The emergence of *Escherichia coli* with CTX-M extended-spectrum beta-lactamases in the United Kingdom

Thesis

THE EMERGENCE OF *ESCHERICHIA COLI* WITH CTX-M EXTENDED-SPECTRUM BETA-LACTAMASES IN THE UNITED KINGDOM

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ABSTRACT

In the past decade, multi-resistant *Escherichia coli* with CTX-M enzymes have rapidly become the leading producers of extended-spectrum β-lactamases (ESBLs) worldwide. *E. coli* with CTX-M-15 β-lactamases are the most common, and are now endemic in many UK hospitals and in the community. Their epidemiology is complex, including five major epidemic clones as defined by pulsed-field gel electrophoresis, as well as many unrelated producers. This study sought to characterise, at a molecular level, *E. coli* isolates with CTX-M ESBLs and to investigate their nationwide dissemination, so as to identify the basis for the clinical success of these organisms. The molecular mechanisms responsible for the multi-drug resistance phenotype of representative isolates with CTX-M enzymes were explored and compared, as were their phylogenetic and virulence backgrounds. The genetic support and environment of the various bla_{CTX-M} genes were also investigated.

The multi-drug resistance of *E. coli* with CTX-M enzymes was principally encoded by single plasmids, generally self-transferable. Among producers of CTX-M-15 enzyme, and regardless of their host strain's epidemic status, these plasmids were closely-related (IncFII) and encoded most often bla_{TEM-1}, bla_{OXA-1} along $bla_{CTX-M-15}$, as well as *aac(6')-Ib-cr*, *aac(3)-IIa* and *tet*(A). The most prevalent UK clone (A) expressed a lower level of CTX-M-15 enzyme than most other producers, probably owing to an IS*26* element located downstream of the gene's normal promoter. Its CTX-M-15-encoding plasmid was not self-transferable *in-vitro*, but carried twelve genes effecting resistance to eight classes of antibiotics, as well as genes for virulence determinants. Most *E. coli* with CTX-M enzymes, including the major epidemic clones, belonged to the virulence-associated phylogenetic group B2 or D, but did not harbour more virulence determinants than B2 isolates with non-CTX-M ESBLs. Although related, three slightly distinct virulence profiles were apparent for clonal and non-clonal isolates with CTX-M-15 enzymes.

In conclusion, CTX-M ESBLs have rapidly spread in the UK among virulent *E. coli* isolates, aided by horizontal transfer of multi-resistance plasmids, as well as by clonal spread of epidemic producer strains. Their dissemination worryingly undermines the success of antibiotic therapy, especially in community patients, where few oral options remain.

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General abbreviations

A	Adenine
AFLP	Amplified Fragment Length Polymorphism
ARMRL	Antibiotic Resistance Monitoring and Reference Laboratory
BAP	Bacterial alkaline phosphatase
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BSAC	British Society for Antimicrobial Chemotherapy
C	Cytosine
cfu	Colony-forming unit(s)
CLSI	Clinical and Laboratory Standards Institute
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
Dig	Digoxigenin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediamine-tetra-acetic acid
EMBL	European Molecular Biology Laboratory
ESBL	Extended-spectrum β-lactamase
etc	*Et cetera*
EUCAST	European Committee for Antibiotic Susceptibility Testing
g	Gravitational force
G	Guanine
HPA	Health Protection Agency
I	Intermediate
IEF	Isoelectric focusing
ISO	IsoSensitest Agar
LB	Luria-Bertani
log	Logarithmic
LPS	Lipopolysaccharide
MH	Mueller-Hinton
MIC	Minimal Inhibitory Concentration

MCS	Multiple cloning site
MRSA	Meticillin-resistant *Staphylococcus aureus*
N	Unspecified nucleotide
NA	Nutrient agar
NB	Nutrient broth
OMP	Outer membrane protein
ORF	Open reading frame
PAI	Pathogenicity-associated island
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
pI	Isoelectric point
QRDR	Quinolone resistance determining region
R	Resistant
RAPD	Random Amplifiable Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RT	Reverse transcriptase
S	Susceptible
SDS	Sodium Dodecyl Sulphate
T	Thymine
TBE	Tris-borate EDTA
Tm	Melting temperature
UK	United Kingdom
UTI	Urinary tract infection
UV	Ultra-violet
v	Volume
VF	Virulence factor
vs	*Versus*
w	Weight

Unit abbreviations

°C	Degree Celsius
%	Per cent
bp	Base pair
cm	Centimetre
F	Faraday
h	Hour
kb	Kilobase pair
L	Litre
mA	Milliampere
mBar	Millibar
MDa	Megadalton
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimeter
mM	Millimolar
mol	Mole
nm	Nanometre
Ω	Ohm
rpm	Revolutions per minute
sec	Second
U	Unit (for enzymatic activity)
µg	Microgram
µL	Microlitre
µM	Micromolar
µm	Micrometre
V	Volt
W	Watt

Antibiotic abbreviations

AMK	Amikacin
AMX	Amoxicillin
AMP	Ampicillin
AUG	Augmentin
AZT	Aztreonam
CTX	Cefotaxime
FOX	Cefoxitin
CPM	Cefepime
CPD	Cefpodoxime
CAZ	Ceftazidime
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLA	Clavulanic acid
CLX	Cloxacillin
COL	Colistin
ETP	Ertapenem
FOS	Fosfomycin
GEN	Gentamicin
IPM	Imipenem
MEM	Meropenem
MIN	Minocycline
NAL	Nalidixic acid
NIT	Nitrofurantoin
OFL	Ofloxacin
OXA	Oxacillin
PIP	Piperacillin
RIF	Rifampicin
STR	Streptomycin
SUL	Sulbactam
SFM	Sulfamethoxazole
TZB	Tazobactam
TET	Tetracycline
TIG	Tigecycline
TOB	Tobramycin
TMP	Trimethoprim

INTRODUCTION

The need to distinguish between pathogenic bacteria (causing disease) and commensal bacteria (living in symbiosis on or within an organism without causing injury) has led to the creation of a classification system known as taxonomy. Bacterial taxonomy is in constant flux as new species are discovered, and also as new technologies are developed and used. Precise identification of bacteria has become essential for the clinical microbiologist, not only for treatment issues, but also for epidemiological and surveillance studies.

1. The Enterobacteriaceae

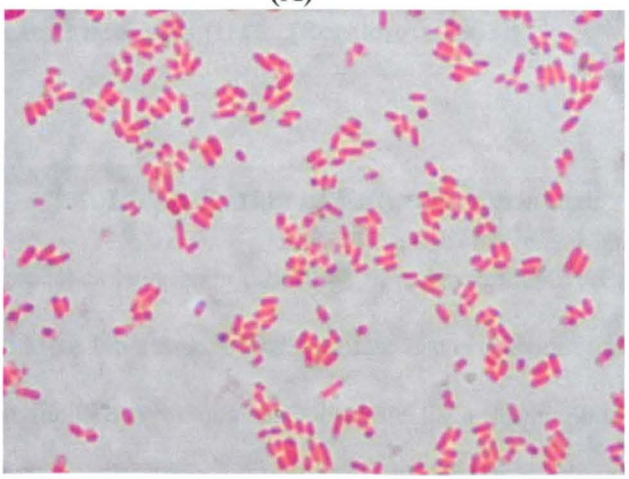
Enterobacteriaceae are one of the commonest families of bacteria encountered in medical microbiology. Members of this family (*e.g. Escherichia coli*) share a number of morphological and biochemical properties, which include:

- Being short (4-5 microns) Gram-negative bacilli not forming spores (Figure 1)
- Possessing peritrichous flagella, if motile
- Being facultative anaerobes: growing both aerobically and, by fermentation of sugars, anaerobically (often associated with gas production)
- Generally displaying optimum growth at 37°C, although some species grow better at 25-30°C
- Being catalase-positive and cytochrome c oxidase-negative
- Being nitrate reductase-positive

Members of the Enterobacteriaceae are closely related to each other and their genomes share a high degree of homology, with a guanosine + cytosine content between 39 and 59%. Depending on the species, Enterobacteriaceae are widely distributed in the environment (plants, soil and water), but are mostly found in the intestines of humans and animals. They are a major component of the normal intestinal flora of humans, and are relatively uncommon at other body sites. Some genera such as *Salmonella* and *Shigella* are inherently pathogenic, while others are known as opportunistic pathogens, and *E. coli*, according to the strain, may fall into either of these categories.

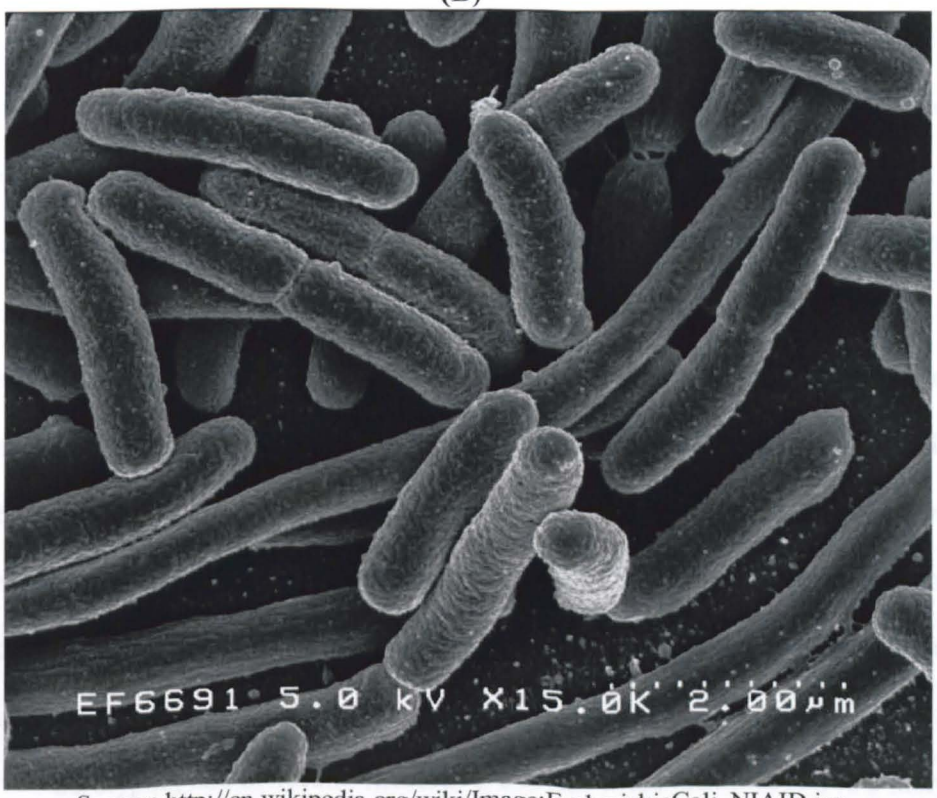
Figure 1. (A) Microscopic observation of *Escherichia coli* following Gram staining; (B) Scanning electron micrograph of *Escherichia coli* adhered to a cover slip

(A)



Source: http://biology.clc.uc.edu/fankhauser/Labs/Microbiology/Gram_Stain

(B)



Source: http://en.wikipedia.org/wiki/Image:EscherichiaColi_NIAID.jpg

Enterobacteriaceae are associated with a wide range of human infections including abscesses, pneumonia, meningitis, septicaemia, and infections of wounds, also infections of the urinary tract and of the intestines. Some species are important causes of nosocomial infections – *e.g. Klebsiella pneumoniae* is a major agent of nosocomial pneumonia and *Escherichia coli* is the major cause of nosocomial urinary tract infections [UTIs, (Ronald, A., 2003)].

1.1. Identification of Enterobacteriaceae

Many different approaches have been described in order to identify Enterobacteriaceae (Farmer, J. J., III *et al.*, 1985). Most laboratories firstly isolate bacteria from clinical materials on to selective agars, which often include a unique carbohydrate as a fermentation substrate (*e.g.* lactose in MacConkey agar). Depending on the colonial appearance after 24 hr incubation, a series of further biochemical screening tests is performed, following confirmation of the absence of cytochrome c oxidase. The most common biochemical pathways examined include the ability to produce acid and gas from various carbon sources such as glucose, lactose, maltose, mannitol, arabinose and citrate, but also the metabolism of urea, indole production and the degradation of various amino-acids (*e.g.* tyrosine, phenylalanine) as well as the production of hydrogen sulphide (H_2S) gas. These tests generally allow identification to species level, although genera such as *Salmonella* or *Shigella* require additional serological testing, usually by agglutination tests. Table 1 summarises key biochemical properties of the genera belonging to those Enterobacteriaceae often isolated from human clinical specimens. Nowadays, clinical laboratories often use commercially available kits such as the API20E system from BioMérieux, which consists of a series of miniaturised and standardised biochemical tests. These time-saving kits usually correctly identify the most common species of Enterobacteriaceae, but may not be as accurate for the identification of new or minor species.

Serotyping and molecular methods are useful when identification to a more advanced level is required, *i.e.* to define strains in research and epidemiological studies. Molecular methods are not often available commercially and protocols may therefore vary between laboratories. Most are based on PCR technology, fragment length polymorphism (e.g. pulsed-field gel electrophoresis) or DNA sequencing, and are more typically used in reference or research laboratories.

Table 1. Main characteristics of Enterobacteriaceae genera frequently responsible for human infections

Genera	Morphological and biochemical properties
Citrobacter spp.	Motile Citrate$^+$; lysine$^-$
Enterobacter spp.	Motile Ferment glucose with production of acid and gas Variable biochemically: citrate$^+$, Voges-Proskauer$^+$
Escherichia spp.	Motile Extremely variable biochemically; indole$^+$ (see page 31)
Hafnia spp.	Motile More active biochemically at 25°C than 37°C
Klebsiella spp.	Mucoid colonies, encapsulated cells, non-motile very active biochemically: ferment most sugars, lysine$^+$ and VP$^+$
Morganella spp.	Motile Very inactive biochemically: citrate$^-$, H_2S^-, gelatinase$^-$, urease$^+$
Proteus spp.	Motile, swarm on blood agar, pungent odour Urease$^+$, H_2S^+, lipase$^+$ and gelatinase$^+$
Providencia spp.	Motile, no swarming H_2S^-, gelatinase$^-$, lipase$^-$ and urease$^-$
Salmonella spp.	Motile except *S. typhi* and *S. paratyphi* Lactose$^-$, sucrose$^-$, H_2S^+
Serratia spp.	Motile Lipase$^+$, DNase$^+$ and gelatinase$^+$
Shigella spp.	Non-motile Biochemically inactive: lysine$^-$, gas$^-$
Yersinia spp.	Motile at 25°C (non-motile at 37°C) More active biochemically at 25°C, urease$^+$

1.2. Clinical significance

Although they are a major component of the normal intestinal flora of humans, species of Enterobacteriaceae can cause a wide range of infections. Data from 1998 (CDR weekly, Communicable Disease report vol. 8(20), 15 may 1998) suggest Enterobacteriaceae account for 80% of clinically-significant Gram-negative bacilli and 50% of all clinically-significant bacteria examined in clinical microbiology laboratories. They cause up to 50% of septicaemia cases, 70% of urinary tract infections and a similar percentage of intestinal infections, according to the same source. Infections due to these organisms are usually divided into two groups: intestinal and extra-intestinal infections. A limited range of species of Enterobacteriaceae are implicated in intestinal infections in humans and animals, predominantly members of the genera *Escherichia*, *Salmonella*, *Shigella* and *Yersinia*. On the other hand, many species of Enterobacteriaceae can cause extra-intestinal infections though *E. coli*, *K. pneumoniae*, *K. oxytoca*, *P. mirabilis*, *E. aerogenes*, *E. cloacae and S. marcescens* are responsible for the vast majority of them. Urinary tract infections are the most common extra-intestinal infections, followed by respiratory, wound, bloodstream and central nervous system infections. Many of these, especially the life-threatening sepsis and meningitis, are hospital-acquired, but *E. coli* is the major agent of community UTIs.

1.3. Antibiotic susceptibility

The antibiotic susceptibility of Enterobacteriaceae can be assessed by several methods, the most commonly-used being disc diffusion and, broth or agar dilution. The basic methodology of antibiotic susceptibility testing itself has hardly been altered since a standardized method was first described in the mid-1960s (Bauer, A. W. *et al.*, 1966), though techniques have been automated to a degree. However, with the appearance of antibiotic resistance, frequently selected following the introduction of a new drug on to the market, interpretation guidelines are constantly being adjusted.

Among Enterobacteriaceae, the frequent antibiotic resistances observed vary depending on the species, but also on its origin. Most species exhibit intrinsic resistance to certain antibiotics (*e.g. Enterobacter aerogenes* is resistant to cephalothin, *Proteus mirabilis* to polymyxins and tetracycline, *Serratia marcescens* to polymyxins).

This intrinsic resistance facilitates identification for the clinical microbiologist (*e.g.* a culture of *Enterobacter* spp. appearing susceptible to cephalothin requires checks for culture purity and repeat test for identification and antibiogram). Finally, in addition to providing the basis for treating patients with infections, antibiograms have also become a significant tool for epidemiological studies, being particularly useful laboratory markers for comparing strains.

2. *Escherichia coli*

The genus *Escherichia* is composed of motile bacteria that conform to the definition of the family Enterobacteriaceae. Although seven species are known (*E. adecarboxylata*, *E. albertii*, *E. blattae*, *E. coli*, *E. fergusonii*, *E. hermannii* and *E. vulneris*), *E. coli* is by far the most common, being one of the most-encountered bacteria in the clinical laboratory. Discovered in 1885 by German pediatrician and bacteriologist Theodore Escherich, *Escherichia coli* has also been used as a model organism for research in microbiology and genetics laboratories for decades. Sequencing of the entire genome of the benign laboratory strain *E. coli* K-12 (4,639,221-bp) was completed in 1997 (Blattner, F. R. *et al.*, 1997), and is now publicly available (http://www.ncbi.nlm.nih.gov).

2.1. Natural habitats

E. coli is most commonly isolated from the intestines of humans and animals, and is the most abundant facultatively anaerobic commensal inhabitant of the large intestines of healthy humans (up to 10^9 cells per gram of faeces) and of many other warm-blooded animals. It colonizes the intestines shortly after birth and remains there throughout life. In the large intestine, *E. coli* assists with waste processing, production of vitamin K, and food absorption. However, *E. coli* is not confined to the mammalian gut, and can be found also in the environment, where it often is an indicator of faecal contamination. As a result of its adaptation to the mammalian intestines, *E. coli* grows best at temperatures around 37°C, rather than the cooler temperatures found in soil and other environments.

2.2. Identification

2.2.1. Biochemical characteristics

Nowadays, most clinical laboratories identify *E. coli* with easy-to-use and time-saving commercial systems, *e.g.* the API20E test kit from BioMérieux (Figure 2). Those kits provide reliable identification in 18-24 hr. Identification to strain level is achieved by serotyping or, increasingly, molecular methods, as detailed below.

2.2.2. Serotyping

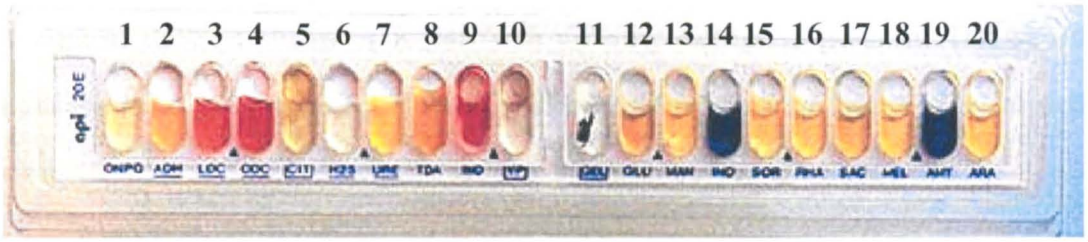
The serologic classification of *E. coli* is based on three types of antigen: O (somatic), H (flagellar) and K (capsular). These antigens are stable and reliable strain characteristics, and are usually identified by agglutination tests. Although complete serotyping of *E. coli* strains is labour-intensive, with more than 170 O antigens, 100 K antigens and 70 H antigens characterised, and over 700 serotypes recognised, it remains particularly useful for epidemiological purposes.

2.2.3. Molecular typing

Pulsed-field gel electrophoresis (PFGE) is used for strain typing of *E. coli* isolates, particularly in reference and research laboratories. Although serotyping and antibiotic susceptibility testing may be sufficient to distinguish between most *E. coli* strains, PFGE remains the gold standard for defining an outbreak strain, detecting fine differences between isolates.

More recently, Multi-Locus Sequence Typing (MLST) has come to be used in order to subtype *E. coli* isolates. MLST entails sequencing internal fragments of a small number of housekeeping genes (usually seven), such as *adk* (adenylate kinase) and *mdh* (malate dehydrogenase) for *E. coli.* Like serotyping and unlike PFGE, MLST assigns definitive types; PFGE and other methods, such as AFLP and RAPD by contrast, are comparative, indicating only the relatedness between several isolates.

Figure 2. API20E strip inoculated with a saline suspension of a pure culture of *E. coli* after 18-hr incubation at 37°C



Source: www.jlindquist.net/generalmicro

	Test	Biochemical property	Result
1	ONPG	β-Galactosidase	+
2	ADH	Arginine dehydratase	-
3	LDC	Lysine decarboxylase	+
4	ODC	Ornithine decarboxylase	+
5	CIT	Citrate use as sole carbon source	-
6	H2S	Hydrogen sulphide production	-
7	URE	Urea hydrolysis	-
8	TDA	Tryptophan deaminase	-
9	IND	Indole production	+
10	VP	Acetoin production	-
11	GEL	Gelatine hydrolysis	-
12	GLU	Glucose fermentation	+
13	MAN	Mannitol fermentation	+
14	INO	Inositol fermentation	-
15	SOR	Sorbitol fermentation	+
16	RHA	Rhamnose fermentation	+
17	SAC	Sucrose fermentation	+
18	MEL	Melibiose fermentation	+
19	AMY	Amygdalin fermentation	-
20	ARA	Arabinose fermentation	+

2.3. Molecular phylogeny and virulence of *E. coli*

It is well known that *E. coli* can co-exist with its host peacefully (non-pathogenic, commensal variants), but it can also cause devastating illness (pathogenic variants). This paradox is due to the existence of different strains of *E. coli* with differing pathogenic potential. This variation is attributable to the presence or not of specific genes encoding virulence factors (VFs), as well as to the capacity of *E. coli* for genetic exchange (*e.g.* by recombination and conjugation...).

Pathogenic *E. coli* strains are usually divided into two groups: those that cause gastro-intestinal diseases and those that cause extra-intestinal infections, although some pathotypes may transcend these boundaries. The ability of a given pathogenic *E. coli* variant to cause disease largely depends on its VF gene repertoire (Siitonen, A., 1994). The most common VFs of *E. coli* include adhesins (promote adhesion), toxins (cause direct injuries to host cells and tissues), siderophores (sequester iron and promote colonisation), secretion systems (stimulate a noxious host inflammatory response), capsules and polysaccharide-coating proteins (help avoiding host defence mechanisms). These factors are generally absent from commensal strains, but are commonly found among both intestinal and extra-intestinal *E. coli* pathotypes (Eisenstein, B. I. and Jones, G. W., 1988; Mobley, H. L. *et al.*, 1994; Orskov, I. and Orskov, F., 1985; Siitonen, A., 1994).

At least, seven major *E. coli* pathotypes are now recognised (Donnenberg, M. S., 2002), including: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic or shiga toxin-producing *E. coli* (STEC or EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), uropathogenic *E. coli* (UPEC) and meningitis-associated *E. coli* (MAEC). Extra-intestinal pathogenic *E. coli* (ExPEC) strains include both UPEC and MAEC pathotypes. In addition, four main phylogenetic groups of *E. coli* are recognised, A, B1, B2 and D, as defined by Multi-Locus Enzyme Electrophoresis (MLEE) and Multi-Locus Sequence Typing [MLST (Clermont, O. *et al.*, 2000; Herzer, P. J. *et al.*, 1990; Lecointre, G. *et al.*, 1998)]. Virulent *E. coli* strains mostly belong to phylogenetic group B2, and to a lesser extent to group D. More specifically, intestinal pathotypes mostly belong to phylogenetic group D, but sometimes also A and B1 if these have acquired sufficient VFs to cause disease.

Extra-intestinal pathogenic *E. coli* are concentrated in group B2 (Johnson, J. R. *et al.*, 2002; Picard, B. *et al.*, 1993). Commensal and environmental strains mostly belong to phylogenetic groups A and B1, respectively.

2.3.1. Adhesive pili

The initial step towards infection, whether intestinal or extra-intestinal, consists of recognising and adhering to the host tissues, and in the case of *E. coli* requires the presence of hair-like structures called pili, which extend from the surface of the bacterium. The best characterised pili of *E. coli* are the P (pyelonephritis) pilus and the type 1 pilus systems, encoded by the *pap* and *fim* genes clusters, respectively. These operon-like structures include genes that encode *(i)* a major rod subunit (*papA* and *fimA*), *(ii)* an adhesin (*papG* and *fimH*) and *(iii)* an adapter (*papEFK* and *fimFG*). The adhesin protein, which is located at the tip of the pilus, facilitates the binding of the invading bacterium to a receptor, usually a carbohydrate or a carbohydrate complex, on the host cell surface (Donnenberg, M. S., 2002).

Type IV pili, also known as fimbriae, are another group of pili, involved in cell interactions and widely distributed among *E. coli* isolates. Their assembly is complex and requires a variety of proteins, which have to be assembled in a co-ordinated manner in order to allow pilin polymerisation, fibre stabilisation and surface translocation. Furthermore, since fimbriae represent a potential target for the host immune system, it is important that their expression remains confined to the first stage of the infection, where they are specifically required.

2.3.2. Siderophores and iron uptake

Human and animal tissues provide all the nutrients necessary for the survival of *E. coli*, but these are more or less available. Iron is an essential cofactor found in all organisms, but its availability for the colonising *E. coli* may easily become a growth-limiting factor, since most of it is sequestered and held within cells (*e.g.* as haemoglobin or ferritin) or is bound to iron-binding proteins such as transferrin and lactoferrin (Donnenberg, M. S., 2002). Like many potential pathogens, *E. coli* produce aerobactins, which are iron-chelating siderophores. These low-molecular weight molecules remove the iron from the iron-binding proteins and transport it into the

bacterial cell. *E. coli* generally uses multiple iron-uptake systems and often will preferentially use the iron from haemoglobin. But, as haemoglobin is rarely present free in tissue fluids, it has been suggested that the toxin haemolysin (see section 2.3.3) may intervene in order to make the iron available (Boehm, D. F. *et al.*, 1990a; Boehm, D. F. *et al.*, 1990b).

2.3.3. Secretion systems and toxins

Toxins are responsible for causing lesions to the host tissues during infections, but may also induce an inflammatory response. Several *E. coli* toxins have been characterised, and different pathotypes produce different types of toxin: *e.g.* STEC produce a shiga-like toxin, most ETEC produce the heat-labile enterotoxin called holotoxin, and UPEC may produce the cytotoxic necrotising factor 1 (Donnenberg, M. S., 2002).

In addition, many Gram-negative pathogens, including *Shigella*, *Yersinia*, *Salmonella*, and also EPEC and EHEC isolates use secretion systems to inject virulence factors into the cytosol of the eukaryotic host cells in order to subvert cellular processes to the advantage of the bacterium. Five major secretion systems have been described to date (numbered I to V), with the best-known secretion substrate being the *E. coli* haemolysin (via the type I system). The type III secretion system also has been extensively characterised and is mostly used by EPEC and EHEC pathotypes. The secretion process, which is activated once the bacteria adhere to the surface of the host cells, allows the transport of VFs across three membranes: the inner and outer membranes of the bacterium, as well as the eukaryotic cell membrane.

The *E. coli* haemolysin, secreted by the type I secretory system, is an important VF involved in infections of the urinary tract and other extra-intestinal sites, and is also produced by the enterohaemorrhagic *E. coli* O157. Haemolysin is a membrane-targeted protein with cytocidal activity, encoded by the *hly* operon. It attacks erythrocytes, releasing the iron bound to haemoglobin, as well as granulocytes, monocytes, endothelial cells and renal epithelial cells. It stimulates the production of cytokines, leading to an inflammatory response (Uhlen, P. *et al.*, 2000).

Other important toxins produced by *E. coli* pathotypes include a wide range of heat-labile and heat-stabile enterotoxins (mostly produced by ETEC), the shiga-like toxins (also known as vero-cytototoxins and secreted by EHEC), the cytolethal distending toxin, and the cytotoxic necrotising factor (CNF).

2.3.4. Capsules and lipopolysaccharides

The various features on the bacterial cell surface, such as flagella, fimbriae, capsules and lipopolysaccharides, are essential components for survival, since they mediate the contact of the bacterium with its direct environment. In addition to "assessing" the environment, these structures often participate in the pathogenesis of infectious diseases. Many pathogenic *E. coli* strains, but especially extra-intestinal pathotypes, have a polysaccharide layer or capsule outside their outer membrane, and/or a complete lipopolysaccharide (LPS) in the outer leaflet of their outer membrane. These traits partly dictate the pathogenic potential of *E. coli.*

Although a capsule's primary function is to mediate the adherence of the bacterium to a given surface, it may also protect the bacterium from non-specific host defence mechanisms by interfering with complement and predatory phagocytes. Four groups of capsules (1-4) have been proposed for *E. coli* (Whitfield, C. and Roberts, I. S., 1999). If intestinal pathogenic *E. coli* isolates possess a capsule, it is usually a group 1 or 4 (*e.g.* K30 and K40), while group 2 and 3 capsules (such as K1 and K5) are mostly associated with extra-intestinal pathogenic *E. coli* (ExPEC) isolates.

LPS is a unique glycolipid present in the outer membranes of Gram-negative bacteria and consists of lipid A inserted in the membrane core, a core oligosaccharide region, and a serotype-specific O-antigen composed of repeating sugar subunits (Orskov, I. *et al.*, 1977). Over 170 antigenically distinct types have been described in *E. coli* so far.

Both capsules and LPS are considered to be VFs that protect *E. coli* against various host defence factors. These structures however also behave as pathogen-associated molecular patterns, thus enhancing the innate immune response.

3. *Escherichia coli*-associated infections

Pathogenic *E. coli* strains are responsible for both gastro-intestinal and extra-intestinal infections. Among the latter, *E. coli* is considered as the "number one" cause of human UTIs, but has been linked also with diseases in virtually every other part of the human body, and also with diseases in a broad array of animal species such as pigs, cattle, birds and sheep (Chiu, N. C. *et al.*, 1989; Farooqui, B. J. *et al.*, 1989). The most common diseases resulting from *E. coli* infections in humans include cystitis and ascending disease that affects the kidneys (*i.e.* pyelonephritis), diarrhoea, dysentery, haemolytic-uremic syndrome, neonatal meningitis and septicaemia.

3.1. Gastro-intestinal tract infections and food-poisoning

Although *E. coli* are the predominant aerobic members of the normal gut flora of humans, ingested pathogenic strains are also responsible for most cases of gastro-intestinal infections. These infections can result in a vast array of diseases with different degree of severity, ranging from mild gastroenteritis to dysentery or enterocolitis, and can subsequently also cause damage at distant body sites.

The five major *E. coli* pathotypes that cause gastro-intestinal infections (EPEC, EHEC, ETEC, EAEC and EIEC) each have specific pathogenic mechanisms. ETEC possess colonization factors that enable the bacteria to bind to corresponding receptors on the intestinal cell membrane, where they secrete powerful enterotoxins, resulting in increased fluid secretion. EHEC serotypes such as *E. coli* O157, which usually are foodborne, attach to the intestinal mucosa and release a verotoxin, also resulting in diarrhoea. In addition, EHEC, which is often also referred to as verocytotoxin-producing *E.* coli, causes haemorrhagic colitis and haemolytic-uraemic syndrome. In contrast, EPEC and EAEC strains do not produce toxins: EPEC use a particular mechanism of adherence to enterocytes, resulting in the destruction of microvilli, whilst EAEC use a distinctive aggregative pattern. Both EPEC and EAEC pathotypes cause diarrhoea, but whereas EPEC is mostly associated with severe infantile gastroenteritis, EAEC generally only cause mild enteritis. Finally, EIEC serotypes attach specifically to the mucosa of the large intestine, invade peripheral cells by endocytosis and spread to adjacent cells, causing tissue destruction and inflammation.

3.2. Urinary tract infections

Aside from its most peripheral sections, the urinary tract is normally a sterile environment. It is also one of the commonest sites of bacterial infections, especially in women. Urinary tract infections are defined by the presence of phagocytes and $>10^5$ bacterial cells per ml of urine. Infections can be confined to the lower urinary tract (cystitis) or may ascend via the urethra to the bladder and kidney, occasionally invading the bloodstream. Most UTIs in both community and hospital practices are due to *E. coli*, where it accounts for up to 90% and 50% of cases, respectively (Bahrani-Mougeot, F *et al.*, 2002; Marrs, Carl F. *et al.*, 2005; Ronald, A., 2003).

Although most of lower UTIs are acute and of short duration, they still contribute to a significant amount of morbidity in the population. Ascending infections are much more serious and, if associated with bacteraemia, have significant mortality. Predisposing factors for UTIs include age (the incidence of UTIs increases with age, but cystitis is also frequent in sexually-active women of child-bearing age), sex (short urethra in females), structural abnormalities of the urinary tract (*e.g.* calculi), catheterization and surgery, but also medical conditions such as diabetes mellitus and immunosuppression.

The subset of *E. coli* causing UTIs in humans is thought to be distinct from commensal *E. coli* strains: although they most often originate in the faecal flora, uropathogenic *E. coli* (UPEC) generally differ from commensal strains in that they possess specific attributes to cause pathogenesis (*e.g.* adhesins, fimbriae, haemolysins, LPS, capsules and siderophores). The ability of strains to cause UTIs may be attributable to their capacity first to colonize the periurethral areas, and then to adhere to the urethral and bladder epithelium using specific pili such as type 1 and P fimbriae, but also Dr adhesins and S fimbriae (Bahrani-Mougeot, F *et al.*, 2002). Other virulence factors significantly found among UPEC isolates in contrast to faecal isolates from healthy individuals include also haemolysin and cytotoxic necrotizing factor 1, iron-chelating siderophores (*e.g.* aerobactin, yersiniabactin), capsules (mostly K1 and K5) and a variety of extracellular polysaccharides (LPS O-antigens) (Donnenberg, M. S., 2002).

3.3. Infections of the central nervous system: meningitis

Bacterial meningitis is a life-threatening infection, which needs urgent effective treatment. It generally arises through the spread of bacteria from the bloodstream to the meninges, but may also result from direct spread of the infection from one individual to another. The vast majority of bacterial meningitis cases are caused by *Neisseria meningitidis*, *Streptococcus pneumoniae* or *Haemophilus influenzae*, and very rarely by *E. coli* (after infancy). Nevertheless, *E. coli* is the commonest cause of meningitis during the neonatal period in developing countries. Mortality and morbidity associated with neonatal *E. coli* meningitis remain high despite advances in antimicrobial chemotherapy, and survivors often sustain neurological sequels (Sik Kim, K, 2002). During or shortly after birth, *E. coli* may colonize external surfaces of the neonate and invade the bloodstream prior to crossing the blood-brain barrier. Predisposing factors include instrumentation (*e.g.* nasogastric incubation or umbilical vessel catheterization), low birth weight, but also the serotype of the *E. coli* isolate involved. Isolates possessing the K1 capsular polysaccharide, which is associated with invasiveness, account for up to 80% of neonatal meningitis-associated *E. coli* [MAEC, (Robbins, J. B. *et al.*, 1974)]. Other virulence factors commonly found among MAEC include OmpA (outer membrane protein A, which is involved in the invasion of brain microvascular endothelial cells), ibe proteins (invasion of brain endothelial cells), and the cytotoxic necrotizing factor 1 (Donnenberg, M. S., 2002).

3.4. Septicaemia

Septicaemia may be described as a systemic illness caused by the spread of microbes and/or their toxins via the bloodstream. Bacteraemia on the other hand, refer to the presence of bacteria in the blood, with or without septic consequences. The nature and the incidence of bacteraemia vary between hospitals, and depend partly on the specialities practiced. *E. coli* is one of the four commonest organisms responsible for septicaemia, along with *Staphylococcus aureus*, *S. epidermidis*, and *Streptococcus pneumoniae*, and it is by far the most frequent Gram-negative bacterium isolated from positive blood cultures.

The most common factors predisposing to *E. coli* (and Gram-negative organisms in general) septicaemia include instrumentation and surgery of the gastro-intestinal and urinary tracts, as well as neutropenia (*e.g.* in oncology and transplant patients), drug-induced immunosuppression and alcoholism. Septicaemia may also arise following the spread of bacteria from other sites, most obviously following UTIs, abdominal infections, gastroenteritis or infections of the central nervous system.

3.5. Intra-abdominal infections

Intra-abdominal infections are usually polymicrobial, though *E. coli* is one of the most common Gram-negative bacilli responsible, together with anaerobes. These infections occur secondary to spontaneous or traumatic gastro-intestinal tract perforation, or after anastomatic disruption with spillage of colon contents and subsequent peritonitis. Intra-abdominal infections due to *E. coli* often result from a perforated viscus (*e.g.* appendix), or may be associated with intra-abdominal abscess, cholecystitis and ascending cholangitis, which result from the obstruction of the biliary system by *e.g.* stones. Liver abscesses can develop as a complication of a biliary tract infection.

4. Preventing and treating infections

The three major strategies used to control infectious diseases consist of immunisation (though vaccines are not available in the case of *E. coli*), antimicrobial chemotherapy and a range of environmental measures such as adequate hygiene and nutrition. These measures are applicable in the community [*E. coli* is able to spread among household members, including pets (Johnson, J. R. and Clabots, C., 2006)], and in hospital settings. Hospitals concentrate infected patients and those vulnerable to infections, hence control measures become even more crucial.

The vast majority of hospital-acquired (nosocomial) infections, whether involving endogenous (*i.e.* organism deriving from the patient's own flora) or exogenous (infecting organism deriving from the hospital environment) pathogens, are caused by *E. coli*, *S. aureus*, *Klebsiella* spp., *Enterobacter* spp. or *Pseudomonas* spp., depending on hospitals. Each of these organisms normally originates from various sources, uses specific routes to cause infections, and causes distinct types of infection even if all lead frequently to septicaemia.

E. coli often originates from the gastro-intestinal tract and is spread by bedpans, urinals, catheters, as well as soiled bedding and via the food chain (Shanson, D. C., 1999a). *E. coli* is not commonly transmitted on hands, like meticillin-resistant *S. aureus* (MRSA) or *Klebsiella* spp. UTIs are the most common type of nosocomial bacterial infections, with catheter-associated UTIs accounting for approximately 40% of all hospital-acquired infections (Stamm, W. E. and Norrby, S. R., 2001). Even though *E. coli* is more associated with community-acquired UTIs, it is also the major cause of nosocomial UTIs, accounting for approximately 80% of uncomplicated infections (Stamm, W. E. and Hooton, T. M., 1993). The causative *E. coli* often come via the patients' own faecal flora, but it is not unusual for the bacteria to originate from the hospital environment (*e.g.* food in the kitchen) or from other infected patients (cross-infection).

Long hospital stays constitute an important risk factor for the development of hospital-acquired UTIs, but the major precipitating factor remains catheterisation of the urinary tract (*e.g.* cystoscopy); at least 40% of patients undergoing these procedures develop an UTI (Shanson, D. C., 1999b). Most *E. coli* infections, regardless if they are community- or hospital-acquired, are avoidable though appendicitis remains an exception and control measures will rely on appropriate hygiene measures, particularly (Dundas, S. and Welsby, P. D., 2002):

- Sterilisation / disinfection of contaminated materials, and appropriate waste disposal
- Use of aseptic techniques in the operating theatre and wards for procedures such as changing of wound dressings, catheterisation, or setting-up intravenous drips
- Adequate preparation of food
- Isolation of infected patients and isolation of vulnerable patients
- Appropriate administration of antibiotic prophylaxis (*e.g.* in surgery or to prevent recurrent UTIs)
- Rotation of antibiotics used to treat patients (in order to discourage the emergence of resistance though options might be limited for multi-drug-resistant bacteria)
- Implementation of strict hygiene measures (*e.g.* use of protective clothing and hand washing for healthcare workers before and after each contact with patients)
- Infection surveillance (enable early recognition of emerging problems such as antibiotic resistance)

Attempts to prevent and control infection may fail and, as a result, antimicrobial treatment may have to be started, or changed (new or additional antibiotic, new dosage, administration by a different route). In severe-ill patients, adequate antibiotic treatment becomes crucial, owing to the risk of complications such as bacteraemia or septic shock, which are often fatal for the patients.

5. Antibiotic modes of action and classes

Most antibiotics in clinical use are derived from natural metabolic products of fungi, actinomycetes or bacteria. All are used to kill or inhibit the growth of micro-organisms. They demonstrate selective toxicity, *e.g.* acting at a target site present in the infecting organism only, and absent from the host. Production of antibiotics is particularly associated with soil and other environmental micro-organisms and may provide a selective advantage in their competition for space and nutrients. However, some agents used as systemic antimicrobials, such as sulphonamides and quinolones, are totally synthetic.

Until the 1930s, prevention was the best mean of protection against bacterial infections, but in 1928, Fleming accidentally discovered what was going to become the first true antibiotic: penicillin. On his return from holidays, he noticed that a contaminant mould (*Penicillium notatum*) showed antibacterial activity on old culture plates smeared with *S. aureus*. However, it was only in 1944 that penicillin finally became commercially available. From this point, research seeking new antimicrobial agents intensified, and still remains on the agenda of many pharmaceuticals companies nowadays due to the constant adaptation of bacteria and to the emergence of new antibiotic resistance mechanisms.

The ideal properties of any antibiotic should include: selectivity for microbial (rather than mammalian) targets, a broad spectrum of activity (though many narrow spectrum drugs have also been developed, and may be more appropriate if the infection-causing organism is known), bactericidal activity, non-toxicity to the host, long plasma half-life, good tissue distribution, oral and parenteral dosing forms, and also no interactions with other drugs, or other side effects.

Many further factors should be considered prior to any antibiotic therapy, including: use of an appropriate drug and administration route, use of the correct dosage, use of a narrow-spectrum antibiotic unless the cause of infection is unclear, preferential use of bactericidal antibiotics if host defence mechanisms are impaired, adequate duration of therapy, as well as the cost.

There are three major ways of classifying antibiotics:

(i) by their chemical structure

Classification based on chemical structure alone may not be practical since there is such diversity.

(ii) by their target site

Although this classification may not allow an accurate prediction of which antibiotic will be active against particular bacterial species, it is useful in the understanding of the molecular basis of antibacterial action. The four main target sites for antibacterial action are cell wall synthesis (*e.g.* penicillin), protein synthesis (*e.g.* tetracyclines), nucleic acid synthesis (*e.g.* quinolones) and metabolic pathways (*e.g.* sulphonamides).These targets greatly differ from their counterparts in human cells, allowing good selective toxicity.

(iii) according to whether they are bacteriostatic or bactericidal

Bacteriostatic antibiotics (*e.g.* tetracycline, chloramphenicol) prevent bacterial multiplication but do not kill bacteria when used alone. If the host defences do not kill the bacteria, then they will multiply again once the drug is withdrawn. On the other hand, bactericidal antibiotics (*e.g.* aminoglycosides, β-lactams) kill bacteria unassisted by the host defence mechanisms. However, they may only have a bacteriostatic action if given in too low a dose. In general, bacteriostatic agents are effective except in immunosupressed patients and in endocarditis.

The following classification of antibiotics is primarily based according to their target site (or mode of action), with special emphasis on antibiotics used for the treatment of *E. coli* infections. Within each group, antibiotics are subdivided based on their chemical structure.

5.1. Inhibitors of cell wall synthesis

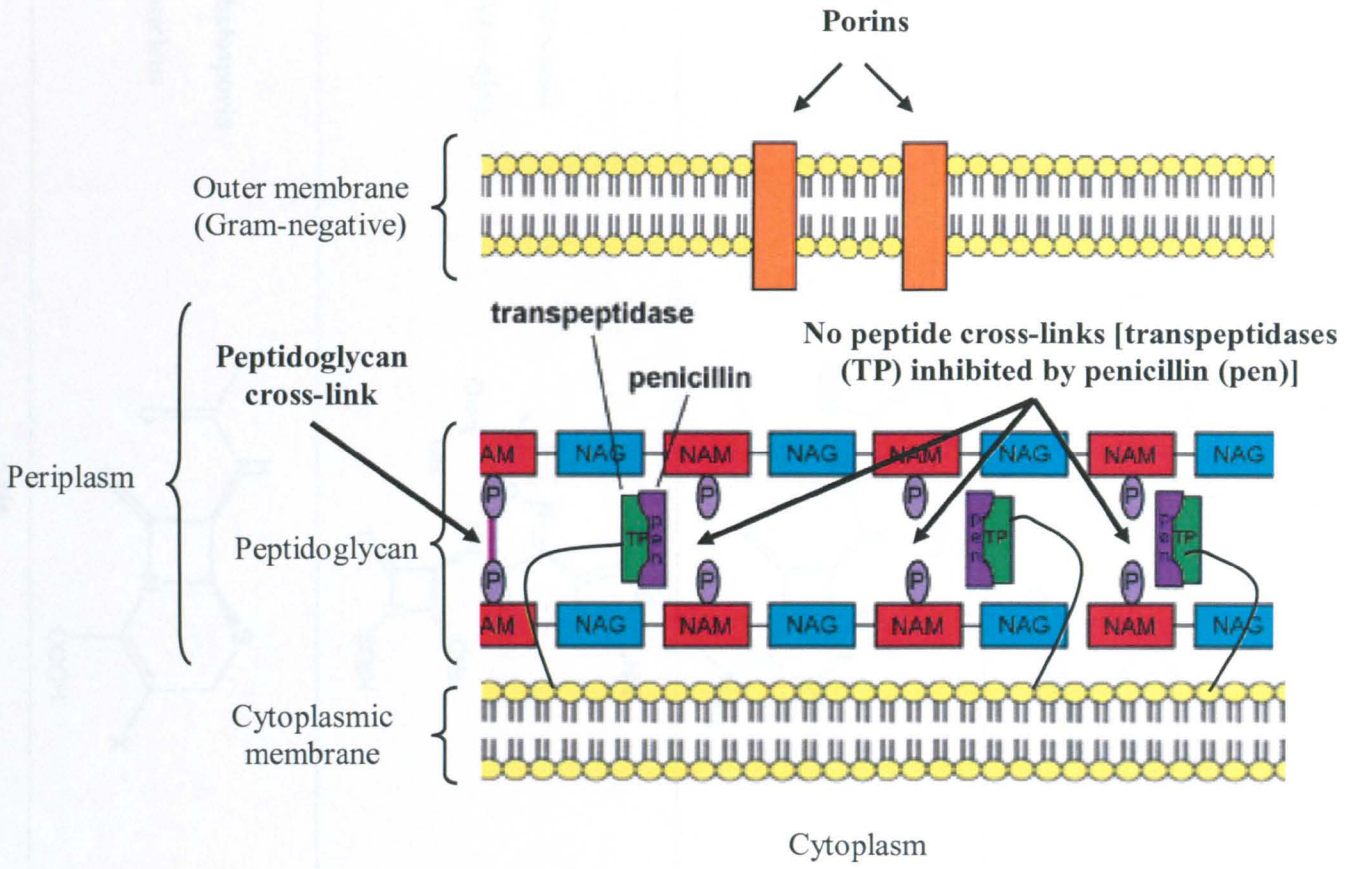
Peptidoglycan and its precursors constitute optimum targets for selective toxicity, since these components are unique to the bacterial cell wall (and to blue-green algae). Peptidoglycan synthesis involves many stages starting in the cytoplasm, proceeding across the cytoplasmic membrane and ending at the cell wall, with each step being a potential target for inhibition. The two major families of antibiotics inhibiting cell wall synthesis are the β-lactams and the glycopeptides, though only the former have useful activity *vs. E. coli* and other Gram-negative bacteria.

5.1.1. β-Lactams

β-Lactams are bactericidal and are the most heavily-used antibacterials in clinical medicine. Many types are registered for clinical use globally, some of which are mainly active *versus* Gram-positive bacteria (*e.g.* penicillin G), while other have primarily been developed against Gram-negative rods such as Enterobacteriaceae (*e.g.* cefotaxime). They specifically bind to and permanently inactivate peptidoglycan carboxypeptidases and transpeptidases [also known as penicillin-binding proteins (PBPs)]. These enzymes are located in the outer leaflet of the cytoplasmic membrane and act in the final stages of cell wall synthesis, cross-linking the peptidoglycan chains that surround the bacterium and maintain its shape and structural integrity. β-Lactams stop bacterial cell wall synthesis and renewal, as illustrated in Figure 3, leading to the accumulation of peptidoglycan precursors in the cytoplasm. Bacterial death results from osmotic lysis, promoted by the activation of the cell's autolytic system, as well as by the failure to integrate new cell wall material. β-Lactams remain inactive against species lacking a cell wall (*e.g. mycoplasma*), as well as against mycobacteria (impenetrable cell wall) or intracellular pathogens (*e.g. Chlamydia*).

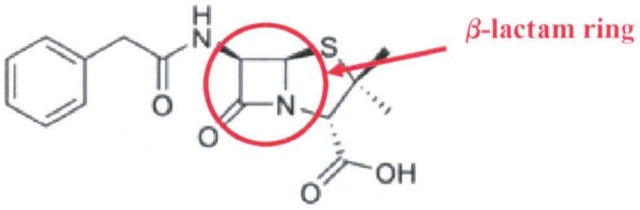
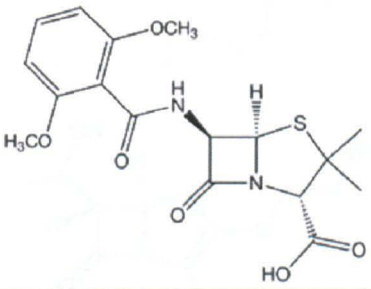
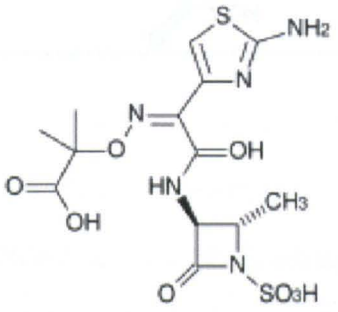
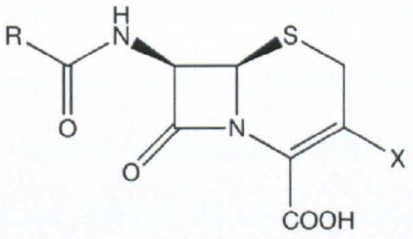
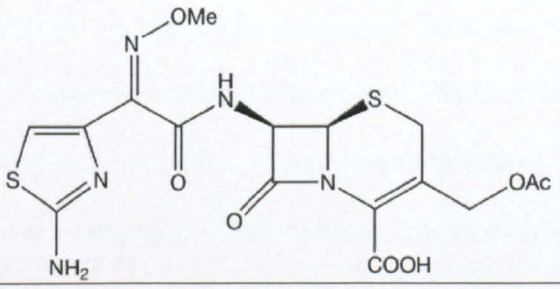
Most β-lactams are administered parenterally, although a few are orally active (*e.g.* amoxicillin). They have generally low toxicity, but may be responsible for allergic reactions in the form of skin rashes or fever and, rarely, fatal anaphylactic shock. β-Lactams comprise several groups of compounds (*e.g.* penicillins, cephalosporins, cephamycins, monobactams and carbapenems) distinguished by the chemical structures of the secondary ring and/or the side chain attached to the β-lactam ring, which is a common feature of all antibiotics of this family (Table 2).

Figure 3. Inhibition of peptidoglycan cross-linking by penicillin[a]



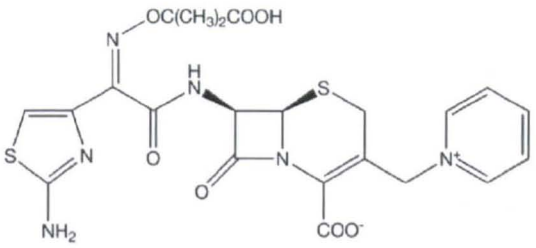
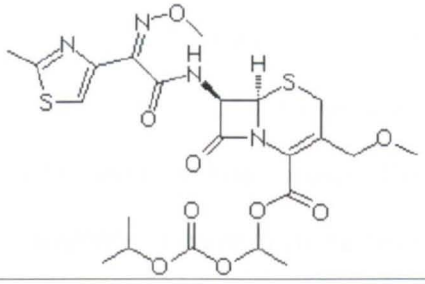
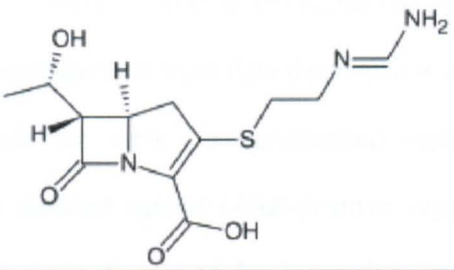
[a] (NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid)

Table 2. Chemical structure of selected β-lactam compounds with different spectrum of activity

	CHEMICAL STRUCTURE (source: http://en.wikipedia.org/wiki/Cephalosporin)
Penicillin G (MW = 334)	
Meticillin (MW = 380)	
Aztreonam (MW = 435)	
Cephalosporin nucleus	
Cefotaxime (MW = 455)	

CHEMICAL STRUCTURE

(source: http://en.wikipedia.org/wiki/Cephalosporin)

Ceftazidime (MW = 547)	
Cefpodoxime (MW = 427)	
Imipenem (MW = 299)	

The history of β-lactams began with Alexander Fleming's discovery of penicillin in the late 1920s. Until the 1950s, β-lactams only comprised the two narrow-spectrum compounds penicillin G (parenteral route of administration) and penicillin V (oral penicillin). The expansion of this major family of compounds occurred in the early 1960s, with the development of the semi-synthetic penicillins (*e.g.* ampicillin, meticillin, oxacillin and amoxicillin), which offer a broader spectrum of activity than penicillins G and V. Meticillin was the first compound to be active against penicillin-resistant *S. aureus*, while ampicillin was mostly aimed against Gram-negative pathogens. Carbenicillin in 1967, and ticarcillin later, were the first β-lactams to show useful clinical activity *vs. P. aeruginosa* (Rolinson, G. N., 1998). Monobactams (*e.g.* aztreonam) are monocyclic β-lactams inactivated by extended-spectrum β-lactamases. They have been discovered in 1978 and have strong activity against susceptible Gram-negative bacteria, but remain inactive *vs.* Gram-positive and anaerobes (Neu, H. C., 1988). In order to overcome the ever growing β-lactam resistance problem, cephalosporins and carbapenems were then developed. Cephalosporins form a class of β-lactams of their own. While the early (first-generation) cephalosporins such as cephalothin or cephazolin were mainly directed against Gram-positive organisms, the second-, third- and fourth-generation compounds were developed for increasing activity against Gram-negative bacteria; each generation of cephalosporins was developed to have a broader spectrum of activity than the previous one. Third-generation cephalosporins are stable to many widespread conventional β-lactamases, such as the TEM-1 enzyme, which confers resistance to penicillin, and have a broad-spectrum of activity including Gram-positive cocci and Gram-negative bacilli (though ceftazidime shows weak activity against staphylococci *in-vitro*). These antibiotics, particularly ceftazidime and cefotaxime, have long been useful in treating hospital-acquired infections. However, their heavy use has rapidly triggered the selection of resistant bacteria, including those producing extended-spectrum β-lactamases (ESBLs). By contrast, carbapenems, which exhibit an exceptionally broad spectrum of activity including Gram-positive, Gram-negative and anaerobic bacteria, are stable against most β-lactamases including AmpC and ESBLs (Zhanel, G. G. *et al.*, 2007). They are not useful against MRSA. Examples of compounds belonging to this class of β-lactams include imipenem, meropenem, ertapenem and doripenem. The successive development of newer β-lactams in order to overcome resistance is reviewed in more detail in section 7.

5.1.2. Glycopeptides

Glycopeptides include vancomycin and teicoplanin. Both are large bactericidal molecules, and are active only against Gram-positive bacteria, since they cannot penetrate the outer membrane of Gram-negative organisms. They act at an earlier stage than β-lactams, by binding to the terminal D-ala-D-ala of pentapeptide chains and inhibiting the transglycosylation reaction, thus preventing incorporation of new subunits into the growing peptidoglycan. Vancomycin and teicoplanin are mainly used for the treatment of infections caused by multi-resistant Gram-positive bacteria, particularly meticillin-resistant *S. aureus* (MRSA) and *S. epidermidis*.

5.1.3. Other inhibitors of cell wall synthesis

Fosfomycin is a fermentation product of a *Streptomyces* spp. It is generally more active against Gram-negative bacilli (Enterobacteriaceae) than against Gram-positive cocci. It interferes with bacterial cell wall synthesis by inhibiting phosphoenolpyruvate transferase, an enzyme involved in handling an early precursor of peptidoglycan. In countries where fosfomycin is marketed (*e.g.* Belgium and Spain, but not in the UK), it is recommended for the treatment of uncomplicated urinary tract infections. Fosfomycin may achieve a synergistic activity against some organisms when used in combination with aminoglycosides or β-lactams.

Other inhibitors of cell wall synthesis include bacitracin and cycloserine, although they have very limited uses, owing to toxicity.

5.2. Inhibitors of protein synthesis

Translation of the messenger RNA (mRNA) into a peptide chain is complex, involving many steps. Several classes of antibiotics inhibit protein synthesis, exploiting the differences between the prokaryotic 70S ribosome and the eukaryotic 80S ribosome. These include aminoglycosides, tetracyclines and chloramphenicol, all of which are useful against *E. coli,* along with macrolides, lincosamides, streptogramines and fusidic acid, which are useful only against Gram-positive bacteria. Some of these compounds inhibit the initiation of protein synthesis, while more target the elongation step.

5.2.1. Aminoglycosides

Aminoglycosides are bactericidal antibiotics, with the most-commonly-used in clinical practice being gentamicin, amikacin and tobramycin. Aminoglycosides irreversibly bind to the 30S subunit of the bacterial ribosome and prevent the formation of the 30S initiation complex known as formylmethionyl-transfer RNA (fmet-tRNA), from which protein synthesis proceeds. As a result, no further initiation can occur. Some aminoglycosides such as streptomycin also causes misreading of mRNA codons, prematurely terminating protein synthesis following insertion of a "wrong" amino-acid (Mims, C. *et al.*, 1999). Aminoglycosides are particularly useful in infections due to Gram-negative bacilli and staphylococci. Combinations with β-lactams are often used to broaden their spectrum of activity to include streptococci and anaerobes, which are not susceptible to aminoglycosides alone. Aminoglycosides must be administered intravenously or intramuscularly for systemic infections. Monitoring of drug levels is essential, especially in patients with renal impairments since the therapeutic window between the serum concentration required for successful treatment and that which becomes nephrotoxic or ototoxic is narrow.

5.2.2. Tetracyclines

Tetracyclines are bacteriostatic antibiotics with large cyclic structures offering many sites for possible chemical substitutions. Although different tetracyclines (*e.g.* tetracycline, minocycline, doxycycline) vary in their pharmacological properties, they all have a relatively similar broad antibacterial spectrum of activity. Like aminoglycosides, tetracyclines inhibit initiation of protein synthesis by binding to the 30S ribosome. However, this interaction is reversible, occurring at a later stage than with aminoglycosides, and acts by preventing the aminoacyl transfer RNA from entering the acceptor sites on the 70S ribosome. Although tetracyclines are active against a wide variety of bacteria including intracellular species, their use is restricted due to widespread resistance (Shlaes, D. M., 2006). Furthermore, tetracyclines, which are usually administered orally, may suppress the normal intestinal flora thus often leading to secondary infections (*e.g.* candidosis).

5.2.3. Chloramphenicol

Chloramphenicol is a broad-spectrum antibiotic with bacteriostatic activity, which acts by blocking the action of peptidyl transferase, thereby preventing peptide bond synthesis. It inhibits bacterial protein synthesis selectively because it has a much higher affinity for the transferase in the 50S subunit of the bacterial ribosome than for the corresponding transferase in the 60S subunit of the mammalian ribosome. Chloramphenicol use is now restricted due to its toxicity (bone marrow suppression).

5.3. Inhibitors of nucleic acid synthesis

The selective toxicity of these agents is a result of the differences between the prokaryotic enzymes targeted and their eukaryotic counterparts. Antibacterial agents of this class include sulphonamides, trimethoprim, quinolones and rifamycins. They intervene at three different stages of nucleic acid synthesis: *(i)* preventing synthesis of precursors (folic acid), *(ii)* inhibiting DNA replication or *(iii)* blocking mRNA synthesis.

5.3.1. Sulphonamides and trimethoprim

By contrast with mammalian cells, many bacteria cannot absorb folic acid and have to synthesise their own. Two major groups of compounds inhibit bacterial folate synthesis: sulphonamides and trimethoprim.

Sulphonamide antibiotics are analogues of para-amino benzoic acid (PABA) and competitively inhibit the formation of dihydropteroic acid, a precursor of tetrahydrofolic acid, by binding to the active site of dihydropteroate synthase. This, in turn, affects the synthesis of purines and pyrimidines, and thus nucleic acid synthesis. Sulphonamides are bacteriostatic, and have long been useful in the treatment of urinary tract infections. They have a broad spectrum of activity, but are primarily used against Gram-negative organisms. Resistance to this aging family of antibiotics is now widespread, thereby its limited use. The most commonly used sulphonamide is sulphamethoxazole, which is often administered in combination with trimethoprim as co-trimoxazole.

Like sulphonamides, trimethoprim is a bacteriostatic antibiotic that competitively inhibits tetrahydrofolic acid synthesis, but intervenes at a later stage, by inhibiting dihydrofolate reductase. It acts as an analogue of the aminohydroxypyrimidine moiety of the folic acid molecule. As just mentioned, trimethoprim is often given in combination with sulphonamides, since both compounds act synergistically against some bacteria (though in the UK, trimethoprim alone is often preferred owing to the toxicity of sulphonamides). Trimethoprim and co-trimoxazole are mainly used for the treatment of urinary tract infections, especially those caused by Gram-negative bacilli. Resistance to trimethoprim alone and to co-trimoxazole is now common.

5.3.2. Quinolones

The quinolones are a large family of synthetic bactericidal agents, with nalidixic acid being the earliest compound. Newer derivatives (fluoroquinolones), such as ciprofloxacin, have improved pharmacological properties and antibacterial activity compared with nalidixic acid. Quinolones inhibit DNA gyrase (topoisomerase IV), an enzyme needed for the replication of DNA, as it breaks and rejoins the strands of bacterial DNA to relieve the stress that occurs during the unwinding of DNA during replication and transcription. Quinolones thereby prevent supercoiling of the bacterial chromosome, and have a broad spectrum of activity. They are mostly used to treat urinary tract and systemic Gram-negative infections, including those caused by *Pseudomonas aeruginosa*. Nevertheless, resistance to quinolones and their derivatives has been expanding in recent years (Livermore, D. M. and Paterson, D. L., 2006).

5.3.3. Rifamycins

Rifamycins are bactericidal antibiotics that specifically bind to bacterial DNA-dependent mRNA polymerase, blocking the synthesis of messenger RNA. Although they have a wide spectrum of activity, they are particularly used for the treatment of *(i)* mycobacterial infections and *(ii)* of severe infections due to Gram-positive organisms. Rifampicin is clinically the most important member of this family. Mutational resistance is common, hence the drugs are mostly used in combination therapy (Murphy, C. K. *et al.*, 2006).

5.4. Miscellaneous

The above classification outlines the most important families of antibiotics, but a few additional antibiotics are also available. These mostly have a narrow spectrum of activity and some are variably available between countries. With regard to infections caused by Gram-negative organisms such as *E. coli*, two additional agents deserve to be mentioned: colistin (polymyxin E) and nitrofurantoin.

5.4.1. Polymyxins

Polymyxins are bactericidal polypeptides that act on the membranes of Gram-negative bacteria, by disrupting the phospholipid structure, thus disturbing osmotic integrity and causing loss of important metabolites. Colistin is clinically the most important polymyxin, but its use is restricted due to its nephrotoxicity. Nevertheless, colistin remains often the "drug of last resort" for the treatment of serious infections caused by *P. aeruginosa* and *Acinetobacter* strains resistant to all other major classes of antibiotics (Arnold, T. M. *et al.*, 2007). It is also used in a nebulised form for treating pulmonary infections caused by *P. aeruginosa* in cystic fibrosis.

5.4.2. Nitrofurantoin

Nitrofurantoin is a synthetic compound administered orally for the treatment of uncomplicated lower urinary tract infections, mostly those caused by coliforms (*i.e. E. coli* and its relatives). It may also be used for prophylaxis of recurrent UTIs. Although its mode of action has not yet been fully elucidated, it has been suggested that nitrofurantoin may interfere with the expression of inducible genes acting at the initiation step of messenger RNA translation (Herrlich, P. and Schweiger, M., 1976). Resistance rarely develops (Karlowsky, J. A. *et al.*, 2003), though therapeutic failure is common, perhaps associated with silent upper UTIs.

6. Antibiotic resistance mechanisms

Since the beginning of the antibacterial era in the 1940s, bacteria have proved capable of adapting and developing resistance to almost every agent. Clinical resistance to an antimicrobial agent occurs when the Minimum Inhibitory Concentration (MIC) of the drug for a given bacterial strain exceeds that which is safely achievable or maintainable in the particular tissue or fluid affected after normal dosage. Bacterial resistance may be either intrinsic (or innate) or acquired (using various means). Intrinsic resistance describes the situation where an entire bacterial species is resistant even before the affected antibiotic has been developed and introduced on to the market (*e.g. Streptococcus pyogenes* is naturally resistant to gentamicin, *S. aureus* to polymyxins). The major reasons for innate drug resistance include lack of penetration of the drug through the cell wall, lack of a suitable cell target, or the consistent production of a drug-destroying enzyme. By contrast, resistance that is selected following the introduction of an antimicrobial agent into the clinic is said to be acquired. Acquired resistance results either from spontaneous single or multiple mutation(s) or from the acquisition of resistance genes encoded by transmissible plasmids or by transposons (colloquially known as "jumping genes").

Mechanisms of antibiotic resistance, whether they are chromosomally-encoded or plasmid-mediated, can be divided into five main categories:

(i) Altered permeability

(ii) Antibiotic inactivation

(iii) Target site alteration

(iv) Metabolic bypass (*i.e.* replacement of a sensitive pathway)

(v) Efflux

These mechanisms are described in more detail below, and are illustrated with examples for each class of antibiotics commonly used for the treatment of *E. coli* infections. Table 3 summarises the most common mechanisms for antibiotic resistance in *E. coli.*

Table 3. Summary of antibiotic resistance mechanisms commonly occurring in clinical *E. coli* isolates

Antibiotic class[a]	Mechanism of antibiotic resistance[b]				
	Altered permeability and uptake	Drug inactivation	Target alteration	Metabolic bypass	Efflux
Aminoglycosides	-/+	++	(streptomycin only)		
β-Lactams	+	++			
Chloramphenicol		+			
Quinolones	+	+/-	++		+/-
Sulphonamides				++	
Tetracyclines	+				++
Trimethoprim				++	

[a] Only antibiotics potentially used to treat infections caused by *E. coli* are listed in this table.
[b] (+/-, rare; +, common; ++, very common)

6.1. Altered permeability

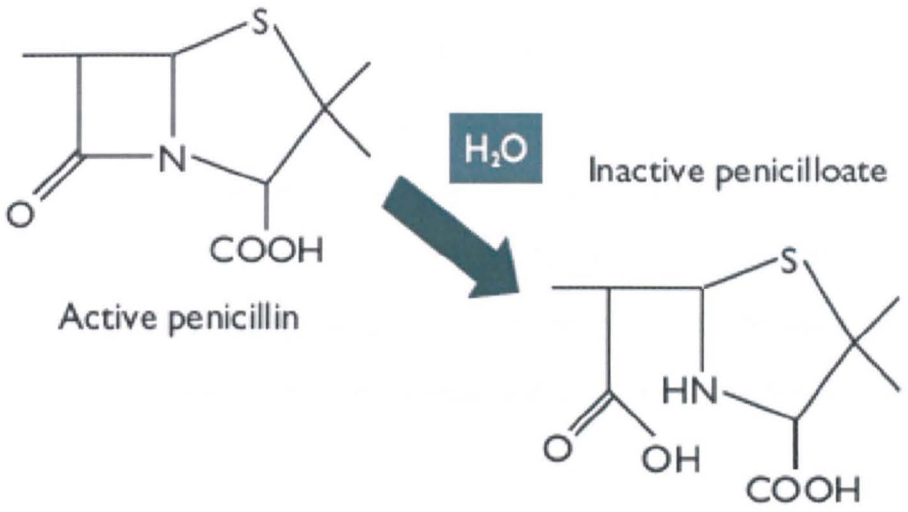
Altered permeability decreases the amount of drug that reaches its target site, and may be responsible for simultaneous resistance to several classes of antibiotics. In Gram-negative bacteria, most antibiotics gain access to their targets by diffusion through channels formed by porin proteins, which are embedded in the outer membrane. Alterations to the genes encoding these porins may result in decreased porin expression or function, and so with reduced uptake of the antibiotic. Since unrelated antibiotics may use the same porins, these alterations may be responsible for cross-resistance. Despite being rarely reported in *E. coli*, resistance to β-lactams by decreased permeability can arise via mutations in the genes encoding its two major porins: OmpC and OmpF (Viveiros, M. *et al.*, 2007). On the other hand, aminoglycosides require active transport system across the cell membrane before they exert their action, and alterations to these energy-dependent transporters may cause aminoglycoside resistance. Growth of *E. coli* under anaerobic conditions reduces the energy potential across the cytoplasmic membrane, thus inducing transient resistance to aminoglycosides.

6.2. Drug inactivation

This is the best-known mechanism of antibiotic resistance, and is very common among many species. Bacteria can produce enzymes that modify or destroy antimicrobials. The best known of these enzymes are β-lactamases, with over 500 variants described in the literature so far (http://www.lahey.org/studies/inc_webt.asp). The most clinically significant of these are produced by Gram-negative organisms. These enzymes hydrolyse the β-lactam ring of penicillins, cephalosporins, monobactams and carbapenems, yielding microbiologically inactive derivatives (Figure 4). Genes encoding β-lactamases are widespread among Gram-positive and Gram-negative organisms, and are found on the chromosome as well as on plasmids. β-Lactamases are discussed in more detail in section 7 of this chapter.

Aminoglycosides and chloramphenicol are other antimicrobial agents susceptible to enzymatic inactivation; tetracycline- and (recently) fluoroquinolone-degrading enzymes have been described too.

Figure 4. Beta-lactamase inactivation of penicillin



(Source: http://www.hpa.org.uk/publications/2005/amr_report_05/3_mdr.htm)

Production of modifying enzymes is far the most important mechanism of acquired resistance to aminoglycosides in *E. coli*. The genes encoding these enzymes are often plasmid-mediated and the type of enzyme produced determines the spectrum of resistance. Hydroxyl and amino groups of aminoglycosides are sites at which these compounds can be inactivated by phosphorylation, adenylylation or acetylation. An aminoglycoside acetyltransferase variant (*aac(6')-Ib-cr*) that acetylates ciprofloxacin and norfloxacin (*i.e.* fluoroquinolones with an unsubstituted piperazyl group) has recently been described. Its action leads to decreased susceptibility only, not frank resistance (Robicsek, A. *et al.*, 2006). Chloramphenicol resistance, which is also most commonly plasmid-mediated, generally results from modification of the drug by chloramphenicol acetyltransferases yielding an inactive product that fails to bind to its ribosomal target.

6.3. Target site alteration

Antibiotic resistance can also arise from alterations to the target site, reducing affinity so that the target is then capable of functioning normally in the presence of antibiotic, or by synthesis of additional target molecules "swamping" the antibiotic (rare, but true in *e.g.* vancomycin intermediary *S. aureus*). Such resistance mechanisms affects many classes of antibiotics, including β-lactams, quinolones, glycopeptides, macrolides or rifamycins, and are most commonly observed among Gram-positive bacteria.

In Gram-negative organisms, target modification is associated mostly with resistance to quinolones (Mims, C. *et al.*, 1999). Quinolone resistance currently is most often chromosomally-encoded, and is usually the result of multiple mutations in the genes encoding DNA gyrase and topoisomerase IV subunits (*e.g. gyrA*, *gyrB*, *parC*...). The activity of quinolones may also be altered by the acquisition of plasmid-encoded Qnr peptides, which interact with the target enzymes, thereby limiting drug access and efficacy. This Qnr-mediated protection mechanism, which is rarer than mutational resistance, leads to reduced susceptibility only, but may predispose the bacterial cell towards full clinical resistance (*e.g.* by chromosomal mutations of the genes coding for DNA gyrase subunits or by decreased uptake).

6.4. Metabolic bypass

Many antibiotics inhibit enzymes involved in specific metabolic pathways. In order to bypass the effect of these drugs, bacteria may acquire a novel drug-resistant metabolic pathway, thus rendering the antibiotic ineffective (Smith, J. T. and Lewin, C. S., 1993). The best example of resistance by metabolic bypass is meticillin-resistant *S. aureus*; it synthesises an additional and altered penicillin-binding protein (PBP) with much lower affinity for β-lactams than the "normal" PBP. Cell wall synthesis then continues even though the "normal" PBPs are inhibited. Resistance to sulphonamides and to trimethoprim is also commonly mediated by metabolic bypass enzymes (Huovinen, P., 2001). Bacteria resistant to these compounds synthesise supplementary dihydropteroate synthase and dihydrofolate reductase enzymes, with little or no affinity for sulphonamides and trimethoprim, respectively, whereas their affinities for their respective substrates (para-aminobenzoic acid and dihydrofolic acid) remain similar to those of their chromosomal counterparts. These enzymes generally are plasmid-mediated (Smith, J. T. and Lewin, C. S., 1993).

6.5. Efflux

Active export via efflux mechanisms is another mean of preventing the antibiotic of reaching its target. *E. coli* has been shown to harbour at least nine distinct chromosomally-encoded proton-dependent efflux pump systems. If over-expressed, these systems may decrease the amount of antibiotic retained within the bacterial cell, thus causing resistance. The genes coding for these pumps are *emrE*, *acrEF*, *emrAB*, *emrD*, *acrAB-tolC*, *mdfABC*, *tehA*, *acrD* and *yhiUV* (Viveiros, M. *et al.*, 2007).

Efflux is commonly associated with resistance to tetracyclines and to quinolones. Tetracycline resistance generally results from the acquisition of new cytoplasmic membrane pumps, encoded by genes carried most often by transposons. Quinolone resistance in *E. coli* may also be due to decreased accumulation of the drug in the cell, to some extent. This is more often due to mutations in the *acrAB* or *mar* genes, which up-regulate efflux and thereby reduce susceptibility to quinolones. This mechanism may lead to cross-resistance or decreased susceptibility to unrelated antibacterial agents.

6.6. Multiple antibiotic resistance mechanisms

It is not uncommon for Gram-negative bacteria (including *E. coli*) to manifest resistance by using a combination of the above mechanisms. For example, high-level quinolone resistance may be the result of a combination of decreased uptake, multiple target alterations, as well as target protection (*e.g.* by mutations in DNA gyrase and by Qnr peptides). The same may be true for carbapenem resistance in Enterobacteriaceae, which may be due to a combination of β-lactamase production and altered permeability (Woodford, N. *et al.*, 2007a).

Resistance to multiple antibiotics on the other hand, arises via plasmids that carry multiple resistance genes, thus limiting treatment options. Multi-drug resistant organisms may drive the clinician to use and overuse the very few unaffected antibiotic, which may then contribute towards the selection of bacteria with an even greater spectrum of resistance.

7. β-Lactamases

Although many other resistance mechanisms can affect the clinical efficacy of β-lactams (*e.g.* mutations to PBPs, acquisition of β-lactam-resistant PBPs, efflux, impermeability), the most significant mechanism in Gram-negative bacteria, particularly in Enterobacteriaceae, is incontestably inactivation by β-lactamases. As illustrated earlier (Figure 4), β-lactamases catalyse the hydrolysis of the β-lactam ring of penicillins, cephalosporins, cephamycins, monobactams and carbapenems, inactivating the antibacterial properties of the drug molecule. These enzymes can be chromosomally-encoded (*i.e.* universal in a given species) or plasmid-mediated (*i.e.* transferable between species). The range of β-lactam substrates varies among β-lactamases.

7.1. Evolution of β-lactamases

Abraham and Chain isolated the first recognised β-lactamase, a chromosomal penicillinase (penicillin-hydrolysing β-lactamase) from *E. coli* in 1940, even before penicillin entered clinical use (Abraham, E. P. and Chain, E., 1940). Early concern however, centred on the spread of β-lactamases in Gram-positive bacteria, particularly *S. aureus*, as the major targets of early β-lactams.

It may be that these enzymes originally protected the bacteria from naturally-occurring β-lactams, long before clinical usage, or that they acted upon β-lactam-like natural regulators of cell wall synthesis. In any event, the spread of β-lactamases, and the inherent resistance of Gram-negative bacteria to early penicillins, forced pharmaceutical companies to seek novel β-lactams (a) able to withstand hydrolysis by staphylococcal penicillinases, and (b) with a broader spectrum of activity to include Gram-negative bacteria. Compounds with activity *vs.* Gram-negative bacteria, including ampicillin and first-generation cephalosporins, were introduced in the early 1960s and, concomitant with their use during the mid-1960s, transferable (*i.e.* plasmid-mediated) broad-spectrum β-lactamases began to be identified in Gram-negative bacteria. These broad-spectrum β-lactamases, include (most importantly) TEM-1 (Datta, N. and Kontomichalou, P., 1965; Sutcliffe, J. G., 1978), TEM-2 (Matthew, M. and Hedges, R. W., 1976; Ambler, R. P. and Scott, G. K., 1978) and SHV-1 (Bradford, P. A., 1999; Levesque, C. *et al.*, 1995; Mercier, J. and Levesque, R. C., 1990).

By the mid-1970s, TEM-1 had become common in Gram-negative bacteria and stability to its hydrolytic activity became the next target of pharmaceutical companies, leading to the development of newer β-lactams with greater stability towards β-lactamases, such as "extended-spectrum" third-generation cephalosporins (*e.g.* cefotaxime and ceftazidime) and carbapenems. In parallel, "suicide-inhibitors" of these β-lactamases (*e.g.* clavulanate, sulbactam and tazobactam) were discovered also in the mid-1970s, and were seen as a major breakthrough in the battle against infections.

The success of these strategies was short-lived, as transferable extended-spectrum β-lactamases (ESBLs) rapidly emerged among Gram-negative bacteria following on the introduction of third-generation cephalosporins in the early 1980s (Turner, P. J., 2005). The heavy use of these β-lactams, especially in hospitals, was shown to select for variants of the TEM and SHV β-lactamases (Knox, J. R., 1995), with mutations expanding their spectrum of activity. Inhibitor-resistant TEM β-lactamase variants also emerged in the late 1980s. Problems for third-generation cephalosporins and β-lactamase inhibitors also arose via the selection of variants, especially *Enterobacter* spp., that hyperproduced their chromosomal AmpC β-lactamases (Paterson, D. L., 2006).

For many years since the 1980s, TEM and SHV variants were the most common ESBL types; more than 150 TEM and 100 SHV variants have now been reported (http://www.lahey.org/studies/webt.asp). However, a newer type of ESBL –known as CTX-M– has been increasingly replacing the TEM and SHV β-lactamases in importance among clinical isolates in the past decade. CTX-M enzymes (described in more depth in section 8) are now the most prevalent ESBLs around the world (Canton, R. and Coque, T. M., 2006).

7.2. Classification of β-lactamases

Two major classification schemes of β-lactamases have been proposed: *(i)* a functional classification based upon substrate profiles as well as susceptibility to suicide inhibitors such as clavulanate (Bush, K. *et al.*, 1995), and *(ii)* a molecular or structural classification (Ambler, R. P., 1980), based on amino-acid sequences alone. Ambler's classification divides β-lactamases into four major classes: A, B, C and D, with class A and C β-lactamases being the most widespread among clinically-significant bacterial species. Ambler's class A, C and D β-lactamases have a serine residue in their active site; this takes part in β-lactam hydrolysis, forming an unstable ester with the opened β-lactam ring. Class B β-lactamases, by contrast, are metallo-β-lactamases that require one or two zinc ions to exert their activity.

7.2.1. Serine β-lactamases

7.2.1.1. Class A β-lactamases

Class A β-lactamases, many of which are plasmid-mediated, are the most common and the best understood β-lactamase types. They occur in both Gram-positive and Gram-negative bacteria, and are very heterogeneous in their structures and kinetic properties, though some motifs are conserved throughout the family. They include the chromosomal β-lactamases of *Klebsiella* spp., staphylococcal penicillinase, the TEM and SHV β-lactamases and their ESBL derivatives, the CTX-M types and the rarer PER, VEB and GES ESBLs, and finally the KPC, IMI and SME carbapenemases.

TEM and SHV type β-lactamases are commonly found in many Gram-negative bacterial species, especially in *Klebsiella* spp. (where SHV is chromosomal and ubiquitous), *E. coli* and *Enterobacter* spp. The amino-acid substitutions responsible for conferring ESBL activity cluster around the active site, changing its configuration to allow access to oxyimino-β-lactams (*i.e.* β-lactams such as cefotaxime and ceftazidime substituted with an oxyimino-aminothiazolyl group, providing stability against non-ESBL TEM and SHV types), but also enhancing their susceptibility to inhibitors such as clavulanic acid. CTX-M ESBLs were named as such for their greater activity against cefotaxime than against other oxyimino-β-lactam substrates such as ceftazidime (Bonnet, R., 2004). They have migrated to plasmids from the chromosomes of *Kluyvera* spp. (commensal organisms that are rarely pathogenic). CTX-M enzymes are not closely related to TEM and SHV β-lactamases, only sharing approximately 40% sequence homology.

Other plasmid-mediated class A ESBLs, including the PER, VEB and GES β-lactamases, are less common and (except PER-2, which occurs in Enterobacteriaceae in Argentina) have mainly been found in *Pseudomonas aeruginosa* (Livermore, D. M. and Woodford, N., 2006). The KPC (highly prevalent in the USA and in Israel), IMI and SME β-lactamase types are effective carbapenemases.

7.2.1.2. Class C β-lactamases

Class C β-lactamases, also known as AmpC enzymes, were first believed to be exclusively chromosomal, but plasmid-borne variants have been identified since the 1980s, reflecting gene escape *e.g.* from the chromosome of *Citrobacter* species. AmpC β-lactamases form a very closely related group, sharing similar structures, particularly within their active sites. They are the chromosomal β-lactamase of most Enterobacteriaceae, and may be inducible (by *e.g.* cefoxitin, ampicillin or imipenem) and are prone to mutational derepression in *Enterobacter* spp., *Citrobacter freundii*, *Morganella morganii*, *Serratia* spp., also *P. aeruginosa*.

Six families of plasmid-mediated AmpC enzymes have been identified (Perez-Perez, F. J. and Hanson, N. D., 2002). These comprise ACC (originating from *Hafnia alvei*), CIT (from *C. freundii*), DHA (from *M. morganii*), EBC (from *E. cloacae*), FOX (maybe from *Aeromonas* spp.) and MOX (maybe from *Aeromonas* spp.).

In contrast to ESBLs (transferable class A β-lactamases hydrolysing oxyimino-cephalosporins and susceptible to clavulanate), AmpC enzymes hydrolyse cephamycins (*e.g.* cefoxitin), but remain inactive *versus* fourth-generation cephalosporins (*e.g.* cefepime). Most importantly, they are not much affected by β-lactamase inhibitors such as clavulanate, owing to very poor affinity (Monnaie, D. and Frere, J. M., 1993).

7.2.1.3. Class D β-lactamases

The class D β-lactamase family was originally defined on phenotypic rather than genotypic criteria, comprising enzymes that preferentially hydrolyse oxacillin, cloxacillin and other anti-staphylococcal penicillins. Class D β-lactamases predominantly comprise the plasmid-mediated oxacillinases (also known as OXA β-lactamases), but also intrinsic chromosomal types found in *Aeromonas* spp., *Pseudomonas* spp. and *Shewanella* spp. (Heritier, C. *et al.*, 2004; Poirel, L. *et al.*, 2004). Amino-acid substitutions in OXA-type β-lactamases can extend their spectrum of activity resulting in ESBL activity, just as in class A. However, while the common ESBLs (CTX-M, TEM and SHV variants) are predominantly found amongst Enterobacteriaceae, OXA-type ESBLs have been found mainly in *P. aeruginosa* and *Acinetobacter* spp (Danel, F. *et al.*, 1997). Another difference compared with common ESBLs is that they are poorly inhibited by clavulanate.

7.2.2. Metallo-β-lactamases (class B)

Some class B β-lactamases show a very broad substrate spectrum (though that from *Aeromonas* spp. is a strict carbapenemase by contrast). Many are capable of hydrolysing all non-monobactam β-lactam antibiotics, including carbapenems, which are generally stable against most other β-lactamases, and which have enjoyed longevity in the clinic since their introduction in the mid-1980s. These enzymes also evade the inhibitory activity of clavulanate and other β-lactamase inhibitors.

Class B β-lactamases, also known as metallo-β-lactamases, require the binding of zinc metal ions to their active site in order to exert their hydrolytic activity.

These β-lactamases include the chromosomal β-lactamase of *Aeromonas* spp., *Chryseobacterium* spp. and *Stenotrophomonas maltophilia*, as well as the plasmid-mediated IMP, VIM, SPM and GIM carbapenemases, amongst which the VIM enzymes are the most prevalent. Although class B β-lactamases display considerable structural variation and do not share any close evolutionary relationships, the active site residues that coordinate the binding of the zinc ions are conserved. The plasmid-mediated types, which pose the greatest threat as they can spread among different species, have been found in Gram-negative enteric organisms, but are commoner in *Pseudomonas* and *Acinetobacter* species. Except for SPM, they are normally encoded within integrons, which may assist their mobility. They have a wide geographic distribution, and are most commonly found in the Far East, Europe and South America.

8. CTX-M extended-spectrum β-lactamases

As mentioned already, extended-spectrum β-lactamases (ESBLs) were first detected in the 1980s [first report from Germany in 1983, (Knothe, H. *et al.*, 1983)], soon after the introduction on to the market of oxyimino "third-generation"-cephalosporins (*e.g.* cefotaxime, ceftazidime, ceftriaxone). ESBLs may be defined as β-lactamases capable of hydrolysing oxyimino-cephalosporins and monobactams, but not cephamycins and carbapenems, and are susceptible to inhibition by β-lactamase inhibitors (the class D ESBLs are a rare exception in this regard). They are frequently encoded by plasmids and, until recently, mostly were derivatives of the TEM-1, TEM-2, or SHV-1 β-lactamases, with changes to the amino-acids around the active site, extending the spectrum of β-lactams susceptible to hydrolysis. These "classical" ESBLs were most commonly produced by *Klebsiella* spp. (and, less so, *Enterobacter* spp.), and were largely found in isolates causing nosocomial infections. Later, in the 1980s, a novel plasmid-mediated ESBL type now known as CTX-M was detected, initially in Japan in 1986 (Matsumoto, Y. *et al.*, 1988) and subsequently in Germany and Argentina [1989, (Bonnet, R., 2004)]. CTX-M ESBLs preferentially hydrolyse cefotaxime rather than ceftazidime, in contrast to most TEM and SHV-type ESBLs.

8.1. Emergence and geographic dissemination of CTX-M enzymes

Although the first CTX-M enzyme was reported in the late 1980s, accounts of this type of extended-spectrum β-lactamase remained very rare throughout the 1990s, except in South America (particularly Argentina) where CTX-M-2 spread widely and rapidly (Power, P. *et al.*, 1999). Otherwise, reports were sporadic in Europe [Germany (Bauernfeind, A. *et al.*, 1990), France (Bernard, H. *et al.*, 1992) and Poland (Gniadkowski, M. *et al.*, 1998)], and also in Asia [Japan, (Ishii, Y. *et al.*, 1995)]. Since around year 2000, however, CTX-M β-lactamases have become a fast-growing group of ESBLs, and have spread rapidly, including in the UK. CTX-M β-lactamases have replaced TEM and SHV derivatives as the predominant ESBLs worldwide, except maybe in the United States (Canton, R. and Coque, T. M., 2006). They have been found among a wide range of species of the Enterobacteriaceae family, including: *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Salmonella* spp, *Citrobacter* spp., *Morganella morganii*, *Shigella* spp., *Proteus* spp, *Serratia* spp. and *Hafnia* spp. There are also a few reports of CTX-M ESBLs production by species such as *P. aeruginosa*, *Acinetobacter* spp. and *Stenotrophomonas maltophilia* (al Naiemi, N. *et al.*, 2006; Celenza, G. *et al.*, 2006).

CTX-M ESBLs are particularly associated with *E. coli* and as a result, the past five years have seen a dramatic change in the epidemiological landscape of ESBL-producing organisms. Nosocomial *Klebsiella* spp. with TEM or SHV ESBLs are now outnumbered in many centres by *E. coli* with CTX-M enzymes, which are fast spreading in hospitals but also, and more worryingly, in the community. CTX-M enzymes are also overtaking TEM and SHV ESBL types in prevalence in *Klebsiella* spp. itself. Moreover, CTX-M ESBLs are not confined to bacterial strains isolated from humans; isolates producing those enzymes have also been detected in pets, farm animals, wild birds, sewage and even in products of the food chain (Canton, R. and Coque, T. M., 2006).

In the UK, the first CTX-M enzyme was reported in 2000. Specifically, a *K. oxytoca* strain, isolated from a stool sample of a six-year-old child in Leeds, was found to produce CTX-M-9, a variant that had previously been described in China and Spain (Alobwede, I. *et al.*, 2003). A retrospective study later revealed that CTX-M enzymes emerged earlier in this country, in the mid 1990s, as these ESBLs were detected among *Salmonella enterica* serotype Virchow isolates (Hopkins, K. L. *et al.*, 2006a). The first hospital outbreak caused by an organism with CTX-M

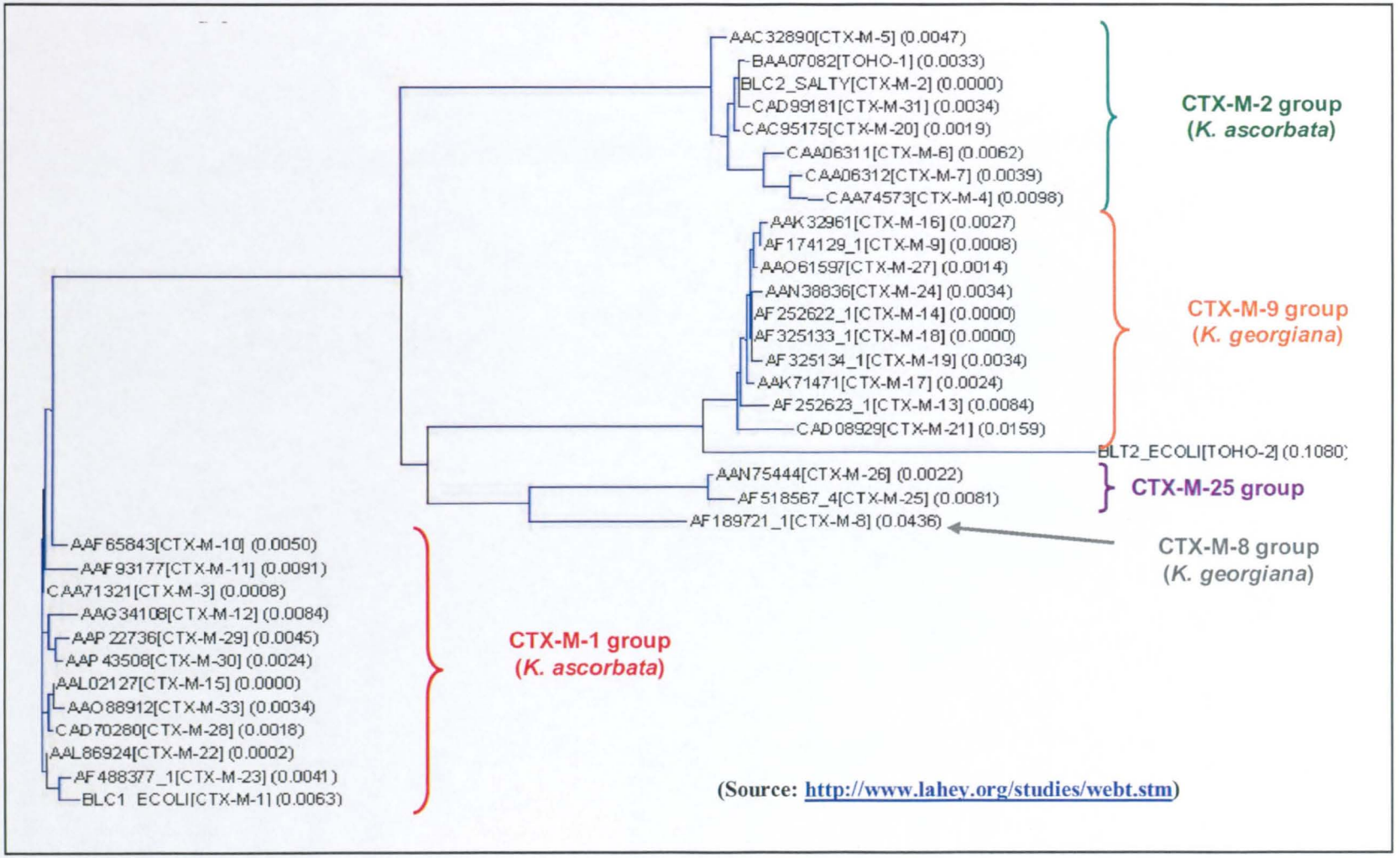
ESBL, which occurred during 2001-2002, was reported in 2003 and involved a *K. pneumoniae* strain with CTX-M-25 at the City Hospital in Birmingham (Brenwald, N. P. *et al.*, 2003). In the same year, four genetically-distinct *E. coli* isolates were shown to produce the CTX-M-15 enzyme, a variant that had been reported previously in Japan, India, France, Poland and Bulgaria (Mushtaq, S. *et al.*, 2003). These isolates were from a survey that examined *c.* 1,000 *E. coli* from in-patients from the UK and Ireland. Since 2003, there has been a sharp and continuing increase in referrals to the Antibiotic Resistance Monitoring Reference Laboratory (ARMRL) of isolates with the CTX-M phenotype, mirroring the global epidemiological scenario.

8.2. Origin and evolution of CTX-M β-lactamases

The CTX-M family comprises well over sixty members (67 on the 11th June 2007, based on amino-acid sequences), with new variants described and added to the literature regularly (http://www.lahey.org/studies/other.asp#table1). On the basis of their amino-acid sequences, these β-lactamases are divided into five distinct phylogenetic groups: group-1, group-2, group-8, group-9 and group-25/26. Figure 5 shows a dendrogram constructed on the basis of the amino-acid sequence alignment for the first 33 CTX-M enzyme variants, illustrating the five distinct CTX-M phylogenetic groups. The vast majority of CTX-M variants belong to phylogenetic groups-1 and -9. Within each phylogenetic group, CTX-M enzymes share at least 95% sequence homology, usually differing by one amino-acid only, while members from different groups share 80 to 90% sequence homology.

Several studies have focused on identifying the sources of CTX-M enzymes (Humeniuk, C. *et al.*, 2002; Olson, A. B. *et al.*, 2005; Poirel, L. *et al.*, 2002b; Rodriguez, M. M. *et al.*, 2004). These have shown that $bla_{CTX\text{-}M}$ genes most likely originated from the chromosomes of various *Kluyvera* spp. (commensal Gram-negative bacilli that are rarely pathogenic). It appeared also that CTX-M enzymes from different phylogenetic groups originated from different species of *Kluyvera*. This association is clear because the chromosomally-encoded β-lactamases of *Kluyvera* spp. share up to 100% amino-acid identity with acquired CTX-M enzymes (*e.g.* for KLUY-1 of *K. georgiana* and CTX-M-14).

Figure 5. Dendrogram showing the relatedness of the first 33 reported CTX-M variants constructed on the basis of amino-acid sequence homology, also indicating the source *Kluyvera* spp.



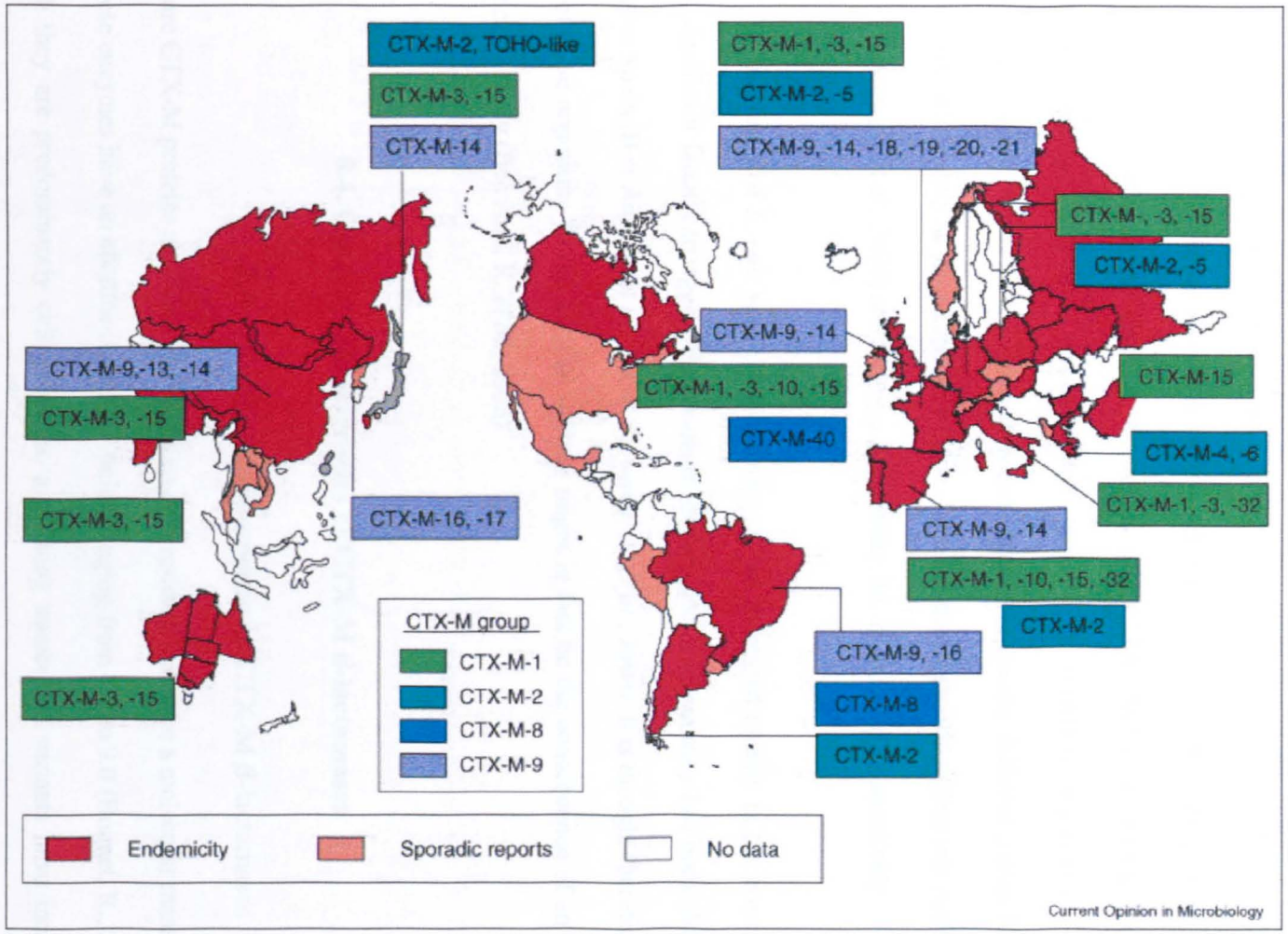
Moreover, the genetic regions flanking acquired bla_{CTX-M} genes exhibit high nucleotide similarity with those surrounding the chromosomal *bla* genes of *Kluyvera* spp. (Canton, R. and Coque, T. M., 2006). In brief, phylogenetic group-1 and group-2 CTX-M enzymes originated from *K. ascorbata* (bla_{KLUA}), while the chromosomally-encoded KLUG β-lactamases of *K. georgiana* appear to be the progenitors of CTX-M enzymes belonging to phylogenetic groups-8 and -9. No progenitor of the CTX-M enzymes from phylogenetic group-25/26 has yet been identified. Furthermore, the chromosomally-encoded bla_{KLUC-1} of *K. cryocrescens* has been shown to share 86% identity with a few bla_{CTX-M} genes from phylogenetic group-1 [$bla_{CTX-M-1}$, $_{-3}$, $_{-10}$, $_{-11}$ and $_{-12}$, (Decousser, J. W. *et al.*, 2001)]; this enzyme may therefore be the progenitor of CTX-M enzymes for a potential sixth phylogenetic group that have not yet emerged (or been recognised) in clinical isolates. It is also worth mentioning that the chromosomal β-lactamases of *Kluyvera* spp. are very weakly expressed (Bonnet, R., 2004), and so do not confer an ESBL phenotype in their original host. The various mechanisms responsible for the escape of bla_{CTX-M} genes from the chromosome of *Kluyvera* spp. and for their expression post mobilisation in their new host are discussed later (section 8.5).

Figure 6 shows the worldwide dissemination of CTX-M ESBLs, with lists of the most prevalent CTX-M variants in various countries. Phylogenetic group-25/26 CTX-M enzymes are the least common variants so far, having been reported only from Canada and from the UK (Munday, C. J. *et al.*, 2004a).

8.3. Global epidemiology of *E. coli* with CTX-M β-lactamases

The sudden emergence of *E. coli* with CTX-M enzymes somehow started in South America with an explosive dissemination of isolates producing CTX-M-2 enzymes in Argentina, which then spread to its neighbouring countries (Canton, R. and Coque, T. M., 2006; Radice, M. *et al.*, 2002). Subsequently, rises in prevalence of isolates with other CTX-M types were observed in South-East Asia and in Europe. Since around 2002, CTX-M enzymes have been reported from all around the world [with maybe the exception of North America, where TEM and SHV mutant ESBLs still remain dominant (Livermore, D. M. *et al.*, 2007)], and were exhibiting an ever growing diversity.

Figure 6. Worldwide dissemination of CTX-M ESBLs[a]



[a] (Canton, R. and Coque, T. M., 2006)

In some countries, such as Spain and Poland, the rise and spread of CTX-M ESBLs is largely explained by horizontal transfer of plasmids encoding $bla_{CTX\text{-}M\text{-}9/\text{-}14}$ and $bla_{CTX\text{-}M\text{-}3}$, respectively (Baraniak, A. et al., 2002b; Velasco, C. *et al.*, 2007). In other countries, such as France and the UK, however, the sudden rise in CTX-M ESBLs is thought to be mostly associated with the dissemination of epidemic strains that produce these enzymes [*e.g.* nationwide dissemination of epidemic clone A with CTX-M-15 β-lactamase in the UK, see section 9, (Lavollay, M. *et al.*, 2006; Woodford, N. *et al.*, 2004)]. As mentioned in section 8.5, another factor contributing to the global spread of CTX-M enzymes is the capacity of $bla_{CTX\text{-}M}$ genes to be mobilised among plasmids, owing to the genetic structures that surround them (*e.g.* insertion sequences). These recombinational events, which may occur within one plasmid or among different plasmids, have been demonstrated recently in Spain and in France, with isolates harbouring plasmids coding for $bla_{CTX\text{-}M\text{-}9}$ (Novais, A. *et al.*, 2006) and $bla_{CTX\text{-}M\text{-}15}$ (Lavollay, M. *et al.*, 2006), respectively.

The incidence of *E. coli* with CTX-M enzymes has increased rapidly in the community and also, significant faecal carriage of these isolates in the general community has been shown in Israel and in Spain (Ben Ami, R. *et al.*, 2006; Valverde, A. *et al.*, 2004). It is thought therefore that the rise of these organisms within hospital settings might in fact be the consequence of an influx from the community (Ben Ami, R. *et al.*, 2006).

8.4. General characteristics of CTX-M β-lactamases

8.4.1. Biochemical properties of CTX-M β-lactamases

The mature CTX-M proteins are made of 291 amino-acid residues and have a molecular mass of 28 kDa. These enzymes have an alkaline isoelectric point, ranging from 7.4 to 9.0 (Bonnet, R., 2004). Although they are predominantly cefotaximases, a growing number of variants have increased activity against ceftazidime owing to mutations (see next section). Overall, CTX-M enzymes show strong hydrolytic activity against all true cephalosporins, including fourth-generation compounds such as cefepime and cefpirome, though not against cephamycins or carbapenems.

They are less active against penicillins compared with TEM and SHV penicillinases. Like other class A ESBLs, CTX-M enzymes are well inhibited by clavulanate and other available β-lactamase inhibitors, which restore the activity of oxyimino-cephalosporins, such as cefotaxime and ceftazidime. Tazobactam is the most active inhibitor, while sulbactam is the least active (Bonnet, R., 2004).

8.4.2. Structure and function of CTX-M β-lactamases

The spectrum of β-lactams hydrolysed by CTX-M enzymes is dictated by the amino-acid conformation of their active site. Residue Ser237 has been shown to be critical for the hydrolysis of cefotaxime (Knox, J. R., 1995); when mutated, both hydrolytic activity against cefotaxime and susceptibility to inhibition by clavulanate are decreased (Gazouli, M. *et al.*, 1998). Various studies have demonstrated that two other amino-acid residues independently play key roles in the evolution of CTX-M enzymes, and especially in expanding their spectrum to include ceftazidime. Pro167 and Asp240 are found in typical CTX-M enzymes with poor catalytic activity against ceftazidime. Increased activity against ceftazidime, as seen in a growing number of variants (sometimes at the cost of losing some activity against cefotaxime) has been associated with substitutions such as Pro167Ser and Asp240Gly.

Overall, at least seven variants from three of the five phylogenetic groups of CTX-M enzymes confer increased resistance to ceftazidime. These include the group-1 variants CTX-M-15, -23 and -42; the group-9 enzymes CTX-M-16, -19, and -27; and finally CTX-M-25 (Bonnet, R., 2004; Munday, C. J. *et al.*, 2004a; Poirel, L. *et al.*, 2001; Poirel, L. *et al.*, 2002a; Stepanova, M. *et al.*, 2005; Sturenburg, E. *et al.*, 2004). CTX-M-15, the commonest CTX-M variant worldwide (Figure 6) is thought to be a ceftazidime-hydrolysing variant of CTX-M-3, differing from its "parent" only by the aspartate to glycine substitution at position 240.

8.4.3. Phenotypic detection of ESBLs

Ceftazidime resistance was for long the primary indicator of ESBL production, as being generally the best substrate for TEM and SHV ESBLs.

However, ceftazidime resistance is not obvious in all organisms with CTX-M ESBLs. As a result, national (BSAC) and international (*e.g.* EUCAST, CLSI) committees have issued revised guidelines on the phenotypic detection of ESBLs. These guidelines stipulate that microbiological laboratories should test for both ceftazidime and cefotaxime resistance, or if a single agent must be used, for resistance to cefpodoxime, as a third-generation cephalosporin readily hydrolysed by all TEM, SHV and CTX-M ESBLs. For isolates found resistant, these third-generation cephalosporins must also be tested in combination with clavulanate to confirm or refute an ESBL mechanism.

8.5. Dissemination and mobilisation of *bla*$_{CTX-M}$ genes

8.5.1. Genetic support and dissemination of *bla*$_{CTX-M}$ genes

Although there have been a very few reports of acquired *bla*$_{CTX-M}$ genes becoming chromosomally-located (Bonnet, R., 2004), these genes are generally found on plasmids in clinical isolates.

These plasmids have been the subject of many studies (Canton, R. and Coque, T. M., 2006). In brief, plasmids harbouring *bla*$_{CTX-M}$ genes were shown to be *(i)* transferable in the vast majority of cases (with transfer frequencies ranging from 10^{-7} to 10^{-2} per donor cell), *(ii)* of both narrow or broad host-ranges, and *(iii)* with sizes varying from 7-kb (Cao, V. *et al.*, 2002) to more than 300-kb (Novais, A. *et al.*, 2006). PCR replicon typing assays (Carattoli, A. *et al.*, 2005) have also shown *bla*$_{CTX-M}$ genes to be associated with plasmids belonging to a wide range of incompatibility groups including IncFI, IncFII, IncI, IncN and IncL/M.

As previously mentioned, CTX-M-15 is the most widespread CTX-M variant worldwide having been detected on every inhabited continents, except Antarctica. CTX-M-15 is also the most prevalent ESBL encountered in many different geographical areas, including in much of Europe (though group-9 CTX-M enzymes have long been the most common in Iberia), India and North Africa. International dissemination of *bla*$_{CTX-M-15}$ may be explained by the fact that this gene has repeatedly been found on epidemic conjugative plasmids, especially of the narrow host-range IncFII incompatibility group. Such plasmids have been detected in clonal and non-clonal *E. coli* clinical isolates from Canada, France and North Africa (Canton, R. and Coque, T. M., 2006).

By contrast $bla_{CTX\text{-}M\text{-}9}$, one of the most prevalent CTX-M-encoding alleles in Spain, has regularly been linked to epidemic broad host-range plasmids [*e.g.* IncP-1α plasmids, (Novais, A. *et al.*, 2006)]. Although very rarely reported so far, these plasmids may be responsible for the spread of CTX-M ESBLs to species other than Enterobacteriaceae (*e.g. P. aeruginosa* or *Acinetobacter* spp.).

Finally, a given $bla_{CTX\text{-}M}$ gene may be found on different genetic supports in a same geographical area, suggesting that $bla_{CTX\text{-}M}$ genes are mobilisable between plasmids. There have also been reports of $bla_{CTX\text{-}M}$ genes being mobilised from plasmids to chromosomes (Yagi, T. *et al.*, 1997). Mobilisation of $bla_{CTX\text{-}M}$ genes most often results from recombinational events involving *e.g.* transposable elements. It may involve different plasmids; *e.g.* $bla_{CTX\text{-}M\text{-}15}$ was found on mosaic plasmids among clonally-related *E. coli* isolates in the Paris area (Lavollay, M. *et al.*, 2006). Mobilisation also may happen within the same plasmid; *e.g.* $bla_{CTX\text{-}M\text{-}9}$ has been found on plasmids with similar restriction profiles, belonging to the same incompatibility group, but located within highly variable integrons in a tertiary-care hospital in Madrid (Novais, A. *et al.*, 2006).

8.5.2. Genetic environment and mobilisation of $bla_{CTX\text{-}M}$ genes

Three different mechanisms have been described for the original mobilisation of the $bla_{CTX\text{-}M}$ genes from *Kluyvera* spp., involving distinct genetic elements. These elements include *(i)* IS*Ecp1*-like insertion sequences, *(ii)* the putative IS*CR1* transposase (Common Region 1), coded by *orf513*, and *(iii)* phage-associated sequences (Canton, R. and Coque, T. M., 2006; Lartigue, M. F. *et al.*, 2004). The last route is the least common, having been reported only once, reflected by the genetic surroundings of the $bla_{CTX\text{-}M\text{-}10}$ gene harboured by distinct plasmids in several species of Enterobacteriaceae in a Spanish hospital (Oliver, A. *et al.*, 2005).

Some $bla_{CTX\text{-}M}$ genes have been found linked to different genetic environments: *e.g.* $bla_{CTX\text{-}M\text{-}10}$ has been found in association with both IS*Ecp1* and with the phage-related sequences just mentioned (Canton, R. and Coque, T. M., 2006). Moreover, $bla_{CTX\text{-}M}$ genes of the same phylogenetic group may be associated with different mobilisation mechanisms: while $bla_{CTX\text{-}M\text{-}9}$ is associated with IS*CR1*, $bla_{CTX\text{-}M\text{-}14}$ has mainly been found downstream of IS*Ecp1*.

IS*Ecp1*-like elements have repeatedly been shown to be responsible for the mobilisation of several bla_{CTX-M} alleles (*e.g.* ORFs encoding CTX-M-1, -2, -3, -14, -15, -19 enzymes). These insertion sequences, which are usually located between 42 and 266-bp upstream of the bla_{CTX-M} gene, can mobilize structurally-unrelated genes by a one-ended transposition mechanism (Canton, R. and Coque, T. M., 2006). On the other hand, the IS*CR1* element participates in gene mobilisation by a rolling-circle replication mechanism. This element is embedded within highly variable class 1 integrons (Garcia, A. *et al.*, 2005), and has been found in association with several antibiotic resistance genes including bla_{CTX-M}, bla_{CMY}, bla_{DHA-1}, and *qnrA*. The IS*Ecp1*-like family of mobilisable elements provides also a promoter responsible for high-level expression of enzymes such as CTX-M-14, -17, 18 and -19 (Poirel, L. *et al.*, 2005); this also applies for the ISCR1 element, which is able to promote the expression of distinct antibiotic resistance genes including $bla_{CTX-M-9}$, the plasmid-mediated quinolone resistance gene *qnrA* and the trimethoprim resistance gene dfr_{A10} (Rodriguez-Martinez, J. M. *et al.*, 2006).

Finally, dissemination of bla_{CTX-M} genes in both nosocomial and community settings may also have been influenced by association with transposable elements. Both $bla_{CTX-M-2}$ and $bla_{CTX-M-9}$ alleles have repeatedly been found within complex class 1 integrons associated with transposons of the Tn*21* family. The Tn*21* family is disseminated worldwide in both environmental and clinical Gram-negative bacteria, often on large conjugative plasmids (Canton, R. and Coque, T. M., 2006). The bla_{CTX-M} genes linked to IS*Ecp1*-like elements also may be part of transposons. For example, the association IS*Ecp1*/$bla_{CTX-M-19}$ has been found within a Tn*1721* transposon (Poirel, L. *et al.*, 2003)].

8.6. Risk factors for infection with CTX-M-producing isolates

Risk factors for nosocomial infection with CTX-M-positive isolates may be regarded as similar as those for infection with bacteria producing other ESBL types. However, since CTX-M enzymes are more prevalent in the community (by contrast with TEM and SHV derivatives), it becomes useful to differentiate the risks of acquiring such an infection within the hospital settings from the risks in the community.

The main risks factors for infection with isolates producing ESBLs (including CTX-M types) in the hospital include prolonged hospitalisation, use of invasive medical devices (*e.g.* urinary catheters), and previous antibiotic treatment (especially with third-generation cephalosporins). Even though outbreaks of infections due to isolates with CTX-M enzymes have been reported in many hospital unit types, patients admitted to intensive care units have a greater risk of acquiring those organisms (Livermore, D. M. and Paterson, D. L., 2006).

For community-acquired infections due to isolates producing CTX-M enzymes, gut flora carriage also represents a risk factor, as does recent antibiotherapy (with cephalosporins, or even quinolones). Many "community-acquired" infections are contracted by people that had recent hospital admission and it becomes hard to distinguish whether the bacteria was truly acquired in the community or during the earlier hospital stay. People over the age of 65, especially females, are at a greater risk of acquiring an infection caused by organisms with CTX-M enzymes (Livermore, D. M. and Paterson, D. L., 2006). Finally, there have been a number of reports of faecal carriage of CTX-M-positive isolates in both hospital and community patients, as well as in healthy volunteers (Munday, C. J. *et al.*, 2004b; Valverde, A. *et al.*, 2004). Colonization of the gut flora often precedes infection, not only for the colonized person, but also for the other patients sharing the same ward.

8.7. Therapeutic options for infections due to isolates with CTX-M enzymes

The emergence of isolates producing CTX-M enzymes has lead clinicians to review the protocols for treatment of infections. Isolates with CTX-M enzymes can be responsible for severe life-threatening infections as well as uncomplicated UTIs. Adequate treatment strategies have therefore become a necessity.

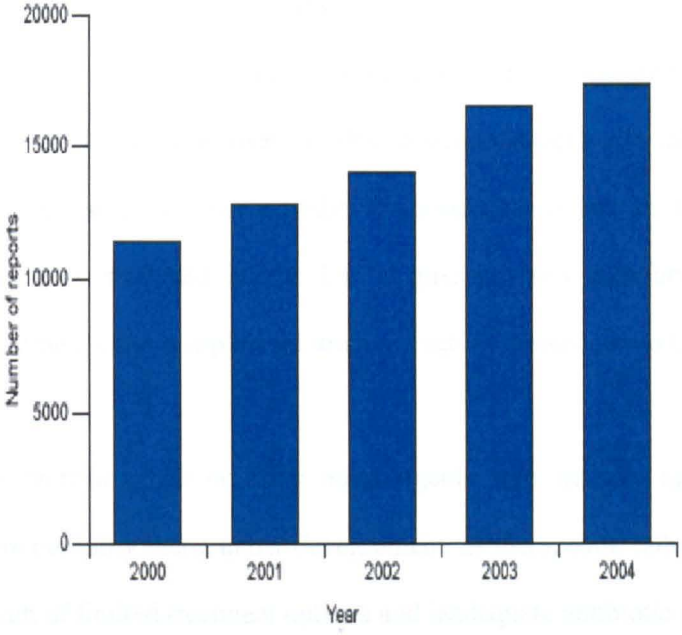
Isolates with CTX-M enzymes should be considered resistant to all penicillins (except temocillin), all cephalosporins (including fourth-generation compounds but not cephamycins) and to aztreonam. Carbapenems and β-lactam/β-lactamase inhibitor combinations are not affected by CTX-M enzymes alone. However, OXA-1 enzyme is often co-produced with CTX-M-15 and is poorly inactivated by β-lactamase inhibitors and confer resistance *e.g.* to piperacillin/tazobactam.

Additionally, a few isolates with CTX-M β-lactamases, principally *Klebsiella* spp., exhibit permeability changes owing to loss of outer membrane proteins; these changes reduce the activity of carbapenems, particularly ertapenem (Mena, A. *et al.*, 2006; Woodford, N. *et al.*, 2007a). More generally, isolates with CTX-M enzymes often are multi-drug resistant, including to aminoglycosides, tetracyclines, sulphonamides, and quinolones (note the parallel increases in prevalence of cephalosporin and quinolone resistant *E. coli* in England and Wales, see Figure 7). Co-resistance means that aminoglycosides and quinolones cannot be recommended as first-line treatments for severe infections caused by isolates with ESBLs, unless susceptibility has been microbiologically demonstrated. The effective treatment options for infections caused by CTX-M-positive isolates, particularly UTIs and bacteraemias, are therefore narrow.

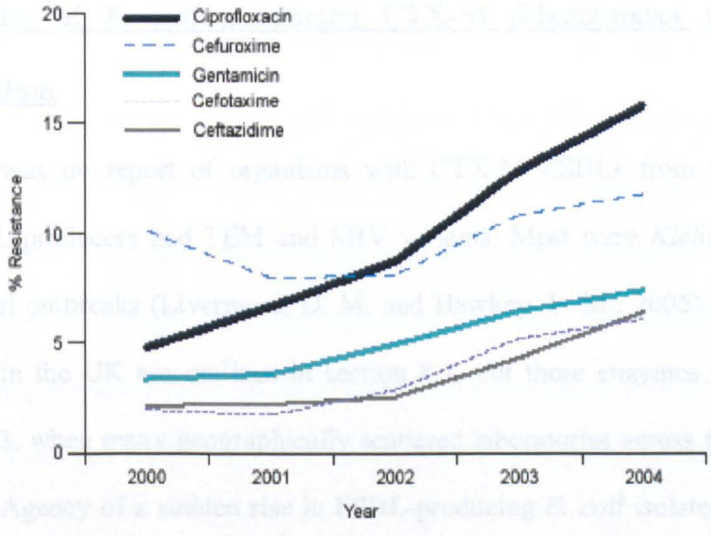
Many studies have demonstrated that inappropriate empirical therapy (*i.e.* that which proves inactive *vs.* the pathogens subsequently identified) is associated with increased mortality among patients with infections (other than UTIs) caused by isolates producing ESBLs such as CTX-M (Paterson, D. L. *et al.*, 2004; Rodriguez-Bano, J. *et al.*, 2006). Currently, the best therapeutic options for hospitalised patients infected (or likely to be infected) with isolates producing CTX-M or other ESBLs are carbapenems, as this class of antibiotics has consistently been associated with better outcomes by comparison with other agents (Rodriguez-Bano, J. and Paterson, D. L., 2006; Rodriguez-Bano, J. *et al.*, 2006). This was illustrated in an international study carried in 2004, involving twelve hospitals from seven countries, showing the use of a carbapenem for the treatment of bacteraemias caused by *Klebsiella* spp. producing ESBLs was associated with a significantly lower mortality than was the use of other agents apparently active *in-vitro* (Paterson, D. L. *et al.*, 2004). However, it is worth stressing that increased prescribing of carbapenems will generate further selective pressure for carbapenemases and other mechanisms of carbapenem resistance (Denton, M, 2007).

Figure 7. (A) Increase of reported cases of *E. coli* bacteraemias, and (B) prevalence of resistance to cephalosporins and ciprofloxacin in *E. coli* from bacteraemias, in England and Wales between 2000 and 2004[a]

(A)



(B)



[a] Source: Health Protection Agency (2006).

Temocillin, a semi-synthetic drug highly stable against most β-lactamases including ESBLs, tigecycline (a glycylcycline compound that overcomes resistance mechanisms to tetracyclines) and polymyxins may also be considered as alternatives, but clinical data versus Enterobacteriaceae producing CTX-M ESBLs are limited for these drugs (Denton, M, 2007). Furthermore, the polymyxin colistin is associated with nephrotoxicity and also, is inactive against some species such as *Proteus* spp. and *Serratia* spp.

Treatment options are even more problematic for non-hospitalised patients since carbapenems are not available for oral therapy. Oral β-lactam/β-lactamase inhibitor combinations and quinolones may be considered if susceptibility to these drugs is proven, but many isolates are resistant. Fosfomycin (not marketed in the UK at present) and nitrofurantoin may also be considered for the treatment of uncomplicated urinary tract infections due to CTX-M-producing *E. coli* isolates.

Finally, it is worrying that no truly novel agents with activity against multi-resistant Enterobacteriaceae are currently being under development, as therapeutic failure and mortality will only increase as a result of limited treatment options and inadequate antibiotic prescribing.

9. The rise of *E. coli* producing CTX-M β-lactamases in the United Kingdom

Prior to 2000, there was no report of organisms with CTX-M ESBLs from the UK, and as elsewhere, most ESBL producers had TEM and SHV variants. Most were *Klebsiella* spp., often involved in nosocomial outbreaks (Livermore, D. M. and Hawkey, P. M., 2005). Early reports of CTX-M type ESBLs in the UK are outlines in section 8.1, but these enzymes reached a major milestone by mid-2003, when many geographically scattered laboratories across the UK informed the Health Protection Agency of a sudden rise in ESBL-producing *E. coli* isolates responsible for urinary tract infections, most often in community patients (Livermore, D. M. and Hawkey, P. M., 2005). Since then, *E. coli* isolates producing CTX-M enzymes have been high on the agenda of the Antibiotic Resistance Monitoring and Reference Laboratory (ARMRL) and the Health Protection Agency, reported or referred by an ever-growing number of clinical microbiology laboratories from across the UK. Most of these isolates are from urinary infections, with minorities from

bacteraemias or other sites. Even though the referred isolates represented only a fraction of all the producers, it was clear that the incidence of CTX-M enzymes was increasing year-on-year (Livermore, D. M. and Hawkey, P. M., 2005). A survey undertaken by the HPA in 2004, covering South-East England (Potz, N. A. *et al.*, 2006), revealed the majority of cephalosporins-resistant Enterobacteriaceae to be *E. coli* with CTX-M enzymes. This was the first report showing these organisms overtaking *Klebsiella* spp. with TEM and SHV ESBLs and *Enterobacter* spp. with derepressed AmpC, as the leading cephalosporin-resistant Gram-negative group in part of the UK. CTX-M enzymes were not only dominant among *E. coli* isolates, but also among *Klebsiella* spp., and were scattered in *Enterobacter* spp. They were also found in few isolates of *Citrobacter* spp., *Morganella* spp and *Proteus* spp. At the molecular level, the majority (*c.* 90%) of *E. coli* isolates referred during the 2003-2004 period had group-1 CTX-M β-lactamases, mostly CTX-M-15 (Woodford, N. *et al.*, 2004). CTX-M-3, -9 and -14 variants were also detected, but more rarely. An early investigation of CTX-M-positive *E. coli* in 2004 showed that isolates with CTX-M-15 enzyme included five major serotype O25 epidemic clones (designated A-E), as defined by pulsed-field gel electrophoresis (PFGE), as well as many unrelated producers (Woodford, N. *et al.*, 2004). Epidemic clone A was the most prevalent, being nationally distributed and recovered from over forty different centres across the UK (see Figure 8), and being dominant in some, such as in Preston, Southampton or Telford. Epidemic clones B-E were much more localised, and most areas across the UK had diverse producers of CTX-M-15 enzyme. Interestingly, it was also shown that most representative of clone A required lower cephalosporin MICs compared with other producers of CTX-M-15 β-lactamase. Preliminary examination indicated an IS*26* insertion element, located between $bla_{\text{CTX-M-15}}$ and its normal promoter, which is provided by IS*Ecp1*, was probably responsible for lowering resistance level to these compounds (Woodford, N. *et al.*, 2004). This molecular feature has since then become an important epidemiological tool, facilitating recognition of the clone.

Finally, *E. coli* with CTX-M enzymes have spread rapidly across the UK, mirroring what happened in South America a decade earlier (though different CTX-M enzyme variants were involved) and it is now a concern that CTX-M ESBLs might one day become as widespread in *E. coli* as TEM-1 (Livermore, D. M. and Hawkey, P. M., 2005).

Figure 8. Geographic dissemination of *E. coli* epidemic clone A in the United Kingdom, based on referrals to ARMRL[a]

[a] Centres where epidemic clone A with CTX-M-15 β-lactamase is dominant are circled in blue colour (Source: HPA, 2006).

10. Aims of the project

The sudden rise of *E. coli* producing ESBLs in 2003/2004 was alarming, with sharp rises in the number of *E. coli* isolates exhibiting a CTX-M phenotype. To a lesser extent, the same pattern was seen for *Klebsiella* and *Enterobacter* spp. There was a rise also in clinical microbiology laboratories across the UK referring such isolates.

The main objective of this project therefore consisted in exploring the emergence and the dissemination of *E. coli* producing CTX-M enzymes in the UK. It especially aimed at identifying the factor(s) contributing to the epidemiological success of the five major national epidemic clones. In order to pursue this objective, the antibiotic resistance mechanisms of clonal and non-clonal CTX-M-positive *E. coli* isolates, and their genetic context, were rigorously compared using various molecular methods. Additionally, the virulence potentials of these organisms were assessed and compared, also being compared to that of *E. coli* isolates with other ESBL types.

Much emphasis was directed towards the five major national epidemic clones and especially towards epidemic clone A, as being the most clinically successful, yet also the least resistant to cephalosporins. In this context, it became important to draw a complete and detailed map of the ESBL-encoding plasmid from epidemic clone A, so as to evaluate whether it played any role in the epidemiological success of its host.

Finally, as the CTX-M family was growing and diversifying fast, this study also included an *in-vitro* evolution investigation of CTX-M-3 β-lactamase, a variant that has long been regarded as the parent of CTX-M-15. This part of the study was undertaken in order to assess the ease with which CTX-M-3 could evolve the broader spectrum of activity associated with CTX-M-15.

MATERIALS & METHODS

1. Bacterial isolates and growth conditions

1.1. Clinical isolates and reference strains

A total of 176 clinical isolates of *E. coli* were used in this study, most of which produced CTX-M extended-spectrum β-lactamases (n = 156), as shown by PCR in a previous study (Woodford, N. *et al.*, 2004). These isolates were selected from a large collection of isolates referred by various hospitals and laboratories from across the UK to the Antibiotic Resistance Monitoring & Reference Laboratory in Colindale. The isolates with CTX-M enzymes investigated here were selected as illustrating the epidemiological situation in the UK at the time of the study (July 2004 - December 2006). They included hospital isolates (*ca.* 50% confirmed by senders) as well as isolates from the community (*ca.* 25% confirmed); clonal (32/156) and non-clonal (124/156) *E. coli* isolates, as determined previously by PFGE (Woodford, N. *et al.*, 2004); and also isolates producing various types of CTX-M β-lactamases, most of them group-1 CTX-M enzymes (109/156) according to PCR (Woodford, N. *et al.*, 2006). At least half the isolates with CTX-M enzymes were known to have been isolated from urine samples, and approximately 10% from blood cultures. Table 4 details the variety of clinical isolates included in this study.

The different reference strains used in this study are listed in **Appendix 1**. Most of these isolates were employed as control strains in a variety of experiments, including antibiotic susceptibility testing, detection of antibiotic-resistance genes by PCR, detection of virulence factor genes by PCR, plasmid extraction, estimation of plasmid size, and characterisation of β-lactamases by IEF. *E. coli* K-12 DH5α and J-53-2 strains were used as recipient strains for resistance transfer studies.

1.2. Media and growth conditions

E. coli isolates were grown aerobically overnight (16 - 24 hours) at 37°C. The different types of media used in this study and their suppliers are summarised in **Appendix 2**. Unless specified, bacteria were generally grown on agar.

Table 4. Overview of clinical isolates studied

Clonal isolates[a] with CTX-M-15-like enzymes	Representative isolates[b] of strain A	13
	Representative isolates[b] of strain B	3
	Representative isolates[b] of strain C	9
	Representative isolates[b] of strain D	3
	Representative isolates[b] of strain E	4
Non-clonal isolates[a] with CTX-M enzymes	Isolates with group-1 CTX-M enzymes	77
	Isolates with group-9 CTX-M enzymes	42
	Isolates with unusual CTX-M enzymes	5
Isolates with non-CTX-M type ESBLs[c]		20
Total number of isolates		176

[a] As defined by PFGE.
[b] These isolates were selected to represent each of the five major UK epidemic *E. coli* strains (A-E) with CTX-M-15-like β-lactamase. For each given strain, representatives from different centres were selected for study.
[c] According to antibiotic resistance phenotypes and to PCR for the detection of bla_{CTX-M} genes.

If grown in liquid media, cultures were incubated overnight at 37°C on an orbital shaker (New Brunswick Scientific, Edison, N.J., USA) at a rotation speed of 100 rpm. If antibiotic selection was required, *e.g.* to ensure that a strain conserved a specific resistance phenotype, discs impregnated with the appropriate antibiotic were firmly applied to the surface of the agar plate using sterile forceps. For long term storage of bacterial strains, loopfuls of freshly grown *E. coli* cultures (*e.g.* from a NA plate) were re-suspended in 2 mL vials containing 1 mL 10% glycerol, and were then transferred to the freezer at -70°C.

2. Apparatus and chemicals

Equipment and chemicals used in this study are detailed in the relevant sections. The list of suppliers can be found in **Appendix 3** and **Appendix 4**.

3. Identification of *E. coli* isolates

E. coli isolates were identified biochemically using API20E kits (BioMérieux, La Balme-les-Grottes, France) according to the manufacturer's instructions.

4. Antibiotic susceptibility testing

Minimal Inhibitory Concentrations (MICs) of antibiotics were usually determined by agar dilution following the British Society for Antimicrobial Chemotherapy (BSAC) guidelines, which are reviewed on a yearly basis (Andrews, J. M., 2006).

4.1. Antibiotics

Antibiotics used in this study and their suppliers are listed in **Appendix 5**. When stored at -70°C, antibiotic stock solutions were thawed for at least 30 min at room temperature prior to use. Clavulanate and tazobactam were used at a final concentration of 4 mg/L in β-lactamase inhibition studies, while EDTA was used at 320 mg/L.

4.2. Incorporation of antibiotics into agar

Bottles containing 40 ml agar were melted in a steamer and kept at 56°C prior to the addition of antibiotic(s). With the exception of ertapenem, imipenem and meropenem, which were incorporated into Mueller-Hinton (MH) agar (Oxoid, Basingstoke, UK) due to its higher zinc content (Walsh, T. R. *et al.*, 2002), most antibiotics were prepared in IsoSensitest agar (ISO, Oxoid Ltd, Basingstoke, UK) using a two-fold serial dilution. Table 5 summarises the ranges of antibiotic concentrations tested versus Gram-negative bacteria as a standardised method.

4.3. Cell suspensions and inoculation

Single colonies of clinical isolates were sub-cultured on to fresh nutrient agar and incubated overnight at 37°C. Cell suspensions with an opacity of 0.5 on McFarland's scale were prepared from freshly grown cultures in IsoSensitest broths using an API densitometer (BioMérieux). This cell density corresponds to approximately 10^4 to 10^5 colony-forming units (cfu) per spot when 0.3 µL amounts are dispensed with a multipoint inoculator (Mast Diagnostics, Merseyside, UK). Agar plates were incubated for 16 h at 37°C. Nine reference strains (see **Appendix 7**, which also summarises their expected MICs), used as controls, were tested in parallel using the same methodology.

4.4. Reading of Minimum Inhibitory Concentrations (MICs)

MICs were read as the lowest concentrations of antibiotic to inhibit visible bacterial growth using the Sorcerer - version 2.1 (Perceptive Instruments Ltd, Haverhill, UK). MICs were interpreted according to BSAC guidelines, except for minocycline and sulbactam where CLSI guidelines were adopted in the absence of published criteria from the BSAC.

Table 5. Concentration range and MIC breakpoints (mg/L) of antibiotics routinely tested by ARMRL[a] *versus E. coli*

Antibiotic	Concentration range	Breakpoints		
		R >	I	S ≤
Amikacin	0.5 - 64	16	16	8
Ampicillin	0.5 - 64	16	16	8
Augmentin	0.125 - 64	16	16	8
Aztreonam	0.125 - 64	1	-	1
Cefotaxime	0.125 - 256	1	-	1
Cefotaxime + Clavulanate	0.06 - 32	1	-	1
Cefoxitin	1 - 64	8	-	8
Cefpirome	0.125 - 64	1	-	1
Cefpirome + Clavulanate	0.06 - 32	1	-	1
Ceftazidime	0.125 - 256	2	-	2
Ceftazidime + Clavulanate	0.06 - 32	2	-	2
Ciprofloxacin	0.125 - 32	1	1	0.5
Colistin	0.5 - 32	4	-	4
Ertapenem	0.125 - 16	2	-	2
Gentamicin	0.125 - 32	4	4	2
Imipenem	0.06 - 32	4	-	4
Imipenem + EDTA	0.03 - 16	4	-	4
Meropenem	0.06 - 32	4	-	4
Minocycline	0.125 - 32	8	8	4
Piperacillin	1- 64	16	-	16
Piperacillin + Tazobactam	1- 64	16	-	16
Sulbactam	1 - 32	8	8	4

[a] Breakpoints changed while thesis in preparation. Version 5 criteria were used throughout data analysis and retained here (Andrews, J. M., 2006).

Antibiotic	Concentration range	Breakpoints		
		R >	I	S ≤
Tigecycline	0.25 - 16	2	-	1
Tobramycin	0.125 - 32	4	4	2

4.5. MIC determinations by E-test®

MICs were determined using E-test strips (BioStat, Stockport) when those of only a few antibiotics ($\leq$6) for a small number of isolates were required. E-test were also used to determine the MICs of antibiotics absent from the standard battery of compounds tested by agar dilution; *e.g.* fosfomycin, nalidixic acid, nitrofurantoin, ofloxacin and rifampicin.

Cell suspensions were prepared to a density of 0.5 McFarland from freshly grown cultures as described above (section 4.3). This inoculum resulted in semi-confluent growth after overnight incubation at 37°C. The complete surface of an ISO plate was inoculated using a sterile swab and left to dry prior to application of the E-test antibiotic strips using sterile forceps, making sure no air bubbles were trapped under the strips. Plates were incubated 16 to 24 h at 37°C. MICs were determined following the manufacturer's instructions and interpreted according to BSAC guidelines.

5. Detection of antibiotic resistance genes by Polymerase Chain Reaction

Table 6 summarises the various antibiotic resistance genes investigated and sought by PCR in this study.

5.1. PCR reaction

Template DNA was obtained by lysing two to three colonies from a NA plate in 100 µL tissue culture water (Sigma-Aldrich, Poole). Following a short vortex (Vortex Whirlmixer™, Fisons Scientific Equipment, Herts) and centrifugation (IEC Micromax RF micro-centrifuge, Thermoquest Scientific Equipment, Basingstoke) at 10,000 x g for 15 sec, 2 µL of the supernatant, containing the released DNA, were added to 23 µL of PCR reaction mix (*Taq* DNA polymerase kit [Invitrogen BV, Renfrew, UK]). The PCR reaction mix contained: 1 x *Taq* buffer, 0.05% W-1 solution, 1.5 mM $MgCl_2$, 1U *Taq* DNA polymerase, 0.2 mM of each dNTP [dATP, dCTP, dGTP and dTTP (Invitrogen BV)], 0.5 µM of each primer [(Sigma Genosys, Haverhill), see **Appendix 6**] and tissue culture water, up to 23 µL.

Table 6. List of antibiotic resistance genes sought by PCR, and antibiotic-resistance mechanisms implicated

Genes	Antibiotic class affected	Resistance mechanisms
bla_{TEM} (all ORFs)	β-lactams	Antibiotic inactivation
bla_{SHV} (all ORFs)		Antibiotic inactivation
$bla_{OXA\text{-}1\text{-}like}$		Antibiotic inactivation
$bla_{CTX\text{-}M}$ (Group-1, -2, -8, -9 and -25/26)		Antibiotic inactivation
bla_{CIT}, bla_{MOX}, bla_{DHA}, bla_{ACC}, bla_{EBC}, bla_{FOX}		Antibiotic inactivation
aac3-IIa	Aminoglycosides	Acetylation of antibiotic
aac(6')-Ib-cr		Acetylation of antibiotic
armA		Methylation of antibiotic
QRDRs of *gyrA*, *gyrB*, *parC*	Quinolones	Target alteration
qnrA, *qnrB* and *qnrS*		Protection of antibiotic target
aac(6')-Ib-cr		Acetylation of antibiotic
tet(A) and *tet*(B)	Tetracyclines	Efflux pump

Amplification was performed in 0.5 mL polypropylene microfuge tubes (Anachem, Luton) or in 0.2 mL low profile 96-well PCR plates (ABgene, Epsom), using a Techne Genius (Techne, Cambridge) or Hybaid PCR Express/Px2 thermocycler (Thermo Life Sciences, Basingstoke). For each PCR, one or more isolates known to carry the gene(s) of interest was/were tested in parallel as positive controls, while tissue culture water was used as a negative control. PCR reactions were stopped with the addition of 10 µL loading buffer (25% [w/v] Ficoll, 50 mM Na_2EDTA, 0.25% [w/v] bromophenol blue).

5.2. PCR conditions and primers

PCR conditions for DNA amplification were dictated by the melting temperature (Tm) of the primers used, as well as the length of the DNA fragment to be amplified. As a rule, the annealing temperature was approximately 2°C below the lowest Tm of the primers used in order to avoid non-specific amplification. The duration of the DNA extension step was directly proportional to the length of the DNA fragment to be amplified. Typical PCR conditions were:

- Initial denaturation at 94°C for 5 min
- 30 to 35 cycles of 94°C for 30 sec; (lowest Tm - 2°C) for 30 sec; 72°C for 60 sec (if DNA fragment ≤1.5 kb)
- Final extension at 72°C for 7 min

Appendix 6 summarises the annealing temperature of the different primers used in this study. In some cases (*e.g.* primers with similar Tm and PCR products of different sizes), two or more genes were amplified at once, in the same reaction.

5.3. Agarose gel electrophoresis of PCR products

PCR amplicons were analysed in agarose gels (size: 165 x 120 x 7 mm) of various concentrations, ranging from 0.7 to 2.5% (w/v), depending on the PCR product size; the shorter the product, the more concentrated the gel. Agarose (Helena BioSciences, Sunderland) gels were prepared with 0.5 x TBE (40 mM Tris-borate, pH 8.0, 1 mM Na_2EDTA [Invitrogen BV]). PCR product/loading buffer mixtures were loaded into the agarose gel wells, along with a 123-bp DNA ladder

(Invitrogen BV), which was used as a DNA size standard. Gels were electrophoresed in 0.5 x TBE buffer at 120 V for approximately 80 min and then stained with a 1 mg/mL ethidium bromide solution. DNA products were visualised under UV light and photographed using the IN Genius Syngene Bio Imaging system (Syngene, Cambridge).

6. Phylogenetic typing of *E. coli*

Template DNA was obtained by lysing bacterial cells in tissue culture water. Phylogenetic typing of clinical isolates was performed using a triplex PCR as described previously (Clermont, O. *et al.*, 2000). PCR products were electrophoresed in 2% agarose gels and visualised under UV light. Results were interpreted as shown in Figure 9.

7. Detection of virulence factor genes

The presence of 32 known or suspected virulence factor genes (Table 7) of extra-intestinal pathogenic *E. coli*, as well as a pathogenicity-associated island marker (PAI), were sought using six multiplex PCR assays (I-VI), according to a method described previously (Johnson, J. R. and Stell, A. L., 2000). All six assays required the same PCR conditions: initial denaturation at 95°C for 12 min, followed by 30 cycles of 94°C for 30 sec, 63°C for 30 sec and 72°C for 3 min, ending with a final extension step at 72°C for 10 min. PCR reactions were undertaken in 0.2-mL low-profile 96-well PCR plates, as described in section 5.1. PCR products were electrophoresed in 2% agarose gels and visualised under UV light. Tissue culture water was used as the negative-control. *E. coli* positive-control isolates (Table 7, Figure 10 and **Appendix 1**), namely 2H16, 2H25, J96, L31, PM9 and V27 (Johnson, J. R. and Stell, A. L., 2000), were gifts from Dr James R Johnson, MD (Minneapolis, MN, USA). The prevalence of virulence factor genes among the different populations of isolates tested was compared using Fisher's exact test (GraphPad: http://www.graphpad.com/quickcalcs/index.cfm).

Figure 9. Interpreting PCR-based phylogenetic typing results for *E. coli*

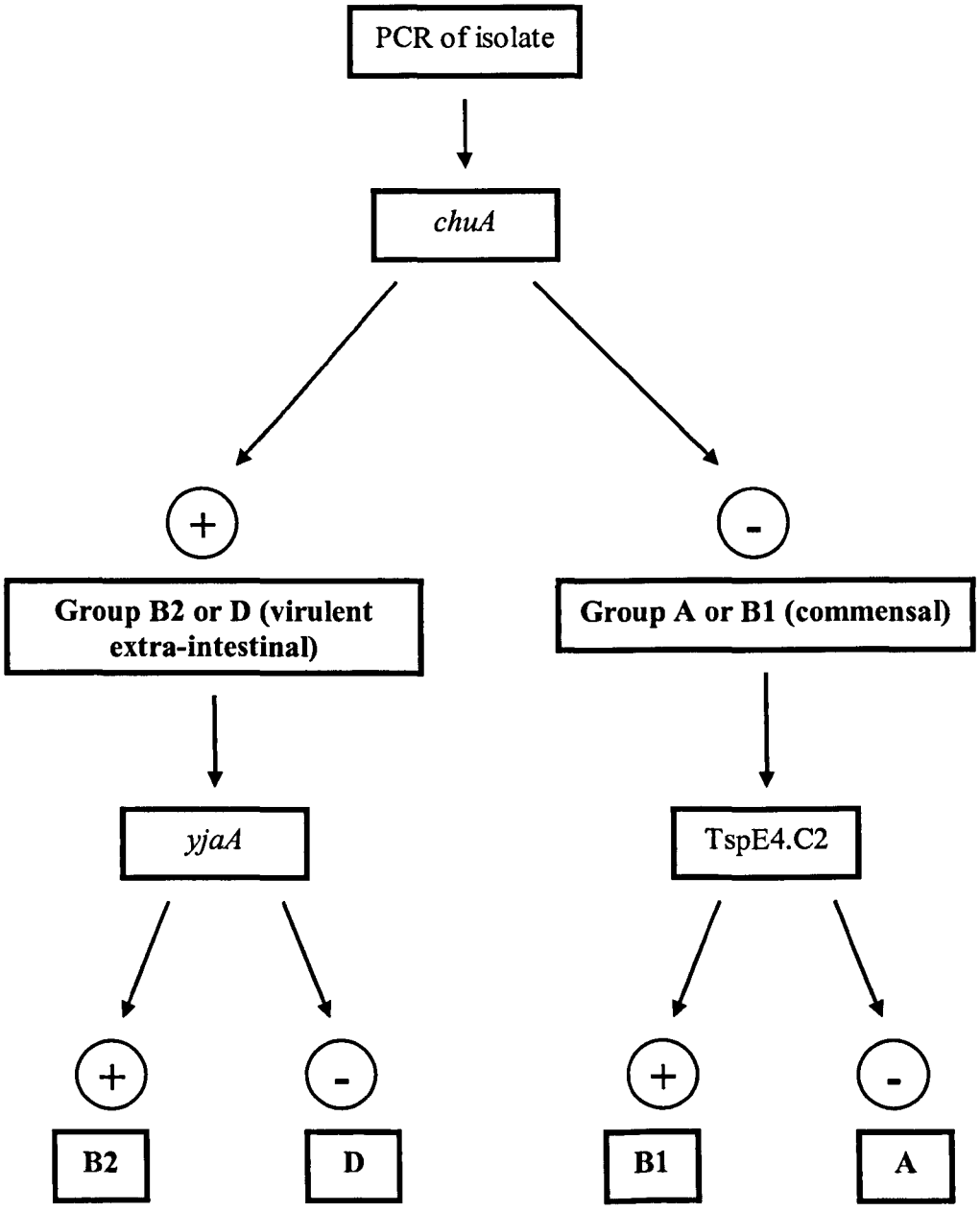
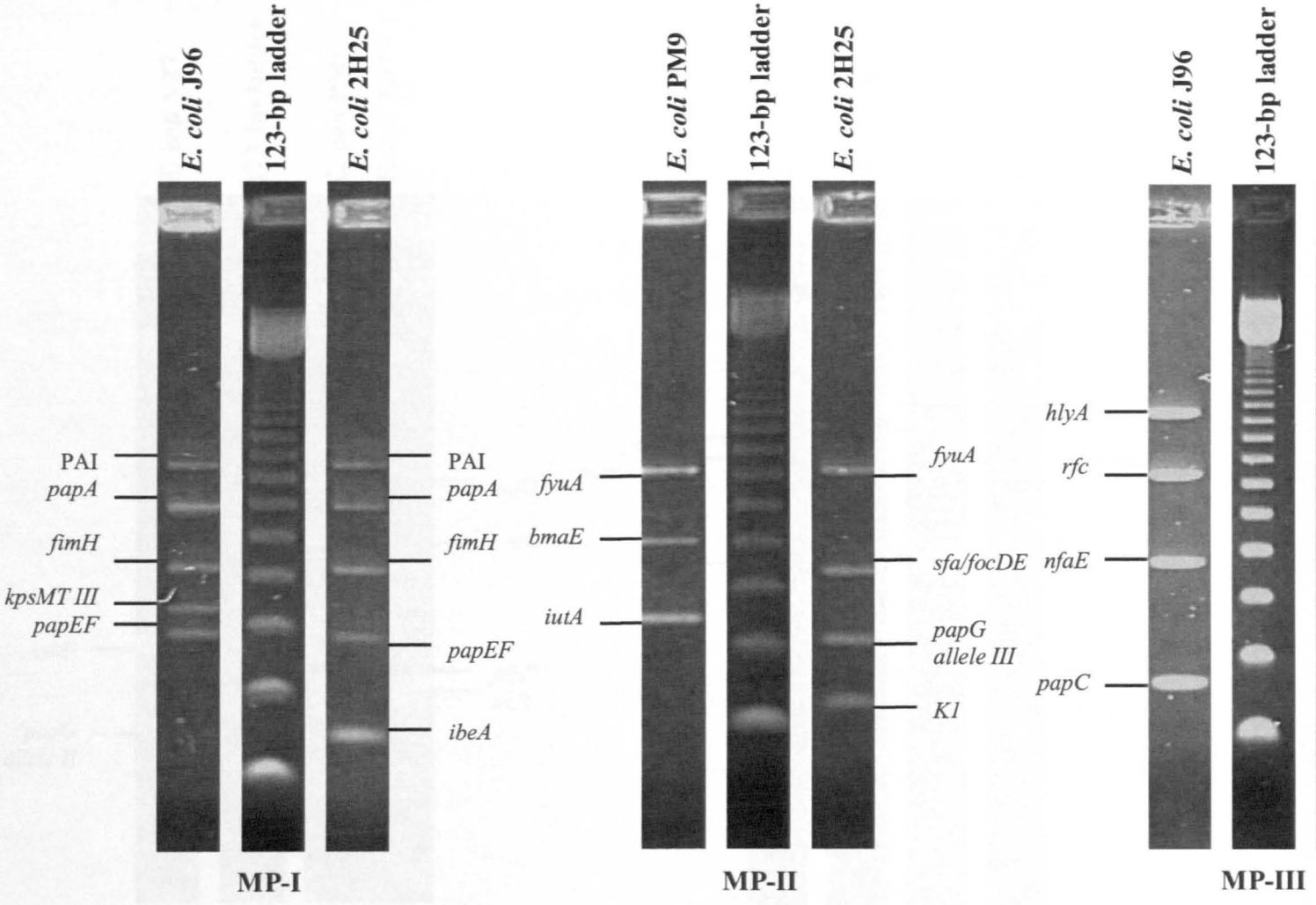


Table 7. List of virulence factor genes investigated in this study, and their respective *E. coli* positive-control strains

Multiplex	Gene	Function & comments	Product size (bp)	Positive-control strain
I	*ibeA*	Invasion of brain endothelium	170	2H25
	papEF	Minor tip pilins; connect PapG to PapA	336	J96
	kpsMT III	Group III capsular polysaccharide synthesis	392	J96
	fimH	D-mannose-specific adhesion, type 1 fimbriae	508	J96
	papA	Major structural subunit of P fimbrial shaft; defines F antigen	720	J96
	malX	Pathogenicity-associated island (PAI) marker	930	J96
II	*kpsMT* K1	K1-specific group II *kpsM*	153	2H25
	papG allele III	Cystitis-associated (pap-2)	258	2H25
	iutA	Ferric aerobactin receptor (iron uptake and transport)	300	PM9
	sfa/focDE	Central region of *sfa* (S fimbriae) and *foc* (F1C fimbriae) operons	410	2H25
	bmaE	Blood group M-specific adhesin	507	PM9
	fyuA	*Yersinia* siderophore receptor (ferric yersiniabactin uptake)	880	PM9
III	*papC*	Pilus assembly; central region of *pap* operon	200	J96
	kpsMT II	Group II capsular polysaccharide synthesis	272	B1-1-1
	papG allele I	J96-associated *papG* variant (rare)	474	B1-1-1
	nfaE	Non-fimbrial adhesin	559	J96

Multiplex	Gene	Function & comments	Product size (bp)	Positive-control strain
	rfc	O4 LPS synthesis	788	J96
	hlyA	α-Haemolysin	1177	J96
IV	*papG* allele II	Pyelonephritis-associated *papG*	190	V27
	traT	Surface exclusion; serum survival outer membrane protein	290	2H25
	focG	Pilus tip molecule; F1C fimbriae (sialic acid-specific)	360	2H25
	cdtB	Cytolethal distending toxin	430	V27
	cvaC	ColV; on plasmid	680	PM9
	gafD	N-acetyl-D-glucosamine-specific fimbriae adhesin	952	PM9
V	*kpsMT* K5	K5 specific group II *kpsM*	159	L31
	sfaS	Pilus tip adhesin; S-fimbriae (sialic acid-specific)	240	L31
	cnf1	Cytotoxic necrotizing factor 1	498	L31
	afa/draBC	Dr-antigen-specific adhesin operon	559	2H16
	papG II,III	Gal(α1-4)Gal-specific pilus tip adhesin molecule	1070	2H16
	papG I	Gal(α1-4)Gal-specific pilus tip adhesin molecule	1190	J96
VI	*usp*	Uropathogenic-specific protein (bacteriocin)	440	V27
	uidA	β-D-glucuronidase	508	V27
	ompT	Outer membrane protein T (protease)	559	J96

Figure 10. Agarose gel electrophoresis showing amplicons from virulence factor multiplex PCR (MP) I-VI of positive-control isolates



MP-IV
E. coli V27
123-bp ladder
E. coli PM9
E. coli 2H25
cdtB
papG allele II
gafD
cvaC
focG
traT
MP-V
E. coli J96
123-bp ladder
E. coli L31
E. coli 2H16
papG I
papG II, III
cnf1
papG II,III
afa/draBC
cnf1
sfaS
k5
MP-VI
E. coli V27
123-bp ladder
E. coli J96
uidA
usp
ompT
uidA

8. Detection of integrons, insertion sequence elements and addiction system genes

Integrons were sought by PCR using primers specific for type I, II and III integrases [**Appendix 6**, (Mazel, D. *et al.*, 2000)]. Cycling conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 5 minutes.

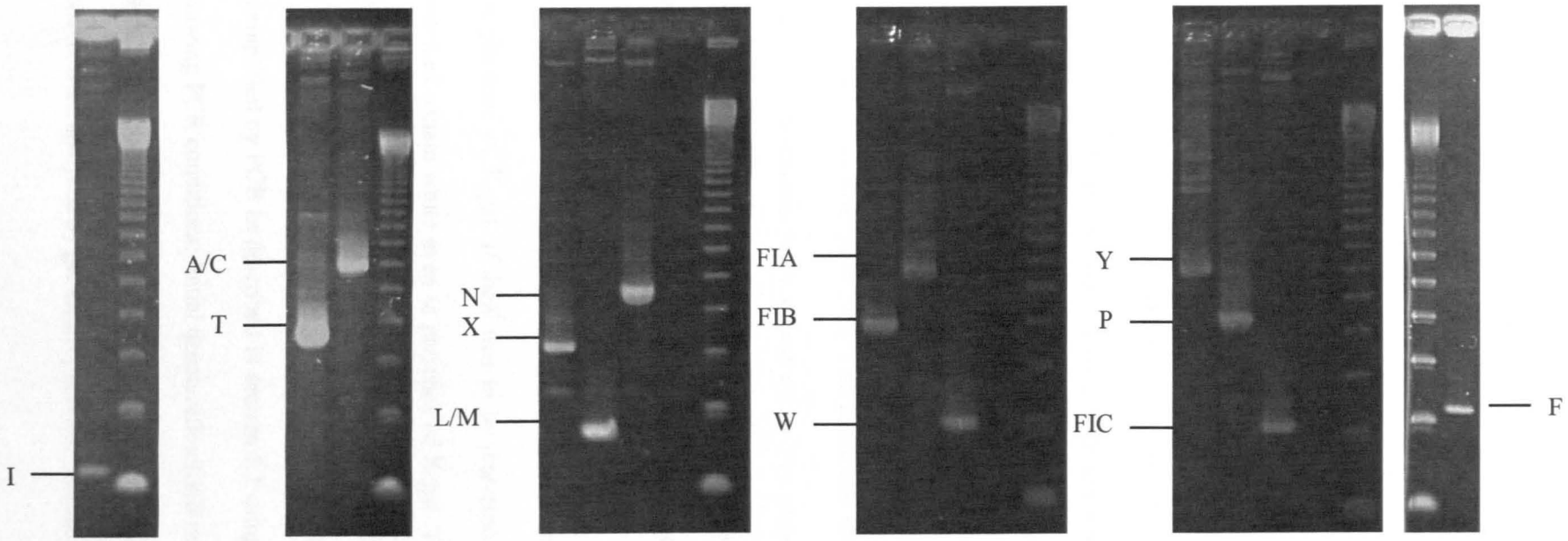
Gene cassettes carried by class I integron were investigated using the Expand Long Template PCR system (Roche Diagnostics, Burgess Hill) with the primer pair int-5'CS and int-3'CS (Levesque, C. *et al.*, 1995), as well as primers specific for genes likely to be integron-mediated (**Appendix 6**). With the amplification of long sequences requiring high-quality DNA, template DNA was obtained using the MagNA Pure Compact Nucleic Acid Isolation Kit 1 (Roche Diagnostics). PCR conditions when using the Expand Long Template PCR system were as follows: 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 10 min of extension at 68°C for a total of 30 cycles. A final extension of 10 minutes finalised the reaction. Amplicons were electrophoresed in 1% agarose gels and visualised under UV light.

Insertion sequences, such as IS*Ecp1* and IS*26*, and the plasmid addiction system *ccdA/ccdB* were sought by conventional PCR as described earlier in section 5.1, with the primer pairs prom+/preCTX-M-3B, IS26-CTX-M for/IS26-CTX-M rev, and ccdA/ccdB, respectively (see **Appendix 6**).

9. Plasmid incompatibility grouping

Template DNA was obtained using the MagNA Pure Compact Nucleic Acid Isolation Kit 1 (Roche Diagnostics, Burgess Hill). Plasmid incompatibility grouping was performed by a PCR-based replicon typing method (Carattoli, A. *et al.*, 2005), with the assistance of Dr Katie Hopkins (Laboratory of Enteric Pathogens, Health Protection Agency - Centre for Infections, London, UK). PCR products were electrophoresed in 2% agarose gels and visualised under UV light.

Figure 11. Agarose gel electrophoresis of positive-control replicons amplified by PCR for plasmid incompatibility grouping



10. Cloning of PCR products using the TA Cloning® kit

Antibiotic resistance genes to be fully sequenced, *i.e.* including the primer binding sites, which are not always covered when directly sequencing PCR products, were cloned using the TA Cloning® kit with pCR®2.1 (Invitrogen, Paisley, UK), used according to the manufacturer's instructions. This kit provided a very rapid and efficient mean of cloning *Taq*-polymerase-amplified PCR products into a plasmid vector. PCR amplicons to be cloned were first purified using the GENECLEAN®Turbo kit (Q-BIOgene, Cambridge, UK) as instructed by the manufacturer. To 2 µL of linearised PCR®2.1 vector in 1 x ligation buffer, were added 2 µL of purified PCR product as well as 1 µL T4 DNA ligase, in a final volume of 10 µL made up with PCR-quality water. Cloning reactions were incubated overnight at 14°C prior to transformation. On the following day, 2 µL volumes of the ligation mixtures were added to vials containing 50 µL of pre-thawed OneShot® competent *E. coli* cells. These cells were then held on ice for 30 minutes, heat-shocked at 42°C for 30 sec and immediately returned onto ice. BHI broths (250 to 500 µL) were added to each vial, and after 60 min incubation at 37°C with horizontal shaking (100 rpm), 10 to 200 µL of the transformant cell suspensions were spread on to NA plates supplemented with 100 mg/L ampicillin and 50 mg/L 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, Invitrogen). To select clones with $bla_{CTX\text{-}M}$ genes, CTX at 2 mg/L was included in the selective NA. The agar plates were then incubated overnight at 37°C.

Vector pCR®2.1 has an inducible *lacZ* gene, which is responsible for a blue coloration of colonies in the presence of X-gal. If *lacZ* was to be disrupted, *e.g.* by the insertion of a PCR product, the colonies remain white even in presence of X-gal. This colorimetric reaction allowed rapid screening for potentially successful clones, and its effectiveness was enhanced by incubating agar plates at 4°C for 2 to 3 h after the overnight incubation at 37°C. The DNA inserts from white colonies were amplified by PCR as described in section 5.1 using the M13 primer pair (**Appendix 6**) and the following PCR conditions: initial denaturation for 5 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 50°C and 3 min at 72°C, ending with a final extension at 72°C for 7 min. PCR products were analysed by gel electrophoresis, purified and sequenced.

11. Sequencing

11.1. Purification of DNA samples using commercial kits

Prior to sequencing, DNA products were purified using one of two commercial kits, depending on the nature of the DNA product:

- For PCR products (up to 10 kb): GENECLEAN®Turbo kit (Q-BIOgene, Cambridge)
- For any other DNA products (up to 300 kb), either in solution or in agarose gel: GENECLEAN®SPIN kit (Q-BIOgene)

11.2. Sequencing reactions

Both strands of the purified DNA samples were sequenced in a microtitre sequencing plate using the Beckman Coulter CEQ 8000 system (Beckman Coulter, High Wycombe). Approximately 50 to 500 ng of DNA was used per sequencing reaction. Following an initial DNA denaturation step at 96°C for 3 min, 0.64 µL of primer (1µg/µL) and 8 µL of DTCS Quick Start Solution (Beckman Coulter) were added per reaction, with sterile water added up to a final volume of 20 µL. The following conditions were used for the sequencing reactions: 30 cycles of 96°C for 20 sec, 50°C for 20 sec, and 60°C for 4 min, with a final holding step at 4°C. Each sequencing reaction was stopped by the addition of:

- 2 µL 0.1 M Na_2EDTA (Sigma-Aldrich) pH 8.0
- 2 µL of 3 M sodium acetate (Sigma-Aldrich) pH 5.2
- 1 µL of 20 mg/mL glycogen (Beckman-Coulter)
- 60 µL of 95% ice-cold ethanol

The microtitre plates were then centrifuged at 5,000 x *g* for 30 min at 4°C in a plate centrifuge (Allegra™ X-22R, Beckman-Coulter) and the DNA pellets were washed twice with 200 µl 70% ethanol by centrifugation at 5,000 x *g* for 10 min each time. The plates then were briefly spun upside down for 15 sec at 500 x *g* in order to remove any residual traces of ethanol, and left to dry at room temperature for about 30 minutes.

The dried DNA pellets were re-suspended in 40 µL of sample loading solution and left at room temperature for 15 minutes. Finally, the microtitre plates were loaded inside the Beckman Coulter CEQ 8000 system following the manufacturer's instructions.

11.3. Sequence analysis

DNA sequence chromatograms were imported into BioNumerics (version 3.5, Applied Maths, St. Marten-Latem, Belgium) for editing and analysis. Multiple sequence alignments were obtained using the BioEdit Sequence Alignment Editor (version 7.0.1). The online BLAST (Basic Local Alignment Search Tool) tool (http://www.ncbi.nlm.nih.gov/BLAST/) was used for comparing edited sequences with those held in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html).

11.4. Sequencing of plasmids pEK499

Plasmid pEK499 (the $bla_{CTX\text{-}M\text{-}15}$-encoding plasmid from representative isolates A_1 of epidemic clone A) was sequenced by MWG Biotech AG (Ebersberg, Germany). The 2- to 3-kb fraction of the randomly sheared plasmid was cloned using the pGEM-Teasy vector system (Promega GmbH, Mannheim) and transformed into *E. coli* DH10b. DNA inserts were sequenced by dye terminator chemistry and sequences were assembled using the Staden Package software (http://staden.sourceforge.net). Combinatorial and directed PCR assays, and walking reads on selected clones were used to assemble the sequences and to fill-in gaps. Plasmid sequence compilation and annotation were accomplished in collaboration with Dr Anthony Underwood (Health Protection Agency, Centre for Infections, Bioinformatics Unit, Colindale). Genes were predicted and annotated using BASys web-server (http://wishart.biology.ualberta.ca/basys/cgi/submit.pl) and compared with other plasmids sequences using the Artemis Comparison tool (http://www.webact.org). The online databases GenBank, EcoGene database of *E. coli*, and the Insertion Sequence database (http://www-is.biotoul.fr/) were also exploited for plasmid sequence analysis.

12. Endonuclease digestion of DNA

Endonuclease digestion of DNA, regardless of its type (total DNA, PCR amplicons or plasmids), was undertaken overnight in 0.5 mL polypropylene microfuge tubes according to the enzyme supplier's instructions (Table 8). A typical reaction contained approximately 10 µL of DNA preparation, 2 µL of enzyme buffer (x10 concentrated), 1 to 10 U of restriction enzyme and PCR-quality water up to a final volume of 20 µL. Reactions were stopped by the addition of 5 µL volumes of loading buffer and the restricted DNA was visualised under UV light following agarose gel electrophoresis (0.8 to 2% agarose gels, 80 to 120 V for 90 to 150 min) and staining in 1 mg/mL ethidium bromide.

13. Nucleic acid extraction

13.1. Extraction of total genomic DNA

Chromosomal DNA from *E. coli* clinical isolates with CTX-M enzymes was extracted according to the method of Pitcher (Pitcher, D., 1989). Bacteria were grown overnight at 37°C in 5 mL LB broths, shaken at 100 rpm on an orbital shaker platform. One-and-a-half mL volumes of the bacterial cultures were centrifuged for 1 min at 10,000 x g to harvest cells, which were then re-suspended in 100 µL of TE buffer (10mM Tris, 1mM Na_2EDTA, pH 8.0). Five hundred µL volumes of GES reagent (5 M guanidium thiocyanate, 0.1 M Na_2EDTA, 0.5% w/v Sarkosyl®) were added to the cell suspensions and tubes were gently mixed in order to lyse the cells. Two hundred and fifty µL volumes of 7.5 M ammonium acetate (Sigma-Aldrich) were then added, and tubes were left on ice for 10 min. Following the addition of 500 µl of phenol: chloroform: isoamyl alcohol [(25: 24: 1), Sigma-Aldrich], the tubes were vigorously shaken by hand for 10 minutes to ensure the two phases (phenol and aqueous phases) mixed well. After a 10 min centrifugation at 10,000 x g, 350 µL of the upper aqueous phase was transferred to a clean microfuge tube containing 875 µL ice-cold 100% ethanol, then gently mixed and kept at -20°C for at least 1 h, allowing precipitation of the DNA.

Table 8. List of restriction enzymes[a] used in this study

Enzyme	Restriction site	Optimal temperature for activity	Purpose of use
*Apa*I	GGGCC / C	25°C	Plasmid RFLP, digestion of total DNA, excision of DNA insert from pCTX-M-3 recombinant vector
*Bam*HI	G / GATCC	37°C	digestion of total DNA
*Eco*RI	G / AATTC	37°C	Digestion of pBBR1MCS-2
*Fok*I	GGATG /	37°C	PCR-RFLP of *aac(6')-Ib*
*Hind*III	A / AGCTT	37°C	Plasmid RFLP
*Hpa*I	GTT / AAC	37°C	Plasmid RFLP and plasmid cross-hybridization studies
*Nde*I	CA / TATG	37°C	PCR-RFLP of *aac(6')-Ib*
*Nla*IV	GGN* / N*CC	37°C	PCR-RFLP of CIT-type AmpC
*Pst*I	CTGCA / G	37°C	Plasmid RFLP
*Sma*I	CCC / GGG	25°C	Excision of DNA insert from pCTX-M-3 recombinant vector
*Xba*I	T / CTAGA	37°C	PFGE of *E. coli* genomic DNA

[a] Manufactured and supplied by New England BioLabs (Hitchin, UK)
* N = A or C or G or T

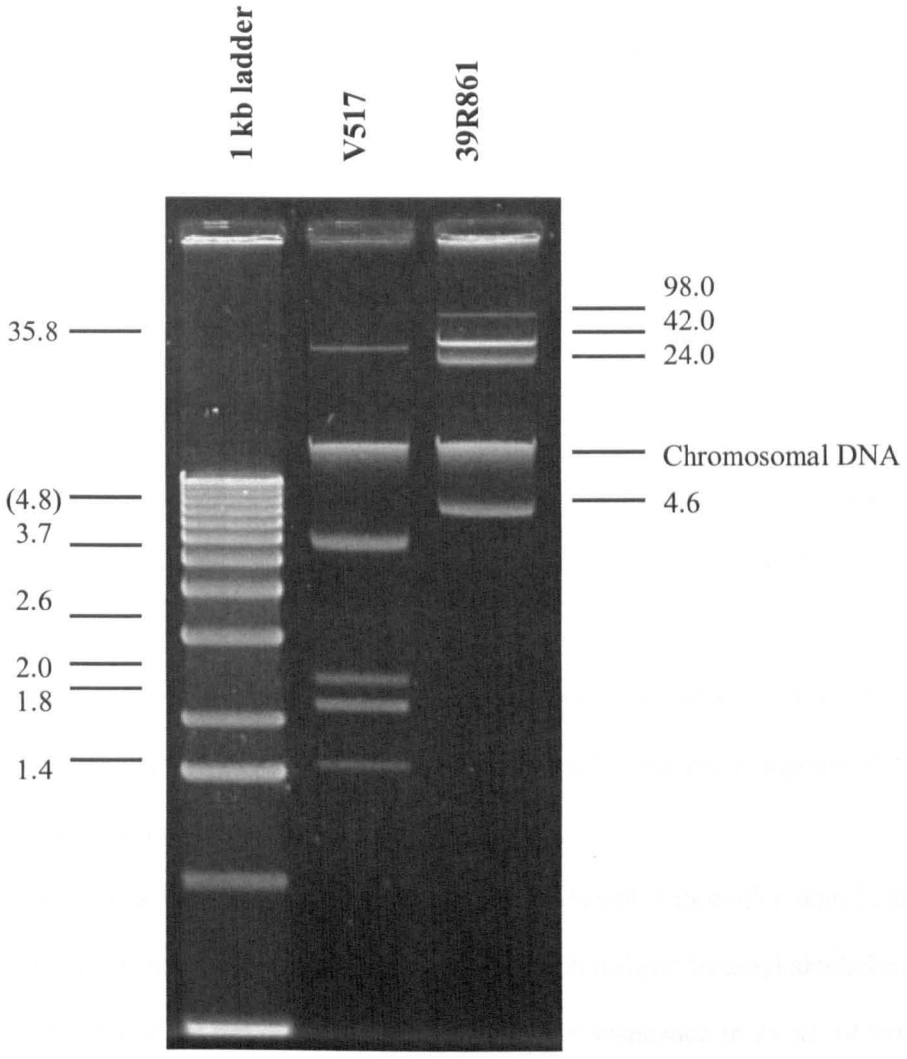
Chromosomal DNA was spooled on to a plastic loop and washed with a few drops of ammonium acetate (7.5 M) / 100% ethanol / water (2:7:1) mix, followed with a few drops of ice cold 100% ethanol. DNA pellets were eventually dissolved in 100 µL of TE buffer and kept overnight at 4°C, before use for enzymatic restriction.

13.2. Extraction of plasmids

13.2.1. Extraction of plasmids by alkaline lysis

Plasmids from *E. coli* clinical isolates were extracted by alkaline lysis, as originally described by Birnboim and Doly (Birnboim, H. C. and Doly, J., 1979). Plasmids from control *E. coli* strains V517 (NCTC 50193) and 39R861 (NCTC 50192) were extracted in parallel and used as molecular size markers (Figure 12, **Appendix 1**). Briefly, loopfuls (rice grain-size) of freshly grown bacterial cultures from NA were re-suspended in 100 µL volumes of solution I (25% w/v sucrose, 10 mM Tris-HCl pH 8.0, 1 mM Na_2EDTA and 10 mg/ml lysozyme) in 1.5 mL microfuge tubes. These tubes were left to stand at room temperature for 5 min. Bacterial cells were then lysed with the addition of 200 µL of solution II [0.2 M NaOH, 1% w/v sodium dodecyl sulphate (SDS)]. Proteins, as well as chromosomal DNA were precipitated by the addition of 150 µL of solution III (3 M potassium acetate, pH 4.8). The tube contents were then mixed by inversion, centrifuged for 10 min at 13,400 x *g* and the supernatants were transferred to fresh 1.5 mL microfuge tubes. One volume (approximately 400 µL) of phenol: chloroform: isoamyl alcohol [(25: 24: 1), Sigma-Aldrich] was added and the samples were mixed by flicking the tubes. Following centrifugation at 13,400 x *g* for 10 min, the upper aqueous layers were carefully removed to clean tubes. Plasmid DNA was then precipitated by the addition of 2 volumes of ice-cold 100% ethanol (approximately 400 µL). After 2 min incubation at room temperature, plasmid DNA was harvested by centrifugation at 13,400 x *g* for 5 min. Pellets were dried at room temperature or at 37°C for at least 30 min and re-suspended in 39 µL of tissue culture water, to which was added 1 µL of RNAse A (1 mg/mL stock, previously heated at 80°C for 10 min).

Figure 12. Agarose gel electrophoresis (0.8%) of plasmids from *E. coli* control strains V517 and 39R861[a] extracted by alkaline lysis



[a] Plasmid sizes indicated are in megadaltons (MDa), with 1 MDa of double stranded DNA equivalent to 1.51 kb.

For gel electrophoresis analysis, 10 µL of loading buffer were added to 20 µL of plasmid preparation (0.8% agarose gel, 2 h 30 min at 80V). The remaining 20 µL of plasmid preparation were kept aside at -20°C for further manipulations such as enzymatic restriction or electroporation. The plasmids preparations were then loaded on to agarose gels, a 1-kb DNA ladder (Invitrogen BV) was used as DNA size standard, along with the plasmid preparations from reference *E. coli* strains V517 and 39R861. For gels requiring Southern blotting and hybridization studies, a Dig-labelled DNA molecular-weight marker II [λ *Hind*III digest (0.12 – 23.1 kb), Roche Diagnostics GmbH, Mannheim] was also loaded onto the agarose gels.

13.2.2. Extraction of plasmids by a modified alkaline lysis protocol

To minimise chromosomal DNA contamination of plasmid preparations (for *e.g.* plasmid cross-hybridization studies), the alkaline lysis protocol described in section 13.2.1 was slightly modified, as described below:

- Following the addition of solution II, the tubes were incubated 1 h at 56°C
- After the addition of solution III and prior to the 10 min centrifugation at 13,400 x *g*, the tubes were incubated on ice for 15 min
- Supernatants from the first centrifugation were decanted through a double thickness of medical gauze prior to addition of the phenol: chloroform: isoamyl alcohol mixture
- At the end of the protocol, plasmid DNA was re-suspended in 24 µL of tissue culture water, to which was added 1 µL of pre-heated (80°C for 10 min) 1 mg/mL RNAse A

Gel electrophoresis analysis was performed as with plasmids extracted by conventional alkaline lysis, *i.e.* in low-concentration agarose gel (usually 0.8%), using 10 µL plasmid preparations, to which 5 µL volumes of loading buffer were added.

13.2.3. Extraction of plasmids using commercial kits

Most commercially available kits for plasmid extraction, such as those used in this study, are based on the alkaline lysis method. Depending on the size of the plasmids, two different kits were utilised (following the manufacturer's instructions): *(i)* the Rapid Plasmid Miniprep System (Marligen

Biosciences, Ijamsville, USA) for extraction of plasmid vectors of ≤45 kb (*e.g.* pBBR1MCS-2), or *(ii)* the High Purity Plasmid Miniprep System (Marligen Biosciences) for plasmids of > 45 kb (*e.g.* pEK499)

13.3. Extraction of total RNA from bacteria

Total RNA from *E. coli* clinical isolates was extracted using the RNeasy® Mini kit (Qiagen Ltd, Crawley) according to the manufacturer's instructions. An on-column DNase treatment using the RNase-free DNase Set (Qiagen Biosciences) was performed in order to remove any traces of DNA contamination. RNA quantification and purity assessment were carried out using a UNICAM UV 500 spectrophotometer (Thermo Electron Corporation, Hemel Hempstead, UK). The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}), knowing that an absorbance of 1 unit at 260 nm corresponded to 40 µg of RNA per mL. The ratio of absorbance readings at 260 nm and 280 nm (A_{260} / A_{280}) provided an estimate of the purity of the RNA preparations. A pure RNA extract has a ratio between 1.9 and 2.3.

14. Reverse transcription and reverse transcriptase (RT)-PCR

RT-PCR was performed in order to assess visually and to compare the expression of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{OXA\text{-}1}$ in various *E. coli* clinical isolates. RT-PCR was carried out in two steps: (*i*) reverse transcription and (*ii*) standard PCR. Messenger RNA (mRNA) from the total RNA extract was first reverse transcribed into cDNA with an oligo-dT primer [(Sigma Genosys), since mRNA possesses a poly-A tail] using the Omniscript™ RT kit (Qiagen Biosciences) following the recommendations of the supplier. This reverse transcription step was followed by a standard PCR using the primers of interest (*e.g.* group-1 CTX-M or OXA-1-like primers). The expression levels of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{OXA\text{-}1}$ genes were assessed visually in 2% agarose gels (electrophoresed at 140 V for 90 min) under UV light.

15. Isoelectric Focusing (IEF) of β-lactamases

15.1. Preparation of crude bacterial cell extracts by sonication

Two or three colonies of an overnight bacterial culture on NA were inoculated into 10 mL nutrient broths, which were incubated at 37°C for 16 to 24 h on an orbital shaker platform (100 rpm). One hundred µL volumes of the bacterial culture were then transferred to fresh 10 mL nutrient broths and the cells were grown into logarithmic (log) phase by incubating at 37°C for approximately 4 h with agitation (100 rpm on an orbital shaker). The cells were then harvested by centrifugation (CR412 refrigerated benchtop centrifuge, Jouan Inc, San Francisco, USA) at 5,000 x *g* for 10 min and re-suspended in 5 ml of sterile phosphate buffer (10 mM, pH 7.0) in disposable glass vials. These cell suspensions were kept on ice until sonication, when the bacterial cell walls were disrupted by immersion of a sonication probe (Ultrasonic Disintegrator, MSE Scientific Instruments Ltd., Crawley) into the cell suspensions. Two 30 sec-burst at 150 W, with a 30 sec interval to allow cooling of the vials, were applied to disrupt bacterial cell walls. The vials were cooled in plastic cups filled with iced water during the whole process. Residual cells were removed by centrifuging for 30 minutes at 5,000 x *g* in a CR412 Bench Top centrifuge at 4°C. The supernatants were then divided into 1 mL aliquots and stored at -20°C until required.

15.2. Preparation of acrylamide gel for IEF

A rubber gasket was positioned in between two glass plates (230 x 115 mm), one of which was covered "inside" with a plastic sheet, ensuring that no air bubbles were trapped underneath. Since polyacrylamide sticks to glass but not to plastic, this assisted the subsequent removal of one glass plate without breaking the gel. Two plastic supports were then positioned on each side of the glass plates, and were tightly held by bulldog clips. The acrylamide gel was prepared as follows. To 27 ml of acrylamide stock solution [25 g sucrose, 59.5 mL of 30% Acrylagel (BDH, VWR International, Poole, UK), 25 mL of 2% bis-acrylagel (BDH) and 165.5 mL of sterile distilled water] were added: 1 mL ampholine pH range 5.0 - 8.0 (Amersham Biosciences), 1 mL ampholine pH range 3.5 - 9.5 (Amersham Biosciences) and 0.6 mL of 0.1 mg/mL riboflavin solution (Sigma-Aldrich). The mixture was then degassed for 10 minutes using a vacuum pump, and 90 µL of

N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma-Aldrich) and 45 µL of freshly-prepared 100 mg/mL ammonium persulphate (Sigma-Aldrich) were finally added. The acrylamide gel mixture was then slowly poured at a constant speed (to prevent the formation of bubbles) between the two glass plates via a 50-mL syringe using a 5-cm long needle. The gel was left to set for at least 2 h before all the clips were removed and the plastic-covered glass plate peeled off, leaving the acrylamide gel on one glass plate, ready to be loaded.

15.3. Enzyme focusing

The β-lactamase activity of the crude bacterial cell extracts was checked by mixing one drop (*c.* 5 µL) of 0.5 mM nitrocefin (BD Biosciences, Oxford, UK) with one drop of extract. Nitrocefin (yellow) is a colorimetric substrate of β-lactamases and becomes red following hydrolysis. The speed at which the reaction occurs is a good indicator of the amount of β-lactamase present in the cell extract. Furthermore, it was essential to work with cell extracts that contained relatively similar enzymatic activities. The ideal change in colour took place between 30 and 60 sec. If a cell extract showed stronger β-lactamase activity, it was diluted with phosphate buffer.

IEF electrode strips (Amersham Biosciences AB, Uppsala, Sweden) were placed at the top and bottom ends of the gel using a pair of forceps; the anode strip was soaked with 1 M phosphoric acid and the cathode strip with 1 M sodium hydroxide. Small pieces of filter paper (10 x 5 mm, up to 20 pieces per gel) were positioned approximately 20 mm from the anionic end of the gel, and 20 µL of crude cell extracts obtained by sonication of the test isolates were applied on each piece. Cell extracts from control isolates producing TEM-1 (pI 5.4), OXA-10 (pI 6.1), SHV-1 (pI 7.6) or SHV-5 (pI 8.2) β-lactamase were also loaded in parallel (**Appendix 1**). The loaded acrylamide gel was then transferred on to the cooling platform of the electrophoresis unit (Flat bed apparatus FEB 3000, Pharmacia Fine Chemicals, Uppsala, Sweden) set to a temperature of approximately 4°C, and which was covered with 1% Triton-X-100 (a non-ionic detergent, Sigma-Aldrich) beforehand to ensure good contact between the glass plate/gel and the platform. A drop of lysed blood was applied to each end of the gel in order to indicate correct migration of the samples. The gel was then electrophoresed at a constant power of 15 W until the two drops of blood met, usually after 50

to 60 min. All the filter papers were then removed using forceps and the gel was electrophoresed for further 10 min, prior to the detection of β-lactamases.

15.4. Gel developing

After removing the electrode strips with a scalpel, the gel was overlaid with 0.5 mM nitrocefin. The presence of β-lactamases (red bands on the gel) was recorded on graph paper. Their pI values were estimated by comparing the distance they had migrated with the distances migrated by the control enzymes with known pI values.

15.5. Detection of AmpC β-lactamases

If the presence of AmpC β-lactamases was suspected (*e.g.* owing to the resistance to the β-lactam/clavulanic acid combination and to cefoxitin), samples were loaded in duplicate on two halves of an IEF gel. Following conventional electrophoresis, one half was overlaid with 0.5 mM nitrocefin as described in the previous section, while the second half was first overlaid with cloxacillin (0.3 mM, Sigma-Aldrich), a competitive inhibitor of AmpC enzymes. After 5 min incubation at room temperature, the second half of the gel was then also overlaid with nitrocefin (double strength, *i.e.* 1 mM) and results were recorded on graph paper. AmpC enzymes were only revealed in the absence of cloxacillin, since this antibiotic inhibits hydrolysis of nitrocefin.

16. Molecular typing by pulsed-field gel electrophoresis (PFGE)

16.1. DNA preparation

E. coli isolates were grown overnight at 37°C on NA. Organisms were re-suspended in 1 mL Salt-EDTA (SE) buffer (75 mM NaCl, 25 mM Na_2EDTA, pH 7.5) to a turbidity of 2.3 to 2.8 McFarland using an API densitometer. For each isolate, blocks of 2 x 6 mm were prepared by mixing equal volumes (*e.g.* 400 µL) of bacterial suspension with molten 2% low-gelling agarose (FMC BioProducts, Rockland, USA) in SE buffer at 50 to 56°C, prior to dispending in appropriate moulds. Moulds were kept at 4°C until the agarose had set. The agarose plugs were then transferred

to bijou bottles containing 3 mL of buffer A [6 mM Tris, 100 mM EDTA, 1 M NaCl, 0.5% Sarkosyl® (BDH), 1 mM $MgCl_2$, 0.5 mg/mL lysozyme, pH 7.5] and were incubated overnight at 37°C on an orbital shaker (300 rpm). Buffer A was replaced on the next day with buffer B (1% Sarkosyl®, 0.5 M Na_2EDTA, 0.5 mg/mL proteinase K, pH 9.5) and the blocks were re-incubated overnight in a water-bath at 56°C with gentle agitation. The plugs were then washed four times for 30 min, each time with 3 mL TE buffer (10 mM Tris, 10 mM Na_2EDTA, pH 7.5) at 4°C, prior to digestion.

16.2. Digestion of genomic DNA in agarose plugs

Genomic DNA was digested with *Xba*I. Agarose strips of 2 x 1 mm in size were cut from the blocks using a scalpel and were pre-incubated in 100 µL *Xba*I enzyme buffer at 4°C for at least 30 min. The restriction buffer was then replaced with 100 µL of fresh buffer containing 20 units of *Xba*I enzyme and the agarose plugs were incubated at 37°C for at least 4 h (preferably overnight), prior to electrophoresis and analysis.

16.3. PFGE gel electrophoresis

Digested plugs were loaded into wells of a 1.2% pulsed-field certified agarose gel containing 2.04 g agarose (BioRad, Hemel Hempstead) in 170 mL 0.5 x TBE; the gel dimensions were 206 x 140 mm. A lambda (λ) concatamer ladder (BioRad), previously treated at 52°C for 8 min, was loaded in parallel to the test samples and was used as a size standard. The wells were sealed with molten agarose and the gel was transferred into a contour-clamped homogeneous-field CHEF DRII electrophoresis apparatus (BioRad), then covered with pre-cooled (4°C) 0.5 x TBE buffer, and electrophoresed under the following conditions: 5 sec initial switching time, 35 sec final switching time, 6 V/cm, for 30 h at 12°C. After electrophoresis, the gel was stained for 90 min in 1 µg/mL ethidium bromide and de-stained for 60 min in distilled water. DNA fingerprints were visualised under UV light and photographed using the IN Genius Syngene Bio Imaging system.

16.4. Data analysis

PFGE data were analysed with the BioNumerics software (version 3.5). Banding patterns for test isolates were normalised against the λ ladder and dendrograms were constructed using the Dice product-moment correlation, with 1% position tolerance, 0.5% optimisation and the unweighted pair group method (UPGMA). Isolates sharing at least 85% homology in banding patterns were considered to belong to a same strain (Tenover, F. C. *et al.*, 1995; Woodford, N. *et al.*, 2004).

17. Hybridization studies

Hybridization studies were undertaken in order to detect $bla_{CTX\text{-}M}$ genes in plasmid extracts as well as in extracts of total DNA from clinical isolates of *E. coli*.

17.1. Southern blotting

Digested and undigested plasmid DNA, as well as digested total DNA, were electrophoresed in 0.8% agarose gels and then transferred on to Hybond™-N-membranes (Amersham Biosciences UK Limited, Chalfont) by vacuum blotting under a constant suction of 45 to 60 mBar, using a LKB VacuGene XL system (Pharmacia Fine Chemicals, Uppsala). A Dig-labelled DNA molecular-weight marker II (Roche Diagnostics GmbH), acting as a DNA size standard as well as a positive control for hybridization using digoxigenin (Dig)-labelling, was also loaded in the agarose gels and transferred to the nylon membrane. Once the gel was correctly positioned on top of the nylon membrane with the vacuum applied, the DNA present in the agarose was fragmented for 4 min with 0.25 M HCl, and then denatured for 3 min with 1.5 M NaCl / 0.5 M NaOH, prior to 3 min neutralisation with 1.5 M NaCl / 0.5 M Tris / 1 mM Na_2EDTA, pH 7.2. Between each step, the liquid was carefully soaked up from the gel with tissue paper. Following this treatment, the DNA was transferred for approximately 60 min on to the membrane by pouring sufficient SSC solution (3 M NaCl, 0.3 M tri-sodium citrate) to completely cover the gel. The transfer of DNA on to the blot was checked under UV light, using a dual intensity UV transilluminator (Ultra-Violet Products, Cambridge). Membranes covered with DNA were finally baked at 80°C for at least 2 h

(preferably overnight), or cross-linked using a Stratalinker® 2400 UV crosslinker (Stratagene, La Jolla, USA), prior to hybridization.

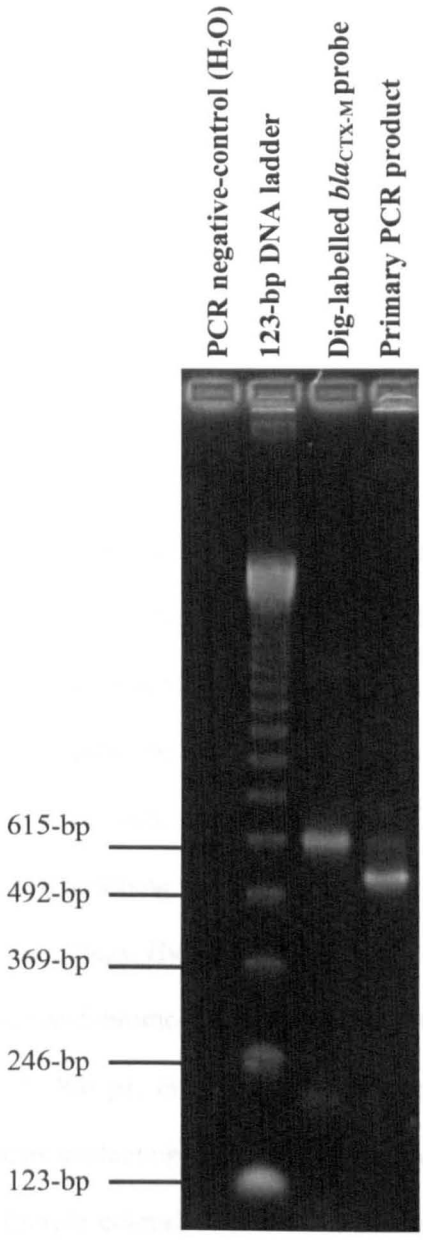
17.2. Preparation of digoxigenin-labelled DNA probes

Dig-labelled probes for the detection of *bla*$_{CTX-M}$ genes were derived from PCR products. A primary PCR using the primer pair CTX-M MA1/ CTX-M MA2 (**Appendix 6**) was carried out as described in section 5, amplifying a DNA sequence portion common to all *bla*$_{CTX-M}$ allele variants. The incorporation of Dig-11-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) into the *bla*$_{CTX-M}$ DNA probe was accomplished in a secondary PCR, in which the volume of dTTP was reduced in favour of Dig-labelled dUTP (2:1 ratio of dTTP:dUTP volumes). Secondary PCR reactions were undertaken in 0.5 mL microfuge tubes using 1 µL of primary PCR product as template. The secondary PCR reaction mixture consisted of 1 x *Taq* polymerase buffer, 0.05% W-1 buffer, 2 mM $MgCl_2$, 0.3 mM dATP, 0.3 mM dCTP, 0.03 mM dGTP, 0.2 mM dTTP, 0.1 mM Dig-11-dUTP, 1 µg of each primer and 1 U *Taq* DNA polymerase in a final volume of 100 µL. PCR conditions were identical for both primary and secondary reactions, with an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 60 sec, ending with a final extension at 72°C for 5 min. PCR products were analysed by gel electrophoresis on a 2% agarose gel run at 120 V for 90 min and visualised under UV light. Incorporation of Dig-11-dUTP into the secondary product retarded its migration and thus it appeared larger than the unlabelled primary PCR product (see Figure 13). Following the incorporation of digoxigenin, secondary PCR products were purified using the GENECLEAN®SPIN kit (Q-BIOgene) and stored at -20°C until required.

17.3. Hybridization with Dig-labelled DNA probes and stringency washes of nylon membranes

Nylon membranes with fixed DNA were placed in HYBAID™ HB-OV-BS hybridization bottles (Thermo Life Sciences, Basingstoke) and incubated at 70°C with 20 mL hybridization solution for at least 1 h under constant and low-speed rotation in a Shake 'n' Stack hybridization oven.

Figure 13. Gel electrophoresis (2% agarose) of primary and secondary (Dig-labelled probe) *bla*$_{CTX-M}$ PCR products for hybridization studies



The hybridization solution consisted of 4 x SSC, 0.02% SDS, 0.1% Sarkosyl and 1 x buffer III from the Dig Wash and Block set (Roche Diagnostics GmbH, Mannheim). The hybridization of the membrane with a denatured DNA probe (30 µL of DNA probe in 10 mL hybridization solution denatured 3 min at 95°C) was undertaken overnight at 70°C under constant rotation. Hybridized membranes were then washed twice at low stringency (2 x SSC / 0.1% SDS, at room temperature) for 5 min, followed by further two washes at high stringency (0.1% SSC / 0.1% SDS, at 70°C) for 15 min.

17.4. Detection of DNA/DNA hybrids

All the steps involved in the detection of hybrids were undertaken at room temperature. Following 30 min incubation with Blocking buffer (Dig Wash and Block set, Roche Diagnostics GmbH), nylon membranes were soaked in 20 mL Washing buffer (Dig Wash and Block set, Roche Diagnostics GmbH) supplemented with 4 µL anti-digoxigenin antibody-conjugate (Fab fragments bound to alkaline phosphatase, Roche Diagnostics GmbH) for 30 min. The membranes were then rinsed for 3 min in developing buffer (Dig Wash and Block set, Roche Diagnostics GmbH) and transferred into plastic bags. Hybrids were detected by a colorimetric reaction involving nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate solution (NBT/BCIP, Roche Diagnostics GmbH, 400 µL in 10 mL Developing buffer), a substrate of alkaline phosphatase enzyme. Plastic bags containing NBT/BCIP were sealed and kept in the dark until the appearance of hybrid bands (purple colour) of sufficient intensity on the blot. The membranes were finally rinsed with distilled water and dried at 70°C for 15 min.

17.5. Plasmid cross-hybridization studies

A Dig-labelled *Hpa*I-digested-pEK499 probe was produced in order to estimate the relative homology between various other plasmids and pEK499 itself. Plasmids encoding CTX-M enzymes were transferred into a laboratory strain of *E. coli* DH5α by electroporation (section 18.2), and subsequently extracted by modified alkaline lysis and digested with *Hpa*I. pEK499 restriction

fragments were random-primed labelled with Dig-11-dUTP using the DIG High Prime DNA Labelling and Detection Starter Kit I for colour detection with NBT/BCIP (Roche Diagnostics GmbH) following the manufacturer's procedure. The test plasmids, also digested with *Hpa*I, were electrophoresed at 80 V for 150 min in 0.8% agarose gels, then transferred on to nylon membranes and hybridized overnight with the Dig-labelled pEK499 fragments. Hybrids were revealed by colorimetric reaction with NBT/BCIP. It was assumed that the amount of digested DNA fragments of a given plasmid hybridised by the Dig-labelled reference pEK499 was proportional to the relatedness of both these plasmids.

18. Transfer of cefotaxime resistance

18.1. Transfer of cefotaxime resistance by conjugation

Conjugative transfer of CTX resistance from clinical isolates with CTX-M enzymes (donor strains) to the cefotaxime-susceptible, rifampicin-resistant *E. coli* K-12 strain J53-2 (NCTC 50006) recipient strain was carried out by a filter-mating method. Donor and recipient strains were grown overnight at 37°C in 5 mL Brain Heart Infusion (BHI) broths. The cells were then sub-cultured into 5 mL amounts of fresh BHI broths and grown up to log phase, then harvested by centrifugation (10 min at 5,000 x *g*) and re-suspended in 1 mL amount of phosphate-buffered saline (PBS). Small squares (20 x 20 mm) of sterile Hybond™-N-membrane were firmly applied on to antibiotic-free NA plates using sterile forceps and 100 µL amounts of each separate bacterial suspension (controls), as well as 100 µL of a 1:1 donor / recipient strain mixture, were inoculated on top of each piece of membrane. The plates were incubated overnight at 37°C, and the bacterial growth from each filter was then collected and re-suspended in 1 mL of PBS. The cell suspensions (donor, recipient and mixture of both strains) then were diluted in ten-fold steps up to 10^{-7} with PBS and 100 µL of each dilution (neat to 10^{-7}) were spread on to NA supplemented with either 200 mg/L rifampicin to suppress the donor strains, or 2 mg/L CTX to suppress the recipient strain, or 200 mg/L rifampicin and 2 mg/L CTX to suppress both parent strains. Viable cell counts were carried out in parallel by inoculating each bacterial dilution on to non-selective media (NA). All plates

were incubated for 16 to 24 h at 37°C. Conjugation frequencies were calculated by dividing the number of transconjugants obtained by the number of input donor cells.

18.2. Transfer of cefotaxime resistance to *E. coli* K-12 strain DH5α by electroporation

18.2.1. Preparation of electrocompetent *E. coli* DH5α cells

E. coli K-12 DH5α was grown up to log phase in 50 mL of LB broth. The culture was then chilled on ice and the cells were harvested by centrifugation for 10 min at 5,000 x *g* at 4°C. The cells were then re-suspended in 50 mL of ice-cold sterile distilled water, and repeatedly centrifuged and re-suspended in decreasing ice-cold water volumes of successively 50, 25, 10 and 1 mL. After a final centrifugation, the cells were re-suspended in 1 mL ice-cold sterile 10% glycerol solution, and were divided into 100 µL aliquots for storage at -70°C until required.

18.2.2. Transformation of competent *E. coli* DH5α cells

Competent *E. coli* DH5α cells were transformed by electroporation using a Gene-Pulser™ (Bio-Rad, Hemel Hempstead). Five µL of plasmid extract (prepared as described in section 13.2.1) were added to 100 µL of pre-thawed competent cells. The mixtures were then transferred into pre-cooled (-20°C) Gene Pulser® cuvettes (Bio-Rad) and electroporated at 2,500 V, 25 µF and 200 Ω. In parallel, competent cells were electroporated also in absence of DNA extracts as a (negative) control measure. Soon after electroporation, the cells were transferred to small bijoux containing 1 mL BHI, and were incubated for approximately 1 h at 37°C with constant shaking (100 rpm). Volumes of 50, 100 and 200 µL were spread onto NA plates supplemented with 2 mg/L CTX, to select CTX-resistant transformants.

19. Fitness studies

The growth rates of native *E. coli* DH5α and *E. coli* DH5α transformed with pEK499 were compared in order to determine whether the carriage of pEK499 was associated with a fitness cost.

Both organisms were grown overnight in 10 mL amounts of nutrient broth (NB) at 37°C with constant agitation (100 rpm). Afterwards, 1 mL from each culture was transferred to sterile 250-mL conical flasks containing 100 mL NB and, after a quick agitation, 1 mL was rapidly sampled from each flask and was serially diluted to 10^{-8} with NB allowing viable counts on drug-free NA plates. Similar measurements were taken after 1, 2, 4, 8, 24 and 32 h incubation of the cultures at 37°C.

20. Outer membrane protein profiles

Outer membrane proteins (OMPs) from *E. coli* clinical isolates were characterised using a method adapted from Chart (Chart, H., 1994a; Chart, H., 1994b).

20.1. Extraction of outer membrane proteins (OMPs)

Bacteria were grown to log phase (4 h at 37°C, with shaking) by transferring 5 mL of overnight 10-mL LB cultures into pre-warmed (37°C) conical flasks containing 100 mL LB. The cells then were harvested by centrifugation (30 min at 5,000 x *g*) and re-suspended in 5 mL of ice-cold 25 mM Tris-HCl, pH 7.5 containing 1mM Na_2EDTA. These cell suspensions were chilled on ice and sonicated as described in section 15.1. The resulting cell extracts were transferred to pre-cooled centrifuge tubes containing 25 mM Tris-HCl (pH 7.4) buffer and residual cells were removed by 30 min centrifugation at 4°C at 5,000 x *g*. The cell envelopes were collected from the supernatants (and separated from cytoplasmic materials) by centrifugation for 60 min at 45,000 x *g* in a Sorvall ultracentrifuge (Kendro Laboratory Products, Bishop's Stortford). The resulting pellets were re-suspended in 20 mL of 25 mM Tris-HCl (pH 7.4) and cytoplasmic membranes were solubilised by adding 1 mL of Sarkosyl and shaking for 30 min at 200 rpm at room temperature. OMPs were finally sedimented by centrifugation at 45,000 x *g* for 60 min at 4°C, re-suspended in 200 µL 25 mM Tris-HCl (pH 7.4) and stored at -20°C until analysis by SDS-PAGE.

20.2. OMP profiles analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

OMPs were resolved in 12.5% polyacrylamide gels using the ATTO (Model AE-6530) minigel system (Genetic Research Instrumentation, Braintree, UK) powered by a Bio-Rad model 1000/500 power supply. The 12.5% separation (resolving) gel was prepared by mixing 5 mL of 1.5 M Tris-HCl (pH 8.8), 0.4% SDS buffer with 6.7 mL distilled water, 8.3 mL 30% acrylamide / 0.8% bis-acrylamide (37.5:1, Severn Biotech Ltd, Kidderminster, UK), 60 µL freshly prepared 10% ammonium persulphate solution and 60 µL TEMED. After approximately 45 min, allowing the polymerisation of the acrylamide, the separation gel was overlaid with a 4% acrylamide stacking gel consisting of 2.5 mL of 0.5 M Tris-HCl (pH6.8), 0.4% SDS, 6 mL distilled water, 1.5 mL 30% acrylamide / 0.8% bis-acrylamide solution, 30 µL 10% ammonium persulphate and 20 µL TEMED. A 12-well comb was inserted in the stacking gel, which was left to polymerise for 30 min.

Prior to electrophoresis, the OMP extracts and the SDS-PAGE Molecular Weight Standard ladder, High Range (Bio-Rad) required denaturation. This was achieved by adding 5 µL of protein preparation to 15 µL of solubilisation buffer (62.5 mM Tris-HCl, 10% glycerol, 5% β-mercaptoethanol, 3% SDS, 0.01% bromophenol blue, pH 6.8), which was then incubated for 5 min at 100°C. The protein samples and the ladder, also mixed with solubilisation buffer in a 1:4 ratio, were also denatured for 45 min at 100°C in presence of 8 M urea in order to improve the resolution (separation of proteins). Eight µL of the denatured samples and ladder (± urea) were then loaded into the wells of the stacking gel and electrophoresed at a constant current of 50 mA in 200 mL running buffer (25 mM Tris-HCl, 250 mM glycine, 0.1% SDS) for approximately 45 min. Acrylamide gels were stained overnight in a coomassie blue solution [0.025% (w/w) coomassie brilliant blue R-250 (BDH), 50% (v/v) methanol, 10% (v/v) acetic acid] with gentle agitation, and de-stained in repeated changes of 10% (v/v) acetic acid until OMP profiles appeared with a satisfactory contrast (3 to 5 h de-staining were usually required).

21. Measurement of CTX-M enzymes specific activity

21.1. Preparation of bacterial cell extracts

Crude bacterial cell extracts were prepared by sonication as described earlier. These were used to measure the specific activity of CTX-M β-lactamases from various clinical isolates and their respective CTX-resistant-derived transformants. Estimation of CTX-M specific activity was achieved by assessing both the cefotaxime hydrolysis rate and the protein content of the sonicates.

21.2. Determination of protein concentration

Protein concentrations in cell extracts were measured using the Bio-Rad Protein Assay Reagent kit (Bio-Rad, Hemel Hempstead, UK), which is based on the method of Bradford (Bradford, M. M., 1976). To 100 µL of each cell extract (undiluted and diluted 1/5 and 1/10) and to 100 µL of standard bovine serum albumin (BSA, Sigma-Aldrich) at 50, 100, 150 and 200 µg/mL, were added 5 mL of dye reagent. After a quick vortex and 5 min incubation at room temperature, absorbances were measured at 595 nm using the UNICAM UV 500 spectrophotometer. For each sample, measurements were made in triplicate. Protein concentrations in cell extracts were calculated against the calibration curve generated from the results obtained with the BSA standards.

21.3. Cefotaxime hydrolysis assay

Cefotaxime hydrolysis was assessed using the UNICAM UV 500 spectrophotometer. Ten to 100 µL amounts of cell extracts were added to 1 mL of pre-warmed (37°C) CTX solution (50 µM final concentration in 10 mM phosphate buffer, pH 7.0) in 1-cm light-path quartz cuvettes. Changes in absorbance were monitored over 5 min at 255 nm against a blank (cell extract in antibiotic-free phosphate buffer), starting as soon as the cell extracts was added. A decrease in absorbance indicated hydrolysis of CTX (Lorian, V., 1996).

21.4. Determination of CTX-M specific activity

The amount of CTX hydrolysed / min / mL of cell extract was determined using the Beer-Lambert law:

$$\boxed{Abs_{255} = e_{255} \times l \times c}$$

Abs_{255} = absorbance at 255 nm (optical density unit)

e_{255} = molar extinction coefficient ($M^{-1}cm^{-1}$)

l = path length (1 cm)

c = concentration (M)

The amount of CTX hydrolysed / mL of cell extract over 5 min was directly proportional to the decrease in absorbance at 255 nm over the same period of time. The CTX-M specific activity (mol of CTX hydrolysed / min / mg of protein) for a given cell extract was calculated by dividing the amount of CTX hydrolysed / min / mL of cell extract by the protein concentration of the cell extract.

22. *In-vitro* evolution of ceftazidimase activity of CTX-M-3 β-lactamase

In order to investigate the evolution of ceftazidimase activity by the CTX-M-3 β-lactamase, we compared the frequencies of emerging ceftazidime resistance in isogenic wild-type and hypermutable CTX-M-3-producing *E. coli* strains, and sequenced the mutant $bla_{CTX\text{-}M}$ alleles following selection pressure. This was achieved by cloning $bla_{CTX\text{-}M\text{-}3}$ into a vector subsequently transferred to isogenic strains of *E. coli*. The resulting recombinant clones were each exposed to four x the CAZ MIC for the original isolate producing CTX-M-3 enzyme, and mutations frequencies were recorded. The $bla_{CTX\text{-}M}$ genes from randomly-selected mutants were then sequenced.

22.1. Vector preparation

The 5,144 bp low-copy number plasmid pBBR1MCS-2 (Kovach, M. E. *et al.*, 1995) was used as the vector, and was extracted using the Rapid Plasmid Miniprep System (Marligen Biosciences) according to the manufacturer's protocol. This vector, which encodes kanamycin resistance, was

then digested overnight at 37°C (as described in section 12) with *Eco*RI, which has a unique restriction site in the multiple cloning site (MCS). Following gel electrophoresis analysis (0.8% agarose, 100 V for 120 min), the linearised vector was purified with the GENECLEAN®SPIN kit (Q-BIOgene), then de-phosphorylated (at the 5'-end) in a 20 µL reaction using bacterial alkaline phosphatase (BAP, Invitrogen BV) to prevent self-religation, and finally purified one more time with the GENECLEAN®SPIN kit. The resulting linearised and de-phosphorylated pBBR1MCS-2 vector was stored at -20°C until required.

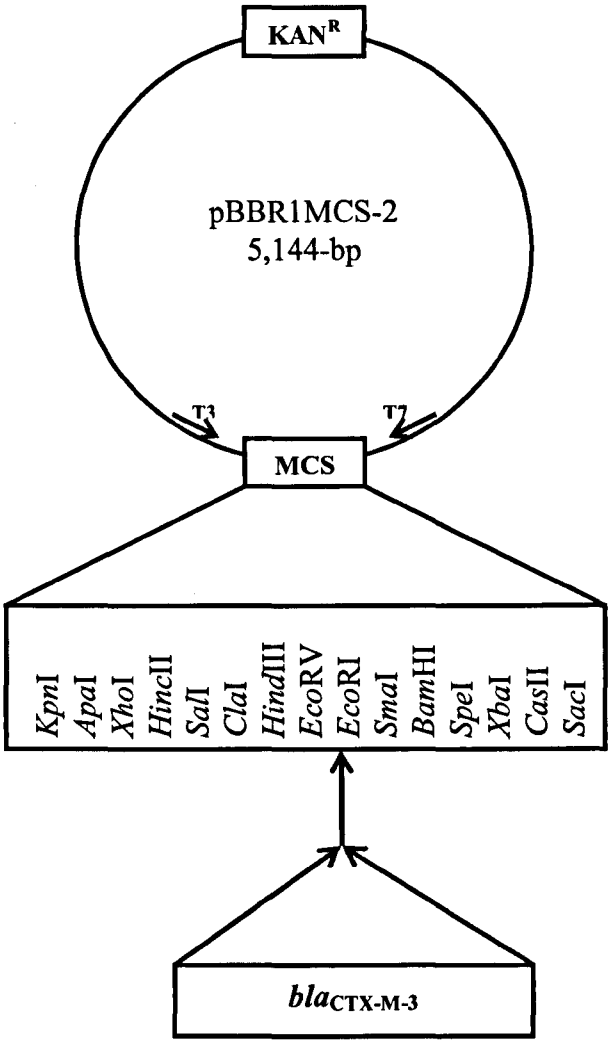
22.2. Amplification of *bla*$_{CTX-M-3}$

The *bla*$_{CTX-M-3}$ gene was amplified from an *E. coli* clinical isolate by PCR with the primer pair prom+ / preCTX-M-3B (Poirel, L. *et al.*, 2002a) using the following PCR conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 25 sec, 52°C for 40 sec and 72°C for 90 sec, with a final extension at 72°C for 7 min. The PCR product was purified using the GENECLEAN®Turbo kit (Q-BIOgene) and stored at -20°C until required.

22.3. Ligation of *bla*$_{CTX-M-3}$ into pBBR1MCS-2 and transformation

Prior to ligation, the *bla*$_{CTX-M-3}$ PCR product obtained as described in section 22.2 first required to be blunt-ended, and this was achieved with DNA Polymerase I, Large (Klenow) Fragment [New England BioLabs (NEB), Hitchin], used as instructed by the supplier. Following a purification step using the GENECLEAN®SPIN kit (Q-BIOgene), the blunt-ended amplicon was then treated with T4 Polynucleotide kinase (NEB) following the supplier's instructions, and was finally purified again. The *bla*$_{CTX-M-3}$ gene was ligated into the MCS of pBBR1MCS-2 (Figure 14) using 1 U of T4 DNA ligase (NEB) as advised by the supplier, in a 20 µL reaction incubated overnight at 14°C. The recombinant vector, designated pCTX-M-3, was transformed into electrocompetent isogenic *E. coli* wild-type 1411 (WT) and hypermutable MutS-negative 1413 (*mutS*) strains (Miller, K. *et al.*, 2002). Preparation of electrocompetent WT and *mutS* cells, and electroporation were as described in section 18.2. Transformants were selected overnight at 37°C on NA supplemented with kanamycin at 50 mg/L and cefotaxime at 2 mg/L.

Figure 14. Construction of pCTX-M-3



22.4. Confirmation of cloning of *bla*$_{CTX-M-3}$

Ligation of *bla*$_{CTX-M-3}$ into pBBR1MCS-2 and transfer of the recombinant vector into *E. coli* WT and *mutS* strains were confirmed by extracting plasmids from at least three colonies of each strain from the selective plates, using the Rapid Plasmid Miniprep System (Marligen Biosciences). The plasmids were then digested overnight at 25°C with *Apa*I to linearise the vector, and with both *Apa*I and *Sma*I enzymes to excise the insert (see Figure 14). The restriction products were analysed by gel electrophoresis (1% agarose gel, 100 V for 120 min) and visualised under UV light. The sizes of the DNA fragments were checked against the 1 kb DNA ladder. Insertion of DNA into the MCS of pBBR1MCS-2 was further confirmed by PCR using the primer pair T3 / T7 (**Appendix 6**), and the presence of *bla*$_{CTX-M-3}$ was sought using prom+ and preCTX-M-3B primers. Finally, expression of the CTX-M-3 phenotype by the recombinant clones was assessed by antibiotic susceptibility testing, as described earlier in section 4.

22.5. Mutant selection and sequencing of *bla*$_{CTX-M-3}$

Ceftazidime-resistant mutants of the pCTX-M-3-carrying WT and *mutS* strains were selected from three independent cultures in quadruple experiments on NA containing ceftazidime (CAZ) at 4 mg/L for the WT and 6 mg/L for *mutS*. The methodology has been detailed elsewhere (Miller, K. *et al.*, 2002). After 18 h incubation at 37°C, CAZ-resistant colonies were counted, and mutation frequencies were calculated, taking plate counts of viable bacteria on drug-free agar as the denominator. Both strands of the *bla*$_{CTX-M}$ gene from *(i)* the parental WT and *mutS* recombinant strains and, *(ii)* randomly selected WT and *mutS* mutants, were sequenced using the primer pair T3/T7.

23. *In-vitro* development of high-level ceftazidime resistance in isolate Eo499

A similar approach to that described previously in paragraph 21.4 of this section was used in order to promote the loss of IS*26* from the promoter region of *bla*$_{CTX-M-15}$ in clinical isolate Eo499, a representative of UK epidemic *E. coli* strain A (also referred to as A_1). Since this insertion element

was suspected of reducing the expression of CTX-M-15 enzyme, the $bla_{CTX\text{-}M\text{-}15}$-positive Eo499-derived transformant was exposed to 4 x CAZ MIC (*i.e.* 16 mg/L). In order to induce the SOS-response and thus promote mutations, ciprofloxacin at ¼ x MIC (*i.e.* 0.008 mg/L) was also incorporated into the selective plates (Vila, J., 2006). Mutation frequencies were recorded and randomly selected CAZ-resistant mutants were then screened by PCR for the presence of IS*26*. In parallel, $bla_{CTX\text{-}M\text{-}15}$ and its normal promoter were amplified by PCR from clinical isolates Eo499 (IS*26*-positive) and Eo516 (IS*26*-negative) using the primer pair prom+ / preCTX-M-3B. Amplicons were cloned into PCR®2.1 and the resulting clones had their CTX and CAZ MICs determined using E-test® strips.

RESULTS

Chapter I. Molecular characterisation of *Escherichia coli* epidemic clones A-E with CTX-M-15-like beta-lactamases

1. Introduction

ARMRL started to monitor *E. coli* with CTX-M enzymes in 2003, and since then, more than 2,000 clinical isolates with a CTX-M phenotype have been referred, from approximately 180 centres across the UK. Until 2005, those referred isolates were typed by PFGE in order to plot the spread of clones of *E. coli* with CTX-M β-lactamases in the UK. Most of the 2,000 isolates were also tested for antibiotic susceptibility and were confirmed as CTX-M-producers by PCR (Woodford, N. *et al.*, 2006). About 85% of those subjected to this analysis produced a group-1 CTX-M enzyme, usually CTX-M-15. PFGE of 772 clinical isolates with group-1 CTX-M β-lactamases identified five major epidemic clones at the national level (representing approximately 30% of the referred isolates), as well as many unrelated producers (Woodford, N. *et al.*, 2004). The five clones were designated A, B, C, D and E. PCR and DNA sequencing showed that all five clones and most of the unrelated isolates produced CTX-M-15-like enzymes (mostly CTX-M-15 itself). The present study firstly focused on the five epidemic clones A-E, since these were prevalent at the national level. Non-clonal *E. coli* isolates with CTX-M-15-like β-lactamases, as well as isolates with different variants of CTX-M enzymes are discussed in subsequent chapters.

Table 9 summarises the MIC ranges and geometric mean MICs for each *E. coli* epidemic clone A-E. All five clones had typical ESBL phenotypes with synergy between clavulanic acid and third-generation cephalosporins and, had a CTX-M phenotype, with cefotaxime and cefpodoxime MICs exceeding those of ceftazidime. Most clone A isolates required lower MICs of third-generation cephalosporins, particularly ceftazidime, which is normally a good substrate for CTX-M-15 enzymes. Interestingly, clone A isolates sometimes even appeared susceptible to ceftazidime by the disc diffusion method.

Table 9. MIC ranges and geometric mean (GM) MICs (mg/L) for *E. coli* epidemic clones A-E with CTX-M-15-like β-lactamases

	R>[a]	Clone A (n=119)		Clone B (n=13)		Clone C (n=47)		Clone D (n=11)		Clone E (n=14)	
		Range	GM	Range	GM	Range	GM	Range	GM	Range	GM
AMP	**16**	64 - >256	78	64 - >256	101.6	64 - >256	81	64 - >256	161	64 - >256	106
AMX / CLA	**16**	8 - 64	16	16 - 128	16	8 - 32	22.6	16 - 32	16	16 - 32	16
CTX	**1**	8 - 256	57	8 - >256	82.3	16 - >256	76.1	64 - >256	161	64 - >256	93.4
CTX/CA	**1**	≤0.06 - 8	0.08	≤0.06 - 1	0.23	≤0.06 - 0.25	0.1	0.125 - 1	1	≤0.06 - 0.5	0.16
CAZ	**2**	0.5 - 64	5	4 - 128	69	1 - 128	32	32 - >64	55.8	8 - >64	36.8
CAZ / CLA	**2**	≤0.125 - 4	0.18	0.25 - 2	0.6	≤0.125 - 2	0.27	0.25 - 1	0.6	0.125 - 1	0.4
CPD	**1**	32 - >256	58.4	32 - >256	152	256	256	256	256	256	256
CPD / CLA	**1**	0.5 - 2	0.7	2	2	1 - 2	1.5	1 - 2	1.5	1 - 2	1.5
FOX	**8**	4 - 64	9.9	8 - 16	12.7	8 - 32	12.22	8 - 16	10.1	8 - 32	16
PIP	**16**	64 - >256	77.5	64 - >256	97	64 - >256	81	64 - >256	181	64 - >256	106
PIP / TZB	**16**	8 - >256	25.2	4 - 32	29.3	2 - 256	17.5	8 - 64	16	4 - >64	22.6
ETP	**2**	≤0.125 - 0.5	0.08	0.06 - 0.125	0.09	0.06 - 0.125	0.1	0.06 - 0.125	0.07	0.03 - 0.064	0.05

[a] BSAC breakpoints defining resistance (Andrews, J. M., 2007)

	R>[a]	Clone A (n=119)		Clone B (n=13)		Clone C (n=47)		Clone D (n=11)		Clone E (n=14)	
		Range	GM	Range	GM	Range	GM	Range	GM	Range	GM
IPM	**4**	≤0.125 - 0.5	0.13	0.125 - 0.25	0.125	≤0.125 - 0.5	0.16	0.125 - 0.5	0.125	0.125 - 0.5	0.125
MEM	**4**	≤0.06 - 0.5	0.05	0.03 - 0.064	0.05	0.03 - 0.064	0.06	0.03 - 0.064	0.04	0.03 - 0.125	0.05
CIP	**1**	0.25 - >256	12.1	8 - 256	16	8 - 128	10.8	8 - 128	43.5	8 - 256	18
AMK	**16**	1 - 32	14.6	4 - 16	8	2 - 64	5	4 - 16	16	4 - 256	11.7
GEN	**4**	0.5 - 8	2.4	0.5 - 256	41.2	1 - 64	32.6	32 - 256	58.7	4 - >32	29.3
MIN	**8**	1 - >32	32	4 - 16	8.8	0.5 - 8	2	4	4	1 - 32	5.3
COL	**4**	≤0.5 - 4	0.5	≤0.5 - 1	0.5	≤0.5 - 8	0.6	≤0.5 - 1	0.5	≤0.5 - 1	0.5
TMP	**2**	256	256	256	256	≤0.25 - 32	0.7	256	256	0.25	0.25
NIT	**32**	8	8	8	8	4 - 16	5.7	8 - 16	9.2	8	8
FOS	**128**	0.5 - 2	0.8	0.5 - 1	0.8	0.5 - 1	0.6	0.5 - 1	0.6	0.5 - 1	0.6

All five clones were multi-drug resistant and among the drugs tested, only carbapenems, cephalosporin/clavulanic acid combinations, nitrofurantoin and fosfomycin retained good activity. Overall, the five epidemic clones had similar antibiograms with only a few minor exceptions, most of which concerned epidemic clone A. In addition to requiring lower MICs of third-generation cephalosporins, clone A was also exceptional in being gentamicin-susceptible and more minocycline-resistant, in contrast to clones B-E. Although trimethoprim was only tested on a few representative isolates of each clone (A, n = 17; B, n = 2; C, n = 6; D, n = 5; E, n = 2), only clones A, B, D, as well as few clone C isolates appeared resistant to that drug.

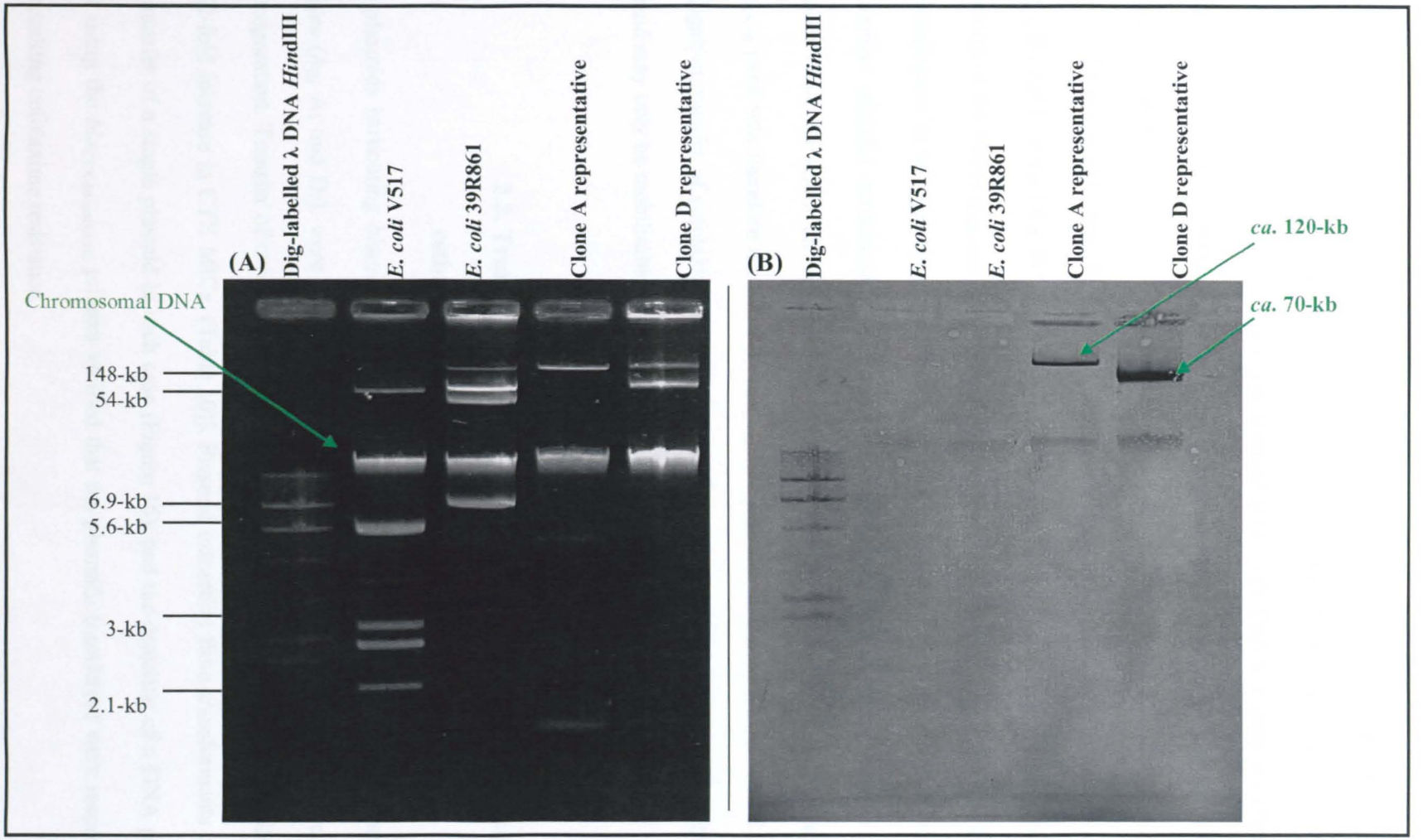
These antibiograms formed the background for the molecular investigation of the different resistance mechanisms present in epidemic clones A-E. In particular, the study focused on locating the genes encoding CTX-M β-lactamases in representative isolates of the five epidemic clones, as well as characterising their genetic support, *i.e.* plasmid. To reach this objective, cefotaxime resistance from various representative clonal isolates was first transferred to fully susceptible laboratory strains, and the transferred plasmids were subsequently characterised in detail. In addition, since these five clones were (by definition) clinically successful, I also explored their phylogenetic backgrounds as well as their virulence genotypes, and compared them with those of sporadic ESBL producers.

2. *bla*$_{CTX-M}$ detection and localisation

2.1. Plasmid extraction and hybridization

The plasmid profiles of a representative isolate of each epidemic clones A and D are shown in Figure 15(A). In order to localise the gene encoding CTX-M enzymes in these isolates, the plasmid extracts were blotted on to a nylon membrane and subsequently hybridized with a digoxigenin-labelled *bla*$_{CTX-M}$ probe [Figure 15(B)]. This revealed the gene to be encoded by *ca.* 120-kb and 70-kb plasmids in clones A and D, respectively. The localisation of the *bla*$_{CTX-M}$ genes in representative isolates of strains B, C and E is discussed later in this chapter.

Figure 15. (A) Plasmid profiles of clone A and D representatives; (B) Southern blot hybridized with *bla*$_{CTX-M}$ probe[a]



[a] Plasmids from *E. coli* control strains V517 and 39R861 were used as molecular size markers and the Dig-labelled λ DNA *Hin*dIII was used as control for hybridization.

2.2. Transfer of cefotaxime resistance by conjugation

Conjugative transfer of cefotaxime resistance to *E. coli* J53-2 rif^R (rifampicin MIC > 256 mg/L) was attempted from one representative isolate of each clone A-E referred to as A_1, B_1, C_1, D_1 and E_1, respectively. Rifampicin MICs for A_1-E_1 were 12 to 16 mg/L as determined using E-test strips. Conjugative transfer was successfully achieved from isolates B_1-E_1, but not from A_1, which consistently failed to transfer its cefotaxime resistance in more than five separate attempts. The transfer also failed when using two other clone A representative isolates, each from different UK centres (A_2 and A_3).

The conjugative transfer rates for cefotaxime resistance-encoding plasmids from isolates B_1, C_1, D_1 and E_1 were 3.5×10^{-6}, 3.6×10^{-4}, 7.6×10^{-6} and 1.3×10^{-4} per donor isolate, respectively. Detection of the $bla_{CTX\text{-}M\text{-}universal}$ gene by PCR in the resulting cefotaxime- and rifampicin-resistant transconjugants confirmed the successful transfer of cefotaxime resistance into *E. coli* J53-2. Subsequent plasmid extractions from transconjugants further confirmed the transfer of single plasmids from the donor clinical isolates, which by contrast harboured multiple plasmids. The $bla_{CTX\text{-}M}$ gene was therefore mediated by conjugative plasmids in clone B, C, D and E. Although conjugative transfer of cefotaxime resistance from clone A representatives was not achieved, the plasmid may only be mobilisable (transfer) with the contribution of a helper plasmid.

2.3. Transfer of cefotaxime resistance by electroporation and estimation of the size of plasmids encoding $bla_{CTX\text{-}M}$

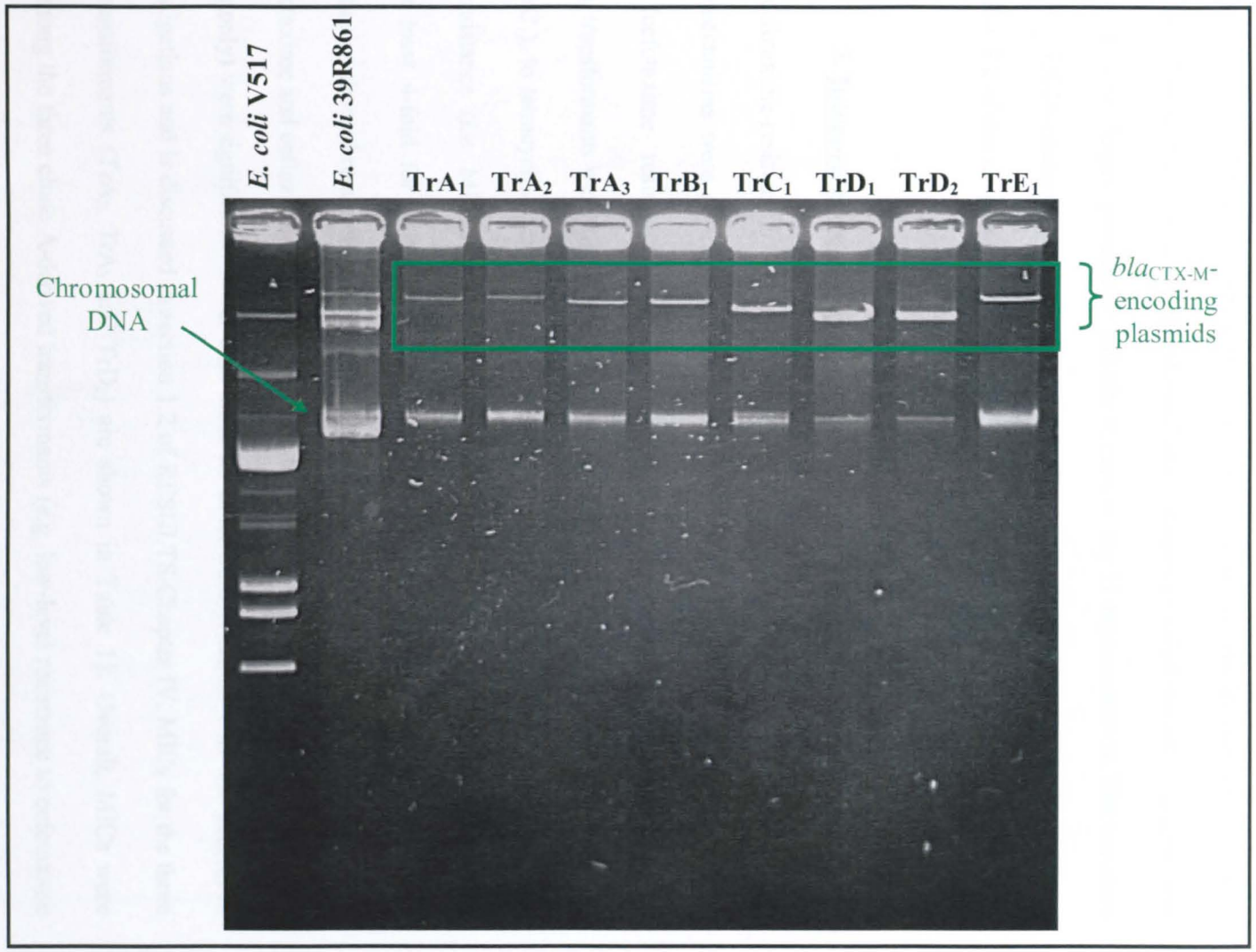
The plasmids harbouring $bla_{CTX\text{-}M}$ from isolates A_1-E_1, as well as from three additional clinical isolates (A_2, A_3 and D_2), were transferred to the fully susceptible *E. coli* K-12 DH5α strain by electroporation. Transfer of cefotaxime resistance was checked by antibiotic susceptibility testing [≥32-fold increase in CTX MICs, (Table 10)]. Plasmid extraction from transformants confirmed the transfer of a single plasmid in each case (Figure 16), and the detection of a DNA product by PCR using the $bla_{CTX\text{-}M\text{-}universal}$ primers verified that the plasmids transferred were responsible for the resulting cefotaxime resistance.

Table 10. MICs (mg/L) for transformants (TrA_1-TrE_1) derived from representative isolates A_1-E_1

	DH5α	Transformants				
		TrA_1	TrB_1	TrC_1	TrD_1	TrE_1
AMP	8	> 64	> 64	> 64	> 64	> 64
AMX / CLA	4	16	16	8	16	16
CTX	≤0.125	4	> 64	16	> 64	256
CTX / CLA	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060
CAZ	≤0.25	1	32	0.5	32	64
CAZ / CLA	0.125	0.125	0.125	0.125	0.25	0.125
PIP	2	> 64	> 64	> 64	64	> 64
PIP / TZB	2	4	4	2	4	16
ETP	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125
IPM	0.125	0.125	0.25	0.125	0.125	0.25
MEM	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060
NAL	0.25	0.25	0.5	0.25	0.5	0.5
CIP	0.03	0.25	0.25	0.03	0.125	0.125
AMK	≤0.5	2	2	0.125	2	4
GEN	≤0.125	≤0.125	16	≤0.125	8	32
TOB	0.25	8	8	0.25	8	32
TET	1	64	64	1	> 64	64
SFM	4	16	4	4	4	nt[a]
TMP	≤0.25	> 32	≤0.25	≤0.25	≤0.25	nt[a]

[a] nt = not tested

Figure 16. Plasmid profiles of cefotaxime-resistant transformants derived from representative clinical isolates of epidemic clones A-E



The bla_{CTX-M} gene was harboured by plasmids with different sizes in the five epidemic *E. coli* clones, suggesting different plasmids were involved. The plasmid sizes ranged from approximately 70-kb in clone D to 150-kb in clone E (*ca.* 120-kb plasmid in clones A and B, and 100-kb in clone C). Figure 16 also shows slight variations in size of the bla_{CTX-M}-encoding plasmid in the three isolates A_1-A_3. Furthermore, the plasmid profiles of eight additional clone A isolates (A_4-A_{11}, Figure 17), all from different UK centres, also appeared slightly different. Although the number of plasmids per isolate was different, ranging from two to five, it is noteworthy that some clone A isolates harboured similarly-sized plasmids. Hybridization studies revealed the bla_{CTX-M} gene was always encoded by the largest plasmid (> 100-kb) in each of the 11 representatives. The variation in size of the CTX-M-encoding plasmids from various isolates of clone A is discussed in more detail in section 8.2 of this chapter.

3. Interpretative reading of antibiograms for transformants TrA_1-TrE_1

MICs for cefotaxime-resistant transformants (Table 10) suggested that additional antibiotic resistance mechanisms were co-transferred with the bla_{CTX-M} gene. In addition to the CTX-M phenotype (cefotaxime resistance, and synergy of third-generation cephalosporins with clavulanate), transformants were also resistant to tobramycin (except TrC_1) and gentamicin (except TrA_1 and TrC_1), to tetracycline (except TrC_1) and to trimethoprim (TrA_1 only). Although formal quinolone resistance (*i.e.* MIC > breakpoint) was not transferred, ciprofloxacin MICs were increased at least 4-fold for most of the transformants (except TrC_1). The clone A-derived transformants also showed reduced susceptibility to sulphamethoxazole.

Cefotaxime and ceftazidime MICs for the transformants derived from clone A and clone C (ceftazidime only) were significantly lower than those for other transformants. This was subject to further investigations and is discussed in section 1.2 of RESULTS-Chapter IV. MICs for the three additional transformants (TrA_2, TrA_3 and TrD_2) are shown in Table 11. Overall, MICs were consistent among the three clone A-derived transformants (*e.g.* low-level resistance to cefotaxime and ceftazidime). MICs for TrD_2 were comparable to those for TrD_1.

Figure 17. Plasmid profiles of various clone A representative isolates

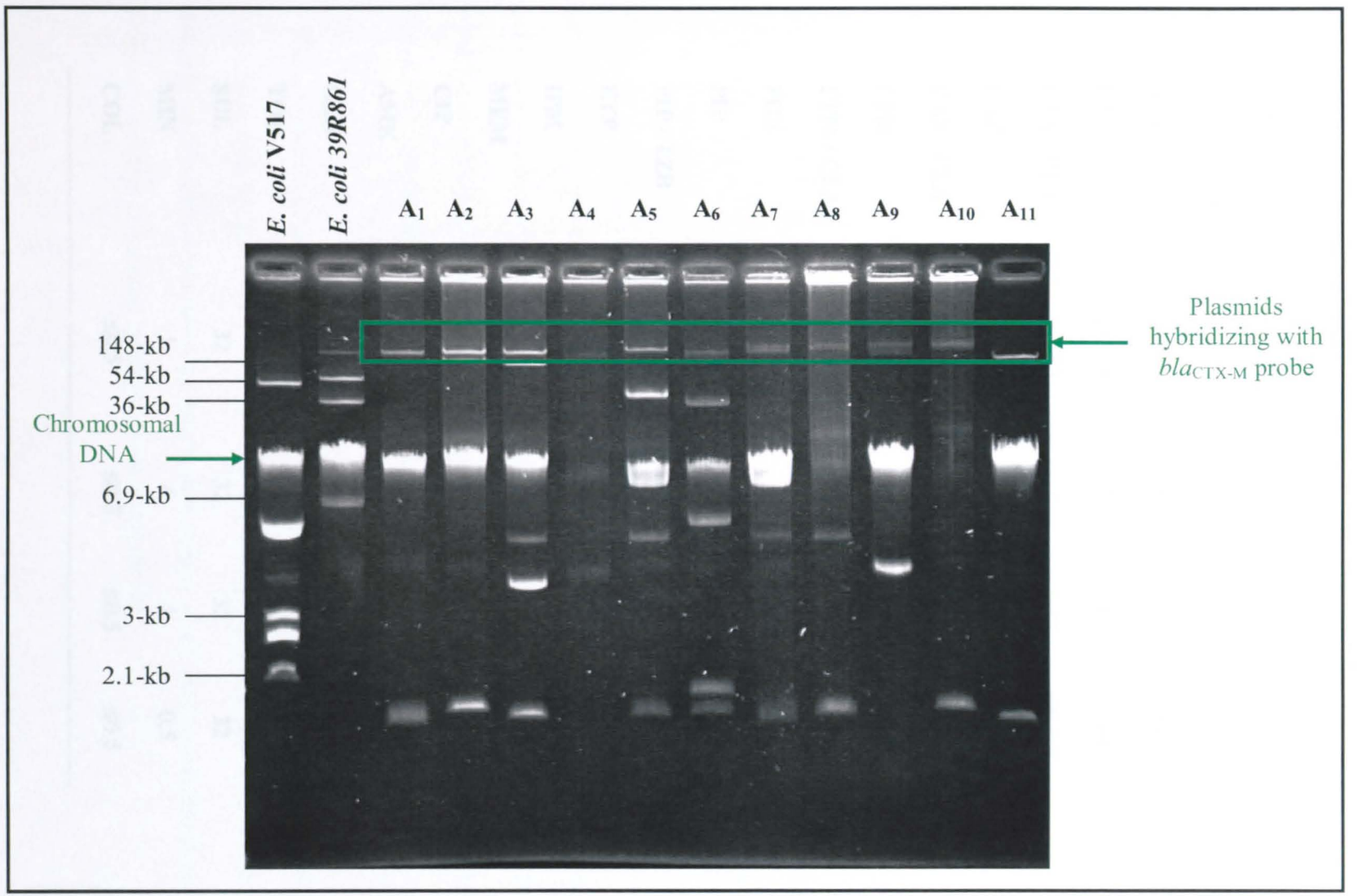


Table 11. MICs (mg/L) for *E. coli* DH5α and for transformants TrA_2, TrA_3 and TrD_2

	Recipient strain (DH5α)	Transformants		
		TrA_2	TrA_3	TrD_2
AMP	8	> 64	> 64	> 64
AMX / CLA	4	16	16	16
AZT	≤0.125	2	1	64
CTX	≤0.125	8	4	> 64
CTX / CLA	≤0.060	≤0.060	≤0.060	≤0.060
CAZ	≤0.25	1	1	32
CAZ / CLA	0.125	0.125	0.125	0.25
CPM	≤0.125	0.5	0.5	16
CPM / CLA	≤0.060	≤0.060	≤0.060	≤0.060
FOX	4	4	4	4
PIP	2	> 64	> 64	> 64
PIP / TZB	2	8	8	4
ETP	≤0.125	≤0.125	≤0.125	≤0.125
IPM	0.125	0.125	0.125	0.125
MEM	≤0.060	≤0.060	≤0.060	≤0.060
CIP	0.03	0.25	0.125	0.25
AMK	≤0.5	1	1	2
GEN	≤0.125	≤0.125	0.25	8
TOB	0.25	4	4	8
SUL	32	32	32	32
MIN	1	4	4	0.5
COL	≤0.5	≤0.5	≤0.5	≤0.5

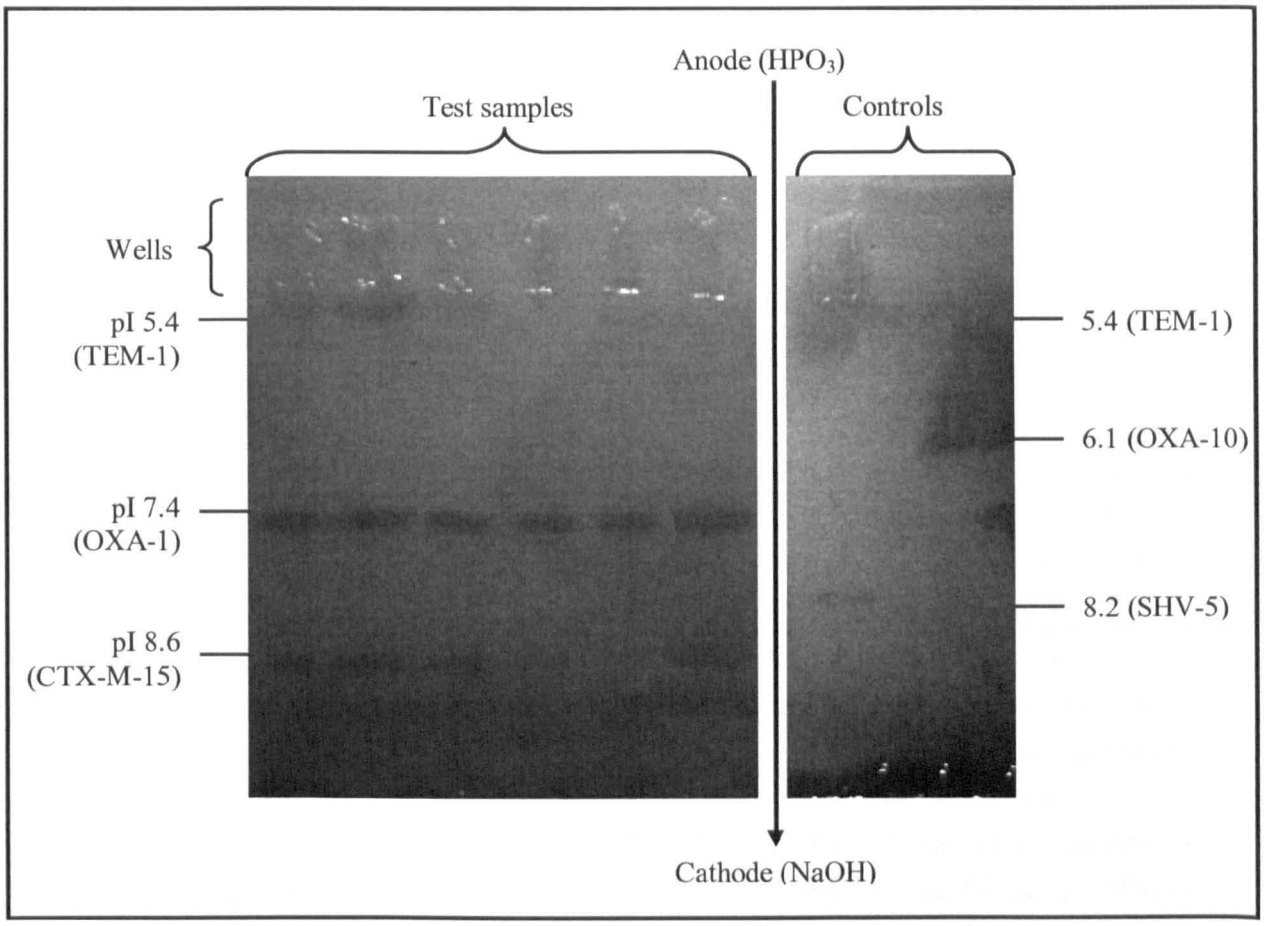
4. Isoelectric focusing (IEF) and identification of beta-lactamases

The β-lactamase enzymes from various representatives of clones A-E were characterised by IEF. Data are summarised in Table 12. With the exception of clone D isolates (referred to ARMRL from a single centre only) and 4/9 clone C isolates, most representatives selected for this investigation originated from different centres.

Overall, the clinical isolates produced either one, two or three β-lactamase(s) according to IEF (see Figure 18) and four different β-lactamases with pI values of 5.4, 7.4, 8.4 and 8.6 were detected, in various combinations, for each isolate. None of the β-lactamases detected among the 30 clonal isolates tested was inhibited by cloxacillin and therefore, none corresponded to the AmpC β-lactamase type. The three clone D isolates consistently produced three β-lactamases (pI values of 5.4, 7.4 and 8.6) and the four isolates of clone E consistently expressed two β-lactamases (pIs of 7.4 and 8.6). In contrast, variations in β-lactamases production were noticeable among clone A, B and C isolates. Clone B isolates always had two β-lactamases in common (pIs of 5.4 and 8.6), but two isolates additionally produced an enzyme with a pI of 7.4. The β-lactamase at pI 8.4 was only detected in 4/9 clone C representatives, all originating from Belfast. These also produced an additional enzyme with a pI of 5.4. The remaining clone C representatives either produced two [1/9, (pIs of 5.4 and 8.6)] or three β-lactamases [4/9, (pIs of 5.4, 7.4 and 8.6)]. Finally, IEF detected only one or two β-lactamase(s) among clone A representative isolates. All had a band at pI 7.4; four had no other enzyme detected, and six additionally produced an enzyme with a pI of 5.4. The remaining clone A representative had two β-lactamases with pIs of 7.4 and 8.6.

The cell extracts of the eight transformants TrA_1-TrE_1 were also tested on IEF; all showed exactly the same β-lactamase profiles as their respective plasmid donor strains. This therefore suggested that the β-lactamases produced by each clinical isolates A_1-E_1 were co-transferred to *E. coli* DH5α, and were thus co-encoded by single plasmids (Figure 16).

Figure 18. Picture of IEF gel for the detection of β-lactamases from six randomly selected clonal isolates (as examples) in absence of cloxacillin[a]



[a] Similar profiles were obtained in presence of cloxacillin at 0.3 mM; proteins migrated from the anode to the cathode as indicated by the arrow.

Table 12. Summary of isoelectric focusing and PCR data for representative isolates of epidemic clones A-E

Clone	Rep.	Origin	No. of bands	pI values of β-lactamases[a]				*bla* gene(s) detected by PCR[b]
A	A_1	Shrewsbury	2	5.4	7.4			bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	A_2	Coventry	2		7.4		8.6	$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	A_3	Southampton	2	5.4	7.4			bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	A_4	London 1	1		7.4			$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	A_5	London 2	1		7.4			$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	A_6	Belfast	1		7.4			$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	A_7	Winchester	2	5.4	7.4			bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	A_8	Southend	2	5.4	7.4			bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	A_9	Milton Keynes	1		7.4			$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	A_{10}	Stevenage	2	5.4	7.4			bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	A_{11}	London 3	2	5.4	7.4			bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
B	B_1	Stoke	3	5.4	7.4		8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	B_2	Birmingham	3	5.4	7.4		8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	B_3	Shrewsbury	2	5.4			8.6	bla_{TEM}, $bla_{CTX\text{-}M}$
C	C_1	Belfast	2	5.4		8.4		bla_{TEM}, $bla_{CTX\text{-}M}$
	C_2	Kingston on Thames	3	5.4	7.4		8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	C_3	Chertsey	3	5.4	7.4		8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	C_4	London city	2	5.4			8.6	bla_{TEM}, $bla_{CTX\text{-}M}$
	C_5	Salisbury	3	5.4	7.4		8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	C_6	Southend	3	5.4	7.4		8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	C_7	Belfast	2	5.4		8.4		bla_{TEM}, $bla_{CTX\text{-}M}$
	C_8	Belfast	2	5.4		8.4		bla_{TEM}, $bla_{CTX\text{-}M}$
	C_9	Belfast	2	5.4		8.4		bla_{TEM}, $bla_{CTX\text{-}M}$
D	D_1	Shrewsbury	3	5.4	7.4		8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	D_2	Shrewsbury	3	5.4	7.4		8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	D_3	Shrewsbury	3	5.4	7.4		8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
E	E_1	Kingston on Thames	2		7.4		8.6	$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	E_2	Portsmouth	2		7.4		8.6	$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	E_3	Chertsey	2		7.4		8.6	$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
	E_4	Papworth	2		7.4		8.6	$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$

[a] pI of TEM-1 is 5.4; pI of OXA-1 is 7.4; pI of CTX-M-3 is 8.4; pI of CTX-M-15 is 8.6.
[b] bla_{SHV} was not detected by PCR in any of the investigated isolates.

In parallel, all 30 clinical isolates detailed in Table 12 and the eight transformants were screened by PCR for the presence of bla_{TEM}, bla_{SHV}, $bla_{OXA\text{-}1\text{-}like}$ and $bla_{CTX\text{-}M}$ (entire ORFs), as these genes encode the most common plasmid-mediated β-lactamases. None of the tested isolates was positive for bla_{SHV} by PCR. On the other hand, the remaining three genes were detected in various combinations among all isolates (Table 12) and the transformants. All $bla_{CTX\text{-}M}$ genes belonged to phylogenetic group 1 (CTX-M-15-like). The bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$ and $bla_{CTX\text{-}M\text{-}group\text{-}1}$ amplicons detected in representative isolates A_1-E_1 were cloned into pCR®2.1 and sequenced fully. Comparing the various combinations of genes detected by PCR to the combinations of enzymes detected by IEF, and with reference to the Lahey clinic database (http://www.lahey.org/studies/other.htm), it was deduced that the β-lactamases with pI values of 5.4, 7.4, 8.4 and 8.6 were encoded by $bla_{TEM\text{-}1}$, $bla_{OXA\text{-}1}$, $bla_{CTX\text{-}M\text{-}3}$ and $bla_{CTX\text{-}M\text{-}15}$, respectively. Although no band at pI 8.6 was detected in the majority of clone A representatives (10/11), the $bla_{CTX\text{-}M}$ gene was consistently detected by PCR. This disparity was investigated further and is discussed in Chapter IV. The low ceftazidime MIC for TrC_1 (Table 10) was considered due to the fact that it produced CTX-M-3 β-lactamase, a variant that does not hydrolyse ceftazidime as efficiently as CTX-M-15. While $bla_{CTX\text{-}M\text{-}3}$ was always linked to $bla_{TEM\text{-}1}$ only, $bla_{CTX\text{-}M\text{-}15}$ was variably linked to $bla_{TEM\text{-}1}$ and / or $bla_{OXA\text{-}1}$.

The geometric mean MIC of the piperacillin/tazobactam combination for the 24 OXA-1-positive clonal isolates was 20.4 mg/L, three-fold higher than that for the six isolates lacking this enzyme (geometric mean MIC = 6.3 mg/L). This may be explained by the fact that OXA enzymes are poorly inhibited by tazobactam, and by the other available β-lactamases inhibitors in general.

5. Resistance mechanisms co-transferred with $bla_{CTX\text{-}M}$

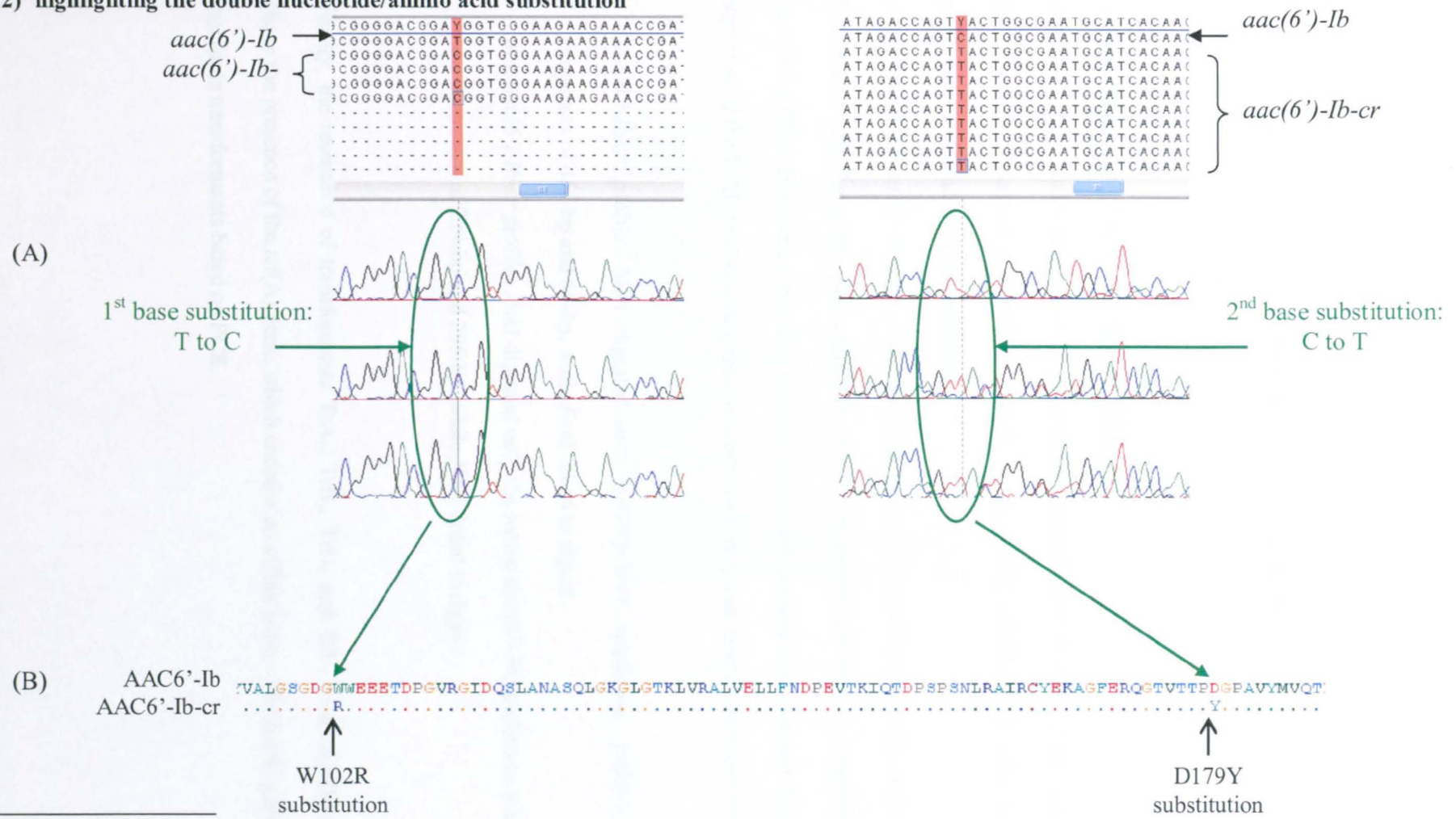
Mechanisms of resistance to antibiotics other than β-lactams were identified by PCR and DNA sequencing. According to the antibiograms of transformants TrA_1-TrE_1 (Table 10), the CTX-M-encoding plasmids were also responsible for resistance or reduced susceptibility to most aminoglycosides (except in TrC_1). DNA sequencing showed that resistance to gentamicin in clone B-, D- and E-derived transformants was encoded by a classical *aac*3-*IIa* gene.

Tobramycin resistance and reduced susceptibility to amikacin in all transformants except TrC_1 was associated with the presence of an *aac*6'-*Ib* gene. This gene was identified by PCR and DNA sequencing among clone A, B, D and E representative isolates. It is noteworthy that clone C representative isolates, which lacked this gene, generally required lower amikacin MICs than clones A, B, D and E representatives (2 *vs.* 8 mg/L).

Interestingly, sequencing of the *aac*6'-*Ib* allele from each positive transformant consistently revealed the presence of the two crucial nucleotide substitutions responsible for Trp102Arg and Asp179Tyr substitutions in the resulting AAC6'-Ib enzyme (Figure 19). The combination of these mutations has previously been shown to broaden the spectrum of activity of the aminoglycoside acetyltransferase to include fluoroquinolones with an unsubstituted piperazinyl group [*i.e.* ciprofloxacin and norfloxacin, (Robicsek, A. *et al.*, 2006)]. The allele variant, designated *aac*6'-*Ib-cr*, was therefore responsible for the four-fold increase in ciprofloxacin MICs for clone A-, B-, D- and E-derived transformants, in addition to the resistance to tobramycin. Acquisition of *aac*6'-*Ib-cr* did not affect the activity of nalidixic acid however, since this antibiotic lacks the relevant piperazinyl group (Table 10).

The other plasmid-mediated amikacin resistance-encoding gene, *armA* (coding for a methylase) was not present among clones A-E representatives and their derived transformants, according to PCR. Additionally, *qnrA*, *qnrB* and *qnrS* genes, which also are plasmid-mediated and reduce the susceptibility to fluoroquinolones, were also sought by PCR, but were not found among the clone A-E representatives and their cefotaxime-resistant transformants. These data support the view that reduced susceptibility to ciprofloxacin in clones A-, B-, D- and E-derived transformants was likely to be solely due to acetylation by AAC6'-Ib-cr.

Figure 19. Partial alignments of (A) nucleotide and (B) amino acid sequences of *aac(6')-Ib-cr* from TrA_1 and of the classical *aac(6')-Ib* allele (GenBank AAV59012)[a] highlighting the double nucleotide/amino acid substitution



[a] Similar results were obtained with TrB_1, TrD_1 and TrE_1.

Long range PCR assays involving separate primer pairings (*i*) aac6'-Ib F / aac3-IIa R, (*ii*) aac3-IIa F / CTX-M-1 M13L, or (*iii*) aac6'-Ib F / CTX-M-1 M13L, suggested *aac3-IIa* was located downstream of *aac(6')-Ib-cr* and upstream of $bla_{CTX\text{-}M\text{-}15}$ in TrD_1. Also, while no amplicon was obtained for TrD_1 using the primer pair aac6'-Ib F / CTX-M-1 M13L (maybe due to the fact that the DNA segment was too long to be amplified, as a result of the insertion of *aac*3-*IIa*), a *ca.* 3-kb DNA fragment was amplified from TrA_1.

Identification of potential *aac*6'-*Ib-cr* variant by PCR-RFLP:

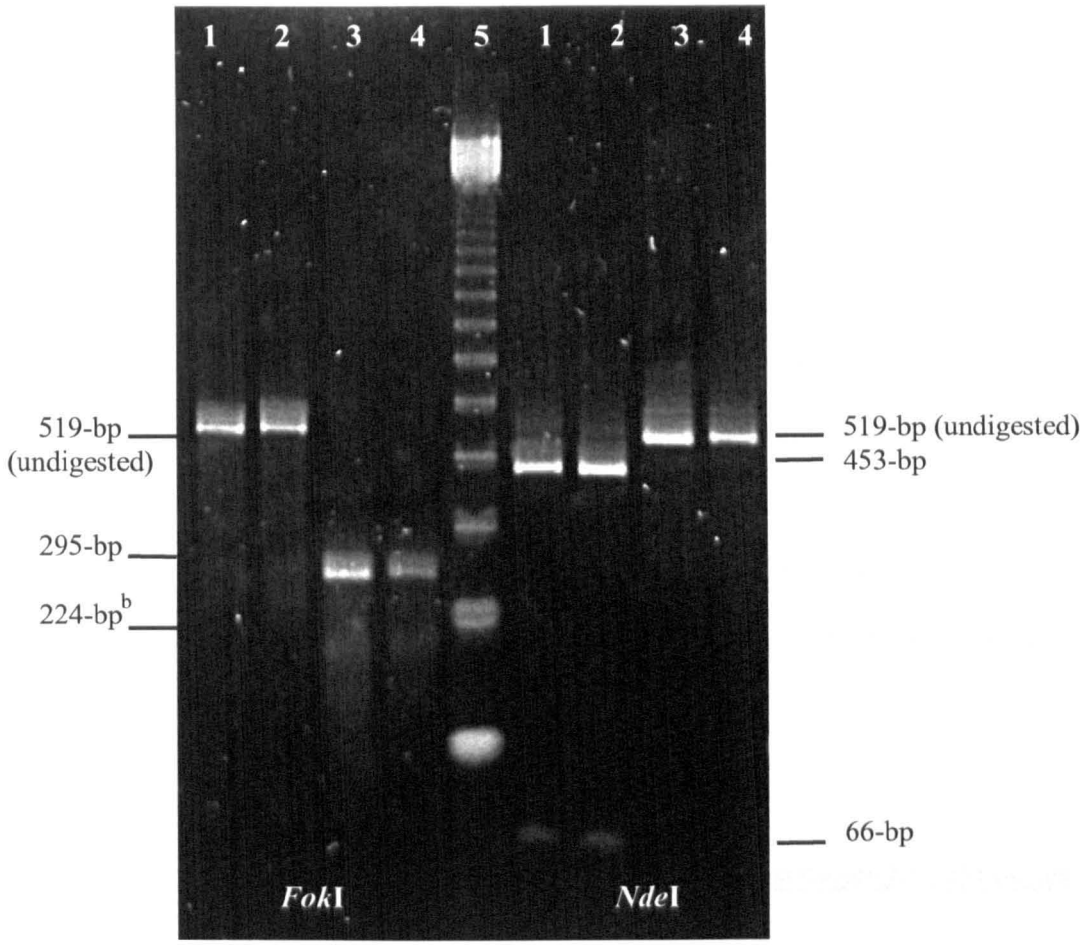
In order to differentiate rapidly potential *aac*6'-*Ib-cr* from classical *aac*6'-*Ib* alleles [approximately 30 variants have been described to date (Robicsek, A. *et al.*, 2006)], a PCR-RFLP using the two restriction enzymes *Fok*I and *Nde*I was developed.

A portion of the *aac*6'-*Ib* gene, which includes the two nucleotides prone for mutations, was first amplified with the primer pair aac(6`)-Ib CR-2F / aac(6`)-Ib CR-2R (see Appendix 6), yielding a 519-bp DNA fragment. Only two possible restriction profiles then resulted from the double digestion of the PCR product using the two restriction enzymes mentioned above (Figure 20):

- *aac(6')-Ib-cr* profile: *Nde*I digested only *aac(6')-Ib-cr* amplicons, yielding two fragments of 453-bp and 66-bp, while *Fok*I failed to digest.
- non-*aac(6')-Ib-cr* profile: *Fok*I digested only the native *aac(6')-Ib* amplicons, yielding two fragments of 224-bp and 295-bp, while *Nde*I failed to digest.

Finally, the resistance of transformants TrA_1, TrB_1, TrD_1 and TrE_1 to tetracycline was attributable to the presence of the *tet*(A) gene, which encodes an efflux pump. The *tet*(B) gene was absent from these transformants based on PCR.

Figure 20. PCR-RFLP assays to distinguish *aac(6')-Ib-cr* from classical *aac(6')-Ib* variants[a]



[a] 1-2, *aac(6')-Ib-cr*; 3-4, classical *aac(6')-Ib* variants; 5, 123-bp ladder.

[b] The 224-bp band resulting from *Fok*I restriction of non-*aac(6')-Ib-cr* alleles appeared only very weakly in every repeated experiment.

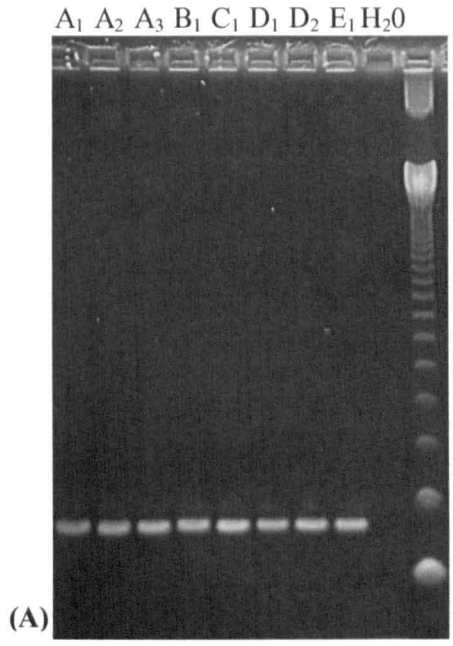
6. Detection of integrons in clone A-E-derived transformants

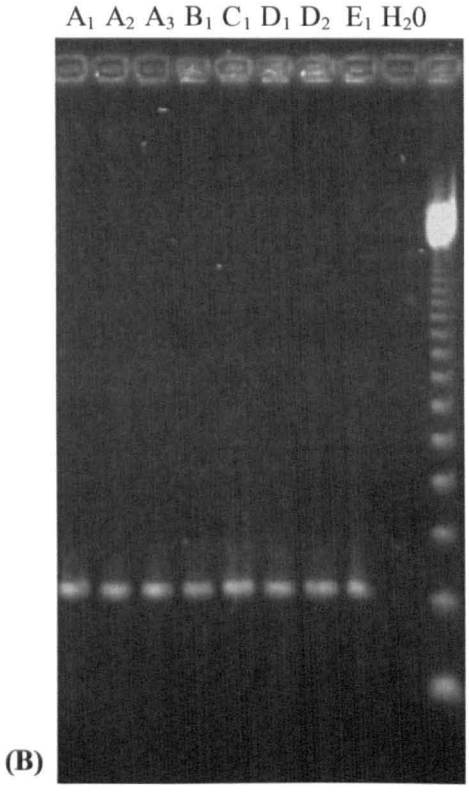
Integrons, which frequently carry antibiotic resistance gene cassettes, were first sought by PCR among all eight transformants using primers specific for *int1*, *int2* and *int3* (type I-III integrase, see Appendix 6). A class I integrase gene was detected in the three clone A-derived transformants only (TrA_1-TrA_3). Neither type II, nor type III integrases were present on the $bla_{CTX\text{-}M}$-encoding plasmids from clones A-E, based on PCR assays. Using the primer pair 5'CS / 3'CS (specific to the 5' and 3' conserved sequences of class I integrons, respectively, see Appendix 6), it was shown that the length of the integron was consistent among all three clone A-derived transformants investigated (*ca.* 1.8-kb). DNA sequencing identified the streptomycin and trimethoprim resistance gene cassettes *aadA5* and dfr_{17}, in all three cases, whilst the presence of *sulI* at the 3' end conserved sequence of the integron explained the higher sulphamethoxazole MIC for TrA_1 (see Table 10, sulphamethoxazole MICs for TrA_2 and TrA_3 were not determined, but both had *sulI* as shown by DNA sequencing).

7. Molecular characterisation of high-level resistance to quinolones

Epidemic clones A-E isolates required ciprofloxacin MICs >8 mg/L. Such levels of resistance to quinolones is not explainable by *aac(6')-Ib-cr* alone, suggesting the presence of multiple chromosomal mutations in the genes encoding DNA gyrase and topoisomerase IV, or, maybe efflux and permeability changes (Robicsek, A. *et al.*, 2006). The quinolone resistance determining regions (QRDRs) of the *gyrA* (190-bp), *parC* (265-bp) and *parE* (265-bp) genes (not *gyrB,* since alterations in this gene are rarely associated with quinolone resistance) from each representative isolates A_1-A_3, B_1, C_1, D_1-D_2 and E_1 were therefore sequenced on both strands. The three amplicons (shown in Figure 21) were 100% identical for all eight representative isolates, harbouring the same multiple nucleotide changes when compared with the fluoroquinolone-susceptible *E. coli* K-12 (GenBank accession NC_000913). These substitutions, some of which were silent, are summarised in Table 13. The double amino acid substitutions in the QRDRs of *gyrA* and *parC* had been described previously, though independently, and are associated with high-level resistance to fluoroquinolones (Everett, M. J. *et al.*, 1996).

Figure 21. Agarose gel electrophoresis of *gyrA* (A, 190-bp amplicon), *parC* (B, 265-bp amplicon) and *parE* (C, 265-bp amplicon) QRDRs PCR products for representative isolates of epidemic clones A-E with a CTX-M-15-like β-lactamase





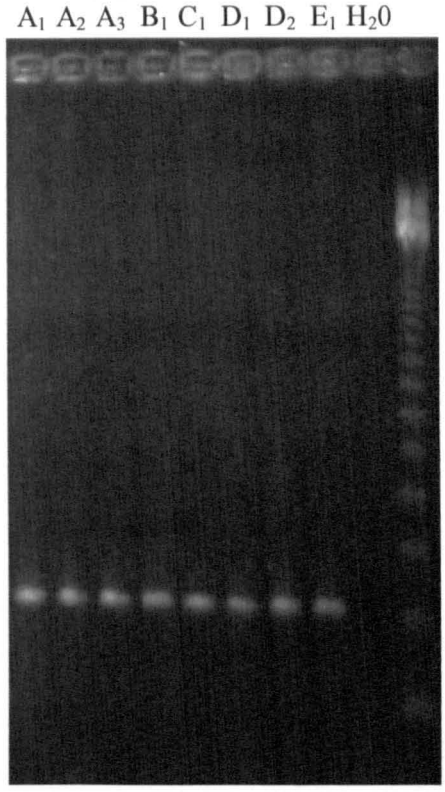


Table 13. Summary of mutations in the quinolone resistance determining region of *gyrA*, *parC* and *parE* genes of epidemic clones A-E representative isolates with CTX-M-15-like β-lactamase

Gene	Nucleotide substitutions	Amino acid substitutions
gyrA	C249T	Ser83Leu
	C256T	Silent mutation
	G260A	Asp87Asn
	C274T	Silent mutation
	T301C	Silent mutation
parC	G240T	Ser80Ile
	A252T	Glu84Val
parE	G1403C	Silent mutation
	C1431T	Silent mutation
	T1448C	Silent mutation
	A1454G	Silent mutation
	C1466G	Silent mutation
	G1475T	Silent mutation

8. Relatedness of *bla*$_{CTX-M}$-encoding plasmids from epidemic clones A-E

8.1. Plasmid incompatibility grouping

According to replicon-based PCR assays, the CTX-M-encoding plasmids extracted from transformants TrA_1-TrE_1 harboured the F-plasmid origin of replication [*repF*, Figure 22(A)] indicative of incompatibility group IncFII. Plasmids from A_1, B_1 and C_1 also carried a second replicon; *repFIA*, which was not present on the CTX-M-encoding plasmids from the clone D and E representatives tested [Figure 22(B)]. This additional replicon did not affect the grouping of the plasmids and all were typed as IncFII, thus sharing a common origin. The assigned incompatibility groups were further confirmed by Dr A. Carattoli (Istituto Superiore di Sanità, Rome, Italy).

8.2. Investigation of plasmid size variations among representative clone A isolates

The slight variations in sizes of the *bla*$_{CTX-M}$-harbouring plasmids from different representatives of clone A (Figure 17), added to the fact that not all of these plasmids encoded TEM-1 β-lactamase (Table 12), led me to undertake comparative studies by restriction analysis, as well as cross-hybridization studies using the *Hpa*I-digested Dig-labelled pEK499 probe (CTX-M-encoding plasmid of representative A_1). The CTX-M-encoding plasmids from five strain A representative isolates (A_1-A_5) were investigated for their degree of similarity. As shown in Figure 17, the *bla*$_{CTX-M}$-harbouring plasmids from isolates A_1 and A_4 appeared to be slightly smaller compared with those from representatives A_2, A_3 and A_5. Moreover, only isolates A_1 and A_3 had been shown to harbour *bla*$_{TEM-1}$. There was therefore no correlation between the presence or absence of *bla*$_{TEM-1}$ and the plasmid size. Although the *Hpa*I restriction profiles of the five plasmids appeared highly similar, none were completely identical [Figure 23(A)]. The plasmid profiles from isolates A_1 and A_3, which both produced TEM-1 β-lactamases, had no additional band in common compared with the plasmid profiles of isolates lacking TEM-1; furthermore, the restriction profiles of plasmids from isolates A_1 and A_4 were not the most related, even though both were smaller than the remaining three plasmids.

Figure 22. Agarose gel electrophoresis of *repF* (A), and *repFIA*, *repFIB* and *repW* (B) PCR products of representative isolates A_1-E_1

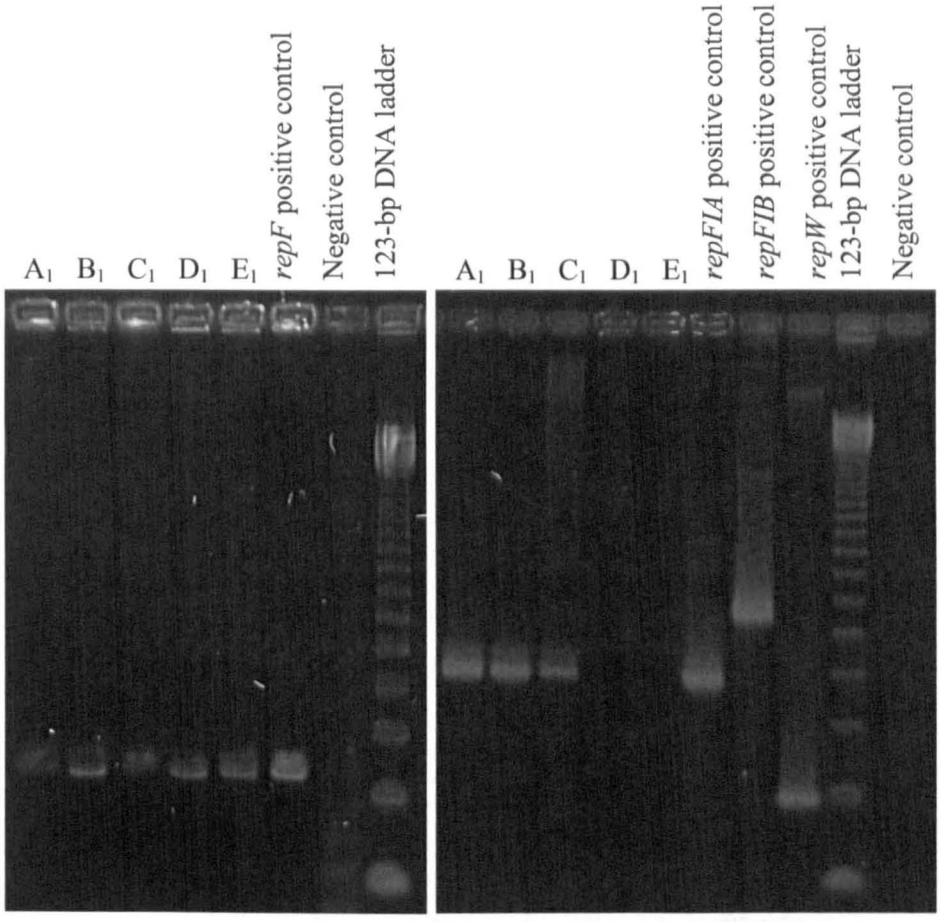
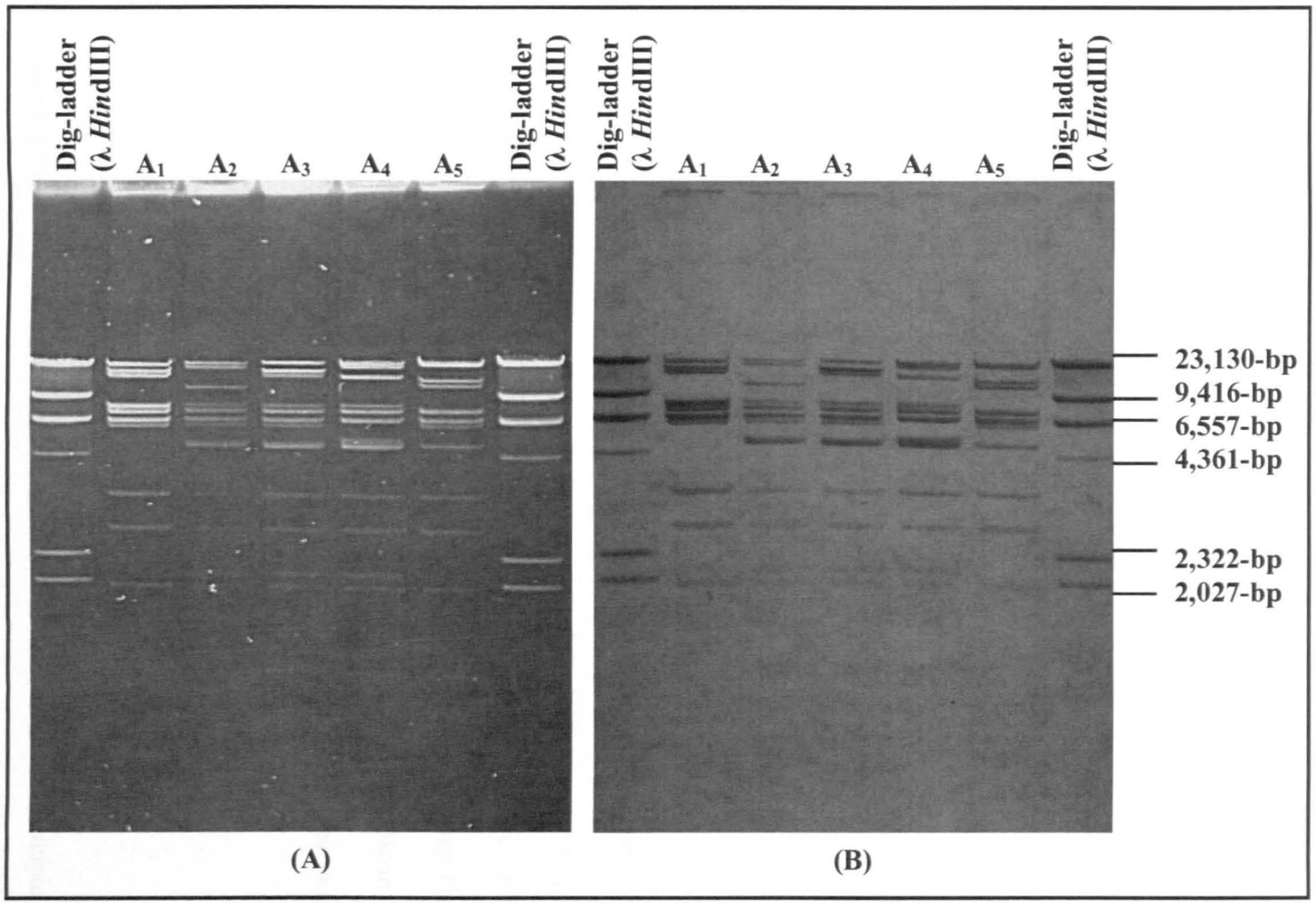


Figure 23. (A) *Hpa*I restriction profiles of the $bla_{CTX\text{-}M\text{-}15}$-harbouring plasmids from isolates A_1-A_5[a], and (B) Southern blot hybridized with dig-labelled *Hpa*I-digested plasmid pEK499 from TrA_1



[a] A_1 = Dig-labelled *Hpa*I-digested pEK499 probe.

Overall, plasmid digests from isolates A_3 and A_4 appeared the most similar and that from A_1 (pEK499, reference plasmid) showed the least similarity to the remaining four profiles. Cross hybridization of each plasmid restriction profiles with the Dig-labelled digested pEK499 [Figure 23(B)] suggested all five plasmids shared a high degree of similarity; every band of each profile hybridized with the probe. Therefore, although the CTX-M-encoding plasmids from various representatives of epidemic clone A presented some minor variations, all appeared highly related. The small variations (*e.g.* loss or gain of bla_{TEM-1}) probably occurred subsequent to the initial spread of clone A isolates.

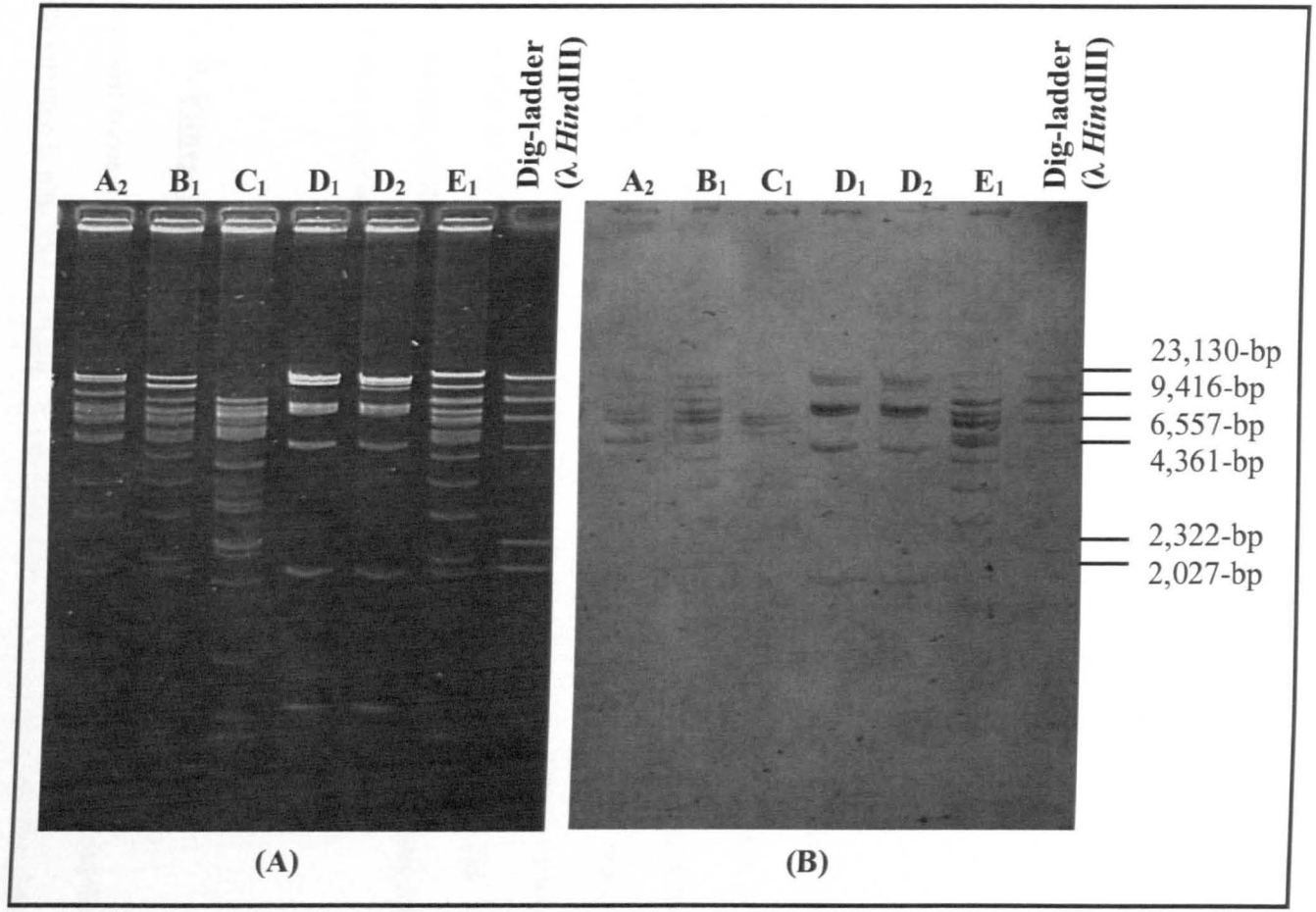
8.3. Homology studies between the plasmids encoding CTX-M enzymes from epidemic clones A-E

In order to estimate the degree of similarity between the CTX-M-encoding plasmids from epidemic clones A-E, plasmids from representative isolates were subjected to *Hpa*I restriction (plasmid RFLP) and cross-hybridization with the Dig-labelled *Hpa*I-digested pEK499 probe (reference plasmid from isolate A_1). Also, since these plasmids belonged to incompatibility group IncFII, a specific part of their *repF* replicon (intervening in the sub-classification of IncFII plasmids, Dr Carattoli, A. personal communication) was sequenced in order to further estimate their degree of relatedness.

8.3.1. Cross-hybridization studies

The CTX-M-encoding plasmids from representatives of clones A, B, D and E had multiple bands in common by size [Figure 24(A)], suggesting therefore some degree of relatedness. On the other hand, the plasmid profile from the clone C representative appeared significantly different. This plasmid, which harboured $bla_{CTX-M-3}$ instead of $bla_{CTX-M-15}$, and encoded less antibiotic resistance compared with the other four plasmids (*e.g.* no resistance to aminoglycosides, nor reduced susceptibility to ciprofloxacin, Table 10), had considerably more restriction fragments. None, or very few of these fragments were shared with the plasmids extracted from clone A, B, D and E representative isolates.

Figure 24. (A) HpaI restriction profiles of the CTX-M-encoding plasmids from representatives of E. coli epidemic clones A-E, and (B) Southern blot hybridized with dig-labelled HpaI-digested plasmid pEK499 from TrA_1



The *HpaI* restriction profiles of the CTX-M-encoding plasmids from isolates D_1 and D_2 were identical, as might be expected since all members of this strain were from a single centre (Shrewsbury). Cross-hybridization with the Dig-labelled pEK499 probe [Figure 24(B)] supported these conclusions, showing a high-level of similarity and relatedness between the plasmids of clones A, B, D and E. Most, or all of the restriction fragments of these plasmids hybridized with the clone A reference plasmid probe. In contrast, only a few restriction fragments from the clone C plasmid hybridized with pEK499, suggesting much less relatedness. However, since the clone C plasmid also belonged to incompatibility group IncFII and was therefore relatively related to pEK499, it was not surprising that a few bands still hybridized with the probe. Also, the positive hybridization with the Dig-labelled pEK499 may have been due to the fact that both plasmids had a few antibiotic resistance genes in common, including $bla_{CTX\text{-}M}$ and $bla_{TEM\text{-}1}$.

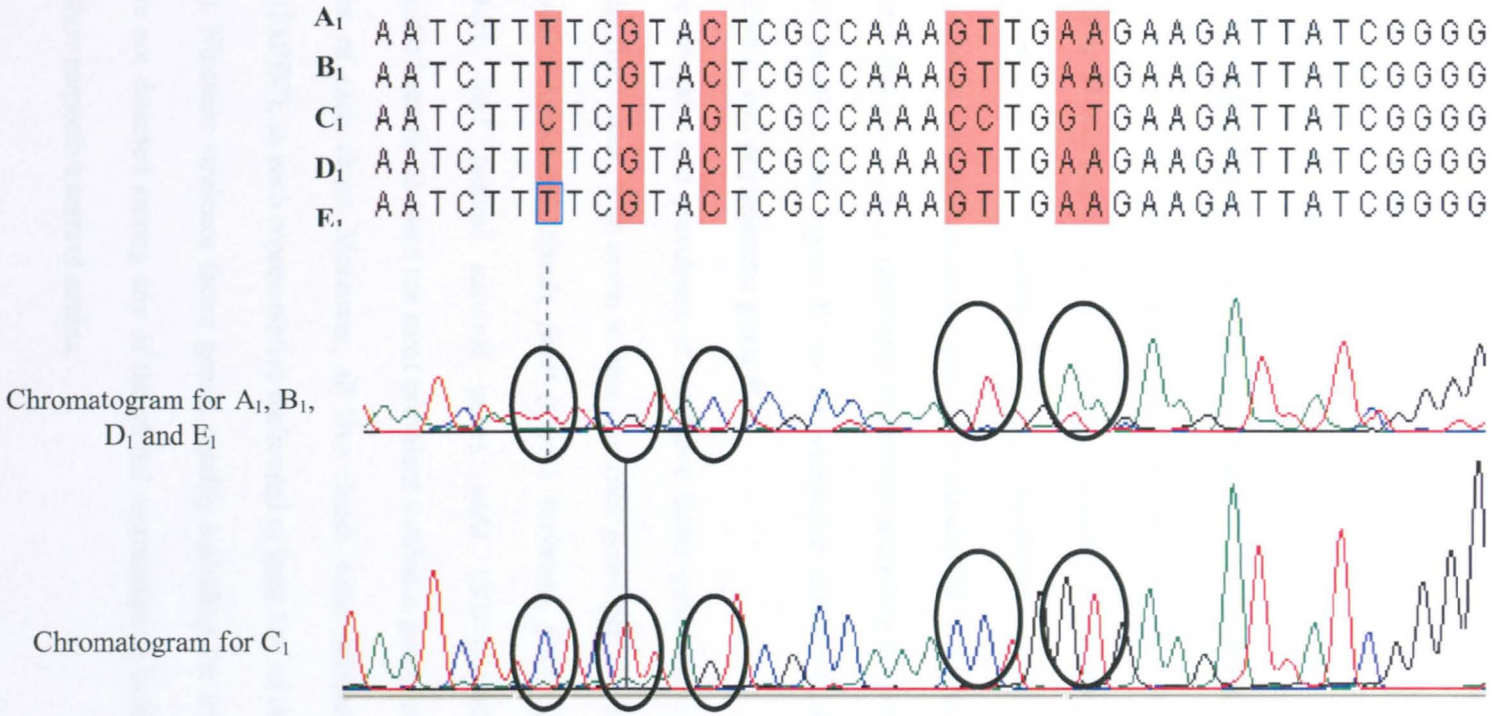
8.3.2. Comparison of *repF* sequences

DNA sequencing of a *c.* 50-bp segment within *repF* revealed that the $bla_{CTX\text{-}M}$-harbouring plasmids from A_1, B_1, D_1 and E_1 were highly related. The DNA sequence of this gene section, which is used in the sub-classification of IncFII plasmids (A. Carattoli and K. Hopkins, personal communication), was identical in all four cases. In contrast, the sequence of this gene fragment was significantly different in the plasmid encoding CTX-M-3 from isolate C_1, presenting seven nucleotide substitutions compared with the sequences from A_1, B_1, D_1 and E_1 (Figure 25). This further confirmed the reduced relatedness of the clone C plasmid to those of the remaining four epidemic clones, even though all belonged to the same incompatibility group.

9. Fitness cost associated with carriage of pEK499

Clone A is the most successful (*i.e.* widespread) *E. coli* lineage with CTX-M-15 enzyme in the UK, and, since this enzyme is plasmid-mediated, it was important to investigate whether harbouring the plasmid was associated with a fitness cost.

Figure 25. Alignment of partial *repF* nucleotide sequences[a] from the bla_{CTX-M}-encoding plasmids of representative isolates A_1-E_1[b]



[a] Relevant to the sub-classification of IncFII plasmids.

[b] Chromatogram colour code: Adenine = green; Cytosine = blue; Guanine = black; Thymine = red; black circles indicate nucleotide changes.

The fitness cost of pEK499 was investigated by comparing the growth rate of *E. coli* DH5α with that of the same strain transformed with pEK499 (TrA_1); the same culture conditions were used in both cases. The growth rates of both organisms were nearly identical (see Figure 26). As a result, although pEK499 was a large molecule (*c.* 120-kb), it was not associated with any major fitness cost under these growth conditions. It is worth noting that pEK499 likewise did not accelerate the growth rate of its host in an antibiotic-free environment.

10. Phylogenetic typing and virulence factor screening

Fourteen clonal *E. coli* clinical isolates (four representatives of clone A, two of clone B, three of clone C, three of clone D and two of clone E) were phylogenetically typed using a previously published method (Clermont, O. *et al.*, 2000) and screened for the presence of 32 virulence factor-associated genes, as well as for a pathogenicity-associated island (PAI, also known as *malX*).

All 14 isolates showed identical phylogenetic profiles according to the triplex PCR (*chuA*-, *yjaA*- and TspE4.C2-positive, see Figure 27 for representative isolates A_1-E_1). This profile is specific for the virulent *E. coli* phylogenetic group B2.

Overall, the diversity and prevalence of virulence factor genes were comparable among representatives of the five clones, with seven to nine virulence genes per isolate on average (Table 14). PAI (Pathogenicity-Associated Island), *fimH* (type 1 fimbriae), *fyuA* (yersiniabactin), *iutA* (aerobactin receptor), *traT* (serum survival gene), *uidA* (β-D-glucuronidase) and *usp* (uropathogenic-specific bactericin) were the most prevalent virulence genes and were detected in most representatives of each clone. Moreover, all five clones were defined as extra-intestinal pathogenic *E. coli* (ExPEC), as each representative harboured at least two of the five ExPEC gene markers (Table 14). Nineteen virulence factor genes, notably including the toxin-encoding genes *hlyA* and *cnf1*, were not detected among any of the clonal representative isolates (Table 14), but were confirmed in their respective control strains.

Figure 26. Logarithmic representation of *E. coli* DH5α and TrA_1 growth rates over 32 hours

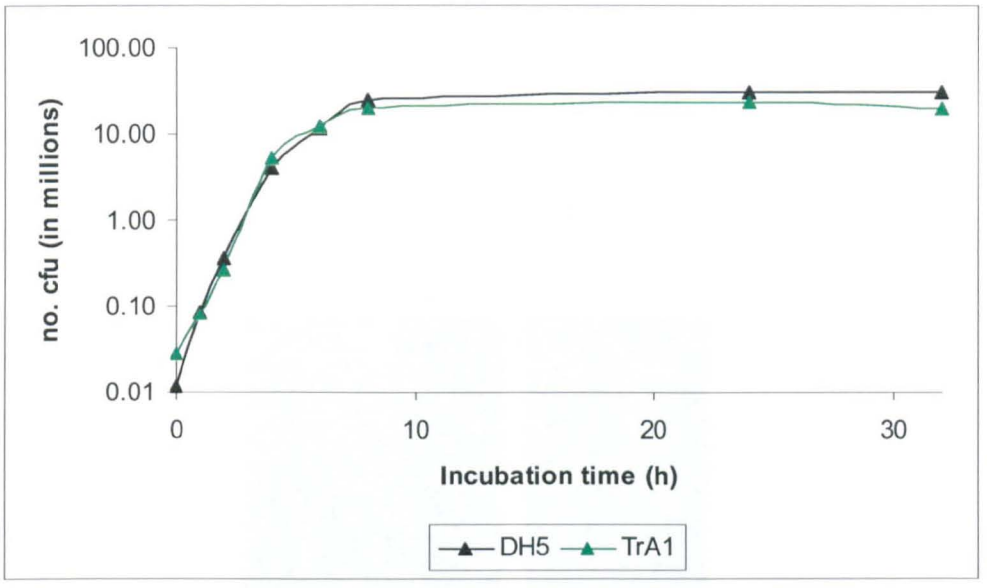


Figure 27. Agarose gel electrophoresis of the phylogenetic typing triplex PCR products of isolates A_1-E_1, taken as representatives of their respective clones.

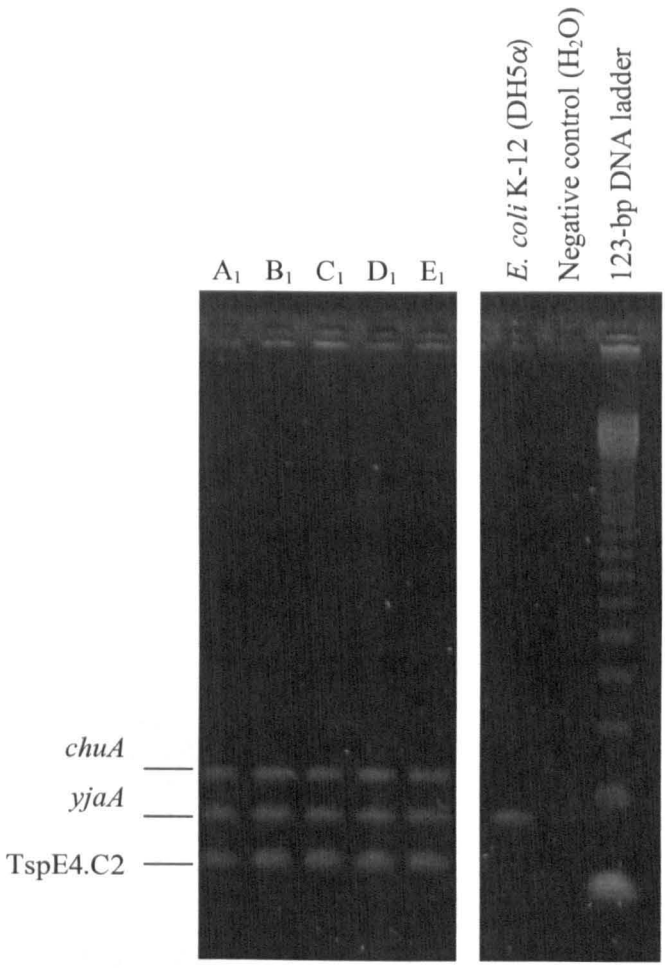


Table 14. Virulence factor gene repertoires[a] of epidemic clones A-E representatives[b]

	Representatives	PAI	*papA*	*fimH*	*kpsMT III*	*papEF*	*ibeA*	*fyuA*	*bmaE*	*sfa/focDE* *iutA*	*papG allele III*	*K1*	*hlyA*	*rfc*	*nfaE*	*papG allele I*	*kpsMT II* *papC*	*gafD*	*cvaC*	*cdtB*	*focG*	*traT*	*papG allele II*	*papG I*	*papG II, III*	*afa/draBC*	*cnf 1*	*sfaS*	*K5*	*ompT*	*uidA*	*usp*
Clone A	A_1			+				+		+												+				+					+	+
	A_2	+		+				+		+												+				+					+	+
	A_3	+		+				+		+												+				+					+	+
	A_4	+		+				+		+												+				+					+	+
Clone D	D_1	+	+	+		+		+		+														+							+	+
	D_2	+	+	+		+		+		+														+							+	+
	D_3	+	+	+		+		+		+														+							+	+
Clone B	B_1			+				+		+							+					+					+		+		+	+
	B_2	+		+				+		+							+												+		+	+
Clone C	C_1			+				+		+							+					+					+		+		+	+
	C_2	+		+				+		+							+												+		+	+
	C_3			+				+		+							+					+					+		+		+	+
Clone E	E_1			+				+		+							+					+					+		+		+	+
	E_2			+				+		+							+					+					+		+		+	+

[a] Key virulence marker genes of ExPEC isolates are highlighted in grey.
[b] + indicates the presence of the corresponding virulence gene.

Based on the key ExPEC markers, three groups of isolates were distinguishable:

(i) Clone A group: isolates harboured *iutA* and *afa/draBC*, but not *papA* nor *kpsMT II*.

(ii) Clone D group: isolates harboured *iutA* and *papA*, but not *afa/draBC* nor *kpsMT II*.

(iii) Clones B-C-E group: isolates harboured *iutA* and *kpsMT II*, but not *afa/draBC* nor *papA*.

Such division of isolates was further enhanced based on the whole set of virulence factor genes screened. While *afa/draBC* was specific to clone A, *papA* and *papEF* were detected in clone D isolates only, and only representatives of clones B, C and E harboured the *kpsMTII*, *sfaS* and *K5* genes. Minor variations were detected within these three groups (*e.g.* for PAI among clone A isolates). This probably was due to loss of DNA from the genome, as shown previously among fluoroquinolone-resistant *E. coli* isolates (Horcajada, J. P. *et al.*, 2005; Soto, S. M. *et al.*, 2006).

11. Further evolution of antibiotic resistance in epidemic clone A

Third-generation cephalosporin/clavulanate combinations retained good activity *vs.* most strain A isolates. However, some clone A isolates from around Preston have acquired a CIT-type plasmid-mediated AmpC β-lactamase (CMY-23), increasing their level of resistance to all β-lactams, except carbapenems (Woodford, N. *et al.*, 2007b). These isolates showed resistance to cephalosporin/clavulanate combinations. In November 2006, a further variant of clone A, also from Preston, and with ertapenem resistance (MIC = 4 mg/L) was referred to ARMRL. PFGE confirmed the isolate, designated A_{12}, was a clone A variant (Figure 28). In addition to bla_{CTX-M}, a bla_{CIT} gene was also detected by PCR in this isolate (Figure 29). A_{12} was isolated from a catheter urine specimen from an 86-year-old female nursing-home resident. She had had a catheter in situ for over four years, but had no recent hospital admissions and minimal exposure to antibiotics, with just one course of oral cephalexin in the preceding 12 months. No other resident had received carbapenems in the home, though they may have done so during periodic hospitalisations. Isolate A_{12} was resistant to most β-lactams, including cephalosporin/clavulanate combinations, to fluoroquinolones, and to aminoglycosides except gentamicin (Table 15).

Figure 28. ***Xba*I-digested genomic DNA profiles of isolates A_1 and A_{12}**

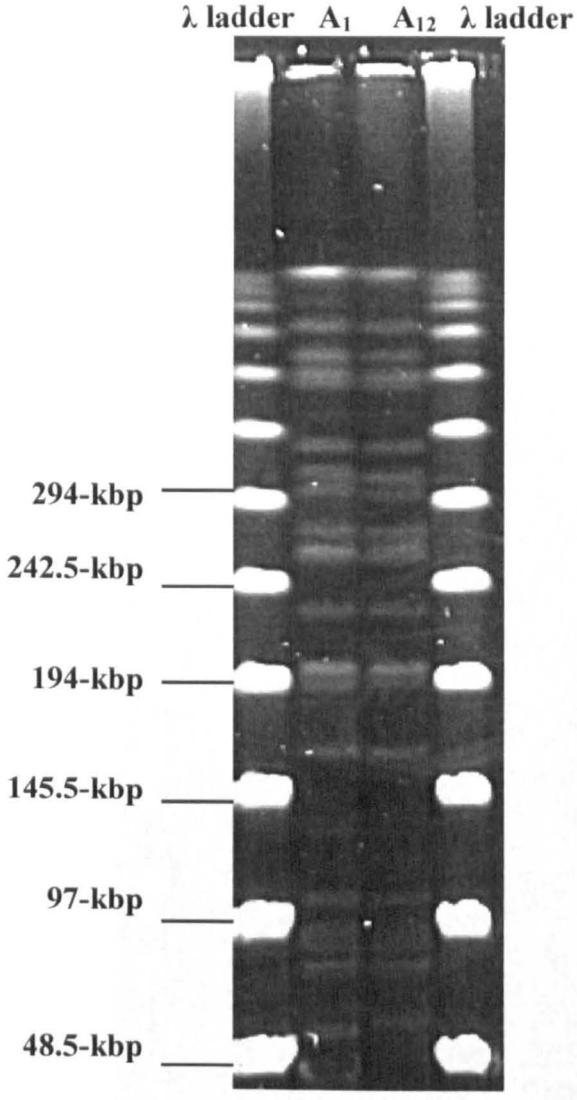


Figure 29. Agarose gel electrophoresis of *bla*$_{CIT}$ and *bla*$_{CTX\text{-}Muniversal}$ PCR products of clinical isolates A_{12} and A_{13}

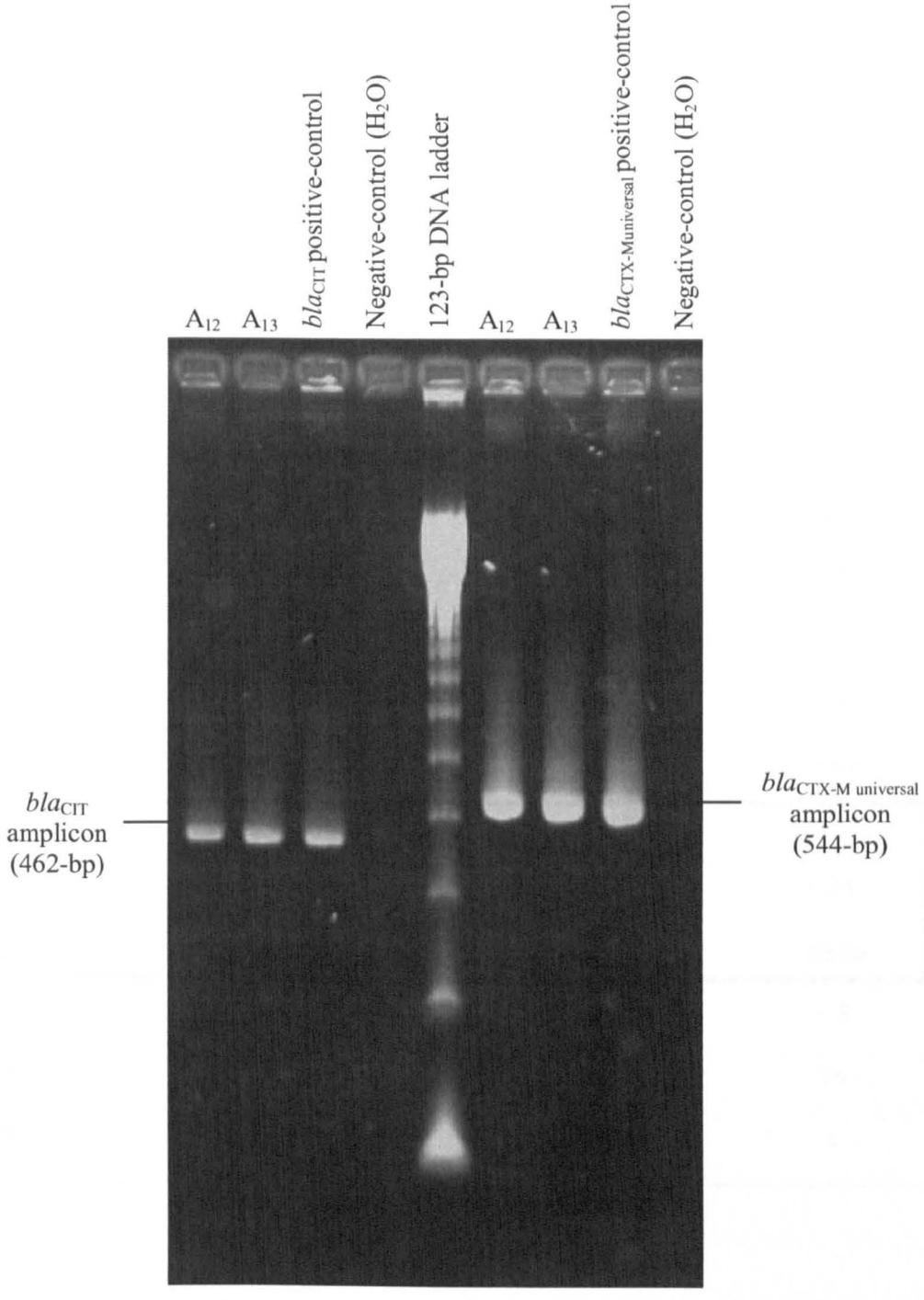


Table 15. MICs (mg/L) for *E. coli* strain A variants A_1, A_{12} and A_{13}

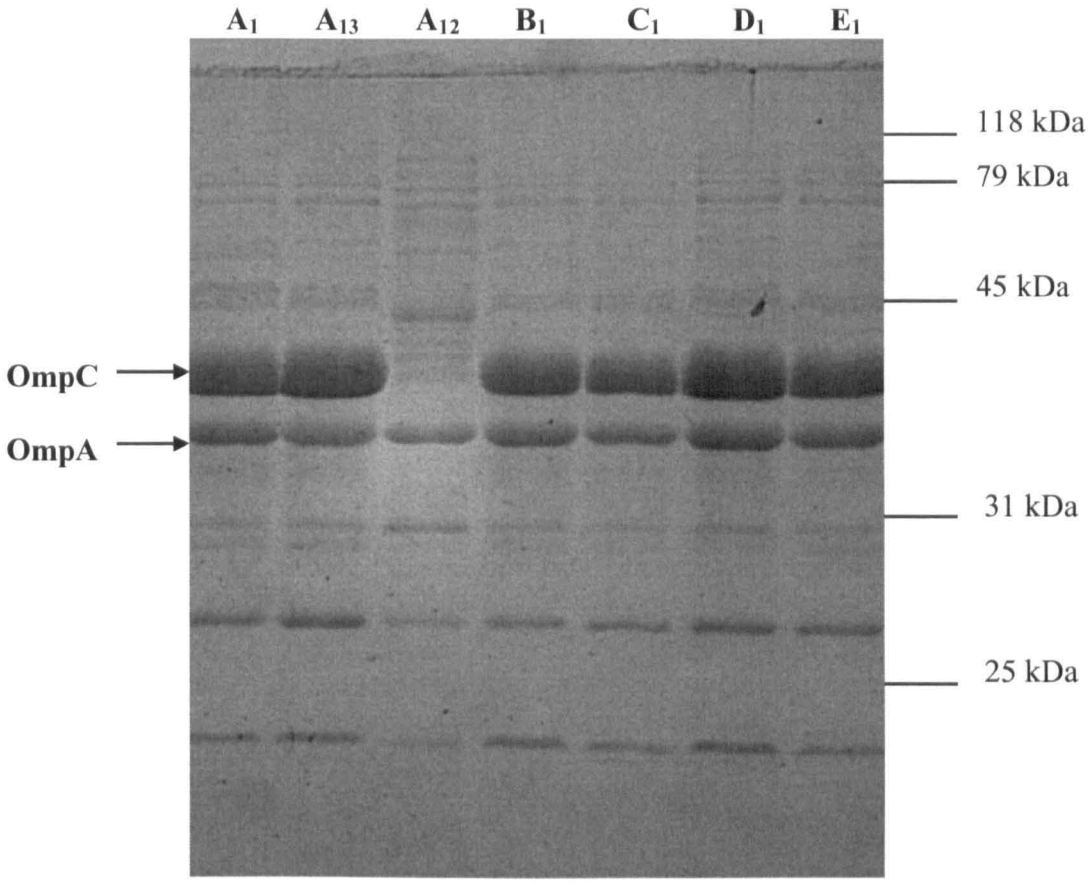
Features	Isolate A_1	Isolate A_{12}	Isolate A_{13}
	CTX-M-15, OXA-1 and TEM-1 OmpC present	CTX-M-15, CMY-23, OXA-1, TEM-1 OmpC absent	CTX-M-15, CMY-23, OXA-1 and TEM-1 OmpC present
AMP	> 64	> 64	> 64
AMX / CLA	16	32	64
CTX	64	64	32
CTX / CLA	0.25	> 32	16
CAZ	8	32	16
CAZ / CLA	0.25	16	16
FOX	8	> 64	> 64
PIP	> 64	> 64	> 64
PIP / TZM	16	64	> 64
ETP	≤0.125	4	≤0.125
IPM	0.125	1	0.25
MEM	≤0.06	0.25	≤0.06
CIP	> 8	> 8	> 8
AMK	16	8	16
GEN	1	1	1

Susceptibility to imipenem and meropenem also was reduced compared with classical *E. coli* strain A isolates such as A_1, and with CMY-23-positive strain A variants, such as A_{13} (Table 15). Carbapenem resistance, which may arise from the association of permeability changes with production of ESBLs or AmpC enzymes, is rarely observed in *E. coli* isolates (Lartigue, M. F. *et al.*, 2007). Also, production of carbapenemase enzymes by A_{12} was very unlikely according to the MICs (imipenem and meropenem MICs not being affected). The outer membrane protein profile of isolate A_{12} was investigated and compared with those of other clone A representatives, as well as with representatives of the other four epidemic clones. SDS-PAGE of bacterial cell extracts (Figure 30) showed loss of OmpC in isolate A_{12} by contrast with isolate A_1, A_{13} and representatives of clones B-E, which all had identical outer membrane protein profiles. OmpF was not detectable on the SDS-PAGE since its expression was not induced under the low osmolarity cultural conditions used (H. Chart, Laboratory of Enteric Pathogens, Centre for Infections, personal communication). OmpA was present in all seven clinical isolates. Ertapenem resistance in isolates A_{12} may therefore be explained by the change in permeability (loss of OmpC), together with the production of acquired AmpC and CTX-M-15 enzymes.

12. Summary

The multi-drug resistance phenotypes of the five major UK epidemic *E. coli* clones were largely encoded by single plasmids harbouring the bla_{CTX-M} gene. These molecules were found to be self-transferable (except for that of epidemic clone A) and to share a range of additional antibiotic resistance genes such as bla_{OXA-1}, bla_{TEM-1}, *aac3-IIa* (except clones A and C) or *aac(6')-Ib-cr* (except clone C). The CTX-M-encoding plasmids from all five clones belonged to incompatibility group IncFII, and most shared high-level homology according to cross-hybridization studies. The representative clone C plasmid however appeared less related to the remaining four; it also harboured fewer antibiotic resistance genes and carried $bla_{CTX-M-3}$ instead of $bla_{CTX-M-15}$. It is nevertheless worth mentioning that some clone C representative isolates (not part of the molecular characterisation study, but undistinguishable by PFGE) required high ceftazidime MICs, suggesting they possibly encoded CTX-M-15 β-lactamase, and not CTX-M-3.

Figure 30. Outer membrane protein profiles of *E. coli* strain A variants A_1, A_{12}, A_{13} and of representative isolates of epidemic clones B-E



High-level resistance to quinolones resulted from the same multiple nucleotide substitutions in all members of the five epidemic clones. Finally, clone A-E isolates belonged to the virulent extra-intestinal phylogenetic group B2. Although similar, three virulence factor profiles were distinguishable, one each for clone A, clone D and the cluster comprising clones B, C and E.

Chapter II. Molecular characterisation of non-clonal *Escherichia coli* isolates with CTX-M-15-like beta-lactamases

1. Introduction

Non-clonal *E. coli* isolates with CTX-M-15-like β-lactamases represented the majority of CTX-M-positive isolates referred to ARMRL, according to PFGE data (approximately 70% of all referred isolates). While not of major concern at the national level, most of these isolates remained a challenge on a smaller scale, often associated with local outbreaks (*e.g.* around Truro). Furthermore, the fact that these diverse CTX-M-producers outnumbered clonal *E. coli* isolates with group-1 CTX-M enzymes further emphasised their significant contribution to the epidemiological success of CTX-M extended-spectrum β-lactamases in the UK.

Non-clonal *E. coli* isolates with CTX-M-15-like enzymes were multi-drug resistant (Table 16) and had similar antibiograms to those of epidemic clone A-E isolates. High-level resistance to both cefotaxime and ceftazidime, with synergy between clavulanate and third-generation cephalosporins was consistent with the possible production of CTX-M-15 β-lactamase (although rarer group-1 CTX-M variants such as CTX-M-23 and CTX-M-42 may also confer this phenotype). The vast majority (*c.* 90%) of non-clonal isolates were highly resistant to ciprofloxacin (MIC >8 mg/L), and *c.* 75% were resistant to at least one aminoglycoside antibiotic (gentamicin or amikacin); resistance to gentamicin (*c.* 75% of isolates) was more common than resistance to amikacin (*c.* 20% of isolates). However, no isolate was fully susceptible to amikacin, and nearly all required amikacin MIC ≥ 2 mg/L. Those genetically diverse isolates were mostly resistant to trimethoprim too, and had reduced susceptibility to minocycline, according to CLSI guidelines (no BSAC breakpoint being defined). As for the clonal isolates, the diverse producers remained completely susceptible to carbapenem antibiotics; most were also susceptible to nitrofurantoin and to fosfomycin.

Table 16. MIC ranges and geometric mean MICs (mg/L) for non-clonal *E. coli* isolates with CTX-M-15-like β-lactamases (n = 242 isolates)

	R>[a]	MIC range	Geom. Mean MIC
AMP	16	64 - >256	69
AMX / CLA	16	32 - >64	26.5
CTX	1	32 - >256	66.6
CTX / CLA	1	≤0.06 - 4	0.1
CAZ	2	1 - 128	57
CAZ / CLA	2	≤0.06 - 2	0.3
CPD	1	256	256
CPD / CLA	1	1 - 2	1.9
FOX	8	4 - 64	14.9
PIP	16	64 - >256	69.2
PIP / TZB	16	2 - >256	39
ETP	2	≤0.125 - 1	0.1
IPM	4	≤0.06 - 1	0.1
MEM	4	≤0.06 - 0.25	0.1
CIP	1	≤0.125 - >256	7
AMK	16	1 - 64	7
GEN	4	0.25 - 128	30
MIN	8	0.5 - >32	6.3
COL	4	≤0.5 - 8	0.7
TMP	2	≤0.25 - >256	47
NIT	32	4 - 256	23.5
FOS	128	0.5 - 256	2.3

[a] BSAC breakpoints defining resistance (Andrews, J. M., 2007)

By interpretative reading of the antibiograms, it was inferred that the same mechanisms of resistance, as seen in epidemic clones A-E, were likely to be involved in the non-clonal isolates; *i.e.* *aac(6')-Ib* (tobramycin resistance), *aac3-IIa* (gentamicin resistance), multiple chromosomal mutations in the QRDRs of *gyrA* and *parC* (ciprofloxacin resistance), in addition to the $bla_{CTX\text{-}M\text{-}15}$ gene.

On this basis, β-lactamases from various randomly-selected isolates were characterised by IEF, and the presence and identity of *bla* genes were confirmed by PCR. Transfer of cefotaxime resistance by electroporation from six non-clonal CTX-M-15 β-lactamase-producers (requiring MICs representative of the non-clonal isolates) to *E. coli* DH5α was attempted to confirm whether $bla_{CTX\text{-}M\text{-}15}$ was plasmid-mediated (at least in those isolates). Additionally, various features of CTX-M-encoding plasmids, such as plasmid maintenance systems and origins of replication, were sought by PCR among representative isolates. Finally, the phylogenetic background and the virulence genotypes of a representative sample were also investigated.

2. Transfer of cefotaxime resistance

Transfer of cefotaxime resistance via electroporation was achieved successfully from all six clinical isolates (designated NC_1, NC_{13}, NC_{14}, NC_{16}, NC_{21} and NC_{34}), representing the diverse *E. coli* producing CTX-M-15-like β-lactamase. The transfer of $bla_{CTX\text{-}M}$ was confirmed by PCR and by antibiotic susceptibility testing by agar dilution. MICs for the recipient, the donor strains (NC), as well as the transformants (TrNC) are shown in Table 17.

MICs for the selected donor isolates were representative of those for non-clonal isolates in general, and appeared very similar to those for the clonal isolates (*e.g.* CTX-M phenotype, multi-drug resistance, carbapenem susceptibility, variable susceptibility to aminoglycosides). MICs for the cefotaxime-resistant transformants revealed that additional mechanisms of antibiotic resistance were transferred along with the CTX-M phenotype. These included mechanisms of resistance to gentamicin (except for $TrNC_{16}$) and to tobramycin, as well as reduced susceptibility to amikacin and ciprofloxacin.

Table 17. MICs (mg/L) for *E. coli* DH5α, representative non-clonal *E. coli* isolates with CTX-M-15-like β-lactamases, and their transformants

	DH5α	NC_1	$TrNC_1$	NC_{13}	$TrNC_{13}$	NC_{14}	$TrNC_{14}$	NC_{16}	$TrNC_{16}$	NC_{21}	$TrNC_{21}$	NC_{34}	$TrNC_{34}$
AMP	8	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
AMX / CLA	4	16	16	16	16	16	16	16	16	16	16	16	16
CTX	≤0.125	>256	>256	>256	256	>256	256	64	32	256	64	>256	>256
CTX / CLA	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	0.125	≤0.060	≤0.060	≤0.060
CAZ	≤0.125	64	32	32	16	32	32	32	16	32	16	64	32
FOX	4	8	8	8	4	16	4	8	4	8	4	8	8
PIP	≤1	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
PIP / TZB	≤1	8	4	4	4	16	4	8	2	16	4	4	4
ETP	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125
IPM	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
MEM	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060
CIP	0.03	>8	0.125	>8	0.125	>8	0.25	>8	0.125	>8	0.25	>8	0.125
TOB	0.25	>32	16	ND[a]	8	ND[a]	16	>32	8	ND[a]	16	ND[a]	16
AMK	≤0.5	8	4	8	2	4	2	16	2	16	4	4	2
GEN	0.25	>32	32	>32	16	>32	32	1	0.25	>32	32	>32	16
MIN	1	4	4	4	2	4	4	4	2	4	4	2	4
SFM	4	ND[a]	4	ND[a]	4	ND[a]	4	ND[a]	8	ND[a]	8	ND[a]	4
COL	≤0.5	≤0.5	≤0.5	1	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	1	≤0.5	1	≤0.5

[a] ND = not determined

3. Characterisation of β-lactamases by IEF

The β-lactamase enzymes from 12 randomly selected non-clonal isolates (designated NC_1-NC_{12}), most of which originated from different centres across the UK, were characterised by IEF. These isolates were known to produce CTX-M-15 β-lactamases, as previously shown by PCR and DNA sequencing (N. Woodford, personal communication). IEF results are summarised in Table 18.

As with most clonal representatives, the non-clonal isolates investigated in this study produced up to three β-lactamases in various combinations; the approximate pI values for each enzymes were 5.4, 7.4 or 8.6. PCR confirmed these enzymes to be of TEM, OXA-1-like and CTX-M types, respectively and DNA sequencing of a representative of each PCR product identified TEM-1, OXA-1 and CTX-M-15. Most isolates (7/12), and also the NC_1-derived transformant, produced TEM-1, OXA-1 and CTX-M-15 enzymes. Four isolates lacked bla_{TEM-1} and the bla_{OXA-1} gene was absent from one isolate only. None of the 12 isolates produced AmpC type enzymes as no band was inhibited by cloxacillin on the IEF gels.

4. Plasmid characterisation

Since clonal and non-clonal isolates had comparable antibiograms and produced similar β-lactamases according to IEF, it was hypothesised that closely-related multi-resistance plasmids were spreading horizontally among clonal and non-clonal *E. coli* isolates. The features of the CTX-M-encoding plasmids of clone A-E representatives were therefore sought by PCR among the non-clonal representatives. These features included the *aac(6')-Ib-cr* gene, as well as the *repF* and *repFIA* replicons and the plasmid maintenance system *ccdA/ccdB*. All these markers had been detected on pEK499, the $bla_{CTX-M-15}$-encoding plasmid of clonal isolate A_1.

Table 18. Summary of IEF and PCR results for non-clonal representative isolates with CTX-M-15-like enzymes

Isolates	Origin	No. of bands	pI values of β-lactamases[a]			*bla* genes detected by PCR
NC_1	Fairfield	3	5.4	7.4	8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
NC_2	Bristol	2		7.4	8.6	$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
NC_3	Bury	2		7.4	8.6	$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
NC_4	Eastbourne	2	5.4		8.6	bla_{TEM}, $bla_{CTX\text{-}M}$
NC_5	Belfast	3	5.4	7.4	8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
NC_6	Hull	3	5.4	7.4	8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
NC_7	Manchester	3	5.4	7.4	8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
NC_8	Newcastle upon Tyne	2		7.4	8.6	$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
NC_9	Nottingham	2		7.4	8.6	$bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
NC_{10}	Shrewsbury	3	5.4	7.4	8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
NC_{11}	Shrewsbury	3	5.4	7.4	8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$
NC_{12}	London	3	5.4	7.4	8.6	bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{CTX\text{-}M}$

[a] pI of TEM-1 is 5.4; pI of OXA-1 is 7.4; pI of CTX-M-15 is 8.6.

4.1. Detection of *aac(6')-Ib-cr*

MICs for the transformants derived from the non-clonal representative isolates indicated the potential presence of *aac(6')-Ib-cr* on the CTX-M-encoding plasmids as tobramycin resistance (≥2-fold increase in MIC) and reduced susceptibility to amikacin (≥4-fold increase in MIC) and ciprofloxacin (≥4-fold increase in MIC) were co-transferred. This was confirmed by PCR-RFLP and DNA sequencing (Figure 31). The *aac(6')-Ib* allele was sought by PCR using the primer pair aac(6`)-IbF/aac(6`)-IbR (Appendix 6) among a total of 27 non-clonal clinical isolates with CTX-M-15-like β-lactamases and was detected in 24 of them. Both strands of the *aac(6')-Ib* amplicon from ten randomly-selected representatives (including NC_1) were sequenced and the allele always corresponded to the *aac(6')-Ib-cr* variant. Some of these *aac(6')-Ib-cr*-positive isolates dated from year 2000, *i.e.* prior to the first report of this allele variant in *E. coli* isolates from China (Robicsek, A. *et al.*, 2006). The wide occurrence of *aac(6')-Ib-cr* in clonal as well as non-clonal producers of CTX-M-15-like enzymes may reflect the dissemination of closely-related groups of plasmids among these isolates.

4.2. Detection of *repF* and *repFIA* replicons

Since the bla_{CTX-M}-encoding plasmids from representatives of clone A-E harboured *repF*, and in some cases *repFIA* as well, both these replicons were also sought by PCR with the primer pairs FrepB-FW/FrepB-RV (*repF*) and FIA-FW/FIA-RV (*repFIA*) among the 27 non-clonal representatives previously screened for *aac(6')-Ib*. Results are summarised in Table 19. Apart from two isolates, most of the unrelated isolates with CTX-M-15 enzymes investigated harboured the *repF* replicon and were therefore inferred to be carrying IncFII plasmids. Additionally, 17/27 encoded *repFIA*. Both *repF* and *repFIA* replicons were alleged to be harboured by the bla_{CTX-M}-encoding plasmids, as previously found in most of the UK clonal isolates, as well as by many other $bla_{CTX-M-15}$-harbouring plasmids worldwide (Canton, R. and Coque, T. M., 2006). Six of the remaining ten representatives had a modified *repFIA* replicon according to PCR. These amplicons, which appeared much shorter than the usual 462-bp fragment on gel electrophoresis (Figure 32), suggested that the plasmids encoding CTX-M-15 enzymes from those isolates maybe belonged to a different sub-group of IncFII plasmids.

Figure 31. Partial nucleotide sequence and chromatogram of *aac(6')-Ib-cr* from non-clonal clinical isolate NC_1 showing the critical double base substitution

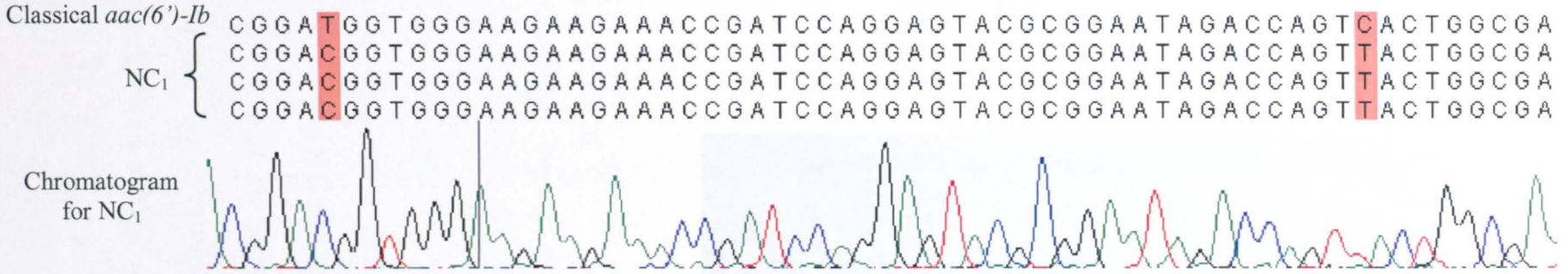
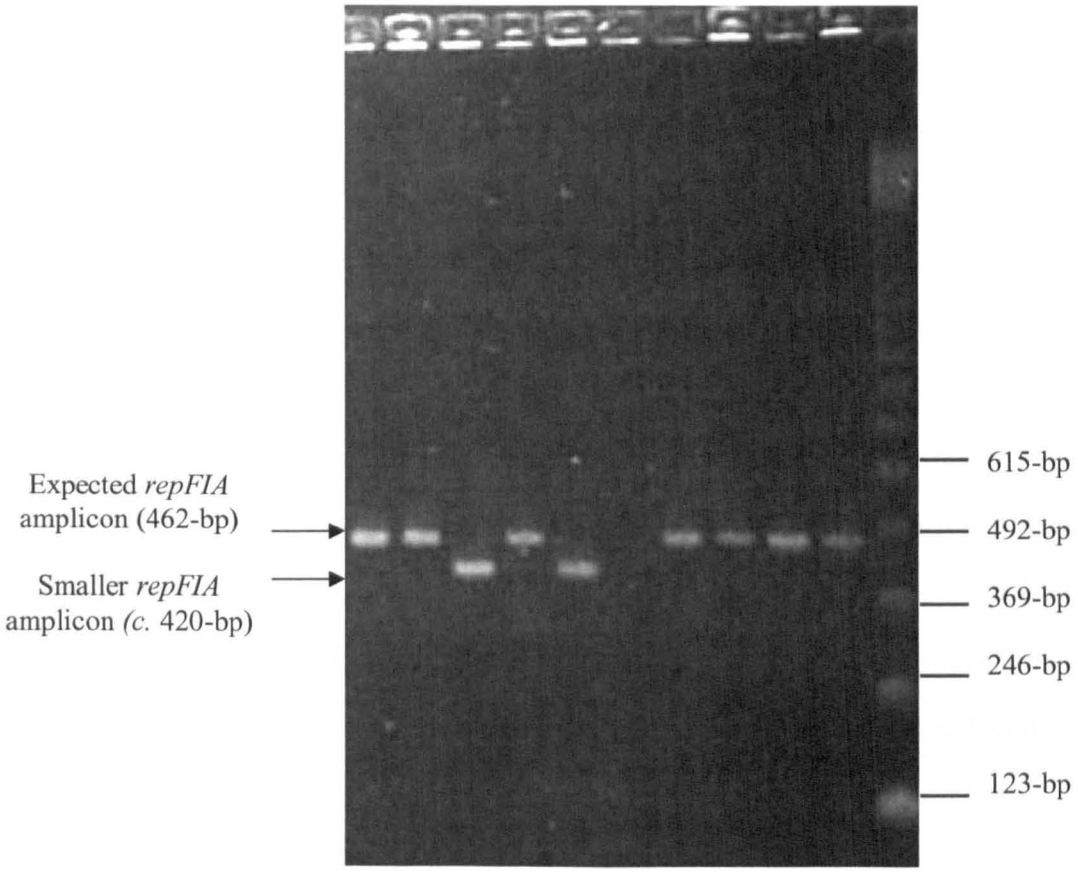


Figure 32. Agarose gel electrophoresis of *repFIA* PCR products from randomly-selected non-clonal isolates with CTX-M-15-like enzyme, showing two different types of amplicon



Among the four *repFIA*-negative isolates, two harboured the *repF* replicon and two lacked the *repF* replicon. This suggested that the CTX-M-encoding plasmids from the two latter isolates did not belong to plasmid incompatibility group IncFII. Interestingly, one of these isolates was shown not to carry the *aac(6')-Ib* gene. This gene was detected in the second *repF*-negative isolate, but it remains unknown whether it corresponded to the *aac(6')-Ib-cr* allelic variant or not (not tested by PCR-RFLP, nor sequenced).

Sequencing of randomly-selected *repF* amplicons:

The 50-bp portion of the *repF* replicon, critical for the classification of IncFII plasmids, was sequenced from six randomly selected diverse producers of CTX-M-15-like enzyme (representatives NC_1, NC_2, NC_{13}, NC_{16}, NC_{21}, and NC33). The *repF* DNA sequence from all six isolates was identical to the corresponding section of *repF* from the CTX-M-encoding plasmids of epidemic clones A, B, D and E (see Figure 25). This therefore suggested that these isolates shared closely related IncFII plasmids (surely, harbouring the same bla_{CTX-M} genes as found in the clonal representatives). Interestingly, some of these plasmids additionally carried a *repFIA* replicon, belonging to either of the two different types, and consequently dividing these IncFII plasmids into at least two different sub-groups.

4.3. Detection of *ccdA/ccdB* plasmid maintenance system

Following the complete DNA sequencing of the CTX-M-15-encoding plasmid of representative clone A isolate A_1 (pEK499), discussed in detail later in Chapter V of the results section, it was found that pEK499 harboured the F-plasmid-derived *ccdA/B* addiction system. This encodes a stable toxin and an unstable antitoxin, militating against plasmid loss (Dao-Thi, M. H. *et al.*, 2002). This key plasmid attribute was therefore also sought by PCR, using the primer pair ccdA/ccdB (Appendix 6), among the 27 unrelated clinical isolates listed in Table 19. The *ccdA/ccdB* plasmid addiction system was found constantly among all the isolates, which harboured an IncFII plasmid (*i.e.* 25/27 isolates), and was absent from the two *repF*-negative isolates.

Table 19. Summary of plasmid features detected among non-clonal representative isolates

	Isolate origin (year of isolation)	*aac(6')-Ib*	*repF*	*repFIA*	*ccdA/B*	*qnrA/B/S*
NC_1	Farifield (2003)	+	+	short	+	absent
NC_2	Bristol (2003)	+	+	short	+	absent
NC_3	Bury (2003)	+	+	+	+	absent
NC_4	Eastbourne (2003)	absent	absent	absent	absent	absent
NC_5	Belfast (2004)	+	+	+	+	absent
NC_6	Hull (2004)	+	+	+	+	absent
NC_7	Manchester (2003)	ND	ND	ND	ND	ND
NC_{12}	London (2003)	ND	ND	ND	ND	ND
NC_{13}	Stoke (2004)	+	+	+	+	absent
NC_{14}	Salisbury (2004)	+	+	+	+	absent
NC_{15}	Birmingham (2004)	+	+	short	+	absent
NC_{16}	Southampton (2004)	+	+	+	+	absent
NC_{17}	Winchester (2004)	absent	+	absent	+	absent
NC_{18}	Southampton (2004)	+	absent	absent	absent	absent
NC_{19}	Reading (2004)	+	+	short	+	absent
NC_{20}	Winchester (2004)	+	+	+	+	absent
NC_{21}	Shrewsbury (2004)	+	+	+	+	absent
NC_{22}	Southend (2004)	+	+	+	+	absent
NC_{23}	South Tyneside (2004)	+	+	+	+	absent
NC_{24}	Birmingham (2004)	+	+	+	+	absent
NC_{25}	Swansea (2004)	+	+	short	+	absent
NC_{26}	Huddersfield (2005)	+	+	short	+	absent
NC_{27}	Stoke (2005)	+	+	absent	+	absent
NC_{28}	Stafford (2005)	+	+	+	+	absent
NC_{29}	Truro (2006)	absent	+	+	+	absent
NC_{30}	Birmingham (2000)	+	+	+	+	absent
NC_{31}	Birmingham (2001)	+	+	+	+	absent
NC_{32}	Birmingham (2000)	+	+	+	+	absent
NC_{33}	Birmingham (2000)	+	+	+	+	absent
NC_{34}	Belfast (2004)	ND	+	+	+	absent

+ = gene detected by PCR; ND = not determined; Short = gene detected by PCR, but smaller amplicon than expected (*c.* 420-bp, instead of 462-bp as found with *e.g.* plasmid pEK499).

4.4. Detection of *qnrA*, *qnrB* and *qnrS*

The 27 diverse producers with CTX-M-15-like enzyme, including NC_1, were also screened by PCR for the three plasmid-mediated *qnr* genes (*qnr*A, *qnrB* and *qnrS*), which encode decreased susceptibility to quinolones. None of the 27 isolates harboured such genes based on PCR assays. Decreased susceptibility to ciprofloxacin, as observed with the NC_1-derived transformant, was therefore anticipated to be due to AAC(6')-Ib-cr (though other unknown genes cannot be ruled out).

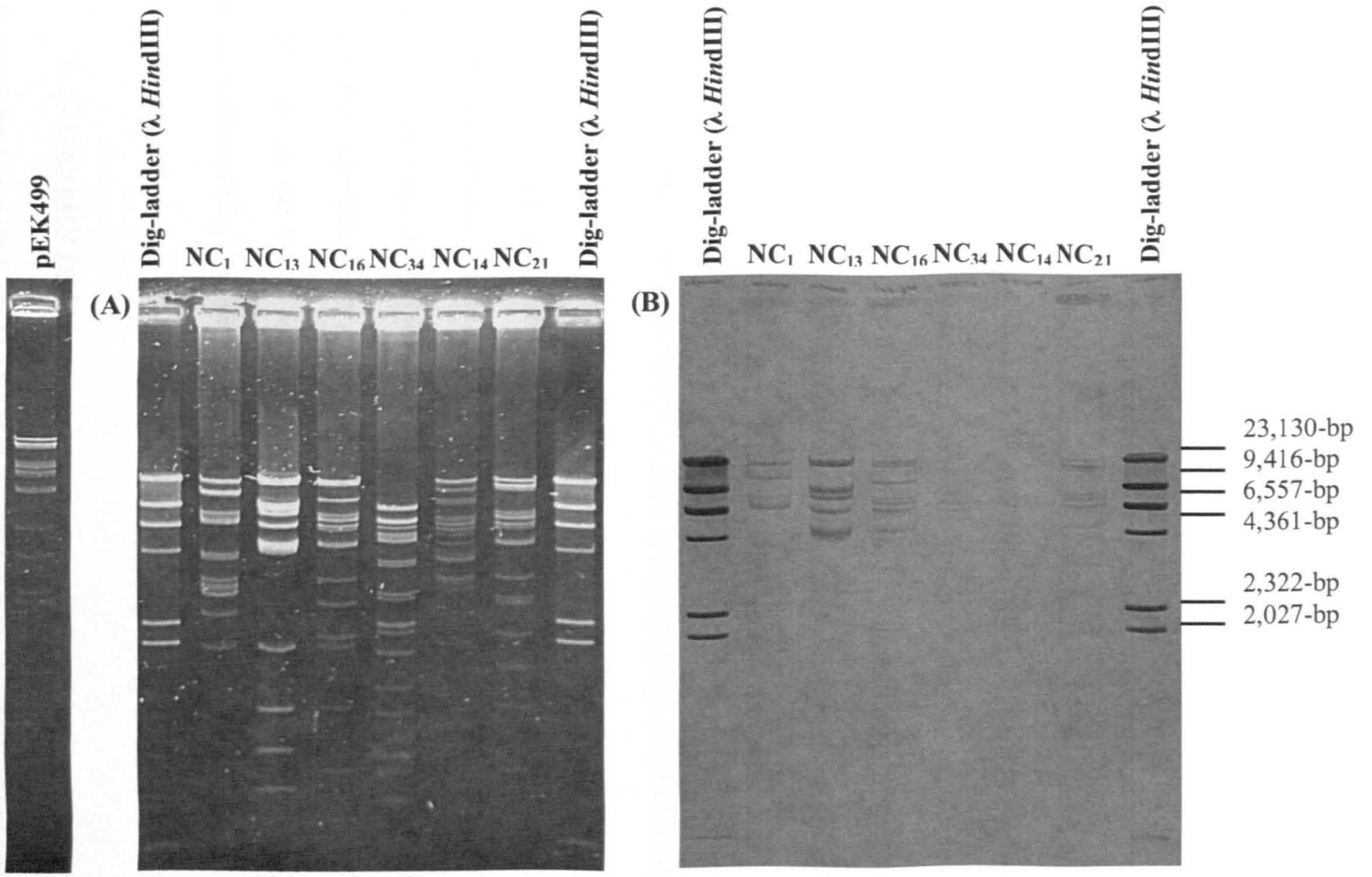
Overall, most of the unrelated producers of CTX-M-15-like enzymes were shown to share several plasmid-mediated features. These features, which were also found among most of the CTX-M-encoding plasmids of clonal isolates, included the *repF* and *repFIA* origins of replication, the *aac(6')-Ib-cr* gene and the F-plasmid-derived addiction system *ccdA/B*. This suggests that closely-related plasmids encoding CTX-M-15 β-lactamases, and belonging to incompatibility group IncFII, are spreading among unrelated *E. coli* isolates, regardless of their genetic background, as defined by PFGE profiles.

5. Plasmid cross-hybridization

Cefotaxime resistance was transferred from six non-clonal representative isolates (NC_1, NC_{13}, NC_{14}, NC_{16}, NC_{21} and NC_{34}) into *E. coli* K-12 DH5α by electroporation. Most of these isolates are listed in Table 19; NC_{34} was a non-clonal isolate from the same centre where clonal representative C_1 was isolated from (Belfast). Following confirmation of $bla_{CTX\text{-}M}$ transfer by PCR, the CTX-M-encoding plasmids were extracted from the transformants by modified alkaline lysis and were then digested with *Hpa*I for cross-hybridization studies with the Dig-labelled *Hpa*I-restricted pEK499 probe.

All six plasmid restriction profiles appeared different [Figure 33(A)], and that of NC_{34} was found to be the least related. Although diverse, the restriction profiles of the CTX-M-encoding plasmids from isolates NC_1, NC_{13}, NC_{14}, NC_{16} and NC_{21} still shared a few bands in common, suggesting some relatedness.

Figure 33. (A) *Hpa*I restriction profiles of plasmids encoding CTX-M ESBLs in selected non-clonal representative isolates[a] and (B) Southern blot hybridized with the Dig-labelled *Hpa*I-digested plasmid pEK499 from TrA_1



[a] The *Hpa*I restriction profile of pEK499 is shown as control.

By contrast, the profile of the $bla_{CTX\text{-}M}$-harbouring plasmid of NC_{34} appeared more related to that from clonal isolate C_1 (isolated at the same centre) than to those from the remaining five non-clonal representatives (isolated at different centres). Cross-hybridization of plasmid digests [Figure 33(B)] suggested the CTX-M-encoding plasmids from non-clonal isolates NC_1, NC_{13}, NC_{16} and NC_{21} were related to that of epidemic clone A, pEK499. Most of their restriction fragments hybridized to the probe. On the other hand, the digested plasmids from isolates NC_{14} and NC_{34} had fewer fragments in common with pEK499. This was very much expected for the NC_{34} plasmid since its restriction profile was noticeably different from that of pEK499. However, the NC_{14} CTX-M-encoding plasmid was anticipated to be much more related to pEK499, considering their similar restriction profiles. The poor hybridization of pEK499 to this digested plasmid may reflect technical imprecision and in the absence of confirmation, this result will therefore not be discussed further.

6. Molecular characterisation of high-level resistance to quinolones

The *gyrA* and *parC* QRDRs from five randomly-selected ciprofloxacin-resistant (MIC >8 mg/L) non-clonal isolates with CTX-M-15-like enzymes were sequenced. Multiple mutations among these gene sections were found to contribute towards resistance to quinolones in epidemic clones A-E. All five clinical isolates exhibited the same double amino acid substitutions in both QRDRs, namely Ser83Leu and Asp87Asn in *gyrA*, and Ser80Ile and Glu84Val in *parC*. These mutations were also found among the clonal isolates and were associated with increased MICs of ciprofloxacin (see results section, Chapter I, section 7).

7. Phylogenetic typing

Fifty-six non-clonal isolates with CTX-M-15-like β-lactamases from different locales across the UK (including most of isolates NC_1-NC_{34}) were phylogenetically grouped. Half of these (n=28) were confirmed urine isolates; the origin of 22/56 (*c.* 40%) was not communicated; the remaining six were isolated from blood (n=4) or wound (n=2). For comparison purposes, a further 20 *E. coli* isolates with non-CTX-M type ESBLs phenotypes [requiring MICs correlating with the production of TEM- or SHV-type ESBLs (ceftazidime MIC > cefotaxime MIC)] were also typed. These were

isolated from blood samples as part of a bacteraemia survey (R. Hope, ARMRL, Centre for Infections, personal communication), each from a different UK centre. Overall, the majority of unrelated *E. coli* isolates with CTX-M-15-like β-lactamase belonged to virulent phylogenetic groups, most often B2 (60%) and to a much lesser extent D (11%). More than a quarter of this group of isolates (28%) belonged to the commensal phylogenetic group A, and 4% were typed as group B1. Similarly, the vast majority of isolates with non-CTX-M type ESBLs belonged to a virulent phylogenetic group (75% of group B2 isolates and 15% of group D); none belonged to phylogenetic group B1 and 10% were typed as commensal isolates of group A.

8. Detection of virulence factor genes

Subsequent to phylogenetic typing, the 56 non-clonal isolates with group-1 CTX-M enzymes and the 20 isolates with non-CTX-M type β-lactamases were screened for the presence of 32 virulence-associated genes, as well as for the PAI. Twenty-four out of the 32 virulence factor genes were detected in at least one isolate. Table 20 summarises the prevalence of these 24 virulence genes detected and of the PAI. Overall, both sets of isolates exhibited similar aggregate virulence score (number of virulence factor genes per isolates) ranging from 2 to 12 genes per isolates for non-clonal CTX-M-producers (average of 6.43 genes/isolate) and from 2 to 14 genes per isolates for non-CTX-M ESBL-producers (average of 6.32 genes/isolate). Among these, only *papEF* was not found among any of the non-clonal isolates with CTX-M-15-like β-lactamases, while *kpsMTIII*, *rfc*, *nfaE*, *focG* and *afa/draBC* were absent from those with TEM- or SHV-type ESBLs. The eight virulence factor genes absent from all 56 clinical isolates, but detected among their respective positive-control strains, included *bmaE*, *K1*, *hlyA*, *papG allele I*, *papG I*, *gafD*, *cvaC* and *cdtB*. The proportion of ExPEC isolates among the two populations of isolates was not significantly different (67% ExPEC isolates among unrelated isolates with CTX-M enzymes *vs.* 79% among TEM- or SHV-type ESBL-producers; p value = 0.0792). Similarly to the group of clone B, C and E representatives (but not clones A and D), the vast majority of ExPEC isolates from the non-clonal CTX-M-producers, as well as those from the producers of non-CTX-M-type ESBLs, were defined as such by the presence of the two key virulence markers *iutA* and *kpsMTII*.

Table 20. Prevalence of virulence factor genes among non-clonal isolates with CTX-M-15-like enzymes and among isolates with non-CTX-M-type ESBLs[a]

Virulence gene	prevalence of factor (% of total)		*p* value
	non-clonal isolates with CTX-M-15-like enzymes (n=56)	isolates with non-CTX-M-type ESBLS (n=20)	
afa/draBC[b]	**17 (31%)**	**0 (0%)**	**<0.0001**
cnf1	2 (3%)	1 (5%)	0.7209
fimH	52 (92%)	19 (95%)	0.5679
focG	1 (2%)	0 (0%)	0.4975
fyuA	40 (72%)	13 (65%)	0.5392
ibeA	1 (2%)	1 (5%)	0.4448
iutA[b]	48 (86%)	16 (80%)	0.4464
K5	**19 (34%)**	**18 (90%)**	**<0.0001**
kpsMTII[b]	**20 (36%)**	**17 (85%)**	**<0.0001**
kpsMTIII	2 (3%)	0 (0%)	0.2462
nfaE	1 (2%)	0 (0%)	0.4975
ompT	6 (11%)	2 (10%)	1
PAI	**16 (29%)**	**12 (60%)**	**<0.0001**
papA[b]	3 (5%)	2 (10%)	0.2828
papC[b]	4 (7%)	3 (15%)	0.2582
papEF	**0 (0%)**	**7 (35%)**	**<0.0001**
papG allele II	3 (5%)	2 (10%)	0.2828
papG allele III	1 (2%)	1 (5%)	0.4448
papG II,III	1 (2%)	2 (10%)	0.033
rfc	1 (2%)	0 (0%)	0.4975
sfa/focDE[b]	2 (3%)	2 (10%)	0.0818
sfaS	4 (7%)	1 (5%)	0.5679
traT	48 (86%)	15 (75%)	0.1528
uidA	53 (95%)	18 (90%)	0.2828
usp	32 (57%)	14 (70%)	0.0776

[a] Significant differences in prevalence are highlighted in bold font.
[b] Key virulence gene marker of ExPEC isolates.

The most prevalent virulence factor genes among both populations of isolates included the PAI (mostly in isolates with non-CTX-M ESBLs), *fimH*, *fyuA*, *iutA*, *kpsMTII*, *traT*, *afa/draBC* (among non-clonal CTX-M-producers only), *uidA* and *usp*. This combination of virulence traits was found among approximately 30% of diverse isolates with CTX-M-15-like β-lactamases and 60% of isolates with non-CTX-M-type ESBLs. Overall, the diverse CTX-M-producers showed much more diversity in their virulence profiles. The *afa/draBC* virulence gene, which encodes a urinary tract adhesin, was found among the unrelated CTX-M-producing isolates only. Furthermore, this virulence gene was specifically found in isolates belonging to a virulent phylogenetic group (B2 or D). On the other hand, isolates with TEM- or SHV-type ESBLs showed less variation in their virulence profiles, often exhibiting the same repertoire of nine virulence-associated genes (*fimH*, *fyuA*, *iutA*, *K5*, *kpsMTII*, PAI, *traT*, *uidA* and *usp*). PAI, *papEF*, *kpsMTII* and *K5* were found to be significantly more common among these isolates than in diverse isolates with CTX-M-15-like β-lactamases.

9. Summary

Non-clonal isolates with CTX-M-15-like β-lactamase were multi-drug resistant, exhibiting a similar phenotype to that of epidemic clones A-E. The $bla_{\text{CTX-M}}$ gene was mediated by IncFII plasmids among the vast majority of these non-clonal isolates, and was linked to various other antibiotic resistance genes such as $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$ and *aac(6')-Ib-cr*. It was also shown that the plasmids encoding CTX-M ESBLs in the majority of non-clonal isolates shared additional features in common with those of the clonal isolates; *e.g.* the origins of replication and the plasmid addiction system *ccdA/ccdB*. Cross-hybridization studies strongly suggested that closely-related plasmids were spreading between clonal and non-clonal isolates. Finally, most of these producers of CTX-M-15-like enzymes belonged to an extra-intestinal virulent phylogenetic group (mostly B2). They did not appear to carry more virulence factor genes than isolates producing non-CTX-M ESBLs, but a collection of eight virulence traits, including the urinary tract adhesin-encoding gene *afa/draBC*, was consistently exhibited by many of the non-clonal CTX-M-producers.

Chapter III . Molecular characterisation of *Escherichia coli* isolates with phylogenetic group-2, -8 and -9 CTX-M beta-lactamases

1. Introduction

The majority of $bla_{CTX\text{-}M}$ genes detected among clinical isolates referred to ARMRL between 2003 and 2006 were phylogenetically typed by PCR (Woodford, N. *et al.*, 2006). Among *E. coli* isolates, group-1 CTX-M β-lactamases (mostly CTX-M-15) were overwhelmingly the predominant type recorded, representing approximately 85% of all CTX-M enzymes. Most of the remaining $bla_{CTX\text{-}M}$ genes detected encoded group-9 CTX-M enzymes, while only a few sporadic isolates harboured group-2 (n=8) or group-8 (n=2) -encoding $bla_{CTX\text{-}M}$ genes. None of the referred *E. coli* clinical isolates was found to carry a $bla_{CTX\text{-}M\text{-}25/26\text{-}like}$ gene.

Table 21 summarises the geometric mean MICs for isolates with group-2, -8 or -9 CTX-M β-lactamases. The CTX-M phenotype was evident among the three groups of isolates, with high-level cefotaxime resistance and synergy between clavulanate and third-generation cephalosporins. Ceftazidime MICs for these isolates were low (especially when compared with those for producers of CTX-M-15 β-lactamases). While approximately 85% of *E. coli* isolates with group-1 CTX-M enzymes required ceftazidime MICs above the BSAC breakpoint (2 mg/L), only *c.* 28% of group-9 CTX-M producers were resistant to that antibiotic. Similarly, ciprofloxacin resistance (MIC >1 mg/L) was not as common among isolates with CTX-M-9-like enzymes (44%) as it was among isolates with CTX-M-15-like enzymes (95%). Isolates with group-2 or -9 CTX-M enzymes were variably resistant to aminoglycosides (*c.* 50% and 33% were resistant to gentamicin, respectively), and most remained susceptible to amikacin. Both isolates with group-8 CTX-M enzymes were susceptible to aminoglycosides. Carbapenems alone retained their full activity against all these isolates.

Table 21. MIC ranges and geometric mean (GM) MIC (mg/L) for *E. coli* isolates with group-2, -8 or -9 CTX-M enzymes

	R>[a]	Isolates with group-2 CTX-M enzymes (n=8)		Isolates with group-8 CTX-M enzymes (n=2)		Isolates with group-9 CTX-M enzymes (n=127)	
		Range	GM	Range	GM	Range	GM
AMP	16	> 64	64	> 64	64	64 - >256	66.2
AUG	16	8 - 32	19.5	8 - 16	11.3	4 - 64	14.4
CTX	1	32 - >256	64	64	64	16 - >256	79.6
CTX / CLA	1	0.125 - 1	0.2	0.06 - 0.25	0.1	≤0.06 - 4	0.2
CTZ	2	2 - 16	4	1 - 4	2	0.5 - 64	1.9
CTZ / CLA	2	0.25 - 2	0.6	0.25 - 0.5	0.4	≤0.06 - 4	0.3
CPM	1	16 - 64	22.6	16	16	1 - 64	8.6
CPM / CLA	1	≤0.06 - 0.125	0.1	0.125	0.125	≤0.06 - 0.25	0.1
FOX	8	8 - 64	13.1	8 - 64	22.6	2 - 64	14
PIP	16	64	64	64	64	64 - 256	64.7
PIP / TZB	16	2 - 64	4.6	4 - 8	5.7	2 - 64	4.3
ETP	2	0.125	0.125	0.125	0.125	≤0.125 - 0.5	0.14
IPM	4	0.125 - 0.5	0.14	0.125	0.125	≤0.06 - 0.5	0.18
MEM	4	0.06	0.06	0.06	0.06	0.06	0.06
CIP	1	≤0.125 - >8	1.2	8	8	≤0.125 - >8	0.9
AMK	16	1 - 16	2.4	1 - 4	2	1 - >64	2.3
GEN	4	0.5 - >32	6.6	0.5	0.5	0.5 - >32	2.7
MIN	8	1 - 32	4	1 - 2	1.4	0.25 - 32	5.2
COL	4	≤0.5 - 2	0.5	≤0.5 - 1	0.7	≤0.5 - 4	0.6

[a] BSAC breakpoints defining resistance (Andrews, J. M., 2007)

Overall, although many of the antibiotics tested against *E. coli* isolates with group-2, -8 or -9 CTX-M enzymes showed reduced efficacy, these organisms did not appear as resistant as the group-1 CTX-M-producers (*e.g.* lower MICs of piperacillin/tazobactam, ciprofloxacin...*etc*). Moreover, antibiograms of isolates with CTX-M-9-like enzymes were more variable, especially regarding ceftazidime, ciprofloxacin and gentamicin.

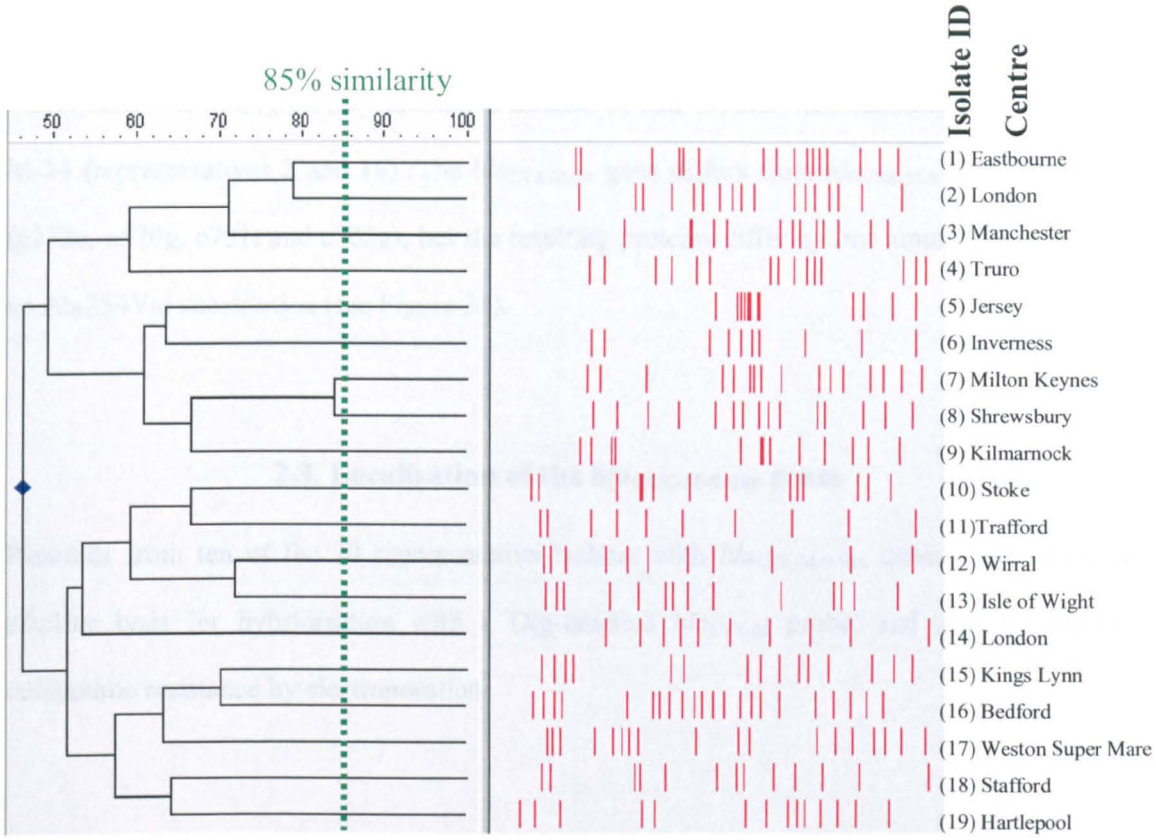
The molecular epidemiology, as well as the molecular characterisation of antibiotic resistance mechanisms and virulence genes among representative isolates with group-2, -8 or -9 CTX-M β-lactamases was investigated in detail. Transfer of cefotaxime resistance by conjugation and electroporation was attempted, in addition to plasmid profiling, and to localization of the bla_{CTX-M} genes. Randomly selected bla_{CTX-M} genes were cloned and fully sequenced; their genetic environment is discussed in the next chapter (Chapter IV).

2. *E. coli* isolates with CTX-M-9-like β-lactamases

2.1. Molecular epidemiology

Group-9 CTX-M enzymes were the second most common CTX-M types detected among *E. coli* isolates in the UK. Although not as prevalent as those with group-1 CTX-M, their host organisms were still widely referred to ARMRL from across the UK and therefore, it became important to understand their epidemiology. A representative sample of twenty clinical isolates, with each one being selected from a different UK centre, was subjected to PFGE in order to identify potential national epidemic clones; the digestion profiles for 19 isolates were analysed with the BioNumerics software as shown in Figure 34 (digestion of genomic DNA for one isolate was not successful). All 19 genomic *Xba*I-digestion profiles shared less than 85% similarity, indicating that none of the investigated isolates were clonally related. Most isolates shared between 50 and 70% similarity in their genomic digestion profiles. Although these isolates might have been involved in local outbreaks, it seemed unlikely there was a major national epidemic clone with CTX-M-9-like enzyme in the UK.

Figure 34. Cluster analysis of PFGE fingerprints of representative *E. coli* isolates with CTX-M-9-like enzymes



2.2. DNA sequencing of selected *bla*$_{CTX\text{-}M\text{-}9\text{-}like}$ genes

The complete *bla*$_{CTX\text{-}M}$ open reading frame from the 20 representative isolates with CTX-M-9-like enzymes was amplified, and four of these were cloned into pCR2.1 and sequenced fully. Two corresponded to *bla*$_{CTX\text{-}M\text{-}9}$ (representative isolates 11 and 19) and the remaining two encoded CTX-M-14 (representatives 2 and 16). The *bla*$_{CTX\text{-}M\text{-}14}$ gene differs from *bla*$_{CTX\text{-}M\text{-}9}$ by four nucleotides (g372a, a570g, c701t and c702g), but the resulting proteins differ by one amino acid only, namely an Ala234Val substitution (see Figure 35).

2.3. Localisation of the *bla*$_{CTX\text{-}M\text{-}9\text{-}like}$ genes

Plasmids from ten of the 20 representative isolates with *bla*$_{CTX\text{-}M\text{-}9\text{-}like}$ genes were extracted by alkaline lysis for hybridization with a Dig-labelled *bla*$_{CTX\text{-}M}$ probe, and also for transfer of cefotaxime resistance by electroporation.

2.3.1. Plasmid extraction and hybridization studies

The majority of isolates with CTX-M-9-like β-lactamases investigated here harboured very few plasmids [Figure 36(A)]; most only had one or two. A similarly-sized plasmid of approximately 150-kb was present in all ten representatives. However, hybridization of the *bla*$_{CTX\text{-}M}$ probe to the plasmid extracts was inconclusive with regards to the localisation of the *bla*$_{CTX\text{-}M\text{-}9\text{-}like}$ genes. The probe did not recognise any of the plasmids [Figure 36(B)], nor did it hybridize with the chromosomal DNA. Hybridization of the plasmids extracts from group-9 CTX-M-producers with the *bla*$_{CTX\text{-}M}$ probe was repeated twice more, but no positive results were obtained, except with the Dig-labelled λ-*Hin*dIII DNA (positive-control), which was successfully detected in each case. Since no *bla*$_{CTX\text{-}M}$ gene was detected by hybridization, it was inferred that these genes were possibly harboured by very large plasmids (>200-kb), which are generally not efficiently extracted by conventional alkaline lysis, a problem exacerbated by the often low copy-number of such plasmids. In support of this, the *bla*$_{CTX\text{-}M}$ probe hybridized to the DNA left in some loading wells [Figure 36(B)].

Figure 35. Amino acid sequence alignment of CTX-M-9 and CTX-M-14 enzymes

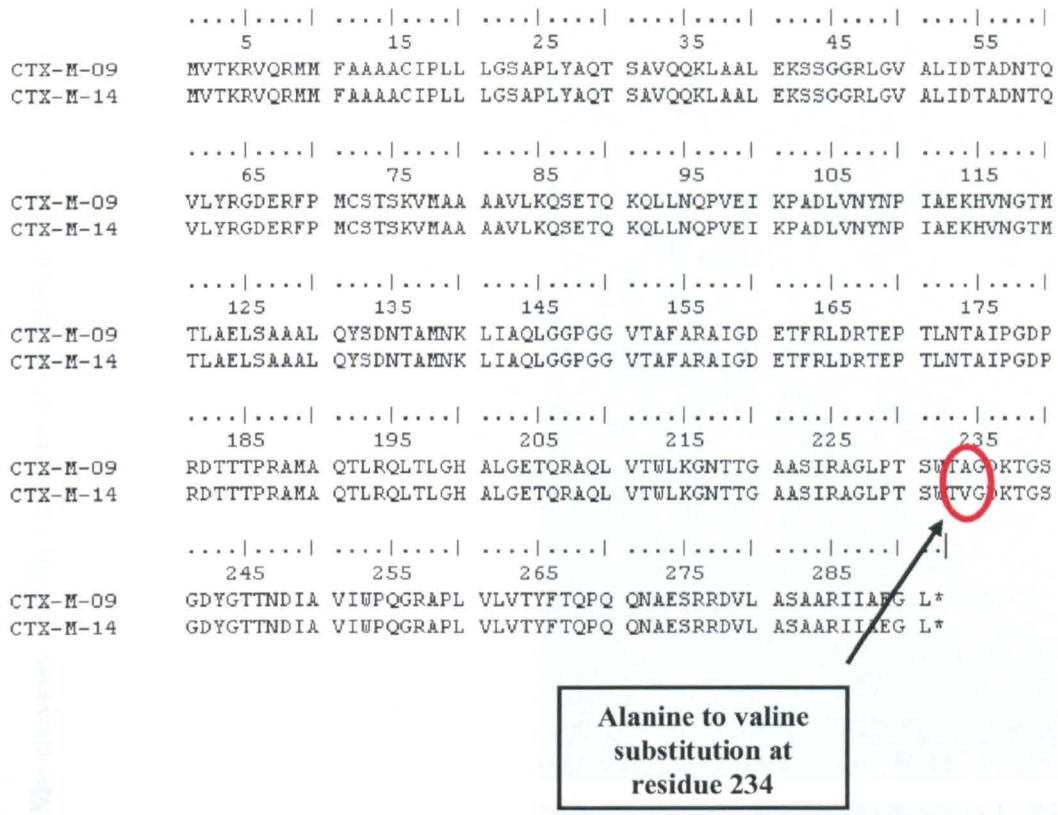
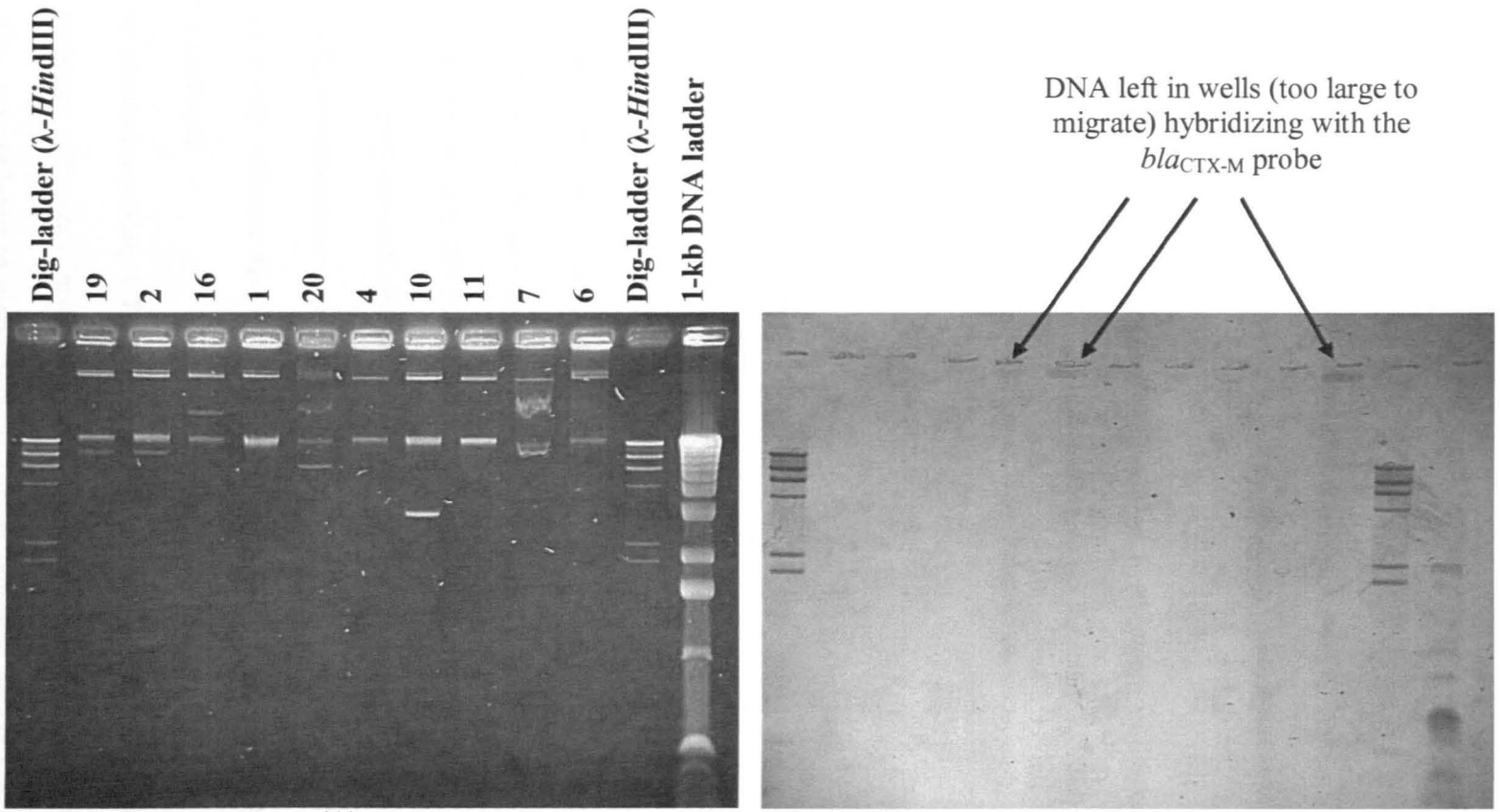


Figure 36. (A) Plasmid extracts of representative clinical isolates with CTX-M-9-like enzymes and (B) Southern blot of plasmid extracts hybridized with the Dig-labelled *bla*$_{CTX-M}$ probe



2.3.2. Cefotaxime resistance transfer

Since hybridization studies did not successfully localise the $bla_{CTX\text{-}M\text{-}9like}$ genes, conjugative transfer of cefotaxime resistance from one CTX-M-9-producer (isolate 19) and one CTX-M-14-producer (isolate 16) was attempted.

The transfer was successful in both cases, and was confirmed both by PCR and by antibiotic susceptibility testing of the resulting transconjugants. The conjugative transfer frequencies were low however; 3.5×10^{-5} and 6×10^{-8} for isolates 19 and 16, respectively. Both $bla_{CTX\text{-}M\text{-}9}$ and $bla_{CTX\text{-}M\text{-}14}$ genes were therefore plasmid-mediated. Furthermore, as the transfer rates of these vectors were low and since the $bla_{CTX\text{-}M\text{-}9\text{-}like}$ genes were not detected by hybridization of plasmids extracted by alkaline lysis, those $bla_{CTX\text{-}M}$ genes were likely to be encoded by very large low-copy number plasmids.

2.4. Antibiotic susceptibility testing of transconjugants

MICs for the transconjugants derived from representative clinical isolates 16 and 19 (respectively named TC16 and TC19) are summarised in Table 22.

The CTX-M phenotype was evident among both the plasmid donors and their derived transconjugants. Clinical isolate 16 was resistant to ciprofloxacin and to minocycline, but these were not co-transferred into *E. coli* J53-2 along with the CTX-M phenotype. Except for drugs affected by the CTX-M-9 enzyme itself, isolate 19 remained susceptible to most of the antibiotics tested. Moreover, MICs of ceftazidime were relatively low reflecting the fact that CTX-M-9-like enzyme variants are poorly effective against ceftazidime, though still responsible for reducing susceptibility to this antibiotic.

Overall, the plasmids encoding CTX-M enzymes from both isolates 16 and 19 did not appear to encode resistance to classes of antibiotics other than those affected by the CTX-M-9 or -14 β-lactamases. Also, susceptibility to piperacillin/tazobactam indicated no OXA-type enzyme was likely to be co-encoded with the $bla_{CTX\text{-}M\text{-}9\text{-}like}$ gene (by contrast with most isolates producing CTX-M-15 β-lactamases).

Table 22. MICs (mg/L) for *E. coli* strain J53-2, representative clinical isolates 16 and 19 and their derived cefotaxime-resistant transconjugants

	Clinical isolates		Recipient	Transconjugants	
	16 (CTX-M-14)	19 (CTX-M-9)	J53-2	TC16 (CTX-M-14)	TC19 (CTX-M-9)
AMP	> 64	> 64	4	> 64	> 64
AUG	8	16	4	8	8
CTX	256	64	≤0.125	> 64	32
CTX / CLA	0.25	0.125	≤0.060	≤0.060	0.125
CAZ	1	2	≤0.125	2	1
CAZ / CLA	0.25	0.25	≤0.060	0.125	0.25
FOX	32	8	4	4	8
PIP	64	> 64	≤1.0	> 64	> 64
PIP / TZB	4	2	≤1.0	≤1.0	2
ETP	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125
IPM	0.125	0.125	0.125	0.125	0.25
MEM	≤0.06	≤0.06	≤0.060	≤0.060	≤0.060
CIP	> 8	≤0.125	≤0.125	≤0.125	≤0.125
AMK	1	1	1	2	1
GEN	2	0.5	0.5	1	1
TOB	ND[a]	ND[a]	0.5	2	1
MIN	32	1	2	1	0.5
COL	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5

[a] ND = not determined

2.5. Additional antibiotic resistance mechanisms

2.5.1. β-Lactamase production

The production of additional β-lactamases by the 20 representative isolates with CTX-M-9-like enzymes was sought by IEF and confirmed by PCR. Results are summarised in Table 23.

IEF identified two types of β-lactamase with approximate pI values of 5.4 and 8.0 among the 20 representative isolates. All 20 isolates had a band at *c.* pI = 8.0, which surely corresponded to the CTX-M-9-like enzyme; CTX-M-9 and CTX-M-14 β-lactamases have very close pI values (they differ by one un-ionised amino acid only, http://www.lahey.org/studies/other.htm): 8.0 and 8.1, respectively, and such small variation might not be distinguishable by IEF. Half the investigated isolates produced an additional enzyme with a pI value of 5.4, probably TEM-1.

All 20 isolates were screened in parallel by PCR for the presence of $bla_{\text{OXA-1-like}}$, bla_{SHV} (all ORFs) and bla_{TEM} (all ORFs) genes. While none of the isolates had $bla_{\text{OXA-1-like}}$ or bla_{SHV}, bla_{TEM} was detected in the ten isolates with the pI 5.4 band, thus correlating with the IEF results.

2.5.2. Resistance to aminoglycosides

According to antibiotic susceptibility testing, gentamicin was variably active against isolates with CTX-M-9-like enzymes, and amikacin remained active against 19/20 (amikacin MIC ≤8 mg/L).

Both *aac(6')-Ib* and *aac3-IIa* genes were sought by PCR among all 20 representatives and results are summarised in Table 23, along with amikacin and gentamicin MICs. One isolate only (4) had reduced susceptibility to amikacin (MIC = 16 mg/L), and PCR confirmed this representative to harbour the *aac(6')-Ib* gene. The remaining 19 isolates remained fully susceptible to amikacin, and tested negative for *aac(6')-Ib*. Furthermore, it is worth mentioning that representative isolate 4 was likely to harbour the classical *aac(6')-Ib* gene, as indicated by PCR-RFLP, and not the *aac(6')-Ib-cr* variant, which encodes the aminoglycoside acetyltransferase cross-reacting with ciprofloxacin.

Table 23. Summary of IEF and PCR results, and of aminoglycosides MICs for representative *E. coli* isolates with CTX-M-9-like β-lactamases

	IEF results			PCR results			MICs (mg/L)	
Isolate ID	no. of bands	pI values[a]		*bla*$_{TEM}$	*aac(6')-Ib*	*aac3-IIa*	AMK	GEN
1	1		8.0				2	1
2[b]	1		8.0			+	2	>32
3	2	5.4	8.0	+			2	1
4	1		8.0		+		16	8
5	2	5.4	8.0	+		+	2	>32
6	1		8.0				8	4
7	2	5.4	8.0	+		+	1	>32
8	1		8.0				2	0.5
9	2	5.4	8.0	+		+	2	>32
10	1		8.0				2	2
11[c]	2	5.4	8.0	+			4	4
12	1		8.0			+	2	>32
13	1		8.0				2	4
14	2	5.4	8.0	+			1	0.5
15	2	5.4	8.0	+		+	4	32
16[b]	1		8.0				1	0.5
17	2	5.4	8.0	+		+	2	>32
18	2	5.4	8.0	+		+	1	>32
19[c]	1		8.0				1	2
20	2	5.4	8.0	+		+	2	>32

[a] pI of TEM-1 is 5.4; pI of CTX-M-9 and of CTX-M-14 is *c.* 8.0
[b] Isolates with CTX-M-14 enzyme
[c] Isolates with CTX-M-9 enzyme

Gentamicin resistance was more common than amikacin resistance among these isolates; 9/20 had the gentamicin resistance-encoding gene *aac3-IIa* based on PCR, and these results correlated with their high gentamicin MICs (> 32 mg/L). Most of the remaining 11 isolates, which tested negative for *aac3-IIa*, required gentamicin MICs ≤2 mg/L only.

2.6. Phylogenetic typing and screening for virulence factor genes

Twenty-four randomly-selected clinical *E. coli* isolates with CTX-M-9-like β-lactamases from across the UK were phylogenetically typed, and their virulence factor profiles were examined. Nine of these (isolates 2, 4, 5, 10, 14-17 and 19) had been investigated previously for antibiotic resistance mechanisms.

The majority of isolates (17/24, 71%) belonged to a virulent extra-intestinal phylogenetic group (B2 or D); 12 (50%) belonged to phylogenetic group D and five (21%) to phylogenetic group B2. Six (25%) were typed as commensal group A isolates and one (4%) belonged to the non-virulent phylogenetic group B1.

On average, *E. coli* producing CTX-M-9-like β-lactamases harboured seven virulence factor genes per isolate. Phylogenetic group B2 and D isolates harboured more virulence-associated genes than their commensal counterparts (from groups A and B1). Isolates from phylogenetic groups B2 and D had on average 10 and 7 virulence genes per isolate, respectively, compared with four and three for phylogenetic groups A and B1 isolates. Thirteen of the 24 isolates (54%) were defined as ExPEC; predictably this trait was significantly less common among commensal isolates with CTX-M-9 enzymes (2/7, 29%) than among their virulent counterparts [11/17, 65% (p value < 0.0001)]. Most ExPEC isolates (10/13, 77%) were defined as such by the presence of *iutA* and *kpsMTII* (Table 24); *afa/draBC* (also a marker of ExPEC isolates) was detected in six isolates producing CTX-M-9 β-lactamases (25%).

Table 24. Prevalence of virulence factor genes among *E. coli* isolates (n=24) with CTX-M-9-like enzymes

Virulence gene	Prevalence of gene (% of total)
afa/draBC[a]	6 (25%)
bmaE	1 (4%)
cnf1	2 (8%)
fimH	22 (92%)
focG	1 (4%)
fyuA	19 (79%)
gafD	1 (4%)
ibeA	1 (4%)
iutA[a]	13 (54%)
K1	1 (4%)
K5	12 (50%)
kpsMTII[a]	13 (54%)
kpsMTIII	0 (0%)
nfaE	0 (0%)
ompT	3 (13%)
PAI	4 (17%)
papA[a]	1 (4%)
papC[a]	6 (25%)
papEF	2 (8%)
papG allele II	2 (8%)
papG allele III	2 (8%)
papG I	1 (4%)
papG II, III	4 (17%)
rfc	0 (0%)
sfa/focDE[a]	2 (8%)
sfaS	2 (8%)
traT	18 (75%)
uidA	21 (88%)
usp	5 (21%)

[a] Key virulence gene marker of ExPEC isolates

Seven of the thirty-three virulence factor genes sought by PCR were not detected among any of the 24 *E. coli* producing group-9 CTX-M enzymes (Table 24). These were: *cdtB*, *cvaC*, *hlyA*, *kpsMTIII*, *papG* allele I, *nfaE* and *rfc*. Overall, isolates belonging to phylogenetic groups B2 or D showed very similar virulence profiles. The most prevalent virulence genes (≥ 50% prevalence) included *fimH*, *fyuA*, *iutA*, *K5*, *kpsMTII*, *traT* and *uidA*. All these were found together, sometimes along with additional virulence genes, in six isolates (25%), always from a virulent phylogenetic background (B2 or D).

3. *E. coli* isolates with group-2 CTX-M β-lactamases

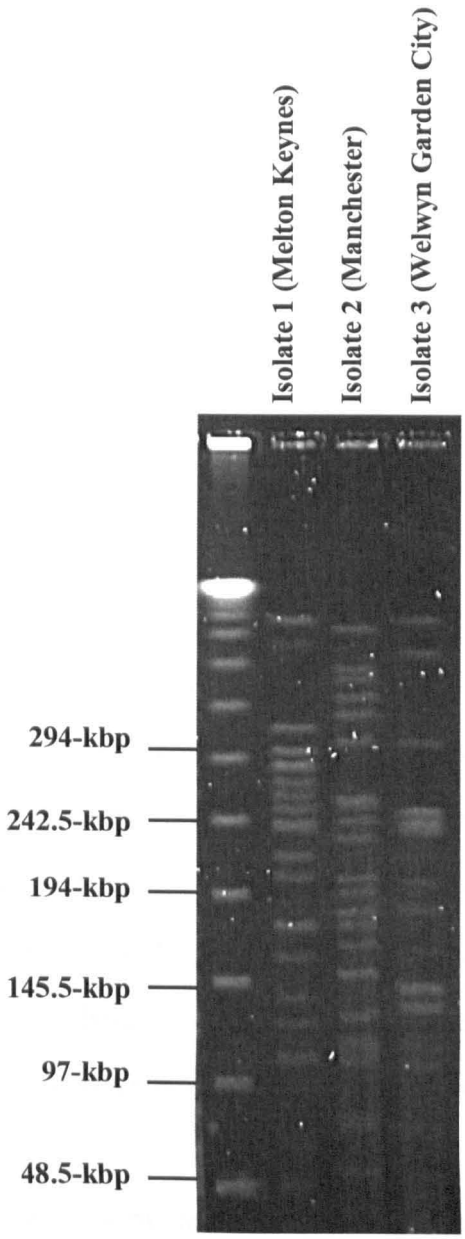
At the time of this investigation, ARMRL had been referred only three *E. coli* isolates with group-2 CTX-M β-lactamases, each from a different centre (Milton Keynes, Manchester and Welwyn Garden City). Five more isolates (from four new centres: Jersey, Hereford, Birmingham and Sutton-in-Ashfield) have since been referred, but these were not included in the molecular and biochemical investigations.

All eight isolates with group-2 CTX-M enzymes had antibiograms consistent with the production of CTX-M ESBLs, and all remained susceptible to carbapenems (Table 21). The three representative isolates investigated here were also resistant to gentamicin (MIC ≥16 mg/L). One isolate only remained fully susceptible to ciprofloxacin (MIC = 0.25 mg/L), while one was resistant (MIC > 8 mg/L), and the last showed reduced susceptibility (MIC = 1 mg/L) to this antibiotic.

3.1. PFGE of *Xba*I-digested genomic DNA

*Xba*I-digested genomic DNA profiles of the three *E. coli* representatives with CTX-M-2-like ESBLs (Figure 37) shared less than 85% similarity according to a BioNumerics analysis, indicating that they were not clonally related.

Figure 37. PFGE of *Xba*I-digested genomic DNA of clinical *E. coli* isolates with CTX-M-2-like enzymes



3.2. DNA sequencing of *bla*$_{CTX\text{-}M\text{-}2\text{-}like}$ genes

The entire *bla*$_{CTX\text{-}M}$ genes from the three isolates were amplified, cloned into the pCR2.1 vector and sequenced. All three DNA sequences were 100% identical and corresponded to the classical *bla*$_{CTX\text{-}M\text{-}2}$ allele.

3.3. Localisation of the *bla*$_{CTX\text{-}M\text{-}2}$ genes

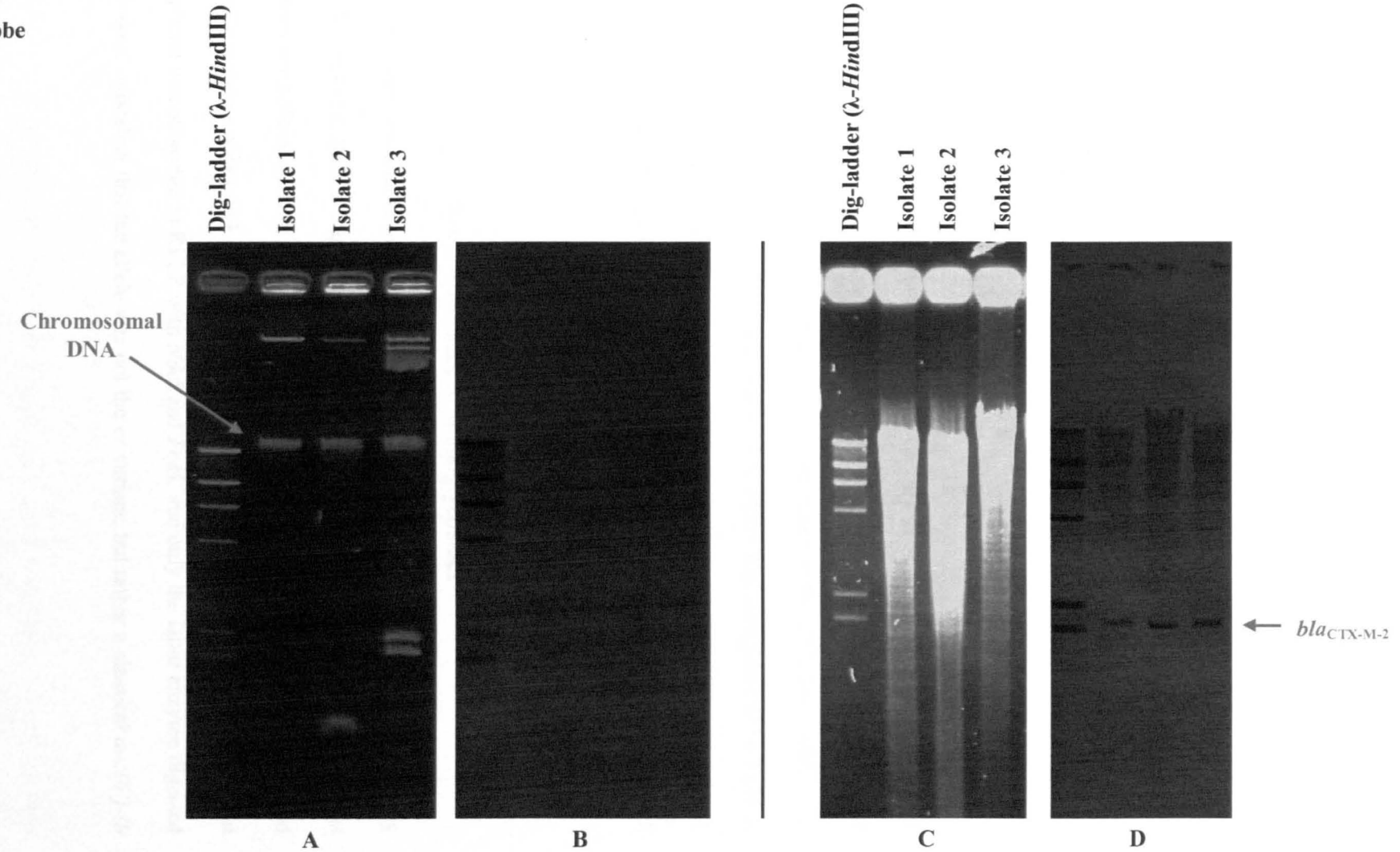
Plasmids were extracted by alkaline lysis for hybridization and for cefotaxime resistance transfer studies.

3.3.1. Plasmid extraction profiles and hybridization studies

The three CTX-M-2-producing isolates had different plasmid profiles. None of the plasmids hybridized with the dig-labelled *bla*$_{CTX\text{-}M}$ probe [Figure 38 (A and B)]. This suggested the *bla*$_{CTX\text{-}M\text{-}2}$ genes were harboured either by very large plasmids (>200-kb) or by the bacterial chromosome. However, the probe did not hybridize strongly enough to the chromosomal DNA to unequivocally support chromosomal carriage of the genes. Therefore, it remained most likely that the *bla*$_{CTX\text{-}M\text{-}2}$ genes were carried by very large plasmids, which are prone to shearing during the alkaline lysis procedure (the sheared fragments then migrate with the sheared chromosomal DNA, causing the weak hybridization).

Since hybridization of plasmid extracts was not convincing, the total genomic DNA (including both chromosome and plasmids) from the three isolates was also extracted. It was then digested with *Bam*HI (there is no *Bam*HI restriction site within the *bla*$_{CTX\text{-}M\text{-}2}$ gene) for hybridization purposes [Figure 38 (C and D)]. This experiment confirmed the presence of the *bla*$_{CTX\text{-}M}$ genes in the genome of the three isolates, as the probe strongly hybridized to restriction fragments at approximately 2.1-kb in each case. Additional and very weak hybridization signals detected were likely to be associated with partially digested DNA fragments containing the *bla*$_{CTX\text{-}M}$ gene.

Figure 38. (A) Plasmid extracts of representative *E. coli* isolates with CTX-M-2 ESBLs and (B) Southern blot of plasmid extracts hybridized with Dig-labelled bla_{CTX-M}; (C) *Bam*HI-digested total genomic DNA of representative *E. coli* isolates with CTX-M-2 ESBLs and (D) Southern blot hybridised with Dig-labelled bla_{CTX-M} probe



3.3.2. Cefotaxime resistance transfer

Transfer of cefotaxime resistance by conjugation was not achieved in three separate attempts, nor was transfer by electroporation of plasmids extracted by alkaline lysis. This might been explained by *(i)* the plasmids being non-conjugative and/or *(ii)* plasmids shearing during the extraction procedure (due to their size), leaving no viable material to be transferred even by electroporation.

3.4. Additional antibiotic resistance mechanisms

3.4.1. Additional β-lactamase production

Production of additional β-lactamases among the three CTX-M-2-producing isolates was sought by IEF. All three bacterial cell extracts showed a similar band at *c.* pI = 7.9, which according to the literature (http://www.lahey.org/studies/other.htm) was consistent with CTX-M-2 ESBL. In addition, isolate 1 had a second β-lactamase with a pI of 5.4, which was anticipated to be TEM-1. None of the three isolates harboured bla_{SHV} or $bla_{OXA\text{-}1\text{-}like}$ genes based on PCR assays. Isolate 1 was positive for bla_{TEM}, and this gene was absent from the remaining two isolates, correlating with IEF results.

3.4.2. Resistance to aminoglycosides

All three isolates were resistant to gentamicin (MIC ≥16 mg/L), but remained fully or moderately susceptible to amikacin (MICs comprised between 1 and 4 mg/L). Gentamicin resistance was associated with *aac3-IIa* in all three cases as shown by PCR. The *aac(6')-Ib* gene was also detected by PCR, but in isolate 1 only (amikacin MIC for isolates 1 =4 mg/L, *vs.* 1 mg/L for isolates 2 and 3). The amplicon was subjected to RFLP with *Nde*I and *Fok*I, and only the latter enzyme digested the PCR product, indicating that the allele was not the *cr* variant, but rather a classical *aac(6')-Ib* variant.

3.5. Phylogenetic typing and screening for virulence factor genes

The three CTX-M-2-producing isolates investigated appeared completely unrelated, each belonging to a distinct phylogenetic group: B1 (isolate 1), A (isolate 2) and D (isolate 3).

Both the isolates typed as belonging to commensal groups (isolates 1 and 2) harboured only two of the 33 virulence factor genes sought by PCR; specifically *fimH* and *traT*. The virulent isolate 3 had nine virulence genes: *cvaC*, *fimH*, *fyuA*, *ibeA*, *iutA*, *K1*, *kpsMTII*, *traT* and the pathogenicity-associated island. This isolate met the criteria for an extra-intestinal pathogenic *E. coli*, since it harboured both the ExPEC markers *iutA* and *kpsMTII*.

4. *E. coli* isolates with unusual CTX-M enzymes

Two very unusual *E. coli* isolates with CTX-M enzymes were referred to ARMRL during this investigation. One had a group-8 CTX-M β-lactamase and the other had both a group-1 and a group-9 CTX-M enzymes. These were investigated in detail, and compared with more typical CTX-M-producers from the UK.

4.1. *E. coli* isolate with group-8 CTX-M β-lactamase

The isolate with a group-8 CTX-M enzyme had a typical CTX-M phenotype, *i.e.* high-level resistance to cefotaxime and synergy of clavulanate with oxyimino-cephalosporins. It was also resistant to ceftazidime, but only at a low level (MIC =4 mg/L). This isolate remained susceptible to carbapenems and to aminoglycosides, but was highly resistant to ciprofloxacin (MIC > 8 mg/L).

PFGE showed this isolate was unrelated (<85% homology) to any of the five major epidemic *E. coli* strains producing CTX-M-15 ESBLs, as expected.

4.1.1. Antibiotic resistance mechanisms

DNA sequencing revealed the isolate harboured $bla_{\text{CTX-M-40}}$, a variant described only once before in an *E. coli* isolate dating from 1999, also from a UK patient (Hopkins, K. L. *et al.*, 2006b). IEF and PCR assays showed the isolate did not produce additional β-lactamase enzymes, such as TEM,

SHV or OXA-1-like enzymes. The *aac(6')-Ib* and *aac3-IIa* genes, which are respectively responsible for resistance to tobramycin and gentamicin, were absent from this isolate based on PCR assays. This finding correlated with the antibiotic susceptibility testing results, which indicated susceptibility to all aminoglycosides.

4.1.2. Localisation of $bla_{CTX\text{-}M\text{-}40}$

Plasmid extraction by alkaline lysis revealed that this isolate harboured at least eight plasmids ranging in size from approximately 1-kb to 150-kb. Transfer of cefotaxime resistance by conjugation or by electroporation was unsuccessful despite three separate attempts at each method. Moreover, none of the eight plasmids, nor the chromosomal DNA hybridized with the $bla_{CTX\text{-}M}$ probe. Nevertheless, the presence of the $bla_{CTX\text{-}M}$ gene in the genome of this isolate was confirmed by PCR, and by hybridization of its *Bam*HI-digested total genomic DNA, in which the gene was harboured by a digested DNA fragment of approximately 2.1-kb. All this suggested $bla_{CTX\text{-}M\text{-}40}$ was possibly mediated by a very large plasmid (>200-kb).

4.1.3. Phylogenetic typing and screening for virulence genes

The CTX-M-40-producing *E. coli* isolate belonged to the virulent phylogenetic group D and was defined as an ExPEC. It harboured seven of the 33 virulence factor genes investigated, namely *afa/draBC*, *fimH*, *fyuA*, *iutA*, *traT*, *uidA* and *usp*.

4.2. *E. coli* isolate with two CTX-M β-lactamases

This unusual *E. coli* isolate, which harboured two $bla_{CTX\text{-}M}$ genes (encoding group-1 and group-9 CTX-M enzymes) according to PCR, was a multi-drug resistant organism with a typical CTX-M phenotype. It required a cefotaxime MIC above 256 mg/L, which was decreased to 0.5 mg/L in presence of clavulanate at 4 mg/L. The isolate was also highly resistant to ceftazidime (MIC =64 mg/L), a feature associated with a few CTX-M variants such as CTX-M-15. Other antibiotics to which the isolate was resistant included ciprofloxacin (MIC >8 mg/L), gentamicin (MIC >32

mg/L) and minocycline (MIC =32 mg/L). It had reduced susceptibility to amikacin (MIC =4 mg/L), and remained susceptible to carbapenems, as did most *E. coli* isolates with CTX-M ESBLs.

The *Xba*I-digested genomic DNA profile of this isolate was less than 85% similar to those of the five major UK epidemic strains with CTX-M-15 ESBLs, indicating that it did not belong to any of the major clones.

4.2.1. Antibiotic resistance mechanisms

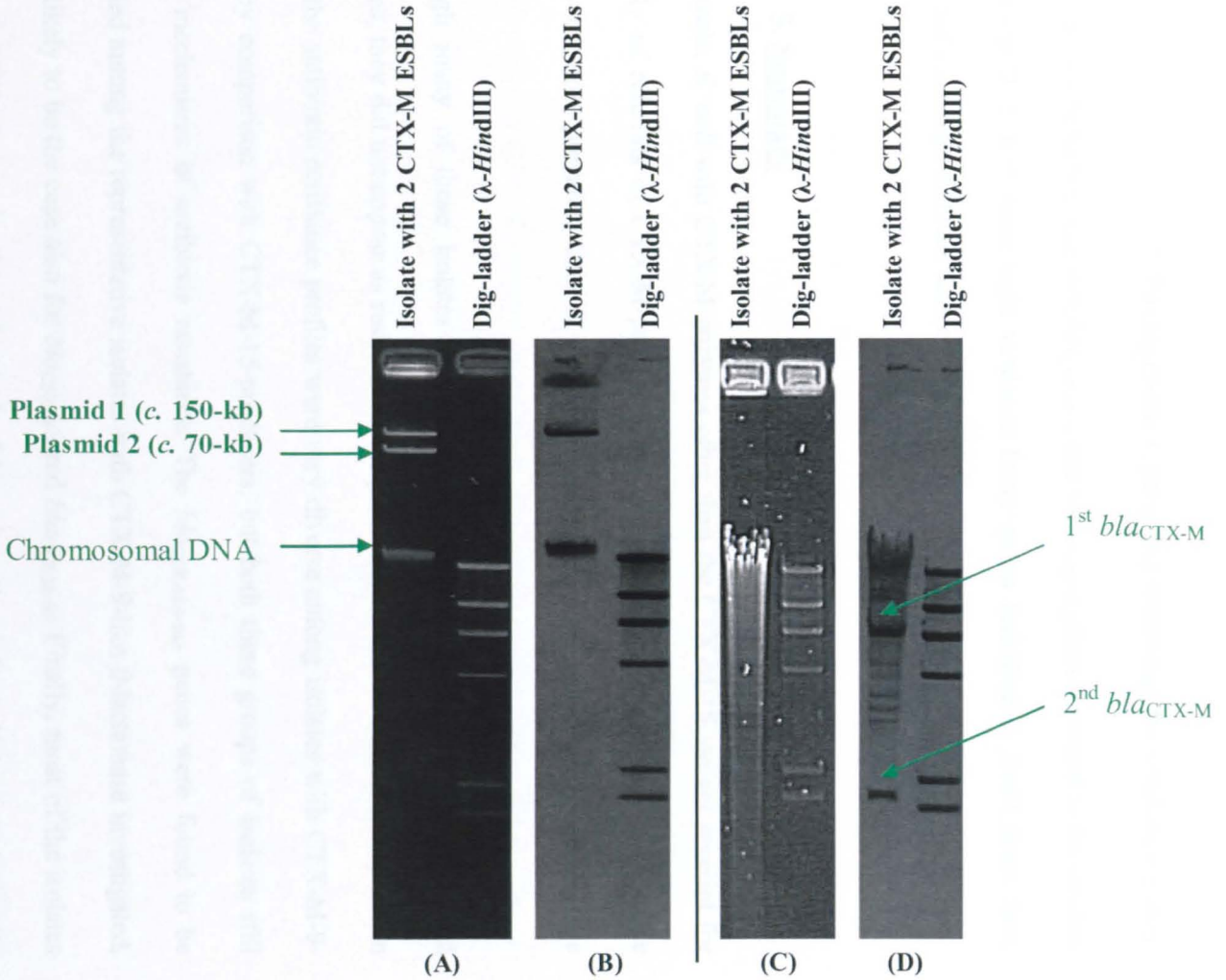
Both $bla_{\text{CTX-M}}$ genes were amplified and cloned into the pCR2.1 vector. DNA sequencing indicated this isolate was producing simultaneously the group-9 enzyme CTX-M-14 and the group-1 enzyme CTX-M-15. IEF indicated the isolate had four β-lactamases in total, with approximate pI values of 5.4, 7.4, 8.1 and 8.6. The latter two corresponded to CTX-M-14 and CTX-M-15 enzymes, respectively. PCR assays confirmed this isolate also harboured the bla_{TEM} and $bla_{\text{OXA-1-like}}$ genes, but not bla_{SHV}, correlating with IEF results. Synergy between tazobactam and piperacillin was poor (MIC =16 mg/L), correlating with the presence of an OXA-type enzyme.

Gentamicin resistance was associated with the presence of an AAC3-IIa enzyme, as PCR detected the presence of the *aac3-IIa* gene. Also, PCR-RFLP and DNA sequencing showed this isolate harboured the *aac(6')-Ib-cr* variant, which encodes the ciprofloxacin-modifying aminoglycosides acetyltransferase variant.

4.2.2. Localisation of $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$

Plasmid extraction by alkaline lysis indicated the isolate harboured at least two plasmids of *c.* 70- and 150-kb. Only the latter hybridized with the Dig-labelled $bla_{\text{CTX-M}}$ probe [Figure 39(A and B)]. Conjugative transfer of cefotaxime resistance was not attempted with this isolate. The $bla_{\text{CTX-M-15}}$ gene only was successfully transferred to *E. coli* DH5α by electroporation, as confirmed by PCR. Transfer of $bla_{\text{CTX-M-14}}$ by electroporation was not achieved after three separate attempts. Based on hybridization of the plasmid extract of the cefotaxime-resistant transformant, $bla_{\text{CTX-M-15}}$ was mediated by the *c.* 150-kb plasmid, and was not linked to $bla_{\text{CTX-M-14}}$.

Figure 39. (A) Plasmid extracts of the *E. coli* isolate with two CTX-M ESBLs and (B) Southern blot of its plasmid extract hybridized with the Dig-labelled bla_{CTX-M} probe; (C) *Bam*HI-digested total genomic DNA of the *E. coli* isolate with two CTX-M ESBLs and (D) Southern blot hybridised with the Dig-labelled bla_{CTX-M} probe



The presence of two bla_{CTX-M} genes among the genome of this isolate was confirmed by hybridizing its *Bam*HI-digested total genomic DNA with the Dig-labelled bla_{CTX-M} probe [Figure 39(C and D)]. Two strong signals at *c.* 2.1- and 6.5-kb indicated this isolate clearly harboured two bla_{CTX-M} genes. Although $bla_{CTX-M-14}$ was not successfully localised, it appeared that this gene was possibly mediated by a very large plasmid (>200-kb), prone to shearing under the alkaline conditions during plasmid extraction.

4.2.3. Phylogenetic typing and screening for virulence genes

The *E. coli* clinical isolate harbouring both $bla_{CTX-M-14}$ and $bla_{CTX-M-15}$ genes belonged to the virulent phylogenetic group D. It harboured eight virulence factor genes (*afa/draBC*, *fimH*, *fyuA*, *iutA*, *KpsMTII*, *traT*, *uidA* and *usp*) and was defined as an ExPEC.

5. Summary

On a national scale, *E. coli* with CTX-M enzymes other than the CTX-M-15 variant account for about 15% only of referrals of CTX-M-positive isolates. Nevertheless, at a local level, these organisms may be more prevalent than *E. coli* isolates with CTX-M-15-like enzymes in some centres.

Although many of these isolates were resistant to β-lactams, aminoglycosides and fluoroquinolones, they did not appear as resistant as the producers of CTX-M-15-like enzymes in general. Also, the antibiotic resistance profiles were very diverse among isolates with CTX-M-9-like enzymes by comparison with CTX-M-15-producers, but both these groups of isolates still shared similar mechanisms of antibiotic resistance. The $bla_{CTX-M-9-like}$ genes were found to be plasmid-mediated among the representative isolates with CTX-M-9-like β-lactamase investigated, and it seemed likely to be the case also for $bla_{CTX-M-2}$ and $bla_{CTX-M-40}$. Finally, most of the isolates with non-group-1 CTX-M enzymes investigated in this study belonged to virulent phylogenetic groups, and most shared similar virulence profiles.

Chapter IV . Genetic environment of *bla*$_{CTX-M}$ genes

CTX-M ESBLs are divided into five phylogenetic groups (group-1, -2, -8, -9 and -25/26). Enzyme variants from each of these five groups have been reported in the UK (see below), indicating different and independent introductory events took place:

- Group-1 CTX-M enzymes: *e.g.* CTX-M-15, first reported in 2001 in *E. coli* isolates from London, Newcastle-upon-Tyne and Belfast (Mushtaq, S. *et al.*, 2003)
- Group-2 CTX-M enzymes: *e.g.* CTX-M-2, first reported in 2006 in *E. coli* strains isolated in 2004/2005 from Milton Keynes and Manchester (Karisik, E. *et al.*, 2006b)
- Group-8 CTX-M enzymes: *e.g.* CTX-M-40, first described from an *E. coli* strain isolated in 1999 (Hopkins, K. L. *et al.*, 2006b)
- Group-9 CTX-M enzymes: *e.g.* CTX-M-9, first reported in a *K. oxytoca* isolate from 2000 in Leeds (Alobwede, I. *et al.*, 2003)
- Group-25/26 CTX-M enzymes: *e.g.* CTX-M-25, first described in a *K. pneumoniae* isolate involved in a hospital outbreak in Birmingham in 2001 (Brenwald, N. P. *et al.*, 2003)

In this study, only CTX-M enzymes from the *E. coli* clinical isolates referred to ARMRL were investigated. These included the CTX-M-2, -3, -9, -14, -15 and -40 variants, representing the phylogenetic group-1, -2, -8 and -9. Until now, no isolates with phylogenetic group-25/26 CTX-M enzymes had been referred to ARMRL and thus, none was investigated; indeed enzymes from this phylogenetic group are extremely rare, having been reported twice only, in Canada and in the UK (Brenwald, N. P. *et al.*, 2003; Munday, C. J. *et al.*, 2004a).

It has now been established that *bla*$_{CTX-M}$ genes have escaped from the chromosome of various *Kluyvera* species; *e.g.* the plasmid-mediated *bla*$_{CTX-M-2}$ gene shares >95% sequence homology with the chromosomally-encoded *bla*$_{KLUA}$ of *K. ascorbata* (Humeniuk, C. *et al.*, 2002) and the KLUY-1 β-lactamase of *K. georgiana* exhibits complete amino acid identity with the CTX-M-14 variant (Olson, A. B. *et al.*, 2005).

Exploring the genetic surroundings of bla_{CTX-M} genes has played a major role in the discovery of their origin, as well as in the understanding of the mobilization mechanisms involved in their escape. Such investigation may also play a significant role for epidemiological purposes. The genetic environments of $bla_{CTX-M-2}$, $bla_{CTX-M-3}$, $bla_{CTX-M-9}$, $bla_{CTX-M-14}$, $bla_{CTX-M-15}$ and $bla_{CTX-M-40}$ in various representative UK isolates were therefore examined.

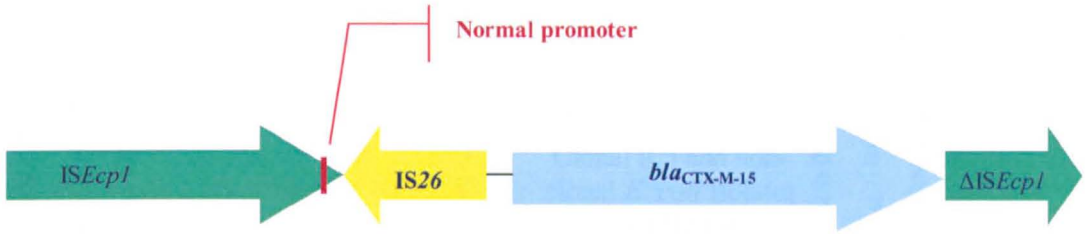
1. Genes encoding group-1 CTX-M enzymes

The genetic surroundings of the group-1 CTX-M-encoding genes $bla_{CTX-M-3}$ and $bla_{CTX-M-15}$ were investigated. These genes have previously been shown to be linked to IS*Ecp1*-like insertion sequence elements (Karim, A. *et al.*, 2001; Lartigue, M. F. *et al.*, 2004; Woodford, N. *et al.*, 2004), which, as well as being involved in the initial mobilization, also provides a strong promoter, allowing their expression (Karim, A. *et al.*, 2001). The spacer region between IS*Ecp1* and the $bla_{CTX-M-3/-15}$ genes was therefore examined among representatives *E. coli* isolates. It should be added that *E. coli* epidemic clone A had previously been shown to harbour an IS*26* element, located between the $bla_{CTX-M-15}$ gene and the promoter provided by IS*Ecp1* [see Figure 40, (Woodford, N. *et al.*, 2004)] and so its impact on the level of expression of $bla_{CTX-M-15}$ was also explored. Whilst this insertion was always present in epidemic clone A isolates typed by PFGE, it was not specific to that clone. Rather, this element had also been detected at that same position among a few clonally related *E. coli* isolates from a single centre (Hope, R., personal communication).

1.1. IS*Ecp1*-$bla_{CTX-M-3/-15}$ spacer region

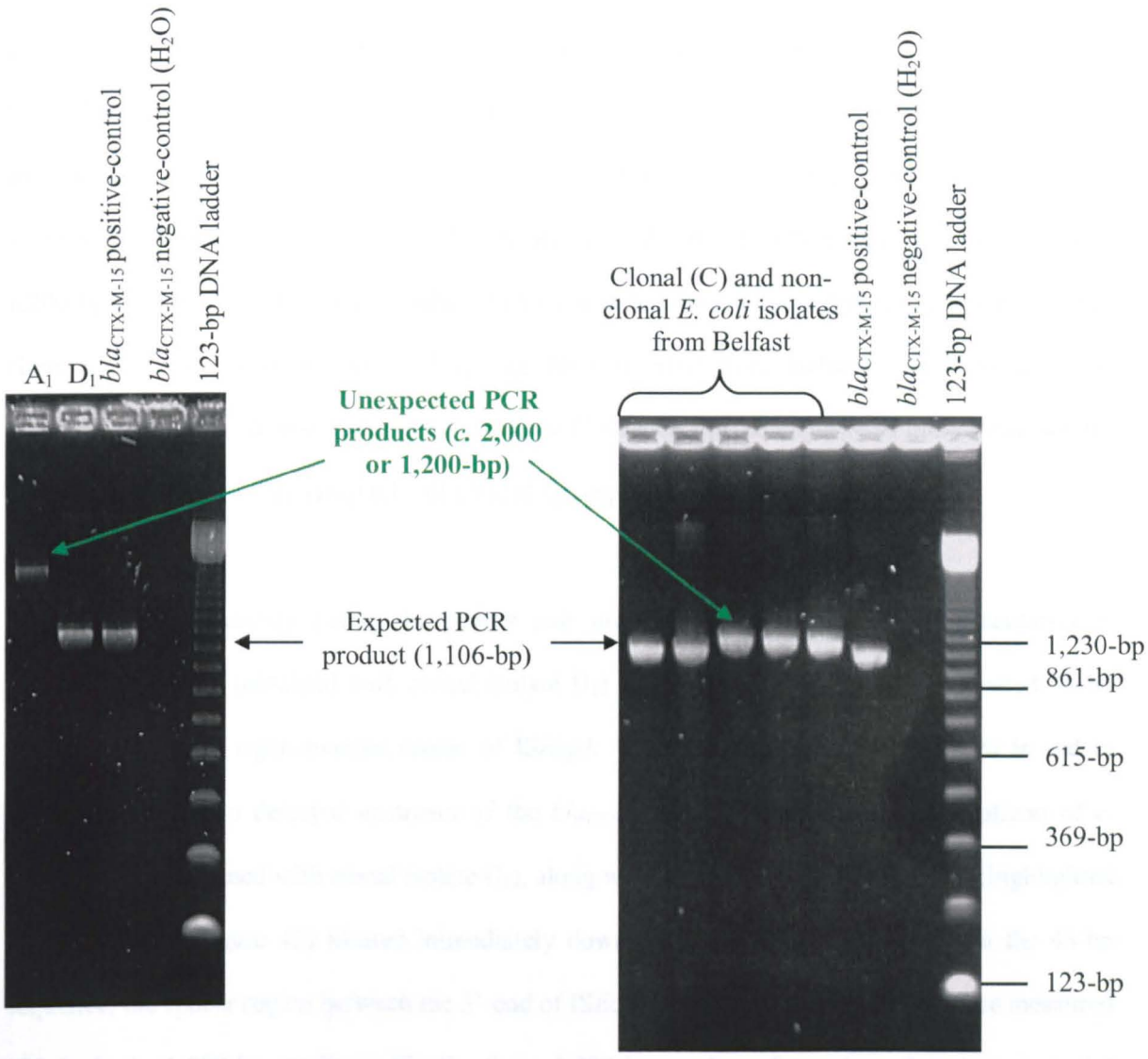
While investigating β-lactamase production by IEF, and more especially, when confirming the presence of bla_{CTX-M} genes by PCR among various clonal and non-clonal *E. coli* isolates using the primer pair CTX-Morf F (prom+)/CTX-Morf R (preCTX-M-3B), three different types of PCR product with sizes of *c.* 1.1, 1.2 and 2-kb were obtained (Figure 41). This led to further investigation.

Figure 40. Schematic representation of the genetic environment of *bla*$_{CTX-M-15}$ in UK *E. coli* epidemic clone A[a]



[a] (Woodford, N. *et al.*, 2004).

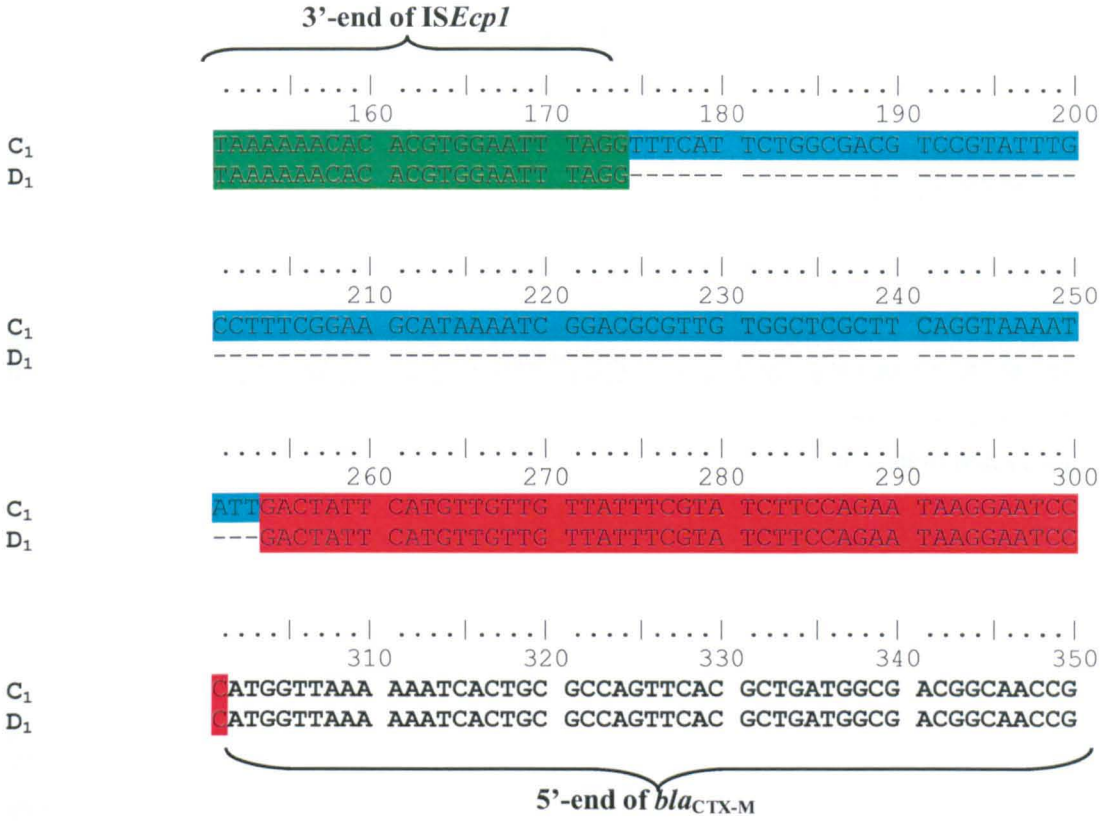
Figure 41. Agarose gel electrophoresis showing the different types of *bla*$_{CTX-M}$ ORF PCR products obtained with various clonal and non-clonal *E. coli* isolates producing CTX-M-15-like β-lactamases



The primer pair prom+/preCTX-M-3B was designed to anneal to the 3'-end of insertion sequence IS*Ecp1* (located upstream of $bla_{CTX\text{-}M\text{-}3/\text{-}15}$) and immediately downstream of $bla_{CTX\text{-}M\text{-}3/\text{-}15}$, respectively. The expected PCR product of 1,106-bp included the entire $bla_{CTX\text{-}M}$ ORF (Poirel, L. *et al.*, 2002a). This specific amplicon size was obtained with the vast majority of *E. coli* isolates with group-1 CTX-M enzymes investigated here. However, epidemic clone A isolates yielded a much longer PCR product, of approximately 2,000-bp, owing to the insertion of IS*26* between $bla_{CTX\text{-}M\text{-}15}$ and its normal promoter provided by IS*Ecp1* (see Figure 40), as also previously shown by DNA sequencing (Woodford, N. *et al.*, 2004). Finally, a third type of PCR product of approximately 1,200-bp was obtained in a small number of cases; this was detected in a few *E. coli* isolates (either clone C or non-clonal isolates), all having been referred from Belfast City Hospital only. Nevertheless, it should be stressed that most clone C isolates and the majority of non-clonal isolates referred from Belfast City Hospital still yielded the expected 1,106-bp PCR product.

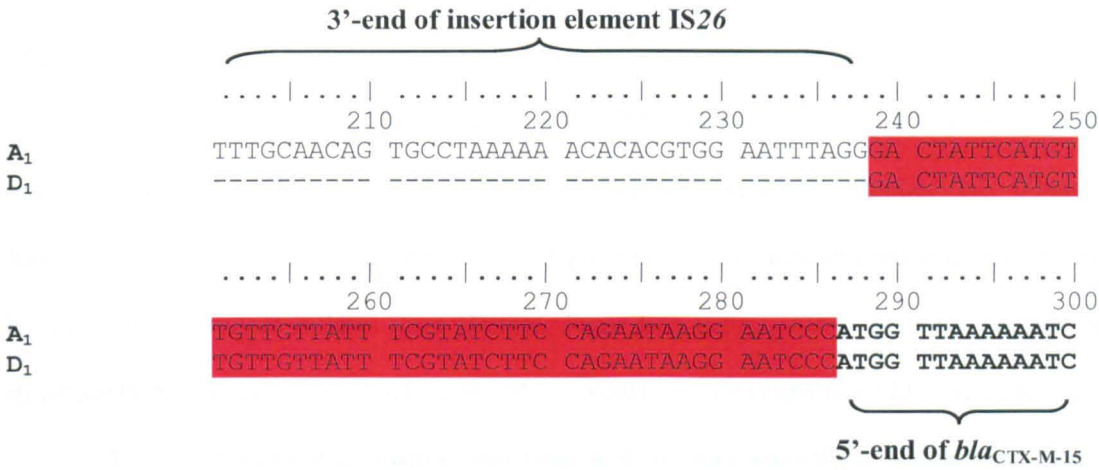
DNA sequencing (using the primer pair prom+/preCTX-M-3B) of a representative *c.* 1,100-bp amplicon (obtained with clonal isolate D_1) revealed that $bla_{CTX\text{-}M\text{-}15}$ was located 48-bp downstream of the right-inverted repeat of IS*Ecp1*. This 48-bp sequence (highlighted in red in Figure 42) was also detected upstream of the $bla_{CTX\text{-}M}$ gene in a representative amplicon of *c.* 1,200-bp (*e.g.* obtained with clonal isolate C_1), along with an additional 80 nucleotides (highlighted in light blue in Figure 42) located immediately downstream of IS*Ecp1* and upstream the 48-bp sequence; the spacer region between the 3'-end of IS*Ecp1* and the $bla_{CTX\text{-}M}$ gene therefore measured 128-bp in the 1,200-bp amplicon. Finally, the *c.* 2,000-bp amplicon from clone A isolates (*e.g.* A_1) also harboured the same 48-bp link upstream of $bla_{CTX\text{-}M\text{-}15}$ (highlighted in red in Figure 43), with; the IS*26* element (*c.* 800-bp) located immediately upstream of this link, moving $bla_{CTX\text{-}M\text{-}15}$ further away from its normal promoter, located at the 3'-end of IS*Ecp1*.

Figure 42. Partial DNA sequence alignment of the 3'-end of the IS*Ecp1*-*bla*$_{CTX-M}$ spacer regions from representative clonal isolates C_1 and D_1[a]



[a] The 48-bp link is highlighted in red; the additional 80-bp found in the 1,200-bp amplicon are highlighted in light blue; IS*Ecp1* is highlighted in green.

Figure 43. Partial alignment of the DNA sequences upstream of *bla*$_{CTX-M}$ in representative clonal isolates A_1 and D_1



The 48-bp sequence spacer region between IS*Ecp1* and the $bla_{CTX\text{-}M\text{-}15}$ gene was the most common among the investigated *E. coli* isolates with group-1 CTX-M enzymes in the UK. This link had been widely reported upstream of $bla_{CTX\text{-}M\text{-}15}$ worldwide [*e.g.* among producers isolated in Cameroon, Canada or India (Boyd, D. A. *et al.*, 2004; Gangoue-Pieboji, J. *et al.*, 2005; Karim, A. *et al.*, 2001)]. By contrast the 128-bp link, which has been described previously in association with either $bla_{CTX\text{-}M\text{-}3}$ or $bla_{CTX\text{-}M\text{-}15}$ (Baraniak, A. *et al.*, 2002a) has until now, only been reported among isolates from Poland (*e.g.* plasmid pCTX-M-3, GenBank accession number: NC_004464). This link has now also been found among CTX-M-positive UK isolates, but remains rare and seems to be confined to clinical isolates from Belfast. These two IS*Ecp1*-$bla_{CTX\text{-}M}$ spacer regions certainly illustrate two independent (initial) mobilization events of their respective $bla_{CTX\text{-}M}$ gene.

The 128-bp link was always associated with isolates showing a β-lactamase band at pI = 8.4 according to IEF (*e.g.* isolate C_1), *i.e.* encoding CTX-M-3 rather than CTX-M-15. Nevertheless, one non-clonal isolate, also from Belfast, had a β-lactamase band at pI = 8.6 corresponding to CTX-M-15, together with the 128-bp link. Otherwise, all the investigated clone C representatives with β-lactamases at pI = 8.6 harboured the widespread 48-bp link. Therefore, it seems that representatives of UK epidemic clone C had independently gained either of two distinct genetic supports (*i.e.* plasmids) harbouring $bla_{CTX\text{-}M}$ genes, one maybe related to plasmid pCTX-M-3 from Poland, the other to the widespread UK plasmids with the 48-bp link. This conclusion was supported by the fact that the CTX-M-encoding plasmid from isolate C_1 differed slightly from the plasmids from the other four clonal representatives (see section 8.3.1 and Figure 24), which harboured the classical 48-bp spacer link.

Finally, the third type of amplicon (*c.* 2,000-bp) was found among all PFGE-defined clone A isolates investigated in this study, but was not specific to these. A few clonally related isolates, as shown by PFGE (n = 26), from a centre that had also referred members of the epidemic clone A, have been shown by PCR to harbour the IS*26* element 48-bp upstream of $bla_{CTX\text{-}M}$ (Woodford, N. and Hope, R., personal communications). The repercussions of IS*26* in clone A isolates are discussed in the next section of this chapter.

1.2. Impact of IS*26* in *E. coli* epidemic clone A isolates

As previously mentioned, strain A has an IS*26* element between $bla_{CTX\text{-}M\text{-}15}$ and its normal promoter, which is provided by IS*Ecp1*. Most other producers of CTX-M-15 enzyme, such as strain D isolates have no such interruption. It was also noted that third-generation cephalosporin MICs and especially those of ceftazidime for the majority of strain A isolates were lower compared with those for other more conventional producers of CTX-M-15 enzymes, including members of epidemic clone D (see geometric mean MICs in Table 9). The bases for these associations were therefore investigated, with attempts to measure the level of CTX-M-15 expression, which was anticipated to be decreased contingent on the IS*26* insertion. In this part of the study, epidemic clone D representative isolates D_1-D_3 were used as controls (representing classical producers of CTX-M-15 enzyme), for comparison purposes with clone A representative isolates A_1-A_3. The CTX-M expression level in these isolates was quantified by RT-PCR and visually assessed on agarose gels. In addition, the specific activity of CTX-M-15 enzyme in the representatives A_1-A_3 and D_1 was biochemically measured by spectrophotometric assays. Selection pressure with high-level ceftazidime was also used in an attempt to remove the IS*26* element from the promoter-$bla_{CTX\text{-}M}$ link in transformant TrA_1. Finally, the DNA section immediately upstream of $bla_{CTX\text{-}M\text{-}15}$ in representative isolates A_1-A_3, which includes IS*26*, was sequenced.

Representatives A_1 and A_3 were typical strain A isolates, requiring low cefotaxime, ceftazidime and cefepime MICs (Table 25) compared with the three strain D representatives (D_1-D_3); the third strain A representative (A_2) was atypical, requiring cephalosporin MICs similar to those for D_1-D_3 and having a strong pI 8.6 band on IEF (see Figure 44). Transformants of all three strain A representatives, including that derived from A_2, required low cephalosporin MICs compared with those derived from the three strain D representatives.

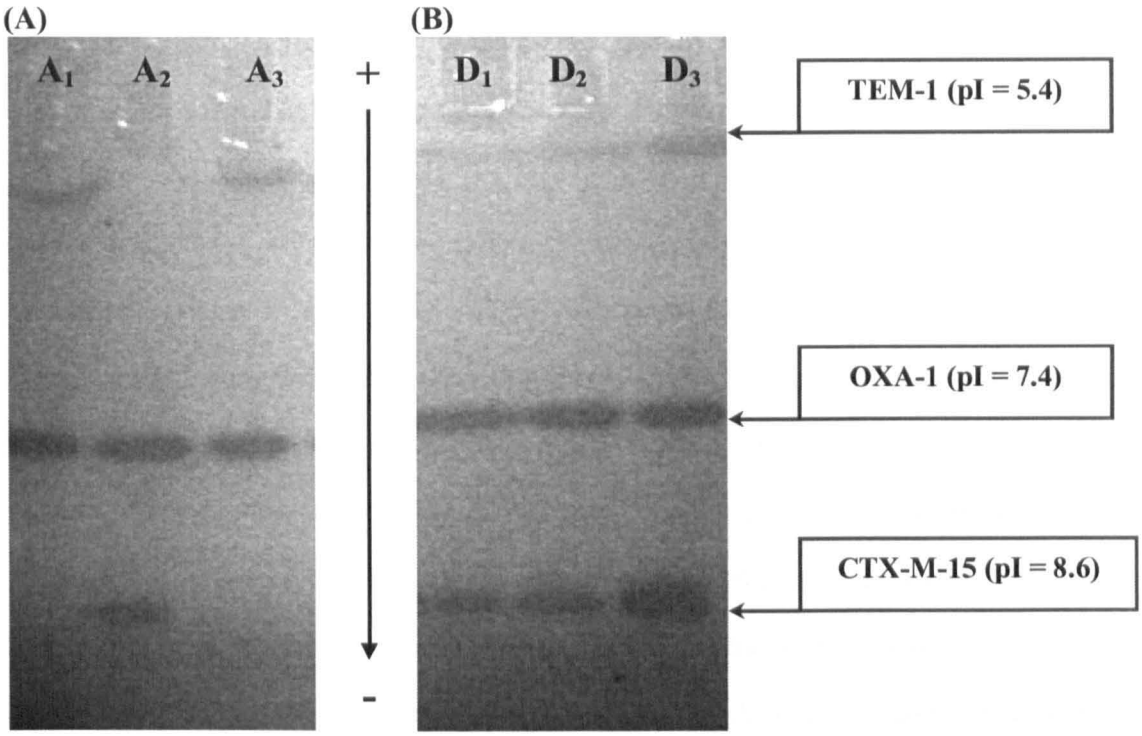
Following these patterns, cefotaximase specific activity was 6- to 15-fold lower in clinical isolates A_1 and A_3 than in D_1-D_3, but only about two-fold lower in case of A_2 (Table 25).

Table 25. Cefotaximase specific activity (μmoles CTX hydrolysed/min/mg protein) and MICs (mg/L) for clinical isolates and their transformants

Isolate	Cefotaximase specific Activity	MICs											
		AMP	AUG	CTX	CTX + CLA	CAZ	CAZ + CLA	CPM	CPM + CLA	IPM	MEM	PIP	PIP + TZM
Recipient strain:													
DH5α	ND[a]	8	4	≤0.125	≤0.060	≤0.250	0.125	≤0.125	≤0.060	0.125	≤0.060	2	2
Clinical isolates:													
A_1	1.65	> 64	32	32	≤0.060	4	0.25	4	≤0.060	0.125	≤0.060	> 64	16
A_2	4.58	> 64	16	> 64	0.125	32	0.25	32	≤0.060	0.25	≤0.060	> 64	32
A_3	1.18	> 64	16	16	≤0.060	2	0.25	1	≤0.060	0.125	≤0.060	> 64	16
D_1	11.16	> 64	32	> 64	0.25	32	1	64	1	0.25	≤0.060	> 64	32
D_2	9.17	> 64	16	> 64	0.125	64	0.25	64	≤0.060	0.125	≤0.060	> 64	32
D_3	11.94	> 64	16	> 64	0.125	64	0.5	> 64	≤0.060	0.125	≤0.060	> 64	32
Transformants:													
TrA_1	1.08	> 64	16	4	≤0.060	1	0.125	0.25	≤0.060	0.125	≤0.060	> 64	4
TrA_2	1.83	> 64	16	8	≤0.060	1	≤0.060	0.5	≤0.060	0.125	≤0.060	> 64	8
TrA_3	1.19	> 64	16	4	≤0.060	1	0.125	0.5	≤0.060	0.125	≤0.060	> 64	8
TrD_1	24.87	> 64	16	> 64	≤0.060	32	0.25	16	0.125	0.125	≤0.060	> 64	8
TrD_2	17.37	> 64	16	> 64	≤0.060	32	0.25	16	≤0.060	0.125	≤0.060	> 64	4
TrD_3	16.09	> 64	16	> 64	≤0.060	16	0.25	16	≤0.060	0.125	≤0.060	> 64	8

[a] ND = not determined

Figure 44. Isoelectric focusing of β-lactamases from strain A (A) and strain D (B) representative isolates[a]



[a] The arrow indicated the direction of protein migration.

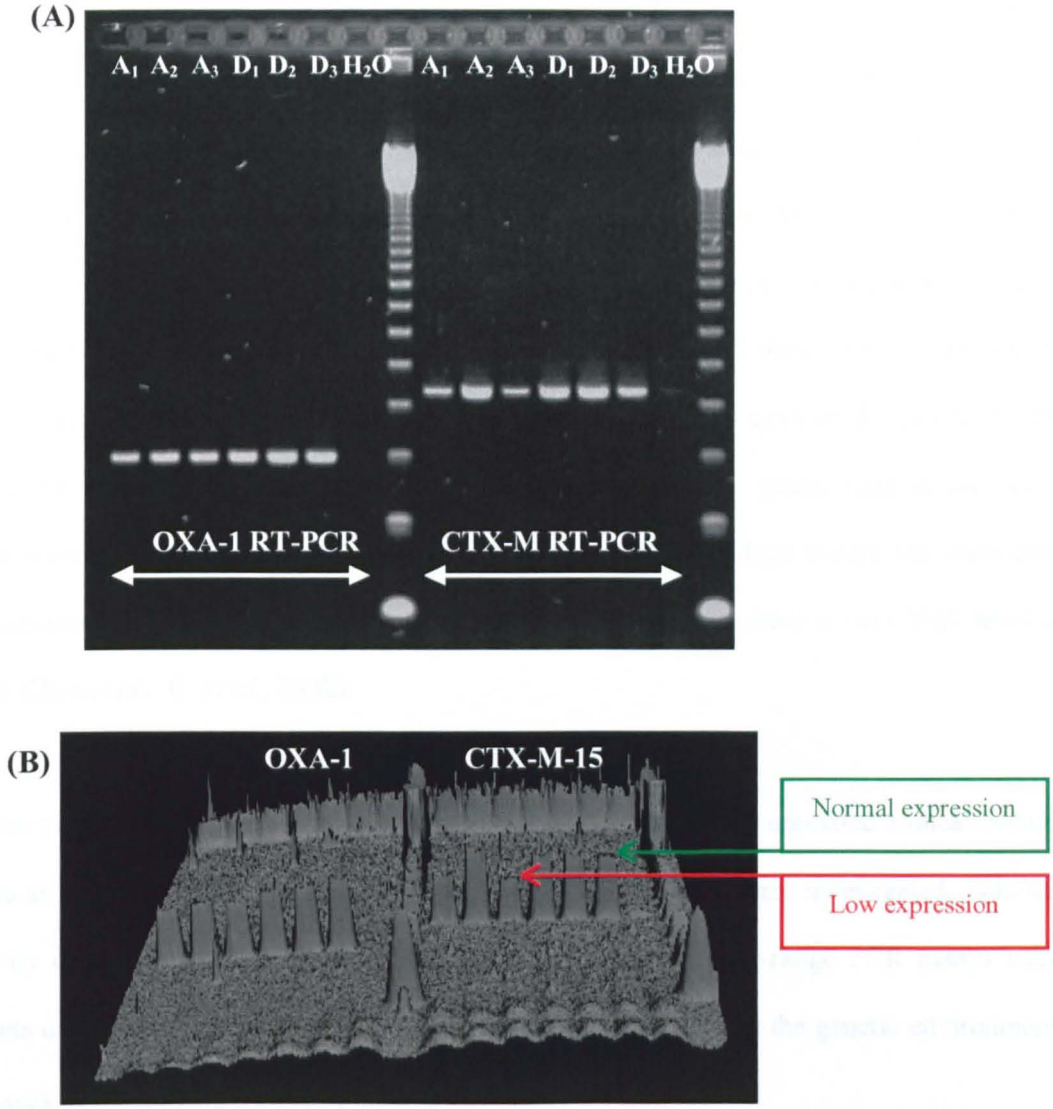
Transformants derived from all three strain A isolates, including that of A_2, had 9- to -23-fold lower cefotaximase specific activity compared with transformants of isolates D_1-D_3.

Additionally and as previously mentioned (see results - Chapter I, section 4), the CTX-M-15 β-lactamase was hardly detectable by IEF in isolates A_1 and A_3 but was obvious in A_2 and in D_1-D_3 (Figure 44). The OXA-1 β-lactamase band was equally intense in all six isolates. Correlating with these IEF results, RT-PCR indicated a decrease in the level of CTX-M expression in isolates A_1 and A_3. The $bla_{CTX\text{-}M\text{-}15}$ band reflecting mRNA presence was of lower intensity for isolates A_1 and A_3 compared with that for isolates A_2 or D_1-D_3, whereas the $bla_{OXA\text{-}1}$ band, which was used as control, was equally intense for all six isolates (Figure 45). Finally, all three strain A representative isolates A_1-A_3 had the IS*26* element between $bla_{CTX\text{-}M\text{-}15}$ and its normal promoter. DNA sequencing indicated no differences immediately upstream of $bla_{CTX\text{-}M\text{-}15}$ among these three strain A isolates.

Attempts to remove the insertion sequence by selective pressure using high-level ceftazidime alone or in presence of ciprofloxacin (an inducer of the SOS response) at ¼ x MIC were unsuccessful. High-level ceftazidime-resistant mutants of TrA_1 were obtained, but all those examined still harboured IS*26* between $bla_{CTX\text{-}M\text{-}15}$ and its promoter (as detected by PCR using the primer pair IS*26*-CTX-M for/IS*26*-CTX-M rev). The mechanisms of high-level ceftazidime resistance in those mutants was not investigated further, but may have involved changes in membrane permeability.

Overall, antibiotic susceptibilities, CTX-M specific activities, IEF and RT-PCR results, all correlated and indicated that expression of CTX-M-15 β-lactamase in two of the three strain A isolates tested was lower than in the strain D isolates used as controls. This probably explained the lower resistance to third-generation cephalosporins of most strain A isolates, and IS*26*, located between $bla_{CTX\text{-}M\text{-}15}$ and its normal promoter, may be responsible for this decrease in enzyme expression. However, CTX-M-15 expression was sometimes up-regulated (such as in isolate A_2) for reasons that remain yet unclear, raising MICs of third-generation cephalosporins. Trans-acting factors are thought to intervene in this phenomenon.

Figure 45. (A) RT-PCR agarose gel electrophoresis picture evaluating OXA-1 and CTX-M-15 mRNA levels and (B) three-dimensional representation of mRNA levels[a]



[a] Peak heights are related to mRNA quantity.

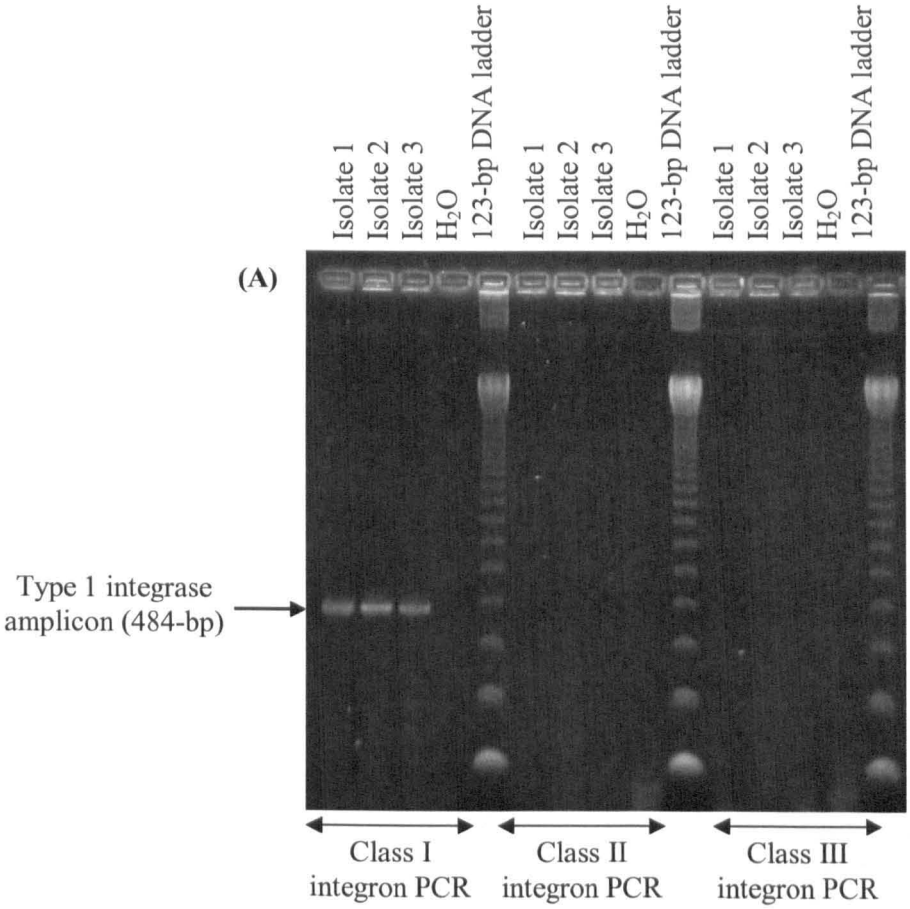
2. Genes encoding group-2 CTX-M enzymes

Genes encoding group-2 CTX-M enzymes comprise at least eight different variants (http://www.lahey.org/studies/other.htm). $bla_{CTX\text{-}M\text{-}2}$ is the most common allele of this phylogenetic group and has long been the predominant $bla_{CTX\text{-}M}$ variant detected among CTX-M-positive Enterobacteriaceae in Argentina (Bonnet, R., 2004). A number of studies looking at the genetic environment of $bla_{CTX\text{-}M\text{-}2\text{-}like}$ genes have shown they were most likely to have originated from the chromosome of *K. ascorbata* (Humeniuk, C. *et al.*, 2002). Most $bla_{CTX\text{-}M\text{-}2\text{-}like}$ genes, including $bla_{CTX\text{-}M\text{-}2}$ itself, have been described in unusual class I integrons and were located upstream of *orf3*, a gene also present downstream of the chromosomal $bla_{KLUA\text{-}1}$ gene of *K. ascorbata*. By contrast, a few other members of this phylogenetic group of $bla_{CTX\text{-}M}$ genes, such as $bla_{CTX\text{-}M\text{-}5}$, have been shown to be associated with an IS*Ecp1-like* element; even here though the immediate genetic surroundings of both $bla_{CTX\text{-}M\text{-}5}$ and $bla_{KLUA\text{-}1}$ were found to share a very high level of homology (Humeniuk, C. *et al.*, 2002).

For this study, the genetic surroundings of $bla_{CTX\text{-}M\text{-}2}$ in the three unrelated clinical isolates referred to as isolate 1, isolate 2 and isolate 3 in Chapter III, section 3 were investigated. This was achieved by conventional PCR in the first instance, and then by long-range PCR assays using various sets of primers (in accordance with previously published data on the genetic environments of $bla_{CTX\text{-}M\text{-}2}$), and finally by DNA sequencing.

No PCR product was obtained following amplification using primers specifically binding to the 3' end of IS*Ecp1* (forward primer) and to $bla_{group\text{-}2\ CTX\text{-}M}$ genes (reverse primer). The three clinical isolates were shown on the other hand to carry class I integrons (Figure 46), whereas neither class II nor class III integrons were detected by PCR. The DNA sequences located between the 5' and 3' conserved sequences of these class I integrons were then amplified using the primer pair int5'-CS/int-3'CS, and PCR products were subsequently sequenced.

Figure 46. Agarose gel electrophoresis of Class I-III integron PCR products of *E. coli* isolates 1-3 harbouring the $bla_{CTX\text{-}M\text{-}2}$ gene



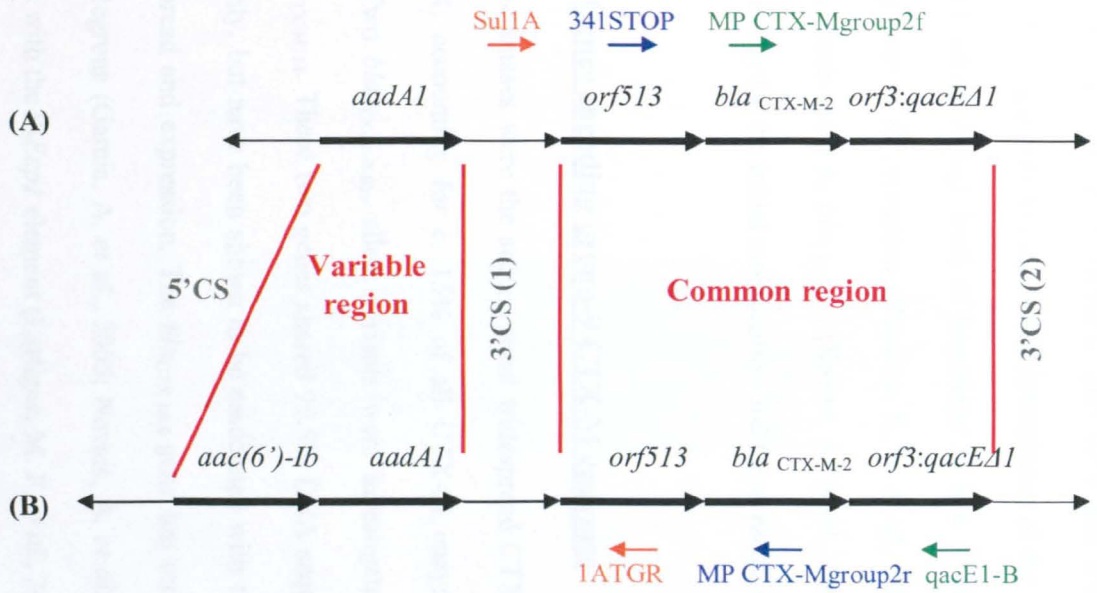
Similar *c.* 1-kb amplicons were obtained for isolates 2 and 3 using primers int5'-CS and int3'-CS, while isolate 1 showed a *c.* 1.8-kb PCR product. DNA sequencing showed none of these amplicons to contain the $bla_{CTX\text{-}M\text{-}2}$ gene. Rather, the integrons from all three isolates were found to carry the gene cassette *aadA1*; that from isolate 1 additionally had *aac(6')-Ib* at its 5'-end.

It was then postulated that the CTX-M-2-encoding gene was mediated by large and complex class I integrons, as often found in the past (Arduino, S. M. *et al.*, 2002; Power, P. *et al.*, 2005; Valverde, A. *et al.*, 2006), and in which the 3' conserved sequence has been duplicated. The resulting integron then becomes too long to be amplified successfully by conventional PCR. In order to overcome this, various pairings of primers (selected according to the published literature) were used to create a map of the whole integrons; the primer pairs used were as following (primer binding sites are indicated in Figure 47):

- Sul1A/1ATGR (shown in orange in Figure 47) to amplify from *sulI* to *orf513*
- 341STOP/MP-CTX-Mgroup2r (shown in blue in Figure 47) to amplify from *orf513* to $bla_{CTX\text{-}M\text{-}2}$
- MP-CTX-Mgroup2f/qacE1-B (shown in green in Figure 47) to amplify from $bla_{CTX\text{-}M\text{-}2}$ to *qacEΔ1*

The PCR reactions using the primer pairs listed above were positive with all three isolates suggesting that the $bla_{CTX\text{-}M\text{-}2}$ gene was mediated by complex class I integrons indeed. These integrons shared a common 3'CS(1)-3'CS(2) region, which harboured the gene cassettes *orf513* and $bla_{CTX\text{-}M\text{-}2}$ (Figure 47). These complex integrons also encoded either *aadA1* only (isolates 2 and 3) or *aac(6')-Ib* and *aadA1* (isolate 1) in their variable region (5'-3'CS). Both these types of integron have been described previously, among various Gram-negative clinical strains isolated in Argentina (Arduino, S. M. *et al.*, 2003).

Figure 47. Schematic representation of the *bla*$_{CTX-M-2}$-harbouring class I integrons in isolates 2 and 3 (A), and in isolate 1 (B)



3. Genes encoding group-8 CTX-M enzymes

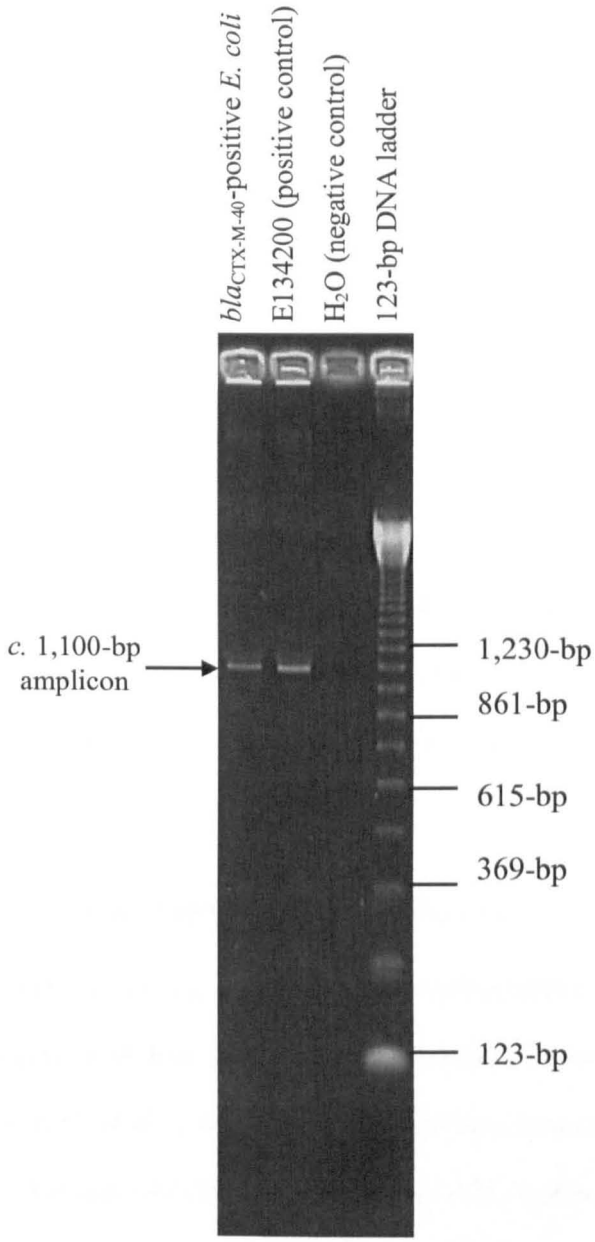
During this study, a single *E. coli* clinical isolate with a group-8 CTX-M enzyme (CTX-M-40) was referred to ARMRL [in 2004, (see Chapter III, section 4.1)]. This allele, found once previously in an *E. coli* isolate from 1999 (E134200), was then found to be associated to an IS*Ecp1-like* element (Hopkins, K. L. *et al.*, 2006b).

A PCR assay using the primers prom+ (specific to the 3'-end of IS*Ecp1*) and CTX-Mgp8-R (specific to 3'end of $bla_{\text{group-8 CTX-M}}$) yielded a *c.* 1,100-bp amplicon (Figure 48), similar to that obtained with the positive control (isolate E134200). This suggested $bla_{\text{CTX-M-40}}$ was also associated with IS*Ecp1* in the isolate referred to ARMRL. This view was confirmed by DNA sequencing, which showed that $bla_{\text{CTX-M-40}}$ was located 66-bp downstream of the 3'-end of IS*Ecp1*, and that its genetic environment shared a high-level of homology to that of $bla_{\text{KLUG-1}}$, the chromosomal β-lactamase-encoding gene of *K. georgiana* (Hopkins, K. L. *et al.*, 2006b). As previously observed with other $bla_{\text{CTX-M}}$ genes such as $bla_{\text{CTX-M-15}}$ (Karim, A. *et al.*, 2001), the IS*Ecp1* element was thought to be responsible for the initial mobilization and the expression of $bla_{\text{CTX-M-40}}$.

4. Genes encoding group-9 CTX-M enzymes

CTX-M-9-like β-lactamases were the second most widespread CTX-M variants found in *E. coli* isolates in the UK, accounting for *c.* 15% of all CTX-M enzymes (N. Woodford, personal communication). Two $bla_{\text{CTX-M-9-like}}$ allele variants were investigated during this study, namely $bla_{\text{CTX-M-9}}$ and $bla_{\text{CTX-M-14}}$. These two genes shared 99.5% DNA sequence homology, differing by four nucleotides only, but have been shown to be associated with two distinct genetic structures facilitating their spread and expression. The $bla_{\text{CTX-M-9}}$ gene has repeatedly been reported within complex class I integrons (Garcia, A. *et al.*, 2005; Novais, A. *et al.*, 2006), while $bla_{\text{CTX-M-14}}$ has usually been linked with the IS*Ecp1* element (Lartigue, M. F. *et al.*, 2004).

Figure 48. Agarose gel electrophoresis of IS*Ecp1*-*bla*$_{\text{group-8 CTX-M}}$ PCR product



In view of these previous data, class I integrons and the association of IS*Ecp1* with $bla_{CTX\text{-}M\text{-}9\text{-}like}$ were sought by PCR among the 20 representative isolates with CTX-M-9-like β-lactamases previously mentioned in Chapter III of results, section 2. Class I integrons were sought with the int1-f and int1-r primers, while the IS*Ecp1*-$bla_{CTX\text{-}M\text{-}9\text{-}like}$ link was searched using the primer pair prom+/CTX-M M9L. Results are summarised in Table 26.

Half the screened isolates (10/20) harboured a class I integron. These included both representatives 11 and 19, which have been shown to encode $bla_{CTX\text{-}M\text{-}9}$ genes but not the two with CTX-M-14 enzymes (see Chapter III, section 2.2). Also, the association IS*Ecp1*-$bla_{CTX\text{-}M\text{-}9\text{-}like}$ was detected among most of the screened isolates (75%, 15/20), including representatives 2 and 16, shown previously to encode $bla_{CTX\text{-}M\text{-}14}$ (see Chapter III, section 2.2), though not in the two confirmed by sequencing to produce the CTX-M-9 enzyme. These finding agree with previous findings reporting $bla_{CTX\text{-}M\text{-}9}$ to be integron-mediated and, $bla_{CTX\text{-}M\text{-}14}$ to be associated with the IS*Ecp1* element. Class I integrons nevertheless were detected in a few isolates harbouring the association IS*Ecp1*-$bla_{CTX\text{-}M}$ (representatives 7, 8, 13, 17 and 18). Although these integrons were not characterised, they were not expected to mediate the $bla_{CTX\text{-}M\text{-}9\text{-}like}$ gene.

4.1. Genetic environment of $bla_{CTX\text{-}M\text{-}9}$

The genetic environment of the $bla_{CTX\text{-}M\text{-}9}$ gene in representative isolates 11 and 19 was investigated. This was done with three separate long PCR assays, in accordance with a previously described method (Sabate, M. *et al.*, 2002), using the following primers pairs:

- Reaction 1: 341stop/1ATGR, amplifying from *orf513* to $bla_{CTX\text{-}M\text{-}9}$
- Reaction 2: 1stopPR/03000SR, amplifying from $bla_{CTX\text{-}M\text{-}9}$ to *orf1005*
- Reaction 3: CTX-M M9U/int3'-CS, amplifying from $bla_{CTX\text{-}M\text{-}9}$ to the 3'-conserved sequence of class I integron

Reaction 1 yielded the expected *c.* 900-bp DNA fragment with both isolates 11 and 19 agreeing with data from the literature, which report $bla_{CTX\text{-}M\text{-}9}$ as being located downstream of *orf513*.

Table 26. Results summary for class I integron and IS*Ecp1-bla*$_{CTX\text{-}M\text{-}9\text{-}like}$ PCR assays[a]

Isolate ID	CTX-M variant[b]	class I integron	ISEcp1-*bla*$_{CTX\text{-}M\text{-}9\text{-}like}$
1	ND[c]	+	
2	CTX-M-14		+
3	ND[c]		+
4	ND[c]	+	
5	ND[c]		+
6	ND[c]		+
7	ND[c]	+	+
8	ND[c]	+	+
9	ND[c]		+
10	ND[c]		+
11	CTX-M-9	+	
12	ND[c]		+
13	ND[c]	+	+
14	ND[c]		+
15	ND[c]		+
16	CTX-M-14		+
17	ND[c]	+	+
18	ND[c]	+	+
19	CTX-M-9	+	
20	ND[c]	+	

[a] + indicates a positive PCR result.
[b] All 20 CTX-M variants belonged to phylogenetic group-9.
[c] ND = not determined.

No amplification product was obtained in reaction 2 suggesting there was no *orf1005* located downstream of $bla_{CTX\text{-}M\text{-}9}$; this was in contrast to various reports in the literature (Novais, A. *et al.*, 2006; Sabate, M. *et al.*, 2002). Finally, a 1.5-kb PCR product was obtained with both isolates 11 and 19 in reaction 3, confirming $bla_{CTX\text{-}M\text{-}9}$ was integron-mediated. In summary, these data suggested that the $bla_{CTX\text{-}M\text{-}9}$ gene in representative isolates 11 and 19 was linked to *orf513*, also known as CR1 (Common Region 1), within class I integrons. The CR1 element has been shown previously to mobilise and promote the expression of various antibiotic resistance genes, including $bla_{CTX\text{-}M}$ genes such as $bla_{CTX\text{-}M\text{-}9}$ and $bla_{CTX\text{-}M\text{-}2}$ (Rodriguez-Martinez, J. M. *et al.*, 2006).

4.2. Genetic environment of $bla_{CTX\text{-}M\text{-}14}$

With preliminary PCR data showing the presence of an IS*Ecp1*-$bla_{CTX\text{-}M}$ association in both the representative isolates 2 and 16 (confirmed to produce CTX-M-14 β-lactamase), it was inferred that $bla_{CTX\text{-}M\text{-}14}$ was linked to IS*Ecp1*, which correlated with previous reports from the literature (Lartigue, M. F. *et al.*, 2004). As seen with $bla_{CTX\text{-}M\text{-}15}$, the IS*Ecp1* element was thought to be responsible for the initial mobilisation and the expression of $bla_{CTX\text{-}M\text{-}14}$. Also, this association was detected by PCR among the majority of representative isolates with group-9 CTX-M enzymes investigated in this study (15/20); this may possibly reflect a dominance of the CTX-M-14-type enzyme *vs.* the CTX-M-9-type among UK *E. coli* isolates with phylogenetic group-9 CTX-M β-lactamases, though this was not investigated further.

5. Summary

The $bla_{CTX\text{-}M}$ genes of UK *E. coli* clinical isolates were found to be linked either to IS*Ecp1* (*e.g.* $bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}40}$), or to CR1 (also known as *orf513*) within complex class I integrons ($bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}9}$). Both these elements have been shown previously to promote the mobilisation and the expression of $bla_{CTX\text{-}M}$ genes.

Chapter V. Complete nucleotide sequence of pEK499, the multi-drug resistance plasmid of UK *Escherichia coli* epidemic clone A with CTX-M-15 beta-lactamase

1. Introduction

In the past decade, CTX-M type enzymes have rapidly become the most common plasmid-mediated ESBLs around the globe, reported among a number of various genera of Enterobacteriaceae, but predominantly in *E. coli*. CTX-M-15 β-lactamase, the most common variant across Europe (except probably in Iberia) and the Far East, is often encoded by self-transferable plasmids belonging to incompatibility group FII (Canton, R. and Coque, T. M., 2006; Lavollay, M. *et al.*, 2006). Very few bla_{CTX-M}-harbouring plasmids have been completely sequenced so far and data for only two are available publicly (accession numbers NC_005327 and NC_004464, http://www.ncbi.nlm.nih.gov). Consequently, very little is known about the degree of relatedness of these bla_{CTX-M}-encoding plasmids, although most have been shown in this work and in the literature, to share a number of characteristics such as the antibiotic resistance determinants bla_{TEM-1}, bla_{OXA-1} and *aac(6')-Ib*.

As this study showed, $bla_{CTX-M-15}$ is also often mediated by IncFII plasmids in the UK, including in the national *E. coli* epidemic clones (see Chapter I), and it is often linked to bla_{TEM-1}, bla_{OXA-1} and *aac(6')-Ib-cr*. Most of these plasmids were found to be self-transferable, except for that of *E. coli* epidemic clone A, the most widespread lineage in the UK, having been referred to ARMRL from over 50 different centres. This study firstly focused on the molecular characterisation of the resistance mechanisms of clone A to the major classes of antibiotics (see Chapter I), finding that most of these were co-encoded on a single plasmid of *c.* 120-kb. Therefore, it was decided to characterise this plasmid further, aiming at better understanding its role in the epidemiological success of clone A. The CTX-M-15-encoding plasmid of representative isolate A_1 (plasmid pEK499) was therefore sequenced fully, by MWG Biotech AG (Ebersberg, Germany) and was annotated in collaboration with Dr Anthony Underwood (HPA-Centre for Infections, Bioinformatics Unit, Colindale).

2. General molecular characteristics of plasmid pEK499

Plasmid pEK499 was a double-stranded circular DNA molecule of 117,536 nucleotides precisely. It encoded 185 predicted genes based on analysis using the BASys web-server. As previously determined by PCR, plasmid pEK499 belonged to incompatibility group IncFII; it harboured genes encoding for the *repA/B/E* initiation replication protein system, specific to plasmids of the IncFII group. Overall, 140 of the 185 predicted genes encoded proteins with known functions, and involved in a wide range of biological processes (Figure 49), including:

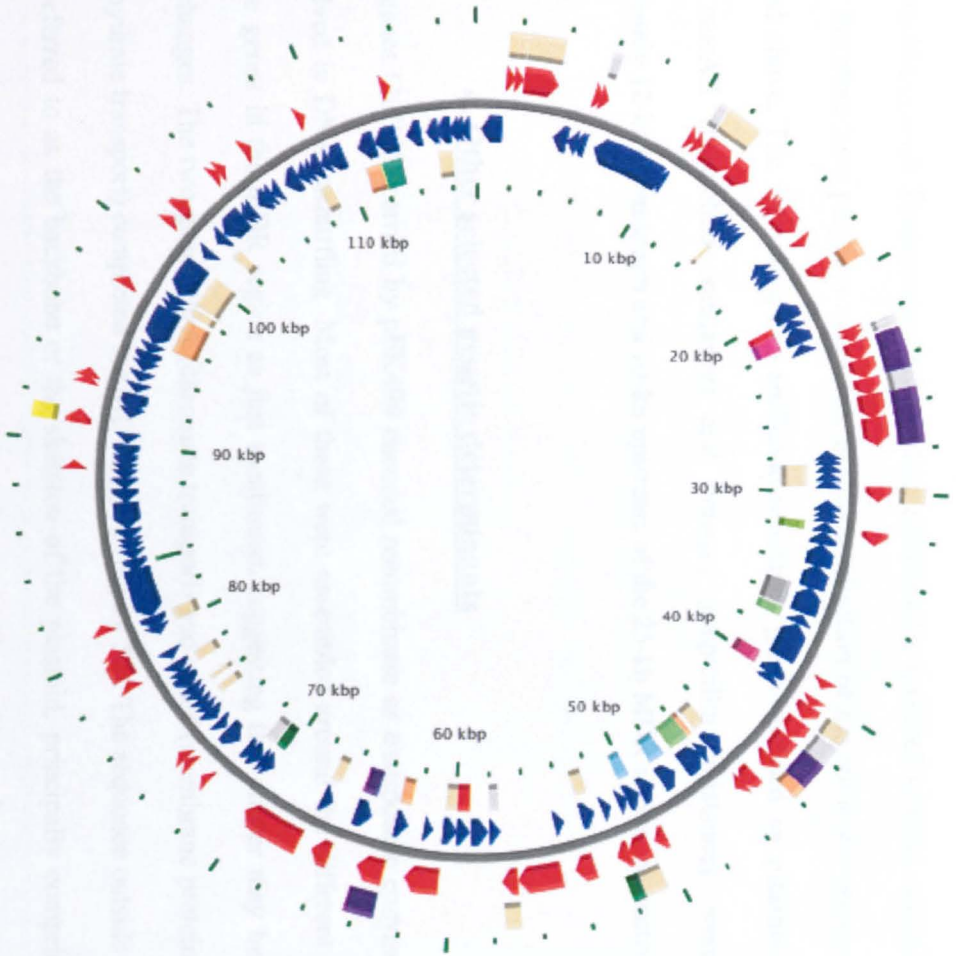
- DNA replication, recombination and repair mechanisms
- Transcription and translation regulation
- Post-translational modifications (chaperones)
- Carbohydrate transport and metabolism
- Amino acid transport and metabolism
- Inorganic ion transport and metabolism
- Cell cycle control and chromosome partitioning
- Energy production and conversion
- Co-enzyme metabolism
- Cell envelope and outer membrane biogenesis

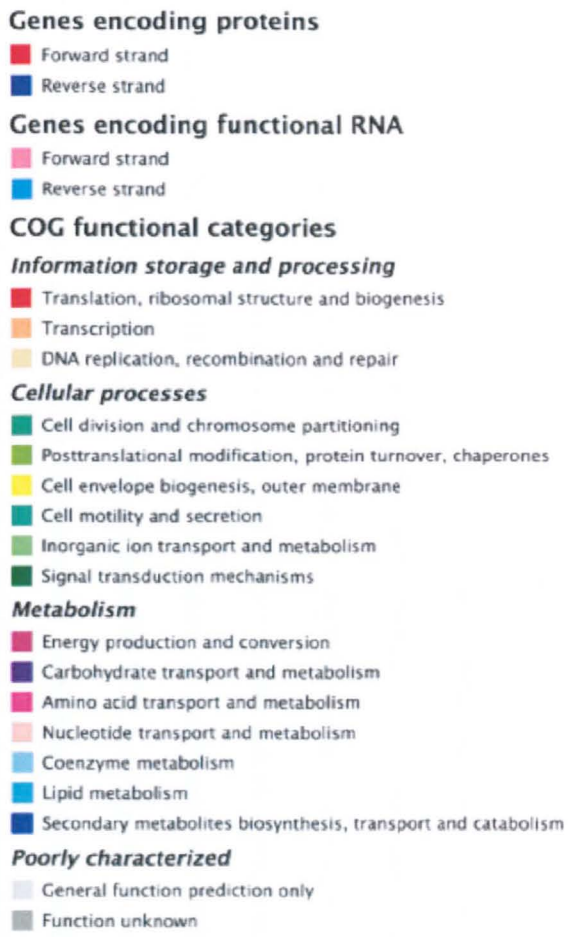
None of the remaining 45 predicted genes, encoding proteins with unknown functions, displayed conserved domains according to the Conserved Domain Database search tool (version 2.11, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

3. Antibiotic resistance determinants

As previously demonstrated (see MICs for TrA_1, Table 10), plasmid pEK499 encoded multi-drug resistance. It affected the activity of most β-lactam antibiotics, except cephamycins and carbapenems, aminoglycosides (except gentamicin), ciprofloxacin, tetracycline, trimethoprim and sulphamethoxazole.

Figure 49. Genetic map of plasmid pEK499 (117,536-bp) from clonal representative isolate A_1





DNA sequencing of the entire plasmid revealed that it carried eleven known antibiotic resistance genes, affecting the activity of eight major classes of antibiotics (Table 27). Nine of these genes were clustered in a *c.* 25-kb "Multi-Drug Resistance" (MDR) region as shown in Figure 50, and included the $bla_{CTX\text{-}M\text{-}15}$ gene (resistance to third-generation cephalosporins) as well as $bla_{OXA\text{-}1}$ (resistance to penicillins and β-lactam/β-lactamase inhibitor combinations), *aac(6')-Ib-cr* (resistance to tobramycin and reduced susceptibility to ciprofloxacin), *catB4* (chloramphenicol resistance), *mphA* (macrolide resistance) and *tet*(A) (tetracycline resistance). It was also confirmed that the association of $bla_{CTX\text{-}M\text{-}15}$ with IS*Ecp1* was interrupted by the introduction of the insertion sequence IS*26*. The 1.8-kb class I integron, initially detected by PCR (see Chapter I) and shown to carry dfr_{17} (trimethoprim resistance), *aadA*5 (streptomycin resistance) and *sulI* (sulphonamide resistance), was also part of the MDR cluster. This MDR region also incorporated Tn*3*- and Tn*1721*-like transposons, as well as four copies of the insertion element IS*26* (including that adjacent to $bla_{CTX\text{-}M\text{-}15}$). Transposons and insertion elements are involved in DNA rearrangements, and may therefore have played a major role in the mobilisation of the antibiotic resistance genes mentioned above. The remaining two antibiotic resistance genes encoded by plasmid pEK499, namely *macAB* (macrolide resistance) and $bla_{TEM\text{-}1}$ (ampicillin resistance), were located approximately 12-kb downstream and 10-kb upstream of the 25-kb MDR region, respectively.

4. Other selected genetic determinants

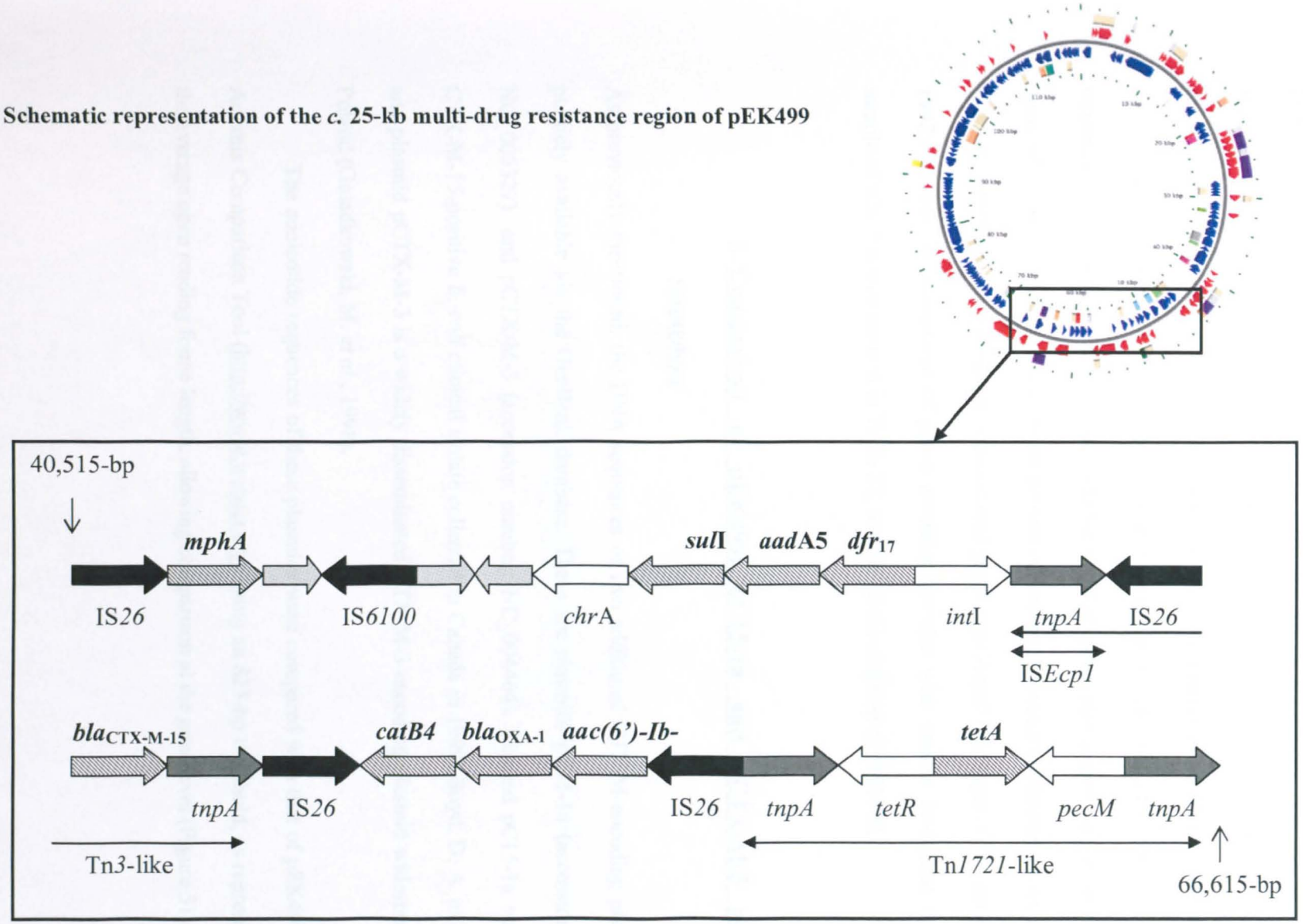
Several genes (30/185) carried by pEK499 encoded recombinase or transposase enzymes, which are involved in DNA reshuffling. Most of these were assembled around the different antibiotic resistance genes in the MDR region as just mentioned, suggesting this cluster may be prone to genetic changes. The two genes *chrA* (chromate transport) and *pecM* (membrane protein involved in carbohydrate transport) completed the MDR region (Figure 50). The sequence outside the MDR region, referred to as the backbone or the skeleton of the plasmid, principally comprised genes involved in plasmid replication and maintenance, as well as those encoding proteins with unrecognised functions.

Table 27. Selected genes encoded by plasmid pEK499 and their function[a]

Gene	Function	
aac(6')-Ib-cr	Aminoglycoside and ciprofloxacin resistance	Within 25-kb MDR region
aadA5	Streptomycin and spectinomycin resistance	
$bla_{CTX\text{-}M\text{-}15}$	β-Lactam resistance	
$bla_{OXA\text{-}1}$	β-Lactam resistance	
catB4	Chloramphenicol resistance	
mphA	Macrolide resistance	
sulI	Sulphonamide resistance	
dfr_{17}	Trimethoprim resistance	
tet (2 genes)	Tetracycline resistance	
chrA	Chromate transport	
pecM	Carbohydrate transport and metabolism	
macAB (3 genes)	Macrolide resistance	Outside MDR region
$bla_{TEM\text{-}1}$	β-Lactam resistance	
traT	Serum survival gene (anti-phagocytosis)	
vagC/vagD	Virulence associated genes	
ccdA/ccdB	F-plasmid-derived addiction system	
pemK/pemI	R100-plasmid-derived addiction system	
FTR1	Inorganic ion (Fe^{2+}/Pb^{2+}) transport and metabolism	
icc	Phosphorus metabolism	
resA	Cytochrome c maturation	
sopA/sopB	Plasmid partitioning	
klcA	Post-conjugation anti-restriction protein	
rep (3 genes)	IncFII plasmid replication initiation system	
tra (14 genes)	Genes involved in conjugative transfer	
ugp (4 genes)	Carbohydrate transport and metabolism	
araQ	L-arabinose transport system permease protein	
yjgB	Zinc-dependent alcohol dehydrogenase	

[a] Antibiotic resistance genes are highlighted in grey.

Figure 50. Schematic representation of the *c.* 25-kb multi-drug resistance region of pEK499



The backbone of plasmid pEK499 shared a number of genes with plasmid R100, including many genes with undefined functions, as well as the *pemK/pemI* system (R100-plasmid-derived addiction system) and part of the *tra* operon (plasmid conjugative transfer). The backbone of plasmid pEK499 also carried the F-plasmid-derived addiction system *ccdA/ccdB*. Both plasmid addiction systems encode a stable toxin and an unstable antitoxin, which militate against plasmid loss, and therefore ensure the maintenance of the plasmid, even in the absence of antibiotic pressure.

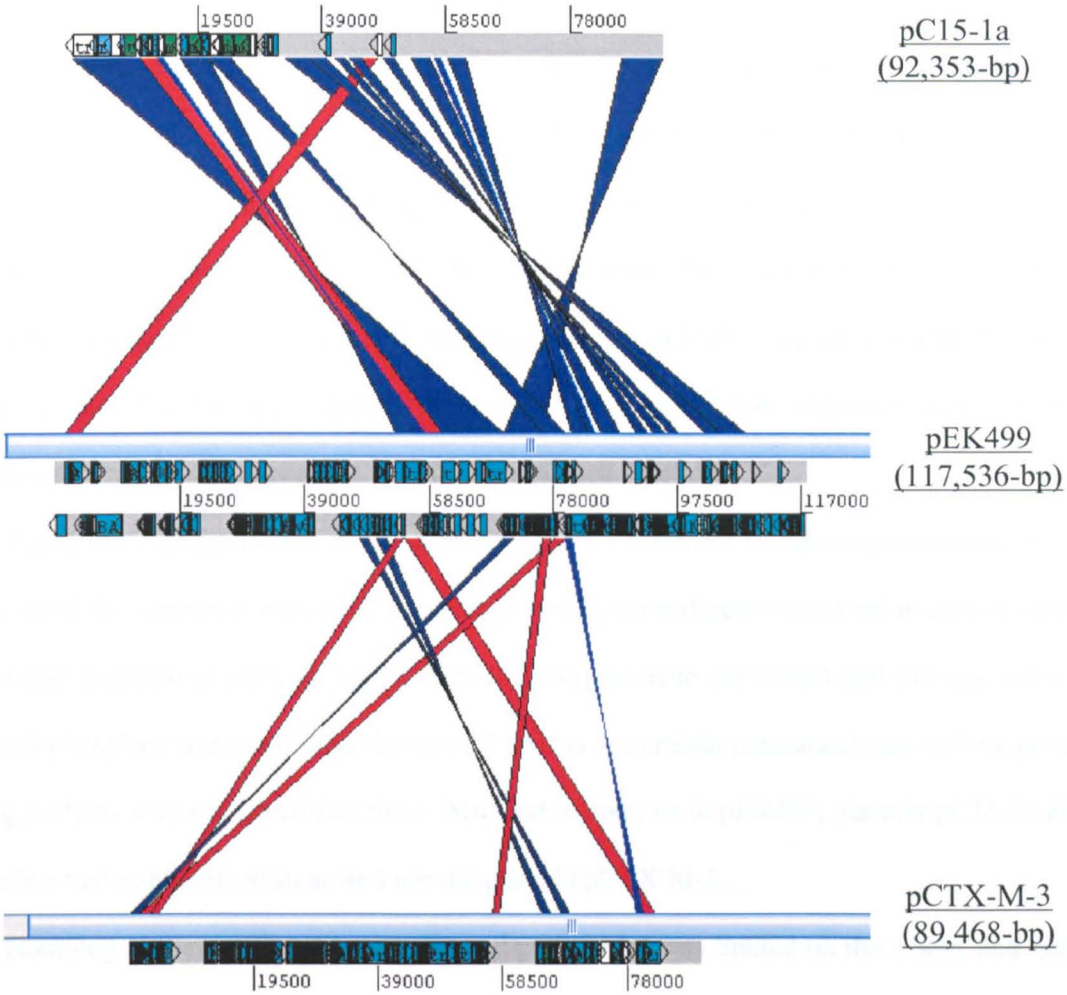
Besides antibiotic resistance genes and transposons, plasmid pEK499 also harboured virulence-associated sequences. These included the *traT* gene, also known as the serum survival gene, which encodes on outer membrane protein enhancing resistance to phagocytosis (Aguero, M. E. *et al.*, 1984), and the pathogenicity-associated genes *vagC/vagD* (Pullinger, G. D. and Lax, A. J., 1992). Finally, an assortment of genes encoding proteins with various biological functions, a sample of which is summarized in Table 27, completed the skeleton of pEK499.

5. Comparison of pEK499, pC15-1a and pCTX-M-3 nucleotide sequences

As previously mentioned, the DNA sequences of two additional CTX-M-encoding plasmids are publicly available via the GenBank database. These are plasmids pC15-1a (accession number: NC_005327) and pCTX-M-3 (accession number: NC_004464). Plasmid pC15-1a was from a CTX-M-15-positive *E. coli* clinical isolate collected in Canada in 1999 (Boyd, D. A. *et al.*, 2004), and plasmid pCTX-M-3 is a widely disseminated CTX-M-3-encoding plasmid widespread across Poland (Gniadkowski, M. *et al.*, 1998).

The nucleotide sequences of these plasmids were compared with that of pEK499 with the Artemis Comparison Tool (http://www.webact.org) using an 825-bp threshold, as representative of the average open reading frame length, allowing comparison at the gene level (Figure 51).

Figure 51. Schematic representation of nucleotide sequence homologies[a] between plasmids pEK499, pC15-1a and pCTX-M-3



[a] Using a 825-bp threshold; homologous sequences are shown in red (same orientation) and blue (opposite orientation).

On the whole, plasmid pEK499 appeared to have more genes in common with pC15-1a than with pCTX-M-3. The vast majority of these corresponded to antibiotic resistance genes, including $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, $bla_{TEM\text{-}1}$, *aac(6')-Ib-cr* and *tet*(A), but also to transposase-encoding genes (*tnpA*), and to insertion sequence elements such as IS*26*. More generally, most of the similarities between pEK499 and pC15-1a were clustered in a *c.* 50-kb region, which included the MDR region as well as a part of the pEK499 skeleton found to share some level of homology with plasmid R100. However, the genes within this 50-kb region, most of which were located within transposons, were arranged differently in the two plasmids. Like pEK499, plasmid pC15-1a belonged to incompatibility group IncFII, thus suggesting that both plasmids descended from a common ancestor. Finally, both plasmids were found to carry remnant sequences from plasmid R100, most of which encoded proteins with uncharacterised functions.

About half the genes present on plasmid pEK499 were not found on plasmid pC15-1a; these included the virulence-associated genes *vagC/vagD*, several genes involved in carbohydrate transport and metabolism such as *kdgt* (2-keto-3-deoxygluconate permease) and the *ugp* operon (glycerol-3-phosphate transport), also the *macAB* operon (macrolide resistance) and various genes encoding proteins with undefined functions. Also and in contrast to pEK499, plasmid pC15-1a did not encode a toxin-antitoxin system, and nor did plasmid pCTX-M-3.

Homology between plasmids pEK499 and pCTX-M-3 was limited on the whole, and their similarities mainly consisted of transposase-encoding genes as well as the $bla_{CTX\text{-}M}$ gene, though plasmid pCTX-M-3 carried $bla_{CTX\text{-}M\text{-}3}$ and not $bla_{CTX\text{-}M\text{-}15}$. The known antibiotic resistance genes mediated by plasmids pEK499, pCTX-M-3 and pC15-1a are summarised in Table 28.

Table 28. Summary of known antibiotic resistance genes carried by pEK499, pCTX-M-3 and pC15-1a

Antibiotic resistance genes	pEK499	pCTX-M-3	pC15-1a
aac3-IIa		+	+
aac(6')-Ib-cr	+		+
aadA2[a]		+	
aadA5[a]	+		
$bla_{CTX\text{-}M\text{-}3}$		+	
$bla_{CTX\text{-}M\text{-}15}$	+		+
$bla_{OXA\text{-}1}$	+		+
$bla_{TEM\text{-}1}$	+	+	+
catB3			+
catB4	+		
dfr_{12}[a]		+	
dfr_{17}[a]	+		
macAB	+		
mphA	+	+	
sulI[a]	+	+	
tet(A) and *tet*(R)	+		+

[a] Class I integron-mediated genes.

6. Summary

Overall, it appeared that plasmid pEK499 was much more related to pC15-1a than to pCTX-M-3, correlating with data reported earlier in Chapter I and Chapter IV. Firstly, plasmid cross-hybridization studies showed that the CTX-M-encoding plasmids from *E. coli* epidemic clones A, B, D and E were highly similar, but were less so to that of epidemic clone C (or representative isolate C_1 at least). Secondly, the CTX-M-encoding plasmid from isolate C_1 was shown to carry the exact same IS*Ecp1*-bla_{CTX-M} link as that previously found on plasmid pCTX-M-3 only. As a result, the plasmid of isolate C_1 and pCTX-M-3 are likely to be more related to each other, than to those of the remaining four epidemic clones, which in contrast were more close to plasmid pC15-1a.

Chapter VI . *In-vitro* evolution of ceftazidime resistance in hypermutable *Escherichia coli* with CTX-M-3 beta-lactamase

1. Introduction

In contrast to TEM- and SHV-type ESBLs, most CTX-M enzyme variants have weak catalytic activity against ceftazidime, but changes to amino acids 167 and 240 are associated with increased ceftazidimase activity (Bonnet, R., 2004; Sturenburg, E. *et al.*, 2004). At least eight variants from three of the five phylogenetic groups of CTX-M enzymes have been shown to confer increased resistance to ceftazidime. These are the group-1 enzymes CTX-M-15, -23, -42 and -54; the group-9 enzymes, CTX-M-16, -19, and -27; and finally, CTX-M-25 (Bae, I. K. *et al.*, 2006; Bonnet, R. *et al.*, 2003; Munday, C. J. *et al.*, 2004a; Poirel, L. *et al.*, 2001; Poirel, L. *et al.*, 2002a; Stepanova, M. *et al.*, 2005; Sturenburg, E. *et al.*, 2004).

CTX-M-15, the commonest CTX-M variant worldwide (Canton, R. and Coque, T. M., 2006), including in the UK (Woodford, N. *et al.*, 2004), is a ceftazidime-hydrolysing variant of CTX-M-3 enzyme, differing from its "parent" only by an aspartate to glycine substitution at position 240 (Poirel, L. *et al.*, 2002a). One factor that may potentially accelerate the evolution of CTX-M β-lactamases such as CTX-M-3 with ceftazidimase activity, is hypermutability, which arises via defects in the methyl-directed, post-replication DNA mismatch repair system, notably in the MutS protein (Chopra, I. *et al.*, 2003; LeClerc, J. E. *et al.*, 1996; Miller, K. *et al.*, 2002).

The *in-vitro* evolution of the ceftazidimase activity of CTX-M-3 β-lactamase was therefore investigated in a hypermutable MutS-negative *E. coli* strain. Also, the rate of emergence of high-level ceftazidime resistance in the hypermutable background was compared with that in an isogenic CTX-M-3-producing wild-type *E. coli* strain, which has a normal mutation frequency.

2. Cloning of $bla_{CTX\text{-}M\text{-}3}$ into pBBR1MCS-2

The $bla_{CTX\text{-}M\text{-}3}$ gene was amplified from *E. coli* clinical representative isolate C_1 (Chapter I) using the primer pair prom+/preCTX-M3B, and was cloned into pBBR1MCS-2; the resulting

recombinant vector was designated pCTX-M-3. The successful insertion of $bla_{CTX\text{-}M\text{-}3}$ into the vector was confirmed by PCR with the primer pair T3/T7 [Figure 52(A)], which bind on either side of the multiple cloning site (MCS), and also by enzymatic restriction as shown in Figure 52 (B and C). The recombinant pCTX-M-3 element was then transformed into isogenic *E. coli* wild-type 1411 (WT) and hypermutable MutS-negative 1413 (Δ*mutS*) strains.

3. Mutation frequencies

Both the WT and Δ*mutS* transformants harbouring pCTX-M-3 had the classical phenotype of CTX-M-3-producers; *i.e.* higher MICS of cefotaxime than ceftazidime (Table 30), with clavulanate restoring full susceptibility to both cephalosporins. As ceftazidime MICs were only 1 and 1.5 mg/L for the pCTX-M-3-harbouring WT and Δ*mutS* strains, respectively, resistant mutants were selected using this third-generation cephalosporin at 4 and 6 mg/L.

The mutation frequencies to ceftazidime resistance for WT and Δ*mutS E. coli* in four independent experiments are summarised in Table 29. Predictably, ceftazidime resistance emerged more readily in the hypermutable background than in the wild-type, with an average mutation frequency approximately 60-fold higher with the Δ*mutS* strain (average mutation frequency of 1.29×10^{-5}, SE 4.9×10^{-6}) than with the WT strain (average mutation frequency of 2×10^{-7}, SE 4.3×10^{-8}).

4. DNA sequences of $bla_{CTX\text{-}M}$ mutants

Both strands of the $bla_{CTX\text{-}M}$ gene from the parental WT and Δ*mutS* recombinant strains, and from three WT (WT1-3) and five Δ*mutS* (*mutS*1-5) randomly selected ceftazidime-resistant mutants were sequenced.

Figure 52. Agarose gel electrophoresis to confirm cloning of *bla*$_{CTX-M-3}$ into pBBR1MCS-2: T3/T7 PCR product (A) and restriction analysis of pBBR1MCS-2 and pCTX-M-3 using *Apa*I only (B) or using *Sma*I and *Apa*I (C)

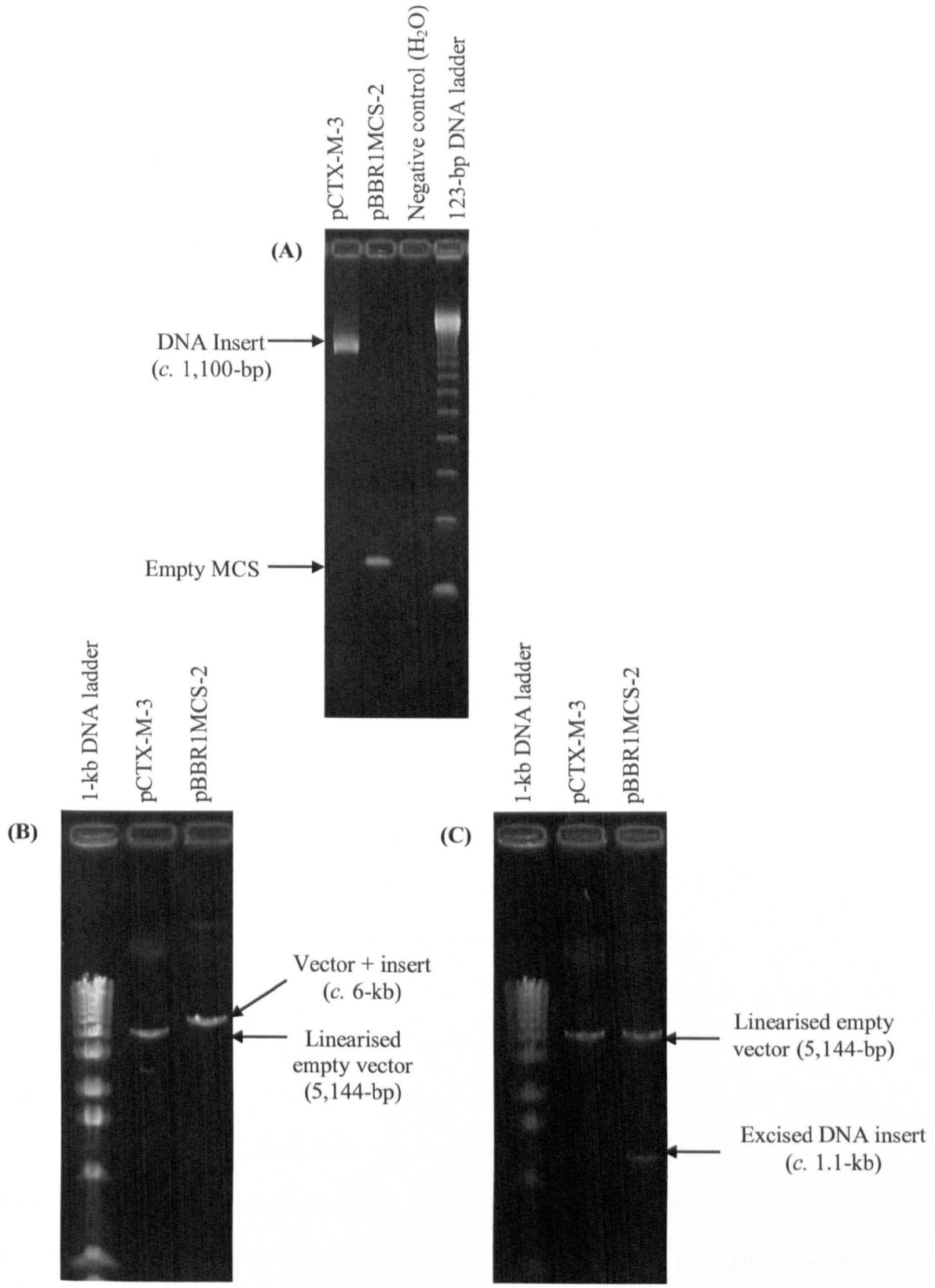


Table 29. Mutation frequencies to ceftazidime resistance for *E. coli* strains 1411 WT and 1413 Δ*mutS* harbouring pCTX-M-3

	1411 WT	1413 Δ*mutS*
Experiment 1	2.88×10^{-7}	1.80×10^{-5}
Experiment 2	8.60×10^{-8}	2.40×10^{-5}
Experiment 3	2.37×10^{-7}	3.90×10^{-6}
Experiment 4	2.00×10^{-7}	5.60×10^{-6}
Mean	2.03×10^{-7}	1.29×10^{-5}
Standard Error	4.3×10^{-8}	4.9×10^{-6}

All eight randomly selected mutants (WT and Δ*mutS*) had single base substitutions at nucleotide 508 or 509 (Figure 53), resulting in amino-acid changes at position 167 (Figure 54).

All five mutants derived in the Δ*mutS* background had the same amino acid substitution: Pro167Ser. This change had been associated with increased ceftazidimase activity in the group-9 enzyme, CTX-M-19 (Poirel, L. *et al.*, 2001), and in laboratory-generated mutants of CTX-M-2 [group-2 CTX-M, (Welsh, K. J. *et al.*, 2005)], though not previously in group-1 enzymes such as CTX-M-3.

Two mutants, both derived in the WT background (WT1-2), had a Pro167Thr substitution corresponding to CTX-M-42 (Stepanova, M. *et al.*, 2005); this substitution is also present in the ceftazidime-hydrolysing group-1 enzyme, CTX-M-23 (Sturenburg, E. *et al.*, 2004). The remaining ceftazidime-resistant WT mutant (WT3) had a Pro167Gln substitution. This change had not been reported at the time of this study in any wild-type CTX-M variant, but was described soon afterwards in a clinical *K. pneumoniae* isolate from Korea (Bae, I. K. *et al.*, 2006) as variant CTX-M-54.

None of the selected $bla_{CTX\text{-}M}$ mutants was found to encode CTX-M-15, which is believed to be the commonest ceftazidimase variant of CTX-M-3.

5. Evolution of antibiotic susceptibilities

All of the randomly-selected mutants (WT1-3 and Δ*mutS*1-5) were clearly resistant to ceftazidime, with MICs increased 32-fold at least (Table 30) by comparison with their respective parent strain. Extraction of the recombinant vectors from ceftazidime-resistant mutants and re-transformation into new cultures of their respective WT or Δ*mutS* host strains was performed to ensure that changes in antibiotic susceptibilities were solely due to the altered $bla_{CTX\text{-}M}$ genes. MICs for the re-transformants, which remained very similar to those for their respective mutant hosts, confirmed that ceftazidime resistance was conferred by the altered β-lactamases encoded by pCTX-M-3. However, variations in MICs between mutants and re-transformants with the same enzyme mutant (*e.g.* cefotaxime MICs for *mutS*1-5 mutants ranged from 8 to 128 mg/L) suggested that other factors, such as levels of enzyme expression levels might also play a role.

Figure 53. Chromatograms of partial *bla*$_{CTX-M}$ sequences from ceftazidime-resistant mutants

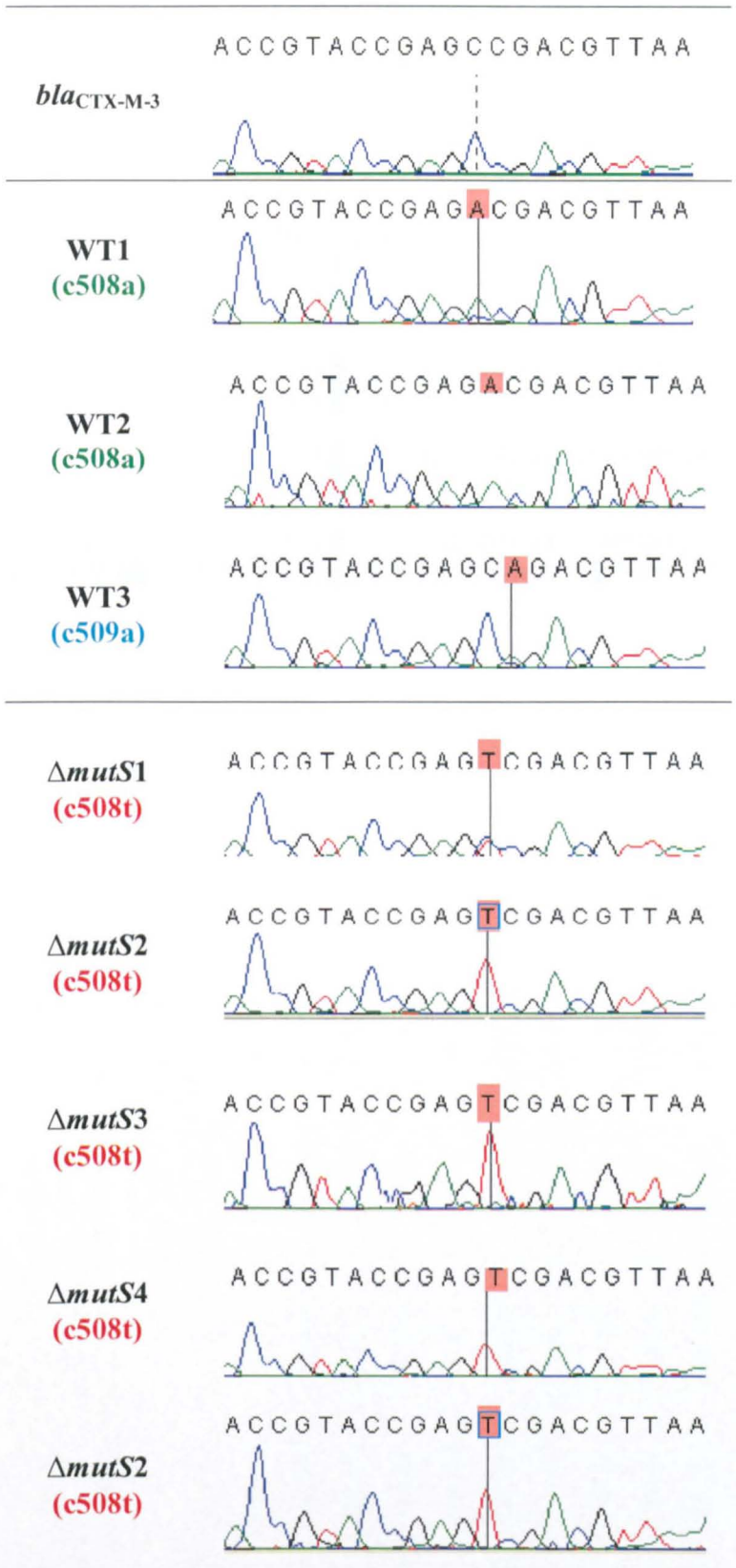
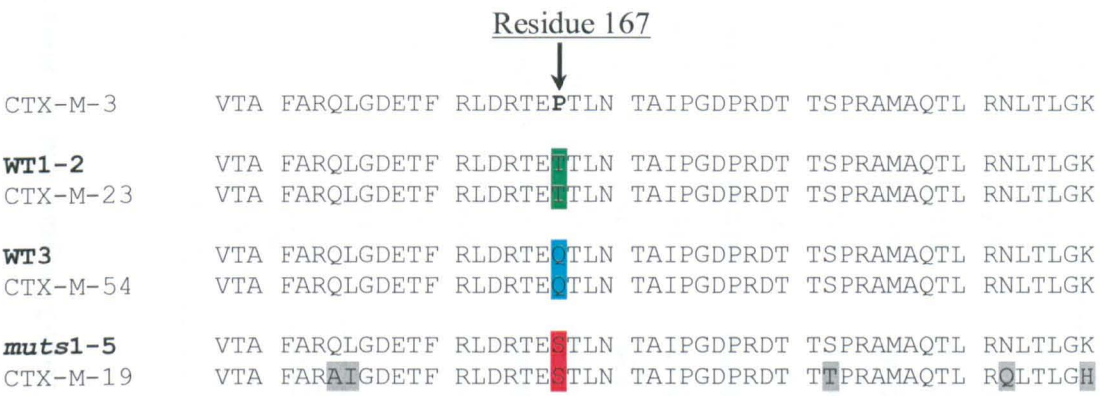


Figure 54. Partial amino acid sequence alignment (residues 148 to 197) of CTX-M-3, -19, -23, -54, and of CTX-M enzymes from ceftazidime-resistant WT and Δ*mutS* mutants showing the different substitutions at position 167[a]



[a] The amino acid changes highlighted in grey correspond to the differences in phylogeny between the group-1 enzyme CTX-M-3 and the group-9 variant CTX-M-19.

Table 30. MIC ranges (mg/L) for host strains, wild-type (1411) and Δ*mutS* (1413) *E. coli* with CTX-M-3 enzyme, and for mutants selected for enhanced ceftazidimase activity and for their derived re-transformants

Organism	1411 (n=1)	1411 pCTX-M-3 (n=1)	1411 mutant (n=2)	1411 re-transformant (n=2)	1411 mutant (n=1)	1411 re-transformant (n=1)	1413 (n=1)	1413 pCTX-M-3 (n=1)	1413 mutant (n=5)	1413 re-transformant (n=5)
Mutation	n/a[a]	Absent	Pro167Thr	Pro167Thr	Pro167Gln	Pro167Gln	n/a[a]	Absent	Pro167Ser	Pro167Ser
AMP	2	> 64	> 64	> 64	> 64	> 64	4	> 64	> 64	> 64
AMX + CLA	2	8	8	4	8	8	4	8	8	4 - 8
CTX	≤0.125	128	16 - 32	8 - 64	32	8	≤0.125	256	8 - 128	4 - 128
CTX + CLA	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060 - 0.125	≤0.060
CAZ	≤0.250	1	32 - 64	32	32	32	≤0.250	1.5	64 - 128	16 - 64
CAZ + CLA	0.125	0.125	1 - 2	1	1	0.5	0.125	0.25	1 - 2	0.5 - 2
CPM	≤0.125	8	4	4 - 8	4	2	≤0.125	16	2 - 8	2 - 16
CPM + CLA	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060
FOX	4	4	4 - 8	4	4	4	8	8	4 - 8	8 - 16
PIP	≤1.0	> 64	> 64	64	> 64	32	≤1.0	> 64	≥64	64
PIP + TZB	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0
ETP	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125
IPM	0.25	0.5	0.25	0.25	0.25	0.25	0.5	0.5	0.25 - 0.5	0.5 - 1
MEM	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060	≤0.060

[a] n/a = not applicable.

Most mutants required higher MICs for ceftazidime than for cefotaxime. This was also observed with other CTX-M variants harbouring changes at position 167, such as CTX-M-19, -23 and -42 (Poirel, L. *et al.*, 2001; Stepanova, M. *et al.*, 2005; Sturenburg, E. *et al.*, 2004). In contrast, cefepime MICs were decreased up to eight-fold compared with strains harbouring the unchanged $bla_{CTX\text{-}M\text{-}3}$. Such an exchange in catalytic activity between ceftazidime and cefepime was previously reported between CTX-M-19 and its progenitor CTX-M-18 (Poirel, L. *et al.*, 2001), and between CTX-M-2 and its *in-vitro* ceftazidimase mutants (Welsh, K. J. *et al.*, 2005). Notably, and perhaps explaining its clinical success, the CTX-M-15 enzyme confers high-level resistance to both cefepime and cefotaxime, as well as ceftazidime.

All of the studied ceftazidime-resistant mutants remained susceptible to carbapenems, and to the combinations of cephalosporin with clavulanate and of piperacillin with tazobactam (the OXA-1 enzyme was not present in these organisms).

6. Summary

Mutations in $bla_{CTX\text{-}M\text{-}3}$ conferring high-level resistance to ceftazidime emerged more readily in a hypermutable background, but also occurred in the wild-type host. All the selected ceftazidime-resistant mutants had changes at residue 167, a site shown previously to affect ceftazidimase activity in various CTX-M enzyme variants. At the time of this study, the Pro167Gln substitution was novel, and has since been found in a clinical *K. pneumoniae* isolate where it was named CTX-M-54 (Bae, I. K. *et al.*, 2006). The presence of this substitution suggests that mutations could play a role in the expansion of substrate ranges for CTX-M enzymes and ESBLs more generally, as others have shown for *e.g.* TEM enzymes (Ellington, M. J. *et al.*, 2006).

More generally, since mutator *E. coli* strains with lesions in *mutS* are known to be present among natural bacterial populations (LeClerc, J. E. *et al.*, 1996), hypermutable *E. coli* may have played and may still play a role in the evolutionary diversification of CTX-M enzymes.

DISCUSSION

Escherichia coli is an ambiguous micro-organism. Some would regard it as "foe", being a major cause of blood-poisoning, gastro-intestinal diseases and of extra-intestinal infections such as those of the urinary tract. Others however would consider *E. coli* mostly as a harmless inhabitant (or "friend") of the human gut, contributing to essential biochemical processes (*e.g.* synthesis of vitamin K). *E. coli* is seen also by many as a reliable laboratory companion, being an exceptionally versatile tool in molecular research. A recent and critical shift is that *E. coli* causing extra-intestinal infections, once considered as among the most susceptible members of the Enterobacteriaceae family, are now becoming among the more antibiotic-resistant. A key element here is the emergence of the CTX-M extended-spectrum β-lactamase family. In the past few years, these ESBLs and their producer strains have rapidly disseminated, not only in the UK, but across the globe.

The emergence and swift diffusion of CTX-M ESBLs has completely altered the landscape of antibiotic resistance in *E. coli*, forcing clinicians to adopt new approaches in terms of antibiotic therapy, and pharmaceutical companies to seek and develop new drugs. It is still not clear when organisms producing CTX-M enzymes first appeared in the UK (producers certainly date back to 1999), but the situation worsened dramatically around year 2003. Since then, reported cases of infection caused by *E. coli* producing CTX-M enzymes, mostly of the urinary tract and many in community patients, have risen exponentially. *E. coli* with CTX-M enzymes are now the most prevalent cephalosporin-resistant Enterobacteriaceae in the UK and represent a major cause for concern in terms of public health (Potz, N. A. *et al.*, 2006).

The epidemiology of *E. coli* with CTX-M enzymes in the UK is complex, as was shown previously by DNA fingerprinting (Woodford, N. *et al.*, 2004). It includes five major epidemic clones (A-E) producing CTX-M-15 enzyme (or CTX-M-3, a close relative, in a few clone C isolates), along with many genetically-diverse producers of CTX-M-15-like enzymes (mostly CTX-M-15 β-lactamase itself), and also a few isolates producing other CTX-M variants (mostly CTX-M-9 and -14). This study sought to characterise current *E. coli* isolates producing CTX-M β-lactamases at the molecular level, so as to identify and better understand the reasons behind their epidemiological success.

1. Multi-drug resistance of clonal and non-clonal *E. coli* with CTX-M-15 β-lactamases

Whether they belonged to one of the five major epidemic clones or not, UK *E. coli* isolates with CTX-M-15-like enzymes exhibited similar multi-drug resistance phenotypes. Major classes of antibiotics, widely utilised for therapy, such as extended-spectrum cephalosporins, aminoglycosides, quinolones, tetracyclines and trimethoprim, were all inactive against those organisms, or had their activity reduced significantly.

Resistance to β-lactams was due to enzymatic inactivation. Most clonal and non-clonal isolates with CTX-M-15 enzymes investigated produced multiple β-lactamases in various combinations, also including TEM-1 and/or OXA-1 β-lactamase(s). CTX-M-15 is one of a few CTX-M variants capable of hydrolysing ceftazidime efficiently (Poirel, L. *et al.*, 2002a); it is believed to be a descendant of CTX-M-3, which differs only by a single amino acid (Asp240Gly). This latter variant, which is poorly effective *vs.* ceftazidime, was detected in four of nine epidemic clone C representatives investigated, all from Belfast. Clone C isolates from other UK centres always had CTX-M-15 enzyme. Other differences between the Belfast isolates with CTX-M-3 enzyme and those with CTX-M-15 are discussed elsewhere.

Resistance to extended-spectrum β-lactams, including third- and fourth-generation cephalosporins (*e.g.* cefotaxime, ceftazidime, cefepime), doubtless resulted from hydrolysis by the CTX-M ESBLs. Of the other β-lactamases found, TEM-1 β-lactamase has been a common cause of resistance to penicillins in *E. coli* isolates since the 1970s (Livermore, D. M., 1995), and a recent pan-European study showed that it remains widespread among *E. coli* isolates responsible for uncomplicated UTIs in the community (Kahlmeter, G. and Menday, P., 2003). The class D OXA-1 β-lactamase preferentially hydrolyses oxacillin and, more importantly, is poorly inactivated by β-lactamase inhibitors. This was illustrated in this study by the MICs of the piperacillin/tazobactam combination, which were three-fold higher at least for clonal isolates harbouring bla_{OXA-1} (geom. mean MIC = 20 mg/L, *i.e.* above the BSAC/EUCAST breakpoint defining resistance) than for those few clonal mutants lacking the gene (geom. mean MIC = 6 mg/L, below the BSAC/EUCAST breakpoint).

Unlike penicillins, oxyimino-cephalosporins, which are poor substrates for OXA-1, remained active in the presence of clavulanate *vs.* isolates producing the CTX-M-15, OXA-1 and/or TEM-1 combination of enzymes. Some *E. coli* epidemic clone A isolates, all referred from Preston, also had a plasmid-mediated AmpC-type β-lactamase, CMY-23, able to withstand inhibition by clavulanic acid and to hydrolyse cephamycins effectively. The accumulation of multiple β-lactamases in *E. coli* is very worrying since β-lactams represent approximately two-thirds of all prescribed antibiotics, and also since cephalosporins [27% of the market shares (by sales) of the leading antibacterial drug classes (Kresse, H *et al.*, 2007)] have long been considered as the drugs of choice for the treatment of many severe infection types.

Among the β-lactams tested here, only carbapenems consistently retained activity *vs. E. coli* isolates with CTX-M-15-like enzymes, whether clonal or diverse and whether or not these also had CMY-23. It is worth adding that previous studies reported that the prevalence of resistance to mecillinam among *E. coli* isolates with CTX-M ESBLs also was relatively low [7.5%, (Potz, N. A. *et al.*, 2006)], and that temocillin, a derivative of ticarcillin highly stable to most β-lactamases (Denton, M, 2007), also retains good *in-vitro* activity against *E. coli* with ESBLs (including CTX-M types), with an 8% resistance rate (Rodriguez-Villalobos, H. *et al.*, 2006). Mecillinam however is only suitable in general for the treatment of lower urinary tract infections, and both it and temocillin have yet to be proved clinically active in infections due to ESBL producers.

Most isolates in this study were resistant, or had reduced susceptibility, to aminoglycosides. In isolates with CTX-M-15 enzymes, this was attributable to the aminoglycoside-modifying enzymes AAC(6')-Ib and AAC(3)-IIa. The latter enzyme was present among the majority of clonal and non-clonal isolates, but not in epidemic clone A, which remained consistently susceptible to gentamicin. The AAC(6')-Ib enzyme, with substrates including tobramycin, kanamycin and amikacin in descending order of preference, was produced by clone A isolates, as well as by most other clonal and non-clonal with CTX-M-15-like enzymes. The exception was that *aac(6')-Ib* was not detected in the clone C representatives from Belfast producing the CTX-M-3 enzyme variant. DNA sequencing of the allele *aac(6')-Ib* from randomly-selected clonal and non clonal producers with CTX-M-15 enzyme revealed it corresponded to the *aac(6')-Ib-cr* variant, recently shown to encode an enzyme also capable of acetylating those

fluoroquinolones that have an unsubstituted amino nitrogen on the piperazinyl group, such as ciprofloxacin and norfloxacin (Robicsek, A. *et al.*, 2006).

UK microbiologists, like those elsewhere, have witnessed also a dramatic increase in the number of quinolone-resistant *E. coli* isolates in recent years. Around 80% of *E. coli* isolates with CTX-M enzymes referred to ARMRL are cross-resistant to these drugs, maybe because successful fluoroquinolone-resistant clones had subsequently acquired CTX-M enzymes. Overall, most clonal and non-clonal isolates with CTX-M-15 enzymes required ciprofloxacin MICs above 8 mg/L and such high-level resistance was due to multiple chromosomal mutations in the Quinolone-Resistance-Determining-Regions of the *gyrA* and *parC* genes, in addition to production of the AAC(6')-Ib-cr enzyme. The plasmid-mediated *qnr* genes, which encode peptides responsible for reduced susceptibility to quinolones (Tran, J. H. *et al.*, 2005), were absent from all the investigated isolates.

Resistance to tetracycline and to trimethoprim in UK *E. coli* isolates with CTX-M-15 enzyme was attributable to efflux [*tet*(A)] and to metabolic bypass (dfr_{17}) mechanisms, respectively.

2. Relatedness of the five major UK *E. coli* epidemic clones with CTX-M-15 β-lactamases

Although PFGE distinguished five major national *E. coli* epidemic clones with CTX-M-15 enzyme in the UK, other typing methods suggest these to be very closely related to one another. All five groups of isolates shared the same serotype [O25, (Woodford, N. *et al.*, 2004)], and also belonged to the same multi-locus sequence type [ST131, (Fox, A., personal communication)]. These data suggest that the five UK epidemic clones share a common ancestor strain.

This present study further supports this view of relatedness: epidemic clones A-E were shown to belong to the same virulence-associated phylogenetic group (B2) and also, to exhibit the same multiple chromosomal mutations conferring high-level resistance to quinolones. Additionally and most importantly, the virulence factor gene pools of those five clones appeared very similar, both quantitatively and qualitatively. However, it should be added that most extra-intestinal pathogenic *E. coli* (ExPEC) isolates belonging to phylogenetic group B2, including 100% of the

clonal isolates and 57% of the non-clonal, generally had similar virulence profiles. The slight variations in the virulence gene patterns of the five epidemic clones suggested evolution in three distinct directions, leading to the emergence of epidemic clone A, clone D and to the B-C-E group of clones, with each lineage acquiring or losing a few virulence genes along their evolutionary route (*e.g. afa/draBC* was specific to clone A among the epidemic lineages). More generally, phylogenetic group B2 ExPEC isolates, defined as diverse by PFGE, but exhibiting similar virulence profiles, may be distant evolutionary mutants of the three early ancestor clones (*i.e.* diverse B2 isolates harbouring *afa/draBC* might come from the same evolutionary route as epidemic clone A).

The five epidemic clones with CTX-M-15 enzyme did not appear to have more potent virulence factors than the diverse producers of CTX-M-15 enzyme belonging to phylogenetic group B2, or than B2 isolates producing other ESBL types. It follows that the reasons behind the epidemiological success of ST131 isolates in the UK remain questionable since none of the virulence factors sought in this study was specific to these isolates. Differential expression of the virulence factor genes sought in this study however cannot be ruled out, and remains a plausible explanation for their success. Carriage of isolates with CTX-M enzymes, including epidemic clone A in the UK, by healthy individuals in the community and in hospitals has been reported in the literature (Loughrey, A. *et al.*, 2007; Munday, C. J. *et al.*, 2004b); this suggests that these organisms (harmless in the gut), might be able to "switch on" their pathogenic potential following transfer to the urinary tract. Other factors besides resistance seem especially likely to underpin the success of UK epidemic clone A, which has unusual susceptibility to gentamicin and lower resistance to cephalosporins.

Another possible reason, which might explain the general success of epidemic clones A-E with CTX-M-15 in the UK, may lie in their chromosome. Their close relationship was demonstrated in this study, and maybe they harbour specifically other factors, either not sought in this study or possibly still unidentified. Those chromosomal traits may be revealed by high-throughput genome sequencing, which is now achievable rapidly and is becoming more affordable. This method may become a very valuable tool in the near future for the rapid comparison of entire

genomes, allowing the identification of new determinants, which may prove to serve those *E. coli* isolates with CTX-M enzymes, and particularly the successful epidemic clones.

3. Plasmids encoding multi-drug resistance in UK *E. coli* isolates with CTX-M-15-like β-lactamases

It is well known that most plasmids are not essential for bacterial growth, but many provide significant selective benefits for their hosts. This study revealed that the $bla_{CTX\text{-}M\text{-}15\text{-}like}$ genes were plasmid-mediated in both clonal and diverse *E. coli* isolates. These plasmids co-encoded all the mechanisms of antibiotic resistance discussed above [TEM-1, OXA-1, AAC(3)-IIa, AAC(6')-Ib-cr, Tet(A) and Dfr_{17}]. Complete DNA sequencing of the plasmid encoding CTX-M-15 enzyme of a representative of epidemic clone A (pEK499) revealed that it encoded twelve antibiotic resistance genes, affecting eight classes of antimicrobials. Such multi-drug resistance plasmids render their host strains easily selectable, particularly in antibiotic-rich environments such as in hospitals, and are likely therefore to play a major role in their epidemiological success. The presence of addiction systems on those multi-drug resistance plasmids furthermore ensures their maintenance, even in the absence of antibiotic pressure (*e.g.* when carried in the guts of healthy people in the community).

Although the plasmids encoding CTX-M-15-like enzymes from clonal isolates varied in size (*c.* 70 to 150-kb), all belonged to incompatibility group IncFII. IncFII plasmids are narrow-host range elements, mostly found among *E. coli* and *Salmonella* spp., but which may cross further genus boundaries following the acquisition of additional replicon(s) (Carattoli, A. *et al.*, 2006). This might possibly explain the common association of CTX-M-15 enzyme with *E. coli*.

The plasmids encoding CTX-M enzymes in epidemic clones B, C, D and E, but not that from clone A, were self-transferable by conjugation *in-vitro*. This may have contributed towards the spread of CTX-M ESBLs among unrelated *E. coli* isolates across the UK, and such a view is supported by several lines of evidence.

(i) Firstly, the plasmids encoding CTX-M-15 enzyme in genetically-diverse isolates from different centres across the UK were also self-transferable.

(ii) Secondly, these plasmids (whether from clonal or non-clonal isolates) had multiple features in common, including several antibiotic resistance genes [$bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{OXA\text{-}1}$, *aac(6')-Ib-cr*], the origin of replication *repF*, and also the plasmid addiction system *ccdA/ccdB*.

(iii) Finally and most importantly, the plasmids encoding CTX-M-15 enzyme in clonal and non-clonal isolates shared a high degree of homology (see plasmid cross-hybridization with pEK499), even when the host strains belonged to different phylogenetic groups.

It was known, prior to this study, that the spread of CTX-M ESBLs in the UK, particularly of CTX-M-15 enzyme, was due in some part to clonal expansion, as illustrated by the nationwide spread of epidemic clone A. This present study has provided further insights in the dominance of these enzymes in the UK. The similar multi-drug resistance phenotype of clonal and non-clonal *E. coli* isolates with CTX-M-15-like ESBLs and, more importantly, the spread of CTX-M enzymes across the UK, appear also to be due to the horizontal spread of closely-related plasmids harbouring the $bla_{CTX\text{-}M\text{-}15}$ gene. Faecal carriage of isolates producing CTX-M enzymes in hospitalised and healthy individuals has been well documented (Miro, E. *et al.*, 2005; Munday, C. J. *et al.*, 2004b; Pallecchi, L. *et al.*, 2004; Valverde, A. *et al.*, 2004), including in the UK (Loughrey, A. *et al.*, 2007). This suggests that the conjugative transfer of plasmids harbouring the $bla_{CTX\text{-}M}$ genes may take place between the different *E. coli* populations inhabiting the human gut.

It remains surprising that plasmid pEK499, which carries $bla_{CTX\text{-}M\text{-}15}$ in the most clinically successful *E. coli* lineage with CTX-M enzyme in the UK (clone A), was non-conjugative *in vitro*, even though this plasmid shared a high degree of homology with self-transferable plasmids from the other epidemic clones. As with the non-conjugative IncFII plasmid pRSB107 (multi-drug resistance plasmid isolated in Germany - accession number: AJ851089), pEK499 harboured the remnants of a conjugative transfer module (Szczepanowski, R. *et al.*, 2005). It remains unknown whether the truncated form of this *tra* operon prevents conjugative transfer of pEK499. Such a condition was found previously among non-conjugative plasmids, such as in the virulence plasmid pWR101 (accession number:AF294823) from a *Shigella flexneri* strain; though this plasmid nevertheless remained mobilisable, *i.e.* able to "parasitize" the conjugative transfer functions of another plasmid (Sansonetti, P. J. *et al.*, 1982), a situation that may apply also for pEK499.

4. Epidemic clone C has acquired independently two distinct plasmids encoding different CTX-M enzymes

UK *E. coli* epidemic clone C, as defined by PFGE (Woodford, N. *et al.*, 2004), was exceptional in that some of its members had CTX-M-15 enzyme, while others produced CTX-M-3. Among the clone C and non-clonal representative isolates investigated, the majority (but not all) of those referred from Belfast had CTX-M-3 enzyme, while CTX-M-15 was expressed by clone C and non-clonal isolates referred from all other participating centres.

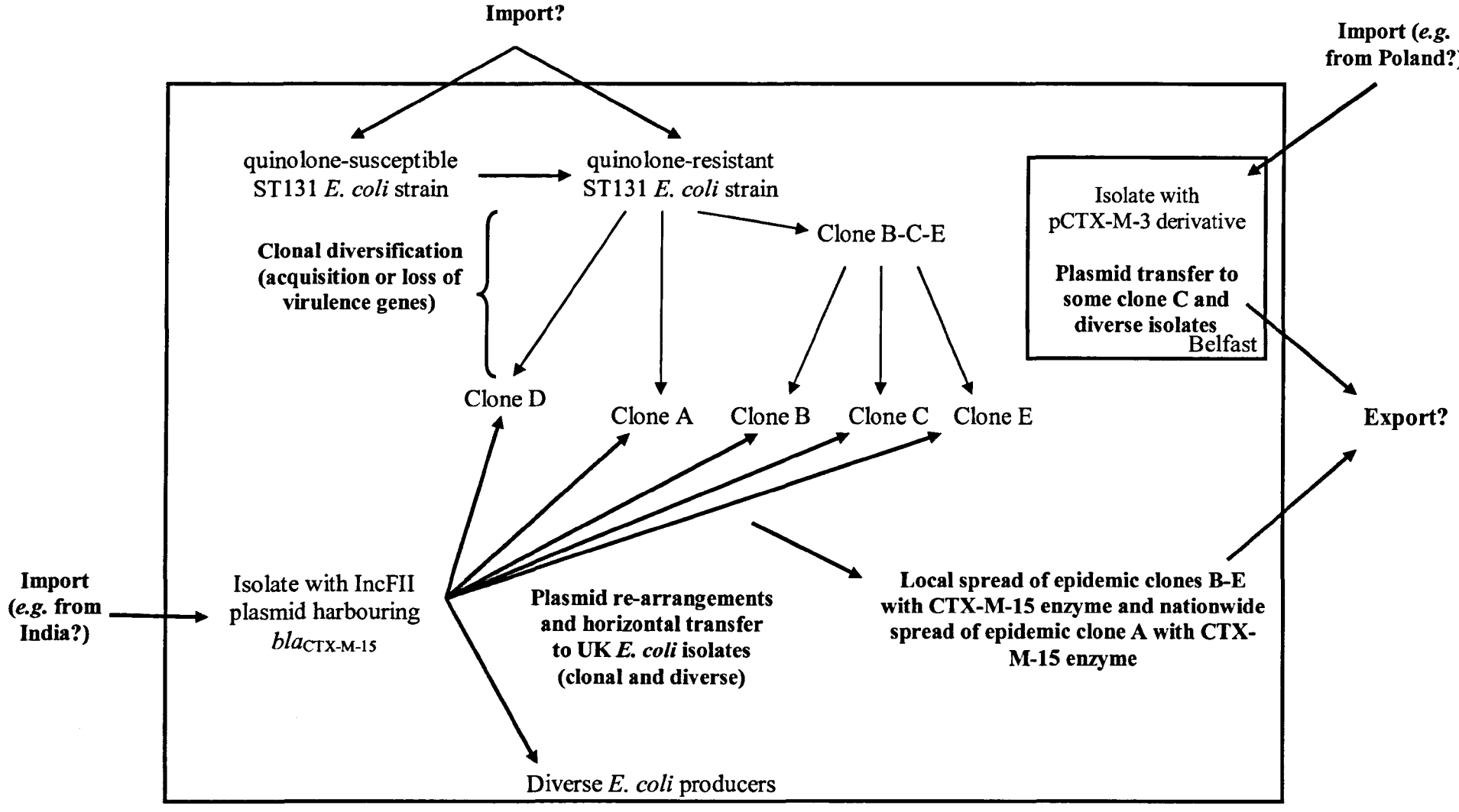
Although they appeared almost indistinguishable by PFGE, clone C isolates sometimes exhibited a few phenotypic variations between them. For instance, ceftazidime MICs were predictably dependent upon the CTX-M variant produced being lower for those with CTX-M-3. Also, clone C isolates that produced the CTX-M-3 enzyme were shown consistently to lack the bla_{OXA-1} gene, thereby explaining lower piperacillin/tazobactam MICs than for most of those with CTX-M-15 enzyme, which generally co-produced the OXA-1 β-lactamase. Clone C isolates additionally exhibited variation at the molecular level. Investigation of the genetic surroundings of their bla_{CTX-M} genes identified two distinct IS*Ecp1*-bla_{CTX-M} links. A 48-bp link was associated with the $bla_{CTX-M-15}$ gene, while $bla_{CTX-M-3}$ was consistently located further downstream from the IS*Ecp1* element, 128-bp apart. The 48-bp link, which was characteristic of all the investigated plasmids encoding $bla_{CTX-M-15}$ in UK isolates and not just those from clone C, was identical to that found on plasmid pC15-1a, isolated from an epidemic *E. coli* strain in Canada (Boyd, D. A. *et al.*, 2004), as well as to the link found in isolates from India (Ensor, V. M. *et al.*, 2006). On the other hand, the 128-bp link has only been reported on plasmid pCTX-M-3, a broad-host range plasmid encoding CTX-M-3 and prevalent among different species of Enterobacteriaceae in Poland since 1998 (Baraniak, A. *et al.*, 2002b). Plasmid pCTX-M-3 also lacks the bla_{OXA-1} and *aac(6')-Ib-cr* genes, as did clone C isolates producing CTX-M-3 enzyme in Belfast.

Consequently, representatives of UK epidemic clone C isolates appeared to have acquired independently two distinct plasmids encoding different CTX-M enzymes. The plasmids themselves carry different genes, but share the same incompatibility group, suggesting a common ancestor. One of these plasmids was related to the plasmids encoding CTX-M-15 enzyme prevalent among other clonal and non-clonal isolates in the UK, as well as to plasmid pC15-1a from Canada, to

which they may also be related, though comparison of DNA sequences revealed low sequence identity. The second plasmid on the other hand, which harboured $bla_{CTX\text{-}M\text{-}3}$, showed less homology to the plasmid encoding CTX-M-15 enzyme from the remaining four epidemic clones as shown by cross-hybridization, but appeared to be possibly related to pCTX-M-3. Since Poland joined the European Union in 2004, there has been an influx of Polish migrants to the United Kingdom, with the Polish community becoming the "dominant" ethnic minority in certain areas (though not in Belfast). Consequently, the plasmid encoding CTX-M-3 enzyme found in epidemic clone C isolates from Belfast may have been "imported" from Poland. Furthermore, the fact that *E. coli* epidemic clone C had acquired two distinct plasmids encoding CTX-M enzymes independently suggests that the host strain was likely to have been established in the UK before the introduction and spread of CTX-M β-lactamases. Acquisition of different CTX-M enzymes is likely to have followed the emergence of quinolone resistance in the ancestral ST131 strain (see Figure 55), since the identical multiple chromosomal mutations in the QRDRs are unlikely to have developed in parallel.

$bla_{CTX\text{-}M\text{-}15}$ differs from $bla_{CTX\text{-}M\text{-}3}$ only by an adenine-to-guanine substitution at position 725. The resulting Asp240Gly change in the CTX-M-15 protein extends the spectrum of good substrates to include ceftazidime (Poirel, L. *et al.*, 2002a). Both enzyme variants have most often been reported from the same geographical areas (Canton, R. and Coque, T. M., 2006), including in the UK, leading to suggestion that $bla_{CTX\text{-}M\text{-}3}$ might have mutated into $bla_{CTX\text{-}M\text{-}15}$. This seems to be the case in Poland, where CTX-M-15 enzyme was coded by minor variants of the widespread pCTX-M-3 plasmid (Baraniak, A. *et al.*, 2002a). Such a close relationship between CTX-M-3 and CTX-M-15 can be discounted for UK strains, allowing the different IS*Ecp1* linkages, as well as the different genes carried and the limited cross-hybridization. It follows that CTX-M-3 and CTX-M-15 were "introduced" or evolved independently in the UK. Similarly and more broadly, even though $bla_{CTX\text{-}M\text{-}3}$ has been detected in isolates from many countries worldwide (Canton, R. and Coque, T. M., 2006), there has been no report in the published literature to my knowledge of this gene being connected to IS*Ecp1* (which is believed to have had a direct role in the initial mobilization process) with the 48-bp link, as is standard for $bla_{CTX\text{-}M\text{-}15}$.

Figure 55. Proposed sequence of events leading to the dissemination of *E. coli* epidemic clones A-E with CTX-M enzymes in the UK.



This suggests that the widespread $bla_{CTX\text{-}M\text{-}15}$ allele is likely to have escaped, and migrated on to a mobile genetic element independently from $bla_{CTX\text{-}M\text{-}3}$.

Nonetheless, CTX-M-3 is able to evolve into an efficient ceftazidime-hydrolysing mutant as inferred by the encoding of both CTX-M-3 and CTX-M-15 by pCTX-M-3-like plasmids in Poland, and as shown here by in vitro selection (Karisik, E. *et al.*, 2006a). However, none of the *in-vitro* selected enzyme mutants was found to correspond to CTX-M-15. Rather, the majority of high-level ceftazidime-resistant mutants raised in this study had CTX-M enzymes harbouring a Pro167Ser substitution, a mutation not reported in any wild-type group-1 CTX-M variant so far. Pro167Ser resulted from a cytosine-to-thymine change, which has been shown previously to be the most likely nucleotide substitution to occur in general (Hastad, O. and Bjorklund, M., 1998).

Overall, I propose that the $bla_{CTX\text{-}M\text{-}15}$ gene (associated with the 48-bp link), which predominates among $bla_{CTX\text{-}M}$ genes worldwide, escaped directly from *Kluyvera* spp., and has been selected independently from $bla_{CTX\text{-}M\text{-}3}$ associated with the 128-bp link. Following its initial escape, the wide occurrence of $bla_{CTX\text{-}M\text{-}15}$ around the globe is largely due to the spread of closely-related plasmids, sometimes acquired by clones with epidemic potential. This dissemination is independent of the escape, spread and subsequent mutation of $bla_{CTX\text{-}M\text{-}3}$.

5. Comparison of antibiotic resistance in *E. coli* with CTX-M-15 enzyme to that in *E. coli* with other CTX-M types

Variants from all five CTX-M phylogenetic groups have been found among UK *E. coli* clinical isolates, illustrating independent introductory and/or evolutionary events. CTX-M-15 has become grossly the most prevalent variant (Woodford, N. *et al.*, 2006), not only in the UK, but also in much of continental Europe, North and Central Africa and India. Based on referrals to ARMRL, CTX-M-9-like enzymes (*e.g.* CTX-M-9 and -14) were the second commonest CTX-M types in the UK (*c.* 10-15%), while other variants, such as CTX-M-2 and CTX-M-40, have been seen only sporadically. The greater ability of CTX-M-15 to hydrolyse ceftazidime efficiently may have contributed to its success, but it is not the only variant with ceftazidimase activity, and other parameters should therefore be considered.

Isolates producing non-CTX-M-15-like variants generally were resistant to fewer antibiotics. In particular, isolates with group-2, -8 or -9 CTX-M enzymes were more susceptible to β-lactam/β-lactamase inhibitor combinations than those with CTX-M-15 (probably because no OXA-type enzyme was present in those isolates), also to ciprofloxacin and to the aminoglycosides amikacin and gentamicin (though epidemic clone A with CTX-M-15 enzyme was also susceptible to this last antibiotic). The multi-drug resistance phenotype of isolates producing CTX-M-15 enzyme may render them more easily selectable (and thus more successful) than those producing other CTX-M variants, thus explaining maybe their dominance in the UK. On a larger scale, the linkage of $bla_{\text{CTX-M-15}}$ with resistances to multiple other classes of antibiotics on self-transferable plasmids may also explain its wide occurrence worldwide. Besides the UK, $bla_{\text{CTX-M-15}}$ has been found also on multi-drug resistance IncFII plasmids in France, Canada, India, North and Central Africa (Canton, R. and Coque, T. M., 2006). It may be that the features of these plasmids, including their fitness cost and addiction systems underpin their success and so that of CTX-M-15.

CTX-M-15 is not predominant or has only recently become the dominant variant in some other countries. In Spain for instance, the group-9 CTX-M-9 and -14 enzymes have long been the most common CTX-M variants, while CTX-M-2 is the most prevalent variant in Israel (Canton, R. and Coque, T. M., 2006; Chmelnitsky, I. *et al.*, 2005), and has also long been the most common type in Argentina (Quinteros, M. *et al.*, 2003). The genes encoding these enzymes have been shown to be mediated by highly transmissible plasmids in Spain and in Israel (Chmelnitsky, I. *et al.*, 2005; Novais, A. *et al.*, 2006) while, in the UK, conjugative transfer of the plasmids encoding these genes was either unsuccessful or much harder to achieve *in vitro*, perhaps explaining the geographic variability in their success. Moreover the isolates with CTX-M-2, -9 or -14 enzymes in Spain or Israel appear resistant to more antibiotics than those in the UK, again suggesting that different plasmids are responsible in locales where these are successful. For example, the majority of isolates producing CTX-M-2 enzymes from Israel and from Argentina co-produce OXA-2 (Chmelnitsky, I. *et al.*, 2005; Power, P. *et al.*, 2005), conferring resistance to β-lactam/β-lactamase inhibitor combinations, as with OXA-1. OXA-2 enzyme was not found in the few isolates with CTX-M-2 enzyme in the UK.

6. Phylogeny and virulence of UK *E. coli* isolates with CTX-M-15 or CTX-M-9-like enzymes

Whether or not the general success of CTX-M-15 enzyme reflects its own activity and that of linked resistances, or the other traits of the host plasmids, it cannot by itself explain the high prevalence of particular producer clones. Their success implies additional parameters, specific to these host strains themselves, such as virulence factors.

Half the isolates with CTX-M-9-like enzymes studied here belonged to phylogenetic group D. This was in contrast with isolates producing CTX-M-15 enzyme; where all of the clonal and *c.* 70% of non-clonal isolates belonged to phylogenetic group B2. Since intestinal *E. coli* pathotypes belong mostly to phylogenetic group D and extra-intestinal pathotypes mainly belong to group B2 (Donnenberg, M. S., 2002), it appears that isolates with CTX-M-15-like enzymes were more likely to cause extra-intestinal infections such as those of the urinary tract than those with CTX-M-9-like enzymes. This inference was further supported by considering only those phylogenetic group B2 isolates that met the definition of ExPEC. These comprised all members of all five epidemic clones A-E with CTX-M-15 enzyme, 57% of non-clonal isolates with CTX-M-15-like enzymes, and 75% of isolates with non-CTX-M ESBLs, but only 21% of those with group-9 CTX-M enzymes. Adding to the fact that producers of CTX-M-9-like enzymes were generally resistant to fewer antibiotics than isolates with CTX-M-15 enzyme, this may also explain the dominance of isolates producing CTX-M-15 enzymes *vs.* producers of group-9 CTX-M enzymes in the UK. Additional factors (not considered in this study) that might "weaken" isolates with CTX-M-9-like enzymes in the UK (*vs.* those with CTX-M-15) cannot be ruled out however. The possible absence of addiction systems on plasmids encoding $bla_{\text{CTX-M-9-like}}$ genes may be responsible for the lower success of their host strains. Also, a low transfer rate or a higher fitness cost associated with the carriage of plasmids encoding $bla_{\text{CTX-M-9-like}}$ genes may be responsible for their low prevalence in this country. The plasmids encoding $bla_{\text{CTX-M-9-like}}$ genes were generally much larger and harder to transfer by conjugation *in vitro* (owing perhaps to a lower copy-number) than those harbouring $bla_{\text{CTX-M-15}}$.

7. Dissemination of CTX-M extended-spectrum β-lactamases in the UK and elsewhere

The emergence and worldwide dissemination of CTX-M ESBLs at the beginning of this century has opened a new era in clinical microbiology and infectious disease; *E. coli* producing CTX-M enzymes represent a major challenge for many microbiologists around the world.

As mentioned earlier, *E. coli* with CTX-M-15 enzyme are widely distributed in many countries besides the UK, including in much of continental Europe (except Iberia, where they nevertheless are spreading), also in North Africa, India and Canada. Here, in the UK, these organisms and their epidemiology have been the subjects of extensive investigations. The situation in France is comparable to that in the UK, *i.e.* the high prevalence of the CTX-M-15 enzyme appears to be the result of clonal expansion of epidemic *E. coli* strains, as well as of the spread of plasmids (Eckert, C. *et al.*, 2004; Lavollay, M. *et al.*, 2006; Leflon-Guibout, V. *et al.*, 2004). More generally, it appears that wherever it is the most common enzyme variant (*e.g.* Canada, France, India, Italy or the UK), CTX-M-15 tends to use more or less the same means for dissemination, combining clonal expansion of successful producer strains [*e.g.* an outbreak caused by an *E. coli* strain harbouring plasmid pC15-1a in Toronto and the surrounding regions, (Boyd, D. A. *et al.*, 2004)] as well as conjugative transfer of multi-drug-resistance plasmids [*e.g.* spread of plasmid pC15-1a among several *E. coli* strains isolated in Western Canada, (Boyd, D. A. *et al.*, 2004)]. In each of these countries, CTX-M-15 was encoded by large self-transferable multi-drug resistance plasmids belonging to incompatibility group IncFII. These plasmids have several features in common, including an R100 backbone as well as a number of antibiotic resistance genes, including $bla_{\text{CTX-M-15}}$ (associated with IS*Ecp1* by a 48-bp link), $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, *aac(6')-Ib-cr* and *tet*(A). This suggests that the major factor behind the worldwide rise of CTX-M-15 ESBL is the spread of related multi-drug resistance plasmids. These plasmids may contribute reciprocally towards the epidemiological success of their host strains.

Epidemiological patterns are different in those countries –Argentina, Israel, Poland and Spain– where CTX-M-15 enzyme is not endemic. In Poland for example, CTX-M-3 has been the dominant CTX-M variant since the late 1990s. Its wide dissemination is mainly associated with the horizontal transfer of a broad-host range plasmid (pCTX-M-3), spreading not only among *E. coli*

isolates, but among various other species of Enterobacteriaceae. Variants of this plasmid coding CTX-M-15 have been documented (Baraniak, A. *et al.*, 2002a), but remain rare, suggesting that the increased ceftazidimase activity of CTX-M-15 (compared with that of *e.g.* CTX-M-3) is not a major selective trait. This suggests, in turn, that the dominance of CTX-M-15 in other countries such as in the UK might not depend on its wider spectrum of activity, but mostly on the features of the multi-drug resistance plasmids by which it is encoded. There have been no reports yet from Poland, to my knowledge, of CTX-M-15 enzyme being encoded by an IncFII plasmid similar to that spreading *e.g.* in the UK (*i.e.* with a 48-bp link); this might explain therefore why CTX-M-15 is not the most common variant in Poland.

The epidemiological scenario in Spain resembled that of Poland in the sense that, until recently, there were no reports of isolates harbouring IncFII plasmids encoding CTX-M-15 enzyme. This might explain why different CTX-M variants have become endemic; specifically CTX-M-9 and CTX-M-14 have long been the most common types observed [as early as 1996, (Canton, R. and Coque, T. M., 2006)], with their wide dissemination principally associated with the spread of multiple epidemic plasmids among unrelated isolates, mostly *E. coli*. Both CTX-M-9 and -14 variants were encoded by highly transmissible multi-drug-resistance plasmids in the Spanish isolates (see section 5). Nevertheless, the epidemiology of CTX-M ESBLs in Spain may be changing, with the rapid spread of CTX-M-15 β-lactamase encoded by an IncFII plasmid reported recently around Madrid (Novais, A. *et al.*, 2007). In Israel, the spread of the locally dominant CTX-M-2 enzyme is the result of both clonal spread and horizontal transfer of plasmids (Chmelnitsky, I. *et al.*, 2005); the precise epidemiology in Argentina, where CTX-M-2 enzyme is also dominant (Quinteros, M. *et al.*, 2003), remains uncertain.

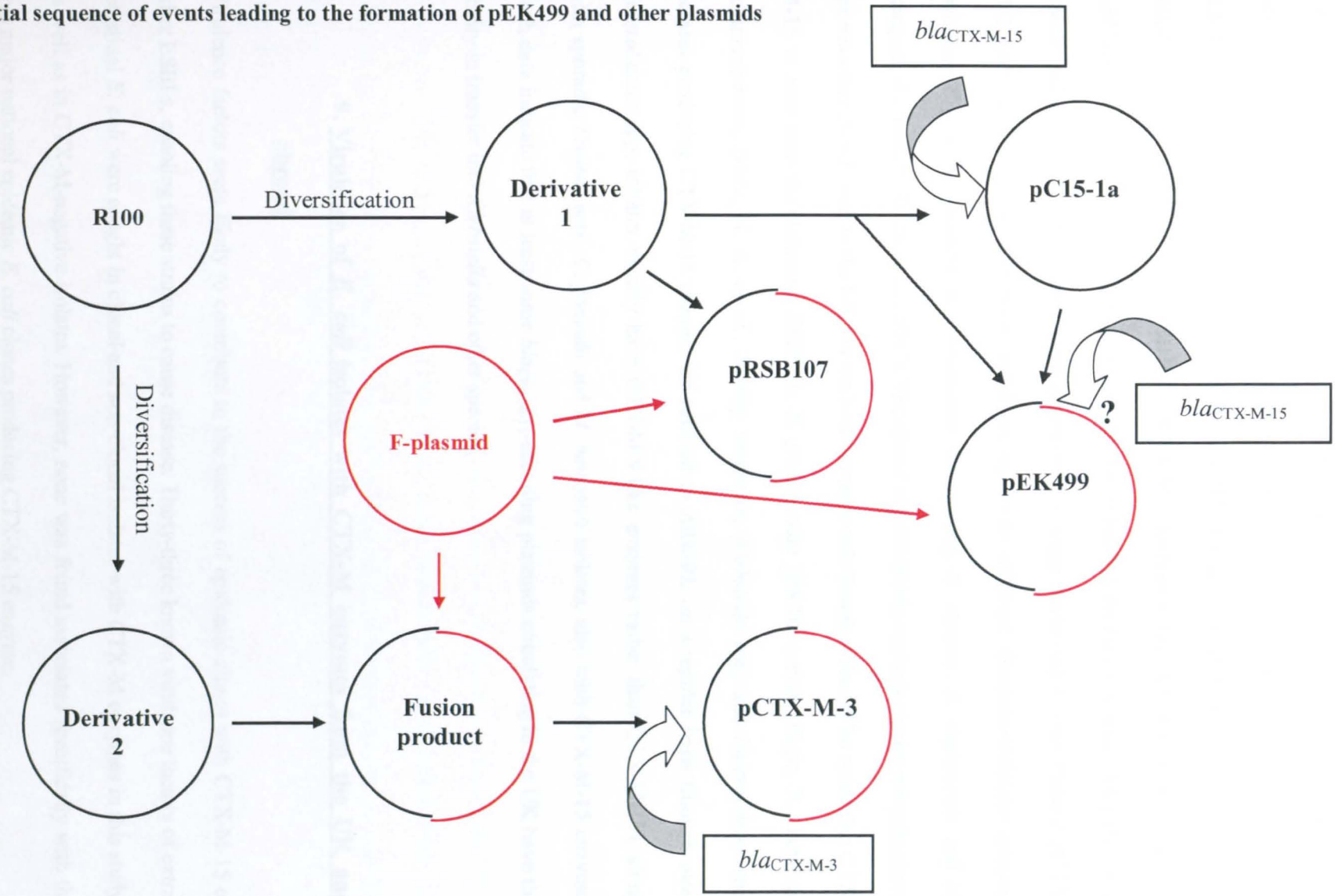
It is worth adding that some $bla_{\text{CTX-M}}$ genes potentially may spread via transposase-mediated lateral transfer (though has not been documented in the UK, or elsewhere), since they are usually located within mobilisable elements such as transposons (*e.g.* $bla_{\text{CTX-M-15}}$ is located within a Tn*21*-like transposon on pEK499). Such mechanisms of dissemination, whilst not proven, are suggested (but not proven) by recent studies in France and in Spain, showing that multiple recombinatorial events involving $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-9}}$ were possible within the same plasmid or among different plasmids (Canton, R. and Coque, T. M., 2006).

8. Multi-drug resistance IncFII plasmids

Although many IncFII plasmids encoding CTX-M-15 enzyme have been identified around the world (*e.g.* in Cameroon, Canada, France, India, Italy, North Africa, Portugal) and have been characterised to various levels, very few only have been completely sequenced. Besides pEK499, pC15-1a (the plasmid widespread in Western Canada) is the only other IncFII plasmid encoding CTX-M-15 enzyme whose entire DNA sequence map is available publicly. Like pEK499 (117,536-bp), pC15-1a (circular molecule of *c.* 92-kb) harboured an R100 backbone and has its antibiotic resistance genes [$bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, $bla_{TEM\text{-}1}$, *aac(6')-Ib-cr*, *aac3-IIa* and *tet*(A)] clustered in a 28.4-kb multi-drug resistance region (MDR) (Boyd, D. A. *et al.*, 2004). Although *aac3-IIa* was absent from pEK499, this gentamicin resistance-encoding gene was widespread in the other epidemic clones (B-E) and non-clonal isolates from the UK. The MDR of pEK499 was approximately 25-kb long, with the antibiotic resistance genes arranged differently to those in the MDR of pC15-1a, though most were located between insertion sequences and transposon-like elements suggestive of multiple recombinatorial re-arrangements. Besides a number of genes encoding proteins with unknown functions, pEK499 additionally carried a class 1 integron (containing the antibiotic resistance gene cassettes *aadA5*, dfr_{17} and *sulI*, and located within its MDR), the plasmid addiction system *ccdA/ccdB* and the virulence-associated genes *vagC/vagD*, all of which were absent from pC15-1a.

Overall, pEK499 appeared to be a mosaic molecule (see Figure 56), sharing some sequence homologies with pRSB107 [a plasmid isolated from activated-sludge bacteria of a wastewater-treatment plant in Germany (Szczepanowski, R. *et al.*, 2005)] as well as pC15-1a. All these three IncFII plasmids harboured the *repF* origin of replication (specific to plasmid R100 and its derivatives). Plasmids pEK499 and pRSB107 also had *repFIA* (found on the F-plasmid), which indicated that both these plasmids were the fusion product of two ancestor plasmids, one of which was related to R100 (and to pC15-1a) and the other to the F-plasmid. Like pEK499, pRSB107 harboured only remnants of the F-homologous DNA-transfer (*tra*) module, which may explain why *in vitro* conjugative transfer of both these plasmids was not successful (see section 3). A possible evolution pathway is shown in Figure 56, but more complex routes cannot be excluded.

Figure 56. Potential sequence of events leading to the formation of pEK499 and other plasmids



The full DNA sequence of pCTX-M-3, the CTX-M-3-encoding plasmid widespread among Enterobacteriaceae across Poland, is also available publicly. Although this plasmid encoded a different CTX-M enzyme and exhibited significantly less sequence identity than pC15-1a with pEK499, it also belonged to incompatibility group IncFII, though to a different subtype than that of pEK499. As with pEK499 (and pRSB107), pCTX-M-3 harboured the two origins of replication *repF* and *repFIA*. The latter might be responsible for extending the range of hosts compared with typical IncFII plasmids, which are generally narrow-host range, preferring *E. coli*. Plasmid pCTX-M-3 and its derivatives have been found among seven different Enterobacteriaceae species, including *E. coli*, *C. freundii*, *K. pneumoniae*, *K. oxytoca*, *E. cloacae*, *S. marcescens* and *M. morganii* (Livermore, D. M. *et al.*, 2007). The spread of pEK499 derivatives among enterobacterial species other than *E. coli* in the UK has not been investigated, though it should be noted that CTX-M-15 is now also the dominant ESBL in *K. pneumoniae* [BSAC survey, Hope, R., personal communication, (Potz, N. A. *et al.*, 2006)]; moreover, *Klebsiella* spp. and *Enterobacter* spp. isolates producing CTX-M-15 enzyme are referred to ARMRL on a regular basis (though most *Enterobacter* spp. isolates usually have CTX-M-9-like enzymes rather than CTX-M-15), along with sporadic *Proteus* spp., *C. freundii* and *M. morganii* isolates, also with CTX-M-15 enzyme. Such data indicate that at least some $bla_{\text{CTX-M-15}}$-encoding plasmids circulating in the UK have the ability to transfer into *Klebsiella* and other species.

9. Virulence of *E. coli* isolates with CTX-M enzymes from the UK and abroad

Virulence factors seem likely to contribute to the success of epidemic clones with CTX-M-15 or other ESBLs, enabling these strains to cause disease. Thirty-three known virulence factors of extra-intestinal *E. coli* were sought in clonal and non-clonal isolates with CTX-M enzymes in this study, as well as in CTX-M-negative isolates. However, none was found associated specifically with the five major national epidemic *E. coli* clones producing CTX-M-15 enzyme.

Elsewhere, data regarding the phylogenetic background and virulence of isolates with CTX-M enzymes are still limited and ambiguous. Most studies, including this one, have suggested that isolates producing CTX-M enzymes are not more virulent than many other CTX-M-negative

isolates of the same virulence-associated phylogenetic groups (B2 or D). Separate studies from the Calgary region in Canada (Pitout, J. D. *et al.*, 2005) and from the Languedoc region in France (Lavigne, J. P. *et al.*, 2007) nevertheless have shown that isolates producing CTX-M-15 enzyme mostly belonged to phylogenetic group B2, whilst those producing CTX-M-9 enzyme generally belonged to group D. Those findings agreed with data from this present study, but were in contrast with figures from another study in France [around Paris, (Branger, C. *et al.*, 2005)] and from a more recent study in Spain (Canton, R. and Coque, T. M., 2006), where isolates producing CTX-M-15 enzyme mostly belonged to phylogenetic group D, whereas isolates with CTX-M-9 generally belonged to the commensal group A, and to a lesser extent to group D. Also, isolates with CTX-M-15 enzymes isolated around Calgary generally had different virulence genotypes compared with UK clonal and non-clonal producers of CTX-M-15 enzyme. In both countries, however, most producers had the same ExPEC markers (*iutA* and *kpsMT*II).

There has been no report, as yet, of particular virulence factors being associated exclusively with isolates producing CTX-M ESBLs. But, it seems plausible that these have additional, unrecognised factors. It is noteworthy too that the urinary tract adhesin Afa/DraBC was associated significantly with production of CTX-M β-lactamase in UK isolates, though it was not present in all producers, but rather in epidemic clone A and in a quarter of diverse producers (with CTX-M-9 or CTX-M-15 enzymes) only. This virulence factor was not found in isolates with CTX-M-9-like or CTX-M-15 enzymes in the study from Canada (Pitout, J. D. *et al.*, 2005). While not more virulent, neither were UK isolates with CTX-M enzymes less virulent than the susceptible controls. Similar findings were also reported from the study in the Calgary region (Pitout, J. D. *et al.*, 2005)..

A recent study using the nematode *Caenorhabditis elegans* as infection model revealed that the virulence potential of UTI-causing ExPEC *E. coli* isolates with CTX-M enzymes belonging to phylogenetic groups B2 or D was relatively low (Lavigne, J. P. *et al.*, 2006). This finding correlated with earlier studies (Johnson, J. R. *et al.*, 2005), which showed *(i)* that UTI-causing antibiotic-resistant strains (typically quinolone-resistant, as with the majority of isolates producing CTX-M enzymes found in the UK) generally had low virulence in the model (Pitout, J. D. *et al.*,

2005), and *(ii)* that there was a decrease in the presence or the expression of some virulence factors, as well as a decreased invasive capacity (Velasco, M. *et al.*, 2001; Vila, J. *et al.*, 2002).

Overall, the virulence potential of *E. coli* isolates producing CTX-M ESBLs remains a complex issue, which requires further investigations. Findings in this study, as well as those from Canada, suggest that the current multi-drug-resistant *E. coli* isolates with CTX-M enzymes may have become more virulent in comparison to early multi-drug-resistant *E. coli* isolates, which lacked CTX-M β-lactamase. This could explain, in some part, the emergence and worldwide success of isolates producing CTX-M enzymes. However, the findings in this study cannot rule out the possibility that the successful epidemic clones producing CTX-M enzymes in the UK might possess additional and specific unidentified factors, or, might up-regulate the expression of particular virulence genes.

10. Routes of spread of *E. coli* isolates producing CTX-M enzymes

Another important question is to identify the sources and routes of propagation of *E. coli* with CTX-M-15 enzyme. Current data, from the UK and abroad, suggest three major possible routes of spread: *(i)* human migration, *(ii)* food and water contamination, and *(iii)* oro-faecal spread between humans.

10.1. Human migration

This study has shown the close similarity of the genetic surroundings of *(i)* $bla_{CTX\text{-}M\text{-}3}$ (128-bp link), *(ii)* $bla_{CTX\text{-}M\text{-}9}$ (complex class I integron structure), and *(iii)* $bla_{CTX\text{-}M\text{-}15}$ (48-bp link) found in UK *E. coli* isolates to those found in *E. coli* strains isolated in Poland, Spain and India, respectively. The migratory flux of people between these countries and the UK is well known, and might partly explain the dissemination of isolates producing CTX-M enzymes. Data from this study thus support (but do not prove) the idea that human migration contributes to the worldwide dissemination of CTX-M enzymes. Whilst it was argued earlier that the five major *E. coli* epidemic clones were likely to have been established in the UK before the emergence and spread of CTX-M enzymes, it seems likely that the plasmids encoding CTX-M-3 and CTX-M-15 enzymes, with resembling

plasmids found elsewhere, reached them from overseas. The source of the plasmid encoding CTX-M-3 enzyme in isolates from Belfast may be pCTX-M-3, or a derivative plasmid from Poland (see section 4), but the source of those encoding CTX-M-15 remains unknown.

10.2. Food chain contamination

A recent investigation of the virulence gene repertoire of *E. coli* isolates from food-producing animals in the USA (Johnson, J. R. *et al.*, 2007) suggested that antibiotic-resistant *E. coli* isolates (particularly those resistant to quinolones, co-trimoxazole and extended-spectrum cephalosporins such as ceftazidime, though not producing CTX-M ESBLs) probably developed in these animals and was subsequently transmitted to humans. The food chain therefore may also play a role in the spread of producers of CTX-M enzymes. There have been a number of reports of *E. coli* isolates with CTX-M enzymes detected in food-producing animals (Duan, R. S. *et al.*, 2006; Meunier, D. *et al.*, 2006), but no epidemiological links have yet been found between those isolates and those found in human clinical specimens. In the UK, isolates with CTX-M enzymes have been recovered from raw food products [mostly raw chicken breasts, (Ensor, V. M. *et al.*, 2007)], though none had the widespread CTX-M-15 variant. Interestingly, most of the food products contaminated with CTX-M-producing *E. coli* were imported from Brazil, Spain, or the Netherlands and the isolates had either CTX-M-2 enzyme (highly prevalent in South America) or CTX-M-9 (very common in Spain). It is worth adding that there have been reports also (from the UK and abroad) of faecal carriage by live animals of *E. coli* isolates producing CTX-M enzymes, further supporting the potential role played by animals and the food chain in the spread of CTX-M enzymes. In a study from Portugal, wild birds were shown to carry *E. coli* isolates with ESBLs, including CTX-M-1 or CTX-14 enzymes (Costa, D. *et al.*, 2006). In an earlier study from Wales, *E. coli* with CTX-M-9-like enzymes have been recovered from calves (Teale, C. J. *et al.*, 2005). In both cases however, no link with the local human epidemiology was evident. Water contamination may constitute another route of dissemination of isolates producing CTX-M enzymes. This was suggested in a recent study by Galvin *et al.*, where they identified three *E. coli* isolates producing CTX-M-3 enzyme (and an unidentified TEM-type β-lactamase) from a wastewater treatment plant in Galway, Republic of Ireland (Galvin, S. *et al.*, 2007).

Food and water contamination by *E. coli* isolates with CTX-M enzymes is now well documented, but it has not yet been shown to have any link to current clinical problems. Consequently, the role of the food chain in the emergence and spread of *E. coli* with CTX-M-15 in the UK still remain to be proven.

10.3. Oro-faecal transmission

Johnson *et al*, demonstrated extensive sharing of a uropathogenic *E. coli* clone among five household members, including the husband, the three children and the pet dog, of a woman with acute cystitis (Johnson, J. R. and Clabots, C., 2006). Following ciprofloxacin therapy, this clone disappeared from the mother's urine and faecal samples, but persisted in the faecal samples of the other family members, who became in turn the reservoir of the virulent *E. coli*, prone for further spread *e.g.* via the oro-faecal route. It is well established that for *E. coli*, gut colonisation precedes infection, and therefore that *E. coli* with CTX-M enzymes may spread via the oro-faecal route. An exceptional characteristic of *E. coli* strains producing CTX-M enzymes is their great penetration in the community, as well as their high prevalence among hospital patients. It becomes therefore important to establish whether initial gut colonisation occurs in healthcare settings or in the community, and more generally, whether isolates producing CTX-M enzymes originated in hospital settings and subsequently escaped in the community, or *vice versa*. Very few studies have been undertaken with regards to this matter. Ben-Ami *et al.* recently demonstrated that isolates producing CTX-M enzymes were not the result of nosocomial emergence at a Tel Aviv hospital (Israel), but rather originated in the community and were subsequently introduced in the hospital settings (Ben Ami, R. *et al.*, 2006). No such work has yet been carried in the UK, but a recent study in Belfast showed gut colonisation in 40% of nursing home residents by *E. coli* epidemic clone A and other strains with CTX-M-15 enzyme (Loughrey, A. *et al.*, 2007). Although it is unclear where the residents acquired such isolates, the high prevalence suggested spread within the nursing homes, presumably via the oro-faecal route.

11. The CTX-M extended-spectrum β-lactamases pandemic

The epidemiological picture of *E. coli* with CTX-M enzymes in the UK resembles that seen in many other countries, but yet it remains unclear how these organisms became a pandemic. Isolates producing CTX-M enzymes have been the subject of many detailed investigations at the local and national levels, but very rarely at the international level. There has been no major study comparing isolates with CTX-M enzymes from different countries, so as to identify *e.g.* how *E. coli* with CTX-M-15 enzymes have disseminated so quickly worldwide, though as discussed earlier, horizontal transfer of closely-related IncFII plasmids appears critical, with these sometimes establishing in strains with local or national epidemic potential. Similarly, $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}9}$ have regularly been found in very similar and complex integron structures in isolates from different countries (Canton, R. and Coque, T. M., 2006; Novais, A. *et al.*, 2006). International dissemination of major epidemic clones or independent selection events in different countries are possible, but remain unproven. An international collaboration investigating the relatedness of different national epidemic strains producing CTX-M enzymes and also comparing their plasmids that encode those ESBLs is therefore becoming more and more of a necessity.

12. What does the future hold?

In the past decade, *E. coli* isolates have accumulated multiple resistance mechanisms including CTX-M β-lactamases, AAC6'-Ib-cr and Qnr protection. Aminoglycosides, β-lactams and quinolones are all affected and, with the exception of carbapenems, the vast majority of antibiotics may no longer be universally relied upon for treating severe infections caused by *E. coli* strains producing CTX-M enzymes. First-line treatment options are having to be re-considered for high risk patients, and those isolates will force the clinical microbiologist to rely more and more on accurate antibiotic susceptibility testing. The emergence and swift dissemination of oxyimino-cephalosporin resistance in *E. coli* worldwide is very worrying and many fear that CTX-M ESBLs might one day become a standard feature of most *E. coli* isolates, just as TEM-1 enzyme did in the 1960s and 1970s.

This may be facilitated, since CTX-M-15 enzyme in UK isolates is generally co-encoded with plasmid addiction systems, ensuring its maintenance and that of co-linked multi-drug resistance, even in the absence of antibiotic pressure.

The accumulation of antibiotic resistance in *E. coli* isolates with CTX-M enzymes seems likely to evolve even further. The accumulation of multiple β-lactamases (CTX-M-15, TEM-1, OXA-1 and CMY-23) as well as the loss of OmpC, responsible for resistance to ertapenem (and reduced susceptibility to other carbapenems) was illustrated in this study in an *E. coli* epidemic clone A isolate. This represents a very worrying prospect for the future as it happened in the most successful UK epidemic clone, and is even more worrying since it happened in absence of antibiotic pressure (the patient had received only one course of cephalexin in the preceding year). Development of resistance to carbapenems may become more frequent as their use increases, driven by them being the only treatment options available for severe infections caused by multi-drug resistant isolates with CTX-M enzymes.

Pharmaceutical companies will have to respond quickly therefore to this shift in *E. coli* by seeking new treatment alternatives, ideally with novel modes of action, and suitable for use in community patients. Oral therapy options for community-acquired infections caused by *E. coli* with CTX-M enzymes are presently very limited. Potential new alternatives include the carbapenems sulopenem and tebipenem, as well maybe as third-generation cephalosporin/β-lactamase inhibitor combinations (Ranbaxy is currently developing cefpodoxime/sulbactam and cefixime/clavulanate, but these will only be available for the Indian market). Even among intra-venous antibiotics there are few options available to treat infections caused by *E. coli* with CTX-M enzymes, potentially including, asides from carbapenems, also temocillin and tigecycline. But how long before *E. coli* develops or acquires new antibiotic resistance mechanisms to these agents?

Finally, seeking novel agents to treat infections due to multi-drug resistant organisms such as those *E. coli* with CTX-M enzymes, or trying to make better use of antibiotics (*e.g.* by rotation, though the options are very limited) might not be the only solutions to the problem. Discovering new antibiotics may just lead to discovering new resistance mechanisms (ideally a mid- to long-term problem though), and rather than looking for solutions (and creating new problems), it would be more useful to look for ways of preventing these problems happening in the first place ("better

preventing a disease than treating it!"). Vaccination (*e.g.* against virulent phylogenetic group B2 *E. coli* isolates specifically) and improved infection control measures (to prevent the transmission of potential pathogens to vulnerable individuals) might be more useful (and more economical) in our battle against the dissemination of organisms producing CTX-M ESBLs.

CONCLUSIONS

E. coli has always been a major cause of extra-intestinal infections, principally of the urinary tract but sometimes leading to septicaemia. It is also a major component of the mixed flora of intra-abdominal sepsis. Until recently, the species was one of the more susceptible Enterobacteriaceae generally leaving many treatment options. In the past decade however, the resistance of *E. coli* isolates towards antibiotic therapy has increased worryingly, particularly with respect to third-generation cephalosporins and to quinolones. The emergence and wide dissemination of CTX-M ESBLs worldwide accounts for most of the resistance to third-generation cephalosporins.

The present study followed from earlier work by Woodford *et al.*, that reported community and hospital spread of *E. coli* isolates with CTX-M ESBLs (principally CTX-M-15) in the UK (Woodford, N. *et al.*, 2004). Five major national epidemic clones (A-E, with A being the most widespread across the country) were identified by DNA fingerprinting, along with many genetically-diverse producers of CTX-M-15 enzymes. This study aimed to characterise multi-drug resistant *E. coli* isolates with CTX-M enzymes at a molecular level, and to investigate the reasons behind their swift clinical success in the UK.

1. Summary of key findings

- Regardless of the type, CTX-M ESBLs in UK isolates of *E. coli* were encoded by large and, generally, self-transferable plasmids (non-self-transmissible in epidemic clone A). These plasmids largely determined the overall multi-drug resistance phenotypes of their host strains, except to quinolones, where resistance was due mostly to multiple chromosomal mutations in the QRDR of the genes encoding the DNA gyrase and topoisomerase IV enzymes. These QRDR mutations were conserved among the five major clones with CTX-M-15 enzyme, suggesting that they ante-dated the divergence of these strains from a potential common ancestor.
- Regardless of the clonality of their host strains, the plasmids encoding $bla_{CTX\text{-}M\text{-}15}$ were highly homologous and belonged to the IncFII incompatibility group. They usually also carried $bla_{TEM\text{-}1}$, $bla_{OXA\text{-}1}$, *aac(6')-Ib-cr*, *aac3-IIa*, *tet*(A), as well as the plasmid addiction system *ccdA/ccdB*. The relatedness of these plasmids indicated that the wide dissemination of the

CTX-M-15 enzyme in the UK was fundamentally associated with horizontal spread, with these plasmids reaching the epidemic clones A-E along with many other, more diverse, lineages.

- I propose the existence of a successful ST131 quinolone-resistant epidemic *E. coli* strain in the UK prior to the local emergence of CTX-M-15 enzyme. The evolution and diversification of this ancestor strain into the five major epidemic clones A-E was then followed by the acquisition of the plasmids encoding CTX-M enzymes. Evidence for this sequence of events is that the plasmids encoding CTX-M-15 enzyme in clone A varied from those found in members of the other four clones, also in that two different types of plasmids encoding CTX-M enzymes were found independently in clone C isolates, but virulence genotypes of all five epidemic clones were related and the chromosomal mutations for quinolone resistance were consistent among all their members.
- The potentially major role played by the plasmids encoding CTX-M-15 enzyme in the epidemiological success of their hosts was illustrated by the complete DNA sequence map of pEK499, which encoded twelve antibiotic resistance genes affecting eight classes of antimicrobials, along with virulence genes (*vagC*/*vagD* and *tra*T). This plasmid shared many features with other IncFII plasmids encoding CTX-M-15 enzyme characterised from around the world, notably in Canada, India, France, Spain, and North and Central Africa. I propose that the widespread $bla_{\text{CTX-M-15}}$ gene, which is associated with a 48-bp link to IS*Ecp1*, escaped directly from its source (*i.e. Kluyvera* spp.) prior to its mobilization on to the IncFII plasmid, and was not a mutational product of $bla_{\text{CTX-M-3}}$, which is also associated with IS*Ecp1*, but generally with a 128-bp link. This view was supported by the observation that CTX-M-3 did not readily yield CTX-M-15 mutants during selection studies, though the fact it can do so is illustrated by the work of Baraniak *et al.* (2002), in Poland.
- Different representatives of *E. coli* epidemic clone C were shown to have acquired independently two different IncFII plasmids, encoding either CTX-M-3 or CTX-M-15 enzymes. Plasmid cross-hybridization studies indicated a low degree of homology between the plasmid encoding CTX-M-3 enzyme and pEK499. The former plasmid had several features in common with an epidemic broad-host range plasmid widespread among enterobacterial species across Poland: pCTX-M-3 (same incompatibility sub-type, self-transferability, 128-bp link

between *bla*$_{CTX-M-3}$ and IS*Ecp1*, *bla*$_{TEM-1}$, lack of *bla*$_{OXA-1}$ and lack of *aac(6')-Ib-cr*). It is proposed that both pCTX-M-3 (or a derivative) and an ancestral *bla*$_{CTX-M-15}$-encoding plasmid were imported into the UK from abroad, and were subsequently acquired by the pre-existing successful *E. coli* clones.

- Finally, investigation of the virulence genotypes of the multi-drug resistant *E. coli* with CTX-M enzymes suggested that they may be more virulent than previous multi-drug resistant *E. coli* without CTX-M enzymes, investigated in past studies prior to the appearance of these ESBLs. This might explain the emergence and clinical success of *E. coli* isolates producing CTX-M enzymes worldwide. The dominance of isolates with CTX-M-15 enzyme *vs.* those with CTX-M-9-like enzymes appeared to be attributable to their broader spectrum of antibiotic resistance, as well as being better adapted to cause extra-intestinal infections (higher proportions of ExPEC phylogenetic group B2 isolates). Other factors cannot, however, be excluded.

2. Prospects for further studies

This study focused on the molecular epidemiology and characterisation of *E. coli* isolates with CTX-M β-lactamases in the UK, and enlightened many aspects with regards to their emergence, spread and success. However, the findings also raise new questions, which might be subjects for future research in order to comprehend fully, and eventually, counter the global threat posed by CTX-M ESBLs.

First, we deduced that epidemic clones A-E with CTX-M-15 enzyme shared a common ST131 ancestor, present in the UK prior to the large scale dissemination of CTX-M enzymes. Little is known about this strain. It was not detected in a survey of fluoroquinolone resistance by mutations among *E. coli* isolates collected in 2001 in the British Isles (Mushtaq, S. *et al.*, 2006), and it is unclear whether it was imported in the UK. The dissemination of epidemic clones with CTX-M-15 enzyme has been documented in many other countries and it would be interesting to explore their relatedness to the ST131 types widespread in the UK, explaining whether the pandemic of CTX-M-15 enzyme involves international dissemination of successful *E. coli* clones.

The spread of closely-related multi-drug resistance plasmids among clonal and non-clonal isolates with CTX-M-15 enzyme in the UK was illustrated in this study. Although these plasmids

have undeniably contributed to the epidemiological success of their host strains (*e.g.* by rendering them easily selectable), other factors are surely involved, particularly for the most successful UK epidemic clone (A), which was remarkably susceptible to gentamicin and also required lower third-generation cephalosporin MICs compared with other producers of CTX-M-15 enzyme. High-throughput genome sequencing, which is now fast and affordable, will help identify those factors that promote the dissemination and/or pathogenicity of the major epidemic clones. This study also suggested that certain virulence factor genes might be "turned on" or expressed only under certain conditions; this hypothesis deserves to be pursued further.

Thirdly, an important area that still remains unclear is the mode of transmission of isolates with CTX-M enzymes. Three potential routes of dissemination were suggested in this study, but none has yet been proven to be responsible for the spread of *e.g. E. coli* epidemic clone A, which has been found in well over 50 different centres across the UK (including in Northern Ireland). Screening more animal food products and more human consumables in general for the presence of isolates with CTX-M enzymes may prove to be very revealing. Assessing faecal carriage in patients admitted to hospitals, prior and following their stay, might be helpful too, as might sampling the general population or sewage. Such strategies might clarify also whether isolates with CTX-M enzymes are brought into hospitals from the community, or *vice versa*.

Finally, the penetration of *E. coli* isolates with CTX-M enzymes in the community is well documented. This study indicated that their stability in antibiotic-free environments (*e.g.* healthy household members) may result from the linkage of the $bla_{\text{CTX-M}}$ genes to addiction systems, such as *ccdA/ccdB*, on single plasmids. In order to verify this theory, it would be interesting to estimate the exact prevalence of such association, particularly among isolates recovered from healthy individuals in the community.

Bibliography

Abraham, E. P. and Chain, E. 1940. An enzyme from bacteria able to destroy penicillin. Nature 46.837.

Aguero, M. E., Aron, L., DeLuca, A. G., Timmis, K. N., and Cabello, F. C. 1984. A plasmid-encoded outer membrane protein, TraT, enhances resistance of *Escherichia coli* to phagocytosis. Infect.Immun. 46.740-6.

al Naiemi, N., Duim, B., and Bart, A. 2006. A CTX-M extended-spectrum beta-lactamase in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. J.Med.Microbiol. 55.1607-8.

Alobwede, I., M'Zali, F. H., Livermore, D. M., Heritage, J., Todd, N., and Hawkey, P. M. 2003. CTX-M extended-spectrum beta-lactamase arrives in the UK. Journal of Antimicrobial Chemotherapy 51.470-1.

Ambler, R. P. 1980. The structure of beta-lactamases. Philos.Trans.R.Soc.Lond B Biol.Sci. 289.321-31.

Ambler, R. P. and Scott, G. K. 1978. Partial amino acid sequence of penicillinase coded by *Escherichia coli* plasmid R6K. Proc.Natl.Acad.Sci.U.S.A 75.3732-6.

Andrews, J. M. 2006. BSAC standardized disc susceptibility testing method (version 5). Journal of Antimicrobial Chemotherapy 58.511-29.

Andrews, J. M. 2007. BSAC standardized disc susceptibility testing method (version 6). Journal of Antimicrobial Chemotherapy.

Arduino, S. M., Catalano, M., Orman, B. E., Roy, P. H., and Centron, D. 2003. Molecular epidemiology of *orf513*-bearing class 1 integrons in multiresistant clinical isolates from Argentinean hospitals. Antimicrobial Agents and Chemotherapy 47.3945-9.

Arduino, S. M., Roy, P. H., Jacoby, G. A., Orman, B. E., Pineiro, S. A., and Centron, D. 2002. *bla*(CTX-M-2) is located in an unusual class 1 integron (In35) which includes Orf513. Antimicrobial Agents and Chemotherapy 46.2303-6.

Arnold, T. M., Forrest, Graeme N., and Messmer, Karen J. 2007. Polymyxin antibiotics for gram-negative infections. American Journal of Health-System Pharmacy 64.819-26.

Babini, G. S. 2001. Prevalence and epidemiology of beta-lactam resistant *Klebsiella* in European ICUs. Thesis 2.15.87-90.

Bae, I. K., Lee, B. H., Hwang, H. Y., Jeong, S. H., Hong, S. G., Chang, C. L., Kwak, H. S., Kim, H. J., and Youn, H. 2006. A novel ceftazidime-hydrolysing extended-spectrum beta-lactamase, CTX-M-54, with a single amino acid substitution at position 167 in the omega loop. Journal of Antimicrobial Chemotherapy 58.315-9.

Bahrani-Mougeot, F, N. W. Gunther IV, M. S. Donnenberg, and H. L. Mobley. 2002. Uropathogenic *Escherichia coli*, ed. by Donnenberg, M. S., 239-68. Baltimore, Maryland: Elsevier Science (USA).

Baraniak, A., Fiett, J., Hryniewicz, W., Nordmann, P., and Gniadkowski, M. 2002a. Ceftazidime-hydrolysing CTX-M-15 extended-spectrum beta-lactamase (ESBL) in Poland. Journal of Antimicrobial Chemotherapy 50.393-6.

Baraniak, A., Fiett, J., Sulikowska, A., Hryniewicz, W., and Gniadkowski, M. 2002b. Countrywide spread of CTX-M-3 extended-spectrum beta-lactamase-producing microorganisms of the family Enterobacteriaceae in Poland. Antimicrobial Agents and Chemotherapy 46.151-9.

Bauer, A. W., Kirby, W. M., Sherris, J. C., and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. Tech.Bull.Regist.Med.Technol. 36.49-52.

Bauernfeind, A., Grimm, H., and Schweighart, S. 1990. A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. Infection 18.294-8.

Ben Ami, R., Schwaber, M. J., Navon-Venezia, S., Schwartz, D., Giladi, M., Chmelnitsky, I., Leavitt, A., and Carmeli, Y. 2006. Influx of extended-spectrum beta-lactamase-producing Enterobacteriaceae into the hospital. Clin.Infect.Dis. 42.925-34.

Bernard, H., Tancrede, C., Livrelli, V., Morand, A., Barthelemy, M., and Labia, R. 1992. A novel plasmid-mediated extended-spectrum beta-lactamase not derived from TEM- or SHV-type enzymes. J.Antimicrob.Chemother. 29.590-2.

Birnboim, H. C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7.1513-23.

Blattner, F. R., Plunkett, G., III, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B., and Shao, Y. 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277.1453-74.

Boehm, D. F., Welch, R. A., and Snyder, I. S. 1990a. Calcium is required for binding of *Escherichia coli* hemolysin (HlyA) to erythrocyte membranes. Infect.Immun. 58.1951-8.

Boehm, D. F., Welch, R. A., and Snyder, I. S. 1990b. Domains of *Escherichia coli* hemolysin (HlyA) involved in binding of calcium and erythrocyte membranes. Infect.Immun. 58.1959-64.

Bonnet, R. 2004. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrobial Agents and Chemotherapy 48.1-14.

Bonnet, R., Recule, C., Baraduc, R., Chanal, C., Sirot, D., De Champs, C., and Sirot, J. 2003. Effect of D240G substitution in a novel ESBL CTX-M-27. Journal of Antimicrobial Chemotherapy 52.29-35.

Boyd, D. A., Tyler, S., Christianson, S., McGeer, A., Muller, M. P., Willey, B. M., Bryce, E., Gardam, M., Nordmann, P., and Mulvey, M. R. 2004. Complete nucleotide sequence of a 92-kilobase plasmid harboring the CTX-M-15 extended-spectrum beta-lactamase

involved in an outbreak in long-term-care facilities in Toronto, Canada. Antimicrobial Agents and Chemotherapy 48.3758-64.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal.Biochem. 72.248-54.

Bradford, P. A. 1999. Automated thermal cycling is superior to traditional methods for nucleotide sequencing of *bla*(SHV) genes. Antimicrobial Agents and Chemotherapy 43.2960-3.

Branger, C., Zamfir, O., Geoffroy, S., Laurans, G., Arlet, G., Thien, H. V., Gouriou, S., Picard, B., and Denamur, E. 2005. Genetic background of *Escherichia coli* and extended-spectrum beta-lactamase type. Emerg.Infect.Dis. 11.54-61.

Brenwald, N. P., Jevons, G., Andrews, J. M., Xiong, J. H., Hawkey, P. M., and Wise, R. 2003. An outbreak of a CTX-M-type beta-lactamase-producing *Klebsiella pneumoniae*: the importance of using cefpodoxime to detect extended-spectrum beta-lactamases. Journal of Antimicrobial Chemotherapy 51.195-6.

Bush, K., Jacoby, G. A., and Medeiros, A. A. 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob.Agents Chemother. 39.1211-33.

Canton, R. and Coque, T. M. 2006. The CTX-M beta-lactamase pandemic. Curr.Opin.Microbiol. 9.466-75.

Cao, V., Lambert, T., and Courvalin, P. 2002. ColE1-like plasmid pIP843 of *Klebsiella pneumoniae* encoding extended-spectrum beta-lactamase CTX-M-17. Antimicrobial Agents and Chemotherapy 46.1212-7.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L., and Threlfall, E. J. 2005. Identification of plasmids by PCR-based replicon typing. J.Microbiol.Methods 63.219-28.

Carattoli, A., Miriagou, V., Bertini, A., Loli, A., Colinon, C., Villa, L., Whichard, J. M., and Rossolini, G. M. 2006. Replicon typing of plasmids encoding resistance to newer beta-lactams. Emerg.Infect.Dis. 12.1145-8.

Celenza, G., Pellegrini, C., Caccamo, M., Segatore, B., Amicosante, G., and Perilli, M. 2006. Spread of *bla*(CTX-M-type) and *bla*(PER-2) beta-lactamase genes in clinical isolates from Bolivian hospitals. Journal of Antimicrobial Chemotherapy 57.975-8.

Chart, H. 1994a. Bacterial fractionation and membrane protein characterization, ed. by Chart, H., 1-10. London: CRC Press.

Chart, H. 1994b. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation and resolution of bacterial components, ed. by Chart, H., 21-34. London: CRC Press.

Chiu, N. C., Huang, F. Y., and Tsai, T. C. 1989. Urinary tract infections in children. Zhonghua Min Guo.Xiao.Er.Ke.Yi.Xue.Hui.Za Zhi. 30.225-32.

Chmelnitsky, I., Carmeli, Y., Leavitt, A., Schwaber, M. J., and Navon-Venezia, S. 2005. CTX-M-2 and a new CTX-M-39 enzyme are the major extended-spectrum beta-lactamases in multiple *Escherichia coli* clones isolated in Tel Aviv, Israel. Antimicrobial Agents and Chemotherapy 49.4745-50.

Chopra, I., O'Neill, A. J., and Miller, K. 2003. The role of mutators in the emergence of antibiotic-resistant bacteria. Drug Resist.Updat. 6.137-45.

Clermont, O., Bonacorsi, S., and Bingen, E. 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl.Environ.Microbiol. 66.4555-8.

Clinical and Laboratory Standards Institute. 2006. Performance Standards for Antimicrobial Susceptibillity Testing. 16th Informational Supplement M100-S16 26 - No3.

Costa, D., Poeta, P., Saenz, Y., Vinue, L., Rojo-Bezares, B., Jouini, A., Zarazaga, M., Rodrigues, J., and Torres, C. 2006. Detection of *Escherichia coli* harbouring extended-

spectrum beta-lactamases of the CTX-M, TEM and SHV classes in faecal samples of wild animals in Portugal. Journal of Antimicrobial Chemotherapy 58.1311-2.

Danel, F., Hall, L. M., Gur, D., and Livermore, D. M. 1997. OXA-15, an extended-spectrum variant of OXA-2 beta-lactamase, isolated from a *Pseudomonas aeruginosa* strain. Antimicrobial Agents and Chemotherapy 41.785-90.

Dao-Thi, M. H., Charlier, D., Loris, R., Maes, D., Messens, J., Wyns, L., and Backmann, J. 2002. Intricate interactions within the *ccd* plasmid addiction system. J.Biol.Chem. 277.3733-42.

Datta, N. and Kontomichalou, P. 1965. Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. Nature 208.239-41.

Decousser, J. W., Poirel, L., and Nordmann, P. 2001. Characterization of a chromosomally encoded extended-spectrum class A beta-lactamase from *Kluyvera cryocrescens*. Antimicrobial Agents and Chemotherapy 45.3595-8.

Denton, M. 2007. Enterobacteriaceae. Int.J.Antimicrob.Agents 29, Supplement 3.9-22.

Donnenberg, M. S. Donnenberg, M. S. (ed.) 2002. *Escherichia coli*: virulence mechanisms of a versatile pathogen. Baltimore, Maryland (USA): Academic Press.

Duan, R. S., Sit, T. H., Wong, S. S., Wong, R. C., Chow, K. H., Mak, G. C., Yam, W. C., Ng, L. T., Yuen, K. Y., and Ho, P. L. 2006. *Escherichia coli* producing CTX-M beta-lactamases in food animals in Hong Kong. Microb.Drug Resist. 12.145-8.

Dundas, S. and P. D. Welsby. 2002. Infection control in hospitals, 14-8. Edinburgh: Science press.

Eckert, C., Gautier, V., Saladin-Allard, M., Hidri, N., Verdet, C., Ould-Hocine, Z., Barnaud, G., Delisle, F., Rossier, A., Lambert, T., Philippon, A., and Arlet, G. 2004. Dissemination of CTX-M-type beta-lactamases among clinical isolates of Enterobacteriaceae in Paris, France. Antimicrobial Agents and Chemotherapy 48.1249-55.

Eisenstein, B. I. and Jones, G. W. 1988. The spectrum of infections and pathogenic mechanisms of *Escherichia coli*. Adv.Intern.Med. 33.231-52.

Ellington, M. J., Livermore, D. M., Pitt, T. L., Hall, L. M., and Woodford, N. 2006. Development of extended-spectrum activity in TEM beta-lactamases in hyper-mutable, *mutS Escherichia coli*. Clin.Microbiol.Infect. 12.800-3.

Ensor, V. M., Shahid, M., Evans, J. T., and Hawkey, P. M. 2006. Occurrence, prevalence and genetic environment of CTX-M beta-lactamases in Enterobacteriaceae from Indian hospitals. Journal of Antimicrobial Chemotherapy 58.1260-3.

Ensor, V. M., Warren, R. E., O'Neill, P., Butler, V., Taylor, J., Nye, K., Harvey, M., Livermore, D. M., Woodford, N., and Hawkey, P. M. 2007. Isolation of quinolone-resistant CTX-M-producing *Escherichia coli* from raw chicken meat sold in retail outlets in the West Midlands, UK (P1025). 17th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID).

Everett, M. J., Jin, Y. F., Ricci, V., and Piddock, L. J. 1996. Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. Antimicrobial Agents and Chemotherapy 40.2380-6.

Farmer, J. J., III, Davis, B. R., Hickman-Brenner, F. W., McWhorter, A., Huntley-Carter, G. P., Asbury, M. A., Riddle, C., Wathen-Grady, H. G., Elias, C., Fanning, G. R., and . 1985. Biochemical identification of new species and biogroups of Enterobacteriaceae isolated from clinical specimens. J.Clin.Microbiol. 21.46-76.

Farooqui, B. J., Khurshid, M., and Alam, M. 1989. Urinary tract infection. J.Pak.Med.Assoc. 39.129-31.

Galimand, M., Courvalin, P., and Lambert, T. 2003. Plasmid-mediated high-level resistance to aminoglycosides in Enterobacteriaceae due to 16S rRNA methylation. Antimicrob.Agents Chemother. 47.2565-71.

Galimand, M., Sabtcheva, S., Courvalin, P., and Lambert, T. 2005. Worldwide Disseminated *armA* Aminoglycoside Resistance Methylase Gene Is Borne by Composite Transposon Tn*1548*. Antimicrob.Agents Chemother. 49.2949-53.

Galvin, S., Morris, D., Hickey, P., and Cormican, M. 2007. Occurrence of ESBL-producing *Escherichia coli* in outflow from a wastewater treatment plant. 17th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID).

Gangoue-Pieboji, J., Miriagou, V., Vourli, S., Tzelepi, E., Ngassam, P., and Tzouvelekis, L. S. 2005. Emergence of CTX-M-15-producing enterobacteria in Cameroon and characterization of a *bla*CTX-M-15-carrying element. Antimicrobial Agents and Chemotherapy 49.441-3.

Garcia, A., Navarro, F., Miro, E., Mirelis, B., Campoy, S., and Coll, P. 2005. Characterization of the highly variable region surrounding the *bla*(CTX-M-9) gene in non-related *Escherichia coli* from Barcelona. Journal of Antimicrobial Chemotherapy 56.819-26.

Gazouli, M., Tzelepi, E., Sidorenko, S. V., and Tzouvelekis, L. S. 1998. Sequence of the gene encoding a plasmid-mediated cefotaxime-hydrolyzing class A beta-lactamase (CTX-M-4): involvement of serine 237 in cephalosporin hydrolysis. Antimicrobial Agents and Chemotherapy 42.1259-62.

Gniadkowski, M., Schneider, I., Palucha, A., Jungwirth, R., Mikiewicz, B., and Bauernfeind, A. 1998. Cefotaxime-resistant Enterobacteriaceae isolates from a hospital in Warsaw, Poland: identification of a new CTX-M-3 cefotaxime-hydrolyzing beta-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. Antimicrobial Agents and Chemotherapy 42.827-32.

Guardabassi, L., Dijkshoorn, L., Collard, J. M., Olsen, J. E., and Dalsgaard, A. 2000. Distribution and in-vitro transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter* strains. J.Med.Microbiol. 49.929-36.

Hastad, O. and Bjorklund, M. 1998. Nucleotide substitution models and estimation of phylogeny. Mol.Biol.Evol. 15.1381-9.

Heritier, C., Poirel, L., and Nordmann, P. 2004. Genetic and biochemical characterization of a chromosome-encoded carbapenem-hydrolyzing ambler class D beta-lactamase from *Shewanella algae*. Antimicrobial Agents and Chemotherapy 48.1670-5.

Herrlich, P. and Schweiger, M. 1976. Nitrofurans, a group of synthetic antibiotics, with a new mode of action: discrimination of specific messenger RNA classes. Proc.Natl.Acad.Sci.U.S.A 73.3386-90.

Herzer, P. J., Inouye, S., Inouye, M., and Whittam, T. S. 1990. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli*. J.Bacteriol. 172.6175-81.

Hopkins, K. L., Batchelor, M. J., Liebana, E., Deheer-Graham, A. P., and Threlfall, E. J. 2006a. Characterisation of CTX-M and AmpC genes in human isolates of *Escherichia coli* identified between 1995 and 2003 in England and Wales. Int.J.Antimicrob.Agents 28.180-92.

Hopkins, K. L., Deheer-Graham, A., Threlfall, E. J., Batchelor, M. J., and Liebana, E. 2006b. Novel plasmid-mediated CTX-M-8 subgroup extended-spectrum beta-lactamase (CTX-M-40) isolated in the UK. Int.J.Antimicrob.Agents 27.572-5.

Horcajada, J. P., Soto, S., Gajewski, A., Smithson, A., Jimenez de Anta, M. T., Mensa, J., Vila, J., and Johnson, J. R. 2005. Quinolone-resistant uropathogenic *Escherichia coli* strains from phylogenetic group B2 have fewer virulence factors than their susceptible counterparts. J.Clin.Microbiol. 43.2962-4.

Humeniuk, C., Arlet, G., Gautier, V., Grimont, P., Labia, R., and Philippon, A. 2002. Beta-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrobial Agents and Chemotherapy 46.3045-9.

Huovinen, P. 2001. Resistance to trimethoprim-sulfamethoxazole. Clin.Infect.Dis. 32.1608-14.

Ishii, Y., Ohno, A., Taguchi, H., Imajo, S., Ishiguro, M., and Matsuzawa, H. 1995. Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class A beta-lactamase isolated from *Escherichia coli*. Antimicrobial Agents and Chemotherapy 39.2269-75.

Johnson, J. R. and Clabots, C. 2006. Extensive sharing of uropathogenic *Escherichia coli* clones among household members of a woman with acute cystitis (C2-73). 46th Interscience Conference on Antimicrobial Agents and Chemotherapy.

Johnson, J. R., Kuskowski, M. A., O'Bryan, T. T., Colodner, R., and Raz, R. 2005. Virulence genotype and phylogenetic origin in relation to antibiotic resistance profile among *Escherichia coli* urine sample isolates from Israeli women with acute uncomplicated cystitis. Antimicrobial Agents and Chemotherapy 49.26-31.

Johnson, J. R., Oswald, E., O'Bryan, T. T., Kuskowski, M. A., and Spanjaard, L. 2002. Phylogenetic distribution of virulence-associated genes among *Escherichia coli* isolates associated with neonatal bacterial meningitis in the Netherlands. J.Infect.Dis. 185.774-84.

Johnson, J. R., Sannes, M. R., Croy, C., Johnston, B., Clabots, C., Kuskowski, M. A., Bender, J., Smith, K. E., Winokur, P. L., and Belongia, E. A. 2007. Antimicrobial drug-resistant *Escherichia coli* from humans and poultry products, Minnesota and Wisconsin, 2002-2004. Emerg.Infect.Dis. 13.838-46.

Johnson, J. R. and Stell, A. L. 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J.Infect.Dis. 181.261-72.

Kahlmeter, G. and Menday, P. 2003. Cross-resistance and associated resistance in 2478 *Escherichia coli* isolates from the Pan-European ECO.SENS Project surveying the antimicrobial susceptibility of pathogens from uncomplicated urinary tract infections. Journal of Antimicrobial Chemotherapy 52.128-31.

Karim, A., Poirel, L., Nagarajan, S., and Nordmann, P. 2001. Plasmid-mediated extended-spectrum beta-lactamase (CTX-M-3 like) from India and gene association with insertion sequence IS*Ecp1*. FEMS Microbiol.Lett. 201.237-41.

Karisik, E., Ellington, M. J., Pike, R., Livermore, D. M., and Woodford, N. 2006a. Development of high-level ceftazidime resistance via single-base substitutions of *bla*(CTX-M-3) in hyper-mutable *Escherichia coli*. Clin.Microbiol.Infect. 12.803-6.

Karisik, E., Ellington, M. J., Pike, R., Livermore, D. M., and Woodford, N. 2006b. Uncommon CTX-M enzyme variants in the United Kingdom. 16th European Congress of Clinical Microbiology and Infectious diseases.

Karlowsky, J. A., Thornsberry, C., Jones, M. E., and Sahm, D. F. 2003. Susceptibility of antimicrobial-resistant urinary *Escherichia coli* isolates to fluoroquinolones and nitrofurantoin. Clin.Infect.Dis. 36.183-7.

Knothe, H., Shah, P., Krcmery, V., Antal, M., and Mitsuhashi, S. 1983. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection 11.315-7.

Knox, J. R. 1995. Extended-spectrum and inhibitor-resistant TEM-type beta-lactamases: mutations, specificity, and three-dimensional structure. Antimicrobial Agents and Chemotherapy 39.2593-601.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., and Peterson, K. M. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166.175-6.

Kresse, H, Belsey, M. J., and Rovini, H. 2007. The antibacterial drugs market. Nature reviews 6.

Lartigue, M. F., Poirel, L., and Nordmann, P. 2004. Diversity of genetic environment of *bla*(CTX-M) genes. FEMS Microbiol.Lett. 234.201-7.

Lartigue, M. F., Poirel, L., Poyart, C., Reglier-Poupet, H., and Nordmann, P. 2007. Ertapenem resistance of *Escherichia coli*. Emerg.Infect.Dis. 13.315-7.

Lavigne, J. P., Blanc-Potard, A. B., Bourg, G., Moreau, J., Chanal, C., Bouziges, N., O'callaghan, D., and Sotto, A. 2006. Virulence genotype and nematode-killing properties of extra-intestinal Escherichia coli producing CTX-M beta-lactamases. Clin.Microbiol.Infect. 12.1199-206.

Lavigne, J. P., Marchandin, H., Delmas, J., Moreau, J., Bouziges, N., Lecaillon, E., Cavalie, L., Jean-Pierre, H., Bonnet, R., and Sotto, A. 2007. CTX-M beta-lactamase-producing Escherichia coli in French hospitals: prevalence, molecular epidemiology, and risk factors. J.Clin.Microbiol. 45.620-6.

Lavollay, M., Mamlouk, K., Frank, T., Akpabie, A., Burghoffer, B., Ben Redjeb, S., Bercion, R., Gautier, V., and Arlet, G. 2006. Clonal dissemination of a CTX-M-15 beta-lactamase-producing *Escherichia coli* strain in the Paris area, Tunis, and Bangui. Antimicrobial Agents and Chemotherapy 50.2433-8.

LeClerc, J. E., Li, B., Payne, W. L., and Cebula, T. A. 1996. High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. Science 274.1208-11.

Lecointre, G., Rachdi, L., Darlu, P., and Denamur, E. 1998. *Escherichia coli* molecular phylogeny using the incongruence length difference test. Mol.Biol.Evol. 15.1685-95.

Leflon-Guibout, V., Jurand, C., Bonacorsi, S., Espinasse, F., Guelfi, M. C., Duportail, F., Heym, B., Bingen, E., and Nicolas-Chanoine, M. H. 2004. Emergence and spread of three clonally related virulent isolates of CTX-M-15-producing *Escherichia coli* with variable resistance to aminoglycosides and tetracycline in a French geriatric hospital. Antimicrobial Agents and Chemotherapy 48.3736-42.

Levesque, C., Piche, L., Larose, C., and Roy, P. H. 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrobial Agents and Chemotherapy 39.185-91.

Livermore, D. M. 1995. beta-Lactamases in laboratory and clinical resistance. Clin.Microbiol.Rev. 8.557-84.

Livermore, D. M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G. M., Arlet, G., Ayala, J., Coque, T. M., Kern-Zdanowicz, I., Luzzaro, F., Poirel, L., and Woodford, N. 2007. CTX-M: changing the face of ESBLs in Europe. Journal of Antimicrobial Chemotherapy 59.165-74.

Livermore, D. M. and Hawkey, P. M. 2005. CTX-M: changing the face of ESBLs in the UK. Journal of Antimicrobial Chemotherapy 56.451-4.

Livermore, D. M. and D. L. Paterson. 2006. Extended-spectrum beta-lactamases in resistance. London: Current Medicine Group Ltd.

Livermore, D. M. and Woodford, N. 2006. The beta-lactamase threat in Enterobacteriaceae, *Pseudomonas* and *Acinetobacter*. Trends Microbiol. 14.413-20.

Lorian, V. 1996. beta-lactams, ed. by Lorian, V., 557-61. New York: Williams & Wilkins.

Loughrey, A., Rooney, P, O'Leary, M., McCalmont, M., Warner, M., Karisik, E., Donaghy, P., Smyth, B., Woodford, N., and Livermore, D. M. 2007. Prevalence of an epidemic ESBL-producing *Escherichia coli* strain in Long Term Care Facilities in Belfast. 17th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID).

Macrina, F. L., Kopecko, D. J., Jones, K. R., Ayers, D. J., and McCowen, S. M. 1978. A multiple plasmid-containing *Escherichia coli strain*: convenient source of size reference plasmid molecules. Plasmid 1.417-20.

Mammeri, H., Van De, Loo M., Poirel, L., Martinez-Martinez, L., and Nordmann, P. 2005. Emergence of plasmid-mediated quinolone resistance in *Escherichia coli* in Europe. Antimicrob.Agents Chemother. 49.71-6.

Marrs, Carl F., Zhang, Lixin, and Foxman, Betsy. 2005. *Escherichia coli* mediated urinary tract infections: Are there distinct uropathogenic *E. coli* (UPEC) pathotypes? FEMS Microbiology Letters 252.183-90.

Matsumoto, Y., Ikeda, F., Kamimura, T., Yokota, Y., and Mine, Y. 1988. Novel plasmid-mediated beta-lactamase from *Escherichia coli* that inactivates oxyimino-cephalosporins. Antimicrobial Agents and Chemotherapy 32.1243-6.

Matthew, M. and Hedges, R. W. 1976. Analytical isoelectric focusing of R factor-determined beta-lactamases: correlation with plasmid compatibility. J.Bacteriol. 125.713-8.

Mazel, D., Dychinco, B., Webb, V. A., and Davies, J. 2000. Antibiotic resistance in the ECOR collection: integrons and identification of a novel aad gene. Antimicrobial Agents and Chemotherapy 44.1568-74.

Mena, A., Plasencia, V., Garcia, L., Hidalgo, O., Ayestaran, J. I., Alberti, S., Borrell, N., Perez, J. L., and Oliver, A. 2006. Characterization of a large outbreak by CTX-M-1-producing *Klebsiella pneumoniae* and mechanisms leading to in vivo carbapenem resistance development. J.Clin.Microbiol. 44.2831-7.

Mercier, J. and Levesque, R. C. 1990. Cloning of SHV-2, OHIO-1, and OXA-6 beta-lactamases and cloning and sequencing of SHV-1 beta-lactamase. Antimicrobial Agents and Chemotherapy 34.1577-83.

Meunier, D., Jouy, E., Lazizzera, C., Kobisch, M., and Madec, J. Y. 2006. CTX-M-1- and CTX-M-15-type beta-lactamases in clinical *Escherichia coli* isolates recovered from food-producing animals in France. Int.J.Antimicrob.Agents 28.402-7.

Miller, K., O'Neill, A. J., and Chopra, I. 2002. Response of *Escherichia coli* hypermutators to selection pressure with antimicrobial agents from different classes. Journal of Antimicrobial Chemotherapy 49.925-34.

Mims, C., J. Playfair, I Roitt, D. Wakelin, and R. Williams. 1999. Antimicrobial Agents and chemotherapy, ed. by crowe, L., 411-40. London: Mosby.

Miro, E., Mirelis, B., Navarro, F., Rivera, A., Mesa, R. J., Roig, M. C., Gomez, L., and Coll, P. 2005. Surveillance of extended-spectrum beta-lactamases from clinical samples and faecal carriers in Barcelona, Spain. Journal of Antimicrobial Chemotherapy 56.1152-5.

Mobley, H. L., Island, M. D., and Massad, G. 1994. Virulence determinants of uropathogenic *Escherichia coli* and *Proteus mirabilis*. Kidney Int.Suppl 47.S129-S136.

Monnaie, D. and Frere, J. M. 1993. Interaction of clavulanate with class C beta-lactamases. FEBS Lett. 334.269-71.

Munday, C. J., Boyd, D. A., Brenwald, N., Miller, M., Andrews, J. M., Wise, R., Mulvey, M. R., and Hawkey, P. M. 2004a. Molecular and kinetic comparison of the novel extended-spectrum beta-lactamases CTX-M-25 and CTX-M-26. Antimicrobial Agents and Chemotherapy 48.4829-34.

Munday, C. J., Whitehead, G. M., Todd, N. J., Campbell, M., and Hawkey, P. M. 2004b. Predominance and genetic diversity of community- and hospital-acquired CTX-M extended-spectrum beta-lactamases in York, UK. Journal of Antimicrobial Chemotherapy 54.628-33.

Murphy, C. K., Mullin, Steve, Osburne, Marcia S., van Duzer, John, Siedlecki, Jim, Yu, Xiang, Kerstein, Kathy, Cynamon, Michael, and Rothstein, David M. 2006. In Vitro Activity of Novel Rifamycins against Rifamycin-Resistant *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 50.827-34.

Mushtaq, S., Ellington, M. J., Livermore, D. M., and Woodford, N. 2006. Mechanisms of high-level ciprofloxacin resistance in twenty-five clinical isolates of *Escherichia coli* collected in the British Isles. 16th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID).

Mushtaq, S., Woodford, N., Potz, N., and Livermore, D. M. 2003. Detection of CTX-M-15 extended-spectrum beta-lactamase in the United Kingdom. Journal of Antimicrobial Chemotherapy 52.528-9.

Neu, H. C. 1988. Aztreonam: the first monobactam. Med.Clin.North Am. 72.555-66.

Novais, A., Canton, R., Moreira, R., Peixe, L., Baquero, F., and Coque, T. M. 2007. Emergence and dissemination of Enterobacteriaceae isolates producing CTX-M-1-like

enzymes in Spain are associated with IncFII (CTX-M-15) and broad-host-range (CTX-M-1, -3, and -32) plasmids. Antimicrobial Agents and Chemotherapy 51.796-9.

Novais, A., Canton, R., Valverde, A., Machado, E., Galan, J. C., Peixe, L., Carattoli, A., Baquero, F., and Coque, T. M. 2006. Dissemination and persistence of *bla*(CTX-M-9) are linked to class 1 integrons containing *CR1* associated with defective transposon derivatives from Tn*402* located in early antibiotic resistance plasmids of IncHI2, IncP1-alpha, and IncFI groups. Antimicrobial Agents and Chemotherapy 50.2741-50.

Oliver, A., Coque, T. M., Alonso, D., Valverde, A., Baquero, F., and Canton, R. 2005. CTX-M-10 linked to a phage-related element is widely disseminated among Enterobacteriaceae in a Spanish hospital. Antimicrobial Agents and Chemotherapy 49.1567-71.

Olson, A. B., Silverman, M., Boyd, D. A., McGeer, A., Willey, B. M., Pong-Porter, V., Daneman, N., and Mulvey, M. R. 2005. Identification of a progenitor of the CTX-M-9 group of extended-spectrum beta-lactamases from *Kluyvera georgiana* isolated in Guyana. Antimicrobial Agents and Chemotherapy 49.2112-5.

Orskov, I. and Orskov, F. 1985. *Escherichia coli* in extra-intestinal infections. J.Hyg.(Lond) 95.551-75.

Orskov, I., Orskov, F., Jann, B., and Jann, K. 1977. Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. Bacteriol.Rev. 41.667-710.

Pallecchi, L., Malossi, M., Mantella, A., Gotuzzo, E., Trigoso, C., Bartoloni, A., Paradisi, F., Kronvall, G., and Rossolini, G. M. 2004. Detection of CTX-M-type beta-lactamase genes in fecal *Escherichia coli* isolates from healthy children in Bolivia and Peru. Antimicrobial Agents and Chemotherapy 48.4556-61.

Paterson, D. L. 2006. Resistance in gram-negative bacteria: Enterobacteriaceae. Am.J.Infect.Control 34.S20-S28.

Paterson, D. L., Ko, W. C., Von Gottberg, A., Mohapatra, S., Casellas, J. M., Goossens, H., Mulazimoglu, L., Trenholme, G., Klugman, K. P., Bonomo, R. A., Rice, L. B., Wagener, M. M., McCormack, J. G., and Yu, V. L. 2004. Antibiotic therapy for *Klebsiella pneumoniae* bacteremia: implications of production of extended-spectrum beta-lactamases. Clin.Infect.Dis. 39.31-7.

Perez-Perez, F. J. and Hanson, N. D. 2002. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J.Clin.Microbiol. 40.2153-62.

Picard, B., Journet-Mancy, C., Picard-Pasquier, N., and Goullet, P. 1993. Genetic structures of the B2 and B1 *Escherichia coli* strains responsible for extra-intestinal infections. J.Gen.Microbiol. 139.3079-88.

Pitcher, D. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Letters of Applied Microbiology 8.151.

Pitout, J. D., Laupland, K. B., Church, D. L., Menard, M. L., and Johnson, J. R. 2005. Virulence factors of Escherichia coli isolates that produce CTX-M-type extended-spectrum beta-lactamases. Antimicrobial Agents and Chemotherapy 49.4667-70.

Poirel, L., Decousser, J. W., and Nordmann, P. 2003. Insertion sequence IS*Ecp1B* is involved in expression and mobilization of a *bla*(CTX-M) beta-lactamase gene. Antimicrobial Agents and Chemotherapy 47.2938-45.

Poirel, L., Gniadkowski, M., and Nordmann, P. 2002a. Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum beta-lactamase CTX-M-15 and of its structurally related beta-lactamase CTX-M-3. Journal of Antimicrobial Chemotherapy 50.1031-4.

Poirel, L., Heritier, C., and Nordmann, P. 2004. Chromosome-encoded ambler class D beta-lactamase of *Shewanella oneidensis* as a progenitor of carbapenem-hydrolyzing oxacillinase. Antimicrobial Agents and Chemotherapy 48.348-51.

Poirel, L., Kampfer, P., and Nordmann, P. 2002b. Chromosome-encoded Ambler class A beta-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum beta-lactamases. Antimicrobial Agents and Chemotherapy 46.4038-40.

Poirel, L., Lartigue, M. F., Decousser, J. W., and Nordmann, P. 2005. IS*Ecp1B*-mediated transposition of *bla*(CTX-M) in *Escherichia coli*. Antimicrobial Agents and Chemotherapy 49.447-50.

Poirel, L., Naas, T., Le, Thomas, I, Karim, A., Bingen, E., and Nordmann, P. 2001. CTX-M-type extended-spectrum beta-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. Antimicrobial Agents and Chemotherapy 45.3355-61.

Poirel, L., Said, Z., Cattoir, V., Souusy, C., and Nordmann, P. 2006. First identification of plasmid-mediated quinolone resistant determinant *qnrB*-like in Europe in an *Enterobacter cloacae* isolate co-expressing a *qnrS* determinant. 46th Interscience Conference on Antimicrobial Agents and Chemotherapy.

Potz, N. A., Hope, R., Warner, M., Johnson, A. P., and Livermore, D. M. 2006. Prevalence and mechanisms of cephalosporin resistance in Enterobacteriaceae in London and South-East England. Journal of Antimicrobial Chemotherapy 58.320-6.

Power, P., Galleni, M., Di Conza, J., Ayala, J. A., and Gutkind, G. 2005. Description of In*116*, the first *bla*(CTX-M-2)-containing complex class 1 integron found in *Morganella morganii* isolates from Buenos Aires, Argentina. Journal of Antimicrobial Chemotherapy 55.461-5.

Power, P., Radice, M., Barberis, C., de Mier, C., Mollerach, M., Maltagliatti, M., Vay, C., Famiglietti, A., and Gutkind, G. 1999. Cefotaxime-hydrolysing beta lactamases in *Morganella morganii*. Eur.J.Clin.Microbiol.Infect.Dis. 18.743-7.

Pullinger, G. D. and Lax, A. J. 1992. A *Salmonella dublin* virulence plasmid locus that affects bacterial growth under nutrient-limited conditions. Mol.Microbiol. 6.1631-43.

Quinteros, M., Radice, M., Gardella, N., Rodriguez, M. M., Costa, N., Korbenfeld, D., Couto, E., and Gutkind, G. 2003. Extended-spectrum beta-lactamases in enterobacteriaceae in Buenos Aires, Argentina, public hospitals. Antimicrobial Agents and Chemotherapy 47.2864-7.

Radice, M., Power, P., Di Conza, J., and Gutkind, G. 2002. Early dissemination of CTX-M-derived enzymes in South America. Antimicrobial Agents and Chemotherapy 46.602-4.

Robbins, J. B., McCracken, G. H., Jr., Gotschlich, E. C., Orskov, F., Orskov, I., and Hanson, L. A. 1974. *Escherichia coli* K1 capsular polysaccharide associated with neonatal meningitis. N.Engl.J.Med. 290.1216-20.

Robicsek, A., Strahilevitz, J., Jacoby, G. A., Macielag, M., Abbanat, D., Park, C. H., Bush, K., and Hooper, D. C. 2006. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat.Med. 12.83-8.

Rodriguez, M. M., Power, P., Radice, M., Vay, C., Famiglietti, A., Galleni, M., Ayala, J. A., and Gutkind, G. 2004. Chromosome-encoded CTX-M-3 from *Kluyvera ascorbata*: a possible origin of plasmid-borne CTX-M-1-derived cefotaximases. Antimicrobial Agents and Chemotherapy 48.4895-7.

Rodriguez-Bano, J., Navarro, M. D., Romero, L., Muniain, M. A., de Cueto, M., Rios, M. J., Hernandez, J. R., and Pascual, A. 2006. Bacteremia due to extended-spectrum beta - lactamase-producing *Escherichia coli* in the CTX-M era: a new clinical challenge. Clin.Infect.Dis. 43.1407-14.

Rodriguez-Bano, J. and Paterson, D. L. 2006. A change in the epidemiology of infections due to extended-spectrum beta-lactamase-producing organisms. Clin.Infect.Dis. 42.935-7.

Rodriguez-Martinez, J. M., Poirel, L., Canton, R., and Nordmann, P. 2006. Common region *CR1* for expression of antibiotic resistance genes. Antimicrobial Agents and Chemotherapy 50.2544-6.

Rodriguez-Villalobos, H., Malaviolle, V., Frankard, J., de Mendonca, R., Nonhoff, C., and Struelens, M. J. 2006. In vitro activity of temocillin against extended spectrum beta-lactamase-producing Escherichia coli. Journal of Antimicrobial Chemotherapy 57.771-4.

Rolinson, G. N. 1998. Forty years of beta-lactam research. Journal of Antimicrobial Chemotherapy 41.589-603.

Ronald, A. 2003. The etiology of urinary tract infection: traditional and emerging pathogens. Dis.Mon. 49.71-82.

Sabate, M., Navarro, F., Miro, E., Campoy, S., Mirelis, B., Barbe, J., and Prats, G. 2002. Novel complex sul1-type integron in *Escherichia coli* carrying $bla_{CTX-M-9}$. Antimicrobial Agents and Chemotherapy 46.2656-61.

Saladin, M., Cao, V. T., Lambert, T., Donay, J. L., Herrmann, J. L., Ould-Hocine, Z., Verdet, C., Delisle, F., Philippon, A., and Arlet, G. 2002. Diversity of CTX-M beta-lactamases and their promoter regions from Enterobacteriaceae isolated in three Parisian hospitals. FEMS Microbiol.Lett. 209.161-8.

Sandvang, D., Aarestrup, F. M., and Jensen, L. B. 1997. Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica Typhimurium* DT104. FEMS Microbiol.Lett. 157.177-81.

Sansonetti, P. J., Kopecko, D. J., and Formal, S. B. 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect.Immun. 35.852-60.

Shanson, D. C. 1999a. Hospital infection, ed. by Shanson, D. C., 430-55. London: Butterworth Heinemann.

Shanson, D. C. 1999b. Hospital-acquired urinary tract infections, ed. by Shanson, D. C., 437. London: Butterworth Heinemann.

Shlaes, D. M. 2006. An update on tetracyclines. Curr.Opin.Investig.Drugs 7.167-71.

Siitonen, A. 1994. What makes *Escherichia coli* pathogenic? Ann.Med. 26.229-31.

Sik Kim, K. 2002. Meningitis-associated *Escherichia coli*, ed. by Donnenberg, M. S., 269-86. Baltimore, Maryland: Elsevier Science (USA).

Smith, J. T. and Lewin, C. S. 1993. Mechanisms of antimicrobial resistance and implications for epidemiology. Vet.Microbiol. 35.233-42.

Soto, S. M., Jimenez de Anta, M. T., and Vila, J. 2006. Quinolones induce partial or total loss of pathogenicity islands in uropathogenic *Escherichia coli* by SOS-dependent or -independent pathways, respectively. Antimicrobial Agents and Chemotherapy 50.649-53.

Stamm, W. E. and Hooton, T. M. 1993. Management of urinary tract infections in adults. N.Engl.J.Med. 329.1328-34.

Stamm, W. E. and Norrby, S. R. 2001. Urinary tract infections: disease panorama and challenges. J.Infect.Dis. 183 Suppl 1.S1-S4.

Stepanova, M., Shevchenko, O., and Edelstein, M. 2005. *In vivo* evolution and emergence of a new CTX-M beta-lactamase with "ceftazidimase" activity in a hypermutable clinical strain. 45th ICAAC, Washington DC, USA.

Sturenburg, E., Kuhn, A., Mack, D., and Laufs, R. 2004. A novel extended-spectrum beta-lactamase CTX-M-23 with a P167T substitution in the active-site omega loop associated with ceftazidime resistance. Journal of Antimicrobial Chemotherapy 54.406-9.

Sutcliffe, J. G. 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc.Natl.Acad.Sci.U.S.A 75.3737-41.

Szczepanowski, R., Braun, S., Riedel, V., Schneiker, S., Krahn, I., Puhler, A., and Schluter, A. 2005. The 120 592 bp IncF plasmid pRSB107 isolated from a sewage-treatment plant encodes nine different antibiotic-resistance determinants, two iron-acquisition systems and other putative virulence-associated functions. Microbiology 151.1095-111.

Teale, C. J., Barker, L., Foster, A. P., Liebana, E., Batchelor, M., Livermore, D. M., and Threlfall, E. J. 2005. Extended-spectrum beta-lactamase detected in *E. coli* recovered from calves in Wales. Vet.Rec. 156.186-7.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H., and Swaminathan, B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J.Clin.Microbiol. 33.2233-9.

Threlfall, E. J., Rowe, B., Ferguson, J. L., and Ward, L. R. 1986. Characterization of plasmids conferring resistance to gentamicin and apramycin in strains of *Salmonella typhimurium* phage type 204c isolated in Britain. J.Hyg.(Lond) 97.419-26.

Tran, J. H., Jacoby, G. A., and Hooper, D. C. 2005. Interaction of the plasmid-encoded quinolone resistance protein Qnr with *Escherichia coli* DNA gyrase. Antimicrobial Agents and Chemotherapy 49.118-25.

Turner, P. J. 2005. Extended-spectrum beta-lactamases. Clin.Infect.Dis. 41 Suppl 4.S273-S275.

Uhlen, P., Laestadius, A., Jahnukainen, T., Soderblom, T., Backhed, F., Celsi, G., Brismar, H., Normark, S., Aperia, A., and Richter-Dahlfors, A. 2000. Alpha-haemolysin of uropathogenic *E. coli* induces Ca2+ oscillations in renal epithelial cells. Nature 405.694-7.

Valverde, A., Canton, R., Galan, J. C., Nordmann, P., Baquero, F., and Coque, T. M. 2006. In*117*, an unusual In0-like class 1 integron containing *CR1* and *bla*(CTX-M-2) and associated with a Tn*21*-like element. Antimicrobial Agents and Chemotherapy 50.799-802.

Valverde, A., Coque, T. M., Sanchez-Moreno, M. P., Rollan, A., Baquero, F., and Canton, R. 2004. Dramatic increase in prevalence of fecal carriage of extended-spectrum beta-lactamase-producing Enterobacteriaceae during nonoutbreak situations in Spain. J.Clin.Microbiol. 42.4769-75.

Velasco, C., Romero, Luisa, Martinez, Jose Manuel Rodriguez, Rodriguez-Bano, Jesus, and Pascual, Alvaro. 2007. Analysis of plasmids encoding extended-spectrum [beta]-lactamases (ESBLs) from *Escherichia coli* isolated from non-hospitalised patients in Seville. International Journal of Antimicrobial Agents 29.89-92.

Velasco, M., Horcajada, J. P., Mensa, J., Moreno-Martinez, A., Vila, J., Martinez, J. A., Ruiz, J., Barranco, M., Roig, G., and Soriano, E. 2001. Decreased invasive capacity of quinolone-resistant *Escherichia coli* in patients with urinary tract infections. Clin.Infect.Dis. 33.1682-6.

Vila, J. 2006. Quinolone resistance and virulence in uropathogenic *Escherichia coli*. 16th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID).

Vila, J., Simon, K., Ruiz, J., Horcajada, J. P., Velasco, M., Barranco, M., Moreno, A., and Mensa, J. 2002. Are quinolone-resistant uropathogenic *Escherichia coli* less virulent? J.Infect.Dis. 186.1039-42.

Viveiros, M., Dupont, M., Rodrigues, L., Couto, I., Davin-Regli, A., Martins, M., Pages, J. M., and Amaral, L. 2007. Antibiotic Stress, Genetic Response and Altered Permeability of E. coli. PLoS.ONE. 2.e365.

Walsh, T. R., Bolmstrom, A., Qwarnstrom, A., and Gales, A. 2002. Evaluation of a new Etest for detecting metallo-beta-lactamases in routine clinical testing. Journal of Clinical Microbiology 40.2755-9.

Welsh, K. J., Barlow, M., Tenover, F. C., Biddle, J. W., Rasheed, J. K., Clark, L. A., and McGowan, J. E., Jr. 2005. Experimental prediction of the evolution of ceftazidime resistance in the CTX-M-2 extended-spectrum beta-lactamase. Antimicrobial Agents and Chemotherapy 49.1242-4.

Whitfield, C. and Roberts, I. S. 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol.Microbiol. 31.1307-19.

Woodford, N., Dallow, J. W., Hill, R. L., Palepou, M. F., Pike, R., Ward, M. E., Warner, M., and Livermore, D. M. 2007a. Ertapenem resistance among *Klebsiella* and *Enterobacter* submitted in the UK to a reference laboratory. Int.J.Antimicrob.Agents 29.456-9.

Woodford, N., Fagan, E. J., and Ellington, M. J. 2006. Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum (beta)-lactamases. Journal of Antimicrobial Chemotherapy 57.154-5.

Woodford, N., Reddy, S., Fagan, E. J., Hill, R. L., Hopkins, K. L., Kaufmann, M. E., Kistler, J., Palepou, M. F., Pike, R., Ward, M. E., Cheesbrough, J., and Livermore, D. M. 2007b. Wide geographic spread of diverse acquired AmpC beta-lactamases among *Escherichia coli* and *Klebsiella* spp. in the UK and Ireland. Journal of Antimicrobial Chemotherapy 59.102-5.

Woodford, N., Ward, M. E., Kaufmann, M. E., Turton, J., Fagan, E. J., James, D., Johnson, A. P., Pike, R., Warner, M., Cheasty, T., Pearson, A., Harry, S., Leach, J. B., Loughrey, A., Lowes, J. A., Warren, R. E., and Livermore, D. M. 2004. Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum beta-lactamases in the UK. Journal of Antimicrobial Chemotherapy 54.735-43.

Yagi, T., Kurokawa, H., Senda, K., Ichiyama, S., Ito, H., Ohsuka, S., Shibayama, K., Shimokata, K., Kato, N., Ohta, M., and Arakawa, Y. 1997. Nosocomial spread of cephem-resistant *Escherichia coli* strains carrying multiple Toho-1-like beta-lactamase genes. Antimicrobial Agents and Chemotherapy 41.2606-11.

Zhanel, G. G., Wiebe, R., Dilay, L., Thomson, K., Rubinstein, E., Hoban, D. J., Noreddin, A. M., and Karlowsky, J. A. 2007. Comparative review of the carbapenems. Drugs 67.1027-52.