

Comparative genomics of *bla*_{CTX-M}
plasmids from veterinary and human
Escherichia coli and methods for their
identification and differentiation

By

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The *bla*_{CTX-M} gene confers resistance to penicillins and cephalosporins and is now the most widely disseminated plasmid mediated Extended Spectrum β -lactamase (ESBL). Plasmids harbouring *bla*_{CTX-M} have been recovered from both human and animals isolates, with increasing evidence for the transmission between hosts which is a major public health concern. The aim of this study was to investigate the relationship of *bla*_{CTX-M} plasmids from UK human and veterinary *E. coli* isolates with plasmids previously sequenced around the world, and develop molecular markers to identify and differentiate plasmids.

Molecular markers were first established as a suitable method for identifying plasmids by studying the prevalence of IncK pCT-like plasmids, which were found to be associated with 30% of CTX-M-14 producers in the UK, with plasmids mobilising the gene between unrelated isolates from cattle, turkeys and humans. Seven *bla*_{CTX-M} plasmids, belonging to four incompatibility groups, from *E. coli* were isolated, fully sequenced and annotated. The only human sequenced plasmid was pH19 the first IncZ *bla*_{CTX-M-14} to be sequenced. The plasmids sequenced from animals included pSAM7 from cattle, the first IncX4 plasmid to be sequenced with *bla*_{CTX-M-14b} in a novel transposition unit. Plasmid pCH01 was isolated from a chicken isolate and harboured the *bla*_{CTX-M-3} and is the first IncA/C group CTX-M to be sequenced. The four IncI1 γ *bla*_{CTX-M-1} plasmids from chicken (pCH02 and pCH03), cattle (pCT01) and turkey (pT01), are the first ST3 and IncI1 γ *bla*_{CTX-M-1} plasmids to be sequenced. Comparative analysis found UK plasmids shared approximately 70-99% sequence coverage with previously published sequences from different hosts and bacterial species around the world. This demonstrated that plasmids in the UK were closely related to plasmids found elsewhere, and no genetic characteristics were identified why these plasmid could not exist in either human or animals isolates, with the main differences observed in the inserted resistance regions. In all plasmids the *bla*_{CTX-M} was associated with *ISEcp1*, and included a novel *bla*_{CTX-M-14b} and *bla*_{CTX-M-3} transposition unit in pSAM7 and pCH01 respectively. In 6/7 plasmids the *ISEcp1-bla*_{CTX-M} was not associated with any other resistance regions, inserting as separate events. Molecular markers were designed from the comparative analysis between plasmids that were capable of both identifying and differentiating plasmids belonging to the same incompatibility group. Five groups were identified for IncX4, eight for IncZ, B and K, 12 for IncI1 γ and 14 for IncA/C. Markers were used in screening of field isolates to identify similar plasmids, with novel combinations being observed, not previously identified *in silico*. These markers represent a new non-sequencing based tool to identify and characterise plasmids further, benefitting the study of plasmids and their epidemiology.

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Contributions of others

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Abbreviations

Abbreviation	Definition
°	Degree
°C	Degrees centigrade
μ F	Microfarads
μ g	Micrograms
μ l	Microlitres
μ m	Micrometers
μ M	Micromolar
3'	3 prime end
5'	5 prime end
<i>aac</i>	Aminoglycoside acetyltransferase
<i>aad</i>	Aminoglycoside adenylytransferase
AGP	Antimicrobial growth promoters
AHVLA	Animal Health and Veterinary Laboratory Agency
AT	Antitoxin
ATP	Adenosine triphosphate
<i>bla</i>	β lactamase
BLAST	Basic local alignment search tool
bp	Base pair
BPU	Biological production unit
BSA	Bovine serum albumin
BSAC	British Society for Antimicrobial Chemotherapy
C	C terminus (carboxyl)
<i>cat</i>	Chloramphenicol acetyltransferase
cm	Centimetre
<i>cml</i>	Chloramphenicol efflux
CMY	Cephamecinase
CTX-M	Cefotaximase
dA	Deoxyribonucleotide Adenine
dC	Deoxyribonucleotide Cytosine
dG	Deoxyribonucleotide Guanine
DAEC	Diffuse adhering <i>E. coli</i>
<i>dfr</i>	Dihydrofolate reductase
DNA	Deoxyribonucleotide nucleic acid
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
EAEC	Enterocaggregative <i>E. coli</i>
EDTA	Ethlenediaminetetraacetic acid

EHEC	Enterohemorrhagic <i>E. coli</i>
EIEC	Enteroinvasive <i>E. coli</i>
emPCR	Emulsion PCR
EPEC	Enteropathogenic <i>E. coli</i>
ESBL	Extendend spectrum β lactamase
ETEC	Enterotoxigenic <i>E. coli</i>
ExPEC	Extraintestinal pathogenic <i>E. coli</i>
<i>floR</i>	Florfenicol resistance
g	grams
<i>groESL</i>	chaperoning
H ₂ O	Water
HGT	Horizontal gene transfer
IMP	IMP metallo β lactamase
Inc	Incompatability group
IS	Insertion sequence
kb	Kilo base pair
KPC	<i>K. Pneumoniae</i> carbapenemase
kV	kilo Volts
L	Litre
LB-G	Luria Bertani minus glucose
LEE	Locus of enterocyte effacement
LGT	Lateral gene transfer
M	Molar
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
MAUVE	Mauve genome alignment software
MDR	Multidrug resistant/resistance
MEGA	Molecular evolutionary genetic analysis
mg	Milligram
MGE	Mobile genetic element
min	Minute
ml	Millilitre
MLST	Multi locus sequence typing
mM	Millimolar
mm	Millimeter
MPF	Mating pair formation
N	N terminus (amino)
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NCM	Non <i>bla</i> CMY-2 plasmids
NDM	<i>bla</i> NDM-1 plasmids
NDM-1	New Dehli metallo- β -lactamase
ng	Nanograms
orf/ORF	Open reading frame
OXA	Oxacillinase
PAI	Pathogenicity island
PBRT	PCR based replicon typing
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

<i>pecM</i>	MDR transporter
PFGE	Pulse field gel electrophoresis
pMLST	Plasmid multilocus sequence typing
pmol	Picomole
<i>qacE1</i>	Quaternary ammonium compound resistance gene
<i>qnr</i>	Fluoroquinolone resistance
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Rotations per minute
SCC	Sodium chloride, trisodium citrate
SDS	Sodium dodecyl sulfate
sec(s)	Seconds
SFE	Safe Food Era
SHV	Sulhydryl variable
SNP	Single nucleotide polymorphism
SPATE	Serine protease autotransporters of Enterobacteriaceae
ST	Sequence type
<i>sul</i>	Sulphonamide resistance gene
T4SS	Type IV secretion system
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TEM	Temoneria
<i>tet</i>	Tetracycline resistance gene
Tn	Transposon
TX	Toxin
U	Enzyme unit
UID	Unique Identification number
UK	United Kingdom
UPEC	Uropathogenic <i>E. coli</i>
USA	United States of America
UV	Ultra violet
V	Volts
VIM	Veron integron encoded metallo- β -lactamase
WT	Wild type
x	Times
ZnSO ₄	Zinc Sulfate
Ω	Ohms

Chapter 1

Introduction

1.1 *Escherichia coli*

Escherichia coli (*E. coli*) was first described as *Bacterium coli commune* by Theodor Escherich in 1885, but reclassification led to the organism being named after its discoverer. *Escherichia coli* is a Gram negative, facultatively anaerobic bacilli organism which is capable of using multiple carbohydrate sources, including lactose, in the presence and absence of oxygen (Gray *et al.*, 1966; Silva, Toledo and Trabulsi, 1980). *E. coli* is part of the *Enterobacteriaceae* family, which includes pathogenic species such as *Salmonella*, *Yersinia*, *Klebsiella* and *Shigella* (Jones, 1988). *E. coli* are commonly found in the intestinal tract of warm blooded animals including humans, cattle and poultry, where they are typically commensals, making up part of the naturally occurring gut flora (Berg, 1996; Eckburg *et al.*, 2005).

1.1.1 Commensal *E. coli*

E. coli is one of the first organisms to colonise the large intestine, occurring within hours of birth, but only makes up around 0.1% of the normal human intestinal flora (Penders *et al.*, 2006; Eckburg *et al.*, 2005). *E. coli* has an important role in protecting the gut from pathogenic strains, such as *E. coli* O157, as well as other pathogens (Hudault, Guignot and Servin, 2001; Leatham *et al.*, 2009). This protective effect is likely due to *E. coli* outcompeting its competitors through an increased utilisation of gluconate, providing a growth advantage (Sweeney *et al.*, 1996). Although not pathogenic in nature commensal *E. coli* can become opportunistic pathogens if the host is immune compromised, their intestinal tract is damaged, or acquire virulence determinants (Kaper, Nataro and Mobley, 2004; Tenailon *et al.*, 2010).

1.1.2 Pathogenic *E. coli*

1.1.2.1 Intraintestinal pathogenic *E. coli*

Despite the fact that most *E. coli* are commensals and as such non pathogenic, some strains have pathogenic potential and may be responsible for intraintestinal and extraintestinal

infections (Kaper, Nataro and Mobley, 2004; Nataro and Kaper, 1998; Johnson *et al.*, 2003). Pathogenic *E. coli* have the potential to cause serious infections possibly leading to death as well as incumbent economic costs, which have been estimated at over one billion dollars and over 8 million cases a year in humans in the USA (Russo and Johnson, 2003). There are six main intrainestinal pathotypes of *E. coli* which usually result in enteric diarrhoeal disease, which can be accompanied by fever, which results from the damage and destruction of the cells within the intestine (Kaper, Nataro and Mobley, 2004; Nataro and Kaper, 1998). Each of the pathotypes has its own mechanism for disrupting the intestine, whether it be through attachment and interference with cellular process or through the production of toxins. Enteropathogenic *E. coli* (EPEC) adhere to and destroy the microvillar structure, causing attachment, effacement and cytoskeletal rearrangements. Enterohemorrhagic *E. coli* (EHEC), produce shiga toxin (Stx), Stx causes the degradation of ribosomal RNA resulting in cell death. Enterotoxigenic *E. coli* (ETEC) bind in the small bowel and produce heat labile (LT) and heat stable (ST) enterotoxins, leading to watery diarrhoea. Enterogagregative *E. coli* (EAEC), adhere in the small and large bowel in biofilm like structures and secrete cytotoxins and enterotoxins. Enteroinvasive *E. coli* (EIEC) cause alterations to the actin filaments, altering the cellular morphology. Diffusely adherent *E. coli* (DAEC) trigger changes in growth of the cell causing finger-like projections which wrap around the bacteria (Kaper, Nataro and Mobley, 2004; Nataro and Kaper, 1998).

1.1.2.2 Extraintestinal pathogenic *E. coli*

Extraintestinal Pathogenic *E. coli* (ExPEC) are responsible for several extraintestinal infections, including urinary tract infections (UTI), pneumonia, septicemia and meningitis (Russo and Johnson, 2000; Johnson and Russo, 2002). UTI's include cystitis, uncomplicated pyelonephritis and catheter associated UTI, which are caused by uropathogenic *E. coli* (UPEC), and are responsible for 90% of UTI's in humans (Brzuszkiewicz *et al.*, 2006; Orskov and Orskov, 1985; Johnson *et al.*, 2003). *E. coli* also can cause meningitis/sepsis, known as MNEC, which are the most common cause of Gram

negative neonatal meningitis with a mortality of 15-40% (Kaper, Nataro and Mobley, 2004; Orskov and Orskov, 1985).

1.1.2.3 Emergence of pathogenic *E. coli*

Pathogenic *E. coli* have emerged from commensals which have acquired mobile elements harbouring virulence factors; these include transposons, pathogenicity islands (PAI), bacteriophages and plasmids (Kaper, Nataro and Mobley, 2004). Comparative genomics of commensal and pathogenic strains show they share similar genomes, however pathogenic *E. coli* have acquired additional genetic elements "Virulence DNA", as seen with UPEC, EHEC, and EPEC (Kaper, Nataro and Mobley, 2004; Brzuszkiewicz *et al.*, 2006; Brzuszkiewicz *et al.*, 2011; Crossman *et al.*, 2010). One of the best examples is the introduction of the bacteriophage carrying the *stx* gene in *E. coli* O157, formerly an EPEC strain which has changed to become an EHEC strain through this acquisition (Reid *et al.*, 2000). EHEC *E. coli* O157 infections can lead to hemolytic uremic syndrome (HUS), which causes the destruction of red blood cells and kidney failure resulting in deaths to the vulnerable (Karmali *et al.*, 1985). A similar event occurred in *E. coli* O104:H4 responsible for the 2011 outbreak in Germany, originally a EAEC, the isolate acquired the *stx* gene from a bacteriophage forming an isolate with both enteroaggregative and hemorrhagic traits (EAHEC) (Rasko *et al.*, 2011; Brzuszkiewicz *et al.*, 2011). The attaching and effacing characteristic associated with EPEC's, are linked to a PAI known as "locus of enterocyte effacement" (LEE) which encodes intimin, and a type III secretion system (Jerse *et al.*, 1990). *E. coli* pathotypes have the ability to adhere to cells, allowing the colonisation of sites not normally inhabited by *E. coli* (Kaper, Nataro and Mobley, 2004).

1.1.3 Epidemiology of *E. coli*

Both commensal *E. coli* and pathogenic *E. coli* are shed in the faeces of the host and consequently the transmission is typically through the faecal oral route. This can occur via direct contact, or through the consumption of contaminated food and water (Dev, Main and Gould, 1991; Money *et al.*, 2010; Bettelheim and Lennox-King, 1976; Pinegar and

Cooke, 1985). The occurrence of *E. coli* is approximately 90% in humans and 56% in wild animals (Penders *et al.*, 2006; Gordon and Cowling, 2003). Hosts often have several *E. coli* strains at one time, with some persisting for months to years, whilst others are more transient lasting a few days to weeks (Sears *et al.*, 1956; Caugant, Levin and Selander, 1981). Strains of *E. coli* frequently move between occupants of households, with studies identifying 14 unique isolates, of which a UPEC was found to be dominant and present in 3-4 occupants at any one time (Johnson and Clabots, 2006; Johnson, Clabots and Kuskowski, 2008; Bailey *et al.*, 2010).

E. coli has been grouped into four phylogroups (A, B, C and D) based on the genes *chuA* and *yjaA* (Clermont, Bonacorsi and Bingen, 2000). The phylogroups A (40.5%) and B2 (25.5%) are the most predominant in humans, while B1 (41%) and A (22%) are more common in animals (Tenailon *et al.*, 2010; Duriez *et al.*, 2001). Domesticated animals have been found to have a far lower strain diversity than wild animals, which may be due to the inhabited environment. B2 strains have been found to be favoured in animals, due to the gastro environment, and this phylogroup has also been identified as more virulent in humans (Gordon and Cowling, 2003; Escobar-Paramo *et al.*, 2006; Nowrouzian, Adlerberth and Wold, 2006). An example of *E. coli* being related to a specific niche is the EHEC O157:H7, which is highly prevalent in cattle (60%), and consequently outbreaks are often related to beef products (Hancock *et al.*, 1998; McEvoy *et al.*, 2003). Certain pathotypes have been associated with locations, with ETEC strains common in the developing world and in travellers to these countries (Sears and Kaper, 1996; Qadri *et al.*, 2005; Mendez Arancibia *et al.*, 2009).

1.1.4 Determining the relationship of *E. coli*

It is important to study the relationship between *E. coli* isolates especially those causing disease, to determine their epidemiology, which can be carried out using several methods. One of the original methods was based on antimicrobial variations to determine the serotype of the isolate based on the H and O antigens, the genes for the H and O

antigens were later used to develop a PCR-based method (Orskov and Orskov, 1992; Clermont *et al.*, 2007). Methods have now been developed to compare and differentiate isolates based on their genetic characteristics. Multi locus sequencing typing (MLST), compares the sequence of seven loci, termed housekeeping genes, which generates an sequence type (ST) (Enright and Spratt, 1999; Wirth *et al.*, 2006). Other PCR based methods include repetitive sequence based polymerase chain reaction (rep-PCR) and enterobacterial repetitive intergenic consensus (ERIC) PCR, which differentiate between isolates (Dombek *et al.*, 2000; McLellan, Daniels and Salmore, 2003). The DNA of isolates can be compared using Pulse Field Gel Electrophoresis (PFGE), which is the macro digestion of chromosomal DNA followed by bioinformatic comparison (Arbeit *et al.*, 1990).

1.2 Antimicrobials

Antimicrobials have been in use since the early 1900's, with both synthetic compounds such as Salvarsan and sulfonamide's, and naturally occurring compounds including penicillin being some of the first to be introduced (Zaffiri, Gardner and Toledo-Pereyra, 2012). Antimicrobials have various targets by which they mediate their inhibitory effects, which can be either bactericidal or bacteriostatic. Targets include inhibition of cell wall synthesis, membrane integrity, DNA and RNA replication, enzymatic processes, transcription and protein synthesis (Kohanski, Dwyer and Collins, 2010). Infections caused by Gram negative organisms are commonly treated with β -lactams (penicillin, cephalosporins and carbapenems) aminoglycosides (gentamicin, kanamycin), quinolones (naladixic acid and ciprofloxacin), tetracyclines, trimethoprim and amphenicols (chloramphenicol and florfenicol). The targets and mechanisms of these antibiotics are shown in Table 1.1.

Table 1.1 Antimicrobial targets and mechanisms

Class	Target	Mechanism	References
β -lactams	Penicillin binding proteins (PBP) (transpeptidase) Cell wall synthesis inhibition	β -lactams are analogues of the of terminal D-alanyl D-alanine, which when bound by the PBP transpeptidase block hydrolysis and formation of the cross link peptide bond. This inhibits cell wall formation and maintenance.	Wise and Park, 1965; Bugg and Walsh, 1992; Holtje, 1998; Josephine, Kumar and Pratt, 2004
Quinolones	Topoisomerase complex DNA synthesis inhibition	Bind to topoisomerase proteins II and IV during the uncoiling of DNA causing cleavage of the DNA in the topoisomerase:DNA complex, leading to cell death.	Lewin, Howard and Smith, 1991; Chen <i>et al.</i> , 1996; Drlica and Malik, 2003; Emmerson and Jones, 2003
Aminoglycosides	30S ribosome Protein synthesis inhibition	Bind the 16S rRNA of the 30S ribosomal subunit and affects the matching of the aminoacyl tRNA to the correct codon in the mRNA causing mistranslation of the protein.	Davis, 1987; Wright, 2003
Phenicol	50S ribosome Protein synthesis inhibition	Binds to the 50S subunit, blocking the translocation of peptidyl tRNA, and inhibiting peptidyl transferase and the elongation of the peptide chain.	Jardetzky, 1963
Tetracyclines	30S ribosome Protein synthesis inhibition	Bind to the 30S subunit of the ribosome, preventing the entry of aminoacyl tRNAs into the ribosome.	Schnappinger and Hillen, 1996; Chopra and Roberts, 2001
Trimethoprim	Dihydrofolate reductase DNA synthesis inhibition	Binds and inhibits dihydrofolate reductase, required for the reduction of dihydrofolic acid, important for purine and thymidylate synthesis.	Hitchings, 1973
Sulphonamides	Dihydropteroate synthetase DNA synthesis inhibition	Binds and inhibits dihydropteroate synthetase which catalyses the conversion of dihydropteroate diphosphate and p-aminobenzoic acid (PABA) into dihydropteroic acid a precursor for dihydrofolic acid	Henry, 1943

1.3 Resistance to antimicrobials

Resistance to antimicrobials was present long before their therapeutic use, and may have resulted from the need of antimicrobial producers to protect themselves (D'Costa *et al.*, 2006; Wright and Poinar, 2012). Shortly after antibiotics were introduced into the clinic, resistant isolates were observed, particularly in hospitals, with resistance correlating to antimicrobial use (Weinstein, 2001). The therapeutic use of different classes of antimicrobial, led to multidrug resistant (MDR) *E. coli* and *Salmonella* spp., which were first identified in the 1960's (Watanabe, 1963; Levy, 2001). Resistant isolates are also selected in the environment when antimicrobial pressure is applied, as susceptible isolates are either inhibited or killed. Antibiotic resistance can be either intrinsic or acquired. Intrinsic resistance occurs within the isolate and typically as the result of step-wise mutations over time increasing efflux or decreased permeability, or reducing regulation of genes (Aleksun and Levy, 2007; Cox and Wright, 2013; Honore, Nicolas and Cole, 1986). Acquired resistance involves resistance genes being obtained from an exogenous source, through mobile genetic elements carrying resistance to β -lactams, aminoglycosides, and chloramphenicols which are examples of acquired resistance (Aleksun and Levy, 2007; Levy and Marshall, 2004; van Hoek *et al.*, 2011). There are four main mechanisms of resistance (i) drug inactivation, (ii) alterations to the drug target site, (iii) pathway alteration and (iv) reduced accumulation of antibiotics in the cell (Hawkey, 2000; Wright, 2010).

1.3.1 Antimicrobial resistance through drug inactivation

Inactivation of the antimicrobial occurs through the production of enzymes which either destroy the compound or chemically modify it. Antibiotics belonging to the β -lactams, aminoglycosides and phenicols are inactivated by such enzymatic reactions. Aminoglycosides are inactivated by the enzymes acetyltransferase (acetylation), phosphotransferase (phosphorylation) and nucleotidyltransferase (adenylation) (Davies and

Wright, 1997). These modifications alter the antibiotics ability to bind ribosomes, allowing protein synthesis to continue (Davies and Wright, 1997). Chloramphenicols can also be inactivated through an chloramphenicol acetyltransferase (CATS) also preventing binding to the ribosome (Schwarz *et al.*, 2004). One of the most studied examples of drug inactivation is that of the β -lactamases which include penicillinase, cephalosporinase, and carbapenemase, all of which hydrolyse the β -lactam ring of their respective substrates inactivating the molecule, these will be discussed in detail later in this introduction (Jacoby and Sutton, 1985; Jacoby and Munoz-Price, 2005).

1.3.2 Antimicrobial resistance through target alteration

The alteration of an antimicrobials target is another effective method for gaining resistance. Alterations to topoisomerase and ribosomes give resistance to fluoroquinolones and tetracyclines and aminoglycosides respectively (Alekhun and Levy, 2007; Levy and Marshall, 2004). Mutations to the GyrA and ParC/GrlA of topoisomerases, which possess the DNA binding domains, prevent fluoroquinolones from binding and increase resistance (Drlica and Malik, 2003).

1.3.3 Antimicrobial resistance through metabolic alteration

Metabolic pathway alteration is another mechanism used to mediate resistance particularly to the sulfonamide and trimethoprim antibiotics. Both of these antimicrobials target enzymes in the metabolic pathways involved in the production of tetrahydrofolic acid (required for thymine production). Certain bacterias can acquire genes which produce proteins which are recalcitrant to drug inhibition (Skold, 2001). Another example of metabolic pathway alteration is the pentapeptide like protein, produced by the *qnr* gene, which protects the topoisomerase genes from inhibition by fluoroquinolones (Tran and Jacoby, 2002).

1.3.4 Antimicrobial resistance through reduced accumulation and efflux

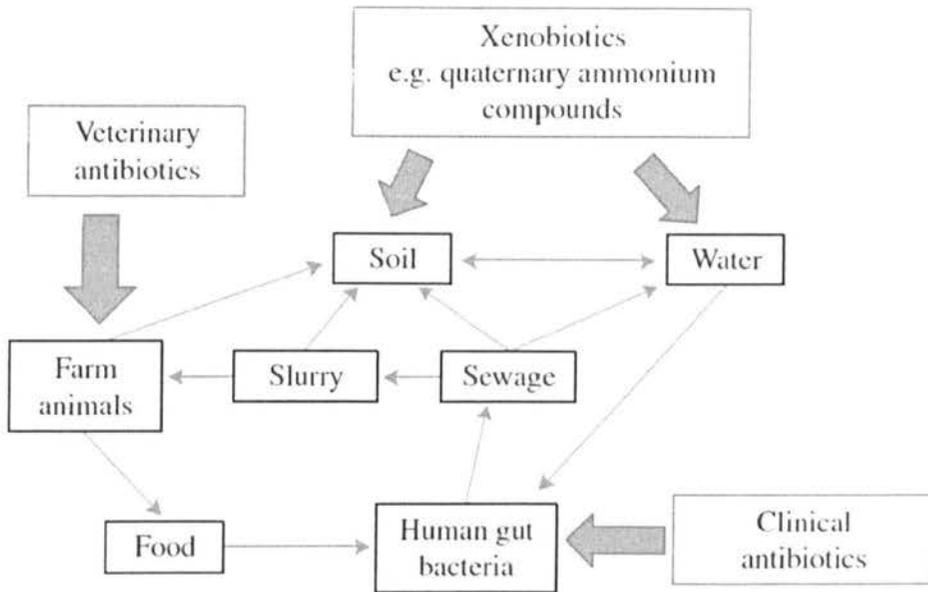
Another mechanism of resistance is to reduce the entry and accumulation of antimicrobials in the cell. Efflux is used to reduce the concentrations of tetracyclines, chloramphenicols and fluoroquinolones in the cell (Levy, 1992; Levy and Marshall, 2004; Alekshun and Levy, 2007). Resistance to tetracyclines is typically mediated through efflux, membrane spanning proteins which export tetracyclines out of the cell, and are regulated by the transcription repressor *tetR*, which is inactivated by the presence of tetracycline and expresses the *tetA* efflux gene (Chopra and Roberts, 2001). Resistance to chloramphenicols can also occur through efflux, coded by the *cmlA* gene, which gives a high level of resistance (George and Hall, 2002; Schwarz *et al.*, 2004). Drug transporters belong to different protein families, including major facilitator (MF), staphylococcal small multidrug (SMR), ATP binding cassette (ABC), resistance nodulation division (RND) and multidrug and toxic compounds extrusion (MATE). The RND proton/drug antiporters are widespread in Gram negative organisms such as *E. coli* and include the AcrA, AcrB and TolC proteins which form the RND pump (Li and Nikaido, 2009; Tikhonova and Zgurskaya, 2004). AcrAB-TolC has been found to have affinity for substrates such as aminoglycosides, and have been shown to have a role in the resistance to β -lactams (Mazzariol, Cornaglia and Nikaido, 2000). ABC drug efflux proteins reduce the accumulation of quinolones in the bacterial cell (Alvarez *et al.*, 2008). Resistance through efflux is often due to the over expression of membrane transporter complexes (Murakami and Yamaguchi, 2003; Li and Nikaido, 2004; Li and Nikaido, 2009).

1.4 Antimicrobial resistance gene mobilization

Antimicrobial resistance genes are present in bacteria from a wide range of ecological niches including humans, animals and environment, and their numbers are increasing as result of the use and misuse of antimicrobials, contributing to the resistance gene pool, the "resistome" (Davies and Davies, 2010; D'Costa *et al.*, 2006; Stokes and

Gillings, 2011). Resistance is not only spread by isolates, but transferred by mobile genetic elements (MGE) within those isolates, which move between different hosts from different environments as shown in Figure 1.1 (Davies and Davies, 2010; Stokes and Gillings, 2011; Hawkey and Jones, 2009).

Figure 1.1 Flow of antibiotic resistance



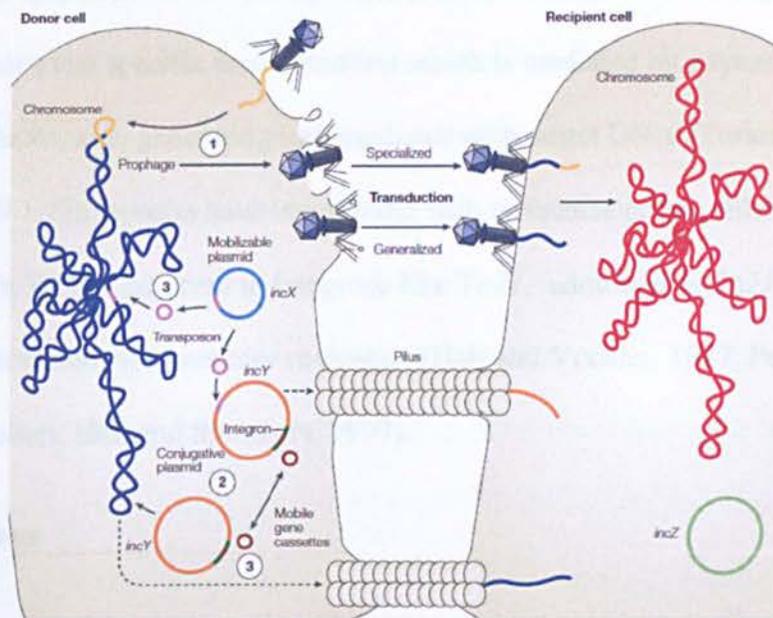
Routes of resistance gene transmission between veterinary, human and environmental isolates image from Hawkey and Jones (2009).

1.4.1 The genetics of resistance gene mobilization

For resistance to be acquired by an isolate, the genes need to be mobile and this is where their mobilization plays an important role in dissemination. The concept of lateral gene transfer (LGT) also known as horizontal gene transfer (HGT) is one of the main causes for the rapid dissemination of resistance genes (Frost *et al.*, 2005; Walsh, 2006; Davies and Davies, 2010; Roy Chowdhury *et al.*, 2011). LGT has the ability to alter the ecological niche of microorganisms, through the transfer of DNA providing alternative phenotypes, and approximately 25% of the DNA in *E. coli* is thought to have been acquired through LGT (Ochman, Lawrence and Groisman, 2000). For successful LGT to occur two main factors need to be overcome; (i) the successful mobilization of the

resistance gene and (ii) stable integration into a new host (Walsh, 2006; Stokes and Gillings, 2011). The mechanisms for resistance gene mobilization include conjugation, transformation and transduction, which are mediated through plasmids, bacteriophage, transposon, integrative and conjugative elements, insertion sequences, integrons and naked DNA, shown in Figure 1.2 (Frost *et al.*, 2005; Walsh, 2006; Davies and Davies, 2010; Partridge, 2011; Stokes and Gillings, 2011).

Figure 1.2 Methods of lateral gene transfer (LGT)



Methods of transfer between cells, (1) Transduction mediated through bacterial phage which package chromosomal (yellow) DNA, which can be transferred to a recipient cells upon infection. (2) Conjugative plasmids and integrative conjugative elements (ICE) which transfer DNA through a pilus and can either integrate into the chromosome or be extra chromosomal. (3) Transposons can be found on plasmids and the chromosome, and can be transferred on plasmids and ICE into recipient cells through the pilus and can remain on the plasmid or integrate into the chromosome image from Frost *et al* (2005).

The mobile nature of these elements means they can interact with each other to form diverse and complex mobile elements, which in turn can transfer MDR phenotypes (Walsh, 2006; Toleman and Walsh, 2011; Davies and Davies, 2010; Partridge, 2011).

1.4.2 Transposons

Transposons are some of the most ubiquitous genes in nature, being present before the use of clinical antibiotics, but resistance genes were quickly associated with them after clinical introduction (Smith, 1967; Barkay and Wagner-Dobler, 2005; Aziz, Breitbart and Edwards, 2010). Transposons are constructed from two components, the transposase *tnpA* and the recombinase *tnpR* which are flanked by inverted repeats (Grinsted, de la Cruz and Schmitt, 1990; Liebert, Hall and Summers, 1999). Transposons can be either complete such as the Tn3 and Tn21 or unit transposons such as the Tn5053 (Partridge, 2011). Transposons have site specific recombination which is mediated by a tyrosine or serine active site at the *res* site, generating intermediates with target DNA (Yurieva and Nikiforov, 1996). Transposons have been found with resistance genes, either directly such as *bla*_{TEM-1} with Tn3, or adjacent to integrons like Tn21, additionally Tn21 has also been found to be associated with mercury resistance (Hall and Vockler, 1987; Partridge and Hall, 2005; Liebert, Hall and Summers, 1999).

1.4.3 Integrons

First described by Stokes and Hall (1989), integrons were found to have two key features. The first is that integrons have a site specific integration function having an integrase and insertion sites *attC* and *attI*, and the second is that integrons can acquire multiple gene cassettes (Stokes and Hall, 1989). Integrons are not mobile, but are mobilised by adjacent MGEs such as insertion sequences and transposons, which often then transfer onto plasmids (Stokes and Hall, 1989; Holmes *et al.*, 2003). Gene cassettes integrate by single stranded recombination using a 59 base element which captures a gene cassette, of which there are over a 100, by forming a loop that can be reformed for exchange between integrons (Bouvier, Demarre and Mazel, 2005; Bouvier *et al.*, 2009; Recchia and Hall, 1995; Hall and Collis, 1995; Partridge *et al.*, 2009).

1.4.4 Insertion sequences

Insertion sequences (IS) consist of a transposase flanked by inverted repeats (IR), and there are over 1500 have been recognised, which are grouped into families including IS 1, 2, 3, 4 and 61, most of which are found in *Enterobacteriaceae* (Siguier, Filee and Chandler, 2006; Mahillon and Chandler, 1998; Frost *et al.*, 2005; Partridge, 2011). IS mobilise adjacent genes through failing to recognise the right inverted repeat (IR_R) and instead utilize an alternative (IR_{alt}) if this lies the other side of a resistance gene then the gene is mobilised with the IS (Poirel, Decousser and Nordmann, 2003; Mahillon and Chandler, 1998; Partridge, 2011). Direct repeats range in size from 2-14 bp are generated when an insertion sequence inserts into a target site (Mahillon and Chandler, 1998). ISs also have the ability to control the expression of genes adjacent acting as a promoter for gene, forming the -35 and -10 hexamers, a good example of this is ISEcp1 commonly found upstream of *bla*_{CTX-M} (Mahillon and Chandler, 1998; Poirel, Decousser and Nordmann, 2003; Poirel *et al.*, 2005). The DDE motif of IS transposases, catalyses the nucleophilic activity of the transposase allowing it to attack DNA, in a similar mechanism to those with phosphoryltransferase activity, and may be related to enzymes found in retroviruses (Mahillon and Chandler, 1998).

1.4.5 Transduction

Transduction is the transfer of DNA by independently replicating bacteriophages (viruses) (Frost *et al.*, 2005). Large phages can "accidentally" package host DNA in the viral capsid on formation of a new phage, this can in turn be spread to a new isolate (Canchaya *et al.*, 2003; Frost *et al.*, 2005). Phages involved in LGT tend to integrate into the chromosome and do not cause lysis of the cells. As bacteriophages tend to have specific targets the transmission of genes is limited to the species in which they infect (Canchaya *et al.*, 2003). Phages play an important role in the control of bacterial populations and are common in sewage treatment facilities which present an excellent opportunity to capture DNA from host genomes. The resistance genes *bla*_{TEM} and *bla*_{CTX-M-G9} and the virulence

gene *stx* have been recovered from phage in sewage treatment plants (Colomer-Lluch, Jofre and Muniesa, 2011; Muniesa and Jofre, 1998).

1.4.6 Conjugative elements

Conjugative elements such as integrative conjugative elements (ICE) and the variant conjugative transposons (CTn) possess the characteristics of plasmids, transposons and phages; and are important in genetic recombinations, having been found to transfer DNA between different species and genera (Burrus and Waldor, 2004; Salyers *et al.*, 1995; Toleman and Walsh, 2011). Unlike integrons, transposon and insertion sequences, ICE possess their own transfer and replication genes (Walsh, 2006; Frost *et al.*, 2005). The first ICE elements to be identified were Tn916 and SXT identified in *Enterococcus faecalis* and *Vibrio cholerae* respectively (Beaber, Hochhut and Waldor, 2002; Scott and Churchward, 1995). ICE's integrate into the host to ensure their maintenance using a recombinase and the *attP* and *attB* sites, integration prevents replication, which is regulated by phage C1 homologues (Scott *et al.*, 1994; Hochhut, Marrero and Waldor, 2000; Beaber, Hochhut and Waldor, 2002; Salyers *et al.*, 1995). Conjugation occurs as a single stranded molecule, that is cleaved allowing for its integration into the host (Scott *et al.*, 1994; Scott and Churchward, 1995; Salyers *et al.*, 1995). ICE's have been found to play an important role in the diversity of genetic elements, responsible for mobilization, rearrangements and changes in the host genome. This includes the movement of resistance genes and virulence genes which can alter the niche of isolates (Frost *et al.*, 2005; Toleman and Walsh, 2011; Walsh, 2006; Burrus and Waldor, 2004).

1.5 Plasmids

Plasmids are extra-chromosomal DNA made up of functional modules that are organised into a stable self replicating entity (Frost *et al.*, 2005; Lederberg, 1952). Plasmids do not alter the genome through integration like other LGT mechanisms, and represent some of the most important MGE's (Ochman, Lawrence and Groisman, 2000; Frost, Ippen-Ihler and Skurray, 1994; Smillie *et al.*, 2010). Plasmids carry resistance,

virulence and metabolic genes capable of altering the ecological niche in which an isolate inhabits. The commensal *Bacillus anthracis* becomes a deadly pathogen through the acquisition of the toxin encoding plasmid pX01, while plasmids in the *Rhizobium* species allow for nitrogen fixation and invasion of plants (Okinaka *et al.*, 1999; Crossman, 2005). Plasmids were present in bacteria before the clinical use of antibiotics, but have since been identified as resistance vectors, with some of first work on resistance (R) plasmids conducted by Watanabe (1963) (Watanabe, 1963; Datta and Hughes, 1983; Jones and Stanley, 1992).

1.5.1 Plasmid replication

Plasmids replicate by one of three mechanisms which are theta (θ), rolling circle (RC) or strand displacement. θ involves the strand displacement of a parent strand, followed by the formation of a primer RNA which is elongated by DNA synthesis, through the binding of the replication initiation protein (Rep) (del Solar *et al.*, 1998). Strand displacement is similar to θ , but requires three proteins needed for initiation and replication RepA, B and C (Scherzinger *et al.*, 1991). RC is unidirectional in nature with both replication of leading and lagging strands being uncoupled, and is mainly observed in small (<10 kb) plasmids (del Solar, Moscoso and Espinosa, 1993; Khan, 1997).

1.5.2 Control of replication

Plasmids vary in their copy number, being either low or high copy number, which is controlled by various mechanisms to ensure a balanced system, which affects the initiation of replication (del Solar *et al.*, 1998). As a result of these mechanisms, two plasmids with the same replicon type are unable to coexist in the same cell in a process known as incompatibility (Novick, 1987). Mechanisms of initiator inhibition occurs through antisense RNA, protein interaction and modulation of DNA sites for initiator proteins. Antisense RNA can bind to primer RNA or that of the mRNA of the Rep protein which prevents translation of Rep (Wagner, Altuvia and Romby, 2002; Novick, 1987).

Antisense RNA can also bind to mRNA and prevent entry to the ribosome inhibiting translation (del Solar and Espinosa, 1992). Control by iterons is mediated through several proposed systems the first is titration, where the Rep protein is titrated by the iterons (Tsutsui *et al.*, 1983). The other suggested method is known as "handcuffing", where Rep binds two iterons simultaneously, causing initiation of replication if the copy number of plasmids is low (McEachern *et al.*, 1989; del Solar *et al.*, 1998).

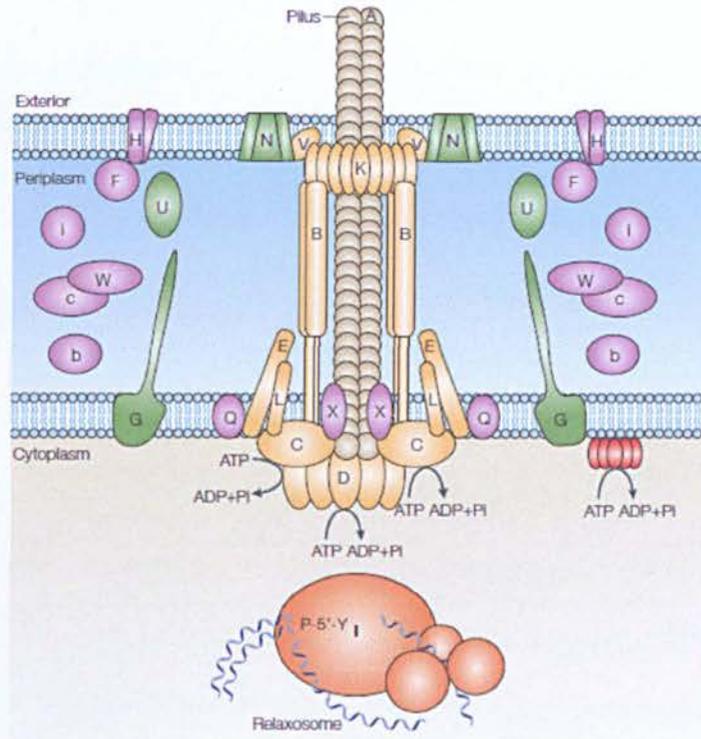
1.5.3 Plasmid mobility and conjugation

The ability of plasmids to move between both bacterial isolates and across species boundaries, is one of the key features to their success as vectors of LGT (Frost *et al.*, 2005; de la Cruz and Davies, 2000; Halary *et al.*, 2010; Smillie *et al.*, 2010). Three systems are required for a plasmid to be self transmissible. The first is the formation of a transfer structure (transferome), the formation of the DNA processing unit also known as the relaxosome and the coupling of these two units by a coupling factor, shown in Figure 1.3 (Smillie *et al.*, 2010). Plasmids which can be mobilized but are themselves not self-transmissible, typically carry the genes required for the production of the relaxosome but not the transferome, however they can be mobilized using the transfer genes encoded and produced by other plasmids.

1.5.3.1 The plasmid transferome

Plasmid DNA is transferred through a pilus, referred to as a sex pilus, which is a member of the Type IV secretion system (T4SS), and is closely related to VirB4 associated with pathogenicity, Figure 1.3 (Lawley *et al.*, 2003; Frank *et al.*, 2005). When bacterial isolates come together they form the membrane associated mating pair formation (MPF) (Smillie *et al.*, 2010; Lawley *et al.*, 2003). The T4SS tip is formed by the products of the *traA*, *L*, *E*, *K*, *C* and *G* genes, which is recognised as a target by bacteriophage. However the tip alone is not sufficient to form the MPF, and is required to be extended further by the products of *traB*, *F*, *H*, *W*, *V*, and *trbCF* genes, aided by *traP* which stabilise the pilus

Figure 1.3 Plasmid conjugation system



An example of the conjugative systems for the transfer of plasmids and ICE, capital letters indicate Tra proteins and lower case are Trb proteins, pink genes are required for pilus assembly, green are for mating pair formation (Frost *et al.*, 2005). The TraD is important for the coupling of the transferome to the relaxosome which is NikB bound to the plasmids DNA (Smillie *et al.*, 2010), Image from Frost *et al* (2005).

(Frank *et al.*, 2005; Lawley *et al.*, 2003; Frost, Ippen-Ihler and Skurray, 1994; Schandel *et al.*, 1987; Anthony *et al.*, 1999). Plasmids of the IncI complex also have the *pil* loci which although not needed for conjugation, enhances the conjugation in the liquid environment and adherence to eukaryotic cells. The *pil* also contains the *pilV* shufflon region, which can undergo rearrangements altering host specificity (Gyohda, Funayama and Komano, 1997; Komano, Kim and Yoshida, 1995; Dudley *et al.*, 2006).

1.5.3.2 The relaxosome

Plasmid DNA needs to be prepared before transfer, which is carried out in the relaxosome complex (de la Cruz *et al.*, 2010; Smillie *et al.*, 2010). The plasmid origin of transfer, *oriT*, is bound by the relaxase protein NikB, cleaving the *oriT* at the *nic* site, unwinding the DNA, which can be transferred with NikB attached (Smillie *et al.*, 2010; de

la Cruz *et al.*, 2010). The Type IV coupling protein (T4CP), brings together the T4SS and the relaxosome, allowing for DNA to be transferred through the channel formed by T4SS (Beranek *et al.*, 2004; Mihajlovic *et al.*, 2009). The T4CP and the relaxosome together form the MOB, of which six different families have been identified, the MOB is required for the mobilization of plasmids, even if T4SS is absent (Francia *et al.*, 2004).

1.5.3.3 Conjugation incompatibility

A bacterial host that contains a plasmid will have mechanisms to prevent plasmids of the same incompatibility group from entering the cell. Surface exclusion prevents the formation of the pilus between two hosts with the same plasmid, the TraT and TraS proteins are important in these roles (Anthony *et al.*, 1999; Manning, Morelli and Achtman, 1981; Perumal and Minkley, 1984). TraG recognises the TraS protein expressed in the membrane of the potential recipient, and prevents the unwinding of DNA (Manning, Morelli and Achtman, 1981; Anthony *et al.*, 1999). TraT is an outer membrane lipoprotein which reduces the stabilization of mating pairs (Perumal and Minkley, 1984).

1.5.4 Plasmid stability and maintenance

Plasmids are foreign DNA, and in most cases are not necessary for the survival of the host as they carry accessory genes, and it has even been shown that the expression of foreign proteins encoded on plasmids effects the metabolic growth of bacteria (Bentley *et al.*, 1990). Consequently unless a gene present on the plasmids is necessary for survival such as a resistance gene in an isolate under antimicrobial pressure, the plasmids may be lost. To compensate plasmids have evolved stability and addiction systems to aid their stable inheritance and persistence.

1.5.4.1 Plasmid partitioning

Plasmid partitioning is a common feature of low copy number plasmids which is used in their maintenance during cell division (Bignell and Thomas, 2001; Gerdes, Moller-

Jensen and Bugge Jensen, 2000). Partitioning of plasmids involves the ParA and ParB proteins, ParB binds to the *parS* site on the plasmid as a dimer which in turn bind to centromeres in the cell (Bignell and Thomas, 2001; Mori *et al.*, 1989; Watanabe *et al.*, 1989). Significant variations have been found in the phylogeny of the ParA and ParB genes varying between plasmids of different incompatibility groups, which may have a role in plasmid incompatibility (Gerdes, Moller-Jensen and Bugge Jensen, 2000). ParA is a ATPase which produces ATP for ParB to localise plasmids to locations in the cell, which increase in number during cell division (Koonin, 1993; Gerdes, Moller-Jensen and Bugge Jensen, 2000; Bignell and Thomas, 2001).

1.5.4.2 Post segregational killing (PSK)

Post segregational killing (PSK) also known as addiction systems, maintain the presence of plasmids through either restricting growth (bacteriostatic) or survival of the host (bactericidal) (Pedersen, Christensen and Gerdes, 2002). Addiction systems are based on a two component system which include a toxin (TX) and antitoxin (AT), the TX has a longer half life than the AT, which needs to constantly be expressed from the plasmid encoded gene. Addiction systems are classed by the mechanism of control exerted by the antitoxin and is grouped into A, B and C types (Yarmolinsky, 1995). Type A are the classical proteic systems, of which CcdB and CcdA (toxin:antitoxin) and RelE and RelB are examples (Gerdes, 2000). The CcdB TX binds to the gyrase protein GyrA, which traps the GyrA in a DNA cleaving phase causing degradation of the chromosome resulting in death of the cell. CcdA (AT) binds to CcdB preventing the interaction with GyrA and protecting the chromosome (Gerdes, 2000; Bernard and Couturier, 1992; Bernard *et al.*, 1993). The RelE (TX) works in a different way to CcdB, by breaking down codons in the A site of the ribosome, preventing protein synthesis (Gronlund and Gerdes, 1999). Type B are the restriction modification (RM) systems, the TX is a restriction enzyme such as EcoRI and the AT is methyltransferase. The bacterial chromosome is degraded by the EcoRI toxin, cleaving the DNA at specific restriction sites. These sites are protected from

degradation by the TX through methylation by the methyltransferase AT (Naito, Kusano and Kobayashi, 1995). RM systems are extensively present in HGT vectors which have an impact on shaping their hosts (Kobayashi, 2001). Type C are the antisense RNA regulated system, of which the TX Hok and AT Sok is an example. Hok attacks the cell membrane resulting in its degradation and influx of periplasmic proteins including RNase I which cleaves RNA. The Sok RNA binds to the mRNA of Hok, preventing its translation, Sok mRNA is degraded by RNase and so constantly needs to be expressed to inhibit Hok (Gerdes, Rasmussen and Molin, 1986; Gerdes *et al.*, 1997).

1.5.5 Analysis of plasmids

Due to plasmids being vectors for both virulence and resistance genes it is important to characterise and compare them to study their epidemiology. Several techniques for plasmid typing have been developed based on both the genotypic and phenotypic properties of plasmids.

1.5.5.1 Incompatibility

One of the first methods to characterise plasmids was the utilization of plasmid incompatibility, with plasmids belonging to the same incompatibility group unable to co-exist in the bacterial host (Novick, 1987; Novick *et al.*, 1976). Plasmids were grouped by their incompatibility (Inc), which is caused by conflicts in replication systems and so were also referred to as replicons (Novick, 1987; Datta and Hedges, 1971; Davison, 1984). Couturier *et al* (1988) devised a method of replicon typing using hybridization, designing 19 DNA probes, for the replication regions of plasmids to overcome some issues with incompatibility testing (Couturier *et al.*, 1988; Bergquist, Saadi and Maas, 1986).

The first PCR based replicon typing scheme was proposed in 1996 but covered only four plasmids groups IncN, W, P and Q (Gotz *et al.*, 1996). In 2005 a more comprehensive scheme was devised by Carattoli *et al* (2005a), with primers developed for FIA, FIB, FIC, III1, III2, II-I γ , L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA (Carattoli

et al., 2005a). This method proved to be successful method of tracking the transmission of plasmids coding resistance genes (Carattoli *et al.*, 2005a; Johnson and Nolan, 2009; Marcade *et al.*, 2009).

1.5.5.2 Sizing and RFLP analysis of plasmids

Plasmids are also compared by their physical properties, which are a product of their genetics, which can also be used for the comparison of isolates. The size and number of different plasmids in a bacterial cell can be determined by gel electrophoresis with rapid methods published (Kado and Liu, 1981; Barton, Harding and Zuccarelli, 1995). Similar to PFGE for isolates, restriction fragment length polymorphism (RFLP) cleaves DNA at restriction sites creating fragments that form a comparative profile, RFLP has been used to analyse resistant plasmids of *Salmonella* in the UK, and plasmids of *Vibrio anguillarum* (Hopkins *et al.*, 2007; Batchelor *et al.*, 2005; Pedersen, Tiainen and Larsen, 1996).

1.5.5.3 pMLST

Similarly to MLST of isolates, pMLST uses specific loci in plasmids which have continuous and stable divergence. Currently four pMLST schemes have been developed for the incompatibility groups IncI1 γ , IncHI2, IncF and IncN which have been associated with resistance (Garcia Fernandez *et al.*, 2007; Garcia-Fernandez *et al.*, 2011; Garcia-Fernandez *et al.*, 2008; Villa *et al.*, 2010). Sequencing of the amplified loci identifies individual alleles which can be used for determining phylogeny and comparing with other plasmids through the use of databases.

1.5.5.4 Plasmid molecular markers

Molecular markers like pMLST identify specific plasmid loci, but do not require sequencing of the amplicon. Several plasmid types have had these markers developed including the IncK pCT plasmid, IncA/C plasmids pYR1, pSN254 and pIP1202 and the

IncX4 plasmid pSAM7 (Cottell *et al.*, 2011; Welch *et al.*, 2007; Stokes *et al.*, 2013). Cottell *et al* (2011) sequenced the pCT plasmids and designed four molecular markers based on specific loci to identify this and similar plasmids. These markers have been used to show that pCT-like plasmids are present in unrelated isolates from cattle, turkeys and humans in the UK, covered in chapter 3 (Stokes *et al.*, 2012; Dhanji *et al.*, 2012; Cottell *et al.*, 2011). Welch *et al* (2007) used 15 markers designed from sequencing three IncA/C plasmids pIP1202, pYR1 and pSN254, these markers have been used to analyse *Salmonella* isolates from food producing species (Welch *et al.*, 2007). Markers have also been developed by Stokes *et al* (2013) to not only identify a particular IncX4 plasmid, pSAM7, but to differentiate between other IncX4 plasmids currently sequenced, this will be covered in more detail in chapter 5 (Stokes *et al.*, 2013).

1.6 β -lactamases

Resistance to penicillin was identified soon after its introduction in the 1940s, with an enzyme termed penicillinase found to confer resistance (Abraham and Chain, 1988; Woodruff and Foster, 1945). Penicillinase was one of the first described β -lactamases which hydrolyses the β -lactam ring via a serine ester mechanism (Vu and Nikaido, 1985). Multiple β -lactamases were first identified by Richmond and Sykes in 1973, based on their physiological roles, and this led to classification schemes being developed, of which two are widely accepted (Richmond and Sykes, 1973). The first was by Ambler (1980) where β -lactamase are grouped into four different classes A, B, C and D corresponding to their amino acid sequence (Ambler, 1980). Class A cover the majority of known β -lactamases, class B are metallo β -lactamases, C are cephalosporinases and D are cloxacillinases (Ambler, 1980). Later Bush *et al* (1995) devised a classification scheme based on enzyme functionality (Bush, Jacoby and Medeiros, 1995). This scheme has three major groups, group 1 are cephalosporinases, poorly inhibited by clavulonic acid, group 2 includes penicillinases, cephalosporinases and broad spectrum β -lactamases which are inactivated by site directed β -lactamase inhibitors such as clavulanic acid, and is further subdivided.

Finally group 3 which are metallo β -lactamase, these enzymes are capable of hydrolysing penicillins, cephalosporins and carbapenems, and are poorly inhibited by any β -lactam containing molecules (Bush, Jacoby and Medeiros, 1995; Bush and Jacoby, 2010).

β -lactamase (*bla*) genes are found on chromosomes and on MGE's such as plasmids and transposons. The AmpC cephalosporinases are found on the chromosomes of many *Enterobacteriae*, and can either be produced constitutively like the class A β -lactamases *bla*_{SHV} from *Klebsiella* isolates, or are inducible such as the chromosomal class C β -lactamases from *Enterobacter aerogenes* and *Pseudomonas aeruginosa* (Livermore, 1995; Philippon, Arlet and Jacoby, 2002). The *bla* genes found on plasmids have chromosomal origins, with the *bla*_{SHV-1} gene on plasmids originating from the AmpC of *Klebsiella pneumoniae* (Livermore, 1995; Haeggman, Lofdahl and Burman, 1997).

1.6.1 Broad spectrum β -lactamases

Many plasmid mediated β -lactamases give rise to either broad spectrum or extended spectrum phenotypes, in which the β -lactamases have hydrolytic activities to multiple compounds. Matthew (1979) defined broad spectrum β -lactamases, as those being capable of hydrolysing both cephaloridine and benzylpenicillin (Matthew, 1979). Plasmid-mediated genes under the title of broad spectrum included, *bla*_{TEM-1}, *bla*_{TEM-2} and *bla*_{SHV-1}, with *bla*_{TEM-1} being found in 30-50% of *E. coli* in the early 1970s (Livermore, 1995).

1.6.2 Extended spectrum β -lactamases

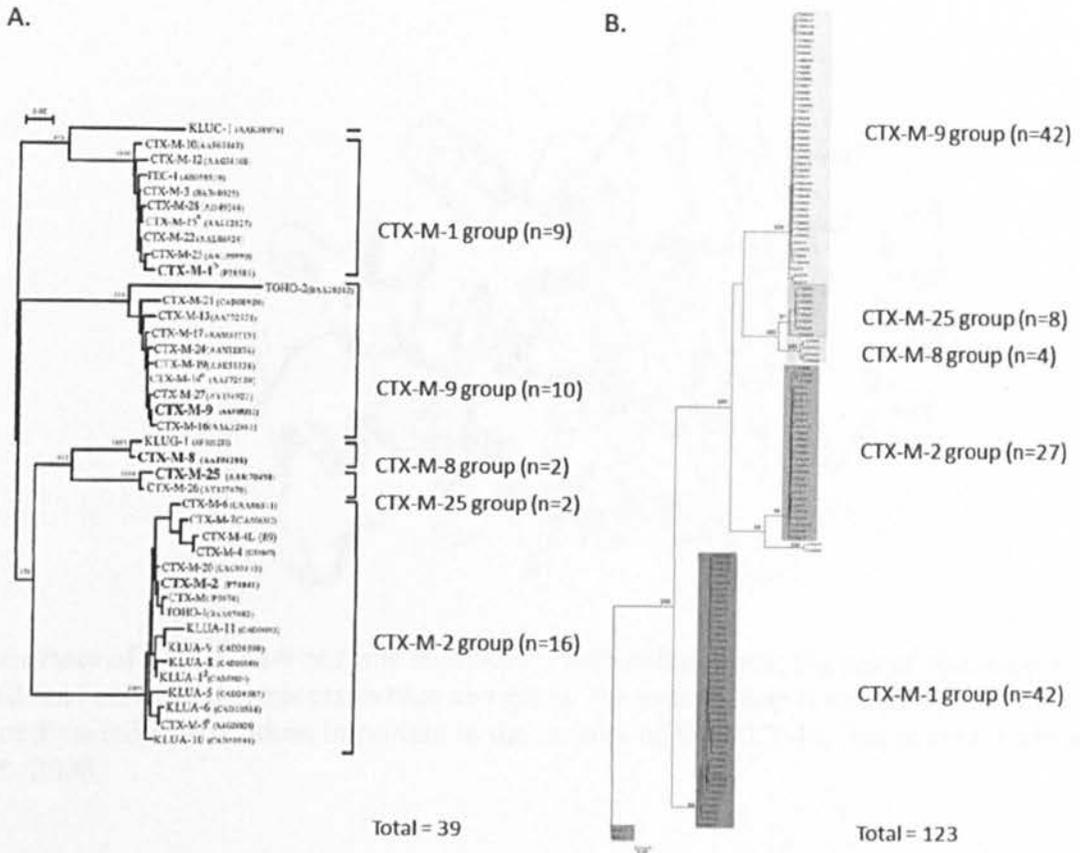
The emergence of broad spectrum β -lactamases drove the development of the oxyimino cephalosporins, which soon became the drugs of choice to treat resistant pneumonia, intra-intestinal and urinary tract infections (Livermore, 2008). Resistance to these antibiotics was first identified in 1982, when Kliebe *et al* (1985) reported a SHV-2 capable of hydrolysing oxyimino cephalosporins, this was followed by CTX-1, a three amino acid variation of TEM-1 and later re-classed as TEM-3 (Kliebe *et al.*, 1985; Sirot *et*

al., 1987). The rapid expansion of variants of TEM and SHV hydrolysing cefotaximases and ceftazidimases led to the term extended spectrum β -lactamases (ESBL) being used by Jarlier *et al* (1988). There are now a number of enzymes which confer an ESBL phenotype, some of these are variants of broad spectrum enzymes such as TEM-52, SHV-12, CMY-2 and OXA-10, while others appear to have evolved independently such as PER-1 which shares only 25% homology with TEM and SHV, class A β -lactamases VEB-1, BES-1, GES and the metallo β -lactamases VIM-1 and IMP also have an important role in resistance (Paterson and Bonomo, 2005; Bradford, 2001).

1.7 The *bla*_{CTX-M} genes and CTX-M

Cefotaximases (CTX-M), termed due to their higher activity towards cefotaxime over ceftazidime, are ESBLs with similar activity to variants of TEM and SHV, but have a different evolutionary history, sharing approximately 40% amino acid homology (Bonnet, 2004). The emergence of ESBL's unrelated to TEM or SHV occurred at a similar time in two parts of the world. The first reported was FEC-1, identified in *E. coli* isolated from a dog used in studies of β -lactams in Japan in 1986 (Matsumoto *et al.*, 1988). Shortly after in 1987, an *E. coli* isolate from a child in Germany, was found to have a high cefotaxime MIC, and was found to have a cephalosporinase termed CTX-M-1, which was transferred on the pMVP-3 plasmid (Bauernfeind, Grimm and Schweighart, 1990). During the late 1980's and early 1990s there was wide-spread occurrence of strains which were resistant to cefotaxime. MEN-1 identified in France was found to be CTX-M-1 and a variant of Toho-1 found in Japan (Barthelemy *et al.*, 1992; Ishii *et al.*, 1995). While CTX-M-3 recovered from isolates in Poland was found to vary from the FEC-1 in Japan by two amino acid substitutions (Gniadkowski *et al.*, 1998). The number of CTX-M variants has expanded greatly in the last 10 years with over 140 CTX-M types identified, distributed among 5 major groups CTX-M-1, 2, 8, 9 and 25 based on their amino acid sequence (www.lahey.org/studies/other.asp) as shown in Figure 1.4 (Bonnet, 2004; Canton, Gonzalez-Alba and Galan, 2012). Each CTX-M group has multiple enzyme variants

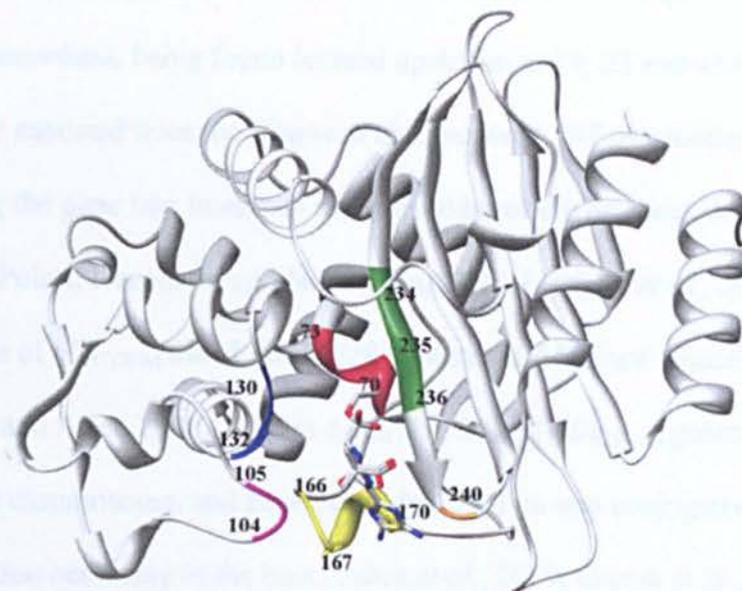
Figure 1.4 Phylogeny of *bla*_{CTX-M} genes 2004-2012



Dendrograms of the 5 CTX-M groups showing increase in numbers identified between (A.) 2004 and (B.) 2012 (Bonnet, 2004; Canton, Gonzalez-Alba and Galan, 2012).

which have evolved through point mutations in the genes resulting in amino acid substitution, which can alter the hydrolytic activity (Novais *et al.*, 2008). CTX-M-15 one of the most prevalent CTX-M's, differs from CTX-M-3 by a substitution at amino acid 240 from asparagine to glycine, and CTX-M-14 differs from CTX-M-9 by an amino acid substitution at position 231 from alanine to valine (Pai *et al.*, 2001; Poirel, Gniadkowski and Nordmann, 2002). Substitutions can result in alterations to the omega loop, shown in Figure 1.5, changing the activity towards substrates (Poirel *et al.*, 2001). Substitutions in CTX-M-18 (alanine 231 valine) and CTX-M-19 (proline 167 serine) have greater activity towards ceftazidime than cefotaxime, and changes to serine at 167, or glycine at 240 in CTX-M-1, also increase activity to ceftazidime, while alanine to valine at 77 and asparagine to serine at 106 increased the activity towards cefotaxime (Novais *et al.*, 2008).

Figure 1.5 Structure of CTX-M enzymes



Structure of a CTX-M-9 enzyme in complex with ceftazidime, the active site is shown in red and conserved elements in blue and green the omega loop is shown in yellow, numbers indicate residues important in the activity of the CTX-M, image from Delmas *et al.*, 2008.

1.7.1 Origins of *bla*_{CTX-M} genes

The *bla*_{CTX-M} genes originated on the chromosomes of *Kluyvera* spp., with each CTX-M group originating from either *Kluyvera ascorbata*, *Kluyvera georgiana* or *Kluyvera cryocrescens*. The group 1 CTX-M's (-1,-3 and -15) cluster with KLUC-1 from *Kluyvera cryocrescens* and group 2 CTX-M's originated from the KLUA produced by *Kluyvera ascorbata* (Decousser, Poirel and Nordmann, 2001; Humeniuk *et al.*, 2002; Lartigue *et al.*, 2006). The group CTX-M 8 and 9 both originate from *Kluyvera georgiana* from the KLUG-1 and KLUY-1 genes respectively (Poirel, Kampfer and Nordmann, 2002; Olson *et al.*, 2005). The genetic environment surrounding the *bla*_{CTX-M} genes further supports *Kluyvera* genomes as the origins of *bla*_{CTX-M}.

1.7.2 The *bla*_{CTX-M} genetic environment

The genetic environment surrounding *bla*_{CTX-M} has been important for the rapid dissemination of these resistance gene. Insertions sequences such as *ISEcp1*, *ISCR1* and *IS26* have been important in mobilising the genes between transposons, plasmids and

chromosomes (Bonnet, 2004; Livermore *et al.*, 2007; D'Andrea *et al.*, 2013; Canton, Gonzalez-Alba and Galan, 2012). *ISEcp1* was involved in the escape of the *bla_{KLUA}* gene from *Kluyvera ascorbata*, being found located upstream at 19, 22 and 43 bp (Lartigue *et al.*, 2006). Once captured from the *Kluyvera* chromosome, *ISEcp1* continues to mobilize *bla_{CTX-M}* moving the gene into integrons and plasmids, which has been demonstrated with *in vitro* studies (Poirel, Decousser and Nordmann, 2003; Lartigue *et al.*, 2006). Examining the environments of *bla_{CTX-M}* found that 23/28 isolates had *ISEcp1* adjacent to *bla_{CTX-M}* (Eckert, Gautier and Arlet, 2006). Within a cell the *ISEcp1-bla_{CTX-M}* genes move between plasmids and the chromosome, and have been identified on non conjugative plasmids supporting insertion occurring in the host (Fabre *et al.*, 2009; Garcia *et al.*, 2005; Cao, Lambert and Courvalin, 2002). *ISEcp1* not only has a role in the mobilisation of *bla_{CTX-M}* but also supplies the *bla_{CTX-M}* promoter having both -35 and -10bp for its expression, and depending on the position of *ISEcp1* in relation to *bla_{CTX-M}* can affect the level of expression (Karim *et al.*, 2001; Ma, Siu and Lu, 2011). Other MGE's have been involved with the mobilization including IS26 and in *ISCR1* (Saladin *et al.*, 2002; Eckert, Gautier and Arlet, 2006; Canton, Gonzalez-Alba and Galan, 2012; Toleman and Walsh, 2011). Due to the mobile natures of MGE's, interactions between them are common, and both *ISCR1* and *ISEcp1* have been associated with integrons which often carry multiple resistance gene cassettes (Walsh, 2006; Partridge, 2011; Arduino *et al.*, 2002; Valverde *et al.*, 2006). These cassettes often confer resistance to compounds such as aminoglycosides, trimethoprim, tetracyclines, sulphonamides and phenicols, which have been associated with CTX-M isolates and aid in their co-selection (Bonnet, 2004; Livermore *et al.*, 2007; Recchia and Hall, 1995; Toleman, Bennett and Walsh, 2006; Machado *et al.*, 2005).

1.7.3 Plasmids and *bla_{CTX-M}*

The *bla_{CTX-M}* genes have become the most widely disseminated plasmid β -lactamases, being found all over the world in isolates from humans, animals and the environment (Bonnet, 2004; Canton and Coque, 2006; Cao, Lambert and Courvalin, 2002;

Garcia *et al.*, 2005; Hawkey and Jones, 2009). Plasmids range in size from 7kb to 430kb and are found in numerous bacterial species such as *E. coli*, *Salmonella spp.*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Citrobacter freundii* and *Proteus vulgaris* (Bonnet, 2004). Nearly all plasmid replicon types have been found to harbour *bla*_{CTX-M} genes, with IncFI, FII, HI2, I1 γ , N, A/C, L/M and K being some of the most prevalent plasmids, which are conjugative plasmids shown in Table 1.2 (Carattoli *et al.*, 2005a; Carattoli, 2009; Carattoli, 2011; Hopkins *et al.*, 2006; Marcade *et al.*, 2009).

Table 1.2 CTX-M and *bla* genes associated with replicon type

Replicon Group	Resistance Genes
F	<i>bla</i> _{CMY-2} , <i>bla</i> _{CTX-M-1,-2,-3,-9,-14,-15,-24,-27} , <i>bla</i> _{TEM-1}
A/C	<i>bla</i> _{CMY-2} , <i>bla</i> _{CTX-M-2,-3,-14,-15,-56} , <i>bla</i> _{SHV-2,-5,-12} , <i>bla</i> _{TEM-3,-21,-24} , <i>bla</i> _{IMP-4,-8,-13} , <i>bla</i> _{VIM-4} , <i>bla</i> _{VEB-1}
L/M	<i>bla</i> _{CTX-M-1,-3,15,-42} , <i>bla</i> _{TEM-3,-10} , <i>bla</i> _{SHV-5} , <i>bla</i> _{IMP-4,-8}
II	<i>bla</i> _{CMY-2,-7,-21} , <i>bla</i> _{CTX-M-1,-2,-3,-9,-14,-15,-24} , <i>bla</i> _{SHV-12} , <i>bla</i> _{VIM-1}
HI2	<i>bla</i> _{CTX-M-2,-3,-9,-14} , <i>bla</i> _{SHV-12} , <i>bla</i> _{IMP-4} , <i>bla</i> _{VIM-1}
N	<i>bla</i> _{KPC-2} , <i>bla</i> _{CTX-M-1,-3,-15,-32,-40} , <i>bla</i> _{VIM-1}

Table adapted from Carattoli (2009).

Some *bla*_{CTX-M} genes are associated more frequently with some incompatibility groups than others, for example IncHI2 plasmids, similar to the avian plasmid IncHI2 pAPEC-O1-R, have been found with *bla*_{CTX-M-2} and ₋₉ genes in humans and poultry in France (Garcia Fernandez *et al.*, 2007). The same plasmid group has also been associated with *bla*_{CTX-M-9} and class 1 integrons with CR1 transposons Tn402 and Tn21 (Novais *et al.*, 2006; Rogers, Sidjabat and Paterson, 2011; Nicolas-Chanoine *et al.*, 2008). The *bla*_{CTX-M-15} gene is particularly associated with IncF plasmids, which has been aided in its success by the O25:ST131 pandemic clone (Mshana *et al.*, 2009; Woodford *et al.*, 2009). IncK plasmids have been found harbouring the *bla*_{CTX-M-14} gene in the UK, Europe and Asia, in unrelated isolates (Valverde *et al.*, 2009; Stokes *et al.*, 2012; Cottell *et al.*, 2011). The *bla*_{CTX-M-1} gene has been found on IncI1 γ plasmids, which have also been found associated with *bla*_{CTX-M-14} and ₋₁₅ in Europe from both *E. coli* and *Salmonella* (Garcia-Fernandez *et al.*, 2008; Cloeckart *et al.*, 2010; Leverstein-van Hall *et al.*, 2011).

1.7.4 Sequencing of *bla*_{CTX-M} plasmids

The number of *bla*_{CTX-M} plasmid being sequenced is constantly growing as a result of new sequencing technologies and the interest in the relationship between the resistance gene and plasmid. Sequencing has provided insights into plasmid backbones, the genes carried and resistance regions, some of which are shown in Table 1.3. Some plasmids are sequenced based on the *bla*_{CTX-M} genes they carry such as those by Smet *et al* (2010b) who looked at *bla*_{CTX-M-15}, while others look at the plasmids carried by a clonal strain such as those sequenced by Woodford *et al* (2009). The sequencing of *bla*_{CTX-M} plasmids has revealed that plasmids have high levels of homology to non *bla*_{CTX-M} plasmids sequenced previously which may be their progenitors. Currently few *bla*_{CTX-M} plasmids from animals have been sequenced, with pCT, pEC_Bactec and pSAM7 being some examples, however other non-CTX-M plasmids from animals have been sequenced which prove useful in comparisons (Cottell *et al.*, 2011; Smet *et al.*, 2010b; Stokes *et al.*, 2013).

1.7.5 CTX-M plasmid epidemiology

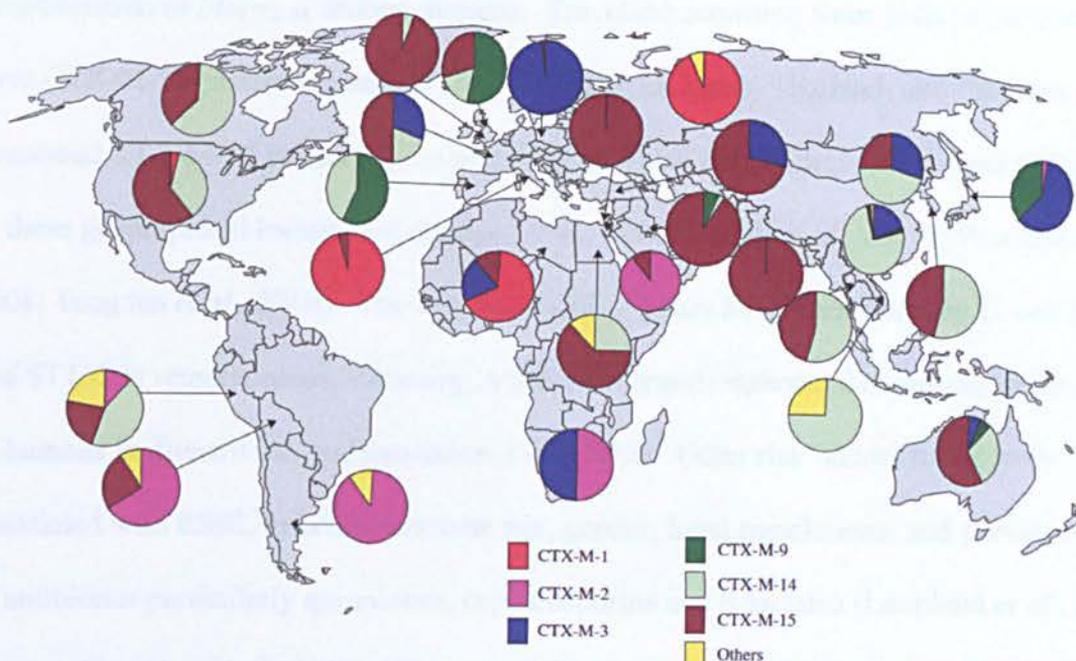
Infections caused by ESBL-producing isolates, particularly CTX-M, increase the mortality, morbidity and economic cost around the world (Hawkey and Jones, 2009; Paterson and Bonomo, 2005; Pitout and Laupland, 2008). CTX-M-producing isolates are found in humans, animals, the environment and food (Hawkey and Jones, 2009; Smet *et al.*, 2010a; Carattoli, 2008). The numbers of ESBL infections and resistance isolates is on the rise due to pressures from the use of antibiotics in both humans and animals, increasing population and greater industrialisation (Davies and Davies, 2010; Stokes and Gillings, 2011). The spread of resistant isolates, plasmids and MGEs is likely to be complex with many potential reservoirs and pressures affecting dissemination (Hawkey and Jones, 2009; Stokes and Gillings, 2011). The once dominant *bla*_{TEM} and *bla*_{SHV} genes have now been surpassed by the CTX-M-producing isolates, being present in all inhabited continents around the world, with their dissemination and distribution shown in Figure 1.6 (Bonnet,

Table 1.3 Sequenced *bla*_{CTX-M} plasmids

Plasmid Name	Source	Country	Size (bp)	Replicon type	Resistance genes	Notes	Reference
pC15-1a (AY458016)	<i>E. coli</i> Human	Canada	92,353	IncFII	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{OXA-1} , <i>bla</i> _{TEM-1} , <i>tetA</i> , <i>aac</i> (6')-Ib and <i>aac</i> (3)-II	Sequencing revealed that this plasmid was largely related to R100, which had undergone 28.4kb insertion containing integrons and the resistance genes, it was similar to <i>bla</i> _{CTX-M-15} plasmid in India.	Boyd <i>et al</i> (2004), Karim <i>et al</i> (2001)
pCTX-M3 (AF550415)	<i>Citrobacter freundii</i> Human	Poland	89,468	IncL/M	<i>bla</i> _{CTX-M-3} , <i>bla</i> _{TEM-1} , <i>aacC2</i> , <i>armA</i> , <i>aadA2</i> , <i>dfrA12</i> and <i>sulI</i>	Comparisons of this plasmid showed links to pEL60 plasmid carried by the plant pathogen <i>Erwinia amylovora</i> , which may have undergone insertions by the MDR regions.	Golebiewski <i>et al</i> (2007)
pKP96 (EU195449)	<i>K. pneumoniae</i>	China	67,850	IncN	<i>bla</i> _{CTX-M-24} , <i>qnrA1</i> , <i>aac</i> (6')-Ib-cr and <i>sulI</i>	Backbone is similar to R46 from <i>Salmonella typhimurium</i> .	Shen <i>et al</i> (2008)
pEK499 (EU935739)	<i>E. coli</i> ST131 Human	UK	117,536	IncFII/FIA	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{OXA-1} , <i>aac</i> (6')-Ib-cr, <i>mph(A)</i> , <i>catB4</i> and <i>tetA</i> and an integron in this region carried <i>aadA5</i> and <i>dfrA7</i>	Has resistances to 8 classes of antimicrobials, and has multiple addiction systems, similar to plasmids sequenced by Smet <i>et al</i> (2010b).	Woodford <i>et al</i> (2009), Smet <i>et al</i> (2010b)

Plasmid Name	Source	Country	Size (bp)	Replicon type	Resistance genes	Notes	Reference
pEK204 (EU935740)	<i>E. coli</i> ST131 Human	UK	93,732	IncI1 γ	<i>bla</i> _{CTX-M-3} and <i>bla</i> _{TEM-1}	Sequence suggests a Tn3 transposon inserted into a pCollb-P9 plasmid, and has some similarity to the insert in pCTX-M3	Woodford <i>et al</i> (2009), Golebiewski <i>et al</i> (2007)
pEC_B24 (GU371926)	<i>E. coli</i> Human	Belgium	73,801	IncFII	<i>bla</i> _{CTX-M-15} and <i>bla</i> _{TEM-1}	Shares a similar backbone to pC15-1a isolated in Canada and has several inheritance genes.	Smet <i>et al</i> (2010b)
pEC_L8 (GU371928)	<i>E. coli</i> Human	Belgium	118,525	IncFII and FIA	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{OXA-1} , <i>aac6'-lb-cr</i> , <i>tetA</i> and <i>catB4</i>	Have several addiction systems and a complete <i>tra</i> operon, and was found to be similar to pEK499.	Smet <i>et al</i> (2010b)
pEC_Bactec (GU371927)	<i>E. coli</i> Horse	Belgium	92,970	IncI1 γ	<i>bla</i> _{CTX-M-15} and <i>bla</i> _{TEM-1}	Shared 90% similarity with the IncI1 γ plasmid pEK204 from humans in the UK.	Smet <i>et al</i> (2010b), Woodford <i>et al</i> (2009)
pCT (FN868832)	<i>E. coli</i> Cattle	UK	93,629	IncK	<i>bla</i> _{CTX-M-14}	This plasmid was isolated from <i>E. coli</i> from cattle in the UK but was also found to be similar to IncB and IncI1 γ plasmids from Europe and Asia.	Cottell <i>et al</i> (2011)
pNDM-MAR (JN420336)	<i>K. pneumoniae</i> Human	Morocco	267,242	IncH	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{NDM-1} and <i>qnrB1</i> .	pNDM-MAR differed significantly from other NDM-1 plasmids previously sequenced	Villa <i>et al</i> (2012)

Figure 1.6 Worldwide CTX-M distribution



The global distribution of the main CTX-M types with respective prevalence image from Hawkey and Jones (2009).

2004; Livermore *et al.*, 2007; Canton, Gonzalez-Alba and Galan, 2012). CTX-M-1, 3, 15 and CTX-M-14 are some of the most prevalent CTX-Ms in the world in both isolates from humans and animals.

1.7.6 *bla*_{CTX-M} genes in isolates from humans

CTX-M producing isolates are found in both community and hospital patients with 72% of the producers in the community and the remaining 28% in hospital (Laupland *et al.*, 2008). The community and hospital environments allow for clonal spread and LGT between a myriad of isolates and bacterial species. A study by Valverde *et al* (2004) of hospital patients over a 12 year period found an increase in ESBL producers from 0.3% to 11.8% in inpatients, and 0.7% to 5.5% in outpatients, 3.7% of healthy individuals were ESBL carriers, indicating that the community may be more important in dissemination (Valverde *et al.*, 2004). Healthy individuals were also found to be carriers of ESBL isolates, of which 86% were CTX-M producers which had increased 10 fold over a 5 year period to 6%, implying wide dissemination in the community (Nicolas-Chanoine *et al.*,

2013). International travel has been recognised as a risk factor for the intercontinental dissemination of *bla*_{CTX-M} among humans. Travellers returning from India were found to have CTX-M-15-producing isolates, and travellers to, Japan, Thailand, and Pakistan, possessed CTX-M-14-producing isolates both of which are the prevailing types of CTX-M in these geographical locations (Laupland *et al.*, 2008; Dhanji *et al.*, 2011c; Freeman *et al.*, 2008; Tangden *et al.*, 2010). The *bla*_{CTX-M-1} and *bla*_{CTX-M-15} genes have been found in *E. coli* ST440 and ST131 in remotes areas, including Antarctic research stations, demonstrating the role of humans in dissemination (Hernandez *et al.*, 2012). Other risk factors found to be associated with ESBL infections include age, gender, local populations, and previous use of antibiotics particularly quinolones, cephalosporins and β -lactams (Laupland *et al.*, 2008; Nicolas-Chanoine *et al.*, 2012). The main CTX-M types identified in human diseases are CTX-M-15 dominant in Europe, Middle East and USA, CTX-M-14 in the USA and Asia, CTX-M-1 in Russia, Africa and Europe and CTX-M-2 in South Africa and South America (Hawkey and Jones, 2009; Livermore *et al.*, 2007; Canton and Coque, 2006).

1.7.6.1 The *bla*_{CTX-M-15} gene in isolates from humans

CTX-M-15-producing isolates are particularly common in the UK, being identified in over 38% of *E. coli* CTX-M producers (Woodford *et al.*, 2004; Karisik *et al.*, 2008). A common clone O25:ST131 has been associated with CTX-M-15 being found in both hospital and community settings, with another four key CTX-M-15 producing strains in circulation (Karisik *et al.*, 2008; Dhanji *et al.*, 2011a). This *E. coli* CTX-M-15 clone has been found to be pandemic, and the *bla*_{CTX-M-15} gene has facilitated its spread, with non CTX-M isolates being less prevalent (Karisik *et al.*, 2008; Rogers, Sidjabat and Paterson, 2011; Nicolas-Chanoine *et al.*, 2008). Other isolates found with CTX-M-15 belong to the virulent B2 phylogroup even those not associated with epidemics or pandemics isolates (Karisik *et al.*, 2008; Woodford *et al.*, 2004; Woodford *et al.*, 2009). Plasmids have been shown to disseminate the *bla*_{CTX-M-15} between isolates that are unrelated isolates in the UK (Mushtaq *et al.*, 2003). CTX-M-15 has been found in human community and nosocomial

isolates across the world including Bulgaria, Germany, Poland, France, Spain, Italy, India, Canada and Japan (Livermore *et al.*, 2007; Boyd *et al.*, 2004; Karim *et al.*, 2001; Mshana *et al.*, 2009; Rogers, Sidjabat and Paterson, 2011).

1.7.6.2 The *bla*_{CTX-M-1} gene in isolates from humans

Although not as wide spread as some of the other CTX-Ms, CTX-M-1 has been found in both healthy and unhealthy humans, and is prevalent in Western Europe (Cloeckaert *et al.*, 2010; Leverstein-van Hall *et al.*, 2011). CTX-M-1 has been found in 10 of 34 ESBL producing isolates from healthy patients in Switzerland and unrelated *E. coli* UTI infections in Austria (Geser *et al.*, 2012; Prelog *et al.*, 2008). IncN and IncL/M plasmids in Spain were found to disseminate *bla*_{CTX-M-1}, with IncN plasmids bearing *bla*_{CTX-M-1} also being identified in Denmark and the Czech Republic in *E. coli* and *Salmonella* of human and animal origins (Novais *et al.*, 2007; Dolejska *et al.*, 2013; Moodley and Guardabassi, 2009). IncII γ plasmids belonging to ST3 have been identified with *bla*_{CTX-M-1} in *Salmonella* spp from patients in France (Cloeckaert *et al.*, 2010). Some institutions have reported CTX-M-1 to be the dominant enzyme, being identified in 51/53 of the *E. coli* ESBL isolates recovered between 1999-2003, of which most were from UTI patients (Brigante *et al.*, 2005).

1.7.6.3 The *bla*_{CTX-M-3} gene in isolates from humans

Of all the group 1 CTX-Ms, CTX-M-3 has been identified extensively in *Enterobacteriaceae* isolates from Poland, which appears to be one of the hotspots for this enzyme, with one of the first sequenced CTX-M-bearing plasmids isolated in Poland (Gniadkowski *et al.*, 1998; Baraniak *et al.*, 2002; Golebiewski *et al.*, 2007). CTX-M-3 has also been reported in clinical isolates from China, Korea and India (Hawkey, 2008; Ho *et al.*, 2007; Kim *et al.*, 2005; Karim *et al.*, 2001).

1.7.6.4 The *bla*_{CTX-M-14} genes in isolates from humans

CTX-M-14 is one of the most prevalent CTX-M enzymes around the world, being identified in Europe, USA and Asia. IncK plasmids in Europe have been found to have a significant role in disseminating the *bla*_{CTX-M-14} plasmids, being identified in 15-19% of UK human isolates, and linked to A, B1 and D phylogroups (Valverde *et al.*, 2009; Stokes *et al.*, 2012; Dhanji *et al.*, 2012). CTX-M-14-producing isolates of the clonal O25:ST131 have been found throughout Spain, and some instances were present in 57.1% of ESBL isolates, the ST101 strain has also been found to have a role in dissemination (Blanco *et al.*, 2009; Mora *et al.*, 2011). In Asia IncF plasmids have been found to play a big role in the dissemination of CTX-M-14 in Korea, but have also been found in China (Kim *et al.*, 2011; Ho *et al.*, 2012; Bae *et al.*, 2008; Chanawong *et al.*, 2002). CTX-M-14 is one of the few CTX-M to be reported in the USA, being prevalent in *E. coli* and *Klebsiella pneumoniae* (Hanson *et al.*, 2008).

1.7.7 *bla*_{CTX-M} genes in isolates from animals

CTX-M-producing isolates are not restricted to humans, and have been found in livestock, domesticated animals and wild animals, which may act as reservoirs of resistance (Smet *et al.*, 2010a; Carattoli, 2008; Knezevic and Petrovic, 2008; Phillips *et al.*, 2004). Antibiotics are not only used in the treatment of bacterial infections, but also as antimicrobial growth promoters (AGPs), which maintain MDR isolates, as seen with the withdrawal of AGP's in Scandinavia which resulted in a decrease in the numbers of resistant *E.coli* and *Salmonella* (Bengtsson and Wierup, 2006; Mathew *et al.*, 1999). Since 2000 there has been a dramatic increase in studies reporting ESBL producing bacteria from food producing animals, companion animals and wild animals (Smet *et al.*, 2010a; Carattoli, 2008).

1.7.7.1 *bla*_{CTX-M} genes in isolates from food producing animals

The prevalence of ESBL isolates at some farms can be as high as 60-100%, with *bla*_{CTX-M-1, -2, -14, -15, TEM-52, and -106} genes having been identified, demonstrating the presence of ESBLs at the farm level (Mesa *et al.*, 2006; Smet *et al.*, 2008). Studies have found that the use of ceftiofur and cefquinome commonly used in veterinary medicine may select for CTX-M-producing *E. coli*, over animals treated with amoxicillin, and that once colonised are readily shed into the environment (Cavaco *et al.*, 2008; Jorgensen *et al.*, 2007; Horton *et al.*, 2011).

Poultry have particularly been identified as having CTX-M-producing isolates. One of the most common types is CTX-M-1 which has been found in broilers and layers from the Netherlands, Belgium, France, Spain, and the UK (Dierikx *et al.*, 2013; Smet *et al.*, 2008; Girlich *et al.*, 2007; Cloeckaert *et al.*, 2010; Randall *et al.*, 2011; Cortes *et al.*, 2010). In France and the Netherlands, *bla*_{CTX-M-1} are associated with Inc11 γ plasmids (Cloeckaert *et al.*, 2010; Dierikx *et al.*, 2013). Other CTX-M's include CTX-M-15 isolates in Asia, Belgium and the UK, CTX-M-14 in Japan, UK and Spain and CTX-M-9 in Belgium and Portugal (Hiroi *et al.*, 2012; Randall *et al.*, 2011; Stokes *et al.*, 2012; Brinas *et al.*, 2003; Riano *et al.*, 2006; Smet *et al.*, 2008; Costa *et al.*, 2009; Kojima *et al.*, 2005). The ESBL isolates are typically found in healthy animals or those without symptoms (6.3%), but are also present in sick animals (Brinas *et al.*, 2003; Zheng *et al.*, 2012).

Cattle isolates have also been found to harbour *bla*_{CTX-M} genes, with enzymes from groups 1, 2 and 9 being identified. CTX-M-1 and -15 have been found in France on plasmids similar to those in humans (IncF) but are not associated with the ST131 clone, whereas IncN plays a role for CTX-M-1 in Denmark (Meunier *et al.*, 2006; Madec *et al.*, 2012; Madec *et al.*, 2008; Garcia-Fernandez *et al.*, 2011). Cattle isolates have been found with *bla*_{CTX-M-14} IncK and IncFII plasmids in the UK and Hong Kong respectively (Liebana

et al., 2006; Stokes *et al.*, 2012; Ho *et al.*, 2012). CTX-M-2 producing isolates have also been found in cattle from Japan (Shiraki *et al.*, 2004).

Swine are another reservoir of *bla*_{CTX-M} genes, and particularly prevalent in Europe with CTX-M-1 being identified in Spain, Denmark and Czech Republic (Dolejska *et al.*, 2013; Cortes *et al.*, 2010; Wu *et al.*, 2008; Moodley and Guardabassi, 2009). In China CTX-M-15, -22 and SHV-2 have been found in isolates from pigs (Tian *et al.*, 2009). Ceftiofur treatment has been found to correlate with the increasing numbers of CTX-M-1 isolates in swine, whilst the withdrawal reduced the number of ESBL isolates, but had no effect on numbers of non ESBL isolates (Ageroso and Aarestrup, 2013; Jorgensen *et al.*, 2007).

1.7.7.2 *bla*_{CTX-M} genes in isolates from companion animals

Companion animals such as dogs, cats and horses which have regular contact with humans have increased opportunity for acquisition of both isolates and plasmids. Healthy dogs in Portugal, Italy, Germany, Spain, Denmark and Tunisia have been found to carry CTX-M-1, -15 and -14 isolates and CTX-M-1 has been reported in cats from Italy and Chile (Pomba *et al.*, 2009; Carattoli *et al.*, 2005b; Sallem *et al.*, 2013; Moreno *et al.*, 2008; Ewers *et al.*, 2010). One of the few sequenced CTX-M plasmids from animals is the IncI1 γ pEC_Bactec recovered from a horse, which shares 90% homology with pEK204 recovered from human ST131 (Smet *et al.*, 2010b).

1.7.7.3 *bla*_{CTX-M} genes in isolates from wild animals

Wild animals have been found with *bla*_{CTX-M} genes, which may act as zoonotic reservoirs. Seagulls have been found to be carriers of CTX-M-1, -14 and -32 isolates in France and Portugal, with isolates recovered in France being related to humans these resistances are likely to have been acquired from contact with human waste (Bonedahl *et al.*, 2009; Poeta *et al.*, 2008). In the USA coastal birds have been found to harbour isolates with *bla*_{CTX-M-15} genes (Poirel *et al.*, 2012). Rats in Germany have been found with the

ST131 pandemic clone, carrying the *bla*_{CTX-M-9} gene, the strain was found to be related to human strains suggesting community based spread (Guenther *et al.*, 2010). Wild animals with limited interaction with humans have been found to have ESBL isolates with 10% of wild boar isolates harbouring CTX-M-1 (Poeta *et al.*, 2009).

1.7.8 *bla*_{CTX-M} genes in isolates from food and the environment

It has long been known that food is a source of antimicrobial resistance and resistant isolates (Corpet, 1988). Food from Tunisia has been found to be contaminated with CTX-M-1 isolates from turkey and beef products, and CTX-M-14-producing isolates from chicken meat (Jouini *et al.*, 2007). Raw chicken in France was reported to be contaminated with CTX-M-1-producing *Salmonella* Virchow, with *bla*_{CTX-M-1} carried on plasmids similar to those in humans (Weill *et al.*, 2004; Cloeckaert *et al.*, 2010). Two studies found that 30-37% of chicken meat imported into the UK were contaminated with CTX-M-2 isolates, which is an uncommon enzyme in the UK (Warren *et al.*, 2008; Dhanji *et al.*, 2010). A study by Leverstein-van Hall *et al.* (2011) has produced some of the most conclusive evidence to date showing a link between animals, food and humans, identifying similar plasmids carrying the CTX-M-1 on IncI1 γ (ST7) plasmids in *E. coli* (Leverstein-van Hall *et al.*, 2011). Food has been identified as a vector for ESBL-producing isolates, being found to be shared between diners at the sites of foodborne disease outbreaks (Lavilla *et al.*, 2008).

ESBL isolates have also been recovered from water, with O25:ST131 CTX-M-14 found in the river Thames, and CTX-M-14, TEM-52 and CMY-1 recovered from rivers in Korea (Dhanji *et al.*, 2011b; Kim, Kang and Lee, 2008). Additionally the clonal CTX-M-15 ST131 strain has been recovered from sewage treatment, and waters have been found to be contaminated with ESBL isolates from human faecal matter (Dolejska *et al.*, 2011b; Machado *et al.*, 2009).

1.7.9 *bla*_{CTX-M} gene transmission between animals and humans

The presence of *bla*_{CTX-M} in food producing animals and on food products poses a significant risk for the transmission of isolates and plasmids between animals and humans, with evidence supporting food borne outbreaks (Lavilla *et al.*, 2008). Several plasmid incompatibility groups bearing CTX-M genes have been found to be shared between unrelated isolates recovered from humans and animals. Highly similar *bla*_{CTX-M-1} IncI1 γ plasmids have been found in human and poultry isolates from France and the Netherlands, which has been aided by pMLST, with possible links to the food chain (CloECKaert *et al.*, 2010; Leverstein-van Hall *et al.*, 2011). In Denmark the same IncN plasmids bearing *bla*_{CTX-M-1} have been found in isolates from pigs and farm workers as determined by RFLP, and in the Czech Republic IncN plasmids from cattle and horses were present in humans also determined by pMLST (Moodley and Guardabassi, 2009; Dolejska *et al.*, 2013). The *bla*_{CTX-M-14} genes have been found to be transferred between human and animal isolates on IncK and IncFII plasmids. In the UK pCT-like plasmids have been found to be associated with unrelated isolates from cattle, turkeys and humans (Stokes *et al.*, 2012). This plasmid was originally detected in cattle but has since been found in human isolates which has been aided by pCT molecular markers (Liebana *et al.*, 2006; Cottell *et al.*, 2011; Stokes *et al.*, 2012). A plasmid similar to the IncFII pHK01 in Hong Kong have been found disseminated among humans, cattle and turkey isolates (Ho *et al.*, 2012). In France *Salmonella* from human and poultry isolates were found to have the same *bla*_{CTX-M-9} IncHI2 plasmids (Garcia Fernandez *et al.*, 2007). The detection of plasmid transmission between isolates from humans and animals has benefitted from techniques such as RFLP, pMLST and molecular markers, and could benefit from further molecular markers expanding the number of plasmids which can be identified and simplifying their identification.

1.8 Hypotheses and aims

Plasmids have been identified as highly effective vectors for the dissemination of *bla*_{CTX-M} genes and have been found in *E. coli* isolates from both humans and animals. The increasing number of published plasmid sequences has provided a greater depth of knowledge of both the genetic features of plasmids and the resistance genes carried. As the comparative analysis of these plasmid grows it is becoming clear that plasmids are likely to share common ancestors which have evolved via MGEs, recombination, insertions and deletions to produce new lineages. The numbers of CTX-M isolates from humans and animals in the UK is increasing, and the role that food and animal interaction has to play in this is not fully understood and warrants further investigation, identifying any ways in which transmission can be interrupted.

Group 1 and 9 *bla*_{CTX-M} genes are the most prevalent *bla*_{CTX-M} genes in the UK, and in this study plasmids from animal and human isolates with these *bla*_{CTX-M} genes were selected to be sequenced. The *bla*_{CTX-M-14} gene has, as mentioned above, been found on IncK and IncF plasmids in Europe and Asia, but is also prevalent in cattle and poultry isolates in the UK. Plasmid molecular markers have been designed to identify the *bla*_{CTX-M-14} pCT plasmids which have been used to study the epidemiology of pCT-like plasmids and led to the identification of related plasmids such pH19. Other plasmid vectors were found to carry the *bla*_{CTX-M-14} gene, which included the untypable IncX4 plasmid pSAM7. The IncA/C plasmid is not commonly associated with *bla*_{CTX-M}, being typically found with *bla*_{CMY-2} ESBL gene. Carriage of *bla*_{CTX-M-3} was found on a IncA/C plasmid, a vector becoming associated with *bla*_{NDM-1} and warranted further investigation. IncI γ plasmids are one of the most common *bla*_{CTX-M-1} vectors in animal isolates in Europe with ST3 and ST7 being some of the predominant sequence types. These are also prevalent in the animal population in the UK, with possible transmission to humans.

The hypotheses of this study are (i) that CTX-M-bearing plasmids from animal and human isolates in the UK are related to other plasmids found around the world, and share key features which enhance their dissemination. (ii) Plasmid molecular markers are effective methods to investigate the epidemiology of plasmids and can detect transmission between human and animal isolates. (iii) that molecular markers can be further expanded to differentiate between closely related plasmids providing new molecular tools to investigate plasmid epidemiology and relationship.

The specific aims of this study are as follows:

1. Apply previously designed molecular markers (pCT) to demonstrate their ability and benefits in examining plasmid dissemination between humans and animals.
2. Isolate, extract and sequence seven CTX-M plasmids from human and animal origins.
3. Fully annotate and compare sequenced CTX-M plasmids to identify key structural features and their relationship to other published CTX-M and non CTX-M plasmids from around the world.
4. Develop molecular markers capable of identifying the CTX-M plasmids sequenced in this study.
5. Develop molecular markers capable of differentiating between plasmids that are closely related and of the same, or similar, incompatibility groups.
6. Apply molecular markers to *E. coli* field isolates to see the prevalence of plasmids of these types and possible related plasmids.

Chapter 2

Methods and

Materials

2.1 Bacteriological methods

2.1.1 Bacterial strain selection

Bacterial isolates used throughout this study came from the Animal Health and Veterinary Laboratories Agency (AHVLA) culture collection, the SafeFoodEra (SFE) consortium culture collection, and from general practice and hospital submission to public health Wales. *E. coli* JIE143 isolated from blood of a patient in Sydney in 2006 (Partridge *et al.*, 2011) and plasmid pCT in *E. coli* DH5 α from Cottell *et al* (2011) (Cottell *et al.*, 2011). A comprehensive list of strains used in chapter 3 are shown in appendix I, chapter 4 and 7 are shown in appendix II and chapter 5 are shown in appendix III. The presence of ESBL encoding genes in these isolates was identified by either PCR, sequencing or microarray previously at AHVLA. Plasmids from wild-type isolates were further studied in laboratory *Escherichia coli* DH10B (Invitrogen) and *Salmonella enterica* serovar Typhimurium 26R (AHVLA) strains. A list of the wild types and plasmids used for sequencing is shown in Table 2.1. Isolates within the AHVLA and SFE collection have been grown on selective medias CHROMagar CTX and CHROMagar ECC in which *E. coli* isolates produce blue colonies and other *Enterobacteriaceae* produce mauve or white colonies, in the case of CHROMagar CTX, cephalosporin sensitive Gram negatives and positives are inhibited (Randall *et al.*, 2009).

2.1.2 Bacterial growth conditions

All bacterial isolates were stored in LB-G 40% glycerol stocks at -80°C and routinely grown on Luria-Bertani without glucose (LB-G) agar (biological productions unit , BPU) supplemented with cefotaxime added to the media, and incubated aerobically at 37°C overnight (~16-18 hours). Typically overnight broth cultures were grown in 5 ml LB-G broth (BPU) supplemented with the appropriate antibiotic, incubated at 37°C, 200 rpm for 18 hours, unless otherwise stated. To maintain the presence of ESBL plasmids isolates were grown on LB agar or in LB broth supplemented with 4 μ g/ml

Table 2.1 Wild-type isolates and plasmids used for sequencing

Isolate	Species	Source	Isolation Date	Plasmid name	Plasmid Inc	Size (bp)	CTX-M
CH01	<i>E. coli</i>	Chicken	2006	pCH01	A/C	160,357	<i>bla</i> _{CTX-M-3}
CH02	<i>E. coli</i>	Chicken	2006	pCH02	I1 γ	75,796	<i>bla</i> _{CTX-M-1}
CH03	<i>E. coli</i>	Chicken	2010	pCH03	I1 γ	105608	<i>bla</i> _{CTX-M-1}
CT01	<i>E. coli</i>	Cattle	2008	pCT01	I1 γ	117577	<i>bla</i> _{CTX-M-1}
T01	<i>E. coli</i>	Turkey	2006	pT01	I1 γ	111,318	<i>bla</i> _{CTX-M-1}
H19	<i>E. coli</i>	Human	2007	pH19	IncZ	99,362	<i>bla</i> _{CTX-M-14}
SAM7	<i>E. coli</i>	Cattle	2008	pSAM7	IncX4	35,341	<i>bla</i> _{CTX-M-14b}
SAM7-2	<i>Enterobacter cloacae</i>	Cattle	2012	pSAM7-2	IncX4	35,341	<i>bla</i> _{CTX-M-14b}

cefotaxime (Sigma-Aldrich). Conjugative strains were initially selected on Rambach agar (BPU) supplemented with 1 μ g/ml cefotaxime and 100 μ g/ml rifampicin (Sigma-Aldrich). Transformed strains were initially selected on LB-G agar supplemented with 2 or 8 μ g/ml cefotaxime.

2.1.3 Antibiotic susceptibility testing

The antimicrobial resistance phenotypes were determined using the disc diffusion method. Colonies grown agar overnight at 37°C on LB-G were resuspended in physiological saline to a density of 0.5 using McFarland standards and was diluted 1:100 in physiological saline and swabbed on to an Iso-sensitest agar across 4 axis. A disc was added to the plate containing nalidixic acid (NA, 30 μ g), tetracycline (TE, 10 μ g), neomycin (N, 10 μ g), ampicillin (AMP, 10 μ g), ceftazidime (CAZ, 30 μ g), cefotaxime (CTX, 30 μ g), chloramphenicol (C, 10 μ g), streptomycin (S, 25 μ g), sulphamethoxazole/trimethoprim (SXT, 25 μ g), amikacin (AK, 30 μ g), gentamicin (CN, 10 μ g), amoxycillin/clavulanic acid (AMC, 30 μ g), sulphonamides (S3, 300 μ g), apramycin (APR, 15 μ g), and ciprofloxacin (CIP, 1 μ g) (OXOID), and incubated aerobically at 37°C. The diameter of inhibition was compared to the breakpoints set out by the British Society of Antimicrobial Chemotherapy (BSAC) (Andrews, 2001).

2.1.4 Bacterial conjugation

2.1.4.1 Plasmid transfer in liquid

The recipient for liquid conjugations was plasmid-free rifampicin resistant *Salmonella enterica* Typhimurium 26R (AHVLA). Liquid conjugations were carried out using a recipient to donor ratio of 10:1, using overnight cultures of both the recipient and donor grown in LB-G broth. To 9 ml broth LB-G 900 µl of the recipient and 100 µl of the donor was added and incubate stationary at 37°C for 5 hours. After the incubation the cultures were agitated and 100 µl of neat and 1/10 serial dilutions in 0.1M phosphate buffered saline (PBS) were pipetted onto Rambach agar with 100 µg/ml rifampicin and 1 µg/ml cefotaxime, followed by incubation at 37°C for 18 hours.

2.1.4.2 Plasmid transfer on solid surface

Solid conjugations used the same recipient and donor ratio as liquid conjugations, 1 ml of the mixture was pipetted onto a Isopore 0.22 µm polycarbonate membrane filter (Millipore GTTP02500) placed on a LB-G agar plate and incubated at 37°C for 5 hours. After the incubation the filter membranes were removed under sterile conditions and placed into 5 ml 0.1M PBS, vortexed, and centrifuged for 5 minutes at 6,000 rpm, the supernatant was removed and the pellet re-suspended in 1 ml 0.1M PBS. The re-suspension was serially diluted and 200 µl of neat, 10^{-1} , 10^{-2} and 10^{-3} was pipetted onto Rambach agar with 100 µg/ml rifampicin and 1 µg/ml cefotaxime, followed by incubation at 37°C for 18 hours

2.2 Molecular Methods

2.2.1 Polymerase chain reaction (PCR)

Typical PCR reactions were carried out using the GeneAmp 9700 thermocycler (Applied Biosystems) using HotStar Taq Master Mix Kit (QIAGEN 203445) and unless stated reactions were carried out in volumes of 25 µl. All reaction started with a 15 minute

denaturation step at 95°C and a final extension step of 10 minutes at 72°C. Numbers of cycles, temperatures and periods for the denaturing, annealing and extension steps vary for each PCR. Relevant negative and positive controls were used in all PCR reactions.

2.2.2 Gel electrophoresis

Amplified DNA from PCR reactions and plasmid DNA was resolved by gel electrophoresis using gels varying from 0.8-2% agarose, made with 1x TAE buffer (40mM Tris-acetate and 1mM EDTA) (BPU) or 1x TBE (100mM Tris, 90mM boric acid 2mM EDTA) (BIORAD 161-0733). Gels were run in 1x TAE or 0.5x TBE using a PowerPac 300 (BIORAD) at 80-120V constant voltage for 60-120 minutes. Gels were stained in 1 µg/ml ethidium bromide (Sigma-Aldrich) and viewed under ultraviolet light and imaged using an GeneSnap imaging system (Syngene).

2.2.3 DNA extraction

2.2.3.1 Crude lysis

Colonies were picked using a sterile swab from an agar plate from overnight incubation at 37°C and re-suspended in 1 ml 0.1M PBS. The re-suspension was incubated at 95°C for 15 minutes, followed by centrifuge for 5 minutes at 13,000 rpm; the aqueous supernatant was removed and stored at -20°C until use.

2.2.3.2 Array lysis

Colonies grown on LB-G agar overnight at 37°C were resuspended in 400 µl bacterial lysis buffer (100mM Tris HCl, 0.05% Tween 20) to which 20 µl of proteinase K (20 mg/ml (Ambion)) was added and incubated at 60°C for 2 hours at 550 rpm, after incubation the proteinase K was inactivated by heating to 95°C for 15 minutes, followed by centrifugation at 13,000 rpm for 5 minutes and removed and retained the supernatant.

2.2.4 Plasmid extraction

2.2.4.1 Phenol chloroform extraction

Plasmid DNA was extracted for sizing by gel electrophoresis using a modified method of Kado and Liu (1981). Aliquots (1 ml) of overnight culture, were centrifuged at 13,000 rpm for 10 minutes and the supernatant discarded. The pellet was re-suspended in 200 µl lysis buffer (3% SDS, 0.6% Tris and 0.03M NaOH) and incubated at 55°C for 35 minutes. Phenol:chloroform:isoamyl alcohol 25:24:1 (Sigma-Aldrich UK cat P2069) (400 µl aliquot) was added, vortexed, and centrifuged at 13,000 rpm for 40 minutes. The aqueous supernatant was removed and stored at -20°C.

2.2.4.2 Takahashi alkaline lysis

Plasmid DNA for RFLP was extracted using a modified method described by Takahashi and Nagano (1984). In brief 3 ml of overnight culture was centrifuged at 13,000 rpm, the supernatant was removed and the pellet re-suspended in 200 µl suspension buffer (2mM Tris, 40mM EDTA). Aliquots (400 µl) of lysis buffer (2% SDS, 50mM Tris, 0.2M NaOH) was added, inverted (x10) and incubated at room temperature for 5 minutes. After incubation 300 µl of neutralization buffer (3M sodium acetate (NaOAc) pH 5.3) was added, inverted (x15) and incubated on ice for 15 minutes. Centrifuged at 13,000 rpm for 30 minutes and 700 µl of the supernatant removed, to which 700 µl chloroform (Sigma-Aldrich 472476) was added, inverted (x10) and centrifuged at 13,000 rpm for 30 minutes. An aliquot (500 µl) of the aqueous layer was removed and 1 ml of absolute ethanol added, inverted (x10) and incubated at -20°C for 20 minutes. Centrifuged at 13,000 rpm for 45 minutes at 4°C, and removed the supernatant, the pellet was re-suspended in 120 µl elution buffer (10mM Tris, 2mM EDTA). Aliquots (350 µl) of elution buffer and 50 µl of precipitation buffer (1M NaOAc, 10mM Tris and 2mM EDTA), were added followed by 1 ml absolute ethanol and incubated at -20°C for 30 minutes. Centrifuged at 13,000 rpm for

45 minutes, discarded the supernatant and re-suspended the pellet in 50 μ l H₂O, store DNA at -20°C.

2.2.4.3 Hi-Speed Plasmid Midi prep

The whole plasmid DNA from the isolates was extracted using HiSpeed plasmid Midi kit (QIAGEN) following the manufacturers protocol. Aliquots (150 ml) of overnight culture grown in LB-G + 4 μ g/ml cefotaxime, were centrifuged at 6,000 g for 15 minutes at 4°C and the supernatant discarded. The pellet was re-suspended in 6 ml buffer P1, and 6 ml lysis buffer P2 was added, inverted (x6) and incubated at room temperature for 5 minutes. Aliquots (6 ml) of neutralization buffer P3 was added and inverted (x6). The lysate was poured onto a QIAfilter cartridge and incubated for 10 minutes. A HiSpeed Midi Tip was equilibrated with 4 ml QBT, the lysate was filtered through the QIAfilter cartridge directly into HiSpeed Midi Tip. The lysate was allowed to pass through the HiSpeed Midi Tip, and washed with 20 ml QC buffer, the DNA was eluted using 5 ml QF buffer. The eluted DNA was passed through a QIAprecipitator, which was washed with 2 ml 70% ethanol, the QIAprecipitator was dried by passing through air. The DNA was eluted from the QIAprecipitator by adding 1 ml H₂O, which was repeated, DNA stored at -20°C.

2.2.4.4 Large construction kit

High yield plasmid DNA for sequencing was extracted from *E. coli* DH10 transformed with plasmids using the QIAGEN large construct kit (QIAGEN) following the manufactures instructions. In brief 500 ml of bacterial culture grown in LB-G + 4 μ g/ml cefotaxime for 18 hours at 37°C and 225 rpm. Bacteria were collected by centrifugation at 6,000 g for 15 minutes, the supernatant was removed and re-suspended in 20 ml buffer P1, and 20 ml lysis buffer P2 was added and inverted (x6) and incubated for 5 minutes at room temperature. Aliquots (20 ml) of chilled neutralization buffer P3 was added and inverted (x6), followed by incubation on ice for 10 minutes. The lysate was

centrifuged at 20,000 g for 30 minutes at 4°C, the lysate was filtered through a 70 µm cell strainer (BD Falcon 35235) and filter paper (Millipore). DNA was precipitated by adding 36 ml isopropanol and inverting (x10) followed by centrifugation at 15,000 g for 30 minutes at 4°C, the supernatant was discarded. The DNA pellet was washed with 5 ml of 70% ethanol and centrifuged at 15,000 g for 15 minutes at 4°C, and the supernatant was discarded. The DNA pellet was re-suspended in 9.5 ml EX buffer, 200 µl ATP-dependent Exonuclease and 300 µl ATP solution (100 mM) and incubated at 37°C for 1 hour. The QIAGEN-tip 500 was prepared by adding 10 ml QBT. To the digestion 10 ml QS was added, and applied to the QIAGEN-tip 500, which was washed with 30 ml QC (x2). DNA was eluted using 15 ml QF buffer at 65°C, and precipitated with 10.5 ml isopropanol and inverted (x10) followed by centrifugation at 15,000 g for 30 minutes at 4°C. The DNA pellet was washed with 5 ml 70% ethanol and centrifuged at 15,000 g for 15 minutes at 4°C, allowed to air dry and re-suspended in 1 ml TE buffer, DNA was stored at -20°C.

2.2.5 Transformation

Purified plasmid DNA was transformed into *E. coli* ElectroMAX DH10B (Fmcra Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 recA1 endA1 araD139 Δ (ara, leu)7697 galU galK λ rpsL nupG) (Invitrogen). The ElectroMAX DH10B cells were thawed and 25 µl were added to a Gene Pulser Cuvettes 0.2 cm (BIORAD) to which 50-100 ng of DNA was added. DNA was transformed using GenePulser II electroporator (BIORAD) using the conditions 2.0 kV, 200 Ω and 25 µF suggested by the manufacturer. The cells were added to 600 µl SOC media (Invitrogen) and incubated at 37°C for 1 hour at 225 rpm, a 100 µl of the transformed cells was spread on LB-G agar + 4 µg/ml cefotaxime and incubated at 37°C for 18 hours.

2.3 Molecular typing

2.3.1 Pulse field gel electrophoresis (PFGE)

The relationship of field isolates was determined using an adapted method from the PulseNet protocol using the *S. Braenderup* (H9812) control (Ribot *et al.*, 2006). Bacteria were swabbed from an overnight culture on LB-G + 4 µg/ml cefotaxime agar and re-suspended in 2 ml suspension buffer (100mM Tris and 100mM EDTA) to an OD 610 of between 3.9 and 4.5 using a DENSIMAT (biomerieux). To an 200 µl aliquot of the cell suspension, a 10 µl of 20 mg/ml proteinase K (Ambion) was added. This was followed by the addition of 200 µl of molten SeaKem agarose (1% SeaKem agarose (Lonza), 1% SDS in TE buffer), and was cast into plug molds. The plugs were digested in 5 ml lysis buffer (50mM Tris, 50mM EDTA, 1% Sarcosyl) with 25 µl proteinase K (20 mg/ml) and incubated at 54°C at 175 rpm for 2 hours. The lysis buffer was discarded and the plugs washed with 15ml H₂O at 50°C for 15 minutes (x2), and then washed with 15ml TE buffer at 50°C for 15 minutes (x4). The plugs were cut into approximately 3 mm slices using a sterile scalpel, and digested with 200 µl restriction digest buffer (20 µl enzyme buffer (D), 5 µl *Xba*I (Promega), 2 µl BSA and 173 µl H₂O) and incubated at 37°C for 2 hours. The digestion was stopped by adding TE buffer (100 µl), plugs were run on 150 ml 1% SeaKem agarose made in 0.5x TBE buffer (BIORAD), in 2.2L of 0.5x TBE buffer with 50 µM thiourea. PFGE was run using CHEF-DR III variable angle system (BIORAD) and the conditions; initial switch time 2.2secs, final switch time 54.2secs, volts/cm 6, included angle 120° run for 19.5 hours at 14°C. Gels were stained in 1 µg/ml ethidium bromide in 1x TBE (BPU) and visualised using the GeneSnap imaging system (Syngene). Gels were analysed using Dice coefficient in Bionumerics v5.10 (Applied Maths).

2.3.2 DNA microarray

2.3.2.1 Labelling the probes

The DNA probes were labelled by PCR before being used in hybridization of the array using the following PCR reaction. PCR reactions were carried out in a volume of 10 μ l, which included 1 μ l dNTP mix (1mM dACG; 0.65mM dTTP (Promega)), 1 μ l 10x Therminator buffer (NEB), 0.1 μ l Therminator DNA polymerase (2U/ μ l (NEB M0261L)), 0.35 μ l Biotin-16-dUTP (Roche), 1 μ l of virulence and antimicrobial resistance primer mix (120 virulence genes and 153 antimicrobial resistance genes, Identibac, Alere) and 6.55 μ l template DNA. PCR conditions were 96°C for 5 minutes, then 62°C for 20secs, 72°C for 40secs, 96°C for 60secs for 40 cycles.

2.3.2.2 Array hybridization

Using the labelled DNA probes from the PCR reaction (10 μ l aliquot) (2.3.2.1), 90 μ l hybridization buffer:3DNA +0.5% BSA was added and incubated at 95°C for 5 minutes. The array was prepared by adding 250 μ l Hybridization buffer:3DNA + 0.5% BSA + 10% glycerol and incubated at 55°C for 5 minutes at 550 rpm, this was discarded and the DNA probes (100 μ l aliquot) were added to the array and incubated at 55°C for 60 minutes at 550 rpm. The DNA probes were discarded and array washed with 250 μ l 2xSCC (300 mM NaCl and 30 mM trisodium citrate), 0.01% Triton and incubated at 40°C for 5 minutes at 550 rpm, discarded and array washed with 250 μ l 2xSCC incubated at 40°C for 5 minutes at 550 rpm, discard and array wash with 250 μ l 0.2xSCC (30 mM NaCl and 3 mM trisodium citrate) and incubated at 30°C for 5 minutes at 550 rpm. The wash was discarded and to each tube 100 μ l Poly-HRP Streptavidin and blocking solution was added and incubated at 30°C for 15 minutes at 550 rpm. Streptavidin blocking solution was prepared by adding 5 μ l streptavidin to 495 μ l 1M PBS, 20 μ l of this is added to 80 μ l blocking reagent (6xSSPE (900 mM NaCl, 60 mM sodium phosphate and 6 mM EDTA) 0.05% Tween 20 + 1% Roche blocking powder). The streptavidin was discarded and the

array tube washed with 250 µl 2xSCC, 0.01% Triton and incubated at 30°C for 5 minutes at 550 rpm, discarded and 250 µl 2xSCC added and incubated at 20°C for 5 minutes at 550 rpm, discarded and 250 µl 0.2xSCC added and incubated at 20°C for 5 minutes at 550 rpm. The wash was discarded and 100 µl seramun green was added and incubated at room temperature for 10 minutes and then discarded. Images of the array strips were taken using the array imager from Clondiag and results analysed using Gene Spring (Agilent Technologies).

2.3.3 Multi locus sequence typing (MLST)

The MLSTs of field isolates were determined using an adapted protocol from the MLST database at ERI, University College Cork (http://mlst.ucc.ie/mlst/dbs/Ecoli/documents/primersColi_html) (Wirth *et al.*, 2006). PCR reactions were carried out in 25 µl, with 12.5 µl HotStarTaq Master Mix (QIAGEN), 10 pmol of primers, 2 µl of boiled DNA made up to 25 µl with H₂O. Conditions were 15 min at 95°C and 30 cycles of 94°C for 1 min, annealing of 54°C for *fumC* and *icd*, 56°C for *adk*, 60°C for *gyrB* for 1 min, extension at 72°C for 2 min. Other reactions were carried out in 50 µl volumes. Conditions were 15 min at 95°C, 30 cycles of 94°C for 1 min, annealing of 64°C for *mdh*, and 56°C for *purA* and *RecA* for 1 min, extension at 72°C for 1 min. PCR products were sequenced and analysed using the MLST website <http://mlst.ucc.ie/mlst/dbs/Ecoli/> (Wirth *et al.*, 2006).

2.3.4 *E. coli* phylogenetic group PCR

The phylogroup of *E. coli* was determined by PCR using the primers described by Clermont *et al* (2000). Standard PCR reaction reagents and the primers in Table 2.2, with the conditions of 94°C for 30secs, 55°C for 30secs, 72°C for 30secs for 30 cycles, products were resolved by gel electrophoresis. Relevant controls were supplied by the HPA.

Table 2.2 Primers for the *E. coli* phylogenetic group

Primer	Sequence (5'-3')	Amplicon (bp)
ChuA.1	GACGAACCAACGGTCAGGAT	279
ChuA.2	TGCCGCCAGTACCAAAGACA	
YjaA.1	TGAAGTGTCAGGAGACGCTG	211
YjaA.2	ATGGAGAATGCGTTCCTCAAC	
TspE4C2.1	GAGTAATGTCGGGGCATTCA	152
TspE4C2.2	CGCGCCAACAAAGTATTACG	

Primers from Clermont *et al* (2000)

2.3.5 CTX-M typing

Typing of the *bla*_{CTX-M-9} group was performed by PCR amplification of the gene using the primers CTX-M-9 FW ATGGTGACAAAGAGAGTGCAAC and CTX-M-9 RV TTACAGCCCTTCGGCGATG using the conditions denaturing 94°C for 30s, annealing 60°C for 30s and extension for 72°C for 30 cycles (Batchelor *et al.*, 2005). Amplicons were resolved by gel electrophoresis before being sent for sequencing, sequences were compared to those in GenBank, to determine the *bla*_{CTX-M}.

2.3.6 Replicon typing

2.3.6.1 Plasmid-based replicon typing (PBRT)

PBRT was carried out for the replicon types IncK, B/O and IncI γ using the method set out by Carattoli *et al* (2005a). Standard PCR reaction reagents and the primers in Table 2.3, with the conditions of 94°C for 60secs, 60°C for 30secs, 72°C for 60secs for 30 cycles, products resolved by gel electrophoresis. Known controls were used and amplicons were resolved by gel electrophoresis. Shaded in grey are the combination of primers used for pCT-like plasmids (Stokes *et al.*, 2012).

Table 2.3 Replicon primers and conditions

Primer	Sequence (5'-3')	Amplicon size (bp)
IncK/B FW	GCGGTCCGGAAAGCCAGAAAAC	160
IncK RV	TCTTTCACGAGCCCGCCAAA	
IncKMSFW	CAGGATCCGGGAAGTCAGAAAAC	165
IncK RV	TCTTTCACGAGCCCGCCAAA	
IncB/O RV	TCTGCGTTCGCCAAGTTCGA	159
IncI1γ FW	CGAAAGCCGGACGGCAGAA	139
IncI1γ RV	TCGTCGTTCGCCAAGTTCGT	

Carattoli *et al* (2005a)

2.3.6.2 Commercial kit

Replicon typing was performed using the commercial PCR based replicon typing kit (DIATHEVA) following the manufacturers protocol. In brief 8 separate multiplex reactions were performed using separate primer mixes M1 (HI1, HI2, I1), M2 (L/M, N, I2, BO), M3 (FIB, FIA, W), M4 (P, FIC), M5 (T, A/C, FIIS), M6 (U, X1, R, FIIK), M7 (Y, X2, K) and M8 (HIB, FIB and FII) using the positive controls supplied. PCR reactions were carried out as follows 23.8 µl of primer mix, 1 µl DNA and 0.2 µl DNA polymerase, using the conditions 95°C for 10 minutes, 95°C for 60sec, 60°C for 30sec, 72°C for 60sec for 30 cycles and a final extension at 72°C for 5 minutes.

2.3.7 Plasmid molecular markers and *bla*_{CTX-M} environment for pCT

2.3.7.1 pCT molecular markers

The presence of pCT like plasmids was tested by PCR using the pCT molecular marker primers by Cottell *et al* (2011) in Table 2.4, using standard PCR reagents and the conditions shown (Cottell *et al.*, 2011). Transformed pCT in *E. coli* DH5α was used as positive control and *S. Typhimurium* 26R used as the negative control in PCR reactions; amplicons were resolved by gel electrophoresis.

Table 2.4 pCT molecular marker primers

Primer	Sequence (5'-3')	Denaturing (°C/secs)	Annealing (°C/secs)	Extension (°C/secs)	Cycles	Amplicon size (bp)
Sigma FW	ACAGCGTCTTCTCGTATCCA					1289
Sigma RV	GTTCTTCCAGCTGACGTAAC					
pCT Rci FW	AAGGTCATCTGCAGGAGT	94/120	51/120	72/120	30	945
pCT Rci RV	GTGTGCGCAGCAACAATA					
pilN FW	GACAGGCAGAGAACACCAGA					627
pilN RV	ATGCTGTTCCACCTGATGAG					
pCT 008 FW	CATTGTATCTATCTTGTGGG	94/30	53/30	72/30	30	428
pCT 009 RV	GCATTCCAGAAGATGACGTT					

2.3.7.2 *nikB* sequencing

The *nikB* gene was amplified by PCR using the primers *nikB* FW 5'-CGTGCMTGCCGTGARCTT-3' and *nikB* RV 5'-TCCCAGCCATCCWTCACC-3' using the conditions 94°C for 60sec, 53°C for 30sec, and 72°C for 60sec for 30 cycles (Cottell *et al.*, 2011). The amplicons were sequenced and sequences compared using Lasergene (DNASTAR).

2.3.7.3 *bla*_{CTX-M-14} environment PCR

The environment of the *bla*_{CTX-M-14} in pCT plasmids was investigated by three PCR's in Table 2.5, with the conditions shown. Transformed pCT in *E. coli* DH5 α was used as a positive control and *S. Typhimurium* 26R used as the negative control in PCR reactions, amplicons resolved by gel electrophoresis.

2.3.8 Plasmid molecular markers

All of the plasmid molecular markers designed in this study were harmonised to run under the same PCR conditions as multiplex reactions, relevant controls were used for all PCR's. PCR molecular marker conditions were 25 μ l reactions using 2x HotStar master mix (QIAGEN) 1-2 μ l of DNA from crude lysates, 10pmol of each primer made up to 25 μ l with H₂O. PCR conditions were 95°C for 15 minutes, followed by 94°C for 60secs, 57.5°C for 30secs, 72°C for 120secs for 25 cycles and final extension of 72°C for 10 minutes. Primers for pSAM7 are shown in Table 2.6 (Stokes *et al.*, 2013), for pH19 in Table 2.7, for Incl1 γ in Table 2.8 and pCH01 in Table 2.9. Transformed pSAM7, pCH01, pCH02, pCH03, pCT01, pT01 and pH19 in *E. coli* D10B were used as positive controls and *E. coli* DH10B and/or *S. Typhimurium* 26R used as the negative control in PCR reactions; amplicons were resolved by gel electrophoresis.

Table 2.5 Primers for the *bla*_{CTX-M-14} environment of pCT

Primer	Sequence (5'-3')	Denaturing (°C/secs)	Annealing (°C/secs)	Extension (°C/secs)	Cycles	Amplicon size (bp)	References
ISEcp1 FW	GCAGGTCTTTTCTGCTCC					527	Karim <i>et al</i> (2001)
ISEcp1 RV	ATTTCGGAGCACCGTTTGC	94/60	51/30	72/90	30		
CTX-M-9 FW	ATGGTGACAAAGAGAGTGCAAC					1288	Batchelor <i>et al</i> (2005), Cottell <i>et al</i> (2011)
Pseudo RV	AACATTCGGCCGTTACAGC						
CTX-M-9 RV	TTACAGCCCTTCGGCGATG	94/120	51/120	72/180	30	2169	Batchelor <i>et al</i> (2005), Cottell <i>et al</i> (2011)
ISEcp1Up FW	CAAATTGATCCCCTCGTC						

Table 2.6 pSAM7 molecular markers

Primer	Sequence (5'-3')	Multiplex	Amplicon (bp)
Rpilx5 FW	CTTAGTTCATTTGTGAATGCC		1060
Rplix5 RV	GAAAGTGTTGATGCTGTGAT		
Rpir FW	CAGTGTGGATTTTGAGCAT	Multiplex	774
Rpir RV	GCCCCTATTGTATAAAGATTCA	1	
RhicA FW	CCAGTTTTCCATACAGGACA		350
RhicA RV	GTTGCATATCTATAGGGGATG		
Hyex FW	CAAAGGGAGGGTGTGAAT		841
Hyex RV	GGAATGGCGATACAAACA	Multiplex	
Smet FW	CGATGGCCTTAAGACCTT	2	471
Smet RV	CGGACACGGTATTTGTTG		

Table 2.7 pH19 molecular markers

Primer	Sequence (5'-3')	Multiplex	Amplicon (bp)
mYqik FW	GTAAAACCATCAGCCGAAG		1207
mYqik RV	CCTGTTCAGCGAGATTTTC		
mHyp-Hyp FW	CCTTTAATGTCTCTTCCGGT	Multiplex	745
mHyp-Hyp RV	GTATTCAATGAGCGGCAG	1	
mArdA FW	GCTGTATACGTTGGAACCTG		463
mArdA RV	CGTCAATAAAGGTGAAGGAG		
mH19 FW	GGACATGAAAGACCAACGTA		846
mH19 RV	CTCTTCACCATCTGTTTCGAG		
mYdeA FW	GAGAGTGCTGATTGTGCTG	Multiplex	551
mYdeA RV	GGACGCTACTTTTCATTTATCTG	2	
mH90 FW	GTATTAGTAGCCCGGAAACAA		269
mH90 RV	GACTTTATTTATTATGGGGAGCT		

Table 2.8 Inc11y molecular markers

Primer	Sequence (5'-3')	Multiplex	Amplicon (bp)
mTnp-Tnp FW	CATTTTCAGCGTGACATC		1180
mTnp-Tnp RV	GCCAATCTCAGTTGAAATAGT		
mISORF FW	CACATCAATGATGAACGC	Multiplex	740
mISORF RV	CGTATGGTTCTGGAAAGTC	1	
mShuf FW	GCAGACCTACAACATAGCTAAA		406
mShuf RV	CTCAGCATGTAATTGGTACAA		
mRetron FW	CTGCCTGATGAACTTGAGA		1316
mRetron RV	GCGTTGAAAGTCAGAACAA		
mYgaA FW	CAATATCAGGTTCCCGTG	Multiplex	576
mYgaA RV	CCGGAGAATATCTGTGATATC	2	
mPIN FW	CTCTATGCCGCACCTAAT		316
mPIN RV	CGTCTTGCTCACCTTGAG		
mCib FW	CAGCAGAAAGAGAATGAGAATA		1414
mCib RV	CCTTTTGTA ACTCCACAGC		
mYbcA FW	GAAGCATTTCAGTACGCC	Multiplex	827
mYbcA RV	CCTGCAGGGAATTGTAAT	3	
mTraD FW	CCACTACTCTGGATGACGA		492
mTraD RV	GTCTCCGGTGAATCCATT		

Table 2.9 pCH01 molecular markers

Primer	Sequence (5'-3')	Multiplex	Amplicon (bp)
mTnIA FW	CCTTGATTCACATGGTGT		1142
mTnIA RV	GTTTCCCATCAAGCTTGA		
mGroEL FW	CAAGGTCGGCAAGGAAG	Multiplex	680
mGroEL RV	CTTCTTTTCCTTCATCTCG	1	
mACH2 FW	CTATGCCGGTAAACAGCA		352
mACH2 RV	CGTCCATTTATCCCCCTT		

Primer	Sequence (5'-3')	Multiplex	Amplicon (bp)
mAERO FW	CCAATAACCCATTAAGTCAATC		905
mAERO RV	GCTAGTTAACTGAGCGACAGA		
mSens FW	CAACCAATTAAGACGTCG	Multiplex 2	649
mSens RV	GTAAGGCCTTTACGTAATTCA		
mACH3 FW	GATCAAAAGCAAGAACCGT		210
mACH3 RV	CTAAATAGGTCTCCAGGTGATG		
mHQ FW	CGAGAGCTTCCCAAAGAG		1151
mHQ RV	CGAAGGTAGAACTGCGCT		
mTraB FW	CCAGAAGAACATGGAATTG	Multiplex 3	706
mTraB RV	GTCAAAGGCTTCGGAAAC		
mACH1 FW	GAGTGATTTATACGAACCGC		427
mACH1 RV	CAGGACAGCTTCTTCAAGAG		

2.3.9 pSAM7 *bla*_{CTX-M-14} environment PCR

Standard PCR reaction reagents was used, using the primers by Stokes *et al* (2013) in Table 2.10 with the conditions of 94°C for 60secs, 56.5°C for 30secs, 72°C for 60secs for 30 cycles, products resolved by gel electrophoresis (Stokes *et al.*, 2013). Transformed pSAM7 in *E. coli* DH10B was used as a positive control and *E. coli* DH10B and *S. Typhimurium* 26R used as negative controls in PCR reactions; amplicons were resolved by gel electrophoresis.

Table 2.10 Primers for the *bla*_{CTX-M-14} environment of pSAM7

Primer	Sequence (5'-3')	Amplicon (bp)
ISEC	GAAAAGCGTGGTAATGCT	739
CTXISEC	GCACCTGCGTATTATCTGC	
CTXSMETH	GTCGTGGACTGTAGGTGATA	767
Smet RV	CGGACACGGTATTTGTTG	
ISECHIC	GCAAATTGGATATTGTAGCA	1098
RhicA FW	CCAGTTTTCCATACAGGACA	

2.3.10 pH19 *bla*_{CTX-M-14} genetic environment

Standard PCR reaction reagents and the primers in Table 2.11, with the conditions of 94°C for 60secs, 57°C for 30secs, 72°C for 60secs for 30 cycles, products resolved by gel electrophoresis. Transformed pH19 in *E. coli* DH10B was used as a positive control and *E. coli* DH10B as a negative control; amplicons were resolved by gel electrophoresis.

Table 2.11 Primers for the *bla*_{CTX-M-14} environment of pH19

Primer	Sequene (5'-3')	Amplicon (bp)
H19YB2ISEC	CAGCCACAGAAATCACAGC	321
H19ISP2YB	CATGTCGTATTTGGCTTCTTT	
H19ISP2CTX	GGGTCATCTCTTGCTAAAGTCA	683
CTXISEC	GCACCTGCGTATTATCTGC	
H19IS92CTX	CGTTTAATGACCAGCACAGTC	509
H19CTX2IS9	GCTCAAAGGCAATACGACC	
H19ISP22IS9	GTGCTGCGGAAATGATCA	1018
H19IS92ISP2	CATTAAACGCGTATTCAGGC	

2.3.11 Restriction fragment length polymorphism (RFLP)

Using plasmid DNA extracted by the Takahashi and Nagano method (2.2.4.2), 4.5 µg of plasmid DNA was digested for 1 hour 30 minutes at 37°C in a 30 µl reaction with 3 µl 10x buffer H, 0.3 µl BSA and 2 µl of *Pst*I (10 U/µl), followed by inactivation at 65°C for 15 minutes. *Eco*RI digestion were carried out as with *Pst*I, using *Eco*RI (12 U/µl) instead and 10x buffer H and inactivated as with *Pst*I (Ma *et al.*, 2002). Transformed pCT in *E. coli* DH5α was used as a positive control and *S. Typhimurium* 26R used as the negative control. DNA restriction fragment profiles were resolved by gel electrophoresis, run on a 0.8% gel at 36V for 20 hours, stained and visualised. Gels were analysed using Dice coefficient in Bionumerics v5.10 (Applied Maths).

2.3.12 Plasmid size and content

2.3.12.1 S1 Nuclease PFGE

Plasmid sizing was carried out using an adapted method of Barton, Harding and Zuccarelli (1995). Bacterial colonies were taken from a previously inoculated LB-G agar plate incubated at 37°C, and re-suspended in 1 ml EC buffer (1M NaCl, 100mM EDTA, 6mM Tris HCl, 0.5% Brij 58, 0.2% deoxycholate, 0.5% N-lauroylsarcosine). Aliquots (500 µl) of the suspended cells were added to 500 µl of molten 1% SeaKem Gold agarose (Lonza) in EC buffer, pipetted into a mold and cooled. Plugs were incubated for 1 hour at 37°C in 1 ml EC buffer with 40 µg/ml RNase and 2 mg/ml lysozyme. Plugs were incubated overnight at 50°C in 150 µl 0.5M EDTA 1% N-lauroylsarcosine 1mg/ml proteinase K. Plugs were treated for 2 hours with 1mM phenylmethylsulfonyl fluoride in TE buffer (10mM Tris, 1mM EDTA) (1 ml) at 37°C (x2), plugs were then washed in 1 ml TE for 1 hour at 37°C (x2). Plugs were cut into 3 mm slices and washed with 1 ml 10mM Tris, and digested with 200 µl of S1 nuclease 1U, 50mM NaCl, 30mM NAOAc, 5mM ZnSO₄ for 45 minutes at 37°C, the digestion was stopped by adding 100 µl ES buffer (0.5M EDTA, 1% N-lauroylsarcosine). Plugs were run on a 1.2% agarose gel in 0.5x TBE, using a pulse field gel electrophoresis CHEF-DR III variable angle system (BIORAD) initial switch time of 45secs for 14 hours, and a final switch of time 25secs for 6 hours at 210V (6.3V/cm). DNA markers were used to determine the size of the plasmid, Lambda Ladder PFGE marker 45.5-1018.5 kb (New England Biolabs (NEB) N03040S), Low range PFGE marker 2.03-194.0 kb (NEB N0350S) and MidRange I PFGE marker 15-291 kb (NEB N3551S).

2.3.12.2 Plasmid Profiling

Plasmid content was examined using DNA extracted by phenol-chloroform (2.2.4.1), Hi-speed Maxi kit (2.2.4.3) and large construct kit (2.2.4.4) using an adapted method of Kado and Liu (1981) (Kado and Liu, 1981). *E. coli* K12 (AHVLA) or

S. Typhimurium 26R was used as a marker for the chromosomal band. *E. coli* 39R (AHVLA) which contains plasmids of 148,000 bp, 63,400 bp, 36,000 bp and 6,900 bp in size and supercoiled DNA Ladder (2-16 kb) (Sigma-Aldrich D5292) were used to estimate plasmid sizes. DNA was resolved by gel electrophoresis on a 0.8% 1x TBE gel run at 150V for 5 hours 30 minutes at 19°C before being stained and visualised.

2.4 Sequencing

2.4.1 Next generation sequencing (NGS) - Roche 454 GS-FLX sequencing

Plasmid DNA extracted using the Large construct kit (QIAGEN) as described was used for sequencing on the 454 GS-FLX (Roche). Next generation sequencing was conducted at the AHVLA central sequencing unit. A shotgun library was prepared from 500 ng of plasmid DNA using the 454 Rapid Library kit (Roche) following the manufacturers protocol. In brief the plasmid DNA was fragmented, and the ends were enzymatically repaired. This was followed by the ligation of adapter sequences. The strands selected based on size and then separated into single stranded DNA. The amplification is known as emulsion-based clonal amplification (emPCR) and involves the immobilization of the DNA library onto capture beads, the beads and DNA are immersed in emulsion oil and the PCR reagents were added to form "Micro-reactors" in which PCR extends the DNA. After the PCR reaction micro reactors were broken open and added to the PicoTiterPlate (Roche) which contains wells with beads and PCR reagents, each dNTP was added separately and light signal recorded. DNA reads were assembled *de novo* using Newbler v2.3 software (Roche) to form contigs.

2.4.2 Chain termination sequencing - ABI sequencing

Sequencing of PCR products was performed by the AHVLA sequencing unit, using the ABI 3730 and chain termination sequencing. In brief the PCR reaction was run on the PCR product incorporating dideoxynucleotides (ddATP, ddGTP, ddCTP and ddTTP) each labelled with a different fluorescent dye, incorporation results in the termination of

extension. The fragments from the PCR reaction are resolved by capillary electrophoresis and the fluorescent signal recorded. DNA sequences were further analysed using SeqMan (DNASTAR).

2.4.3 Plasmid closure

To fully assemble plasmid contigs generated by NGS, PCR was used to close the sequence gaps between contigs. Both standard and long range PCR were used to close the gap.

2.4.3.1 Short range PCR

Short range PCR were carried out as standard PCR reactions using the conditions 94°C for 60secs, 57.5-62°C for 30secs, 72°C for 240secs for 25 cycles and the primers used are shown in Table 2.12

2.4.3.2 Long range PCR

Long range PCR was carried out using Elongase (Invitrogen 10480-010), carried out in 36 µl reaction volumes consisting of 1 µl 10mM dNTPs, 1 µl DNA solution, 2 µl 10pmol primers 4 µl buffer A, 6 µl buffer B (Invitrogen), 2 µl Elongase made up to 36 µl . Conditions are listed in the table for each primer pair, PCR consisted of an initial denaturing at 94°C for 30secs, followed by 94°C for 30secs, 57.5-62°C for 30secs, 68°C for 900secs for 30 cycles and with a final extension at 68°C for 420secs. The primers used are shown in Table 2.12.

Table 2.12 Primers used in plasmid gap closure

Primer	Sequence (5'-3')	Denaturing °C/secs	Annealing °C/secs	Extension °C/secs	Cycles	Short or Long range PCR
CH01BFG1	GCTAAATCGTCAGAATAGAGTT	94/30	57.5/30	68/900	30	Long
CH01BRG1	GTCAGGATAGGATTGAATTT					
CH01CF	GATGTCACGCTGAAAATGCC	94/60	58/30	72/240	25	Short
CH01BR	CCATGTGTAAATTGCGTCAGG					
CH01BF	CCCTAACTTGGCGTCAGACCAT	94/30	57.5/30	68/900	30	Long
CH01AR	GATAAAAATCGACAGTGCGG					
CH02AF	GGGTGAGCCATTGAGGTAAGTC	94/60	58/30	72/240	25	Short
CH02DR	CCTGGCAGTAGTGGGCATC					
CH02BF	CGTTTTCCAGTTGGACAAGTAGC	94/60	61.5/30	72/240	25	Short
CH02AR	CGGACATTGAATCGGCTTGTA					
CH02BFG1	GACAAGTAGCTGTTGCTC	94/30	57.5/30	68/900	30	Long
CH02BRG1	CTTACAAGTGAAGAACTG					
CH03AFG1	GATTTTGGTCATCTCAATAA	94/30	53.5/30	68/900	30	Long
CH03ARG1	CAACAAGATCCGTTTATTT					
pCT01 EF	CCAAAGGGTGAGCCATTCA	94/60	60/30	72/240	25	Short
pCT01 FR	GCCCTTTCTTGTGAACAGAA					
pCT01 FF	GGTACTGTTCCGGGCTGATGG	94/60	62/30	68/900	30	Long
pCT01 ER	CTGGCAGTAGTGGGCATC					
pCT01FEEFG1	CAGGAAAGCGCCAACCTGTG	94/60	61.5/30	72/240	25	Short
pCT01FEFRG1	CCGGTACGTGGAGGACTTTAG					

Primer	Sequence (5'-3')	Denaturing °C/secs	Annealing °C/secs	Extension °C/secs	Cycles	Short or Long range PCR
CT1Con F	CAGATCCTCCATTACCTCC	94/60	57/30	72/240	25	Short
CT1Con R	GTGTAAACAACAAGGGCAT					
pT01 CR	CAGGTACTTATGGAGTGCG	94/60	58/30	72/240	25	Short
pT01 BF	CGTATTTGTACCATTTGCGT					
pT01 CF	GATGGTCGCCTTTATACTGGTGA	94/60	58/30	72/240	25	Short
pT01 AR	GTGAGGTGCATATTCCGC					
pT01 AF	CAAAGGGTGAGCCATTCA	94/60	58/30	72/240	25	Short
pT01BR	CCATTACCTCCAGATGCATA					
pH19-F	GTTCCCGCTGTGGACTTT	94/60	62/30	72/240	25	Short
pH19-R	GTATGCAGGATGCGACCG					
pSAM7 FW	GCACGCATTA AAAAGCCTTAT	94/60	59/30	72/240	25	Short
pSAM7 RV	GGCAGATTAACAACAGATTCAA					
pSAM7-2 FW	GAGTGGGGATCAAGTTTACG	94/60	59/30	72/240	25	Short
pSAM7-2 RV	CTTCCGTATGTTTCATGATTC					

2.5 Bioinformatics

2.5.1 Annotation

Fully assembled and closed plasmids were annotated using several methods to maximise the coverage. Annotated files and GenBank files were created in both Sequin (NCBI) and Artemis.

2.5.1.1 RAST

Plasmids were annotated initially using the Rapid Annotation using Subsystem Technology (RAST) <http://rast.nmpdr.org/>. RAST identifies both protein encoding genes, rRNA genes and tRNA genes, predicts their functions and assigns them to subsystems using a library of known genes and subsystems. The output is visualised in the SEED environment allowing the annotation and subsystems to be visualised and exported as an excel file for further analysis and comparison (Aziz *et al.*, 2008).

2.5.1.2 Artemis

Further annotation of the plasmids was carried out using Artemis: Genome Browser and Comparison available from Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk/resources/software/artemis/>) (Rutherford *et al.*, 2000; Carver *et al.*, 2008). Open reading frame (ORF) are determined in all six frames with predicted proteins restricted to ≥ 50 amino acids in length and can be compared directly using the NCBI database GenBank <http://www.ncbi.nlm.nih.gov/genbank/>. ORF are predicted by identifying the start and stop codons and their respective promoters.

2.5.1.3 BLASTn

Initial BLASTn searches were conducted of the assembled plasmids to identify related plasmids using the BLASTn megablast algorithm

(http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome)

(Altschul *et al.*, 1990; Zhang *et al.*, 2000). The annotation from the GenBank plasmids were used to create a database containing each ORF and its corresponding DNA sequence. The database was created in a FAS (.fas) format which was compiled in Notepad, Excel and Word, with the format shown below.

```
>ORF(#1)
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>ORF(#2)
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
>ORF(#3)
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
```

The database was created using the command line function below using the BLAST-2.2.25+.exe executable file.

```
makeblastdb -in Name of database.fas -parse_seqids -dbtype nucl
```

The database was used in a standalone format using the BLAST-2.2.25+.exe (<http://www.ncbi.nlm.nih.gov/books/NBK1762/>) run through the command line, using the BLAST algorithm to compare each ORF to the sequenced plasmids in this study. BLAST identifies homologous sequences in a heuristic methods, finding matches between two short sequences, which is followed by local alignments to compare base pair matching. The command line used is shown below. Highlighted are the requested output information, which is summarised below.

```
blastn.exe -task dc-megablast -query Name of queried plasmid.fas -db Name of Database.fas -outfmt "6 qacc sacc pident positive ppos length mismatch gapopen qstart qend sstart send qseq sseq evalue bitscore" -out Name of Output.txt
```

qacc = Query name	sacc = Sequence name
pident = Percentage of identical matches	positive = Number of positive scoring matches
ppos = Percentage of positive scoring	length = Alignment length
mismatch = Number of mismatch	gapopen = Number of gap openings
qstart = Query start	qend = Query end
sstart = Subject start	send = Subject end
qseq = Query sequence	sseq = Subject sequence
evalue = Expect value	bitscore = Bit score

The output .txt file can be opened in Excel, and provides the following information such as start and stop position, percentage identity, number of mismatches and sequence of the query. Each ORF sequence and translated protein was searched and checked with BLAST.

2.5.1.4 Insertion sequences

Insertion sequences were identified using the IS finder website (<https://www-is.biotoul.fr/>) a database of insertion sequences and transposons and their respective repeats (Siguier *et al.*, 2006).

2.5.2 Sequence comparisons

2.5.2.1 MAUVE

Multiple plasmid alignments were conducted using Mauve 2.3.1 (<http://asap.ahabs.wisc.edu/software/>) using ProgressiveMauve (Darling, Mau and Perna, 2010). Mauve uses multiple local alignments as anchors by a seed and extended hashing method, followed by pairwise alignment without gaps, this penalizes the alignment anchoring in repetitive regions providing an accurate and rapid alignment of sequences. Mauve was used to identify common and uncommon regions shared between plasmids which can be visualised through the Mauve window.

2.5.2.2 Artemis Comparison Tool (ACT)

Plasmids sequences were compared using ACT (Sanger institute), and WebAct (<http://www.webact.org/WebACT/home>) was used to generate the pairwise comparison files (Abbott *et al.*, 2005; Abbott, Aanensen and Bentley, 2007). Comparison files were opened and analysed using ACT (<http://www.sanger.ac.uk/resources/software/act/>) to visualise the comparison of plasmids, images were exported as JPEGs for further annotation (Carver *et al.*, 2005; Carver *et al.*, 2008).

2.5.2.3 BLASTn

Plasmid comparisons were carried out as for the annotation as previously described, with plasmids similar to those sequenced in this study being identified. A database of the ORFs of the sequenced plasmids pH19, pSAM7, pCH01, pCH02, pCH03, pCT01 and pT01 was created as before and used to compare against selected panels of plasmids of their respective types, as performed in 2.5.1.3. Comparison allowed for the presence or absence of ORFs to be identified, their percentage similarity and also the sequence was extracted and analysed in Excel.

2.5.2.4 Additional software

Additional manipulation of sequences and proteins was carried out using several software packages including Lasergene (DNASTAR), Ugene (<http://ugene.unipro.ru/>) (Okonechnikov *et al.*, 2012), and ExPASy (<http://web.expasy.org>)

2.5.2.5 Phylogenetic analysis - MEGA 5.1

The phylogeny of both genes and plasmids (generated from selected genes which have been concatenated). FAS sequence files were aligned using MEGA 5.10 (<http://www.megasoftware.net/>) using the Clustal W algorithm (Tamura *et al.*, 2011). Phylogenetic trees were constructed from the alignment files using neighbour joining with 1000 bootstrap repeats.

2.5.3 *In silico* PCR

2.5.3.1 *In silico* plasmid molecular marker PCR

Plasmid molecular markers were identified through comparisons using BLASTn and Mauve, as described above. Primers designed for the regions to be used as molecular markers (Table 2.6-2.9) and their products were checked against plasmids from GenBank using BLASTn, and Sci Ed central primer design v5.01 (Science and Educational Software). The *in silico* screening of these molecular marker primers was carried out on

respective plasmids using BLASTn, and Sci Ed central primer design v5.01 to predict whether amplicons would be produced, sequences from GenBank plasmids predicted to produce amplicons were extracted for phylogenetic analysis using MEGA 5.10 and the neighbour joining methods with 1000 bootstrap repeats.

2.5.3.2 *In silico* PCR for IncI1 γ pMLST

In silico PCR for IncI1 γ pMLST was performed using BLASTn and Sci Ed central using the primers by Garcia-Fernandez *et al* (2008) shown in Table 2.13 (Garcia-Fernandez *et al.*, 2008). Sequences were analysed using the pMLST website (http://pubmlst.org/perl/bigfdb/bigfdb.pl?db=pubmlst_plasmid_seqdef&page=sequenceQuery) to identify the plasmid ST type (Jolley and Maiden, 2010).

Table 2.13 Primers for IncI1 γ pMLST

Primer	Sequence (5'-3')	Amplicon size (bp)
repII FW	CGAAAGCCGGACGGCAGAA	142
repII RV	TCGTCGTTCCGCCAAGTTCGT	
ardA FW	ATGTCTGTTGTTGCACCTGC	501
ardA RV	TCACCGACGGAACACATGACC	
trbA FW	CGACAAATGCTTCCGGGGT	883
pndC RV	CGAATCCCTCACCATCCAG	
sogS FW	TTCCGGGGCGTAGACAATACT	291
sogS RV	AACAGTGATATGCCGTCGC	
pilL FW	CCATATGACCATCCAGTGCG	316
pilL RV	AACCACTATCTCGCCAGCAG	

2.5.3.3 *In silico* PCR for IncX plasmids

In silico PCR for IncX1-4 plasmid typing was performed using BLASTn and Sci Ed central using the primers by Johnson *et al* (2012a) shown in Table 2.14 (Johnson *et al.*, 2012a).

Table 2.14 Primers for IncX1-4 typing

Primer	Sequence (5'-3')	Amplicon size (bp)
X1 FW	GCTTAGACTTTGTTTTATCGTT	461
X1 RV	TAATGATCCTCAGCATGTGAT	
X2 FW	GCGAAGAAATCAAAGAAGCTA	678
X2 RV	TGTTGAATGCCGTTCTTGTCCAG	
X3 FW	GTTTTCTCCACGCCCTTGTTCA	351
X3 RV	CTTTGTGCTTGGCTATCATAA	
X4 FW	AGCAAACAGGGAAAGGAGAAGACT	569
X4 RV	TACCCCAAATCGTAACCTG	

2.5.3.4 *In silico* PCR for Welch IncA/C primers

In silico PCR for Welch IncA/C plasmid PCR was performed using BLASTn and Sci Ed central using the primers by Welch *et al* (2007) shown in Table 2.15 (Welch *et al.*, 2007).

Table 2.15 Primers for Welch IncA/C PCR

Primer	Sequence (5'-3')	Amplicon size (bp)
R1F	AGCACGATAGCTTGTGAGTTCC	1440
R1R	AGCAGATAAGAAGGCGATGACC	
R2F	CAACCCCTTACCAGCTTTGAAC	1752
R2R	AGCAGATAAGAAGGCGATGACC	
R3F	ATGCCACATGGGTAGACATCAC	1640
R3R	GAATGCATAACGACGAGTTTGG	
R4F	CGTATTTCTCGTCGCTACATGC	1339
R4R	AGTAGCGGAATCGATCCAGAAG	
R5F	GAACGTGCTTGATGGTTTCTTG	1764
R5R	CTGCTCCACATGATCTACTGGG	
R6F	GGACGTCATCTAACCCCTGTTC	1868
R6R	AGCAGCTCTACGCCTTTACGTC	
R7F	CAGCACAAACATCTTCCCAGAC	1552
R7R	GGGTAACACCGCCA ACTCTTAC	
R8F	GAAAGCGCAACAACACAAAGAC	1703
R8R	TGACTACTCTTGCCAGCTTTGC	
R9F	GTTCAA ACTCACGCTGCAA AAC	1661
R9R	ATACCGCAGACGGAAAGAGAAG	
R10F	AGAATAGCCGCGTCATAGAAG	1537
R10R	AAAAGGCGTACCGACAAGAGAG	
R11F	ATCGAGGGAGTGTTCTCTGAC	1762
R11R	CAAGGCTGAGGGTTCCTATCAC	
R12F	TGCTCCAGAAAAGCAGAGTCAC	1601
R12R	CCGGGACAAATTACAGGAGAA	

Chapter 3

The Identification of pCT-like Plasmids in Unrelated *E. coli* Isolates from Humans, Turkeys and Cattle in England and Wales Using Molecular Markers

3.1 Introduction

The *bla*_{CTX-M-14} gene was first reported in clinical isolates from Asia in 2001, having been found in *E. coli* and *Klebsiella pneumoniae* in China and Korea, and additionally in *Shigella sonnei* in Korea (Chanawong *et al.*, 2002; Pai *et al.*, 2001). The *bla*_{CTX-M-14} gene differs from the *bla*_{CTX-M-9} by 4 nucleotides leading to an amino acid substitution from alanine to valine at 231 (Chanawong *et al.*, 2002; Pai *et al.*, 2001). Since being discovered in Asia, *bla*_{CTX-M-14} has become one of the most dominant CTX-M types, being found all over the world, particularly in western Europe and Asia (Canton and Coque, 2006; Livermore *et al.*, 2007; Hawkey and Jones, 2009). The *bla*_{CTX-M-14} gene is not the most prevalent CTX-M in clinical isolates in the UK, with *bla*_{CTX-M-15} being the most dominant gene in communities and hospitals (Lau *et al.*, 2008; Woodford *et al.*, 2004; Livermore and Hawkey, 2005; Tarrant, MacGowan and Walsh, 2007). However *bla*_{CTX-M-14} has been identified in animals in the UK (Randall *et al.*, 2011; Horton *et al.*, 2011; Teale *et al.*, 2005), and in human and animal isolates in Europe and Asia (Blanc *et al.*, 2006; Bou *et al.*, 2002; Brinas *et al.*, 2003; Dutour *et al.*, 2002; Kim *et al.*, 2011). The *bla*_{CTX-M-14} gene has been associated with several plasmid replicon types including IncK, FII, FIB, I1 γ , HI2, B and A/C (Carattoli, 2009; Carattoli, 2011).

The first *bla*_{CTX-M} gene reported in humans in the UK was the group 9 CTX-M gene *bla*_{CTX-M-9}, isolated from *Klebsiella oxytoca* from a child's stool sample in 2000 (Alobwede *et al.*, 2003). Several years later in 2005 the first report of *bla*_{CTX-M} in UK animals was published, with the group 9 CTX-M gene, *bla*_{CTX-M-14}, being isolated from calves with scouring like disease in Wales (Teale *et al.*, 2005). A longitudinal study of this farm over a 8 month period found the proportion of CTX-M isolates to increase from 64.6 to 92.7% in calves between visit 1 and 3, and from 3.3 to 23.8% in milking cows over the same time period, visit 3 also identified environmental CTX-M isolates (Liebana *et al.*, 2006). The *bla*_{CTX-M-14} gene was found to be carried on a transmissible 65 MDa IncK plasmid which was found to move between *E. coli* isolates as determined by PFGE (Liebana *et al.*, 2006).

The association of *bla*_{CTX-M-14} with the IncK replicon type has been observed in humans in Spain, Portugal, Australia and France and in calves in the Netherlands (Valverde *et al.*, 2009; Machado *et al.*, 2013; Marcade *et al.*, 2009; Zong *et al.*, 2008; Hordijk *et al.*, 2013). In Spain these IncK plasmids, termed pRYC105, were found to be distributed evenly among A, B1 and D phylogroups, which may have a role in their dissemination (Valverde *et al.*, 2009).

The plasmid involved in the dissemination on the UK cattle farm was fully sequenced by Cottell *et al.* (2011) and designated pCT (FN868832) (Cottell *et al.*, 2011). Sequencing of the plasmid revealed that it harbored only one resistance gene which was present downstream of *ISEcp1* and upstream of *IS903D* as previously reported (Poirel, Decousser and Nordmann, 2003; Eckert, Gautier and Arlet, 2006; Cottell *et al.*, 2011). Sequence comparisons found the IncK plasmid to be related to the IncB plasmid pR3521 and IncI γ plasmids (Papagiannitsis *et al.*, 2011; Cottell *et al.*, 2011). No virulence genes were identified, the plasmid had putative RNA polymerase sigma factor gene and it was noted that this plasmid backbone lacked any addiction systems (Cottell *et al.*, 2011). Targets were identified on the pCT backbone which could be used as molecular markers to detect the pCT plasmid. These markers included the genes for *pilN*, sigma factor, shufflon recombinase and a hypothetical gene specific to the pCT plasmid (pCT008-009) (Cottell *et al.*, 2011). Cottell *et al.* (2011) also designed primers to amplify the *nikB* gene for sequence analysis. Early applications of these markers by the author found similar plasmids in human isolates in Asia and Australia, but could only be found in cattle in the UK (Cottell *et al.*, 2011). Further studies of this plasmid have found it to be stable in the bacterial population both with and without antibiotic pressure, or the presence or absence of the *bla*_{CTX-M-14} gene. It was also observed that the presence of this plasmid had no effect on the fitness of the host (Cottell, Webber and Piddock, 2012).

3.1.1 Hypothesis and aims

The hypothesis of this study was that pCT plasmids were widespread in the UK, and were likely to be found in both humans and animals, with possible transmission between hosts. The aims of this study were to (i) demonstrate the benefits of pCT molecular markers in epidemiological studies, (ii) investigate the impact of pCT-like plasmids in the dissemination of the *bla*_{CTX-M-14} genes in the UK, (iii) investigate the prevalence of pCT-like plasmid in human and other animal populations and (iv) observe any clonal relationship and genotypes of pCT-like *E. coli* field isolates.

3.2 Methods and Materials

Bacterial isolates used in this study were recovered from routine surveillance studies by AHVLA, general practice and hospital submissions to public health Wales, and isolates from SFE collection. pCT transformed into *E. coli* DH5 α was supplied by J. Cottell, a complete list of isolates is provided in appendix I. CTX-M-1, -3, -14 and -15 isolates were screened using the pCT molecular markers as described in 2.3.7.1. CTX-M-14 isolates with two or more molecular markers were studied further; this included the isolation of *bla*_{CTX-M-14} plasmid by conjugation as described in 2.1.4.1. Transconjugants were investigated for the presence of a plasmid by plasmid profiling as stated in 2.3.12.2, and *bla*_{CTX-M-9} PCR followed by sequencing as described in 2.3.5. The conjugated plasmids were re-tested for pCT markers (2.3.7.1), *bla*_{CTX-M-14} genetic environment analysed (2.3.7.3), replicon determined using the PBRT primers stated in 2.3.6.1 and the *nikB* gene sequenced as described in 2.3.7.2. Plasmids were accurately sized through their linearization using S1 nuclease as described in 2.3.12.1. RFLP was utilized to analyse the plasmids relationship further, DNA was extracted using the Takahashi method (2.2.4.2) and digested with EcoRI and PstI as described in 2.3.11. The AMR and virulence genotype of the *E. coli* wild type isolates with pCT-like plasmids were analysed by microarray, with DNA extracted by array lysis as stated in 2.2.3.2, followed by array

hybridization as stated in 2.3.2.2. The clonal relationship of the *E. coli* with pCT-like plasmids was determined by PFGE as described in 2.3.1.

3.3 Results

3.3.1 Screening veterinary and human *E. coli* isolates for pCT plasmids

To investigate the prevalence of *bla*_{CTX-M-14} pCT-like plasmids in human and veterinary sources a panel of isolates was examined. The pCT markers were used to screen 70 CTX-M-14 producing *E. coli* field isolates from cattle (MSC, n=33), turkeys (MST, n=9) sheep (MSS, n=2) and humans (MSH, n=26), isolated between 2006 and 2010. Screening identified a total of 25/70 (31.4%) isolates having two or more pCT molecular markers (sigma factor, shufflon rci, pilN and pCT008-009). Four molecular markers were detected in 22/25 (88%), three markers in 1/25 (4%) and two markers in 2/25 (8%) of wild type isolates, shown in Table 3.1. The isolates were distributed across England and Wales, as shown in Figure 3.1, and were collected from hospital, general practice submissions, and farm surveillance visits. Plasmid profiling of these isolates revealed multiple plasmids, which included plasmids ranging from 90-100 kb in size which is consistent with the 93,629 bp pCT plasmid.

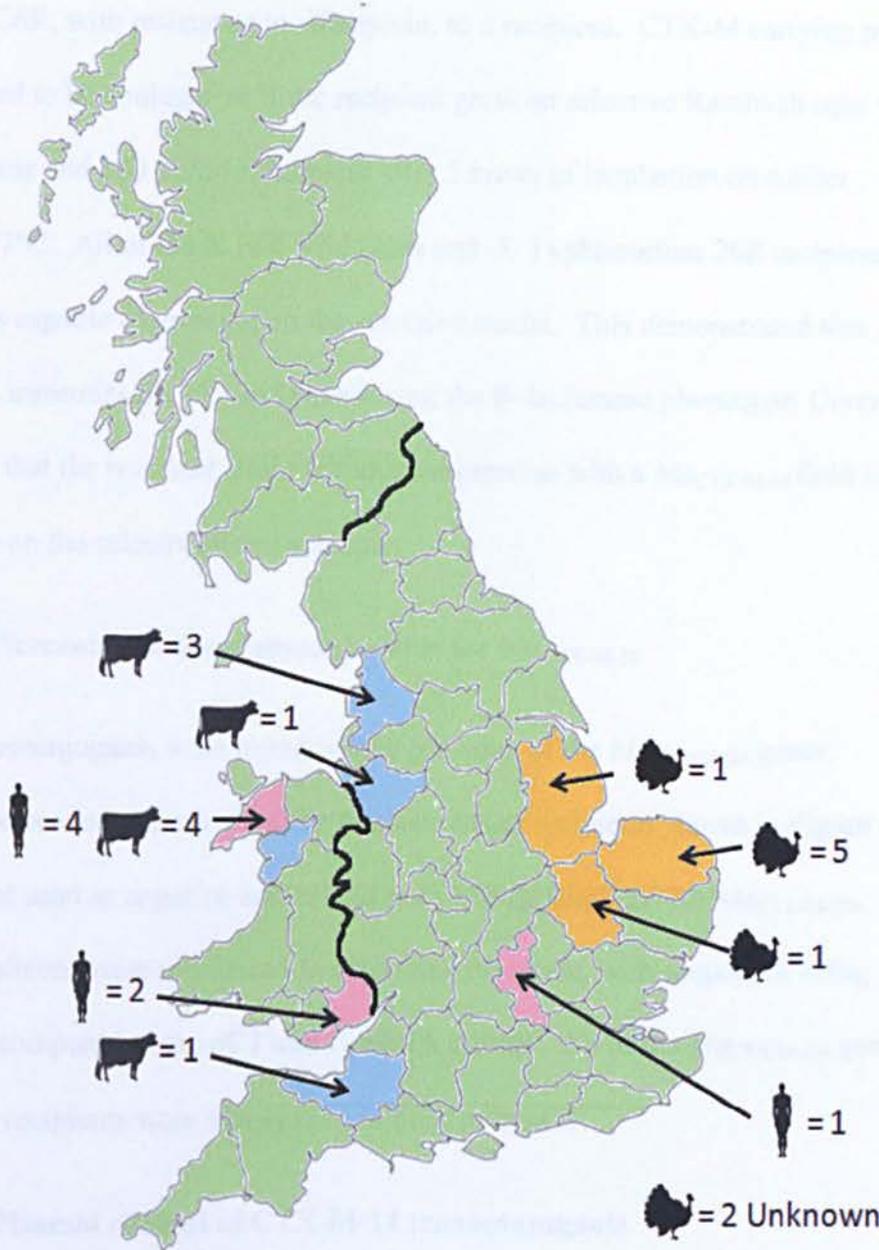
To examine the prevalence of pCT-like plasmids in non CTX-M-14 producing isolates, and the specificity of the markers a panel of non CTX-M-14 producing isolates were investigated. The panel included a total of 37 isolates including CTX-M-1 from cattle (n=7), turkeys (n=5) and chickens (n=15), CTX-M-3 from cattle (n=1) and chicken (n=1) and CTX-M-15 from cattle (n=8). Five (13.5%) CTX-M-1 isolates had at least one pCT genetic markers, (two cattle isolates had two markers and three chicken isolates had one marker. Additionally two of the CTX-M-15 producing isolates had 2 pCT genetic markers.

Table 3.1 pCT screening of CTX-M-14 *E. coli* field isolates

Isolate ID	Host Species	Isolation date	Sample type	Location	pCT multiplex PCR ¹	No. Plasmids	Plasmid size (kb)
MSC1	Cattle	2010	Faeces	Middlewich	S, R, N, P	3	95, 5, 4
MSC2	Cattle	2010	Faeces	Caernarfon	S, R, N, P	3	90, 70, 5
MSC3	Cattle	2010	Faeces	Pwllheli	S, R, N, P	3	95, 6, 5
MSC4	Cattle	2010	Faeces	Lancashire	S, R, N, P	3	90, 30, 20
MSC5	Cattle	2010	Faeces	Lancashire	S, R, N, P	4	120, 90, 4, 2
MSC6	Cattle	2006	Faeces	North Wales	S, R, N, P	6	150, 90, 70, 50, 6, 4
MSC7	Cattle	2008	Faeces	Lancashire	S, R, N, P	3	90, 6, 5
MSC8	Cattle	2006	Caecum	North Wales	S, R, N, P	6	150, 95, 60, 45, 6, 4
MSC9	Cattle	2006	Faeces	Somerset	S, R, N, P	4	110, 90, 6, 4
MSH1	Human	2007	Urine	Carmathen	S, R, N, P	4	90, 65, 7, 3
MSH2	Human	2007	Urine	Cardiff	S, R, N, P	3	90, 50, 35
MSH3	Human	2007	Urine	Newport	S, R, N, P	4	100, 80, 8, 5
MSH4	Human	2007	Unknown	Bangor	S, R, N, P	2	100, 95
MSH5	Human	2007	Urine	Bangor	S, N,	4	110, 95, 30, 4
MSH6	Human	2007	Urine	Bangor	S, N,	4	110, 95, 30, 4
MSH7	Human	2009	Urine	Buckinghamshire	S, R, N	ND	ND
MST1	Turkey	2006	Faeces	Norfolk	S, R, N, P	3	110, 90, 12
MST2	Turkey	2006	Faeces	Norfolk	S, R, N, P	4	90, 80, 8, 4
MST3	Turkey	2006	Faeces	Unknown	S, R, N, P	ND	ND
MST4	Turkey	2006	Faeces	Norfolk	S, R, N, P	3	110, 90, 70
MST5	Turkey	2006	Faeces	Lincolnshire	S, R, N, P	5	110, 90, 70, 10, 4
MST6	Turkey	2006	Faeces	Norfolk	S, R, N, P	ND	ND
MST7	Turkey	2006	Faeces	Unknown	S, R, N, P	ND	ND
MST8	Turkey	2006	Faeces	Norfolk	S, R, N, P	ND	ND
MST9	Turkey	2006	Faeces	Peterborough	S, R, N, P	ND	ND

S = Sigma factor (1,289 bp), R = shufflon recombinase (945 bp) N = pilN (627 bp) and P = pCT008-009 (428 bp), ND= Not determined

Figure 3.1 Distribution of pCT positive CTX-M-14 isolates from humans, cattle and turkeys



Map of the UK showing the distribution of *E. coli* field isolates in England and Wales with humans isolates shown in pink, cattle in blue and turkeys in orange.

3.3.2 Characterization of the *bla*_{CTX-M-14} plasmids

The plasmids from the 25 CTX-M-14 *E. coli* field isolates with two or more pCT markers were investigated further to determine the extent of the relationship between them, and the pCT plasmids, which was carried out by transfer into the same host background.

3.3.2.1 Conjugations

Plasmids were investigated for their ability to conjugate using a plasmid free *S. Typhimurium* 26R, with resistance to rifampicin, as a recipient. CTX-M carrying plasmids were determined to be conjugative if the recipient grew on selective Rambach agar with 1 µg/ml cefotaxime and 100 µg/ml rifampicin after 5 hours of incubation on a filter membrane at 37°C. All of the *E. coli* wild types and *S. Typhimurium* 26R recipients yielded isolates capable of growing on the selective media. This demonstrated that all isolates carried transmissible plasmids conferring the β- lactamase phenotype. Control strains showed that the recipient strain without conjugation with a *bla*_{CTX-M-14} field isolate could not grow on the selective Rambach agar.

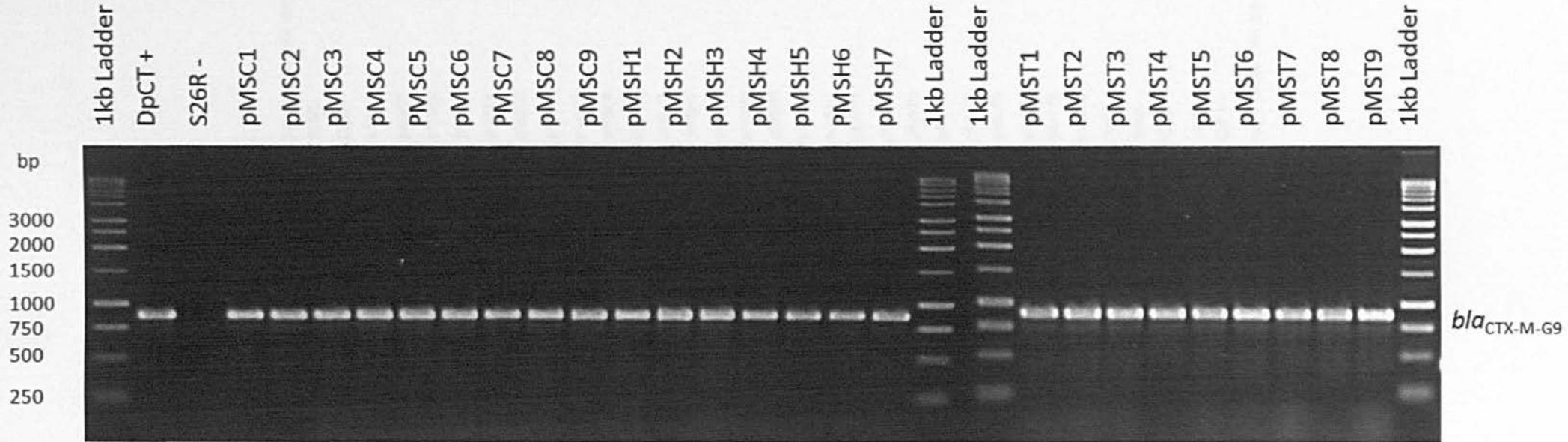
3.3.2.2 Screening of the transconjugants for *bla*_{CTX-M-14}

The transconjugants were tested for the presence of the *bla*_{CTX-M-G9} genes. Amplicons were produced from all of the transconjugant recipients shown in Figure 3.2, and the recipient used as negative control did not yield an amplicon for *bla*_{CTX-M-G9}. The *bla*_{CTX-M-G9} amplicons were sequenced to determine the allele, with sequences being assembled and compared to the pCT and Genbank entries. All of the *bla*_{CTX-M-G9} genes in the conjugative recipients were *bla*_{CTX-M-14}, as present in pCT.

3.3.2.3 Plasmid content of CTX-M-14 transconjugants

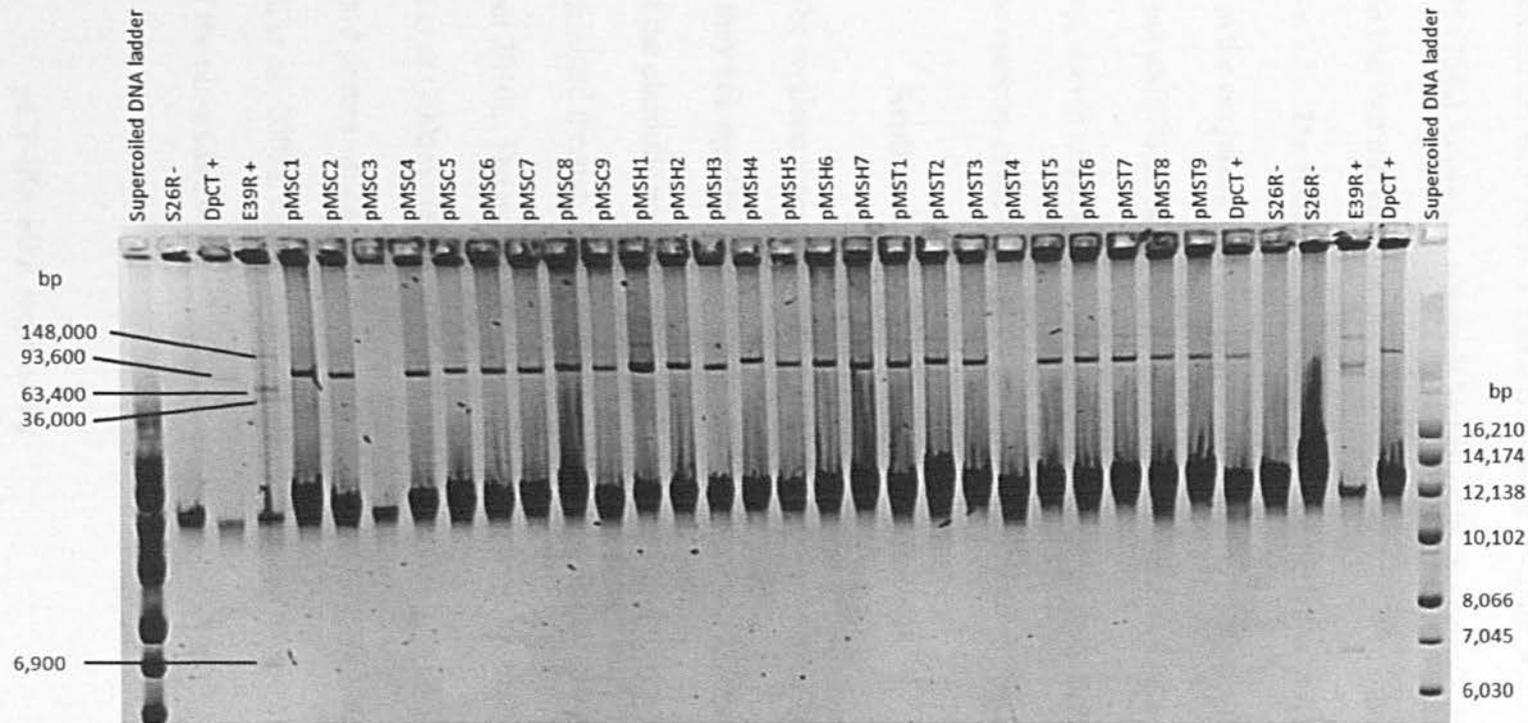
To determine whether a plasmid carrying the *bla*_{CTX-M-14} had been transferred by conjugation, the transconjugants were screened for plasmid content. With the exception of pMST4 all of the transconjugants had a plasmid of approximately 85-110 kb in size, shown in Figure 3.3. The observation that pMST4 post conjugation was positive for the *bla*_{CTX-M-14} but lacked a plasmid suggesting the possibility that the plasmid may have integrated into the chromosome. In addition the field isolate had all the pCT markers, supporting the presence of a similar plasmid.

Figure 3.2 CTX-M-G9 PCR of transconjugants



CTX-M-G9 PCR of transconjugants, DpCT+ is the positive control and S26R- is the negative control, which is the recipient used in conjugations. DNA resolved on 1.5% agarose gel.

Figure 3.3 Plasmid content of CTX-M-14 transconjugants



Plasmid content of the transconjugant strains after conjugation with the field isolates. Supercoiled DNA ladder, DpCT+ and E39R+ are positive controls with known sizes, and plasmid free S26R- was used as a marker for the chromosomal band. DNA resolved on 0.8% agarose gel.

3.3.2.4 pCT molecular marker testing of the transconjugants

To ensure that the plasmids which conjugated into the *Salmonella* recipient carried the pCT markers, and that the markers were not on the chromosome, the transconjugants were re-examined with the pCT markers. Testing with the pCT molecular markers showed that 19/25 (76%) *bla*_{CTX-M-14} plasmids had all of the markers, 3/25 (12%) had 3 markers, 2/25 (8%) had two markers and one conjugant had one (4%) marker, as shown in Figure 3.4 (Table 3.2). The recipient was negative for all pCT markers and so the presence of the markers in the conjugant was due to plasmid transfer. With the exception of pMSC2, pMST4 and pMST6 all of the conjugative plasmids had the same markers present in the wild types, shown in Table 3.2. Due to the presence of these plasmids with the pCT molecular markers in the conjugants they were described as "pCT-like" plasmids.

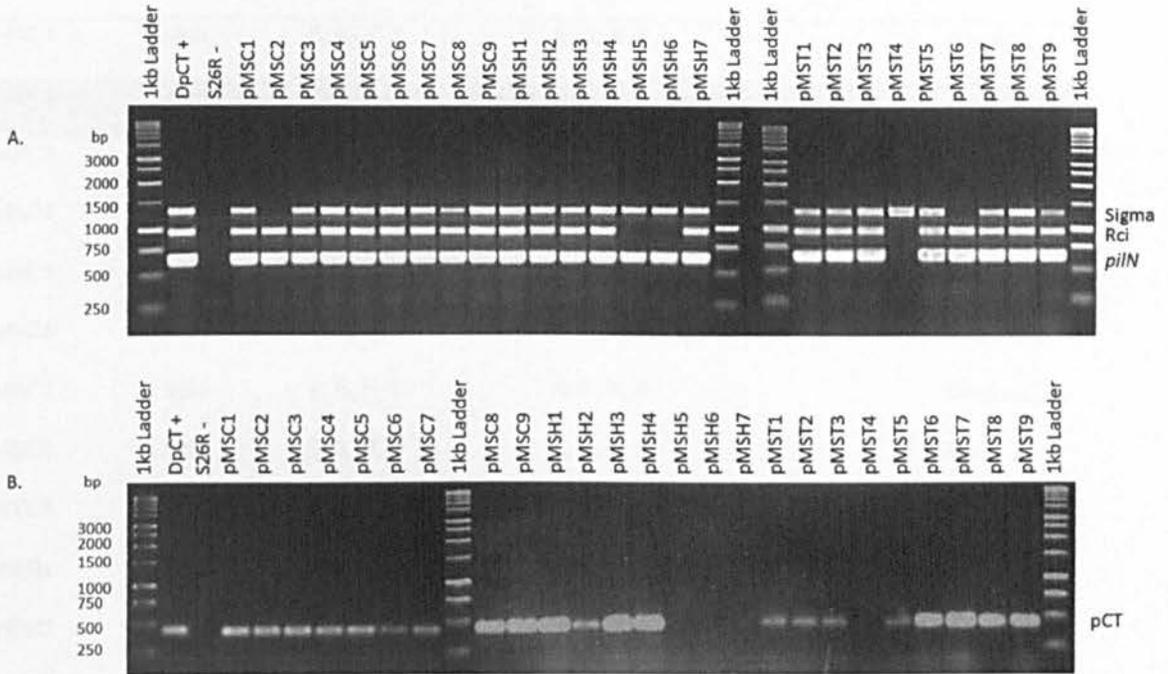
3.3.2.5 Replicon typing of the pCT-like plasmids

The replicon type of the conjugative pCT-like plasmids was examined to determine whether they belonged to the IncK incompatibility group, as does pCT. IncK replicon typing of the plasmids previously had not yielded a replicon type, and the IncK type was only determined through complete sequencing (Dr J Cottell personal communication 8th September 2010). The use of the PCR primers for the IncK replicon type described by Carattoli *et al* (2005a) yielded no product, which is likely to be due to base mismatches in the forward primer which was redesigned based on the sequence of pCT (FN868832) (Carattoli *et al.*, 2005a; Cottell *et al.*, 2011). The newly designed primer yielded a product for all of the plasmids except for four plasmids pMST4 and three from humans, pMSH5, 6 and 7.

3.3.2.6 pCT-like *nikB* sequencing

The *nikB* gene from the plasmids was amplified and sequenced to compare its sequence with the pCT plasmids. All of the transconjugants produced an amplicon with

Figure 3.4 pCT molecular markers of the CTX-M-14 transconjugants



(A) pCT multiplex PCR (sigma factor (sigma), shufflon recombinase (Rci), pilN) on the transconjugants and (B) pCT008-009 simplex (pCT), DpCT+ is the positive control and S26R- is the negative control, which is the recipient used in conjugations. DNA resolved on 1.5% agarose gel.

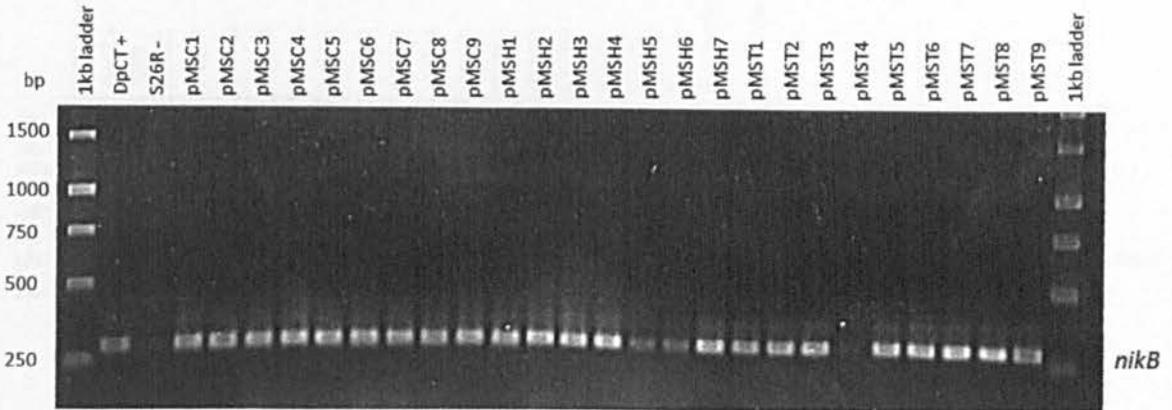
the exception of pMST4, Figure 3.5. Sequencing and analysis of the amplicons revealed that all of the plasmids apart from pMSH5 and pMSH6 had the same sequence with no polymorphisms detected. The sequence of *nikB* from pMSH5 and pMSH6 varied from pCT by 9 nucleotide substitutions which were 81 (T>C), 90 (C>T), 96 (T>G), 129 (A>C), 130 (T>G), 216 (A>G), 222 (C>G), 231 (T>C) and 240 (A>G). The sequence of these two plasmids when compared to GenBank was found to be identical to that of pHUSEC41-1 (HE603110) which harboured *bla*_{TEM-1}, *sul2* and *strAB* and was sequenced from a *bla*_{CTX-M-15} EHEC isolate in Germany (Kunne *et al.*, 2012).

Table 3.2 pCT molecular markers in *E. coli* field isolates and transconjugants

Isolate ID	Host Species	Field isolate pCT multiplex PCR*	<i>Salmonella</i> Typhimurium 26R transconjugant pCT Multiplex PCR	<i>bla</i> _{CTX-M}
MSC1	Cattle	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MSC2	Cattle	S, R, N, P	R, N, P	<i>bla</i> _{CTX-M-14}
MSC3	Cattle	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MSC4	Cattle	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MSC5	Cattle	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MSC6	Cattle	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MSC7	Cattle	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MSC8	Cattle	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MSC9	Cattle	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MSH1	Human	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MSH2	Human	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MSH3	Human	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MSH4	Human	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MSH5	Human	S, N,	S, N,	<i>bla</i> _{CTX-M-14}
MSH6	Human	S, N,	S, N,	<i>bla</i> _{CTX-M-14}
MSH7	Human	S, R, N	S, R, N	<i>bla</i> _{CTX-M-14}
MST1	Turkey	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MST2	Turkey	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MST3	Turkey	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MST4	Turkey	S, R, N, P	S	<i>bla</i> _{CTX-M-14}
MST5	Turkey	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MST6	Turkey	S, R, N, P	R, N, P	<i>bla</i> _{CTX-M-14}
MST7	Turkey	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MST8	Turkey	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}
MST9	Turkey	S, R, N, P	S, R, N, P	<i>bla</i> _{CTX-M-14}

S = Sigma factor (1,289 bp), R = shufflon recombinase (945 bp) N = pilN (627 bp) and P = pCT008-009 (428 bp). Shaded are isolates that had different markers in the *E. coli* field isolate and the transconjugant.

Figure 3.5 *nikB* amplicons from pCT-like transconjugants



nikB PCR on the transconjugants, DpCT+ is the positive control and S26R- the negative control, which is the recipient used in conjugations. DNA resolved on 1.5% agarose gel.

3.3.2.7 The *bla*_{CTX-M-14} genetic environment

The genetic environment surrounding the *bla*_{CTX-M-14} was investigated to determine whether it was indistinguishable from pCT and those previously published.

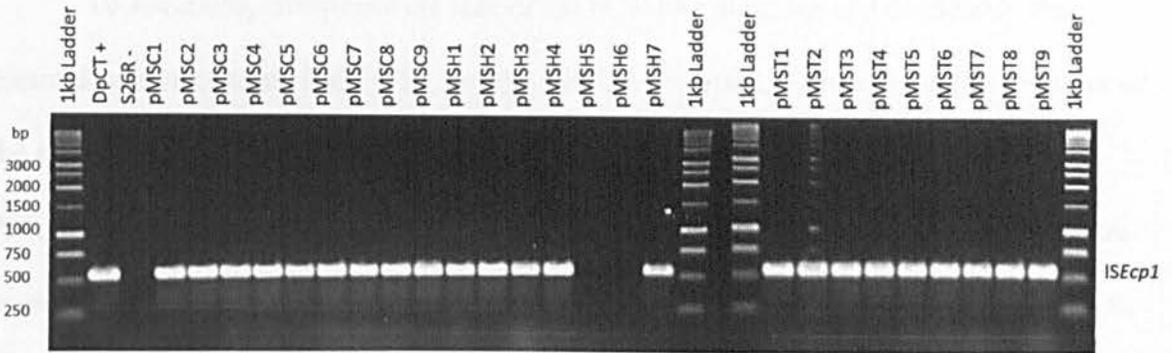
3.3.2.7.1 *ISEcp1* PCR on transconjugants

The transconjugants with the pCT-like plasmids were investigated for presence of the insertion sequence *ISEcp1* which is present upstream of *bla*_{CTX-M-14} in pCT. All of the pCT like transconjugants produced amplicons for *ISEcp1* with the exception of the plasmids pMSH5 and pMSH6, as shown in Figure 3.6

3.3.2.7.2 Location of *bla*_{CTX-M-14}

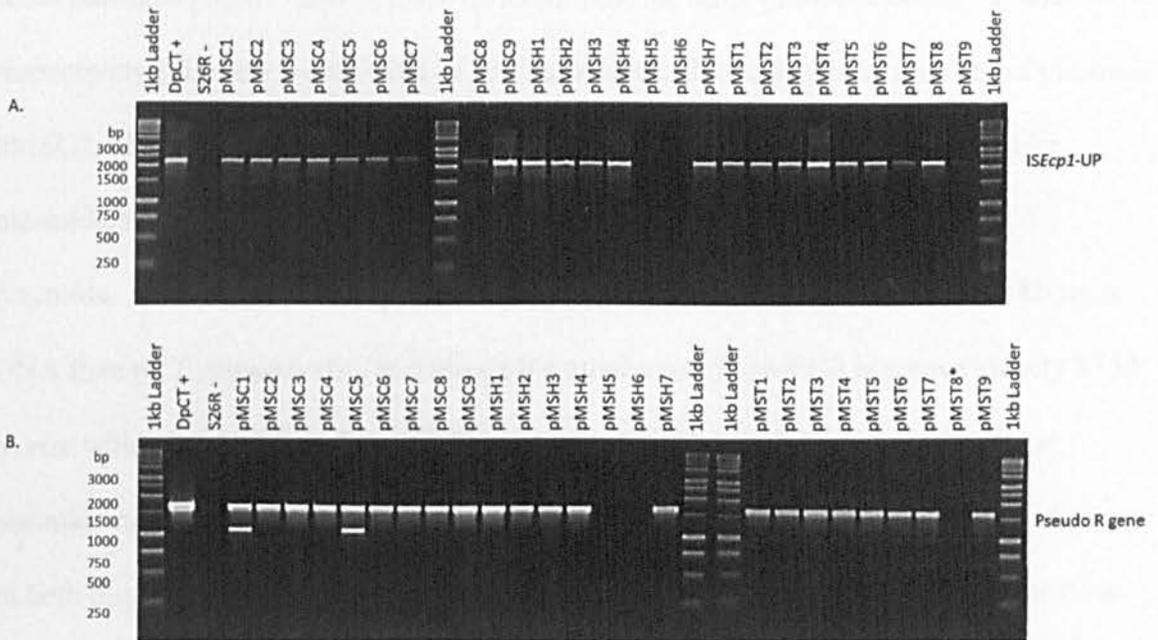
After ascertaining the presence of *ISEcp1* in the plasmids, it was determined whether *bla*_{CTX-M-14} was located downstream of *ISEcp1* and upstream of the pseudo R gene as in pCT, this was investigated by PCR, as shown in Figure 3.7. All of the pCT-like plasmids had *bla*_{CTX-M-14} downstream of *ISEcp1* and upstream pseudo R except for pMSH5 and 6.

Figure 3.6 *ISEcp1* PCR on transconjugants



ISEcp1 PCR on the transconjugants, DpCT+ is the positive control and S26R- is the negative control, which is the recipient used in conjugations. DNA resolved on 1.5% agarose gel.

Figure 3.7 *bla*_{CTX-M-14} environment PCR



The genetic environment PCR from (A) *ISEcp1* to *bla*_{CTX-M-14}, (B) *bla*_{CTX-M-14} to Pseudo R, DpCT+ is the positive control and S26R- the negative control, which is the recipient used in conjugations. DNA resolved on 1.5% agarose gel. * = Pseudo R repeated for pMST8 and was positive.

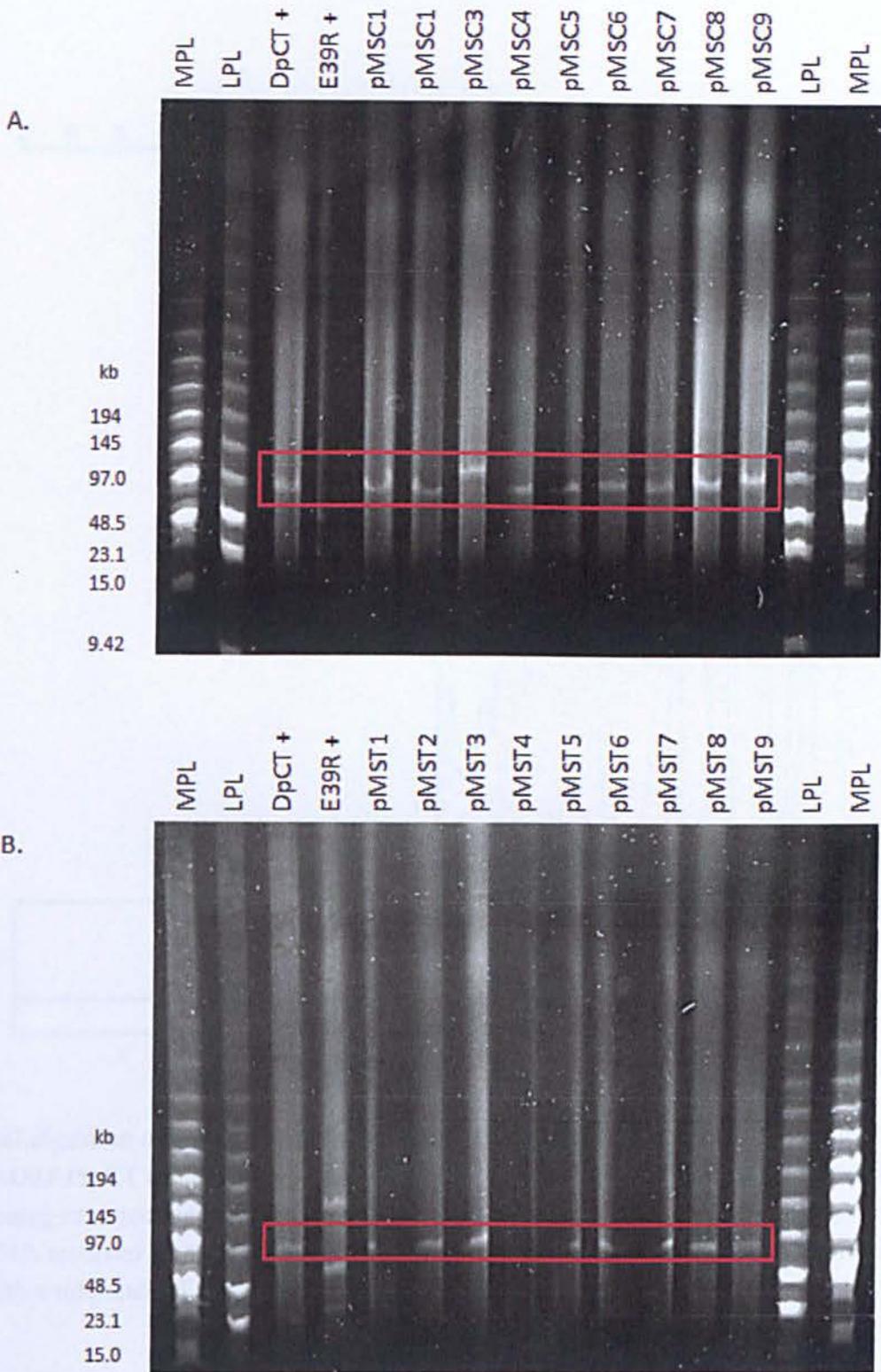
3.3.2.8 Plasmid sizing of pCT-like plasmids

To accurately determine the size of the pCT-like plasmids and to identify if a plasmid was present in pMST4, S1 nuclease PFGE was used. Through the linearization of the plasmids, the size of the plasmids can be accurately determined using lambda and PFGE ladders. The pCT-like plasmids were found to have plasmids which ranged in size from 85-110 kb and as with plasmid profiling no plasmid was observed in pMST4, the S1 nuclease for cattle and turkey pCT plasmids are shown in Figure 3.8.

3.3.2.9 Restriction fragment length polymorphism of pCT-like plasmids

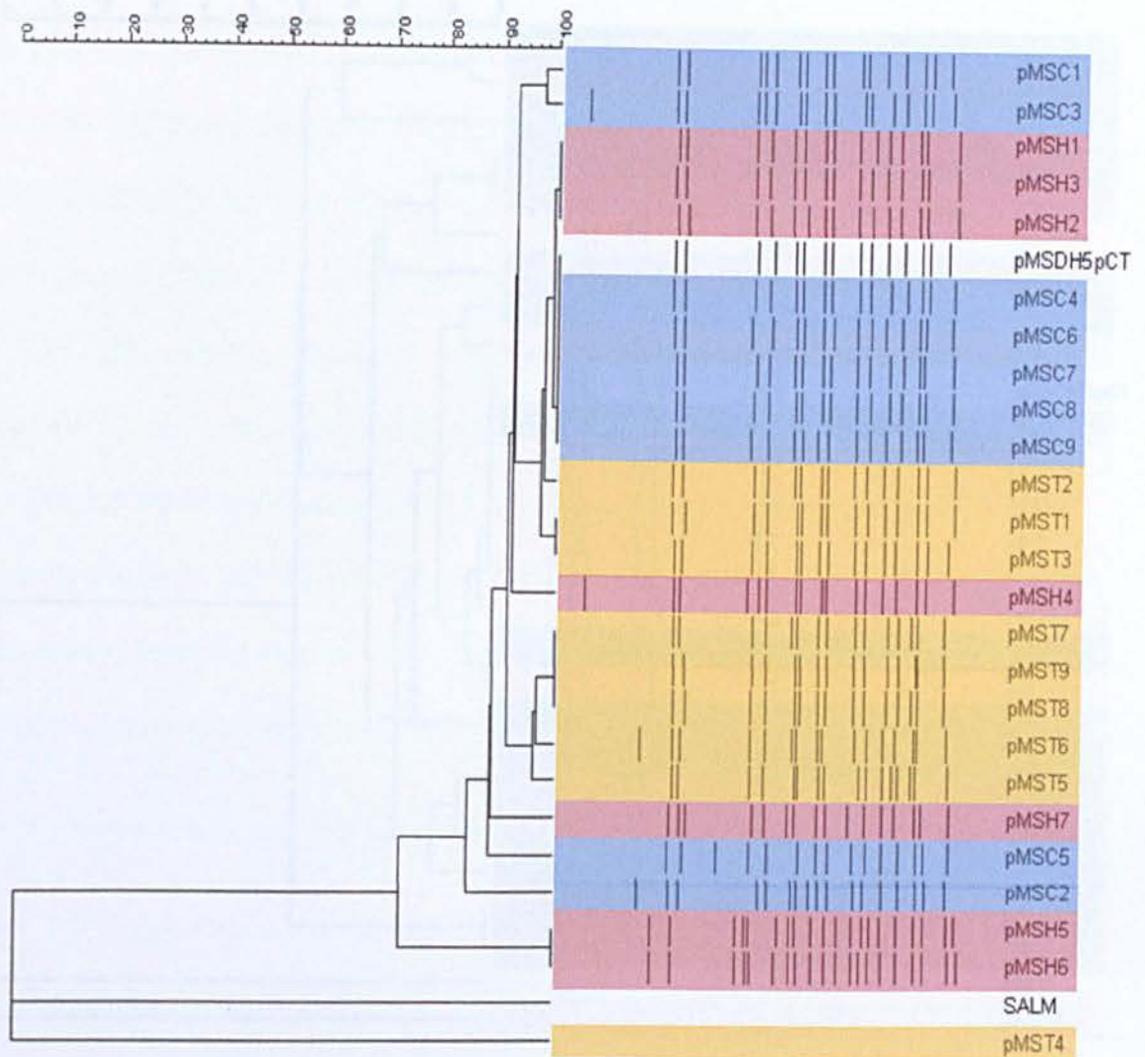
To analyse the plasmids further restriction fragment length polymorphism (RFLP) was performed using two restriction enzymes *PstI* and *EcoRI*. The majority of plasmids had a homology of >80% for *PstI* (Figure 3.9) and >75% for *EcoRI* (Figure 3.10), in both cases plasmids pMSH5 and pMSH6 differed from the other plasmids having 70% and 60% respectively (Figure 3.9 and 3.10). The *EcoRI* digest also highlighted a cluster of plasmids pMSC2, pMSC3 and pMSH4, which shared 68% similarity with the other pCT-like plasmids, these plasmids correspond to those with differing size to that of the pCT plasmids. The large plasmids pMSC3 and pMSH4 have an estimated 15 and 10 kb more DNA than pCT respectively. In contrast the other plasmid pMSC2 is approximately 85 kb in size which is 10 kb less than pCT, but groups with the two larger plasmids, these plasmids may represent pCT-like plasmids that have undergone deletions and insertions. In both digests the DNA extracted from the pMST4 transconjugant yielded no restriction profiles, as did the plasmid free *Salmonella* recipient control.

Figure 3.8 S1 nuclease PFGE of pCT-like plasmids from cattle and turkeys



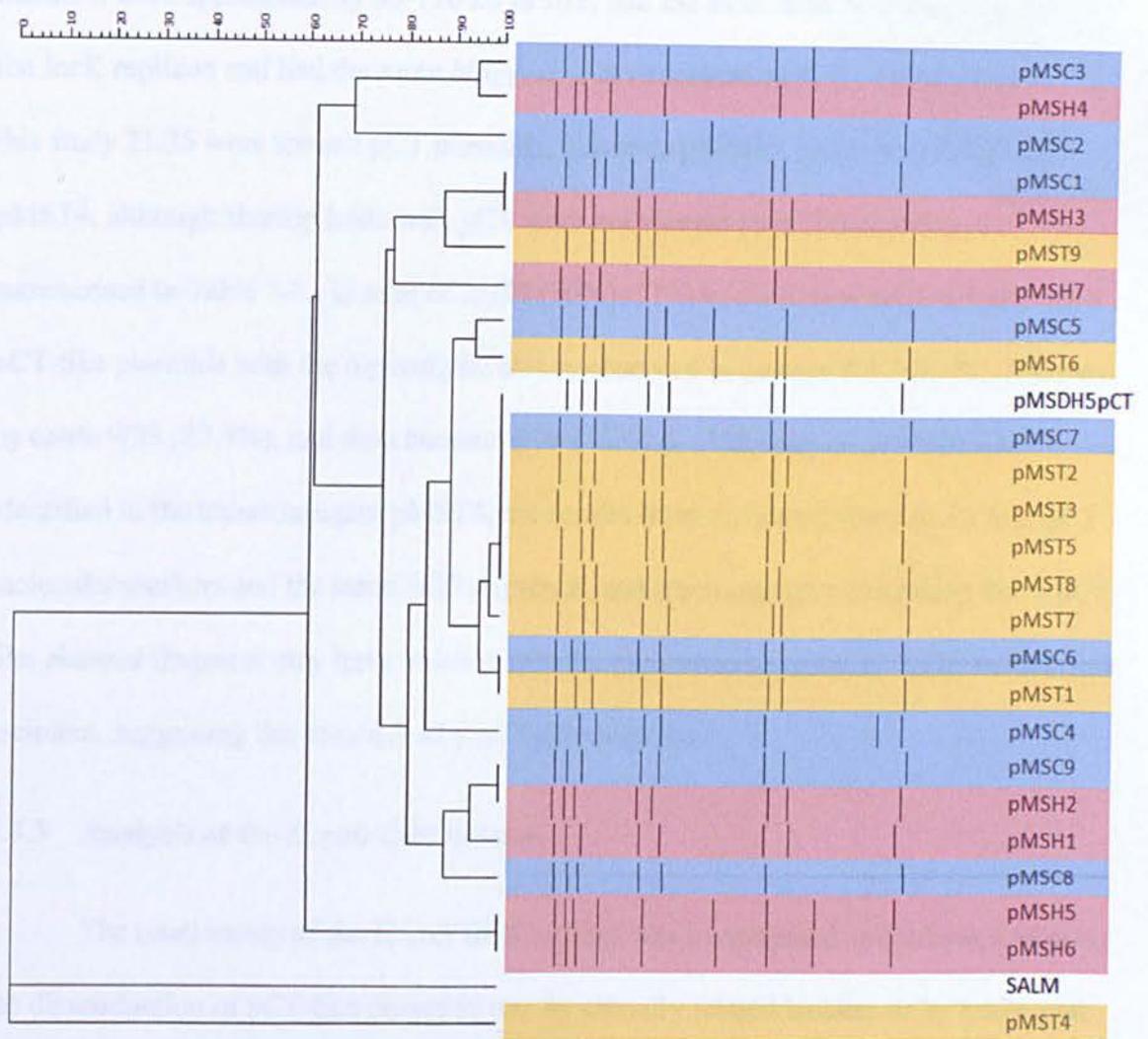
S1 nuclease PFGE of plasmids from (A) cattle (B) turkeys. MPL (MidRange PFGE marker) and LPL (Low Range PFGE marker) are commercial markers of known size and DpCT+ and E39R+ are isolates with plasmids of known size. DNA resolved by PFGE electrophoresis on 1.2% agarose gel.

Figure 3.9 *Pst*I RFLP analysis of pCT-like plasmids



*Pst*I digestion of 4.5 µg of plasmid DNA from transconjugants with 20U of *Pst*I. pMSDH5pCT used as a control for the pCT plasmids and SALM used as plasmid free, conjugation recipient control, humans shown in red, cattle in blue and turkeys in orange. DNA resolved on a 0.8% agarose gel, analysed in bionumerics 5.10 using DICE coefficient with a tolerance of 1.0%, scale shows % similarity.

Figure 3.10 *EcoRI* RFLP analysis of pCT-like plasmids



EcoRI digestion of 4.5 µg of plasmid DNA for transconjugants with 24U of *EcoRI*. pMSDH5pCT used as a control for the pCT plasmids and SALM used as plasmid free, conjugation recipient control, humans shown in red, cattle in blue and turkeys in orange. DNA resolved on a 0.8% agarose gel, analysed in bionumerics 5.10 using DICE coefficient with a tolerance of 1.5%, scale shows % similarity.

3.3.2.10 pCT-like plasmids analysis

Plasmids were defined as pCT-like if they harboured three or more pCT molecular markers, were approximately 85-110 kb in size, had the same *nikB* sequence, positive for the IncK replicon and had the same *bla*_{CTX-M-14} environment as pCT. Of the plasmids in this study 21/25 were termed pCT plasmids, plasmids pMSH5, pMSH6, pMSH7 and pMST4, although sharing traits with pCT were not classed as pCT-like plasmids, summarised in Table 3.3. In total of 21/70 (30%) CTX-M-14 *E. coli* isolates harbored a pCT-like plasmids with the highest prevalence observed in turkeys 8/9 (88.9%), followed by cattle 9/33 (27.3%), and then humans 4/26 (15.4%). Although no plasmid was identified in the transconjugant pMST4, the results from the parents having all four pCT molecular markers and the same *nikB* sequence, and transconjugant indicating that a pCT-like plasmid fragment may have inserted into the chromosome of the *S. Typhimurium* 26R recipient, suggesting the presence of a pCT-like plasmid.

3.3.3 Analysis of the *E. coli* field isolates

The relationship of the *E. coli* field isolates was investigated to determine whether the dissemination of pCT-like plasmids was by clonally related isolates or by horizontal transfer, and to determine the resistance and virulence gene profiles associated with pCT-like plasmids.

3.3.3.1 Clonal relationship of *E. coli* field isolates with pCT-like plasmids

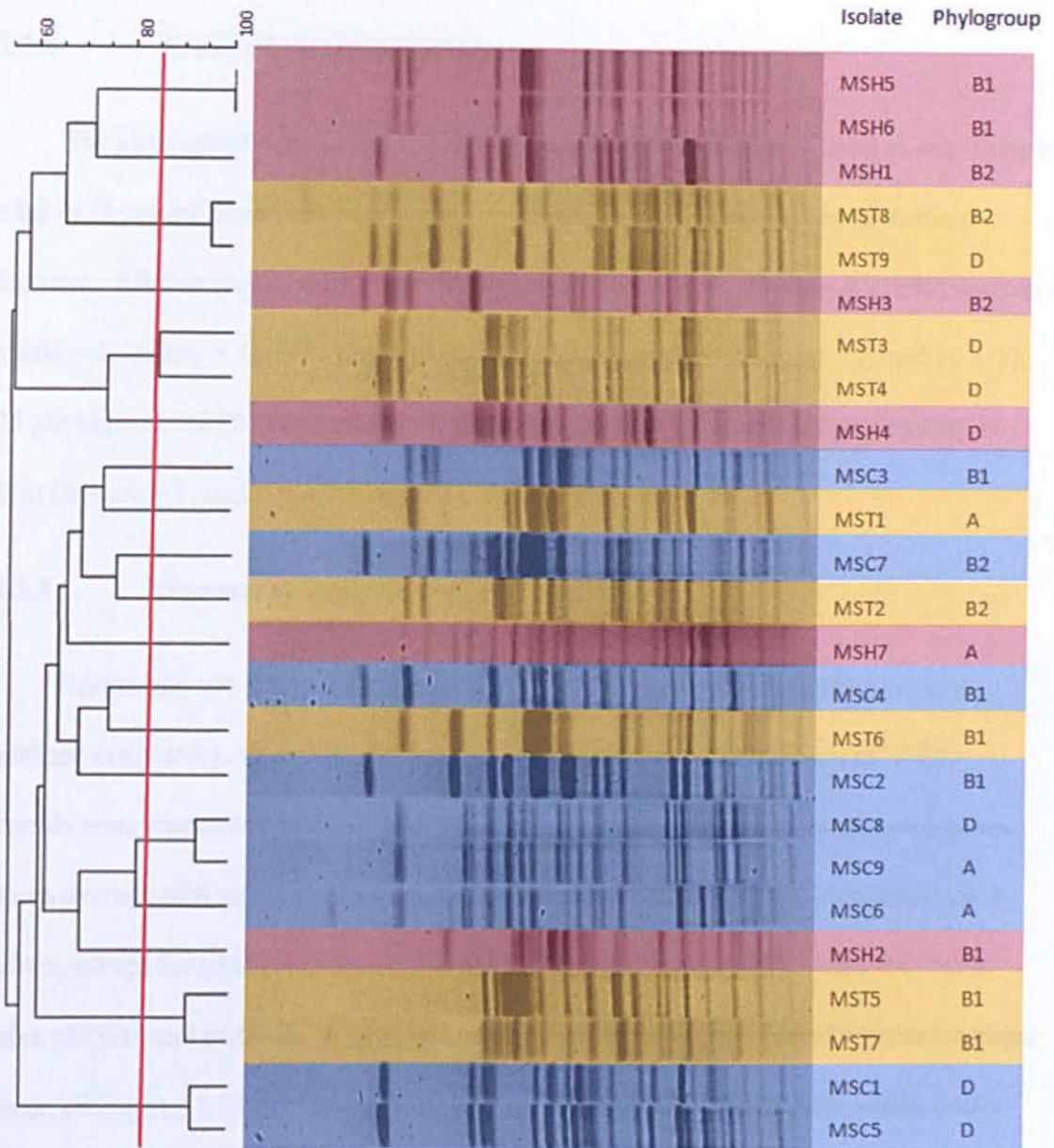
The clonal relationship of the 25 *E. coli* field isolates with pCT-like or similar plasmids was investigated using *Xba*I PFGE as set out by PULSENET. The digestion and resolution of chromosomal DNA was analysed using bionumerics to determine the relationship. Analysis identified 19 unique PFGE profiles using a cut off at 85%, which included 6 clusters, shown in Figure 3.11. The field isolates MSH5 and MSH6 were found to be 100% identical, with MSC1 and MSC5, MST8 and MST9, MST3 and MST4, MST5 and MST7, and MSC8 and MSC9 all being clustered together (Figure 3.11). No

Table 3.3 Summary of the pCT-like plasmids

Plasmid ID	Host Species	S26R TC pCT Multiplex PCR	<i>bla</i> _{CTX-M-14}	<i>ISEcp1</i>	<i>ISEcp1-bla</i> _{CTX-M-9}	<i>bla</i> _{CTX-M-9-pseudo}	Plasmid Size (kb)	IncK replicon	<i>nikB</i> PCR
pMSC1	Cattle	S, R, N, P	+	+	+	+	90	+	+
pMSC2	Cattle	R, N, P	+	+	+	+	85	+	+
pMSC3	Cattle	S, R, N, P	+	+	+	+	110	+	+
pMSC4	Cattle	S, R, N, P	+	+	+	+	90	+	+
pMSC5	Cattle	S, R, N, P	+	+	+	+	95	+	+
pMSC6	Cattle	S, R, N, P	+	+	+	+	95	+	+
pMSC7	Cattle	S, R, N, P	+	+	+	+	95	+	+
pMSC8	Cattle	S, R, N, P	+	+	+	+	95	+	+
pMSC9	Cattle	S, R, N, P	+	+	+	+	95	+	+
pMSH1	Human	S, R, N, P	+	+	+	+	95	+	+
pMSH2	Human	S, R, N, P	+	+	+	+	95	+	+
pMSH3	Human	S, R, N, P	+	+	+	+	95	+	+
pMSH4	Human	S, R, N, P	+	+	+	+	105	+	+
pMSH5	Human	S, N,	+	-	-	-	100	-	+(9)
pMSH6	Human	S, N,	+	-	-	-	100	-	+(9)
pMSH7	Human	S, R, N	+	+	+	+	100	-	+
pMST1	Turkey	S, R, N, P	+	+	+	+	95	+	+
pMST2	Turkey	S, R, N, P	+	+	+	+	95	+	+
pMST3	Turkey	S, R, N, P	+	+	+	+	95	+	+
pMST4	Turkey	S	+	+	+	+	ND	-	+(FI)
pMST5	Turkey	S, R, N, P	+	+	+	+	95	+	+
pMST6	Turkey	R, N, P	+	+	+	+	95	+	+
pMST7	Turkey	S, R, N, P	+	+	+	+	100	+	+
pMST8	Turkey	S, R, N, P	+	+	+	+	95	+	+
pMST9	Turkey	S, R, N, P	+	+	+	+	95	+	+

Summary of pCT-like plasmids, those shaded in grey were not classed as pCT-like plasmids. S26R TC is the *Salmonella* Typhimurium 26R transconjugant. FI, field isolate.

Figure 3.11 *Xba*I PFGE and phylogrouping of *E. coli* field isolates



*Xba*I PFGE of the 25 *E. coli* field isolates containing pCT-like plasmids, humans shown in red, cattle in blue and turkeys in orange, DNA was resolved on a 1% gel and *Salmonella* Braenderup was used to align profiles and banding patterns, analysis was performed in bionumerics 5.10 using the DICE coefficient with a tolerance of 1.5%, scale shows % similarity. The red line indicates the 85% cut off and phylogroups are shown on the right.

association between *E. coli* isolates from different hosts was identified, and with the exception of the clusters the *E. coli* were unrelated supporting plasmid transfer between isolates.

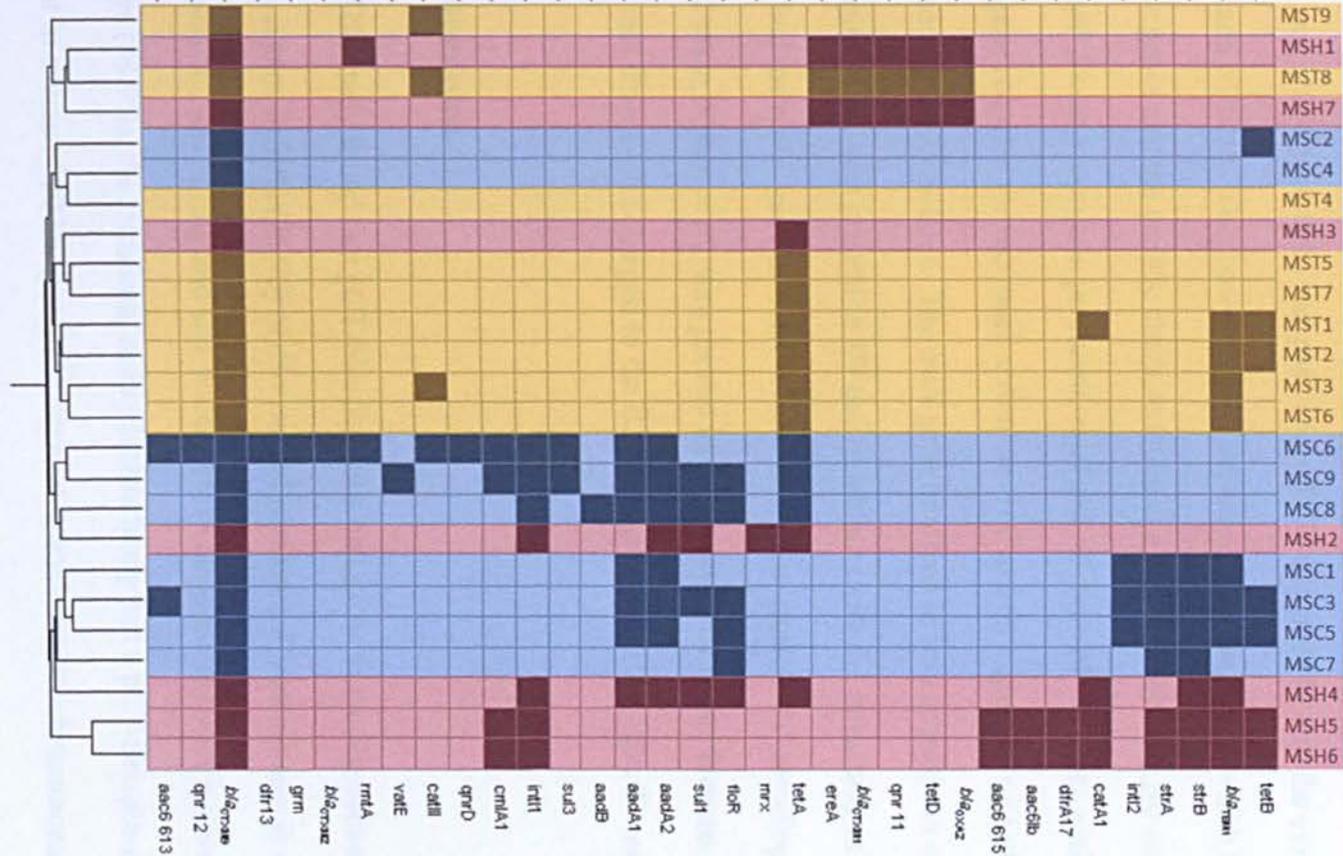
3.3.3.2 *E. coli* phylogenetic groups

The phylogenetic groups of the *E. coli* field isolates was determined to see if any of the B2 or D groups were present as these are associated with virulent extraintestinal infections. All four phylogroups were identified, 4/25 were phylogroup A (16%) (human = 1, cattle = 2, turkey = 1), 9/25 phylogroup B1 (36%) (human = 1, cattle = 2, turkey = 1), 5/25 phylogroup B2 (20%) (human = 2, cattle = 1, turkey = 2) and 7/25 phylogroup D (28%) (human = 1, cattle = 3, turkey = 3), shown in Figure 3.11.

3.3.3.3 Microarray analysis of *E. coli* field isolates

Resistance and virulence genes in the field isolates were determined using a combined microarray, to see the virulence and resistance genotype which pCT-like plasmids were associated with. *E. coli* field isolates had between 1-15 resistance genes with an average of 6 genes per isolate. All of the isolates had different resistance gene profiles, except for pMSC4 and pMST4, pMSH3, pMST5 and pMST7, and the clonal strains pMSH5 and pMSH6. *E. coli* isolates from turkeys had the fewest resistance genes with an average of 3.33 per isolate, followed by cattle and human isolates which had a similar number of resistance genes of 7.44 and 7.57 respectively, shown in Figure 3.12. In addition to *bla*_{CTX-M-14}, three isolates two from humans and one from cattle also had the β -lactamase genes *bla*_{CTX-M-G1} and *bla*_{OXA-2} while the *bla*_{TEM-1} gene was present in 10 isolates (human = 3, cattle = 3, turkey = 4). The integrase gene for class 1 integrons was present in 7 isolates, four human and three cattle, and integrase for class 2 integrons in three cattle isolates. The *tetA* tetracycline resistance gene was the second most prevalent to *bla*_{CTX-M-14} which was present in 12 isolates (human = 3, cattle = 3, turkey = 6), followed by the chloramphenicol genes *catIII* (n = 4), *cmlA* (n = 4), *floR* (n = 6) and *catA* (n = 4).

Figure 3.12 *E. coli* field isolates resistance genes



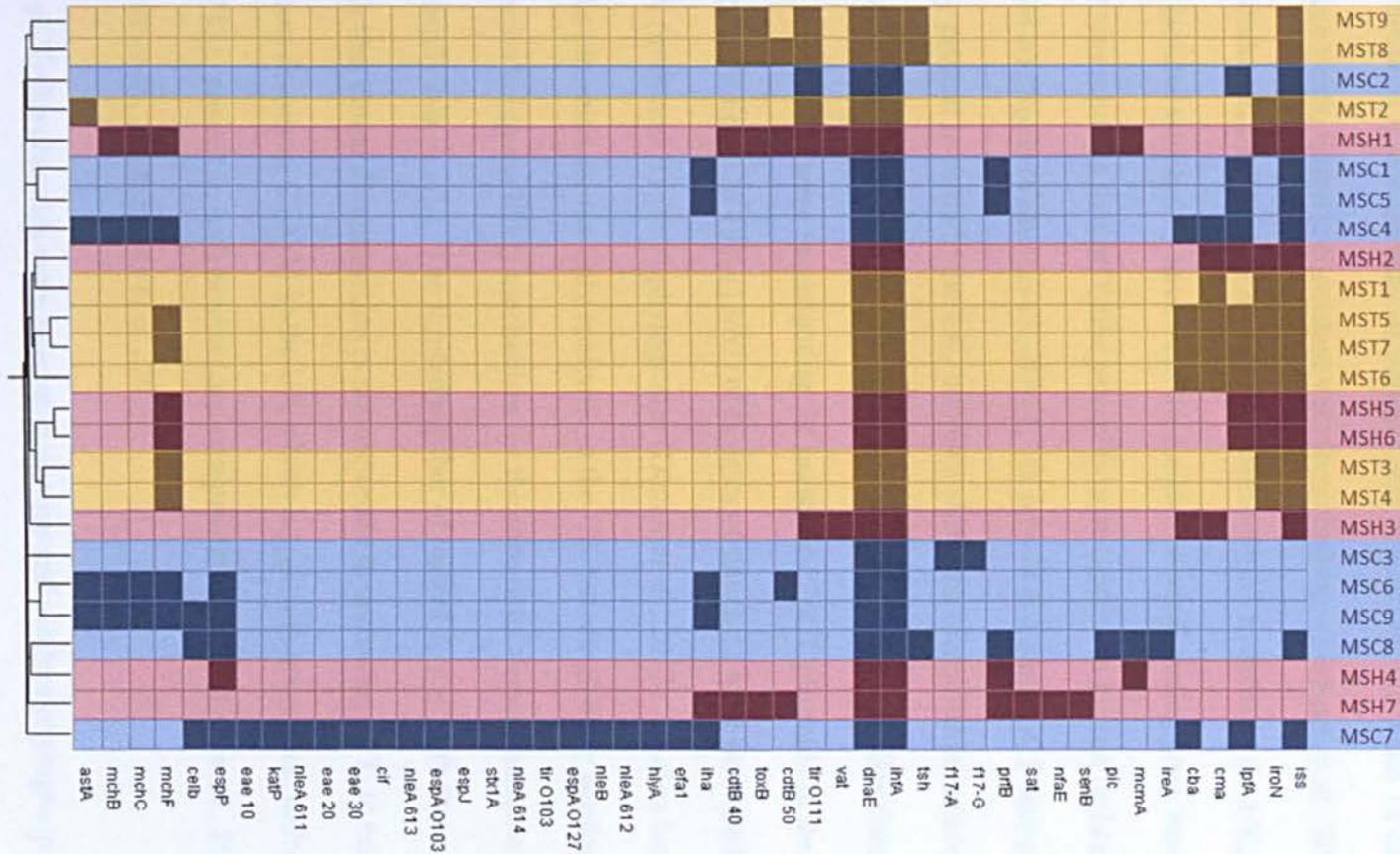
Resistance microarray analysis performed on *E. coli* field isolates with pCT like plasmids using Genespring with values adjusted in line with the controls, humans shown in red, cattle in blue and turkeys in orange. Squares filled in black indicate the presence of the gene, which are shown below.

The average number of virulence genes determined by microarray was 7.88 per isolate, with the lowest number of virulence genes being observed in isolates from turkeys with 4.56 per isolate. Similarly to the results for the resistance genes cattle and human isolates had a similar number of virulence genes with 5.37 and 5.71 respectively as shown in Figure 3.13. All of the isolates had diverse virulence gene profiles with the exception of pMSC1 and pMSC5, pMST3 and pMST4, pMST5 and pMST7 and the clonal isolates pMSH5 and pMSH6. This however does exclude one cattle isolate which had a total of 25 virulence genes and the genetic characteristics of a EHEC, including the *stx* gene for shiga toxin, *espP* for an exoprotein precursor, *espJ* and *cif* type III effectors, and *eae* for intimin. The *iss* gene required for increased serum survival and an important trait of ExPEC *E. coli* was present in 20/25 isolates. The *iroN* gene involved in iron acquisition was found in 10/25 isolates, and was present in 7/9 turkey isolates with the remaining in humans, 9/25 isolates had the *mchF* gene for ABC transporters. The cytolethal distending toxin B gene *cdtB* and toxin B gene *toxB* were present in four isolates two from humans and two from turkeys, one from each belonged to the B2 phylogroup, and the other *E. coli* from turkey was D.

3.4 Discussion

This study found that pCT-like plasmids had a role in the dissemination of the *bla*_{CTX-M-14} gene in 30% (21/70) of diverse and unrelated *E. coli* isolates from cattle, turkeys and humans in England and Wales. pCT-like plasmids were particularly prevalent in turkeys (88.9%), the remaining turkey isolate likely had a pCT-like plasmid, of which a fragment in excess of 22 kb appears to have integrated into the chromosome. This assumption is made based on the presence of the sigma factor marker and the *bla*_{CTX-M-14} in the same genetic environment as pCT in the transconjugant, but the absence of any plasmid. The integration of *bla*_{CTX-M-15} genes from plasmids into the chromosome has been reported before in *S. Concord* in children adopted from Ethiopia (Fabre *et al.*, 2009). The pCT-like plasmids had the pCT molecular markers with all four in 19/21 and

Figure 3.13 *E. coli* field isolates virulence genes



Virulence microarray analysis performed on the *E. coli* field isolates with pCT like plasmids using Genespring with values adjusted in line with the controls, humans shown in red, cattle in blue and turkeys in orange. Squares filled in black indicate the presence of the gene, *ihfA* and *dnaE* are both positive controls, which are shown below.

2/21 having three, all had RFLP profiles >75% similarity, IncK replicon, the same *nikB* sequence and *bla*_{CTX-M-14} environment. The plasmids were identified in unrelated *E. coli* isolates with 17/19 clusters having pCT-like plasmids, which were distributed across England and Wales. Liebana *et al* (2006) reported that pCT was transmissible having spread between *E. coli* isolates on a cattle farm, being found in 9 and 18 unrelated isolates over three visits, which is consistent with these findings (Liebana *et al.*, 2006). The initial study conducted by Cottell *et al* (2011) identified pCT in cattle in the UK, but not in any human isolates which may have been the result of a small testing pool, however in the study it was reported that pCT-like plasmids were present in human isolates from China, Australia and Spain (Cottell *et al.*, 2011). As seen in this study pCT-like plasmids were not only present in cattle in the UK, but have also been identified in isolates from turkeys and importantly humans in the UK (Stokes *et al.*, 2012). At a similar time to this study, Dhanji *et al* (2012) were investigating the presence of pCT plasmids in the UK human population finding it in 19% (13/67) of isolates of which 11 were unrelated and belonged to the A (n=6) and B1 (n=7) phylogroups (Dhanji *et al.*, 2012). Studies have shown that pCT-like plasmids are not only epidemic in the UK but are globally pandemic with conjugation being the likely mechanism for the successful dissemination of pCT and consequently *bla*_{CTX-M-14}. The limited number of related *E. coli* with these plasmids suggests that clonal dissemination is not important in spreading pCT, in contrast *bla*_{CTX-M-15} the most dominant CTX-M in the UK which is typically associated with clonal *E. coli* O25:ST131, present in both humans and companion animals (Lau *et al.*, 2008; Nicolas-Chanoine *et al.*, 2008; Ewers *et al.*, 2010).

pCT-like plasmids were present in diverse and virulent phylogroups B2 (n = 5) and D (n = 6) which are associated with ExPEC infections in humans (Johnson *et al.*, 2003; Kanamaru *et al.*, 2006). These phylogroups have been found in farm animals, and have been associated with *bla*_{CTX-M-14} genes, which further supports the possible transmission between humans and animals (Moulin-Schouleur *et al.*, 2007; Pitout *et al.*, 2005; Sidjabat

et al., 2009; Wu *et al.*, 2012; Valat *et al.*, 2012). Virulence genes associated with increased serum survival and iron utilization were prevalent in isolates from humans and turkeys while ABC transporters were found in cattle isolates, which have been observed in animal isolates previously (Wu *et al.*, 2012). The pCT-like plasmids was identified in an isolate with the genetic characteristics of a EHEC isolate from cattle, the serotype of this isolate was not determined but there is a high prevalence of EHEC O157 shed from cattle in the UK and has been found in beef products (Low *et al.*, 2005; Chapman *et al.*, 2001). In addition to *bla*_{CTX-M-14} other resistance genes present in the field isolates, which included aminoglycosides and chloramphenicol resistance genes present in human and cattle isolates but not in turkeys, which out of all isolates had the fewest resistance genes. The multidrug resistance genes profiles of these isolates suggest that co-selection through treatment with different antimicrobials may occur with these isolates, complicating treatment options in the clinic and veterinary environments.

Animals have been found to be reservoirs for resistance, and increasingly for ESBL genes, which is likely to be the case with pCT-like plasmids in the UK, being found in 40% of unrelated animal isolates from two food producing animal sources (Smet *et al.*, 2010a; Carattoli, 2008). The transmission of resistance between humans and animals may occur by several methods including direct contact, consumption of contaminated food or through contamination of the environment such as water sources. Only within the last few years have studies appeared looking at the transmission of CTX-M plasmids between animal and human isolates, with reports coming from Europe and Asia. In Denmark IncN plasmids have been shown to have a role in the dissemination of *bla*_{CTX-M-1} between *E. coli* on pig farms, between pigs and farm workers (Moodley and Guardabassi, 2009). IncN plasmids have also been found to have a role in the Czech Republic disseminating *bla*_{CTX-M-1} plasmids between cattle, horses, pigs and humans as determined by IncN pMLST and RFLP (Dolejska *et al.*, 2011a; Dolejska *et al.*, 2011c; Dolejska *et al.*, 2013; Garcia-Fernandez *et al.*, 2011). The *bla*_{CTX-M-1} has also been found to be spread by IncI γ

plasmids between poultry and human *E. coli* and *Salmonella* isolates (Cloeckaert *et al.*, 2010; Girlich *et al.*, 2007; Leverstein-van Hall *et al.*, 2011), this CTX-M and plasmid combination is also present in broilers in the UK (Randall *et al.*, 2011). While IncK plasmids have a role in the dissemination of *bla*_{CTX-M-14} in the UK and Europe, in Asia the IncFII plasmid type appears to have a dominant role in the dissemination of *bla*_{CTX-M-14} (Kim *et al.*, 2011; Ho *et al.*, 2012). The transmission of *bla*_{CTX-M-14} IncFII pHK01 between animals and humans was the dominant mechanisms of dissemination in Hong Kong with pHK01-like plasmids identified in 36.2% of animals and 39.2% of humans while Inc11 γ was found in 20.7% of animals and 11.8% of humans, and of note IncK plasmids were also identified in this study (Ho *et al.*, 2011a; Ho *et al.*, 2012).

The impact of antibiotic usage in animals and its effect on the rise of resistance in man has been documented and recommended that their use should be tightly regulated (Teale, 2002; Hunter *et al.*, 2010; Smet *et al.*, 2010a). The therapeutic use of cephalosporins such as ceftiofur has been shown to select for ESBL isolates in pigs and poultry, and the withdrawal of antibiotic treatment reduced the numbers of resistance isolates (Cavaco *et al.*, 2008; Dutil *et al.*, 2010). The presence of resistance isolates and plasmids in food producing animals increases their numbers at both the farm level and in the abattoir, increasing the risk of food contamination (Horton *et al.*, 2011; Randall *et al.*, 2011; Smet *et al.*, 2008). The study by Leverstein-van Hall *et al.* (2011) showed the complete flow from farm to fork, using pMLST and other techniques to find similar Inc11 γ plasmid in the food chain (Leverstein-van Hall *et al.*, 2011). Food has been associated with ESBL isolates with *bla*_{CTX-M-1} identified on beef and turkey meat samples, and *bla*_{CTX-M-14} on chicken (Jouini *et al.*, 2007). Meat imported into the UK has been associated with CTX-M-2 and -8 which are uncommon CTX-M's in the UK but are prevalent in South America the source of the imported meat; also chicken meat of unknown origin was found to have *bla*_{CTX-M-14} (Dhanji *et al.*, 2010; Warren *et al.*, 2008). Although the likely route of transmission of resistance between animals and humans is through the food chain, there is

also the possibility that resistance could be transmitted from humans to animals. Multidrug resistance integrons have been found in higher numbers among companion animals and intensively reared farm animals than wild animals, suggesting that isolates move from humans where they have a high prevalence to animals (Escobar-Paramo *et al.*, 2006). Scavenging animals such as seagulls have been found to harbor the *bla*_{TEM-52}, CTX-M-1, CTX-M-15 and CTX-M-14 genes in France and Portugal, and isolates from those in France matched sequence types and phylogroups with those found in humans (Poeta *et al.*, 2008; Bonnedahl *et al.*, 2009). CTX-M-9-producing *E. coli* belonging to the pandemic clonal strain O25:ST131 were recovered from feral rats in Germany, which is likely to have been acquired through human shedding into the environment such as water sources which in turn contaminates the land that farm animals and crops are grown (Guenther *et al.*, 2010; Dhanji *et al.*, 2011b; Zarfel *et al.*, 2013).

The pCT molecular markers proved to be highly beneficial tools in the study of the epidemiology of plasmids. In this study alone they have identified the presence of plasmids in isolates of human and animal origin supporting their transmission. Of the 37 non CTX-M-14 isolates tested only three had one pCT marker, with four isolates having two markers. This supports the association of the IncK, pCT plasmid backbone with the *bla*_{CTX-M-14} which appears to be a successful combination. Dhanji *et al* (2012) noted that of the non IncK isolates tested, the *pilN*, *nikB* and sigma factor were present in nine isolates, this study also commented on the absence of shufflon recombinase which was said to be specific to pCT, however we found it in six non-CTX-M-14 isolates (Dhanji *et al.*, 2012; Cottell *et al.*, 2011). The use of plasmid molecular markers has enabled the identification of plasmid dissemination between human and animal isolates in the UK. Although the association of these plasmids could have been made over a lengthy comparative process, pCT markers in this study gave an initial method which was built upon and the relationship of plasmids further investigated by other molecular techniques, which has been undertaken by others (Stokes *et al.*, 2012; Dhanji *et al.*, 2012). If these markers had been published

earlier the pandemic spread of this plasmid could have been identified, and possibly tracked, observing the movement of plasmids between Spain, China, Australia, the Netherlands and the UK (Valverde *et al.*, 2009; Chanawong *et al.*, 2002; Zong *et al.*, 2008; Liebana *et al.*, 2006; Hopkins *et al.*, 2006). Just as pMLST schemes have further improved the way in which researchers study plasmids there is a place for plasmid molecular markers such as those designed for pCT and future markers developed in studying plasmids.

Chapter 4

**Sequencing of the first IncZ Plasmid
with *bla*_{CTX-M-14} from an Human Isolate
and the Development of Molecular
Markers for Incompatibility groups
IncZ, B and K**

4.1 Introduction

The *bla*_{CTX-M-14} gene is becoming one of the dominant CTX-M genes in Europe and Asia being found in both human and animal *E. coli* isolates (Chanawong *et al.*, 2002; Kim *et al.*, 2011; Valverde *et al.*, 2009). This ESBL encoding gene has been associated with several plasmid vectors, with IncFII and IncK groups being frequently identified (Cottell *et al.*, 2011; Valverde *et al.*, 2009; Kim *et al.*, 2011; Ho *et al.*, 2012). The full sequence of the *bla*_{CTX-M-14} IncFII plasmids pTN48 and pHK01, IncL/M pJEG011 and the IncK pCT plasmids have aided the understanding of the backbone and genetic characteristics of these vectors (Billard-Pomares *et al.*, 2011; Ho *et al.*, 2011a; Espedido *et al.*, 2013). These vectors have been found to be both multidrug resistant and virulent such as the pTN48 plasmids, where as other others have few or no virulence genes, with *bla*_{CTX-M-14} being the only resistance genes as in pCT.

The full sequencing of pCT, was used to design PCR molecular markers to identify pCT-like plasmids (Cottell *et al.*, 2011). The application of these markers found pCT-like plasmids to be present in Asia, Australia, Spain and throughout the UK in isolates from humans, turkeys and cattle (Cottell *et al.*, 2011; Stokes *et al.*, 2012; Dhanji *et al.*, 2012). Plasmid pCT contained the *bla*_{CTX-M-14} resistance gene only, and no known virulence genes were identified, and the authors commented on the absence of notable addiction systems within the plasmid (Cottell *et al.*, 2011). As this plasmid backbone had been found to disseminate successfully with the absence of addiction systems, it was speculated that the *pil* locus may play a role in the persistence in environments such as slurry due to the absence of selective pressure (Cottell *et al.*, 2011).

The application in the UK of the molecular markers to identify pCT-like plasmids (chapter 3), identified a *bla*_{CTX-M-14} plasmid, pMSH6, and termed pH19 in this chapter, from a human *E. coli* isolate which had similar characteristics to pCT (Stokes *et al.*, 2012). Plasmid pH19 was found to have two of the four pCT markers (sigma and *pilN*), and

despite being a similar size to pCT had only a 65% relationship by RFLP, had an alternative *nikB* allele and the IncK replicon was absent.

The IncK pCT plasmid belongs to the incompatibility group I complex of plasmids which includes IncI γ , K, B and Z, which are all regulated by RNA transcripts, controlling copy number and replication (Praszkier *et al.*, 1991; Nikoletti *et al.*, 1988). Although the *repA* of IncI γ , K and B are closely related, the product of the *repA* gene of IncZ shares higher similarity with the replication proteins of IncFII plasmids (Kato and Mizobuchi, 1994). Members of the IncI complex have been associated with CTX-M genes, particularly IncI γ with group 1 CTX-Ms *bla*_{CTX-M-1} and ₃ (Dahmen, Haenni and Madec, 2012; Dhanji *et al.*, 2011d). IncK plasmids have been associated with the group 9 CTX-Ms being carried by pCT-like vectors (Stokes *et al.*, 2012; Dhanji *et al.*, 2012; Cottell *et al.*, 2011). Just as with IncI γ and IncK, the IncB plasmids have also been found to be associated with several β -lactamases, in both human and animal isolates. IncB plasmids have been found with *bla*_{TEM-1}, *bla*_{ACC-4} and *bla*_{SCO-1} in humans, while *bla*_{CMY-2} and *bla*_{CTX-M-1} genes have been found in animal isolates (Kunne *et al.*, 2012; Papagiannitsis *et al.*, 2011; Rodriguez *et al.*, 2009; Hiki *et al.*, 2013).

The IncK pCT plasmids have already been found in both animal and human isolates. However the pH19 identified in screening did not fit the exact profile of pCT, which may restrict its dissemination. The similarities between pCT and pH19 warranted further investigation, to determine the relationship with pCT and other plasmids, and to see if possible sources could be identified for this plasmid. The limited sequence data on plasmids associated with *bla*_{CTX-M-14} required the full sequencing of pH19, which would be used to determine the genotype and would further add to the knowledge base for ESBL vectors.

4.1.1 Hypotheses and aims

Due to the similarities with pCT, it was hypothesised that pH19 (pMSH6) may be distantly related, and may share a common ancestor or be another plasmid belonging to the IncI complex, and that similar plasmids can be differentiated using molecular markers. The aims of this study were to (i) extract the pH19 plasmid DNA from the H19 isolate, sequence and annotate the plasmid to determine its genotype and features. (ii) Compare plasmid pH19 with other plasmid previously sequenced, to determine the relationship between plasmids. (iii) Design molecular markers capable of identifying pH19 and differentiating between similar plasmids, which can be used as epidemiological tools. (iv) Screen a panel of *E. coli* field isolates for the prevalence of pH19 and similar plasmids, and to identify a possible reservoir.

4.2 Methods and materials

The H19 *E. coli* field isolate was isolated from a human urine sample submitted from general practice, to Public Health Wales in 2007, and was identified during the pCT-like plasmid screening covered in chapter 3 (Stokes *et al.*, 2012). The sequence type of H19 was determined using MLST as described in 2.3.3. Total DNA was extracted from H19 using the QIAGEN Hi-Speed plasmid midi prep as described in 2.2.4.3, which was then transformed into *E. coli* DH10B (2.2.5). Transformants were checked for the presence of a single CTX-M plasmid by plasmid profiling as described in 2.3.12.2 and using the group 9 CTX-M PCR (2.3.5). The antimicrobial sensitivities of the H19 field isolate and the pH19 transformant were determined by disc diffusion as described in 2.1.3. Plasmid content and sizes were determined by S1 nuclease for both the H19 field isolate and pH19 transformant (2.3.12.1), and replicons were determined using a commercial kit for the field isolate (2.3.6.2) and by PBRT for the transformant using the IncB and IncK primers as described in 2.3.6.1. The transferability of pH19 was determined by liquid conjugation as stated in 2.1.4.1. Plasmid DNA for sequencing was extracted from the

pH19 transformant using the QIAGEN large construct kit following the protocol (2.2.4.4), performed by M. AbuOun and H. Preedy. pH19 was sequenced on a Roche 454 GS FLX (2.4.1) with closure of the sequence by PCR using the primers in Table 2.12, using the conditions in 2.4.3.1. The plasmid was annotated using several programs including RAST (2.5.1.1), Artemis (2.5.1.2) and BLASTn using the annotations from plasmids pCT (FN868832), pHUSEC41-1 (HE603110), pR3521 (GU256641), pO26-Vir (FJ386569) and pPWD4_103 (HQ114284) as described in 2.5.1.3. The genetic environment of the *bla*_{CTX-M-14} gene in both the field isolate and transformant were tested by PCR as stated in 2.3.10. Plasmids from GenBank were compared with pH19 using MAUVE (2.5.2.1), ACT (2.5.2.2) and BLASTn (2.5.1.3) to determine the relationship between plasmids and identify potential molecular marker candidates. The phylogeny of the plasmids was analysed using concatenated sequence compiled from open reading frames present in all plasmids which was analysed as described in 2.5.2.5. The screening of the pH19 molecular markers in 2.3.8, Table 2.7 were performed against plasmids from GenBank *in silico* as described in 2.5.3.1, with the phylogeny of the markers analysed as stated in 2.5.2.5. The pCT molecular markers (2.3.7.1; Table 2.4) were also tested *in silico* against the GenBank plasmids as described in 2.5.3.1. The pH19 markers were screened against a panel of 136 ESBL *E. coli* isolates from the SFE collection (appendix II) which included 98 group 1 CTX-M isolates from cattle (n=27), chicken (n=29), humans (n=27) and turkeys (n=15) and 38 group 9 CTX-M isolates from cattle (n=19), humans (n=9) and turkeys (n=10). DNA was extracted by crude lysis (2.2.3.1) and PCR's run as stated in 2.3.8 (Table 2.7) using *E. coli* DH10B and *S. Typhimurium* 26R as negative controls and pH19 transformant, H19 field isolate, pCT transformant and pCT field isolate as positive controls.

4.3 Results

4.3.1 Analysis of the H19 *E. coli* field isolate

4.3.1.1 Reduced antimicrobial sensitivities of H19

Antimicrobial sensitivities were determined by the disc diffusion method with the H19 field isolate being found to have reduced susceptibilities to nalidixic acid, tetracyclines, neomycin, ampicillin, cefotaxime, ceftazidime, chloramphenicol, streptomycin, sulphonamides and ciprofloxacin. This result implies that H19 encodes for multiple drug resistance and could complicate treatment options and persist by co-selection.

4.3.1.2 Genotypic analysis of H19

The H19 isolate was analysed by antimicrobial resistance (AMR) and virulence DNA microarray, which supported the phenotype with the resistance genes *cmlA1*, *aac6-1b*, *dfrA17*, *catA1*, *strA*, *strB*, *tetB*, *bla_{TEM-1}* and *bla_{CTX-M-G9}* being identified. H19 was found to belong to the ST602 which is part of the ST446 complex.

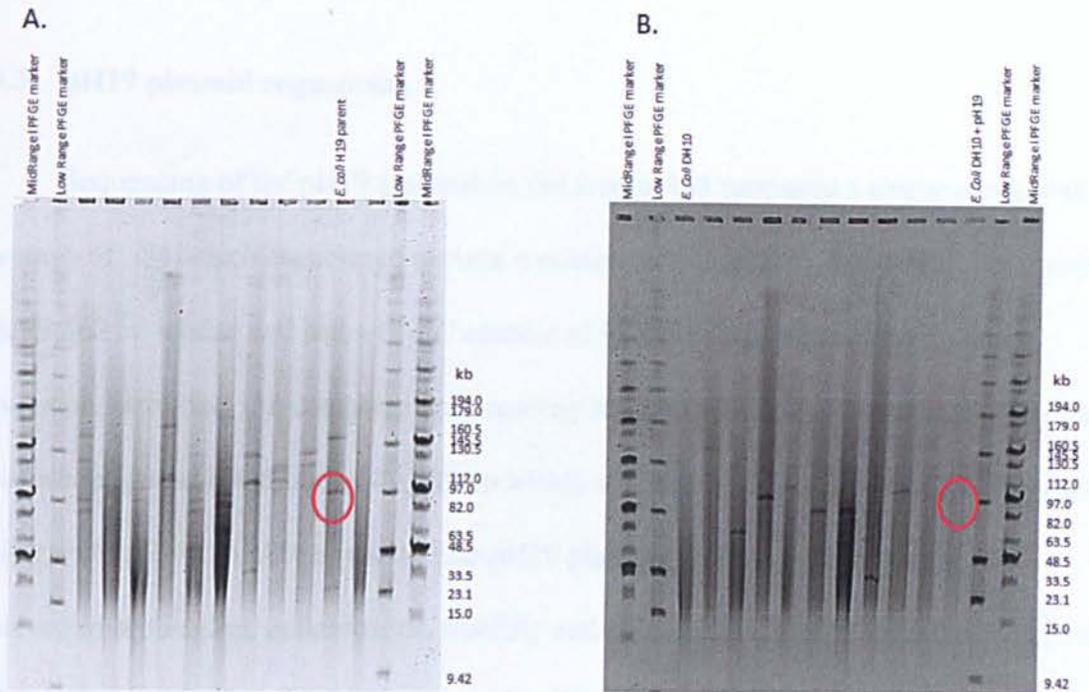
4.3.1.3 Plasmid analysis of H19

The H19 was found to have multiple replicon types including B/O, FIB, A/C and FII, determined using the commercial PBRT kit. However only three plasmids were identified by S1 nuclease with sizes of 25kb, 97kb and 150kb, as shown in Figure 4.1.

4.3.2 Analysis of the pH19 plasmid

The extracted plasmid DNA for pH19 was successfully transformed into *E. coli* DH10 (DH10+pH19), which was confirmed by PCR and plasmid profiling. The pH19 transformant was tested for its antimicrobial sensitivities as previously for the field isolate, and was found to confer resistance to neomycin, ampicillin, cefotaxime, ceftazidime and chloramphenicol. This result suggests that the resistance for nalidixic acid, tetracyclines,

Figure 4.1 S1 Nuclease PFGE sizing of H19 *E. coli* field isolate plasmids and pH19



S1 nuclease PFGE of (A) the *E. coli* H19 field isolate and (B) transformed *E. coli* DH10 with pH19, *E. coli* DH10 used for transformations as a negative control, the pH19 plasmid is circled in red. Plasmid digested with 8U of S1 nuclease for 45 minutes, DNA bands were resolved 1% agarose gel. MidRange and Low Range PFGE markers were used for sizing.

streptomycin, sulphonamides and ciprofloxacin were from genes either present on the genome of H19 or carried on other plasmids. Replicon typing performed using the PBRT identified the pH19 as an IncB plasmid, and sizing determined the plasmid to be approximately 95kb corresponding to the 97kb plasmid in the field isolate (Figure 4.1).

4.3.2.1 Transferability of pH19

The plasmid had previously been shown to conjugate from the field isolate (Chapter 3) but to ensure that the plasmid was self-transmissible, conjugations were carried out using the pH19 transformant. Plasmids were transferred by liquid conjugations using a rifampicin resistant *S. Typhimurium* 26R as the recipient, conjugations were considered to have occurred by the presence of red colonies on Rambach agar supplemented with 100 µg/ml rifampicin and 1 µg/ml cefotaxime, with the recipient only used as a negative

control. Single red colonies were selected and inoculated further on LB-G agar supplemented with 4 µg/ml cefotaxime.

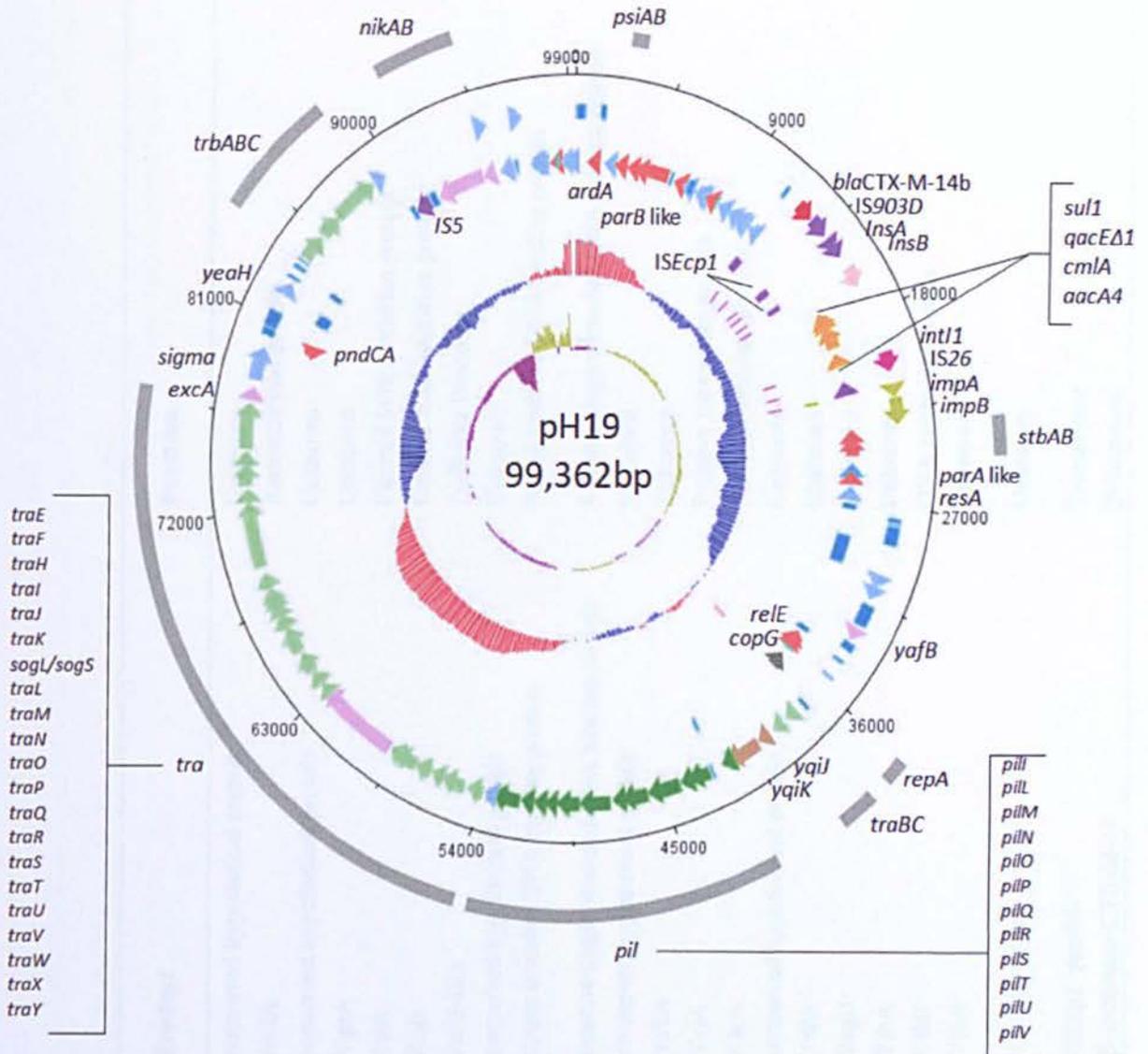
4.3.3 pH19 plasmid sequencing

Sequencing of the pH19 plasmid on the Roche 454 produced a single contig with a coverage of >74 which was closed to form a continuous sequence. The complete sequence was 99,362 bp in size and had a G + C content of 52.76%. Sequence analysis and annotation identified 136 complete open reading frames (orf), with the plasmid being made up of a contiguous backbone of 89,089 bp which contains a 10,273 bp multidrug resistant region with a G + C 51.8% content. The pH19 plasmid backbone contained genes involved in replication, maintenance, stability and conjugation, as shown in Figure 4.2, a full gene list can be found in Table 4.1, and will be discussed in detail below.

4.3.3.1 Analysis of the pH19 replication region

The replication region of the pH19 shares close similarity with that of IncZ plasmids pECOED and pEI545 (Praszkier *et al.*, 1991; Touchon *et al.*, 2009). This contradicts the IncB plasmid result from the replicon typing performed on the transformant. Despite the overall sequence being similar to IncB and IncK plasmids, the *repA* gene produces a protein 292 amino acids in length, sharing 99% identity with the RepA from pECOED and pEI545 and 100% identity with the RepA proteins from the IncFII plasmid group (Kato and Mizobuchi, 1994; Praszkier *et al.*, 1991; Touchon *et al.*, 2009). The pH19 plasmid has a truncated RNAII with only 726/790 bp present in the region used for replicon typing of IncB plasmids by Carattoli *et al.* (2005a), which is also found in pR3521, pO26-vir, and pMU707 (Carattoli *et al.*, 2005a; Papagiannitsis *et al.*, 2011; Fratamico *et al.*, 2011).

Figure 4.2 Plasmid map of pH19



Circular plasmid map of pH19. Shown are the coding regions in the pH19 plasmid (complemented in Table 4.1) with the outer arrowed circle denoting the positive orientation and the inner arrow circle denoting the minus orientation strand. Green and pink arrows indicate genes involved in conjugation, with pale green for *tra* genes and bright green for *pil* genes. Purple indicate insertion sequences and mobile elements while shades of orange indicate resistance genes. Red arrows apply to genes associated stability and plasmid maintenance. Pale blue arrows indicate putative genes or those named but without known function, cyan blue arrows indicate hypothetical genes. The outer graph shows the GC plot and the inner graph shows the GC skew.

Table 4.1 Open reading frames present in pH19

Gene	ORF	Direction	Location	Length (bp)	Protein length (aa)	Product	Function
<i>orf1</i>	1	Forward	21-518	498	166	conserved hypothetical protein	Unknown
<i>ardA</i>	2	Reverse	634-1134	501	167	ArdA	Antirestriction protein
<i>orf3</i>	3	Forward	1176-1430	255	85	conserved hypothetical protein	Unknown
<i>ygaA</i>	4	Reverse	1596-2192	597	199	YgaA	Unknown
<i>psiA</i>	5	Reverse	2189-2908	720	240	PsiA	Plasmid SOS inhibition protein
<i>psiB</i>	6	Reverse	2905-3342	438	146	PsiB	Plasmid SOS inhibition protein
<i>parB-like</i>	7	Reverse	3394-5358	1965	655	ParB-like	ParB-like Homolog
<i>orf8</i>	8	Reverse	5411-5644	234	78	conserved hypothetical protein	Unknown
<i>ssb</i>	9	Reverse	5702-6229	528	176	single stranded DNA binding protein	Single stranded DNA binding protein
<i>putative ssb</i>	10	Reverse	6226-6432	207	69	putative single stranded DNA binding protein	Putative single stranded DNA binding protein
<i>orf11</i>	11	Reverse	6591-6836	246	82	conserved hypothetical protein	Unknown
<i>ycaA</i>	12	Reverse	6999-7190	192	64	YcaA	Unknown
<i>ycjA</i>	13	Reverse	7187-7609	423	141	YcjA	Putative membrane protein
<i>klcA</i>	14	Reverse	7656-8081	426	142	KlcA	Stable inheritance protein
<i>orf15</i>	15	Reverse	8329-8496	168	56	conserved hypothetical protein	Unknown
<i>yfdA</i>	16	Reverse	8496-9110	615	205	YfdA	Unknown
<i>ycgB</i>	17	Reverse	9155-9589	435	145	YcgB	Unknown
<i>yfcA</i>	18	Reverse	9603-9824	222	74	YfcA	Unknown
<i>yfbE</i>	19	Reverse	9825-10508	684	228	YfbE	DNA methylase protein
<i>yfbA</i>	20	Reverse	10585-10889	305	NA	YfbA	Unknown
<i>yfaB truncated</i>	21	Reverse	10893-11114	222	NA	YfaB truncated	Unknown
<i>ISEcp1 partial</i>	22	Forward	11115-11607	493	NA	ISEcp1 partial	Transposase
<i>bla_{CTX-M-14}</i>	23	Forward	11857-12732	876	292	β -lactamase CTX-M-14	β -lactamase

Gene	ORF	Direction	Location	Length (bp)	Protein length (aa)	Product	Function
<i>IS903D</i>	24	Forward	12767-13735	969	323	IS903D transposase	Transposase
<i>ISEcp1 partial</i>	25	Reverse	13792-14223	432	144	ISEcp1 partial	Transposase
<i>insA</i>	26	Forward	14279-14554	276	92	InsA	Transposase
<i>insB</i>	27	Forward	14473-14976	504	168	InsB	Transposase
<i>ISEcp1 partial</i>	28	Reverse	14992-15347	356	NA	ISEcp1 partial	Transposase
<i>orf29</i>	29	Forward	15548-16579	1032	344	Methyl accepting aerotaxis	Putative methyl accepting domain protein
<i>sul1</i>	30	Reverse	16732-17064	246	82	Sul1 truncated	Sulphonamide resistance
<i>qacE1</i>	31	Reverse	16971-17318	348	116	Quaternary ammonium compound resistance	Putative Quaternary ammonium resistance
<i>cmlA</i>	32	Reverse	17510-18769	1260	420	Chloramphenicol resistance	Chloramphenicol resistance
<i>aac6</i>	33	Reverse	19036-19590	555	185	Aminoglycoside resistance	Aminoglycoside resistance
<i>int1</i>	34	Forward	19770-20729	960	320	Integrase Int1	Integrase
<i>IS26</i>	35	Reverse	20620-21339	720	240	IS26 transposase	Transposase
<i>impC truncated</i>	36	Forward	21388-21507	120	NA	ImpC truncated	DNA/UV repair
<i>impA</i>	37	Forward	21504-21941	438	146	ImpA DNA repair	DNA/UV repair
<i>impB</i>	38	Forward	21941-23215	1275	425	ImpB	DNA/UV repair
<i>stbB</i>	39	Reverse	23217-23633	417	139	StbB	Partitioning protein
<i>stbA</i>	40	Reverse	23626-24660	1035	345	StbA	Partitioning protein
<i>ybiA</i>	41	Reverse	25020-25328	309	103	YbiA	Putative chaperonin
<i>parA-like</i>	42	Reverse	25415-26092	678	226	ParA-like	ParA homolog
<i>resA</i>	43	Reverse	26239-27018	780	260	Resolvase	Site specific resolvase
<i>orf44</i>	44	Reverse	27184-27471	288	96	conserved hypothetical protein	Unknown
<i>orf45</i>	45	Forward	27546-27740	195	65	hypothetical protein	Unknown
<i>orf46</i>	46	Forward	27779-27898	120	40	hypothetical protein	Unknown
<i>orf47</i>	47	Forward	27955-28818	864	288	conserved hypothetical protein	Unknown

Gene	ORF	Direction	Location	Length (bp)	Protein length (aa)	Product	Function
<i>orf48</i>	48	Reverse	28843-30342	1500	500	conserved hypothetical protein	Unknown
<i>ydfA</i>	49	Forward	30610-30864	255	85	YdfA	Unknown
<i>ydeA</i>	50	Forward	30864-31454	591	197	YdeA	Unknown
<i>orf51</i>	51	Forward	31667-32719	1053	351	conserved hypothetical protein	Unknown
<i>yafB</i>	52	Forward	32895-33497	603	201	YafB	Conjugation repressor FinO
<i>orf53</i>	53	Forward	33588-33743	156	52	hypothetical protein	Unknown
<i>orf54</i>	54	Forward	33743-34009	267	89	conserved hypothetical protein	Unknown
<i>orf55</i>	55	Reverse	34200-34475	276	92	conserved hypothetical protein	Unknown
<i>orf56</i>	56	Forward	34504-34620	117	39	conserved hypothetical protein	Unknown
<i>relE</i>	57	Reverse	34581-34856	276	92	RelE toxin-antitoxin	RelE toxin-antitoxin
<i>copG</i>	58	Reverse	34856-35134	279	93	CopG transcriptional regulator	Transcriptional regulator
<i>orf59</i>	59	Reverse	35183-35350	168	56	conserved hypothetical protein	Unknown
<i>repA4</i>	60	Forward	35349-35480	132	44	RepA4	Putative replication protein
<i>repA1</i>	61	Reverse	36039-36917	879	293	RepA1	Replication initiator protein
<i>orf62</i>	62	Forward	37066-37254	189	63	conserved hypothetical protein	Unknown
<i>traB</i>	63	Forward	37620-38261	642	214	TraB	Conjugal transcription antitermination factor
<i>traC</i>	64	Forward	38402-39064	663	221	TraC	Transfer Conjugal protein
<i>yqiJ</i>	65	Forward	39331-39951	621	207	YqiJ	Inner membrane protein - Adhesin like
<i>yqiK</i>	66	Forward	39978-41672	1695	565	YqiK	Inner membrane protein - Adhesin like
<i>pill</i>	67	Forward	41678-41995	318	106	PilI	Conjugative transfer protein
<i>orf68</i>	68	Reverse	42291-42422	132	44	conserved hypothetical protein	Unknown
<i>orf69</i>	69	Forward	42479-42595	117	39	conserved hypothetical protein	Unknown
<i>pilL</i>	70	Forward	42673-43743	1071	357	PilL	Lipoprotein
<i>pilM</i>	71	Forward	43747-44184	438	146	PilM	Pilus biogenesis protein
<i>pilN</i>	72	Forward	44216-45835	1620	540	PilN	Formation outer membrane protein
<i>pilO</i>	73	Forward	45856-47151	1296	432	PilO	Pilin accessory protein

Gene	ORF	Direction	Location	Length (bp)	Protein length (aa)	Product	Function
<i>pilP</i>	74	Forward	47141-47599	459	153	PilP	Pilus biogenesis protein
<i>pilQ</i>	75	Forward	47702-49210	1509	503	PilQ	Pilus biogenesis protein ATPase
<i>pilR</i>	76	Forward	49212-50306	1095	365	PilR	Integral membrane protein
<i>pilS</i>	77	Forward	50368-50904	537	179	PilS	Major pilin subunit
<i>pilT</i>	78	Forward	50949-51434	486	162	PilT	Pilin transglycosylase
<i>pilU</i>	79	Forward	51450-52076	627	209	PilU	Pilus prepilin protein (peptidase)
<i>pilV</i>	80	Forward	52094-53455	1362	454	PilV	Minor pilin subunit
<i>orf81</i>	81	Forward	53689-53841	153	51	putative recombinase	Putative recombinase
<i>traE</i>	82	Forward	53928-54749	822	274	TraE	Conjugal transfer protein
<i>traF</i>	83	Forward	54851-56053	1203	401	TraF	Conjugal transfer protein
<i>traH</i>	84	Forward	56157-56615	459	153	TraH	Conjugal lipoprotein
<i>traI</i>	85	Forward	56612-57448	837	279	TraI	Conjugal lipoprotein
<i>traJ</i>	86	Forward	57432-58580	1149	383	TraJ	Plasmid transfer ATPase
<i>traK</i>	87	Forward	58577-58867	291	97	TraK	Conjugal transfer protein
<i>sogL</i>	88	Forward	58931-62992	4062	1354	SogL	DNA primase
<i>sogS</i>	89	Forward	59120-62992	3873	1291	SogS	DNA primase regulator
<i>traL</i>	90	Forward	63009-63359	351	117	TraL	Conjugal transfer protein
<i>traM</i>	91	Forward	63371-64066	696	232	TraM	Conjugal transfer protein
<i>traN</i>	92	Forward	64077-65051	975	325	TraN	Conjugal transfer protein
<i>traO</i>	93	Forward	65115-66386	1272	424	TraO	Conjugal transfer protein
<i>traP</i>	94	Forward	66383-67096	714	238	TraP	Conjugal transfer protein
<i>traQ</i>	95	Forward	67093-67623	531	177	TraQ	Conjugal transfer protein
<i>traR</i>	96	Forward	67670-68068	399	133	TraR	Conjugal transfer protein
<i>traS</i>	97	Forward	68125-68376	252	84	TraS	Transfer protein
<i>traT</i>	98	Forward	68396-69109	714	238	TraT	Conjugal transfer protein
<i>traU</i>	99	Forward	69404-72448	3045	1015	TraU	Nucleotide binding protein

Gene	ORF	Direction	Location	Length (bp)	Protein length (aa)	Product	Function
<i>traV</i>	100	Forward	72448-73068	621	207	TraV	Conjugal transfer protein
<i>traW</i>	101	Forward	73191-74231	1041	347	TraW	Conjugal lipoprotein
<i>traX</i>	102	Forward	74228-74797	570	190	TraX	Conjugal transfer protein
<i>traY</i>	103	Forward	74872-77037	2166	722	TraY	Integral membrane protein
<i>excA</i>	104	Forward	77125-77778	654	218	ExcA	Surface exclusion
<i>orf105</i>	105	Forward	78052-79638	1587	529	Sigma factor	Putative sigma factor
<i>pndA</i>	106	Reverse	79776-79928	153	51	PndA	Plasmid stability protein
<i>pndC</i>	107	Reverse	79783-80076	294	98	PndC	Plasmid stability protein
<i>orf108</i>	108	Reverse	80092-80211	120	40	conserved hypothetical protein	Unknown
<i>orf109</i>	109	Forward	80180-80500	321	107	conserved hypothetical protein	Unknown
<i>orf110</i>	110	Forward	80588-80779	192	64	conserved hypothetical protein	Unknown
<i>orf111</i>	111	Forward	80791-81159	369	123	conserved hypothetical protein	Unknown
<i>orf112</i>	112	Forward	81163-81378	216	72	conserved hypothetical protein	Unknown
<i>orf113</i>	113	Reverse	81576-82199	624	208	conserved hypothetical protein	Unknown
<i>yeaA</i>	114	Forward	82357-82824	468	156	YeaA	Putative endonuclease
<i>orf115</i>	115	Forward	82974-83150	177	59	conserved hypothetical protein	Unknown
<i>orf116</i>	116	Reverse	83213-83509	297	99	conserved hypothetical protein	Unknown
<i>orf117</i>	117	Forward	83530-83715	186	62	conserved hypothetical protein	Unknown
<i>orf118</i>	118	Forward	83879-84010	132	44	conserved hypothetical protein	Unknown
<i>trbA</i>	119	Forward	84141-85457	1317	439	TrbA	Regulation
<i>trbB</i>	120	Forward	85454-86578	1125	375	TrbB	ATPase
<i>trbC</i>	121	Forward	86559-88862	2304	768	TrbC	Putative prepillin
<i>orf122</i>	122	Forward	88976-89269	294	98	conserved hypothetical protein	Unknown
<i>orf123</i>	123	Forward	89329-89559	231	77	putative iron regulated outer membrane protein	Putative iron regulated outer membrane protein
<i>orf124</i>	124	Reverse	89657-89896	240	80	conserved hypothetical protein	Unknown
<i>IS5 tnpA</i>	125	Reverse	89878-90858	981	327	IS5 transposase	Transposase

Gene	ORF	Direction	Location	Length (bp)	Protein length (aa)	Product	Function
<i>orf126</i>	126	Reverse	90899-91252	354	118	conserved hypothetical protein	putative TonB/TolA
<i>nikB</i>	127	Reverse	91356-94067	2712	904	NikB	Relaxase
<i>nikA</i>	128	Reverse	94079-94498	420	140	NikA	Relaxome component
<i>yggA</i>	129	Forward	94644-94979	336	112	YggA	Putative
<i>ydiA</i>	130	Reverse	95064-95912	849	283	Ydia	Putative
<i>orf131</i>	131	Reverse	95954-96118	165	55	conserved hypothetical protein	Unknown
<i>orf132</i>	132	Forward	96144-96257	114	38	hypothetical protein	Unknown
<i>orf133</i>	133	Forward	96261-96458	198	66	conserved hypothetical protein	Unknown
<i>ygeA</i>	134	Forward	96492-96743	252	84	YgeA	Putative
<i>ydgA</i>	135	Reverse	96774-96995	222	74	YdgA	Putative
<i>orf136</i>	136	Reverse	97019-97909	891	297	putative transposase	Putative transposase
<i>ccgAII</i>	137	Reverse	97906-98370	465	155	CcgAII	CcgAII
<i>orf138</i>	138	Reverse	98279-98398	120	40	conserved hypothetical protein	Unknown
<i>ydfB</i>	139	Reverse	98472-98738	267	89	YdfB	Unknown
<i>ydfA</i>	140	Reverse	98830-99264	435	145	YdfA	PndC like post segregational killing

4.3.3.2 Analysis of the pH19 transfer region

The pH19 plasmid contains a large transfer region of 39,418 bp which is made up of the *tra* (transfer operon) and *pil* (pilus) genes and the smaller (4,721 bp) *trbABC* region (Yoshida, Kim and Komano, 1999; Komano *et al.*, 1990; Komano *et al.*, 2000). The *tra* region is in the same conformation as other plasmids including pR3521, pCT and pECOED. The *traB* and *traC* are followed by the adhesion genes *yqiK* and *yqiJ*, which are upstream of the *pil* locus which includes, *pilI, L, M, N, O, P, Q, R, S, T, U* and *V* (Antao, Wieler and Ewers, 2009; Papagiannitsis *et al.*, 2011). The remaining *tra* locus follows downstream from the *pil* genes, and includes the genes *traE, F, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X,* and *Y*, within the *tra* locus is the *sogL* coding the DNA primase (Merryweather *et al.*, 1986). As with other closely related plasmids there is an absence of the *traG, pilJ* and *pilK* genes. The conjugation regulation genes *trbABC* are separated from the *tra* genes by several hypothetical genes, the surface exclusion gene *excA*, addiction system genes *pndCA* and the endonuclease gene *yeaH* (Furuya and Komano, 1994; Mnif *et al.*, 2010). Other genes involved in plasmid transfer include the *nikBA* genes which form the relaxome for the unwinding of plasmid DNA and *yafB* coding for a FinO conjugation repressor (Furuya, Nisioka and Komano, 1991; Furuya and Komano, 1995).

4.3.3.3 Analysis of the pH19 stability regions

Several mechanisms are present in the plasmid pH19, to aid both its persistence and stability. Contained within the backbone are the *pndCA* genes coding for a post segregation killing system (Mnif *et al.*, 2010), which was complemented by a putative *relE* gene, however the antitoxin *relB* gene was not identified and may be regulated by a hypothetical gene or *copG* which lies adjacent to *relE* (Gotfredsen and Gerdes, 1998). Additional stability genes were identified in the backbone of pH19 which were the stability/partitioning genes *stbA (parM) stbB* and a *parA*-like homologue (Guynet *et al.*,

2011). These genes were contained within what was termed the stability and hypothetical region which was similar to those found in IncI1 γ plasmids and pECOED (CU928147) as shown in Figure 4.3 (Fricke *et al.*, 2011; Touchon *et al.*, 2009). However some of the hypothetical genes differ and the *yagA*, *cib* and *imm* in IncI1 γ plasmids has been replaced by a large hypothetical gene in pH19. The only other report of this hypothetical gene is in the IncI1 γ plasmid pPWD4_103 (HQ114284) downstream of *yafB* (Johnson *et al.*, 2011). Genes aiding persistence and stability of pH19 were identified such as the antirestriction genes *ardA* and *klcA* and the SOS inhibition genes *psiAB* (Delver *et al.*, 1991; Althorpe *et al.*, 1999; Serfiotis-Mitsa *et al.*, 2010).

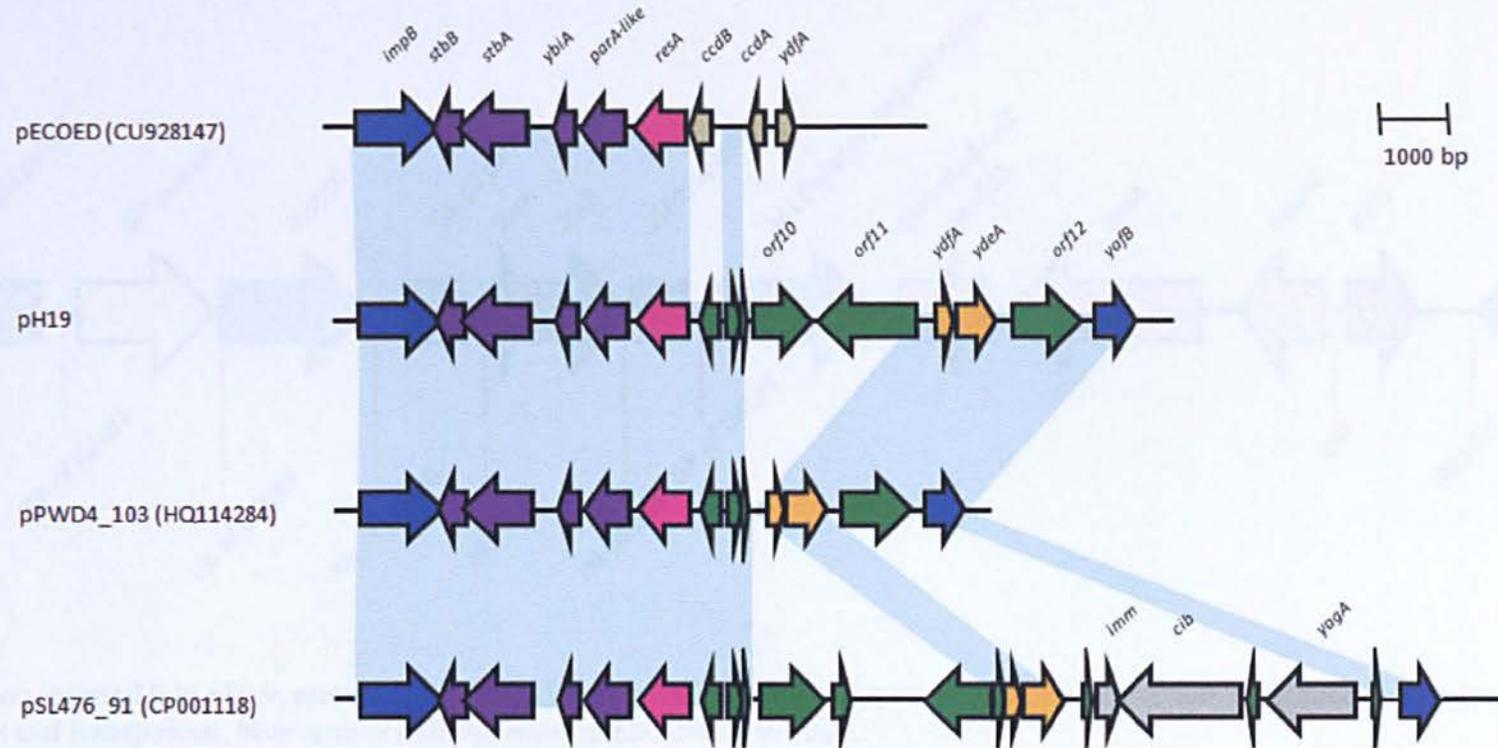
4.3.3.4 Analysis of the pH19 resistance region

All of the resistance genes present in pH19 were clustered in a 10,273 bp resistance region. The region consisted of a *bla*_{CTX-M-14} which was associated with the *ISEcpI* insertion sequence and *IS903D*, which has been previously reported; no *iroN* gene was identified downstream (Poirel, Decousser and Nordmann, 2003; Eckert, Gautier and Arlet, 2006). Downstream of *IS903D* was a methyl accepting aerotaxis protein, which is part of *Tn1721*, this was followed by a class 1 integron which contained the resistance genes *aacA4*, *cmlA*, *qacE Δ 1* which had truncated *sulI* as shown in Figure 4.4.

4.3.3.4.1 The *bla*_{CTX-M-14} gene in pH19

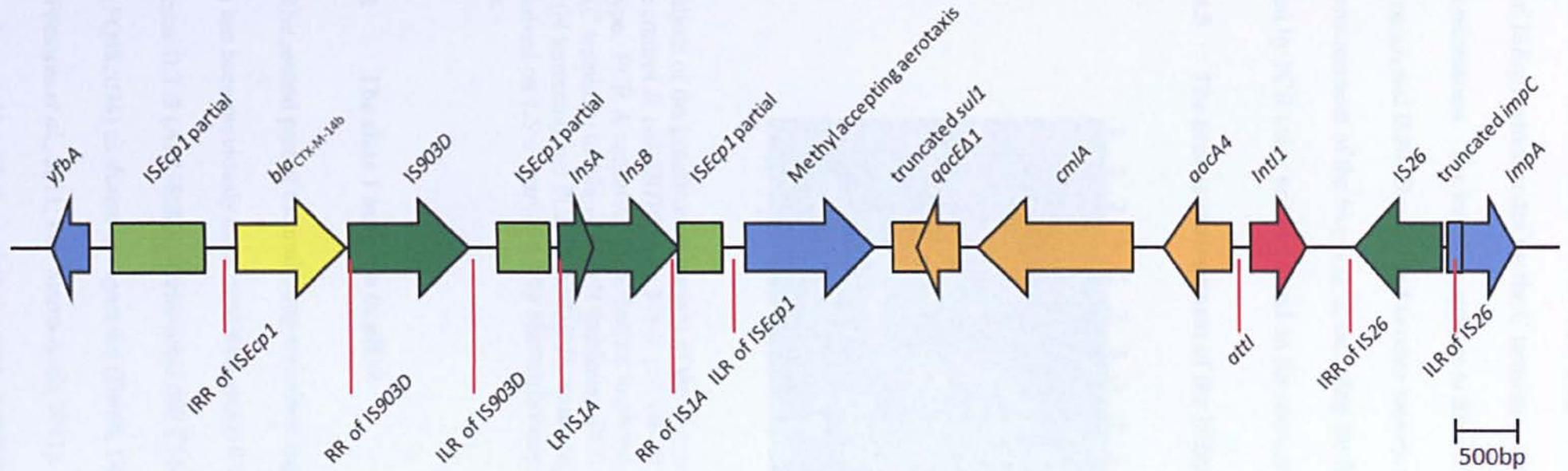
The *bla*_{CTX-M-14} was located 42 bp downstream of the inverted right repeat of the *ISEcpI* transposon, and 2 bp upstream of the inverted right repeat of *IS903D* transposon. Unlike the *ISEcpI-bla*_{CTX-M-14} seen in other isolates and plasmids such as pCT, the *ISEcpI* transposon had been disrupted by the insertion of *IS1* (*insA* and *insB*). The insertion of *IS1* into *ISEcpI* has been observed before in *Escherichia coli* C1635 (JF701188) isolated from a Tunisian hospital (Ben Slama *et al.*, 2011). In C1635 the insertion of *IS1* resulted in *ISEcpI* being fragmented into two sections, and remained in the same orientation (Ben Slama *et al.*, 2011). The insertion of *IS1* into the pH19 *ISEcpI* occurred at the same

Figure 4.3 The stability region in pH19, pECOED and IncI1 plasmids



Comparison of the stability region present in pH19 to that present in the other IncZ plasmid pECOED, and the two IncI1 γ plasmids pSL476_91 and pPWD4_103. Green arrows indicate hypothetical genes, purple arrows indicate stability associated genes, pink indicates resolvase, other genes are indicated by separate colours and labelled. Areas highlighted in blue show homologous regions, drawn to scale.

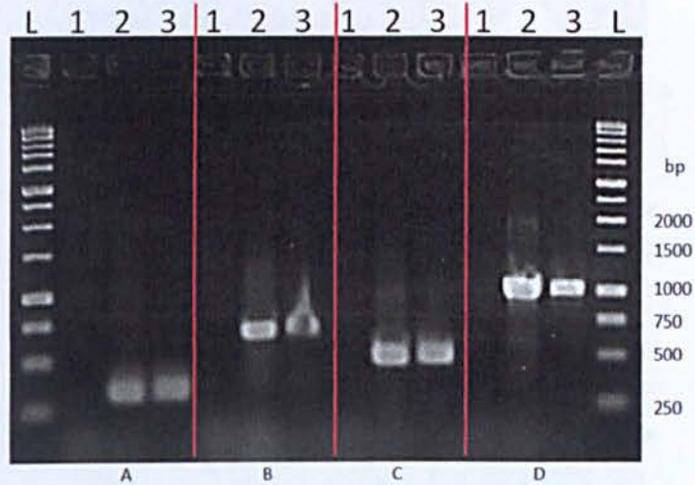
Figure 4.4 The class 1 integron and resistance region in pH19



The resistance region inserted into pH19, arrows indicate ORF's with their orientation, yellow and orange indicate resistance genes, green arrows indicate insertion sequences and transposons, blue arrows indicate other genes, drawn to scale.

position as in C1635 but resulted in *ISEcp1* fragmenting into three sections. A 493 bp section of *ISEcp1* which codes for the C terminus, remains upstream of *bla*_{CTX-M-14} in the negative orientation. The insertion appears to have caused a change in the orientation of the *bla*_{CTX-M-14} and *IS903D* compared to other reports of this genetic environment. The genetic environment of the *bla*_{CTX-M-14}, including the fragmentation of the *ISEcp1* was confirmed by PCR using primers based on the sequenced pH19, shown in Figure 4.5.

Figure 4.5 The genetic environment of the *ISEcp1*-*bla*_{CTX-M-14}-*IS903D* in pH19

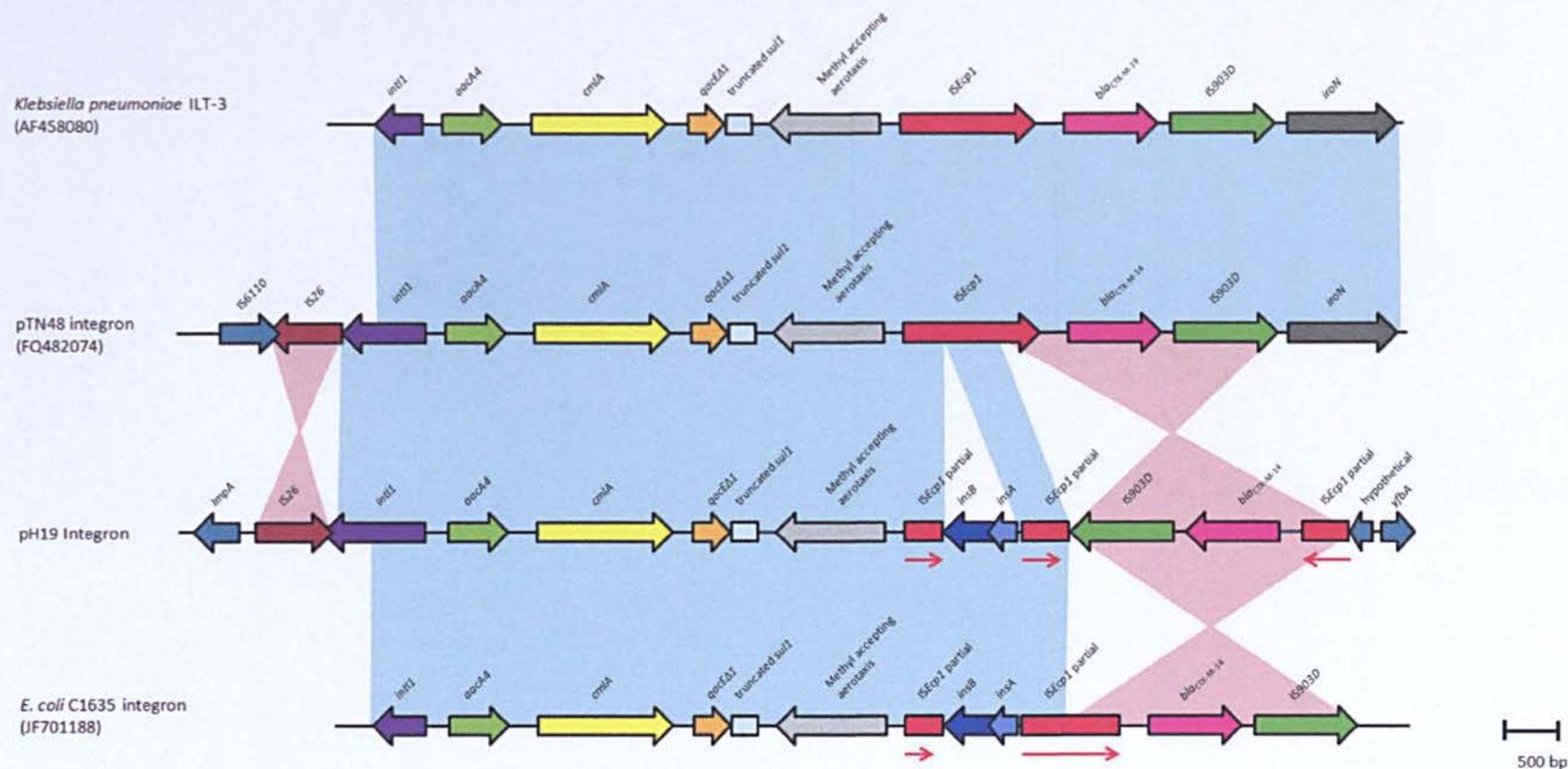


PCR analysis of the genetic environment of the *bla*_{CTX-M-14} gene in pH19. **Lane 1** is the negative control *E. coli* DH10, **lane 2** is *E. coli* DH10 + pH19 and **lane 3** is H19 field isolate type. PCR **A** conserved hypothetical to partial *ISEcp1* (C terminus), PCR **B** partial *ISEcp1* (C terminus) to *bla*_{CTX-M-14} (N terminus), PCR **C** *bla*_{CTX-M-14} (C terminus) to *IS903D* (N terminus) and PCR **D** *IS903D* (C terminus) to partial *ISEcp1*. PCR products were resolved on 1.5% agarose gel by electrophoresis, a 1kb ladder was used as a marker for sizes.

4.3.3.4.2 The class 1 integron in pH19

The second part of the multidrug resistance region is the class 1 integron, a similar integron has been previously associated with group 9 CTX-M genes in *Klebsiella pneumoniae* ILT-3 (AF458080), *Escherichia coli* C1635 and the *bla*_{CTX-M-14} plasmid pTN48 (FQ482074) as shown in Figure 4.6 (Poirel, Decousser and Nordmann, 2003; Billard-Pomares *et al.*, 2011; Ben Slama *et al.*, 2011). The class 1 integron contains the Integrase gene *intI1*, with the resistance gene cassettes *aacA4*, *cmlA1*, *qacEA1* and a *sulI*

Figure 4.6 Comparison of integrons associated with group 9 CTX-M genes



The CTX-M group 9 genes associated with integrons, including that of the *Klebsiella pneumoniae* ILT-3, pTN48, pH19 and *E. coli* C1635. Genes drawn as compared with pH19, boxed arrows indicate the orientation of the gene, square boxes indicate truncated or partial genes, arrows indicate the orientation of partial ISEcp1, blue areas show regions of homology and red shows areas are homology in the reverse orientation, drawn to scale.

gene truncated from the insertion of *qacEΔ1*. Apart from the changes to insertion and inversion of the *ISEcp1* the MDR is identical to that found in ILT-3, C1635 and pTN48. This includes the putative methyl accepting aerotaxis protein located upstream of the truncated *sull*, this protein appears to be linked to the transposon Tn1721. Downstream of the *IntI* in the class 1 integron is IS26 (Figure 4.6). This may be part of the MDR region, but is not found in this orientation with the other class 1 integrons mentioned, as is the case with pTN48 (Figure 4.6). This suggest that the insertion of IS26 occurred after the class 1 integron had inserted, whether it has a role in the mobilization is unknown. The insertion of IS26 occurred upstream of the of *impC* gene causing the loss of the C terminus of *impC*.

4.3.3.5 Analysis of additional MGE and accessory genes in pH19

The pH19 had undergone other insertions which are not related to any resistance or virulence genes. This includes the transposon IS5D (89,878-90,858 bp) identified inserted between two conserved hypothetical genes, both of which were present in other IncI complex plasmids. pH19 also harboured the UV damage DNA repair genes with complete *impB* and *impA*, with truncated *impC* missing the N terminus as result of the insertion of IS26 (Runyen-Janecky, Hong and Payne, 1999). A potential virulence factor in the form of a putative iron regulated outer membrane protein being present at 89,329-89,559 bp (*orf118*) was also identified. A conserved motif for the TonB/TolA protein was identified in a conserved hypothetical gene (90,899-91,252 bp) and may have a role in energy transduction (Keller, Brinkman and Larsen, 2007).

4.3.4 Comparison of pH19 with other plasmids of the IncI complex

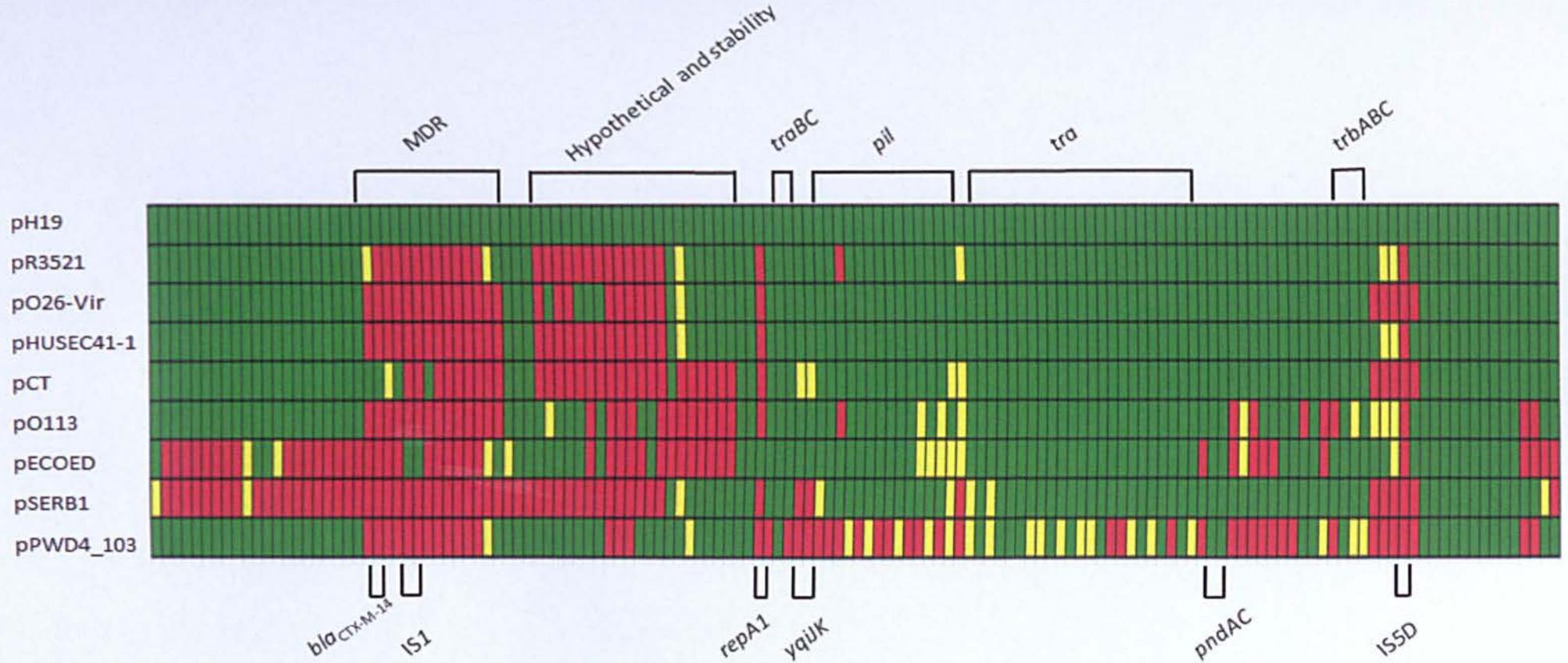
The complete pH19 plasmid sequence was compared to sequences in the GenBank database using the BLASTn algorithm to identify similar plasmids. These searches showed that pH19 had high levels of nucleotide coverage with several plasmids belonging to the IncI complex, including pR3521, pO26-Vir , pHUSEC41-1, pCT, pO113, pECOED, pSERB1, and pPWD4_103, shown in Table 4.2, which were selected for further

Table 4.2 Plasmids compared with pH19

Plasmids	Accession number	% Coverage with pH19	% Coverage with pH19 no MDR	Size (bp)	Replicon type	Bacterial host	Source	Location	Resistance and Virulence	Date of Isolation or submission	Reference
pH19	NA	NA	NA	99,632	Z	<i>E. coli</i>	Human	UK	<i>bla</i> _{CTX-M-14} , <i>cmlA1</i> , <i>aacA4</i> <i>qacE1</i>	2007	NA
pR3521	GU256641	78	85	110,416	B	<i>E. coli</i>	Human	Greece	<i>sul2</i> , <i>strA</i> , <i>strB</i> <i>bla</i> _{TEM-1} <i>bla</i> _{ACC-4} , and <i>bla</i> _{SCO-1}	2002	Papagiannitsis <i>et al</i> (2011)
pO26 -Vir	FJ386569	77	86	168,100	B	<i>E. coli</i>	Human	Canada	<i>toxB</i> , <i>espS</i> , <i>hlyCABD</i>	1977	Fratamico <i>et al</i> (2011)
pHUSEC41-1	HE603110	77	86	91,492	B	<i>E. coli</i>	Human	Germany	<i>sul2</i> , <i>strA</i> , <i>strB</i> and <i>bla</i> _{TEM-1}	2001	Kunne <i>et al</i> (2012)
pCT	FN868832	75	80	93,269	K	<i>E. coli</i>	Cattle	UK	<i>bla</i> _{CTX-M14}	2004	Cottell <i>et al</i> (2011)
pO113	AY258503	74	83	165,548	K	<i>E. coli</i>	Human	Australia	<i>hylC</i> , <i>A</i> , <i>B</i> and <i>D</i> , <i>lldP</i> , <i>espP</i> , <i>epeA</i> , <i>saa</i> , <i>iha</i> , and <i>subBA</i>	2002	Leyton <i>et al</i> (2003)
pECOED	CU928147	65	71	119,594	Z	<i>E. coli</i>	Human	France	None	2008	Touchon <i>et al</i> (2009)
pSERB-1	AY686591	57	63	67,034	K	<i>E. coli</i>	Human	USA	None	2006	Dudley <i>et al</i> (2006)
pPWD4_103	HQ114284	30	33	103,297	Inc11y	<i>E. coli</i>	Pig	USA	<i>hph</i> , <i>aacC4</i> , <i>strA</i> <i>strB</i>	2007	Johnson <i>et al</i> (2011)

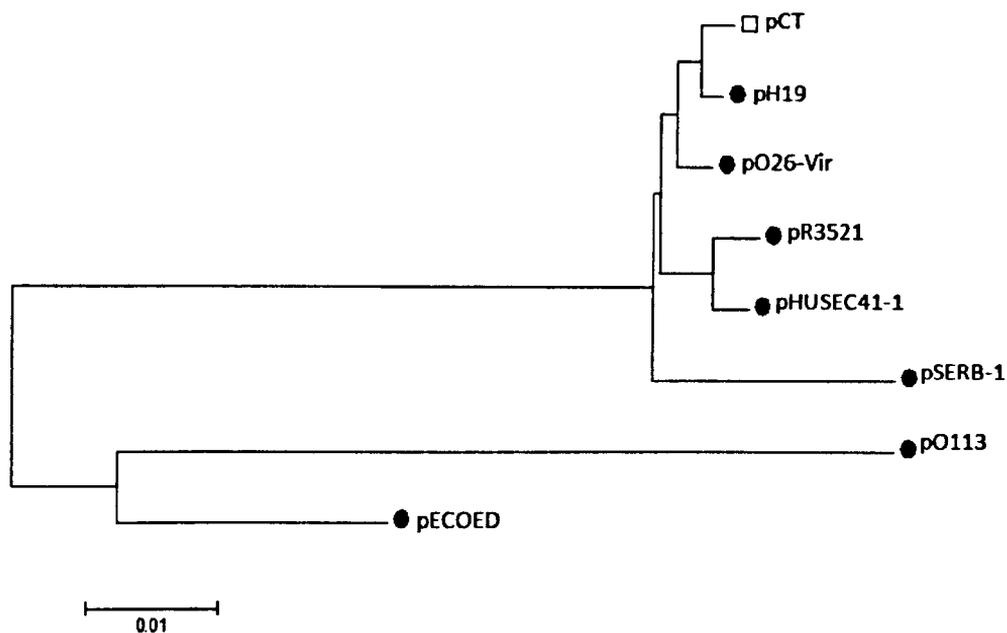
comparisons. All of the plasmids, with the exception of the plasmid pPWD4_103, share a similar core backbone which consists of the 22 *tra*, 12 *pil* and three *trb* conjugation genes present in pH19 (37,620-88,862 bp). Comparisons of the *tra* operon showed a high level of similarity with pHUSEC41 (100%), pO26-vir (99%), pR3521 (99%), pCT (95%), pSERB1 (90%), pO113 (90%), and pECOED (88%). Other genes commonly present in plasmids included the UV damage repair genes *impCAB*, the relaxome genes *nikAB*, DNA primase genes *sogL* and *sogS*, *pndAC*, *ydiA*, *yggA*, *ygeA* and *ydgA* genes. The orfs of pH19 was compared to the other plasmids using the BLASTn algorithm to generate a heat map showing the presence or absence of the ORF's as shown in Figure 4.7. As can be seen from the comparison of pH19 with other plasmids belonging to the IncI1γ complex (Figure 4.7), the main difference between pH19 and the core backbone present in other plasmids is the insertion of the MDR region and the stability region (23,217-32,719 bp). The *stbAB* and *parAB*-like genes present in the stability region were also identified in pO113, pECOED and pPWD4_103. The presence of two hypothetical gene regions, one from 33,588-35,134 bp which includes the *relE* and *copG* genes and the other from 88,976-91,252 bp which is where the IS5D transposon has inserted, vary between plasmids (Figure 4.7). The phylogentic analysis of the GenBank plasmids with the exception of pPWD4_103, identified that pCT and pH19 shared the most similarity, followed by pO26-Vir, pHUSEC41-1, pR3521, pSERB1 pECOED and pO113 (Figure 4.8).

Figure 4.7 Heat map of pH19 ORF comparisons with IncB, K and Z plasmids



Heat map generated from the comparisons of orf's from pH19 with the IncB plasmids pR3521, pHUSEC41-1 and pO26-Vir. The IncK plasmids pCT, pO113 and pSERB-1, IncZ plasmid pECOED and Inc1 γ plasmid pPWD4_103. Green indicates the presence of the orf, red indicates the absence of the orf, yellow indicates <80% of the length of the pH19 orf.

Figure 4.8 Phylogeny of the core backbone of IncB, K and Z plasmids

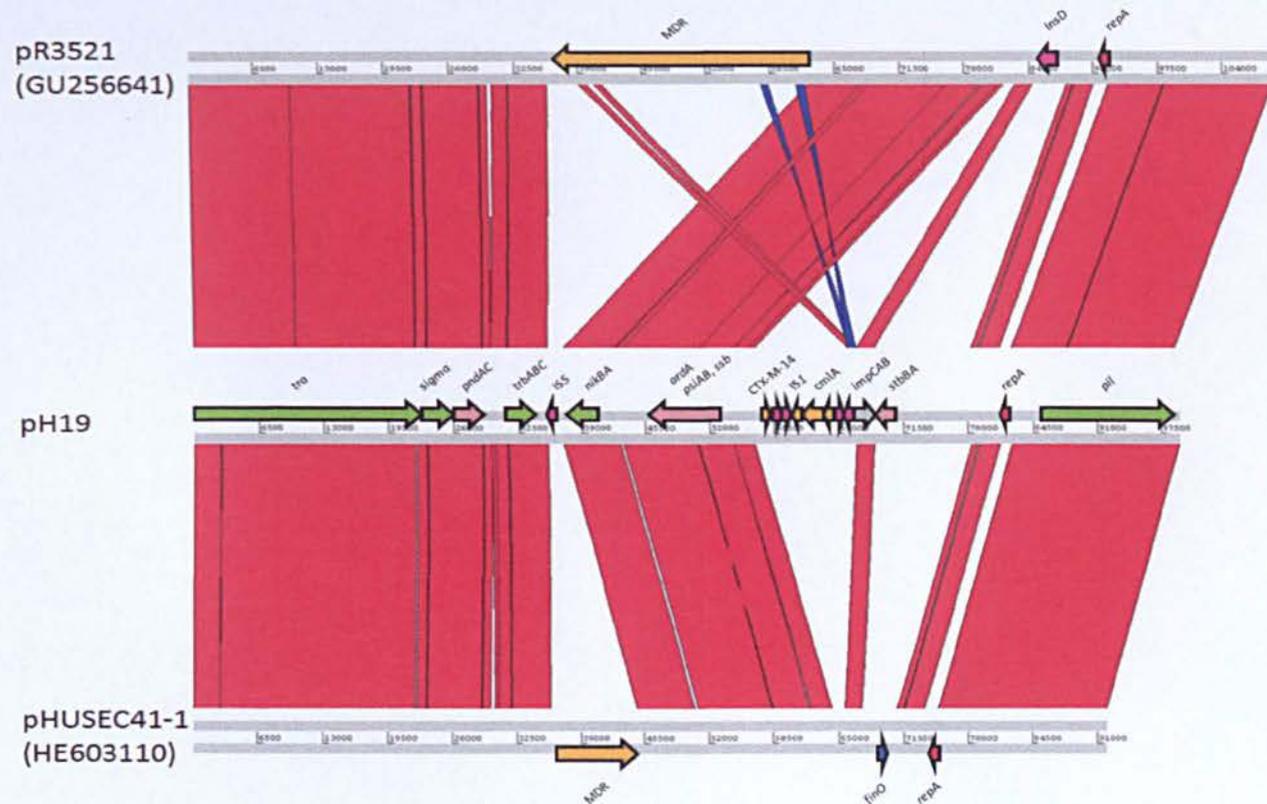


Dendrogram of the plasmids backbone for pH19, pR3521, pO26-Vir, pHUSEC41-1, pO113, pECOED and pSERB1, square indicates plasmid from cattle, circle indicates human plasmids. The backbone was constructed from the concatenated gene sequences. Phylogeny carried out using neighbour joining method with 1000 bootstrap repeats. The scale indicates substitution per base.

4.3.4.1 Comparison with the IncB plasmids pR3521 and pHUSEC41-1

pH19 shares a high level of nucleotide coverage with the plasmids pR3521 (85%) and pHUSEC41-1 (86%), which is predominantly associated with plasmid conjugation genes, as shown by the red regions shared between plasmids in Figure 4.9 (Papagiannitsis *et al.*, 2011; Kunne *et al.*, 2012). The IncB pR3521 was derived from an *E. coli* isolate from a Greek patient with a urinary tract infection (Papagiannitsis *et al.*, 2011). This plasmid is similar in size to pH19 but has a larger multidrug resistance region (26,382 bp), which has inserted between the *trbC* and *nikB* genes (Figure 4.9). This region includes the resistance genes *sul2*, *strA*, *strB* and *aacC2*, *bla*_{TEM-1}, and the carbapenemase genes *bla*_{ACC-4}, and *bla*_{SCO-1}. These resistance genes are associated with four MGE's IS26, IS*Kpn11*, IS*Kpn12*, Tn2 and a non functional IS*Ecp1*.

Figure 4.9 Artemis comparison tool analysis of pH19 with the IncB plasmids pR3521 and pHUSEC41-1



Artemis comparison tool analysis of the DNA sequence for pR3521 and pHUSEC41-1 with pH19. Green arrows are genes involved in conjugation, magenta arrows are MGE's, orange arrows are resistance genes and regions (MDR), pale red arrows are stability genes and regions, grey arrows are UV repair genes and red are the *rep* genes, blue arrows are other genes. Red areas show DNA homology between plasmids, blue is homologous DNA in the reverse orientation, regions of no colour show non homologous DNA.

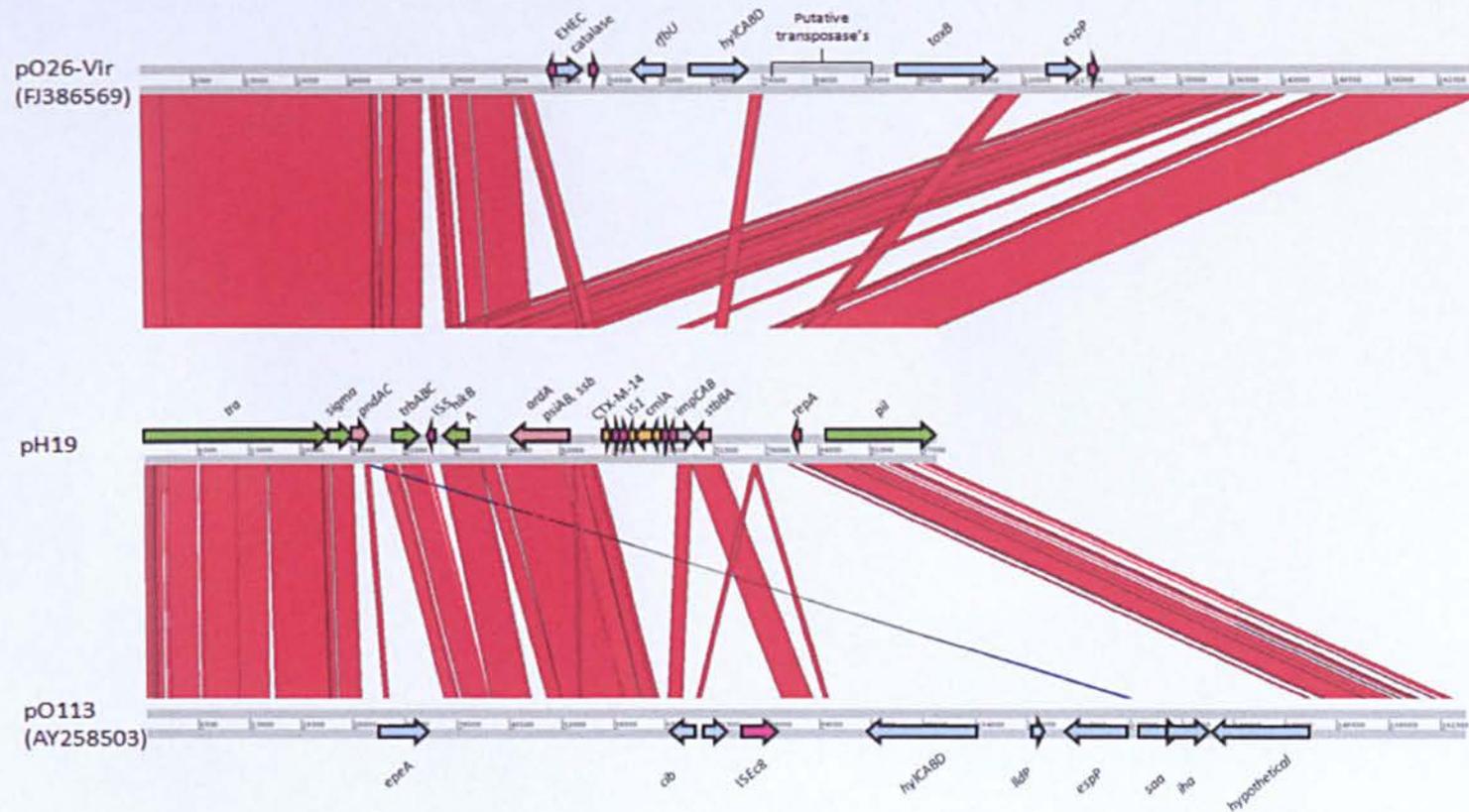
The IncB pHUSEC41-1 was recovered from an entero-aggregative shiga toxin producing *E. coli* O194:H4 (EAHEC) isolated from a child with hemolytic-uremic syndrome in Germany in 2001 (Kunne *et al.*, 2012). pHUSEC41-1 has fewer resistant genes than pR3521 but still harbours *sul2*, *strA*, *strB* and *bla*_{TEM-1} for resistance to sulphonamides, streptomycin and penicillins (Figure 4.9). The resistance genes in pHUSEC41-1 have inserted downstream of the *trbC* gene, in the same location as pR3521 (Papagiannitsis *et al.*, 2011; Kunne *et al.*, 2012). Despite being in a virulent host, no virulence genes were attributed to this plasmid. The location of the MDR insertion in both pR3521 and pHUSEC41-1 between the *trbC* and *nikB* genes, suggests that this region may act as a possible "hot spot" for insertions, which may also apply to pH19.

4.3.4.2 Comparison with the large virulence plasmids pO26-Vir and pO113

The two large virulence plasmids pO26-Vir (168kb) and pO113 (165kb) share a similar backbone with pH19 with nucleotide coverage's of 86% and 83% respectively, which have undergone the insertion of multiple virulence genes, but not resistance genes, as shown in Figure 4.10 (Fratamico *et al.*, 2011; Leyton *et al.*, 2003). As with pHUSEC41-1, the IncB plasmid pO26-Vir was also isolated from the shiga toxin producing *E. coli* O26:H11 recovered from a child with diarrhoea in Canada in 1977 (Fratamico *et al.*, 2011). In addition to the common backbone shared with pH19, pO26-Vir also has the virulence genes *toxB* located upstream of the conjugative gene *traB* and the serine protease gene, *espP* lies upstream of *nikB*. A large region containing the hemolysin transport genes *hylC*, *A*, *B* and *D* is upstream of the *stbAB* genes in the pH19 backbone (Figure 4.10). As mentioned above pO26-vir lacks any resistance genes and despite five plasmids present in this isolate, only resistance to tetracyclines was identified (Fratamico *et al.*, 2011).

Plasmid pO113 was isolated from a child with haemolytic uremic syndrome caused by *E. coli* EHEC O113:H21 in Australia (Leyton *et al.*, 2003). Comparisons of the

Figure 4.10 Artemis comparison tool analysis of pH19 with the large virulence plasmids pO26-Vir and pO113



Artemis comparison tool analysis of the DNA sequences of pO26-Vir and pO113 with pH19. Green arrows are genes involved in conjugation, magenta arrows are MGE's, orange arrows are resistance genes and regions (MDR), pale red arrows are stability genes and regions, grey arrows are UV repair genes, red are the *rep* genes and blue arrows are other genes. Red areas show DNA homology between plasmids, blue is homologous DNA in the reverse orientation, regions of no colour shows non homologous DNA.

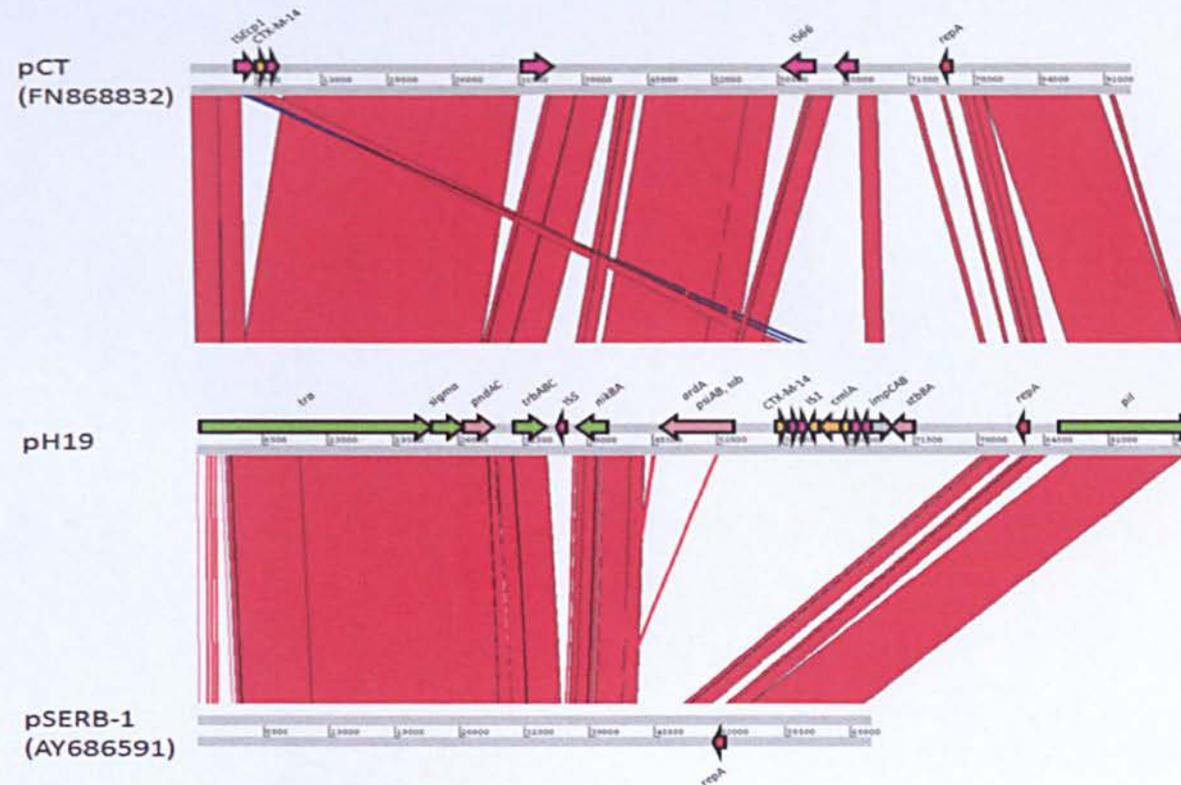
replication regions of IncK and IncB plasmids with pO113 showed a higher similarity with IncK plasmids, and is classed as IncK in this study. pO113 shares the core backbone of pH19, which has undergone insertion of virulence regions as indicated in Figure 4.10. This includes a 10.7kb insert between the *pndC* and *trbA* genes harbouring the *epeA* gene coding for a SPATE like protein. The *imm* and *cib* genes for immunity and production of colicin respectively are located downstream of the *ydeA* gene, as observed in IncII γ plasmids, this region also includes the *stbB*, *stbA*, *ybiA* and *parA*-like present in pH19 but are absent in pO26-Vir. Downstream of the stability region lies the addiction system genes *ccdA* and *ccdB* followed by the hemolysin transport genes *hylC*, *A*, *B* and *D*, and the virulence genes *lldP*, *espP*, *saa*, *iha*, and *subBA* (Leyton *et al.*, 2003).

4.3.4.3 Comparison with the IncK plasmids pCT and pSERB-1

Covered extensively in chapter 3, the pCT plasmid was isolated from *E. coli* from cattle in 2004, and was the first *bla*_{CTX-M-14} isolated from an animal in the UK (Teale, 2002; Cottell *et al.*, 2011). Although IncK, pCT shares a high level of nucleotide coverage (80%) with pH19 as shown by the homologous regions in Figure 4.11. The region from *ardA* gene to the truncated *yfbA* of pH19 is absent in pCT, having been replaced by several hypothetical genes and the IS66 transposon. pCT harbours a single resistance gene which was in the *ISEcpI-bla*_{CTX-M-14}-*IS903D* transposition unit, which is similar to that present in pH19 when the *IS1* insertion is taken into consideration. pCT shares the majority of the *tra* and *pil* genes with pH19, however the pCT also codes the pilV shufflon proteins A and D', shufflon recombinase and four hypothetical genes which are absent in pH19 and may have an effect on its environmental persistence (Cottell *et al.*, 2011).

Comparison of the published partial sequence of pSERB-1 with pH19, show that the plasmids share a similar backbone (63%), with the *tra*, *pil*, *pndC* and *trbABC* genes present in both plasmids (Dudley *et al.*, 2006). There are variations in the *traE*, *traF*, *traH* and *traI* genes with coverages ranging from 65-80%, additionally pSERB-1 has

Figure 4.11 Artemis comparison tool analysis of pH19 with the IncK plasmids pCT and pSERB-1



Artemis comparison tool analysis of the DNA sequences of pCT and pSERB-1 with pH19. Green arrows are genes involved in conjugation, magenta arrows are MGE's, orange arrows are resistance genes and regions (MDR), pale red arrows are stability genes and regions, grey arrows are UV repair genes, red are the *rep* genes and blue arrows are other genes. Red areas show DNA homology between plasmids, blue is homologous DNA in the reverse orientation, regions of no colour shows non homologous DNA.

the *traG* gene (25,553-26,137 bp) which is absent in pH19 and other IncB and IncK plasmids, shown in Figure 4.11. The variation in the *tra* genes suggests that pSERB-1 is closely related to the IncI1 γ plasmids compared to IncB and K. Although no resistance or virulence genes were identified in pSERB-1, the *pilS* gene was found to be important in the adherence of bacteria to eukaryotic cells (Dudley *et al.*, 2006).

4.3.4.4 Comparison with the IncZ plasmid pECOED and the IncI1 γ pPWD4_103

The only other fully sequenced IncZ plasmid found to have similarity to pH19 was pECOED, identified in *E. coli* ED1a in a human faeces sample from France (Touchon *et al.*, 2009). The replication region of pECOED shares the highest similarity with pH19, including the RNAI (93%) and *repA* gene (98.9%). In addition to the similarity observed in between pH19 and pECOED (71%), pECOED also carries numerous plasmid stability mechanisms coded by the genes *yacABC*, *doc/phd* and *ccdA/ccdB* as shown in Figure 4.12 (Touchon *et al.*, 2009). pECOED possesses bactericidal genes including *imm*, *cib*, *mcbG* and *mcbF*, all of which have been inserted downstream of the *repA1*. With the exception of pPWD4_103, pECOED shares the highest number of genes in the stability and hypothetical region with pH19. Despite the additional stability and bactericidal genes no resistance genes were identified on pECOED and are likely to maintain the plasmid when not under selective pressure (Touchon *et al.*, 2009; Johnson *et al.*, 2011).

The incompatibility group IncI1 γ plasmid pPWD4_103 shared the least similarity (33%) with pH19, and was isolated from *E. coli* in pigs in the USA (Johnson *et al.*, 2011). The conjugal backbone of the pPWD4_103 and pH19 plasmids shared limited similarity, with the *pilM*, *pilO*, *pil*, *pilR*, *pilU*, *traR*, *traS* and *traX* of pH19 absent in pPWD4_103, with coverage's of between 67.5-81.25% remaining for the other genes, as shown in Figure 4.12. pPWD4_103 had the highest coverage of the stability and hypothetical region present in

pH19, this may suggest that this area is mobile, or remains from an ancestor. Additionally the genes *psiAB*, *ssb*, *parB*-like, *klcA*, *impABC*, *stbAB* and *parA*-like were shared between the plasmids. pPWD4_103 lacks any virulence genes but has the resistance genes *hph* (hygromycin B), *aacC4*, *strA* and *strB*, which are flanked by the transposons IS91 and IS26, and have inserted downstream of the *repZ* (Johnson *et al.*, 2011).

4.3.5 Development of molecular markers to differentiate between IncB, K and Z plasmids.

4.3.5.1 *In silico* testing of the pCT molecular markers on IncB, K and Z plasmids

The pH19 plasmid was originally identified through the application of the pCT markers during a screening process for pCT-like plasmids (Cottell *et al.*, 2011; Stokes *et al.*, 2012). The markers were applied *in silico* to the plasmids belonging to the IncI complex compared in this study to determine whether they would have been identified. The results of the *in silico* analysis show that 5/9 IncI plasmids (pH19, pR3521, pHUSEC-41, pO26-Vir and pSERB-1) produced the same PCR products (sigma and pilN), only the Rci was present in pECOED and only the sigma factor marker was found in pO113, none of the markers for pCT were present in pPWD4_103 shown in Table 4.3.

These results could be further complicated by the PBRT, with the IncB primers identifying both IncB and IncZ plasmids as the same replicon (Carattoli *et al.*, 2005a). Differing only by the *repA* gene, the replication control region remains so similar that IncZ and IncB plasmids are incompatible (Praszkier *et al.*, 1991; Kato and Mizobuchi, 1994). The core backbone of the IncZ and IncB plasmids remains very similar, producing the same results for screening with the pCT molecular markers.

Table 4.3 *In silico* testing of molecular markers for pCT against sequenced plasmids

Plasmid	Molecular marker				No.
	pCT	Sigma	Rci	PilN	
pH19	0	1	0	1	2
pR3521	0	1	0	1	2
pHUSEC41-1	0	1	0	1	2
pO26-Vir	0	1	0	1	2
pECOED	0	0	1	0	1
pCT	1	1	1	1	4
pO113	0	1	0	0	1
pSERB1	0	1	0	1	2
pPWD4_103	0	0	0	0	0

Presence of markers is shown by 1 with a green background and absence shown by 0 and a red background.

4.3.5.2 Molecular markers for the differentiation of IncB, K and Z plasmids

Comparison of pH19 and the other IncB, K and Z plasmids was used to design PCR- based molecular markers that could be used to differentiate plasmids belonging to these replicon types than that achieved by the pCT markers. The molecular markers selected for this differentiation were all present in the pH19 plasmid, so testing *in vitro* could be performed, as analysis on the GenBank plasmids would be performed *in silico*. Comparative analysis identified the *ardA* gene (mArdA) for the antirestriction protein, and the *yqiK* gene which codes for an adhesin like inner membrane protein (mYqiK) (Althorpe *et al.*, 1999; Antao, Wieler and Ewers, 2009). The *ydeA* gene coding for a conserved hypothetical protein (mYdeA), and regions coding for the hypothetical genes designated mH90, mH19 and mHyp-Hyp in this study, were also used as molecular markers.

4.3.5.3 *In silico* testing of the pH19 molecular markers

The pH19 markers were tested *in silico* against the nine plasmids, two IncZ plasmids pH19 and pECOED, three IncB plasmids pR3521, pHUSEC41-1, pO26-vir, three IncK plasmids pCT, pO113 and pSERB-1 and the most closely related IncI γ plasmid, pPWD4_103. The results of the *in silico* testing are shown in Table 4.4, the mArdA marker was the most predominant marker, present in seven plasmids. The next most predominant were mYqiK and mHyp-Hyp markers both present in six plasmids. The mH90 marker was found to be present in four plasmids, while the mYdeA marker was identified in just two plasmids. The mH19 marker was found to be specific to the pH19 plasmid which was confirmed by BLASTn searches of GenBank. The pH19 markers were capable of differentiating between all three IncK plasmids, the three IncB plasmids were differentiated into two groups, with pR3521 and pHUSEC41-1 being in the same group and both IncZ plasmids were differentiated. Marker combinations were found to be present in single incompatibility groups, and not found associated with other replicons. A binary system was used to assign a number to each molecular marker based on the prevalence, with lower numbers assigned to more prevalent markers. The binary system was as follows, mArdA (n=1), mYqiK (n=2), mHyp-Hyp (n=4), mH90 (n=8), mYdeA (n=16) and mH19 (n=32) based on their occurrence. This would allow a unique identification number (UID) to be assigned to a specific combination of molecular markers, allowing the cataloging of plasmids for use with any future database developed (Table 4.4). As can be seen from Table 4.4 all of the plasmids in this study with the exception of pHUSEC41-1 and pR3521 can be differentiated between using the pH19 molecular marker scheme.

Table 4.4 *In silico* testing of the pH19 molecular markers with the GenBank plasmids

Plasmid	Source	Replicon type	Markers						Number of markers	UID
			mArd A	mYde A	mHyp- Hyp	mH90	mH19	mYqiK		
pH19	Human	Z	1	1	1	1	1	1	6	63
pR3521	Human	B	1	0	1	1	0	1	4	15
pHUSEC41-1	Human	B	1	0	1	1	0	1	4	15
pO26-Vir	Human	B	1	0	1	0	0	1	3	7
pECOED	Human	Z	0	0	0	1	0	1	2	10
pCT	Cattle	K	1	0	1	0	0	0	2	5
pO113	Human	K	1	0	0	0	0	1	2	3
pSERB1	Human	K	0	0	1	0	0	0	1	4
pPWD4_103	Pig	Incl1 γ	1	1	0	0	0	0	2	17

Presence of molecular markers are shown by 1 with a green background and absence shown by 0 and a red background, UID = unique ID number. Molecular markers are mArdA = 463 bp, mYdeA = 551 bp, mHyp-Hyp = 745 bp, mH90 = 269 bp, mH19 = 846 bp, and mYqiK = 1,207 bp

4.3.5.4 Evaluation of the molecular markers

The mYdeA marker was present in only pH19 and pPWD4_103 and absent in the remaining plasmids due to the presence of the 9,000 bp region termed the stability hypothetical region (23,159-32,158 bp), which contains the stability genes including *ydeA*. Although the *ydeA* gene is present in pO113 and pECOED it lacks the same 3' end present in pH19 preventing the primers from binding. The absence of the stability and hypothetical region in pR3521, pHUSEC41-1, pO26-Vir, pCT and pSERB-1 resulted in the absence of the *ydeA* gene and mYdeA marker. Additional IncI1 γ plasmids were found to have the mYdeA marker, and implies a close relationship between these regions.

The mH90 marker is based on a conserved hypothetical gene, which is the first (*orf122*, 88,976-89,269 bp) of a cluster of four conserved hypothetical genes located between *trbC* and *nikB*, and in pH19 is the site of the insertion of IS5. These four conserved hypothetical genes are present in pR3521, and both the first and last (*orf126* 90,899-91,252 bp) genes of this cluster were also present in pHUSEC41-1 and pECOED. The two internal genes, a putative iron regulated outer membrane protein (*orf123*) and conserved gene (*orf124*) were incomplete. This region seems to be an area associated with insertion as both pHUSEC41-1 and pR3521, have inserts into this region, with the insert in pHUSEC41-1 occurring in the putative iron regulated outer membrane protein gene. The absence of this region of conserved hypothetical genes in pO26-Vir, pCT, pO113, pSERB-1 and pPWD4_103, results in the absence of the mH90 marker.

The absence of the mHyp-Hyp marker, based on two conserved hypothetical genes (*orf108* and *orf109*), in pECOED, pO113 and pPWD4_103, is due to several events. Plasmid pECOED plasmid has one of the conserved hypothetical gene (81,576-82,199 bp), but lacks the other hypothetical gene. The region of conserved genes between the *pndC* and *trbA* genes is missing in pPWD4_103 having been replaced by the *finQ* gene coding for the fertility inhibition of F plasmids (Ham and Skurray, 1989). In pO113, two copies

of *orf108* and *orf109* were present, however in both circumstances only 88.4% of *orf108* and 95.5% of *orf109* remain. The first instance of these genes has been truncated as the result of the insertion of *epeA* linked to transposons, the second occurrence is in the reverse orientation and is truncated as a result of the insertion of *espP* (Leyton *et al.*, 2003). If the insertions of the virulence genes had not occurred at these positions it is predicted that the amplicon for this molecular marker would have been produced.

The adhesion gene *yqiK* which is utilised as a molecular marker (mYqiK) is absent in pCT, pSERB-1 and pPWD4_103. The annotation of pCT did not identify the *yqiJ* and *yqiK* genes, typically present between the *traC* and *pilI* genes in other plasmids (Cottell *et al.*, 2011; Antao, Wieler and Ewers, 2009). However these are remnants of these genes, between 91,760-91,903 bp in pCT, with the N terminus of *yqiJ* and C terminus of *yqiK* overlapping by 9 bases. This may suggest that these genes were present in this backbone at one time and a deletion may have formed the pseudogene missing the N terminal as reported (Cottell *et al.*, 2011). This is further supported as the pSERB-1 which lacks these genes between the *traC* and *pilI* and any remnants. pPWD4_103 appears to have a deletion in the *pil* locus between *traC* and *pilK* which is where the *yqiJ* and *yqiK* genes are located in pH19.

The most prevalent of all the markers selected was the mArdA molecular marker which was only absent in pECOED and pSERB-1. There appears to be two regions deleted in the pECOED when compared to pH19 and other plasmids, which are located between *impA* and *ycaA*, and a conserved hypothetical and *ydgA*. The latter of these region includes the *ardA* gene which is used as the marker and the *psiAB* and *ssb* genes. As with pECOED, pSERB-1 also appears to have a deletion in a similar region between genes *ccgAII* and *yafB*, this may be due to the pSERB-1 only being a partial sequence, or a true deletion as three hypothetical genes and *yagA* are also absent from this plasmid.

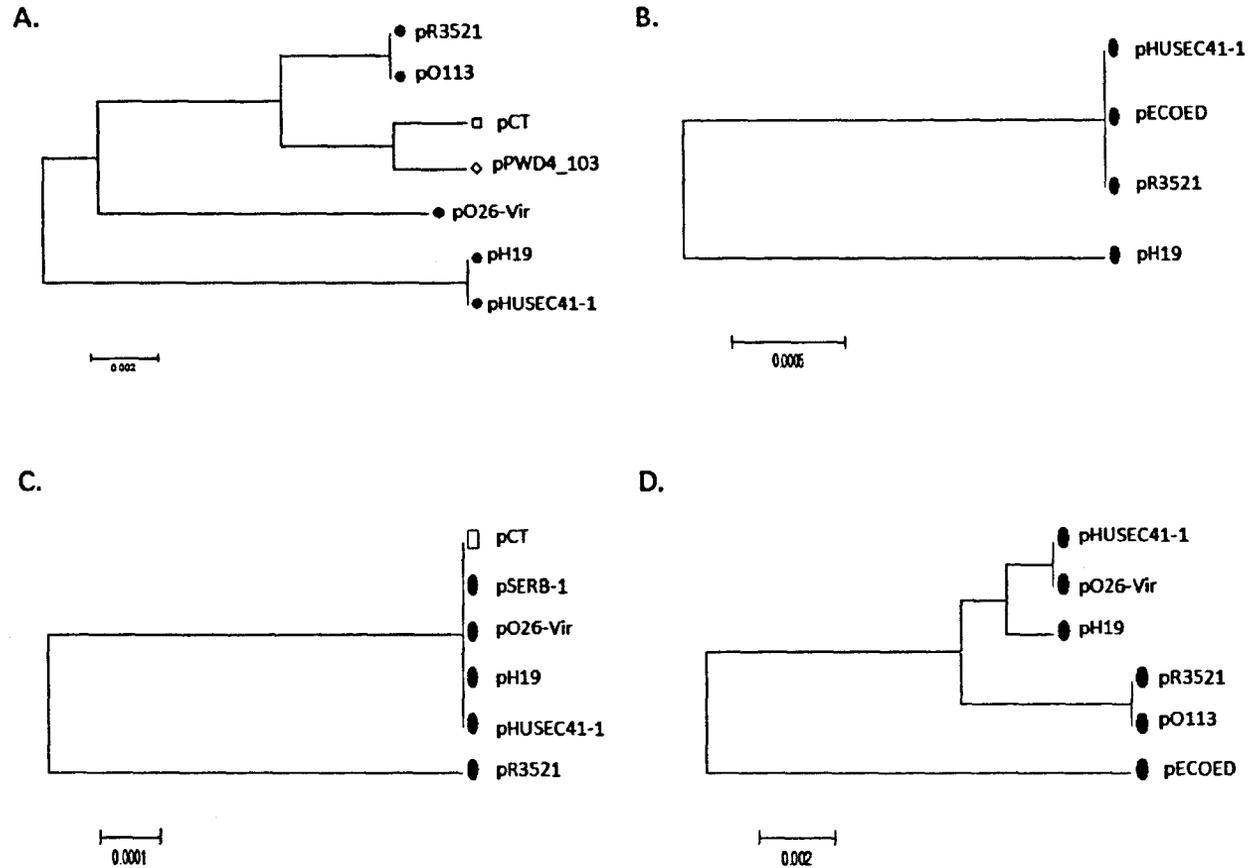
4.3.5.5 Amplicon sequence analysis

Although not required for initial differentiation, the sequencing of the molecular markers could be used to further differentiate plasmids, with multiple alleles identified for mArdA, mH90, mHyp-Hyp and mYqiK markers as shown in Figure 4.13. This is similar to pMLST used for some plasmids such as IncI1γ and IncN (Garcia-Fernandez *et al.*, 2011; Garcia-Fernandez *et al.*, 2008). The mArdA marker was the most discriminative separating the seven plasmid amplicons into five alleles, pR3521 had the same allele as pO113, as did pH19 and pHUSEC41-1. Analysis of the mYqiK marker identified four alleles among the six plasmid amplicons, both pHUSEC41-1 and pO26-Vir had the same alleles as did pR3521 and pO113. The mH90 marker distinguished pH19 from the other three plasmids which all had the same allele. Comparison of the mHyp-Hyp marker distinguished pR3521 from the other 5 plasmids. Sequencing of the amplicons was capable of differentiating pR3521 and pHUSEC-41, both of which had the mArdA, mHyp-Hyp, mH90 and mYqiK molecular markers. All of these markers with the exception of mH90, have different alleles.

4.3.6 Screening for IncB, K and Z plasmids using the pH19 molecular markers

In total 136 *E. coli* field isolates were screened using the pH19 molecular markers, including 98 group 1 CTX-M-producing isolates from cattle (n=27), chicken (n=29), humans (n=27) and turkeys (n=15) and 38 group 9 CTX-M-producing isolates from cattle (n=19), humans (n=9) and turkeys (n=10). Screening of the group 1 CTX-M-producing isolates identified that 24/98 isolates had no ampilcons (human n=15, cattle n=5, chicken n=2 and turkey n=2). One amplicon was present in 18/98 field isolates (human n=5, cattle n=10, chicken n=1 and turkey n=2), while 51/98 had 2 markers (human n=5, cattle n=10, chicken n=25 and turkey n=11). Three amplicons were present in three isolates (human n=1, cattle n=1 and chicken n=1), four amplicons was the highest number observed in a

Figure 4.13 Phylogenetic comparison of the alleles for the mArdA, mH90, mHyp-Hyp and mYqiK molecular markers



Phylogenetic comparison of the *in silico* amplicons from the molecular markers (A.) mArdA, (B.) mH90, (C.) mHyp-Hyp and (D.) mYqiK aligned using MEGA5 by neighbour joining with 1000 bootstrap repeats. Circles represent human plasmids, square are cattle plasmids and diamond is a porcine plasmid, scale is substitution per base.

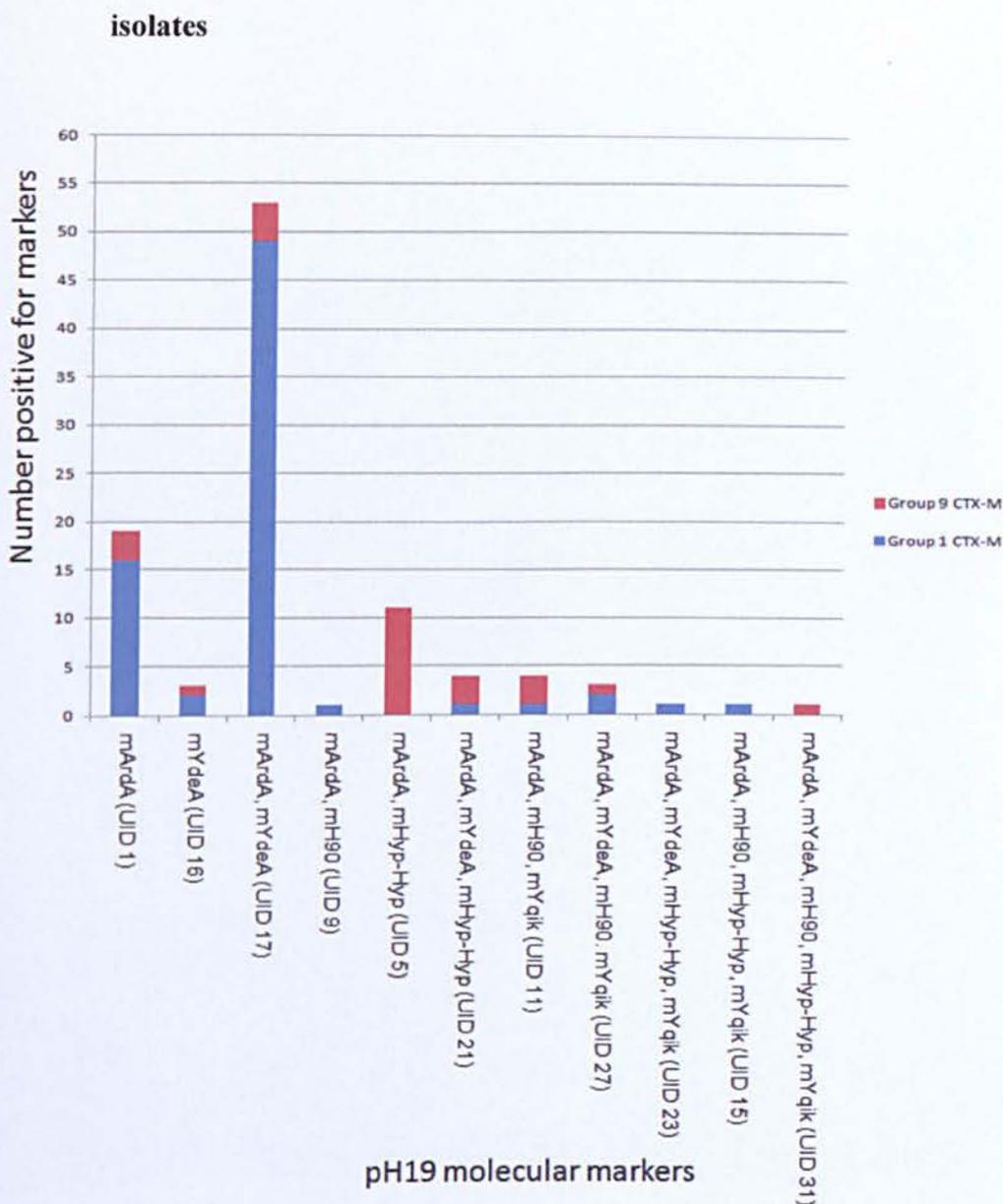
single isolate, being identified in two isolates (human n=1 and cattle n=1). The screening of the group 9 CTX-M field isolates showed that 11/38 isolates had no amplicons (human n=4 and cattle n=7), one marker was identified in four isolates (human n=3 and cattle n=1). Two markers were found in 15/38 isolates (human n=2, cattle n=4 and turkey n=9), three markers in 6/38 isolates (cattle n=5 and turkey n=1). Four markers were found in one cattle isolate while five markers were found in another.

The pH19 molecular markers were present individually and in combination with other markers in the field isolates, a total of 11 combinations were observed. The mArdA marker was the most prevalent of all the markers 97/136 isolates, and in all but one combination, the second most common was the mYdeA marker present in 66/136, as shown in Table 4.5. Figure 4.14 shows the combinations of markers and their occurrence. Of note was that the mYqiK marker was only ever present with two or more markers. The H19 marker was shown to be specific to the pH19 plasmid as it was not present in any of the field isolates tested. The 136 field isolates were screened for the presence of the incompatibility groups using the replicon typing scheme by Carattoli *et al* (2005a) for the IncK, IncB and IncI1 γ replicons (Carattoli *et al.*, 2005a). Replicon typing identified 61

Table 4.5 Prevalence of pH19 molecular markers in *E. coli* CTX-M field isolates

Group 1 CTX-M								
Source	No. of isolates	No. of positive	mYqiK	mHyp-Hyp	mArdA	mH19	mYdeA	mH90
Cattle	27	22	2	1	21	0	11	3
Chicken	29	27	1	1	26	0	27	0
Human	27	12	2	1	12	0	6	2
Turkey	15	13	0	0	12	0	12	0
Group 9 CTX-M								
Cattle	19	12	5	3	11	0	9	5
Human	9	5	0	2	5	0	0	0
Turkey	10	10	0	10	10	0	1	0
Total	136	101	10	18	97	0	66	10

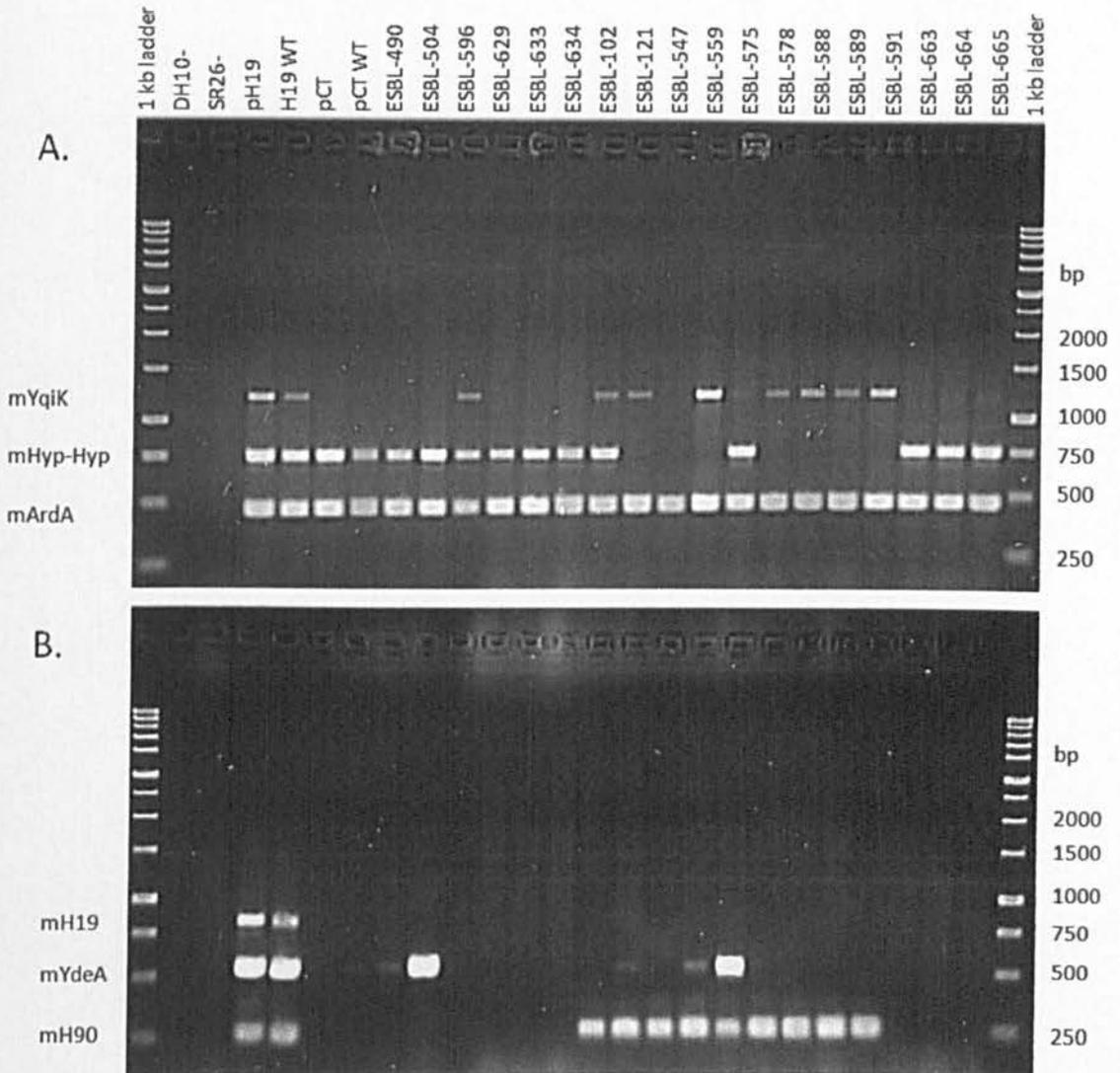
Figure 4.14 Combinations of pH19 molecular markers in *E. coli* CTX-M field



The combinations of pH19 molecular markers observed in screening of the CTX-M-1 and CTX-M-9 field isolates.

IncI γ plasmids, 9 IncB, 11 IncK and three IncK/IncB plasmids. A panel of 18 field isolates belonging to incompatibility groups IncK n=6, IncB n=9 and 3 IncK/B, of which seven also had the IncI γ replicon type (IncK n=5 and IncB n=2), were retested using the molecular markers for pH19. The results of the molecular marker screening are shown in Figure 4.15 and Table 4.6.

Figure 4.15 pH19 molecular marker screening of IncB and IncK *E. coli* CTX-M field isolates



Multiplex PCR screening of the 18 *E. coli* field isolates with **(A)** multiplex 1 (mYqiK, mHyp-Hyp and mArdA) and **(B)** multiplex 2 (mH19, mYdeA and mH90). DH10- is *E. coli* DH10 negative control, S26R- *S. Typhimurium* 26R negative control, pH19 is pH19 transformed into *E. coli* DH10 positive control, H19 WT is *E. coli* field isolate positive control, pCT is pCT transformed into *E. coli* DH10 positive control and pCT WT is pCT *E. coli* field isolate positive control. Figure 4.15 complements Table 4.6, PCR products resolved on a 1.5% agarose gel by electrophoresis.

Table 4.6 pH19 molecular marker screening of IncB and IncK *E. coli* CTX-M field isolates

Isolate	Source	CTX-M group	Molecular markers							Replicon	IncII γ	UID
			mYqiK	mHyp-Hyp	mArdA	mH19	mYdeA	mH90				
E. coli DH10 S. Typhimurium 26R	NA	NA	0	0	0	0	0	0	0	NA	NA	NA
DH10 + pH19	Human	G9	1	1	1	1	1	1	1	B	ND	63
<i>E. coli</i> H19	Human	G9	1	1	1	1	1	1	1	B	ND	63
DH10 + pCT	Cattle	G9	0	1	1	0	0	0	0	K	ND	5
<i>E. coli</i> C159	Cattle	G9	0	1	1	0	1	0	0	ND	Y	21
ESBL-490	Cattle	G9	0	1	1	0	1	0	0	K	Y	21
ESBL-504	Cattle	G9	0	1	1	0	1	0	0	K	N	21
ESBL-596	Chicken	G1	1	1	1	0	0	0	0	K	Y	7
ESBL-629	Turkey	G9	0	1	1	0	0	0	0	K	Y	5
ESBL-633	Turkey	G9	0	1	1	0	0	0	0	K	Y	5
ESBL-634	Turkey	G9	0	1	1	0	0	0	0	K	Y	5
ESBL-102	Human	G1	1	1	1	0	0	1	1	B	N	15
ESBL-121	Human	G1	1	0	1	0	1	1	1	B	Y	27
ESBL-547	Cattle	G1	0	0	1	0	0	1	1	B	N	9
ESBL-559	Cattle	G9	1	0	1	0	1	1	1	B	N	27
ESBL-575	Cattle	G9	1	1	1	0	1	1	1	B	Y	29
ESBL-578	Cattle	G9	1	0	1	0	0	1	1	B	N	11
ESBL-588	Cattle	G9	1	0	1	0	0	1	1	B	N	11
ESBL-589	Cattle	G9	1	0	1	0	0	1	1	B	N	11
ESBL-591	Cattle	G1	1	0	1	0	0	1	1	B	N	11
ESBL-663	Turkey	G9	0	1	1	0	0	0	0	K/B	N	5
ESBL-664	Turkey	G9	0	1	1	0	0	0	0	K/B	N	5
ESBL-665	Turkey	G9	0	1	1	0	0	0	0	K/B	N	5

The pH19 markers identified 6/18 potential pCT-like plasmids (UID 5), due to the presence of both mArdA and mHyp-Hyp, consistent with the pCT plasmid from *in silico* and *in vitro* testing. All of these isolates were group 9 CTX-M and originating from UK turkeys, three isolates had the IncK and three had IncK/B replicon types. Two isolates had the mYdeA marker in addition to mArdA and mHyp-Hyp, of which one tested positive for the IncII γ replicon, which was likely to have attributed this marker. The pCT field isolate also has this marker combination, with other plasmids contributing the mYdeA marker. A group 1 CTX-M chicken isolate was positive for mArdA, mHyp-Hyp and mYqiK (UID 7),

along with the IncK replicon this may suggest a pCT-like plasmid that has retained the *yqiK* gene suspected of being deleted in pCT.

A human group 1 CTX-M-producing isolate from the Netherlands had the same marker combination as pR3521 and pHUSEC41-1 plasmids (UID 15), both of which were recovered from human isolates (Kunne *et al.*, 2012; Papagiannitsis *et al.*, 2011). Similarly a group 9 CTX-M cattle isolate from the UK had the same marker combination and mYdeA (UID 29), however this may be attributed to the IncI1 γ plasmid identified in this isolate, as is the case with the pCT field isolate. The markers mYqiK, mArdA and mH90 were identified in 4/18 isolates (UID 11), this combination is similar to pHUSEC41-1 and pR3521 (UID 15) with mHyp-Hyp missing, and similar to pECOED (UID 10) with mArdA in addition. The mHyp-Hyp marker may have been absent due to the insertion of another gene as observed with the virulence genes in pO113 (Leyton *et al.*, 2003). Two isolates also had the same markers and mYdeA (UID 27), with one found in a human isolate from the Netherlands which was positive for the IncI1 γ replicon type. The screening of the molecular markers also revealed a combination of markers that had not been observed in combination *in silico*, with a group CTX- M-1 cattle isolate having the mH90 and mArdA together, with the presence of an IncB replicon type.

The presence of an IncI1 γ plasmid in some strains could potentially contribute additional markers, particularly the mArdA and mYdeA, both of which have been found to be present in the closely related IncI1 γ plasmids. The mYdeA marker has not been found to be associated with any other IncK, IncB or IncZ plasmid apart from pH19 from *in silico* testing, and is therefore important in identifying pH19 and the stability and hypothetical region. The mArdA marker is present in IncK, B and Z plasmids, and is important for differentiation in both PCRs and amplicon analysis. Isolation of the plasmid by conjugation or transformation would be recommended for isolates positive for either mArdA or mYdeA, especially if the IncI1 γ replicon type has been identified in the isolate.

4.4 Discussion

The pH19 plasmid was harboured in a MDR *E. coli* isolated in 2007 from a patient with a urinary infection, and was identified as part of the pCT-like study (Stokes *et al.*, 2012). The plasmid was found to confer resistant to neomycin, ampicillin, cefotaxime, ceftazidime and chloramphenicol. The sequencing of the 99,362 bp pH19 plasmid identified 136 open reading frames and a single resistance region. Although being typed as IncB plasmid by PBRT, sequencing identified that the plasmids belonged to the IncZ group, with the *repA* genes being unrelated (Praszkier *et al.*, 1991; Carattoli *et al.*, 2005a). The RNAI involved in the control of replication and incompatibility of both IncB and IncZ plasmids shares such similarity that the plasmids are unable to coexist in the same cell (Praszkier *et al.*, 1991). This RNAI is a small antisense molecule that prevents the formation of the pseudoknot required for the translation of *repA* (Praszkier *et al.*, 1991; Praszkier and Pittard, 2002). This stem loop RNA is also important for the stability of plasmids as aminoglycosides targeting this RNA have caused the loss of IncB plasmids (Thomas *et al.*, 2005). Despite IncZ plasmids having a different *repA* gene to that of IncB plasmids, with a IncFII origin, with the RNAI still functioning (Kato and Mizobuchi, 1994). It is likely that changes to the pseudoknot have occurred over time to allow similar plasmids to coexist in the same bacterial host.

Analysis of pH19 found it to be related to several plasmids belonging to the IncI complex including IncB, K, Z and IncI1 γ , with homologous nucleotide coverages ranging from 63-85% (Bradley, 1984). This suggests that pH19 shares a common ancestor with plasmids of the IncI complex due to their susceptibility to the same bacteriophage, with pH19 undergoing an exchange of the replication region with an IncFII plasmid such as R100 (Bradley, 1984; Kato and Mizobuchi, 1994). pPWD4_103 had the highest coverage with pH19 (33%) of any IncI1 γ plasmid, this suggests an early divergence of pH19 from the IncI1 γ group, with limited similarity observed in the *pil* and *tra* genes (Johnson *et al.*, 2011). The 51 kb conjugative region (*tra*, *pil* and *trb*) present in pH19 was highly

conserved among the IncB, K and Z plasmids, with nucleotide coverages ranging between 88-100%. These regions are important for the conjugative transfer of pH19 and the *pil* genes and may have a role in liquid transfer and possibly adhesion to eukaryotic cells (Komano *et al.*, 1990; Dudley *et al.*, 2006).

After transfer of pH19 into a new host its persistence is aided by two stability regions, the first of which contained the genes *ardA*, *klcA*, *psiAB* and *ssb*, which are common in IncB, K and I1 γ plasmids (Althorpe *et al.*, 1999; Serfiotis-Mitsa *et al.*, 2010; Shereda, Bernstein and Keck, 2007; Johnson *et al.*, 2011). However pECOED, the other IncZ plasmid in this study, lacked these stability genes which had been replaced with multiple colicin related genes (Touchon *et al.*, 2009). The absence of these genes in pECOED may suggest that pH19 has recently converted to the IncZ incompatibility from either an IncB or IncK plasmid. The second stability region in pH19 consisted of the genes *stbAB*, *resA*, *ydfA* and *ydeA* and was similar to that found in IncI1 γ plasmids, with additional hypothetical genes found in p53638_75 (CP001065) from *E. coli* and pKPX-2 (AP012056) from *Klebsiella pneumoniae* (Huang *et al.*, 2013). The *yagA*, *cib* and *imm* genes present in this region in IncI1 γ plasmids have been replaced by a conserved hypothetical gene (*orf51*) which was also identified in pPWD4_103, the only other Genbank entry for this gene. This stability region is partially present in pO113 and pECOED, suggesting that it is a remnant of the shared common ancestor.

The pH19 MDR consisted of a class 1 integron with the gene cassettes *cmlA*, *aacA4*, *qacEA1* and truncated *sull* which has undergone the insertion of ISEcp1-*bla*_{CTX-M-14}-IS903. Integrons have long been associated with resistance genes carried within gene cassettes, and have been found with a high frequency in multiple bacterial species (Stokes and Hall, 1989; van Essen-Zandbergen *et al.*, 2007). The *bla*_{CTX-M-14} gene has also been found in numerous isolates from both human and animals around the globe (Hordijk *et al.*, 2013; Voets *et al.*, 2012; Hansen *et al.*, 2013; Kameyama *et al.*, 2012; Tamang *et al.*, 2013). The association of group 9 CTX-M genes with the same class 1 integron present in

pH19 have been reported before in the IncFII plasmid pTN48, *E. coli* C1635 and *Klebsiella pneumoniae* ILT-3 (Poirel, Decousser and Nordmann, 2003; Billard-Pomares *et al.*, 2011; Ben Slama *et al.*, 2011). As observed in pH19, the *ISEcp1* in C1635 has undergone insertion by *IS1* into the *ISEcp1* at the same location as in pH19. Insertion of transposons and insertion sequences into the *ISEcp1* has been reported before, with *IS1* inserted into *ISEcp1-bla_{CTX-M-3}*, *IS10* in *ISEcp1-bla_{CTX-M-14}* and truncation of *ISEcp1* by *IS26* (Eckert, Gautier and Arlet, 2006; Ben Slama *et al.*, 2011). The insertion of *IS1* into *ISEcp1* is likely to cause its inactivation and change in orientation when compared to similar integrons (Poirel, Decousser and Nordmann, 2003; Ben Slama *et al.*, 2011). However the presence of *bla_{CTX-M-14}* in the class 1 integron, allows for both antimicrobial co-selection and also provides the opportunity to be mobilised by adjacent transposon such as *Tn21* or *Tn1721*. With both a similar integron and *repA* present in the IncFII plasmid pTN48 to those found in pH19 could indicate a recombination between two such plasmids resulting in the conversion of an IncB or K plasmid into an MDR IncZ (Billard-Pomares *et al.*, 2011; Kato and Mizobuchi, 1994).

The findings of this study and of others have clearly identified that the core plasmid backbone, irrespective of replicon type be it IncK, B or Z, is largely conserved (Papagiannitsis *et al.*, 2011; Cottell *et al.*, 2011). Studies have found this backbone to be associated with resistance genes as observed in both pR3521 and pHUSEC41-1 (Papagiannitsis *et al.*, 2011; Kunne *et al.*, 2012). These plasmids have undergone the insertion of resistance genes downstream of *trbC*, with both plasmids having *sul2*, *strA*, *strB* and *bla_{TEM-1}*, and additionally *aacC2*, *bla_{ACC-4}*, and *bla_{SCO-1}* in pR3521 (Papagiannitsis *et al.*, 2011; Kunne *et al.*, 2012). Equally these plasmid backbones are capable of being vectors for virulence with both pO26-Vir and pO113 carrying hemolysin, catalase and serine protease genes (Leyton *et al.*, 2003; Fratamico *et al.*, 2011). There appears to be a "trade off" with these plasmids, carrying either resistance or virulence genes, and currently have not been identified as carrying both (Papagiannitsis *et al.*, 2011; Kunne *et al.*, 2012;

Leyton *et al.*, 2003; Fratamico *et al.*, 2011). This is in contrast to the IncFII plasmids pTN48 and pHK01 which have both resistance and virulence regions (Billard-Pomares *et al.*, 2011; Ho *et al.*, 2011a).

Studies have already documented the presence of β -lactamase resistance genes on both IncB and IncK plasmids, with a clear link present between IncK and *bla*_{CTX-M-14}, shown in the UK, Spain and Asia (Stokes *et al.*, 2012; Valverde *et al.*, 2009; Cottell *et al.*, 2011; Zong *et al.*, 2008). IncB plasmids have been found to harbour β -lactamase genes including *bla*_{TEM-1}, *bla*_{CTX-M-1}, *bla*_{CTX-M-14} and *bla*_{CMY-2} isolated from both humans and animals (Rodriguez *et al.*, 2009; Leverstein-van Hall *et al.*, 2011; Kunne *et al.*, 2012; Hiki *et al.*, 2013; Hordijk *et al.*, 2013; Ho *et al.*, 2012; Zong *et al.*, 2008). There is increasing evidence for the transmission of plasmids between animal to human isolates, including IncK, N and I1 γ (Leverstein-van Hall *et al.*, 2011; Stokes *et al.*, 2012; Moodley and Guardabassi, 2009; Dhanji *et al.*, 2012; Dolejska *et al.*, 2011c; Machado *et al.*, 2009). Husbandry practices, contaminated feed and contamination of the environment all play a role in the introduction and maintenance of *bla*_{CTX-M} into the animal population (Snow *et al.*, 2012; Teale, 2002; Machado *et al.*, 2009; Dhanji *et al.*, 2011b) This highlights the importance of studying the epidemiology of plasmids between humans, animals and the food chain. The classification of plasmids belonging to the IncI complex by PBRT, can occasionally yield misleading results with both IncB and IncZ replicons being grouped as IncB plasmids. Comparative analysis in this study also suggests that plasmids share a higher level of similarity than first thought, and so would benefit from further differentiation. The six molecular marker designed in this study were capable of distinguishing 8/9 plasmids compared in this study by direct PCR, and amplicon sequence analysis could distinguish all of the plasmids, in a similar approach to pMLST (Garcia-Fernandez *et al.*, 2011; Garcia-Fernandez *et al.*, 2008). Plasmids belonging to the IncB, K and Z replicon groups did not share the same marker profile, and all but two IncB plasmids could be distinguished. In combination with PBRT, this marker scheme improves on both

characterization and differentiation (Carattoli *et al.*, 2005a). Although the markers mArdA and mYdeA can be identified in some IncI1 γ plasmids, they are important markers in identifying the stability regions present in pH19, and are absent in other IncK, B and Z backbones. These markers can be removed from the typing scheme without directly affecting the discrimination between plasmids. The presence of these markers in IncI1 γ further highlights the ancestral relationship of the IncI plasmid complex. The classification of IncZ plasmids could be aided by designing markers for the *repA* gene, however this would also identify IncFII plasmids and consequently would only be beneficial if isolated in a plasmid free background. The use of UID numbers for molecular marker combinations means that a database could be created such as those used by MLST and pMLST, allowing researches to enter their marker profiles to compare with others internationally. By using a global approach to screening for these plasmids their extent of dissemination, routes of transmission and reservoirs can be determined.

The *in vitro* testing identified 101/136 isolates as having one or more markers, closer investigation of some isolates suggested the presence of some plasmids previously published. Isolates with marker profiles similar to the *in silico* profiles of the human associated plasmids pR3521 and pHUSEC41-1 (UID = 15) were observed in a human and a cattle isolates. Numerous turkey isolates had a profile identical to pCT (UID = 5), which is similar to the previous findings by Stokes *et al* (2012). Combinations of markers were identified in isolates, which had not been found from *in silico* testing, this potentially highlights new plasmid backbones which warrant further investigation, and again adds to the benefits of the markers in identifying plasmids. Although pH19 was not detected in any other isolates, the H19 isolate itself was found in an unrelated patient, spreading the plasmid through clonal dissemination (Stokes *et al.*, 2012). Dissemination of plasmid in this way has been reported before for those with *bla*_{CTX-M-15} genes, with *E. coli* ST131, in the UK and around the world (Mora *et al.*, 2011; Dhanji *et al.*, 2011a; Coque *et al.*, 2008).

The molecular markers can not only be applied to plasmids but can also be used to study the isolates as well, possibly being used to ascertain when isolates obtained plasmids, and monitor horizontal transmission across bacterial genera. In turn this can be used to potentially identify reservoirs and routes of dissemination, allowing the implementation of control. Such applications are important as non pathogenic isolates can become problematic by acquiring plasmid mediated resistance or virulence both of which have been associated with the IncI complex backbones. This study has described the first IncZ plasmid associated with *bla*_{CTX-M}, which has been recovered from a multidrug resistant human *E. coli* isolate. The self transmissible nature of pH19 which confers resistance to three antimicrobial classes, and its possible combination with virulence genes seen in pO113 and pO26-Vir is of major concern, potentially limiting treatment options for virulent isolates. The markers presented in this study present a novel method for the further characterization of plasmids belonging to the IncI complex and may be beneficial in the further understanding of their epidemiology.

Chapter 5

**Development and Application of
Molecular Markers for IncX4
Plasmids Bearing *bla*_{CTX-M} Found in
Human and Veterinary
Enterobacteriaceae.**

5.1 Introduction

The *bla*_{CTX-M-14} gene is one of the most widely disseminated CTX-M genes with IncK and IncFII plasmids having roles in its spread in Europe and Asia respectively, as previously discussed in chapters 3 and 4 (Stokes *et al.*, 2012; Valverde *et al.*, 2009; Kim *et al.*, 2011; Ho *et al.*, 2012). A plasmid backbone, that until 2011 had not been associated with *bla*_{CTX-M}, belongs to the incompatibility group IncX (Partridge *et al.*, 2011). IncX plasmids have been studied extensively, with R6K being one of the first due to its ability to confer resistance to penicillins (Kontomichalou, Mitani and Clowes, 1970). Multiple IncX plasmids including pR485 and pR487 were identified through incompatibility studies with R6K (Kontomichalou, Mitani and Clowes, 1970; Hedges *et al.*, 1973). The IncX (R6K) plasmids were also found to confer susceptibility to bacteriophage X-2 in *E. coli*, *Salmonella enterica* and *Serratia marcescens* which binds to the pilus, produced by R6K (Bradley *et al.*, 1981; Coetzee *et al.*, 1988). The presence of the IncX plasmids also enhances *Salmonella* phage conversion, through binding to the pilus (Brown *et al.*, 1999).

IncX plasmids were present in the bacterial population before the clinical use of antibiotics (Jones and Stanley, 1992). However IncX plasmids are increasingly being associated with resistance genes across the world such as *ISEcp1-bla*_{CTX-M-15} in Australia, Tn3 associated *bla*_{TEM-52} in Denmark and the Netherlands, *oxqAB* in Denmark and both *bla*_{TEM-1} and *qnrS1* in the Czech Republic and Poland (Partridge *et al.*, 2011; Bielak *et al.*, 2011; Sorensen *et al.*, 2003; Dolejska *et al.*, 2011a; Literak *et al.*, 2010a). IncX plasmids have also been shown to have a role in the formation of bacterial biofilms with some plasmids such as R485 and pOLA52 having Type III biofilm cassettes, flanked by transposons (Burmolle *et al.*, 2012; Norman *et al.*, 2008).

The detection of IncX plasmids by PBRT was limited to plasmids belonging to a subdivision of IncX referred to as the IncX2, and typified by R6K (Carattoli *et al.*, 2005a). Consequently IncX1 plasmids such as R485 were not identified by PBRT, and although

presumed to be more ubiquitous than IncX2, their role in the dissemination of resistance could not easily be determined (Jones, Osborne and Stanley, 1993; Couturier *et al.*, 1988). The full genome sequencing of isolates and novel plasmid vectors soon identified other IncX plasmids that were neither IncX1 or IncX2 (Johnson *et al.*, 2012a). This included plasmids pIncX-SHV from *Klebsiella pneumoniae* classed as an IncX3, and pCROD2 from *Citrobacter rodentium* belonged to the IncX4 group (Garcia-Fernandez *et al.*, 2012; Petty *et al.*, 2010; Johnson *et al.*, 2012a). Johnson *et al.* (2012a) identified the limitations of PBRT to detect the increasing number of variants of IncX plasmids and devised a new typing scheme. The new scheme was based on the phylogentic relationship of the *taxC* gene which codes for the plasmid nickase (Johnson *et al.*, 2012a). This gene was found to be conserved within a IncX subgroup but unrelated to the other subgroups, four sets of primers were designed based on the sequence to type plasmids IncX1-4 (Johnson *et al.*, 2012a).

The first and currently only IncX plasmid with *bla*_{CTX-M} was isolated from an *E. coli* from a patient's blood sample in Australia in 2006 (Partridge *et al.*, 2011). Conjugations using the field isolate identified a transmissible 35 kb plasmid carrying *bla*_{CTX-M-15}. The PBRT typing of the conjugate failed to yield a replicon type for the plasmid and so sequencing was performed. The plasmids designated pJIE143 (JN194214) was a *pir* (π protein for initiation of replication) related plasmid with a single resistance gene in the transposition unit, *ISEcp1-bla*_{CTX-M-15-orf477} (Partridge *et al.*, 2011). During the routine screening and conjugative studies of ESBL plasmids performed by AHVLA, a conjugative plasmid bearing *bla*_{CTX-M-14} was identified that could not be typed by PBRT, which was termed pSAM7 (L. Randall personal communication, July 15 2010).

5.1.1 Hypotheses and aims

The hypothesis of this study was that pSAM7 is related to IncX plasmids, which are un-typable by PBRT, and that similar plasmids can be identified and differentiated

using molecular markers. The aims of this study were to (i) extract the pSAM7 plasmid DNA from the SAM7 isolate, sequence and annotate the plasmid to determine its genotype and features. (ii) Compare plasmid pSAM7 with other plasmid previously sequenced, to determine the relationship between plasmids. (iii) Design molecular markers capable of identifying pSAM7 and differentiating between similar plasmids, which can be used as epidemiological tools. (iv) Screen a panel of *E. coli* field isolates for the prevalence of pSAM7 and similar plasmids, and to identify a possible reservoir.

5.3 Methods and materials

The SAM7 (pSAM7) *E. coli* field isolate, was isolated from cattle faeces in 2008 and *Enterobacter cloacae* ECR528 (pSAM7-2) was isolated from waste milk in 2012 during routine surveillance studies at AHVLA. Identity of isolates was determined by matrix assisted laser desorption and ionisation time of flight (MALDI-TOF) at AHVLA (O. Diribie personal communication July 2012). Sequence types of *E. coli* in this study were determined by MLST as described in 2.3.3. Total DNA was extracted from SAM7 using the QIAGEN Hi-Speed plasmid midi prep as described in 2.2.4.3, which was then transformed into *E. coli* DH10B (2.2.5). Transformants were checked for the presence of a single CTX-M plasmid by plasmid profiling as described in 2.3.12.2 and using the group 9 CTX-M PCR (2.3.5). DNA extraction and transformations for *Enterobacter cloacae* ECR528 (pSAM7-2) were performed by M. AbuOun and H. Preedy. The antimicrobial sensitivities of the SAM7 and ECR528 wild types and the pSAM7 and pSAM7-2 transformants were determined by disc diffusion as described in 2.1.3. Plasmid content and sizes were determined by S1 nuclease for both the field isolates and transformants (2.3.12.1), replicons were determined using a commercial kit for the field isolates (2.3.6.2). The transferability of pSAM7 and pSAM7-2 was determined by solid conjugations as stated in 2.1.4.2, as were isolates positive for pSAM7 markers. Plasmid DNA for sequencing was extracted from the pSAM7 transformant using the QIAGEN large construct kit as described in 2.2.4.4, and sequenced on a Roche 454 GS FLX (2.4.1)

with closure of the sequence by PCR using the primers in Table 2.11, using the conditions in 2.4.3.1, pSAM7-2 was extracted and sequenced by M. AbuOun and H. Preedy. The plasmid was annotated using several programs including RAST (2.5.1.1), Artemis (2.5.1.2) and BLASTn using the annotations from plasmids pJIE143 (JN194214), pBS512_33 (CP001059) and pCROD2 (FN543504) as described in 2.5.1.3. *In silico* IncX PCR was performed on the pSAM7 plasmid as described in 2.5.3.3. Plasmids from GenBank were compared with pSAM7 using MAUVE (2.5.2.1), ACT (2.5.2.2) and BLASTn (2.5.1.3) to determine the relationship between plasmids and identify molecular marker candidates. The phylogeny of the plasmids was analysed using concatenated sequence compiled from open reading frames present in all plasmids which was analysed as described in 2.5.2.5. The screening of molecular markers in 2.3.8, Table 2.6 were performed against plasmids from GenBank *in silico* as described in 2.5.3.1, with the phylogeny of the markers analysed as stated in 2.5.2.5. *E. coli* field isolates with small ESBL encoding plasmids from the SFE collection (D Mevius personal communication, February 12 2012, appendix III) were screened using the pSAM7 markers using the conditions in 2.3.8, Table 2.6, in total 42 isolates with *bla*_{CTX-M-1} (human n=9, cattle n=7, poultry n=5, pig n=4), *bla*_{CTX-M-14} (human n=2, cattle n=5), *bla*_{CTX-M-15} (cattle n=2), *bla*_{TEM-52} (poultry n=7) or *bla*_{SHV-12} (human n=1) were screened. Plasmids in isolates positive for three or more markers were conjugated (2.1.4.2) and re-tested for pSAM7 markers. The genetic environment of the *bla*_{CTX-M-14b} gene was analysed using the primers in 2.3.9 (Table 2.10) and the size of the plasmids determined by S1 nuclease as stated in 2.3.12.1. The relationship of *E. coli* isolates was determined by PFGE as stated in 2.3.1

5.3 Results

5.3.1 Analysis of SAM7 *E. coli* and *Enterobacter cloacae* field isolates

5.3.1.1 Antimicrobial sensitivity testing of field isolates

The reduced antimicrobial sensitivities of *E. coli* SAM7 and *Enterobacter cloacae* ECR528 were determined by disc diffusion. SAM7 was found to have reduced sensitivity to tetracycline, neomycin, ampicillin, cefotaxime, ceftazidime, streptomycin, trimethoprim, sulphonamides, and amoxicillin with clavulanic acid. ECR528 was only found to be resistant to β -lactams with reduced sensitivities to ampicillin, cefotaxime and ceftazidime.

5.3.1.2 Plasmid analysis of field isolates

Both SAM7 and ECR528 were analysed for plasmid content by both replicon typing and S1 nuclease PFGE for plasmid sizing. SAM7 was positive for three replicons which were IncI1 γ , FIB and P, no IncX replicon was detected, three plasmids of 35, 100 and 180 kb in size were identified by S1 nuclease PFGE. ECR528 tested negative for all replicons and only a single plasmid was identified by S1 nuclease PFGE of 35 kb in size. These results suggested that the 35 kb plasmid in both isolates was the vector for the *bla*_{CTX-M-14b} gene.

5.3.2 Analysis of the pSAM7 and pSAM7-2 plasmids

The extracted plasmids pSAM7 from *E. coli* SAM7 and pSAM7-2 from *Enterobacter cloacae* were successfully transformed into *E. coli* DH10 (DH10+pSAM7 and pSAM7-2), which was confirmed by plasmid profiling and PCR. As with the parent strains, the transformants were tested by disc diffusion, both transformants were only found to have resistance to β -lactams, including ampicillin, cefotaxime and ceftazidime. Plasmid analysis by S1 nuclease demonstrated that both transformants had plasmids of 35 kb in size.

5.3.2.1 Transferability of pSAM7 and pSAM7-2 plasmids

The transferability of pSAM7 and pSAM7-2 plasmids from the transformants was investigated by conjugation using a plasmid free, rifampicin-resistant *S. Typhimurium* 26R as the recipient. Plasmids were transferred by liquid conjugations, which were considered to have occurred by the presence of red colonies on Rambach's agar supplemented with 100 µg/ml rifampicin and 1 µg/ml cefotaxime, with the recipient only used as a negative control, being unable to grow on cefotaxime. Single red colonies were selected and inoculated onto LB-G agar supplemented with 4 µg/ml cefotaxime. Plasmids pSAM7 and pSAM7-2 were found to be conjugative and capable of permitting growth on selective media.

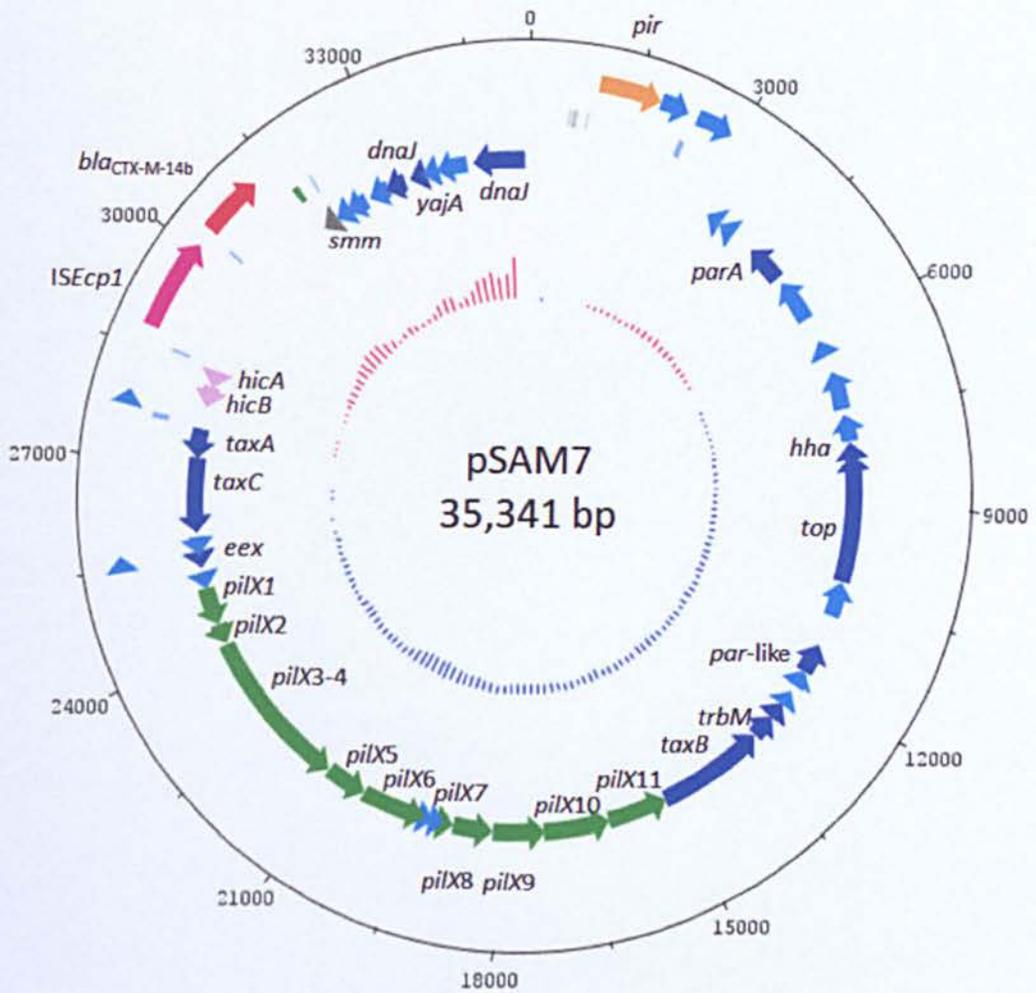
5.3.3 pSAM7 and pSAM7-2 plasmid sequencing

Sequencing of the plasmids and pSAM7 and pSAM7-2 both yielded a single contig with >240 fold coverage after *de novo* assembly, contigs were closed to form a continuous sequence by PCR. The complete sequence was 35,341 bp in length and had a G+C content of 41.8%. Sequence comparison of the two plasmids pSAM7 and pSAM7-2 found that they were identical with no single nucleotide polymorphisms (SNPs) detected and was termed pSAM7. Sequence analysis and annotation identified 51 complete open reading frames, which were arranged in a 32,095 contiguous backbone which contains a 3,246 bp resistance insert. The pSAM7 plasmid backbone contained genes involved in replication, stability and conjugation as shown in Figure 5.1, and will be described in detail, a full gene list is presented in Table 5.1.

5.3.3.1 Analysis of the pSAM7 replication region

The replication of pSAM7 like other IncX plasmids is mediated through iterons, repetitive 22 bp sequences which act as the binding site for the π protein for initiation of replication coded by the *pir* gene (Stalker and Helinski, 1985; Saxena *et al.*, 2010). These iterons form three sites for replication known as *oriVa*, *oriV β* and *oriV γ* the latter of which

Figure 5.1 Plasmid map of pSAM7



Circular plasmid map of pSAM7. Shown are the coding regions in the pSAM7 plasmid (complemented in Table 5.1) with the outer arrowed circle denoting the positive orientation and the inner arrow circle denoting the negative orientation strand. Green and arrows indicate genes involved in conjugation, dark blue represent other known genes, orange represents the *pir* replication protein gene light blue are hypothetical genes, pink genes are the addition system, magenta represents the transposon gene *ISEcp1* and in red is the *bla_{CTX-M-14}* resistance gene, the graph shows the GC plot.

Table 5.1 Open reading frames present in pSAM7

Gene	ORF	Direction	Location	Length (bp)	Protein length (aa)	Product	Function
<i>pir</i>	1	Forward	967-1839	873	291	Replication protein	Plasmid replication
<i>orf2</i>	2	Forward	1852-2256	405	135	Conserved hypothetical protein	Unknown
<i>orf3</i>	3	Forward	2391-2918	528	176	Conserved hypothetical protein	Unknown
<i>orf4</i>	4	Reverse	3225-3440	216	72	Conserved hypothetical protein	Unknown
<i>orf5</i>	5	Reverse	3502-3753	252	84	Conserved hypothetical protein	Unknown
<i>parA</i>	6	Reverse	4185-4844	660	220	ParA	Plasmid partitioning
<i>orf7</i>	7	Reverse	4949-5689	741	247	Resolvase	Resolvase
<i>orf8</i>	8	Reverse	6113-6307	195	65	Conserved hypothetical protein	Unknown
<i>orf9</i>	9	Reverse	6667-7323	657	219	Conserved hypothetical protein	Unknown
<i>orf10</i>	10	Reverse	7427-7891	465	155	H-NS like	Nucleotide binding protein
<i>hha</i>	11	Reverse	7907-8113	207	69	Hha	Regulator
<i>topIII</i>	12	Reverse	8110-10263	2154	718	Top III	Topoisomerase
<i>orf13</i>	13	Reverse	10268-10843	576	192	Conserved hypothetical protein	Unknown
<i>par-like</i>	14	Reverse	11369-11881	513	171	Putative Par	Plasmid partitioning
<i>orf15</i>	15	Reverse	11860-12168	309	103	Conserved hypothetical protein	Unknown
<i>orf16</i>	16	Reverse	12271-12525	255	85	Conserved hypothetical protein	Unknown
<i>trbM-like</i>	17	Reverse	12531-12836	306	102	KikA like	Host specificity
<i>Orf18</i>	18	Reverse	12820-13239	420	140	Putative lipoprotein	Unknown
<i>taxB</i>	19	Reverse	13251-15113	1863	621	TaxB	DNA transfer
<i>pilx11</i>	20	Reverse	15100-16128	1029	343	PilX11	ATPase
<i>pilx10</i>	21	Reverse	16130-17248	1119	373	PilX10	Conjugal transfer protein
<i>pilx9</i>	22	Reverse	17241-18149	909	303	PilX9	Membrane spanning protein
<i>pilx8</i>	23	Reverse	18146-18832	687	229	PilX8	Conjugal transfer protein
<i>pilx7</i>	24	Reverse	18825-18953	129	43	PilX7	Conjugal transfer protein
<i>orf25</i>	25	Reverse	18966-19169	204	68	Conserved hypothetical protein	Unknown

Gene	ORF	Direction	Location	Length (bp)	Protein length (aa)	Product	Function
<i>orf26</i>	26	Reverse	19171-19341	171	57	Conserved hypothetical protein	Unknown
<i>pilx6</i>	27	Reverse	19347-20441	1095	365	PilX6	Conjugal transfer protein
<i>pilx5</i>	28	Reverse	20453-21169	717	239	PilX5	Type IV pilus
<i>pilx3-4</i>	29	Reverse	21181-23928	2748	916	PilX3-4	ATPase
<i>pilx2</i>	30	Reverse	23947-24273	327	109	PilX2	Type IV pilus
<i>pilx1</i>	31	Reverse	24287-24907	621	207	PilX1	Transglycolase
<i>orf32</i>	32	Reverse	24910-25086	177	59	Conserved hypothetical protein	Unknown
<i>eex</i>	33	Reverse	25264-25479	216	72	Eex	Surface exclusion protein
<i>orf34</i>	34	Reverse	25476-25769	294	98	Conserved hypothetical protein	Unknown
<i>orf35</i>	35	Forward	25537-25662	126	42	Conserved hypothetical protein	Unknown
<i>taxC</i>	36	Reverse	25846-27075	1230	410	TaxC	Nickase
<i>taxA</i>	37	Reverse	27079-27582	504	168	TaxA	DNA transfer
<i>orf38</i>	38	Forward	27805-27930	126	42	Conserved hypothetical protein	Unknown
<i>hicB</i>	39	Reverse	27943-28290	348	116	HicB	Antitoxin protein
<i>hicA</i>	40	Reverse	28287-28535	249	83	HicA	Toxin protein
<i>ISEcp1 tnpA</i>	41	Forward	28819-30081	1263	421	ISEcp1 TnpA	Transposon
<i>bla_{CTX-M-14b}</i>	42	Forward	30331-31206	876	292	CTX-M-14	β -lactamase
<i>smm</i>	43	Reverse	31608-31829	222	74	S-methylmethioninepermease	Unknown
<i>orf44</i>	44	Reverse	31868-32005	138	46	Conserved hypothetical protein	Unknown
<i>orf45</i>	45	Reverse	32081-32446	366	122	Conserved hypothetical protein	Unknown
<i>orf46</i>	46	Reverse	32522-32863	342	114	Conserved hypothetical protein	Unknown
<i>dnaJ partial</i>	47	Reverse	32823-33182	360	120	DnaJ Truncated	Chaperone protein
<i>yajA</i>	48	Reverse	33275-33502	228	76	YajA	Unknown
<i>orf49</i>	49	Reverse	33489-33740	252	84	Conserved hypothetical protein	Unknown
<i>orf50</i>	50	Reverse	33737-34279	543	181	Conserved hypothetical protein	Unknown
<i>dnaJ</i>	51	Reverse	34384-35262	879	293	DnaJ	Chaperone protein

has 7 repeats flanked either side by *oriVa* and *oriVβ* at considerable distance approximately 3.5 kb (Saxena *et al.*, 2010; Saxena, Abhyankar and Bastia, 2010). All of these iterons are required to bind to the π protein to initiate replication, these proteins also act as regulators by the handcuffing method through binding to the iterons as dimers (Saxena *et al.*, 2010; Bowers, Kruger and Filutowicz, 2007). The three *oriV* regions in pSAM7 were near identical to those found in plasmid pJIE143 with the exception of *oriVγ3* as shown in Table 5.2 (Partridge *et al.*, 2011). The *topIII* gene for topoisomerase for the unwinding of DNA was present in pSAM7 as was the gene for *dnaJ* a chaperone involved in the activation of the π protein.

Table 5.2 Iterons in pSAM7 and pJIE143

<i>oriV</i>	pJIE143	pSAM7	Location (bp)
<i>oriVγ1</i>	AAACATGAGAGCTTCCTCGGTT	AAACATGAGAGCTTCCTCGGTT	603-624
<i>oriVγ2</i>	AAACATGATAACTTCCTCGGTT	AAACATGATAACTTCCTCGGTT	625-646
<i>oriVγ3</i>	AAACATGAT <u>TA</u> ACTTCCTCGGTT	AAACATGAG <u>GA</u> GCTTCCTCGGTT	647-668
<i>oriVγ4</i>	AAACATGAGAGCTTCCTCGGTT	AAACATGAGAGCTTCCTCGGTT	669-690
<i>oriVγ5</i>	AAACATGATAACTTCCTCGGTT	AAACATGATAACTTCCTCGGTT	691-712
<i>oriVγ6</i>	AAACATGAGAGCTTCCTCGGTT	AAACATGAGAGCTTCCTCGGTT	713-734
<i>oriVγ7</i>	AAACATGAGAAGATACTCGGTT	AAACATGAGAAGATACTCGGTT	873-894
<i>oriVa</i>	TAAAATCGTCGCTGCATATGTC	TAAAATCGTCGCTGCATATGTC	27718-27739
<i>oriVβ</i>	AAAATTGAGGTC	AAAATTGAGGTC	1832-1843

Differences in sequence are shown in bold underlined red letters.

5.3.3.2 Analysis of the pSAM7 transfer region

The mobility of IncX plasmids has been studied using the archetype plasmid R6K, finding two key elements, the Dtr_X which is involved in conjugative DNA processing and Mpf_X involved in pilus synthesis and assembly (Nunez, Avila and delaCruz, 1997). The Mpf_X in pSAM7 is made up of 11 *tra* (*pil*) genes which are involved in the assembly of the pilus which is a target for X-2 phage (Bradley *et al.*, 1981; Coetzee *et al.*, 1988; Nunez, Avila and delaCruz, 1997; Norman *et al.*, 2008). There are two ATPase proteins PilX3-4 and PilX11, two type IV like components PilX2 and PilX5, and the PilX1 is a transglycolase. The Dtr_X consists of Tax A, B and C proteins of which TaxC is the relaxase protein, pSAM7 also has an Eex protein, which is a putative surface exclusion

protein which shares 49% identity to TraG on IncQ plasmids (Avila, Nunez and de la Cruz, 1996; Norman *et al.*, 2008).

5.3.3.3 Analysis of the pSAM7 stability region

The pSAM7 plasmid contains two stability mechanisms. The first is the putative partitioning proteins ParA, resolvase and a ParB-like protein which are also present in pJIE143 with ParB sharing 95% similarity (Partridge *et al.*, 2011). These proteins are also present in other IncX plasmids including pOLA52 and pCROD2 (Norman *et al.*, 2008; Petty *et al.*, 2010). The second is a proteic class addiction system which was recently identified by Jorgensen *et al.* (2009) which is involved in the persistence of the plasmids (Jorgensen *et al.*, 2009). The *hicA* genes present in pSAM7 produces a 83 amino acid toxin protein (HicA) which binds and cleaves mRNA causing a bacteriostatic state, similar to RelE. The larger HicB antitoxin protein binds to HicA preventing RNA cleavage, and is capable of resuscitating HicA affected cells (Jorgensen *et al.*, 2009). The pSAM7 plasmid also contains KikA like protein (TrbM) related to that present on IncN plasmids, this protein may have a role in the host range in which these plasmids can persist, and causes reversible growth inhibition (Holcik and Iyer, 1996; Hengen, Denicourt and Iyer, 1992). The presence of this protein in *Klebsiella oxytoca* caused a reduction in cell viability, however in *E. coli* no change in the viability was observed (Holcik and Iyer, 1996).

5.3.3.4 Analysis of the pSAM7 resistance region

A single resistance gene was identified on the pSAM7 backbone, the ESBL gene *bla*_{CTX-M-14b}. The *bla*_{CTX-M-14b} differs from *bla*_{CTX-M-14a} by three nucleotide substitutions at 372 A>G, 569 G>A and 702 G>A all of which are synonymous mutations. It has been hypothesised that these two genes have diverged on different plasmid backbones with *bla*_{CTX-M-14a} on IncK plasmids and *bla*_{CTX-M-14b} on IncHI2 plasmids (Navarro *et al.*, 2007). The progenitor of the *bla*_{CTX-M-9} gene is from the chromosome of *Kluyvera georgiana* with KLUY-2, varying from CTX-M-14 by one amino acid substitution at 269 from arginine >

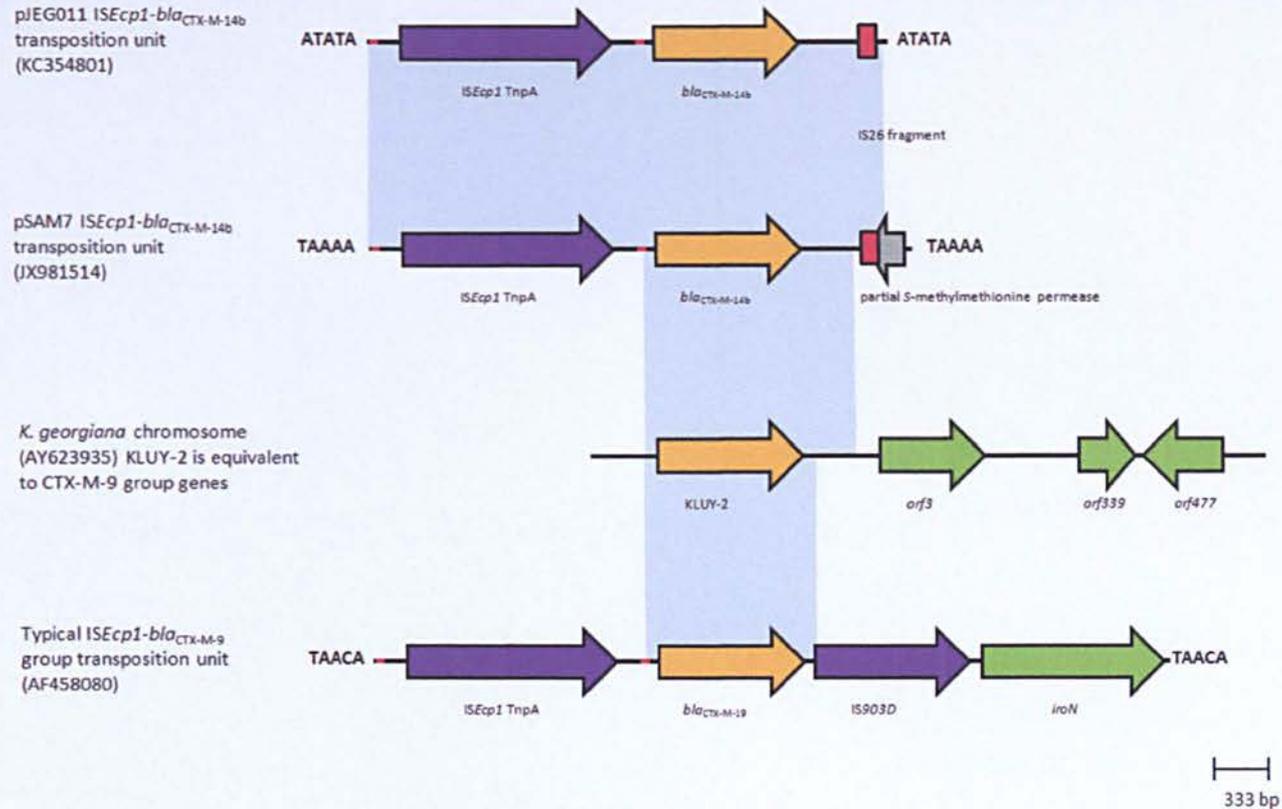
proline (Olson *et al.*, 2005). In pSAM7 the *bla*_{CTX-M-14b} gene was located downstream of *ISEcp1*, as previously reported for the *bla*_{CTX-M-G9} genes (Poirel, Decousser and Nordmann, 2003; Eckert, Gautier and Arlet, 2006). However *IS903* was absent downstream of the *bla*_{CTX-M-14} gene, replaced by fragments of S-methylmethionine permease and *IS26* genes, which form a unique transposition unit.

The *bla*_{CTX-M-14b} insertion unit has inserted 97 bp upstream of *hicA* between 28,628-31,873 bp and the 5 bp direct repeats TAAAA have been formed at 28,628-28,632 and 31,869-31,873 bp respectively from the *ISEcp1* insertion. Typically the *ISEcp1* transposition units carrying *bla*_{CTX-M-9} group genes contain a 920 bp region which corresponds to the *Kluyvera georgiana* chromosome (AY623935), with *IS903* 2 bp downstream. In pSAM7 a region matching 1,256 bp of the *Kluyvera georgiana* is present which is followed by 108 bp of the N terminal part of *IS26* followed by a 222 bp hypothetical gene of which 177 bp is an internal fragment of the S-methylmethionine permease gene, as shown in Figure 5.2. A similar transposition unit was found in the IncL/M plasmid pJEG011 (JX981514) isolated from *K. pneumoniae* recovered in Australia, but only 73 bp of the S-methylmethionine permease gene is present (Espedido *et al.*, 2013).

5.3.3.5 Analysis of the pSAM7 accessory genes

The H-NS-like nucleotide binding protein and a *hha* regulator present in pSAM7, have motifs which bind DNA and have roles in both packaging of DNA, gene expression and may increase pSAM7 stability (Tendeng and Bertin, 2003). pSAM7 had 20 conserved hypothetical genes in the backbone which have unknown functions, but which are present in other plasmids such as pJIE143, pBS512_33 and pCROD2 (Partridge *et al.*, 2011; Petty *et al.*, 2010).

Figure 5.2 Comparison of the *bla*_{CTX-M-14b} genetic environment of pSAM7



Comparison of the *ISEcp1* transposon units in pSAM7, pJEG011, a typical *ISEcp1-bla*_{CTX-M-9} group transposition unit and with the chromosome of *Kluyvera georgiana*. Purple arrows indicates the transposon *ISEcp1* and *IS903D*, orange indicates the *bla*_{CTX-M-9} genes, green arrows are other genes associated with the insertion or genetic environment, red boxes are the IS26 fragment and the grey arrow indicates the S-methylmethionine gene. Direct repeats are shown by the 5 bases at the end of the sequence, red lines are the inverted repeats for *ISEcp1*, blue regions show homology between transposon units, drawn to scale.

5.3.4 Comparison with other IncX4 plasmids

The complete sequence of pSAM7 was compared to sequenced plasmids in GenBank database using the BLASTn algorithm to identify similar plasmids. The searches identified pSAM7 to be similar to pJIE143 (93%), pJEG012 (85%), pBS512_33 (84%), pSH696_34 (83%), pCROD2 (80%) and pUMNF18_32 (73%), as shown in Table 5.3. All of the plasmids had the core backbone genes which consists of the 11 *pilX taxA, B* and *C, hha* and the histone like gene, *yajA* and *dnaJ*. Comparison of the *tra* region with the other plasmids had high identities pJIE143 (99%), pJEG012 (99%), pBS512_33 (99%), pSH696_34 (97%), pCROD2 (95%) and pUMNF18_32 (97%), the pSAM7 *tra* region also had 76% identity with the plasmid pJARS35 from *Yersinia pestis*., This plasmid only shares 23% coverage with pSAM7 (Eppinger *et al.*, 2012). The annotated pSAM7 plasmid was used to create a database of all the open reading frames each ORF was compared with the plasmids in Table 5.3, the output of the ORF comparison was shown as a heat map in Figure 5.3.

5.3.4.1 Phylogeny of IncX4 plasmids

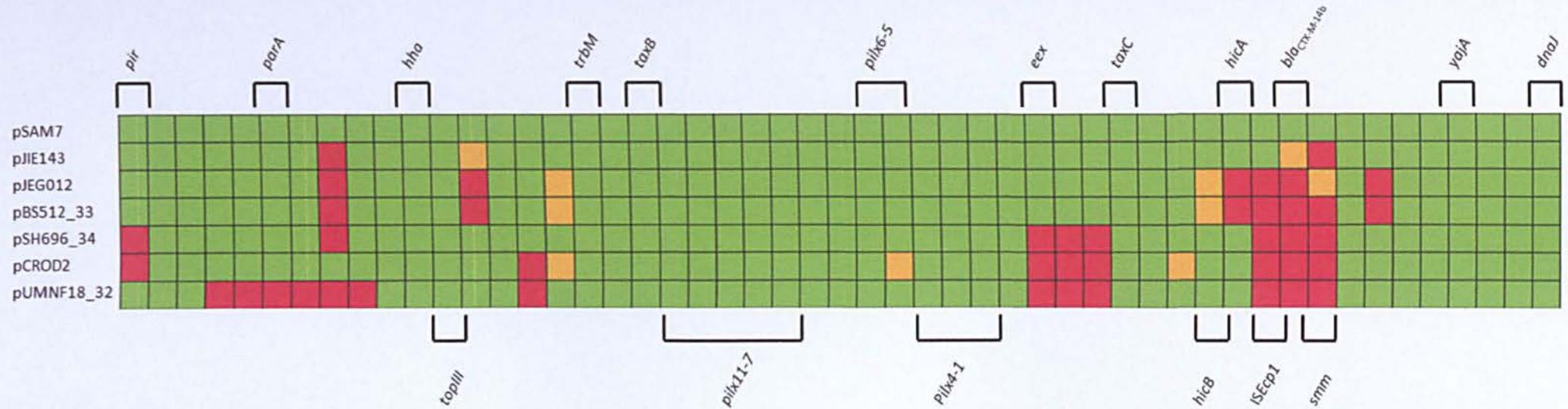
The phylogeny of the IncX4 plasmids was compared using a concatenation of backbone genes as shown in Figure 5.4. The phylogenetic relationship showed that pSAM7 and pJIE143 were the most closely related with 99% coverage and only 41/17916 nucleotide polymorphisms. pBS512_33 and pJEG012 were also grouped together which only had 6 SNPs between them. pCROD2, pSH696_34 and pUMNF18_32 all fall into a separate cluster, pCROD2 shares 97% identity with pSAM7, pSH696_34 shares 98% and pUMNF18_32 shares 97%.

Table 5.3 Plasmids compared with pSAM7

Plasmids	Accession number	% coverage with pSAM7	% coverage with pSAM7 without insert	Size (bp)	Replicon	Bacterial species	Source	Location	Resistance genes	Isolation or submission date	Reference
pSAM7	JX981514	NA	NA	35,341	X4	<i>E. coli</i> <i>Enterobacter cloacae</i>	Cattle	UK	<i>bla</i> _{CTX-M-14b}	2008 2012	Stokes <i>et al</i> (2013)
pJIE143	JN194214	93	97	34,345	X4	<i>E. coli</i>	Human	Australia	<i>bla</i> _{CTX-M-15}	2006	Partridge <i>et al</i> (2011)
pJEG012	KC354802	85	93	41,919	X4	<i>Klebsiella pneumoniae</i>	Human	Australia (Egypt)*	<i>bla</i> _{OXA-9} , <i>bla</i> _{TEM-1} <i>aacA4</i> and <i>aadA1</i>	2010	Espedido <i>et al</i> (2013)
pBS512_33	CP001059	84	93	33,103	X4	<i>Shigella boydii</i>	Human	USA	None	2008	Rasko <i>et al</i> (unpublished)
pSH696_34	JX258654	83	91	33,765	X4	<i>Salmonella enterica</i> Hedielberg	Turkey	USA	None	2000	Han <i>et al</i> (2012)
pCROD2	FN543504	80	88	39,265	X4	<i>Citrobacter rodentium</i>	Rodent	USA	None	1976	Petty <i>et al</i> (2010)
pUMNF18_32	CP002895	73	81	32,487	X4	<i>E. coli</i>	Pig	USA	None	2006	Shepard <i>et al</i> (2012)

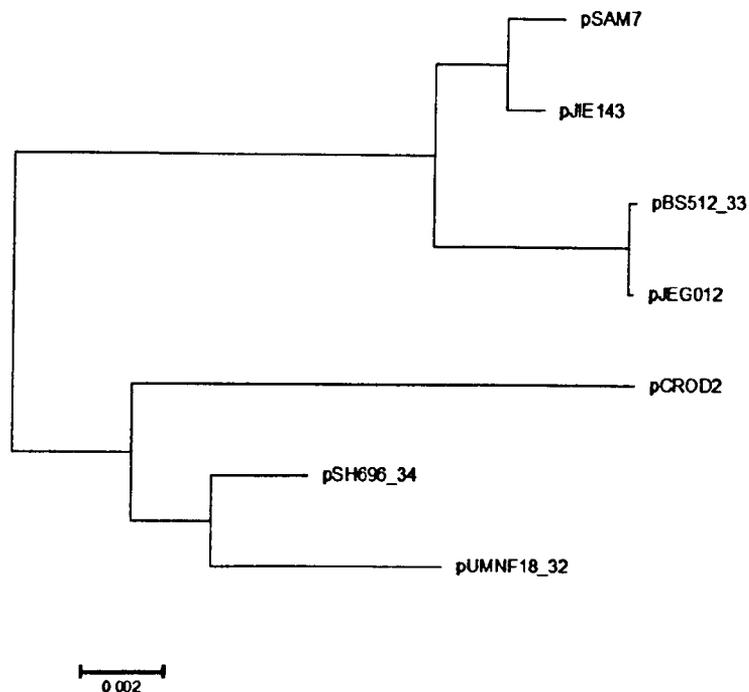
* = Locations in brackets are countries that the host had links to or recent travel

Figure 5.3 Heat map of pSAM7 ORF comparison with IncX4 plasmids



Heat map of all of the ORFs of pSAM7 compared to the IncX4 plasmids pJIE143, pEG012, pSH512_33, pSH696_34, pCROD2 and pUMNF18_32. Green indicates the presence of the ORF, orange indicates less than 80% of the ORF present and red is the absence of the ORF. Data generated using stand alone BLASTn.

Figure 5.4 Phylogeny of the IncX4 plasmids backbone



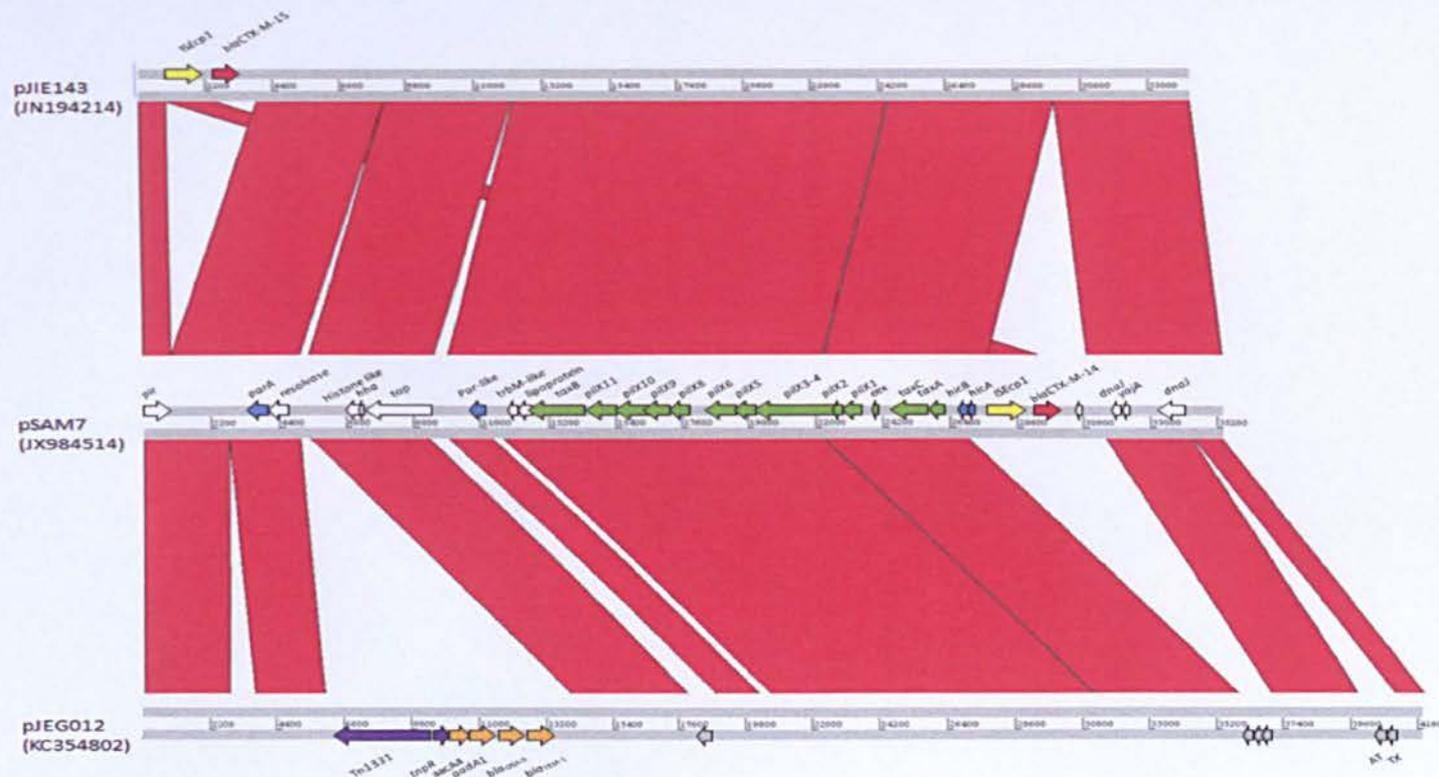
Dendrogram of the IncX4 plasmids using a concatenation of backbone genes, aligned using Clustal W MEGA 5.0 using neighbour joining with 1000 bootstrap repeats, scale indicates substitutions per base.

5.3.4.2 Comparison of pSAM7 with pJIE143 and pJEG012

Of the IncX4 plasmids in this study, pJIE143 is the most closely related to pSAM7 and is the only other plasmid to have a *bla*_{CTX-M} gene, as shown in Figure 5.5. The plasmid pJIE143 was isolated from *E. coli* ST131, from a patient in Australia with bacteraemia. pJIE143 could not be typed by PBRT and as a result was sequenced, the findings found it to be a *pir* type IncX plasmid. pJIE143 carries the common *ISEcp1*-*bla*_{CTX-M-15-orf477} transposition unit, which has inserted downstream of the *pir* gene and is flanked by GGATA direct repeats (Partridge *et al.*, 2011).

The removal of both of the transposition units from pSAM7 and pJIE143 found that these plasmids were highly similar with a nucleotide coverage of 99%. Closer examination revealed that apart from the insertion of the *bla*_{CTX-M} transposition units, the only other differences were two insertions in pSAM7. The first was between positions 6,061-6,377

Figure 5.5 Artemis comparison tool analysis of pSAM7 with pJIE143 and pJEG012



Artemis comparison tool analysis of the DNA sequences of pJIE143 and pJEG012 with pSAM7. Red regions indicate areas of DNA homology between sequences, spaces indicate unique sequence to that plasmid. Genes involved in conjugation are shown by green arrows, *ISEcp1* is shown by yellow arrows and *bla_{CTX-M}* by red arrows, stability genes are shown by blue arrows and other genes are shown by white arrows. Purple arrows indicate transposons, orange arrows indicate resistance genes and grey arrows indicate hypothetical genes not present in pSAM7.

bp which is in a conserved hypothetical gene in pJIE143 (positions 8,971-9,222), which was flanked by the nearly perfect direct repeat sequences GGACAGAATCACCTGTATGTC (6,040-6,060) and GGACAAAATGACCTGTATGTC (6,377-6,397). The other insertion is between positions 10,489-10,925 of pSAM7, which form part of a hypothetical coding sequence (10,268-10,843). This region is also present in the IncX4 plasmids pSH696_34 and the IncX1 plasmids pE001 (JF776874) and pDKX1-TEM-52 (JQ269336) (Han *et al.*, 2012; Bielak *et al.*, 2011; Johnson *et al.*, 2012a).

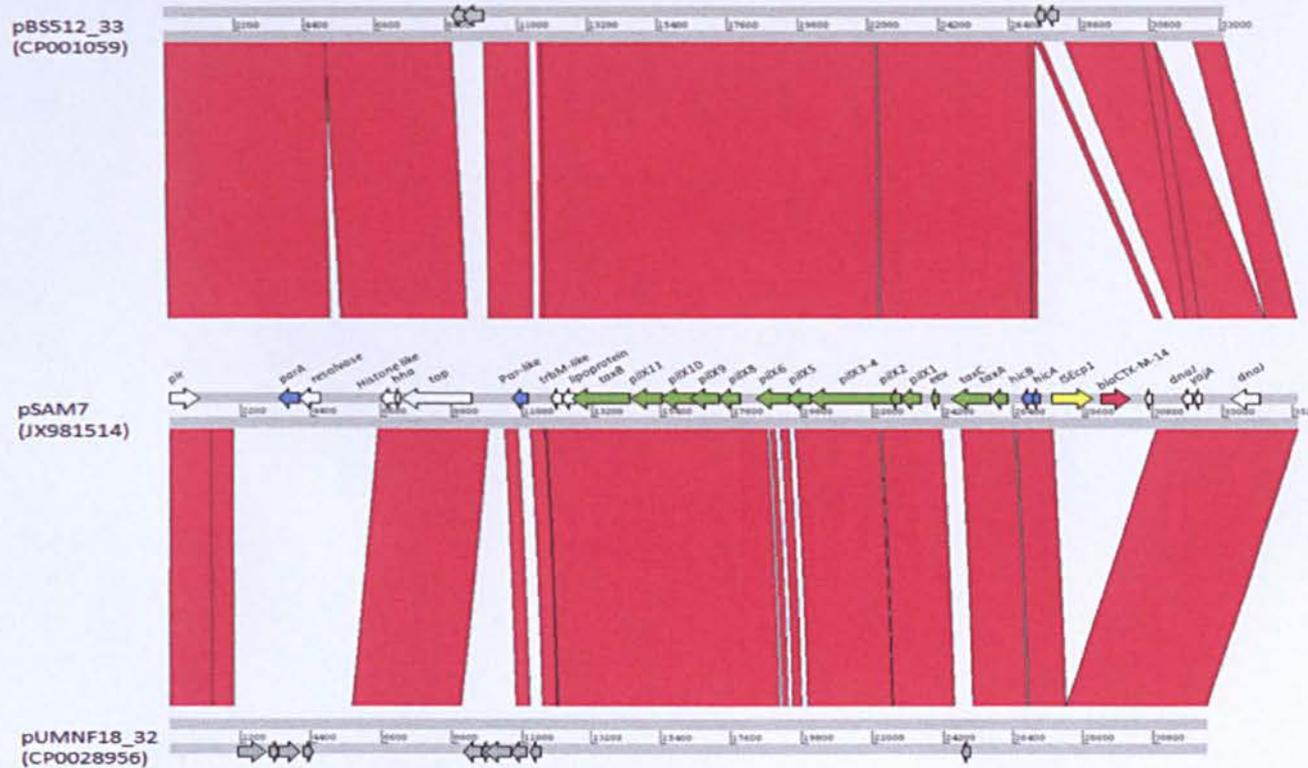
The plasmid pJEG012 was harboured in a OXA-48 producing *Klebsiella pneumoniae* isolate which resulted in the death of four patients in Australia, it was first identified in a traveller from Egypt but was transient through the intensive care unit (Espedido *et al.*, 2013). There are two β -lactamase genes present on pJEG012, *bla*_{OXA-9} and *bla*_{TEM-1} which were part of a Tn1331 that also contains the aminoglycoside resistance genes *aacA4* and *aadA1*, this Tn1331 unit has inserted downstream of the putative resolvase (Espedido *et al.*, 2013). There is a high degree of similarity between pJEG012 and pSAM7, after taking into account the resistance insertions, as shown in Figure 5.5 through homologous regions in red, however the addiction system *hicAB* is absent in pJEG012. A sequence complementing 159 bp of the internal part of *hicB* is present downstream of *taxA* which is the same region as pSAM7, and may suggest that the insertion of hypothetical genes at this site has caused the loss of *hicAB*. pJEG012 has two genes coding for a putative antitoxin-toxin system which may demonstrate the importance for IncX4 plasmids to have an addiction system. Neither of the two insertions found in pSAM7 are present in pJEG012.

5.3.4.3 Comparison of pSAM7 with pBS512_33 and pUMNF18_32

One of the first IncX4 plasmids to be sequenced was through the whole genome sequencing of *Shigella boydii* CDC 3083-94 causing dysentery in a human (Rasko *et al*, unpublished) This plasmid has no known resistance or virulence genes, and yet shares a high level of nucleotide coverage with the pSAM7 backbone, as shown by the homologous red regions in Figure 5.6. The 6,061-6,377 bp insertion in pSAM7 is absent in pBS512_33 as with pJIE143, however a 59 bp of the 3' of the second insertion sequence is present in the pBS512_33, suggesting the presence of this insertion previously which may have been lost. pBS512_33 also had several hypothetical genes which are absent in pSAM7 these were found downstream of *top* and *taxA*. As with pJEG012 the *hicAB* genes are missing in pBS512_33 but the same 159 bp of *hicB* is present, supporting the loss of this system.

Full genome sequencing of the isolate UMNF18, an ETEC isolated from pigs identified a IncX4 plasmids pUMNF18_32 (Shepard *et al.*, 2012). This plasmid has the lowest similarity with pSAM7 of all the plasmids, with only 81% coverage of the backbone, and has no resistance or virulence genes, as shown by the homology in Figure 5.6, with hypothetical genes indicated. pUMNF18_32 has a region of three hypothetical genes and a *parA*, located downstream of the *pir*, which shares limited similarity with that of *parA* in pSAM7 with the products sharing only 36% amino acid identity. Several other hypothetical genes present in pUMNF18_32, have replaced the *orf15* of pSAM7, and may represent an insert. As with the other plasmids the insertion between 6,061-6,377 bp in pSAM7 is absent, but 355/437 bp of the pSAM7 10,489-10,925 insert is present in a hypothetical gene (8,500-9,129). The *hicAB* addiction system genes are present in pUMNF18_32.

Figure 5.6 Artemis comparison tool analysis of pSAM7 with pBS512_33 and pUNMF18_32



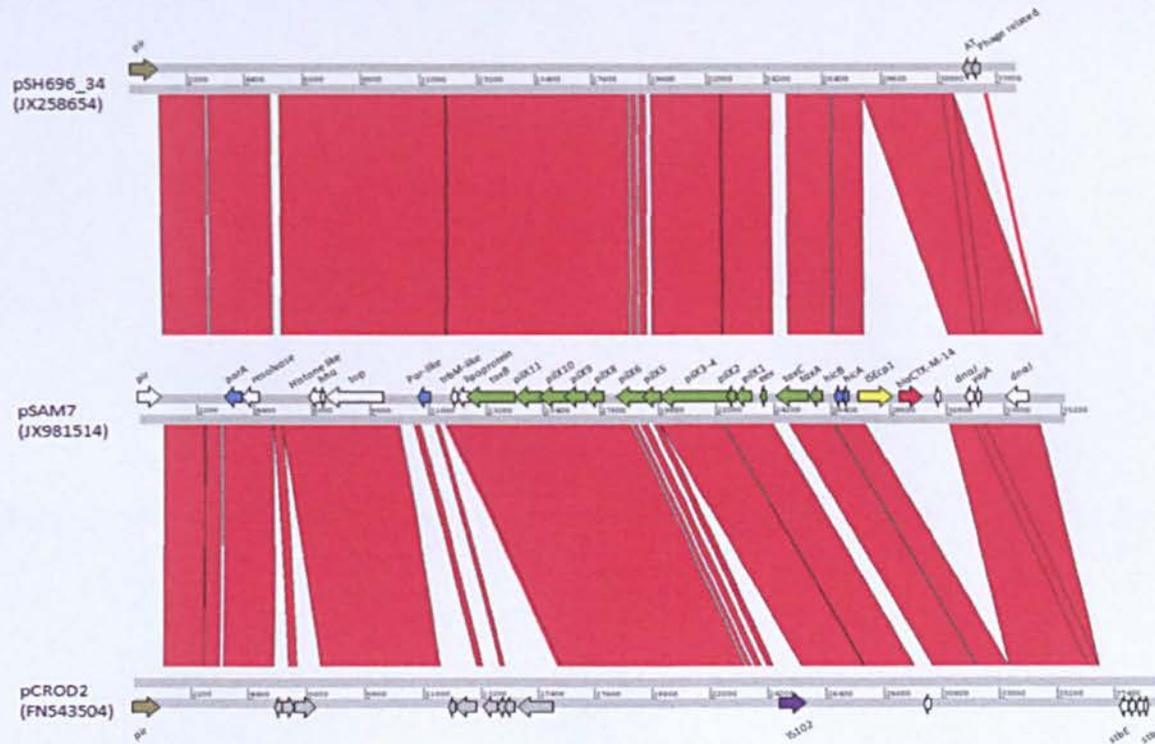
Artemis comparison tool analysis of the DNA sequences of pBS512_33 and pUMNF18_32 with pSAM7. Red regions indicate areas of DNA homology between sequences, spaces indicate unique sequence to that plasmids. Genes involved in conjugation are shown by green arrows, *ISEcp1* is shown by yellow arrows and *bla_{CTX-M}* by red arrows, stability genes are shown by blue arrows, other genes are shown by white arrows. Purple arrows indicate transposons, orange arrows indicate resistance genes and grey arrows indicate hypothetical genes not present in pSAM7.

5.3.4.4 Comparison of pSAM7 with pSH696_34 and pCROD2

Although pSH696_34 and pCROD2 are both *pir* IncX4 plasmids, the π protein of these two plasmids and pSAM7 share only 29% amino acid identity with a coverage of 55%. Despite this difference in *pir* pSH696_43 shares 91% similarity with pSAM7, as shown by the red regions shared in Figure 5.7. The 6,061-6,377 bp insert is absent, however the 10,489-10,925 bp insert is present located in a hypothetical gene (73-657 bp), which is also present in pSAM7. In addition to the *hicAB* genes, pSH696_34 also has putative toxin antitoxin genes which are present in pJEG012 although one is labelled as phage related, both are located downstream of *dnaJ*. As with pBS512_33 and pUMNF18_32 no virulence or resistant genes were present on pSH696_34.

The plasmid pCROD2 was isolated from *Citrobacter rodentium*, which caused colitis and transmissible colonic hyperplasia in mice as identified by studies in 1976 (Barthold *et al.*, 1976). Sequencing of this isolate found similarities to *E. coli* and identified the IncX4 plasmid pCROD2 (Petty *et al.*, 2010). The pCROD2 plasmid differs from pSAM7 at several regions, downstream of the resolvase are several hypothetical genes present in pCROD2 that are absent in pSAM7, as shown in Figure 5.7 by the arrows and absence in homology. Downstream of *orf16*, six hypothetical genes are present in pCROD2. Additionally the insertion sequence IS102 has inserted into the *pilX5* gene, which may result in the inability of this plasmid to conjugate. As well as having the *par* genes, pCROD2 also has the *hicAB* addiction system and the *stbED* stability genes, the absence of any resistance genes in this plasmid may require it to have more stability mechanisms to maintain its presence.

Figure 5.7 Artemis comparison tool analysis of pSAM7 with pSH696_34 and pCROD2



Artemis comparison tool analysis of the DNA sequences of pSH696_34 and pCROD2 with pSAM7. Red regions indicate areas of DNA homology between sequences, spaces indicate unique sequence to that plasmids. Genes involved in conjugation are shown by green arrows, *ISEcp1* is shown by yellow arrows and *bla_{CTX-M}* by red arrows, stability genes are shown by blue arrows, other genes are shown by white arrows. Purple arrows indicate transposons, orange arrows indicate resistance genes and grey arrows indicate hypothetical genes not present in pSAM7.

5.3.5 Development of molecular markers for pSAM7 and IncX4 plasmids

IncX plasmids have recently been re-classed by Johnson *et al* (2012a) into four groups IncX1-4. All of the plasmids in this study fall into the IncX4 group, as predicted from *in silico* testing (Johnson *et al.*, 2012a). This new grouping scheme amplified the *taxC* gene which varies between the IncX groups, however sequence comparisons performed in this study have identified differences between the IncX4 plasmids. Molecular markers were designed to identify pSAM7-like plasmids and investigate their dissemination, and additionally to further differentiate IncX4 plasmids.

Four molecular markers were selected to identify pSAM7 and differentiate between IncX4 plasmids and another to identify the unique *bla*_{CTX-M-14b} transposition unit. The regions selected as molecular markers surrounded the *pir* (Rpir), *pilx5* (RpilX5), *hicA* (RhicA) genes and a hypothetical region spanning *orf13-orf14* which encompasses one of the pSAM7 insertions (Hyex). An additional marker was selected for transposition unit based on the presence of the S-methylmethionine permease fragment termed Smet. The molecular markers were tested against the other IncX4 plasmids *in silico* to identify their presence or absence. The RpilX5 marker was present in all IncX4 plasmids as designed which can be used to identify IncX4 plasmid in the same way the *taxC* gene is used by Johnson *et al* (2012a). Rpir and RhicA markers were present in five plasmids, with Rpir in pSAM7, pJIE143, pJEG012, pBS512_33 and pUMNF18_32. RhicA was present in pSAM7, pJIE143, pSH696_34, pCROD2 and pUMNF18_32. The Hyex marker was the most specific to pSAM7 of all the markers only present in two plasmids pSAM7 and pSH696_34 which produces an amplicon of 841 bp. BLASTn searches of these primers identified that a larger amplicon of 1,754 bp could be produced in some plasmids such as pE001 and pDKX1-TEM-52 both of which are IncX1 plasmids. The markers for pSAM7 were capable of differentiating the seven IncX4 plasmids into five groups. The Smet marker for the novel *bla*_{CTX-M-14b} transposition was only found to be produced in pSAM7, and BLASTn searches demonstrated that it was unique for this plasmid. The pSAM7

molecular markers were also tested *in silico* with the IncX plasmids listed by Johnson *et al* (2012a) to identify if any amplicons were predicted to be produced (Johnson *et al.*, 2012a). The results found that none of the plasmids belonging to groups IncX1, X2 and X3 produced amplicons, and that the markers were specific for plasmids belonging to the IncX4 group. The results of the *in silico* testing are shown in Table 5.4, each marker was assigned a number which can be used in a binary format so each marker combination could have a unique ID number (UID), RpilX5 = 1, Rpir = 2, RhicA = 4 and Hyex = 8, Smet was not assigned a number due to it being for a transposition unit (Table 5.4). The use of a UID number in this way could facilitate in establishing a database to compare plasmids globally between researchers.

5.3.5.1 Evaluation of the molecular markers

The RpilX5 as designed was found in all of the plasmids, however this marker could be used to further differentiate the IncX4 plasmids through sequencing the amplicons. Sequence analysis of the RpilX5 amplicons differentiates the seven products into five clusters, of which pSAM7 and pJIE143 are identical as are pBS512_33 and pJEG012, the SNPs range from 11-17 compared to pSAM7, as shown in Figure 5.8.

Both pSH696_34 and pCROD2 fail to produce an amplicon for the Rpir marker, this is due to an alternative *pir* gene in these plasmids which produces a different π protein. The five plasmids that have the Rpir marker, can also be further differentiated by the sequence analysis of the marker, being divided into four clusters of which only pSAM7 and pJIE143 are identical, as shown in Figure 5.8.

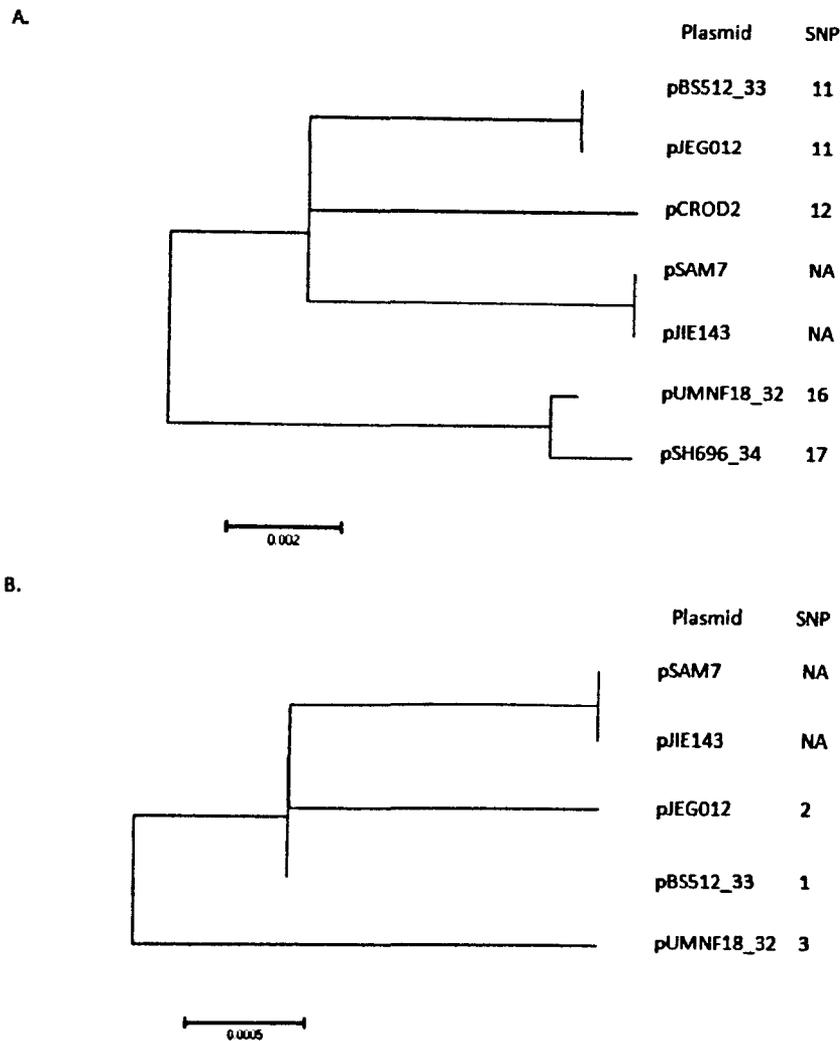
The RhicA marker is absent in pJEG012 and pBS512_33, both of which lack the *hicAB* genes however there is a 159 bp remnant of *hicB*. This remnant suggest that *hicB* was present as might have been *hicA*, the loss of *hicA* may have occurred first preventing any detriment to the cell, followed by a later deletion in the antitoxin gene. Analysis of the amplicons from the five plasmids showed there were two clusters, pSAM7, pJIE143 and

Table 5.4 *In silico* testing of the pSAM7 molecular markers

Plasmid	Host	Source	Marker					UID
			RpilX5	Rpir	RhicA	Hyex	Smet	
pSAM7	<i>E. coli</i> *	Cattle	1	1	1	1	1	15
pJIE143	<i>E. coli</i>	Human	1	1	1	0	0	7
pJEG012	<i>K. pneumoniae</i>	Human	1	1	0	0	0	3
pBS512_33	<i>Shigella boydii</i>	Human	1	1	0	0	0	3
pSH696_34	<i>Salmonella enterica</i>	Turkey	1	0	1	1	0	13
pCROD2	<i>Citrobacter rodentium</i>	Mouse	1	0	1	0	0	5
pUMNF18_32	<i>E. coli</i>	Pig	1	1	1	0	0	7

The presence of the marker is shown by a 1 with a green background, and 0 with a red background is absence of the marker. UID = unique ID number is shown on the right.* =Also found in *Enterobacter cloacae* from cattle

Figure 5.8 Phylogeny of the RpilX5 and Rpir marker amplicons



Dendrogram of (A) RpilX5 amplicons and (B) Rpir amplicons aligned using Clustal W MEGA 5.0 using neighbour joining with 1000 bootstrap repeats, scale indicates substitutions per base. Number of SNPs are indicated on the right.

pCROD2 in one and pUMNF18_32 and pSH696_34 in another cluster, with only one SNP difference.

The Hyex marker was the most specific of all the markers present in only pSAM7 and pSH696_34, and sequence analysis of the amplicons found them to be identical. The failure to yield amplicons from pJIE143, pUMNF18_32, pCROD2, pBS512_33 and pJEG012 has occurred as a result of three possible events. Firstly the *orf13* in pSAM7 seems to have been formed from the insertion of 437 bp sequence (10,489-10,925)

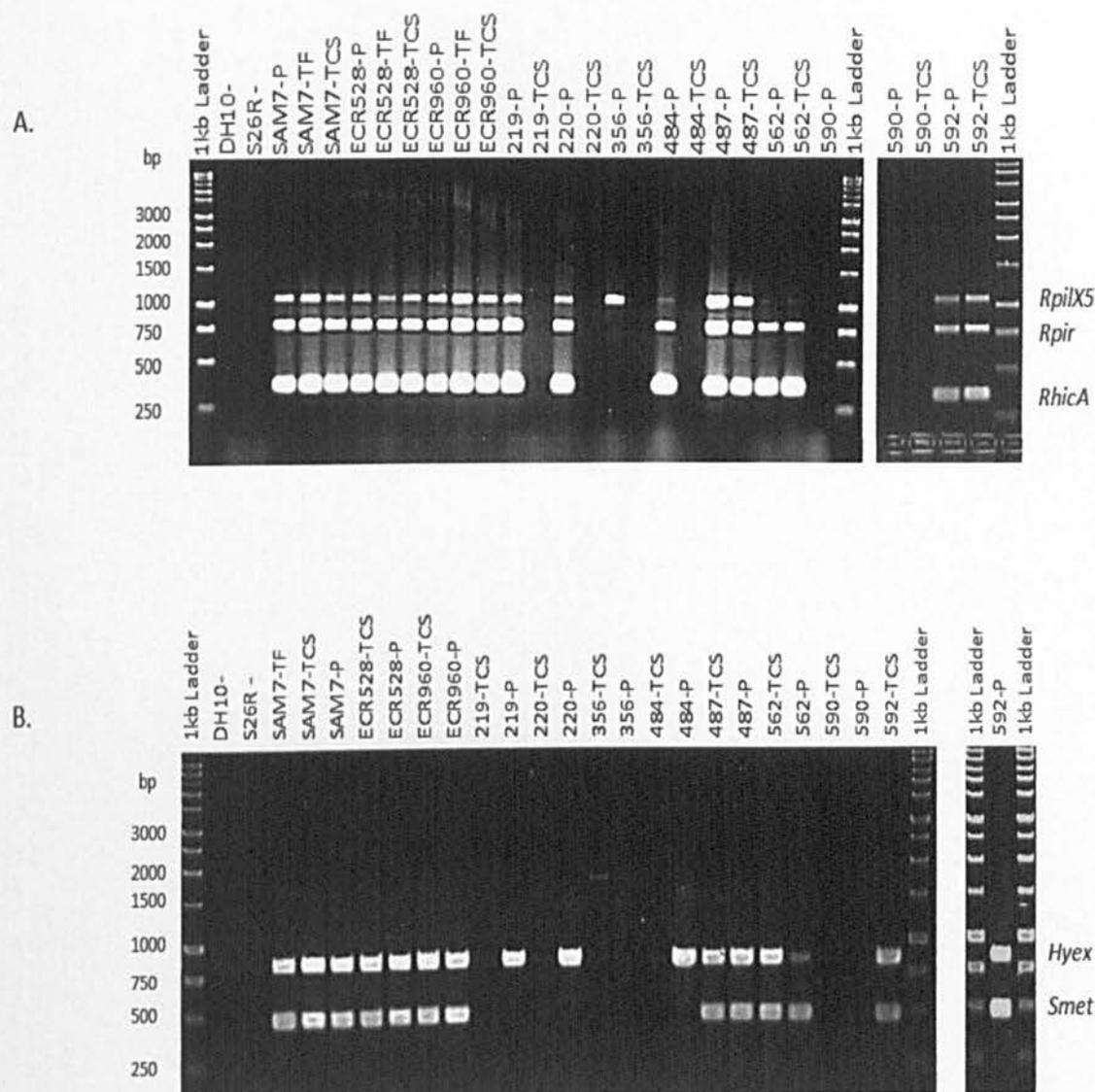
correlating to a hypothetical gene, and creating the binding sites for the Hyex marker primers. Plasmids pBS512_33 and pJEG012 lack *orf13*, and as a result the forward primer fails to bind and no product is predicted. The most similar plasmid pJIE143, has a 222 bp fragment which correspond to the C terminus of *orf13*, however no pSAM7 sequence insert is present in pJIE143 and so the complete *orf13* is not formed resulting in the forward Hyex primer failing to bind. Initially pUMNF18_32 and pCROD2 were predicted to yield a product, however closer inspection proved otherwise. Both plasmids have the *orf13*, and 27/55 bp of the insert sequence outside of this open reading frame. However, sequence alterations result in 3 base mismatches in the forward primer, and so its predicted at 57.5°C a product would fail to be produced. The presence of 27 bp of the inserted sequence may suggest that it was present but the insertion of three hypothetical genes in pUNMF18_32 and one in pCROD2 may have caused the loss of the remainder of the insert. In addition 3 bp mismatches for the reverse primer binding site in pUNMF18_32 and 10 mismatches in pCROD2 prevent amplicons from being produced.

5.3.6 Screening for *E. coli* field isolates for pSAM7-like and IncX4 plasmids

The pSAM7 molecular markers were used to screen *E. coli* field isolates to identify both pSAM7-like plasmids and IncX4 plasmids. As IncX4 plasmids have been found with both *bla*_{CTX-M-14b} and *bla*_{CTX-M-15}, selecting plasmids based on CTX-M genes was decided against, instead plasmid were selected based on size. The pSAM7 molecular markers primers were combined into two multiplex's, the first included RpiIX5, Rpir and RhicA and the second Hyex and Smet (*bla*_{CTX-M-14b} transposition unit). Isolates from the SAFEFOODERA study were selected based on small (30-50 kb) ESBL plasmids (D Mevius personal communication, February 12 2012). A panel of 42 *E. coli* isolates which included isolates with *bla*_{CTX-M-1} (human n=9, cattle n=7, poultry n=5, pig n=4), *bla*_{CTX-M-14} (human n=2, cattle n=5), *bla*_{CTX-M-15} (cattle n=2), *bla*_{TEM-52} (poultry n=7) or *bla*_{SHV-12} (human n=1), which had been determined previously by PCR and array analysis. Screening identified six isolates which had two or more pSAM7 markers. Conjugations of

these field isolates were carried out using the *Salmonella enterica* Typhimurium 26R, and retested using the pSAM7 molecular markers as shown in Figure 5.9, and Table 5.5. The JIE143 *E. coli* strain harbouring the pJIE143 plasmid was also tested and produced the amplicons for RpilX5, Rpir and RhicA as predicted by *in silico* testing.

Figure 5.9 pSAM7 molecular marker screening of field isolates and transconjugants



The pSAM7 molecular marker screening of field isolates and transconjugants for **(A)** RpilX5, Rpir and RhicA multiplex and **(B)** Hyex and Smet duplex. DH10- and S26R- are plasmid free *E. coli* DH10B used in transformations and *Salmonella* conjugant recipients used for negative controls, SAM7-P is the field isolate and SAM7-TF is the *E. coli* DH10B transformed with pSAM7 as positive control. TCS denotes a transconjugant and P is the field isolate. DNA resolved on a 1.5% agarose gel with a 1 kb ladder used as a size marker.

Table 5.5 Results of pSAM7 Screening of field isolates and transconjugants

Isolate	Species ^a	Host	Origin	Year	CTX-M	ST	pSAM7 markers ^b					bla _{CTX-M-14}
							<i>Rpir</i>	<i>Rpil</i>	<i>RhicA</i>	<i>Hyex</i>	<i>Smet</i>	TU ^c
SAM7	<i>E. coli</i>	Cattle	UK	2008	14	10	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>
ECR528	<i>E. cloacae</i>	Cattle	UK	2012	14	-	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>
ESBL487	<i>E. coli</i>	Cattle	UK	2008	14	117	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>
ESBL562	<i>E. coli</i>	Cattle	UK	2007	14	559	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>
ESBL592	<i>E. coli</i>	Cattle	UK	2008	14	2177	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>Y</u>
ESBL219	<i>E. coli</i>	Human	Germany	unknown	1	ND	Y	Y	Y	Y	N	ND
ESBL220	<i>E. coli</i>	Human	Germany	unknown	1	ND	Y	Y	Y	Y	N	ND
ESBL484	<i>E. coli</i>	Cattle	UK	2007	1	ND	Y	Y	Y	Y	N	ND

^aConfirmed by MALDI-TOF

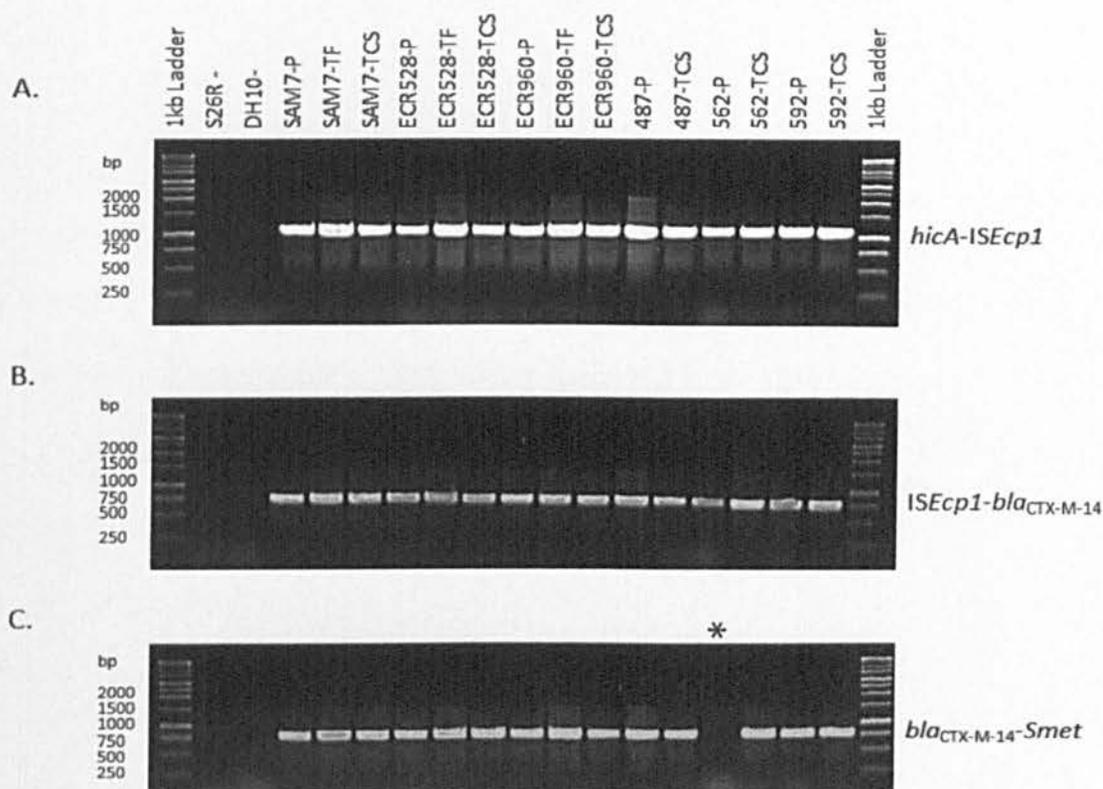
^bpSAM7 transformed into DH10 was used a positive control and *Salmonella* Typhimurium strain 26R was used as negative control in PCR. Y, amplicon obtained; Y, amplicon also obtained from transconjugant; N, no amplicon.

^cISEcp1-bla_{CTX-M-14} transposition unit found in pSAM7.

ND = Not determined

Three of the transconjugants from the six isolates retained the RpilX5, Rpir, RhicA and Hyex pSAM7 markers, which were 487, 562 and 592 (Figure 5.9). These transconjugants also had the *bla*_{CTX-M-14b} transposition marker, Smet (Figure 5.9). The environment surrounding the *bla*_{CTX-M-14b} gene was determined by PCR to be the same as pSAM7, which was also carried out for the plasmids from *Enterobacter cloacae* (ECR528 and ECR960). PCR reactions from the *hicA* to *ISEcp1*, *ISEcp1* to *bla*_{CTX-M-14b} and *bla*_{CTX-M-14b} to Smet showed that all conjugated plasmids had the same genetic environment as pSAM7 as shown in Figure 5.10.

Figure 5.10 *bla*_{CTX-M-14b} genetic environment of field isolates and transconjugants



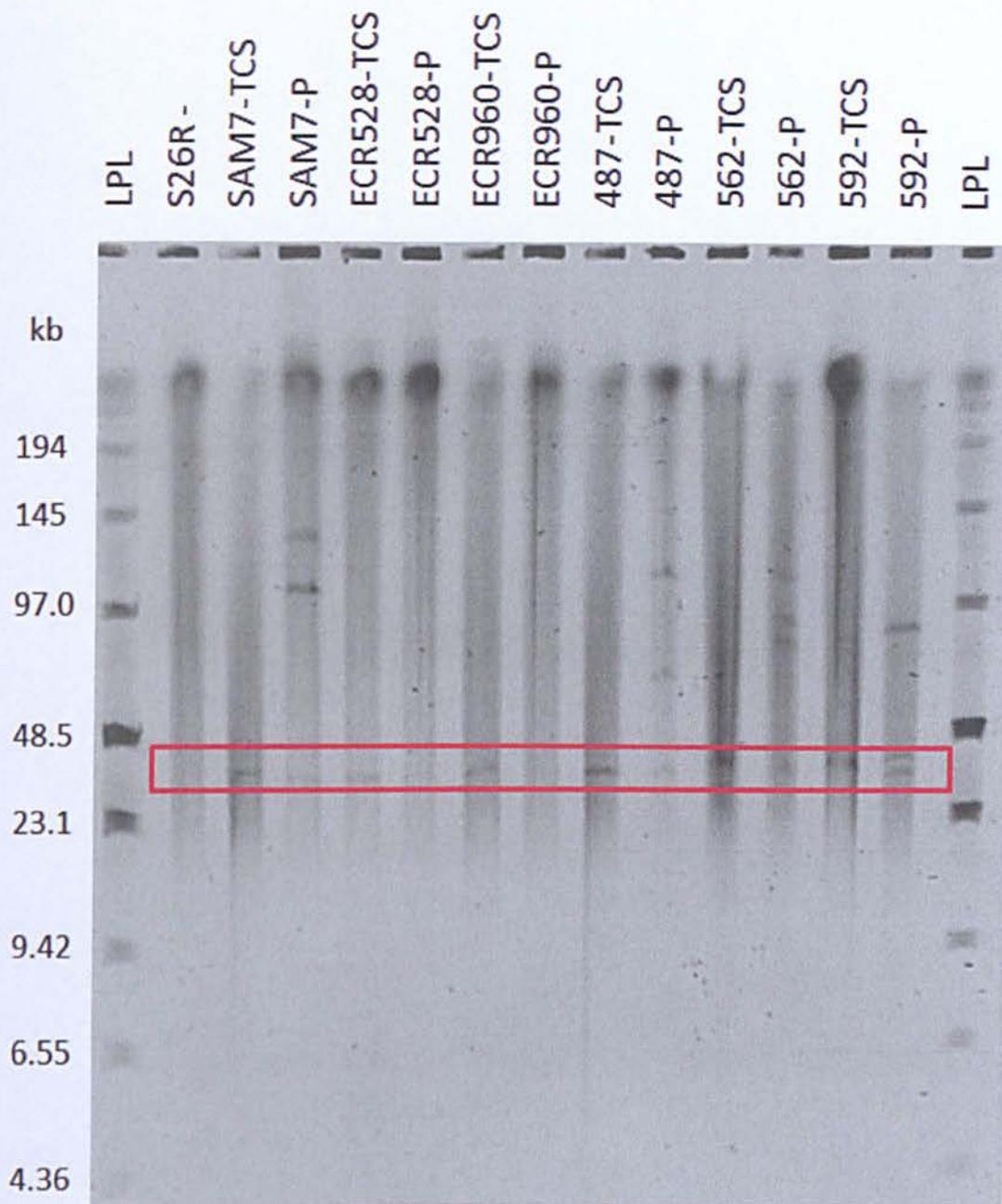
The *bla*_{CTX-M-14b} environment screening of the field isolates and transconjugants for (A) *hicA* to *ISEcp1* (B) *ISEcp1*-*bla*_{CTX-M-14} (C) *bla*_{CTX-M-14} to *Smet*. DH10- and S26R- are plasmid free *E. coli* DH10B used in transformations and *Salmonella* conjugant recipients used for negative controls, SAM7-P is the field isolate and SAM7-TF is the *E. coli* DH10B transformed with pSAM7 a positive control. TCS denotes a transconjugant and P is the field isolate. DNA resolved on a 1.5% agarose gel with a 1 kb ladder used as a size marker. * The PCR was repeated and was positive.

The plasmids in the transconjugants were determined to be the same as pSAM7 at approximately 35kb by S1 nuclease, as shown in Figure 5.11. To determine whether pSAM7-like plasmids were moving between isolates, the relationship of the field isolates was investigated further by XbaI PFGE and MLST, the field isolate for JIE143 was also included in the PFGE comparison. All isolates were found to be unrelated at 85% cut off apart from 487 and 562 which share 92.5% similarity, as shown in Figure 5.12. The MLST results for the isolates revealed that all were unrelated, with each having a different sequence type (ST) (Figure 5.12).

5.4 Discussion

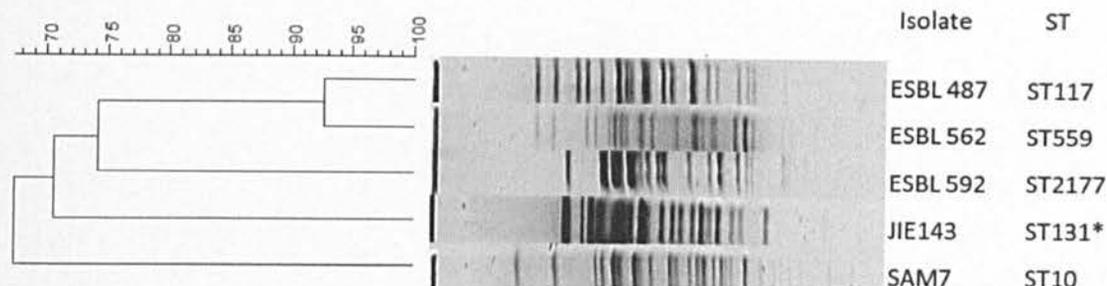
Identical pSAM7 plasmids were found in clonally unrelated *E. coli* and *Enterobacter cloacae* isolates from cattle in the UK. Sequencing and annotation of pSAM7 found it to have 51 ORFs, which included a single resistance gene, *bla*_{CTX-M-14b} in a novel transposition unit. Sequencing of pSAM7 demonstrated that it belonged to the newly classed IncX4 group of plasmids, after classic PBRT failed to detect any replicons in the transconjugants (Johnson *et al.*, 2012a). Comparative genomics identified that pSAM7 was closely related to the only other *bla*_{CTX-M} IncX4 plasmids pJIE143, which was sequenced as a result of the same PBRT issues experienced with pSAM7. These two plasmids had a sequence coverage of 99% once both *bla*_{CTX-M} inserts had been removed, with only two main differences, both of which were inserts in pSAM7. The site of the *ISEcp1-bla*_{CTX-M-15-orf477} insertion occurred 17 bp downstream of *pir* and formed the direct repeats of GGATA, of note this same 5 bp sequence is present downstream of *pir* in pSAM7. The insertion of *ISEcp1-bla*_{CTX-M-14b-Smet} in pSAM7 occurred 92 bp upstream of *hicA* forming the direct repeats TAAAA, this same 5 bp sequence is also present in the same location in pJIE143. Why both the *ISEcp1* inserts occurred at different locations is unknown at this time and warrants further investigation.

Figure 5.11 S1 nuclease of pSAM7-like plasmids



S1 nuclease of field isolates and transconjugants to size plasmids. S26- is the recipient plasmid free control and LPL is the low range PFGE ladder for size comparison. TCS denotes a transconjugant and P is the field isolate.

Figure 5.12 PFGE and MLST of pSAM7-like *E. coli* field isolates



*Xba*I PFGE and MLST of pSAM7-like field isolates. Restriction profiles were analysed by bionumerics 5.10 using Dice-coefficient with a tolerance of 2.0%, scale indicates % similarity. MLST was performed as described in Chapter 2, *= ST sequenced by Partridge *et al* (2011).

The similar IncX4 plasmids pBS512_33 and pJEG012 have the GGATA sequence 17 bp downstream of the *pir*, and pCROD2 and pSH696_34 have TAAAA located 92 bp upstream of *hicA*, with pUMNF18_32 having both of these 5 bp sequences at their respective locations. Potentially these IncX4 plasmids could also acquire *bla*_{CTX-M} and other ESBL genes aiding their dissemination, and so further differentiation of such plasmids would be beneficial. The presence of such similar plasmids in isolates from humans and animals, in different global locations with no clear epidemiological link is cause for concern. This observation supports the suggestion that this plasmid backbone has been in the bacterial population for some time, before acquisition of resistance genes as reported for other plasmids, and is emerging as a vector for the *bla*_{CTX-M} genes (Datta and Hughes, 1983; Jones and Stanley, 1992). pSAM7 appears to be a highly stable plasmid, as over a 4 year period an identical plasmid was found in two different bacterial species. These highly similar plasmids are mobile and have been found in unrelated isolates with SAM7 being in *E. coli* ST10 and JIE143 in ST131; the presence in both human and animal isolates suggest these plasmids could move between hosts, as is the case for IncK and IncN plasmids (Partridge *et al.*, 2011; Stokes *et al.*, 2013; Stokes *et al.*, 2012; Moodley and Guardabassi, 2009).

The *bla*_{CTX-M-14b} transposition unit in pSAM7 is novel, however the IncL/M plasmid pJEG011 possessed a near identical unit, although shorter with 3095/3246 bp present. This *Klebsiella pneumoniae* isolate also possessed the IncX4 plasmids pJEG012 which has a coverage 85% (93% without insert) with pSAM7, and itself confers an MDR phenotype with *aacA4*, *aadA1*, *bla*_{OXA-9} and *bla*_{TEM-1} inserted in Tn1331 (Espedido *et al.*, 2013). The presence of a similar plasmid pJEG012 to pSAM7 and transposition unit in pJEG011 to pSAM7, in the same isolates may have resulted in the combination of the two mobile genetic elements to produce a pSAM7-like plasmid found in the UK (Walsh, 2006; Frost *et al.*, 2005). The presence of IS26 fragments and a gene coding for S-methylmethionine permease which is found in the chromosomes of *E. coli* DH1 (AP012030) and K12 (CP000948), suggests at some point *bla*_{CTX-M-14b} may have been incorporated into the chromosome or other plasmids, and several insertion events have occurred to create this transposition unit (Partridge, 2011).

The limitations of PBRT to detect the growing variations of IncX plasmids was noted by Johnson *et al* (2012a), who designed new primers to group plasmids IncX1-4 to address this issue (Carattoli *et al.*, 2005a; Johnson *et al.*, 2012a). At the time of this study, the new primers designed to group IncX plasmids had not been published and consequently at this time the IncX4 plasmids could still not be identified, making it a target for molecular marker design (Johnson *et al.*, 2012a). The detailed comparison of IncX4 plasmids identified several molecular markers which could be used to detect pSAM7 and serve to further differentiate plasmids of the IncX4 group. The molecular markers selected in this study differentiated the seven plasmids into five different groups, and plasmids within the same group could be further distinguished through the sequencing of either the RpiIX5, Rpir or RhicA amplicons. This multiplex marker scheme therefore allows for the further differentiation of IncX4 plasmids identified using the primers designed by Johnson *et al* (2012a). The screening of the pSAM7 markers on field isolates, identified three other *E. coli* isolates with pSAM7-like plasmids all in the UK cattle. This suggests that cattle

may act as a reservoir for IncX4 *bla*_{CTX-M-14b} plasmids, as for IncK *bla*_{CTX-M-14} in cattle and turkeys in the UK (Stokes *et al.*, 2013; Stokes *et al.*, 2012). Three other isolates were found with the RpilX5, Rpir, RhicA and Hyex however the conjugated plasmids carrying the *bla*_{CTX-M-1} genes were not on IncX plasmids, it is likely that these isolates possessed IncX4 plasmids which were not associated with ESBL genes as is the case with plasmids pBS512_33, pCROD2, pUNMF18_32 and pSH696_34 which were identified as a result of complete genome sequencing (Petty *et al.*, 2010; Shepard *et al.*, 2012; Han *et al.*, 2012).

IncX plasmids appear to have an animal reservoir, with 4/7 IncX4 plasmids in this study being found in isolates from animals including, cattle, pig, turkey and mouse. Additionally IncX plasmids have been found with *bla*_{TEM-52}, *bla*_{TEM-1}, *qnrS1* and *oxqAB* genes all of which have been found in isolates from animals across Europe and now *bla*_{CTX-M-14b} has also been found in cattle (Bielak *et al.*, 2011; Sorensen *et al.*, 2003; Dolejska *et al.*, 2011a; Literak *et al.*, 2010a; Stokes *et al.*, 2013). The staphylococcal resistance gene *cfr* has also been identified in *E. coli* isolates from animals, in an environment similar to that of pCROD2 and pBS512_33, giving resistance to multiple compounds (Wang *et al.*, 2012). It is not just ESBL genes associated with IncX plasmids that are being identified, but also carbapenemase producing genes *bla*_{KPC} and *bla*_{NDM} which have been found on these backbones, and is of major clinical concern. A MDR *E. coli* isolate recovered from a patient in Germany with recent travel to Yemen, was found to have the *bla*_{NDM-7} a new variant of NDM-1 found on an IncX3 plasmid (Gottig *et al.*, 2013). *Klebsiella pneumoniae* have also been found with a IncX plasmids with a IncX3 plasmid, pKpS90 harbouring *bla*_{KPC-2} and a novel IncX5 group harbouring *bla*_{KPC-5} (Kassis-Chikhani *et al.*, 2013; Chen *et al.*, 2013). IncX plasmids have not only been associated with resistance genes but also with genes important in biofilm production identified in plasmids pOLA52 and R485 possibly contributing to the virulence of the host (Norman *et al.*, 2008; Burmolle *et al.*, 2012). Virulence genes have also been found on IncX plasmids, as studies of virulence plasmids in *Salmonella enterica* were found to be

incompatible with an IncX plasmid pOG670 (Olsen *et al.*, 2004). These resistance and virulence genes have been mobilised by insertion sequences and transposons which have an important role in their insertion such as Tn1331, Tn3, IS1, ISEcp1, and IS26 (Espedido *et al.*, 2013; Burmolle *et al.*, 2012; Norman *et al.*, 2008; Partridge *et al.*, 2011; Wang *et al.*, 2012). As the number of resistance and virulence genes associated with IncX plasmids increases, the need for improved methods of detection and differentiation grows, allowing the impact on dissemination to be investigated. The pSAM7 molecular markers presented in this study will facilitate in such future investigations into IncX4 plasmids.

Chapter 6

**Comparison of a Veterinary *bla*_{CTX-M-3} IncA/C Plasmid from the UK
with Plasmids Isolated in USA,
Europe, Asia and Emerging Vectors
for the *bla*_{NDM-1}**

6.1 Introduction

The broad host range IncA/C plasmids have been isolated from numerous bacterial species including *E. coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Aeromonas hydrophila*, *Photobacterium damsela* and *Yersinia* spp., recovered from humans and animals around the world (Fernandez-Alarcon, Singer and Johnson, 2011; Welch *et al.*, 2007; Doublet *et al.*, 2012; Del Castillo *et al.*, 2013; Kim *et al.*, 2008). These plasmids have been found particularly in farm animals in the USA, and fish in Asia (Fernandez-Alarcon, Singer and Johnson, 2011; Welch *et al.*, 2007; Glenn *et al.*, 2011; Lindsey *et al.*, 2009; Johnson *et al.*, 2012c). IncA/C plasmids tend to be large in size (>120 kb) compared to other resistance plasmids such as Inc11 γ , IncN and IncX (Fricke *et al.*, 2009; Sekizuka *et al.*, 2011).

Some evidence supports the origins of IncA/C plasmids in the aquatic environment with plasmids being identified in pathogens associated with fish such as *Aeromonas* spp, *Photobacterium damsela*, *Salmonella* spp., *Edwardsiella ictaluri* and *Yersinia ruckeri* (Welch *et al.*, 2007; Del Castillo *et al.*, 2013; Kim *et al.*, 2008; Fricke *et al.*, 2009; Welch *et al.*, 2009). Plasmid pRA1 considered the ancestor of IncA/C plasmids was isolated from *Aeromonas hydrophila* from a fish in 1971, and was resistant to tetracycline and sulphonamides, both of which are water soluble (Fricke *et al.*, 2009). In addition to fish and farm animals, clinical isolates have been found with IncA/C plasmids (Johnson *et al.*, 2012b; Dierikx *et al.*, 2010; Veldman *et al.*, 2010; Garcia *et al.*, 2011; Chouchani *et al.*, 2012).

The resistance genes *floR* and *bla*_{CMY-2} are commonly found on IncA/C plasmids, conferring resistance to florfenicol and chloramphenicol through efflux, and β -lactams via hydrolysis respectively (Braibant *et al.*, 2005; Barlow and Hall, 2002; Wu *et al.*, 1999; Zhao and Hu, 2013). Both of these resistance genes have been found in *E. coli* and *Salmonella* spp. from food producing farm animals including cattle, pigs and poultry

(Fernandez-Alarcon, Singer and Johnson, 2011; Call *et al.*, 2010; Folster *et al.*, 2012). Integrons and transposons have an important role in the MDR phenotype expressed by many IncA/C plasmids and also resistance to heavy metals such as mercury, with Tn21 adjacent to the *mer* operon (Liebert, Hall and Summers, 1999; Fernandez-Alarcon, Singer and Johnson, 2011; Welch *et al.*, 2007; Call *et al.*, 2010; McIntosh *et al.*, 2008; Roy Chowdhury *et al.*, 2011; Toleman and Walsh, 2010). IncA/C MDR plasmids have been recovered from bacterial species of major concern for public health, including pIP1202 from *Yersinia pestis* with similar plasmids found in *Vibrio cholerae* O139 in China (Welch *et al.*, 2007; Pan *et al.*, 2008). IncA/C plasmids have been associated with the emerging resistant gene *bla*_{NDM-1}, with such plasmids being found in *E. coli*, *Klebsiella pneumoniae*, and the soil organism *Providencia stuartii* (Sekizuka *et al.*, 2011; Carattoli *et al.*, 2012; McGann *et al.*, 2012). The *bla*_{NDM-1} gene confers resistance to carbapenems and contributes to treatment failures through cephalosporin and carbapenem use (Sidjabat *et al.*, 2011; Walsh *et al.*, 2011). Sequencing of IncA/C plasmids has found a high level of similarity with variations being the result of MDR insertions and deletions (Fernandez-Alarcon, Singer and Johnson, 2011; Welch *et al.*, 2007; Doublet *et al.*, 2012; Del Castillo *et al.*, 2013; Call *et al.*, 2010; Carattoli *et al.*, 2012).

The *bla*_{CTX-M-3} gene is part of the group 1 CTX-M genes, and has been found throughout Asia and Europe, often in association with *ISEcp1* (Gniadkowski *et al.*, 1998; Poirel, Gniadkowski and Nordmann, 2002; De Champs *et al.*, 2000; Kirchner *et al.*, 2011; Wang *et al.*, 2003; Karim *et al.*, 2001). Several *bla*_{CTX-M-3} plasmids have been fully sequenced, which has provided an insight into the plasmid backbones that harbours this gene. Plasmids include the IncL/M pCTX-M3 (AF550415) from *Citrobacter freundii* in Poland and pCTX-M360 (EU938349) from *Klebsiella pneumoniae* in China, IncFII pHK23a (JQ432559) from *E. coli* from a pig in Hong Kong, IncI1γ pEK204 (EU935740) from an *E. coli* clinical isolate in the UK, and IncF pKPX-2 (AP012056) isolated from a NDM-1 *Klebsiella pneumoniae* in Taiwan (Golebiewski *et al.*, 2007; Zhu *et al.*, 2009; Ho

et al., 2013; Woodford *et al.*, 2009; Huang *et al.*, 2013). To date the only IncA/C plasmid bearing *bla*_{CTX-M} to be sequenced is the pKPHS3(CP003225) isolated from *K. pneumoniae* in China which has the *bla*_{CTX-M-14} gene (Liu *et al.*, 2012).

6.1.1 Hypotheses and aims

The hypotheses of this study was (i) that the *bla*_{CTX-M-3} IncA/C plasmids from *E. coli* isolated from a chicken in the UK is closely related to plasmids from around the world, and (ii) that IncA/C plasmids can be further differentiated by molecular markers. The aims of this study were to (i) extract the pCH01 plasmid DNA from the CH01 isolate, sequence and annotate the plasmid to determine its genotype and features, (ii) compare plasmid pCH01 with other plasmids previously sequenced, to determine the relationship between plasmids, and (iii) design molecular markers capable of identifying pCH01 and differentiating between similar plasmids, which can be used as epidemiological tools.

6.2 Methods and materials

The CH01 *E. coli* field isolate, was isolated from chicken faeces during routine surveillance screening by AHVLA in 2006. The sequence type of CH01 was determined using MLST as described in 2.3.3. Total DNA was extracted from CH01 using the QIAGEN Hi-Speed plasmid midi prep as described in 2.2.4.3, which was then transformed into *E. coli* DH10B (2.2.5). Transformants were checked for the presence of a single CTX-M plasmid by plasmid profiling as described in 2.3.12.2. The antimicrobial sensitivities of the CH01 field isolate and the pCH01 transformant were determined by disc diffusion as described in 2.1.3. Plasmid content and sizes were determined by S1 nuclease for both the CH01 field isolate and pCH01 transformant (2.3.12.1), replicons were determined using a commercial kit for the wild type (2.3.6.2). The transferability of pCH01 was determined by liquid and solid conjugations as stated in 2.1.4.1 and 2.1.4.2. Plasmid DNA for sequencing was extracted from the pCH01 transformant using the QIAGEN large construct kit following the protocol (2.2.4.4), and sequenced on a Roche

454 GS FLX (2.4.1) with closure of the sequence by PCR using the primers in Table 2.12, using the conditions in 2.4.3.1 and 2.4.3.2. The plasmid was annotated using several programs including RAST (2.5.1.1), Artemis (2.5.1.2) and BLASTn using the annotations from plasmids pR55 (JQ010984), pIP1202 (CP000603), pAR060302 (FJ621588), pAPEC1990_61 (HQ023863) and pSN254 (CP000604) as described in 2.5.1.3. Plasmids from GenBank were compared with pCH01 using MAUVE (2.5.2.1), ACT (2.5.2.2) and BLASTn (2.5.1.3) to determine the relationship between plasmids and identify molecular marker candidates. The phylogeny of the plasmids was analysed using concatenated sequence compiled from open reading frames present in all plasmids which was analysed as described in 2.5.2.5. The screening of molecular markers in 2.3.8, Table 2.9 were performed against plasmids from GenBank *in silico* as described in 2.5.3.1, with the phylogeny of the markers analysed as stated in 2.5.2.5. The Welch markers were also tested *in silico* as described in 2.5.3.1, using the primers in 2.5.3.4 Table 2.15.

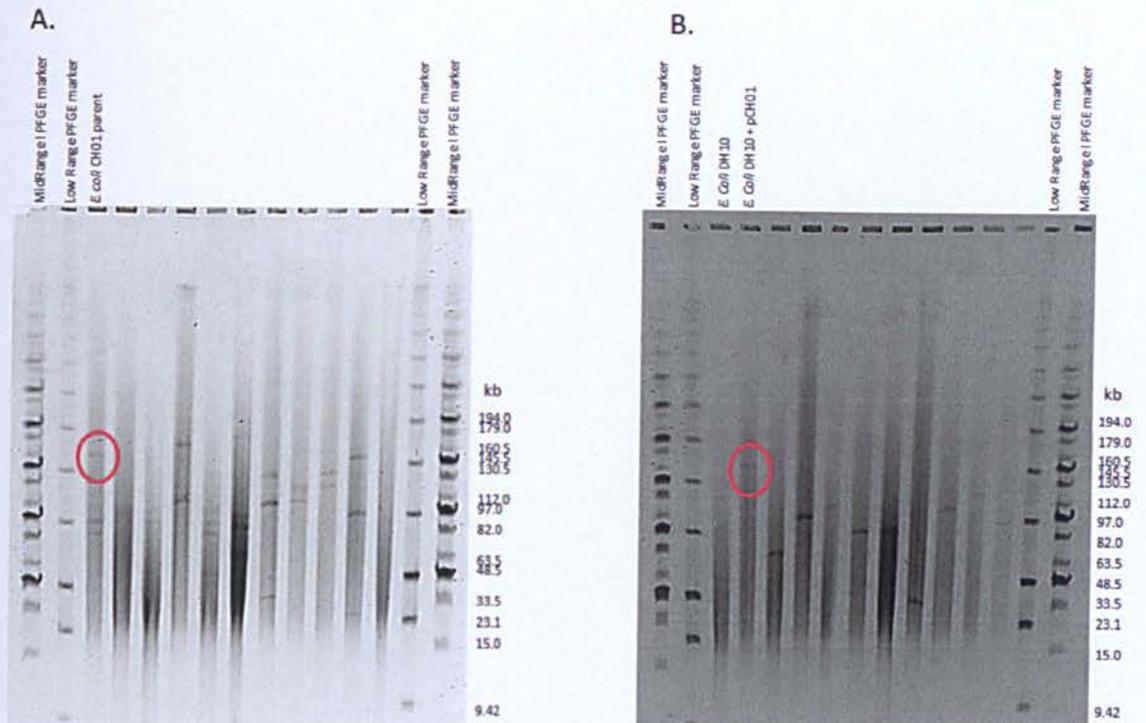
6.3 Results

6.3.1 Analysis of the *E. coli* CH01 field isolate

6.3.1.1 Antimicrobial sensitivity and plasmids of CH01

Antimicrobial sensitivities were determined using the disc diffusion method, with CH01 found to have reduced sensitivity to tetracycline, ampicillin, ceftazidime, cefotaxime, aminoglycosides and sulphonamide compounds. Plasmid analysis of the field isolate showed four plasmids ranging in size from 82, 95, 130 and 160 kb with the repicon types IncI1 γ , FIB, A/C, P, K and FII identified as shown in Figure 6.1. The CH01 *E. coli* was found to belong to ST350 by MLST.

Figure 6.1 Plasmid sizing of CH01 field isolate and pCH01 transformant



S1 nuclease PFGE of (A) the *E. coli* CH01 field isolate and (B) transformed *E. coli* DH10 with pCH01, *E. coli* DH10 used for transformations as a negative control, pCH01 plasmids are circled in red. Plasmids digested with 8U of S1 nuclease for 45 minutes, DNA bands were resolved on a 1% agarose gel. MidRange and Low Range PFGE markers were used for sizing.

6.3.2 Analysis of the pCH01 plasmid

The extracted pCH01 plasmid was transformed into *E. coli* DH10 by electroporation, and the antimicrobial sensitivities of the pCH01 transformant were determined as for the field isolate. The transformant had reduced sensitivity to tetracycline, ampicillin, ceftazidime, cefotaxime, aminoglycosides and sulphonamides, the same as the wild type CH01. The size of the pCH01 was determined to be 160 kb which corresponded to the 160 kb plasmid in the wild type (Figure 6.1).

6.3.2.1 pCH01 plasmid transferability

pCH01 was investigated to see if it was self transmissible, conjugations using the plasmid transformant were conducted. Liquid and solid conjugations were carried out

using a plasmid free and rifampicin resistant *Salmonella enterica* Typhimurium 26R as a recipient. Conjugations were considered to have occurred if red colonies grew on Rambach supplemented with 100 µg/ml rifampicin and 1 µg/ml cefotaxime which inhibits growth of the recipient negative control. Colonies were then grown on LB-G with 4 µg/ml cefotaxime, to confirm the selective growth. pCH01 was found to transfer in both solid and liquid conjugations.

6.3.3 pCH01 plasmid sequencing

pCH01 DNA was sequenced by 454 sequencing, and had a >42 fold coverage, the *de novo* assembly produced 3 contigs which were closed using PCR with primers designed based on the contig sequences. The sequenced pCH01 plasmid was 160,357 bp in size and had a G+C content of 52.87%. Annotation and analysis of the plasmids identified 196 complete open reading frames (orfs), of which 96 were hypothetical and 18 were putative genes, with the plasmid being made up of a 124,031 bp contiguous backbone which had undergone four insertions, the position and functions of genes are shown in Figure 6.2 and listed in Table 6.1.

6.3.3.1 Analysis of the pCH01 replication region

The replication of the IncA/C plasmids is mediated through iterons as seen with plasmids belonging to IncP incompatibility group (Llanes *et al.*, 1994). The IncA/C plasmid pRA1 was found to have 13 direct repeats which make up the iterons involved in replication, which are located downstream of *repA* (Llanes *et al.*, 1994; Llanes *et al.*, 1996). pCH01 has a single replication gene (*repA*) coding a 366 amino acid replication protein, and is part of the IncA/C₂ subdivision. The RepA IncA/C₂ plasmids vary from pRA1 by 6 amino acid substitutions (98% identity), at 88 S>T, 92 Q>H, 161 T>N, 352 T>M, 361 R>P and 363 S>R (Fricke *et al.*, 2009). The majority of the MDR IncA/C plasmids belong to the IncA/C₂, as opposed to the product of the *repA* used for PBRT (Carattoli *et al.*, 2005a; Doublet *et al.*, 2012).

Table 6.1 Open reading frames present in pCH01

Gene	ORF	Direction	Location	Length (bp)	Protein Length (aa)	Product	Function
<i>orf1</i>	1	Forward	323-862	540	179	conserved hypothetical	Unknown
<i>orf2</i>	2	Forward	867-1154	288	95	conserved hypothetical	Unknown
<i>repA</i>	3	Forward	1139-2239	1101	366	RepA	Replication protein
<i>orf4</i>	4	Forward	2585-2866	282	93	conserved hypothetical	Unknown
<i>orf5</i>	5	Reverse	3276-3709	432	143	conserved hypothetical	Unknown
<i>orf6</i>	6	Reverse	3888-4841	954	317	phosphoadenosine phosphosulfate reductase family	Paps reductase
<i>orf7</i>	7	Forward	5110-5718	609	202	conserved hypothetical	Unknown
<i>orf8</i>	8	Forward	5723-6244	522	173	conserved hypothetical	Unknown
<i>orf9</i>	9	Forward	6247-6768	522	173	conserved hypothetical	Unknown
<i>orf10</i>	10	Forward	6865-7338	474	157	conserved hypothetical	Unknown
<i>orf11</i>	11	Forward	7304-7642	339	112	conserved hypothetical	Unknown
<i>orf12</i>	12	Forward	7647-8501	855	284	conserved hypothetical	Unknown
<i>sppA</i>	13	Forward	8501-9460	960	319	SppA	Serine peptidase
<i>dsbA-like</i>	14	Forward	9476-10336	861	286	DsbA-like	Thioredoxin like
<i>orf15</i>	15	Forward	10370-10798	429	142	conserved hypothetical	Unknown
<i>orf16</i>	16	Forward	10855-11214	360	119	conserved hypothetical	Unknown
<i>orf17</i>	17	Forward	11214-11660	447	148	conserved hypothetical	Possible ABC type siderophore
<i>orf18</i>	18	Forward	11657-12175	519	172	putative membrane protein	Formate dependent nitrite reductase
<i>orf19</i>	19	Forward	12178-12405	228	75	conserved hypothetical	Unknown
<i>orf20</i>	20	Forward	12392-13249	858	285	conserved hypothetical	Unknown
<i>orf21</i>	21	Forward	13480-14007	528	175	nuclease domain protein	Nuclease domain protein
<i>hup</i>	22	Forward	14065-14337	273	90	DNA binding protein HU	DNA binding protein HU-beta
<i>orf23</i>	23	Forward	14425-14718	294	97	conserved hypothetical	Putative plasmid related protein
<i>orf24</i>	24	Reverse	14745-15047	303	100	DNA binding domain	Cro like protein
<i>orf25</i>	25	Reverse	15052-15402	351	116	conserved hypothetical	68% to toxin antitoxin RelE

Gene	ORF	Direction	Location	Length (bp)	Protein Length (aa)	Product	Function
<i>orf26</i>	26	Forward	15565-16113	549	182	conserved hypothetical	Transcriptional regulator
<i>orf27</i>	27	Forward	16454-16648	195	64	conserved hypothetical	Unknown
<i>orf28</i>	28	Forward	16659-17030	372	123	conserved hypothetical	Unknown
<i>orf29</i>	29	Forward	17023-17493	471	156	conserved hypothetical	Unknown
<i>orf30</i>	30	Reverse	17508-17843	336	111	conserved hypothetical	Unknown
<i>orf31</i>	31	Reverse	17940-18428	489	162	conserved hypothetical	Unknown
<i>orf32</i>	32	Forward	18431-18928	498	165	conserved hypothetical	Unknown
<i>orf33</i>	33	Forward	19490-20422	933	310	conserved hypothetical	Unknown
<i>orf34</i>	34	Forward	20496-21959	1464	487	ATPase domain protein	ATPase
<i>orf35</i>	35	Forward	22115-22444	330	109	conserved hypothetical	Unknown
<i>orf36</i>	36	Forward	22449-22841	393	130	conserved hypothetical	Unknown
<i>orf37</i>	37	Forward	22890-23084	195	64	conserved hypothetical	Unknown
<i>orf38</i>	38	Forward	23078-24262	1185	394	putative membrane protein	Unknown
<i>orf39</i>	39	Forward	24274-24621	348	115	conserved hypothetical	Unknown
<i>orf40</i>	40	Forward	24618-25046	429	142	conserved hypothetical	Unknown
<i>orf41</i>	41	Forward	25039-25602	564	187	conserved hypothetical	Unknown
<i>orf42</i>	42	Forward	26186-26731	546	181	conserved hypothetical	Unknown
<i>parB-like</i>	43	Forward	26848-27786	939	312	ParB-like	ParB like
<i>orf44</i>	44	Forward	27943-28221	279	92	conserved hypothetical	Unknown
<i>orf45</i>	45	Forward	28466-28828	363	120	conserved hypothetical	Unknown
<i>dcm-1</i>	46	Forward	28821-30065	1245	414	DNA (cytosine-5-)-methyltransferase	DNA (cytosine-5-)-methyltransferase
<i>orf47</i>	47	Forward	30079-30528	450	149	conserved hypothetical	Unknown
<i>orf48</i>	48	Forward	30510-30821	312	103	conserved hypothetical	Unknown
<i>parA</i>	49	Forward	30995-31780	786	261	ParA	Partitioning
<i>parB</i>	50	Forward	31784-32965	1182	393	ParB	Partitioning
<i>orf51</i>	51	Forward	33008-33286	279	92	conserved hypothetical	Unknown

Gene	ORF	Direction	Location	Length (bp)	Protein Length (aa)	Product	Function
<i>IS4</i>	52	Forward	33397-34740	1344	447	IS4 related transposon	Transposase
<i>orf53</i>	53	Reverse	34769-35437	669	222	conserved hypothetical	Unknown
<i>orf54</i>	54	Reverse	35475-35654	180	59	conserved hypothetical	Unknown
<i>orf55</i>	55	Forward	35958-36335	378	125	conserved hypothetical	Unknown
<i>orf56</i>	56	Forward	36328-36609	282	93	conserved hypothetical	Unknown
<i>orf57</i>	57	Forward	36551-37258	708	235	conserved hypothetical	Thmidylate kinase
<i>orf58</i>	58	Forward	37251-37757	507	168	conserved hypothetical	Unknown
<i>orf59</i>	59	Forward	37742-38074	333	110	conserved hypothetical	Unknown
<i>orf60</i>	60	Forward	38083-38583	501	166	conserved hypothetical	Unknown
<i>dcm-2</i>	61	Forward	38587-40014	1428	475	DNA (cytosine-5-)-methyltransferase	DNA (cytosine-5-)-methyltransferase
<i>orf62</i>	62	Forward	40014-40670	657	218	conserved hypothetical	Unknown
<i>orf63</i>	63	Forward	40609-40872	264	87	conserved hypothetical	Unknown
<i>orf64</i>	64	Forward	40876-41094	219	72	conserved hypothetical	Unknown
<i>orf65</i>	65	Forward	41188-41805	618	205	conserved hypothetical	Unknown
<i>orf66</i>	66	Forward	41806-42012	207	68	conserved hypothetical	Unknown
<i>orf67</i>	67	Forward	42017-42316	300	99	conserved hypothetical	Unknown
<i>orf68</i>	68	Reverse	42408-42896	489	162	conserved hypothetical	Unknown
<i>topB</i>	69	Reverse	42911-45103	2193	730	topoisomerase III	DNA topoisomerase III
<i>orf70</i>	70	Reverse	45103-45336	234	77	conserved hypothetical	Unknown
<i>orf71</i>	71	Reverse	45318-45950	633	210	putative lipoprotein	Putative lipoprotein
<i>traI</i>	72	Forward	46103-49081	2979	992	TraI	Conjugative relaxase
<i>traD</i>	73	Forward	49078-50943	1866	621	TraD	Conjugative coupling factor
<i>orf74</i>	74	Forward	50954-51538	585	194	conserved hypothetical	Unknown
<i>orf75</i>	75	Forward	51495-52124	630	209	putative conjugal coupling factor	Putative conjugal coupling factor
<i>orf76</i>	76	Forward	52134-52580	447	148	conserved hypothetical	Unknown
<i>orf77</i>	77	Forward	52590-52967	378	125	conserved hypothetical	Unknown

Gene	ORF	Direction	Location	Length (bp)	Protein Length (aa)	Product	Function
<i>orf78</i>	78	Forward	52967-53629	663	220	conserved hypothetical	Unknown
<i>orf79</i>	79	Forward	53810-53953	144	47	conserved hypothetical	Unknown
<i>orf80</i>	80	Forward	53953-54330	378	125	conserved hypothetical	Unknown
<i>traL</i>	81	Forward	54474-54755	282	93	TraL	Conjugative protein
<i>traE</i>	82	Forward	54752-55378	627	208	TraE	Pilus assembly protein
<i>traK</i>	83	Forward	55362-56279	918	305	TraK	Conjugative protein
<i>traB</i>	84	Forward	56276-57592	1317	438	TraB	Pilus assembly protein
<i>traV</i>	85	Forward	57589-58167	579	192	TraV	Pilus assembly protein
<i>traA</i>	86	Forward	58171-58563	393	130	TraA	Pilin subunit protein
<i>orf87</i>	87	Forward	58764-64295	5532	1843	conserved hypothetical	Unknown
<i>dsbC</i>	88	Forward	64444-65151	708	235	DsbC thiol:disulfide interchange protein	thiol:disulfide interchange protein
<i>traC</i>	89	Forward	65148-67595	2448	815	TraC	Conjugative protein
<i>orf90</i>	90	Forward	67610-67927	318	105	conserved hypothetical	Unknown
<i>traF</i>	91	Forward	67924-68454	531	176	TraF	Conjugative peptidase
<i>traW</i>	92	Forward	68417-69682	1266	421	TraW	Pilus assembly protein
<i>orf93</i>	93	Forward	69679-70350	672	223	diguanylate phosphodiesterase domain containing	EAL domain
<i>traU</i>	94	Forward	70275-71354	1080	359	TraU	Pilus assembly protein
<i>traN</i>	95	Forward	71458-74268	2811	936	TraN	Stabilization protein
<i>orf96</i>	96	Reverse	74307-75167	861	286	conserved hypothetical	Unknown
<i>orf97</i>	97	Reverse	75290-75931	642	213	conserved hypothetical	Unknown
<i>orf98</i>	98	Forward	76225-76545	321	106	conserved hypothetical	Unknown
<i>orf99</i>	99	Forward	76783-77034	252	83	conserved hypothetical	Unknown
<i>orf100</i>	100	Forward	77254-78222	969	322	ATPase	ATPase
<i>orf101</i>	101	Forward	78233-79141	909	302	conserved hypothetical	Unknown
<i>ssb</i>	102	Forward	79205-79732	528	175	Ssb	Single stranded DNA binding protein
<i>bet</i>	103	Forward	79785-80816	1032	343	putative DNA recombination protein	DNA recombination protein

Gene	ORF	Direction	Location	Length (bp)	Protein Length (aa)	Product	Function
<i>orf104</i>	104	Forward	80879-81889	1011	336	conserved hypothetical	Putative endonuclease
<i>orf105</i>	105	Forward	81886-82026	141	46	conserved hypothetical	Unknown
<i>orf106</i>	106	Forward	82077-83366	1290	429	conserved hypothetical	Cobalamine biosynthesis
<i>orf107</i>	107	Forward	83451-85247	1797	598	von willebrand factor type A protein	Von Willebrand factor type A protein
<i>orf108</i>	108	Forward	85309-85644	336	111	conserved hypothetical	Unknown
<i>orf109</i>	109	Forward	85909-86448	540	179	conserved hypothetical	Unknown
<i>orf110</i>	110	Forward	86539-87039	501	166	conserved hypothetical	Unknown
<i>ISEcp1</i>	111	Forward	87228-88490	1263	420	ISEcp1 transposase	Transposase
<i>bla_{CTX-M-3}</i>	112	Forward	88743-89618	876	291	β -lactamase CTX-M-3	β -lactmase
<i>orf477</i>	113	Reverse	89665-89997	333	110	Orf477	Unknown
<i>orf114</i>	114	Forward	90047-90970	924	307	conserved hypothetical	DNA modification methylase
<i>orf115</i>	115	Forward	91036-91260	225	74	2Fe-2S binding domain protein	Ferredoxin
<i>orf116</i>	116	Forward	91322-91711	390	129	conserved hypothetical	Unknown
<i>orf117</i>	117	Forward	91701-92153	453	150	conserved hypothetical	Unknown
<i>orf118</i>	118	Forward	92158-92433	276	91	conserved hypothetical	Unknown
<i>orf119</i>	119	Forward	92491-92682	192	63	conserved hypothetical	Unknown
<i>orf120</i>	120	Forward	92751-93104	354	117	conserved hypothetical	Type II topoisomerase
<i>IS186B</i>	121	Reverse	93279-94391	1113	370	IS186B transposons	Transposase
<i>orf122</i>	122	Forward	94646-94990	345	114	conserved hypothetical	Unknown
<i>orf123</i>	123	Forward	95068-95370	303	100	conserved hypothetical	hydrofolate links
<i>dcm-3</i>	124	Forward	95448-97073	1626	541	DNA (cytosine-5-)-methyltransferase	DNA (cytosine-5-)-methyltransferase
<i>orf125</i>	125	Forward	97134-97565	432	143	conserved hypothetical	Unknown
<i>orf126</i>	126	Forward	97639-98193	555	184	conserved hypothetical	Unknown
<i>orf127</i>	127	Forward	98426-98812	387	128	conserved hypothetical	Unknown
<i>orf128</i>	128	Forward	98900-101353	2454	817	conserved hypothetical	Putative phosphoadenosine phosphosulfate reductase
<i>orf129</i>	129	Forward	101555-101758	204	67	conserved hypothetical	Unknown

Gene	ORF	Direction	Location	Length (bp)	Protein Length (aa)	Product	Function
<i>ybaA</i>	130	Forward	101830-102435	606	201	YbaA	5' nucleotidase
<i>orf131</i>	131	Forward	102428-102697	270	89	conserved hypothetical	Unknown
<i>orf132</i>	132	Forward	102678-102929	252	83	conserved hypothetical	Unknown
<i>orf133</i>	133	Forward	102919-103560	642	213	conserved hypothetical	Pyruvate/2 oxoglutarate dehydrogenase
<i>ngrC</i>	134	Forward	103602-104486	885	294	putative septum formation inhibitor	Putative septum formation inhibitor
<i>orf135</i>	135	Forward	104691-104837	147	48	conserved hypothetical	Unknown
<i>orf136</i>	136	Forward	104944-105330	387	128	conserved hypothetical	Unknown
<i>orf137</i>	137	Forward	105478-107235	1758	585	putative DNA primase	DNA primase
<i>orf138</i>	138	Forward	107222-107500	279	92	conserved hypothetical	Unknown
<i>orf139</i>	139	Forward	107561-107812	252	83	conserved hypothetical	Unknown
<i>orf140</i>	140	Forward	107822-107938	117	38	conserved hypothetical	Unknown
<i>orf141</i>	141	Forward	108032-108433	402	133	conserved hypothetical	Unknown
<i>orf142</i>	142	Forward	108547-109272	726	241	conserved hypothetical	Unknown
<i>rhs-like</i>	143	Forward	109405-110079	675	224	Rhs like protein	N terminus
<i>orf144</i>	144	Reverse	109813-110574	762	253	conserved hypothetical	Unknown
<i>orf145</i>	145	Forward	110836-111117	282	93	conserved hypothetical	Unknown
<i>orf146</i>	146	Forward	111416-111952	537	178	conserved hypothetical	Unknown
<i>int</i>	147	Reverse	111955-112965	1011	336	Phage integrase	Recombinase/integrase
<i>put</i>	148	Forward	112970-113839	870	289	putative exonuclease	Exonuclease
<i>orf149</i>	149	Forward	113836-114327	492	163	conserved hypothetical	Unknown DNA replication terminus site binding protein
<i>ter</i>	150	Forward	114644-115519	876	291	replication terminus site binding protein	protein
<i>orf151</i>	151	Reverse	115730-117307	1578	525	methyl group accepting protein (Aero)	Methyl-accepting chemotaxis protein
<i>IS26 truncated</i>	152	Forward	117619-117915	297	98	IS26 truncated	Transposase
<i>IS26</i>	153	Reverse	117861-118583	723	240	IS26 transposon	Transposase
<i>tnpM</i>	154	Reverse	118775-119383	609	202	TnpM transposon modulator	Transposon Modulator

Gene	ORF	Direction	Location	Length (bp)	Protein Length (aa)	Product	Function
<i>int1</i>	155	Reverse	119328-120341	1014	337	Int1 integron	Integrase
<i>aadA</i>	156	Forward	120372-121277	906	301	AadA aminoglycoside resistance adenylyltransferase	Streptomycin 3' adenylyltransferase
<i>aacC</i>	157	Forward	121441-122343	903	300	AacC aminoglycoside resistance 3-N-acetyltransferase	Aminoglycoside 3-acetyltransferase
<i>groS</i>	158	Forward	122430-122870	441	146	GroES	Chaperonin
<i>groL</i>	159	Forward	122926-124563	1638	545	GroEL	Heat shock protein
<i>insE</i>	160	Forward	124752-126281	1530	509	InsE transposon	Transposase
<i>insF</i>	161	Forward	126549-127784	1236	411	InsF transposon	Transposase
<i>qacE</i>	162	Forward	128002-128349	348	115	qacE quaternary ammonium compound resistance	Multidrug efflux
<i>sul1</i>	163	Forward	128316-129182	867	288	Sul1 dihydropteroate synthase	Sulphonamide resistance
<i>pac</i>	164	Forward	129310-129810	501	166	Pac puromycin N acetyltransferase	ABC like
<i>orf165</i>	165	Forward	129834-129941	108	35	conserved hypothetical	Unknown
<i>istB</i>	166	Reverse	129986-130771	786	261	IstB transposition protein for IS1326	Transposase
<i>istA</i>	167	Reverse	130758-132281	1524	507	IstA transposition protein for IS1326	Transposase
<i>tniB</i>	168	Reverse	132383-133243	861	286	TniB	Transposition protein
<i>tniA</i>	169	Reverse	133246-134961	1716	571	TniA	Transposition protein
<i>urf2M</i>	170	Reverse	135000-135707	708	235	urf2M	Unknown
<i>merE</i>	171	Reverse	135704-135940	237	78	MerE	Mercury resistance
<i>merD</i>	172	Reverse	135937-136299	363	120	MerD	Mercury regulator
<i>merA</i>	173	Reverse	136317-138011	1695	564	MerA	Mercuric reductase
<i>merC</i>	174	Reverse	138063-138485	423	140	MerC	Mercury transport protein
<i>merP</i>	175	Reverse	138521-138796	276	91	MerP	Mercury transport protein
<i>merT</i>	176	Reverse	138810-139214	405	134	MerT	Mercury transport protein
<i>merR</i>	177	Forward	139232-139666	435	144	MerR	Mercury regulator
<i>orf178</i>	178	Forward	140669-140911	243	80	relaxase	Putative relaxase
<i>tetR</i>	179	Reverse	140943-141620	678	225	TetR tetracycline resistance	Tetracycline repressor protein
<i>tetA</i>	180	Forward	141624-142898	1275	424	TetA	Tetracycline efflux

Gene	ORF	Direction	Location	Length (bp)	Protein Length (aa)	Product	Function
<i>pecM</i>	181	Reverse	142930-143814	885	294	PecM	PecM like
<i>Tn21 tnpA</i>	182	Forward	144279-146099	1821	606	Tn21-like transposase	Transposase
<i>kfrA</i>	183	Forward	146270-147310	1041	346	KfrA	Chromosome segregation
<i>orf184</i>	184	Forward	147310-147588	279	92	conserved hypothetical	Unknown
<i>uvrD</i>	185	Forward	147597-149108	1512	503	UvrD	Putative DNA helicase
<i>traF</i>	186	Forward	149231-150259	1029	342	TraF	Pilin signal peptidase
<i>traH</i>	187	Forward	150261-151694	1434	477	TraH	Pilus assembly protein
<i>traG</i>	188	Forward	151707-155321	3615	1204	TraG	Pilus assembly protein
<i>orf189</i>	189	Forward	155359-155721	363	120	putative membrane protein	Putative permease
<i>orf190</i>	190	Forward	156435-156707	273	90	Ner-like DNA binding protein	Ner-like DNA binding protein
<i>orf191</i>	191	Forward	156707-157240	534	177	transglycosylase	Transglycosylase
<i>orf192</i>	192	Forward	157032-157859	828	275	ABC-type polar amino acid transport	ABC-type polar amino acid transport
<i>orf193</i>	193	Forward	157856-158407	552	183	conserved hypothetical	Unknown
<i>orf194</i>	194	Reverse	158467-158886	420	139	H-NS-like DNA binding protein	H-NS DNA binding protein
<i>orf195</i>	195	Reverse	158888-159181	294	97	conserved hypothetical	Unknown
<i>stbA</i>	196	Reverse	159198-160181	984	327	StbA	ParM like

6.3.3.2 Analysis of the pCH01 transfer region

The transfer proteins involved in the IncA/C conjugations have not been fully characterised and their functions are still unknown. The pCH01 *tra* locus, coding the conjugative genes are separated into two sections *tra* 1 and *tra* 2. The first is located downstream of the topoisomerase, and is 28,166 bp in size (orf72-98). The *tra* 1 contains the genes *traI*, *D*, *L*, *E*, *K*, *B*, *V*, *A*, *C*, *F*, *W*, *U* and *N* which are involved in pilus assembly and stabilization. Also located within the *tra* 1 region are 8 hypothetical genes, *dsbC* and a diguanylate phosphodiesterase gene. The second is located downstream of the putative helicase gene *uvrD*, 74,962 bp away from the first, and contains *traF*, *H*, and *G* for pilus assembly and pilin signal peptidase. The separation of the *tra* loci is a common feature of IncA/C plasmids and the conjugative genes in IncA/C plasmids have been compared to those in integrative and conjugative elements (ICE) (Fernandez-Alarcon, Singer and Johnson, 2011; Wozniak *et al.*, 2009). BLAST searches of ICE SXT/R391 showed that most of the conjugative genes are present in IncA/C plasmids, and the similarities extend to hypothetical genes located throughout the *tra* loci (Wozniak *et al.*, 2009). ICEs have been found in numerous bacteria including *Vibrio cholerae* and *Photobacterium damsela*, just like many of the IncA/C plasmids they have been found to be vectors for antibiotic, mercury and quaternary ammonium resistance (Rodriguez-Blanco, Lemos and Osorio, 2012; Osorio *et al.*, 2008). Despite little work on the conjugative regions, studies have shown that IncA/C plasmids are conjugative, just as pCH01 is, although the presence of *bla*_{CMY-2} may reduce the rate of conjugation (Poole *et al.*, 2009; Carattoli *et al.*, 2002).

6.3.3.3 Analysis of the pCH01 stability regions

pCH01 and other IncA/C plasmids have few stability or addiction genes. The plasmid partitioning genes *parA* and *parB* were identified in pCH01 along with *parA*-like, *parB*-like and a putative *parM* genes (Davis, Martin and Austin, 1992; Davis *et al.*, 1996). pCH01 carries three different DNA (cytosine-5)-methyltransferase genes which contribute

to restriction modification systems and has a role in repair of mismatch 5-methylcytosine (Korba and Hays, 1982; Lieb, 1987). The *ssb* gene coding for single stranded binding protein which protects the plasmid DNA from degradation was located upstream of *bet* a DNA recombination gene. pCH01 had the *kfrA* gene which has been found to have a role in the presence of maintaining plasmids and preserving a minimum copy number in IncP plasmids (Adamczyk *et al.*, 2006). A putative gene (*orf25*) coding a protein with 68% amino acid identity to the antitoxin:toxin protein RelE was also identified in pCH01 and other IncA/C plasmids, which may imply a putative addiction system.

6.3.3.4 Analysis of the pCH01 resistance regions

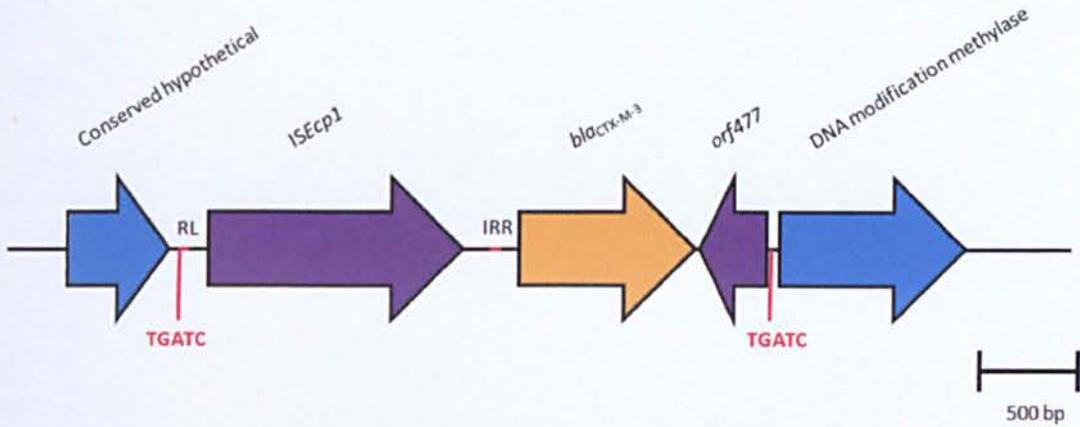
The pCH01 plasmid contains two separate resistance regions, the first harbours the *bla*_{CTX-M-3} and the second is the larger MDR insert, linked to the *mer* operon.

6.3.3.4.1 The *bla*_{CTX-M-3} gene in pCH01

The *bla*_{CTX-M-3} belongs to the group one CTX-M enzymes, and varies from *bla*_{CTX-M-1} by 10 nucleotide substitutions, and from *bla*_{CTX-M-15} by a single nucleotide substitution from A>G at position 725 causing a change in the protein at 242 D>G. The *bla*_{CTX-M-3} lies downstream of the *ISEcp1* and upstream of *orf477* as part of a 2,968bp transposon (87,042-90,009 bp); similar environments have been reported before (Eckert, Gautier and Arlet, 2006; Golebiewski *et al.*, 2007). However the distance between *ISEcp1* and *bla*_{CTX-M-3} in the pCH01 transposition unit, is 82 bp shorter than those previously reported, and shares greater homology with *bla*_{CTX-M-15} environments such as those found in pJIE143, pEC_L46, pEK516 and pC15-1a (Partridge *et al.*, 2011; Smet *et al.*, 2010b; Woodford *et al.*, 2009; Boyd *et al.*, 2004). This may suggest that the transposition unit was previously *bla*_{CTX-M-15} and has mutated to *bla*_{CTX-M-3} or vice versa, or was captured in a separate event, however the transposition unit present in pCH01 has not been reported before. This transposition unit inserted downstream of a conserved hypothetical gene (86,539-87,039 bp) which is also present in several other IncA/C plasmids and is flanked by the direct

repeats TGATC (87,037-87,041 and 90,010-90,014 bp), as shown in Figure 6.3. In pCH01 the *bla*_{CTX-M-3} gene is not associated with any other resistance genes, transposons or integrons unlike pH19 and other plasmids such as pKPSH3.

Figure 6.3 The *bla*_{CTX-M-3} gene present in pCH01

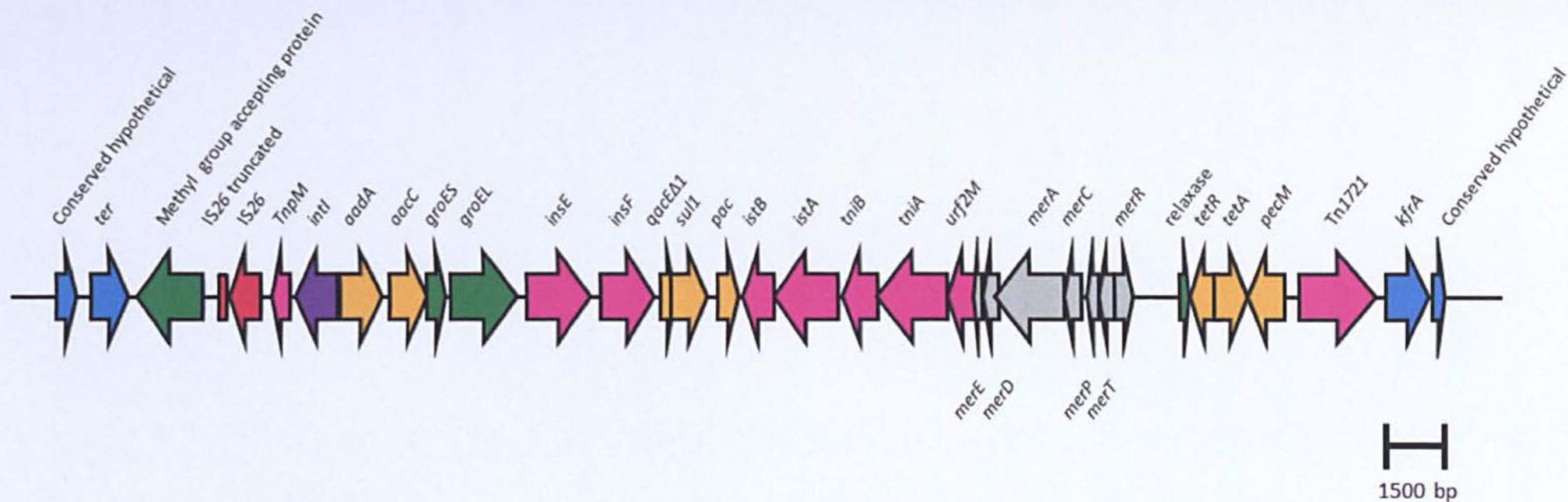


The *ISEcp1-bla*_{CTX-M-3}-*orf477* insertion in pCH01, purple arrows are the *ISEcp1* and *orf477*, the orange arrow is the *bla*_{CTX-M-3} genes, drawn to scale.

6.3.3.4.2 The MDR region in pCH01

The second resistance region is the large MDR region (30,590 bp), located between *ter* and *kfrA* (115,544-146,133 bp), shown in Figure 6.4. Located downstream of a methyl group accepting protein, IS26 and a transposon modulator is the *ISCR16* which has been observed in several other plasmids including those that belong to the IncA/C incompatibility group including pSN254, pAR060302 and peH4H (Fernandez-Alarcon, Singer and Johnson, 2011; Welch *et al.*, 2007; Call *et al.*, 2010; Toleman, Bennett and Walsh, 2006). The *ISCR16* consists of *intI1*, *aadA*, *aacC*, *groES/groEL* *insE* (*ISCR16*), *insF*, *qacE1* and *sulI* (Toleman, Bennett and Walsh, 2006). The heat shock genes allow bacterial growth at higher temperatures (42°C), which may have a role in persistence in the environment (Fayet, Ziegelhoffer and Georgopoulos, 1989). Downstream of the *ISCR16* was the puromycin resistance gene *pac*, which was followed by a region closely related to the Tn21 *mer* operons (AF071413), but lacks the TnpA of this transposon. *IS1326* (*istB*

Figure 6.4 MDR region of pCH01



The MDR insertion present in pCH01, magenta arrows indicate mobile elements, purple are integrons and red are IS26, orange arrows are resistance genes, and grey arrows are the *mer* operon, green and blue arrows are other genes, drawn to scale.

and *istA*), *tniB* and *tniA* are located downstream of *pac* and upstream of the *mer* operon (Liebert, Hall and Summers, 1999). The *mer* operon consists of *merE*, *merD*, *merA*, *merC*, *merP*, *merT*, and *merR* and confers resistance to mercury in the environment converting Hg²⁺ ions into volatile compounds (Barrineau *et al.*, 1984; Nascimento and Chartone-Souza, 2003). The concluding part of the MDR region contains the tetracycline resistance genes *tetR* and *tetA* which are upstream of the *pecM* and Tn1721. The *pecM* gene has been found in numerous bacterial isolates including *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Proteus mirabilis* and belongs to the EamA family, encoding a drug/metabolite transporters (Jack, Yang and Saier, 2001). The association of *tetR*, *tetA* and *pecM* with Tn1721 has been seen before in the plasmids pK1HV (HF545434), pWSH1 (DQ464880) and the IncA/C plasmid pR148 (Del Castillo *et al.*, 2013). Downstream from IS1326 in this MDR region shares a high level of homology with that of the chromosome of *E. coli* 042 from a child with diarrhoea in Peru isolated in 1983 (Chaudhuri *et al.*, 2010).

6.3.3.5 Analysis of additional MGE and accessory genes in pCH01

In addition to those associated with the resistance regions two additional insertion sequences were identified. The first was located downstream of *orf51* (33,008-33,286, IS4MS), 447 amino acids in length and has a 88% identity to that of IS4 which is predominantly found in *Shewanella baltica*, an aquatic organism suggesting a possible link between pCH01 and the aquatic environment (Caro-Quintero *et al.*, 2012). This transposon, like others has 50 bp repeats which flank the gene, which are located from 33,331-33,380 bp and an inverted repeat between 34,700-34,749 bp. The other insertion sequence was IS186B which was located in the reverse orientation between *orf120* and *orf122* at position 93,108-94,446 bp. This transposon has also been found in other resistance plasmids including the IncL/M pNDM-HK and IncFII pEC_B24 as well as bacterial chromosomes (Smet *et al.*, 2010b; Ho *et al.*, 2011b). pCH01 had several accessory genes which may have a role in metabolism and in transcriptional regulation.

Firstly the *sppA* gene codes for a serine peptidase involved in the breakdown of peptides involved in signalling (Wang *et al.*, 2008). The product of *dsbA* has a thioredoxin like domain involved in redox signalling in the cell and *dsbC* present in the *tra 1* region has a role of a thiol:disulfide interchange (Lasica and Jagusztyn-Krynicka, 2007). Hypothetical genes coding for proteins with a phosphoadenosine phosphosulfate reductase activity and ferredoxin domains were also present. Additionally several genes encoding transcriptional regulators belonging to the nucleoid associated proteins were identified in pCH01. These included H-NS, HU-beta and Ner-like DNA binding proteins, and a cro-like protein (Doyle *et al.*, 2007; Yun *et al.*, 2010; Acebo *et al.*, 1998).

6.3.4 Comparison of pCH01 with IncA/C plasmids

The complete sequence of pCH01 plasmid was compared by searching GenBank using BLASTn, which identified similar plasmids, and 23 were used for further comparisons. A range of plasmids were selected having been isolated from *E. coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Yersinia ruckeri* and *pestis*, *Photobacterium damsela*, *Salmonella enterica*, *Aeromonas hydrophila*, and *Providencia stuartii* (Table 6.2). These plasmid hosts have been isolated from across the globe in both clinical and veterinary isolates. All of the plasmids ranged in size from 105,974-195,560 bp and had at least two antimicrobial resistance genes. For further comparison of plasmids they will be divided into three groups, which are those plasmids lacking *bla*_{CMY-2} (NCM), those bearing *bla*_{CMY-2} (CMY) insertion as Fernandez-Alarcon *et al* (20114) and those with *bla*_{NDM-1} (NDM).

Table 6.2 IncA/C plasmids compared in this study

Plasmid	Accession	% Coverage pCH01	% Coverage with no insert	Size	Rep	Host	Source	Location	Date	Resistance genes	Reference
pCH01	NA	NA	NA	160,357	A/C	<i>E. coli</i>	Chicken	UK	2006	<i>bla_{CTX-M-3}</i> , <i>aadA</i> , <i>aadC</i> , <i>sul1</i> , <i>tetRA</i> , <i>pac</i> , <i>pecM</i>	
pEA1509	FQ203354	87	99	162,202	A/C	<i>Enterobacter aerogenes</i>	Human	France	2004	<i>bla_{TEM-121}</i> , <i>aacA4</i> , <i>dfrA1</i> , <i>emeR</i> , <i>sul</i>	Diene <i>et al</i> (2013)
pR55	JQ010984	86	99	170,810	A/C	<i>Klebsiella pneumoniae</i>	Human	France	1969	<i>bla_{OXA-21}</i> , <i>floR</i> , <i>catA</i> , <i>aadB</i> , <i>sul1</i>	Doublet <i>et al</i> (2012)
pTC2	JQ824049	86	99	180,184	A/C	<i>Providencia stuartii</i>	Mouse	France	2008	<i>mphA</i> <i>mrx</i> <i>mphR</i> <i>bla_{VIM-1}</i> , <i>aacA7</i> , <i>dfrA1</i> , <i>aadA1</i> <i>bla_{SHV-5}</i> , <i>sul1</i> , <i>aadA1</i> , <i>dfrA12</i> , <i>aphA1</i>	Drieux <i>et al</i> (2013)
pYR1	CP000602	76	99	158,038	A/C	<i>Yersinia ruckeri</i>	Fish	USA	1996	<i>strAB</i> , <i>tetRA</i> , <i>sul2</i> , <i>dhfr1</i>	Welch <i>et al</i> (2007)
pR148	JX141473	85	98	165,906	A/C	<i>Aeromonas hydrophila</i>	Fish	Thailand	2012	<i>bla_{OXA-10}</i> , <i>aadA1</i> , <i>sul1</i> , <i>catA2</i> , <i>sul1</i> , <i>pecM</i> , <i>tetA</i>	Del Castillo <i>et al</i> (2013)
pNDM-1_dok01	AP012208	83	98	195,560	A/C	<i>E. coli</i>	Human	Japan	2009	<i>bla_{NDM-1}</i> , <i>bla_{TEM-1}</i> , <i>mph2</i> , <i>armA</i> , <i>aadA2</i> , <i>dfrA12</i> , <i>bla_{CMY-2}</i> , <i>mel</i> , <i>msr(E)</i> , <i>sul1</i>	Sekizuka <i>et al</i> (2011)
pIP1202	CP000603	85	96	182,913	A/C	<i>Yersinia pestis</i>	Human	Madagascar	1995	<i>bla_{SHV-1}</i> , <i>strAB</i> , <i>aadA</i> , <i>aphA7</i> , <i>tetRA</i> , <i>catA1</i> , <i>sul1</i> , <i>sul2</i> , <i>qacE1</i>	Welch <i>et al</i> (2007)
pP99-018	AB277723	75	96	150,157	A/C	<i>Photobacterium damsela</i>	Fish	Japan	1999	<i>aphA7</i> , <i>tetA</i> , <i>catA1</i> , <i>sul2</i>	Kim <i>et al</i> (2008)
pP91278	AB277724	71	91	131,520	A/C	<i>Photobacterium damsela</i>	Fish	USA	1991	<i>tetA</i> , <i>sul2</i>	Kim <i>et al</i> (2008)
pPG010208	HQ023861	71	89	135,803	A/C	<i>E. coli</i>	Cattle	Chile	2004	<i>floR</i> , <i>tetA</i> , <i>strB</i> , <i>msr</i> , <i>mph</i> , <i>sul2</i>	Fernandez-Alarcon <i>et al</i> (2011)
pAR060302	FJ621588	84	89	166,530	A/C	<i>E. coli</i>	Cattle	USA	2002	<i>bla_{CMY-2}</i> , <i>florR</i> , <i>tetA</i> , <i>strAB</i> , <i>aac(3)-Iva</i> <i>aadA</i> <i>aadB</i> , <i>sul1</i> <i>sul2</i>	Call <i>et al</i> (2010)
pSN254	CP000604	84	89	176,473	A/C	<i>Salmonella Newport</i>	Fish	USA	2000	<i>bla_{CMY-2}</i> , <i>bla_{CMY-2}</i> , <i>floR</i> , <i>strAB</i> , <i>tetA</i> , <i>aadA</i> , <i>aacC</i> , <i>sul1</i> , <i>sul2</i>	Welch <i>et al</i> (2007)

Plasmid	Accession	% Coverage pCH01	% Coverage with no insert	Size	Rep	Host	Source	Location	Date	Resistance genes	Reference
pUMNK88	HQ023862	80	89	160,573	A/C	<i>E. coli</i>	Pig	USA	2007	<i>bla</i> _{CMY-2} , <i>florR</i> , <i>tetA</i> , <i>strAB</i> , <i>sul2</i>	Fernandez-Alarcon <i>et al</i> (2011)
pAM04528	FJ621587	75	89	158,195	A/C	<i>Salmonella Newport</i> <i>Salmonella enterica dublin</i>	Human	USA	1998	<i>bla</i> _{CMY-2} , <i>bla</i> _{CMY-2} , <i>floR</i> , <i>strAB</i> , <i>tetA</i> , <i>sul1</i> , <i>sul2</i>	Call <i>et al</i> (2010)
pSD_174	JF267651	81	88	173,673	A/C	<i>Salmonella enterica dublin</i>	Cattle	USA	2011	<i>bla</i> _{CMY-2} , <i>bla</i> _{CMY-2} , <i>floR</i> , <i>strAB</i> , <i>tetA</i> , <i>sul1</i> , <i>sul2</i>	Han <i>et al</i> (2012)
pMR0211	JN687470	76	87	178,277	A/C	<i>Providencia stuartii</i>	Human	Afghanistan	2011	<i>bla</i> _{NDM-1} , <i>bla</i> _{OXA-10} , <i>aac(6')</i> , <i>armA</i> , <i>aadA</i> , <i>msr(E)</i> , <i>mph(E)</i> , <i>qnrA1</i> , <i>sul1</i> , <i>floR</i> , <i>tetA</i> , <i>strBA</i> , <i>sul2</i> , <i>bla</i> _{CMY-6}	McGann <i>et al</i> (2012)
pNDM10505	JF503991	74	87	166,744	A/C	<i>E. coli</i>	Human	Canada	2010	<i>bla</i> _{NDM-1} , <i>bla</i> _{CMY-6} , <i>rmtC</i> , <i>bleMBL</i> , <i>aphA6</i> , <i>aac(3')</i> , <i>aacA4</i> , <i>sul1</i>	Boyd <i>et al</i> (Unpublished)
pNDM-KN	JN157804	73	87	162,746	A/C	<i>Klebsiella pneumoniae</i>	Human	Kenya	2009	<i>bla</i> _{CMY-6} , <i>bla</i> _{NDM-1} , <i>rmtC</i> , <i>cmlA7</i> , <i>aadA1</i> , <i>ereC</i> , <i>arr-2</i> , <i>sul1</i>	Carattoli <i>et al</i> (2012)
pNDM10469	JN861072	73	87	137,813	A/C	<i>Klebsiella pneumoniae</i>	Human	Canada	2010	<i>bla</i> _{NDM-1} , <i>bla</i> _{CMY-6} , <i>emtC</i> , <i>bleMBL</i> , <i>aac(6')-Ib</i> , <i>sul1</i>	Boyd <i>et al</i> (Unpublished)
pAPEC1990_61	HQ023863	82	86	161,081	A/C	<i>E. coli</i>	Turkey	USA	1995	<i>bla</i> _{CMY-2} , <i>florR</i> , <i>tetA</i> , <i>strAB</i> , <i>sul2</i>	Fernandez-Alarcon <i>et al</i> (2011)
pRA1	FJ705807	68	85	143,963	A/C	<i>Aeromonas hydrophila</i>	Fish	Japan	1971	<i>tetA</i> , <i>sul2</i>	Fricke <i>et al</i> (2009)
peH4H	FJ621586	67	65	148,105	A/C	<i>E. coli</i>	Cattle	USA	2002	<i>bla</i> _{CMY-2} , <i>bla</i> _{CMY-2} , <i>floR</i> , <i>tetA</i> , <i>strAB</i> , <i>aadA</i> , <i>aph-3'</i> , <i>sul1</i> , <i>sul2</i>	Call <i>et al</i> (2010)
pKPHS3	CP003225	44	48	105,974	A/C	<i>Klebsiella pneumoniae</i>	Human	China	2011	<i>bla</i> _{CTX-M-14} , <i>bla</i> _{TEM-1} , <i>strAB</i> , <i>tetA</i> , <i>cmlA9</i> , <i>dfrA12</i> , <i>rmtB</i> , <i>aadA</i> , <i>sul1</i> , <i>sul2</i>	Liu <i>et al</i> (2012)

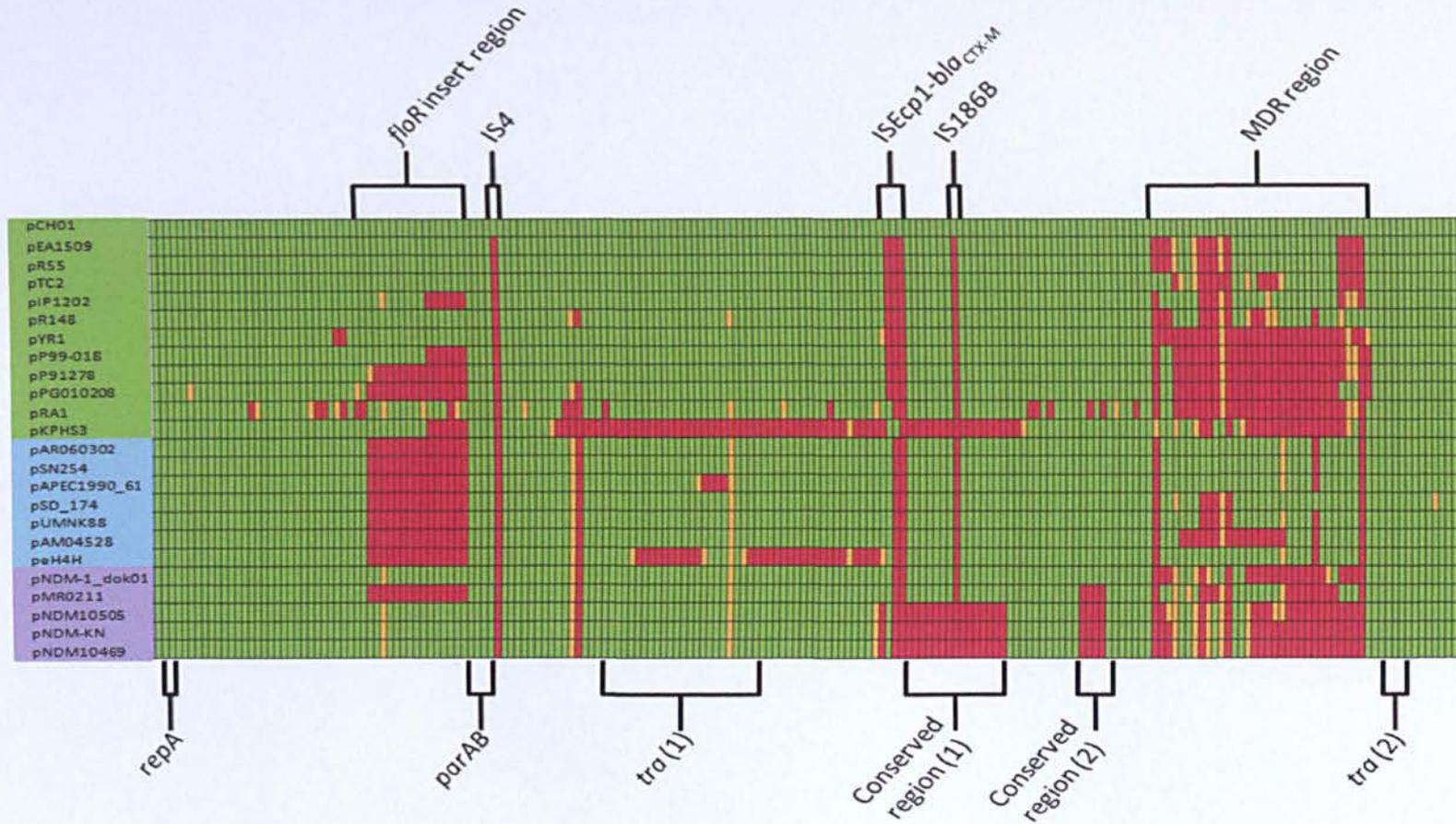
Plasmids used for comparisons in this study, those highlighted in green are NCM, blue CMY and purple NDM

Initial comparisons showed that pCH01 shared a high level of nucleotide similarity with the other IncA/C plasmids with coverages between 85-99% (excluding pEH4H and pKPHS3 due to deletions), this suggest that the IncA/C plasmid backbone is very stable and conserved. The individual orfs of pCH01 were compared with the plasmids in Table 6.2 using BLASTn, the results of which were used to devise a heat map, shown in Figure 6.5. The phylogeny of the IncA/C plasmids was also determined using a concatemer of sequences present in all plasmids with the exception of pKPHS3 which has a large deletion, shown in Figure 6.6. The results of both the orf comparison and phylogeny showed the presence of three apparent lineages, which were NCM, CMY and NDM plasmids, which were found to group together with some exceptions. Two main areas of interest were noted in pCH01 which were the truncated *rhs*, which is present in other IncA/C plasmids and *orf63* and *64*. The *rhs* gene present in other IncA/C, has undergone deletion in pCH01, with only 675 bp of the 5' of *rhs* truncated to a 225 amino acids protein, compared to the *rhs* of 4,146 bp and 1,382 amino acids in pSN254 (Welch *et al.*, 2007). The deletion also resulted in the loss of two hypothetical genes downstream of *rhs* and 86 bp of the C terminus of a third. The *rhs* genes have been found to be dynamic and undergo rearrangements which may have been the case with pCH01 (Jackson *et al.*, 2009). The other main difference observed that all CMY and NDM plasmids lacked the complete *orf63* and *64*, these orfs have likely been deleted as 70bp of the N terminus of *orf63* remains and the last 47 bp of the 93 bp downstream of *orf64* is homologous with other plasmids.

6.3.4.1 Comparison of pCH01 with non *bla*_{CMY-2} (NCM) plasmids

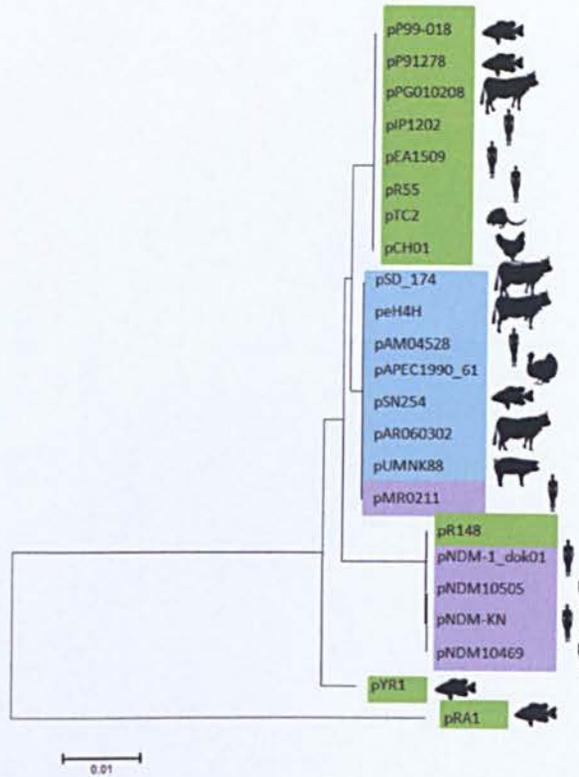
Comparison of plasmids pCH01 with pEA1509, pR55, pTC2, pYR1, pR148, pIP1202, pP99-018, pP91278, pPG010208, pRA1 and pKPHS3 showed a high level of homology (89-99%, 85% for pRA1 and 48% for pKPHS3) among plasmids, as shown in

Figure 6.5 Heat map of pCH01 orf comparisons with GenBank IncA/C plasmids



Heat map of all of the ORFs of pCH01 compared to the IncA/C plasmids. Green indicates the presence of the ORF, orange indicates less than 80% of the ORF present and red is absent. Data generated using stand alone BLASTn.

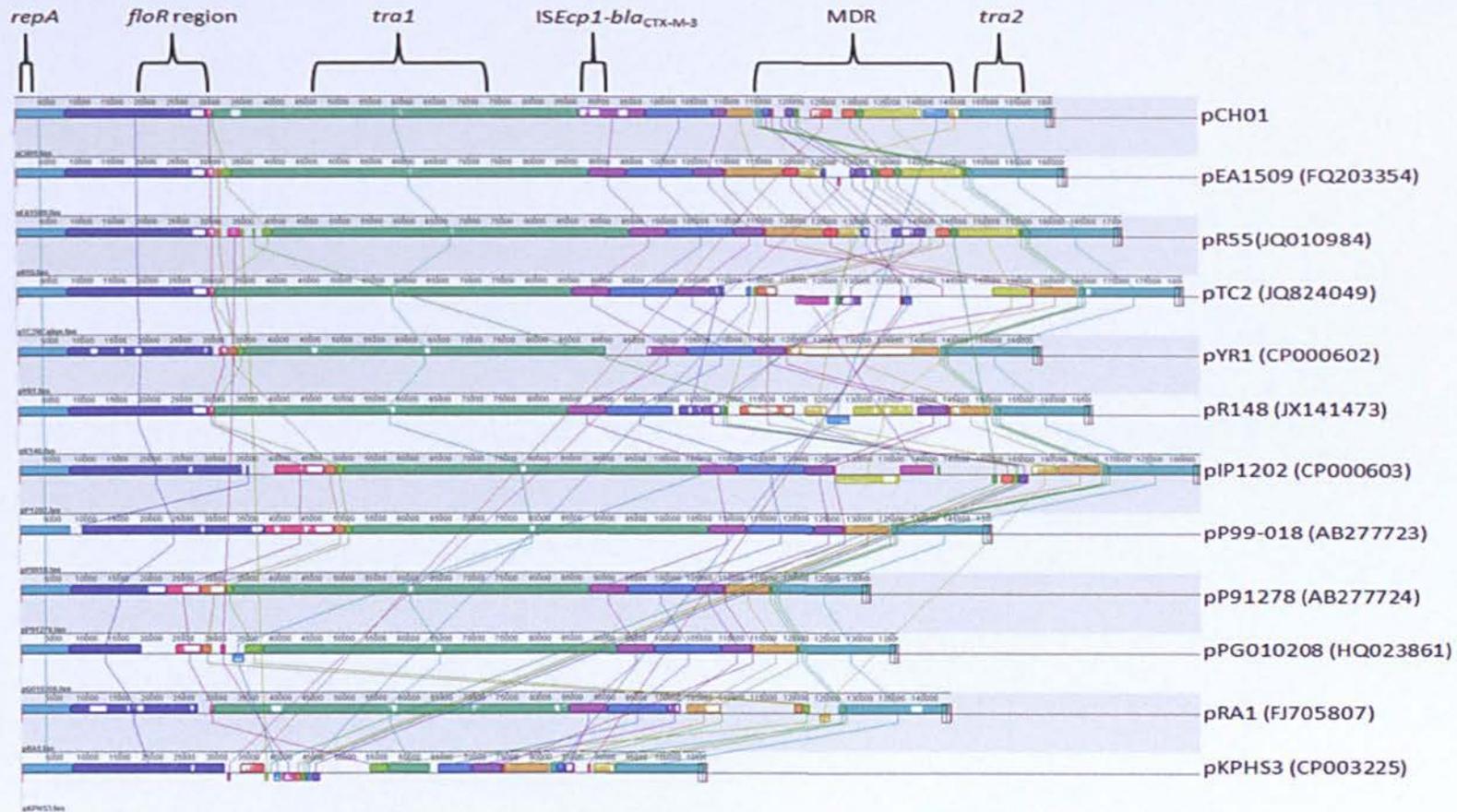
Figure 6.6 Phylogeny of IncA/C plasmids



Dendrogram of the phylogeny of the backbone of IncA/C backbones created using Neighbour joining with 1000 bootstrap replicates. Plasmids in green are NCM, blue CMY and purple NDM, scale is substitution per base.

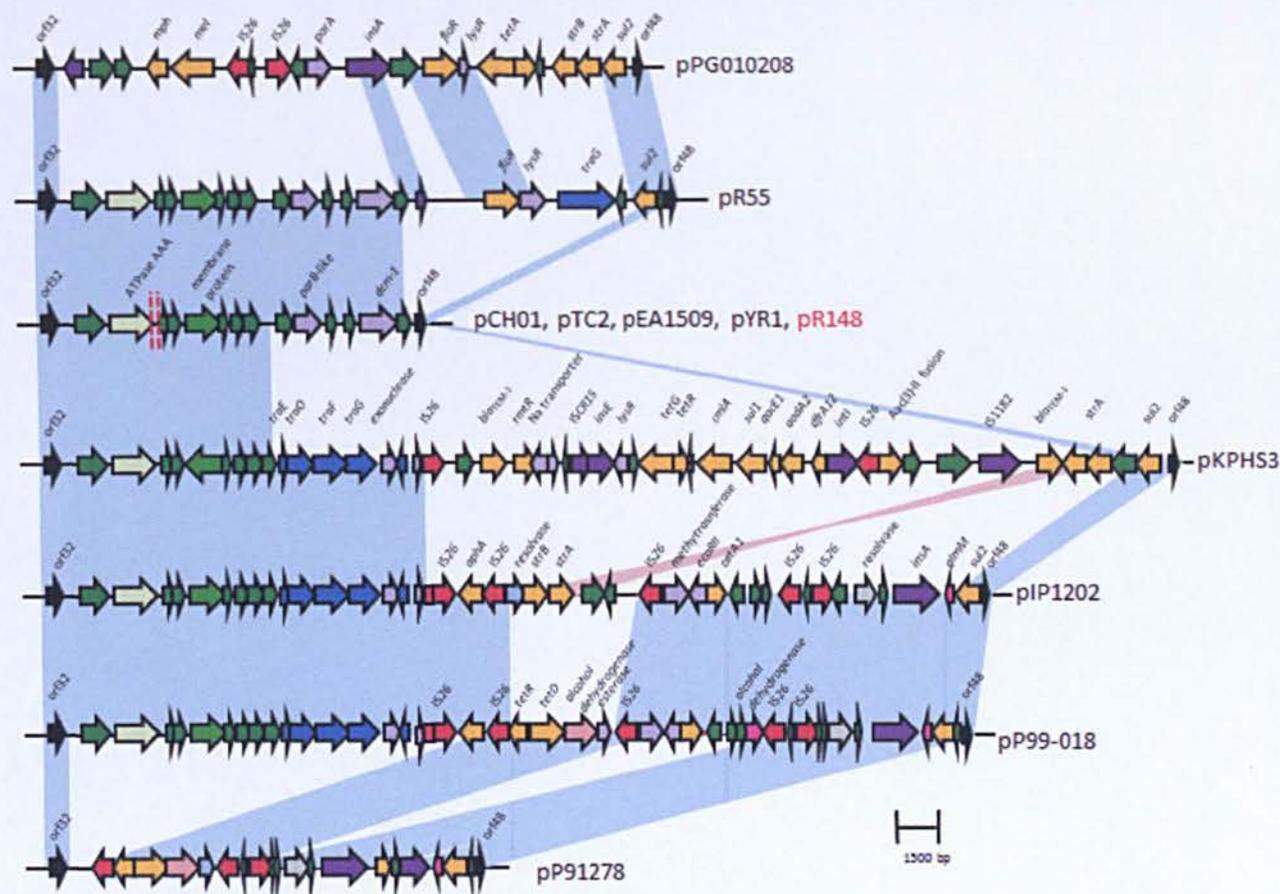
Figure 6.7, with most sharing the same core backbone which had evolved through the insertion and deletion of regions, such as multidrug resistance regions and heavy metal resistance. Three hotspots have previously been highlighted in IncA/C plasmids as sites of integration of resistance regions (Fernandez-Alarcon, Singer and Johnson, 2011). One of these sites lies downstream of multiple hypothetical genes approximately 17 kb downstream of the *repA* gene. This particular hotspot is the site for the insertion of the *floR* region typically found in the CMY plasmids (Fernandez-Alarcon, Singer and Johnson, 2011; Fricke *et al.*, 2009; Call *et al.*, 2010). In pCH01 there is a 12,391 bp region between *orf33* and *orf47*, containing genes for ATPase domain proteins, putative membrane protein, parB like, DNA methyltransferase and 11 conserved hypothetical proteins, as shown in Figure 6.8.

Figure 6.7 MAUVE alignment of non *bla*_{CMY-2} IncA/C plasmids with pCH01



MAUVE 2.3.1 alignments of the DNA from the NCM plasmids, pCH01, pEA1509, pR55, pTC2, pYR1, pR148, pIP1202, pP99-018, pP91278, pG10208, pRA1 and pKPHS3. Blocks of colour indicate homology between regions of conserved DNA, absence of colour indicate sequences unique to that plasmid.

Figure 6.8 Comparisons of the *floR* region of NCM IncA/C plasmids



Comparison of the NCM *floR* region, green arrows are hypothetical genes, orange are resistance genes, purple arrows are mobile elements and red are IS26. Blue arrows are conjugative genes, grey, pale purple, pale green, and pale red genes are other genes. Red dashed lines show a deletion in this region on plasmids pR148. Blue regions show homology between sequences and red areas show homology in the reverse orientation, drawn to scale.

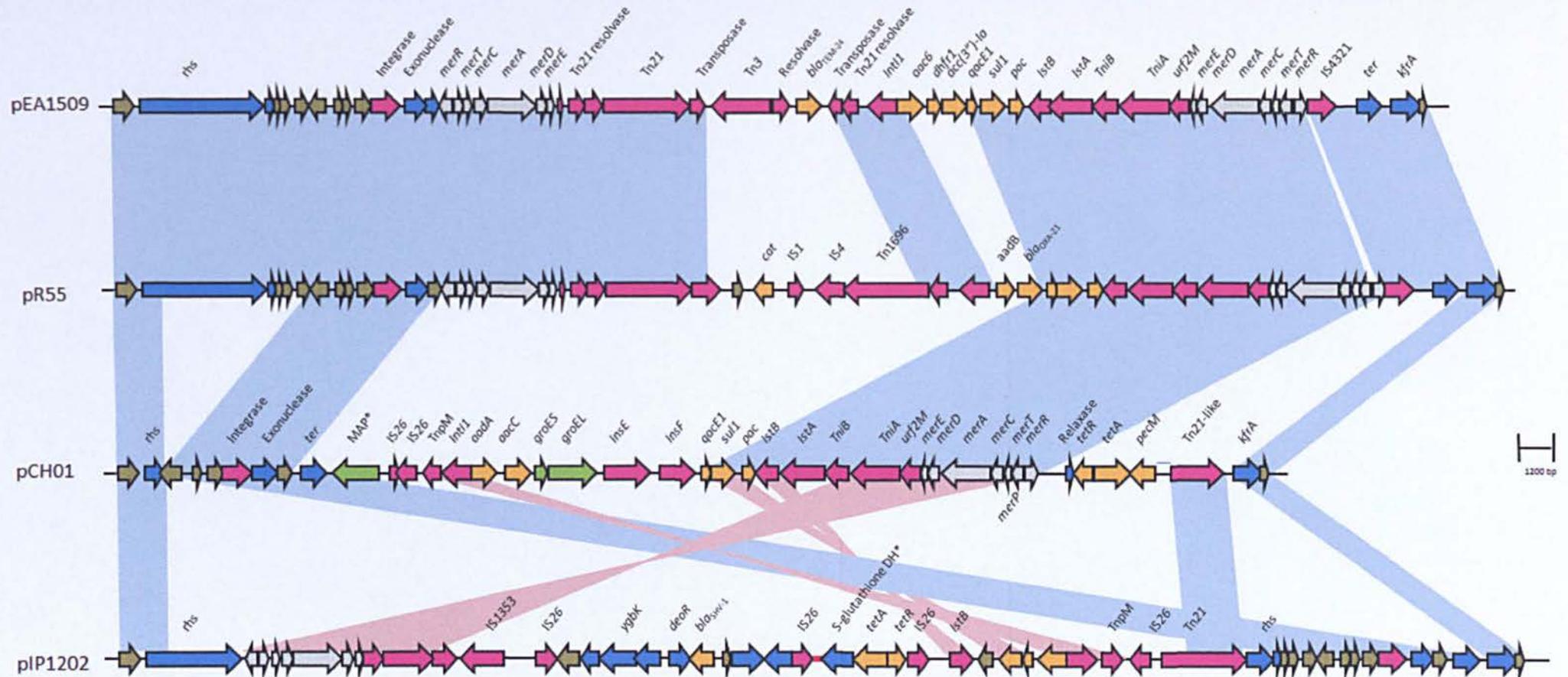
This *floR* region is absent in its entirety in the NCM plasmid pP91278. pPG010208 has a similar *floR* insert present to those in CMY plasmids, with additional *mph* and *mel* genes upstream conferring macrolide resistance (Figure 6.8) (Fernandez-Alarcon, Singer and Johnson, 2011; Kim *et al.*, 2008). pR55 also has an ISCR2 linked *floR* resistance region which has inserted into this region however unlike pPG010208, none of the genes present in pCH01 have been lost, and as the insert differs from that present in pPG010208 and CMY is likely to have been the result of a separate event (Figure 6.8) (Doublet *et al.*, 2012). pP91278 has a insertion containing the resistance genes *tetRD*, flanked by IS26, a putative dihydrofolate reductase, and *sul2*. This insert also contains putative alcohol dehydrogenase, esterase and ArsR regulator, and shares a high level of homology with the inserted region in pP99-018 (Figure 6.8) (Kim *et al.*, 2008). Plasmids pKPHS3, pIP1202 and pP99-018 share the first 7,180 bp of this region with pCH01, with the remaining *orf42-47* being absent. Located downstream of *orf41* (25,039-25,602) in these plasmids lie the conjugation genes *traE*, *O*, *F*, and *G*, an endonuclease, and a fertility inhibition *fipA* gene (Figure 6.8). Downstream of these genes in pKPHS3 are multiple resistance genes including *bla_{TEM-1}*, *rmtR*, *tetGR*, and a class 1 integron with gene cassettes for *cmlA*, *sul1*, *qacE1*, *aada* and *dfrA12*. Located downstream of the integron is an *aacC* fusion gene, *bla_{TEM-1}*, *strAB*, and *sul2*. Included in this MDR were the insertions sequences IS26, ISCR15, *insE*, *intI* and IS1182 (Figure 6.8) (Liu *et al.*, 2012). Plasmids pIP1202 and pP99-018 share more homology than pKPHS3, with an IS26 flanked *aphA* gene downstream of the conjugation genes (Kim *et al.*, 2008; Welch *et al.*, 2007). Both plasmids then have an unrelated insert with a *strAB* linked to a putative resolvase and hypothetical genes in pIP1202, and *tetDR* with a alcohol dehydrogenase and an esterase in pP99-018, in both circumstances these are flanked by IS26, and may represent a recombination event substituting these genes. After these inserts both plasmids share the same sequence carrying the IS26, methyltransferase, *ecoRII*, *catA1*, IS26, IS26, resolvase, *insA*, *glmM* and *sul2* before aligning with *orf48* of pCH01 (Figure 6.8). The loss of the hypothetical and

transfer genes in pP91278 compared to pP99-018, may have occurred through the IS26-*tetDR* inserting into a different location, this was followed by the insertion of methyltransferase, *ecoRII* and *catA1* mobilised by IS26 in pP99-018, absent in pP91278 (Kim *et al.*, 2008). The insertion of multidrug resistances into this region highlights it as a possible 'hotspot' for the insertions, and is the likely cause for deletion of *orf33-47* present pCH01.

The second site observed as a hotspot for integration in the NCM is located downstream of a conserved hypothetical gene, and is termed as the MDR region in this study. The plasmids pEA1509 and pR55 share a similar MDR region, both of which have an initial *mer* operon sharing only 82% homology with that present in pCH01, and is downstream of Tn21, as shown in Figure 6.9 (Diene *et al.*, 2013; Doublet *et al.*, 2012). Separating the class 1 integrons is two different insertions with *bla*_{TEM-24} with a Tn3 present in pEA1509 and a *catA1* with IS1, 4 and Tn1696 in pR55. The cassettes present in the integrons vary as well with pEA1509 having *aac6*, *dhfr1* and *aac(3'')-Ib*, while pR55 has *aadB* and *bla*_{OXA-10} (Figure 6.9) (Diene *et al.*, 2013; Doublet *et al.*, 2012). From *qacE1* the remaining portion of the integron and the MDR region is highly homologous with that of pCH01, including the presence of the *mer* operon, with the exception of the *tetAR*, *pecM* and Tn21 like transposon in pCH01. The insertion of the MDR region has occurred at different locations in plasmids pEA1509 and pR55 compared to pCH01, which may have been a result of the partial deletion of the *rhs*. The MDR region is found located downstream of *ter* in pCH01 and upstream of *ter* in pEA1509 and pR55.

The MDR region in the *Yersinia pestis* plasmid pIP1202, shares some similarities with that of pCH01, however the MDR appears to be in the opposing orientation to that of pCH01 (Welch *et al.*, 2007). Both plasmids share a similar *mer* operon which is located at the 5' end of the MDR in pIP1202, and 3' in pCH01, consequently the integron is in

Figure 6.9 Comparison of the MDR regions of the NCM IncA/C plasmids pEA1509, pR55, pCH01 and pIP1202



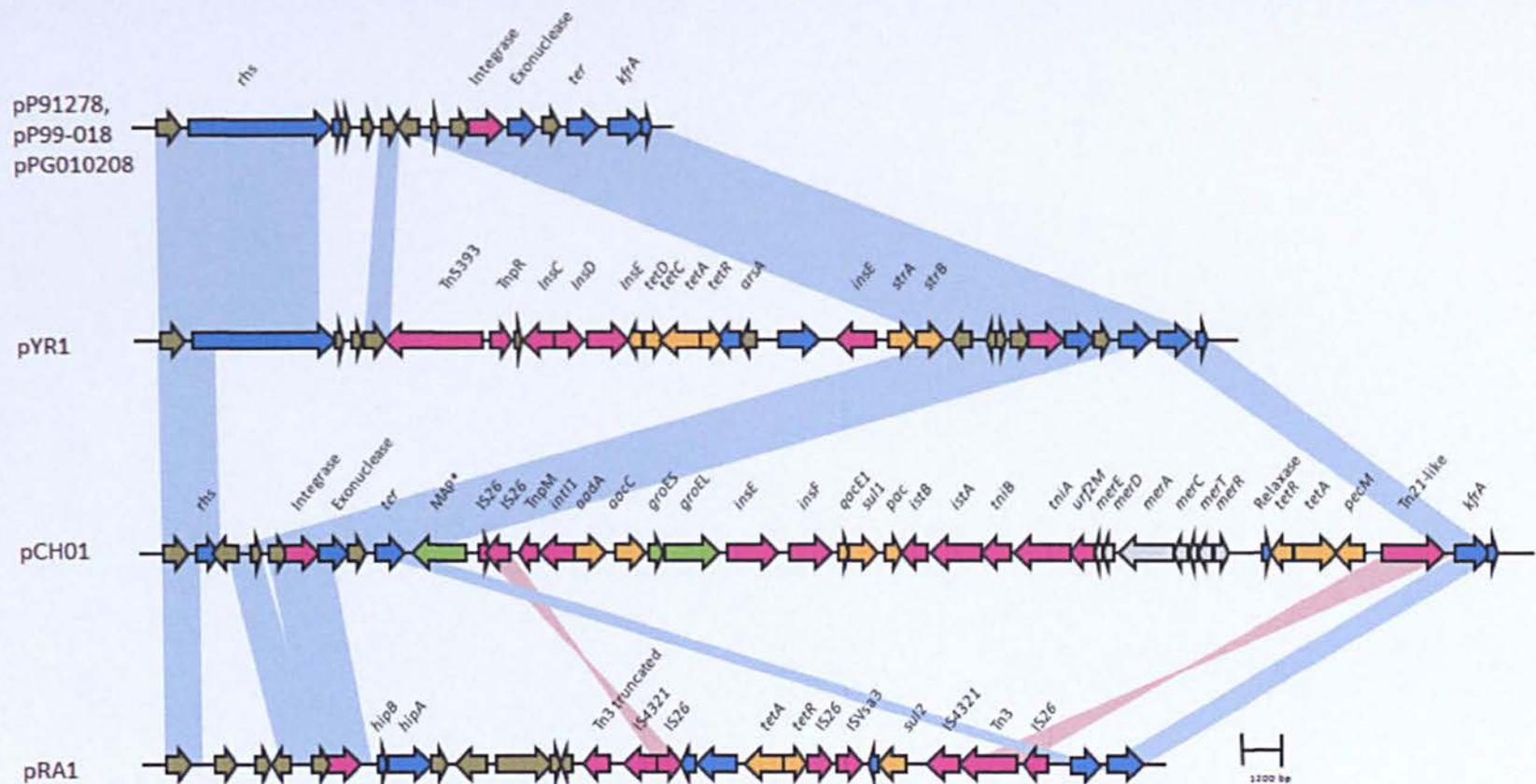
Comparison of the MDR regions of pEA1509, pR55, pCH01 and pIP1202. Magenta arrows are mobile elements, olive arrows are hypothetical genes, orange arrows are resistant genes, blue and green arrows are known genes, grey arrows are the *mer* operon genes. Blue areas show homology between sequences and red areas show homology in the reverse orientation, drawn to scale.

opposing end in the reverse orientation. The cassettes in the integron are similar, with pIP1202 lacking the *aacC* gene and the *groESL* insertion (Figure 6.9). Located between the *mer* operon and integron are several accessory genes, which may have roles in metabolic functions, and resistance genes *tetAR* and *bla_{SHV-1}* (Welch *et al.*, 2007).

Despite the plasmids pP99-108, pP91278 and pPG010208 having resistance genes in the *floR* region, all three plasmids lack any insertions in the MDR region, as shown in Figure 6.10 (Fernandez-Alarcon, Singer and Johnson, 2011; Kim *et al.*, 2008). However all of these plasmids have the *rhs* and the hypothetical genes absent in pCH01. This may indicate that these plasmids originate in hosts that have not been exposed to selective antimicrobial pressures or a diverse gene pool. The plasmid pYR1, which like pP99-018 and pP29178 was also isolated from the a fish pathogen has an insertion in the MDR however it does not consist of a class 1 integron or *mer* operon as in pCH01 and other plasmids, but contains *tetACDR* and *arsA*, flanked by *InsE* and *strAB*. These genes are associated with a Tn5393 transposon, *insD* and *insC*, which have had a role in the insertion of these resistance genes (Figure 6.10) (Welch *et al.*, 2007).

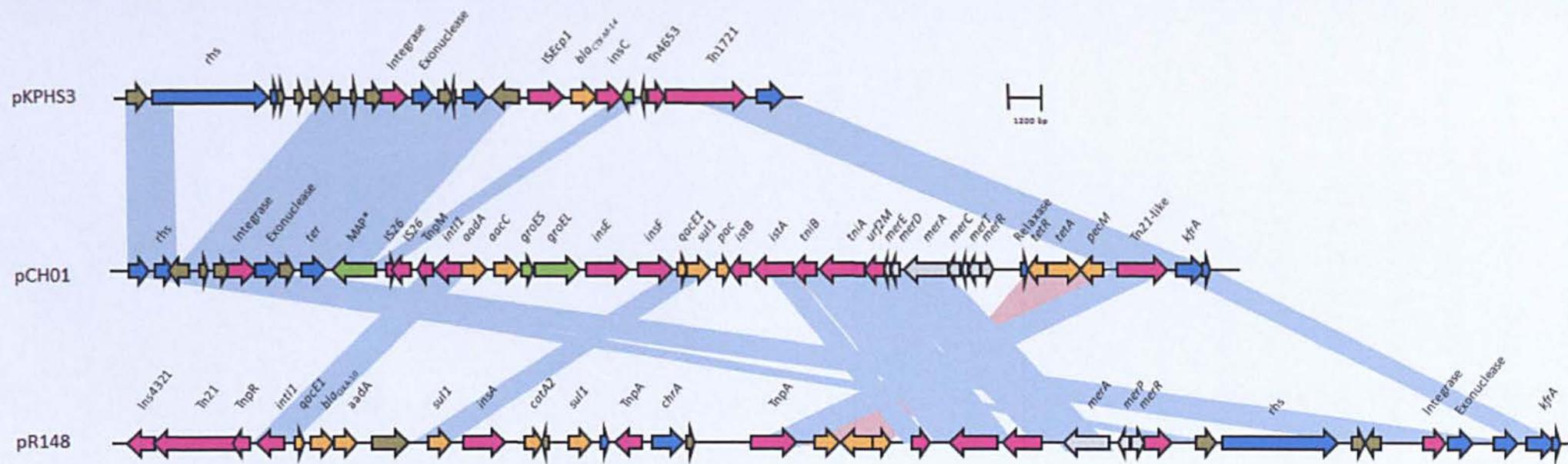
The two resistance genes present in the archetype plasmid pRA1, are both located in the MDR region. pRA1 lacks the *rhs* gene with the insertion appearing to start downstream of a putative integrase (Figure 6.10) (Fricke *et al.*, 2009). The transcriptional regulators *hipBA* are present as are resistance genes, *tetAR* flanked by IS26, and *sul2* flanked by IS*Vsa-3* on the 5' and IS4321 on the 3'. The MDR region appears to share little homology with pCH01, other than the insertion sequences. Plasmid pKPHS3 also lacks any integron in the MDR, and contains only the *bla_{CTX-M-14}* linked to *ISEcp1* which are upstream of Tn4653 and Tn1721, and have inserted between *ter* and *kfr*, as shown in Figure 6.11 (Liu *et al.*, 2012).

Figure 6.10 Comparison of the MDR regions of the NCM IncA/C plasmids pP91278, pP99-018, pPG010208, pYR1, pCH01 and pRA1



Comparison of the MDR regions of pP91278, pP99-018, pPG010208, pYR1, pCH01 and pRA1. Magenta arrows are mobile elements, olive arrows are hypothetical genes, orange arrows are resistant genes, blue and green arrows are known genes, grey arrows are the *mer* operon genes. Blue areas show homology between sequences and red areas show homology in the reverse orientation, drawn to scale.

Figure 6.11 Comparison of the MDR regions of the NCM IncA/C plasmids pKPHS3, pCH01 and pR148



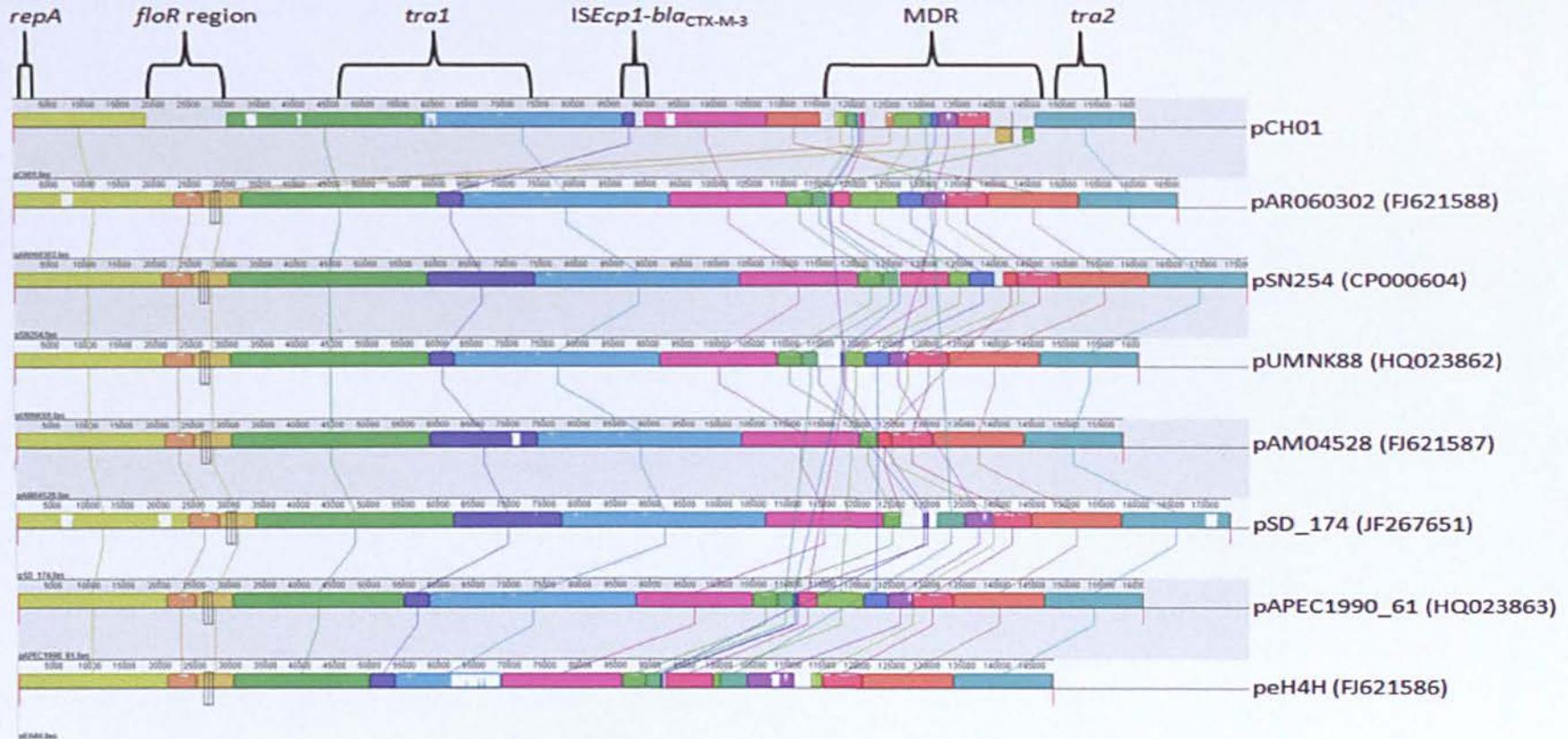
Comparison of the MDR regions of pKPHS3, pCH01 and pR148. Magenta arrows are mobile elements, olive arrows are hypothetical genes, orange arrows are resistant genes, blue and green arrows are known genes, grey arrows are the *mer* operon genes. Blue areas show homology between sequences and red areas show homology in the reverse orientation, drawn to scale.

Comparisons found that the MDR region in NCM plasmids inserts between the exonuclease and the *ter* gene, however in pR148 isolated from *Aeromonas hydrophila*, the MDR insertion has occurred upstream of the *rhs* gene (Figure 6.11) (Del Castillo *et al.*, 2013). The MDR consists of an Tn21 transposon with a class 1 integron, containing the *qacE1*, *bla*_{OXA-10}, *aadA* and *sul1*, downstream is a *insA* linked to *catA2* and *sul1*. pR148 is the only other IncA/C compared in this study that has the *tetAR* and *pecM* resistance genes linked to Tn21 like transposon, which is present in the reverse orientation. In pCH01 this insert has occurred downstream of the *mer* operon, where, as in pR148, it has occurred in the *istB* truncating the gene (Del Castillo *et al.*, 2013). This supports that the insertion of the *pecM*, *tetAR* genes in the MDR region are a separate event.

6.3.4.2 Comparison of pCH01 with the *bla*_{CMY-2} (CMY) plasmids

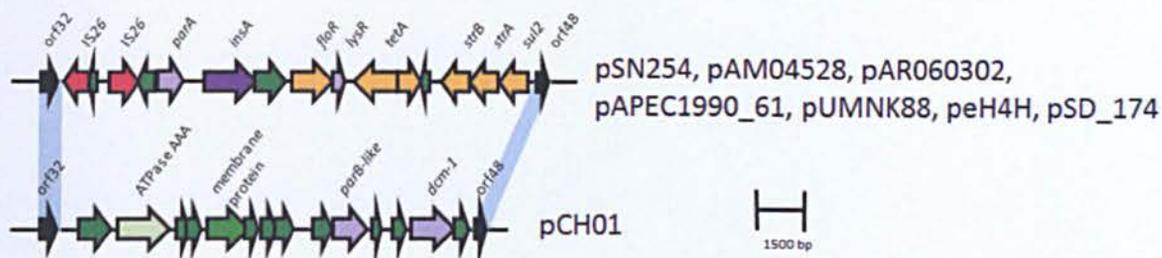
The CMY IncA/C plasmids pAR060302, pSN254, pUNMK88, pAM04528, pSD_174, pAPEC1990_61 and pEH4H compared in this study have all come from either *E. coli* or *Salmonella enterica* recovered in the USA, with only one isolated from a human host, with the remaining plasmids found in cattle (n=3), pig (n=1), fish, (n=1) and turkey (n=1) (Call *et al.*, 2010; Welch *et al.*, 2007; Fernandez-Alarcon, Singer and Johnson, 2011; Han *et al.*, 2012). The pCH01 backbone was shared with the CMY plasmids (65-89% coverage), with variations at three main regions, which have been noted for the insertions of *floR*, MDR region and the *bla*_{CMY-2}, as shown in Figure 6.12 (Call *et al.*, 2010; Welch *et al.*, 2007; Fernandez-Alarcon, Singer and Johnson, 2011; Han *et al.*, 2012). All of the CMY-2 plasmids have an insertion downstream of a hypothetical gene present at 18,431-18,928 bp (*orf32*) in pCH01 which contains IS26-hypothetical-IS26-hypothetical-resolvase-*InsA*(ISCR2)-hypothetical-*floR*-*lysR*-*tetA*-*tetR*-hypothetical-*strB*-*strA*-*sul2*, shown in Figure 6.13 (Fernandez-Alarcon, Singer and Johnson, 2011; Call *et al.*, 2010; Toleman, Bennett and Walsh, 2006; Toleman and Walsh, 2010). This insertion has caused the loss of a 13,603 bp region (*orf33-47*) which is present in pCH01 and other plasmids.

Figure 6.12 MAUVE comparison of the CMY IncA/C plasmids with pCH01



MAUVE 2.3.1 alignments of the DNA of CMY plasmids, pCH01, pAR060302, pSN254, pUMNK88, pAM04528, pSD_174, pAPEC1990_61 and peH4H. Blocks of colour indicate homology between regions of conserved DNA, absence of colour indicate sequences unique to that plasmid.

Figure 6.13 Comparisons of the *floR* region of the CMY and NCM IncA/C plasmids

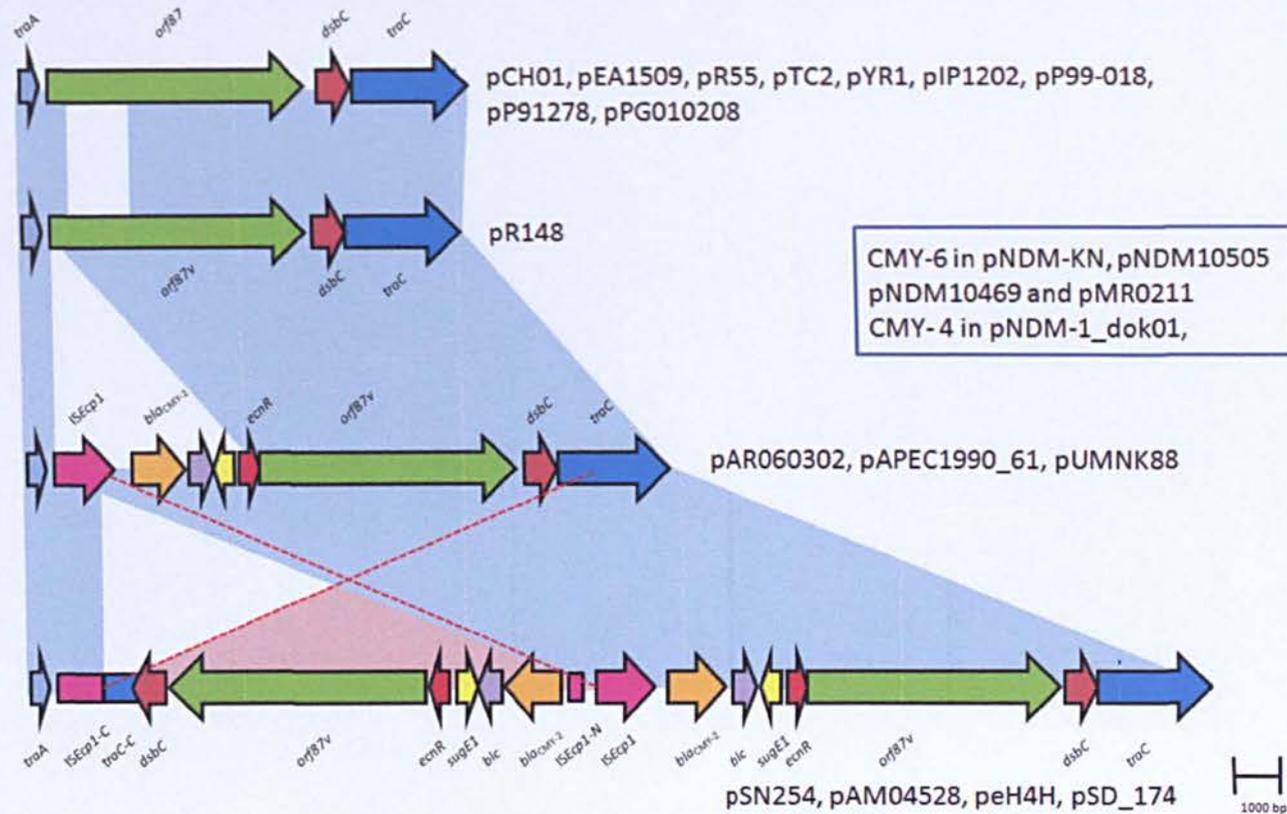


Comparison of the *floR* region of the CMY plasmids and pCH01 backbone. Green arrows are hypothetical genes, orange are resistance genes, purple arrows are mobile elements and red are IS26. Pale purple are other genes Blue areas show homology between sequences, drawn to scale.

The *floR* insertion is also present in the same location in NCM pPG010208 and NDM pMR0211 plasmids, which may suggest that the *floR* region was present in the IncA/C backbone before *bla*_{CMY-2} was acquired (Fernandez-Alarcon, Singer and Johnson, 2011; McGann *et al.*, 2012).

The *bla*_{CMY-2} region inserted downstream of *traA* and upstream of large conserved hypothetical in all of these plasmids except for pAPEC1990_61 in which *bla*_{CMY-2} inserted downstream of *traL* due to a loss of several conjugation genes, as shown in Figure 6.14 (Call *et al.*, 2010; Welch *et al.*, 2007; Fernandez-Alarcon, Singer and Johnson, 2011; Han *et al.*, 2012). In pSN254, pAM04528, pSD_174 and peH4H the insert is duplicated including the large conserved hypothetical and *dsbC* which are in the reverse orientation upstream of the insert in single copies, with an additional *ISEcp1* present downstream of *traA* which is in the forward sense direction, and may have been the result of the *ISEcp1* inserting in the reverse orientation. Separating the region found in the single copy and double copy is a 309 bp sequence which relates to the N terminus of *ISEcp1* in the reverse sense. Call *et al* (2010) suggested that the event had occurred through transposition rather than homologous exchange due to the repeats of ATTCCCTA at the 3' and ATTCCTTA at the 5' (Call *et al.*, 2010).

Figure 6.14 Comparison of the *bla*_{CMY-2} regions of NCM, CMY and NDM IncA/C plasmids



Comparison of the *bla*_{CMY-2} regions NCM, CMY and NDM plasmids. Blue arrows are *tra* genes, magenta arrows are *ISEcp1*, orange are the *bla*_{CMY-2} genes (*bla*_{CMY-6} in NDM plasmids), green arrows are hypothetical genes, brown arrows *dsbC* gene, yellow *sugE1* and pale purple *bic*. Blue areas show homology between sequences and red areas show homology in the reverse orientation, drawn to scale.

A variation was observed in a large conserved hypothetical genes (*orf87* in pCH01) present in NCM, CMY and NDM plasmids (Figure 6.14). This gene ranges from 5,532 bp in NCM to 5,487 bp in CMY and NDM plasmids (termed *orf87v*), both genes share the same first 430 bp, however after this each gene has a unique sequence of 1,403 bp and 1,358 bp respectively. BLAST searches found that the genes in NCM and CMY plasmids were specific to their respective plasmids, although the *orf87v* was identified in the NCM pR148. This difference in the sequence of the conserved genes results in 78% amino acid identity between the hypothetical proteins. The presence of *orf87v* may be a precursor to acquiring the *ISEcp1-bla_{CMY-2}-blc-sugE1* as all of the *bla_{CMY-2}* plasmids contain this form of the conserved gene.

The MDR inserts of pSN254, peH4H, pAPEC1990_61 and pAR060302 are all highly similar to that of pCH01, especially when compared to the NCM plasmids, as shown in Figure 6.15 (Welch *et al.*, 2007; Call *et al.*, 2010; Fernandez-Alarcon, Singer and Johnson, 2011). Plasmids all have the same class 1 integron and *mer* operon however all of these have the insertion of *insG* downstream of *istA* which is absent in pCH01. With the exception of peH4H, these plasmids have a truncated TniA due to the insertion of IS26, *urf2* and a *mer* operon sharing only 82% coverage with pCH01, followed by IS4321 downstream. peH4H differs from the other plasmids as it has an insert downstream of IS26 and *kanR*, *merE*, *merD*, and *merA* which is truncated by *bla_{TEM-1}* (Call *et al.*, 2010). The heat shock *groES* and *groEL* genes are present adjacent to ISCR16, in the integrons of the pSN254, peH4H, pAPEC1990_61 and pAR060302 plasmids (Welch *et al.*, 2007; Call *et al.*, 2010; Fernandez-Alarcon, Singer and Johnson, 2011). This integron also has a 288 bp conserved hypothetical gene, which has been truncated to 85 bp in pCH01 due to the insertion of IS1326 (*istB*, *istA*), which may suggest that its insertion may be a separate event. The MDR insertion in these plasmids has occurred downstream of a hypothetical gene *orf139*, and includes the IS4321 and transposon Tn21 at the 5' end of the MDR region. Plasmid peH4H has a deletion of approximately 22 kb from pCH01 including *traD*

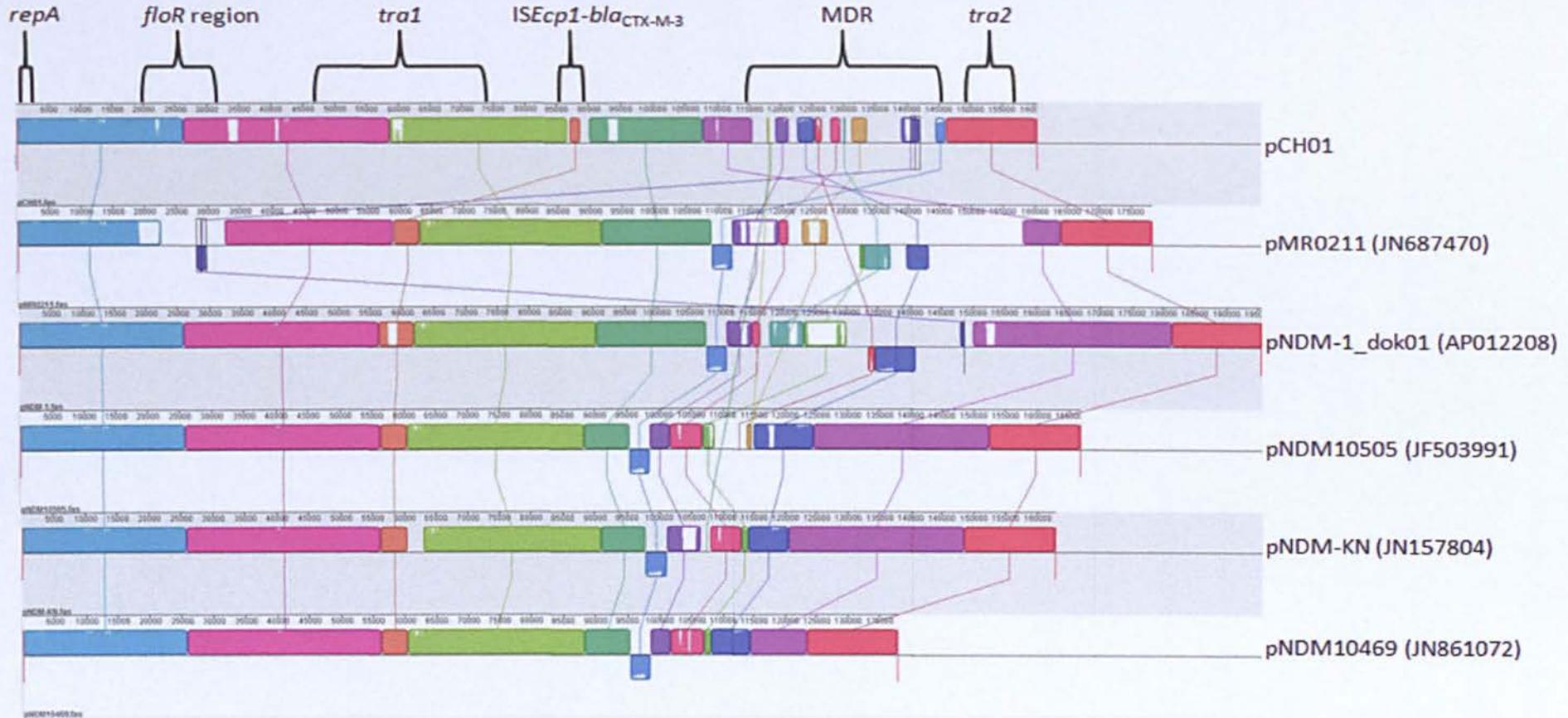
to *traK* and *traC* to *orf110* (Call *et al.*, 2010). The MDR in pUMNK88 isolated from *E. coli* from pigs is similar to pSN254 and peH4H, but lacks the *groESL*, *insE* and *insD* and has different gene cassettes with *aadA* and *aacC* replaced by *cmlA* (Fernandez-Alarcon, Singer and Johnson, 2011).

The pSD_174 plasmid has a similar integron to that of the other plasmids, also having IS4321 and Tn21 upstream but lacks *aacC*, *groES*, *groEL*, *InsE* and *InsF* in the integron (Figure 6.14). pSD_174 is the only *bla*_{CMY-2} plasmid to share the same *mer* operon as pCH01, suggesting that this MDR has evolved separately to that of pSN254 and peH4H. The only *bla*_{CMY-2} plasmid isolated from a human isolate was pAM04528, apart from the *floR* region and the 2 copies of *bla*_{CMY-2} no other resistance genes were present (Liu *et al.*, 2012). Inserted downstream of *orf139* was the IS4321 and a truncated Tn21 which had lost the C terminus due to the insertion of IS26 upstream of the same *mer* operon as other *bla*_{CMY-2} plasmids which shares 82% identity with pCH01, suggesting that this *mer* operon has come from an unrelated transposon, likely to be related to Tn1696 (Call *et al.*, 2010).

6.3.4.3 Comparison of pCH01 with *bla*_{NDM-1} (NDM) plasmids

The NDM plasmids, pNDM-1_dok01, pMR0211, pNDM10505, pNDM-KN and pNDM10469 have all been identified in clinical isolates from humans, and have been identified in *E. coli*, *Klebsiella pneumoniae* and *Providencia stuartii* (Sekizuka *et al.*, 2011; McGann *et al.*, 2012; Carattoli *et al.*, 2012). The NDM plasmids like the other IncA/C plasmids share a high level of similarity between pCH01 and the NDM plasmids, with pNDM-1_dok01 having a coverage of 98% and the remaining plasmids 87%, as shown in Figure 6.16. All of the NDM plasmids have *bla*_{CMY-6}, with *CMY-4* in pNDM-1_dok01 inserted downstream of *traA*, and have the variant of *orf87*, as with the CMY plasmids. With the exception of pMR0211 all of the NDM plasmids have the same *floR*

Figure 6.16 MAUVE comparison of the NDM plasmids with pCH01



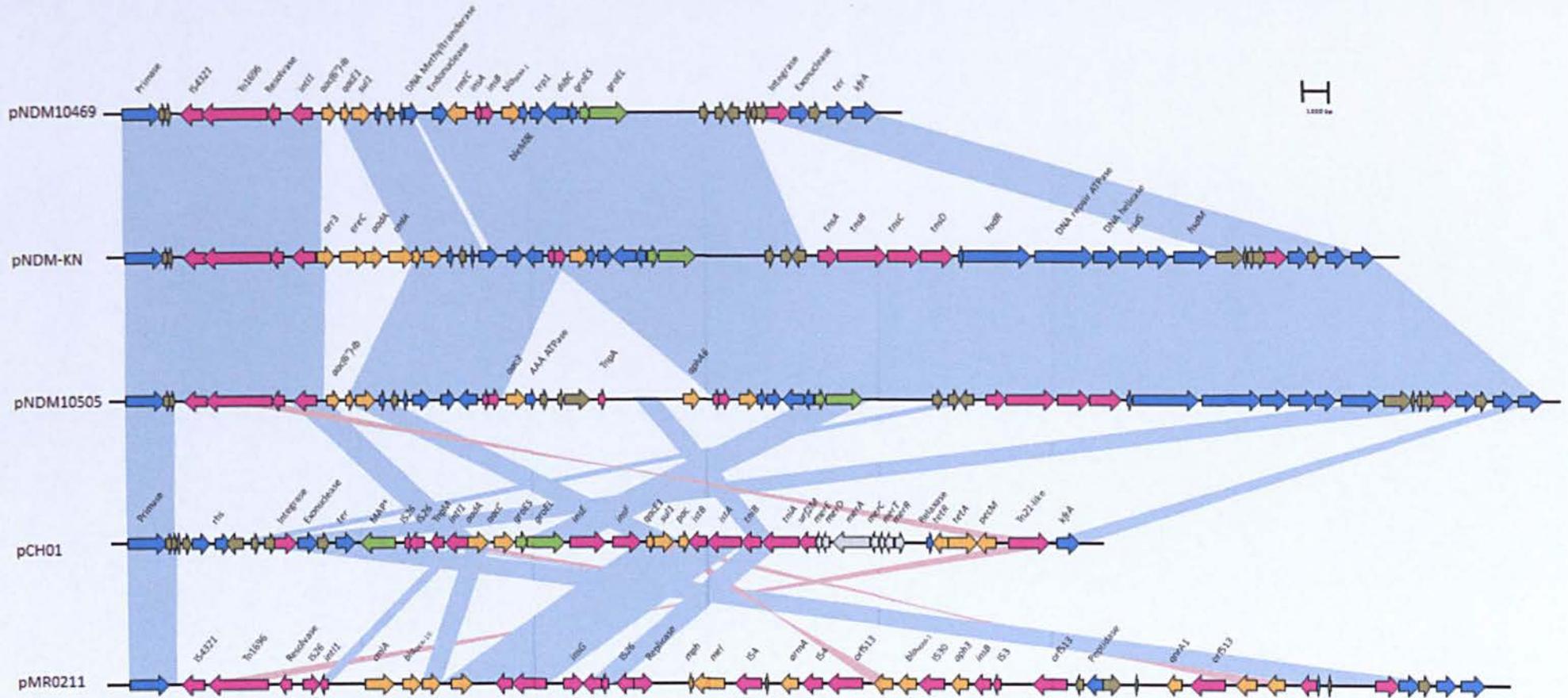
MAUVE 2.3.1 alignments of the pCH01 with the NDM plasmids, pMR0211, pNMD-1_dok01, pNDM10505, pNDM-KN and pNDM10469. Blocks of colour indicate homology between regions of conserved DNA, absence of colour indicate sequences unique to that plasmid.

region as pCH01, pMR0211 is the only plasmids which has *floR* resistance region along with *strAB*, *tetRA* and *sul2* (McGann *et al.*, 2012).

The NDM plasmids pNDM10505 and pNDM-KN have very similar MDR regions, which have inserted downstream of *orf139*, as shown in Figure 6.17 (Carattoli *et al.*, 2012). The MDR begins with IS4321 and Tn1696 followed by a class 1 integron, pNDM10505 has the cassettes *aac(6'')-Ib*, *qacE1* and *sul1*, and pNDM-KN has the cassettes *arr3*, *ereC*, *aadA*, *cmlA*, *qacE1* and *sul1*. Inserted downstream of the *insB* in pNDM10505, lies *aac3*, AAA ATPase and hypothetical genes with a truncated *tniA*, and *aphA6* (Carattoli *et al.*, 2012). The *bla*_{NDM-1} gene is located downstream of *insA* and *insB*, which has been duplicated in these plasmids, and is associated with *bleMBL*, *trp1*, *dsbC* and *cutA1*, commonly found with *bla*_{NDM-1}, which is followed by *groESL*. pNDM-KN and pNDM10505 also have the large transposon *tnsA*, *B*, *C* and *D* which is upstream of genes for DNA repair ATPase, DNA helicase, *hsdS* and *hsdM*. pNDM10469 has sequence coverage of 87% with pNDM10505, and the MDR region is similar, however the *aac3*, AAA ATPase, *aphA6*, and the large transposon linked to the DNA repair genes is absent (Carattoli *et al.*, 2012). There is limited similarity between the MDR insertions of these plasmids and pCH01, with the only homology observed between insertion sequences. These plasmids also have the insertion of *groES* and *groEL* however they share only 86% and 90% identity with those in pCH01 and pSN254 respectively, and the insertion sequences *InsE* and *InsF* are absent. These NDM plasmids lack *orfs108-129* present in all other plasmids compared in this study and the *orfs140-143* have also been lost due to the insertion of the MDR regions into the IncA/C backbone.

The plasmid pMR0211 isolated in Afghanistan from a human infected with *Providencia stuartii*, has a 54 kb MDR insert downstream of hypothetical gene (*orf139*) and has limited similarity to that of the other NDM plasmids (Figure 6.17) (McGann *et al.*, 2012). The MDR insert contains IS4321, Tn1696 and a class 1 integron, which contains the cassettes *cmlA*, *bla*_{OXA-10}, *aadA*, *qacE1* and *sul1*. pRM0211 shares the

Figure 6.17 Comparison of the MDR regions of pCH01 and the NDM plasmids pNDM10469, pNDM-KN, pNDM10505, and pMR0211



Comparison of the MDR regions of pCH01, pNDM10469, pNDM-KN, pNDM10505, and pMR0211. Magenta arrows are mobile elements, olive arrows are hypothetical genes, orange arrows are resistant genes, blue and green arrows are known genes, grey arrows belong to the *mer* operon. Blue areas show homology between sequences and red areas are homology in the reverse orientation, drawn to scale.

insertion sequences *istB*, *istA* and *tniB* with pCH01, but has additional insertion sequences *insG*, IS26 and IS4 accompanied by the resistance genes *mph*, *mel* and *armA*. The *bla*_{NDM-1} gene is in the reverse orientation to other NDM plasmids, and is downstream of IS30 which is upstream of *aph3*. pMR0211 has the remnants of another class 1 integron which has the gene cassettes *aadA* and *sul1*, but the *intI1* is truncated, with *qnrA1* (McGann *et al.*, 2012).

pNDM-1_dok01 isolated in Japan from a human *E. coli* infection, shares the most coverage of any NDM plasmid with 98% (Figure 6.16) (Sekizuka *et al.*, 2011). Like the other NDM and CMY plasmids, pNDM-1_dok01 has *ISEcp1-bla*_{CMY-4}-*blc-sugE*, and the *orf87v*. The 73 kb MDR insertion which has inserted downstream of *orf138* and is similar to those found in other NDM plasmids, but shares little similarity with pCH01 (Figure 6.17), with variations to the gene cassettes carried in the class 1 integron, being *dfrA12*, *aadA2*, *qacE1* and *sul1*. The MDR contains IS5, IS1 and IS903, which have the resistance genes *armA*, *mel* and *mph2*, following this is *InsE*, *groEL* and *groES* located downstream of the integron. The *bla*_{NDM-1} gene lies upstream of IS903, but no obvious transposon is present upstream, *bla*_{TEM-1} follows and is associated with a Tn3 transposon, the remaining insert is made up of hypothetical genes and Tn7 (Sekizuka *et al.*, 2011).

6.3.5 Development of the IncA/C molecular markers

Two PCR methods for the identification of IncA/C plasmids are currently in use, with the most common being PBRT using primers for the *repA* gene, and the other using primers for 12 regions have been developed (Carattoli *et al.*, 2005a; Welch *et al.*, 2007). Comparison of pCH01 with the other IncA/C plasmids in this study was used to design molecular markers capable of identifying pCH01 in combination and differentiating the IncA/C plasmids. In total 9 molecular markers were selected to identify the IncA/C plasmids. The molecular markers selected were genes encoding the methyl-accepting chemotaxis protein (mAERO), EAL domain containing protein (mSenS), mTraB,

mGroEL, the IS4 related transposon (mTnIA) and the hypothetical genes mHQ (PAPS reductase), mACH1, mACH2 and mACH3. These markers were selected based on their presence in the pCH01 plasmid allowing for their design and *in vitro* testing.

6.3.5.1 *In silico* testing of the pCH01 IncA/C molecular markers

The pCH01 primers and sequence of molecular markers were tested *in silico* against pCH01 and the 23 GenBank plasmids (Table 6.2) compared in this study, results shown in Table 6.3. The mACH1 marker was found to be the most prevalent (22/24), present in all plasmids except pYR1 and pRA1. Markers mTraB and mSenS were both present in 21/24 plasmids, with mTraB absent in pAPEC1990_61, pKPHS3 and pRA1, and mSenS absent in peH4H, pRA1 and pKPHS3. The mHQ marker was present in 19/24 plasmids, being absent in pNDM10505, pNDM-KN, pNDM10469, pKPHS3 and pRA1. The mACH2 marker was found to be in approximately half of the plasmids (11/24), with the mGroEL marker identified in 9/24 plasmids. The mACH3 marker was present in 8/24 plasmids, while the mAERO marker was present in three plasmids, pKPHS3, pTC2 and pCH01, the mTnIA marker was found to be specific to pCH01. The markers were found to differentiate the 24 IncA/C plasmids into 14 groups, with *in silico* testing results shown in Table 6.3.

The markers were assigned a unique number, with lower numbers assigned to those markers which are most prevalent so that, mACH1 = 1, mTraB = 2, mSenS = 4, mHQ = 8, mACH2 = 16, mGroL = 32, mACH3 = 64, mAERO = 128 and mTnAI = 256. Using a binary based system for the input of markers a unique ID (UID) number is generated for each combination of markers (Table 6.3). This system could be applied to a future database for IncA/C plasmids thereby allowing users to input their plasmid typing results and compare them with other researchers around the world.

Table 6.3 *In silico* marker results for pCH01 and GenBank plasmids

Plasmid	m TnAI	m AERO	m GroEL	m ACH1	m ACH2	m ACH3	m TraB	m HQ	m SenS	UID
pCH01	1	1	1	1	1	1	1	1	1	511
pEA1509	0	0	0	1	1	1	1	1	1	95
pR55	0	0	0	1	1	1	1	1	1	95
pTC2	0	1	0	1	1	1	1	1	1	223
pYR1	0	0	0	0	1	1	1	1	1	94
pR148	0	0	0	1	1	0	1	1	1	31
pNDM-1_dok01	0	0	1	1	1	0	1	1	1	63
pIP1202	0	0	0	1	0	1	1	1	1	79
pP99-018	0	0	0	1	0	1	1	1	1	79
pP91278	0	0	0	1	0	1	1	1	1	79
pPG010208	0	0	0	1	0	0	1	1	1	15
pAR060302	0	0	1	1	0	0	1	1	1	47
pSN254	0	0	1	1	0	0	1	1	1	47
pUMNK88	0	0	0	1	0	0	1	1	1	15
pAM04528	0	0	0	1	0	0	1	1	1	15
pSD_174	0	0	0	1	0	0	1	1	1	15
pMR0211	0	0	0	1	0	0	1	1	1	15
pNDM10505	0	0	1	1	1	0	1	0	1	55
pNDM-KN	0	0	1	1	1	0	1	0	1	55
pNDM10469	0	0	1	1	1	0	1	0	1	55
pAPEC1990_61	0	0	1	1	0	0	0	1	1	45
pRA1	0	0	0	0	1	0	0	0	0	16
peH4H	0	0	1	1	0	0	1	1	0	43
pKPHS3	0	1	0	1	0	0	0	0	0	129

Results of the *in silico* testing of the marker against the GenBank plasmids, presence of the marker is denoted by a 1 with a green background, and absence by 0 with a red background, the UID is shown in the right column. Plasmids in green are NCM, blue CMY and purple NDM.

6.3.5.2 Evaluation of the pCH01 IncA/C markers

The mACH1 was the most common marker, which was present in 22/24, the absence of the mACH1 marker in pYR1 is due to the insertion of two hypothetical genes downstream of *orf27* (16,454-16,648) causing the loss of two hypothetical 16,659-17,030 and 17,023-17,493 genes present in the other plasmids in this study. In pRA1 no insertions could be identified and it is likely that *orf29* has been lost as a result of a deletion, as 89 bp of the N terminus of the *orf28* are present and there is a deletion of the 3' end of *orf30*.

The mTraB marker was absent in three plasmids, the first of which was pRA1, which has a *traB* that shares only 81% homology at the sequence level and 90% at the amino acid level, consequently the primers are predicted not to yield a product. In pAPEC1990_61 the insertion of *ISEcp1-bla_{CMY-2}*, occurred downstream of *traE*, due to a deletion of *traK*, *traB*, *traV* and *traA* genes, which may have been present before the insert or caused by the insert. The large deletion of over 60 kb in pKPHS3 resulted in the loss of numerous conjugation genes and conserved hypothetical genes, including *traB*.

As observed with the marker mTraB, mSenS was also absent in three plasmids. The same deletion in pKPHS3 resulting in the loss of mTraB, also resulted in the loss of mSenS, located between *orf60* and *orf131* in pKPHS3 is an integrase and IS3 which may have caused this substantial loss in the plasmid. In pRA1, the EAL gene differs from that of pCH01 and other IncA/C plasmids, sharing 75% sequence homology and 73% amino acid identity, and so the primers are predicted not to produce an amplicon. The loss of the EAL gene in peH4H has been caused as was the loss of mTraB in pAPEC1990_61, by the insertion of the *ISEcp1-bla_{CMY-2}-blc-sugE* and two hypothetical genes, causing the loss of several hypothetical genes from 67,610-86,448.

The *in silico* testing for the mHQ marker (based on PAPS reductase), demonstrated its absence in the NDM plasmids pNDM10505, pNDM10469 and pNDM-KN which lack the region present in pCH01 from 87-101 kb containing 12 hypothetical genes, putative 2Fe-2S binding domain protein and DNA cytosine-5 methyl transferase, which includes the mHQ marker. The mHQ marker is missing from pKPHS3, due to the same deletion causing the absence of mSenS and mTraB. Both pCH01 and pRA1 have the 2,454 bp putative phosphoadenosine phosphosulfate reductase, used as the mHQ marker target however as with mTraB and mSenS, the gene varies at the nucleotide level with 93% homology and 96% amino acid identity, consequently the marker is not predicted to be amplified.

The CMY plasmids contain the *floR* insert in the *floR* region which has resulted in the loss of *orf33-47* present in pCH01 and the NCM plasmids, which contains the target for the mACH2 marker, the same *floR* insert is also seen in the NCM pPG010208 and NDM pMR0211. The insertion of MDR regions in the NCM plasmids pIP1202, pP99-018, pP91278, and pKPHS3, downstream of *orf41* has also resulted in the loss of the mACH2 marker.

pCH01 contains the *ISCR16* which includes the *groEL* and *groES* genes in a class 1 integron, used for the mGroEL marker. These chaperonin genes are also present in CMY plasmids pAPEC1990_61, pAR06030, peH4H and pSN254, the *ISCR16* is absent in the other NCM and CMY markers. Although pUMNK88 is a CMY plasmid the transposable region contains an additional insertion of a integrase associated *cmlA1* and *aadA2*, which has inserted 809 bp into the *aadA* gene truncating the N terminus, possibly resulting in the loss of *groES* and *groEL*. pSD_174, and pAM04528 lack the mGroEL marker, due to the absence of the gene cassettes. The NDM plasmids pNDM-KN, pNDM-1_dok01, pNDM10505 and pNDM10469 all have the *groEL* gene, however it shares only 89.88% and 92% homologies at the nucleotide and amino acid level respectively, the mGroEL marker was designed to identify these genes which could be further differentiated by sequence analysis.

The mACH3 marker shares the discriminative characteristics of both the mHQ and mACH2 markers, in that only NCM plasmids had this marker, being present in only 8/24. The area downstream of *orf63* (40,014-40,670), appears to have undergone a deletion, with 70bp of *orf63* remaining in the CMY and NDM plasmids. This is also the case with the NCM pR148, and pPG010208 with 70 bp and 99 bp of *orf63* present respectively. However in pRA1 the marker is absent due to a larger deletion, which is the same case for pKPHS3.

The mAERO marker was found to be present in pCH01 and two other plasmids pKPHS3 and pTC2 where it lies upstream of the MDR region containing 12 antimicrobial resistance genes and upstream of *ISEcpI*-*bla*_{CTX-M-14} respectively. In both plasmids the methyl group accepting protein are upstream of IS26 which is likely to mobilise this protein, however the regions surrounding the methyl accepting chemotaxis protein are highly diverse. As this marker appears to be linked to a transposon, the absence of this insertion is the likely cause for the absence of this marker in other plasmids.

Despite the mTnIA marker being linked to a transposon found in pCH01, it has not been found on any plasmid, and has only been found within *Shewanella baltica* and other aquatic bacterial genomes. IS4MS shares 78% and 88% nucleotide and amino acid identity respectively, with those recovered from *Shewanella baltica*, and the markers are not predicted to amplify any of these insertion sequences.

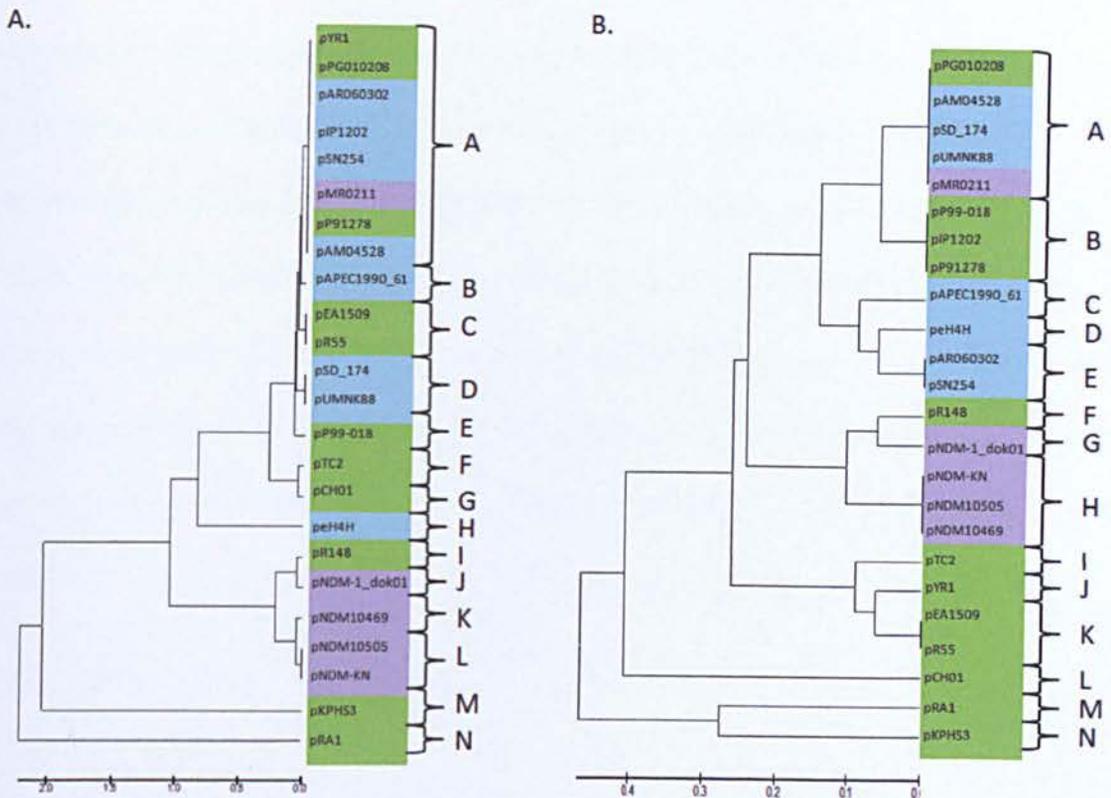
6.3.5.3 Amplicon sequence analysis

The sequence analysis of the pCH01 molecular markers could further be used to differentiate the plasmids with different alleles for the markers mACH1, mACH2, mGroEL, mHQ, mSenS and mTraB. The markers mACH1, mGroEL, mHQ and mTraB all had 2 alleles while mSenS had three and mACH2 had four. In this study the only plasmids that could be further separated by phylogenetic analysis of the markers was pPG010208 from pUMNK88, pAM04528, pSD_174 and pMR0211, with different alleles for mSenS and mTraB.

6.3.5.4 Comparison with the markers by Welch *et al* (2007)

A study by Welch *et al* (2007) in which three IncA/C plasmids pYR1, pIP1202 and pSN254 were sequenced, designed 12 PCRs (R1-12) for these plasmids, all of which were run in simplex (Welch *et al.*, 2007). The *in silico* testing of these markers against the 24 plasmids compared in this study, identified 14 marker combinations (groups), of which 14 plasmids were spread among four groups, shown in Figure 6.18. One group contained 8

Figure 6.18 Comparison of the Welch (R1-12) and pCH01 IncA/C markers



Dendrogram showing the groups for the (A.) Welch markers (R1-12) and (B.) pCH01 markers, letters indicate the groups, phylogeny generated using UPMGA using MEGA 5.10.

plasmids while the other three had two plasmids each. The group containing the 8 plasmids could be further subdivided into 4 groups using the pCH01 markers, however these groups included three plasmids which were separated into a further two groups using the Welch markers (Figure 6.18). All of the other plasmids had the same level of differentiation using either the pCH01 or Welch markers.

It was noted during the *in silico* testing that three of the regions selected as targets for the Welch markers, R3, R10 and R11 were absent in pCH01. As a result these areas which provide a significant level of differentiation were not identified in pCH01, and were not selected as markers as *in vitro* testing could not be carried out. These three regions were removed from the *in silico* testing to compare the level of differentiation of pCH01 markers with the absence of these regions. The results found that for the Welch markers, 8

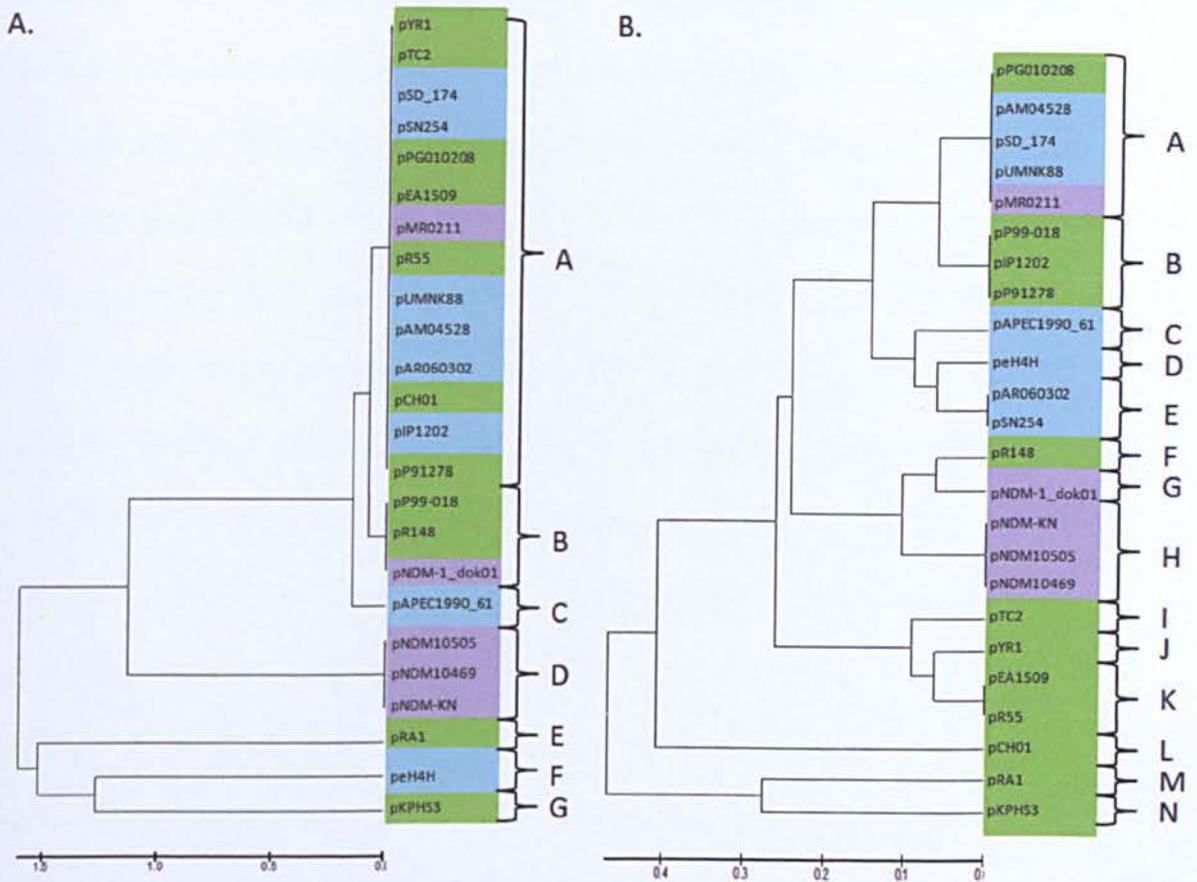
groups were identified for the 24 plasmids, of which 20 were in 3 groups, one group contained 14 plasmids, and the other six plasmids split among two groups, as shown in Figure 6.19. The group containing 14 plasmids were differentiated into 8 groups by pCH01 markers. One of the other two groups containing multiple plasmids was differentiated to the same level with pCH01 markers, and the other group could be further differentiated although one of the plasmids was grouped with another plasmid group. All non grouped plasmids were differentiated equally by both the pCH01 or Welch markers. The absence of regions R3 (part of the *floR* insert), R10 (*rhs* deletion) and R11 (variation upstream of *ter*) demonstrates that the pCH01 markers are more discriminative than Welch markers.

6.4 Discussion

pCH01 was harboured in *E. coli* isolate from chicken faeces in the UK and was found to be multidrug resistant and self transmissible. The reduced susceptibility to cephalosporins, aminoglycosides, sulphonamides and tetracyclines was attributed to pCH01, suggesting this isolate had no other resistances on the chromosome or plasmids. pCH01 was large (160,357 bp) and of the 196 orfs, 96 were hypothetical and 18 putative genes. Other genes were involved in replication, stability, conjugation, resistance and MGE's. The resistance genes were located in two parts of the plasmid, the first was the *ISEcp1-bla_{CTX-M-3}*, and with the exception of some NDM-1 plasmids and pYR1, the genetic environment around the *ISEcp1-bla_{CTX-M-3}-orf477* insertion is homologous with other IncA/C plasmids and is not associated with any other resistance genes. In pYR1, an insertion of *arsR*, *insB* flanking *cysZ* and *dhfR1* had inserted into *orf110* (Welch *et al.*, 2007). This may suggest that the area of IncA/C plasmids acts as a 'hot spot' for MGE's. The other resistant region is a large composite of insertion sequences, transposons and integrons which appears to have evolved over time, the MDR included a *ISCR16*, with *groESL*, and resistance genes, *aadA*, *aacC*, *qacEΔ1*, *sul1*, *pac*, *mer* operon, *tetR*, *tetA* and *pecM*. The *tra* locus of pCH01 was functional, with pCH01 being able to transfer from

Figure 6.19 Comparison of the Welch (without R3, R10 and R11) and pCH01

IncA/C markers



Dendrogram showing the groups for the (A.) Welch markers without the R3, R10 and R11 markers and (B.) pCH01 markers, letters indicate the groups, phylogeny generated using UPMGA using MEGA 5.10.

parent and transformant and like other plasmids was closely related to ICEs (Wozniak *et al.*, 2009; Fernandez-Alarcon, Singer and Johnson, 2011; Fricke *et al.*, 2009).

Comparisons of the IncA/C plasmids showed them to share the same core backbone constituted of the *tra* locus, replication gene, stability genes and hypothetical genes. The variation seen among the IncA/C plasmids is caused by the insertion of resistance regions which appeared to occur in three main locations (Fernandez-Alarcon, Singer and Johnson, 2011; Welch *et al.*, 2007; Call *et al.*, 2010; Doublet *et al.*, 2012; Del Castillo *et al.*, 2013; Lindsey *et al.*, 2011a). The first location for the insertion was located downstream of *orf32* which is termed the *floR* region due to the presence of the insert containing *floR* which

replaced *orf33-47* in pCH01. All of the *bla*_{CMY-2} containing plasmids had the *floR* region, which may suggest that insertions have co-evolved, the presence of the *floR* region in pPG0101208 could indicate a possible progenitor to these plasmids (Fernandez-Alarcon, Singer and Johnson, 2011). The NDM pMR0211 plasmid, also has the *floR* region which is absent in the other NDM plasmids, suggesting that this plasmid may share a similar progenitor to pPG010208 (McGann *et al.*, 2012). The *floR* region has undergone other insertions, but not with the *floR* containing insert but other MDR inserts as described in pKPHS2, pIP1202, pP99-018 and pP91278 (Liu *et al.*, 2012; Welch *et al.*, 2007; Kim *et al.*, 2008). Both the CMY and NDM plasmids have a variation in a large hypothetical gene which lie downstream of the insertion site for *bla*_{CMY-2}. In plasmids pSN254, pAM04528, peH4H and pSD_174 this insert is duplicated in the reverse orientation including the hypothetical gene. All NCM have the same *orf87* as pCH01, with the exception of pR148, which has the same *orf87v* as CMY plasmids and as with pPG010208 for CMY plasmids, may have been the progenitor for some NDM plasmids, with both lacking the *floR* region (Del Castillo *et al.*, 2013).

The MDR region of the IncA/C plasmids shows the most variation between plasmids, and is present in 21/24 plasmids. The MDR consists of either a transposon *Tn21* (9), *Tn1721* (1) or *Tn1696* (3) linked to a class 1 integron (n=14) with multiple gene cassettes. In some incidences the MDR is associated with *mer* operon (9/24) which is mobilised by the transposon. MDR regions not containing transposons, typically have resistance genes flanked by insertion sequences such as IS26 or IS4321. The *bla*_{NDM-1} gene is mobilised by either *insAB* (*ISkpn14*) or IS30 which have inserted into the MDR region. A study by Lindsey *et al* using an array of 286 genes from IncA/C plasmids found that the backbone of the IncA/C plasmids was highly conserved, with the MDR regions being the most variable with ISCR2 and ISCR16 having a particular role in variations (Lindsey *et al.*, 2011a). The MDR region present in pCH01 has homology with two plasmids, the integron (*int1-istA*) is closely related to that found in pSN254, pAR060302, pAPEC1990_61, and

the later part of the MDR is related to pR55, which has the same insertions and transposons and a related *mer* operon, suggesting these plasmids share a similar origin. The combination of MDR, and ISCR's with ICE which share significant homology with the *tra* loci of the IncA/C may represent the origins of these plasmids (Wozniak *et al.*, 2009; Osorio *et al.*, 2008; Toleman and Walsh, 2011; Toleman and Walsh, 2010; Toleman, Bennett and Walsh, 2006).

IncA/C plasmids have been found to have an impact on the genetics of the host cell. *In vitro* experiments using the IncA/C plasmids pR55 and pRA1 have found increases in the transfer frequency of *Salmonella* genomic island 1 (SGI1), an integrative mobile element made up of complex resistance genes, integrons and transposons, and may have a role in the evolution of these regions (Douard *et al.*, 2010; Kiss, Nagy and Olasz, 2012; Doublet *et al.*, 2005). The transcriptome of IncA/C plasmids was investigated by Lang *et al.* (2012), with findings supporting that under non selective growth, much of pAR060302 was transcriptionally inactive, but found a putative toxin and antitoxin system (*cro*-like and *RelE* like in pCH01) and H-NS were highly transcribed, as were resistance genes (Lang *et al.*, 2012). These findings suggested that the burden of the large IncA/C plasmids is reduced by the numerous transcriptional regulators such as *cro*, H-NS, HU beta and *Ner* like found in pCH01 (Doyle *et al.*, 2007; Yun *et al.*, 2010; Acebo *et al.*, 1998; Lang *et al.*, 2012). These transcriptional regulators may have a particular role in the persistence of IncA/C plasmids, which have few stability genes, with *parAB* being identified and a putative addiction system, however three *dcm* genes are present which protect plasmids from restriction enzymes (Korba and Hays, 1982). It has been found that some level of antibiotic use is required to maintain IncA/C plasmids in generations >2400, whether that is cephalosporin, amphenicol or other (Subbiah *et al.*, 2011).

The markers designed for pCH01 and the other IncA/C plasmids were capable of differentiating the 24 plasmids used in this study into 14 different groups, the same level as the 12 primer pairs from Welch *et al.* (2007). This demonstrated that both the pCH01 and

Welch markers had a similar level of differentiation, however the removal of the three primers for the regions absent in pCH01 from the Welch scheme, identified only 8 groups, showing that the pCH01 markers have a higher level of differentiation. As the pCH01 markers are designed to be used as three multiplex's, it may offer a more accessible approach for screening for IncA/C plasmids. Specific marker combinations were observed with closely related plasmids, and with relation to drug resistance insertions (*floR* and MDR). Comparisons of concatenated backbones showed that the non *bla*_{CMY-2} plasmids were closely related, with pYR1 and pRA1 being the most distantly related of all the plasmids. pR148 was closely related to the *bla*_{NDM-1} which may indicate it as being a progenitor to the NDM plasmids, particularly as it has the large hypothetical variant (*orf87v*), and shares the same *repA* gene (99.18% to that of pCH01). The NCM plasmids were differentiated into 9 separate marker profiles, although the profile of pPG010208 which contains the *floR* region was in the same group as some CMY plasmids. The molecular markers generated four profiles for the CMY plasmids, which all shared a highly conserved backbone. The NDM plasmid pMR0211 also shared the same profile as CMY plasmids, which was also seen in the similarity of the backbone. The main marker for differentiation of this group was the mGroEL, which is inserted into the class 1 integron by *insE* and *insF* and is also present in pCH01. The NDM plasmids were all closely related with the exception of pMR0211, with two marker profiles. It is likely that as with pPG010208, pMR0211 evolved with the *bla*_{CMY-2} separately from NCM and NDM plasmids. The molecular markers designed based on the pCH01 backbone would be beneficial in identifying separate lineages, to plasmid epidemiology.

IncA/C plasmids have become vectors for both resistance to cephalosporins and florfenicol through the carriage of *bla*_{CMY-2} and *floR* genes respectively, which are commonly found together in farm animals (Fernandez-Alarcon, Singer and Johnson, 2011; Call *et al.*, 2010; Welch *et al.*, 2007; Han *et al.*, 2012). These CMY IncA/C plasmids in animals may have been selected through the use of third generation cephalosporins such as

ceftiofur, or have been co-selected through the use of florfenicols, tetracyclines and aminoglycosides (Singer, Patterson and Wallace, 2008; Cavaco *et al.*, 2008; Jorgensen *et al.*, 2007). Although frequently found in animal isolates, CMY plasmids have also been recovered from humans around the world (Welch *et al.*, 2007; Lindsey *et al.*, 2009; Hopkins *et al.*, 2006; Verdet *et al.*, 2009; Doublet *et al.*, 2004). The association of *bla*_{CTX-M} genes with IncA/C plasmids appears less common than *bla*_{CMY-2}. As with pCH01, the *bla*_{CTX-M-3} gene has previously been found on an IncA/C plasmid in *E. coli* isolated from a human blood sample in Spain (Novais *et al.*, 2007). Other *bla*_{CTX-M} genes found with the IncA/C backbone include *bla*_{CTX-M-2} in France and the UK, *bla*_{CTX-M-14} in France and China, and *bla*_{CTX-M-15} in France and Spain (Marcade *et al.*, 2009; Hopkins *et al.*, 2006; Liu *et al.*, 2012; Novais *et al.*, 2012). Other ESBL genes associated with IncA/C plasmids include *bla*_{VEB-1,IMP-4,IMP-13} and *vim-4*, demonstrating their ability to be versatile vectors for resistance (Carattoli, 2009).

The IncA/C plasmid backbone appears to have an aquatic origin with one of the earliest plasmids being found in *A. hydrophila* in 1971, and have additionally been recovered from *P. damsela*, *Salmonella enterica* and *Y. ruckeri* from fish (Fricke *et al.*, 2009; Kim *et al.*, 2008; Welch *et al.*, 2007). However an IncA/C plasmid sequenced in 2012 was from a human *K. pneumoniae* recovered in 1969, which contradicts this origin, but may have been acquired from a fish pathogen (Doublet *et al.*, 2012). pCH01 may have links to the aquatic environment with IS4MS being identified, closely linked to the *Shewanella baltica* (Caro-Quintero *et al.*, 2012). IncA/C plasmids are not restricted to tight species boundaries being found in more than 9 different species, recovered from different sources. This ability to move between bacterial species and their association with resistance genes make them serious public health concerns (Welch *et al.*, 2007; Call *et al.*, 2010; Fernandez-Alarcon, Singer and Johnson, 2011; Johnson and Lang, 2012b; Fricke *et al.*, 2009). IncA/C plasmids have a higher association with pathogenic *E. coli* (3%) than commensals (1%) which is significant when considering the MDR nature of these plasmids

and the complications it could have in treating infections (Johnson *et al.*, 2007). The emergence of *bla*_{NDM-1} encoding IncA/C, is of major concern due to resistance to carbapenems, considered one of the last antibiotics of use, and signifies the importance of these MDR vectors (Walsh *et al.*, 2011; Sekizuka *et al.*, 2011; McGann *et al.*, 2012; Carattoli *et al.*, 2012). The markers proposed in this study could benefit in both identifying and differentiating among the IncA/C plasmids aiding the epidemiological study of these potentially threatening vectors.

Chapter 7

Comparative Genomics of Incl1γ Plasmids from Animals and Humans and Molecular Markers for their Differentiation

7.1 Introduction

IncI γ plasmids belong to the IncI complex which are defined by their susceptibility to bacteriophages If1 and PR64FS, and includes the plasmids IncI γ , B, K and Z (Bradley, 1984; Coetzee, Sirgel and Lecatsas, 1980). The IncI γ plasmids predate the clinical use of antibiotics and are likely to be progenitors for some of the resistance plasmids found today (Jones and Stanley, 1992; Datta and Hughes, 1983). As with other plasmid backbones, the IncI γ plasmids are established vectors for the transmission of resistance genes (Carattoli, 2009; Carattoli, 2011; Smet *et al.*, 2010b). The dissemination of resistance genes by IncI γ is aided by the two conjugation loci which code for both the thin pilus and the thick pilus, involved in plasmid transfer on both solid surfaces and in liquids (Komano *et al.*, 2000; Yoshida, Kim and Komano, 1999). The IncI γ plasmid backbone has been associated with several ESBL resistance genes, including *bla*_{CTX-M-1, -15, -9, 14}, *bla*_{CMY-2, CMY-7}, *bla*_{TEM-52} and *bla*_{SHV-12} have been isolated from humans, cattle, poultry, turkeys and pets from the UK, France, Italy, Spain, USA and the Netherlands (Garcia-Fernandez *et al.*, 2008; Hopkins *et al.*, 2006; Marcade *et al.*, 2009; Leverstein-van Hall *et al.*, 2011; Cloeckaert *et al.*, 2010; Cloeckaert *et al.*, 2007; Carattoli, 2009; Carattoli, 2011). Several ESBL IncI γ plasmids have now been sequenced including the *bla*_{CTX-M-3} pEK204 isolated from human *E. coli* O25:ST131 in the UK, and the *bla*_{CTX-M-15} plasmids pEC_Bactec from equine *E. coli* in Belgium and pESBL-EA11 from human shiga toxin producing *E. coli* O104:H4 in USA with links to the German EHEC O104:H4 outbreak in May 2011, (Mellmann *et al.*, 2011), all of which harbour group 1 CTX-M (Woodford *et al.*, 2009; Smet *et al.*, 2010b; Ahmed *et al.*, 2012; Mellmann *et al.*, 2011). Additionally *bla*_{CMY-2} plasmids pNF1358 from *Salmonella* Thompson from a human and pCVM29188_101 from a chicken *Salmonella* Kentucky, both from the USA have been sequenced (Dunne *et al.*, 2000; Fricke *et al.*, 2009). IncI γ plasmids have also been sequenced due to their association with virulent ETEC isolates in swine (Johnson *et al.*, 2007), where they have been found in higher numbers in pathogenic strains than

commensals (Johnson *et al.*, 2011; Johnson *et al.*, 2007). This may be due to the isolates harbouring IncI1 γ plasmids having an increased adhesion to eukaryotic cells (Dudley *et al.*, 2006). The sequencing of IncI1 γ plasmids and their recognition as vectors of resistance led to the development of a plasmid multi locus typing scheme (pMSLT) based on five loci, *repII*, *ardA*, *trbA*, *sogS* and *pil*, in which the alleles form a sequence type (ST) of which there are now over 125 ST's (Garcia-Fernandez *et al.*, 2008). IncI1 γ pMLST has allowed researchers to improve their epidemiological understanding of plasmids in both animal and human populations.

The *bla*_{CTX-M-1} gene has been associated with several plasmid backbones including N, I1 γ , FII and L/M, with IncN and I1 γ appearing to be the more prevalent vectors (Carattoli, 2009; Carattoli, 2011). IncN plasmids have been found with *bla*_{CTX-M-1} in dairy cattle and horses in the Czech Republic and in cattle from Denmark (Garcia-Fernandez *et al.*, 2011; Dolejska *et al.*, 2013; Dolejska *et al.*, 2011c). A study by Moodley *et al.* (2009) found that IncN plasmids moved between unrelated isolates from pigs and farm workers on Danish farms (Moodley and Guardabassi, 2009). The *bla*_{CTX-M-1} gene is also commonly found on IncI1 γ plasmids, and as with IncN plasmids is particularly prevalent in animals which suggest that animals may act as reservoir for the *bla*_{CTX-M-1} IncN and IncI1 γ plasmids (Carattoli, 2008). In the Netherlands and France, IncI1 γ plasmids have been found with *bla*_{CTX-M-1} in *E. coli* from cattle and poultry, with ST3 and ST7 being common sequence types (Hordijk *et al.*, 2013; Dierikx *et al.*, 2013; Leverstein-van Hall *et al.*, 2011; Madec *et al.*, 2011; Cloeckaert *et al.*, 2010). Evidence supports the movement of these plasmids from animals to human, with the food chain likely to play a role (Dierikx *et al.*, 2013; Leverstein-van Hall *et al.*, 2011; Madec *et al.*, 2011; Girlich *et al.*, 2007; Cloeckaert *et al.*, 2010; Garcia-Fernandez *et al.*, 2008). IncI1 γ ST3 plasmids have also been identified in poultry in both Italy and Poland, where other STs are also present (Accogli *et al.*, 2013). The likelihood of transmission of ST3 plasmids between animals and humans seems to be increasing as isolates from pets in France and Tunisia have been found with these plasmids

(Dahmen, Haenni and Madec, 2012; Grami *et al.*, 2013). In the UK IncI1 γ plasmids have mainly been found to harbour the *bla*_{CTX-M-3} in humans, while *bla*_{CTX-M-1} and *bla*_{CTX-M-15} have been found on IncI1 γ plasmids isolated from chickens and turkeys (Dhanji *et al.*, 2011d; Randall *et al.*, 2011).

7.1.1 Hypotheses and aims

The hypothesis of this study was that *bla*_{CTX-M-1} IncI1 γ plasmids in UK farm animals (chickens, turkeys and cattle) are related to IncI1 γ plasmids around the world, which could be further differentiated by molecular markers. The aims of this study were to (i) extract the IncI1 γ plasmids DNA from the IncI1 γ isolates, sequence and annotate the plasmids to determine their genotypes and features. (ii) Compare IncI1 γ plasmid with other plasmid previously sequenced, to determine the relationship between plasmids. (iii) Design molecular markers capable of identifying IncI1 γ and differentiating between similar plasmids, which can be used as epidemiological tools. (iv) Compare the IncI1 γ molecular markers to the IncI1 γ pMLST. (v) Screen a panel of *E. coli* field isolates for the prevalence of the IncI1 γ plasmids and similar plasmids and to identify possible reservoirs.

7.2 Methods and materials

The *E. coli* field isolates CH02 and CH03 were both isolated from chicken faeces in 2006 and 2010 respectively, CT01 isolated from cattle faeces in 2008 and T01 isolated from turkey faeces in 2006, all samples were recovered as part of routine surveillance by AHVLA. The sequence types of CH02, CH03, CT01 and T01 were determined using MLST as described in 2.3.3. Total DNA was extracted from CH02, CH03, CT01 and T01 using the QIAGEN Hi-Speed plasmid midi prep as described in 2.2.4.3, which was then transformed into *E. coli* DH10B (2.2.5). Transformants were checked for the presence of a single CTX-M plasmid by plasmid profiling as described in 2.3.12.2. The antimicrobial sensitivities of the CH02, CH03, CT01 and T01 field isolates and the pCH02, pCH03, pCT01 and pT01 transformants were determined by disc diffusion as described in 2.1.3.

Plasmid content and sizes were determined by S1 nuclease for both the CH02, CH03, CT01 and T01 field isolates and the pCH02, pCH03, pCT01 and pT01 transformants (2.3.12.1), replicons were determined using a commercial kit for the field isolates (2.3.6.2) and using the PBRT primers for IncI1 γ as described in 2.3.6.1. The transferability of pCH02, pCH03, pCT01 and pT01 was determined by liquid and solid conjugations as stated in 2.1.4.1 and 2.1.4.2. Plasmid DNA for sequencing was extracted from the pCH02, pCH03, pCT01 and pT01 transformant using the QIAGEN large construct kit as following the protocol (2.2.4.4), and sequenced on a Roche 454 GS FLX (2.4.1) with closure of the sequence by PCR using the primers in Table 2.12, using the conditions in 2.4.3.1 and 2.4.3.2. The plasmids were annotated using several programs including RAST (2.5.1.1), Artemis (2.5.1.2) and BLASTn using the annotations from plasmids pColIb-P9 (AB021078), pSL476_91 (CP001118), R64 (AP005147), pEK204 (EU935740) and pEC_Bactec (GU371927) as described in 2.5.1.3. Plasmids from GenBank were compared with pCH02, pCH03, pCT01 and pT01 using MAUVE (2.5.2.1), ACT (2.5.2.2) and BLASTn (2.5.1.3) to determine the relationship between plasmids and identify molecular marker candidates. The phylogeny of the plasmids was analysed using concatenated sequence compiled from open reading frames present in all plasmids which was analysed as described in 2.5.2.5. The screening of molecular markers in 2.3.8, Table 2.8 were performed against plasmids from GenBank *in silico* as described in 2.5.3.1, with the phylogeny of the markers analysed as stated in 2.5.2.5. The pMLST of IncI1 γ plasmid was performed *in silico* for pCH02, pCH03, pCT01 and pT01 and GenBank plasmids as described in 2.5.3.2. The IncI1 γ markers were screened against a panel of 136 ESBL *E. coli* field isolates from the SFE collection (appendix II) which included 98 group 1 CTX-M isolates from cattle (n=27), chicken (n=29), humans (n=27) and turkeys (n=15) and 38 group 9 CTX-M isolates from cattle (n=19), humans (n=9) and turkeys (n=10). DNA was extracted by crude lysis (2.2.3.1) and PCR's run as stated in 2.3.8 (Table 2.8) using *E. coli* DH10B and *Salmonella* Typhimurium 26R as negative controls and pCH03 and pCT01

transformants, CH03 and CT01 field isolates as a positive control. *In vitro* comparison of the IncI1 γ markers were performed using IncI1 γ plasmids with known pMLST (n = 17), which were transferred by liquid conjugation (2.1.4.1), and tested for both IncI1 γ replicon and IncI1 γ markers as described previously. Phylogeny of plasmids using pMLST for both GenBank and those with known pMLST were carried out using concatenated sequenced loci and were compared by neighbour joining method as described in 2.5.2.5.

7.3 Results

7.3.1 Analysis of the IncI1 γ *E. coli* field isolates

7.3.1.1 Antimicrobial sensitivity testing of *E. coli* field isolates

The results of the antimicrobial sensitivities of testing the CH02, CH03, CT01 and T01 *E. coli* field isolates are shown in Table 7.1. Isolate CH02 had reduced susceptibility to tetracyclines, ampicillin, ceftazidime, cefotaxime, streptomycin, gentamicin, amoxicillin/clavulanic acid and sulphonamides. CH03 had reduced susceptibility to tetracyclines, ampicillin, ceftazidime, cefotaxime, amoxicillin/clavulanic acid and sulphonamides. Isolate CT01 has reduced susceptibility to tetracyclines, ampicillin, ceftazidime, cefotaxime, chloramphenicol, streptomycin, sulphamethoxazole/trimethoprim, gentamicin, amoxicillin/clavulanic acid, sulphonamides and apramycin. Isolate T01 had reduced susceptibility to tetracyclines, ampicillin, ceftazidime, cefotaxime, sulphamethoxazole/trimethoprim, amoxicillin/clavulanic acid and sulphonamides. This demonstrated that all of the isolates were multidrug resistant, limiting treatment options and aiding their dissemination through co-selection through the use of different classes of antibiotics.

7.3.1.2 Plasmid analysis of the IncI1 γ harbouring *E. coli* field isolates

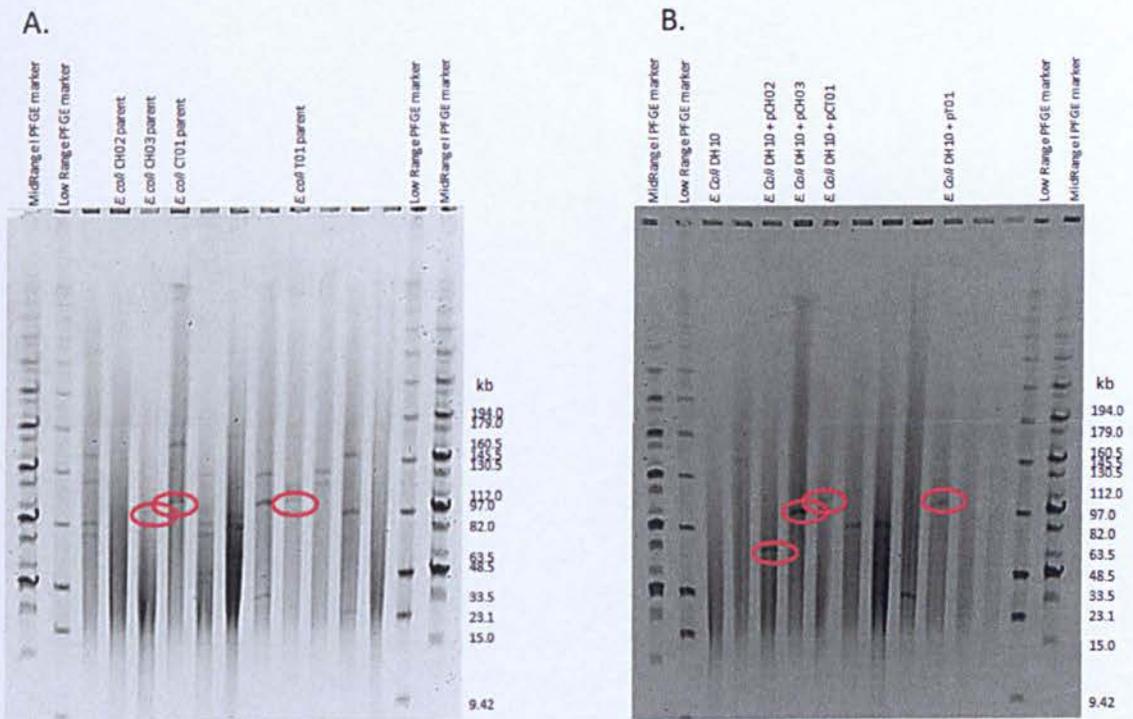
The IncI1 γ field isolates were analysed for plasmid content by replicon typing and size, shown in Figure 7.1. The CH02 isolate produced amplicons for the plasmids IncI1 γ ,

Table 7.1 Reduced antimicrobial susceptibility of field isolates and transformants of sequenced IncI γ plasmids

Isolate	Reduced sensitivities of field isolate	Reduced sensitivities of IncI γ transformants
CH02/pCH02	TE, AMP, CAZ, CTX, S, CN, AMC, S3	TE, AMP, CTX, S3
CH03/pCH03	TE, AMP, CAZ, CTX, S, AMC, S3	TE, AMP, CTX, S3
CT01/pCT01	TE, AMP, CAZ, CTX, C, S, SXT, CN, AMC, S3, APR	AMP, CTX, SXT, S3
T01/pT01	TE, N, AMP, CAZ, CTX, SXT, AMC, S3	AMP, CTX, SXT, S3

NA = Nalidixic acid, TE = Tetracycline, N = Neomycin, AMP = Ampicillin, CAZ = Ceftazidime, CTX = Cefotaxime, C = Chloramphenicol, S = Streptomycin, SXT = Sulphamethoxazole/Trimethoprim, CN = Gentamicin, AMC = Amoxicillin/clavulanic acid, S3 = Sulphonamides and APR = Apramycin. Reduced susceptibility was determined using BSAC breakpoints

Figure 7.1 Plasmid sizing of field isolates and transformants with IncI γ plasmids



S1 nuclease PFGE of (A) the *E. coli* CH02, CH03, CT01 and T01 field isolates and (B) transformed *E. coli* DH10 with pCH02, pCH03, pCT01 and pT01, *E. coli* DH10 used for transformations as a negative control, the IncI γ plasmids are circled in red. Plasmids digested with 8U of S1 nuclease for 45 minutes, DNA bands were resolved on a 1% agarose gel. MidRange and Low Range PFGE markers were used for sizing.

FIB, P, A/C and FII, however no plasmids were identified by S1 nuclease which suggest enzyme degradation of the DNA. The CH03 isolate had amplicons for IncI1 γ , FIB, P, A/C and FII, two plasmids were identified by S1 nuclease, which were 95 kb and 100 kb in size. The cattle isolate CT01 had amplicons for IncI1 γ , FIB and FII, and S1 nuclease identified two plasmids of 110 kb and 170 kb in size. As with the CT01 isolate, T01 has the IncI1 γ , FIB and FII amplicons and had three plasmids at 100 kb, 110kb and 120kb. This implies that the genotype of the *E. coli* has been altered through multiple plasmid acquisitions which may also be vectors for resistance genes or provide opportunities for rearrangements between MGE's.

7.3.2 Analysis of the pCH02, pCH03, pCT01 and pT01 plasmids

7.3.2.1 Antimicrobial sensitivity testing of the pCH02, pCH03, pCT01 and pT01 plasmids

As with the parental field isolates, the reduced antimicrobial susceptibilities of the IncI1 γ plasmids transformed into *E. coli* DH10B were determined by disc diffusion, shown in Table 7.1. Both pCH02 and pCH03 had reduced susceptibilities to tetracycline, ampicillin, cefotaxime and sulphonamides. Plasmids pCT01 and pT01 also had the same reduced susceptibilities to ampicillin, cefotaxime, sulphamethoxazole/trimethoprim and sulphonamides. This suggests that these resistances are conferred by the IncI1 γ plasmids, and the other resistances present in the field isolates (Table 7.1), are conferred by genes on the chromosome or other plasmids.

7.3.2.2 IncI1 γ plasmid typing and sizing

The plasmids in the transformants were analysed for the IncI1 γ replicon type using PBRT and size by S1 nuclease. All of the transformants with pCH02, pCH03, pCT01 and pT01 were positive for the IncI1 γ replicon type. S1 nuclease of the transformants revealed

they all had a single plasmid, pCH02 was found to be 70 kb in size, pCH03 was 95 kb in size, pCT01 110kb in size and pT01 was 100 kb in size, as shown in Figure 7.1

7.3.2.3 Transferability of the plasmids pCH02, pCH03, pCT01 and pT01

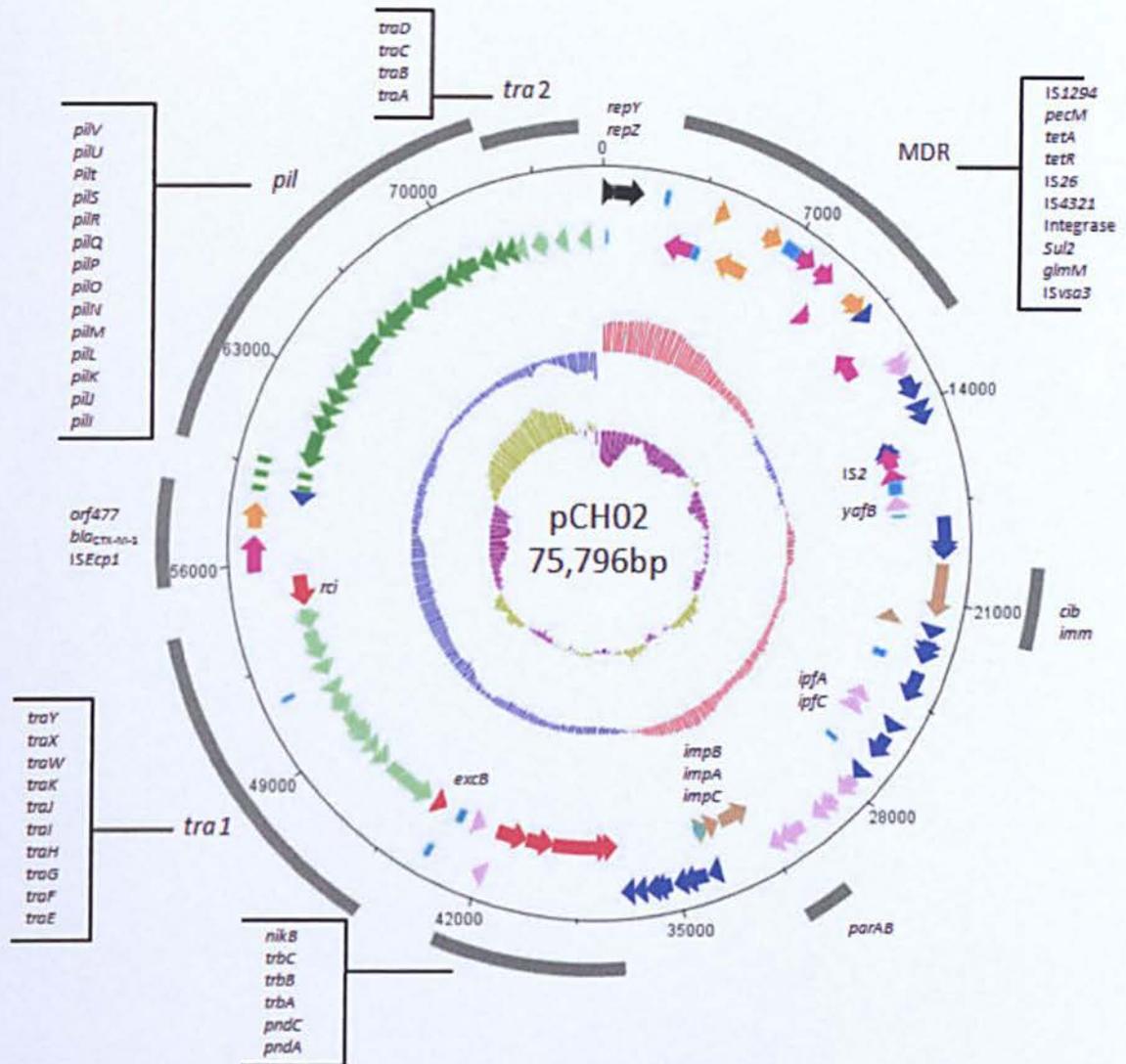
The plasmids were investigated to see if they were self transmissible, conjugations using the plasmid transformants were conducted. Liquid and solid conjugations were conducted using the plasmid-free and rifampicin-resistant *S. Typhimurium* 26R as a recipient. Conjugations were considered to have occurred if pink colonies grew on Rambach's media supplemented with 100 µg/ml rifampicin and 1 µg/ml cefotaxime which the recipient negative control was unable to. Colonies were then grown on LB-G with 4 µg/ml cefotaxime to confirm selective growth. pCH02 failed to transfer in both solid and liquid conjugations, pCT01 only transferred in solid conjugations, and both pCH03 and pT01 transferred in liquid and solid conjugations.

7.3.3 Sequencing of the IncI1γ plasmid

The sequencing of the plasmids produced different coverage for each plasmid, pCH02 had >213 fold coverage, pCH03 had >48 fold coverage, pCT01 had >128 fold coverage and pT01 had > 50 fold coverage. Newbler 2.3 assembly of the sequence for pCH03 produced a single contig, while three contigs were produced for each of the plasmids pCH02, pCT01 and pT01, with contigs closed by PCR and ABI sequencing. Plasmid pCH02 was the smallest of all the IncI1γ plasmids at 75,796 bp in length with a GC content of 49.36%, the sequence appeared to be a contiguous backbone of 62,038 bp which had undergone three insertions, pCH02 had 109 ORFs, of which 14 were hypothetical, shown in Figure 7.2. pCH03 was 105,608 bp in size with a GC content of 50.74%, the sequence appeared to be a contiguous backbone of 92,918 bp which has undergone two insertions, pCH03 had 149 ORFs of which 18 were hypothetical, as shown in Figure 7.3. pCT01 was 117,577 bp in size with a GC content of 50.77%, the sequence appeared to be a contiguous backbone of 92,919 bp which has undergone five insertions,

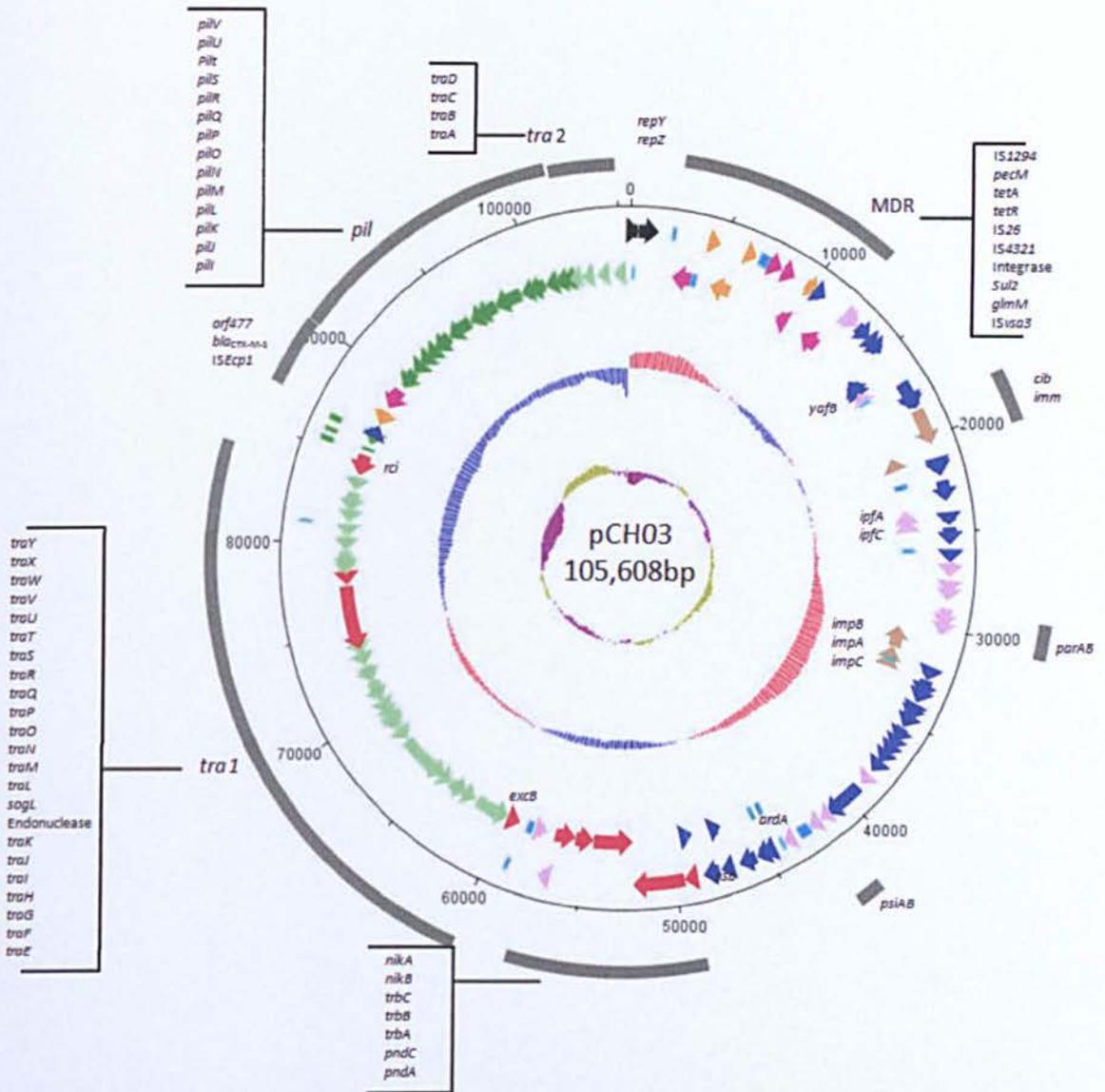
pCT01 had 152 ORFS of which 20 were hypothetical, as shown in Figure 7.4. The pT01 plasmids was 111,318 bp in size with a GC content of 50.77%, the sequence appeared to be a contiguous backbone of 92,918 bp which had undergone three insertions, 149 ORFs were identified of which 20 were hypothetical, as shown in Figure 7.5. Table 7.2 shows the orfs present in all four plasmids and complements Figures 7.2-5. Plasmid contained genes involved in replication, stability, conjugation and resistance which will be discussed in detail.

Figure 7.2 Plasmid map of pCH02



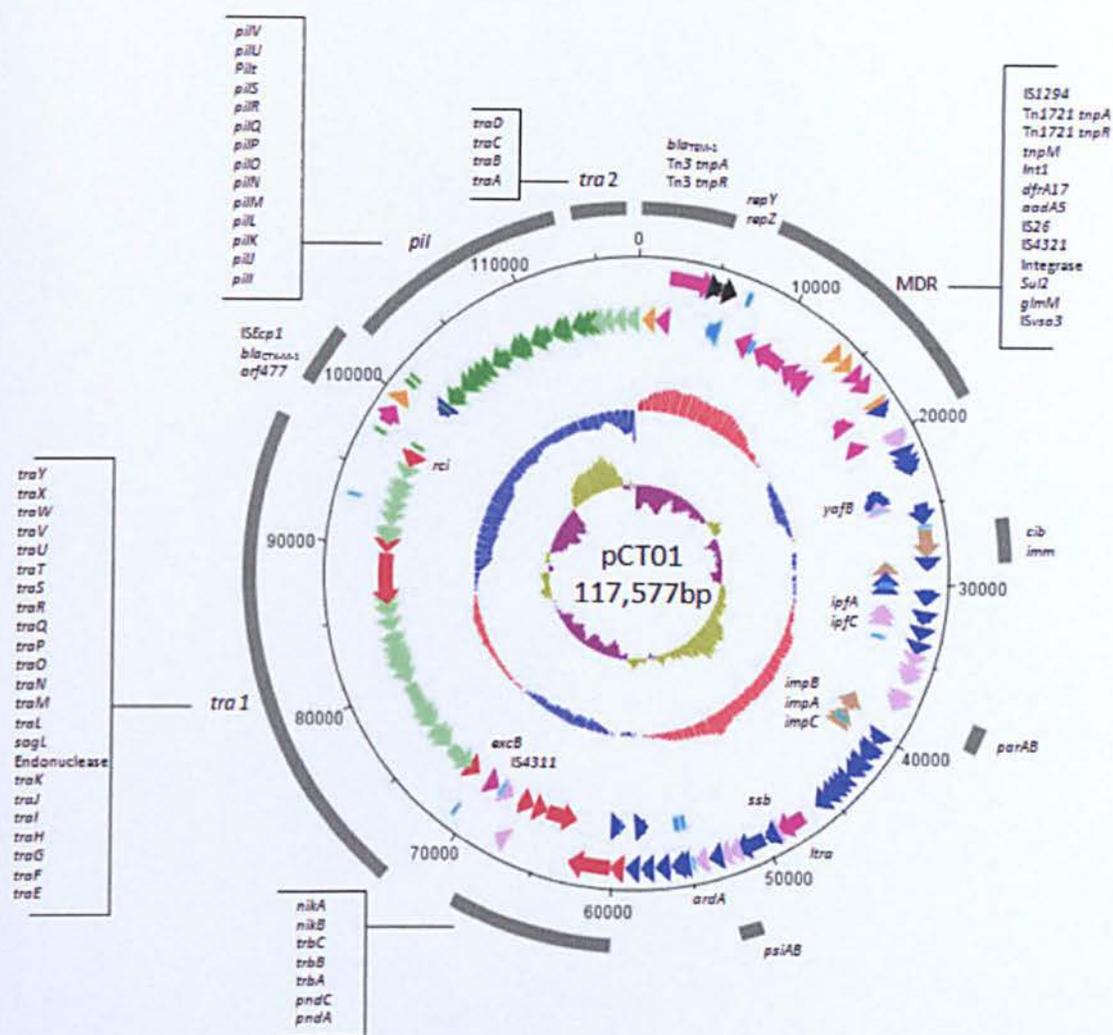
Circular plasmid map of pCH02. Shown are the coding regions in the pCH02 plasmid (complemented in Table 7.2) with the outer arrowed circle denoting the positive orientation and the inner arrow circle denoting the negative orientation. Green arrows indicate genes involved in conjugation with the light green indicating the *tra* loci and the darker green the *pil* loci. Red arrows are other genes involved in conjugation, orange arrows indicate resistance genes, and magenta arrows are mobile elements, light pink are genes involved in stability, brown are accessory genes, dark blue are known genes and cyan are hypothetical genes. The outer graph shows the GC plot and the inner graph shows the GC skew.

Figure 7.3 Plasmid map of pCH03



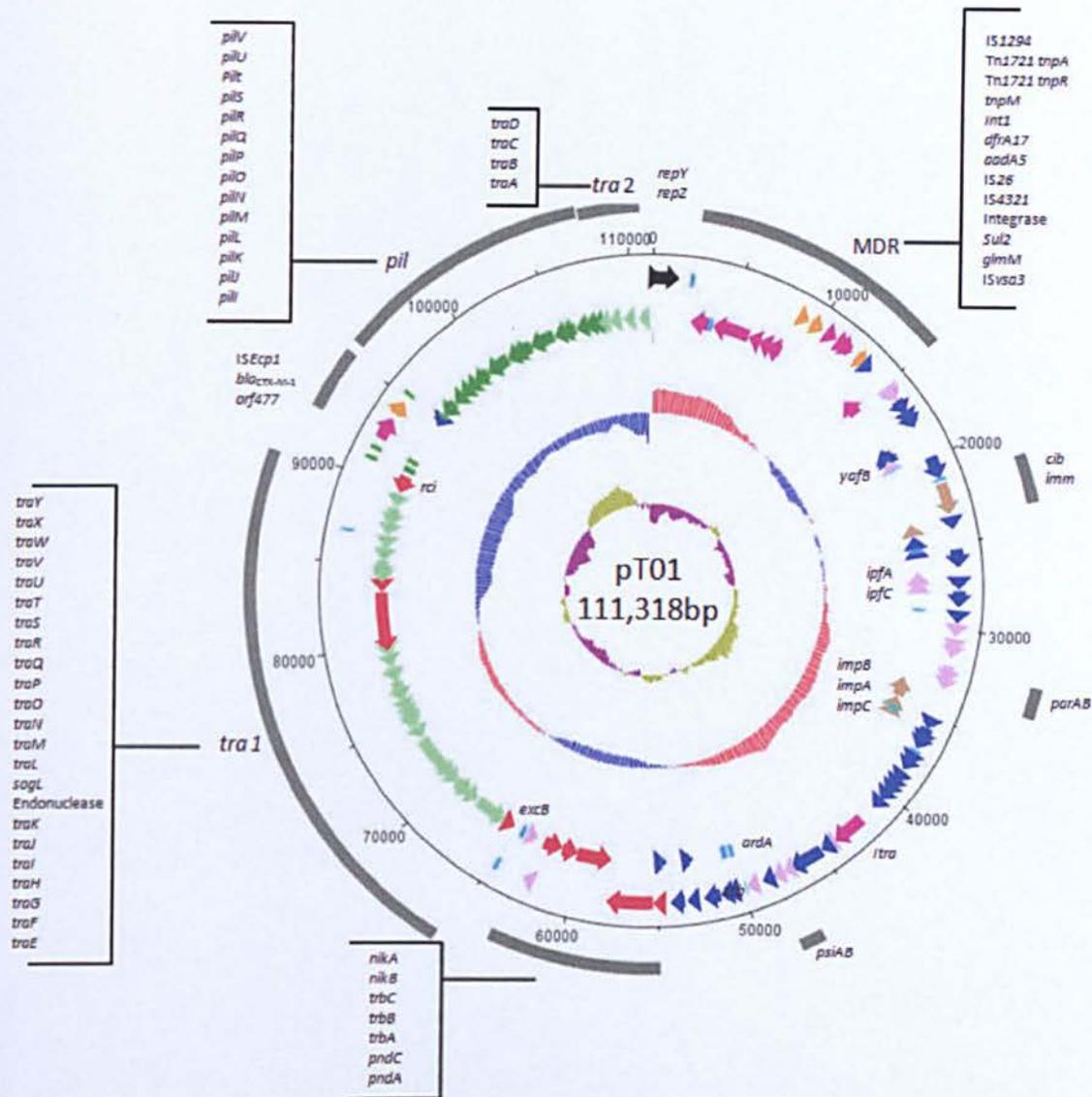
Circular plasmid map of pCH03. Shown are the coding regions in the pCH03 plasmid (complemented in Table 7.2) with the outer arrowed circle denoting the positive orientation and the inner arrow circle denoting the negative orientation. Green arrows indicate genes involved in conjugation with the light green indicating the *tra* loci and the darker green the *pil* loci. Red arrows are other genes involved in conjugation, orange arrows indicate resistance genes, and magenta arrows are mobile elements, light pink are genes involved in stability, brown are accessory genes, dark blue are known genes and cyan are hypothetical genes. The outer graph shows the GC plot and the inner graph shows the GC skew.

Figure 7.4 Plasmid map of pCT01



Circular plasmid map of pCT01. Shown are the coding regions in the pCT01 plasmid (complemented in Table 7.2) with the outer arrowed circle denoting the positive orientation and the inner arrow circle denoting the negative orientation. Green arrows indicate genes involved in conjugation with the light green indicating the *tra* loci and the darker green the *pil* loci. Red arrows are other genes involved in conjugation, orange arrows indicate resistance genes, and magenta arrows are mobile elements, light pink are genes involved in stability, brown are accessory genes, dark blue are known genes and cyan are hypothetical genes. The outer graph shows the GC plot and the inner graph shows the GC skew.

Figure 7.5 Plasmid map of pT01



Circular plasmid map of pT01. Shown are the coding regions in the pT01 plasmid (complemented in Table 7.2) with the outer arrowed circle denoting the positive orientation and the inner arrow circle denoting the negative orientation. Green arrows indicate genes involved in conjugation with the light green indicating the *tra* loci and the darker green the *pil* loci. Red arrows are other genes involved in conjugation, orange arrows indicate resistance genes, and magenta arrows are mobile elements, light pink are genes involved in stability, brown are accessory genes, dark blue are known genes and cyan are hypothetical genes. The outer graph shows the GC plot and the inner graph shows the GC skew

Table 7.2 Open reading frames present in the IncI1 γ plasmids

Gene	ORF	Direction	Location				Length (bp)	Protein Length (aa)	Product	Function
			pCH02	pCH03	pCT01	pT01				
<i>bla</i> _{TEM-1}	1	Reverse	NP	NP	229-1089	NP	861	286	TEM-1	β -lactam hydrolysis
<i>tnpR</i> Tn3	2	Reverse	NP	NP	1272-1829	NP	558	185	Tn3 Resolvase	Resolvase
<i>tnp</i> Tn3	3	Forward	NP	NP	1991-4996	NP	3006	1001	Tn3 Transposase	Transposon
<i>orf4</i>	4	Reverse	128-247	128-247	5081-5200	128-247	120	39	Conserved hypothetical	Unknown
<i>repY</i>	5	Forward	378-467	378-467	5331-5420	378-467	90	29	RepY	Replication regulator
<i>repZ</i>	6	Forward	410-1486	410-1486	5363-6439	410-1486	1077	358	RepZ	Replication protein
<i>orf7</i>	7	Forward	2182-2394	2182-2394	7135-7347	2182-2394	213	70	Conserved hypothetical	Unknown
<i>IS1294</i>	8	Reverse	2509-3717	2509-3717	7462-8670	2509-3717	1209	402	IS1294 transposase	Transposon
<i>orf9</i>	9	Reverse	3698-3970	3698-3970	8651-8923	3698-3970	273	90	Conserved hypothetical	Unknown
<i>pecM</i>	10	Forward	4087-4620	4061-4795	NP	NP	735	244	PecM	Multidrug Efflux
<i>tetA</i>	11	Reverse	4652-5926	4827-6101	NP	NP	1275	424	TetA	Tetracycline Efflux
<i>tetR</i>	12	Forward	5930-6607	6105-6782	NP	NP	678	225	TetR regulator	Regulator
<i>orf13</i>	13	Forward	6721-7274	6896-7449	NP	NP	555	184	Putative recombinase	Recombinase
<i>tnp</i> Tn1721	14	Reverse	NP	NP	8943-11414	3990-6461	2472	823	Tn1721 Transposase	Transposon
<i>tnpR</i>	15	Reverse	NP	NP	11418-11978	6465-7025	561	186	Tn1721 resolvase	Resolvase
<i>tnpM</i>	16	Reverse	NP	NP	12154-12762	7201-7809	609	202	Transposon modulator	Transposon modulator
<i>intI</i>	17	Reverse	NP	NP	12707-13720	7754-8767	1014	337	IntI integrase	Integrase
<i>dfrA17</i>	18	Forward	NP	NP	13752-14351	8799-9398	600	199	Dfr17	Trimethoprim resistance
<i>aadA5</i>	19	Forward	NP	NP	14482-15270	9529-10317	789	262	AadA5	Aminoglycoside resistance

Gene	ORF	Direction	Location				Length (bp)	Protein Length (aa)	Product	Function
			pCH02	pCH03	pCT01	pT01				
<i>IS26</i>	20	Forward	7291-8013	7466-8188	15454-16176	10501-11223	723	240	IS26 transposase	Transposase
<i>IS4321</i>	21	Forward	8132-8821	8294-8983	16295-16984	11342-12031	690	229	Tuncated IS4321	Transposase
<i>orf22</i>	22	Reverse	9003-9179	9165-9341	17166-17342	12213-12389	177	58	Putative integrase	Integrase
<i>sul2</i>	23	Forward	9509-10324	9671-10485	17672-18487	12719-13534	816	271	Sul2	Sulphonamide resistance
<i>glmM</i>	24	Forward	10411-10713	10572-10874	18574-18876	13621-13923	303	100	GlmM	Phosphoglucosamine mutase
<i>ISvs3</i>	25	Reverse	10889-12025	11050-12186	19052-20188	14099-15235	1137	378	ISvs3 transposase	Transposase
<i>yacA</i>	26	Forward	12392-12661	12553-12822	20555-20824	15602-15871	270	89	YacA	Antitoxin ribbon helix-helix fold protein
<i>yacB</i>	27	Forward	12658-12939	12819-13100	20821-21102	15868-16149	282	93	YacB	Toxin RelE
<i>yacC</i>	28	Forward	12979-13827	13140-13988	21142-21990	16189-17037	849	282	YacC	Exonuclease
<i>yadA</i>	29	Forward	13944-14417	14105-14578	22107-22580	17154-17627	474	157	YadA	Unknown
<i>orf30</i>	30	Forward	14458-14577	14619-14738	22621-22740	17668-17782	120	39	Conserved hypothetical	Unknown
<i>yaeA</i>	31	Forward	14552-14785	14713-14946	22715-22948	17762-17995	234	77	YaeA	Unknown
<i>yaeB</i>	32	Reverse	14894-15074*	15055-15324	23057-23326	18104-18373	270	89	YaeB	Unknown
<i>IS2 orfB</i>	33	Reverse	15085-15990	NP	NP	NP	906	301	IS2 orfB transposase	Transposon
<i>IS2 orfA</i>	34	Reverse	15948-16313	NP	NP	NP	366	121	IS2 orfA transposase	Transposon
<i>yafA</i>	35	Reverse	16496-17029	15321-15854	23323-23817	18370-18864	495	164	YafA	Unknown
<i>yafB</i>	36	Reverse	17046-17648	15871-16473	23873-24475	18920-19522	603	200	YafB	FinO like fertility inhibition
<i>orf37</i>	37	Reverse	17805-17924	16630-16749	24632-24751	19679-19798	120	39	Conserved hypothetical	Unknown
<i>yagA</i>	38	Forward	18002-19348	16827-18173	24829-26175	19876-21222	1347	448	YagA	Unknown
<i>orf39</i>	39	Forward	19357-19521	18182-18346	26184-26348	21231-21395	165	54	Putative resolvase	Unknown
<i>cib</i>	40	Forward	19627-21507	18452-20332	26453-28334	21501-23381	1881	626	colicin Ib protein	Colicin
<i>imm</i>	41	Reverse	21525-21872	20350-20697	28352-28699	23399-23746	348	115	colicin Ib immunity	Colicin immunity

Gene	ORF	Direction	Location				Length (bp)	Protein Length (aa)	Product	Function
			pCH02	pCH03	pCT01	pT01				
<i>ybaA</i>	42	Forward	21991-22311	20816-21136	28818-29138	23865-24185	321	106	YbaA	Unknown
<i>ybbA</i>	43	Reverse	22359-22949	21184-21774	29186-29776	24233-24823	591	196	YbbA	Unknown
<i>ydfA</i>	44	Reverse	22949-23206	21774-22031	29776-30033	24823-25080	258	85	YdfA	Unknown
<i>orf45</i>	45	Reverse	23191-23358	22016-22183	30018-30185	25065-25232	168	55	Conserved hypothetical	Unknown
<i>orf46</i>	46	Reverse	23360-23509	22185-22334	30187-30336	25234-25383	150	49	Conserved hypothetical	Unknown
<i>ybcA</i>	47	Forward	23500-24558	22325-23383	30327-31385	25374-26431	1059	352	YbcA	Unknown
<i>ibfA</i>	48	Reverse	24734-25078	23559-23903	31561-31905	26607-26951	345	114	IbfA	Abortive infection protein
<i>ibfC</i>	49	Forward	25268-25414	24093-24239	32095-32241	27141-27287	147	48	IbfC	Abortive infection protein
<i>ybeA</i>	50	Forward	25602-25811	24426-24635	32428-32637	27475-27684	210	69	YbeA	Unknown
<i>ybeB</i>	51	Forward	25942-26823	24766-25647	32768-33649	27815-28696	882	293	YbeB	Unknown
<i>orf52</i>	52	Reverse	27026-27220	25850-26044	33852-34046	28899-29093	195	64	Conserved hypothetical	Unknown
<i>ybfA</i>	53	Forward	27295-27747	26119-26571	34121-34572	29168-29620	453	150	YbfA	Unknown
<i>resA</i>	54	Forward	27749-28528	26573-27352	34574-35353	29622-30401	780	259	ResA site specific resolvase	Resolvase/Integrase
<i>orf55</i>	55	Forward	28708-29352	27532-28176	35533-36177	30581-31225	645	214	ParA homolog	ATPase like partitioning
<i>orf56</i>	56	Forward	29439-29747	28263-28571	36264-36572	31312-31620	309	102	Putative chaperonin	YbiA Unknownaperone
<i>parA</i>	57	Forward	30161-31141	28985-29965	36986-37965	32034-33014	981	326	ParA	ParA ATPase
<i>parB</i>	58	Forward	31134-31550	29958-30374	37958-38374	33007-33423	417	138	ParB	ParB recombinase
<i>impB</i>	59	Reverse	31552-32826	30376-31650	38376-39650	33425-34699	1275	424	ImpB	DNA polymerase V UmuC
<i>impA</i>	60	Reverse	32826-33263	31650-32087	39650-40087	34699-35136	438	145	ImpA	UV protection
<i>impC</i>	61	Reverse	33260-33508	32084-32332	40084-40332	35133-35381	249	82	ImpC	Dini DNA damage
<i>orf62</i>	62	Reverse	33505-33669	32329-32493	40329-40493	35378-35542	165	54	Conserved	Unknown

Gene	ORF	Direction	Location				Length (bp)	Protein Length (aa)	Product	Function
			pCH02	pCH03	pCT01	pT01				
<i>yccA</i>	63	Forward	33619-33888	32443-32712	40443-40712	35492-35761	270	89	YccA	Unknown
<i>orf64</i>	64	Reverse	33771-33884	NP	40595-40708	35644-35757	114	37	Conserved hypothetical	Unknown
<i>yccB</i>	65	Forward	33902-34828	32726-33652	40726-41652	35775-36701	927	308	YccB	Unknown
<i>ycdA</i>	66	Forward	34825-35136	33649-33960	41649-41960	36698-37009	312	103	YcdA	Unknown
<i>orf67</i>	67	Forward	35213-35896	34037-34720	42037-42720	37086-37769	684	227	Putative DNA methylase	DNA methylase
<i>yceA</i>	68	Forward	35897-36118	34721-34942	42721-42942	37770-37991	222	73	YceA	Unknown
<i>yceB</i>	69	Forward	36130-36564	34954-35388	42954-43388	38003-38437	435	144	YceB	Unknown
<i>ycfA</i>	70	Forward	36615-37003	35439-36209	43439-44209	38488-39258	771	256	YcfA	Unknown
<i>ycgA</i>	71	Forward	NP	36184-36654	44184-44654	39233-39703	471	156	YcgA	Unknown
<i>ycgB</i>	72	Forward	NP	36627-37052	44627-45052	39676-40101	426	141	YcgB	Putative antirestriction
<i>ycgC</i>	73	Forward	NP	37099-37521	45099-45521	40148-40570	423	140	YcgC	Unknown
<i>orf74</i>	74	Forward	NP	NP	45518-45709	40567-40758	192	63	Conserved hypothetical	Unknown
<i>ycaA</i>	75	Forward	NP	37780-38127	45780-46127	40829-41176	348	115	YcaA	Unknown
<i>ltra</i>	76	Forward	NP	NP	47131-49032	42180-44081	1902	633	Retro trranscriptase	Reverse transcriptase
<i>ssb partial</i>	77	Forward	NP	38479-39006*	49227-49667	44276-44716	441	146	Ssb Stability protein partial	Single stranded binding protein
<i>ykfF</i>	78	Forward	NP	39037-39297	49698-49958	44747-45007	261	86	YkfF	Unknown
<i>ycjA</i>	79	Forward	NP	39350-41314	50011-51975	45060-47024	1965	654	YcjA	ParB like domain
<i>psiB</i>	80	Forward	NP	41369-41803	52030-52464	47079-47513	435	144	PsiB	Plasmid SOS inhibition B
<i>psiA</i>	81	Forward	NP	41800-42519	52461-53180	47510-48229	720	239	PsiA	Plasmid SOS inhibition A
<i>ygaA</i>	82	Forward	NP	42516-43112	53177-53773	48226-48822	597	198	YgaA	Unknown

Gene	ORF	Direction	Location				Length (bp)	Protein Length (aa)	Product	Function
			pCH02	pCH03	pCT01	pT01				
<i>ardA</i>	83	Forward	NP	43575-44075	54236-54736	49285-49785	501	166	ArdA	Antirestriction protein
<i>orf84</i>	84	Forward	NP	44059-44202	54720-54863	49769-49912	144	47	Conserved hypothetical	Unknown
<i>orf85</i>	85	Reverse	NP	44215-44514	54876-55175	49925-50224	300	99	Conserved hypothetical	Unknown
<i>orf86</i>	86	Reverse	NP	44536-44781	55272-55481	50321-50530	210	69	Conserved hypothetical	Unknown
<i>orf87</i>	87	Reverse	NP	44783-45001	55444-55662	50493-50711	219	72	Conserved hypothetical	Unknown
<i>ydfA</i>	88	Forward	NP	44804-45238	55465-55899	50514-50948	435	144	YdfA	Unknown
<i>ydfB</i>	89	Forward	NP	45330-45596	55991-56257	51040-51306	267	88	YdfB	Unknown
<i>ydgA</i>	90	Forward	NP	45661-46602	56322-57263	51371-52312	942	313	YdgA	Unknown
<i>ygeA</i>	91	Reverse	NP	46865-47116	57526-57777	52575-52826	252	83	YgeA	Helix turn Helix domain
<i>ydhA</i>	92	Forward	NP	47236-47571	57897-58232	52946-53281	336	111	YdhA	Unknown
<i>ydiA</i>	93	Forward	NP	47696-48544	58357-59205	53406-54254	849	282	YdiA	Methyltransferase
<i>yggA</i>	94	Reverse	NP	48629-48964	59290-59625	54339-54674	336	111	YggA	Unknown
<i>orf95</i>	95	Reverse	NP	48954-49070	59615-59731	54664-54780	117	38	Conserved hypothetical	Unknown
<i>nikA</i>	96	Forward	NP	49198-49530	59859-60191	54908-55240	333	110	NikA	Relaxosome component
<i>nikB</i>	97	Forward	37002-37293	49541-52240	60202-62901	55251-57950	2700	899	NikB	Nickase
<i>trbC</i>	98	Reverse	37290-39621	52237-54567	62898-65228	57941-60277	2331	776	TrbC	Conjugal transfer
<i>trbB</i>	99	Reverse	39614-40684	54560-55630	65221-66291	60270-61340	1071	356	TrbB	Protein disulfide isomerase
<i>trbA</i>	100	Reverse	40703-41911	55649-56857	66310-67518	61359-62567	1209	402	TrbA	Conjugal transfer
<i>pndC</i>	101	Forward	42064-42348	57010-57294	67671-67955	62720-63004	285	94	PndC	Post segregational killing
<i>pndA</i>	102	Reverse	42427-42678	57373-57624	68034-68285	63083-63334	252	83	PndA	Post segregational killing
<i>orf103</i>	103	Reverse	43337-43513	58283-58459	68944-69120	63993-64169	177	58	Conserved hypothetical	Unknown

Gene	ORF	Direction	Location				Length (bp)	Protein Length (aa)	Product	Function
			pCH02	pCH03	pCT01	pT01				
<i>orf104</i>	104	Reverse	43542-43670	58488-58616	69148-69243*	64197-64325	129	42	Conserved hypothetical	Unknown
<i>IS4311 tnpA</i>	105	Reverse	NP	NP	69286-70173	NP	888	295	IS4311 transposase	Transposases
<i>orf106</i>	106	Forward	43905-44114	58851-59060	70824-71033	64560-64769	210	69	Conserved hypothetical	HEAT repeats
<i>excB</i>	107	Reverse	44186-44629	59132-59614	71105-71587	64841-65324	483	160	ExcB	Surface exclusion protein
<i>traY</i>	108	Reverse	44908-47078	59853-62023	71826-73996	65563-67733	2172	723	TraY	Conjugal transfer integral membrane protein
<i>traX</i>	109	Reverse	47175-47759	62120-62704	74093-74677	67830-68414	585	194	TraX	Conjugal transfer
<i>traW</i>	110	Reverse	47788-48265	62733-63935	74706-75908	68443-69645	1203	400	TraW	Lipoprotein
<i>traV</i>	111	Reverse	NP	63902-64516	75875-76489	69612-70226	615	204	TraV	Conjugal transfer
<i>traU</i>	112	Reverse	NP	64516-67560	76489-79533	70226-73270	3045	1014	TraU	Nucleotide binding protein
<i>traT</i>	113	Reverse	NP	67650-68450	79623-80423	73360-74160	801	266	TraT	Pilus protein
<i>traS</i>	114	Reverse	NP	68434-68622	80407-80595	74144-74332	189	62	TraS	Conjugal transfer
<i>traR</i>	115	Reverse	NP	68686-69090	80659-81063	74396-74800	405	134	TraR	Conjugal transfer
<i>traQ</i>	116	Reverse	NP	69141-69668	81114-81641	74851-75378	528	175	TraQ	Conjugal transfer
<i>traP</i>	117	Reverse	NP	69668-70372	81641-82345	75378-76081	705	234	TraP	Conjugal transfer
<i>traO</i>	118	Reverse	NP	70372-71661	82345-83634	76081-77370	1290	429	TraO	Conjugal transfer
<i>traN</i>	119	Reverse	NP	71664-72647	83637-84620	77373-78356	984	327	TraN	Conjugal transfer
<i>traM</i>	120	Reverse	NP	72658-73350	84631-85323	78367-79059	693	230	TraM	Conjugal transfer
<i>traL</i>	121	Reverse	NP	73347-73694	85320-85667	79056-79403	348	115	TraL	Signal peptide
<i>sogL</i>	122	Reverse	NP	73712-77479	85685-89452	79421-83188	3768	1255	DNA primase	DNA primase
<i>orf123</i>	123	Reverse	NP	77568-780118	89541-90092	83277-83827	552	183	Plasmid Endonuclease	Endonuclease
<i>traK</i>	124	Reverse	48317-48607	78133-78423	90107-90397	83842-84132	291	96	TraK	Conjugal transfer

Gene	ORF	Direction	Location				Length (bp)	Protein Length (aa)	Product	Function
			pCH02	pCH03	pCT01	pT01				
<i>traJ</i>	125	Reverse	48604-49752	78420-79568	90394-91542	84129-85277	1149	382	TraJ	ATP binding
<i>traI</i>	126	Reverse	49749-50567	79565-80383	91539-92357	85274-86092	819	272	TraI	Lipoprotein
<i>traH</i>	127	Reverse	50564-51022	80380-80838	92354-92812	86089-86547	459	152	TraH	Lipoprotein
<i>orf128</i>	128	Forward	51254-51403	81070-81219	93044-93193	86779-86928	150	49	Conserved hypothetical	Unknown
<i>traG</i>	129	Reverse	51417-52001	81233-81817	93207-93791	86942-87526	585	194	TraG	Histidine phosphatase domain
<i>traF</i>	130	Reverse	52061-53263	81877-83079	93851-95053	87586-88788	1203	400	TraF	Conjugal transfer
<i>traE</i>	131	Reverse	53348-54172	83164-83988	95138-95962	88873-89697	825	274	TraE	Conjugal transfer
<i>shfrci</i>	132	Reverse	54323-55477	84139-85293	96113-97267	89848-91002	1155	384	Shufflon DNA recombinase	DNA recombination
<i>shfC</i> [#]	133	Forward	59299-59550	85358-85609	97332-97582	91650-91901	251	NA	ShfC	Pilus tip
<i>shfC</i> [#]	134	Reverse	59084-59302	85606-85824	97579-97797	91898-92116	219	72	ShfC'	Pilus tip
<i>ISEcpI</i> [#]	135	Forward	55771-57033	87293-88168	98060-99322	92379-93641	1263	420	ISEcpI	Transposon
<i>bla</i> _{CTX-M-1} [#]	136	Forward	57321-58196	86974-87246	99610-100485	93929-94804	876	291	CTX-M-1	β-lactam hydrolysis
<i>orf477</i> [#]	137	Reverse	58243-58515	86693-86901	100532-100804	94851-95123	273	90	orf477	Unknown
<i>shfB</i> [#]	138	Forward	58588-58796	86693-86901	100877-101085	95196-95404	209	NA	ShfB	Pilus tip
<i>shfB</i> [#]	139	Reverse	58798-59050	86440-86691	101087-101337	95406-95657	251	NA	ShfB'	Pilus tip
<i>shfA</i> [#]	140	Forward	59572-59794	85846-86068	101357-101579	91053-91276	223	NA	ShfA'	Pilus tip
<i>pilV</i> truncated [#]	141	Reverse	NP	86065-86419	NP	91273-91629	357	118	PilV-T	Pilus tip
<i>pilV</i> [#]	142	Reverse	59791-61218	88456-89718	101576-102999	95651-96740*	1425	474	PilV	Pilus tip
<i>pilU</i>	143	Reverse	61218-61874	91030-91686	102999-103655	96740-97396	657	218	PilU	Peptidase
<i>pilT</i>	144	Reverse	61859-62419	91671-92231	103640-104200	97381-97941	561	186	PilT	Transglycolase
<i>pilS</i>	145	Reverse	62429-63043	92241-92855	104210-104824	97951-98565	615	204	PilS	prepillin
<i>pilR</i>	146	Reverse	63061-64158	92873-93970	104842-105939	98583-99680	1098	365	PilR	Integral membrane
<i>pilQ</i>	147	Reverse	64171-65724	93983-95536	105952-107505	99693-101246	1554	517	PilQ	ATP binding

Gene	ORF	Direction	Location				Length (bp)	Protein Length (aa)	Product	Function
			pCH02	pCH03	pCT01	pT01				
<i>pilP</i>	148	Reverse	65735-66187	95547-95999	107516-107968	101257-101709	453	150	PilP	Pilus assembly
<i>pilO</i>	149	Reverse	66174-67469	95986-97281	107955-109250	101696-102991	1296	431	PilO	Pillin accessory
<i>pilN</i>	150	Reverse	67462-69144	97274-98956	109243-110925	102984-104666	1683	560	PilN	Pilus biosynthesis
<i>pilM</i>	151	Reverse	69158-69595	98970-99407	110939-111376	104680-105117	438	145	PilM	Conjugal transfer
<i>pilL</i>	152	Reverse	69595-70662	99407-100474	111376-112443	105117-106184	1068	355	PilL	Pilus chaperone
<i>pilK</i>	153	Reverse	70696-71289	100508-101101	112477-113070	106218-106811	594	197	PilK	Pilus chaperone
<i>pilJ</i>	154	Reverse	71286-71582	101098-101394	113067-113363	106808-107104	297	98	PilJ	Conjugal transfer
<i>pilI</i>	155	Reverse	71758-72057	101570-101869	113539-113838	107280-107579	300	99	PilI	Pilus assembly
<i>traD</i>	156	Reverse	72174-72725	101986-102537	113955-114506	107696-108247	552	183	TraD	Conjugal transfer
<i>traC</i>	157	Reverse	72898-73581	102710-103393	114679-115362	108420-109103	684	227	TraC	Conjugal transfer
<i>traB</i>	158	Reverse	73835-74368	103647-104180	115616-116149	109357-109890	534	177	TraB	Conjugal transfer
<i>traA</i>	159	Reverse	74810-75097	104622-104909	116591-116878	110332-110619	288	95	TraA	Conjugal transfer

* Size of gene varies, NP = *orf* not present, # rearrangements occur to the order of these genes

7.3.3.1 Analysis of the IncI1 γ replication region

The replication of the IncI1 γ plasmids is mediated through the replication region. This includes the RepZ replication protein coded by the *repZ* gene, and the two regulators RepY (*repY*) and the antisense control region (Inc) (Asano *et al.*, 1999; Hama *et al.*, 1990). As with other members of the IncI family, plasmids are controlled through the antisense RNA which prevents the production of a pseudoknot (Nikoletti *et al.*, 1988). The translation undergoes both positive and negative regulation by RepY and Inc respectively. RepY enables the translation of *repZ* by inducing the formation of the pseudoknot by binding to the stem loop, and it is the process of translation as opposed to the protein which is required for *repZ* translation (Asano, Moriwaki and Mizobuchi, 1991; Asano *et al.*, 1999). The Inc RNA acts in *trans* and binds to two sites of *repZ* mRNA which is the target site of Inc RNA and additionally the ribosomal binding site (Asano *et al.*, 1999). The stem loops sequence is present from 302-352 bp in pCH02, pCH03 and pT01, and 5,255-5,305 bp in pCT01 with the Inc region spanning 1-418 bp and 5,059-5,476 bp respectively (Nikoletti *et al.*, 1988).

7.3.3.2 Analysis of the IncI1 γ plasmid transfer region

The transfer regions in the IncI1 γ plasmids range in size from 57,290 bp in pCT01 (59,589-116,878 bp), 55,712 bp in pCH03 (49,198-104,909 bp) and pT01 (54,908-110,619 bp) and typically consists of 48 ORFs. The transfer region in pCH02 is smaller due to a deletion at only 38,105 bp in size (37,002-75,106 bp). The IncI1 γ transfer region consists of four elements (i) *traABCD* regulator region, (ii) *pil* genes of the type IV system, (iii) *tra/trb* conjugation genes and (iv) *oriT* and *nikAB* (Sampei *et al.*, 2010; Komano *et al.*, 2000). The regulator genes are located downstream of the *pil* region, with *traB* and *C* essential for liquid conjugation, and for the formation of the thin pilus (Kim, Funayama and Komano, 1993). The type IV pilus is coded by the 14 *pil* genes, *pilI, J, K, L, M, N, O, P, Q, R, S, T, U* and *V* (Yoshida, Kim and Komano, 1999; Sampei *et al.*, 2010). This

region is not needed for conjugation on solid surfaces but is important for conjugations in liquid (Yoshida, Kim and Komano, 1999). Located at the C terminus of the *pilV* gene is the shufflon region, a multi inversion system which alters the pilus tip and can affect host specificity, through the generation of seven C terminal segments A, A', D', C, C', B', and B (Komano *et al.*, 1994; Komano, Kim and Yoshida, 1995). The shufflon region is present in pCH02, pCH03, pCT01 and pT01 which has undergone various rearrangements between the plasmids and is the location of the insertion of the *bla*_{CTX-M-1} which will be discussed later.

The *tra* region consists of 22 genes, *traE, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X, and Y* which form a large transfer operon, required for plasmid conjugative transfer (Komano *et al.*, 2000; Sampei *et al.*, 2010). The *traF, G, L* and *N* code for signal peptides, *traJ* and *U* nucleotide binding proteins and *traH, I* and *W* are lipoproteins (Komano *et al.*, 2000). The *tra* genes *K, M, O, P, Q, R, X, and Y* are all integral membrane proteins contributing to the formation of the pilus. The transfer region contains the *trbABC* genes which are essential for conjugation as found by Furuya and Komano (1996), *trbB* is similar to protein disulfide isomerase (Furuya and Komano, 1996). Plasmids pCH03, pCT01 and pT01 all have a complete *tra* operon, however a 14,910 bp deletion (75,144-90,053 bp in pCT01) has occurred in pCH02 causing the loss of *traV, U, T, S, R, Q, P, O, N, M, and L, sogL* and the plasmid endonuclease; the *nikA* gene has also been lost in a separate deletion. The transfer region also contains the DNA processing genes *nikAB* which are located at the start of the transfer region. NikA binds to the DNA at the *OriT*, which is then bound by NikB forming the relaxosome, NikB cleaves the DNA for transfer through the pilus (Furuya, Nisioka and Komano, 1991; Furuya and Komano, 1995). As well as the conjugation genes, the transfer region also contains a DNA primase gene *sogL* which facilitates the transfer of single stranded DNA between cells during conjugation (Merryweather *et al.*, 1986). Inc11 γ plasmids in this study were found to have the surface exclusion gene *excB*. ExcB has a role in recognising the TraY protein in the cell membrane

and restricts mating with the cells containing the same plasmid (Furuya and Komano, 1994; Sakuma *et al.*, 2013). The Addiction system genes *pndAC* are also present in the transfer region, located downstream of the *trbABC* genes (Furuya and Komano, 1996).

7.3.3.3 Analysis of the IncI1 γ plasmid stability regions

The IncI1 γ plasmids have several genes which contribute to the stability of the plasmid and isolate. The IncI1 γ plasmids appear to have two partitioning systems the first is the *parAB* genes which code for an ATPase and recombinase respectively, which localise the plasmids during cell division (Gerdes *et al.*, 2004). Upstream of the *parAB* genes is a *resA* gene and *parA* homolog which code for a recombinase and putative ATPase respectively, which are likely to have a role in the partitioning of the plasmids. The *psiAB* plasmid SOS inhibition genes are present, which are important for the establishment of the plasmid in a new host cell, and are transferred early in the conjugative process (Althorpe *et al.*, 1999; Bagdasarian *et al.*, 1986). Two addiction systems are present, the first is the PndAC coded by the *pndAC* genes and is an antisense RNA regulated system, the *pndA* produces the toxin gene which targets the cell membrane causing cell lysis, the antitoxin is the RNA molecule from the *pndC* gene, which inhibits the translation of *pndA* mRNA (Mnif *et al.*, 2010; Furuya and Komano, 1996). The second putative addiction system is coded by the *yacB* gene. The translated protein has similarity to the RelE the toxin of the classical proteic systems, which represses translation, however the antitoxin protein RelB could not be identified, instead located upstream was the gene *yacA* which coded a protein with the COG3905 domain which RelB belongs to (Gronlund and Gerdes, 1999; Gotfredsen and Gerdes, 1998). Two phage protection genes were also identified in the plasmid, *ibfAC*, which inhibit the growth of phages (Furuichi, Komano and Nisioka, 1984). The single stranded binding protein gene *ssb* was also present on the IncI1 γ plasmids, and is required for stable DNA synthesis (Howland *et al.*, 1989; Bates *et al.*, 1999). The *ardA* gene part of the leading strand, prevents the activity of restriction enzymes such as EcoK1, preventing degradation of the plasmid (Del'ver *et al.*,

2002; Althorpe *et al.*, 1999; Nekrasov *et al.*, 2007). As seen in the IncZ plasmid, the IncI1 γ plasmids also have the *yafB* gene which is a homolog of the FinO protein that encodes a repressor of bacterial conjugation (Frost, Ippen-Ihler and Skurray, 1994). Plasmids pCH03, pCT01 and pT01 all have the stability genes described above however a 16,121 bp deletion in the pCH02 plasmids, has caused the loss of *ssb*, *psiAB*, *ardA*, and *nikA*.

7.3.3.4 Analysis of IncI1 γ Resistance regions

The IncI1 γ plasmid backbone of pCH02, pCH03 and pT01 appeared to be a suitable vector for resistance genes with two resistance insertions, which included the MDR region and the *bla*_{CTX-M-1}. The pCT01 plasmid also had the separate insertion of *bla*_{TEM-1}.

7.3.3.4.1 The *bla*_{CTX-M-1} genes in the IncI1 γ plasmids

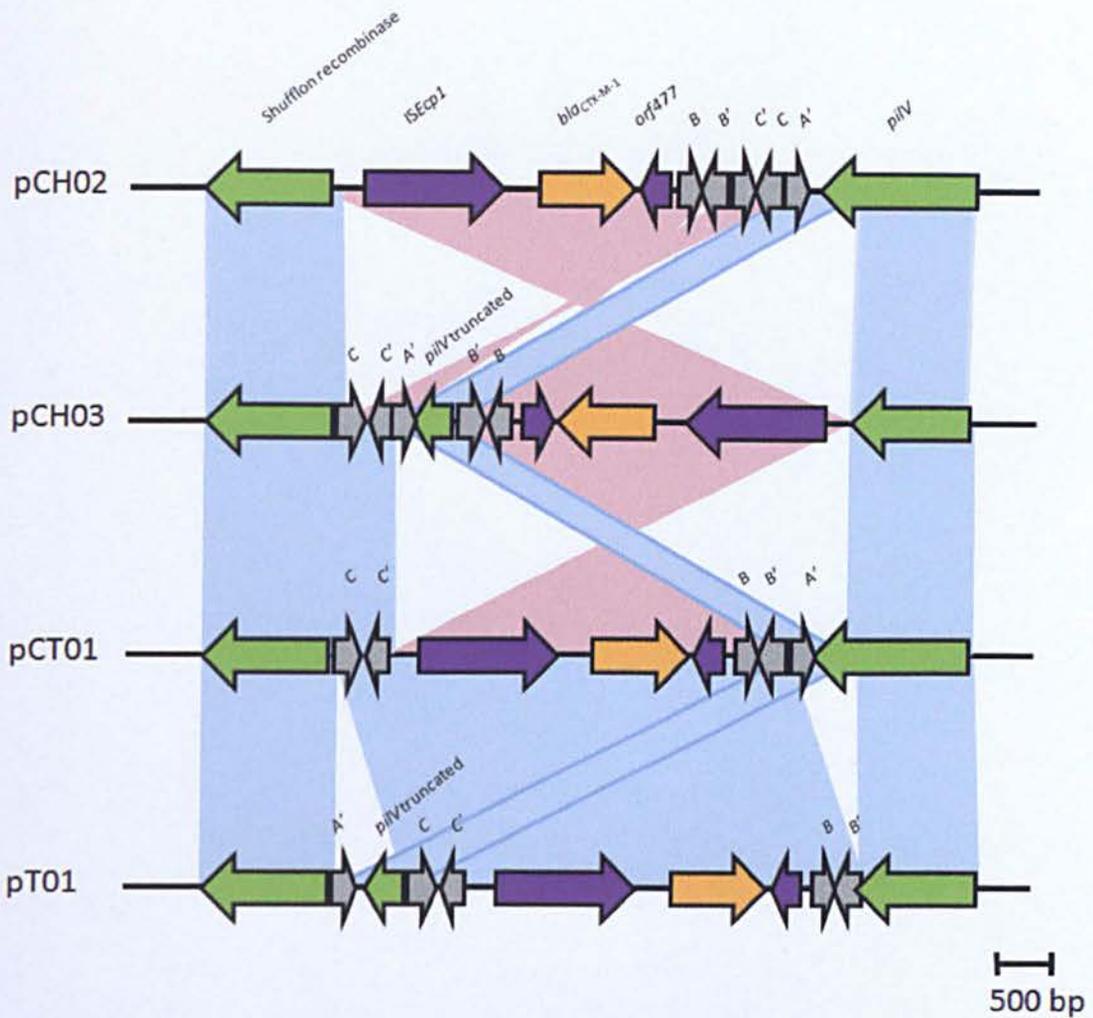
The *bla*_{CTX-M-1} gene was first identified in Germany, and was found on plasmid pMVP-3, its isoelectric point indicated it was unrelated to TEM and SHV and was designated CTX-M the first use of this designation (Bauernfeind, Grimm and Schweighart, 1990; Bauernfeind *et al.*, 1996). All of the sequenced IncI1 γ plasmid in this study had the *bla*_{CTX-M-1} gene which was part of a 3,014 bp *ISEcpI* mediated insert with the 5 bp direct repeats of AAAAA located at either end of the insertion unit. The insert is located in pCH02 between 55,580-58,593 bp, in pCH03 between 86,896-89,909 bp, in pCT01 between 97,869-100,882 bp and in pT01 between 92,188-95,201 bp. The *bla*_{CTX-M-1} is located 81 bp downstream of the inverted right repeat of *ISEcpI*, and *orf477* is located 46 bp downstream of *bla*_{CTX-M-1}. This *bla*_{CTX-M-1} environment has been reported in clinical isolates from France between 2001 and 2002 in both *E. coli* and *Klebsiella pneumoniae* and in both horse and cattle isolates from Germany (Eckert, Gautier and Arlet, 2006; Schink *et al.*, 2013). The pCTX1261 (HF549090) *bla*_{CTX-M-1} plasmid in cattle from Germany was found on an IncI1 γ plasmid, as were those in this study (Schink *et al.*, 2013).

The *bla*_{CTX-M-1} has inserted into the shufflon region in all four plasmids located downstream of the shufflon recombinase gene, (*rci*). Due to the nature of the shufflon region the environment surrounding the genes varies (Komano, Kim and Yoshida, 1995; Komano *et al.*, 1994). In plasmids pCH02, pCT01 and pT01 inserts have occurred in shufflon B which has resulted in its fragmentation, shown in Figure 7.6. However the insert varies in distance from the *rci* gene in pCH02 being 107 bp, in pCT01 606 bp and 1,190 bp in pT01, which is likely due to rearrangements in the shufflon region, or separate insertion events. The shufflon region is located downstream of the insert in pCH02, downstream of the *shfC* and *shfC'* region in pCT01, and in the same location in pT01, however there has been rearrangements to the *pilV* and *shfA*. The insert in pCH03 has occurred in the reverse orientation to the other plasmids and is located 35 bp upstream of the *pilV* gene which has undergone the same rearrangement as observed in pT01 (Figure 7.6).

7.3.3.4.2 The *bla*_{TEM-1} gene in pCT01

In addition to the *bla*_{CTX-M-1}, plasmid pCT01 also had the *bla*_{TEM-1} gene. As seen before this gene was located adjacent to the transposon Tn3 in a 4,948 bp mobile genetic element located between 82-5,029 bp, which was upstream of a conserved hypothetical gene and *repY* (Partridge and Hall, 2005; Sutcliffe, 1978). The Tn3 insertion is identical to that found on the IncF plasmid pAPEC-O2-R (AY214164) from an avian *E. coli*, and shares 99% identity with that found in the first the Tn3-*bla*_{TEM} (V00613) sequenced in 1976 (Johnson *et al.*, 2005; Sutcliffe, 1978). A similar Tn3 unit (97% identity) was also identified in the IncI γ plasmid pEK204 (EU935740) isolated from *E. coli* ST131 from the UK which also harbours the *bla*_{CTX-M-3}, and the IncB (member of the IncI complex) plasmid pR3521 (GU256641) from *E. coli* (Woodford *et al.*, 2009; Papagiannitsis *et al.*, 2011).

Figure 7.6 The *bla*_{CTX-M-1} insertion unit in plasmids pCH02, pCH03, pCT01 and pT01



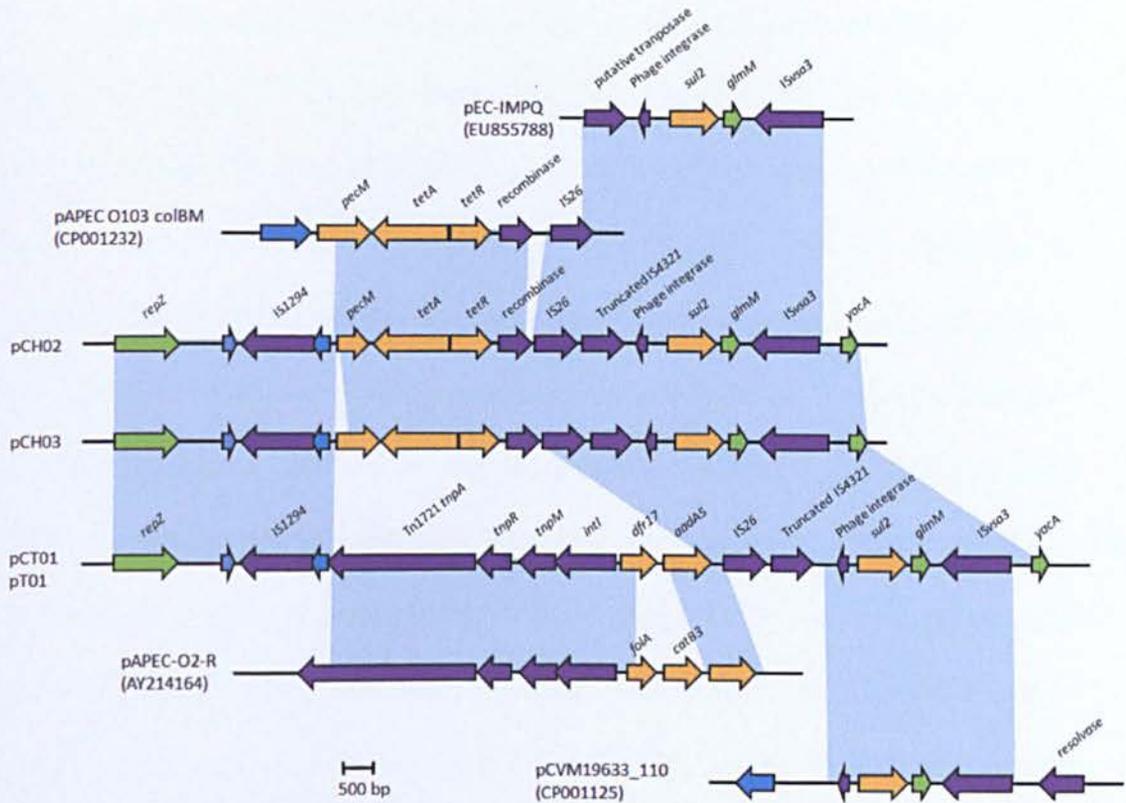
The *ISEcp1*-*bla*_{CTX-M-1}-*orf477* insertion units in pCH02, pCH03, pCT01 and pT01 with rearrangements to the shufflon region, shown by grey arrows. Purple arrows are the *ISEcp1* and *orf477*, orange arrows are the *bla*_{CTX-M-1} genes. Blue regions show sequence homology, and red show inverted sequence homology, drawn to scale.

7.3.3.4.3 The MDR regions in the Inc11 γ plasmids

The MDR region in pCH02 and pCH03 has not been published before in GenBank and seems to be a composite of various mobile elements. The insertion in pCH02 is 9,515 bp located between 2,338-11,852 bp and in pCH03 is 9,676 bp located between 2,338-12,013 bp. In both plasmids the MDR region is located 851 bp downstream of the *repZ* gene, which is where the right inverted repeat of IS1294 is located upstream of a hypothetical which is likely to have inserted as part of another element as the left inverted

repeat of *IS1294* is absent. The first resistance gene is a *pecM* like gene, and in pCH03 this gene is 201 bp larger than that in pCH02, this suggest this gene may have been captured in a separate event, apart from this difference the remaining MDR is identical in both plasmids. Downstream of *pecM* is the tetracycline resistance genes *tetAR* which are upstream of a putative recombinase, this region is similar to a large MDR region in pAPEC-O103-ColBM (CP001232) a IncFIIA/FIB hybrid plasmid isolated from a chicken with ExPEC as shown in Figure 7.7 (Johnson *et al.*, 2010). Located downstream of the recombinase is the transposase *IS26* which has inserted into the N terminus of *IS4321* truncating the transposase. The *sul2* gene is downstream of a putative phage integrase, and upstream of the phosphoglucosamine mutase gene *glmM*, the final transposase is *ISvsa3* which is in the reverse orientation with the left inverted repeat located at 11,803-11,858 bp in pCH02 and 11,964-12,013 bp in pCH03. The *IS26* to *ISvsa3* region was also identified in the IMP producing IncHI2 plasmid pEC-IMPQ (EU855788) from *Enterobacter cloacae* (Figure 7.7) (Chen *et al.*, 2009). The MDR in pCT01 and pT01 are identical and as with pCH02 and pCH03, have not been published before in GenBank. The MDR of both pCT01 and pT01 share the same start as plasmid pCH02 and pCH03 being located 851 bp downstream of *repZ* and the first 1,714 bp which includes the *IS1294*. However the identical 12,725 bp insertions in pCT01 and pT01 located between 7,291-20,015 bp and 2,338-15,062 bp respectively vary with respect to resistance genes. The MDR shares with pCH02 and pCH03, the last 4,593 bp, although both pCT01 and pT01 have an additional 14 bp downstream of *IS26* (Figure 7.7). Downstream of the *IS1294* is the transposon Tn1721 comprising of the *tnpA*, *tnpR* and *tnpM* which is linked to a integrase gene *intI1* which was associated with *dfrA17* and *aadA5* gene cassettes. The transposon linked to the integron most closely matches the IncF plasmid pAPEC-O2-R (AY214164) from an avian *E. coli*, however the integron has the *folA* and *catB3* genes which are absent in pCT01 and pT01 (Johnson *et al.*, 2005). The pCT01 and pT01 integron has also been identified in other plasmids such as the IncFII-FIB pTN48

Figure 7.7 Comparison of the MDR regions in pCH02, pCH03, pCT01 and pT01



Comparison of the MDR regions of pCH02 and pCH03 with pAPEC O103 colBM and pEC-IMPQ, and pCT01 and pT01 with pCH03, pAPEC-O2-R and pCVM19633_110. Purple arrows are mobile elements, orange arrows are resistance genes, blue arrows are known genes and green arrows are hypothetical genes. Blue regions are areas of homology, drawn to scale.

(FQ482074), which also has the genes *qacE1* and *sul1* and the IncF pEK499 (EU935739) which also has *sul1* and *chrA* (Woodford *et al.*, 2009; Billard-Pomares *et al.*, 2011). This may suggest that the integron in pCT01 and pT01 had more resistance genes but the insertion of IS26 has caused their loss. Downstream of *aadA5* is the IS26 transposon which is homologous with pCH02 and pCH03. The phage integrase linked to the *sul2*, *glmM* and *ISvsa3* has also been identified in pCVM19633_110 (CP001125) isolated from *Salmonella* Swarzengrund on dried chili in Thailand in 2002 (Fricke *et al.*, 2011).

7.3.3.5 Analysis of additional MGE's and accessory genes

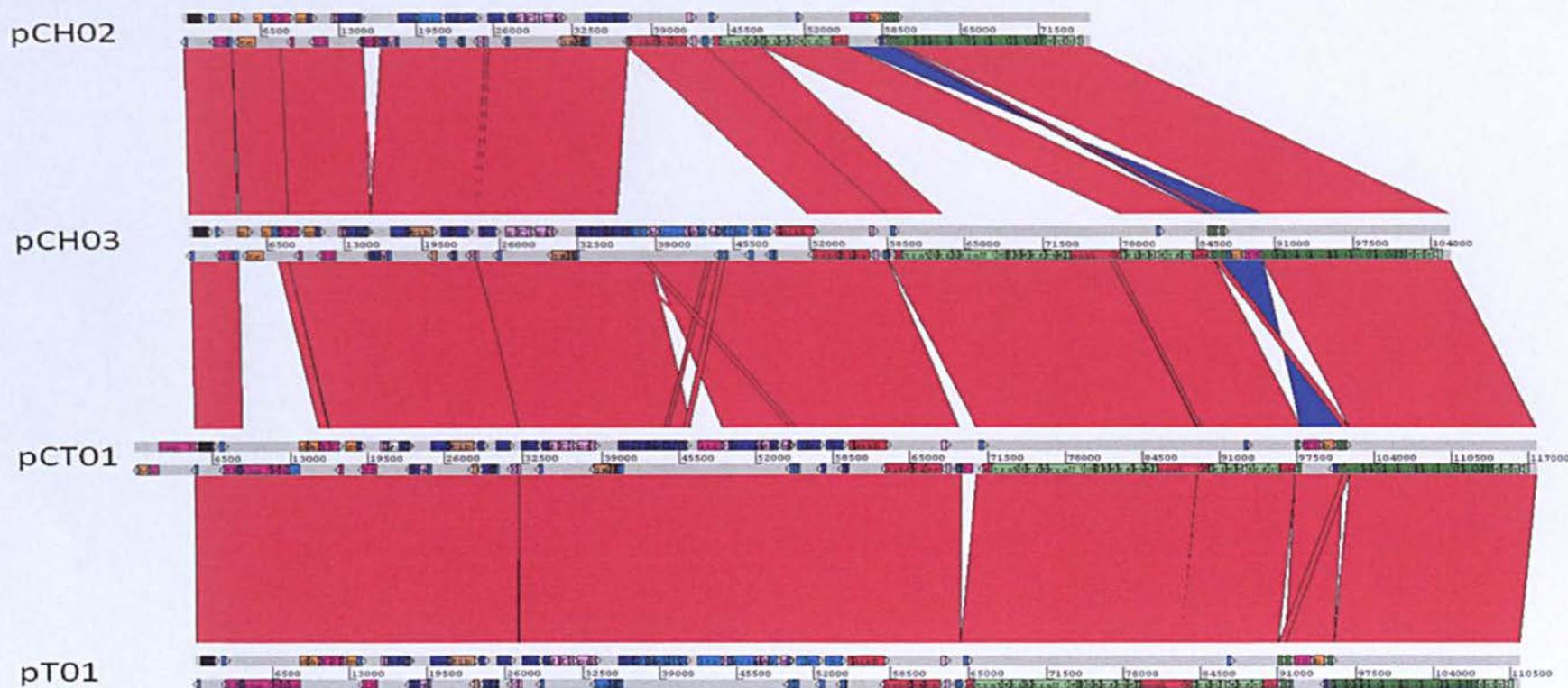
Apart from the insertion sequences and transposons associated with resistance genes several other insertion were identified. In plasmid pCH02, the insertion sequence IS2 was identified downstream of the *yeaA* gene located between 15,085-16,313 bp which has resulted in the loss of the C terminus of the *yeaB* gene. Plasmid pCT01 had the insertion of IS3411 located 69,244-70,553 bp, downstream of a conserved hypothetical gene, however, it was not associated with any resistance genes. Both pCT01 and pT01 both have a 2,661 bp insertion located between 46,518-49,178 bp in pCT01 and 41,567-44,227 bp in pT01, both of which are present 1,003 bp downstream of *ycaA*. Within this insertion is a 1,902 bp gene (*Itra*) encoding a 633 amino acid RNA reverse transcriptase related to phage, the only other entry in GenBank of this sequence is present in the plasmid pEK499 (16,631-18,532 bp) which differ by only a single nucleotide which is synonymous (Woodford *et al.*, 2009). This insertion is likely to be the result of a bacteriophage which has integrated, although this is the only foreign DNA that could be identified.

The IncI1 γ plasmids in this study have the colicin gene *cib*, and immunity gene *imm* and are likely to be colicigenic plasmids. Colicins kill susceptible bacteria in the environment that do not encode the immunity genes, reducing environmental competition (Ayala, Krane and Hartl, 1994; Males and Stocker, 1980). As with plasmids R64 and pColIb-P9, the IncI1 γ plasmids all contain the *impCAB* operon, which is involved in the survival after exposure to UV radiation and DNA damage (Runyen-Janecky, Hong and Payne, 1999; Khmel *et al.*, 1981). Both of these gene regions are likely to enhance the survival of the isolate harbouring the plasmids and so the plasmids themselves will persist, contributing to the resistome.

7.3.4 Comparison of the IncI1γ plasmids pCH02, pCH03, pCT01 and pT01

Plasmids pCH02, pCH03, pCT01 and pT01 were compared using BLASTn and ACT. Plasmid pCH02 had a coverage without insertions with pCH03, pCT01 and pT01 of 66%, 66% and 66% respectively. pCH03 had a coverage with pCH02, pCT01 and pT01 of 99%, 100% and 100% respectively. pCT01 had a coverage with pCH02, pCH03 and pT01 of 99%, 100% and 100% respectively. pT01 had a coverage with pCH02, pCH03 and pCT01 of 99%, 100% and 100% respectively. All of the plasmids were highly similar with the exception of pCH02 which has two large deletions of 16,121 bp between 35,828-51,948 bp in pCH03 and 14,870 bp of the *tra* region between 63,211-78,080 bp in pCH03, as shown by the DNA homology in Figure 7.8. Both of these deletions have led to lower coverage with the IncI1γ plasmids sequenced in this study. Plasmids pCH03 and pCH02 share the same MDR region located in the same position, with variation seen in the *pecM* gene, pCH02 also has the IS2 insert which is absent in pCH03. The shufflon region of these plasmids also varies, with *pilV* in pCH03 in two fragments which may suggest the pilus tip segment was rearranging at the time of sequencing, while in pCH02 the *pilV* is intact, the *ISEcp1-bla_{CTX-M-1}-orf477* insert is also in the reverse orientation in pCH03. Plasmids pCT01 and pT01, have a coverage of 100% suggesting they are closely related. Both plasmids share the same MDR insert, and also a reverse transcriptase unique to these plasmids and pEK499 (Figure 7.8). The main difference observed were the Tn3 transposon linked to *bla_{TEM-1}* and *IS4311* in pCT01 that are absent in pT01. Also observed was a rearrangement in the shufflon region of the plasmids, with the *pilV* truncated in pT01 as in pCH03 suggesting a possible pilus rearrangement. Plasmids were analysed *in silico* for their pMLST, plasmid pCH03, pCT01 and pT01 all belonged to ST3, however pCH02 could not be typed as only the *pilL*, *repII* and *trbA* genes were present, however the alleles of these markers indicated that pCH02 could belong to ST3, supported by the close sequence homology with pCH03.

Figure 7.8 Artemis comparison tool analysis of the IncI1 γ plasmids pCH02, pCH03, pCT01 and pT01



Artemis comparison tool analysis of the DNA sequences pCH02, pCH03, pCT01 and pT01. Green arrows indicate genes involved in conjugation with the light green indicating the *tra* loci and the darker green are the *pil* loci. Red arrows are other genes involved in conjugation, orange arrows indicate resistance genes, and magenta arrows are mobile elements, light pink are genes involved in stability, brown are accessory genes, dark blue are known genes and cyan are hypothetical genes. Red regions are homologous sequence and blue regions are homologous areas in the reverse orientation, white regions show areas of no homology.

7.3.5 Comparisons with GenBank plasmids

Plasmids pCH02, pCH03, pCT01 and pT01 were compared with 16 GenBank plasmids pNF1358 from *Salmonella enterica* Thompson, pSL476_91 from *Salmonella enterica* Heidelberg, pColIB-P9 from *Shigella sonnei*, pCVM29188_101 from *Salmonella enterica* Kentucky, pSH1148_107 from *Salmonella enterica* Heidelberg, pND11_107 from *E. coli*, pEC_Bactec from *E. coli*, pSE11-1 from *E. coli*, pEK204 from *E. coli*, pND12_96 from *E. coli*, pESBL-EA11 from *E. coli*, pCS0010A_95 from *Salmonella enterica* Kentucky, pPWD4_103 from *E. coli*, pUNMF18_IncI1 from *E. coli*, pUMNK88_91 from *E. coli*, and R64 from *Salmonella enterica* Typhimurium. The coverage with these plasmid varied between plasmids but ranged between 70-97%, excluding pUMNF18_IncI1, as shown in Table 7.3.

The ORFs of each of the plasmids pCH02, pCH03, pCT01 and pT01 were used to generate a database which could be used to screen for each ORF against the GenBank plasmids using the BLASTn algorithm. The results of these BLASTn screens were used to create a heat map for the presence, partial presence or absence of the ORF. All of the ORFs present in pT01 were also present in pCT01, as were those of pCH02 in pCH03, with the exception of IS2. The heat map comparisons of plasmids pCH03 (Figure 7.9) and pCT01 (Figure 7.10) with those from GenBank revealed that IncI1 γ plasmids share a highly similar *tra* and *pil* loci involved in solid and liquid conjugations. Plasmid pCH02 as mentioned has a deletion in the *tra* loci, and pUMNF18_IncI1 also has a large deletion to both the *tra* and *pil* loci, eight plasmids also lacked the *traD* gene. The genes *ybcA* to a conserved hypothetical region, which includes the infective abortive genes *ibfAC* was absent in 12 plasmids. The region of genes from *yacA* to *yaeA* which includes the putative addiction system *yacAB* was absent in 9 plasmids. The partitioning genes *parAB* were absent in five plasmids and the *impCAB* genes were absent in three plasmids. All of the plasmids had the *ardA* and *psiAB* genes and the *yafB* (*finO* homolog) except for pSE11-1 which only had 60.53% of the gene present. pSE11-1 also

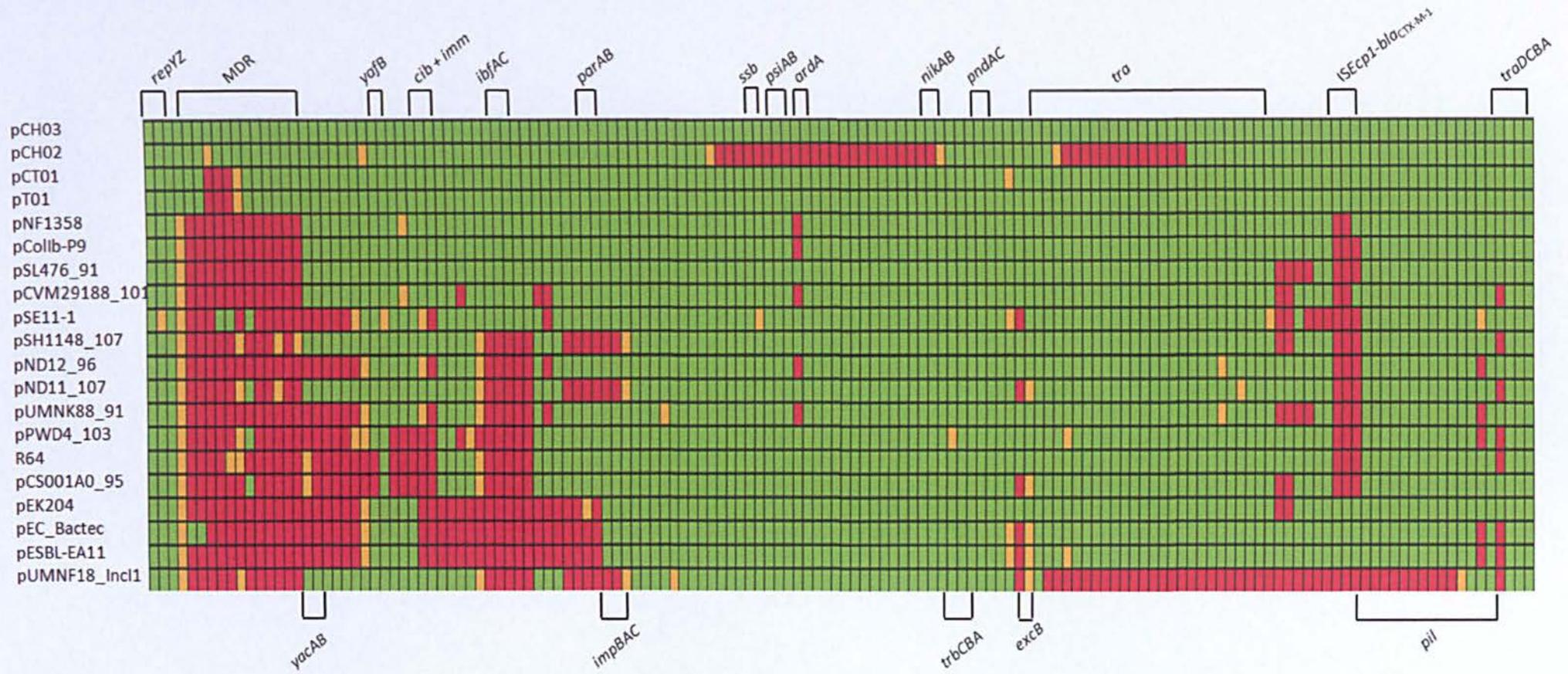
Table 7.3 GenBank plasmids compared with pCH02, pCH03, pCT01 and pT01

Plasmid	Accession	% coverage with no inserts				Size (bp)	Rep	Host	Source	Location	Date	Resistance genes	References
		pCH02	pCH03	pCT01	pT01								
CH02	NA	NA	66	66	66	75,796	I1γ	<i>E. coli</i>	Chicken	UK	2006	<i>bla</i> _{CTX-M-1} , <i>tetRA</i> , <i>sul2</i> , <i>pecM</i>	This study
CH03	NA	99	NA	100	100	105,608	I1γ	<i>E. coli</i>	Chicken	UK	2010	<i>bla</i> _{CTX-M-1} , <i>tetRA</i> , <i>sul2</i> , <i>pecM</i>	This study
CT01	NA	99	100	NA	100	117,577	I1γ	<i>E. coli</i>	Cattle	UK	2008	<i>bla</i> _{CTX-M-1} <i>bla</i> _{TEM-1} , <i>dfrA17</i> , <i>aadA5</i> <i>sul2</i>	This study
T01	NA	99	100	100	NA	111,318	I1γ	<i>E. coli</i>	Turkey	UK	2006	<i>bla</i> _{CTX-M-1} , <i>dfrA17</i> , <i>aadA5</i> , <i>sul2</i>	This study
pNF1358	DQ017661	96	97	97	97	99,331	I1γ	<i>Salmonella</i> Thompson	Human	USA	1996	<i>bla</i> _{CMY-2}	Dunne <i>et al</i> (2000)
pCollb-P9	AB021078	96	97	97	97	93,399	I1γ	<i>Shigella</i> <i>Sonnei</i>	NA	Japan	NA	NA	Sampei <i>et al</i> (1998)*
pSL476_91	CP001118	95	97	97	97	91,374	I1γ	<i>Salmonella</i> Heidelberg	Turkey	USA	2003	NA	Fricke <i>et al</i> (2011)
pCVM29188_101	CP001121	92	94	94	94	101,461	I1γ	<i>Salmonella</i> Kentucky	Chicken	USA	2003	<i>bla</i> _{CMY-2}	Fricke <i>et al</i> (2009)
pSE11-1	AP009241	84	89	89	89	100,021	I1γ	<i>E. coli</i>	Human	Japan	2008	<i>tetAR</i>	Oshima <i>et al</i> (2008)
pSH1148_107	JN983049	82	88	88	88	106,833	I1γ	<i>Salmonella</i> Heidelberg	Human	USA	2008	<i>aacC</i> , <i>aadA</i> , <i>sul1</i>	Han <i>et al</i> (2012)

Plasmid	Accession	% coverage with no inserts				Size (bp)	Rep	Host	Source	Location	Date	Resistance genes	References
		pCH02	pCH03	pCT01	pT01								
pND12_96	HQ114282	82	87	87	87	92,290	I γ	<i>E. coli</i>	Pig	USA	2007	NA	Johnson <i>et al</i> (2011)
pND11_107	HQ114281	80	86	86	86	107,138	I γ	<i>E. coli</i>	Pig	USA	2007	<i>aadA2, cmlA, aadA1, sul2</i>	Johnson <i>et al</i> (2011)
pUMNK88_91	CP002731	80	86	86	86	90,868	I γ	<i>E. coli</i>	Pig	USA	2007	NA	Shepard <i>et al</i> (2012)
pPWD4_103	HQ114284	77	84	84	84	103,297	I γ	<i>E. coli</i>	Pig	USA	2007	<i>hph, aac(3), strAB</i>	Johnson <i>et al</i> (2011)
R64	AP005147	76	84	84	84	120,826	I γ	<i>Salmonella</i> Typhimurium	Human	Japan	1966	<i>tetDCAR strAB</i>	Sampei <i>et al</i> (2010)
pCS0010A_95	HQ114283	75	83	83	83	95,175	I γ	<i>Salmonella</i> Kentucky	Chicken	USA	2007	NA	Johnson <i>et al</i> (2011)
pEK204	EU935740	72	81	81	81	93,732	I γ	<i>E. coli</i>	Human	UK	2009	<i>bla_{CTX-M-3}, bla_{TEM-1}</i>	Woodford <i>et al</i> (2009)
pEC_Bactec	GU371927	70	79	79	79	92,970	I γ	<i>E. coli</i>	Horse	Belgium	2010	<i>bla_{CTX-M-15} bla_{TEM-1}</i>	Smet <i>et al</i> (2010b)
pESBL-EA11	CP003290	70	79	79	79	88,544	I γ	<i>E. coli</i>	Human	Germany USA	2011	<i>bla_{CTX-M-15} bla_{TEM-1}</i>	Ahmed <i>et al</i> (2012)
pUMNF18_IncII	AGTD0100 0002	49	49	48	49	69,065	I γ	<i>E. coli</i>	Pig	USA	2006	<i>aadA1, aadA2, cmlA</i>	Shepard <i>et al</i> (2012)

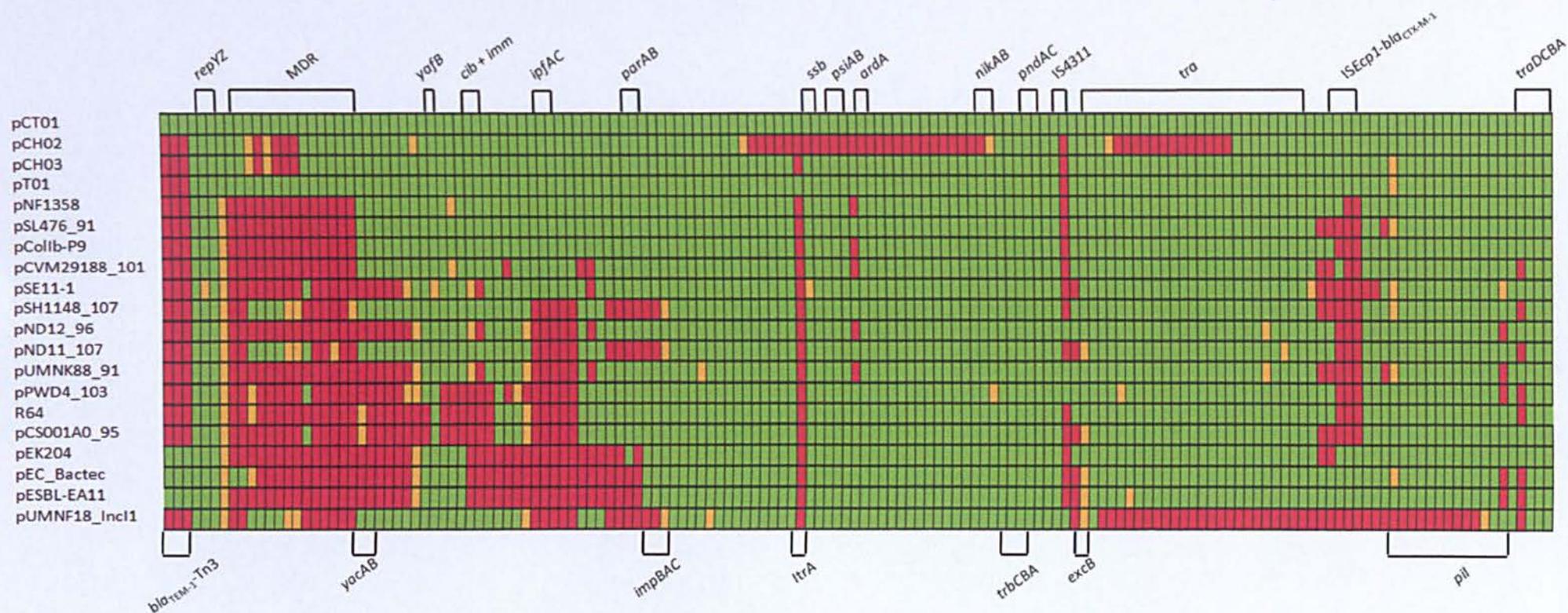
* Date submitted to GenBank and unpublished.

Figure 7.9 Heat map of pCH03 ORF comparison with GenBank IncI1 γ plasmids



Heat map of all of the ORFs of pCH03 compared to the IncI1 γ plasmids. Green indicates the presence of the ORF, orange indicates less than 80% of the ORF present and red is absent. Data generated using stand alone BLASTn.

figure 7.10 Heat map of pCT01 ORF comparison with GenBank IncI1γ plasmids



Heat map of all of the ORFs of pCT01 compared to the IncI1γ plasmids. Green indicates the presence of the ORF, orange indicates less than 80% of the ORF present and red is absent. Data generated using stand alone BLASTn.

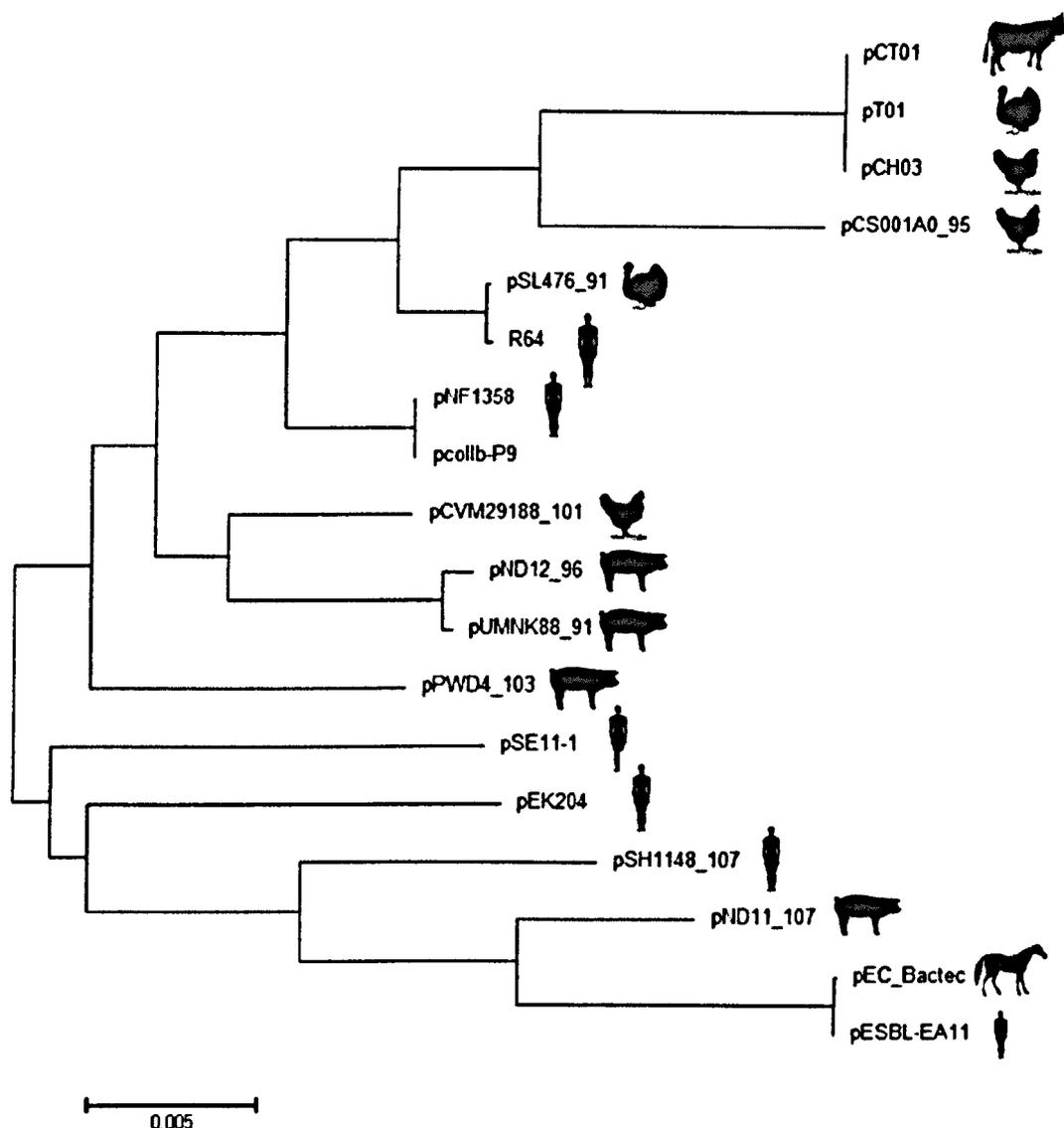
had a variation in the *repY* gene, with only 64.44% of the gene present, all the other plasmids had the *repY* and *repZ* genes present. The MDR regions of pCH02, pCH03, pCT01 and pT01 were unique to these plasmids when compared to the GenBank IncI1 γ plasmids in this study, the integrase gene *IntI* was identified in pSH1148_107, pND11_107 and pUMNF18_IncI1 and the tetracycline resistance genes *tetRA* was present in pSE11-1. The *bla*_{TEM-1-Tn3} ORFs were identified in pEC_Bactec, pEK204 and pESBL-EA11, which coincidentally also all have *bla*_{CTX-M} genes.

The phylogeny of the plasmids was compared using a concatemer of sequences present in all plasmids with the exception of pCH02 and pUMNF18_IncI1 due to the substantial deletions, in these plasmids. The *pilV* gene and shufflon region were also excluded due to rearrangements to this region. The phylogenetic comparison of the plasmids identified that pCH03, pCT01 and pT01 belonged to the same group with pCS001A0_95 being the most closely related, as shown in Figure 7.11. pNF1358 and pColb-P9, and pEC_Bactec and pESBL-EA11 were in the same cluster, with pSL476_91 and R64, and pND12_96 and pUMNK88_91 closely related (Figure 7.11). Plasmids were compared in more detail using Artemis comparison tool (ACT), with pCT01 being used as a representative plasmid for those sequenced in this study.

7.3.5.1 Comparison of pCT01 with the human plasmids pColIb-P9, pEK204 and pESBL-EA11

Plasmid pColIb-P9 was one of the first IncI1 γ plasmids to be worked on, isolated from *Shigella sonnei* in Japan (Sampei and Mizobuchi, unpublished), this plasmid has no known resistance genes or virulence genes. pColIb-P9 shares a high similarity with pCT01 sharing the *tra*, *pil* and stability genes including those between *yacA* and *yaeB* as shown by the regions of homology in Figure 7.12. The removal of the insertions from pCT01 produced a coverage of 97% with pColIb-P9, and may indicate this plasmid as a progenitor to the pCH02, pCH03, pCT01 and pT01.

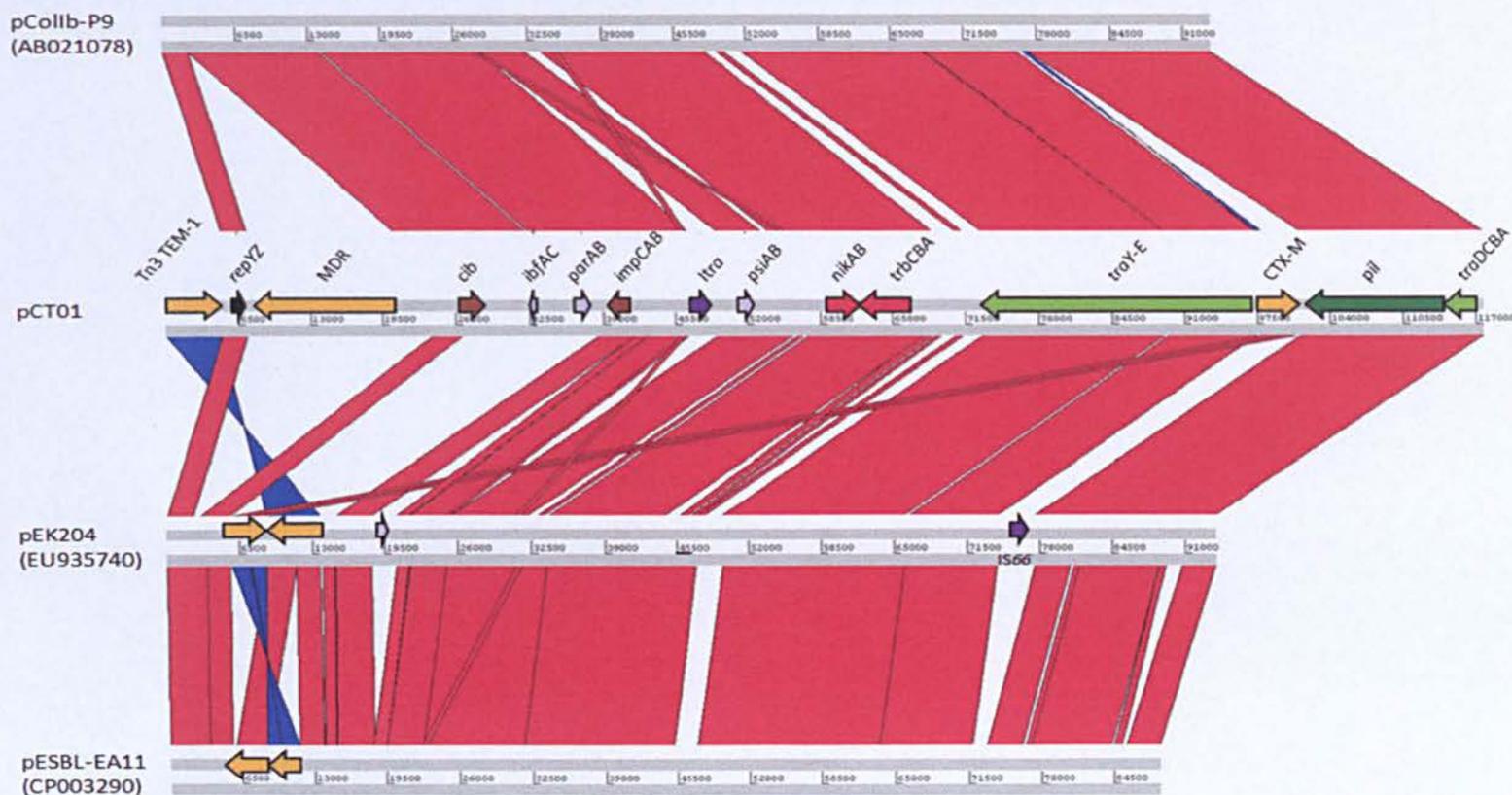
Figure 7.11 Phylogenetic relationship of IncI1y plasmids



Dendrogram of phylogenetic relationship of IncI1y plasmids based on a concatenation of sequences from each plasmid, aligned using neighbour joining method with 1000 bootstrap repeats in MEGA5, the scale indicates substitutions per base.

Isolated in the UK from *E. coli* O25:ST131 from a human patient, plasmid pEK204 was 93,732 bp in size and was predicted to have 112 ORFs (Woodford *et al.*, 2009). pEK204 shared the *tra* and *pil* genes with pCT01 and had a coverage of 81% with the inserts removed, however it lacked the MDR present in pCT01 as shown in Figure 7.12. The genes *yacA* to *yaeB* are absent in pEK204, which are downstream of *repZ* and the MDR in pCollb-P9 and pCT01 respectively. pEK204 has two inserts *bla*_{TEM-1} linked to Tn3 which is likely to have inserted into a pCollb-P9 backbone, resulting in the loss of

Figure 7.12 Artemis comparison tool analysis of pCT01 with pCollb-P9, pEK204 and pESBL-EA11



Artemis comparison tool analysis of the DNA sequences of pCT01 with pCollb-P9, pEK204 and pESBL-EA11. Green arrows indicate genes involved in conjugation with the light green indicating the *tra* loci and the darker green are the *pil* loci. Red arrows are other genes involved in conjugation, orange arrows indicate resistance genes, and magenta arrows are mobile elements, light pink are genes involved in stability, brown are accessory genes. Red regions are homologous DNA and blue regions are homologous DNA in the reverse orientation, white regions show areas of no DNA homology.

genes between *yagA* and *impB* present in pCT01 and pColIb-P9 backbone (Woodford *et al.*, 2009). This insertion is in a different location to that of pCT01 and in the reverse orientation, and was followed by the insertion of *ISEcp1-bla_{CTX-M-3}* upstream of Tn3.

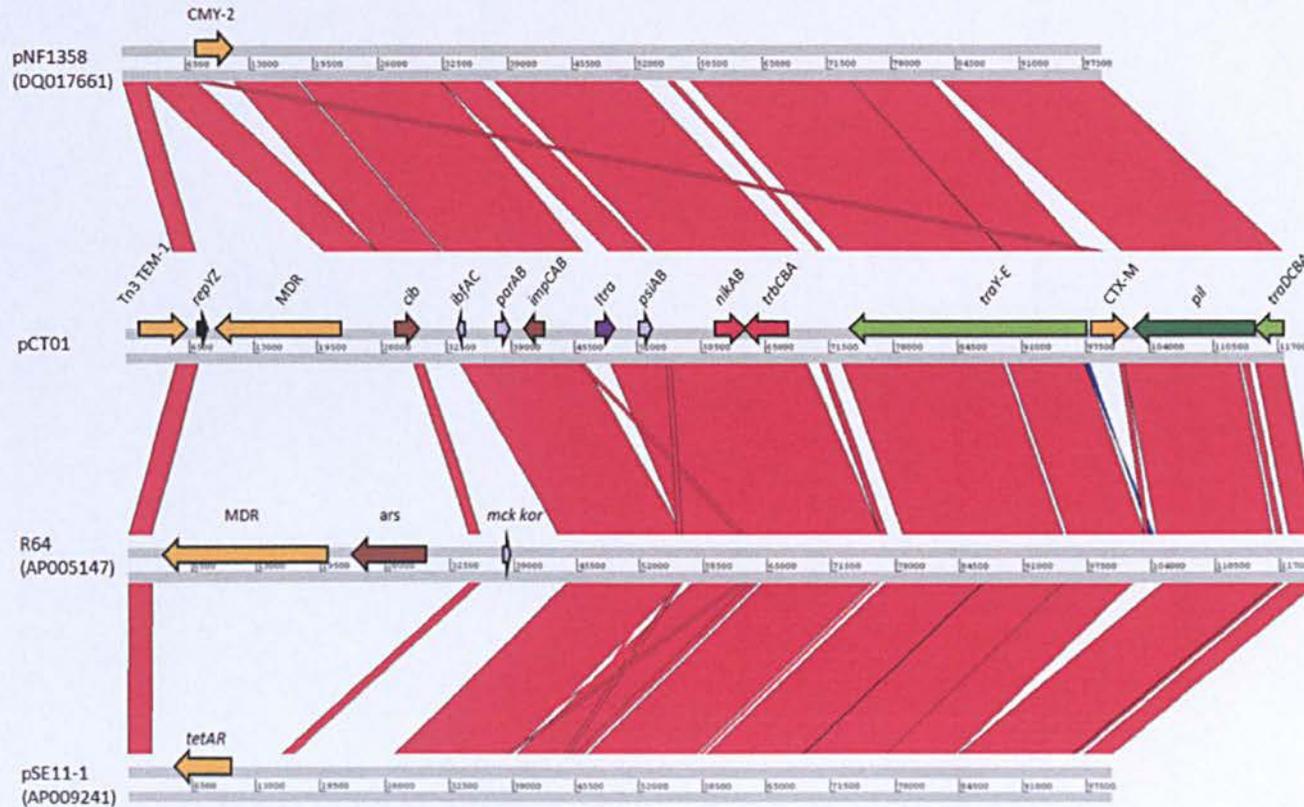
A *stx* positive O104 *E. coli* isolated from a patient in the USA with recent travel to Germany during the EHEC outbreak in 2011, was found to harbour a IncI1 γ *bla_{CTX-M-15}* plasmid, pESBL-EA11 (Ahmed *et al.*, 2012). As with pEK204 this plasmid has lost the colicin genes and *impB*, present in pCT01, and additionally has the Tn3-*bla_{TEM-1}* adjacent to *bla_{CTX-M-15}*, however its located downstream of Tn3 and in the reverses orientation to pEK204. The *pilJ* and *traD* genes are absent in pESBL-EA11, which may have an effect on its ability to conjugate, without inserts pCT01 shares 79% coverage with pESBL-EA11(Figure 7.12).

7.3.5.2 Comparison of pCT01 with the human plasmids pNF1358, R64 and pSE11-1

Plasmid pNF1358 was harboured in a clinical *Salmonella enterica* Thompson isolate submitted to the National Antibiotic Resistance Monitoring System (NARMS) in 1996 in the USA (Dunne *et al.*, 2000; Carattoli *et al.*, 2002). This plasmid confers resistance to the extended spectrum β -lactamases which is mediated through the *ISEcp1-bla_{CMY-2-bla-sugE}* transposition, a common transposition unit seen in IncA/C plasmids, which has inserted downstream of *yafB* (Carattoli *et al.*, 2002). pNF1358 shares 97% coverage with pCT01 (insertions removed), which includes the stability genes and conjugation genes, as shown by the DNA homology in Figure 7.13.

Isolated in 1966 from *Salmonella enterica* Typhimurium, R64 is one of the earliest IncI1 γ plasmids to be studied along with pColIb-P9 (Meynell and Datta, 1966). The full sequence of R64 has recently been reported by Sampei *et al* (2010) revealing its conjugation, stability and resistance regions (Sampei *et al.*, 2010). R64 shares 84% coverage with pCT01 which is predominantly made up of the transfer region (*pil* and *tra*)

Figure 7.13 Artemis comparison tool analysis of pCT01 with pNF1358, R64 and pSE11-1



Artemis comparison tool analysis of the DNA sequenced of pCT01 with pNF1358, R64 and pSE11-1. Green arrows indicate genes involved in conjugation with the light green indicating the *tra* loci and the darker green are the *pil* loci. Red arrows are other genes involved in conjugation, orange arrows indicate resistance genes, and magenta arrows are mobile elements, light pink are genes involved in stability, brown are accessory genes. Red regions are homologous DNA and blue regions are homologous DNA in the reverse orientation, white regions show areas of no DNA homology.

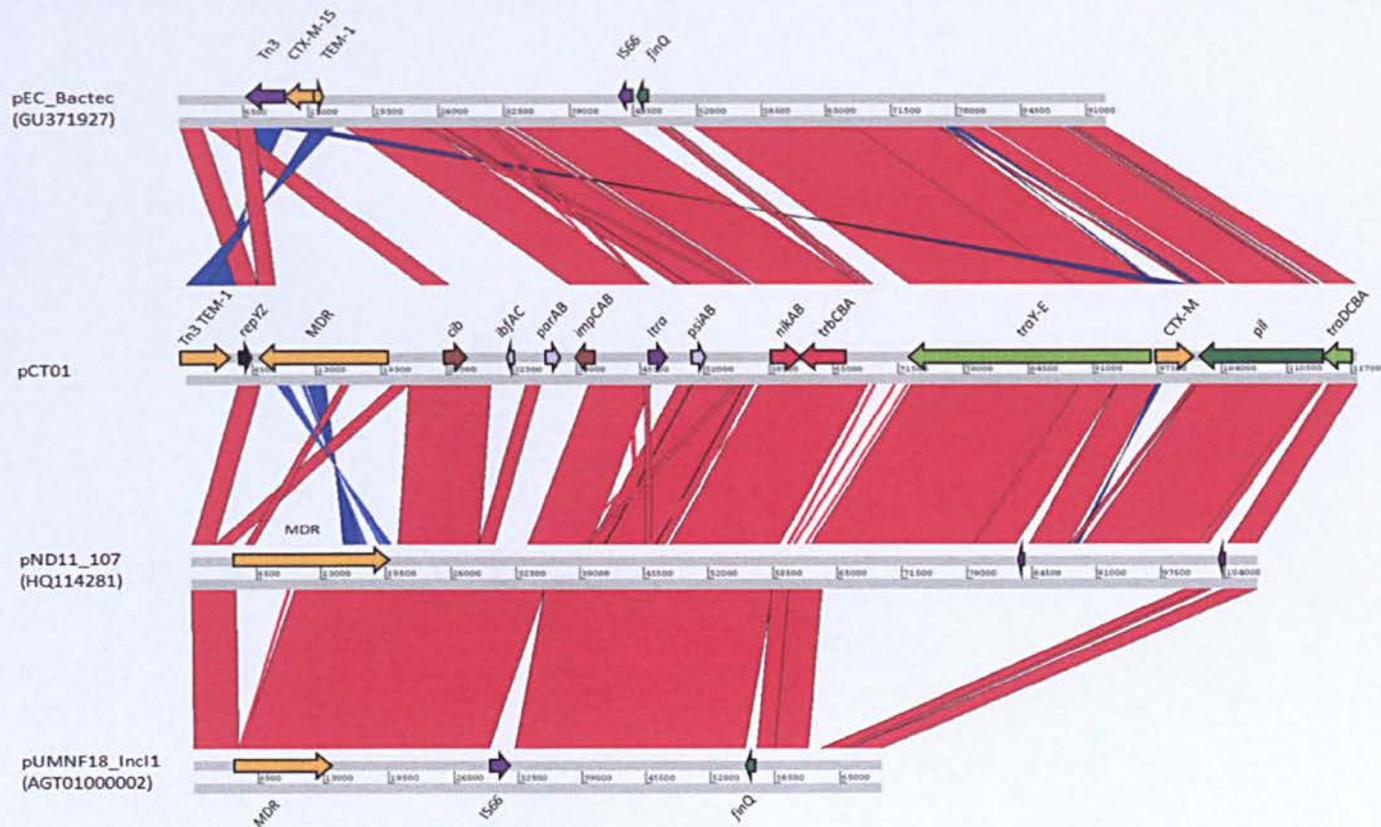
as shown in Figure 7.13. R64 lacks the genes *yacA* to *ybbA*, this may be due to the large (27.7 kb) insertion containing arsenic resistance genes and resistance to tetracycline and streptomycin, located downstream of the *repZ* gene. The tetracycline resistance genes *tetDCAR* were linked to *Tn10* and *strAB* linked to *Tn6082*, which have inserted in a arsenic resistance operon (*ars*) for the exportation of arsenic from the cell. R64 lacks the colicin genes, and additionally has the addiction system genes *mck* and *kor* absent in pCT01.

Sequencing of the commensal *E. coli* isolate from a healthy individual in Japan identified the 100,002 bp plasmid pSE11-1 (Oshima *et al.*, 2008). This plasmid was found to be highly similar to pColIb-P9, and consequently with pCT01 with a coverage of 89% (no insertions). However unlike pCT01 and pColIb-P9, pSE11-1 lacks the genes *yacA* to *yeaA*, and the shufflon region is absent (Figure 7.13). pSE11-1 has a single resistance gene, which is *tetAR* located downstream of IS26. The *repY* of pSE11-1 shares only 64.44% coverage with the IncI1 γ plasmids sequenced in this study, possibly indicating an alternative replication control.

7.3.5.3 Comparison of pCT01 with the animal plasmids pEC_Bactec, pND11_107 and pUNMF18_IncI1

The only IncI1 γ *bla*_{CTX-M} plasmid to be sequenced from an animal is pEC_Bactec which was recovered from *E. coli* isolated from the joint of a horse in Belgium (Smet *et al.*, 2010b). pEC_Bactec shares a similar *tra* and *pil* loci with pCT01 and has a coverage of 79% (no insertions), however the *traD* and *pilJ* genes are absent as in pESBL-EA11, as shown by the red regions in Figure 7.14. pEC_Bactec also has two deletions when compared to pCT01 the first is from *yacA* to *yeaB* and the colicin genes to *parB* which is similar to pEK204 (Smet *et al.*, 2010b). The IS66 adjacent to the fertility inhibition gene *finQ* has inserted upstream of the *pndC* gene. Two resistance genes are present in pEC_Bactec, an Tn3 linked *bla*_{TEM-1}, in which the Tn3 has become the site for the insertion of *ISEcp1-bla*_{CTX-M-15}.

Figure 7.14 Artemis comparison tool analysis of pCT01 with the animal plasmids pEC_Bactec, pND11_107 and pUMNF18_IncI1



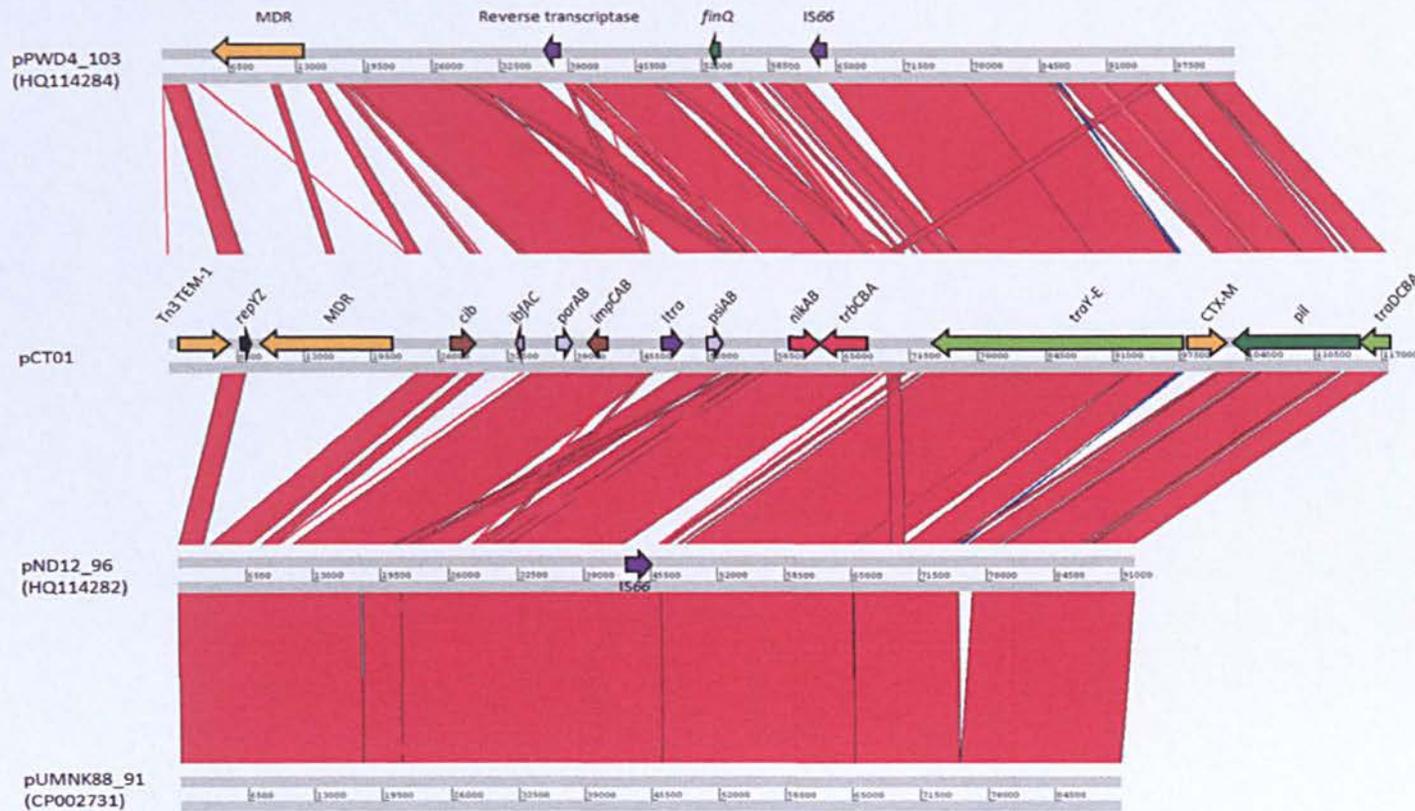
Artemis comparison tool analysis of the DNA sequenced of pCT01 with pEC_Bactec, pND11_107 and pUMNF18_IncI1. Green arrows indicate genes involved in conjugation with the light green indicating the *tra* loci and the darker green are the *pil* loci. Red arrows are other genes involved in conjugation, orange arrows indicate resistance genes, and magenta arrows are mobile elements, light pink are genes involved in stability, brown are accessory genes. Red regions are homologous DNA and blue regions are homologous DNA in the reverse orientation, white regions show areas of no DNA homology.

Isolated from a pig with neonatal diarrhea in the USA, plasmid pND11_107 was 107,138 bp in size and shared a coverage of 86% with pCT01 as shown by the DNA homology in Figure 7.14 (Johnson *et al.*, 2011). pND11_107 had the putative stability genes *yacAB* and the region downstream to *yeaB* absent in other plasmids, but lacks the genes *ybcA* to *ybeC* which includes the infective abortive genes *ibfAC*. In addition the *parAB* and *impCAB* genes are also absent in pND11_107 as in plasmids pSH1148 and pUMNF18_IncI1. A multidrug resistance region has inserted into pND11_107 downstream of the *yacC* which included a class 1 integron *IntI1* with the gene cassettes *aadA2*, *cmlA*, *aadA1*, *qacH* and *sul2*, which are upstream of *Tn21*. A closely related plasmid to pND11_107, pUNMF18_IncI1 was also isolated from pigs in the USA, this plasmid like pCH02 has a large deletion of the *tra* and *pil* loci from *traX* to *pilL* shown by the sequence comparison in Figure 7.14. As with pND11_107 has a MDR insertion downstream of *yacC* of a *Tn21* linked class 1 integron with cassettes *sat*, *aadA2*, *cmlA* and *aadA1*, the *finQ* gene is also present between *trbA* and *pndC*, as in pEC_Bactec, however IS66 is absent (Johnson *et al.*, 2011; Shepard *et al.*, 2012).

7.3.5.4 Comparison of pCT01 with the porcine plasmids pPWD4_103, pND12_96 and pUMNK88_91

Johnson *et al* (2011) established that IncI1 γ plasmids were common in ETEC *E. coli* from pigs in the USA, with pPWD4_103, pND12_96 and pUMNK88_91 being examples (Johnson *et al.*, 2011; Shepard *et al.*, 2012). pPWD4_103 lacks the genes *yacA* to *yeaB* and *yagA* to *ybeB* which includes the colicin genes and *ibfAC*, also absent are the *traD* and *pilJ* genes, and has an overall coverage of 84% with pCT01, as shown by the DNA homology in Figure 7.15. Located downstream of the *psiAB* genes is a putative reverse transcriptase, however the predicted protein shares only 33% identity with that present in pCT01. The MDR region in pPWD4_103 is located downstream of several hypothetical genes which are in turn downstream of *repZ*. It contains IS91, *hph*, *aac(3)-IV*,

Figure 7.15 Artemis comparison tool analysis of pCT01 with the porcine plasmids pPWD4_103, pND12_96 and pUMNK88_91



Artemis comparison tool analysis of the DNA sequence of pCT01 with pPWD4_103, pND12_96 and pUMNK88_91. Green arrows indicate genes involved in conjugation with the light green indicating the *tra* loci and the darker green are the *pil* loci. Red arrows are other genes involved in conjugation, orange arrows indicate resistance genes, and magenta arrows are mobile elements, light pink are genes involved in stability, brown are accessory genes. Red regions are homologous DNA and white regions show areas of no DNA homology.

two copies of *IS26* and *strAB*. The *finQ* gene is also present downstream of *trbA* which is absent in pCT01.

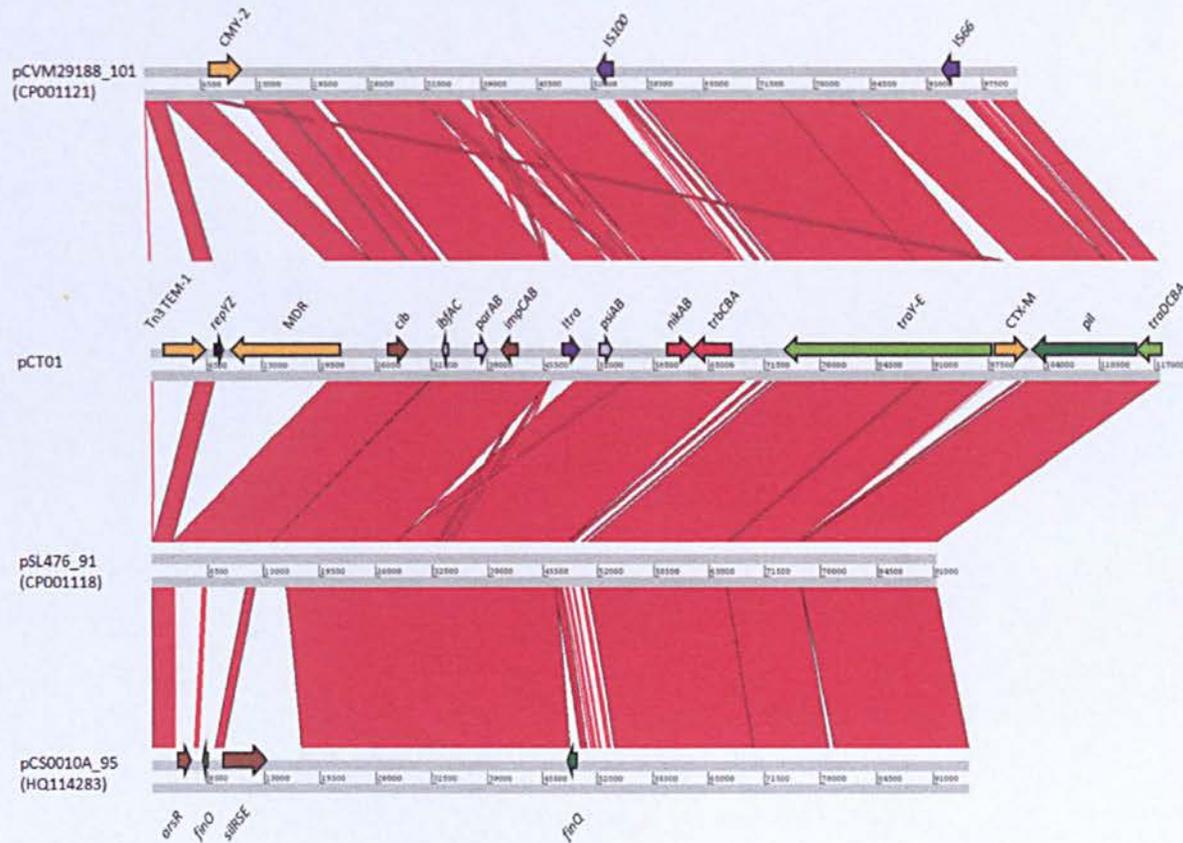
Both pND12_96 and pUMNK88_91 lack any known resistance genes and have coverage's of 87% and 86% respectively (Figure 7.15). As with other IncI1 γ plasmids the *yacA* to *yaeB* genes and the region surrounding the *ibfAC* genes were absent and alternative colicin genes were present in pND12_96. The *tra* and *pil* loci shared a high level of similarity although the *pilJ* was absent and *traH* was truncated by a putative transposase. pUMNK88_91 is highly similar to pND12_96 with a coverage of 100%, however it lacks the *shfC* and *shfC'* region (Shepard *et al.*, 2012; Johnson *et al.*, 2011).

7.3.5.5 Comparison of pCT01 with the avian plasmids pCVM29188_101, pSL476_91 and pCS0010A_95

The plasmid pCVM29188_101 was isolated from *Salmonella enterica* Kentucky in 2003 in the USA from chicken intended for retail meat (Fricke *et al.*, 2009). This plasmid had a single resistance gene which was *bla*_{CMY-2} linked to *ISEcp1*, as present in pNF1358, which was located downstream of *yagA*. pCVM29188_101 shares a coverage of 94% with pCT01 as shown in Figure 7.16, and contains the *yacA* to *yaeB* genes absent in other IncI1 γ plasmids, however the *traD* and *shfC* and *shfC'* region were absent. Two insertions not associated with resistance genes were also present in pCVM29188_101 with *IS100* downstream of the *trb* region and *IS66* downstream of *pilN*.

pSL476_91 was isolated from a turkey in the USA in 2003, and lacks any known resistance genes (Fricke *et al.*, 2011). The backbone of this plasmid shares a high level of similarity with the IncI1 γ plasmids sequenced in this study with 97% coverage (Figure 7.16). This colicin plasmid contains the *yacA* to *yaeB* region, however this plasmid lacks the *shfC*, *shfC'* and *shfA'* region and has a truncated *pilV* which may be the result of rearrangements to the pilus tip. The plasmid pCS0010A_95 was sequenced as part of the ETEC study by Johnson *et al.* (2011), and was harboured in *Salmonella enterica* Kentucky

Figure 7.16 Artemis comparison tool analysis of pCT01 with the avian plasmids pCVM29188_101, pSL476_91 and pCS0010A_95



Artemis comparison tool analysis of the DNA sequence of pCT01 with pCVM29188_101, pSL476_91 and pCS0010A_95. Green arrows indicate genes involved in conjugation with the light green indicating the *tra* loci and the darker green are the *pil* loci. Red arrows are other genes involved in conjugation, orange arrows indicate resistance genes, and magenta arrows are mobile elements, light pink are genes involved in stability, brown are accessory genes. Red regions are homologous DNA and white regions show areas of no DNA homology.

isolated from a healthy chicken in the USA (Johnson *et al.*, 2011). pCS0010A_95 has multiple insertions downstream of the *repZ* gene which confer resistance to heavy metals with the *silRSE* adjacent to IS26 and has the conjugal repressor gene *finO* a homolog of *yafB*. As a result of these insertions the region of genes from *yacA* to *ybeB* is absent which includes colicin and *ibfAC* genes, as with the other avian IncI1 γ plasmids the *shfC*, *shfC'* region is absent, however the *tra* and *pil* loci are well conserved with pCT01 which had a coverage of 83% as shown in Figure 7.16.

7.3.6 Development of IncI1 γ molecular markers

The identification of IncI1 γ plasmids by PCR is carried out using the replicon typing primers, with plasmids being further analysed based on the alleles of five loci to form the IncI1 γ pMLST (Carattoli *et al.*, 2005a; Garcia-Fernandez *et al.*, 2008). In this study we propose a new PCR typing method for IncI1 γ plasmids that does not require sequencing of amplicons. Comparative analysis of the IncI1 γ plasmid pCH02, pCH03, pCT01 and pT01, and plasmids published on GenBank were used to identify potential molecular markers. In total eight genes and regions were selected as molecular markers, selection of the markers was based on the ability to be tested *in vitro* as comparison with other plasmids would be conducted *in silico*. The molecular markers selected were a transposon region (mTnp), mCib, mYbcA, mltra, mYgaA, shufflon region (mShuf), mTraD, and phage integrase like gene (mPIN), as shown in Table 7.4

7.3.6.1 *In silico* testing of the IncI1 γ molecular markers

The primers for the selected molecular markers and the markers themselves were compared to the 16 GenBank IncI1 γ plasmids and the plasmids sequenced in this study (Table 7.3). The most prevalent of all the markers was mYgaA, present in 14/20 plasmids. The markers for mTraD and mShuf were both present in 12/20, the mCib marker was present in 11/20 plasmids, the mYbcA marker was present in 8/20 plasmids. The mTnp

Table 7.4 IncI1y molecular markers

PCR	Marker	plasmid marker location				Size (bp)
		pCH02	pCH03	pCT01	pT01	
Multiplex 1	Tnp-Tnp	7330-8509	7505-8671*	15493-16672	10540-11719	1180
	Shuf	59113-59518	85390-85795	97364-97768	91682-92087	406
Multiplex 2	Itra	NA	NA	47134-48449	42183-43498	1316
	YgaA	NA	42534-43109	53195-53770	48244-48819	576
	PIN	6879-7194	7054-7369	NA	NA	316
Mutlplex 3	Cib	19885-21298	18710-20123	26712-28125	21759-23172	1414
	YbcA	23622-24448	22447-23273	30449-31275	25495-26321	827
	TraD	72222-72713	102034-102525	114003-114494	107744-108235	492

NA = molecular marker not present, * amplicon was 1,167 bp in size

marker was present in 4/20 plasmids all of which were sequenced in this study, mPIN (phage integrase) and mItra (reverse transcriptase) were present in only 2/20 plasmids, and were specific to plasmids sequenced in this study, mPIN in pCH02 and pCH03, and mItra in pCT01 and pT01. The results of the *in silico* marker testing of the plasmids in this study and the GenBank plasmids are shown in Table 7.5. Each molecular marker was given a binary number depending on their prevalence with markers with higher prevalence having lower assigned numbers. Numbers assigned were as follows mYgaA = 1, mCib = 2, mTraD = 4, mShuf = 8, mYbcA = 16, mTnp = 32, mPIN = 64 and mItra = 128. By assigning each marker a number, combinations of markers can be assigned a unique ID (UID) which would facilitate analysis using a database (Table 7.5). The pMLST of plasmids was determined *in silico* for each of the plasmids from GenBank and in this study (Table 7.5).

The molecular markers were capable of distinguishing the 20 IncI1 γ plasmids into 12 separate groups, with five clusters, compared to the 14 groups with four clusters determined by pMLST, as shown in Figure 7.17. Plasmids pSH1148_107 and pUMNF18_IncI1 were in the same cluster (UID = 3), plasmids pSE11-1, pCS0010A_95 and pEK204 were in the same cluster (UID = 5), plasmids pPWD4_103, R64, pEC_Bactec and pESBL-EA11 were in the same cluster (UID = 9), pNF1358 and pColI-P9 were in the same cluster (UID = 30) as were pCT01 and pT01 (UID = 191). This molecular marker scheme offers a non sequencing based method for distinguishing IncI1 γ plasmids into several different groups.

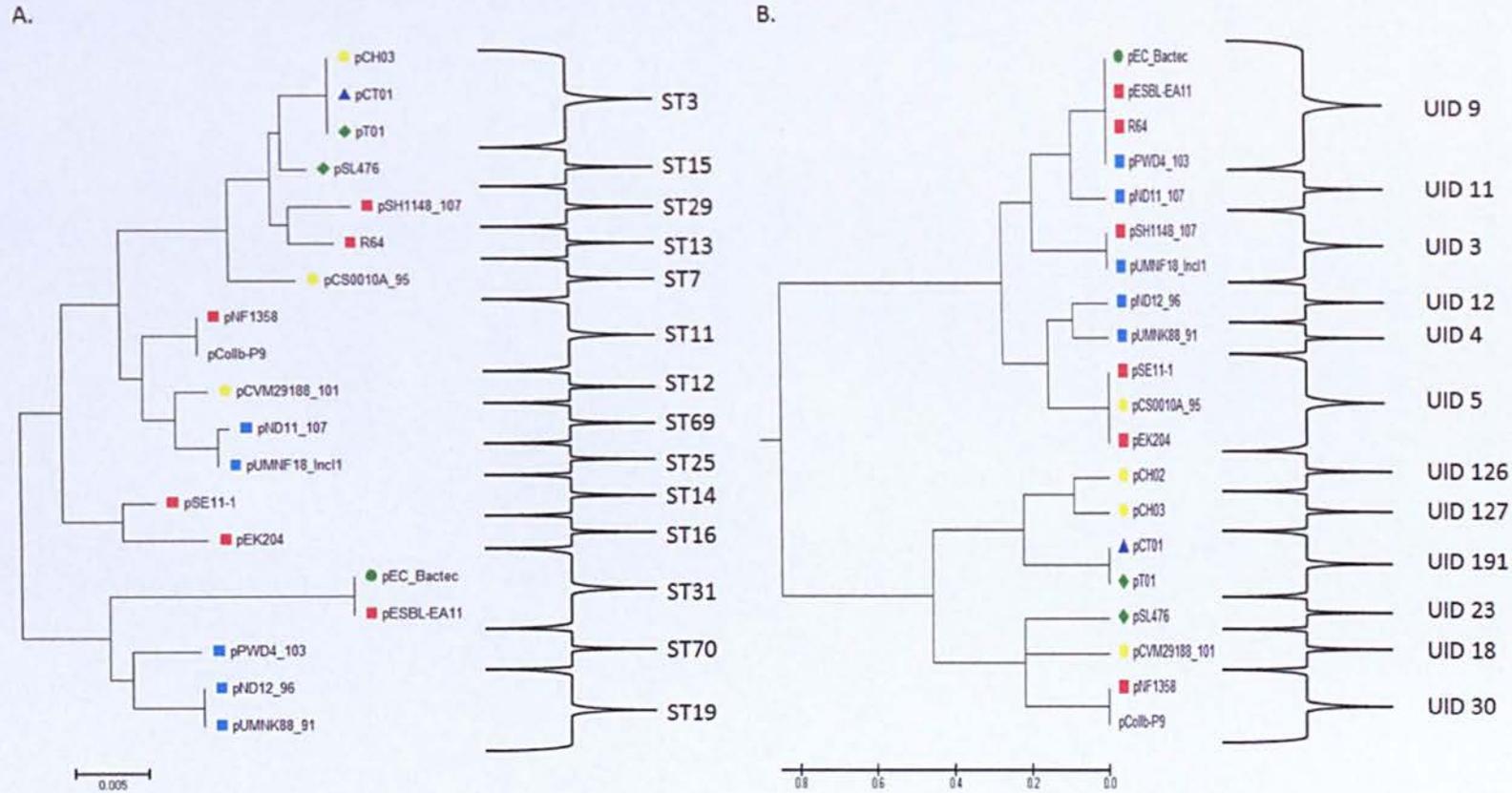
The molecular markers in this study distinguished the three ST3 plasmids into two groups and could class the untypable pCH02 plasmids (suspected ST3). The ST11 plasmids were grouped into the same UID which was 30, and the two ST31 plasmids were both grouped as UID 9, however so were plasmids from ST13 and ST70. ST7, 14 and 16 were all grouped together as UID 5 and ST25 and 29 were UID 3. The molecular markers

Table 7.5 IncI1 γ molecular markers results from *in silico* testing of GenBank plasmids

Name	Source	Molecular markers								Markers	UID	pMLST
		mTnp	mPIN	mItra	mShuf	mCib	mYgaA	mTraD	mYbcA			
CH02	Chicken	1	1	0	1	1	0	1	1	6	126	NA
CH03	Chicken	1	1	0	1	1	1	1	1	7	127	3
CT01	Cattle	1	0	1	1	1	1	1	1	7	191	3
T01	Turkey	1	0	1	1	1	1	1	1	7	191	3
pSL476_91	Turkey	0	0	0	0	1	1	1	1	4	23	15
pNF1358	Human	0	0	0	1	1	0	1	1	4	30	11
pColIb-P9	NA	0	0	0	1	1	0	1	1	4	30	11
pCVM29188_101	Chicken	0	0	0	0	1	0	0	1	2	18	12
pSE11-1	Human	0	0	0	0	0	1	1	0	2	5	14
pSH1148_107	Human	0	0	0	0	1	1	0	0	2	3	29
pND12_96	Pig	0	0	0	1	0	0	1	0	2	12	19
pND11_107	Pig	0	0	0	1	1	1	0	0	3	11	69
pUMNK88_91	Pig	0	0	0	0	0	0	1	0	1	4	19
pPWD4_103	Pig	0	0	0	1	0	1	0	0	2	9	70
R64	Human	0	0	0	1	0	1	0	0	2	9	13
pCS0010A_95	Chicken	0	0	0	0	0	1	1	0	2	5	7
pEK204	Human	0	0	0	0	0	1	1	0	2	5	16
pEC_Bactec	Horse	0	0	0	1	0	1	0	0	2	9	31
pESBL-EA11	Human	0	0	0	1	0	1	0	0	2	9	31
pUMNF18_IncI1	Pig	0	0	0	0	1	1	0	0	2	3	25

The presence of a marker is denoted by a 1 with a green background, and absence of marker by 0 with a red background. UID = unique ID number and pMLST are shown on the right.

Figure 7.17 Comparison of IncI1 γ pMLST and IncI1 γ molecular markers of GenBank plasmids



Comparison of pMLST (A.) and IncI1 γ molecular markers (B.) of Genbank plasmids. pMLST of plasmids was carried out by neighbour joining method of the pMLST alleles of the five genes with 1000 bootstrap replicates, ST shown. IncI1 γ molecular marker dendrogram is based on UPGMA of the presence or absence of the markers, UID shown. Yellow circles are plasmids from chickens, green diamonds plasmids from turkeys, red squares plasmids from humans, blue triangle plasmid from cattle, blue square are plasmids from pigs and green circle is a plasmid from horse.

were capable of distinguishing ST19 into two UIDs and another 3 ST's had unique UIDs. Although not achieving the same level of discrimination to that of pMLST, these results suggest that the IncI1 γ molecular markers offer a comparative level of differentiation when considering this is purely a PCR based non sequencing method.

7.3.6.2 Evaluation of IncI1 γ molecular markers

The molecular markers were evaluated to identify their absence in some plasmids while present in others. The mItra marker was present in only two plasmids, pCT01 and pT01, this region codes for a RNA dependent reverse transcriptase, which is likely to be related to a phage. The DNA sequence for this gene has only been found in one other plasmid published on GenBank, at the time of this study, which was the IncFII *bla*_{CTX-M-15} pEK499 isolated from a human *E. coli* infection. This marker was absent in the other plasmids and is likely to be due to the absence of this phage or insertion.

Similarly to mItra the mPIN molecular marker was also present in two plasmids, being pCH02 and pCH03. This molecular marker is located in the MDR region, located downstream of *tetR* and is for a putative integrase, despite the potential as a mobile element, this integrase has not been associated with any IncI1 γ plasmids previously published in GenBank, and the closest match to this integrase are in plasmids pAPEC-O103-ColBM (CP001232) and plasmid 6/9 (AJ628353), in which it was located between *tetR* and IS26 as in pCH02 and pCH03 (Daly *et al.*, 2005; Johnson *et al.*, 2010). The absence of this marker in the other plasmids is either due to the absence of the MDR region or the integrase as a separate element. The mTnp marker was specific to plasmids sequenced in this study, and corresponds to two transposons IS26 and IS4321. Searches of GenBank identified plasmids which harboured both transposons, however not in the combination observed in the four plasmids sequenced, which appears specific to plasmids from UK animals. The locality of these transposons may be representative of a MDR in circulation in the UK animal population.

The mYbcA marker is absent in 12 plasmids in total, the mYbcA is located in a region of genes which appears to be deleted, or are targeted as sites of insertion for mobile genetic elements. This marker is absent in the plasmids pEK204, pEC_Bactec and pESBL-EA11 due to the insertion of Tn3-*bla*_{TEM-1} and ISEcp1-*bla*_{CTX-M} which has inserted downstream of *ygaA* and upstream of *impB* resulting in the loss of 19 genes present in pCT01, which includes the *ybcA* gene. The insertion of heavy metal resistance genes *silRSE* in pCS0010A_95 and a ABC transporter, *mck* and *kor* in R64 between two conserved genes in pCT01, has caused the loss of *ybcA*. Plasmids pND11_107, pND12_96, pPWD4_103, pUMNF18_IncI1 and pUMNK88_91 have all undergone a deletion between two conserved hypothetical genes (*orf40-46*) with 48 bp of the C terminal of the first remaining and 166 bp of the N terminal of the second remaining, this deletion included the *ybcA* gene.

The mCib marker was absent in plasmids pEK204, pESBL-EA11 and pEC_Bactec due to the same insertion of the Tn3-*bla*_{TEM-1} and ISEcp1-*bla*_{CTX-M} which resulted in the loss of the mYbcA marker. Plasmids pSE11-1, pND12_96 and pUNMK88_91 are all colicin producing plasmids, however they produce ColIa and not ColIb which is the gene used as the molecular marker. The 626 amino acid proteins have a coverage of 100% but an identity of 82%, and the genes share the first 1335 bp (98% identity) however the 3' sequence of the ColIa gene varies, preventing the binding of the reverse primer. Plasmids R64, pPWD4_103 and pCS0010A_95 all lack the colicin gene and immunity gene which are replaced by conserved hypothetical genes related to infection abortive proteins, these may have inserted causing the loss of the colicin genes, or alternatively the colicin genes were acquired.

Rearrangements to the shufflon region in plasmids pSL476_91, pCVM29188_101, pSE11-1, pSH1148_107, pUNMK88_91, pCS0010A_95 and pEK204 have resulted in the loss of the shufflon genes *shfC* and *shfC'* which are used for the mShuf marker. This may be important for plasmid host specificity, through alterations to the pilus tip, possibly

limiting the rearrangements. The large deletion in pUMNF18_IncI1 resulted in the loss of conjugation genes between *traY* and *pilK* which included the shufflon recombinase, *rci*, and shufflon region.

The mTraD marker is absent in eight plasmids, seven have resulted in the loss of the *traD* gene between *pilI* and *traC*. pCVM29188_101, pSH1148_107 and pUMNF18_IncI have a hypothetical gene present which may have caused the loss of *traD*, and pND11_107 has the IS2 transposon at this site causing the loss. Plasmids pPWD4_103, pEC_Bactec, and pESBL-EA11 have lost this gene, with 127 bp sequence between *pilI* and *traC*. The *traD* gene is present in R64, however this gene has no similarity with the *traD* in the other IncI1 γ plasmids, and as a result the primers are predicted not to bind.

The mYgaA is absent in pNF1358, pColIb-P9, pCM29188_101, pND12_96 and pUMNK88_91, all of these plasmids lack the *ygaA* gene which is located between *psiB* and *ardA*. In these plasmids the insertion sequence IS605 is present, which is likely to have caused the loss of the *ygaA*. The presence of this insert in so many plasmids at the same site suggests that it may have happened some time ago and has become a feature of these plasmids, supported by the absence of the complete IS605 left repeat sequence.

7.3.6.3 IncI1 γ marker amplicon sequence analysis

The *in silico* testing of the molecular markers identified that the 20 IncI1 γ plasmids could be distinguished into 12 groups by production or absence of amplicons. However 13 plasmids were grouped into five clusters, based on their marker profiles. To analyse whether these plasmids could be further distinguished, the sequences of the *in silico* amplicons for mShuf, mYbcA, mYgaA and mCib were compared. The markers mYbcA and mShuf both yielded two alleles respectively, mYbcA could distinguish the plasmid pCVM29188_101 from the other plasmids yielding this amplicon. The mShuf amplicon was capable of distinguishing pPWD4_103 from the other plasmids with mShuf.

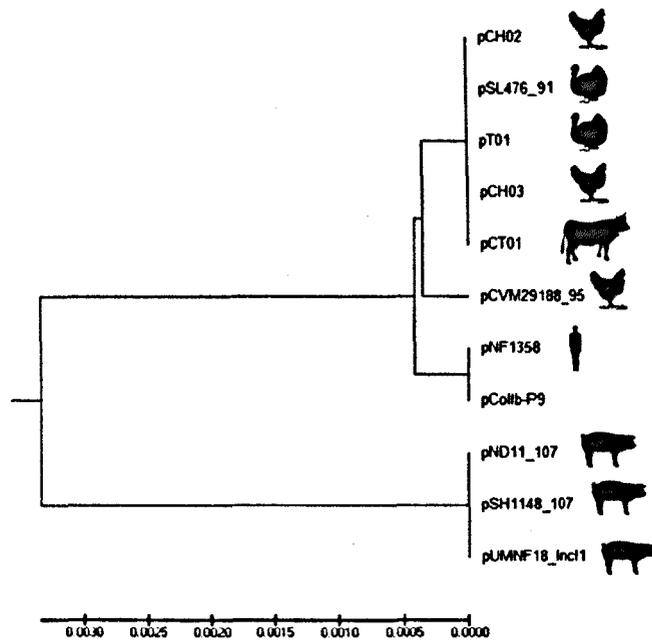
Analysis of the mCib marker identified four alleles, pCH02, pCH03, pCT01, pT01 and pSL476_91 all belonged to a single allele, pCVM29188_101 produced an allele on its own, pNF1358 and pColIb-P9 shared an allele, as did pND11_107, pSH1148_107 and pUMNF18_Inc11, as shown in Figure 7.18. The most discriminating marker by sequence was also the most prevalent being mYgaA, which had five alleles (Figure 7.18). The three plasmids pSE11-1, pCS0010A_95 and pEK204 all of which have the same marker combination (UID 5) can be distinguished from each other based on the sequence of the YgaA amplicon. The UID 9 plasmids, pPWD4_103, pESBL-EA11, pEC_Bactec and R64 could be further distinguished, however pESBL-EA11 and pEC_Bactec shared the same allele, which was the same for pEK204. pUMNF18_Inc11, pSH1148_107 and pND11_107 shared the same allele as is the case with pCS0010A_95, R64, pSL476_91, pT01, pCT01, and pCH03 all shared the same allele, while pSE11-1 and pPWD4_103 were separate alleles.

7.3.7 Screening of the Inc11 γ molecular markers against *E. coli* field isolates

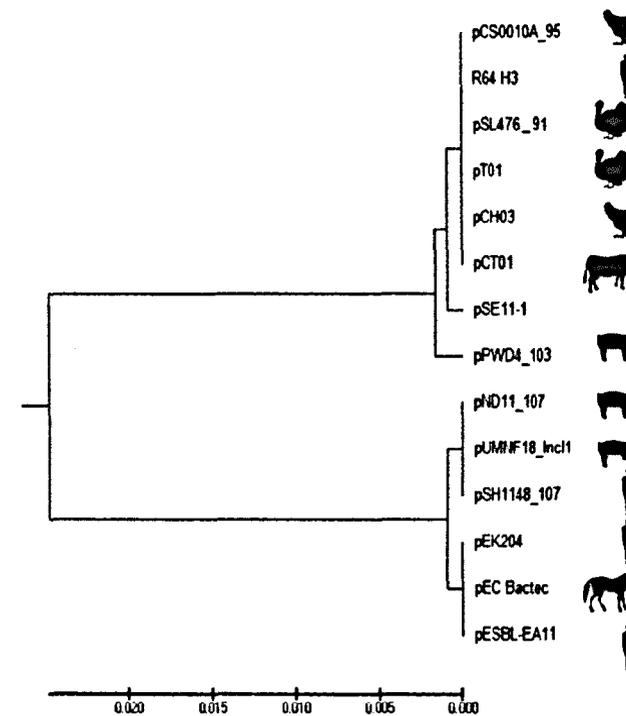
As was conducted for the pH19 molecular markers in chapter 4, the Inc11 γ markers were screened against 136 wild type *E. coli* isolates including 98 group 1 CTX-M isolates from cattle (n=27), chicken (n=29), humans (n=27) and turkeys (n=15) and 38 group 9 CTX-M isolates from cattle (n=19), humans (n=9) and turkeys (n=10). All of the Inc11 γ plasmids sequenced in this study carried group 1 CTX-M genes, and screening of the group 1 CTX-M field isolates identified that 14/98 had no markers, 24/98 had a single marker, 12/98 had two markers, 5/98 had three markers, 5/98 had four markers, 20/98 had five markers, 6/98 had six markers and 12/98 had seven markers. The screening of group 9 CTX-M isolates, identified 11/38 had no markers, 5/38 had one marker, 10/38 had two markers, 7/38 had three markers, 2/38 had four markers, and 2/38 markers had five markers which was the highest number of markers present. The distributions of the markers is shown in Table 7.6

Figure 7.18 Phylogeny of mCib and mYgaA IncI1 γ molecular markers

A.



B.



Phylogeny mCib (A) and mYgaA (B) amplicons aligned using Clustal W MEGA5 using UPGMA, scale indicates substitutions per base.

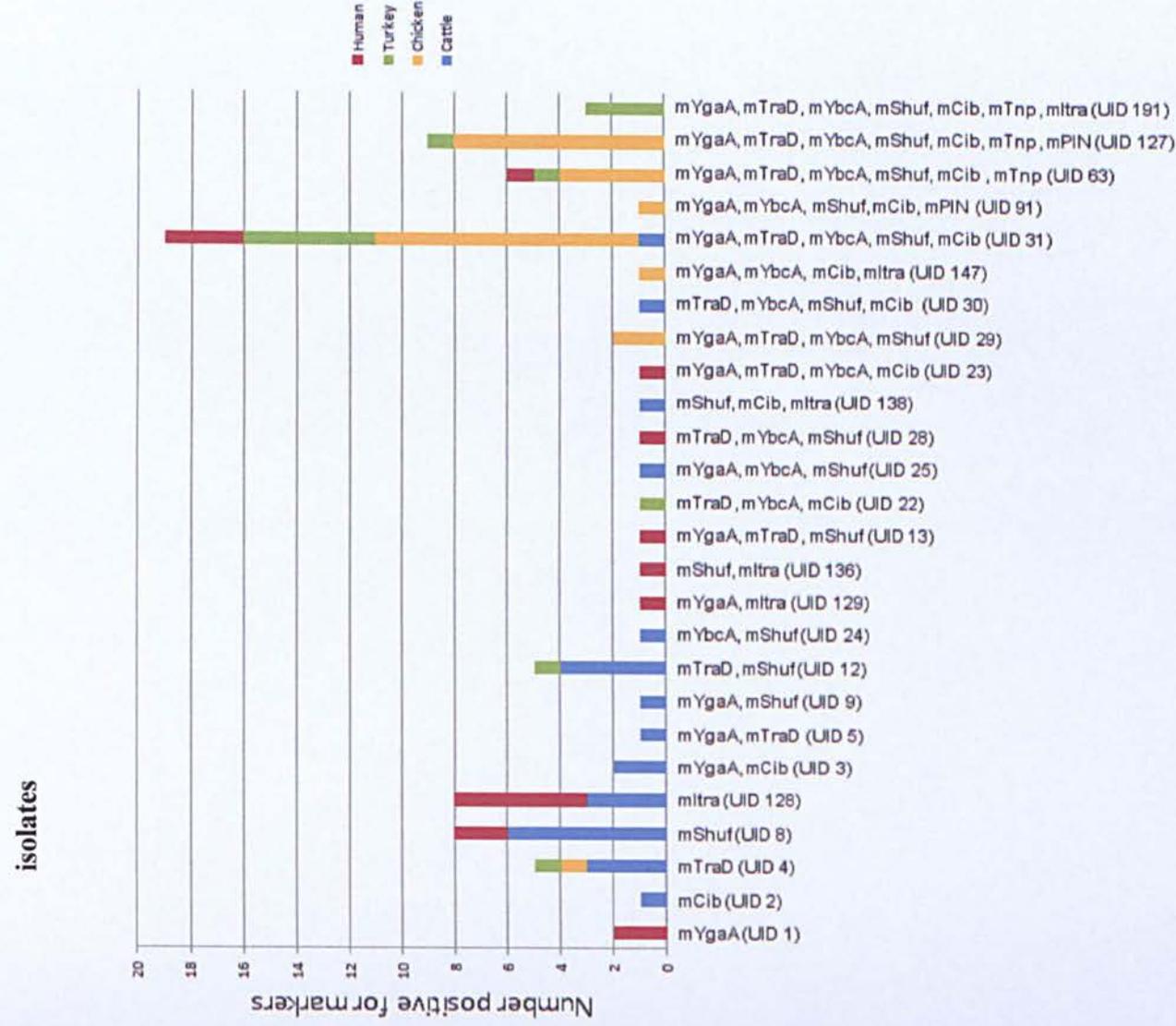
Table 7.6 Prevalence of IncI1 γ molecular markers in field isolates

Source	No. of positives isolates	Molecular marker							
		mTnp	mShuf	mItrA	mYgaA	mPIN	mCib	mYcbA	mTraD
Group 1 CTX-M									
Cattle	26	0	16	4	6	0	6	4	10
Chicken	27	12	25	1	26	9	24	26	25
Turkey	13	5	11	3	10	1	11	11	13
Human	18	1	9	7	9	0	5	6	7
Group 9 CTX-M									
Cattle	11	1	5	1	10	0	7	4	5
Turkey	10	4	4	0	10	0	2	1	0
Human	6	1	3	2	3	0	0	0	2
Total	111	24	73	18	74	10	55	52	62

The IncI1 γ molecular markers were found individually in field isolates (29/136) but were typically found in combinations with other molecular markers, as shown for group 1 CTX-M in Figure 7.19 and group 9 CTX-M Figure 7.20. Combination of markers produced 26 UIDs among the group 1 CTX-M isolates of which 8 had been identified in GenBank plasmids (UID 3, 4, 5, 9,12, 30, 127 and 191), and 16 unique UIDs among the group 9 CTX-M of which three had been identified in Genbank plasmids (UID 3, 9, and 11). The most common combination of markers in the group 1 CTX-M isolates were mYgaA, mTraD, mYcbA, mShuf and mCib, which belong to UID 31 a novel combination not observed in the GenBank plasmids, and was found to be in 19 isolates (Figure 7.19).

The second most prevalent combination belonged to UID 127 (mYgaA, mTraD, mYcbA, mShuf, mCib, mTnp and mPIN) which was present in 8 chicken isolates and one turkey isolate, and corresponds to the plasmid pCH03. The UID 4 corresponding to pUMNK88_91 from pigs was identified in 5 isolates (cattle n = 3, chicken n = 1, turkey n = 1). Isolates with similar markers to pCT01 and pT01 UID 191 were identified in three turkey isolates. The most common combination of markers in group 9 CTX-M isolates

Figure 7.19 Inc11γ molecular marker combinations present in group 1 CTX-M isolates

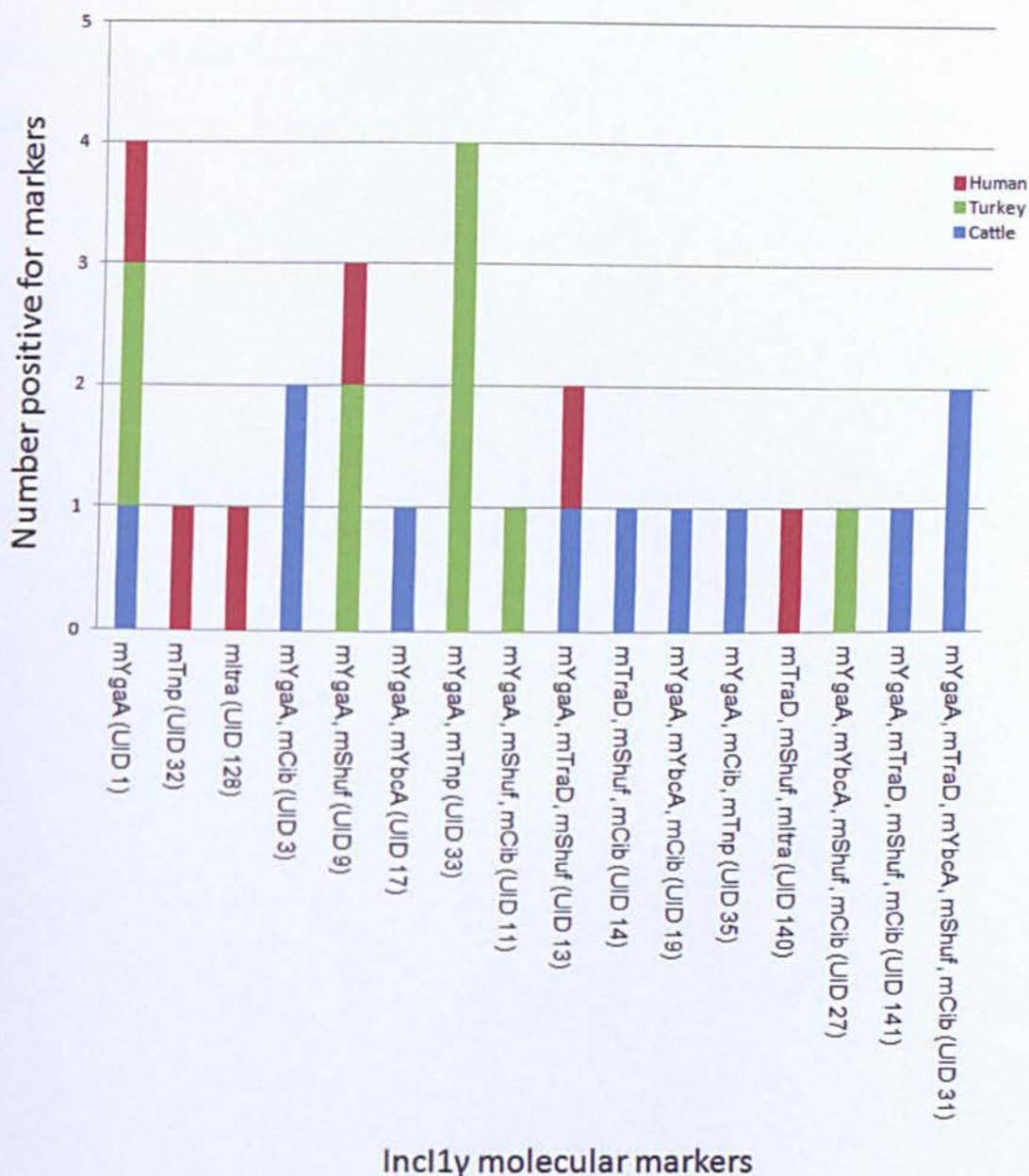


Inc11γ molecular markers

Inc11γ molecular marker combinations from screening the group 1 CTX-M field isolates, UID shown in brackets.

was mYgaA and mTnp (UID 33) which was a novel combination, not seen *in silico* testing, shown in Figure 7.20.

Figure 7.20 IncI1 γ molecular marker combinations present in group 9 CTX-M isolates



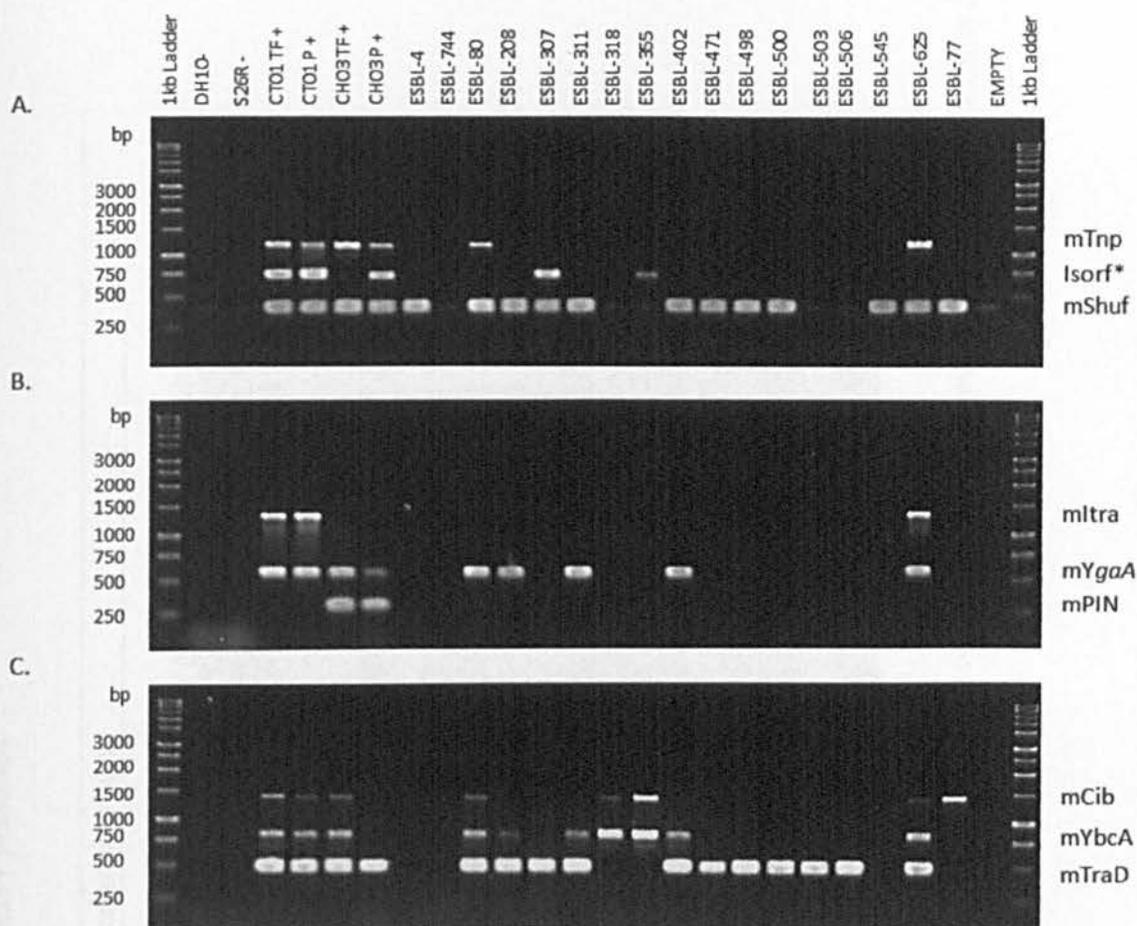
IncI1 γ molecular marker combinations from screening the group 9 CTX-M field isolates, UID is shown in brackets.

Testing of the field isolates identified 111/136 had IncI1 γ markers however replicon typing using the PBRT IncI1 γ primers identified 61/136 isolates as having the IncI1 γ replicon. It could be the case that the IncI1 γ plasmid was not detected by PBRT, due to base mismatching of the primers, or PCR failure, which appears to be the case for 13 isolates. Further analysis of the non IncI1 γ isolates found that, 24/50 had a single

marker and 13/50 had two, of which 10 were either IncK or IncB. It was noted that some cross reactivity was observed with isolates with the K and B replicon which had the mYgaA marker, due to the close relationship between the IncI complex of plasmids. The markers are more specific for IncI γ plasmids with 55/61 isolates having more than one marker.

To compare how the molecular markers proposed in this study discriminated IncI γ plasmids to the sequence based pMLST, a panel of plasmids with known pMLST were tested. Part of the work conducted by the SAFEFOODERA consortium was the pMLST typing of CTX-M IncI γ plasmids, 17 plasmids belonging to nine known ST's were submitted to the pMLST database which were ST 3 (n=2), ST7 (n=3) ST12 (n=2) ST16 (n=2), ST25 (n=1), ST31 (n=2), ST35 (n=1), ST36 (n=2) and ST37 (n=2). The IncI γ plasmids from these isolates were transferred by conjugation, all conjugates tested positive for the IncI γ replicon. The transconjugants were tested with the IncI γ molecular markers, as shown in Figure 7.21, and Table 7.7. The molecular markers were capable of dividing these nine sequence types into 11 UIDs Figure 7.22. The two ST16 plasmids were both classed as UID 4, the two ST31 as UID 8, and two of the three ST7 were classed as UID 31 these results are consistent with the differentiation achieved by pMLST. However the plasmids ST36 and ST37 were both classed as UID 12, which demonstrates that the molecular markers could not differentiate these plasmids as effectively as pMLST, but both ST36 and ST37 plasmids were differentiated from the other IncI γ plasmids. The markers were capable of differentiating the two ST3 plasmids into two different UIDs (UID 61 and 191), one of which was the same as pCT01 and pT01, this may indicate that ST3 in humans and turkeys are unrelated, and requires further investigation. One of the ST7 plasmids was differentiated from the other two ST7 with UID 29 compared

Figure 7.21 IncI1 γ molecular marker screening of known pMLST IncI1 γ plasmids



IncI1 γ molecular marker screening of pMLST IncI1 γ plasmids with multiplex 1 mTnp and mShuf markers (A.), multiplex 2 mltra, mYgaA and mPIN (B.) and multiplex 3 mCib, mYbcA and mTraD (C.). DH10- and S26R- are plasmid free *E. coli* DH10B used in transformations and *Salmonella* conjugant recipients used for negative controls, and DH10 transformed with pCT01 and pCH03, and their field isolates are positive controls. DNA resolved on a 1.5% agarose gel with a 1 kb ladder used as a size marker. * the Isorf marker was removed from screening results and the molecular marker scheme.

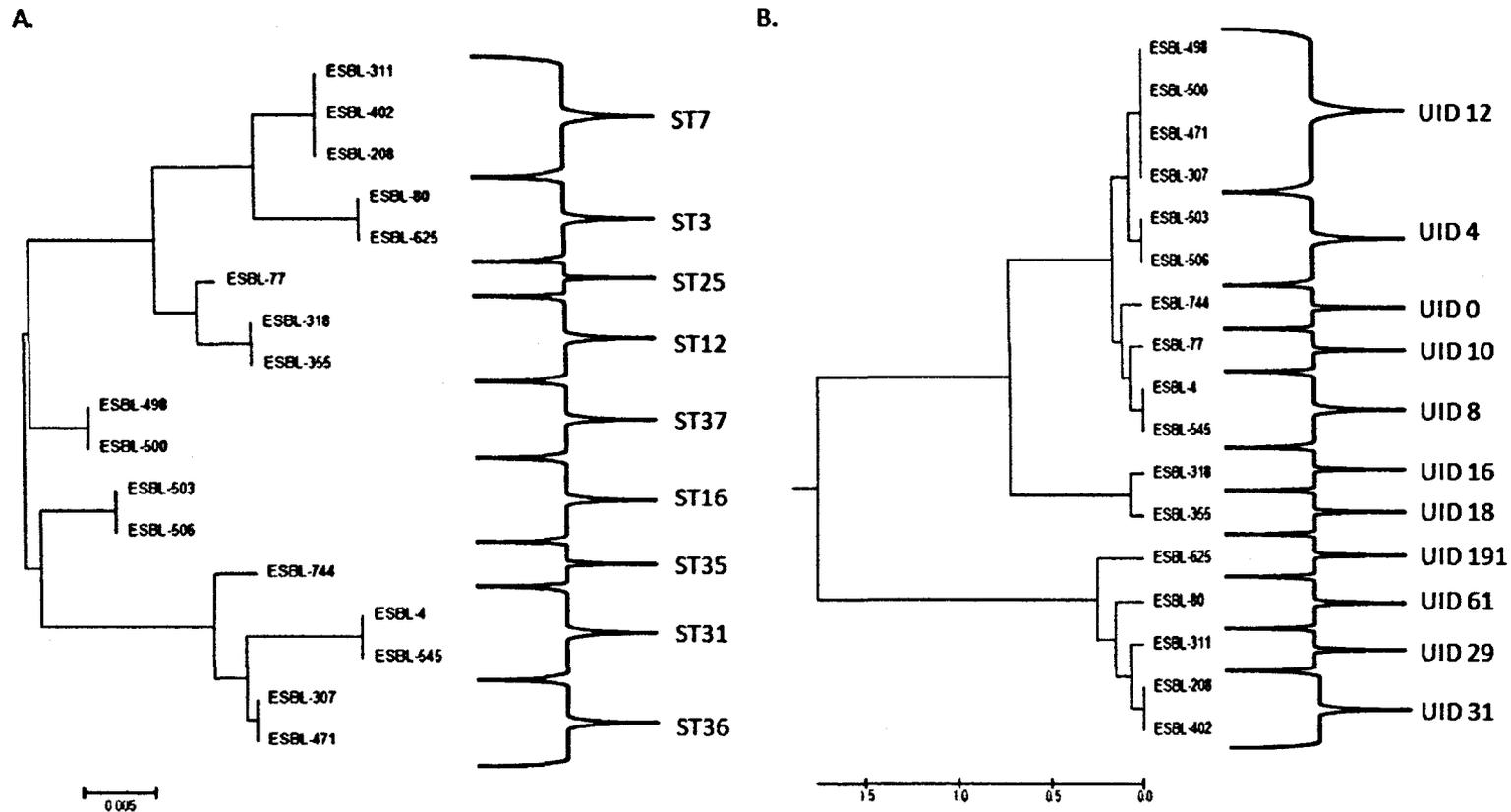
to UID 31, which is the same UID as plasmids from pEC_Bactec and pESBL-EA11 from GenBank. The two ST12 plasmids were discriminated into UID 16 and UID 18, the UID of plasmid pCVM21988_101. ST35 and ST25 both had unique UID and were differentiated from the other IncI1 γ plasmids, consistent with IncI1 γ pMLST.

Table 7.7 IncI1 γ molecular markers results from known pMLST IncI1 γ plasmids

Name	Species	Inc	Molecular markers								pMLST	UID
			mTnp	mPIN	mItra	mShuf	mCib	mYgaA	mTraD	mYcbA		
ESBL-4	Human	IncI1 γ	0	0	0	1	0	0	0	0	31	8
ESBL-744	Human	IncI1 γ	0	0	0	0	0	0	0	0	35	0
ESBL-80	Human	IncI1 γ	1	0	0	1	0	1	1	1	3	61
ESBL-208	Broiler	IncI1 γ	0	0	0	1	1	1	1	1	7	31
ESBL-307	Broiler	IncI1 γ	0	0	0	1	0	0	1	0	36	12
ESBL-311	Broiler	IncI1 γ	0	0	0	1	0	1	1	1	7	29
ESBL-318	Broiler	IncI1 γ	0	0	0	0	0	0	0	1	12	16
ESBL-355	Meat	IncI1 γ	0	0	0	0	1	0	0	1	12	18
ESBL-402	Broiler	IncI1 γ	0	0	0	1	1	1	1	1	7	31
ESBL-471	Cattle	IncI1 γ	0	0	0	1	0	0	1	0	36	12
ESBL-498	Cattle	IncI1 γ	0	0	0	1	0	0	1	0	37	12
ESBL-500	Cattle	IncI1 γ	0	0	0	1	0	0	1	0	37	12
ESBL-503	Cattle	IncI1 γ	0	0	0	0	0	0	1	0	16	4
ESBL-506	Cattle	IncI1 γ	0	0	0	0	0	0	1	0	16	4
ESBL-545	Cattle	IncI1 γ	0	0	0	1	0	0	0	0	31	8
ESBL-625	Turkey	IncI1 γ	1	0	1	1	1	1	1	1	3	191
ESBL-77	Human	IncI1 γ	0	0	0	1	1	0	0	0	25	10

The presence of a marker is denoted by a 1 with a green background, and absence of marker by 0 with a red background. UID = unique ID number and pMLST are shown on the right.

Figure 7.22 Comparison of IncI γ pMLST and IncI γ molecular markers of known pMLST plasmids



Comparison of pMLST (A.) and IncI γ molecular markers (B.) of screening of the known pMLST IncI γ plasmids. pMLST of plasmids was carried out by neighbour joining method of the pMLST alleles of the five genes with 1000 bootstrap replicates, ST shown. IncI γ molecular marker dendrogram is based on UPGMA of the presence or absence of the markers, UID shown.

7.4 Discussion

The plasmids pCH02 and pCH03 from chicken, pCT01 from cattle and pT01 from turkey are the first *bla*_{CTX-M-1} IncI1 γ plasmids to have been sequenced. These plasmids ranged in size from 75,796-117,577 bp with the differences in size due to varying deletions and mobile genetic insertions. The number of ORFs varied between plasmids in accordance with size ranging from 109-152, and GC contents ranging from 49.36-50.77%. All four plasmid harboured the *ISEcp1-bla*_{CTX-M-1}*.orf477* insert, which was located in the shufflon region in all plasmids, which may represent a "hotspot" for the insertion of genetic elements. The insertion in the shufflon region does not appear to have affected the conjugation, as both pCH03 and pT01 are still capable of solid and liquid conjugation. Each plasmid was multidrug resistant, with pCH02 and pCH03 being resistant to cefotaxime, sulphonamides and tetracycline, and pCT01 and pT01 resistant to cefotaxime, trimethoprim, sulphonamides and the gene for resistance to aminoglycoside (aminoglycoside-3'-adenyltransferase) was present. In addition to the resistance genes on the plasmids the field isolates also had various resistances to compounds such as neomycin, chloramphenicols, streptomycin, gentamicin and apramycin, which are likely to be on the chromosome or other plasmids and contribute to the dissemination of the isolate and plasmid through antimicrobial co-selection.

In addition to the *bla*_{CTX-M-1}, a MDR insert was present in all plasmids. Sequence analysis of MDR showed that all of the plasmids had *IS1294* insertion sequence at the start of the MDR, which was likely to have been present in the plasmid backbone, and may have been originally linked to the *pecM* gene upstream of *tetAR* present in pCH02 and pCH03. However the MDR in pCT01 and pT01 varies, the *IS1294* is in the same location but downstream the transposon *Tn1721* has inserted which is linked to a class 1 integron with the *dfra17* and *aadA5* resistance gene cassettes before being homologous with pCH02 and pCH03, with *IS26*, *IS4321* and *sul2* and *IS_{vs}a3* located downstream of the MDR. The genetic relationship of pCH02, pCH03, pCT01 and pT01, demonstrated that they were

closely related and highly conserved, and at one point were likely to share the same plasmid backbone, which had undergone insertions and deletions, particularly evident in pCH02 which had lost two large regions, one containing the stability genes of the leading strand and the other genes of the *tra* loci, the second deletion prevented the pCH02 from self-mobilisation (Bates *et al.*, 1999).

The four sequenced IncI1 γ plasmids in this study all belonged to ST3, with the exception of pCH02 which lacked the *ardA* and *sogS* loci, however the alleles present implied that it would of likely belonged to ST3. IncI1 γ ST3 plasmids have been found previously with *bla*_{CTX-M-1} in livestock animals being present in both poultry and cattle in the Netherlands and France, which implies an animal reservoir for these plasmids (Hordijk *et al.*, 2013; Dierikx *et al.*, 2013; Leverstein-van Hall *et al.*, 2011; Madec *et al.*, 2011; Girlich *et al.*, 2007; Cloeckaert *et al.*, 2010). ST3 plasmids have also been identified in poultry from Italy and Poland (Accogli *et al.*, 2013). Studies have also found evidence that ST3 plasmids are moving between animal isolates to those found in humans. In the Netherlands, the ST3 plasmids have been found in poultry farms in both the poultry and farm workers, and a study found similar plasmids in poultry, retail meat and humans (Dierikx *et al.*, 2013; Leverstein-van Hall *et al.*, 2011). In France ST3 IncI1 γ plasmids are common in isolates from humans but also in poultry as well, suggesting a possible link which may involve the food chain (Cloeckaert *et al.*, 2010). pCT01 and pT01 appear to be closely related to the ST3 plasmids isolated from cattle *Salmonella* in France, which like these two plasmids also had the *dfxA17* and *aadA5* in addition to the *bla*_{CTX-M-1}, this suggest that these plasmids may be epidemic in Europe (Madec *et al.*, 2011). Additionally IncI1 γ ST3 plasmids with resistance to tetracycline, sulphonamides and trimethoprim alongside *bla*_{CTX-M-1} were identified in human isolates from France, indicating possible pCH02, pCH03, pCT01 and pT01 plasmids (Cloeckaert *et al.*, 2010). The likelihood of transmission of ESBL IncI1 γ plasmids between animal and human isolates is increasing, with IncI1 γ ST3 *bla*_{CTX-M-1} plasmids having been found in pets and domestic animals, with

pathological disease in France and Tunisia (Dahmen, Haenni and Madec, 2012; Grami *et al.*, 2013). Although there is evidence for the movement of the plasmids from animals to humans, a study by Dobiasova *et al.* (2013) found isolates from 11 different animal species in a zoo, all carried *bla*_{CTX-M-1} on ST3 IncI1 γ plasmids in unrelated isolates, which may indicate the role of humans or food in transmission (Dobiasova *et al.*, 2013). IncI1 γ plasmids are not only present in livestock animals but have also been isolated from wild mammals and migrating birds in Poland and the Czech Republic (Literak *et al.*, 2010a; Literak *et al.*, 2010b). IncI1 γ plasmids (ST3) have also been recovered with *bla*_{TEM-52} from pigs and *bla*_{CMY-2} from cattle and chickens (Rodrigues *et al.*, 2013; Hopkins *et al.*, 2006; Carattoli *et al.*, 2002; Johnson *et al.*, 2011). The *bla*_{CTX-M-1} IncI1 γ plasmids have been found to be one of the common zoonotic risks to humans from non pathogenic *E. coli* from healthy poultry and pose a significant risk to human health (Bortolaia *et al.*, 2010).

Comparison of the IncI1 γ plasmid from animals in the UK with those published on GenBank revealed a high level of similarity particularly in the *pil* and *tra* loci, with sequence coverage's ranging from 70-97%, and plasmids pNF1358, pColIb-P9 and pSL476_91 being the most closely related. The genes of the leading strand and those of the *tra* and *pil* loci are highly conserved as found by Sampei *et al.* (2010) and Johnson *et al.* (2011). The exceptions to this were pCH02 and pUMNF18_ IncI1 γ which have both undergone deletions to the *tra* and *pil* loci respectively, likely to render these plasmids immobile. Of the remaining plasmids the *pilJ* gene was absent in five and *traD* absent in eight plasmids, the *nikAB* genes for the relaxome and the *trbCBA* also involved in conjugation were present in all the plasmids. The leading strand genes *ardA*, *psiAB* and *ssb* involved in establishing the plasmids in the cell were present in all plasmids except for pCH02, suggesting that the deletion in pCH02 occurred once the plasmid was established in the cell. The conjugation and establishment of IncI1 γ are complemented by stability mechanisms which were present across the IncI1 γ plasmids such as *parAB* (16/20), *pndCA* (20) and *yacAB* (11/20). Variation in the colicin produced was also noted, 14/20 plasmids

were colicingenic, three of which produced ColIa, with the remaining ColIb as are those sequenced in this study. Genes aiding the survival of the host *impCAB* involved in DNA damage repair, were present in all plasmid except three, and the *ibfAC* for abortive infection, present in 9 plasmids.

Three main variations were observed in the IncI1 γ plasmids which may be representative of different lineages. The first is located downstream of the MDR in the plasmids sequenced in this study, but is located downstream of the *repZ* gene in other plasmids. This region consists of the of seven genes, *yacA* to *yaeB* which includes a putative addiction system, this region was absent in 9/20 plasmids. The second is located between two hypothetical genes *orf46-52*, and consists of five genes including *ibfAC*, which was absent in 11/20, including all of those from pigs in the USA. The third region is a region containing the *parAB* and *impCAB* genes involved in plasmid stability and host survival absent in 3/20 plasmids, with the *parAB* absent in a further three plasmids, all of which harboured the *bla*_{CTX-M}. These variations suggest that the plasmids sequenced in this study were most closely related to pColIb-P9, pSL476_91 and pNF1358, which all have these regions and may have diverged as part of the same lineage.

Virulence factors do not appear to be common features of IncI1 γ plasmids as with other plasmids such as those belonging to the IncF (pTN48) group and the closely related IncB (pO113 and pO26-Vir) plasmids, however they appear to be good vectors for resistance, with various resistance genes present on 15/20 plasmids compared in this study (Billard-Pomares *et al.*, 2011; Fratamico *et al.*, 2011; Leyton *et al.*, 2003). The insertion of resistances regions has occurred predominantly downstream of the *repZ*, *yacC* or *yafB* genes.

Three *bla*_{CTX-M} IncI1 γ plasmids have now been fully sequence, The pEC_Bactec from a horse in Belgium being ST31 with *bla*_{CTX-M-15}, the highly similar pESBL-EA11, also being ST31 and having *bla*_{CTX-M-15}, isolated from Stx producing O104 in the USA

(Smet *et al.*, 2010b; Ahmed *et al.*, 2012). The only IncI1 γ *bla*_{CTX-M-3} plasmid sequenced was pEK204 (ST16), isolated from a human in the UK, in the clonal strain O25:ST131 (Woodford *et al.*, 2009). These plasmids all possessed a Tn3 linked *bla*_{TEM-1} which became the site for the *ISEcp1-bla*_{CTX-M-3/-15} mediated insertion. The insertion of Tn3 has resulted in the loss of 19 genes present in pCT01. The insert has occurred downstream of *ygaA* resulting in the loss the colicin genes, infection abortion genes and *parAB*. This substantial loss of approximately 12 kb is likely the reason that these plasmids have some of the lowest coverage's (79-81%) with those sequenced in this study, despite bearing *bla*_{CTX-M} genes. Although pCT01 has a Tn3-*bla*_{TEM-1} it is not associated with the *bla*_{CTX-M-1} gene, having inserted downstream of the *traA* gene. The *bla*_{TEM-1} gene has frequently been found on *bla*_{CTX-M} IncI1 γ plasmids (Dahmen, Haenni and Madec, 2012; Rodrigues *et al.*, 2013; Garcia-Fernandez *et al.*, 2008).

Molecular markers were designed based on the IncI1 γ plasmids sequenced in this study so they could be identified and differentiate between published plasmids. The eight selected markers were capable of differentiating between plasmids pCH02, pCH03, pCT01 and pT01 and could distinguish the 16 GenBank plasmids into 9 groups, which included 4 clusters. The pMLST analysis of these 16 plasmids produces 13 individual ST types, which initially offers a higher level of discrimination than the molecular markers however this technique does require sequencing and analysis compared to presence or absence of amplicons from the molecular markers proposed in this study. Although the molecular marker scheme is not based on sequencing, comparisons of alleles of the mYgaA marker, distinguished the plasmids in two clusters, with the remaining two clusters being indistinguishable even by pMLST. Comparatively the two typing schemes can discriminate between plasmids that the other scheme cannot. Of note pCH02 could not be assigned a ST due to the absence of two loci. However the molecular marker scheme could not only assign a UID, but also differentiate from other plasmids, which is one of the benefits of using a yes/no scheme as the absence or presence is considered in the plasmids

typing. The IncI γ molecular markers differentiate based on changes to the plasmids which have occurred over time, and are consistent being found in multiple plasmids, this could imply divergent lineages of the IncI γ plasmids.

Unlike pMLST, the molecular markers do not require sequencing and so are suitable for screening of large numbers of field isolates. In this study 136 isolates were screened with the IncI γ molecular markers, and 111 were positive for one or more markers, 61 of which were positive for IncI γ replicon. Of the isolates negative for the IncI γ replicon 24 had a single marker, which was typically Itra, which has been previously found on the IncF pEK499, present in the UK in the clonal O25:ST131 and may account for its broad distribution (Woodford *et al.*, 2009). The failure of replicon typing of the field isolates cannot be excluded as the results suggest that 13 plasmids belong to the IncI γ . It was noted that some IncB plasmids produced the mCib marker, as colicin b genes have been found on some IncB plasmids such as pO26-Vir and pO113, however these plasmids can be distinguished by replicon typing. As seen with the pH19 primers (chapter 4) there is some cross reactivity with IncI γ plasmids (mArdA and mYdeA) and this highlights the close relationship between the plasmids of the IncI complex. Only the YgaA marker was identified in the IncK pCT plasmids, and no markers were identified in the IncZ pH19 plasmid. Consequently it would be recommended that isolates positive for the IncI γ molecular markers be further investigated by isolating the plasmids through either conjugation or transformation. Plasmids with known ST were also tested with the molecular markers to reflect an *in vitro* screening of the markers, the 9 ST's were grouped into 11 UID's demonstrating a higher level of differentiation, however as with pMLST, the molecular markers grouped some plasmids together which could be differentiated between by pMLST, and plasmids grouped as the same ST could be differentiated by molecular markers. This suggests that the markers can be used either as an alternative to pMLST or as a complement. The system of assigning each molecular marker a unique number, in much the same ways as alleles are assigned numbers, means combinations can be

compared, and a database could be created so that researchers globally can share and compare results.

IncI1 γ plasmids are effective vectors for the transmission of resistance and potentially contribute to the virulence of their hosts. IncI1 γ plasmids bearing *bla*_{CTX-M-1} genes are particularly prevalent within isolates from livestock, which may be a reservoir for these genes, particularly as most are multidrug resistant, and have stability mechanisms to aid their survival and persistence. Evidence is emerging for the transmission of these plasmids between animal isolates and humans, with the likely involvement of the food chain and domesticated animals and pets. The interest in IncI1 γ plasmids lead to the development of pMLST so that plasmids could be studied in more detail, which has been used to investigate plasmids further (Garcia-Fernandez *et al.*, 2008; Carattoli, 2011). This study presents a new tool to investigate and screen for IncI1 γ plasmids that is not based on sequencing, making it more accessible and applicable for screening of high numbers of isolates, being carried out in just three multiplex reactions. With this tool the epidemiology of plasmids can be further investigated, as seen with the markers for the IncK pCT plasmid and IncX4 pSAM7 plasmid, which have been shown to be beneficial in such investigations (Cottell *et al.*, 2011; Stokes *et al.*, 2012; Stokes *et al.*, 2013).

Chapter 8

General Discussion

8.1 General Discussion

Antibiotics have become one of the main tools clinicians and veterinarians use to combat infectious disease. However the efficacy of antimicrobial agents are constantly being undermined by the evolution of resistant pathogens. Multidrug-resistant Gram-negative bacteria, and in particular *E. coli* are a serious public health concern, in both hospitals and the community, with a possible return to a pre-antibiotic era (Giske *et al.*, 2008; Pitout and Laupland, 2008; Thomson and Bonomo, 2005). The rise of resistance to broad spectrum β -lactams by the TEM and SHV enzymes, through persistent over use, resulted in the development of extended spectrum β -lactams such as the third generation oxyimino cephalosporins. However soon after their introduction resistance to third generation antibiotics emerged with a new class of enzyme being identified namely CTX-M (Bonnet, 2004; Bradford, 2001). CTX-M have now become the most widely disseminated plasmid mediated ESBL's in the world, and are displacing TEM and SHV as the predominant β -lactamase (Bonnet, 2004; Bradford, 2001; Bush, 2010; Canton and Coque, 2006; Hawkey and Jones, 2009; Livermore *et al.*, 2007). Plasmids bearing *bla*_{CTX-M} have been isolated from 26 bacterial species, particularly in members of the *Enterobacteriaceae*, including *E. coli*, *Salmonella enterica* and *Klebsiella pneumoniae* common causes of gastrointestinal, urinary and respiratory infections (Bonnet, 2004; Zhao and Hu, 2013; Carattoli, 2009). Plasmids are also the vectors for the emerging carbapenemase genes *bla*_{KPC} and *bla*_{NDM-1}, which confer resistance to all β -lactams, and cannot be ignored, being one of the most important public health concerns (Carattoli, 2013; Queenan and Bush, 2007). Therefore the studying and understanding of plasmids has an ever increasing in importance.

CTX-M-producing isolates have a significant impact on the outcomes of disease often resulting in delayed treatment or treatment failure, which is particularly evident in UTI's and bacteraemia. Bacteraemia caused by ESBL-producing *E. coli* have been found to increase mortality (60.8%), mostly as a result of treatment failure, in both hospitals and the community (Rodriguez-Bano *et al.*, 2010; Schwaber and Carmeli, 2007; Melzer and

Petersen, 2007). Patients with UTI's have been found to be a risk factor for the dissemination of CTX-M isolates, between members of same household, community and hospitals (Rodriguez-Bano *et al.*, 2006; Rodriguez-Bano *et al.*, 2008). The carriage of CTX-M isolates amongst healthy individuals is also increasing, raising the possibility of dissemination within the community and chances of infection (Nicolas-Chanoine *et al.*, 2013; Valverde *et al.*, 2004). Several risk factors have been associated with ESBL infections which include international travel to areas of high ESBL prevalence, origin of birth, chronic illness, diabetes, previous antibiotic use and urinary tract pathology (Freeman *et al.*, 2008; Tangden *et al.*, 2010; Nicolas-Chanoine *et al.*, 2012; Calbo *et al.*, 2006; Rodriguez-Bano *et al.*, 2006). To manage and treat these infections correct diagnosis, treatment and infection control are vital considerations (Pitout and Laupland, 2008; Paterson and Bonomo, 2005).

Plasmids are the main vector for the transmission of CTX-M enzymes between isolates, with nearly all plasmid groups associated with CTX-M genes (D'Andrea *et al.*, 2013; Canton, Gonzalez-Alba and Galan, 2012; Carattoli, 2009; Carattoli, 2011). Plasmids have long been identified as vectors of multiple resistance genes and their transmission between bacterial species in different environments, which has lead to their further study (Watanabe, 1963; Kruse and Sorum, 1994).

Plasmids were present in the bacterial population before the use of antimicrobials and without resistance genes, and it is likely that these plasmids have become vectors for resistance through the acquisition of MGE's (Datta and Hughes, 1983; Jones and Stanley, 1992). The increase in next generation sequence technology and whole genome sequencing has increased the number of resistance plasmids being fully sequenced. This sequencing information is allowing researchers to gain a greater understanding of the relationship between plasmid backbones and the genes they carry. Comparison of plasmids bearing *bla*_{CTX-M} genes have identified potential progenitors and related plasmids, modified by insertions and deletions. The *bla*_{CTX-M} IncFII plasmids pC15-1a, pEK499,

pEC_B24 and pEC_L8, all from *E. coli* share sequence homology to the plasmids R100, pRSB107 and pIP106 (Boyd *et al.*, 2004; Woodford *et al.*, 2009; Smet *et al.*, 2010b). The IncL/M pCTX-M3 plasmid from Poland, was found to be highly similar to pEL60 isolated from the plant pathogen *Erwinia anylovora*, and the sequence analysis of the IncN plasmid pKP96 suggested it was a heavily modified ancestor of plasmid R46 (Golebiewski *et al.*, 2007; Shen *et al.*, 2008). The same is evident for the IncI1 γ plasmids pEK204 and pEC_Bactec which appear to be related to an ancestor similar to pColIb-P9 which have undergone the insertion Tn3-*bla*_{TEM-1} and ISEcp1-*bla*_{CTX-M} (Woodford *et al.*, 2009; Smet *et al.*, 2010b). These progenitor plasmids may have provided a genetic scaffold and backbone which was modified by the insertion of resistance genes.

To determine the relationship between *bla*_{CTX-M} plasmids from animals and humans in the UK with those around the world, seven *bla*_{CTX-M} plasmids, one from a human *E. coli* isolate and six from animal *E. coli* isolates were sequenced in this study. The plasmids were fully annotated and compared with published plasmids from around the world. The seven plasmids represented four incompatibility groups which included the IncZ pHI9 from a human (chapter 4), IncX4 pSAM7 from cattle (chapter 5), IncA/C pCH01 from a chicken (chapter 6) and four IncI1 γ plasmids pCH02, pCH03, pCT01 and pT01 from chickens, cattle and turkey (chapter 7). These sequenced plasmids represent some of the *bla*_{CTX-M} vectors present in the UK bacterial population. All of the plasmids with the exception of the IncI1 γ pCH02, were found to be self conjugative, being capable of disseminating the *bla*_{CTX-M}. All of these plasmids were isolated from diverse *E. coli* and had a MDR phenotype, except for pSAM7 which was only resistant to β -lactams. This could result in the co-selection of both the *bla*_{CTX-M} plasmids and their hosts through treatment with multiple antimicrobial compounds including aminoglycosides, trimethoprim, chloramphenicols, tetracyclines and sulphonamides. The comparison of the plasmids from each incompatibility group with related plasmids in GenBank revealed that their core backbones were highly conserved, with the most homology observed in the

conjugation and stability genes. This supports the hypothesis that plasmids recovered from isolates in the UK are similar to plasmids from animals and humans around the world, and that the conjugation and stability genes are important in their dissemination.

Plasmid pH19 was identified during the screening for pCT-like plasmids. This IncZ plasmid represents a combination of an IncI complex backbone, such as the IncB, with the replication protein from an IncFII plasmid (Kato and Mizobuchi, 1994; Praszquier *et al.*, 1991). Despite sharing the replication protein with IncFII plasmids no other homology between these plasmids was observed, with pH19 being closely related to IncB and K plasmids. Comparison of the IncB, K and Z plasmids found that they carried either resistance or virulence genes, but not both, which may suggest a cost or burden on the plasmid backbone. EHEC isolates have been found harbouring the closely related virulent (pO26-Vir and pO113) and resistant (pHUSEC41-1) plasmids around the world (Fratamico *et al.*, 2011; Leyton *et al.*, 2003; Kunne *et al.*, 2012). Comparison of pH19 with the IncK pCT plasmid from cattle identified a coverage of 75% compared to the 65% determined by RFLP, which was similar coverage to other IncB and K plasmids. pH19 had undergone one main insertion of a complex class 1 integron which was also found in *E. coli* C1635 and the IncFII plasmid pTN48, it could have been possible that the change in replication protein and acquisition of the MDR occurred at the same time from a plasmid such as pTN48 (Billard-Pomares *et al.*, 2011; Ben Slama *et al.*, 2011). The example of the *bla*_{CTX-M} group 9 environment by Poirel *et al.* (2003), is in the same class 1 integron, all of these isolates are either from France or Tunisia, which may be a reservoir for this integron (Poirel, Decousser and Nordmann, 2003).

The small IncX4 plasmid pSAM7, is part of an expanding group of plasmids being recognised through the new molecular markers proposed by Johnson *et al.* (2012). The pSAM7 plasmid is the second IncX4 *bla*_{CTX-M} plasmid to have been sequenced with *bla*_{CTX-M-15} pJIE143 being the first, and was the first IncX4 plasmid to be sequenced in the UK (Partridge *et al.*, 2011; Stokes *et al.*, 2013). The pSAM7 plasmid had a 99% coverage with

pJIE143 which was isolated from human *E. coli* ST131 in Australia, with the main difference being the *bla*_{CTX-M} gene and inserts. The *ISEcp1-bla*_{CTX-M-14b} insert was novel, however a similar insert was identified in the IncL/M pJEG011 plasmid from *Klebsiella pneumoniae*, which also harbours the IncX4 plasmid pJEG012 (Espedido *et al.*, 2013). Such a combination of plasmids may have lead to the formation of plasmid and insert observed in pSAM7. The IncX4 plasmids shared a coverage of 81-97% suggesting they have likely been in the bacterial population for some time, due to their presence in unrelated animals and human isolates on separate continents.

The IncA/C plasmids have a remarkably high level of homology and are highly conserved, as mentioned by Fernandez-Alarcon *et al* (2011) and appear to be divided into three separate lineages (Fernandez-Alarcon, Singer and Johnson, 2011). The IncA/C plasmids have a broad host range with those sequenced being recovered from 11 bacterial species isolated from humans, cattle, poultry and fish. The pCH01 plasmid was the largest of all the plasmids sequenced in this study, and had a coverage ranging from 85-99% with other IncA/C plasmids. Of all the plasmids in this study, IncA/C plasmids appear to be the most conserved with the variations being due to the insertion of resistance regions. IncA/C plasmids have been found with numerous *bla* genes including *bla*_{OXA}, -_{CMY}, -_{SHV}, -_{CTX-M} and of major health concern *bla*_{NDM-1} (Kumarasamy *et al.*, 2010). Those bearing the *bla*_{CMY} genes seemed to be in a separate lineage to those with *bla*_{NDM-1} and the other IncA/C plasmids. The pCH01 plasmid is the first group 1 CTX-M IncA/C plasmid to be sequenced, bearing the *bla*_{CTX-M-3} gene in a novel environment, in addition to its MDR region conferring resistance to a further three classes of antimicrobial and proposed to confer resistance to mercury and quaternary ammonium compounds. The pCH01 plasmid was most closely related to two plasmids pEA1509 from *Enterobacter aerogenes* and pR55 from *Klebsiella pneumoniae* both isolates from humans, which also shared the highest homology with the MDR regions (Doublet *et al.*, 2012; Diene *et al.*, 2013).

The sequenced IncI1 γ plasmids isolated from animals in the UK, all shared the same plasmid backbone which had undergone deletions and the insertion of various MDR regions. All of the plasmids belonged to the ST3 or were likely to, which is a prevalent plasmid sequence type carrying *bla*_{CTX-M-1} in poultry, cattle and humans in Europe (Cloeckaert *et al.*, 2010; Madec *et al.*, 2011; Dierikx *et al.*, 2013; Leverstein-van Hall *et al.*, 2011). IncI1 γ plasmids are common in animal species, particularly those from swine but are also present in poultry and cattle, with animals acting as a possible reservoir for these plasmids (Johnson *et al.*, 2011). It is likely that the same plasmid IncI1 γ backbone is present in cattle, chicken and turkeys in the UK, and appears to be related to plasmids pCollb-P9, pSL476 and pNF1358 (Dunne *et al.*, 2000; Fricke *et al.*, 2011). The comparative analysis of the IncI1 γ plasmids shows the backbone is conserved in the *tra* and *pil* loci, however there appears to be several lineages, linked to resistance and accessory genes. Despite the IncI1 γ plasmids pEK204, pEC_Bactec and pESBL-EA11 all having *bla*_{CTX-M} genes, these plasmids share the lowest coverage with the plasmids sequenced in this study, but themselves are closely related suggesting they share the same lineage (Woodford *et al.*, 2009; Smet *et al.*, 2010b; Ahmed *et al.*, 2012).

The comparative genomics of the sequenced plasmids in this study with those previously published highlighted the high level of similarity of plasmids from different hosts and locations. It is likely that all plasmids stemmed from ancestral plasmids that have undergone changes throughout multiple passages to form different lineages, these ancestral plasmids are likely to have acquired resistance genes over time as separate events (Datta and Hughes, 1983; Jones and Stanley, 1992; Walsh, 2006; Frost *et al.*, 2005; Davies and Davies, 2010). The findings suggest that as similar plasmids are found in both isolates from humans and animals, and that the plasmids sequenced in this study would be capable of existing in isolates adapted to animals and humans. This study also indicates that the spread of *bla*_{CTX-M} involves at least three key steps. The first is the mobilization of the resistance gene, this occurs through transposons and insertion sequences with *ISEcp1*,

ISCR1 and IS26 being some of the most important (Lartigue *et al.*, 2006; Toleman and Walsh, 2011; Toleman, Bennett and Walsh, 2006; Poirel *et al.*, 2005; Eckert, Gautier and Arlet, 2006). *ISEcp1* was located upstream of the *bla*_{CTX-M} gene in all of the plasmids in this study, and in 6/7 was independent from any other MDR region such as an integron or transposon. This further demonstrates the importance of the *ISEcp1* and other transposons in mobilising the genes not only between plasmids, but also from the chromosomes of bacteria (Fabre *et al.*, 2009; Lartigue *et al.*, 2006). Once *bla*_{CTX-M} has been mobilized it is typically incorporated into a plasmid or chromosome, its incorporation into a plasmid facilitates the spread of *bla*_{CTX-M} in two ways. The first is by providing a relatively stable backbone, with plasmid addiction and partitioning systems and a replicating platform to persist; and secondly the means to disseminate between bacterial isolates and across genera (Gerdes, Moller-Jensen and Bugge Jensen, 2000; Bignell and Thomas, 2001; Mnif *et al.*, 2010). The plasmids are then disseminated in the environment by bacterial isolates which provide the opportunity for plasmids to move between hosts by horizontal transmission. Some bacterial clones are particularly successful in the dissemination of resistance genes and have been termed as "high risk clones" (Woodford, Turton and Livermore, 2011). In particular the pandemic *E. coli* O25:ST131 clone which is typically found carrying *bla*_{CTX-M-15} IncF plasmids. This clone is commonly associated with UTI infections where the use of fluoroquinolones may have a co-selective effect (Rogers, Sidjabat and Paterson, 2011; Coque *et al.*, 2008; Nicolas-Chanoine *et al.*, 2008; Dhanji *et al.*, 2011a). To fully understand the transmission and dissemination of resistance genes these three key steps need further investigation, with plasmids being the focus of this study and should be the subject of further studies.

The sequencing of whole plasmids and their fragments has led to the development of molecular methods for the characterization and epidemiological study of plasmids. Such methods include replicon typing, sequencing of several loci (pMLST) and molecular markers for specific genes or regions (pCT and pSAM7) (Carattoli *et al.*, 2005a; Garcia-

Fernandez *et al.*, 2008; Garcia-Fernandez *et al.*, 2011; Cottell *et al.*, 2011; Stokes *et al.*, 2013). Replicon typing is considered the "gold standard" in plasmid characterization and has greatly advanced the study of plasmid epidemiology, having particularly been applied to plasmids associated with resistance genes, but also screening of pathogenic and commensal isolates (Carattoli *et al.*, 2005a; Carattoli, 2009; Hopkins *et al.*, 2006; Marcade *et al.*, 2009; Lindsey *et al.*, 2011b; Johnson *et al.*, 2007). pMLST has given a deeper insight into plasmid epidemiology, the sequencing of loci allows phylogenetic analysis of plasmids which is particular prevalent in IncI γ plasmids, but also for IncN, F and HI2 (Garcia-Fernandez *et al.*, 2008; Garcia-Fernandez and Carattoli, 2010; Garcia-Fernandez *et al.*, 2011; Villa *et al.*, 2010).

Plasmid molecular markers present a new approach for the characterization of plasmids. Analysis of plasmid genetic backbones has been used to design molecular markers without the need for sequencing or plasmid isolation. The markers developed by Cottell *et al.* (2011) for the IncK *bla*_{CTX-M-14} pCT plasmid from UK cattle, have been used to demonstrate that similar plasmids are present in isolates from cattle, turkeys and humans in the UK (chapter 3), and around the world (Cottell *et al.*, 2011; Stokes *et al.*, 2012; Dhanji *et al.*, 2012). To determine whether plasmid molecular markers are effective in investigating plasmid epidemiology, the pCT markers, which were being developed concurrently, were used to screen diverse *E. coli* for pCT-like plasmids. This resulted in the identification of 21 pCT-like plasmids, which was 30% of all *bla*_{CTX-M-14} isolates and 36% of those from animals. Plasmids were additionally found to have the same replicon, size and RFLP profiles, supporting the hypothesis that plasmid molecular markers can be used to identify the same or highly similar plasmids to those found in Europe and Australia and benefit epidemiological studies (Stokes *et al.*, 2012; Cottell *et al.*, 2011; Valverde *et al.*, 2009; Zong *et al.*, 2008). The pH19 IncZ plasmid (chapter 4) was identified using the pCT markers, which like the IncK pCT belongs to the IncI complex. This demonstrated

the possibility that molecular markers could not only identify specific plasmids but also could be extended to differentiate between related plasmids.

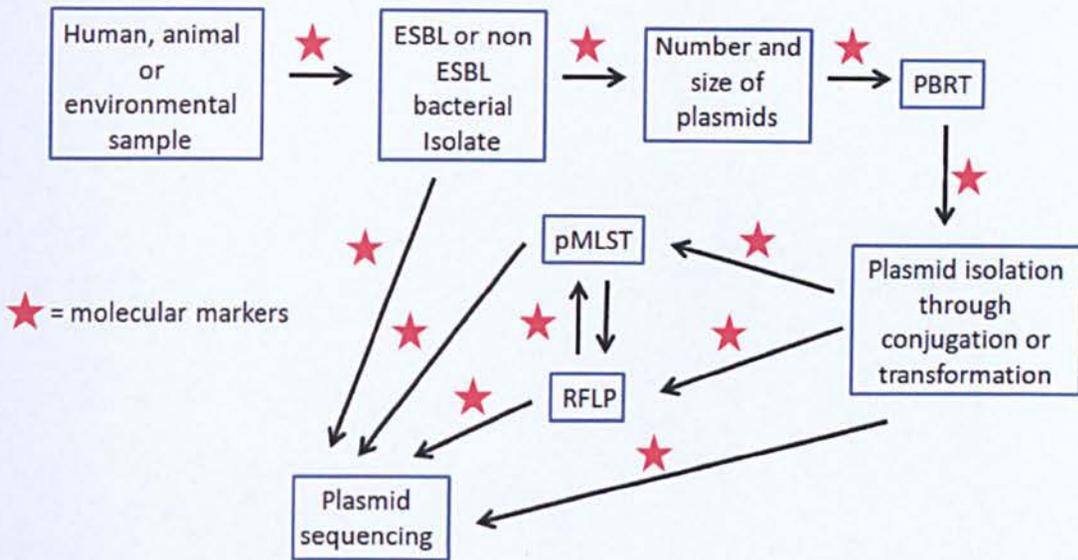
The plasmid content of an isolate can be used to study their epidemiology, but plasmids are increasingly becoming topics of epidemiological research in their own right (Mayer, 1988). Methods have improved on sizing, RFLP and hybridizations to PCR based methods, with replicon typing and pMLST being commonly used. This study and others have already reported on the benefits of plasmid molecular markers, with the identification of pCT plasmids (Stokes *et al.*, 2012; Stokes *et al.*, 2013; Dhanji *et al.*, 2012; Cottell *et al.*, 2011). Molecular markers were designed to identify the plasmids sequenced in this study so that the epidemiology and prevalence of these plasmids can be determined, which would be part of further work. Another aim of the molecular markers designed in this study was to select markers with the ability to differentiate between plasmids of the same incompatibility group or those that were closely related, which would provide new higher resolution tools to differentiate and study plasmids. Molecular markers were designed for all four plasmid incompatibility groups, representative of plasmids in animal and human isolates in the UK. *In silico* testing of the markers with the GenBank plasmids, showed that each Inc group could be subdivided, with eight groups for IncB, K and Z, five groups for IncX4, 14 groups for IncA/C and 12 groups for IncI1 γ . This supports the hypothesis that molecular markers can not only identify specific plasmids but can also be used to differentiate between plasmids of the same incompatibility group. The *in vitro* analysis showed that these molecular markers could identify the sequenced plasmids, and through *in silico* testing differentiate the IncI complex, IncX4, IncA/C and IncI1 γ plasmids. Initial screening with these markers has already identified pSAM7-like plasmids (Stokes *et al.*, 2013), and plasmids relating to pCH03, pCT01 and pT01. Although no pH19 like plasmids were identified, pCT-like plasmids were identified using the pH19 markers. Screening using the IncA/C markers was not carried out at this time due to a redesign of

the molecular markers, but if more time and resources were available this work would have been carried out.

It is proposed that the molecular markers be used in conjunction with PBRT by Carattoli *et al* (2005a) and in the case of IncX plasmids using those from the Johnson *et al* (2012), but could also be used independently if looking specifically for the four plasmids groups (Carattoli *et al.*, 2005a; Johnson *et al.*, 2012). Isolates identified by PBRT for one of the four replicon types, could be further analysed using the respective molecular markers, further differentiating the plasmids into a subdivision which are assigned a UID. It is recommended that for accurate results plasmids be isolated into a known background through transformation or conjugation, this is to avoid some cross reactivity with the IncI complex plasmids and molecular markers. The molecular markers proposed in this study provide researchers with more information than just the replicon type, and saves time and cost with respect to RFLP and pMLST. The conjugative nature of plasmids which makes them successful vectors for resistance also can make them difficult to track when transferred between isolates and across bacterial genera. The molecular markers proposed in this study are likely to have the ability to characterise plasmids in numerous bacterial genera, with *Salmonella enterica* and *Klebsiella pneumoniae* being of particular interest. For example if there was a *bla*_{CTX-M} outbreak in a hospital among *E. coli* isolates, researchers may establish the clonality of the isolates, however if they are unrelated and/or *Klebsiella pneumoniae* isolates started to exhibit the same phenotype, then a transmissible element is likely to be responsible. Currently researchers are restricted to PBRT, sizing, RFLP, pMLST (if possible) and ultimately sequencing. This would result in the plasmids being hard to identify. With the use of molecular markers a further method can be applied to determine plasmid transmission. As these markers screen for plasmid backbones rather than resistance genes they carry, these markers will also identify plasmids which may act a potential vectors for resistance, which could be risk factors and warrant monitoring. Additionally markers could be used alongside PCR for resistance genes for a more targeted

approach. The molecular markers could also be used to observe the acquisition of plasmids by an isolate over a period of time, the possible stages where molecular markers could be used are shown in Figure 8.1.

Figure 8.1 Possible uses of the plasmid molecular markers



Examples of where the molecular markers could be used to identify and study the epidemiology of plasmids.

Each possible combination of molecular markers produces a unique ID (UID), due to each marker being assigned a number in binary format. The UID's could be used to populate a database for the four plasmid incompatibility groups in this study, in a similar way to MLST and pMSLT databases, allowing researchers to compare plasmids around the world aiding epidemiological studies. Unlike pMLST the markers do not require sequencing, which saves time and money, but also makes them accessible to laboratories with a limited knowledge of sequence analysis or sequencing facilities, which is common in governmental and high throughput surveillance laboratories. Although not reliant on sequencing, it was found that sequencing of some markers can be beneficial in distinguishing plasmids with the same marker combination.

Several studies have now provided evidence for the transmission of IncN, K, FII and IncII γ plasmids between humans and animals, (Moodley and Guardabassi, 2009; Ho *et al.*, 2012; Stokes *et al.*, 2012; Leverstein-van Hall *et al.*, 2011; Cloeckaert *et al.*, 2010). Further studies are still needed to provide definitive proof of plasmid transmission for other plasmids groups such as IncX4 and IncA/C, and further expand on IncB, K, Z and IncII γ plasmids, to see the impact of these vectors on resistance dissemination. If plasmid mediated resistance is moving between animals and humans then contaminated food is the most likely route of transmission, following the "farm to fork" notion. Food has already been found to be contaminated with *bla*_{CTX-M} isolates, which, if handled incorrectly or undercooked could pose a threat to human health by either being pathogenic or through horizontal transmission of resistance plasmids (Kola *et al.*, 2012; Silva *et al.*, 2012; Jouini *et al.*, 2007; Warren *et al.*, 2008; Dhanji *et al.*, 2010; Leverstein-van Hall *et al.*, 2011). More work is required to further establish the links between animals, food products and humans which would be benefitted by the marker schemes designed in this study. The data from this work could go towards additional information for mathematical models which are being used to model plasmid mediated gene resistance, aiding the understanding of the dynamics of plasmids transfer (Volkova *et al.*, 2012; Volkova *et al.*, 2013).

Antimicrobial resistance is one of the biggest health problems the world faces, which was emphasised in the Chief Medical Officer annual report published in March 2013 (Department of Health., 2013). Although this study has focused on the *bla*_{CTX-M} genes it must not be forgotten that plasmids are vectors for carbapenemases such as KPC and the metallo β -lactamases NDM-1 (Carattoli *et al.*, 2012; Walsh *et al.*, 2011; Queenan and Bush, 2007). These enzymes confer resistance to all β -lactams, with clinicians often resorting to the use of colistin and tigecycline, drugs considered the last line of defence. This further highlights the importance of identifying and studying the epidemiology of plasmids, routes of transmission and reservoirs. With the desperate need for new antibiotics and limited funding in academia for new compounds, the need to control the

spread rather than treat the problem is growing ever greater (Head *et al.*, 2013; Wise *et al.*, 2011). If animals are implicated in the dissemination of resistance or act as reservoirs for plasmids and mobile elements then the reduction of antibiotic use, and in particular cephalosporins, has been shown to reduce the number of resistant isolates (Dutil *et al.*, 2010; Cavaco *et al.*, 2008; Jorgensen *et al.*, 2007; Agerso and Aarestrup, 2013). Other methods are needed to control infections, this may involve plasmid curing, silencing, improved hygiene, patient management, and what may prove the most important factor; controlling the use of antibiotics (Hunter *et al.*, 2010).

In conclusion this study has sequenced, annotated and compared the first *bla*_{CTX-M-14} IncX4 and IncZ plasmids, the first ST3 *bla*_{CTX-M-1} IncI1 γ and the first group 1 CTX-M IncA/C plasmids, contributing to the knowledge of CTX-M plasmids in the UK and the world. These plasmids were found to be highly similar to those sequenced from animal and human isolates around the world, suggesting that these plasmids all share common ancestors which have evolved over time through insertions and deletions, typically facilitated by MGE's, to form new lineages. The *bla*_{CTX-M} gene in all of the six plasmids from animals were found to have inserted independently of any other MGE. This would hypothesise that the *ISEcp1-bla*_{CTX-M} has inserted as a separate event, into pre-existing plasmids present in the animal population which have acted as suitable vectors for *bla*_{CTX-M} carriage. Plasmids from animals were found to have an MDR phenotype with the exception of pSAM7, which may have aided their persistence through co-selection with other antimicrobials, such as aminoglycosides, sulphonamides and tetracyclines. The MDR integron present in pH19 from a human isolate, has been identified previously which may suggest the acquisition of *bla*_{CTX-M} in humans is due to complex MGE's. This study has shown that molecular markers are effective tools in investigating plasmids identifying the same plasmid or similar plasmids are present in human and animal isolates in the UK, suggesting possible transmission across species. This study proposes new molecular markers which expand on their previous applications being capable of not only identifying

specific plasmids but also differentiating between closely related plasmids, providing a non sequence based PCR to further subdivide plasmid incompatibility groups into separate lineages. This contributes a new tool which can be used to further investigate the epidemiology of plasmids and improve the understanding of routes of transmission and reservoirs which can be targeted to prevent further dissemination.

8.2 Further work

The molecular markers developed in this study would benefit from further work and refinement. Currently the selection of the markers was restricted to targets which could be tested *in vitro* using the plasmids sequenced in this study. To determine whether the level of differentiation for the IncX4, IncA/C, pH19 and IncII γ markers coincided with those from *in silico* testing, the markers should be tested with each plasmid *in vitro* to ensure the stated conditions would yield or fail to yield amplicons. The availability of sequenced GenBank plasmids for *in vitro* testing restricted target selection and marker design, if more plasmids were available then additional markers which may have a higher level of differentiation than those in this study could be identified. An example of this was observed with the IncA/C markers in the study by Welch *et al* (2007), identifying markers in three regions absent in pCH011, which offered an alternative level of differentiation (Welch *et al.*, 2007). Further work would be to obtain and/or sequence more plasmids allowing for the design of molecular markers with an increased differentiation potential.

This study has demonstrated that plasmid molecular markers can be used in the identification, differentiation and epidemiological study of plasmids (Stokes *et al.*, 2012; Stokes *et al.*, 2013). However the markers developed in this study are limited to IncB, K, Z, X4, IncII γ and A/C plasmids. Further work should be undertaken to develop additional molecular markers for plasmids belonging to the IncFII, IncN, IncHI2 and IncL/M, due to their association with resistance genes, which would be the next targets for marker development if this study was continued (Carattoli, 2009; Carattoli, 2011). This would be

carried out by identifying suitable plasmid candidates, sequencing and comparison as performed in this study, followed by *in vitro* testing and field isolate screening.

Microarrays are increasingly being used to investigate the resistance and virulence gene contents of bacterial cells (Geue *et al.*, 2010; Card *et al.*, 2013; Stokes *et al.*, 2012).

Further work would be to develop and adapt plasmid molecular markers into an array based format which could be incorporated into commercial arrays, allowing researchers to not only identify resistance and virulence genes in an isolate but also for plasmid content, in a single assay. This could be extended further to create microarrays for orfs found on the plasmids belonging to the IncI complex, IncI1 γ and IncX4 plasmids as has been previously carried out for IncA/C by Lindsey *et al* (2011a).

To investigate the epidemiology of specific plasmid types belonging to IncB, K, Z I1 γ and A/C, studies such as those conducted for pCT and pSAM7 which be carried out as part of further work (Cottell *et al.*, 2011; Dhanji *et al.*, 2012; Stokes *et al.*, 2012; Stokes *et al.*, 2013). The study would specifically focus on the "farm to fork" notion, to investigate the extent of plasmid carriage at the farm level, retail area and in both community and noscomial infections in the UK, similar to that by Leverstein-van Hall *et al* (2011). As screening in this study focused on *E. coli* isolates, part of continued work would be to extend screening of host species to include *Salmonella* spp and *Klebsiella* spp isolated from the environment, animals, food products and humans. The aims of this continued screening would be to identify the dissemination of plasmids from animals to humans through the food chain, but also the environment, identifying both plasmid transmission across bacterial species and hosts. This would provide yet more information on the involvement of farm animals and the food chain in transmission into the human population. Although *bla*_{CTX-M} bearing plasmids were the focus of this study, comparative genomics of plasmids from the representative incompatibility groups, found that few plasmids were associated with *bla*_{CTX-M}. However 45/58 plasmid compared in this study had at least one resistance gene, which could be used as another criteria for selection. This would fit the

hypothesis raised in this study that animal isolates act as reservoirs for not only resistance genes but also plasmid vectors, which can acquire *bla*_{CTX-M} transposition units. This may suggest that the mere presence of a plasmid is a potential risk factor for the dissemination of resistance genes, and if some backbones are more successful than others then non resistant isolates should also be screened.

In this study the genetic characteristics of seven *bla*_{CTX-M} plasmids was examined in depth. However the investigation into the phenotypic properties of these plasmids was limited to the antimicrobial resistances conferred to the host and transferability of the plasmid. Although the plasmids confer a beneficial resistance phenotype on the host, further studies should be conducted to determine whether any other phenotype is conferred be it beneficial or detrimental to the host. Contributions to the pathogenicity of the host could be investigated using eukaryotic cell culture, to observe levels of adherence and also invasion between hosts with and without plasmids, similar to the study conducted by Dudley *et al* (2006). Various plasmid combinations have also been found to influence the virulence of pathogenic avian *E. coli* strains, which may be the case with the plasmids sequenced in this study (Mellata *et al.*, 2010). Additionally invertebrate models such as *Galleria mellonella* could be used to determine mortality rates caused by infections with plasmid bearing hosts (Leuko and Raivio, 2012). Initial studies using *Galleria mellonella*, not included in this thesis but undertaken as part of this study, showed an 60% increase in mortality between *E. coli* DH10 and *E. coli* DH10 transformed with pCT, and 10% increase in mortality with pCH01. Such studies may aid in the understanding of the functions of the many hypothetical genes present in the four incompatibility group plasmids in this study, which could additionally be investigated using cloning and knock outs.

The metabolism of the host may also be altered by the presence of a plasmid, and should be investigated for those plasmids in the UK (Diaz Ricci and Hernandez, 2000;

Wang *et al.*, 2006). The use of a high throughput system such as Biolog would allow comparisons of the respiration and utilisation of different carbohydrate, nitrogen and phosphate sources (Bochner., 2009). Identifying any changes in metabolism caused by the plasmids, whether it is an increase in the utilization of compounds or a burdening effect. The effects on the growth of a host harbouring plasmids should also be undertaken. This would determine any fitness cost on the host, and through competition experiments observe whether plasmid hosts are outcompeted in the environment (Mellata *et al.*, 2012; Bentley *et al.*, 1990; Haft, Mittler and Traxler, 2009).

The successful dissemination of *bla*_{CTX-M} genes by plasmids is affected by both transferability and persistence. The four plasmids belonging to different incompatibility groups were all found to conjugate, however experiments only included a single bacterial species as a recipient, and the rate of transfer was not determined. Further work should be conducted to include more bacterial species as recipients, such as other *E. coli*, *Salmonella* spp and *Klebsiella* spp which have been found with *bla*_{CTX-M} genes. The conjugation frequency of the plasmids between hosts should also be determined to observe whether plasmids would be transferred in the time frames relevant in the environment, humans and animals, such as transient passage through the digestive tract (Toszeghy *et al.*, 2012). Once transferred into a new host, the persistence of the plasmid is important for continuous dissemination, which should be investigated further for the plasmids in this study. Experiments to determine the persistence of each plasmids in the host under non selective conditions with >100 generations should be conducted, to simulate the environments inhabited by the host, and determine whether selective pressure is required to maintain the plasmids (Cottell, Webber and Piddock, 2012; Subbiah *et al.*, 2011). The persistence of the *bla*_{CTX-M} gene itself should also be monitored concurrently, as it may be that the plasmid persists, but the mobile transposition unit containing *bla*_{CTX-M} may have been lost (Lartigue *et al.*, 2006; Fabre *et al.*, 2009).

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Appendix I Isolates used in the chapter 3 *bla*_{CTX-M-14} pCT-like plasmid screening

Isolate name	Origin	Sample	Isolation date	Location	CTX-M	Inc type	Source
C159/11	Cattle	Faeces	2004	UK	14	K	AHVLA
26-C0343-11-06	Cattle	Caecum	2006	UK	14	ND	AHVLA
17-C0243-04-08	Cattle	Faeces	2008	UK	14	ND	AHVLA
26-C0359-04-08	Cattle	Faeces	2008	UK	14	ND	AHVLA
26-C0346-09-06	Cattle	Faeces	2006	UK	14	K	AHVLA
26-C0399-03-09	Cattle	Faeces	2009	UK	14	ND	AHVLA
27-C0248-09-07	Cattle	Faeces	2007	UK	14	ND	AHVLA
21-C0190-04-08	Cattle	Faeces	2008	UK	14	ND	AHVLA
23-C0346-07-08	Cattle	Faeces	2008	UK	14	ND	AHVLA
23-C0313-09-06	Cattle	Faeces	2006	UK	14	ND	AHVLA
23-C0101-09-06	Cattle	Faeces	2006	UK	14	ND	AHVLA
23-C0645-03-08	Cattle	Faeces	2008	UK	14	ND	AHVLA
23-C0542-10-08	Cattle	Faeces	2008	UK	14	ND	AHVLA
23-C0136-08-06	Cattle	Faeces	2006	UK	14	ND	AHVLA
21-C0097-07-08	Cattle	Faeces	2008	UK	14	ND	AHVLA
23-C0165-08-08	Cattle	Faeces	2008	UK	14	ND	AHVLA
24-C0317-04-08	Cattle	Faeces	2008	UK	14	ND	AHVLA
27-C0010-07-08	Cattle	Faeces	2008	UK	14	F, FIA	AHVLA
143-18B	Cattle	Faeces	2010	UK	14	ND	AHVLA
701-90W	Cattle	Faeces	2010	UK	14	ND	AHVLA
702-11B	Cattle	Faeces	2010	UK	14	ND	AHVLA
702-24B	Cattle	Faeces	2010	UK	14	ND	AHVLA
111-44B	Cattle	Faeces	2010	UK	14	ND	AHVLA
097-41B	Cattle	Faeces	2010	UK	14	ND	AHVLA
205-6B	Cattle	Faeces	2010	UK	14	ND	AHVLA
156-42B	Cattle	Faeces	2010	UK	14	ND	AHVLA
070-54B	Cattle	Faeces	2010	UK	14	ND	AHVLA
023-10B	Cattle	Faeces	2010	UK	14	ND	AHVLA
110-18B	Cattle	Faeces	2010	UK	14	ND	AHVLA
072-6B	Cattle	Faeces	2010	UK	14	ND	AHVLA
293-27B	Cattle	Faeces	2010	UK	14	ND	AHVLA
037-66B	Cattle	Faeces	2010	UK	14	ND	AHVLA
LR12-B2-1	Cattle	Faeces	Unknown	UK	14	ND	AHVLA
GP-EC 6	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 17	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 4	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 23	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 55	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 9	Human	Urine	2007	UK	14	ND	NHWS
H-EC 15	Human	Urine	2007	UK	14	ND	NHWS
H-EC 12	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 13	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 8	Human	Urine	2007	UK	14	ND	NHWS
H-COL 6	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 7	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 8	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 13	Human	Urine	2007	UK	14	ND	NHWS

Isolate name	Origin	Sample	Isolation date	Location	CTX-M	Inc type	Source
GP-EC 15	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 22	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 30	Human	Urine	2007	UK	14	ND	NHWS
GP-EC 33	Human	Urine	2007	UK	14	ND	NHWS
ESBL-715	Human	Urine	2009	UK	14	ND	SFE
ESBL-756	Human	Urine	2009	UK	14	ND	SFE
ESBL-762	Human	Urine	2009	UK	14	ND	SFE
ESBL-783	Human	Urine	2009	UK	14	ND	SFE
ESBL-788	Human	Urine	2009	UK	14	ND	SFE
ESBL-817	Human	Urine	2009	UK	14	ND	SFE
ESBL-831	Human	Urine	2009	UK	14	ND	SFE
ESBL-869	Human	Urine	2009	UK	14	ND	SFE
LR-1-B2-1	Sheep	Faeces	Unknown	UK	14	ND	AHVLA
LR-1-2-1	Sheep	Faeces	Unknown	UK	14	ND	AHVLA
284/2B	Turkey	Faeces	2006	UK	14	ND	AHVLA
317/2B	Turkey	Faeces	2006	UK	14	K	AHVLA
356/5A	Turkey	Faeces	2006	UK	14	FIA, K	AHVLA
362/1B	Turkey	Faeces	2006	UK	14	F, FIA, K	AHVLA
369/3B	Turkey	Faeces	2006	UK	14	K	AHVLA
387/2B	Turkey	Faeces	2006	UK	14	FIA, K	AHVLA
40/1A	Turkey	Faeces	2006	UK	14	FIA, K	AHVLA
41/1B	Turkey	Faeces	2006	UK	14	ND	AHVLA
80/2A	Turkey	Faeces	2006	UK	14	FIA, 11-y, K	AHVLA
26-C0363-09-07	Cattle	Faeces	2007	UK	1	F	AHVLA
LR17/B	Cattle	Faeces	Unknown	UK	1	F, FIA, FIB	AHVLA
LR584/B	Cattle	Faeces	Unknown	UK	1	F, FIA, FIB	AHVLA
LR756/B	Cattle	Faeces	Unknown	UK	1	F, FIA, FIB	AHVLA
17-C0133-02-08	Cattle	Faeces	2008	UK	1	F, FIA	AHVLA
28-C0008-09-08	Cattle	Faeces	2008	UK	1	FIA	AHVLA
23-C0140-09-06	Cattle	Faeces	2006	UK	1	FIA, P	AHVLA
BZ532/P	Chicken	Faeces	2006	UK	1	F, FIB, 11-y, P	AHVLA
BZ521/P	Chicken	Faeces	2006	UK	1	F, FIB, 11-y, P	AHVLA
BZ766/P	Chicken	Faeces	2006	UK	1	F, FIB, 11-y, P	AHVLA
BZ641/P	Chicken	Faeces	2006	UK	1	11-y	AHVLA
BZ1009/P	Chicken	Faeces	2006	UK	1	11-y	AHVLA
BZ1013/S	Chicken	Faeces	2006	UK	1	11-y	AHVLA
BZ696/P	Chicken	Faeces	2006	UK	1	11-y	AHVLA
BZ820/P	Chicken	Faeces	2006	UK	1	11-y	AHVLA
BZ859/P	Chicken	Faeces	2006	UK	1	11-y	AHVLA
BZ951/S	Chicken	Faeces	2006	UK	1	11-y	AHVLA
BZ592/P	Chicken	Faeces	2006	UK	1	11-y	AHVLA
BZ514/P	Chicken	Faeces	2006	UK	1	11-y, P	AHVLA
BZ537/P	Chicken	Faeces	2006	UK	1	11-y, P	AHVLA
BZ551/P	Chicken	Faeces	2006	UK	1	11-y	AHVLA
BZ657/P	Chicken	Faeces	2006	UK	1	ND	AHVLA
184/2B	Turkey	Faeces	2006	UK	1	F	AHVLA
8/3A	Turkey	Faeces	2006	UK	1	FIA, 11-y	AHVLA
122/1A	Turkey	Faeces	2006	UK	1	11-y, k	AHVLA

Isolate name	Origin	Sample	Isolation date	Location	CTX-M	Inc type	Source
71/2B	Turkey	Faeces	2006	UK	1	11-y, k	AHVLA
243/2B	Turkey	Faeces	2006	UK	1	11-y, k	AHVLA
26-C0447-04-07	Cattle	Faeces	2007	UK	3	A/C, P, FIA	AHVLA
BZ693/P	Chicken	Faeces	2006	UK	3	A/C, P	AHVLA
23-C0258-11-06	Cattle	Faeces	2006	UK	15	ND	AHVLA
17-C0088-09-06	Cattle	Faeces	2006	UK	15	F, FIA, 11-y	AHVLA
LR19-B2-1	Cattle	Faeces	Unknown	UK	15	ND	AHVLA
LR38-B2-1	Cattle	Faeces	Unknown	UK	15	ND	AHVLA
LR62-B2	Cattle	Faeces	Unknown	UK	15	ND	AHVLA
LR89-B2	Cattle	Faeces	Unknown	UK	15	ND	AHVLA
LR118-1	Cattle	Faeces	Unknown	UK	15	ND	AHVLA
LR122	Cattle	Faeces	Unknown	UK	15	ND	AHVLA

ND = Not determined, AHVLA = Animal Health and Veterinary Laboratories Agency,

SFE SafeFoodEra, NHWS = National Health Wales

Appendix II Isolates used in the chapter 4 and 7 *bla*_{CTX-M} screening of IncB, K, Z and IncI1 γ

Isolate name	Origin	Sample	Isolation date	Location	CTX-M	Inc type	Source
ESBL-004	Human	Urine	2009	NED	G1	I1 γ	CVI
ESBL-017	Human	Urine	2009	NED	G1	I1 γ	CVI
ESBL-044	Human	Urine	2009	NED	G1	I1 γ	CVI
ESBL-051	Human	Urine	2009	NED	G1	I1 γ	CVI
ESBL-066	Human	Urine	2009	NED	G1	I1 γ	CVI
ESBL-069	Human	Urine	2009	NED	G1	ND	CVI
ESBL-080	Human	Urine	2009	NED	G1	I1 γ	CVI
ESBL-102	Human	Urine	2009	NED	G1	B	CVI
ESBL-121	Human	Urine	2009	NED	G1	I1 γ , B	CVI
ESBL-139	Human	Urine	2009	NED	G1	ND	CVI
ESBL-208	Chicken	Caecum	2006	NED	G1	ND	CVI
ESBL-211	Chicken	Caecum	2006	NED	G1	ND	CVI
ESBL-271	Chicken	Caecum	2007	NED	G1	I1 γ	CVI
ESBL-288	Chicken	Caecum	2008	NED	G1	I1 γ	CVI
ESBL-298	Chicken	Caecum	2008	NED	G1	ND	CVI
ESBL-309	Chicken	Caecum	2008	NED	G1	I1 γ	CVI
ESBL-311	Chicken	Caecum	2008	NED	G1	I1 γ	CVI
ESBL-325	Chicken	Caecum	2008	NED	G1	ND	CVI
ESBL-333	Chicken	Caecum	2008	NED	G1	ND	CVI
ESBL-336	Chicken	Caecum	2008	NED	G1	I1 γ	CVI
ESBL-367	Chicken	meat	2006	NED	G1	I1 γ	CVI
ESBL-379	Chicken	meat	2006	NED	G1	I1 γ	CVI
ESBL-402	Chicken	meat	2007	NED	G1	ND	CVI
ESBL-413	Chicken	meat	2007	NED	G1	I1 γ	CVI
ESBL-416	Chicken	meat	2007	NED	G1	I1 γ	CVI
ESBL-428	Chicken	meat	2006	NED	G1	I1 γ	CVI
ESBL-471	Cattle	meat	2007	NED	G1	I1 γ	CVI
ESBL-485	Cattle	Faeces	2009	UK	G1	K	AHVLA
ESBL-488	Cattle	Faeces	2007	UK	G1	ND	AHVLA
ESBL-492	Cattle	Faeces	2006	UK	G1	I1 γ	AHVLA
ESBL-500	Cattle	Faeces	2006	UK	G1	I1 γ	AHVLA
ESBL-501	Cattle	Lung	2006	UK	G1	ND	AHVLA
ESBL-502	Cattle	Faeces	2009	UK	G1	I1 γ	AHVLA
ESBL-503	Cattle	Faeces	2007	UK	G1	I1 γ	AHVLA
ESBL-506	Cattle	Faeces	2009	UK	G1	ND	AHVLA
ESBL-521	Cattle	Faeces	2006	UK	G1	ND	AHVLA
ESBL-523	Cattle	Faeces	2006	UK	G1	ND	AHVLA
ESBL-526	Cattle	Faeces	2007	UK	G1	I1 γ	AHVLA
ESBL-528	Cattle	Faeces	2008	UK	G1	ND	AHVLA
ESBL-533	Cattle	Faeces	2006	UK	G1	ND	AHVLA
ESBL-535	Cattle	Faeces	2008	UK	G1	I1 γ	AHVLA
ESBL-538	Cattle	Faeces	2006	UK	G1	ND	AHVLA
ESBL-541	Cattle	Faeces	2008	UK	G1	ND	AHVLA
ESBL-544	Cattle	Faeces	2007	UK	G1	I1 γ	AHVLA
ESBL-545	Cattle	Faeces	2009	UK	G1	I1 γ	AHVLA

Isolate name	Origin	Sample	Isolation date	Location	CTX-M	Inc type	Source
ESBL-547	Cattle	Faeces	2007	UK	G1	B	AHVLA
ESBL-557	Cattle	Faeces	2007	UK	G1	ND	AHVLA
ESBL-564	Cattle	Faeces	2008	UK	G1	ND	AHVLA
ESBL-567	Cattle	Faeces	2007	UK	G1	I1γ	AHVLA
ESBL-580	Cattle	Faeces	2007	UK	G1	I1γ	AHVLA
ESBL-586	Cattle	Faeces	2006	UK	G1	ND	AHVLA
ESBL-590	Cattle	Udder	2008	UK	G1	ND	AHVLA
ESBL-591	Cattle	Faeces	2007	UK	G1	B	AHVLA
ESBL-593	Chicken	Caeca	2008	UK	G1	I1γ	AHVLA
ESBL-594	Chicken	Caeca	2008	UK	G1	I1γ	AHVLA
ESBL-596	Chicken	Caeca	2008	UK	G1	I1γ, K	AHVLA
ESBL-597	Chicken	Caeca	2008	UK	G1	I1γ	AHVLA
ESBL-598	Chicken	Caeca	2008	UK	G1	ND	AHVLA
ESBL-600	Chicken	Caeca	2008	UK	G1	ND	AHVLA
ESBL-601	Chicken	Caeca	2008	UK	G1	I1γ	AHVLA
ESBL-602	Chicken	Caeca	2008	UK	G1	I1γ	AHVLA
ESBL-604	Chicken	Caeca	2008	UK	G1	ND	AHVLA
ESBL-605	Chicken	Caeca	2008	UK	G1	I1γ	AHVLA
ESBL-607	Chicken	Caeca	2008	UK	G1	I1γ	AHVLA
ESBL-609	Chicken	Caeca	2008	UK	G1	I1γ	AHVLA
ESBL-613	Chicken	Caeca	2008	UK	G1	I1γ	AHVLA
ESBL-619	Turkey	Faeces	2006	UK	G1	I1γ	AHVLA
ESBL-625	Turkey	Faeces	2006	UK	G1	I1γ	AHVLA
ESBL-626	Turkey	Faeces	2006	UK	G1	I1γ	AHVLA
ESBL-627	Turkey	Faeces	2006	UK	G1	I1γ	AHVLA
ESBL-636	Turkey	Faeces	2006	UK	G1	I1γ	AHVLA
ESBL-637	Turkey	Faeces	2006	UK	G1	I1γ	AHVLA
ESBL-638	Turkey	Faeces	2066	UK	G1	I1γ	AHVLA
ESBL-639	Turkey	Faeces	2006	UK	G1	I1γ	AHVLA
ESBL-640	Turkey	Faeces	2006	UK	G1	ND	AHVLA
ESBL-642	Turkey	Faeces	2006	UK	G1	I1γ	AHVLA
ESBL-647	Turkey	Faeces	2006	UK	G1	I1γ	AHVLA
ESBL-649	Turkey	Faeces	2006	UK	G1	ND	AHVLA
ESBL-650	Turkey	Faeces	2006	UK	G1	ND	AHVLA
ESBL-651	Turkey	Faeces	2006	UK	G1	ND	AHVLA
ESBL-668	Turkey	Faeces	2007	UK	G1	ND	AHVLA
ESBL-693	Human	Urine	2008	UK	G1	ND	HPA
ESBL-696	Human	Urine	2009	UK	G1	ND	HPA
ESBL-703	Human	Urine	2009	UK	G1	ND	HPA
ESBL-711	Human	Urine	2009	UK	G1	ND	HPA
ESBL-716	Human	Urine	2009	UK	G1	ND	HPA
ESBL-727	Human	Urine	2009	UK	G1	ND	HPA
ESBL-733	Human	Urine	2009	UK	G1	ND	HPA
ESBL-740	Human	Urine	2009	UK	G1	ND	HPA
ESBL-744	Human	Urine	2009	UK	G1	ND	HPA
ESBL-748	Human	Urine	2009	UK	G1	ND	HPA
ESBL-757	Human	Urine	2009	UK	G1	ND	HPA

Isolate name	Origin	Sample	Isolation date	Location	CTX-M	Inc type	Source
ESBL-758	Human	Urine	2009	UK	G1	ND	HPA
ESBL-769	Human	Urine	2009	UK	G1	ND	HPA
ESBL-777	Human	Urine	2009	UK	G1	ND	HPA
ESBL-789	Human	Urine	2008	UK	G1	ND	HPA
ESBL-813	Human	Urine	2008	UK	G1	ND	HPA
ESBL-835	Human	Urine	2009	UK	G1	ND	HPA
ESBL-490	Cattle	Faeces	2008	UK	G9	I1γ, K	AHVLA
ESBL-504	Cattle	Faeces	2008	UK	G9	K	AHVLA
ESBL-525	Cattle	Faeces	2008	UK	G9	ND	AHVLA
ESBL-531	Cattle	Faeces	2008	UK	G9	I1γ	AHVLA
ESBL-539	Cattle	Faeces	2006	UK	G9	ND	AHVLA
ESBL-546	Cattle	Faeces	2006	UK	G9	ND	AHVLA
ESBL-559	Cattle	Faeces	2009	UK	G9	B	AHVLA
ESBL-561	Cattle	Faeces	2008	UK	G9	ND	AHVLA
ESBL-562	Cattle	Faeces	2007	UK	G9	I1γ	AHVLA
ESBL-565	Cattle	Faeces	2008	UK	G9	ND	AHVLA
ESBL-568	Cattle	Faeces	2008	UK	G9	B	AHVLA
ESBL-575	Cattle	Faeces	2008	UK	G9	I1γ, B	AHVLA
ESBL-576	Cattle	Faeces	2009	UK	G9	B	AHVLA
ESBL-578	Cattle	Faeces	2008	UK	G9	B	AHVLA
ESBL-581	Cattle	Faeces	2007	UK	G9	I1γ	AHVLA
ESBL-583	Cattle	Faeces	2007	UK	G9	ND	AHVLA
ESBL-585	Cattle	Faeces	2008	UK	G9	I1γ	AHVLA
ESBL-588	Cattle	Faeces	2009	UK	G9	B	AHVLA
ESBL-589	Cattle	Faeces	2007	UK	G9	B	AHVLA
ESBL-623	Turkey	Faeces	2006	UK	G9	K	AHVLA
ESBL-629	Turkey	Faeces	2006	UK	G9	I1γ, K	AHVLA
ESBL-633	Turkey	Faeces	2006	UK	G9	I1γ, K	AHVLA
ESBL-634	Turkey	Faeces	2006	UK	G9	I1γ, K	AHVLA
ESBL-635	Turkey	Faeces	2006	UK	G9	I1γ, K	AHVLA
ESBL-661	Turkey	Faeces	2007	UK	G9	K	AHVLA
ESBL-663	Turkey	Faeces	2007	UK	G9	K/B	AHVLA
ESBL-664	Turkey	Faeces	2007	UK	G9	K/B	AHVLA
ESBL-665	Turkey	Faeces	2007	UK	G9	K/B	AHVLA
ESBL-669	Turkey	Faeces	2007	UK	G9	K	AHVLA
ESBL-756	Human	Urine	2009	UK	G9	ND	HPA
ESBL-762	Human	Urine	2009	UK	G9	ND	HPA
ESBL-783	Human	Urine	2008	UK	G9	ND	HPA
ESBL-788	Human	Urine	2008	UK	G9	I1γ	HPA
ESBL-817	Human	Urine	2009	UK	G9	ND	HPA
ESBL-831	Human	Urine	2009	UK	G9	ND	HPA
ESBL-856	Human	Urine	2009	UK	G9	I1γ	HPA
ESBL-869	Human	Urine	2008	UK	G9	I1γ	HPA
ESBL-878	Human	Urine	2008	UK	G9	ND	HPA

UK = United Kingdom, NED = the Netherlands, G1 = Group 1 CTX-, G9 = Group 9
CTX-M, CVI = Central Veterinary Institute (NED), AHVLA = Animal Health and
Veterinary Laboratories Agency, HPA = Health Protection Agency, ND = Not determined

Appendix III Isolates used in the chapter 5 IncX4 plasmid screening

Isolate name	Origin	Sample	Isolation date	Location	ESBL	Source
ESBL-10	Human	Urine	2009	NED	CTX-M-1	CVI
ESBL-37	Human	Urine	2009	NED	CTX-M-1	CVI
ESBL-64	Human	Urine	2009	NED	CTX-M-1	CVI
ESBL-68	Human	Urine	2009	NED	CTX-M-1	CVI
ESBL-124	Human	Urine	2009	NED	CTX-M-1	CVI
ESBL-152	Pig	Organs	Unknown	GER	CTX-M-1	BfR
ESBL-159	Pig	Organs	Unknown	GER	CTX-M-1	BfR
ESBL-160	Pig	Organs	Unknown	GER	CTX-M-1	BfR
ESBL-168	Cattle	Faeces	Unknown	GER	CTX-M-1	BfR
ESBL-171	Pig	Organs	Unknown	GER	CTX-M-1	BfR
ESBL-173	Cattle	faeces	Unknown	GER	CTX-M-1	BfR
ESBL-188	Cattle	Organs	Unknown	GER	CTX-M-1	BfR
ESBL-218	Human	Unknown	Unknown	GER	CTX-M-1	BfR
ESBL-219	Human	Unknown	Unknown	GER	CTX-M-1	BfR
ESBL-220	Human	Unknown	Unknown	GER	CTX-M-1	BfR
ESBL-222	Human	Unknown	Unknown	GER	CTX-M-1	BfR
ESBL-236	Cattle	Organs	2009	GER	CTX-M-1	BfR
ESBL-277	Poultry	Caecum	2007	NED	CTX-M-1	CVI
ESBL-370	Poultry	Meat	2007	NED	CTX-M-1	CVI
ESBL-442	Poultry	Meat	2006	NED	CTX-M-1	CVI
ESBL-484	Cattle	Faeces	2007	UK	CTX-M-1	AHVLA
ESBL-530	Cattle	Faeces	2007	UK	CTX-M-1	AHVLA
ESBL-590	Cattle	Milk	2008	UK	CTX-M-1	AHVLA
ESBL-603	Poultry	Caeca	2008	UK	CTX-M-1	AHVLA
ESBL-628	Turkey	Faeces	2006	UK	CTX-M-1	AHVLA
ESBL-21	Human	Urine	2009	NED	CTX-M-14	CVI
ESBL-55	Human	Urine	2009	NED	CTX-M-14	CVI
ESBL-487	Cattle	faeces	2008	UK	CTX-M-14	AHVLA
ESBL-516	Cattle	Liver	2008	UK	CTX-M-14	AHVLA
ESBL-562	Cattle	Faeces	2007	UK	CTX-M-14	AHVLA
ESBL-587	Cattle	Intestine	2008	UK	CTX-M-14	AHVLA
ESBL-592	Cattle	Faeces	2008	UK	CTX-M-14	AHVLA
ESBL-497	Cattle	Faeces	2008	UK	CTX-M-15	AHVLA
ESBL-591	Cattle	Faeces	2007	UK	CTX-M-15	AHVLA
ESBL-25	Human	Urine	2009	NED	SHV-12	CVI
ESBL-284	Poultry	Caecum	2008	NED	TEM-52	CVI
ESBL-299	Poultry	Caecum	2008	NED	TEM-52	CVI
ESBL-356	Poultry	Meat	2007	NED	TEM-52	CVI
ESBL-376	Poultry	Meat	2007	NED	TEM-52	CVI
ESBL-411	Poultry	Meat	2007	NED	TEM-52	CVI
ESBL-423	Poultry	Meat	2006	NED	TEM-52	CVI
ESBL-425	Poultry	Meat	2006	NED	TEM-52	CVI
SAM7	Cattle	Faeces	2008	UK	CTX-M-14	AHVLA
ECR528	Cattle	Milk	2012	UK	CTX-M-14	AHVLA
ECR960	Cattle	Milk	2012	UK	CTX-M-14	AHVLA
JIE143	Human	Blood	2006	AUS	CTX-M-15	S Partridge

UK = United Kingdom, NED = the Netherlands, GER = Germany, AUS = Australia, CVI
= Central Veterinary Institute (NED), BfR = Bundesinstitut für Risikobewertung,
AHVLA = Animal Health and Veterinary Laboratories Agency



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