasmid and determine the persistence of this IncX1 plasmid amongst bacteria already harbouring multiple MDR plasmids over a prolonged period.

In conclusion, the work carried out in this thesis demonstrates the ability of these plasmids to exist in commensal bacteria in cattle, implicating them as natural reservoirs for resistant strains, with the ability to transmit these multidrug resistance determinants and persist in a host without imposing metabolic burden to the host and in some cases even increase fitness by conferring resistance to EPIs, which has implications in future treatment options that favour the development of EPIs in future treatment strategies (Lomovskaya et al., 2007).

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APPENDIX

Appendix A

Please see attached table for a comprehensive view of the strains presented in this thesis.

Appendix B

The table below lists the primers used in this study. The primers are listed in the 5' to 3' direction.

Primers used for *E. coli* phylogrouping		
Name	**Sequence**	**Reference**
ChuA.1	F- GACGAACCAACGGTCAGGAT	Clermont et al. (2000)
ChuA.2	R- TGCCGCCAGTACCAAAGACA	
YjaA.1	F- TGAAGTGTCAGGAGACGCTG	Clermont et al. (2000)
YjaA.2	R- ATGGAGAATGCGTTCCTCAAC	
TspE4C2.1	F- GAGTAATGTCGGGGCATTCA	Clermont et al. (2000)
TspE4C2.2	R- CGCGCCAACAAAGTATTACG	

DNA array PCR validation primers		
Name	**Sequence**	**Reference**
qnr	F- GATAAAGTTTTTCAGCAAGAGG	Jacoby et al. (2003)
	R- ATCCAGATCGGCAAAGGTTA	
sul1	F- TCACCGAGGACTCCTTCTTC	Randall et al. (2004)
	R- AATATCGGGATAGAGCGCAG	
sul2	F- GATATTCGCGGTTTTCCAGA	R. Walker, unpublished
	R- CGAATTCTTGCGGTTTCTTT	
sul3	F- ACCGATAGTTTTTCCGATGG	Batchelor et al. (2008)
	R- TGCGGAGATAATCTGCACCT	
tet(A)	F- GCTACATCCTGCTTGCCTTC	Ng et al. (1999)
	R- CATAGATCGCCGTGAAGAGG	
tet(B)	F- TTGGTTAGGGGCAAGTTTTG	Ng et al. (1999)
	R- GTAATGGGCCAATAACACCG	
tet(C)	F- CTTGAGAGCCTTCAACCCAG	Ng et al. (2001)
	R- ATGGTCGTCATCTACCTGCC	
tet(D)	F- AAACCATTACGGCATTCTGC	Ng et al. (2001)
	R- GACCGGATACACCATCCATC	
tet(E)	F- AAACCACATCCTCCATACGC	Ng et al. (2001)
	R- AAATAGGCCACAACCGTCAG	
tet(G)	F- CCGGTCTTATGGGTGCTCTA	Randall et al. (2004)
	R- CCAGAAGAACGAAGCCAGTC	
intI1	F- GGCATCCAAGCAGCAAG	Goldstein et al. (2001)
	R- AAGCAGACTTGACCTGA	
intI2	F- TTATTGCTGGGATTAGGC	Goldstein et al. (2001)
	R- ACGGCTACCCTCTGTTATC	

aadA1-like	F- TATCAGAGGTAGTTGGCGTCAT	Standvang and Aarestrup. (2000)
	R- GTTCCATAGCGTTAAGGTTTCATT	
aadA2-like	F- TGTTGGTTACTGTGGCCGTA	Randall et al. (2004)
	R- GATCTCGCCTTTCACAAAGC	
aadA4-like	F- CAATCCACCTGTTCGGATCT	Batchelor et al. (2008)
	R- AGCAACGTCCTTAGGAGCAA	
blaPSE-1-like	F- GCTTCGCAACTATGACTAC	Randall et al. (2004)
	R- GTTCACCATCCAAGACTC	
cmlA1-like	F- TGTCATTTACGGCATACTCG	Guerra et al. (2003)
	R- ATCAGGCATCCCATTCCCAT	
catA1	F- CGCCTGATGAATGCTCATCCG	Aarestrup et al. (2003)
	R- CCTGCCACTCATCGCAGTAC	
catIII	F- CCTGGAACCGCAGAGAAC	Arcangioli et al. (2000)
	R- CCTGCTGAAACTTTGCCA	
catB3-like	F- GGTACGACTGGGCATCATCT	Batchelor et al. (2008)
	R- TCGAGCCAATACTTGTGCAG	
floR	F- GGAGCAGCTTGGTCTTCAAC	Randall et al. (2004)
	R- AATGAATATCGCCTGCCATC	
dfrA1	F- GTGAAACTATCACTAATGG	Naviaet al. (2003)
	R- TTAACCCTTTTGCCAGATTT	
dfrA7	F- CAGAAAATGGCGTAATCG	Batchelor et al. (2008)
	R- TCAACGTGAACAGTAGACAAA	
dfr12	F- GGTG(G/C)GCAGAAGATTTTTCGC	Naviaet al. (2003)
	R- TGGGAAGGCGTCACCCTC	
dfrA14	F- ATAGCTGCGAAAGCGAAAAA	Batchelor et al. (2008)
	R- CCCTTTTTCCAAATTTGATAGC	
dfrA17	F- CAGAAAATGGCGTAATCG	Batchelor et al. (2008)
	R- TCAACGTGAACAGTAGACAAA	
dfrA19	F- GCGATTTACGCGGATTTCTA	Batchelor et al. (2008)
	R- CAAAGTGAATGCGCTCTTGA	
aac(3)-Ia	F- TTGATCTTTTCGGTCGTGAGT	Frana et al. (2001)
	R- TAAGCCGCGAGAGCGCCAACA	
aac(3)-IVa	F- TCGGTCAGCTTCTCAACCTT	Batchelor et al. (2008)
	R- ACCGACTGGACCTTCCTTCT	
aac(6')-Ib	F- GTTACTGGCGAATGCATCACA	Frana et al. (2001)
	R- TGTTTGAACCATGTACACGGC	
ant(2")-Ia	F- GGGCGCGTCATGGAGGAGTT	Steward et al. (2001)
	R- TATCGCGACCTGAAAGCGGC	
blaDHA-1	F- AACTTTCACAGGTGTGCTGGGT	Perez-Perez and Hanson.(2002)
	R- CCGTACGCATACTGGCTTTGC	
blaACC	F- AACAGCCTCAGCAGCCGGTTA	Perez-Perez and Hanson.(2002)
	R- TTCGCCGCAATCATCCCTAGC	
blaMOX	F- GCTGCTCAAGGAGCACAGGAT	Perez-Perez and Hanson.(2002)
	R- CACATTGACATAGGTGTGGTGC	
blaCMY	F- TGGCCAGAACTGACAGGCAAA	Perez-Perez and Hanson.(2002)
	R- TTTCTCCTGAACGTGGCTGGC	
blaFOX	F- AACATGGGGTATCAGGGAGAT	Perez-Perez and Hanson.(2002)
	R- CAAAGCGCGTAACCGGATTGG	
blaSHV	F- CGGCCCCGCAGGATTGACT	E. Pleydell, unpublished
	R- TCCCGGCGATTTGCTGATTTC	
blaTEM-1	F- TCGTGTCGCCCTTATTCCCTTTTT	E. Pleydell et al. (2007)
	R- GCGGTTAGCTCCTTCGGTCCTC	
blaOXA-1	F- TTGATGCGGAAATAATAGAT	E. Pleydell, unpublished
	R- TGCGGACACAAAAACATA	
blaOXA-2	F- TTCAAGCCAAAGGCACGATAG	Walker et al. (2001)
	R- TCCGAGTTGACTGCCGGGTTG	
blaOXA-7	F- CGTGCTTTGTAAAAGTAGCAG	Walker et al. (2001)
	R- CATGATTTTGGTGGGAATGG	
blaCTX-M-1	F- CGATGTGCAGTACCAGTAA	Batchelor et al.(2005)
	R- TTAGTGACCAGAATCAGCGG	
blaCTX-M-2	F- CGATGTGCAGTACCAGTAA	Batchelor et al.(2005)
	R- TTAGTGACCAGAATCAGCGG	
blaCTX-M-9	F- CGATGTGCAGTACCAGTAA	Batchelor et al. (2005)
	R- TTAGTGACCAGAATCAGCGG	
blaOXA-9	F- CGTCGCTCACCATATCTCCC	Batchelor et al. (2008)
	R- CCTCTCGTGCTTTAGACCCG	

Amplification of gyrA/parC genes		
Name	**Sequence**	**Reference**
qepA	F- AACTGCTTGAGCCCGTAGAT	Kim et al. (2009)
	R- GTCTACGCCATGGACCTCAC	
gyrA	F- AAATCTGCCCGTGTCGTTGGT	Rodríguez-Martínez et al. (2006)
	R- GCCATACCTACGGCGATACC	
parC	F- CTGAATGCCAGCGCCAAATT	Rodríguez-Martínez et al. (2006)
	R- GCGAACGATTTCGGATCGTC	

CTX-M universal primers		
Name	**Sequence**	**Reference**
CTX-M-uni	F- CGATGTGCAGTACCAGTAA	Batchelor et al. (2005)
	R- TTAGTGACCAGAATCAGCGG	

Primers used for the amplification of CTX-M group 1 and 9 genes		
Name	**Sequence**	**Reference**
CTX-M-9	F- GTGACAAAGAGAGTGCAACGG	Sabate et al. (2002)
	R- ATGATTCTCGCCGCTGAAGCC	
CTX-M-1	F- CCCATGGTTAAAAAATCACTGC	Carattoli A. (2008)
	R- CAGCGCTTTTGCCGTCTAAG	

Primers used for the amplification of CTX-M groups		
Name	**Sequence**	**Reference**
CTX-M-1	F- AAAAATCACTGCGCCAGTTC	Woodford et al. (2006)
	R- AGCTTATTCATCGCCACGTT	
CTX-M-2	F- CGACGCTACCCCTGCTATT	Woodford et al. (2006)
	R- CCAGCGTCAGATTTTTCAGG	
CTX-M-9	F- CAAAGAGAGTGCAACGGATG	Woodford et al. (2006)
	R- ATTGGAAAGCGTTCATCACC	

Primers usd for the amplification of IS*Ecp1* and adjacent *bla*CTX gene		
Name	**Sequence**	**Reference**
ISEcp1F	F- TGCTCTGTGGATAACTTGC	Poirel et al. (2002)
CTX-M-15	R- CCGTTTCCGCTATTACAAAC	
CTX-M-9	R- ATGATTCTCGCCGCTGAAGCC	Sabate et al. (2002)

PCR primers fot the identification of plasmid incopatibility groups		
Name	**Sequence**	**Reference**
F_{repB}	F- TGATCGTTTAAGGAATTTTG	Carattoli et al. (2005)
	R- GAAGATCAGTCACACCATCC	
K/B	F- GCGGTCCGGAAAGCCAGAAAAC	
K	R- TCTTTCACGAGCCCGCCAAA	
B/O	R- TCTGCGTTCCGCCAAGTTCGA	
HI1	F- GGAGCGATGGATTACTTCAGTAC	
	R- TGCCGTTCACCTCGTGAGTA	
HI2	F- TTTCTCCTGAGTCACCTGTTAACAC	
	R- GGCTCACTACCGTTGTCATCCT	
I1	F- CGAAAGCCGGACGGCAGAA	
	R- TCGTCGTTCCGCCAAGTTCGT	
X	F- AACCTTAGAGGCTATTTAAGTTGCTGAT	
	R- TGAGAGTCAATTTTTATCTCATGTTTTAGC	
L/M	F- GGATGAAAACTATCAGCATCTGAAG	
	R- CTGCAGGGGCGATTCTTTAGG	
N	F- GTCTAACGAGCTTACCGAAG	
	R- GTTTCAACTCTGCCAAGTTC	
FIA	F- CCATGCTGGTTCTAGAGAAGGTG	
	R- GTATATCCTTACTGGCTTCCGCAG	
FIB	F- GGAGTTCTGACACACGATTTTCTG	
	R- CTCCCGTCGCTTCAGGGCATT	
W	F- CCTAAGAACAACAAAGCCCCCG	
	R- GGTGCGCGGCATAGAACCGT	
Y	F- AATTCAAACAACACTGTGCAGCCTG	
	R- GCGAGAATGGACGATTACAAAACTTT	

P	F- CTATGGCCCTGCAAACGCGCCAGAAA	
	R- TCACGCGCCAGGGCGCAGCC	
FIC	F- GTGAACTGGCAGATGAGGAAGG	
	R- TTCTCCTCGTCGCCAAACTAGAT	
A/C	F- GAGAACCAAAGACAAAGACCTGGA	
	R- ACGACAAACCTGAATTGCCTCCTT	
T	F- TTGGCCTGTTTGTGCCTAAACCAT	
	R- CGTTGATTACACTTAGCTTTGGAC	
FII_s	F- CTGTCGTAAGCTGATGGC	
	R- CTCTGCCACAAACTTCAGC	

Primers used for PCR amplification and sequencing of IncI1 plasmids		
Name	**Sequence**	**Reference**
repI1	F- CGAAAGCCGGACGGCAGAA	Garcia-Fernandez et al. (2008)
	R- TCGTCGTTCCGCCAAGTTCGT	
ardA	F- ATGTCTGTTGTTGCACCTGC	Garcia-Fernandez et al. (2008)
	R- TCACCGACGGAACACATGACC	
trbA	F- CGACAAATGCTTCCGGGGT	Garcia-Fernandez et al. (2008)
pndC	R- CGAATCCCTCACCATCCAG	
SogS	F- TTCCGGGGCGTAGACAATACT	Garcia-Fernandez et al. (2008)
	R- AACAGTGATATGCCGTCGC	
pilL	F- CCATATGACCATCCAGTGCG	Garcia-Fernandez et al. (2008)
	R- AACCACTATCTCGCCAGCAG	