Target Protein Mutation in the Analysis of Bactericidal Antibiotic Action and Resistance

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Abstract

Future antimicrobial drug design and resistance minimisation, requires an understanding of current antimicrobial action and resistance. This study investigated bactericidal action and resistance within two antimicrobial groups; the fluoroquinolones and the aminoglycosides.

The importance of target protein mutation to fluoroquinolome resistance was characterised in an outbreak of fluoroquinolone resistant *Escherichia coli* (n=17), and allowed a direct comparison with previous data deduced solely from *in vitro* laboratory selected mutants. Resistance to ciprofloxacin ranged from (MICs) 0.25→256mg/L, with 7/17 isolates highly resistant (MICs=≥32mg/L). A genotypic analysis by Random Amplified Polymorphic DNA (RAPD) typing, demonstrated that clonal spread of two strains was responsible for the highly resistant isolates. Enterobacterial intergenic concensus (ERIC) PCR typing supported RAPD results whereas PFGE and AFLP analysis were unsuccessful in typing isolates. A sequential increase in resistance was observed in both strains providing a unique opportunity to study resistance acquisition in the clinic. Five isolates (TYPE-I) showed the following increase in resistance: - MICs of ciprofloxacin 8→ 32→ 128→256 mg/L. Gene amplification and sequencing showed that all isolates contained mutations Ser83→Leu and Asp87→Asn/Tyr in DNA Gyrase GyrA, and mutation Ser80→Ile in Topoisomerase IV ParC. Isolates with ciprofloxacin MICs of 128 and 256 mg/L, had the additional mutation Glu84→Lys in ParC. Four isolates (RAPD type II) showed an increase in resistance from a clinically sensitive (MIC=1mg/L) to a highly resistant (MIC=32mg/L) phenotype. The sensitive isolates (n=3) contained mutations Ser83→Leu in GyrA and Ser80→Ile in ParC. With progression to clinical resistance this strain additionally acquired mutations Asp87→Asn in GyrA, and Glu84→Val in ParC. This indicates that selection of high-level resistance is dependent upon second step mutations in both DNA gyrase and topoisomerase IV enzymes, as loss of Ser83 in GyrA and Ser80 in ParC do not, in themselves, confer the highly resistant

phenotype. These results also confirm that DNA Gyrase is the primary quinolone target in *E. coli.*

DNA gyrase mutations Ser83→Leu and Asp87→Asn were created by site-directed mutagenesis to allow the contribution of single amino-acid changes to resistance to be assessed. Expression of the Ser83→Leu mutant was unsuccessful due to multiple primer insertions during PCR stage. The contribution of Asp87→Asn to fluoroquinolone resistance was assessed by expression in a temperature sensitive (Ts) *gyrA* mutant. Resistance to quinolones nalidixic acid, ciprofloxacin, gemifloxacin, moxifloxacin and sparfloxacin increased 3 to 8 fold when compared to the wild-type. Resistance levels were however not clinically significant, confirming that secondary mutations are required to confer the clinically resistant phenotype. Resistance conferred by this mutation demonstrates the importance of this amino acid in drug-target binding.

The role of Ribosomal S12 mutations in aminoglycoside action and resistance was characterised in a step-wise mutation study. The S12 mutation Lys87→Arg confers streptomycin resistance of >1500mg/L in a single step selection; however the role of this S12 mutation in resistance to other aminoglycosides has not been previously characterised. Mutants of *E. coli* NCTC10418 were selected in four steps (I-IV) on aminoglycoside containing plates. Lack of cross-resistance between streptomycin mutants and other aminoglycosides (gentamicin, neomycin, kanamycin, tobramycin), suggested that the streptomycin target may not be shared by other aminoglycosides. Cross-resistance between neomycin, tobramycin and kanamycin mutants, implied that these drugs may share similar targets and binding pockets. PCR amplification and sequencing of the *rpsL* gene encoding the S12 protein demonstrated a Lys87→Glu mutation in streptomycin resistant mutants. Ribosomal S12 mutations were absent in gentamicin, neomycin, kanamycin and tobramycin resistant mutants. Replacement of Lys87 with a glutamic acid residue resulted in a streptomycin MIC of 25mg/L. Mutation in S12 Lys87 to a basic arginine (MIC=1500mg/L) residue rather than an

acidic glutamic acid (MIC=25mg/L) residue results in elevated resistance and hence probably reduced binding. Lack of additional mutations in 2nd, 3rd and 4th step mutants implicated the role of alternative ribosomal proteins/targets in streptomycin action.

The requirement of protein synthesis in bactericidal aminoglycoside action was analysed. Pre-incubation with chloramphenicol significantly reduced streptomycin bactericidal activity, indicating a role of protein synthesis in cell death. Substituting streptomycin (1mg/L) for chloramphenicol gave a similar significant reduction in bactericidal activity, indicating that at sub-lethal concentrations streptomycin acts as a protein synthesis inhibitor, which blocks its own cell death when challenged with a higher concentration. Streptomycin induced cell death thereby requires additional protein synthesis dependent death mechanisms. Stationary phase cells were resistant to drug challenge, supporting this result.

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Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

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Presentations and Publications

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Abbreviations

AFLP	-	amplified fragment length polymorphism
Am/Cl	-	co-amoxiclav
Amox	-	amoxicillin
amp	-	ampicillin
bp	-	base pairs
BSAC	-	British society for antimicrobial chemotherapy
C/Chlor	-	chloramphenicol
cft	-	ceftazidime
cfu	-	colony-forming unit
Cip	-	ciprofloxacin
DM	-	Davis-Mingioli
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleotide tri-phosphate
dsDNA	-	double stranded DNA
E	-	ERIC-type
EDTA	-	ethylenediaminetetraacetic acid
ERIC-PCR	-	enterobacterial intergenic concensus - polymerase chain reaction
Gem	-	gemifloxacin
G/Gent	-	gentamicin
IPTG	-	isopropyl-β-D-thiogalactopyranoside
IST	-	Iso-sensitest
K	-	kanamycin
kb	-	kilobases
LB	-	Lennox L both
LBA	-	Lennox L agar
log	-	logarithm
mar	-	multiple antibiotic resistant
Me	-	methoxy

mer	-	meropenem
MDR	-	multi-drug resistant
MIC	-	minimum inhibitory concentration
min	-	minutes
mox	-	moxifloxacin
mRNA	-	messenger ribonucleic acid
MPC	-	mutant prevention concentration
N	-	neomycin
NB	-	nutrient broth
NC	-	Newcastle
NMR	-	nuclear magnetic resonance
OBC	-	optimum bactericidal concentration
O.D.	-	optical density
OMP	-	outer membrane protein
PAE	-	post-antibiotic effect
PBP	-	penicillin binding protein
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
PFGE	-	pulsed-field gel electrophoresis
QRDR	-	quinolone resistance determining region
R	-	RAPD-type
RAPD	-	random amplified polymorphic DNA
Rep-PCR	-	repetitive extragenic palindromic - polymerase chain reaction
RFLP	-	restriction fragment length polymorphism
RNA	-	ribonucleic acid
rRNA	-	ribosomal ribonucleic acid
s	-	seconds
SOS	-	bacterial stress response
Spar	-	sparfloxacin
ssDNA	-	single stranded deoxyribonucleic acid

S/strep	-	streptomycin
T	-	tobramycin
TBE	-	tris borate EDTA
TE	-	tris EDTA
Tm	-	melting temperature
TopoIV	-	DNA topoisomerase IV
Trim	-	trimethoprim
tRNA	-	transfer RNA
Ts	-	temperature sensitive
U	-	units of enzyme
UD	-	undetermined
UTI	-	urinary tract infection
WT	-	wild type

The standard single and three letter abbreviations are used for amino-acids.

1. Introduction

1.1 An Overview

The development of antibiotics in the 1960s revolutionised medicine to such an extent that the threat posed to man by bacterial infection was believed to be over. This however was a premature prediction, with antibiotic resistance now disabling even the most potent drugs in our antibiotic armoury. The dissemination of resistance pathogens is a major threat to public heath, and now over 40 years since their first development, antibiotic discovery is back on the agenda. The design of novel and more effective antimicrobial agents to combat the multi-resistant pathogens we face, demands a detailed understanding of interactions between the drug and its target site.

As our knowledge grows on how resistance mutations affect these interactions, it will be possible to design drugs which have greater potency and less potential to select resistance. Advances in understanding bacterial physiology and population dynamics shed new light on the way in which antibiotics elicit cell death. The involvement of secondary target pathways, stress response, protein synthesis subsequent to initial target binding may be crucial to cell death. Deciphering which proteins are important in cell death and bacterial resistance, will provide the much needed impetus for future drug discovery.

1.2 Bactericidal Antibiotic Action

After several decades of research into the bactericidal action of existing antibiotics, the mechanisms by which they kill bacteria still remain a mystery. Most researchers focus their attention on understanding the bactericidal mechanism of a single agent or group of agents, due to the fact that mechanism of action studies are complicated and groups are diverse. They may, however, lose insight into mechanisms of cell death which are conserved among diverse groups of drugs such as a requirement for protein synthesis, induction of stress response, and a role of signal transduction.

1.2.1 β-lactams

Penicillins and other β-lactam derivatives bind to penicillin binding proteins (PBPs), which are involved in synthesising and shaping the bacterial cell wall (Tipper & Strominger, 1965). Tipper and Strominger (1965) originally proposed that binding to PBPs, and consequent inhibition of peptidoglycan assembly resulted in a mechanically weakened cell wall, where the internal osmotic pressure and mechanical forces were believed to result in lysis. Subsequent work however has shown that penicillin induced lysis is enzymatically mediated, and involves a set of cellular enzymes the murein hydrolases (autolysins) which hydrolyse peptidoglycan (Rogers & Forsberg,1971).

The discovery that lysis is murein hydrolase dependent does not however explain lethality. Penicillin exerts a potent bactericidal response in the absence of murein hyrolase activity, suggesting that lysis and lethality may be two separate events (Moreillon, 1990). Loss of culture viability occurs shortly after penicillin exposure,

whereas lysis occurs after a lag phase, indicating that lysis is a secondary event. This begs the question as to what is responsible for lethality.

The key to understanding β-lactam cell death could come from looking at the way bacteria respond to antimicrobials at different growth phases. When non-growing or slowly growing bacteria are challenged with β-lactams, they survive (Tuomanen, 1986). The stringent (stress) response encoded by the *relA* gene is also known to have a protective effect from antibiotic action (Cashel *et al.*, 1996). For example, starved bacteria will bind antibiotic but they do not die (Cashel *et al.*, 1996). The accumulation of 3`,5`bispyrophosphate (ppGpp) which is synthesised by ppGpp sythetase I accumulates and shuts down the synthesis of DNA, phospholipids and cell wall peptidoglycan (Metzger *et al.*,1988, Sokawa *et al.*,1968). Antibiotic-induced autolysis is blocked by an uncharacterised defect in autolysin activation. This protection from death or phenotypic tolerance is a source of residual bacteria surviving antimicrobial therapy, and can thereby lead to treatment failure, and promotion of resistance acquisition. It is known that *Escherichia coli* can grow up to two hours in the presence of penicillin, with cells taking the form of filaments increasing in length at an exponential rate (Rolinson, 1980; Gardner, 1940). Removal of antibiotic allows the filaments to divide again, suggesting that the lethality does not occur until lysis (Starka & Moravora, 1967).

It is clear that the binding of penicillins to their target does not directly cause cell death. A second bacterial process to trigger endogenous suicidal enzymes that

dissolve the cell during autolysis is the most likely cause, although the signal and trigger pathway remain unknown (Novak et al., 2000). The identification of a two component regulatory system (VNcRS) which affects bacterial susceptibility to a wide range of antibiotics may shed light on the way bacteria regulate their suicidal autolytic pathways. Novak *et al.* (1999) have demonstrated that a secreted peptide Pep^{27} can be sensed by the VNcRS system which initiates a set of uncharacterised events leading to cell death. After secretion and accumulation of the peptide during late log and stationary phase, it is likely to be sensed by histadine/phosphatase VncS receptors triggering pathways leading to cell death. The stringent response is known to downregulate autolysis in bacteria, and it has been shown that this peptide along with β-lactam agents can relax this response, and thereby may regulate this pathway. The way in which β-lactams feed into this pathway remains to be established. The finding that the heat shock response can inhibit cell lysis but not cell death adds further evidence to the role of the stringent response pathway (Powell & Young,1991).

Peptidoglycan biosyntheis involves a complex system of proteins with specialised functions. It appears that cell lethality is due to regulation by a signal transduction mechanism, the stimuli for which remain to be identified. The whole picture of the bactericidal mechanism of β-lactams is only just being comprehended, and may involve a complex signal transduction pathway leading to eventual cell death.

1.2.2 Trimethoprim

Trimethoprim is a selective inhibitor of the enzyme dihyrofolate reductase which reduces dihyrdofolic acid (DHF) to tetrahydrofolic acid (THF) (Amyes & Smith, 1974). The consequent depletion of tetrahyrofolates which are required for amino-acid (methionine and glycine) and purine synthesis leads to inhibition of DNA and protein synthesis. Like other bactericidal drugs trimethoprim does not elicit a bactericidal response in the absence of protein synthesis, as shown when bacteria are challenged in minimal media or in the presence of chloramphenicol, a protein synthesis inhibitor (Amyes & Smith, 1974).

Bacteria exposed to trimethoprim undergo filamentation suggesting that the SOS response is induced, as the SOS gene *sfiA* codes for a protein that binds to *sfiB* gene product, which is essential for septation. The SOS induction has been shown to contribute to lethality, as SOS deficient strains have increased resistance to trimethoprim (Lewin & Amyes, 1991). The involvement of the SOS response and the requirement for protein synthesis suggest that a higher regulatory signal transduction pathway may be operating in trimethoprim induced cell death.

1.2.3 Aminoglycosides

Aminoglycosides primarily affect the process of translation in prokaryotes, via binding to the 30S ribosomal subunit and consequently inhibiting protein synthesis. The molecular basis for protein synthesis inhibition is due to a number of effects, such as binding to 16S RNA (Fourmy *et al.*,1996), unstable initiation complex formation and

elongation inhibition (Hausner *et al.*, 1988), along with misreading during translation (Gorini,1974). These effects do not however explain why aminoglycosides are lethal to bacterial cells.

Chloramphenicol and other protein synthesis inhibitors which interact with the ribosome exert bacteriostatic action, and ethionine a strong inducer of misreading does not produce a rapid killing effect (Pine, 1978). Binding of streptomycin to ribosomes is reversible and ribosomes are not irreversibly inactivated (Chang & Flaks, 1972). Efflux of antibiotic from cells would therefore be expected after a suspension has been diluted for plating, which would lead to a bacteriostatic rather than a bactericidal effect. Protein synthesis may not therefore be the sole target of aminoglycoside action. Paradoxically it is also suggested that bactericidal activity cannot proceed without protein synthesis (Jawetz *et al.*, 1952).

The finding that streptomycin uptake is irreversible (Muir *et al.*, 1984) led Davis (1987) to propose the most recent and widely accepted hypothesis for aminoglycoside action. The following sequence of events was proposed to account for cell death : 1) antibiotic penetrates through imperfections in the growing membrane, 2) antibiotic binds to ribosomes inducing misreading, 3) misread protein is incorporated into the membrane creating channels, thus permitting influx of antibiotic, initiating the autocatalytic process of increasing influx, misreading and channel formation, 4) antibiotic concentration reaches a level that blocks protein synthesis due to binding to

initiating ribosomes, 5) lethality is due to irreversible uptake and persistent protein synthesis inhibition.

The Davis hypothesis may be widely accepted, however, there are a number of reasons why it is unconvincing. It is firstly unclear why streptomycin uptake is irreversible (Nichols, 1989), and Davis does not propose an explanation. The reason why bactericidal aminoglycosides like streptomycin and gentamicin are bactericidal, whereas compounds which have closely related structures like the aminocyclitols are only bacteriostatic can also not be explained. It also remains controversial whether misreading is required for bactericidal action (Hausner *et al.*, 1988). Some aminoglycoside *rpsL* revertants that have increased sensitivity to streptomycin show no effect on misreading (Dabbs, 1980), and error frequency can also be reduced to normal when streptomycin is removed suggesting that misreading is reversible (Edelmann & Gallant, 1977). A further argument against the Davis hypothesis is that treatment with streptomycin increases the passive permeability of the *E. coli* membrane to several small ions (Busse *et al*, 1992), however the changes are small and the membrane potential remains high meaning that transmembrane channels are highly unlikely.

The fact that aminoglycoside treated cells have enhanced protease activity (Hipkiss & Kogut, 1973) led Busse *et al.*(1992) to propose an explanation as to why aminoglycosides remain in cells. They proposed that rapid proteolysis of mistranslated proteins in the cell membrane cages the antibiotics within cells, leading to prolonged

protein synthesis inhibition. However, sensitive cells treated with streptomycin lose colony forming ability before many of the changes in metabolism implying that binding to a critical target and consequent protein synthesis inhibition do not necessarily constitute the lethal event (Hancock 1981).

Aminoglycosides may have a second target site responsible for the lethal event. Hancock (1981) originally proposed that the *oriC* DNA membrane attachment site was affected in aminoglycoside treated cells. Subsequently Matsunaga *et al.* (1986) demonstrated the ability of hebakacin and related aminoglycosides to inhibit re-initiation of DNA replication and *oriC* membrane attachment. Attachment of *oriC* DNA to the membrane is required for initiation of chromosome replication. The sequential events of membrane damage and inhibition of DNA initiation and protein synthesis may progress synergistically resulting in cell death.

The involvement of stress proteins has also been implicated in aminoglycoside action. Bakker (1992) demonstrated that hygromycin B, a bactericidal aminocyclitol, which does not induce misreading may induce lethality as a side effect. After hygromycin B challenge, *E. coli* produced certain proteins such as EF_{TU} and OmpA with high speed and fidelity. They also showed that streptomycin, gentamicin and neomycin treated cells produced stress proteins postulated to be DnaK and GroEL as well as Lon and other proteases. The role of these proteins in cell death has not been assessed. It is clear that despite a large body of research on aminoglycoside induced cell death, the precise molecular mechanisms of action still remains elusive.

1.2.4 Fluoroquinolones

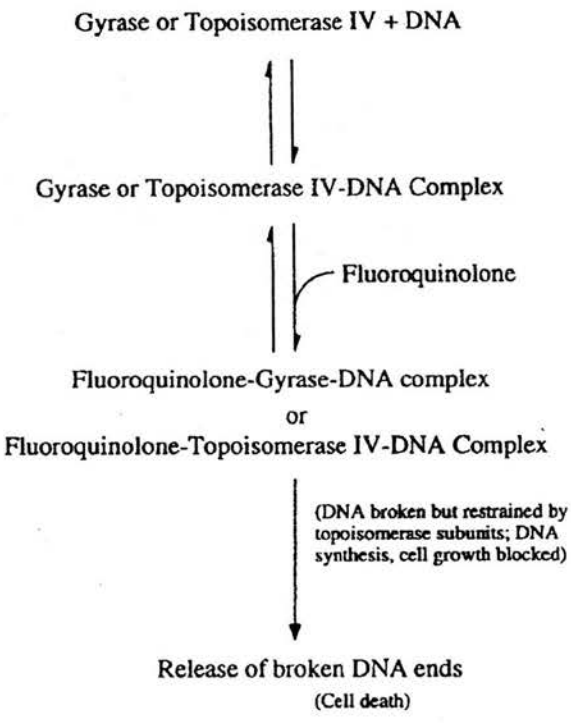
Fluoroquinolones are broad-spectrum potent antimicrobial compounds that target two essential enzymes in the bacterial cell, DNA gyrase and topoisomerase IV (Levine *et al.* 1998). DNA gyrase is constituted of two sub-units encoded by the genes *gyrA* and *gyrB* (Swanberg & Wang, 1987), with topoisomerase IV constituting of *parC* and *parE* (*grlA* and *grlB* in *Staphylococcus aureus*) gene products (Kato *et al.*, 1990). DNA Gyrase introduces negative supercoils into DNA, and thereby relieves the torsional stress which would be expected ahead of transcription and replication complexes (Reece & Maxwell, 1991). Topoisomerase IV (TopoIV) is a decatenating enzyme, unlinking newly replicated DNA, thereby allowing proper chromosome and plasmid segregation. As quinolones inhibit these essential enzymes bacterial growth inhibition is expected, however these drugs are bactericidal, and their death mechanism appears to be much more complex than simple enzyme inhibition.

Before an understanding of the basis of quinolone action can be deduced, it is important to analyse the morphological and physiological effects on bacteria. These include an inhibition of DNA and protein synthesis, (Piddock *et al.*,1990), induction of the SOS response (Piddock & Wise,1987) and cell filamentation (Diver & Wise, 1986). It is likely that DNA damage caused by quinolones activates RecA which turns on the SOS response due to interaction with LexA, consequently resulting in cell filamentation. Piddock and Walters (1992) used mutants of *E. coli* deficient in SOS response to demonstrate that SOS had a role in repair of DNA damage induced by the quinolones. The heat shock response is also induced (Krueger and Walker, 1984),

however its precise significance is unknown. It may have a role in degrading SOS proteins and is likely to be a secondary effect in quinolone action (Walker, 1984).

The physiological effects of quinolones seem diverse, however they can be partly explained by the molecular effects of the drugs on their targets proteins. Snyder and Drlica (1979) demonstrated that inhibition of DNA synthesis correlated with the formation of oxolinic acid/gyrase complexes, leading them to hypothesise that such complexes may block replication folk movement. Complexes are also formed by drug interaction with topoisomerase IV in a similar manner, with analysis of quinolone/ topoisomerase IV complexes indicating that quinolones stimulate DNA cleavage/ religation reactions (Anderson *et al.*, 1998). It is worth noting that due to their different roles in DNA replication, the interaction with gyrase causes a rapid inhibition of DNA synthesis, whereas TopoIV targeting does not lead to a rapid block in DNA replication (Khodursky *et al.*, 1995). These lesions on the DNA consequently block the movement of polymerases. These complexes can be converted to lesions which result in irreversible double stranded breaks in DNA (Figure 1.1) (Marians & Hiasa, 1997).

Figure 1.1: Intracellular action of fluoroquinolones

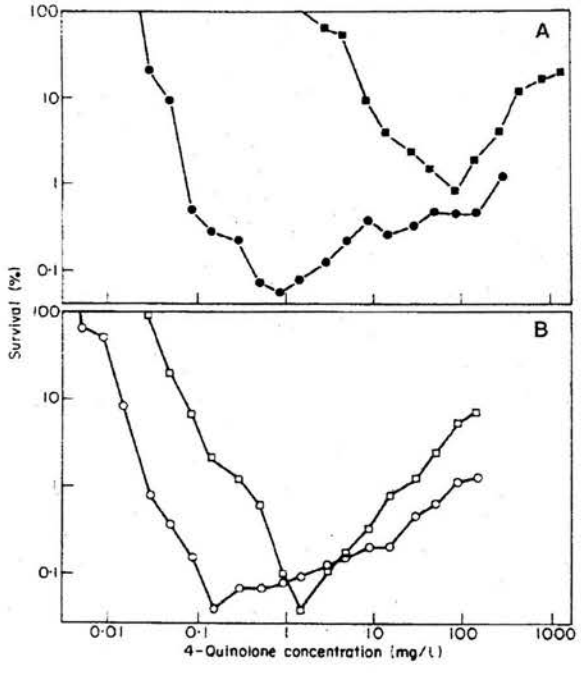


(From Drlica & Zhao, 1997)

It is important not to oversimplify the mechanism of quinolone action. Although we have an understanding of quinolone target interactions, we do not know the precise mechanism of cell death after complex formation. Trapping of the cleaved DNA/topoisomerase complex is only the first in an intricate series of events (notably SOS induction, and illegitimate recombination) leading to cell death. Smith (1986) showed the response of an *E. coli* population when challenged with the quinolone antibiotics (Figure 1.2). This survival curve demonstrates that fluoroquinolones have an optimal bactericidal concentration, above which killing activity decreases. It was hypothesised that RNA and protein synthesis inhibition may account for the second

phase of quinolone activity in which increasing drug concentrations lead to less killing, thereby demonstrating a requirement of protein synthesis in cell death.

Figure 1.2: Optimum bactericidal concentration (Smith, 1986)



Survival of *E. coli* KL16 cells challenged with: — nalidixic acid ■; norfloxacin □ ofloxacin ●; ciprofloxacin O; after 3 h growth.

Smith (1986) proposed three mechanisms of quinolone killing:- Mechanism A (common to all quinolones) requires RNA and protein synthesis and is only effective against dividing bacteria: mechanism B which does not require RNA and protein synthesis and can act on bacteria unable to multiply; mechanism C requires RNA and protein synthesis, however does not require cell division. More recent work by Chen *et al.* (1996) also supports the idea of three mechanisms of cell killing. Mechanism A

would be blocking of replication by the gyrase/quinolone complex, mechanism B may be dissociation of gyrase subunits which constrain the ternary complex, and mechanism C may correlate with trapping of topoisomerase IV complexes on DNA. The events between DNA breakage and cell death remain unresolved.

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1.2.5 The post-antibiotic effect

The method classically used to measure the bactericidal activity of antibiotics on a given cell population is the viable count method (Hanberger *et al.*,1990). This involves removing the antibiotic from cells at time intervals after an antibiotic challenge, plating onto suitable culture medium, and analysis of colony formation. In fact, after antibiotic is removed from a culture, cells can maintain metabolic activity for long periods of time. Bacterial populations begin to recover with time and exponential growth is re-established, this time period is known as the post-antibiotic effect (PAE).

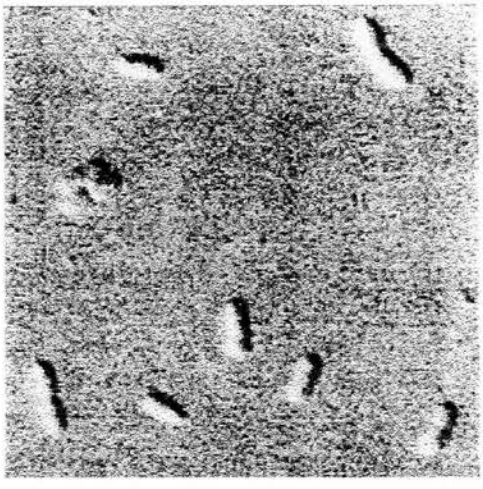
The viable count method does not therefore give a measure of whether a cell is dead, only whether it has lost colony forming ability, and these two are not necessarily related. Post-antibiotic effect has been measured using viable counts (Hanberger *et al.*, 1991), microphysiology (Libby, 1998), bio-illuminescence (Hanberger *et al.*, 1990), however only recent studies utilising flow cytometry have given good morphological data concerning the fate of these bacterial populations (Gottfredsson *et al.*,1998; Wickens *et al.*, 2000). Deductions concerning antibiotic action can be gleaned from an understanding of this effect. If once challenged with an antibiotic,

cells are condemned to death, they cannot divide again. However the fact that post-antibiotic effect exists show that some cells do survive, the questions of how and why are yet to be answered. It may be that cells that survive have not undergone the death pathway and are recoverable once antibiotic is removed, even though they cannot be immediately cultured. Alternatively they may be present in too small numbers for culture.

After antimicrobial treatment (β-lactams, fluoroquinolones, trimethoprim) cells undergo filamentation due possibly to SOS response induction (Diver & Wise, 1986; Rolinson, 1980; Amyes & Smith, 1974). Guan and Burnham (1992) suggested that these filamentous cells divide again after ciprofloxacin removal and proceed to normal growth. Wickens *et al.* (2000) however do not support this early hypothesis and propose that filamentous cells do not divide again. They suggest that growth after antibiotic removal is due to a small proportion of normal sized cells present. As these cells have not filamented it may suggest that SOS response is not yet induced, and therefore, may be a prerequisite for cell death.

Figure 1.3: Filamentous cells post antibiotic treatment

(a)



(b)

Morphological response of *E. coli* KL16 cells to ciprofloxacin. (a) drug free control and (b) 2h incubation with ciprofloxacin at 60mg/L (From Diver & Wise, 1986)

1.3 Bacterial programmed cell death

As seen in the mechanisms of cell death, there is commonality in that they induce a stress response either by activation of SOS proteins or induction of the heat shock response. Induction of these systems may lead to a mechanism of cell death involving bacterial "apoptosis" or "programmed cell death".

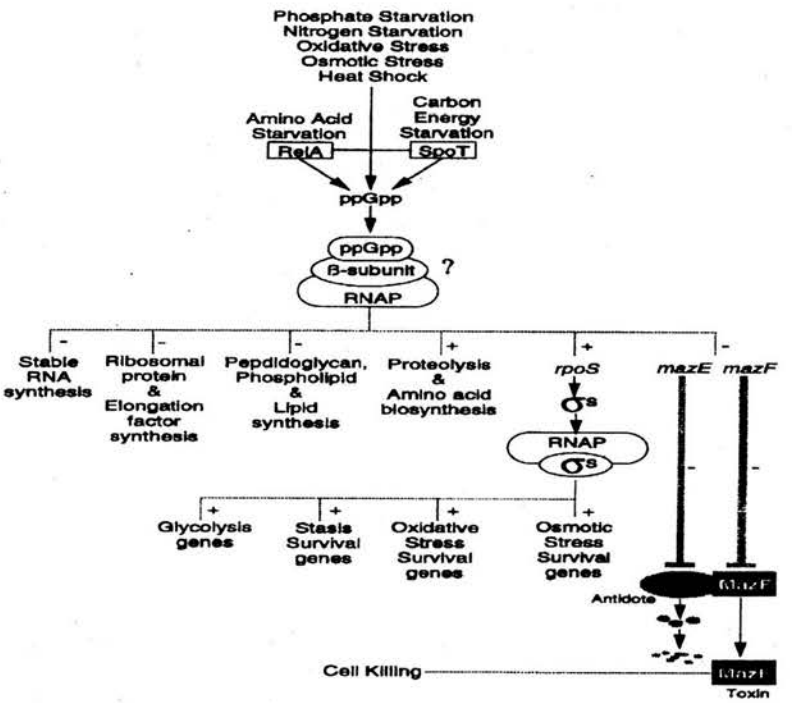
1.3.1 Addiction Modules

The bacterial toxin CcdB encoded by the F plasmid *ccd* locus belongs to a recently discovered programmed cell death system (Jensen & Gerdes, 1995). This toxin kills bacteria in an analogous manner to the quinolone antibiotics via interaction with DNA gyrase, DNA damage and consequent SOS response induction. The presence of these systems can be explained, as they promote plasmid carriage within cells. In plasmid containing cells, both antidote and toxin are present, thereby the toxin is neutralised and the cells are viable. In plasmid cured cells, however, the antidote and toxin are no longer synthesised, and the unstable antidote is degraded by a specific protease allowing the toxin to exert its affect (Gerdes *et al.*, 1997). The cell is therefore addicted to the plasmid, hence the name addiction module.

Plasmid-encoded programmed cell death systems also possess chromosomal analogues, like the MazEF system described by Aizenman *et al.* (1996), consisting of a MazE (antidote) and MazF (toxin) which form an operon with the *relA* gene of the stringent response. MazEF killing is negatively regulated by 3`,5`-bispyrophoshate ppGpp (Aizenman *et al.*, 1996), implying that cell death therefore also associates with

the stringent response. Proteins produced during the stringent response degrade the MazE antidote allowing the toxin to express its function. The negative regulatory effect of ppGpp on the programmed cell death system may be responsible for bacterial survival during post-antibiotic challenge, as seen with ppGpp effect on the stringent response after β-lactam treatment. The stasis response during the bacterial post-antibiotic effect could also therefore modulate this system meaning that bacteria are not subjected to programmed cell death mechanism during this period. It is known that heat shock, starvation and stress response all modulate this system (Figure 1.4).

Figure 1.4: Model for bacterial programmed cell death by the MaEF addiction module



The stringent response blocks further production of MazEF and allows the more stable MazF toxin to express its killing function. (From Nystrom, 1998)

As bacteria are single cells is it difficult to imagine why they should behave in an altruistic fashion, however work by Shapiro (1997) has challenged the conventional view of bacterial colonies composed of identical individuals. Shapiro (1997) demonstrated that bacterial populations constitute multi-cell structures in which individuals may undergo genetic variation. In this context it is understandable how programmed cell death mechanisms may exist. Under a stress (i.e. starvation, antibiotic challenge), death of certain sub-populations may occur which may benefit the population as a whole. The fact that bacteria also produce signals (quorum sensing) in certain environmental conditions, allowing the population to respond supports the hypothesis that bacterial populations have structure (Gray, 1997; Kleerebezem *et al.*, 1997). It may be that mutagenic systems ensure species survival by killing or promoting adaption of a fraction of the bacterial population. The question of whether this is the case after an antibiotic challenge remains an important question for future research.

1.3.2 Free radical production

It has been a long accepted fact that the growth phase of a bacterial population affects its susceptibility to certain antibiotics. Antibiotics generally exert their greatest killing activity when a bacterial population is at log/exponential phase growth, and can have significantly reduced effect when a bacteria are slow growing or in stationary phase (Eng *et al.*, 1991).These differences may be reconciled with a requirement for active protein synthesis in bactericidal drug action, and programmed cell death may play a part in this.

Dodd *et al.* (1997) suggest that the lethal effects associated with stress and chemical challenge can result in self-destruction of cells or "suicide", and in exponentially growing cells suggest that this is due to an oxidative burst occurring after growth arrest. As it is known with the post-antibiotic effect, a cell`s metabolism may be active long after growth arrest, and a build up of free radicals is thought to trigger death. Stationary phase populations may be more resistant to death because of their low metabolism. Lamlertthon *et al.* (1999) supports this hypothesis by demonstrating that free radical scavengers can increase resistance to certain biocides. Bacterial metabolic rate has a considerable effect on cell death, and these new insights shed light into general death mechanism which may be applicable to bacterial reaction to a variety of stresses including antimicrobial challenge.

1.4 Drug-Target Interactions

The interactions between drugs and their molecular targets are complex, and can be heavily dependent upon the structure of the drug. Drugs may also target different enzymes in different bacterial species. Understanding these interactions will aid better drug design, with an emphasis on resistance minimisation.

1.4.1 Quinolones: structure-activity relationships

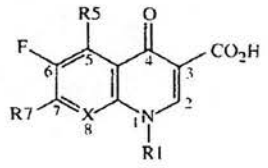
The first quinolone to be synthesised was nalidixic acid (Figure 1.5), discovered as a by-product of the chloroquinine purification process (Lesher *et al.*, 1962). This agent was used extensively to treat urinary tract and enteric infections due to its high potency for *Enterobacteriaceae* (Hooper, 1998). The basic quinolone core has subsequently undergone numerous modifications, leading to quinolones with increased potency, broader spectrum and improved pharmacokinetic profiles.

The most significant discovery in the development of quinolones came when it was discovered that insertion of a fluorine atom at C-6 of the quinolone core enhanced and broadened antimicrobial activity (Koga *et al.*, 1980). The development of newer quinolones with antibacterial activities a thousand times that of nalidixic acid followed (Smith & Lewin, 1988). Structures of quinolones (Figure 1.5) have since developed along two main pathways (Table 1.1): the napththyridones (e.g. gemifloxacin) (containing original nalidixic acid core), and the fluoroquinolones (e.g. ciprofloxacin Figure 1.5) in which a carbon atom is substituted for a nitrogen at position 8 of the nucleus (Cormican & Jones, 1997; Domagala, 1994). All have a fluorine atom at

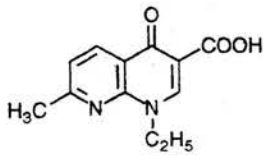
position 6. Modifications of groups N-1, C-6, C-7 and C-8 positions have also been particularly successful in enhancing antimicrobial activity (Chu & Fernandes, 1989) The precise mechanisms by which these substitutions affect potency are unknown, however it is likely that they affect the interaction between drug and target topoisomerase.

Figure 1.5: Structure of fluoroquinolone and napthyridone derivatives

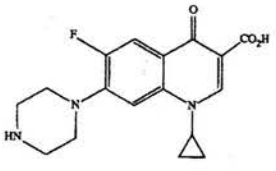
Quinolone core



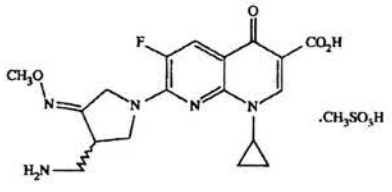
Nalidixic acid



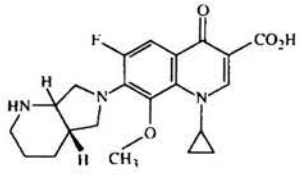
Ciprofloxacin



Gemifloxacin



Moxifloxacin



Sparfloxacin

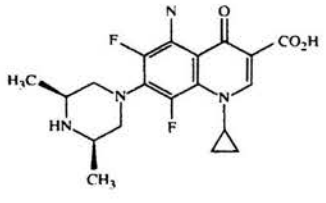


Table 1.1: Quinolone generations and characteristics

Generation	Fluorquinolone Core	Naphthyridone Core	Characteristics
I	flumequine	nalidixic acid	urinary tract infection, Gm -ve
IIa	ciprofloxacin, ofloxacin, levofloxacin	enoxacin	enhanced Gm -ve activity
IIb	grepafloxacin, sparfloxacin	tosufloxacin	more broad spectrum, enhanced activity against pneumococci, two sites of action (targets)
IIIa	moxifloxacin, gatifloxacin, clinafloxacin	trovafloxacin	enhanced Gm +ve activity, respiratory infection,
IIIb	none developed	gemifloxacin	markedly enhanced Gm+ve & Gm-ve activity

Gm +ve = Gram-positive bacteria
Gm -ve = Gram-negative bacteria

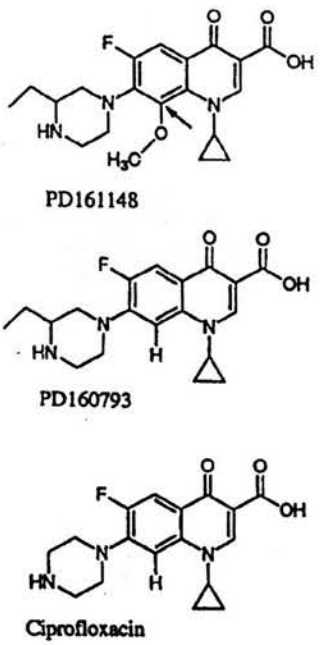
From Ball (2000)

The second generation fluoroquinolones such as ciprofloxacin have a wide spectrum of antimicrobial activity, and are particularly potent against Gram-negative infections such as *Escherichia coli* (Krumpe *et al.*, 1999). They target mainly DNA Gyrase within the bacterial cell. They however are less effective against gram-positive organisms, such as pneumococcal respiratory tract infection (Lee *et al.*, 1991). This has been addressed by the development of agents with enhanced gram-positive

activity such as moxifloxacin (Dalhoff *et al.*, 1996), gatifloxacin (Perry *et al.*, 1999), and gemifloxacin (Cormican & Jones, 1997). The second and third generation fluoroquinolones target two bacterial enzymes, both DNA gyrase and topoisomerase IV. Earlier fluoroquinolones such as ciprofloxacin with primarily gram-negative activity target primarily DNA gyrase, whereas fluorquinolone with enhance gram-positive activity target primarily topoisomerase IV in *E. coli.* DNA gyrase and topoisomerase IV are secondary targets in gram-positive and negative bacteria respectively and may also be dual and equal targets for some drugs (see section 1.5.4).

Recent studies by Dong *et al.* (1998) have found that substituting a C-8-methoxy group can change the target specificity of the quinolone, and make the drug more likely to bind to dual targets. Zhao *et al.*, (1999) consequently demonstrated that a C-8-methoxyl fluoroquinolone was four times more effective against *Mycobacterium tuberculosis* than a C-8-H control and the fluoroquinolone ciprofloxacin (Figure 1.6). These drugs have increased potency against first-step gyrase and topoisomerase IV resistant mutants, and also fewer resistant mutants are selected when bacteria are challenged with a C-8-methoxyl than with a C-8-H derivative. This has important consequences for resistance minimisation. Alovero *et al.* (2000) also found that addition of a benzenesulfonamide group to the C-7 piperazyl ring of ciprofloxacin increase potency against *Streptococcus pneumoniae*, and changes target specificity of the quinolone from DNA gyrase to topoisomerase IV.

Figure 1.6: C-8-methoxy group modification



PD161148 - (C8-Ome) compound PD160793 (C8-H) control. The arrow indicates the C-8 position. (From Zhao *et al.*, 1999)

1.4.2 Quinolone -Topoisomerase Binding

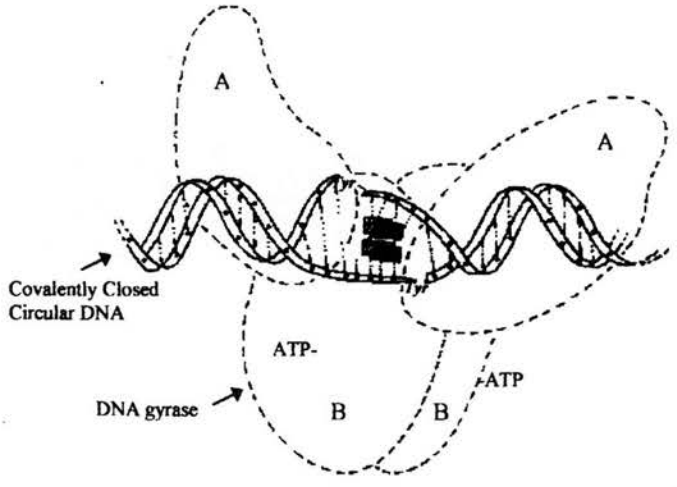
There are several models of how quinolones bind to the cleavable complex formed with DNA Gyrase and Topoisomerase IV, however a clear consensus is yet to be reached. Most research has focused on the interaction between quinolone, DNA and DNA Gyrase, however it is believed that quinolones bind to Topoisomerase IV in a similar manner.

1.4.2.i Interaction between quinolone and DNA gyrase/DNA complex

Before binding studies were carried out, it was believed that quinolones bound directly to GyrA (Gellert *et al.*, 1977), as quinolone resistance mutations occurred in the *gyrA* gene. The occurrence of quinolone resistance mutations in the *gyrB* gene further suggested that the quinolone binding site may encompass both sub-units (Yamagishi *et al.*, 1986).

In 1985 Shen and colleagues carried out binding studies, which suggested contrary to popular belief that quinolones do not bind to gyrase, but rather to DNA. Shen *et al.* (1989) later proposed a co-operative drug binding model, in which drug binds to a single-stranded DNA-binding pocket revealed following DNA cleavage by gyrase. The quinolone drugs are thought to interact with the bases through hydrogen bonds which occur between the 3-carboxy and 4-oxo groups which almost all quinolones possess (Figure 1.7). At least 4 molecules are believed to bind per binding site and ring stacking and hydrophobic interactions contribute to this cooperativity. Shen *et al.* (1989) postulated that gyrase interacts with the C-7 position of the drug, however Maxwell (1992) later pointed out that the C-7 group is variable in the quinolones.

Figure 1.7: Quinolone binding model (Shen *et al.*, 1989)



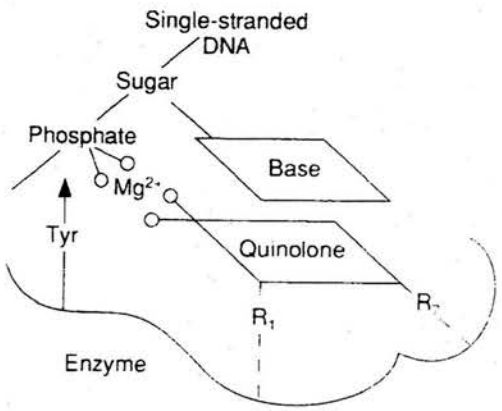
Filled and hatched boxes represent quinolone molecules inside a gyrase-induced single stranded DNA binding site. (From Shen *et al.*, 1989)

A later finding by Critchlow and Maxwell (1996) disputes Shen's model by demonstrating that drugs still bind gyrase with mutations at the active-site tyrosine (Tyr 122 → Ser/Phe in GyrA), despite being unable to cleave DNA. They proposed that a pre-cleavage complex between gyrase, quinolone and DNA exists, which is converted to a perhaps more stable complex following DNA cleavage. Capranico *et al.* (1993) suggest that quinolones intercalate in the space next to the cleaved phosphodiester bond of DNA, with modelling studies by Llorente *et al.* (1996) supporting this hypothesis.

Palumbo *et al.* (1993) proposed an alternative model which involves drug binding to phosphates in DNA via a Mg^{2+} bridge, with interaction with the quinolone by ring

stacking (Figure 1.8). Yoshida *et al.* (1993) believed that drugs bind in a pocket appearing during DNA cleavage and reunion process with GyrA and GyrB sub-units determining binding affinity. It is clear that the precise mechanism of binding between quinolone, topoisomerase and DNA is unknown, and requires the use of high resolution structure studies, made particularly difficult by the large size of DNA gyrase (~ 400 kDa).

Figure 1.8: Quinolone binding model (Palumbo *et al.*, 1993)



Quinolone drugs are though to bind to DNA via a Mg^{2+} bridge between the C-3 and C-4 groups and phosphate groups of the DNA. Interaction between gyrase and drug is proposed to occur via C-7 and N-1 substituents on the quinolone.

1.4.3 Aminoglycosides: structure-activity relationships

The aminoglycoside group of antibiotics are biologically active bacterial secondary metabolites. They are structurally defined by the presence of amino sugars bound by glysosidic linkage to a dibasic cyclitol nucleus. In streptomycin the dibasic cyclitol is streptidine, however in most of the clinically used aminoglycosides subsequently

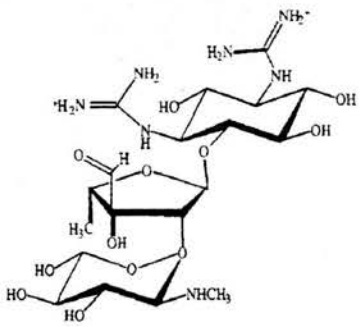
developed, it is 2-deoxystreptamine. Conserved elements of aminoglycosides are rings I and II, and within ring II the amino groups at positions 1 and 3 (Figure 1.9). Drugs with enhanced potency have been developed by substituting the 2-deoxystreptamine ring at positions 4 and 5, as for neomycin, and positions 4 and 6, as for kanamycin, gentamicin and tobramycin (Figure 1.9).

Aminoglycosides are active against a wide range of aerobic gram-negative bacilli, many staphylococci and certain mycobacteria (Edson & Terrell, 1999). Streptomycin has a particular role in the treatment of multi-drug resistant *Mycobacterium tuberculosis*, and gentamicin for treating serious hospital acquired infections caused by *Enterbacteriaceae* and *P. aeruginosa* (Edson & Terrell, 1999). Their severe nephro and ototoxicity limit the clinical use of aminoglycosides (Kahlmeter & Dahlager, 1984).

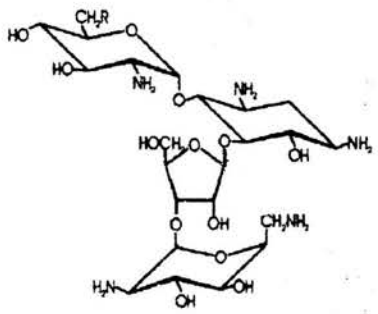
Attempts to make semi-synthetic aminoglycoside derivatives have focussed on drugs which will be able to overcome aminoglycoside modifying enzymes, the main mechanism of aminoglycoside resistance (see section 1.5.3); for example tobramycin lacks a crucial 3`-hydroxyl group, therefore it is no longer a substrate for APH(3`) enzymes. Amikacin has an acetylated N-1 group, which makes it a poor substrate for a number of modifying enzymes.

Figure 1.9 Aminoglycoside structures

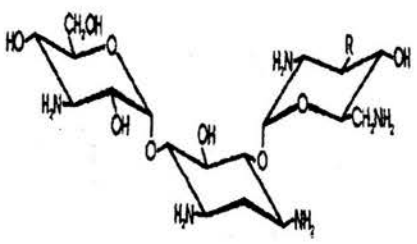
Streptomycin



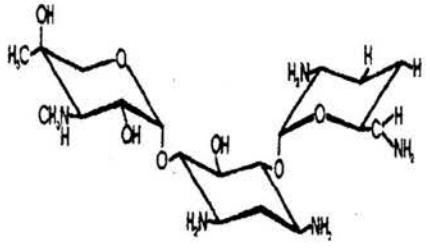
Neomycin R=NH_2



Tobramycin R=H
Kanamycin R=OH



Gentamicin



1.4.4 Aminoglycoside-RNA Binding

The A-site (530 loop) of the 16S ribosomal RNA within the 30S ribosomal complex was identified as a main aminoglycoside target, when Moazed and Noller (1987) demonstrated that aminoglycosides protect the region from binding to chemical

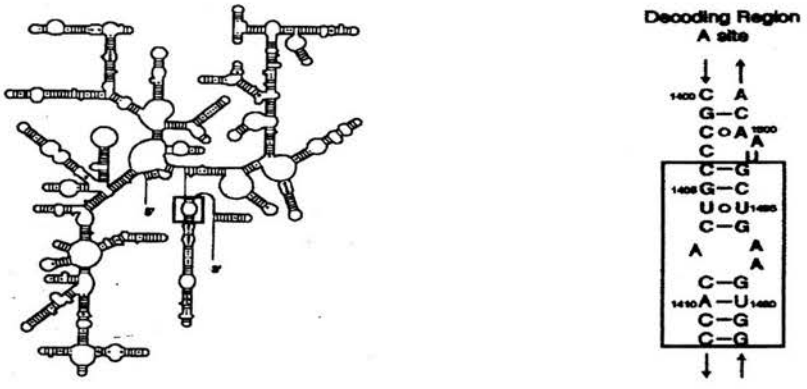
probes. The protected area was implicated in the function of the drug, as the A-site is involved in the decoding process, and therefore this observation corresponded with mechanism of action.

To analyse the precise structural interaction however, X-ray analysis has been performed. Clemons *et al.* (1999) obtained an X-ray structure of the 30S complex of *Thermus thermophilus* ribosomes, giving a 3-dimensional picture of the decoding site, however the precise contacts between antibiotic and target could not be visualised. An alternative approach has been the determination of a NMR structure of a model A-site complexed with the aminoglycoside paromycin (Fourmy *et al.* 1996). The decoding site is an irregular helix that binds antibiotic via its major groove, and contacts the codon-anticodon complex via its minor groove. Aminoglycosides are multiply charged compounds with high flexibility. Their positive charges are attracted to the negatively charged RNA backbone, and their flexibility facilitates binding into the major groove. Binding of antibiotic to RNA induces a conformational change in RNA, switching the A-site into a high affinity state for mRNA-tRNA recognition (Fourmy *et al.*, 1998). This increased affinity may lead to misreading of the genetic code.

Aminoglycoside drug structure determines where the drugs bind on rRNA, with neomycin, gentamicin and kanamycin binding to the A-site of 16S RNA in *E. coli* in a similar manner (Moazed and Noller, 1987). Rings I and II of the neomycin class of antibiotics has been shown to target binding to a unique pocket in the 16S RNA (Fourmy *et al.*, 1996). Alper *et al.* (1998) suggested that the 1,3-hydroxylamine

moiety present in almost all aminoglycosides may also be an important recognition motif for RNA binding.

Figure 1.10: Sequence of the decoding region A-site from *E. coli* rRNA



A-site region is boxed (From Lynch *et al.*, 2000)

In addition to targeting the 16S ribosome there is evidence that the 12S ribosomal subunit is also a target of aminoglycosides, with mutations in ribosomal S12 leading to streptomycin resistance and dependence (LaCoste *et al*, 1977; Funatsu & Wittman, 1972; Momose & Gorini, 1971). The S12 protein has been structurally linked to the A-site (530 region) of the 16S RNA with residues in the 530 stem protected by the S12 protein in chemical protection experiments (Stern *et al.*, 1988). Moazed and Noller (1986) proposed that the structure of the 16S RNA may be stabilised by the ribosomal S12 protein.

1.5 Topoisomerase Mutation

1.5.1 DNA Gyrase

Quinolones are known to target the enzyme DNA gyrase in the bacterial cell (Yoshida *et al.*, 1988). Sequence analysis of DNA from many bacterial species has demonstrated resistance mutations near the active site (Tyr 122 in *E. coli*) in the GyrA protein. This region, at the amino terminus between amino-acids 67 and 106, has been named the quinolone resistance determining region (QRDR). The residues which have been found to change correlating with an increase in MIC of quinolone drugs are as follows: alanine-67, glycine-81, serine-83, alanine-84, aspartic acid-87 and glutamine-106 (Table 1.2) (Reece & Maxwell, 1991), however mutations in serine-83 and aspartate-87 are known to confer the greatest reduction in quinolone susceptibility (Hallett & Maxwell, 1991; Oram & Fisher, 1991; Yoshida *et al.*, 1990).

Mutations at serine-83 to a leucine or tryptophan residue confer the greatest levels of resistance (Table 1.2), and it was therefore implicated that hydrophobic residues at these positions confer the quinolone resistance phenotype (Yoshida *et al.*, 1990). The bulkiness of leucine and tryptophan residues may also destabilise the quinolone gyrase interaction and prevent access to the binding pocket. It has also been proposed that hydrophilic residues serine-83, aspartate-87 and glutamine-106 participate in hydrogen-binding interactions with the drug (Hallett & Maxwell, 1991). The Binding model proposed by Shen *et al.* (1989), suggests that hydrogen bonding occurs between DNA and the drug, whilst enzyme drug interactions occur with the C-7

group of the quinolone (section 1.4.2.i). Mutations may destabilise this quinolone-enzyme interaction.

Table 1.2: Mutations in the QRDR of the *E. coli gyrA* gene

Amino Acid Position	Amino-Acid Exchange	MIC Change* Ciprofloxacin (mg/L)	Reference
67	Ala→Ser	4	Yoshida *et al.*,1990
81	Gly→Cys	8	Yoshida *et al.*,1990
81	Gly→Asp	not detectable	Cambau *et al.*,1993
83	Ser→Ala	4	Hallett & Maxwell, 1991
83	Ser→Leu	32	Yoshida *et al.*,1990
83	Ser→Trp	32	Yoshida *et al.*,1990
84	Ala→Pro	8	Yoshida *et al.*,1990
87	Asp→Asn	16	Yoshida *et al.*,1990
87	Asp→Val	5	Yoshida *et al.*,1990
87	Asp→Tyr	8	Heisig *et al.*,1993
106	Gln→Arg	4	Hallett & Maxwell, 1991
106	Gln→His	4	Yoshida *et al.*,1990

* The change in MIC is given as the factor by which the MIC has changed to the original parent strain. (Note: Above mutations were generated by either site-directed mutagenesis, *in vitro* selection or direct analysis of clinical isolates, thereby MIC values may not be directly comparable)

The GyrB protein of DNA gyrase is also known to have a quinolone resistance determining region (Yoshida *et al.*, 1991), however mutations in this area are thought to be of minor importance, and may be at distant sites from the active site. Mutations have been described at central residues aspartate-426 to asparagine and lysine-447 to glutamate, and have the effects of raising quinolone MICs (Yamagishi *et al.*, 1986). Only a small increase in MICs of nalidixic acid, oxolinic acid and cinoxacin were

detected for both mutations, however the change of aspartate-426 to asparagine conferred fluoroquinolone resistance (Yamagishi *et al.*, 1986). GyrB mutations may lead to resistance due to different contacts with GyrA being formed, and therefore a change in the quinolone binding pocket.

1.5.2 DNA Topoisomerase IV

Kumagai *et al.* (1996) have demonstrated that mutations in DNA topoisomerase IV (topoIV) may be associated with fluoroquinolone resistance. They identified ParC mutations (Homologous to GyrA) among quinolone resistant *E. coli* isolates, at positions equivalent to Glycine-78, Serine-80 and Glutamate-84 comparable to those in the QRDR of GyrA. In *E. coli* ParC mutations contribute to resistance but only in the presence of GyrA mutations and cause no resistance in strains with wild-type GyrA.

ParE mutations have been found in conserved regions of ParE homologous to those in GyrB, however these mutations are not thought to be important in the acquisition of quinolone resistance (Ruiz *et al.*, 1997). The contribution of mutation in ParC depends on drug structure and bacterial species, with ParC mutations selected primarily in gram-positive organisms.

1.5.3 The plasmid dominance test

Resistance conferred by mutations in the *gyrA* gene are recessive to wild-type DNA gyrase (Gellert *et al.*, 1977). When a plasmid with a sensitive gyrase is introduced into

a cell which contains a resistant *gyrA* allele, the resistant phenotype is not seen (Nakamura *et al.*, 1989). This character relates to the mechanism of quinolone action, as cleaved complexes may still form in a cell with a mixture of sensitive and resistant gyrase sub-units. Removal of these complexes would release double strand DNA breaks in the cell, which are lethal even in small numbers (Krasin & Hutchinson, 1977). The recessive nature of gyrase resistance has been used to attribute quinolone resistance to gyrase mutations in a number of bacteria including *E. coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* (Heisig & Weiderman, 1991, Nakamura *et al.*, 1989). Cells that acquire quinolone susceptibility when a WT plasmid is introduced are inferred as *gyrA* mutants. Care must be taken when analysing these tests, as mutations in secondary targets may be partially complemented by plasmid encoded wild-type alleles. This is particularly important as topoisomerase IV is also a drug target, and resistance may be due to mutation in this enzyme.

For topoisomerase IV of *E. coli*, no clear dominance is seen when resistant and sensitive alleles are both present as single copy genes. In *S. aureus*, where topoisomerase IV is thought to be the primary target, wild type ParC and ParE must be over-expressed to confer sensitivity and mutant *parC* alleles introduced on a plasmid can confer resistance (Yamagishi *et al.*, 1996). The difference in dominance between gyrase and topoisomerase IV may be due to the fact that fewer topoisomerase IV cleavage complexes are trapped on the chromosome (Chen *et al.*, 1996). It is clear that the consequences of interactions of quinolones differ between topoisomerase IV and DNA gyrase.

1.5.4 Multiple mutations: primary and secondary drug targets

Deductions from *in vitro* mutation studies have shown that DNA gyrase is the primary target in *Escherichia coli*, with GyrA mutation selected primarily followed by topoisomerase IV mutations at higher quinolone MICs (Chen *et al.*, 1996, Khodursky *et al.*, 1995). Topoisomerase mutations do not confer resistance in the absence of gyrase mutation. Examination of clinical isolates supports *in vitro* mutation studies regarding the location of GyrA and ParC mutations, however, data from isogenic strains showing resistance progression is lacking. Mutation of serine-83 of GyrA protein is associated with moderate level resistance, addition of one or two ParC protein mutations correlates with increased resistance, 3 mutations (two GyrA and one ParC) are associated with high-level resistance and four mutations (two GyrA and two ParC) with very high-level resistance (Table 1.3) (Vila *et al.*, 1996).

Table 1.3: Multiple mutations in GyrA and ParC proteins in clinical *E. coli* isolates (Vila *et al.*, 1996)

GyrA		ParC		MIC (ciprofloxacin) mg/L
Ser83	Asp87	Ser80	Glu84	
-	-	-	-	0.007-0.25
Leu	-	-	-	0.25
Leu	-	Arg	-	1-4
Leu	-	-	Lys	4
Leu	Tyr	-	Lys	8-32
Leu	Asn	Ile	-	8-64
Leu	Asn	Arg	-	16
Leu	Asn	Ile	Val	64
Leu	Tyr	Ile	Lys	128

In *S. aureus*, topoisomerase IV appears to be the primary ciprofloxacin target (Ng *et al.*, 1996; Ferrero *et al.*, 1995), as *S. aureus* DNA gyrase is less susceptible to inhibition than *E. coli* gyrase (Blanche *et al.*, 1996). Thus ParC mutations confer low level resistance, with higher level resistance requiring additional mutations in GyrA. Gyrase mutations do not in themselves confer resistance (Ng *et al.*, 1996). The situation is similar in *Streptococcus pneumoniae* with ciprofloxacin (Munoz & de la Campa, 1996), however sparfloxacin selects GyrA mutants before ParC mutants in the same organism (Pan & Fisher, 1997). It therefore appears that quinolone structure can alter target preference. One group of quinolones i.e. ciprofloxacin selects ParC and ParE mutations suggesting that these drugs act preferentially through topoisomerase IV *in vivo* (Janoir *et al.*, 1996), whereas a second group typified by sparfloxacin selects GyrA mutants in the first step, consistent with gyrase being the primary target *in vivo* (Fukuda & Hiramatsu, 1999). Durham *et al.* (1999) have also found that GyrA mutations can be selected preferentially to ParC mutations in an *in vitro* mutation study, contradicting information from previous authors. This calls into question the way in which *in vitro* mutation studies are performed without standardised criteria, and how relevant they are to the clinical situation.

1.5.5 Contribution of non-target protein mutation to fluoroquinolone resistance

In addition to mutations affecting target enzymes, resistant isolates often show reduced drug accumulation (Everett *et al.*, 1996). The genetic basis of this remains however obscure. In mutants selected *in vitro* Mar-like (multiple antibiotic resistance

conferring) mutations seem to occur as second step mutations preceding further mutations in drug targets (Heisig & Tschorny, 1994; Hooper *et al.*, 1987). There is discrepancy between *in vitro* selected mutants and clinical isolates, only half of which express a Mar phenotype (Oethinger *et al.*, 1998).

Mutations can occur which affect expression of porins (Mortimer & Piddock, 1993), or active efflux of quinolone from the bacterial cell (Okusu *et al.*, 1996). Tavio *et al.* (1999) demonstrated that gyrase mutations were the principal determining factor in high-level fluoroquinolone resistance, however changes in outer membrane proteins (OMPs) and efflux were complimentary in determining quinolone MICs in *in vitro* selected *E. coli* isolates. These mechanisms could facilitate resistance development in the clinic, and facilitate subsequent acquisition of target protein mutations. They also complicate studies of the contribution of individual target protein mutations to quinolone resistance.

1.5.6 Increasing emergence of fluoroquinolone resistance in the clinic

During the late 1980`s there was a general optimism concerning the apparent lack of potential for resistance development to the quinolones in a majority of organisms. Clinically significant resistance was limited to staphylococci and *Pseudomonas aeruginosa,* and even amongst these species, was confined to pathogens causing pyogenic infections in tissue sites where penetration or activity of these agents is less than optimal (e.g. Bone, prostate or abscesses) (Ball, 1990). This picture has however

changed significantly, as it is inevitable with increasing reliance on this group of antimicrobial agents.

Resistance among many other bacterial species including various streptococci, gonococci, enteropathogens and opportunist Gram-negative pathogens (e.g. *Serratia marcescens*) has now been reported (Ball, 1994). Among traditionally highly susceptible enterobacteria such as *Escherichia coli*, for which fluoroquinolone minimum inhibitory concentrations (MICs) are normally < 1 mg/L, resistance has until recently only been very rarely observed (Lopez-Brea & Alarcon, 1990). However the frequent use of norfloxacin and ciprofloxacin in prophylaxis of patients with neutropenia and cirrhosis, as well as in therapy of urinary tract infections seems to be associated with the emergence of resistance in this previously fluoroquinolone susceptible organism (Pena *et al.*, 1995). This may have severe consequences for the treatment of invasive illness and therefore it is imperative that we monitor this emerging trend.

The first reports of emergence of fluoroquinolone resistance in *E. coli* originated from Spain. Pena *et al.* (1995) observed increasing resistance to ciprofloxacin among *E. coli* strains isolated in blood cultures at the Hospital de Bellvitge, Barcelona. The rates of *E. coli* bacteraemia caused by ciprofloxacin resistant strains increased steadily from 0% to 7.5% in 1992. Carratala *et al.* (1995) also performed a similar study in a teaching hospital in Barcelona and showed that 37% of *E. coli* bacteraemia in neutropenic cancer patients was due to quinolone resistant strains, correlating with an

increasing reliance on fluoroquinolones during the study period. Although the reports of rapid increases in the prevalence of highly fluoroquinolone resistant *E. coli* often appear in Spain, the extent of quinolone use in other European countries leaves no room for complacency.

The U.K. is already beginning to experience problems with the emergence of ciprofloxacin resistance in clinical *E. coli* isolates, as reported by Threlfall *et al.* (1997). In the three year period Nov 1993 to Nov 1996, resistance to ciprofloxacin was detected in isolates of *E. coli* from 40 patients with invasive illness or urinary tract infection (MICs ranging from 4-256 mg/L). All the isolates were resistant to at least one additional antimicrobial with the majority being multiply resistant (4 or more antimicrobials). Also in Germany the prevalence of fluoroquinolone resistance among clinical isolates of *E. coli* increased from <1% to 5% between 1990 and 1995 (Kresken *et al.*, 1994). Thomson (1999) has also reported a general trend of increasing fluoroquinolone resistance. The fact that resistance is generally chromosomally encoded has meant that resistance spread has not been as prolific as it could be, however the report by Martinez-Martinez *et al.* (1998), showing that quinolone resistance can be transferred by a plasmid vector thereby giving the potential for rapid spread is of great concern. Although the resistance acquired with this plasmid was low, emergence of high-level resistant mutants was over 100-fold higher in strains with the plasmid compared to wild-type strains.

Epidemiological studies utilising pulsed field gel electrophoresis (PFGE) and PCR-based typing methods have shown that clinical resistance to the fluoroquinolones emerges as a combination of horizontal transfer of clonal strains, and individual selection of resistant isolates (Lehn *et al*, 1996; Oethinger *et al.*, 1996). However there seems to be a prevalence of individually selected resistant strains, with clonal diversity among isolates in individual and separate centres generally unrelated (Lehn *et al.*, 1996).

1.6 Ribosomal protein mutation

Aminoglycosides are known to interact with the 30S ribosomal sub-unit of the prokaryotic ribosome. Much knowledge about drug binding has been recently derived from structural studies of aminoglycoside 16S RNA complexes (Fourmy *et al.*, 1996) Data from mutation studies have also implicated a role for ribosomal protein S12 and 16S RNA in aminoglycoside interaction. It should however be noted that these studies generally utilise streptomycin, and resistance mutation to other aminoglycosides are not well characterised.

1.6.1 S12 protein mutation

The *rpsL* gene of *E. coli* encodes the highly conserved S12 protein of the ribosomal accuracy centre. The ribosomal accuracy centre is a component of the cellular translation apparatus (Kurland & Ehrenberg, 1984), comprising of a ribosomal RNA domain and several polypeptides of the small sub-unit including S12, S4 and S5. The study of mutations in the S12 gene has provided information about aminoglycoside binding and mode of action.

Mutations in the *rpsL* gene, encoding S12 have been found to generate resistance and in some cases dependence on high-levels of streptomycin (≥ 25mg/L) (Funatsu & Wittman, 1972; LaCoste *et al.*, 1977). These mutations map to two specific regions of the polypeptide, centred on residues 41-43 and 85-91, notably the two lysine residues at positions 42 and 87. Mutations to any of four amino acids (lysine, asparagine, threonine of arginine) have been found at position 42, whereas mutation

at position 87 is typically to an arginine residue (Funatsu & Wittman, 1972). The MICs of mutants selected in a single step generally range from 64 → > 4000mg/L (Bryan & van den Elsen, 1977), depending on other genetic mutations present in cells. Clinically significant resistance to streptomycin in *Mycobacterium tuberculosis* is due to *rpsL* mutation at positions 43 and 88 of Lys to Arg/Thr (Finken *et al.*, 1993, Nair *et al.*, 1993). Streptomycin dependent strains may have changes at position Lys87 (Timms *et al.*, 1992). Glutamine in position 42 has also been found in some streptomycin dependent mutants (Funatsu & Wittman, 1972).

Streptomycin resistance mutations are associated with the phenotypic suppression of nonsense mutations (Gorini, 1971), and altered expression of translationally regulated proteins (Redaschi & Bickle, 1996). Streptomycin dependent strains exhibit hyper-accurate translation with general growth impairment (Ruusala & Kurland, 1984), reduced ternary complex formation and reduced translational elongation rate (Bilgen *et al.*, 1992; Bohman *et al.*, 1984). The normal role of S12 seems to maintain some slight imperfections in decoding, the mutant S12 enforces a more precise expression of each codon in a mRNA (Bohman *et al.*, 1984). Streptomycin resistance mutations are likely to antagonise the effect of structural change in S12, restoring the ribosome to its normal position and hence permitting a low level of inaccuracy in translation. Streptomycin dependence mutations in S12 may cause the ribosome to become too demanding in its recognition requirements of aminoacyl tRNA species (Ozaki *et al.*, 1969).

It has been seen how a single amino-acid position mutation can confer high-level resistance, and deductions have been implied for aminoglycoside binding. Chang & Flaks (1972) concluded that *rpsL* mutants have lost a single high affinity binding site in the ribosomal sub-unit, however can maintain low affinity binding sites. However subsequent experiments by Lelong *et al.* (1974), showing that S12 antiserum does not block streptomycin binding and therefore it may be that S12 forms part of a 3-dimensional binding pocket influenced by several ribosomal proteins. Moazed and Noller (1986) implicate S12 in the stabilisation of 16S ribosomal RNA (see section 1.3.4). Mutations in S12 have not been described for the other aminoglycosides, and it would be interesting to understand their relationship with this important target protein.

1.6.2 Mutation in 16S RNA

Structural studies have confirmed that aminoglycosides bind to the 16S ribosomal RNA of the 30S sub-unit. Before structural studies confirmed this, some evidence for 16S RNA as a ribosomal target was deduced from studies on resistant mutants.

Although streptomycin resistance in *E. coli* is usually linked to mutations in the S12 ribosomal protein, Montandon *et al.*, (1985) isolated mutants of *Euglena gracilis* with streptomycin resistant chloroplasts which had a point mutation in (C876T) in the 16S RNA gene. To examine the contribution of 16S mutation to streptomycin resistance in *E. coli* the authors used site-directed mutagenesis to introduce this mutation and found that transformed cells expressed resistance (Montandon *et al.*,

1986). Bonny *et al.* (1991) showed that point mutations in the 912-915 region of 16S RNA can confer resistance in *E. coli*, however these mutations are not generally isolated by natural means. These mutations confirm a role of the 16S rRNA in binding, while S12 mutations may influence the availability of this site (Garvin *et al.*, 1974).

1.6.3 Mechanism of aminoglycoside resistance in the clinic

Unlike the situation with the fluoroquinolones, mutations in target proteins are rarely associated with aminoglycoside resistance in the clinic. An exception to this is streptomycin resistance caused by mutation in the 30S ribosomal segment of *M. tuberculosis*. The most frequent and important mechanism of aminoglycoside resistance are the plasmid mediated aminoglycoside modifying enzymes (Davies & Wright, 1997; Shaw *et al.*, 1993), which catalyse the covalent modification of amino and hydroxyl functions, leading to chemically modified drugs which bind poorly to ribosomes. Resistance in *Enterbacteriaceae* to neomycin, gentamicin, kanamycin emerged as a result of this genetically transferable mechanism (Davies & Wright, 1997). Reduced drug uptake, mostly seen in Pseudomonas spp., is likely to be due to membrane impermeabilisation, but the underlying molecular mechanism is largely unknown (Chambers & Sande, 1995). Active efflux has been evidenced for neomycin, kanamycin and hygromycin A in *Escherichia coli* (Edgar & Bibi, 1997), however its clinical significance is still undetermined (Lynch *et al.*,1997; Nikaido, 1996).

1.7 The Complexity of Mutation Selection

An understanding of the way in which mutants are selected is crucial if we are to minimise drug resistance and preserve our antimicrobial armoury. It is clear that there are many factors involved in selecting resistant mutants, from environmental constraints, to bacterial physiological state. It is also very highly drug structure dependent, with those drugs aimed at targeting more than two enzymes more likely to minimise the emergence of resistance. This is crucial knowledge for future drug design.

1.7.1 Mutation rate

When a bacterial population is subjected to antibiotic challenge, selectively favourable mutations occur which may lead to a visibly resistant phenotype. "Mutation rate" is often defined as the *in vitro* frequency of mutant selection at a given antibiotic concentration.

The analysis of mutation rate is complicated by the fact that mutations in different genes produce similar antibiotic resistance phenotypes. The quinolone resistance mutation rate is a result of a combination of mutations of the genes that encode GyrA, GyrB, ParC, ParE and multi-drug resistance (MDR) systems (Hooper, 1999). With new found knowledge of the way bacterial populations behave, we are grasping the complex factors that interplay to determine the type and rate of mutations selected under antimicrobial pressure.

1.7.2 Mutability

Mutability can be defined as the probability that a mutation will produce an antibiotic resistance phenotype, and is dependent upon the number and structure of genes in which mutations produce resistance. These mutations must be non-lethal, not produce a significant fitness loss, and produce resistance. The probability of this type of mutation occurring will depend on the number of these positions in the target enzymes. An example is in *E. coli* where fluoroquinolone resistance can be conferred by changes at seven different positions in *gyrA* gene, however only 3 in the *parC* gene, and consequently *gyrA* mutants are more prevalent than those in *parC* (Hooper, 1999). In *Streptococcus pneumoniae*, only 2 *gyrA* changes are known, however there are 5 changes in *parC*, and therefore *parC* mutants are more frequently found. Streptomycin resistance results from a change in one or a few nucleotides in the ribosomal S12 gene and therefore spontaneous resistance rate is low (Bonny *et al.*, 1991).

1.7.3 Independent and co-operative mutations

Many genes can be involved in antibiotic action and resistance as antibiotic interacts with the cell via 1) direct antibiotic targets, 2) access and 3) protection pathways. The synthesis and regulatory pathways controlling target expression are thus also susceptible to mutation under antimicrobial selective pressure

1.7.3.i Independent Mutation

Single gene mutations can give rise to antibiotic resistance, as in DNA Gyrase mutations in quinolone resistance (Yoshida *et al.*, 1990). These mutations are target protein structural changes leading to decreased binding affinity of the drug and target, thereby elevating the MIC. Mutations controlling efflux can also increase MIC of the antimicrobial, examples being mutations activating expression of MDR determinants (Kohler *et al.*, 1997).

1.7.3.ii Co-operative mutation

The presence of several lethal targets in the cell would lead to the probability that single mutations in a single drug target would be an infrequent occurrence, as interaction with the other lethal target would ensure cell death. Mutation rate to cefotaxime resistance (*in vitro*) in *Streptococcus pneumoniae* is higher than for penicillin resistance, because cefotaxime has one less penicillin binding protein (Hakenbeck, 1999). Resistance to certain fluoroquinolones in *S. aureus* can also only be conferred when both *gyrA* and *grlA* mutations are present (Deplano *et al*, 1997). It can be deduced from this that antimicrobials that have dual lethal targets are favourable, knowledge that is exploited in the search for new antimicrobial compounds. Mutations in several access pathways may also be required to prevent the action of drugs which can be transported through multiple routes.

1.7.4 Antibiotic concentration

As previously discussed a number of pathways from target, access and detoxification are involved when an antibiotic interacts with a bacterial cell. A single mutation in one of these targets will result in low-level resistance, whereas multiple mutations in several targets will result in high-level resistance. It thereby follows that when challenged with a low antibiotic concentration, single mutations will occur, as in Ser83 in DNA Gyrase after quinolone challenge. Several mutations are required to overcome challenge with high antibiotic concentrations, as seen with multiple mutations in GyrA and ParC, along with efflux to produce a highly resistant phenotype (Tavio *et al.*,1999).

Antibiotic concentration is very important in mutation selection, and a specific MIC is often optimal for selecting specific mutation types and rate. At low antibiotic concentrations variants may not be selected out, however if concentrations are too high both susceptible and variant populations will be suppressed. It is also possible that if bacterial populations are not killed effectively, the stress that the cells are under will increase the mutation rate.

Concentrations are very important as few mutants will be recovered if antibiotic concentrations require a cell to contain two or more concurrent mutations for growth in the presence of drugs (current dosing protocols allow cells to grow after only one resistance mutation) thereby aiding resistance development. With fluoroquinolones the mutation frequency is less than 10^{-7} and so more than 10^{14} bacteria would be required

to find two target mutations. In the clinic bacterial populations may reach 10^{10} cells in an infected individual, but 10^{14} is unlikely. This higher drug concentration required to prevent mutant selection has been termed the "mutant prevention concentration" (MPC) (Drlica, 2001), and may have important implications for future antibiotic therapy regimens. The concentration between the MIC and MPC of an antibiotic is the mutant selection window. As target preference for drugs is very much dependent upon drug structure, understanding the interaction between drugs and targets and altering drugs so that they may target dual enzymes may mean that in future the MPC equals the MIC.

1.7.5 Bacterial physiological state

1.7.5.i Adaptive mutation

Early work on dividing bacteria by Lederberg & Lederberg (1952) lead to the presumption that mutation occurs as a result of errors occurring during the DNA replication process. Recent advances in understanding of bacterial populations has demonstrated that mutation can occur in non-dividing cells (Rosenberg *et al.*, 1996; Shapiro, 1984), a process termed "adaptive" or "stress induced mutation". With this type of mutation, the rate of mutation selection can actually increase under starvation conditions (Shapiro, 1984).

Adaptive mutation has been classically studied using non-lethal selection as it was thought that lethal selection would kill bacteria before they entered starvation. Recent work on emergence of antibiotic resistance has however yielded exciting new results.

Quinolones, which induce the SOS response, increase the rate of emergence of resistance to drugs in *E. coli* (Riesenfeld *et al.*, 1997). Antibiotic challenge also increases the emergence of MDR mutants in *P. aeruginosa* (Alonso *et al.*, 1999), and *E. coli* exposed to streptomycin have a hypermutable phenotype (Ren *et al.*,1999).

This demonstrates the influence of bacterial growth conditions on mutation selection. This stress enhanced mutation is regulated by stress-responsive error prone DNA polymerase V (*umuCD*) and IV (*dinB*) which transiently increase mutation rate (Radman, 1999). Bacteria growing *in vivo* are generally under stress (Foley *et al.*, 1999), contending with starvation, colonisation of hostile niches, host defences and antibiotic challenge. The frequency of mutation *in vivo* is therefore likely to be much higher than that measured by *in vitro* analysis.

1.7.5.ii Mutator strains

Bacterial populations are known to contain a proportion of hypermutable or "mutator" cells, which have a mutation rate increase from 10-10,000 times normal, due to a defective mismatch repair system (Miller, 1996). These bacteria can evade stressful environments such as antimicrobial treatment (Matic *et al.*, 1997), due to their large diversity of alleles. Antibiotic selection of mutator alleles can be the basis of multiple antibiotic resistance in bacteria with mutations in several targets (LeClerc *et al.*, 1996).

Mutators are not expected to be stable in a bacterial population, as they are less fit and may be controlled by programmed cell death systems (Mittenhuber, 1999). It can however been seen how an environment which is fluctuating between stress and no stress may maintain a population of mutators. This is the environment experienced by many pathogenic and epidemic bacterial clones, leading to the hypothesis that these are in fact mutator strains.

1.7.5.iii Pre-existing low-level resistance

The presence of low-level antibiotic resistance mutations is likely to facilitate the acquisition of clinical resistance when exposed to antibiotic challenge. Markham (1999) has shown that inhibition of MDR efflux pumps in *S. pneumoniae* using reserpine, reduces mutation rate and consequently resistance development. It therefore seems that the presence of low-level antibiotic resistance mechanisms might favour the emergence of clinically relevant antibiotic resistant bacteria. Bacteria growing at a high density can also be considered to have low-level resistance to antibiotic challenge (i.e. biofilms), and favour the emergence of resistant mutants. Waters & Davies (1997) have also shown that amino-acid variation within the GyrA sub-unit of DNA gyrase is also associated with natural non-selected resistance to fluoroquinolones.

1.7.6 Bacterial fitness

The reason why mutants usually do not proliferate in a non-selective environment is due to the detrimental effect mutation has on bacterial fitness (Bjorkman *et al.*, 1998),

however in some cases fitness can be increased (Blot *et al.*, 1994). Reduction in fitness can be compensated in time with mutations at other chromosomal loci (Bjorkman *et al.*, 2000). A recent demonstration by Bjorkman *et al.* (2000) that compensatory mutations selected *in vitro* are different from those selected *in vivo*, raises questions about the interpretation of current models of *in vitro* resistance development, and the relevance of these models to the *in vivo* situation. Antibiotic cycling policies rely on resistant organisms being at a disadvantage when antibiotic pressure is removed. The presence of these compensatory mutations increasing fitness may therefore make their benefits obsolete.

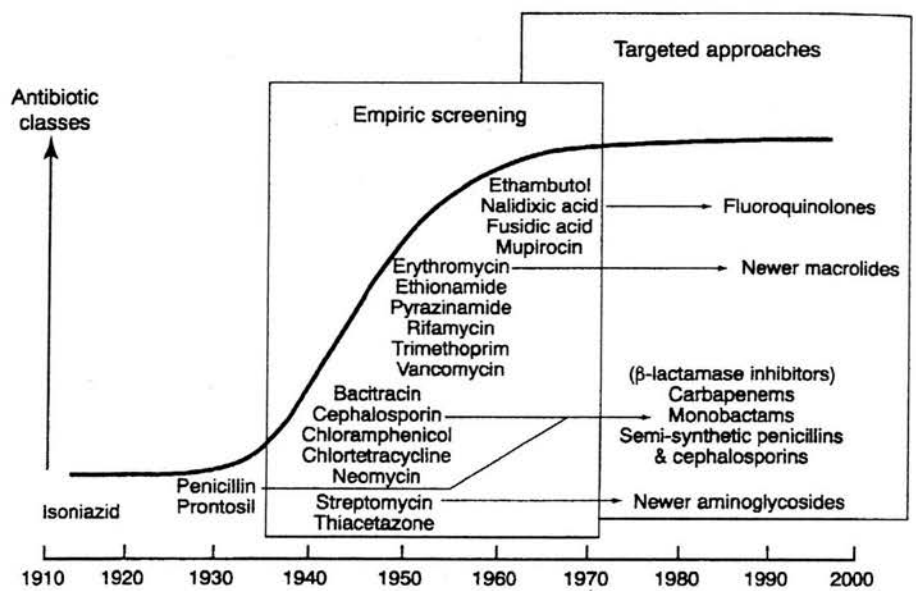
1.7.7 Selective habitat

The environment in which bacteria colonise can determine the variability of a population and the rate at which resistance occurs (Korona *et al.*, 1994). Structured environments such as surfaces allow bacteria to occupy different niches and thereby minimise competition, allowing all alleles capable of surviving selective pressure to grow. Bacterial colonisation during infection is frequently in a heterogeneous environment and involves attachment to various surfaces and host cells. This environment probably allows more types of antibiotic resistant mutant selection than *in vitro* tests in liquid culture medium. The size of the habitat and of the bacterial population also alter mutation selection.

1.8 Drug Discovery: future antibiotic targets

The antibiotics that we have in clinical use today were discovered by random screening campaigns which started in the 1940`s. These antibiotic classes were structurally modified when we had further knowledge of their targets to produce many of the antibiotics of clinical use today (Figure 1.11). Empirical screening and structural modification have limitations, and fail to produce the range of chemicals needed to keep pace with the changing clinical situation. We are now under the threat that drug resistant micro-organisms will overcome all of our current antibiotics, and renewed interest from the pharmaceutical companies has meant the search for the new class (new target) of antibiotic has begun.

Figure 1.11: Time-line of antibiotic discovery



From Knowles (1997)

1.8.1 Microbial Genomics

The range of bacterial genome sequences now available has revolutionised the way in which we can look for new targets. We now have tools which will enable the discovery of essential pathways in bacterial physiology, infection process and antibiotic induced cell death.

A particularly promising technology is that of "DNA microarrays" or "gene chips". This technology can be used to measure the expression patterns of thousand of genes in parallel, providing information about gene function, as well as monitoring changes in gene expression in response to drug treatments. Identification of targets associated with bacterial adaption /survival are crucial. The expression profiles created by micro-arrays serve as "signatures" of the inhibitor used, and in cases where antibiotic action is unknown can incriminate affected pathways of specific target enzymes. The potential of this technology has excited the interest of pharmaceutical and small biotech companies alike, and has the potential to become as essential to research as PCR is now.

1.8.2 Analysis of drug-target interaction

The interaction between drugs and known targets is also of crucial importance in the development of drugs which have reduced resistance potential. As previously mentioned, drugs which simultaneously inhibit more than one target are at an advantage, as mutations in both targets would be required to confer resistance. The technology of site-directed mutagenesis, involving the directed substitution of amino-

acids may prove invaluable in drug-target analysis as it allows the importance of specific amino acids to drug-target interaction and resistance to be measured. Understanding complex drug-target interactions and resistance may lead to the development of antibiotics which can structurally overcome these constraints, is an aid to future drug discovery and resistance minimisation. Revised administration of drugs, using doses at or above the MPC may also reduce the potential for resistance selection.

Aims of this thesis

- To determine the mechanism of fluoroquinolone resistance in clinical *Escherichia coli* isolates, concentrating on the identification of target protein (DNA gyrase and Topisomerase IV) mutation.

- To assess the clonal diversity of clinical *E. coli* isolates, by restriction and PCR based techniques.

- To employ site-directed mutagenesis, to analyse the contribution of individual DNA gyrase (Ser83 and Asp87) mutations to fluoroquinolone resistance.

- To study the role of ribosomal S12 protein mutation, in conferring aminoglycoside resistance, and thereby assess the role of S12 as an aminoglycoside target.

- To investigate the protein synthesis requiring, bactericidal action of the aminoglycoside streptomycin.

2. Materials and Methods

2.1 Bacterial Strains and Plasmids

Bacterial strains used in this study are listed in table 2.1. Fluoroquinolone resistant clinical isolates were collected from hospitals in the Newcastle area (Supplied by Dr. O. Murphy, Freeman Hospital, Newcastle). Plasmids used for site-directed mutagenesis work were pUC18 supplied by Stratagene (UK), and pPH3-gyrA (JmtacA) was a generous gift from Prof. A. Maxwell (University of Leicester).

Table 2.1: Bacterial strains

Bacterial Strain	Characteristics	Source
E. coli NCTC10418	Standard strain	R. Paton (Edinburgh University)
P. aeruginosa NCTC10662	Standard strain	R. Paton (Edinburgh University)
E. coli clinical isolates (n=15) Isolated 1996	Fluoroquinolone resistant	O. Murphy (Freeman Hospital, Newcastle)
E. coli NCTC10418 mutants	Aminoglycoside resistant	This work
Epicurian Coli® XL1-Blue	Supercompetent cells	Stratagene, UK
E. coli KNK453	*gyrA* $^{ts\,(42°C)}$ mutants	A. Maxwell (Leicester University)
E. coli KNK453^{Asp87}	*gyrA*^{ts} Aspartate 87 mutant	This work
E. coli (GyrA mutants)	Ser83 *gyrA* mutation	C. J. Thomson
E. coli JmtacA(Jm109[pH3]	*gyrA* plasmid containing strain	A. Maxwell (Leicester University)

2.2 Materials

2.2.1 Antimicrobial Agents

Antimicrobial Agents, manufacturers and solubility information are listed in table 2.2. Fresh stock solutions of all antibiotics were prepared using sterile distilled water on the day of use.

Table 2.2: Antimicrobial agents and their solvents

Antimicrobial Agent	Solvent	Manufacturer / Supplier
Ampicillin	Water	Sigma-Aldrich
Amoxycillin	Water	CP Pharmaceuticals Ltd.
Amox/Clav (Augmentin)	Water	Smithkline Beecham
Ceftazidime	Water	Glaxo laboratories
Meropenem	Water	AstraZeneca
Trimethoprim	Water + Lactic acid	Sigma-Aldrich
Ciprofloxacin	Water	Bayer plc
Gemifloxacin	Methanol/Water	Smithkline Beecham
Moxifloxacin	Water	Bayer plc
Sparfloxacin	0.5M NaOH	Rhone-Poulenc Rhorer
Nalidixic acid	Water	Sigma-Aldrich
Streptomycin	Water	Sigma-Aldrich
Gentamicin	Water	Sigma-Aldrich
Neomycin	Water	Sigma-Aldrich
Kanamycin	Water	Sigma-Aldrich
Tobramycin	Water	Sigma-Aldrich
Spectinomycin	Water	Sigma-Aldrich
Chloramphenicol	Ethanol/Water	Sigma-Aldrich

2.2.2 Chemicals, buffers and enzymes

All chemicals were purchased from Sigma-Aldrich Company Ltd.(Poole, UK) unless otherwise stated and solutions made up with sterile distilled water. Saline was made up with 0.85% (w/v) NaCl and sterilised before use. Buffers were made according to Data for Biochemical Research (1974). All PCR reagents and restriction enzymes were supplied by Promega unless otherwise stated.

2.2.3. Media

Media used in this study were Nutrient agar (NA), Nutrient Broth No.2 (NB), MacConkey agar, Iso-sensitest agar (IST), Lennox L Broth (LB) and Lennox L (LB) agar (GibcoBRL, Life Technologies Ltd., Paisley, UK), NZY broth [1L: 10g NZ amine (casein hydrolase), 5g yeast extract, 5g NaCl (pH7.5), 12.5mL 1M $MgCl_2$, 12.5mL 1M $MgSO_4$, 20mL 20% (w/v) glucose] and Davis Mingioli medium [1L contains: Dipotassium hydrogen phosphate 14.0g, Potassium dihydrogen phosphate 6.0g, trisodium citrate 0.94g, Magnesium sulphate 0.2g, Ammonium sulphate 2.0g] (Davis & Mingioli, 1950).

All media powders were obtained from Oxoid (Basingstoke, UK) and made up with distilled water according to the manufacturer's instructions. Prior to use all media were sterilised by autoclaving at 121°C and 15psi for 15 minutes to destroy all vegetative cells and spores.

2.3 Bacterial growth and maintenance

Bacterial strains were stored at -70°C in nutrient or Lennox broth (plasmid containing strains) supplemented with glycerol to 10% (w/v). *E. coli* strains were subcultured on MacConkey selective agar, whereas strains used in site-directed mutagenesis work were subcultured on LB agar, supplemented with 50 mg/L ampicillin (plasmid containing strains). Bacteria were routinely grown in liquid culture (NB or LB) overnight at 37°C. Temperature sensitive mutants were routinely grown at 30°C (permissive temperature) on LB agar or in LB broth.

2.4 Bacterial Identification

Speciation of isolates was performed by the API20NE system (BioMerieux, Marcy-l'Etoile, France) according to the manufacturer's instructions.

2.5 Antimicrobial Susceptibility Testing (MIC determination)

Antimicrobial susceptibility testing was performed on Iso-sensitest (IST) agar following the British Society for Antimicrobial Chemotherapy (BSAC) guidelines (Phillips *et al.*, 1991). Minimum inhibitory concentrations (MICs) of antibiotics were determined by agar double dilutions of the antimicrobial agent. Antibiotic stock solutions were freshly prepared, and agar was cooled to 50°C before mixing with antibiotic on plates.

Bacterial strains to be tested were inoculated into nutrient broth and incubated overnight in an orbital shaker at 37°C. One hundred-fold dilutions of overnight cultures were made in sterile saline solution [0.85% NaCl], to give an inoculum size of approximately 10^6 colony forming units (cfu) per spot. Diluted culture was inoculated onto plates with a Denley multi-point inoculator (Denley, Billinghurst, Surrey). After drying, inoculated plates were incubated at 37°C for >16 hours. The MIC of the antibiotic was defined as the lowest concentration of antibiotic to inhibit all visible growth.

2.6 Viable Counts

To determine killing activity of a single antibiotic or antibiotic combination against *E. coli* NCTC10418, dose response and time-kill experiments were performed. The medium used for bacterial growth during and subsequent to antibiotic challenge was generally IST broth and agar; however Davis-Mingioli medium and phosphate buffered saline (PBS) [500mL: NaCl 3.4g, Na_2PO_4 0.715g, KH_2PO_4 0.215g] was substituted in some experiments, when analysing the effects of protein synthesis on cell death. Pre-incubation with antibiotics prior to dose-response and time-kill assays was also performed.

2.6.1 Dose response assay

To analyse killing activity of antibiotics at various concentrations dose response experiments were performed. A single colony of *E. coli* NCTC10418 was inoculated into 10mL NB and grown overnight at 37°C. This culture was diluted 100-fold in pre-

warmed NB and grown for 2 hours shaking until log phase. Antibiotic containing IST broths (9.9mL) were inoculated with 100μL aliquots of this culture and incubated for 3h at 37°C. After incubation 10mL of cold (4°C) NB was added to each tube before centrifugation at 3000rpm for 15 minutes in a Sorvall RT6000D bench-top centrifuge. The pellet was re-suspended in 10mL NB. Aliquots of cultures: neat 10^{-2}, 10^{-4}, 10^{-6} dilutions were used to inoculate viable count plates which were incubated at 37°C overnight. Colonies on plates were counted using an Anderman Colony counter (Anderman, Kingston-upon-Thames, UK). Log_{10} (cfu/mL) was plotted against antimicrobial concentration to assess killing activity.

2.6.2 Time-kill assay

To analyse the killing activity of antibiotics over time, time-kill experiments were performed. *E. coli* NCTC10418 was grown overnight in 10mL NB at 37°C. This culture was subsequently diluted 100 fold in pre-warmed NB and grown to log phase shaking at 37°C. One hundred microliter aliquots of this culture were used to inoculate pre-warmed antimicrobial containing IST broths, which were subsequently incubated at 37°C for periods of 0 to 3 hours. Viable counts were performed at hourly intervals utilising the method described for dose response assays, and percentage bacterial survival (% of viable count at time 0) was plotted against time.

2.7 Selection of aminoglycoside resistant mutants

Aminoglycoside resistant mutants were selected following the previously described protocol by Montandon *et al.* (1986). *E. coli* NCTC10418 was grown overnight at

37°C in NB. Two hundred microliter aliquots of this culture were spread onto IST agar plates containing increasing aminoglycoside (streptomycin, gentamicin, neomycin, tobramycin, kanamycin) concentrations. Plates were incubated at 37°C for 48h, and mutants with the highest level of resistance were used to inoculate plates for subsequent series. Mutants were selected in a total of four series. Antibiotic susceptibility of mutants to aminoglycosides was determined via the agar dilution method (section 2.5).

2.8 Mutation detection via the polymerase chain reaction (PCR)

Mutations in target proteins DNA gyrase *gyrA* gene, Topoisomerase IV *parC* gene and ribosomal S12 protein *rpsL* gene were detected by PCR amplification and sequencing (section 2.13). DNA template for PCR reactions was prepared by boiling (section 2.14). All reactions were prepared in 100μL volumes in a Techne Cyclogene Thermal cycler (Cambridge Biosciences, Cambridge), and all products were analysed by agarose gel electrophoresis (section 2.9).

2.8.1 Amplification of the quinolone resistance determining region (QRDR) of *gyrA*

To identify *gyrA* mutations two oligonucleotide primers, 5′-ATGAGCGACCTTGC GAGAGAAATTACACCG-3′ and 5′-TTCCATCAGCCCTTCAATGCTGATG TCTTC-3′ (Yoshida *et al.*, 1988) were used to amplify a 620bp fragment of *gyrA*. Reactions consisted of: 1×PCR buffer (Advanced Biotechnologies, Dorking, Surrey), 2.5mM $MgCl_2$, 200μM each dNPT (Boerhinger-Mannheim, Mannheim, Germany),

10pmoles each primer (Oswel DNA services Ltd., Southhampton), 10μL of boiled DNA and 2U of *Taq* DNA polymerase (Advanced Biotechnologies, Surrey). Cycling parameter were as follows: i) one cycle of 96°C for 30s, 50°C for 60s and 70°C for 90s; ii) 24 cycles of 96°C for 15s, 50°C for 30s and 70°C for 90s; iii) 70°C for 5 min.

2.8.2 Amplification of the QRDR of *parC*

To identify *parC* mutations two oligonucleotide primers 5′-AAACCTGTTCA GCGCCGCATT-3′ and 5′-GTGGTGCCGTTAAGCAAA-3′ (Vila *et al.*, 1996) were used to amplify the fragment of *parC* from nucleotides 115 to 509 (395bp). Reactions consisted of: 1×PCR buffer (Advanced Biotechnologies), 3mM $MgCl_2$ (Advanced Biotechnologies), 100μM each dNTP (Boehringer-Mannheim, Mannheim, Germany), 25pmoles of each primer (Oswel), 10μL of boiled DNA and 2U of *Taq* DNA polymerase (Advanced Biotechnologies, Surrey). Cycling parameter were as follows: i) 30 cycles of 94°C for 60s, 55°C for 60s and 72°C for 60s.

2.8.3 Amplification of *rpsL* gene of 30S ribosomal sub-unit

Oligonucelotide primers 5′-GCAAAAGCTAAAACCAGGAGC and 5′-GGTTCTCC GTTAAGTAAGGCC were used to amplify a 424bp region of ribosomal protein S12 (primers designed using Primer3 software (http://www.genome.wi.mit.edu/cgi-bin/primer). Reactions consisted of: 1×PCR buffer (Advanced Biotechnologies), 2.5mM $MgCl_2$ (Advanced Biotechnologies), 200μM of each dNTP (Boerhinger-Manheim), 25pmoles of each primer (Oswel), 10μL of boiled DNA and 2U of *Taq* DNA polymerase (Advanced Biotechnologies). Cycling parameters were as follows: i)

1 cycle of 96°C for 30s, 50°C for 60s and 70°C for 90s; ii) 24 cycles of 96°C for 15s, 50°C for 30s and 70°C for 90s; iii) 70°C for 5 min.

2.9 Analysis of DNA by agarose gel electrophoresis

Agarose gel electrophoresis of DNA was typically performed on 1.5-4% agarose gels (Gibco, BRL) in TAE buffer [40mM Tris-acetate (pH8.0), 2mM EDTA]. Electrophoresis was performed on horizontal gels utilising the Bio-Rad Sub-Cell® GT agarose gel electrophoresis system. The DNA samples were mixed with loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (w/v) sucrose] at a ratio of 5:1 prior to gel loading. Samples were electrophoresed at a constant voltage between 70 and 100V, for a time length depending on DNA fragment size (~30-120 min). Samples were electrophoresed alongside a suitable molecular weight marker, generally 100bp DNA ladder (Promega) of λ*Hind*III DNA (GibcoBRL, Life Technologies Ltd., Paisley). Following electrophoresis, gels were stained in a 0.5μg/mL ethidium bromide solution and visualised on a UV trans-illuminator (UV products, Cambridge). Gels were photographed with a polaroid camera containing an orange filter. The Bio-Rad gel doc 2000 system was also latterly used to visualise and photograph gels.

2.10 Restriction of PCR amplicons

Restriction endonuclease cutting of PCR products was performed in volumes between 20 and 100μL. These contained the requisite amount of DNA (usually 1-10μg) and the appropriate restriction buffer (Promega) according to the manufacturer's

instructions. Restriction enzyme was usually present in 2-5 fold excess i.e. 2-5U/μg DNA. Final volumes were made up with distilled water and incubated at 37°C for 1-3 hours. Products were analysed by agarose gel electrophoresis (section 2.9).

2.11 Sizing plasmid DNA

The *gyrA* containing plasmid (pPH3) was linearised for sizing with *Xho*I restriction endonuclease digestion (Promega). Restriction was carried out in total volumes of 20μL containing 5μL of plasmid DNA and 10U of restriction endonuclease according to the manufacturer's instructions. Restrictions were incubated at 37°C for 2 hours. Digested and undigested product was electrophoresed alongside λ/*Hind*III DNA (Promega) for accurate sizing of plasmid DNA.

2.12 PCR purification

PCR products were purified using a QIAquick PCR purification kit (Qiagen Ltd, Crawley, UK) according to the manufacturer's instructions. The purified PCR products were eluted from the column into 30μL of elution buffer to concentrate the DNA.

2.13 DNA sequencing

The primers used for PCR amplification were also used for DNA sequencing, with 3.2pmol of primer required per sequencing sample. The DNA content of each sample to be sequenced was determined as described in section 2.15 to ensure that it

contained 30-90ng of DNA. An ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Warrington, UK) was used following the manufacturer's instructions and sequencing cycles were performed on a Techne Cyclogene Dri-Block Thermal Cycler (Cambridge Biosciences). The cycle sequencing program consisting of 25 repetitions of 96°C for 30 seconds, 45°C for 15 seconds and 60°C for 4 minutes. Extension products were purified by the rapid ethanol precipitation method suggested by the sequencing kit manufacturer. Automatic DNA sequencing was determined by Edman degradation performed on an Applied Biosystems Procise Sequencer. Analysis of sequence data was performed with ABIView version 1 or Chromas Version 1.56 software. All results were compared to the published sequences detailed at the European Bioinformatics institute (http:www2.ebi.ac.uk/fasta3/).

2.14 Extraction of chromosomal DNA

Extraction of *E. coli* chromosomal DNA for use in PCR amplification was typically performed by boiling. A single colony of a bacterial strain was inoculated into NB and grown overnight at 37°C. Bacterial cells were centrifuged at 12,000rpm in a microcentrifuge and re-suspended in 50μL of sterile water. The cells were subsequently boiled for 5 minutes to release the DNA. Cell debris was removed by further centrifugation at 12,000rpm and clear lysate containing DNA was used for subsequent PCR template. Cells obtained directly from fresh agar plates (loopful) were also boiled using the same procedure to prepare template. DNA was also

extracted with the Qiagen DNA extraction kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions.

2.15 DNA quantification

A rapid estimation of DNA concentration of PCR products was performed by electrophoresing 1μL of sample DNA alongside 2μL of Lambda/*Hind*III DNA (Promega), each band of which is known to contain a specified DNA concentration. After electrophoresis and ethidium bromide staining the size and thickness of sample bands was compared with Lambda/*Hind*III bands to estimate DNA concentration.

DNA concentration was also measured using absorbance at A_{260} on a Bio-Rad SmartSpec™ 3000.

2.16 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis was carried out to type the clinical isolates by a modification of the method described by Barrett *et al.* (1994). In brief: strains were grown in 10 mL LB broth at 37°C with vigorous shaking to an optical density (O.D.) of 1.2 at 610nm. Cells were washed twice in 75mM NaCl-25mM EDTA and resuspended in the original volume. One half millilitre of the washed bacterial suspension was carefully mixed with 2% low melting grade agarose (Bio-Rad Laboratories, Herts.), and the mixture was dispensed into 1.5mm thick moulds (Bio-Rad). Plugs were allowed to solidify for 20 minutes and then transferred to tubes containing lysis buffer [50mM Tris (pH8.0), 50mM EDTA (pH8.0), 1%N-

Laurylsarcosine, 1mg of proteinase K/mL] and tubes were incubated at 56°C for 4 days. After lysis the clear plugs were washed 6 times in TE buffer [10mM Tris-1mM EDTA (pH8.0)] with 15 minute incubations at 4°C between each wash. Unless used immediately plugs were stored at 4°C in TE buffer. Before digestion with restriction enzyme, 3mm thick slices were removed from plugs and were washed twice with the appropriate restriction enzyme buffer recommended by the manufacturer. Two restriction enzymes were used to distinguish between strains: *Xba*I and *Not*I (Promega). Restriction digests were carried out in a volume of 100µL with 40U of restriction endonuclease and incubated at 37°C overnight. After restriction the reaction was stopped by the addition of 5×Tris-borate EDTA (TBE) buffer (Sigma) followed by further incubation at 4°C overnight. Restriction fragments were separated by electrophoresis through 1% PFGE agarose (Bio-Rad) in 0.5×TBE buffer at 14°C in a CHEF DR-II apparatus (Bio-Rad). The run time for *Xba*I restrictions was 20 hours at 200V, with a linearly ramped pulse time of 5 to 50 seconds. The run time for *Not*I restrictions was 18 hours at 200V, with a pulse time of 10 to 30 seconds. A Lamda ladder (50-1000kb) (Bio-Rad) was electrophoresed alongside samples. Gels were stained with ethidium bromide (0.5µg/mL) for 15 minutes, and destained in 0.5×TBE buffer for 30 minutes prior to UV visualisation. The gels were photographed with the Gel-Doc 2000 system (Bio-Rad).

PFGE patterns were compared visually, and interpreted by the criteria described by Tenover *et al.*, (1995). Isolates were classified as indistinguishable if they had identical PFGE patterns. Isolates with 3 differing bands were considered closely

related, and with 4 to 6 differing bands "possibly related". Isolates were considered unrelated if they differed by seven or more bands.

2.17 Random amplified polymorphic DNA (RAPD) typing

RAPD typing was performed essentially as described by Berg *et al.* (1994), with PCR amplification of random sequences by short oligonucleotide primers 5'-AAG AGCCCGT and 5'-GCGATCCCCA (Oswel DNA services Ltd., Southampton). Reactions were carried out in 100μL volumes and consisted of: 1×PCR buffer (Advanced Biotechnologies, Dorking, Surrey), 3mM $MgCl_2$ (Advanced Biotechnologies), 0.01% gelatin, 200μM each dNTP (Boehringer-Mannheim, Germany), 200pmoles of each primer (Oswel) and 10μL of a 10 fold dilution of boiled *E. coli* (section 2.14). PCR was carried out in a Techne cyclogene thermal cycler (Cambridge Bioscience, Cambridge) with the following cycling parameters: i) 96°C for 4 min; ii) 40 cycles of 94°C for 1min, 36°C for 1min and 72°C for 2 min and iii) 72°C for 5min. PCR products were separated by agarose gel electrophoresis (section 2.9) and analysed for similarity by visual comparison of bands.

2.18 Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR)

The clonal diversity of *E. coli* isolates was studied by amplification of template DNA with primers for enterobacterial intergenic consensus (ERIC) sequences as described by Dalla-Costa *et al.* (1998). PCR was performed with the following primer

sequences: 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and 5′-AAGTAAGTGACT GGGGTGAGCG-3′ (Dalla-Costa *et al.*, 1998). Each 50μL reaction mixture contained: 1×PCR buffer (Promega), 4mM $MgCl_2$ (Promega), 100μM each dNTP (Promega), 100pmoles of each primer (Interactiva), 50ηg DNA(10μL boiled DNA) (section 2.14), and 2U of *Taq* DNA polymerase (Promega). PCR amplification was carried out in a GeneAmp 9700 PCR system (PE Applied Biosystems, California) with the protocol: i) 95°C for 5min; ii) 35 cycles of 94°C for 45s, 52°C for 60s, 70°C for 10min and iii) 70°C for 20min. PCR products were separated by agarose gel electrophoresis (section 2.9), and banding patterns visually compared.

2.19 Amplified fragment length polymorphism (AFLP)

E. coli isolates were typed by the DNA fingerprinting method AFLP, with a modification of the protocol described by Zhao *et al.* (2000) and the manufacturer's kit (Perkin Elmer/Applied Biosystems Division, Foster City, CA). Genomic DNA was extracted using the Qiagen DNA extraction kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions. The AFLP protocol involved 3 steps 1) digestion of total genomic DNA with restriction endonucleases and ligation of oligonucleotide adaptors, 2) selective amplification of sets of restriction fragments by PCR using fluorescently labelled primers and 3) separation of amplified DNA fragments of denaturing polyacylamide sequencing gels.

Table 2.3: Adaptor and primer sequences used in AFLP

Enzyme	Cut site	Adaptor	Primer Core
***Eco*RI**	G/AATTC	5′-CTCGTAGACTGCGTACC 3′-CTGACGCATGGTTAA	5′-GACTGCGTACCAATTCE
***Mse*I**	T/TAA	5′-GACGATGAGTCCTGAG 3′-CTACTCAGGACTGAT	5′-GATGAGTCCTGAGTAAE

2.19.1 DNA digestion and adaptor ligation

DNA restriction enzyme digestion and ligation of adapters to compatible overhanging ends of cleaved DNAs was performed in a single step. Reactions were performed in 11μL volumes containing: 50ηg DNA, 5U *Eco*RI (Promega), 1U of *Mse*I (Promega), 1×T4 ligase buffer (Promega), 50mM NaCl, 50μg/mL bovine serum albumin (BSA), 1U T4 ligase (Promega), 0.04μM *Eco*RI and 0.4μM *Mse*I specific adaptor pairs. The reactions were incubated overnight at room temperature and then diluted 20× with TE buffer [1mM Tri-HCl. 0.1mM EDTA, pH 8.0]. Products were electophoresed to check for adequate restriction and sufficient DNA.

2.19.2 PCR amplification

*Eco*RI primers were labelled with fluorescent dyes: 6-carboxyfluorescein (FAM;blue), 2′,7′-dimethoxy-4′,5′-dichloro-6-carboxyfluorescein (JOE;green) or N,N,N′,N′-tetramethyl-6-carboxy-rhodamine (TAMRA;yellow). Three primer combinations were used to amplify restricted products: *Eco*RI + 0 JOE/*Mse*I+CG, *Eco*RI + A FAM/ *Mse*I + CA and *Eco*RI + C TAMRA/ *Mse*I + C. PCR reactions were carried out in 20μL volumes and consisted of: 3μL of digested ligation reaction, *Eco*RI (0.05μM)

and *Mse*I (0.25μM), 0.5U *Taq* DNA polymerase (Promega), 1.5mM $MgCl_2$ (Promega), 200μM dNTPs (Promega) and 1×PCR buffer. Temperature cycling was performed on a GeneAmp PCR system 9600 (Perkin-Elmer) using the following protocol: i) 94°C for 2min, 65°C for 30s and 72°C for 2min; ii) 94°C for 20s, 64°C for 30s and 72°C for 2min. Subsequently the annealing temperature was reduced from 63°C to 56°C in increments of 1°C for each cycle. For reactions where *Eco*RI was labelled with FAM or JOE, 23 cycles were performed at the lower annealing temperature (56°C), whereas 25 cycles were performed for reactions containing TAMRA labelled primers. PCR products from single reactions using the same DNA template but different AFLP primer sets were pooled in the following proportion: 5μL FAM, 7.5μL JOE and 10μL TAMRA labelled products. DNA was precipitated by addition of 3 volumes of cold absolute ethanol and incubation at -70°C for 10 min, followed by centrifugation at 12,000rpm in a benchtop centrifuge to obtain a pellet. The pellet was dried and resuspended in 5μL of HPLC grade water ready for electrophoresis.

2.19.3 Electrophoresis

Electrophoresis was performed in 1×TBE buffer (Sigma) at constant voltage (2500V) for 4h at 51°C on an automated DNA sequencer (Perkin-Elmer/Applied Biosystems) equipped with GeneScan Analysis software (version 2.1) (Perkin Elmer/Applied Biosystems).

2.20 Site-directed mutagenesis of DNA gyrase Ser83 and Asp87

Site-directed mutagenesis was carried out with the QuikChange™ Site-directed mutagensis kit according to the manufacturer's instructions (Stratagene, UK). All reagents, competent cells and plasmids were supplied by the stratagene kit unless otherwise stated.

2.20.1 Primer design

Primers used for mutagensis are shown in table 2.4. They were designed to be between 25 and 45 bases in length with a melting temperature (Tm) of greater or equal to 78°C according to the following formula:

$$Tm = 81.5 + 0.41\ (\%GC) - 675/N - \%\ \text{mismatch}$$

where N is the primer length in base pairs. Primers were obtained from Interactiva Biotechnologie (Hybaid Ltd., Middlesex, UK) and were PAGE purified.

Table 2.4: Mutagenic *gyrA* Ser83 and Asp87 primers

Primer	Sequence	Base Change	Amino-acid change
Ser83 5`	CATGGTGACT**T**GGCGGTCTATGACAC	C→G	Ser(TCG)→Leu (TTG)
Ser83 3`	GTCTCATAGACCGCC**A**AGTCACCATG	G→A	Ser(AGC)→Leu(AAC)
Asp87 5`	TCGCGCGTCTAT**A**ACACGATTGTCCG	G→A	Asp(GAC)→Asn(AAC)
Asp87 3`	CGGACAATCGTG**T**TATAGACGGCGGA	C→T	Asp(CTG)→Asn(TTG)

- Mutation sites are shown in bold

2.20.2 PCR amplification

PCR reactions were carried out in a total volume of 50μL prepared in 0.5mL polypropylene tubes (Alpha laboratories) with a Techne Genius Dri-Block cycler (Cambridge Biolabs, Cambs). PCR amplifications consisted of 1×PCR reaction buffer (Stratagene, UK), 5-50ng of dsDNA plasmid template (pH3 *gyrA*, From A. Maxwell), 125 ng of each primer (Interactiva), 100μM each dNTP (Stratagene), and ultrapure water to a final volume of 50μL. *PfuTurbo*™ (Stratagene) was then added at 2.5 U per reaction. The cycling parameters are shown in table 2.5.

Table 2.5: Cycling parameter for plasmid amplification

Segment	Cycles	Temperature	Time
1	1	95°C	30s
2	16	95°C	30s
		55°C	1min
		68°C	16min

To check for sufficient amplification 10μL of the amplified product was electrophoresed on a 1.5% agarose gel (section 2.9).

2.20.3 Digestion with *Dpn*I

PCR Products were digested with 10U of *Dpn*I restriction enzyme per sample reaction. The solution was mixed by pipetting, spun down in a micro-centrifuge for 1min, and then incubated at 37°C for 1h to digest the parental (non-mutated) supercoiled dsDNA.

2.20.4 Transformation of Epicurian Coli ® XL1-Blue supercompetent cells

For transformation into Epicurian Coli ® cells, cells were initially gently thawed on ice for 2min. For each sample to be transformed, 50μL of competent cells were aliquotted into prechilled glass tubes. *Dpn*I treated DNA (1μL) was then added, and the reactions gently swirled before incubation on ice for 30min. The control plasmid pUC18 (0.1ng/μL) was also added to an aliquot of the competent cells to test transformation efficiency. The transformation reactions were heat pulsed for 45s at 42°C in a water bath and then placed on ice for 2min. Preheated NZY broth (0.5mL) was added to each reaction and reactions were then incubated at 37°C for 1h shaking at 225-250rpm. The control and the sample reactions were then plated on LBA agar supplemented with 10% (w/v) X-gal and 0.1mM IPTG. Transformation plates were incubated at 37°C for >16hours. Transformation efficiency was calculated by analysing the number of blue colonies on X-gal plates containing pUC18 transformants.

2.21 Extraction of plasmid DNA

Small scale plasmid extraction was performed using the Hybaid Recovery™ Plasmid Prep kit (Hybaid Ltd., Middlesex, UK) according to the manufacturer's instructions. The extractions yielded approximately 50μg of plasmid DNA.

2.22 Preparation of competent cells

Cells of the *E. coli gyrA* ^{ts} mutant KNK453 (supplied by A. Maxwell, University of Leicester) were made competent ready for transformation with mutageneised plasmid. A single bacterial colony was inoculated into 10mL LB broth and grown overnight at 30°C (permissive temperature) shaking. This culture was then diluted 100 fold in LB broth and grown at 30°C shaking until the O.D at A600 was 0.6. The cells were placed on ice to cool rapidly and centrifuged in a Sorvall RC5B centrifuge (5000g) at 4.2K for 20 minutes at 2°C. Cells were re-suspended in TFI buffer [30mM KoAc, 10mM $CaCl_2$-$2H_2O$, 100mM KCl, 15% glycerol, 50mM $MnCl_2$] by gentle shaking. Cells were re-centrifuged at 4.2K for 8 minutes at 2°C, and resuspended in 4 mL of TFBII buffer [75mM $CaCl_2$, 10mM KCl, 15% glycerol, 10mM Na-MOPs pH 7.0]. Cells were aliquotted into prechilled microfuge tubes and stored at -70°C.

2.23 Transformation of *gyrA* ^{ts} *Escherichia coli*

Competent KNK453 cells were transformed with mutagenised plasmid. Cells were slowly thawed on ice until just defrosted. One hundred microliter aliquots of cells were then dispensed into pre-chilled glass tubes. Approximately 10ng of plasmid DNA was added for the transformation and the tubes were incubated on ice for 30minutes. Samples were the heat shocked at 42°C for 60 seconds, and placed back on ice. One millilitre of pre-warmed LB broth (30°C) was added to each tube, and they were incubated at 30°C for 2h shaking. Control transformations with pUC18 plasmid DNA to test the competent cells were performed in the same manner.

2.24 Analysis of fluoroquinolone resistance phenotype in temperature-sensitive mutants

Expression of plasmid *gyrA* in KNK453 *E. coli* $gyrA^{ts}$ mutants was confirmed by plating transformants at 42°C in the presence and absence of the inducer IPTG. Control plates at 30°C were also performed. Fluoroquinolone resistance conferred by the mutant plasmid was determined by a modification of the susceptibility testing method described in section 2.5. Cells were grown overnight at 30°C in LBA broth. Dilutions were made of cell cultures and used to inoculate antibiotic containing LB amp agar plates, supplemented with 0.1mM IPTG. Cells were grown at 42°C (non-permissive temperature) for >24 hours. Control plates were also incubated at 30°C (permissive temperature). The MIC was determined as the lowest antibiotic concentration to inhibit all visible growth.

3. Results: Clinical fluoroquinolone resistance

3.1 An Overview

An outbreak of ciprofloxacin resistant *Enterobacteriaceae* in a hospital in Newcastle, provided a unique opportunity to study acquisition of fluoroquinolone resistance in the clinic. Fifteen isolates were collected, identified as *Escherichia coli* by the API 20 NE system and designated NC-1-15 for the purpose of this study.

Antibiotic susceptibility to fluoroquinolones and other drug classes was determined by the agar dilution method (see section 2.5). In order to assess whether this outbreak was caused by clonal spread of resistant isolates or individual emergence of resistance, the clonal diversity of isolates was determined by PCR and restriction-based fingerprinting techniques (section 2.16-19). Alterations in target proteins were identified by PCR amplification and DNA sequencing (2.8 & 2.13) of target proteins (DNA gyrase *gyrA*/Topoisomerase IV *parC*).

3.2 Antibiotic Susceptibility

The MICs of ciprofloxacin, sparfloxacin, moxifloxacin and gemifloxacin against the 15 clinical *E. coli* isolates NC-1-15 are shown in Table 3.1.

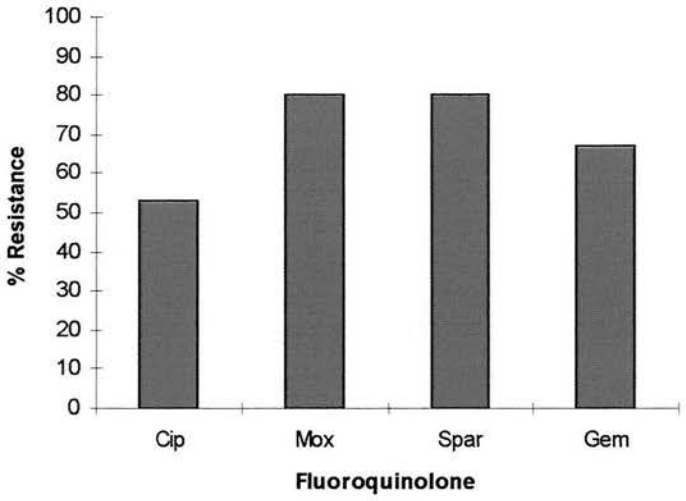
Table 3.1: Fluoroquinolone susceptibility of clinical isolates NC-1-15 (MIC mg/L)

Isolate	Ciprofloxacin	Moxifloxacin	Sparfloxacin	Gemifloxacin
NC-1	128	64	64	128
NC-2	0.25	2	2	1
NC-3	0.25	1	1	1
NC-4	32	2	2	1
NC-5	1	8	4	4
NC-6	1	8	32	8
NC-7	0.015	0.5	0.128	0.128
NC-8	1	8	4	4
NC-9	0.25	1	1	1
NC-10	128	64	64	128
NC-11	32	64	32	32
NC-12	32	64	32	16
NC-13	8	16	8	16
NC-14	256	128	96	256
NC-15	8	32	32	16
NCTC10418	0.015	0.015	0.016	0.015
Break point	2	2	2	2

Cross-resistance was seen between the high-level fluoroquinolone resistant isolates, with ciprofloxacin-resistant isolates being additionally resistant to moxifloxacin, sparfloxacin and gemifloxacin (Table 3.1). Of the clinical isolates, 8/15 (53%) were resistant to ciprofloxacin with MICs ranging from 8 to 256mg/L. Six of these isolates had ciprofloxacin MICs of ≥32mg/L, an unusually high level of fluoroquinolone

resistance in *Escherichia coli.* Twelve isolates (80%) were resistant to moxifloxacin, a gram-positive targeted fluoroquinolone, 12 isolates (80%) to sparfloxacin, and 10 isolates (67%) to gemifloxacin, a new napthyridone derivative (Figure 3.1).

Figure 3.1: Percentage of isolates resistant to ciprofloxacin, sparfloxacin, moxifloxacin and gemifloxacin



Resistance to the fluoroquinolones was high, with a higher percentage of isolates resistant to moxifloxacin and sparfloxacin than to gemifloxacin and ciprofloxacin, possibly due to the difference in target proteins within drugs. These isolates had only been previously exposed to ciprofloxacin in the clinic and therefore the resistance mechanisms seem to be ubiquitous among the fluoroquinolones. The newer fluoroquinolone gemifloxacin, which has been shown to have increased potency against *Enterbacteriaceae* in comparison with ciprofloxacin, did not show enhanced activity against resistant isolates. In fact 67% isolates were resistant to gemifloxacin in comparison with 53% to ciprofloxacin.

The MICs of trimethoprim, amoxycillin, amox/clav, gentamicin, meropenem and ceftazidime were determined to examine the possibility that the isolates had a multi-resistant phenotype (Table 3.2).

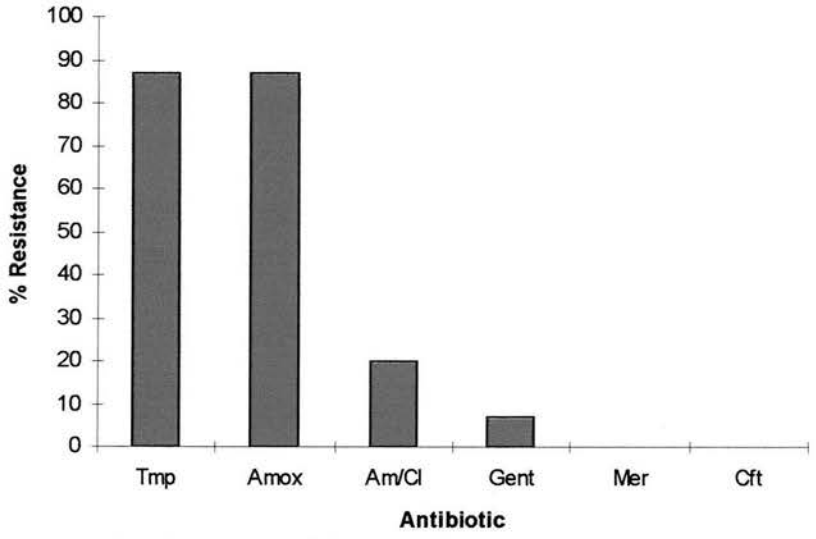
Table 3.2: Susceptibility of clinical isolates NC-1-15 to trimethoprim, amoxycillin, co-amoxiclav, gentamicin, meropenem and ceftazidime

Isolate	Cip	Trim	Amox	Amox/ Clav	Gent	Mer	Cft
NC-1	128	32	128	8	0.5	0.12	1
NC-2	0.25	32	8	4	0.5	0.12	1
NC-3	0.25	32	128	8	0.5	0.12	1
NC-4	32	32	128	16	0.5	0.12	1
NC-5	1	32	128	8	16	0.12	1
NC-6	1	32	128	8	0.5	0.12	1
NC-7	0.015	1	128	16	0.5	0.12	1
NC-8	1	32	128	8	0.5	0.12	1
NC-9	0.25	32	128	32	0.5	0.12	1
NC-10	128	32	128	4	0.5	0.12	1
NC-11	32	32	128	8	0.5	0.12	1
NC-12	32	32	128	8	0.5	0.12	1
NC-13	8	32	128	8	0.5	0.12	1
NC-14	256	32	128	8	0.5	0.12	1
NC-15	8	32	8	4	0.5	0.12	1
NCTC10418	0.06	1	4	4	2	0.12	1
Break point	2	2	8	8	4	4	2

All of the ciprofloxacin resistant isolates showed resistance to, at least one additional antibiotic (trimethoprim), certain isolates to two additional antibiotics (trimethoprim & amoxycillin), and isolate NC-4 showed resistance to three additional antibiotics

(trimethoprim, amoxycillin and amox/clav), thereby demonstrating a multi-resistant phenotype. Ciprofloxacin sensitive isolates NC-9 and 7 were resistant to trimethoprim and amoxycillin. Thirteen (87%) isolates were resistant to trimethoprim (Tmp), 13 isolates (87%) to amoxycillin, 3 isolates (20%) to co-amoxiclav (Amox/Clav) combination, and 1 isolate (7%) to gentamicin (Gent). All isolates were sensitive to meropenem (Mer) and ceftazidime (Cft) (Figure 3.2).

Figure 3.2: Proportion of isolates resistant to trimethoprim, amoxicillin, co-amoxiclav, gentamicin, ceftazidime and meropenem



Am/Cl = co-amoxiclav

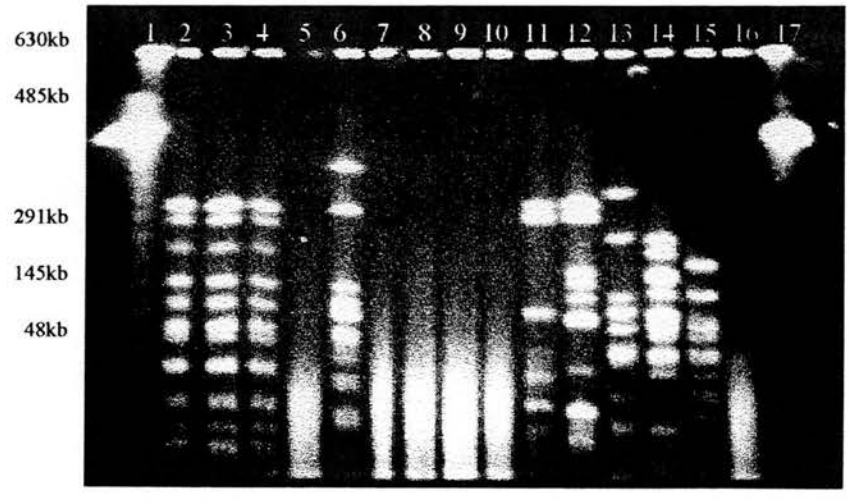
3.3 Isolate diversity

In order to determine whether emergence of resistant isolates was due to 1) independent selection of resistance in individual patients, 2) clonal spread of resistant strains, or 3) a combination of both, the genotypes of the isolates NC-1-15 were determined.

3.3.1 PFGE typing

PFGE analysis was initially performed with restriction enzyme *Xba*I. Figure 3.3 shows pulsotypes of NC-1-15.

Figure 3.3: PFGE of NC-1-15 with *Xba*I

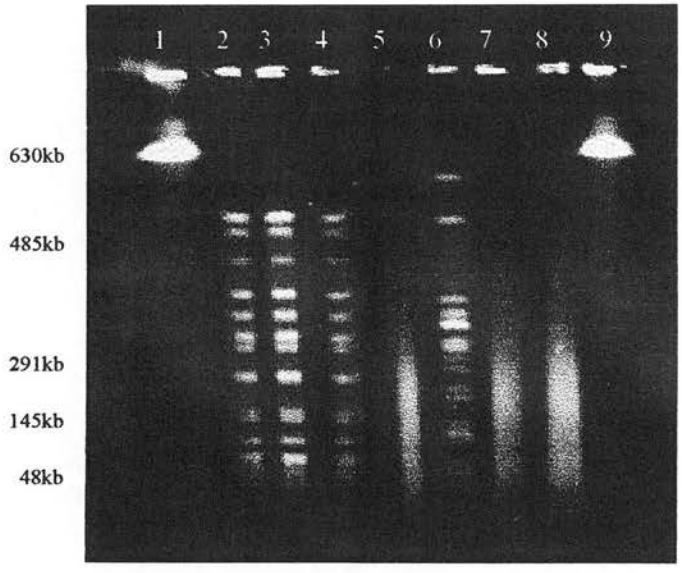


Lane:1 - DNA lambda Ladder; 2 - NC-14; 3 - NC-1; 4 - NC-10; 5 - NC-4; 6 - NC-5; 7 - NC-11; 8 - NC-8; 9 - NC-13; 10 - NC-6; 11 - NC-9; 12 - NC-15; 13 - NC-12; 14 - NC-2; 15 - NC-3; 16 - NC-7; 17 - DNA lambda Ladder

*Xba*I restriction enzyme is the enzyme of choice for typing *Enterobacteriaceae*, however this failed to yield pulsotypes for 6/15 isolates (Figure 3.3). Untypable

isolates produced smeared DNA after agarose gel electrophoresis, indicating the possibility of DNA breakdown during the PFGE procedure prior to restriction. In an attempt to eliminate this effect and type isolates, PFGE parameters were altered. Increased lysis time, increased washing and doubling restriction enzyme concentration, along with use of enzymes to eliminate endonucleases, did not however eliminate the problem of smearing.

Figure 3.4: PFGE of 7 isolates with *Xba*I

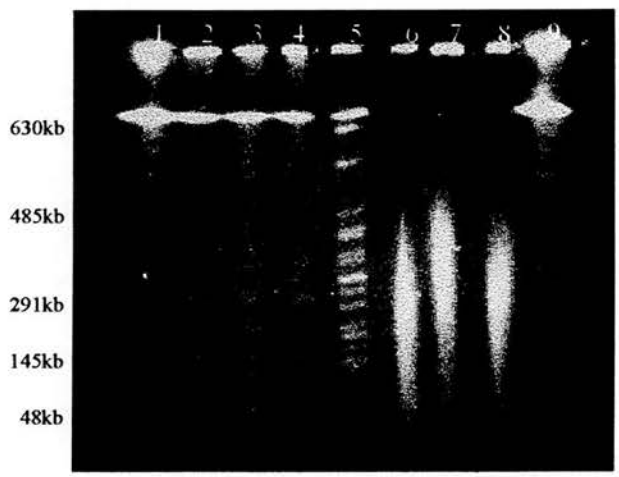


Lane: 1 - DNA lambda ladder; 2 - NC-14; 3 - NC-1; 4 - NC-10; 5 - NC-13; 6 - NC-5; 7 - NC-11; 8 - NC-8; 9 - DNA lambda ladder

Pulsotypes were obtained for 9 isolates with *Xba*I, with isolates NC-14, NC-10 and NC-1 exhibiting identical PFGE restriction patterns (PFGE type: A). The remaining 6 isolates typed were heterogeneous in nature (PFGE types: B-H), differing by 7 of more bands and therefore deemed unrelated (Table 3.3). PFGE restriction with a second enzyme *Not*I was attempted in order to type isolates which could not be typed

by *Xba*I restriction. Interestingly isolates NC-8, NC-13 and NC-11, which produced smeared DNA after *Xba*I restriction, also produced smeared DNA after *Not*I restriction (Figure 3.5). Isolate NC-5 was the only isolate of 7 restricted to produce a PFGE restriction pattern with *Not*I. Isolates NC-14, NC-10 and NC-1 which were typed by *Xba*I restriction were only partially restricted by *Not*I (Figure 3.5). Doubling the restriction enzyme concentration failed to eliminate this problem.

Figure 3.5: PFGE of 7 isolates with *Not*I



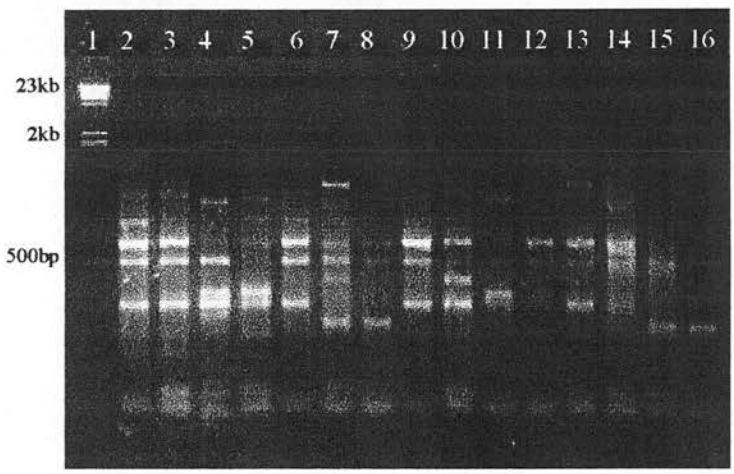
Lane: 1 - DNA ladder; 2 - NC-14; 3 - NC-1; 4 - NC-10; 5 - NC-5; 6 - NC-13; 7 - NC-11; 8 - NC-8; 9 - DNA lambda ladder

The failure to type 6/15 clinical isolates by PFGE led to the use of PCR-based typing methods of RAPD and ERIC-PCR to further differentiate isolates.

3.3.2 RAPD-PCR

DNA from NC-1-15 was amplified using short random primers to give RAPD banding patterns (Figure 3.6).

Figure 3.6: RAPD banding patterns of NC-1-15



Lane: 1 - λ*Hind*III DNA ladder; 2 - NC-1; 3 - NC-13; 4 - NC-3; 5 - NC-7; 6 - NC-10; 7 - NC-11; 8 - NC-6; 9 - NC-14; 10 - NC-12; 11 - NC-2; 12 - NC-15; 13 - NC-4; 14 - NC-9; 15 - NC - 8; 16 - NC-5

Isolates NC-14, NC-10 and NC-1 which had been deemed identical by PFGE, had identical RAPD patterns (RAPD type: RI) A further two isolates NC-4, and NC-13, which had been untypable by PFGE were related to RI isolates with one band difference in RAPD pattern (RAPD type: RI^1). Isolates NC-11, NC-8, NC-6 and NC-5 had identical RAPD patterns (RAPD type: RII). NC-11, NC-8 and NC-6 had been untypable by PFGE, however NC-5 was PFGE type G. The remaining isolates were unrelated (RAPD type: RIII to RVII), supporting results from PFGE typing.

3.3.3 ERIC-PCR typing

A second PCR typing technique was employed to type strains and confirm RAPD results, as PCR based techniques may lack the reproducibility and reliability of PFGE. Clinical isolates were typed by ERIC-PCR, a technique based on the amplification of the intergenic consensus sequence of isolates (Figure 3.7).

Figure 3.7: ERIC-PCR patterns of NC-1-15

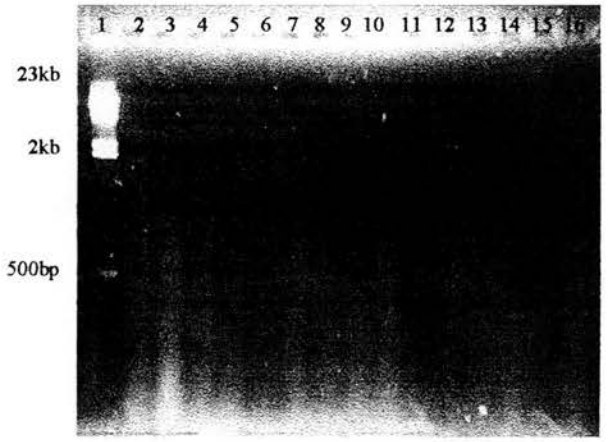
Lane: 1 100bp DNA ladder; 2 - NC-14; 3 - NC-10; 4 - NC-1; 5 - NC-4; 6 - NC-13; 7 - NC-5; 8 - NC-8; 9 - NC-11; 10 - NC-6; 11 - NC-9; 12 - NC-15; 13 - NC-12; 14 - NC-2; 15 - NC - 3; 16 - NC-7

Isolates NC-14, NC-10 and NC-1 produced identical ERIC pattern (ERIC type: EI), supporting results from RAPD-PCR (Type: RI) and PFGE (Type: A). Isolates NC-4 and NC-13 which were also typed Type I by RAPD-PCR, showed 2-3 band variations with ERIC-PCR (ERIC type: I^2 and I^3), demonstrating that they are related but not identical. Isolates NC-11, NC-8 and NC-5 (ERIC type: II) had identical ERIC patterns, supporting RAPD results. Isolate NC-20 which had been identical to NC-8, NC-5 and NC-11 by RAPD-PCR, gave a 1 band difference when typed by ERIC (ERIC type: II^1). All other isolates were unrelated (ERIC type: III to VIII), supporting RAPD and PFGE results.

3.3.4 AFLP typing

To overcome the small discrepancies between ERIC and RAPD-PCR techniques, AFLP typing was performed. AFLP typing was based on restriction of DNA extracts with a mixture of restriction enzymes, adaptor ligation and PCR amplification with fluorescently labelled primers. Figure 3.7 shows the results of DNA digestion with the restriction enzyme mixture, with restricted DNA seen a smear on the agarose gel.

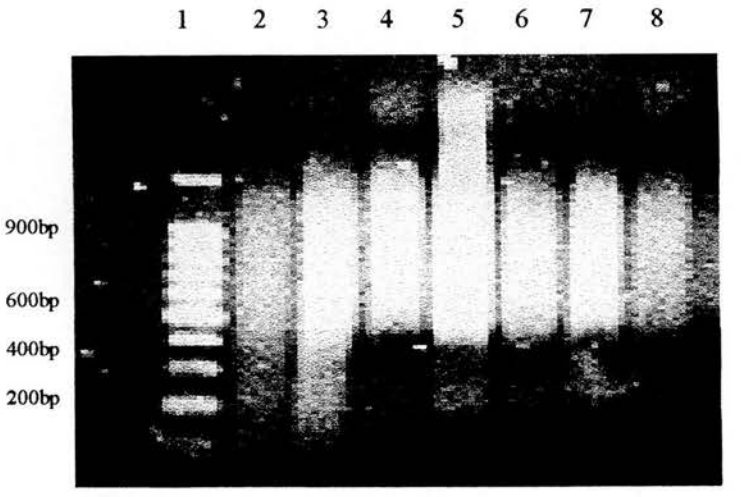
Figure 3.8: Restriction of NC-1-15 with enzyme mix



Lane: 1 - λ*Hind*III DNA ladder; 2 - NC-1; 3 - NC-13; 4 - NC-3; 5 - NC-7; 6 - NC-10; 7 - NC-11; 8 - NC-6; 9 - NC-14; 10 - NC-12; 11 - NC-2; 12 - NC-15; 13 - NC-4; 14 - NC-9; 15 - NC - 8; 16 - NC-5

PCR amplification of restricted products produced a smear of DNA products (Figure 3.8)

Figure 3.9: AFLP PCR products



Lane: 1 - 100bp DNA ladder; 2 - NC-1; 3 - NC-13; 4 - NC-3; 5 - NC-7; 6 - NC-10;7 - NC-11; 8 - NC-6

After running on GeneScan analysis software, no products could be seen, although fluorescent primers could be seen at the bottom of the gel. The reason for this failure is not clear. Figure 3.7 shows that there was adequate restriction of DNA, leading to the theory that there were problems with the PCR amplification step. Products were, however, produced after PCR amplification (Figure 3.9), and therefore it was unexpected that fluorescent products were not detected by GeneScan. The PCR reaction conditions were altered, using alternative *Taq* polymerase, and varying DNA starting concentrations and $MgCl_2$ concentrations along with fresh preparation of all reagents. Theses measures did not however affect the result, with only fluorescent primers seen after GeneScan analysis.

3.3.5 Summary of PFGE, RAPD and ERIC PCR typing results

Table 3.3 shows the results from the PFGE, RAPD and ERIC-PCR typing for NC-1-15.

Table 3.3: Summary of results from PFGE, RAPD and ERIC typing

Isolate	Ciprofloxacin MIC(mg/L)	Type		
		PFGE	RAPD	ERIC
NC-14	256	A	R-I	E-I
NC-10	128	A	R-I	E-I
NC-1	128	A	R-I	E-I
NC-4	32	UD	R-I[1]	E-I[2]
NC-13	8	UD	R-I[1]	E-I[3]
NC-11	32	UD	R-II	E-II
NC-8	1	UD	R-II	E-II
NC-6	1	UD	R-II	E-II
NC-7	0.015	UD	R-III	E-III
NC-9	0.25	B	R-IV	E-IV
NC-15	8	C	R-V	E-V
NC-12	32	D	R-VI	E-VI
NC-2	0.25	E	R-VII	E-VII
NC-3	0.25	F	R-VIII	E-VIII
NC-5	1	G	R-II	E-II

UD = Undetermined

PCR-based typing techniques of RAPD and ERIC-PCR were the most successful in typing isolates. With the exception of isolates NC-15 and NC-12, resistant isolates were typed into two groups with related isolates NC-14, NC-10, NC-1, NC-4 and NC-13 designated TYPE I, and related isolates NC-11, NC-8, NC-6 and NC-5 designated TYPE II, based on PFGE, RAPD and ERIC-PCR. Variations in MICs between related isolates provided the unique opportunity to study resistance

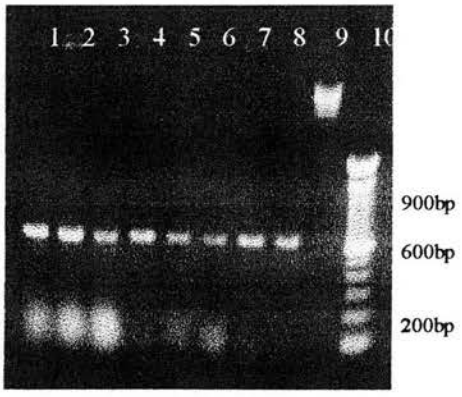
acquisition in isogenic clinical isolates, to compare with data previously derived solely from *in vitro* based studies.

3.4 PCR amplification of the QRDR of *E. coli gyrA* and *parC*

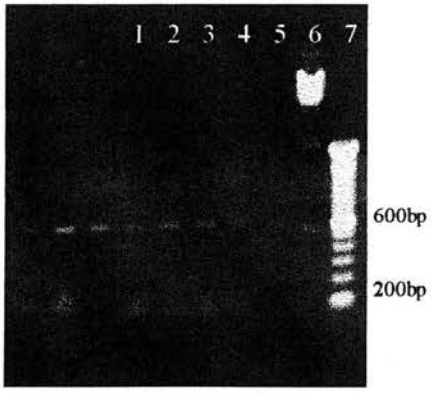
3.4.1 DNA gyrase *gyrA*

Fluoroquinolone resistance conferring mutations are known to occur in a region of DNA gyrase known as the quinolone resistance determining region (QRDR). To screen for mutations occurring within this region a 623bp fragment incorporating the 117bp QRDR was amplified for the 15 clinical isolates NC-1-15 (Figure 3.10).

Figure 3.10: PCR amplification of a 623bp region containing the QRDR of DNA gyrase *gyrA*



Lane: 1 - NC-2; 2 - NC-3; 3 - NC-9; 4 - NC-8; 5 - NC-5; 6 - NC-7; 7 - NC-6; 8 - NC-13; 9 - λ*Hind*III DNA; 10 - 100bp DNA ladder

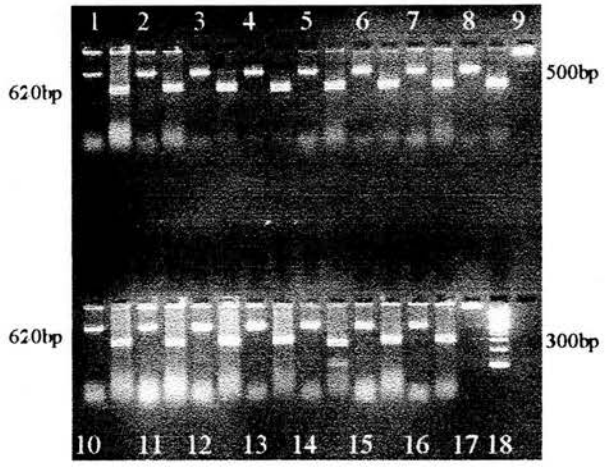


Lane: 1 - NC-4; 2 - NC-15; 3 - NC-12; 4 - NC-14; 5 -NC- 11; 6 - NC-10; 7 - NC-13; 8 - NC-1; 9 - λ*Hind*III DNA; 10 - 100bp DNA ladder

Certain common *gyrA* mutations conferring quinolone resistance, for example resulting in a substitution of amino-acid Ser83 in the QRDR of *gyrA*, result in the loss of a *Hinf*I G/ANTC restriction site. *Hinf*I digestion was therefore used to screen for the presence of this mutation in the clinical isolates.

Restriction fragment length polymorphism's (RFLPs) were carried out on the amplified 623bp amplicons of *E. coli* NC-1-15, with restriction endonuclease *Hinf*I (Figure 3.11).

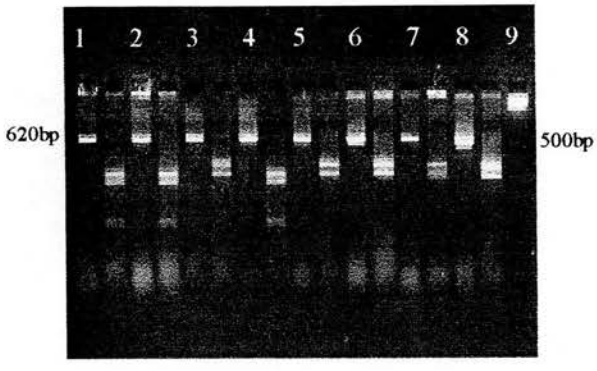
Figure 3.11: Restriction fragment length polymorphism's of the 623bp section of *gyrA* of NC-1-15



Lanes containing unrestricted DNA and size markers have been labelled, corresponding *Hinf*I digested DNA for each isolate follows unrestricted DNA. Lane: 1 - NC-14; 2 - NC-6; 3 - NC-10; 4 - NC-4; 5 - NC-15; 6 - NC-2; 7 NC-11; 8 - NC-1; 9 - λ*Hind*III DNA marker; 10 - NC-9; 11 - NC-12; 12 - NC-5; 13 - NC-8; 14 - NC-7; 15 - NC-13; 16 - NC-3; 17 - λ*Hind*III DNA marker; 18 - 100bp DNA ladder

A control RFLP experiment was performed with 1) quinolone resistant *E. coli* known to possess a GyrA change, 2) quinolone resistant *E. coli* with no GyrA change and 3)sensitive *E. coli.* To allow comparison between restriction fragment patterns these controls were analysed alongside representative isolates of varying ciprofloxacin MICs 0.015, 0.25, 1, 8, and 32 mg/L (Figure 3.12). *Hinf*I restricted DNA was run alongside unrestricted DNA on agarose gels.

Figure 3.12: RFLPs of the 623bp *gyrA* of *E. coli* control strains (with and without Ser83 mutation) and clinical isolates with ciprofloxacin MICs 0.015, 0.25, 1, 8 and 32 mg/L



Lanes containing unrestricted DNA and size markers have been labelled, corresponding *Hinf*I digested DNA follows unrestricted DNA. Lane: 1 - sensitive *E. coli* with no *gyrA* mutation; 2 - resistant *E .coli* without Ser83 mutation; 3 - Ciprofloxacin resistant *E. coli* with a *gyrA* Ser83 mutation; 4 - NC-7 (MIC=0.015mg/L); 5 - NC-11 (MIC= 32mg/L); 6 - NC-13 (MIC=8mg/L); 7 - NC-6 (MIC=1mg/L); 8 - NC-12 (MIC=0.25mg/L); 9 - λ*Hind*III DNA

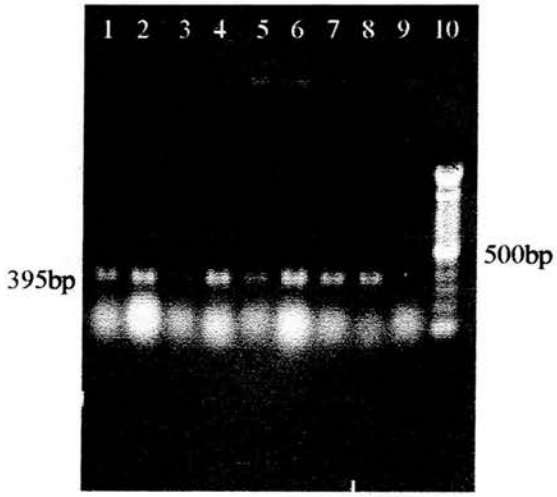
Control *E. coli* isolates containing no *gyrA* mutation at Ser83 produced three fragments upon restriction with *Hinf*I restriction endonuclease, confirming that there was no loss of restriction site (Figure 3.12). Control *E. coli* containing a Ser83 mutation produced only two fragments upon restriction, demonstrating the loss of the *Hinf*I restriction site G/ANTC (Figure 3.12) Fourteen of the 15 clinical isolates showed loss of the *Hinf*I restriction site (2 fragments), indicating the presence of *gyrA* mutations at Ser83 in the QRDR of *gyrA* (Figure 3.11). Only one isolate NC-7 produced three bands, retaining the *Hinf*I restriction site, and correlating with controls with no *gyrA* mutations, indicating that this isolate did not possess the Ser83 *gyrA* mutation. This may be expected as this isolate is highly sensitive to ciprofloxacin with an MIC of 0.015mg/L. However we cannot conclude that ciprofloxacin sensitive

strains with low MICs will contain no Ser83 changes, as these results indicate that strains with ciprofloxacin MICs as low as 0.25mg/L contain *gyrA* changes.

3.4.2 DNA topoisomerase IV *parC*

Fluoroquinolone resistance conferring mutations have been described in the enzyme Topoisomerase IV *parC* in a region equivalent to the QRDR of DNA gyrase *gyrA*. The *parC* gene of NC-1-15 was analysed for mutations by PCR amplification of a 395bp region incorporating the QRDR (Figure 3.13), and subsequent DNA sequencing (see section 3.5.2).

Figure 3.13: PCR amplification of a 395bp region of *parC*



Lane: 1 - NC-14; 2 - NC-13; 3 - NC-4; 4 - NC-1; 5 - NC-10; 6 - NC-11; 7 - NC-8; 8 - no DNA; 9 - 100bp DNA ladder

Figure 3.13 shows *parC* amplicons for eight clinical isolates; *parC* amplicons were obtained for all 15 clinical isolates in the same way. The bands seen below the amplified product are primer dimers, deduced as such as they are still present in the

control sample with no DNA, ruling out non-specific DNA amplification. Unlike *gyrA* mutations *parC* mutations could not be detected by restriction as known amino-acid substitutions do not span a known restriction site, and therefore isolates of interest were subsequently sequenced (Section 3.5.2).

3.5 Sequencing of the Quinolone Resistance Determining Region (QRDR) of *gyrA* and *parC* genes

3.5.1 DNA gyrase *gyrA*

Mutations in the QRDR of *gyrA* of clinically sensitive and resistant isolates were analysed by DNA sequencing. TYPE I isolates NC-13, NC-4, NC-10, NC-1 and NC-14 with related genotypes (Section 3.3), however varying levels of ciprofloxacin resistance (8-256mg/L) were sequenced to analyse progression to high-level resistance. TYPE II isolates NC-8 and NC-11, which had identical genotypes, were also sequenced to analyse progression from a clinically sensitive (1mg/L) to a resistant phenotype (32mg/L). All sequences determined were compared to *E. coli gyrA* in GenBank (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST), with accession number (XO6744), a sequence originally described by Swanberg & Wang (1987). Only the QRDR of each sequence is shown (Figure 3.14 - 3.19) as amino acid mutations were not found outwith this region. An exception to this was isolates NC-10 and NC-1 which had mutations outwith this region, and therefore an extended region of sequence is shown (Figure 3.16).

3.5.1.i WT *gyrA*

The QRDR of *E. coli gyrA* spans the region of amino-acids from Ala67 to Glu106, and mutation hotspots within this region are at Ser83 and Asp87. Mutations outwith the QRDR have not been reported to confer significant effects on resistance levels. The QRDR of the clinical isolates were compared to that of WT *gyrA* QRDR found in GenBank (Figure 3.14).

Figure 3.14: QRDR of WT *E. coli gyrA*

GCT	CGT	GTC	GTT	GGT	GAC	GTA	ATC	GGT	AAA
Ala	Arg	Val	Val	Gly	Asp	Val	Ile	Gly	Lys
67									
TAC	CAT	CCC	CAT	GGT	GAC	**TCG**	GCG	GTC	TAT
Tyr	His	Pro	His	Gly	Asp	**Ser**	Ala	Val	Tyr
						83			
GAC	ACG	ATT	GTC	CGC	ATG	GCG	CAG	CCA	TTC
Asp	Thr	Ile	Val	Arg	Met	Ala	Gln	Pro	Phe
87									
TCG	CTG	CGT	TAT	ATG	CTG	GTA	GAC	GGT	CAG
Ser	Leu	Arg	Tyr	Met	Leu	Val	Asp	Gly	Gln
									106

Mutation hotspots are shown in bold

3.5.1.ii QRDR of DNA gyrase *gyrA* of RAPD type I related isolates

NC-13 and NC-4

The QRDR sequence of NC-13 (Cip MIC=8mg/L) and NC-4 (Cip MIC=32mg/L) were found to be identical (Figure 3.15).

Figure 3.15: *gyrA* QRDR of NC-13 and NC-4

GCT	CGT	GTC	GTT	GGT	GAC	GTA	ATC	GGT	AAA
Ala	Arg	Val	Val	Gly	Asp	Val	Ile	Gly	Lys
67									
TAC	CAT	CCC	CAT	GGT	GAC	TTG	GCG	GTT	TAT
Tyr	His	Pro	His	Gly	Asp	Leu	Ala	Val	Tyr
						83		85	
AAC	ACG	ATT	GTC	CGT	ATG	GCG	CAG	CCA	TTC
Asn	Thr	Ile	Val	Arg	Met	Ala	Gln	Pro	Phe
87				91					
TCG	CTG	CGT	TAC	ATG	CTG	GTA	GAC	GGT	CAG
Ser	Leu	Arg	Tyr	Met	Leu	Val	Asp	Gly	Gln
			100						106

Amino-acid substitutions are shown in red
Silent nucleotide substitutions are shown in blue

The sequence of NC-13 and NC-4 contained two amino-acid mutations; Ser83→Leu and Asp87→Asn. Nucleotide substitutions which did not confer amino-acid alterations were found at amino-acid positions 85, 91 and 100.

NC-1 & NC-10

The QRDR sequence of NC-1 (Cip MIC=128mg/L) and NC-10 (Cip MIC=128mg/L) were identical (Figure 3.16).

Figure 3.16: *gyrA* QRDR of NC-1 and NC-10

GCT	CGT	GTC	GTT	GGT	GAC	GTA	ATC	GGT	AAA
Ala	Arg	Val	Val	Gly	Asp	Val	Ile	Gly	Lys
67									
TAC	CAT	CCC	CAT	GGT	GAC	TTG	GCG	GTT	TAT
Tyr	His	Pro	His	Gly	Asp	Leu	Ala	Val	Tyr
						83		85	
TAC	ACG	ATT	GTC	CGT	ATG	GCG	CAG	CCA	TTC
Tyr	Thr	Ile	Val	Arg	Met	Ala	Gln	Pro	Phe
87				91					
TCG	CTG	CGT	TAC	ATG	CTG	GTA	GAC	GGT	CAG
Ser	Leu	Arg	Tyr	Met	Leu	Val	Asp	Gly	Gln
			100						106
GGT	AAC	TTC	GGT	TCC	ATC	GAC	GGC	GAC	TCT
Gly	Asn	Phe	Gly	Ser	Ile	Asp	Gly	Asp	Ser
				111					
GCG	GCG	GCA	ATG	CGT	TAT	ACG	GAA	ATC	CGT
Ala	Ala	Ala	Met	Arg	Tyr	Thr	Glu	Ile	Arg
CTG	GCG	AAA	ATT	GCC	CAT	GAA	CTG	ATG	GCC
Leu	Ala	Lys	Ile	Ala	His	Glu	Leu	Met	Ala
GAT	CTC	GAA	AAA	GAG	ACG	GTC	GAT	TTC	GTT
Asp	Leu	Glu	Lys	Glu	Thr	Val	Asp	Phe	Val
GAT	AAC	TAT	GAC	GGC	ACG	GAA	AAA	ATT	CCC
Asp	Asn	Tyr	Asp	Gly	Thr	Glu	Lys	Ile	Pro
									156
GAC	GTC	ATG	CCA	ACC	AAA	ATT	CCT	AAC	CTG
Asp	Val	Met	Pro	Thr	Lys	Ile	Pro	Asn	Leu
CTG	GTG	AAC	GGT	TCT	TCC	GGT	ATC	GCC	GTA
Leu	Val	Asn	Gly	Ser	Ser	Gly	Ile	Ala	Val
GGT	ATG	GCA	ACC	AAC	ATC	CCG	CTG	CAC	AAC
Gly	Met	Ala	Thr	Asn	Ile	Pro	Leu	His	Asn
							185		

Amino-acid substitutions are shown in red
Silent nucleotide substitutions are shown in blue

The sequence of NC-1 and NC-10 contained amino-acid mutations: Ser83→Leu, Asp87→Tyr and Pro185→Leu. Non amino-acid altering nucleotide substitutions were found at amino-acid positions 85, 91, 100, 111 and 156.

NC-14

The *gyrA* QRDR of isolate NC-14 (Cip MIC=256mg/L) is shown in figure 3.17.

Figure 3.17: *gyrA* QRDR of NC-14

GCT	CGT	GTC	GTT	GGT	GAC	GTA	ATC	GGT	AAA
Ala	Arg	Val	Val	Gly	Asp	Val	Ile	Gly	Lys
67									
TAC	CAT	CCC	CAT	GGT	GAC	TTG	GCG	GTT	TAT
Tyr	His	Pro	His	Gly	Asp	Leu	Ala	Val	Tyr
						83		85	
TAC	ACG	ATT	GTC	CGT	ATG	GCG	CAG	CCA	TTC
Tyr	Thr	Ile	Val	Arg	Met	Ala	Gln	Pro	Phe
87				91					
TCG	CTG	CGT	TAC	ATG	CTG	GTA	GAC	GGT	CAG
Ser	Leu	Arg	Tyr	Met	Leu	Val	Asp	Gly	Gln
			100						106

Amino-acid substitutions are shown in red
Silent nucleotide substitutions are shown in blue

The sequence of NC-14 contained two mutations: Ser83→Leu and Asp87→Tyr. Non amino-acid altering nucleotide changes were found at positions 85, 91 and 100.

3.5.1.iii The QRDR of DNA gyrase *gyrA* of type II related isolates

NC-8

The *gyrA* QRDR if isolate NC-8 (Cip MIC=1mg/L) is shown in figure 3.18.

Figure 3.18: *gyrA* QRDR of NC-8

GCT	CGT	GTC	GTT	GGT	GAC	GTA	ATC	GGT	AAA
Ala	Arg	Val	Val	Gly	Asp	Val	Ile	Gly	Lys
67									
TAC	CAT	CCC	CAT	GGT	GAC	TTG	GCG	GTT	TAT
Tyr	His	Pro	His	Gly	Asp	Leu	Ala	Val	Tyr
						83		85	
GAC	ACG	ATT	GTC	CGT	ATG	GCG	CAG	CCA	TTC
Asn	Thr	Ile	Val	Arg	Met	Ala	Gln	Pro	Phe
87				91					
TCG	CTG	CGT	TAC	ATG	CTG	GTA	GAC	GGT	CAG
Ser	Leu	Arg	Tyr	Met	Leu	Val	Asp	Gly	Gln
			100						106

Amino-acid substitutions are shown in red
Silent nucleotide substitutions are shown in blue

The sequence of NC-8 contained one amino-acid mutation: Ser83→Leu. Non amino-acid altering nucleotide mutations were found at positions 85, 91 and 100.

NC-11

The *gyrA* QRDR sequence of isolate NC-11 (Cip MIC=32 mg/L) is shown in figure 3.19.

Figure 3.19: QRDR of NC-11

GCT	CGT	GTC	GTT	GGT	GAC	GTA	ATC	GGT	AAA
Ala	Arg	Val	Val	Gly	Asp	Val	Ile	Gly	Lys
67									
TAC	CAT	CCC	CAT	GGT	GAC	TTG	GCG	GTT	TAT
Tyr	His	Pro	His	Gly	Asp	Leu	Ala	Val	Tyr
						83		85	
AAC	ACG	ATT	GTC	CGT	ATG	GCG	CAG	CCA	TTC
Asn	Thr	Ile	Val	Arg	Met	Ala	Gln	Pro	Phe
87				91					
TCG	CTG	CGT	TAC	ATG	CTG	GTA	GAC	GGT	CAG
Ser	Leu	Arg	Tyr	Met	Leu	Val	Asp	Gly	Gln
			100						106

Amino-acid substitutions are shown in red
Silent nucleotide substitutions are shown in blue

The sequence of NC-11 contained two amino-acid mutations: Ser83→Leu and Asp87→Asn. Non amino-acid altering nucleotide substitutions were found at amino-acid positions 85, 91 and 100.

3.5.2 DNA topoisomerase IV *parC*

Sequencing of the QRDR of DNA gyrase revealed that certain isolates with varying MICs has identical *gyrA* mutations, raising the theory that mutations in the fluoroquinolone target DNA topoisomerase IV *parC* gene may be present and responsible for resistance.

Mutations in the QRDR of *parC* of clinically sensitive and resistant isolates were analysed by DNA sequencing. TYPE I isolates NC-13, NC-4, NC-10, NC-1 and NC-14 with related genotype however varying levels of resistance (Cip MIC=8→256 mg/L) were sequenced to analyse the *parC* changes associated with resistance progression. TYPE II isolates NC-8 and NC-11 which had identical genotypes were also sequenced to analyse progression from a clinically sensitive to resistant phenotype (Cip MICs=1→32 mg/L). Isolates NC-9 and NC-7 were sequenced to screen for the presence of *parC* mutations in isolates with MICs of ≤0.25 mg/L, to assess if *gyrA* of *parC* mutations occur primarily because 14/15 isolates were known to contain *gyrA* mutations by restriction analysis.

All sequences determined were compared to *E. coli parC* in GenBank (http://www .ncbi.nlm.nih.gov/cgi-bin/BLAST), with accession number (M58408), a sequence originally described by Kato *et al.* (1990).

3.5.2.i WT *parC*

The QRDR of *E. coli parC* spans an equivalent region to that of *gyrA*, between amino-acids Lys61 and Trp106, encompassing mutation hotspots Ser80 (equivalent to *gyrA* Ser83) and Glu84 (equivalent to *gyrA* Asp87). The QRDR sequences of clinical isolates were compared to the WT *parC* sequence shown in figure 3.20.

Figure 3.20: QRDR of *E. coli* WT *parC*

AAA	AAA	TCG	GCC	CGT	ACC	GTC	GGT	GAC	GTA
Lys	Lys	Ser	Ala	Arg	Thr	Val	Gly	Asp	Val
61									
CTG	GGT	AAA	TAC	CAT	CCG	CAC	GGC	GAT	**AGC**
Leu	Gly	Lys	Tyr	His	Pro	His	Gly	Asp	**Ser**
									80
GCC	TGT	TAT	**GAA**	GCG	ATG	GTC	CTG	ATG	GCG
Ala	Cys	Tyr	**Glu**	Ala	Met	Val	Leu	Met	Ala
			84						
CAA	CCG	TTC	TCT	TAC	CGT	TAT	CCG	CTG	GTT
Gln	Pro	Phe	Ser	Tyr	Arg	Tyr	Pro	Leu	Val
GAT	GGT	CAG	GGG	AAC	TGG				
Asp	Gly	Gln	Gly	Asn	Trp				
					106				

Mutation hotspots are shown in bold

3.5.2.ii QRDR of DNA topoisomerase IV *parC* of RAPD type I related isolates

NC-13

The QRDR of NC-13 (Cip MIC=8mg/L) is shown in figure 3.21.

Figure 3.21: *parC* QRDR of NC-13

AAA	AAA	TCG	GCC	CGT	ACC	GTC	GGT	GAC	GTA
Lys	Lys	Ser	Ala	Arg	Thr	Val	Gly	Asp	Val
61									
CTG	GGT	AAA	TAC	CAT	CCG	CAC	GGC	GAT	ATC
Leu	Gly	Lys	Tyr	His	Pro	His	Gly	Asp	Ile
									80
GCC	TGT	TAT	**GAA**	GCG	ATG	GTC	CTG	ATG	GCG
Ala	Cys	Tyr	**Glu**	Ala	Met	Val	Leu	Met	Ala
			84						
CAA	CCG	TTC	TCT	TAC	CGT	TAT	CCG	CTG	GTT
Gln	Pro	Phe	Ser	Tyr	Arg	Tyr	Pro	Leu	Val
GAT	GGT	CAG	GGG	AAC	TGG				
Asp	Gly	Gln	Gly	Asn	Trp				
					106				

Amino-acid substitutions are shown in red

The sequence of NC-13 contained one amino-acid mutation; Ser80→Ile.

NC-4

The QRDR of NC-4 (Cip MIC=32mg/L) is shown in figure 3.22.

Figure 3.22: *parC* QRDR of NC-4

AAA	AAA	TCG	GCC	CGT	ACC	GTC	GGT	GAC	GTA
Lys	Lys	Ser	Ala	Arg	Thr	Val	Gly	Asp	Val
61									
CTG	GGT	AAA	TAC	CAT	CCG	CAC	GGC	GAT	ATC
Leu	Gly	Lys	Tyr	His	Pro	His	Gly	Asp	Ile
									80
GCC	TGT	TAT	**GAA**	GCG	ATG	GTC	CTG	ATG	GCG
Ala	Cys	Tyr	**Glu**	Ala	Met	Val	Leu	Met	Ala
			84						
CAG	CCG	TTC	TCT	TAC	CGT	TAT	CCG	CTG	GTT
Gln	Pro	Phe	Ser	Tyr	Arg	Tyr	Pro	Leu	Val
GAT	GGT	CAG	GGA	AAC	TGG				
Asp	Gly	Gln	Gly	Asn	Trp				
					106				

Amino-acid substitutions are shown in red
Silent nucleotide substitutions are shown in blue

The sequence of NC-4 contained one amino-acid mutation; Ser80→Ile. Nucleotide substitutions which did not confer amino-acid alterations were found at positions 91 and 104.

NC-1

The QRDR of NC-1 (Cip MIC=128mg/L) is shown in figure 3.23.

Figure 3.23: *parC* QRDR of isolate NC-1

AAA	AAA	TCG	GCC	CGT	ACC	GTC	GGT	GAC	GTA
Lys	Lys	Ser	Ala	Arg	Thr	Val	Gly	Asp	Val
61									
CTG	GGT	AAA	TAC	CAT	CCG	CAC	GGC	GAT	ATC
Leu	Gly	Lys	Tyr	His	Pro	His	Gly	Asp	Ile
									80
GCC	TGT	TAT	AAA	GCG	ATG	GTC	CTG	ATG	GCG
Ala	Cys	Tyr	Lys	Ala	Met	Val	Leu	Met	Ala
			84						
CAA	CCG	TTC	TCT	TAC	CGT	TAT	CCG	CTG	GTT
Gln	Pro	Phe	Ser	Tyr	Arg	Tyr	Pro	Leu	Val
GAT	GGT	CAG	GGG	AAC	TGG				
Asp	Gly	Gln	Gly	Asn	Trp				
					106				

Amino-acid substitutions are shown in red

The sequence of NC-1 contained amino-acid substitutions; Ser80→Ile and Glu84→Lys.

NC-10

The QRDR of isolate NC-10 (Cip MIC=128mg/L) is shown in figure 3.24.

Figure 3.24: *parC* QRDR of NC-10

AAA	AAA	TCG	GCC	CGT	ACC	GTC	GGT	GAC	GTA
Lys	Lys	Ser	Ala	Arg	Thr	Val	Gly	Asp	Val
61									
CTG	GGT	AAA	TAC	CAT	CCG	CAC	GGC	GAT	ATC
Leu	Gly	Lys	Tyr	His	Pro	His	Gly	Asp	Ile
									80
GCC	TGT	TAT	AAA	GCG	ATG	GTC	CTG	ATG	GCG
Ala	Cys	Tyr	Lys	Ala	Met	Val	Leu	Met	Ala
			84						
CAA	CCG	TTC	TCT	TAC	CGT	TAT	CCG	CTG	GTT
Gln	Pro	Phe	Ser	Tyr	Arg	Tyr	Pro	Leu	Val
GAT	GGT	CAG	GGG	AAC	TGG				
Asp	Gly	Gln	Gly	Asn	Trp				
					106				

Amino-acid substitutions are shown in red

The sequence of NC-10 contained amino-acid substitutions; Ser80→Ile and Glu84→Lys.

NC-14

The QRDR of NC-14 (Cip MIC=256mg/L) is shown in figure 3.25.

Figure 3.25: *parC* QRDR of NC-14

AAA	AAA	TCG	GCC	CGT	ACC	GTC	GGT	GAC	GTA
Lys	Lys	Ser	Ala	Arg	Thr	Val	Gly	Asp	Val
61									
CTG	GGT	AAA	TAC	CAT	CCG	CAC	GGC	GAT	ATC
Leu	Gly	Lys	Tyr	His	Pro	His	Gly	Asp	Ile
									80
GCC	TGT	TAT	AAA	GCG	ATG	GTC	CTG	ATG	GCG
Ala	Cys	Tyr	Lys	Ala	Met	Val	Leu	Met	Ala
			84						
CAA	CCG	TTC	TCT	TAC	CGT	TAT	CCG	CTG	GTT
Gln	Pro	Phe	Ser	Tyr	Arg	Tyr	Pro	Leu	Val
GAT	GGT	CAG	GGG	AAC	TGG				
Asp	Gly	Gln	Gly	Asn	Trp				
					106				

Amino-acid substitutions are shown in red

The sequence contained amino-acid substitutions; Ser80→Ile and Glu84→Lys.

3.5.2.iii The QRDR of DNA Topoisomerase IV *parC* of type II related isolates

NC-8

The QRDR of NC-8 (Cip MIC=1mg/L) is shown in figure 3.26.

Figure 3.26: *parC* QRDR of NC-8

AAA	AAA	TCG	GCC	CGT	ACC	GTC	GGT	GAC	GTA
Lys	Lys	Ser	Ala	Arg	Thr	Val	Gly	Asp	Val
61									
CTG	GGT	AAA	TAC	CAT	CCG	CAC	GGC	GAT	ATC
Leu	Gly	Lys	Tyr	His	Pro	His	Gly	Asp	Ile
									80
GCC	TGT	TAT	**GAA**	GCG	ATG	GTC	CTG	ATG	GCG
Ala	Cys	Tyr	**Glu**	Ala	Met	Val	Leu	Met	Ala
			84						
CAG	CCG	TTC	TCT	TAC	CGT	TAT	CCG	CTG	GTT
Gln	Pro	Phe	Ser	Tyr	Arg	Tyr	Pro	Leu	Val
GAT	GGT	CAG	GGG	AAC	TGG				
Asp	Gly	Gln	Gly	Asn	Trp				
					106				

Amino-acid substitutions are shown in red
Silent nucleotide substitutions are shown in blue

The sequence of NC-8 contained one amino acid substitution: Ser80→Ile. A non amino-acid altering nucleotide substitution was present at amino-acid position 91.

NC-11

The QRDR of NC-11 (Cip MIC=32mg/L) is shown in figure 3.27.

Figure 3.27: *parC* QRDR of NC-11

AAA	AAA	TCG	GCC	CGT	ACC	GTC	GGT	GAC	GTA
Lys	Lys	Ser	Ala	Arg	Thr	Val	Gly	Asp	Val
61									
CTG	GGT	AAA	TAC	CAT	CCG	CAC	GGC	GAT	ATC
Leu	Gly	Lys	Tyr	His	Pro	His	Gly	Asp	Ile
									80
GCC	TGC	TAT	GTA	GCG	ATG	GTC	CTG	ATG	GCG
Ala	Cys	Tyr	Val	Ala	Met	Val	Leu	Met	Ala
			84						
CAG	CCA	TTC	TCT	TAT	CGT	TAT	CCG	CTG	GTC
Gln	Pro	Phe	Ser	Tyr	Arg	Tyr	Pro	Leu	Val
GAC	GGG	CAG	GGG	AAC	TGG				
Asp	Gly	Gln	Gly	Asn	Trp				
					106				

Amino-acid substitutions are shown in red
Silent nucleotide substitutions are shown in blue

The sequence of NC-11 contained two amino acid mutations: Ser80→Ile and Glu84→Val. Non amino-acid altering nucleotide substitutions were found at positions 87, 91, 92 , 95, 100, 101 and 102.

3.5.2.iv QRDR of DNA topisomerase IV *parC* of isolates with ciprofloxacin MICs of ≤ 0.25mg/L

NC-7 and NC-9

The QRDR of NC-7 (Cip MIC=0.015mg/L) and NC-9 (Cip MIC=0.25mg/L) is shown in figure 3.28.

Figure 3.28: *parC* QRDR of NC-7 and NC-9

AAA	AAA	TCG	GCC	CGT	ACC	GTC	GGT	GAC	GTA
Lys	Lys	Ser	Ala	Arg	Thr	Val	Gly	Asp	Val
61									
CTG	GGT	AAA	TAC	CAT	CCG	CAC	GGC	GAT	**AGC**
Leu	Gly	Lys	Tyr	His	Pro	His	Gly	Asp	**Ser**
									80
GCC	TGT	TAT	**GAA**	GCG	ATG	GTC	CTG	ATG	GCG
Ala	Cys	Tyr	**Glu**	Ala	Met	Val	Leu	Met	Ala
			84						
CAA	CCG	TTC	TCT	TAC	CGT	TAT	CCG	CTG	GTT
Gln	Pro	Phe	Ser	Tyr	Arg	Tyr	Pro	Leu	Val
GAT	GGT	CAG	GGG	AAC	TGG				
Asp	Gly	Gln	Gly	Asn	Trp				
					106				

Mutation hotspots are shown in bold

The sequences of NC-7 and NC-9 were identical to WT containing no amino-acid or nucleotide substitutions.

3.6 Summary of Progression to High-Level Resistance

In this outbreak resistance can be clearly associated with resistance mutations occurring within target proteins DNA gyrase *gyrA* and Topoisomerase IV *parC*. All isolates except NC-7 (Cip MIC=0.015mg/L) contained *gyrA* mutation at Ser83, however NC-7 and NC-9 did not show a *parC* mutations. The progression to high-level resistance in two strains Type I and II is summarised in Table 3.4.

Table 3.4: Amino-acid alterations associated with progression to high-level fluoroquinolone resistance

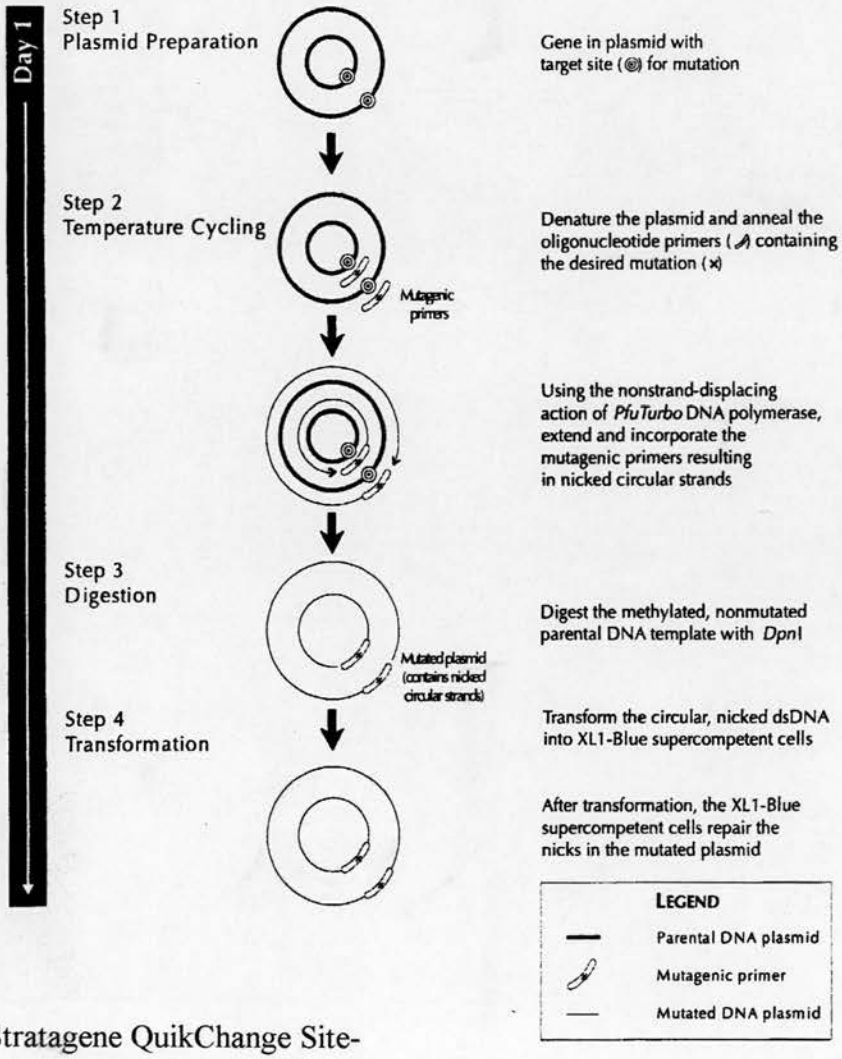
Strain	Ciprofloxacin MIC (mg/L)	Type	GyrA	ParC
14	256	I	Ser83→Leu Asp87→Tyr	Ser80→Ile Glu84→Lys
1	128	I	Ser83→Leu Asp87→Tyr Pro191→Leu	Ser80→Ile Glu84→Lys
10	128	I	Ser83→Leu Asp87→Tyr Pro185→Leu	Ser80→Ile Glu84→Lys
4	32	I	Ser83→Leu Asp87→Asn	Ser80→Ile
13	8	I	Ser83→Leu Asp87→Asn	Ser80→Ile
11	32	II	Ser83→Leu Asp87→Asn	Ser80→Ile Glu84→Val
8	1	II	Ser83→Leu	Ser80→Ile

4. Results: Site-Directed Mutagenesis

4.1 An Overview

Mutations at amino acids Ser83 and Asp87 were introduced into *E. coli* DNA gyrase by *in vitro* site-directed mutagenesis. Mutagenesis was performed with the QuikChange™ kit supplied by Stratagene, UK, and was based on the protocol shown in figure 4.1.

Figure 4.1: Site-directed mutagenesis protocol



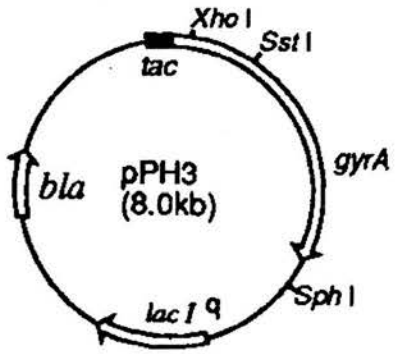
From Stratagene QuikChange Site-Directed Mutagenesis protocol

Mutated plasmids were subsequently transformed into Ts mutant KNK453 *E. coli* (Kreuzer & Cozzarelli, 1979) to analyse resistance phenotype conferred by altered *gyrA* genes.

4.2 Plasmid pPH3

The *gyrA* containing plasmid used for mutagenesis was pPH3; a plasmid with *gyrA* optimally located downstream from a tac promoter (Figure 4.2). Expression of *gyrA* was therefore regulated by the inducer IPTG, in the presence of which cells show an increased level of GyrA expression (Hallett *et al.*, 1990). When expressed in the *gyrA*^{ts} KNK453 strain, growth was not detected at 42°C in the absence of IPTG, whereas induction by IPTG allowed cell growth. Induction of *gyrA* expression by IPTG provided additional control when expressing plasmid in *gyrA*^{ts} cells.

Figure 4.2: pPH3 plasmid map

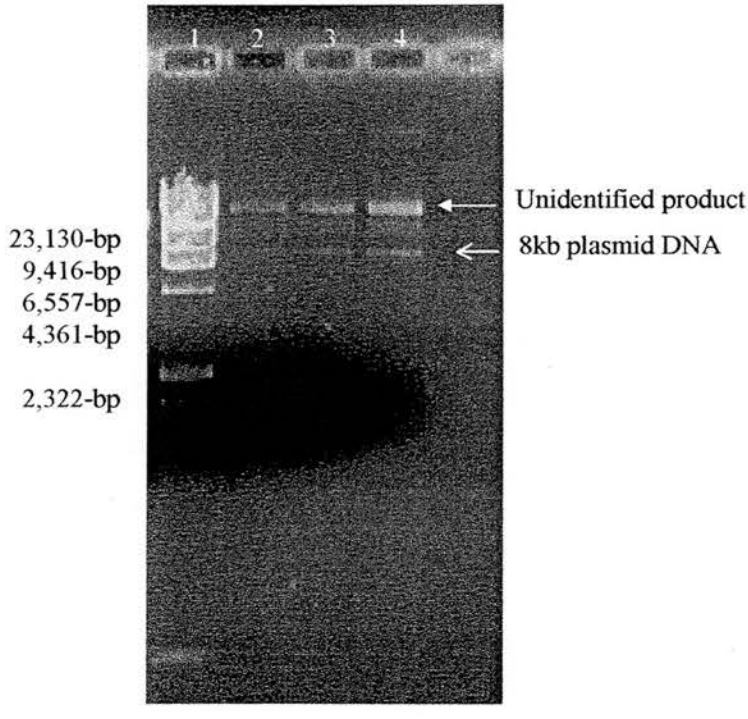


From Hallett *et al.*, 1990

4.3 Extraction of plasmid DNA from JMtacA *E. coli* cells

Plasmid DNA was extracted from JMtacA (JM109[pPH3]) *E. coli* cells using the hybaid Plasmid prep kit (Hybaid, Middlesex, UK). Electrophoresis of plasmid extracts demonstrated a product of 8kb, the expected plasmid size, along with a larger product of >23,000-bp (Figure 4.3) This larger unidentified band was likely as a consequence of plasmid concatermers, or due to the presence of contaminating chromosomal DNA.

Figure 4.3: Plasmid extract pPH3

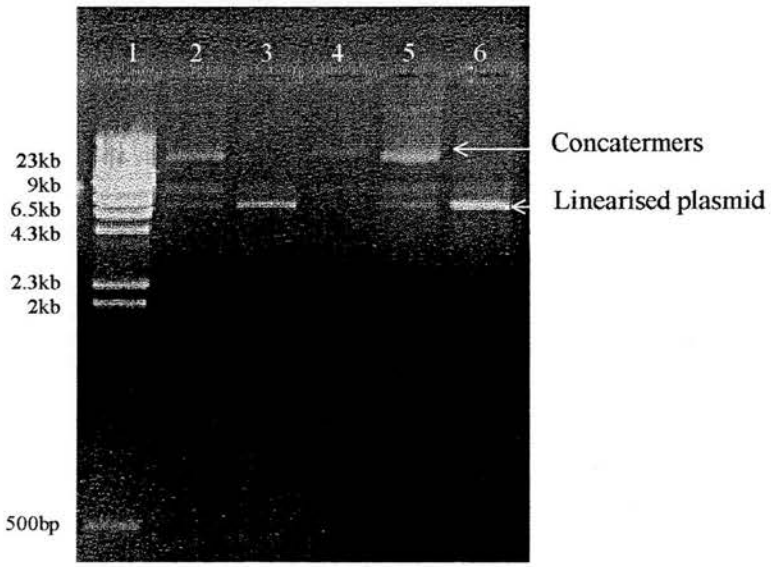


Lane: 1 - λHindIII DNA ladder; 2 - Plasmid DNA (1μL); 3 - Plasmid DNA (2μL); 4 - Plasmid DNA (5μL)

To eliminate possible concatermers and accurately size the plasmid DNA, it was linearised with the restriction endonuclease *Xho*I, which produces a single cut in the plasmid (Figure 4.2). Restriction of two plasmid extracts P1 and P2, yielded single

8kb products, and confirmed plasmid identity (Figure 4.4). Restriction of DNA eliminated the 23kb band, thereby demonstrating that this was plasmid concatermers, and not contaminating chromosomal DNA. The concentration of plasmid in extracts was estimated to be 30ηg/μL utilising comparison with *Hind*III/Lamda DNA.

Figure 4.4: Restriction of pPH3 with *Xho*I



Lane: 1 - λ*Hind*IIIDNA ladder; 2- P1; 3- *Xho*I cut P1; 4 - P2; 5 - *Xho*I cut P2

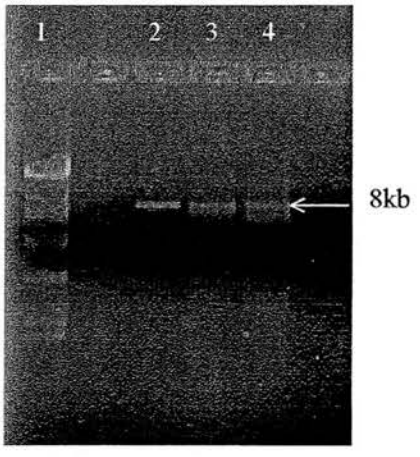
4.4 Verification of the Ts phenotype of *E. coli* KNK453 mutants

E. coli Ts mutants KNK453 (*gyrA43 polA thyA uvrA phxA*) carry a temperature sensitive *gyrA* allele and were found to be viable at 30°C, but not at 42°C. Upon arrival cells were replica plated at 30°C and 42°C; 90% were found to have retained their Ts phenotype. Ts mutants and were stored at -70°C or on plates for further experimentation.

4.5 Mutagenesis of Ser83 and Aps87 amino-acids

PCR amplification of *gyrA* containing plasmid template utilised DNA template concentrations of 15ηg, 30ηg and 60ηg. Initial amplification with Ser83 mutagenic primers was successful (Figure 4.5), however amplification failed with Asp87 mutagenic primers. For Ser83 amplification reactions, the DNA template of 15ηg gave the most specific amplification product (Figure 4.5). As starting concentration was increased from this, PCR specificity decreased (Figure 4.5). Ser83 plasmid products were subsequently transformed into Epicurian Coli® cells (see section 4.6).

Figure 4.5: PCR amplification of pPH3 with Ser83 mutagenic primers

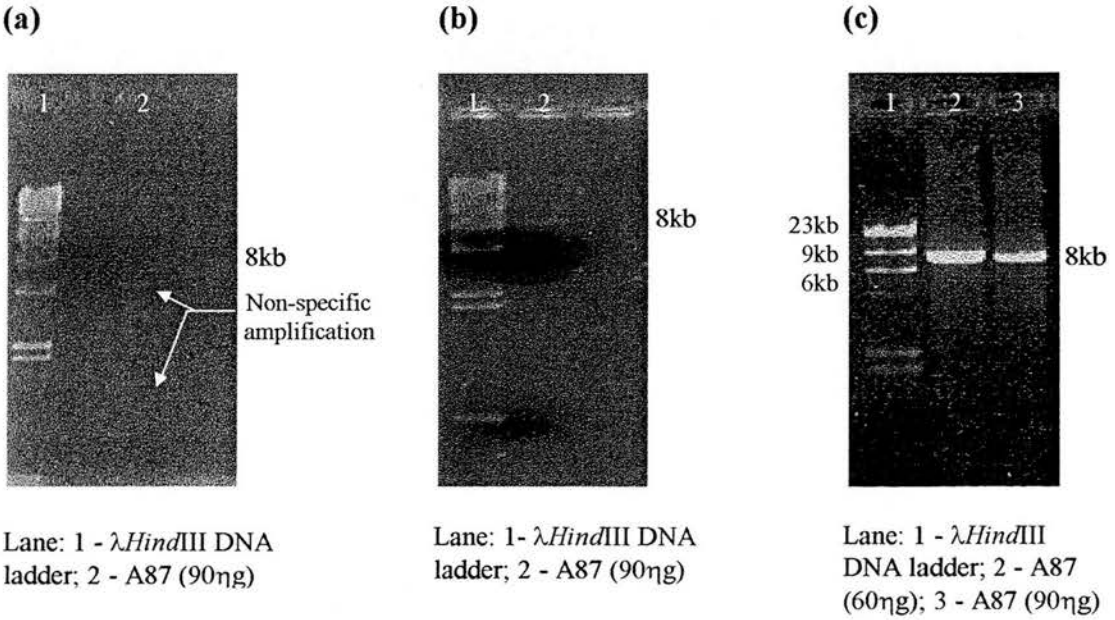


Lane: 1- λ*Hind*III DNA ladder; 2- S83 (15ηg); 3 - S83 (30ηg); 4- S83 (60ηg)

PCR amplification with Asp87 mutagenic primers required further optimisation as PCR amplification with staring template of 15-60 ηg was unsuccessful. Increasing DNA starting concentration to 90ηg was successful however yielded non-specific DNA products (Figure 4.6a). To increase specificity, annealing temperature was increased from 50°C to 52°C; this increased specificity gave product, however DNA

yield remained low (Figure 4.6b). The optimal product was obtained by retaining this increased annealing temperature, decreasing DNA concentration to 60ηg, and increasing the number of PCR cycles (Figure 4.6c).

Figure 4.6: PCR amplification of pPH3 with Asp87 mutagenic primers



Lane: 1 - λ*Hind*III DNA ladder; 2 - A87 (90ηg)

Lane: 1- λ*Hind*III DNA ladder; 2 - A87 (90ηg)

Lane: 1 - λ*Hind*III DNA ladder; 2 - A87 (60ηg); 3 - A87 (90ηg)

Asp87 mutated plasmid products were subsequently transformed into Epicurian Coli® cells (Section 4.6).

4.6 Transformation of Epicurian Coli® XL-1 Blue super-competent cells

Nicked mutant plasmid DNA and control pUC18 plasmid DNA was transformed into Epicurian Coli® (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F' *proAB lacI*^{q} *ZM15* Tn*10*(Tet^{r})] cells. These cells are known to allow repair of the nicks in plasmid created during the DNA restriction step (Figure 4.1). Transformants were selected on LB amp plates, substituted with IPTG and X-gal for pUC18 control.

A control transformation was initially performed using the plasmid pUC18. Transformation with the pUC18 plasmid should optimally yield >250 colonies (10^{8} cfu) with 98% having the blue phenotype (QuikChange protocol). Transformation of cells with pUC18 however yielded only 15 cells with 10 having the blue phenotype, showing that transformation efficiency was 6% of the expected value. This raised questions about the efficiency of the Epicurian Coli® cells. Cells had been stored in the freezer for 6 months, and may have undergone temperature variations during this time and upon arrival. As these cells are extremely sensitive, these factors may have affected their efficiency and ability to uptake plasmid DNA.

Transformation with Asp87 and Ser83 mutant plasmids yielded slowly growing transformants > 48 hours. Transformants should be selected in approximately 16 hours, and therefore there is a possibility that cells seen after 48 hour selection were selected for ampicillin resistance (ampicillin resistance revertants), and selection may not be due to a plasmid containing the *bla* (amp^{R}) resistance gene. Attempts to extract

plasmid from these cells failed, supporting that they may not be carrying the desired plasmid, however did not rule out the possibility that there were faults with the plasmid extraction technique.

As transformation of the mutant plasmid failed in Epicurian Coli®, the transfer of plasmid directly into KNK453 *gyrA*^{ts} *E. coli* cells was attempted. Transformants were selected with low efficiency, with pUC18 control yielding only 20 transformants. Transformation with mutant plasmids yielded ~10 transformants per sample (50% pUC18 control). Plasmid extraction from transformants also failed, however in this system it was also possible to analyse plasmid carriage by the phenotype of the cells in a Ts environment. Transformants were selected on ampicllin containing plates, and transformants replica plated at 30°C and 42°C, in the presence and absence of IPTG. Plasmids extracts transformed were Wild-type (W1, W2), Ser83 (S1, S2) and Asp87 (A1, A2). The results are shown in table 4.1.

Table 4.1: Phenotypic analysis of wild-type, Ser83 and Asp87 *gyrA*^{ts} *E. coli* transformants

Transformant	30°C		42°C	
	+ IPTG	- IPTG	+IPTG	-IPTG
W1	+	+	+	-
W2	+	+	+	-
S1	+	+	-	-
S2	+	+	-	-
A1	+	+	-	-
A2	+	+	-	-
C *gyrA*^{ts}	+	+	-	-

+ = growth
- = no growth

These results indicated that there was successful transformation of WT plasmid into Ts mutants. Plasmid was subsequently extracted from WT transformants. The fact that Asp87 and Ser83 mutant transformants did not grow at 42°C indicated that these cells did not contain plasmid. Over 100 transformants were tested in a the same manner, and all failed to grow at 42°C independent of the presence or absence of IPTG. Stimulation of plasmid *gyrA* expression by IPTG in broth was attempted before plating, however this also failed yield plasmid or a complementary phenotype. The failure to transform into Ts mutants was possibly due to the fact that endonucleases within these cells would break down the nicked plasmid DNA, before repairs to the plasmid could be completed. Epicurian coli® cells are engineered to repair DNA damage and limit DNA breakdown.

As original Epicurian Coli® transformation failure pointed to a loss of efficiency in competent cells, a fresh batch of Epicurian Coli® were obtained for use in subsequent transformation reactions. Transformation of the second batch of Epicurian Coli® with pUC18 yielded 210 cfu out of expected 250 cfu, 87% of the expected value, compared to the 6% of transformation into original Epicurian Coli®. This indicated that original transformation failure had been due to loss of efficiency in competent cells, rather than a problem with plasmid or experimental procedure. Transformation of these cells with control pUC18, WT, Ser83 and Asp87 mutated plasmid also yielded transformants with increased efficiency in comparison with original transformation reactions (Table 4.2).

Table 4.2: Transformation efficiency of Epicurian Coli ® cells

Plasmid (1,5,10ng)	**Number of Transformants**	**% Efficiency**
PUC18	210	100
Ser83-1	59	28
Ser83-5	108	51
Ser83-10	107	51
Asp-87-1	154	73
Asp-87-5	156	74
Asp87-10	51	24

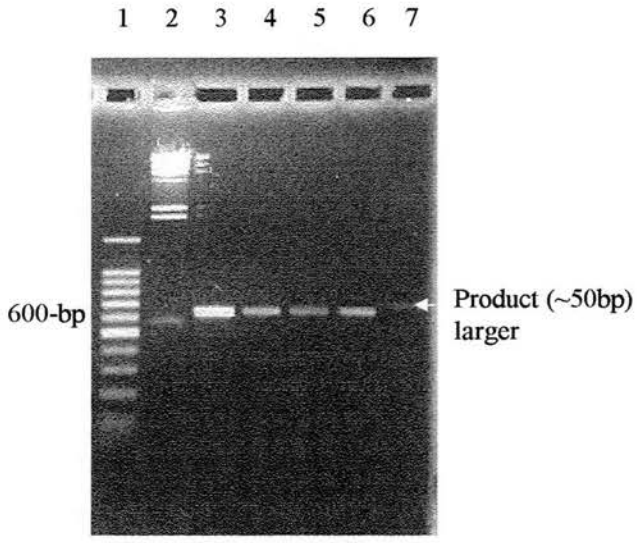
% transformation efficiency was calculated by a comparison of the number of sample transformants with number of control pUC18 transformants. Transformation of pUC18 plasmid was designated 100% efficient.

An 8kb plasmid was extracted from ~60% of transformants from each transformation experiment, and used for subsequent transformation of Ts gyrA mutants (see section 4.8).

4.7 PCR amplification of the QRDR of DNA gyrase *gyrA*

A 620bp region of DNA gyrase *gyrA* incorporating the QRDR of WT, Ser83 and Asp87 plasmid *gyrA* were amplified (Figure 4.7), and sequenced to confirm correct mutation incorporation.

Figure 4.7: *gyrA* amplification of WT, Ser83 and Asp87 plasmid DNA



Lane: 1- 100-bp DNA ladder; λ*Hind*III DNA ladder; WT *gyr*A; 5 - A1 *gyr*A; 6 - S1 *gyr*A; 7 - S2 *gyr*A; S3 *gyr*A

GyrA amplification was performed on twenty plasmids, 7 Ser83, 7 Asp87 and 6 WT transformants, however only 5 amplifications were successful, shown in figure 4.7. Four of the amplification products were of the expected size of ~ 620bp, whereas one product was ~ 50bp larger, suggesting that an insertion has taken place. All 5 products were consequently sequenced.

4.8 Sequencing of plasmid *gyrA* QRDRs

Sequences of the 620bp *gyrA* amplicons of WT, Ser83(S1-3) and Asp87 (A1-2) were compared with *E. coli gyrA* found in GenBank (http://www.ncbi.nlm.nih.gov:80/blast/Blast.cgi) with accession number (X06744.1), a sequence originally determined by Swanberg & Wang (1987). Figures 4.8-4.11, show sequences of the QRDR only, as mutations were not found out with these regions.

4.8.1 Wild-type

The nucleotide and amino acid sequence of the QRDR region of WT plasmid encoded *gyrA* is shown in figure 4.8.

Figure 4.8: Sequence of the QRDR of WT *E. coli gyrA*

GCT Ala 67	CGT Arg	GTC Val	GTT Val	GGT Gly	GAC Asp	GTA Val	ATC Ile	GGT Gly	AAA Lys
TAC Tyr	CAT His	CCC Pro	CAT His	GGT Gly	GAC Asp	**TCG** **Ser** **83**	GCG Ala	GTC Val	TAT Tyr
GAC **Asp** **87**	ACG Thr	ATT Ile	GTC Val	CGC Arg	ATG Met	GCG Ala	CAG Gln	CCA Pro	TTC Phe
TCG Ser	CTG Leu	CGT Arg	TAT Tyr	ATG Met	CTG Leu	GTA Val	GAC Asp	GGT Gly	CAG Gln 106

Mutation hotspots are shown in bold

No mutations were present within the sequence, and therefore this plasmid provided an accurate starting template for mutagenesis, as well as a control for phenotypic analysis of mutant plasmids.

4.8.2 Ser83 mutant

Sequence of Ser83 mutants was expected to contain a Ser83→Leu mutation (TCG→TTG) to confirm successful mutagenesis. Three mutants S1-3 were sequenced, figure 4.9 shows the sequence of mutants S1 and S2, and figure 4.10 shows the sequence of S3.

Figure 4.9: Sequence of Ser83 mutants S1 & S2

GCT Ala 67	CGT Arg	GTC Val	GTT Val	GGT Gly	GAC Asp	GTA Val	ATC Ile	GGT Gly	AAA Lys
TAC Tyr	CAT His	CCC Pro	CAT His	GGT Gly	GAC Asp	**TCG** **Ser** **83**	GCG Ala	GTC Val	TAT Tyr
AAC **Asn** **87**	ACG Thr	ATT Ile	GTC Val	CGC Arg	ATG Met	GCG Ala	CAG Gln	CCA Pro	TTC Phe
TCG Ser	CTG Leu	CGT Arg	TAT Tyr	ATG Met	CTG Leu	GTA Val	GAC Asp	GGT Gly	CAG Gln 106

Mutations are shown in red

These strains contained Asp87→Asn mutations shown in red (Figure 4.9) This result was unexpected as the primer used should have created a Ser83 mutation. This mutation may have occurred naturally, however as there was no quinolone selection pressure this also seems an unlikely scenario. It may be that errors occurred during the PCR step and primer annealing, as this amino acid occurs at the end of the primer sequence.

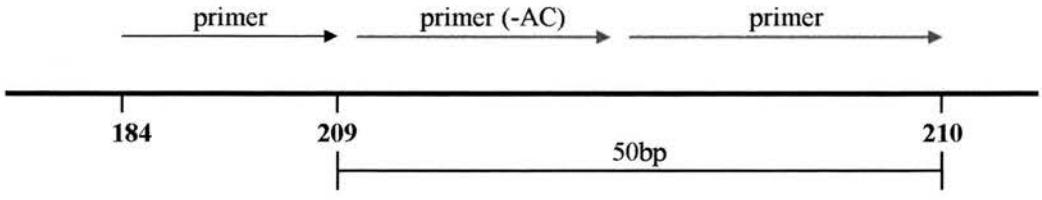
Figure 4.10: Sequence of Ser83 mutant S3

GCT Ala 67	CGT Arg	GTC Val	GTT Val	GGT Gly	GAC Asp	GTA Val	ATC Ile	GGT Gly	AAA Lys
TAC Tyr	CAT His	CCC Pro	CAT His	GGT Gly	GAC Asp	TTG Ser **83**	GCG Ala	GTC Val	TAT Tyr
GAC **Asp** **87**	AC/C Thr	ATG Met	GTG Val	ACT Thr	TGG Trp	CGG Arg	TCT Ser	ATG Met	ACC Thr
ATG Met	GTG Val	ACT Thr	TGG Trp	CGG Arg	TCT Ser	ATG Met	ACA Thr	CGA Arg	TTG Leu
TCC Ser	GCA Ala	TGG Trp	CGC Arg	AGC Ser	CAT His	TCT Ser	CGC Arg	TGC Cys	GTT Val
ATA Ile	TGC Cys	TGC Cys	**TAG** -	ACG Thr	GTC Val	AGG Arg			

Introduced mutations are shown in red ; Altered reading frame is shown in green
Inserted primer sequence is underlined; - = STOP codon

This strain contained the desired mutation of Ser83→Leu (TCG→TTG), however it also contained a 50bp insertion concurrent with the greater product size on the electrophoresis gel (Figure 4.7). This insertion consisted of two additional primer sequences, inserting after amino-acid Asp87 (Figure 4.11), and changed the reading frame of the gene.

Figure 4.11: Map of primer insertions



Primer inserts are shown in green

As the insertion changed the reading frame of the gene and a stop codon was incorporated, *gyrA* expression would be interrupted, thus preventing use of this mutant plasmid for further experimentation. Four separate attempts of Ser83 PCR reactions yielded problems with mutagenesis. Unfortunately this meant that Ser83 mutant plasmid could not be used for subsequent phenotypic analysis.

4.8.3 Asp87 mutant

The expected sequence of A1 for a successfully mutagenised plasmid should contain a mutation of Asp87→Asn (GAC→AAC). The sequence of A1 is shown in figure 4.12.

Figure 4.12: Sequence of Asp 87 mutant A1

GCT Ala 67	CGT Arg	GTC Val	GTT Val	GGT Gly	GAC Asp	GTA Val	ATC Ile	GGT Gly	AAA Lys
TAC Tyr	CAT His	CCC Pro	CAT His	GGT Gly	GAC Asp	**TCG** **Ser** **83**	GCG Ala	GTC Val	TAT Tyr
AAC **Asn** **87**	ACG Thr	ATT Ile	GTC Val	CGC Arg	ATG Met	GCG Ala	CAG Gln	CCA Pro	TTC Phe
TCG Ser	CTG Leu	CGT Arg	TAT Tyr	ATG Met	CTG Leu	GTA Val	GAC Asp	GGT Gly	CAG Gln 106

Introduced mutations are shown in red

This transformant contained the desired mutation of Asp87→Asn (GAC→AAC), and was subsequently expressed in the *gyr*^{ts} system

4.9 Expression of mutant phenotype in a temperature-sensitive system

WT plasmid extracted from JM109 cells, and mutant Asp87 plasmid extracted from Epicurian cells was successfully transformed into competent KNK453 Ts mutant *E. coli* cells. Transformation with pUC18 control yielded 70 transformants, whereas transformation with mutant Asp87 plasmid yielded 50 (71%). The transformants were grown at 30°C and 42°C on LB amp selective agar in the presence and absence of IPTG. The WT cells and Asp87 mutants grew at 42°C in the presence of IPTG. Growth was seen after16 hours and cells were amp selected suggesting that they were plasmid containing. Plasmid was successfully extracted from them.

4.10 Fluoroquinolone MICs

The minimum inhibitory concentrations of nalidixic acid, ciprofloxacin, gemifloxacin, moxifloxacin and sparfloxacin were determined (Table 4.2) using the agar dilution method. Bacteria were grown at 42°C on IPTG containing plates to ensure expression of plasmid *gyrA*.

Table 4.3: MICs of WT and Asp87 mutant containing *E. coli* for a range of quinolones

QUINOLONE	STRAIN		MIC (FOLD) INCREASE (Comparison with WT)
	WT	Asp87	
Nalidixic acid	0.01	2	7
Ciprofloxacin	0.007	0.04	3
Sparfloxacin	0.01	0.03	2
Moxifloxacin	0.05	0.06	<2
Gemifloxacin	0.005	0.04	3

The presence of plasmid encoded Asp87→Asn *gyrA* mutation increased quinolone MICs from < 2-7 fold (Table 4.2). Resistance was greatest with nalidixic acid, with newer fluoroquinolones concurring only 2-3 fold increases in MICs. The levels of resistance concurred remained below levels of clinically significant resistant levels, indicating that this mutation alone could not be responsible for high-level quinolone resistance.

5. Results: Ribosomal protein mutation

5.1 An Overview

Mutations in two conserved regions between amino-acids (41 & 43) and (85 & 91) of *E. coli* ribosomal protein S12 are known to confer high-level streptomycin resistance, with MICs exceeding 1500mg/L. The role of mutations in the S12 protein in conferring resistance to other members of the aminoglycoside group of antibiotics has not been previously characterised.

This work characterised the sequential progression to high-level aminoglycoside resistance via a stepwise mutation study, analysing resistance progression for a range of aminoglycosides. Mutants of *E. coli* NCTC10418 were selected in four steps (Series 1-4) on plates with increasing aminoglycoside (streptomycin, neomycin, gentamicin, kanamycin and tobramycin) concentrations. Cross-resistance between different antibiotics was determined, to analyse whether resistance mechanisms between aminoglycosides are common to all drugs, and hence whether these drugs share common targets. The nucleotide sequence of ribosomal *rpsL*12 encoding S12, was determined for resistant mutants to analyse whether mutation in this target contributes to resistance to aminoglycosides other than streptomycin resistance, and whether the ribosomal S12 protein is a common target for all of the aminoglycosides.

5.2 Selection of aminoglycoside resistant mutants

Mutants of *E. coli* NCTC10418 were selected in four steps (Series 1-4) on plates containing increasing aminoglycoside concentrations. The mutation frequencies and MICs of mutants at each subsequent step is shown in table 5.1.

Table 5.1: MICs and mutation frequencies of aminoglycoside resistant mutants selected in four series (1-4)

Drug	Series (MIC mg/L and mutation frequency)			
	1	2	3	4
Streptomycin (S)	25 (1×10^{-6})	100 (1×10^{-4})	1000 (1×10^{-3})	1500 (1×10^{-3})
Neomycin (N)	10 (1×10^{-6})	25 (1×10^{-6})	50 (1×10^{-6})	50 (1×10^{-6})
Tobramycin (T)	10 (1×10^{-6})	20 (1×10^{-6})	50 (1×10^{-6})	50 (1×10^{-6})
Kanamycin (K)	10 (1×10^{-6})	20 (1×10^{-3})	50 (1×10^{-6})	50 (1×10^{-6})
Gentamicin (G)	2 (1×10^{-5})	4 (1×10^{-6})	10 (1×10^{-6})	10 (1×10^{-6})

Streptomycin resistant mutants were selected with increasing frequency with each selection step (1 in $10^6 \rightarrow 1$ in 10^3), the first mutation being the most infrequent; however after selection of this initial mutant, subsequent mutants were more readily selected (Table 5.1). Resistance to streptomycin was not selected at levels greater than 1000mg/L until the third step, which was unexpected, as previous work has documented streptomycin resistance of greater than 1500mg/L selected in a single step. In this experiment four selection steps were required to achieve this level of resistance. The levels of streptomycin resistance achieved after four series was higher

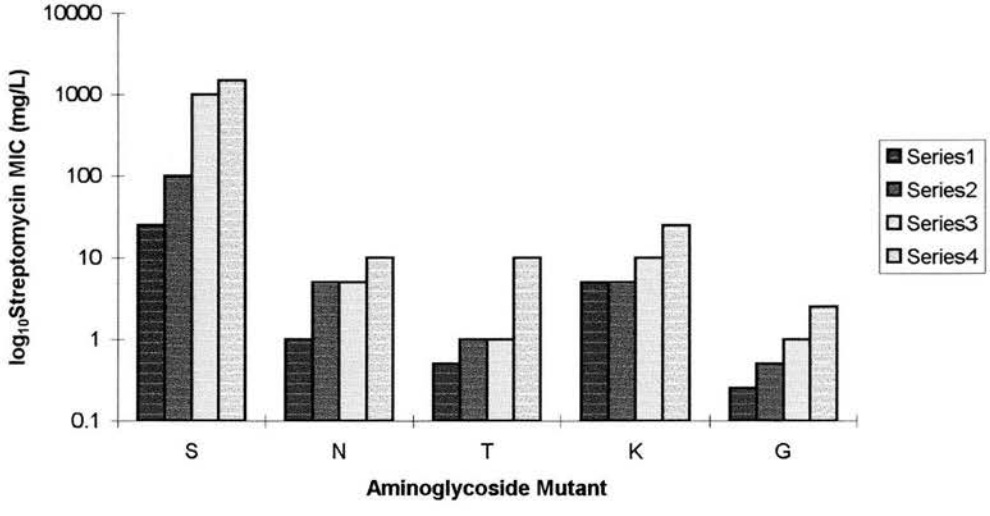
than resistance to the other aminoglycosides. Resistance to kanamycin, tobramycin, and neomycin progressed in a similar manner with each selection step, giving final resistance levels of 50-100mg/L with the 4^{th} step selection. Excluding kanamycin which had a higher mutation frequency on the second step selection (1 in 10^3), mutation frequencies remained low (1 in 10^6) and constant throughout the four selections. Gentamicin resistance levels were the lowest after four selections, at 10mg/L, with mutation frequencies remaining low and constant throughout the four selections.

5.3 Aminoglycoside cross-resistance

The levels of cross-resistance between streptomycin (S), neomycin (N), tobramycin (T), kanamycin (K) and gentamicin (G) resistant mutants were determined (Figures 5.1 to 5.5).

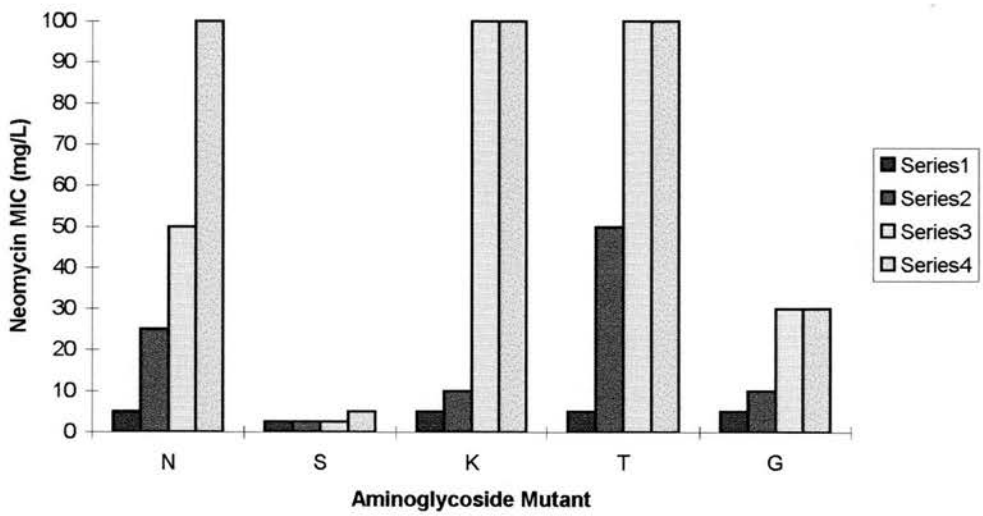
Streptomycin resistance was only seen in mutants previously selected on streptomycin plates, with resistance rising to 1500mg/L within four selection steps (Figure 5.1). Tobramycin, neomycin, gentamicin and kanamycin-selected mutants remained sensitive to streptomycin, indicating that the mutational changes and, therefore, drug targets between of streptomycin appear different to the other aminoglycoside groups. This is also confirmed in figures 5.2 to 5.5, where it can be seen that streptomycin selected mutants remain sensitive to all other aminoglycosides tested.

Figure 5.1: Mutant susceptibility to streptomycin



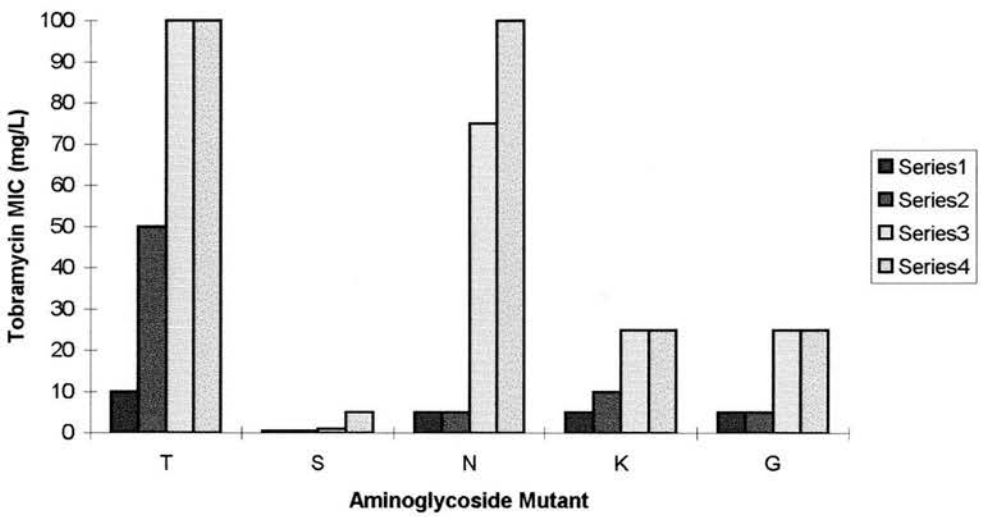
High-level resistance (100mg/L) to neomycin was seen in neomycin, kanamycin and tobramycin selected mutants (Figure 5.2). Gentamicin mutants also showed resistance to neomycin to a level of 30mg/L. Streptomycin mutants remained sensitive to neomycin. Cross-resistance was significant between neomycin, kanamycin and tobramycin and could be due to the fact that these antibiotics have similar structures and therefore share common target proteins.

Figure 5.2: Mutant susceptibility to neomycin



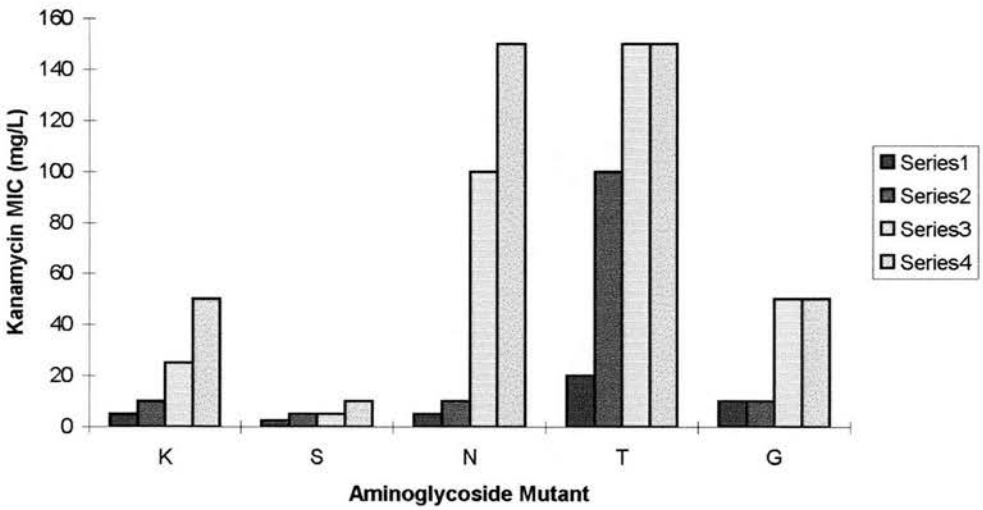
The greatest levels of resistance to tobramycin was seen in tobramycin and neomycin selected mutants, with resistance levels reaching 100mg/L. Gentamicin and kanamycin showed similar levels of resistance (25mg/L), whereas mutants selected by streptomycin remained sensitive (Figure 5.3).

Figure 5.3: Mutant susceptibility to tobramycin



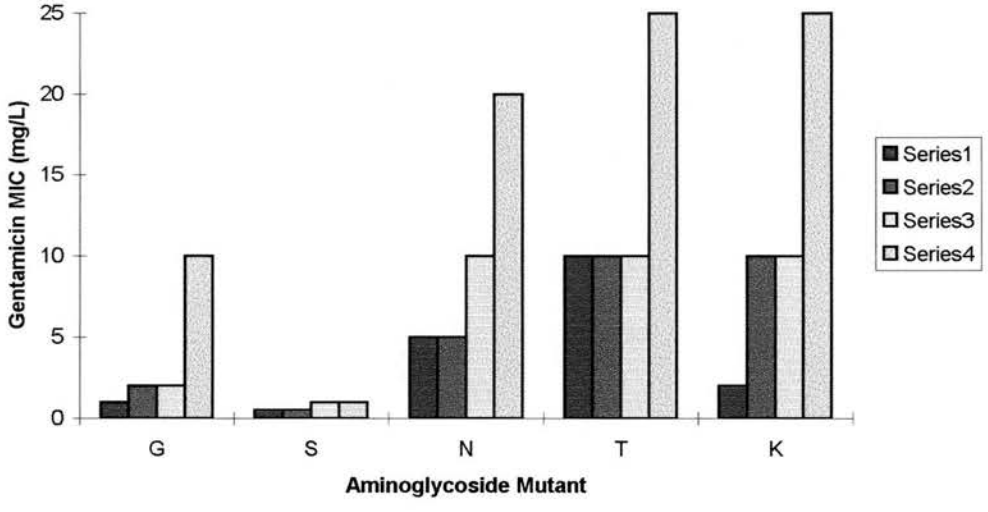
High-level resistance to kanamycin was seen in neomycin, kanamycin and tobramycin and gentamicin mutants (50-100mg/L) (Figure 5.4). Streptomycin mutants remained sensitive. Interestingly, neomycin, tobramycin and gentamicin-selected mutants had greater or equal levels of kanamycin resistance, than mutants selected on kanamycin plates. This again indicates the possibility of shared target proteins and hence resistance mutations conferring aminoglycoside cross-resistance.

Figure 5.4: Mutant susceptibility to kanamycin



Resistance to gentamicin was lower than other aminoglycosides rising to 25mg/L for tobramycin and kanamycin 4th step mutants (Figure 5.5). Gentamicin resistance in gentamicin-selected mutants rose to 10mg/L, a lower level than for neomycin, kanamycin and tobramycin selected mutants. Neomycin, tobramycin and kanamycin may therefore be more effective in selecting mutations that lead to aminoglycoside cross-resistance. Streptomycin selected mutants remained sensitive to gentamicin.

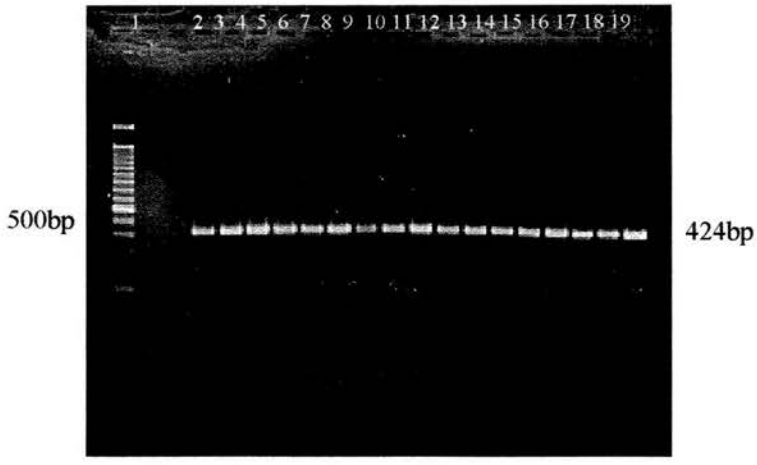
Figure 5.5: Mutant susceptibility to gentamicin



5.4 PCR amplification and sequencing of the *rpsL* 12 gene

The gene encoding ribosomal protein S12 (*rpsL*12) encompassing mutation hotspots (41-43 and 85-91) was amplified by PCR, producing a 424bp PCR amplicon (Figure 5.6).

Figure 5.6: A 424bp *rpsL* amplicon of aminoglycoside resistant mutants



Lane 1: 100bp DNA ladder; 2 - S-100; 3 - S-25; 4 - S-1000; 5 - S-1500; 6 - G-2; 7 - G-1(2^{nd}); 8 - G-1(3^{rd}); 9 - G-10; 10 - N-5; 11 - N-25; 12 - N-50; 13- N-100; 14 - T-10; 15 - T-50; 16 - T-100; 17 - K-5; 18 - K-10; 19; K-50 (see table 5.1)

Mutational changes within the *rpsL* genes were analysed by automated DNA sequencing and compared with the *E. coli* S12 sequence in GenBank with accession number V00355, originally described by Post & Namura, (1980) (Figure 5.7). Mutations have been previously described in the two main areas, which are shown in bold in Figure 5.7.

Figure 5.7: Wild-type *E. coli rpsL* sequence

ATG	GCA	ACA	GTT	AAC	CAG	CTG	GTA	CGC	AAA
Met	Ala	Thr	Val	Asp	Gln	Leu	Val	Arg	Lys
CCA	CGT	GCT	CGC	AAA	GTT	GCA	AAA	AGC	AAC
Pro	Arg	Ala	Arg	Lys	Val	Ala	Lys	Ser	Asn
GTG	CCT	GCG	CTG	GAA	GCA	TGC	CCG	CAA	AAA
Val	Pro	Ala	Leu	Glu	Ala	Cys	Pro	Gln	Lys
CGT	GGC	GTA	TGT	ACT	CGT	GTA	TAT	ACT	ACC
Arg	Ala	Val	Cys	Thr	Arg	Val	Tyr	Thr	Thr
ACT	**CCT**	**AAA**	**AAA**	CCG	AAC	TCC	GCG	CTG	CGT
Thr	**Pro**	**Lys**	**Lys**	Pro	Asn	Ser	Ala	Leu	Arg
	41		**43**						
AAA	GTA	TGC	CGT	GTT	CGT	CTG	ACT	AAC	GGT
Lys	Val	Cys	Arg	Val	Arg	Leu	Thr	Asn	Gly
TTC	GAA	GTG	ACT	TCC	TAC	ATC	GGT	GGT	GAA
Phe	Glu	Val	Thr	Ser	Tyr	Ile	Gly	Gly	Glu
GGT	CAC	AAC	CTG	CAG	GAG	CAC	TCC	GTG	ATC
Gly	His	Asn	Leu	Gln	Glu	His	Ser	Val	Ile
CTG	ATC	CGT	GGC	GGT	**CGT**	**GTT**	**AAA**	**GAC**	**CTC**
Leu	Ile	Arg	Gly	Gly	**Arg**	**Val**	**Lys**	**Asp**	**Leu**
					85		**87**		
CCG	**GGT**	GTT	CGT	TAC	CAC	ACC	GTA	CGT	GGT
Pro	**Gly**	Val	Arg	Tyr	His	Thr	Val	Arg	Gly
	91								
GCG	CTT	GAC	TGC	TCC	GGC	GTT	AAA	GAC	CGT
Arg	Leu	Asp	Cys	Ser	Gly	Val	Lys	Asp	Arg
AAG	CAG	GCT	CGT	TCC	AAG	TAT	GGC	GTG	AAG
Lys	Gln	Ala	Arg	Ser	Lys	Tyr	Gly	Val	Lys
CGT	CCT	AAG	GCT	TAA					
Arg	Pro	Lys	Ala	-					
			123						

Mutation hotspots are shown in bold
- STOP

Sequencing of the *rpsL* gene of S-1500, N-50, K-50, T-50 and G-10 series 4 (S-1500) mutants revealed mutation in the *rpsL* gene of the streptomycin resistant mutant only. The other sequences mimicked that of wild-type *rpsL* (Figure 5.7). The sequence of S-1500 is shown in Figure 5.8.

Figure 5.8: S-1500 streptomycin mutant *rpsL* sequence

ATG Met	GCA Ala	ACA Thr	GTT Val	AAC Asp	CAG Gln	CTG Leu	GTA Val	CGC Arg	AAA Lys
CCA Pro	CGT Arg	GCT Ala	CGC Arg	AAA Lys	GTT Val	GCA Ala	AAA Lys	AGC Ser	AAC Asn
GTG Val	CCT Pro	GCG Ala	CTG Leu	GAA Glu	GCA Ala	TGC Cys	CCG Pro	CAA Gln	AAA Lys
CGT Arg	GGC Ala	GTA Val	TGT Cys	ACT Thr	CGT Arg	GTA Val	TAT Tyr	ACT Thr	ACC Thr
ACT Thr	**CCT Pro 41**	**AAA Lys**	**AAA Lys 43**	CCG Pro	AAC Asn	TCC Ser	GCG Ala	CTG Leu	CGT Arg
AAA Lys	GTA Val	TGC Cys	CGT Arg	GTT Val	CGT Arg	CTG Leu	ACT Thr	AAC Asn	GGT Gly
TTC Phe	GAA Glu	GTG Val	ACT Thr	TCC Ser	TAC Tyr	ATC Ile	GGT Gly	GGT Gly	GAA Glu
GGT Gly	CAC His	AAC Asn	CTG Leu	CAG Gln	GAG Glu	CAC His	TCC Ser	GTG Val	ATC Ile
CTG Leu	ATC Ile	CGT Arg	GGC Gly	GGT Gly	**CGT Arg 85**	**GTT Val**	**GAA Glu 87**	**GAC Asp**	**CTC Leu**
CCG Pro	**GGT Gly 91**	GTT Val	CGT Arg	TAC Tyr	CAC His	ACC Thr	GTA Val	CGT Arg	GGT Gly
GCG Arg	CTT Leu	GAC Asp	TGC Cys	TCC Ser	GGC Gly	GTT Val	AAA Lys	GAC Asp	CGT Arg
AAG Lys	CAG Gln	GCT Ala	CGT Arg	TCC Ser	AAG Lys	TAT Tyr	GGC Gly	GTG Val	AAG Lys
CGT Arg	CCT Pro	AAG Lys	GCT Ala 123	TAA -					

Amino-acid substitutions are shown in red

An amino-acid substitution of Lys87→Glu was found in this sequence (S-1500). To analyse at which point during mutation selection over the four series (1-4) this mutation had occurred, Series 1(25mg/L), 2(100mg/L) and 3(1000mg/L) mutants were sequenced. The Lys87→Glu mutation was present in all four series of mutants (Streptomycin MICs 25→1500mg/L), indicating that this mutation was not responsible for the extremely high-level resistance found in streptomycin-resistant mutants (1500mg/L).

The lack of S12 mutations in tobramycin, gentamicin, kanamycin and neomycin mutants suggested that the S12 protein encoded by *rpsL* may be a target unique to streptomycin, with target preference closely associated with drug structure. The lack of additional mutations in streptomycin resistant mutants, indicated the role of other ribosomal proteins /targets in streptomycin action.

6. Results: Bactericidal antibiotic action

6.1 An Overview

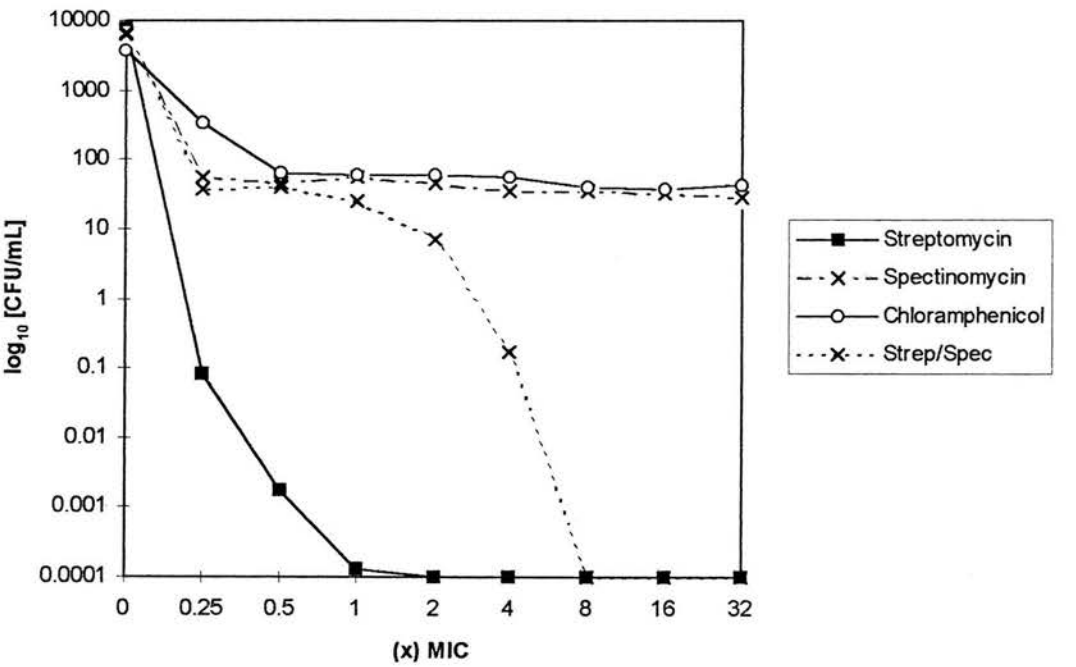
Antibiotics which interfere with bacterial protein synthesis machinery are known to exert both bacteriostatic and bactericidal effects on cells. For example, chloramphenicol which interacts with the bacterial 70S ribosome, is bacteriostatic, as is spectinomycin, an aminocyclitol which is structurally closely related to the aminoglycosides. Streptomycin, an aminoglycoside which interacts with the 30S ribosomal sub-unit, exerts rapid bactericidal activity upon bacterial populations. The reason why the aminoglycosides are bactericidal, whereas other protein synthesis inhibitors are bacteriostatic remains to be determined. The way in which aminoglycosides exert their killing effect in stationary phase populations, under conditions of amino-acid starvation, or when protein synthesis is inhibited by drug intervention may provide important new evidence essential for understanding these differences.

This work investigated streptomycin induced cell death in log phase *E. coli* NCTC10418 populations when challenged with single antibiotics, or antibiotic combinations. The requirement of protein synthesis in streptomycin-induced cell death was additionally analysed by challenging cell populations in minimal media, under various growth conditions, and in the presence of bacteriostatic protein synthesis inhibitors. Both dose-response and time-kill activity were examined, by assays utilising 3-hour incubations of *E. coli* populations with a specific drug or drug combination.

6.2 Activity of streptomycin, spectinomycin, chloramphenicol and a streptomycin/spectinomycin combination against log-phase *E. coli*

The activity of streptomycin, spectinomycin and chloramphenicol were assessed against a log-phase *E. coli* population (Figure 6.1). Streptomycin is thought to paradoxically require protein synthesis for its bactericidal activity. As spectinomycin is a bacteriostatic protein synthesis inhibitor, cells were also challenged with a streptomycin/spectinomycin combination, to examine any possible antagonistic activity caused by spectinomycin halting putative protein synthesis required for cell death (Figure 6.1).

Figure 6.1: Activity of streptomycin, spectinomycin, chloramphenicol, and a streptomycin/spectinomycin combination against log-phase *E. coli*

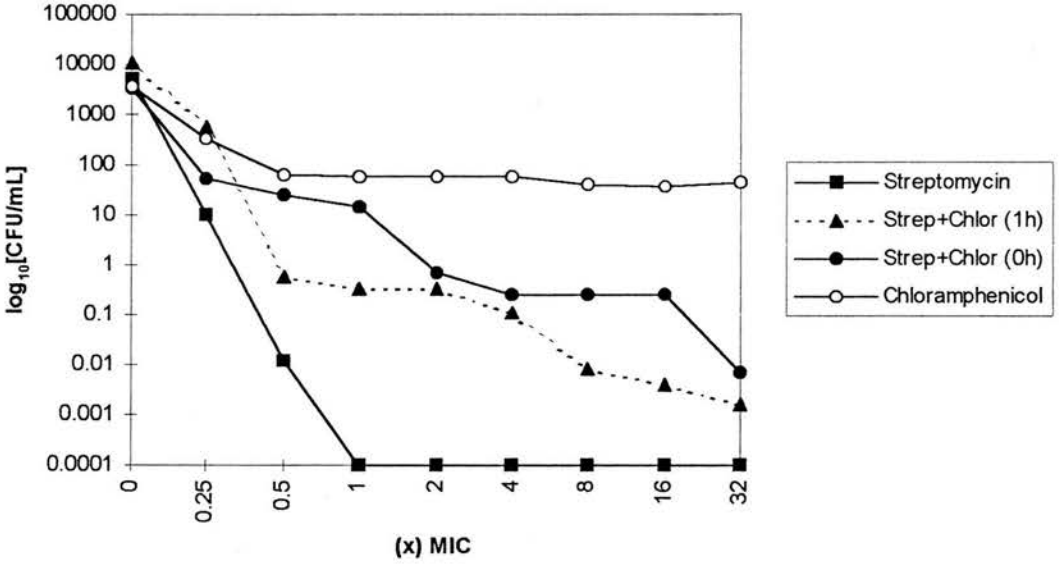


The aminoglycoside streptomycin exerted a rapid bactericidal effect at concentrations below its MIC (10mg/L), with bactericidal activity directly proportional to dose. Chloramphenicol (MIC=2mg/L) and the aminocyclitol spectinomycin (MIC=100mg/L) exerted a principally bacteriostatic effect, with some initial killing in comparison with the original viable count (Figure 6.1). At low doses (below MIC) the streptomycin effect was antagonised by the presence of spectinomycin; however streptomycin can still exert its maximum bactericidal effect at three times higher concentrations than without spectinomycin. This indicates that incubation with this protein synthesis inhibitor did prevent death at low concentrations; however at high streptomycin concentrations, streptomycin may not be reliant on protein synthesis for cell death. Spectinomycin was however only added at time 0h in this experiment, thereby some protein synthesis would have proceeded before spectinomycin exerted its effect. Therefore, although streptomycin still exerted its effect at high concentrations, the antagonistic effect of spectinomycin on streptomycin induced cell death, suggested that there was a requirement for some protein synthesis in cell death.

6.3 Effect of chloramphenicol on streptomycin activity

The activity of streptomycin against *E. coli* in the presence of the bacteriostatic protein synthesis inhibitor chloramphenicol was investigated. Chloramphenicol was added at Time 0h and at 1h prior to streptomycin addition, to analyse the effect of a more prolonged protein synthesis inhibition before streptomycin action (Figure 6.2).

Figure 6.2: Bactericidal activity of streptomycin against *E. coli* treated with chloramphenicol (10mg/L) at time 0h and 1h previous to streptomycin addition

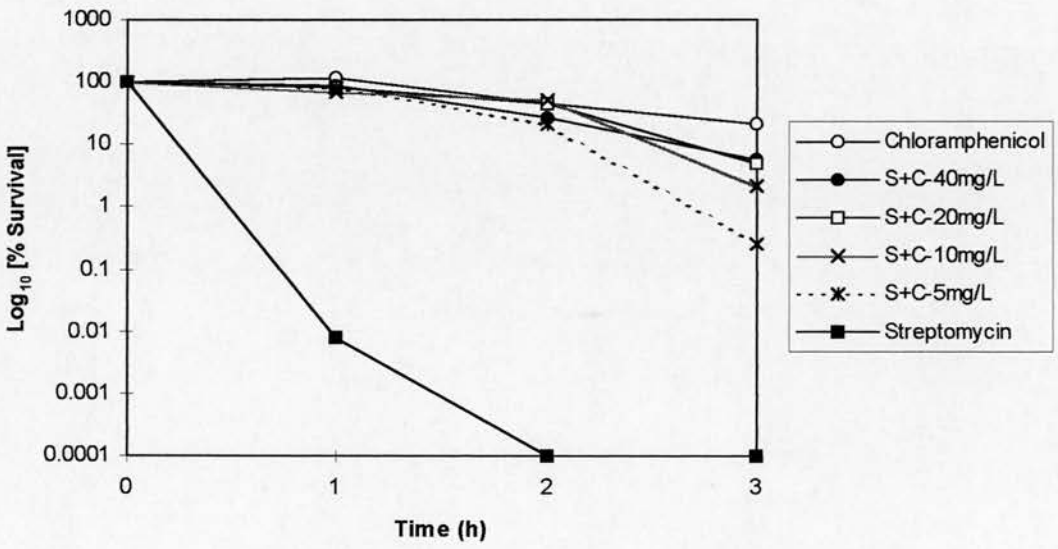


Streptomycin exerted a potent bactericidal activity, with cell populations reduced more than seven fold when treated with 10mg/L (1xMIC) and higher concentrations. A bi-phasic response, as is seen for fluoroquinolones, where cell viability begins to increase after an optimal bactericidal concentration was not seen, with cell death proportional to drug concentration. Chloramphenicol alone produced a bacteriostatic

response, after an initial phase of cell death. Chloramphenicol reduced bactericidal killing 1-2 fold, and pre-incubating cells with chloramphenicol for 1h previous to streptomycin addition had no effect on cell death.

The bactericidal activity of streptomycin (S) in the presence of increasing chloramphenicol (C) concentrations over a three hour time period was assessed (Figure 6.3). Log-phase *E. coli* populations were pre-incubated for 1h with chloramphenicol (5mg/L to 40mg/L), before subsequent addition of 100mg/L streptomycin. This dose was chosen, as it is would give a maximal bactericidal effect.

Figure 6.3: Bactericidal activity of streptomycin against *E. coli* pre-treated with varying chloramphenicol concentrations (5-40mg/L)



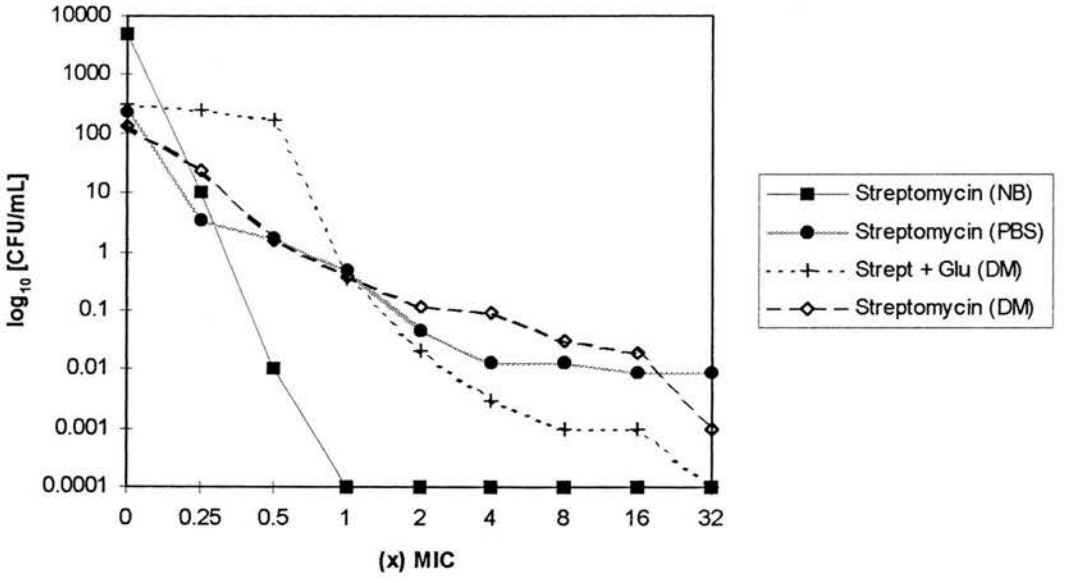
Streptomycin activity was proportionally reduced against *E. coli* pre-incubated with increasing chloramphenicol concentrations, with chloramphenicol-5mg/L providing the least antagonistic effect, and chloramphenicol at 40mg/L being the most

antagonistic. Streptomycin (100mg/L) alone produced rapid bactericidal cell death in 1h with cell population reduced 6-fold after three hours. Chloramphenicol alone (40mg/L), produced a principally bacteriostatic effect, with cell populations decreasing by less than 0.5-fold. These results indicated that streptomycin-induced cell death, required protein synthesis. The contrasting finding between this experiment and that of figure 6.2, may be due to an insufficient chloramphenicol concentration, and therefore incomplete protein synthesis inhibition in the previous experiment.

6.4 Effect of amino-acid starvation on streptomycin activity

The previous findings had indicated that protein synthesis was an integral requirement for streptomycin-induced cell death. To add weight to this hypothesis, streptomycin activity was analysed against cells incubated in media lacking a carbon source. The media used for these challenge experiments were Davis-Mingioli (DM) minimal media and phosphate buffered saline. Cells were also challenged in nutrient broth and Davis-Mingioli medium containing glucose as controls (Figure 6.4).

Figure 6.4: Streptomycin activity against *E. coli* under conditions of amino-acid limitation



The activity of streptomycin against *E. coli* in minimal media (DM and PBS) was reduced 1-2 fold in comparison with cells in carbon-rich media (NB and DM + glucose). This reduction was therefore not significant with killing levels 5-fold, in comparison to the 1-fold reduction seen when streptomycin acts upon

chloramphenicol-treated cells. This result, however, does not necessarily contradict the result found with chloramphenicol antagonism, as amino-acid starvation conditions may still produce residual amino-acids, which upon protein breakdown provide the supply necessary for streptomycin-induced cell death. Breakdown of these residual amino-acids may explain the difference between streptomycin challenge of *E. coli* in amino-acid starvation, compared with that under chloramphenicol treatment.

6.5 The role of streptomycin as a protein synthesis inhibitor - mechanism of bactericidal cell death ?

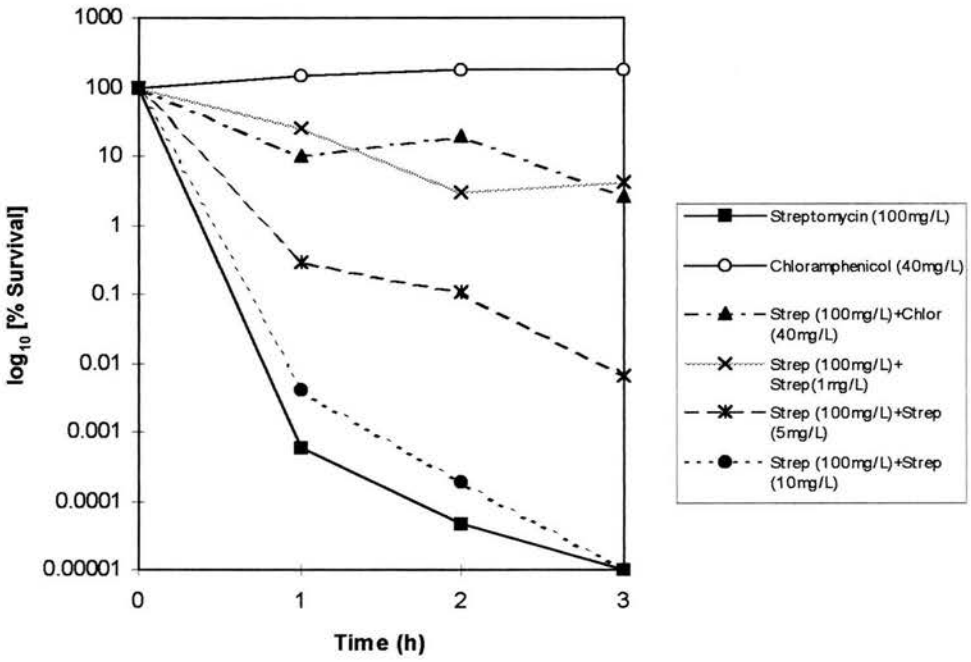
Streptomycin is known to exert effects on ribosomal proteins leading to the inhibition of protein synthesis. The role of this protein synthesis inhibition in cell death is not well characterised. We have seen that other ribosome interacting antibiotics such as chloramphenicol and spectinomycin are bacteriostatic, leading to the hypothesis that although streptomycin interacts with the ribosome and interferes with protein synthesis, it is not the effect on protein synthesis that leads to cell death. The fact that streptomycin requires protein synthesis for bactericidal activity, shown by the antagonistic effects of chloramphenicol, suggests that other mechanisms may be responsible for cell death.

The protein synthesis inhibiting action of streptomycin was investigated in experiments in which *E. coli* populations pre-treated with low streptomycin concentrations, were challenged with a higher bactericidal streptomycin dose. This was based upon the hypothesis that at low concentrations streptomycin would exert its effects as a protein synthesis inhibitor, and therefore consequently could inhibit its own bactericidal protein synthesis requiring mechanism at higher streptomycin concentrations.

Log-phase *E. coli* populations were pre-treated with chloramphenicol (10mg/L) as a control, and various concentrations of streptomycin (10, 5, 1mg/L). Controls

challenging cells with streptomycin and chloramphenicol alone were also performed (Figure 6.5).

Figure 6.5: Bactericidal activity of streptomycin against *E. coli* pre-treated with chloramphenicol (10mg/L) and streptomycin (10, 5, 1mg/L)

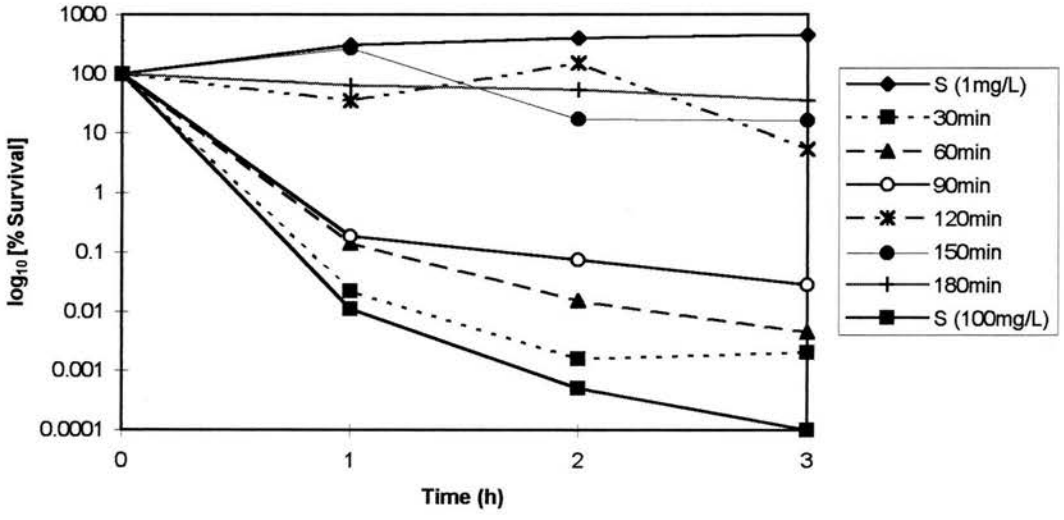


Streptomycin (100mg/L) induced a rapid bactericidal cell death within 1h, with cell viability reduced to <0.0001% at 3h. Pre-incubation with chloramphenicol significantly reduced the bactericidal activity indicating a requirement of protein synthesis in cell death. Substituting 1mg/L streptomycin for chloramphenicol gave a similar significant reduction in bactericidal activity. Pre-incubation with 5 or 10mg/L streptomycin conferred a reduced effect on the killing rate of streptomycin (100mg/L). These results indicated that at sub-lethal concentrations streptomycin acts

as a protein synthesis inhibitor which subsequently blocked its own bactericidal activity.

The length of time protein synthesis needed to be halted before addition of the second streptomycin dose was assessed (Figure 6.6). *E. coli* were pre-incubated with streptomycin (1mg/L) for various time points (30-180min), before subsequent addition of streptomycin (100mg/L).

Figure 6.6: Bactericidal activity of streptomycin (100mg/L) against *E. coli* pre-treated with streptomycin (1mg/L) for 30-180 minutes

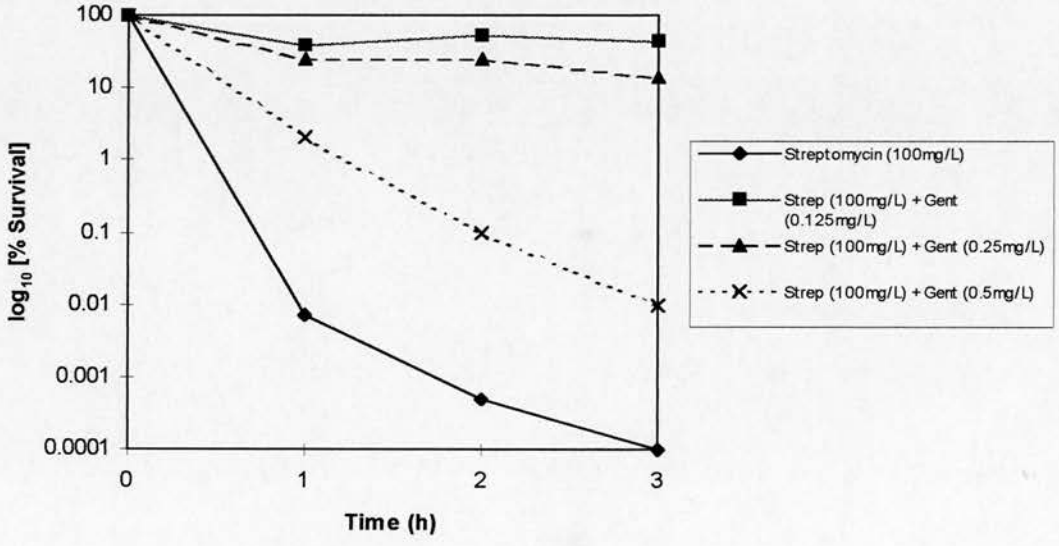


The length of streptomycin (1mg/L) pre-incubation time correlated directly with subsequent inhibition of streptomycin activity. The inhibition of streptomycin killing was most prominent with a pre-incubation time of ≥120minutes. Streptomycin

(100mg/L) alone as expected had rapid bactericidal activity, and streptomycin (1mg/L) exerted a bacteriostatic effect.

In order to rule out the possibility that the three hour pre-incubation with streptomycin was not producing a streptomycin resistant population, leading to the subsequent survival of cells when challenged with a higher bactericidal dose, the experiment was repeated substituting streptomycin pre-incubation for gentamicin pre-incubation. Log-phase *E. coli* cells were pre-incubated with low concentrations of gentamicin (0.125, 0.25 and 0.5mg/L) for 2 hours prior to the addition of a bactericidal streptomycin (100mg/L) dose (Figure 6.7).

Figure 6.7: Bactericidal activity of streptomycin against *E. coli* pre-treated with gentamicin (0.125, 0.25 and 0.5mg/L)



Streptomycin (100mg/L) induced a rapid bactericidal cell death within 1h, with cell viability reduced to <0.0001% at 3h. Substituting gentamicin (0.125mg/L) for

streptomycin (1mg/L) gave a similar significant reduction in bactericidal activity as seen in figure 6.5. Pre-incubation with or 0.25 and 0.5mg/L gentamicin had no effect on the killing rate of streptomycin (100mg/L). These results indicated that at sub-lethal concentrations aminoglycosides act as a protein synthesis inhibitors which subsequently blocked its own cell death when challenged with a higher bactericidal dose. This result demonstrates that pre-incubation with low doses of streptomycin is not creating a population of cells which are more readily resistant to streptomycin, but that it is the effect on protein synthesis inhibition that reduced the bactericidal action of streptomycin against this cell population.

7. Discussion

Analysis of the way in which target proteins mutate to confer antibiotic resistance provides a clearer understanding of bacterial resistance mechanisms. It also sheds light on the important antibiotic-target protein interaction, an understanding of which is crucial if we are to elucidate the complex mechanisms of antibiotic-induced bacterial cell death. The deduction of primary and secondary bacterial targets is of special significance for the future of antimicrobial drug design and resistance minimisation, as is an understanding of pathways of cell death not directly related to the initial drug-target interaction. Indeed there may be commonality between cell death induced by drugs, which have very different initial targets, as there is differentiation in cell death induced by drugs that share target proteins, for example those which target ribosomal protein synthesis machinery. This thesis presents results on mutations in the target proteins of two bacterial drug classes; the fluoroquinolones, which target the enzymes DNA gyrase and DNA topoisomerase IV, and the aminoglycosides, which target the ribosomal proteins. In addition, the role of protein synthesis requiring cell death was examined for the aminoglycoside streptomycin.

7.1 Fluoroquinolone resistance in clinical *E. coli* isolates

Resistance to the fluoroquinolones in clinical *E. coli* isolates has until recently been rarely observed in the U. K.; however, the situation is now an emerging phenomenon of increasing concern. This study characterised the resistance profile of 15 clinical *E. coli* isolates obtained from a hospital in Newcastle in 1996. Of the 15 viable isolates obtained in this study, 8/15 were found to have high-level ciprofloxacin resistance with MICs ranging from 8 to 256mg/L. The MICs of six of these isolates was ≥32mg/L, an unusually high level of quinolone resistance for *E. coli* isolates.

Reports of increasing levels of fluoroquinolone resistance in Europe has been previously limited to Spain, where lack of antibiotic control and increased usage has led to resistance levels rising alarmingly from 0% in 1988 to 7.5% in 1992 in *E. coli* bacteraemias (Pena *et al.*, 1995). Carratala *et al.* (1995), also demonstrated that 37% of bacteraemias in neutropenic cancer patients were due to quinolone resistant strains. This rise in fluoroquinolone resistance demands the use of tight restrictions on prescribing, which Spain lacks. The high-level ciprofloxacin resistance in this study suggests that the U.K may not be exempt from a problem of emerging fluoroquinolone resistance, and support evidence from Threlfall *et al.* (1997), who detected high-level ciprofloxacin resistance in *E. coli* isolates from patients with invasive illness or urinary tract infection (MICs=4-256mg/L) during the period 1994-1997. Taken together, these results suggest that the UK is not exempt from the problem of increasing fluoroquinolone resistance, and raise serious concerns over the future use of ciprofloxacin, and the promotion of fluoroquinolone resistance. It should

however be noted that a majority of *E. coli* strains (95%) isolated in the UK remain sensitive to the fluoroquinolones (Thomson,1999).

Although only 8/15 isolates were clinically resistant, 14/15 isolates showed increased levels of fluoroquinolone resistance, when compared to the control strain *E. coli* NCTC10418. These strains with underlying low to moderate levels of resistance may represent a bacterial population poised to become highly resistant upon further drug treatment. These underlying resistance levels are rarely detected by the techniques employed in the clinical microbiology departments, which rely heavily on breakpoint sensitivities. Indeed MIC breakpoints may provide a false sense of security and thereby contribute to the selective enrichment of antibiotic resistant mutants by encouraging the use of marginal compounds as monotherapy. We may be facing a widespread population of low-level resistance strains, which will pose major problems for future antimicrobial therapy.

In addition to determining the resistance levels of ciprofloxacin, the activity of new second and third generation fluoroquinolones against isolates in this outbreak was also investigated. Many new fluoroquinolones have been modified to enhance potency, and certain substitutions are known to enhance dual target, rather than single target binding. It was, therefore, of particular interest to examine the activity of these new compounds against isolates that already show wide ranging levels of ciprofloxacin resistance. Surprisingly, high-level resistance to the new fluoroquinolones moxifloxacin, sparfloxacin and gemifloxacin was demonstrated in

the Newcastle isolates, with resistance levels correlating closely with ciprofloxacin resistance. In fact a greater percentage of the clinical isolates were resistant to sparfloxacin and moxifloxacin.

These results have important consequences for the future use of the new fluoroquinolones, and highlight their limitations. These new quinolones have not been introduced into clinical practice in the UK. The results of this study thereby indicate that resistance mechanisms between ciprofloxacin and these new drugs are shared. Although the new compounds may have been engineered to have greater potency and hence may slow the initial selection of antibiotic mutants in the clinic (Zhao *et al.*, 1997), they do not appear to be any more active against clinical strains, which have low and high-levels of previously selected fluoroquinolone resistance.

Drugs with a C-8 methoxy substitution such as moxifloxacin have been reported to be more active against strains which have an initial gyrase mutation (Zhao *et al.*, 1999; Dong *et al.*, 1998; Zhao *et al.*, 1997), however this was not the case against the clinical isolates in this study. Moxifloxacin a generation IIIa, gram-positive targeted fluoroquinolone was no more effective against resistant strains than ciprofloxacin. There are however high hopes that third generation quinolones will provide a class of antibiotics, which are effective even against resistant strains. A greater percentage of isolates were resistant to gemifloxacin, a generation IIIb napthyridone, with exceedingly high-levels of gemifloxacin seen in correlation with the levels of ciprofloxacin resistance (256mg/L). The correlation between MIC levels of the

fluoroquinolones suggest that resistance mechanisms between these drugs are ubiquitous, shared, and therefore selecting resistance to one of these antibiotics will inevitably lead to resistance upon treatment with another drug of the same class. This has not been the case for other antimicrobial classes, in which infections resistant to a first generation β-lactam (penicillin), are often successfully treated with a third generation drug (cefotaxime). This is because of the differences in β-lactamases required to overcome these drugs.

Gemifloxacin has been modified to enhance potency, and therefore may be less effective at selecting out bacterial resistance in the first place, as once a drug with increased potency is used, the frequency of mutation selection will be reduced. However, once mutations are selected, the results in this thesis suggest that gemifloxacin, and the newer fluoroquinolones will be no more effective than the second generation ciprofloxacin. The fact that moxifloxacin resistance was generally higher may have been due to the fact that this drug has different target specificity than the other fluoroquinolones, which have increased activity against gram-negative organisms (Ball, 2000; Pestova *et al.*, 2000).

All of the fluoroquinolone resistant isolates were found to be resistant to at least one additional antimicrobial; trimethoprim, the majority were additionally resistant to amoxycillin, with one isolate demonstrating a multi-resistant phenotype, showing resistant to three antimicrobials (trimethoprim, amoxycillin and co-amoxiclav). These results support those of Threlfall and colleagues (1997), who found that the

fluoroquinolone-resistant strains they isolated expressed a multiply resistant phenotype. This raised concerns for the possibility of treatment failure in these *E. coli*. Sensitivity of isolates in the current study to ceftazidime and meropenem, demonstrated that there were however treatment options for patients infected with these organisms. The multiple antibiotic resistant phenotype in these isolates implicated the involvement of plasmid mediated resistance mechanisms, which confer resistance to penicillins and trimethoprim. Changes in outer membrane proteins (OMPs) and efflux, are also known to confer resistance to numerous drug classes (Charvalos *et al.*, 1995; Li *et al.*, 1994; Hooper *et al.*, 1992; Cohen *et al.*, 1989). The MICs of fluoroquinolones, did not, however correlate with resistance against aminoglycosides, suggesting that changes in the OMPs were of minor importance. Cohen *et al.* (1989) demonstrated cross-resistance to fluoroquinolones in multiple antibiotic resistant (Mar) *E. coli*, and Okusu *et al.* (1996) showed that the AcrAB efflux pump plays a major role in the antibiotic resistant phenotype of Mar mutants.

The clonal diversity of strains was determined to assess whether the outbreak had been due to clonal spread of resistant strains, implicating defective infection control measures, or due to individual selection of resistance in patients. A variety of typing techniques were employed to achieve the most accurate result, along with an assessment of the value of restriction and PCR based techniques in typing *E. coli*. Genotyping by PFGE has been shown to be a powerful tool for the study of the genetic diversity of *E. coli* strains from related lineage, and accordingly offers a high level of discriminatory power (Arbeit *et al.*, 1990). Interestingly, several strains

among the fluoroquinolone resistant isolates in this study were nontypable. The exact reasons for this nontypability are unknown. Nontypable strains showed smeared DNA fragments between 40 and 270kb. And there was no indication that these smeared DNA fragments were due to DNAase activity. Typing was attempted utilising two separate enzymes *Xba*I and *Not*I. *Xba*I produced restriction for 9/15 isolates, with 6 remaining untypable. Attempts to restrict these untypable isolates using the enzymes *Not*I also failed, yielding the same pattern of DNA fragment as found when restricted with *Xba*I. Interestingly *Not*I was also not effective in typing strains which has been previously restricted with *Xba*I, yielding only partially restricted DNA fragments. Doubling *Not*I concentration failed to eliminate this problem and for economical reasons, attempts to type with this enzyme were discontinued.

Failure to type fluoroquinolone resistant *E. coli* isolates by PFGE has previously been reported by Oethinger *et al.* (1996), who found that of 23 isolates, only 16 were typable by PFGE. The nontypbable isolates produced smeared DNA fragments similar to those seen in this study. Oethinger *et al.* (1996) found that nontypability of his strains correlated with their high-level fluoroquinolone resistance, whereas the nontypable stains in this study were a mixture of sensitive and resistant isolates. This supports results from other authors, who found nontypability in some stains of fluoroquinolone sensitive *E. coli* (Berg *et al.*, 1994).

The Tenover criteria were used to assess the band differences between restriction patterns (Tenover *et al.*, 1995) generated by PFGE typed isolates. These criteria are

intended for us in clinical hospital laboratories with small sets of isolates (typically no more than 30) which are related to putative disease outbreaks, and therefore were applicable to this study. The interpretation criteria are based on fragment differences expected within PFGE patterns that have undergone a defined number of "genetic events". These may be point mutations that result in the creation or loss of restriction sites, and insertions and deletions of DNA. PFGE that differ by two or three fragments are deemed closely related as fragment differences have arisen due to a single genetic event. Restriction patterns that differ by four to six fragments, are interpreted a possibly related, and PFGE with less than 50% fragments in common are considered unrelated, indicative of three of more genetic events that result in seven of more fragment differences. Based on these criteria the PFGE of 9 of the clinical *E. coli* isolates revealed 7 pulsotypes (Type A-G), with three highly resistant strains NC-14, NC-10 and NC-1 exhibiting identical PFGE restriction patterns (Type A), and the remaining PFGE typed isolates heterogenous in nature (B-G), differing by more than 7 bands.

PFGE has been considered the gold standard of bacterial fingerprinting, however the failure of this technique to type six of the clinical isolates led to the employment of PCR based typing techniques. Unlike PFGE, there are no standardised criteria for the interpretation of PCR generated fingerprints. These techniques additionally suffer from a high sensitivity to reaction conditions, DNA quality and PCR temperature profiles, leading to problems of reproducibility. PCR based techniques have however been successfully employed to type a range of bacteria (Versalovic *et al.*, 1991), and

specifically used in previous cases to type fluoroquinolone resistant Enterobacteriaceae (Oethinger *et al.*, 1996), where PFGE has failed. Short random primers were initially used to amplify genomic fragments by RAPD-PCR, with fingerprints produced for clinical isolates NC-1-15. The correlation between strain diversities of PFGE and RAPD-PCR was found acceptable although incomplete. RAPD-PCR typed strains into seven types (RI-RVII), with isolates NC-14, NC-10 and NC-1, which were deemed identical by PFGE having identical RAPD patterns (RI). A further two isolates NC-4 and NC-13 which were untypable by PFGE were related to RI isolates with one band difference in RAPD pattern (RI^1). Isolates NC-11, NC-8, NC-6 and NC-5 had also identical RAPD patterns (RII). All but one of the high-level resistant isolates were typed within type RI or RII, indicating that clonal spread of resistant isolates was responsible for the high-level resistance observed. Identical isolates showed varying levels of fluoroquinolone resistance, and therefore a combination of clonal spread followed by subsequent further selection of resistance within individual patients was likely to be responsible for the selection of resistance. The treatment regimen, and patient data for these strains were unfortunately unavailable, however it is likely that drug selection was associated with increases in resistance. The remaining isolates were typed as unrelated (RIII to RVIII), supporting results from PFGE.

Due to the lack of standardised interpretation criteria for RAPD results, a second PCR based typing method was employed, which relied upon the amplification of enterobacterial intergenic consensus (ERIC) sequences, as described by Gillings &

Holley (1997). The results from ERIC-PCR typing supported those from RAPD-PCR. Isolates NC-4, and NC-13, which were RAPD type I (RI), showed 2-3 band difference with ERIC-PCR (EI^2 & EI^3), demonstrating a degree of relativity but that the strains were not identical. Isolate NC-11 which was RAPD type II, differed by one band from this type by ERIC. All other isolates were deemed unrelated (EIII to EVII), supporting PFGE and RAPD results.

The small discrepancies between RAPD-PCR and ERIC-PCR based typing methods, led to the use of a new technique, AFLP, which is based on the detection of genomic restriction fragments by PCR amplification, and thereby combines the reliability of RFLP technique with the power of the PCR technique (Vos *et al.*, 1995). This typing method however failed to yield results. The reason for this failure was unapparent, as restriction products were produced, and PCR amplification appeared to produce strong products upon agarose gel electrophoresis, however, after running the genescan analysis, only fluorescent primers were seen on gels. This led to the hypothesis that the PCR stage of the technique had failed, however several attempts at optimisation with a variety of reagents failed to solve this problem. One conclusion from this typing method in comparison with the simple PCR based techniques of RAPD and ERIC was that it was time consuming and costly, and therefore the PCR based typing would be recommended for future use of clinical *E. coli* strains, even though a comparison of two different techniques yielded small discrepancies. Much is dependent upon interpretation of the result, something which can be aided by computer software such as diversity database, however even software is dependent

upon detection of bands by the eye, and therefore is dependent upon the perception of the investigator. The benefits of the PCR-based typing methods employed in this study were the fact that they were user friendly, inexpensive to run, and yielded rapid results.

From a comparison of the types obtained from the three typing methods (PFGE, RAPD/ERIC-PCR) the Newcastle isolates were typed into eight strains, with two stains TYPE-I and TYPE-II of major importance in the dissemination of resistance. The spread of resistant strains and individual emergence of resistance has been previously found by Oethinger *et al.* (1996) who studied *E. coli* strains among hospitalised cancer patients from different geographic locations. Other reports have demonstrated independent emergence of resistance in isolates from a single unit (Lehn *et al.*, 1996), with Kern *et al.* (1994) demonstrating combination of horizontal spread and individual emergence of resistance in *E. coli* isolates. Clonal diversity among the resistant strains from Newcastle was less than that of the non-resistant strains, but the low-level resistance seen between heterogeneous isolates may reflect an increasing background colonisation of strains with low-levels of fluoroquinolone resistance. These subtle epidemiological changes may have developed unrecognised within the background colonisation of the population at large, and it is conceivable that in such a situation, patients at high risk for bacterial infection, and frequently treated with fluoroquinolones are the first in which clinically significant fluoroquinolone resistance would develop. This would fit the hypothesis that resistance development requires

more than one mutational steps, and therefore may be potentially minimised by a change in dosing policy.

The studies performed to date analysing the chromosomal mutations responsible for increases in fluoroquinolone resistance have concentrated on selection of mutant bacteria in an *in vitro* situation, and in individually isolated clinical strains (Tavio *et al.*, 1999; Heisig, 1996; Vila *et al.*, 1996; Heisig & Tschorny, 1994; Yoshida *et al.*, 1990; Nakamura *et al.*, 1989). The existence of this isogenic population of resistant strains with varying levels of fluoroquinolone resistance from Newcastle, provided a unique opportunity to compare the clinical situation with that found *in vitro*.

Preliminary data indicate that highly resistant laboratory generated mutants have impaired viability, as indicated by significantly increased doubling times compared to that of the parent strain, or random clinical isolates carrying a similar *gyrA* or *parC* mutation (Heisig, 1996, Heisig & Tschorny, 1994). Under *in vivo* conditions, i.e., at the site of infection, variations in the global gene expression pattern might also cause alterations in the response to environmental stimuli, and thus affect the type of mutation preferentially selected. As a consequence, mutations selected *in vitro* would differ from those selected *in vivo*, as was observed by Bagel *et al.*, 1999, however there have not been previous opportunities to study resistance selection in the clinic. In an *in vivo* environment, bacteria are constantly under conditions of stress forcing a population of bacteria to mutate, and hence rapidly evolve to cope with this stressful environment. Bjorkman *et al.* (2000) have shown that compensatory mutations

selected *in vivo* can differ form those selected *in vitro*. This is of concern as most of the data to date about the emergence of quinolone resistance come from *in vitro* mutation studies, and therefore raises questions about the data deduced from these studies providing an accurate model of the clinical situation.

The most commonly found change in DNA gyrase, a mutation at amino-acid position Ser83 (Everett *et al.*, 1996; Heisig, 1996; Ruiz *et al.*, 1995), results in the loss of a *Hinf*I restriction enzyme recognition sequence. This allows for fast screening for mutations by digestion of the PCR products with *Hinf*I to detect mutation at codon 82 of 83 (Fisher *et al.*, 1989). This loss was detected in 14/15 (CIP MIC range 0.25 to 256mg/L) clinical isolates in this study, indicating the presence of a Ser83 gyrase mutation. This is in concordance with both *in vitro* and clinical resistance isolates described by other investigators (Tavio *et al.*, 1999, Evertt *et al.*, 1996; Heisig, 1996; Ruiz *et al.*, 1995; Vila *et al.*, 1994), who show that low level resistant isolates most frequently carry a single *gyrA* mutation altering Ser83→Leu. Because several different point mutations may contribute to this change of *Hinf*I recognition sequence and additional mutations cannot be identified in this way, the complete QRDR was sequenced for a selection of low, moderate and high-level resistant isolates. The fact that this mutation was present at such low resistance levels suggested that it was the primarily selected mutation, as *parC* mutations were not present in isolates with an ciprofloxacin MIC as low as 0.25mg/L.

There has been some controversy over the primary and secondary fluoroquinolone target proteins in gram-negative and gram-positive bacteria. The generally accepted view is that in gram-negative bacteria DNA gyrase is the primary ciprofloxacin target (Chen *et al.*, 1996), whereas ciprofloxacin targets topoisomerase IV initially in gram-positive organisms (Ng *et al.*, 1996; Ferrero *et al.*, 1995). There has however been conflicting evidence using clinically selected gram-positive fluoroquinolone resistant bacteria which showed that DNA gyrase mutations were selected primarily (Durham *et al.*, 1999). This brought into question the value of data deduced from *in vitro* mutation studies when analysing primary and secondary fluoroquinolone targets. The data from the clinical isolates in this study support the evidence form *in vitro* mutation studies, indicating that in gram-negative organisms DNA gyrase mutations are selected prior to those in topoisomerase IV, and therefore DNA gyrase was confirmed as the primary fluoroquinolone target.

In addition to confirming DNA gyrase as the primary ciprofloxacin target in *E. coli*, the resistance progression with subsequent additional target mutation acquisition supported data observed in previous *in vitro* mutation studies with gram-negative bacteria (Heisig, 1996; Khodursky *et al.*, 1995; Yoshida *et al.*, 1990). Resistance-levels increased progressively with the accumulation of target enzyme mutations in both TYPE-I and TYPE II isolates, demonstrating the requirement for multiple selection steps for the acquisition of high-level resistance (Cip MIC=>4mg/L) (Table 3.4). The clinically sensitive isolate NC-8 (Cip MIC=1mg/L) contained a single mutation in DNA gyrase (Ser83→Leu) and topoisomerase IV (Ser80→Ile). The

presence of two gyrase mutations in low-level clinical resistant isolates has been previously documented (Truong *et al.*, 1995; Vila *et al.*, 1994), but the presence of a topoisomerase IV mutation at these low levels of resistance is not common (Vila *et al.*, 1996). Mutations affecting the Ser80 and Glu84 amino-acids in ParC are generally associated with high-level resistance (Heisig, 1996; Kumagai *et al.*, 1996; Vila *et al.*, 1996). The fact that mutation was found in topoisomerase IV at such low levels suggested that Topoisomerase IV target may be more equal to DNA gyrase as a fluoroquinolone target in gram-negative bacteria than previously thought. Progression to high-level clinical resistance (Cip MIC=32mg/L) in this strain resulted in second step mutations in each enzyme at DNA gyrase Asp87→Asn and Topoisomerase IV Glu84→Val. This indicated that second step mutations in each enzyme had the important role of conferring high level resistance, rather than mutations in one enzyme being more responsible for resistance than the other. Mutation progression in Type I isolates showed similar results, however in this case two gyrase mutations (Ser83→Leu and Asp87→Asn) and one topoisomerase IV mutation (Ser80→Ile) conferred a ciprofloxacin MIC of 8-32mg/L, with acquisition of the second topoisomerase IV mutation (Glu84→Lys) conferring levels of resistance to >128mg/L.

Isolates NC-13 (Cip MIC=8mg/L) and NC-4 (Cip MIC=32mg/L) had identical target enzyme mutation profiles (GyrA Ser83→Leu; Asp87→Asn; ParC Ser80→Ile) suggesting that resistance mechanisms other than those identified were responsible for the 3-fold difference in ciprofloxacin MIC between the two isolates. This difference

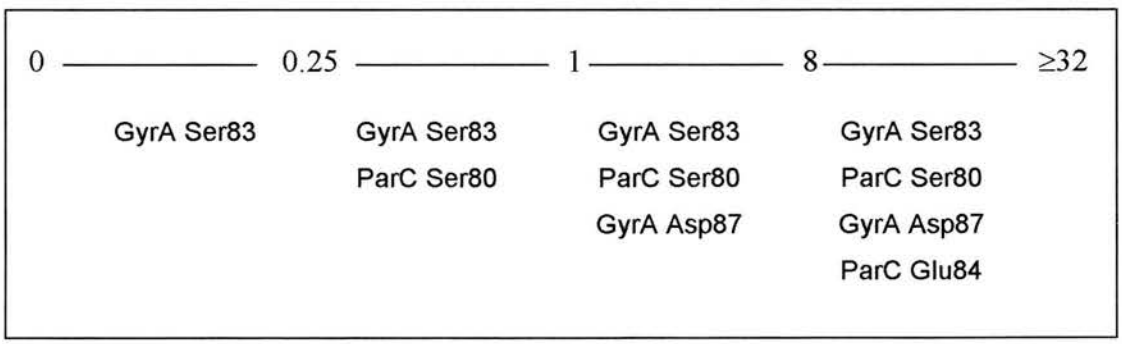
can also be seen between isolate NC-11 (Cip MIC=32mg/L) and isolates NC-14 (Cip MIC=256mg/L), with both isolates containing the four identified target mutations, although in NC-14 Asp87 is replaced with tyrosine rather than asparagine, and Glu84 is replaced with a lysine rather than the valine seen in NC-11. This indicates that the amino-acid replaced may play an important role in determining resistance, rather than just the simple loss of a crucial amino-acid at these positions. As well as changes in amino-acids, resistance mechanisms such as efflux, changes in outer membrane proteins (OMPs), or perhaps alterations in the genes *gyrB* or *parE* may contribute to variation in resistance between strains with the same GyrA and ParC target protein mutations. The fact that strains with four mutations have a range of resistances from 32 to 256mg/L, indeed suggest that these mechanisms may play a major role in resistance acquisition, however the genetic basis of these resistance mechanisms were not investigated in this study.

In *in vitro* selected mutants, non-target gene mutations accumulate in second and third-step mutants upon exposure to the fluoroquinolone and include, but are not limited to, mutations that occur in the *mar* and *sox* regulons (Kern *et al.*, 2000; Bagel *et al.*, 1999). Many high-level fluoroquinolone resistant isolates show a multiple antibiotic resistance (Mar) phenotype and increased tolerance to organic solvents (Conrad *et al.*, 1996; Everett *et al.*, 1996). They accumulate less ciprofloxacin than reference strains, and or lack outer membrane protein OmpF (Everett *et al.*, 1996), however the lack of cross-resistance to gentamicin seen in the clinical isolates in this study does not implicate OMP loss as a major resistance mechanism. Mar mutations

seem to play an important early step in resistance selection *in vitro* (Piddock *et al.*, 1991; Cohen *et al.*, 1988; Hooper *et al.*, 1987), however Manneewannakul & Levy (1996), found that this mutation was present in less than 15% clinical fluoroquinolone resistant *E. coli* isolates. Mar mutations don't therefore seem to be the predominant mutations affecting quinolone resistance in clinical isolates, nonetheless, reduced accumulation has been detected in several clinical isolates (Everett *et al.*, 1996; Oethinger *et al.*, 1996), with the genetic basis remaining obscure. The contribution of mutational changes in the *gyrB* and *parE* genes were not investigated, as they have been previously found to be absent or confer insignificant changes in fluoroquinolone MIC (Tavio *et al.*, 1999; Ruiz *et al.*, 1997; Vila *et al.*, 1994).

Isolate NC-10 which contained a Pro185 mutation in addition to two *gyrA* and two *parC* mutations had a lower MIC than strain NC-14, which did not contain this mutation. This indicated that this mutation was likely to be a back mutation, altering enzyme conformation to confer fluoroquinolone sensitivity. Combining the results from clinical isolates in this study, it was possible to deduce a model for ciprofloxacin resistance progression in the clinic, shown in Figure 7.1.

Figure 7.1: Mutational changes conferring ciprofloxacin resistance (mg/L) in clinical *E. coli*



This model demonstrates the requirement for three target protein mutations to enable strains to overcome the clinically detected resistant breakpoint (Cip MIC=2mg/L), again emphasising the dangers of low level selection. This model predicts the lowest resistance levels conferred by the specified amino-acid alterations. It must be noted that isolates with increased MIC values may have the same pattern of resistance mutations.

DNA gyrase and topoisomerase IV mutations confer resistance to the new fluoroquinolones, gemifloxacin, moxifloxacin and sparfloxacin in a similar manner to that of ciprofloxacin allowing for a 2-fold difference in MIC (Table 7.1). A Second *parC* mutation had a greater effect of increasing MICs of these drugs compared to ciprofloxacin which may reflect a greater ParC specificity of the new compounds. Also a second *gyrA* mutation did not seem to effect these drugs to the same extent as that of gyrase. Third generation fluoroquinolones such as gemifloxacin have enhanced greater potential to target DNA gyrase and topoisomerase IV equally, with certain substitutions enhancing activity against resistant organisms (Zhao *et al.*, 1999).

Table 7.1: Effect of target protein mutation in resistance (MIC mg/L) to new fluoroquinolones, gemifloxacin, moxifloxacin and sparfloxacin

DRUG	GyrA-Ser83	GyrA-Ser83 ParC-Ser80	GyrA-Ser83 ParC-Ser80 GyrA-Asp87	GyrA-Ser83 ParC-Ser80 GyrA-Asp87 ParC-Glu84
Ciprofloxacin	0.25	1	8	≥32
Gemifloxacin	1	4	16	≥32
Moxifloxacin	1	8	16	≥64
Sparfloxacin	1	4	8	≥32

The step-wise nature of resistance development observed in this study has implications for more effective drug design. Compounds that attack both gyrase and topoisomerase equally would be ideal, as there would be no concentration at which mutant having only one resistant target as seen here with ciprofloxacin selection could arise (Ng *et al.*, 1996; Pan *et al.*, 1996). Drugs such as gemifloxacin have been deemed to be more effective against strains which have a first step gyrase mutation for example, and therefore a second mutation would be required to achieve resistance.

A C-8 methoxyl substituent as is found in moxifloxacin, was found to facilitate attack against first-step gyrase mutant against *Mycobacterium tuberculosis* (Dong *et al.*, 1998). This was not the case for *E. coli* in this study, where moxifloxacin MICs were similar to those of ciprofloxacin against low-level resistant mutants, suggesting that the clinical isolates have resistance mechanisms that are not selected *in vitro*. This has implications about the deductions of the effectiveness of these drugs for use *in vivo* against strains which are already ciprofloxacin resistant. The C-8Me substituent increase lethal action against *E. coli*, and also recovery of resistant mutants was three fold less than observed with compounds lacking a methoxyl group at C-8 (Zhao *et al.*, 1997). Clinical isolates of *E. coli* that are highly resistant to C-8 methoxy group have been isolated in this study. Therefore due to the heavy use of fluoroquinolones and sequential acquisition of mutations in DNA gyrase and topoisomerase IV genes, it is unlikely that these new agents will encounter a fully susceptible WT population. The optimism displayed by authors about the potential of these new fluoroquinolones may

not hold true in the clinical environment (Zhao *et al.*, 1997). As newer quinolones are brought into clinical practice it has been suggested that use of older drugs such as ciprofloxacin should be limited as this will limit the use of the newer drugs. This indeed may be the case, as the newer drugs may be less likely to select for resistance, however strains which are already resistant by enzyme mutation will be resistant to the newer fluoroquinolones.

This study has shown that three mutations are required to over come the ciprofloxacin breakpoint in clinical isolates. The question therefore is posed as to why so many isolates now becoming resistant to ciprofloxacin (Threlfall *et al.*, 1997; Goldstein & Acar, 1995; Kern *et al.*, 1994). The fact that 14/15 isolates contained a *gyrA* mutation suggests that the probability is that in the bowel 1st and perhaps 2nd and 3rd mutations persist for long periods of time in the absence of selective pressure. The reasons why these mutants can persist maybe due to compensatory mutations which occur in the clinical environment (Levin *et al.*, 2000), for example defective mutant gyrase may be compensated for by mutation in topoisomerase I (Hooper *et al.*, 1998). The course of quinolone treatment may select for further mutations, which allow resistance to overcome the breakpoint. Susceptibility to fluoroquinolone can no longer be taken for granted, and this study demonstrates the need to prevent further spread of resistant strains, which appear to persist in the absence of selective pressure.

This study has indicated the that 14/15 strains contained low-level resistant mutants, which would not be detected by breakpoint sensitivity testing. The presence of such

low-level resistant mutants may be overcome if we reassess dosing policy for the fluoroquinolones, a consideration if we are to preserve these potent compounds. If bacteria are not killed effectively by a fluoroquinolone, then the stress induced within the bacteria will increase mutation rate (Ren *et al.*, 1999; Phillips *et al.*, 1987), facilitating further selection of resistant isolates. Current dosing protocols allow cells to grow after only one resistance mutation, and thereby aid resistance development, poising the population for further resistance selection. However, as seen in this study, more than three mutations are required to achieve clinically significant resistance. The mutation frequency of fluoroquinolones is less than 10^{-7}, and so more than 10^{14} bacteria would be required to find two consecutive target mutations. In the clinic, bacterial populations may reach 10^{10} cells in an infected individual, but 10^{14} is unlikely. Treating patients with higher drug concentrations would prevent initial selection of single mutations. The engineering of new drugs should also concentrate on lowering the concentration required to prevent mutant selection, by engineering dual targets, therefore at least two consecutive mutations would be required for resistance. However, as we have seen even these engineered changes will not have greater effects against organisms which already possess target site mutations through sequential selection by ciprofloxacin and older generation fluoroquinolones.

7.2 Site-directed mutageniesis

In order to assess the contribution of single amino-acid changes to the phenotype of fluoroquinolone resistance, specific amino-acid mutations were introduced into the fluoroquinolone target protein DNA gyrase by site-directed mutagenesis. The attribution of resistance to specific amino-acid changes has previously been investigated by complementation tests (Heisig & Wiedermann, 1991; Yoshida *et al.*, 1990; Nakamura *et al.*, 1989). These involve the introduction of plasmids conferring sensitive *gyrA* gene into a resistant bacteria, and assessing the decrease in resistance conferred by WT plasmid. Although many authors have attributed resistance to specific mutations in *gyrA* by this manner (Khodursky *et al.*, 1995), this test is not completely reliable. With the discovery of the *parC* target, we cannot rule out the fact that part of the resistance being complemented is due to mutations in the *parC* gene, especially as these are not expressed in the absence of *gyrA* mutation.

As was seen in the clinical isolates, increased fluoroquinolone MICs did not necessarily correlate with mutational changes in the target enzymes DNA gyrase and topoisomerase IV, probably due to the presence of complicating factors such as efflux and outer membrane proteins. The contribution of these factors to resistance can make it difficult to assess the direct role of chromosomal mutations in changing fluoroquinolone MIC. The presence of efflux mechanisms has been demonstrated by the use of efflux pump inhibitors CCCP and reserpine, however these experiments lack reproducibility and cannot be transferred among species (Schmitz *et al.*, 1998; Li *et al.*, 1994). Mutations in OMPs have also been demonstrated (Everett *et al.*, 1996;

Hooper *et al.*, 1992), however they are usually found in the presence of additional target protein mutation changes.

A direct assessment of the contribution of individual target protein alterations to fluoroquinolone resistance was performed by the introduction of the single clinically demonstrated gyrase mutations, Ser83→Leu and Asp87→Asn into WT *gyrA,* expressed on plasmid pPH3. Unfortunately this protocol yielded problems with the PCR annealing of the Ser83 mutant primer, due to double insertions during the PCR stage of the reactions. Due to time constraints when these experiments were performed, this could not be repeated.

A mutation of Asp87→Asn, the second-step gyrase mutation was however successfully introduced into *gyrA* on the plasmid pPH3, allowing the contribution of this mutation in the absence of complicating factors to be assessed. Expression of the plasmid encoded *gyrA* was achieved in a temperature sensitive (Ts) *gyrA* KNK453 strain (Kreuzer & Cozzarelli, 1979) which grew at 30°C, but not at 42°C. The use of Ts strain was necessary as the DNA gyrase sensitive allele has been shown to be dominant to the resistant allele in phenotypic expression studies (Soussy *et al.*, 1993; Power *et al.*, 1992). This is likely a consequence of the fluoroquinolone mechanism of action. They act like poisons and therefore interaction with a sensitive target will form complexes leading to cell death, even if there is also interaction with resistant target. Therefore if the pPH3 plasmid containing mutant *gyrA* was expressed in cells which expressed sensitive *gyrA,* the resistance phenotype would not be conferred. The

plasmid was additionally under the tight control of the tac promoter, which therefore only induced expression of *gyrA* in the presence of the inducer IPTG, providing additional control in the experiment.

Sequencing of the plasmid *gyrA* confirmed the presence of the desired single Asp→Asn mutation at position 87. The contribution to fluoroquinolone resistance conferred by this amino-acid alteration was assessed in the Ts KNK453 mutant at 42°C by calculating MICs for a range of fluoroquinolones. The greatest increase in MIC was seen for the older quinolone nalidixic acid, which showed a 7-fold resistance increase compared to WT, whereas resistance for ciprofloxacin and the new fluoroquinolones gemifloxacin, moxifloxacin and sparfloxacin increased only 2-3 fold in comparison with the WT strain. Acquisition of an Asp87→Asn mutation in the clinical selection model in Figure 7.1, conferred a jump from clinically sensitive to a resistant phenotype (Cip MIC 1mg/L→8mg/L), and was the third target mutation selected. Therefore, the site-directed mutagenesis correlated well with the increase in fluoroquinolone resistance seen with the acquisition of an Asp87 mutation in the clinic, suggesting that at this point in resistance selection the roles of efflux and OMPs had not been important in the clinical isolates. Although the Asp87→Asn mutation was crucial in increasing MIC of clinical isolates to resistance levels above their clinically detected breakpoints, the results of the site-directed mutagenesis, demonstrate that the Asp87→Asn mutation alone does not confer an increase in resistance large enough to be detected by clinical means and therefore is acting cumulatively with other mutations to confer this increase in MIC. As previously

demonstrated mutations in GyrA Ser83 confer less than 0.25 mg/L ciprofloxacin resistance, and single ParC mutations are not seen, therefore clinical resistance by any one mutational change is highly unlikely, confirming results of the predicted model that three target protein mutations are required for clinical resistance.

7.3 Ribosomal protein mutation

Along with investigating the fluoroquinolone target proteins, this thesis presented data on how aminoglycoside target protein S12 can mutate to confer aminoglycoside resistance. Ribosomal S12 protein has been previously implicated in streptomycin resistance (Toivonen *et al.*, 1999; Funatsu & Wittmann, 1972; Ozaki *et al.*, 1969), however the importance of ribosomal S12 protein in conferring resistance to other members of the aminoglycoside family has not been previously assessed.

This study characterised the role of S12 mutation in stepwise progression to resistance for aminoglycosides neomycin, gentamicin, tobramycin and kanamycin in addition to streptomycin, thereby providing insights into specific drug targets and mechanism of resistance acquisition. Aminoglycoside resistant mutants were selected at a low frequency (1×10^{-6}), suggesting the number of possible mutation hotspots for resistance was small. Mutants were selected in four selection steps, with streptomycin resistant mutants conferring the largest increase in resistance after the four step selection. Streptomycin (4^{th} step = MIC 1500mg/L) mutants remained sensitive to all other aminoglycosides tested, indicating that resistance was not due to a membrane permeability change or mutation in a shared aminoglycoside target. The antibiotics neomycin, tobramycin and kanamycin mutants showed cross-resistance, implicating a common target protein and shared mechanism of action. The cross-resistance seen between these drugs could be influenced by their structure, as neomycin, kanamycin, gentamicin and tobramycin have a deoxy-streptamine ring, whereas streptomycin has

a streptidine ring, and therefore indicates the importance this structural determinant alteration in drug binding.

Analysis of the sequence of the entire streptomycin S12 gene, demonstrated a novel Lys87→Glu mutation in all streptomycin resistant mutants (Strep MIC=25-1500mg/L), confirming the importance of ribosomal S12 protein as a streptomycin target. Previous authors have described mutations in areas 41-43 and 85-91 of the S12 protein (Timms *et al.*, 1992; Lui *et al.*, 1989; Funatsu & Wittman, 1972), however a glutamic acid substitution at position 87 of *E. coli rpsL* gene has not been previously demonstrated. A previously described Lys87→Arg mutation can confer resistance levels of 1500mg/L in a single step selection (Timms *et al.*, 1992), however the Lys87→Glu mutation in this study conferred resistance to a level of 25mg/L. Conversion of Lys87 to a basic rather than an acidic residue therefore seems to have a great deal of influence on target site availability and drug binding. Recent NMR studies have demonstrated that aminoglycosides bind to the tRNA site of the 16S ribosomal sub-unit (Moazed & Noller, 1996), with S12 having a suggested role in stabilising the pseudoknot structure of 16S RNA (Stern *et al.*, 1988).

The lack of additional S12 target protein mutations in 2nd (Strep MIC=100mg/L) to 4th step (Strep MIC=1500mg/L) selections, implicated other resistance mechanisms in these mutants. If resistance increase was due to an outer membrane effect, then cross-resistance between the other aminoglycosides would be expected in 3rd and 4th step resistant mutants, which was not demonstrated. A second target protein was therefore

implicated in streptomycin binding, supporting the hypothesis that S12 may either form part of a binding pocket, or control access to a target protein.

S12 mutations were not present in neomycin, kanamycin, tobramycin and gentamicin resistant mutants, demonstrating that S12 is not likely to be the primary target of these antibiotics. Mutations in other ribosomal targets such as 16S rRNA have been implicated in resistance (Montandon *et al.*, 1985). The strong cross-resistance between the drugs suggest a common target, and thus may be a consequence of close structural similarity. This demonstrates the importance of drug structure in target protein determination.

7.4 Bactericidal antibiotic action

Bactericidal drugs such as the fluoroquinolones are known to require protein synthesis to exert their killing activity (Lewin *et al.*, 1991). This requirement has explained why fluoroquinolones have a bi-phasic dose-response curve, with high concentrations of antibiotic inhibiting protein synthesis and thereby decreasing cell death (Smith, 1986). This protein synthesis requirement is not wholly understood, with investigators postulating the possible roles of SOS response proteins in cell death (Piddock *et al.*, 1990; Lewin *et al.*, 1989; Walters *et al.*, 1989). The β-lactam antibiotics are also known to require protein synthesis in cell death (Tuomanen, 1986), and Novak *et al.* (2000) have demonstrated the role of signal transduction cascades in cell death. Although we may understand the complex interaction between drugs and their initial bacterial targets, it is important to look beyond these initial target interactions if we are to understand cell death and consequently design the antimicrobials of the future. This thesis investigated the putative protein synthesis requirement in streptomycin induced cell death, in order to assess whether the aminoglycosides like the fluoroquinolones require protein synthesis for bactericidal action.

The activity of streptomycin in the presence of protein synthesis inhibitors was initially determined, in order to characterise the protein synthesis requirement in cell death. Chloramphenicol and spectinomycin produced a principally bacteriostatic effect against log-phase *E. coli* populations, exerting some initial killing activity. Streptomycin acting alone against a log-phase bacterial population exhibited bactericidal activity below its defined MIC of 10mg/L, with cell death at its maximum

at and above this concentration. Streptomycin activity directly correlated with drug concentration, and no bi-phasic response was demonstrated. Treatment of a log-phase *E. coli* population with a streptomycin/spectinomycin combination resulted in antagonism of streptomycin action, however this antagonism was not demonstrated at high drug concentrations. The protein synthesis inhibition by spectinomycin/chloramphenicol may not have been fast enough to prevent synthesis of proteins required for cell death. The antagonism observed at low drug concentrations, suggested that death required protein synthesis, whereas at high concentrations death progresses independently of protein synthesis.

One may speculate at a dual mechanism of cell death, which would tie in with hypothesis by Hancock (1981) and Matsunaga *et al.* (1986) who have suggested that alternative targets such as DNA synthesis inhibition can be involved in cell death. It is not certain whether all protein synthesis was inhibited by spectinomycin, and pre-incubation with this drug may have been more effective in inhibiting the streptomycin effect. The fact some antagonism was shown suggested a role for protein synthesis in cell death. The bacteriostatic protein synthesis inhibitor chloramphenicol was shown to have a greater effect at antagonising streptomycin action; however, streptomycin was still able to exert its action in the presence of this protein synthesis inhibitor. These results were indicative of more than one mechanism of cell death, raising the question of whether the aminoglycosides share similarity with the fluoroquinolones, which have protein synthesis dependent and independent pathways of killing. To account for the possibility of residual protein synthesis, streptomycin activity was

assessed over a three hour period in the presence of increasing chloramphenicol concentrations. The higher the chloramphenicol concentration, the more bacteriostatic the effect of streptomycin became, demonstrating a clear requirement for protein synthesis in cell death.

The activity of streptomycin against bacteria in amino-acid starvation conditions was subsequently investigated. Streptomycin still elicited cell death against bacteria in these conditions, thereby contradicting results previously demonstrated, that streptomycin required protein synthesis for cell death. Breakdown of proteins within the cell under amino-acid starvation conditions to provide a residual supply of amino-acids may account for this result. This supports the mechanism of bactericidal cell death proposed by Davis (1987), who suggest that the incorporation of mistranslated proteins into the bacterial membrane accounts for cell death. Death would therefore proceed in amino-acid starvation, as this misreading would be increased under these conditions. The Davis hypothesis does not however explain a mechanism of cell death, outwith that of prolonged protein synthesis inhibition.

The action of streptomycin as a protein synthesis inhibitor is well demonstrated (Brimacombe *et al.*, 1988; Hausner *et al.*, 1988), with this thesis providing supporting evidence that streptomycin exerts its effect by interaction with ribosomal protein S12. Additionally the results demonstrated with the antagonism of streptomycin activity in the presence of protein synthesis inhibitors implicate a protein synthesis requirement in cell death. Based upon this knowledge the hypothesis that streptomycin acting as a

protein synthesis inhibitor could inhibit its own cell death was investigated. This study demonstrated that bactericidal streptomycin activity was significantly reduced, when acting upon a bacterial population pre-treated for 3 hours with a low concentration (1mg/L) streptomycin. The low streptomycin concentration (1mg/L) exerted a bacteriostatic effect on cells, and therefore was acting as a protein synthesis inhibitor in a similar manner to chloramphenicol and spectinomycin. Streptomycin pre-incubation inhibited subsequent bactericidal streptomycin dose (100mg/L) in a similar manner to pre-incubation with chloramphenicol. These results suggested that at low concentrations streptomycin can act as a protein synthesis inhibitor, which inhibits a second yet unidentified protein synthesis dependent mechanism of cell death. The action of streptomycin as a protein synthesis inhibitor is therefore separate to the reason for drug lethality. The reason why pre-incubation with slightly higher concentrations of streptomycin (5, 10mg/L) failed to yield the same antagonistic activity is unknown.

An alternative hypothesis to explain why bacteria survive streptomycin challenge after cells have been treated with a low concentration of streptomycin, would be if a bacterial population had adapted and therefore become a more resistant population of hypermutators. Under conditions of stress such as antibiotic treatment, the mutation rates in a bacterial population are increased. Taddei *et al.* (1997) have demonstrated that an antibiotic treated cell population can turn into a population of hypermutators, which is subsequently more resistant to bacterial challenge. This adaption generally takes more than three hours, and therefore makes this an unlikely hypothesis to

explain the result seen in this study. Nonetheless to rule out adaption, an *E. coli* population was pre-treated with gentamicin prior to subsequent streptomycin challenge. Streptomycin induced cell death was inhibited confirming that it is likely the inhibition of protein synthesis rather than a streptomycin adapted population which results in the reduction in streptomycin activity.

The effectiveness of antimicrobials are usually measured by their ability to inhibit or kill bacteria. However, tests of this ability are usually performed on log-phase bacteria or those in rapid growth phase, in media supplying all the necessary nutrients for optimum growth. Clinically, some infected tissues may be walled off quickly by host leukocytes, followed by fibrin deposits, and thus growth of bacteria may be less than optimal, and controlled by the limited access to nutrition. The dormant state of bacteria could be responsible for it escaping antibiotic therapy. Cells *in vivo* are less likely to be killed than cells growing *in vitro* log-phase.

The results of these experiments show that analysing the initial target protein interaction, although of crucial importance for future engineering of more potent antimicrobial compounds, is not sufficient to elucidate the complex mechanisms of cell death. Investigators must focus their energy on understanding complex cellular machinery and feedback cascades which may represent these protein synthesis requiring pathways to provide the future targets of antimicrobial drug design.

7.5 Conclusions

A model for the development of fluoroquinolone resistance due to mutational change in target enzymes DNA gyrase GyrA and Topoisomerase IV ParC in clinical *E. coli* isolates was deduced : GyrA Ser83 (Cip MIC=0.25mg/L) → ParC Ser80 (Cip MIC=1mg/L) → GyrA Asp87 (Cip MIC=8mg/L) → ParC Glu84 (Cip MIC=32mg/L). Three target protein mutations were required to achieve clinical resistance (Cip MIC=≥2mg/L). DNA gyrase was confirmed as the primary fluoroquinolone target in *E. coli*, with first step GyrA mutations demonstrated in the absence of mutations in Topoisomerase IV ParC. Resistance varied between strains with the same target protein mutation profile, demonstrating the additional role of putative efflux mechanisms and changes in outer membrane proteins in conferring resistance. The demonstration of GyrA mutations in 14/15 isolates with ciprofloxacin MICs as low as 0.25mg/L, indicated that these mutations are maintained undetected in the population poised for consequent selection to resistance upon further antimicrobial treatment. The new fluoroquinolones sparfloxacin, moxifloxacin and gemifloxacin were equally ineffective at treating these ciprofloxacin selected resistant isolates. This calls into question the future value of these new compounds, which as demonstrated here are highly unlikely to face a fully susceptible wild-type population.

Site-directed mutagenesis demonstrated that a single Asp87→Asn mutation in DNA gyrase GyrA cannot confer clinically significant levels of resistance, confirming results found with the clinical isolates that resistance requires accumulation of several mutations. The three-fold increase in resistance compared to wild-type seen with the

Asp87→Asn mutation correlated closely with progression seen in the clinic, demonstrating that resistance from changes in OMPs and efflux were unlikely to have a major role in this selection step.

This thesis additionally analysed the role of ribosomal S12 in conferring resistance to the aminoglycoside antibiotics. Ribosomal S12 protein was demonstrated to be involved in streptomycin action, however was unlikely to be a primary target of gentamicin, kanamycin, neomycin and tobramycin. *In vitro* selected streptomycin resistant mutants (Strep MIC=25→!500mg/L) had a novel Lys87→Glu mutation. Previous work had demonstrated a Lys87→Arg mutation conferred resistance to levels of 1500mg/L in a single step selection, and therefore results form this study imply that replacement with a basic rather than acidic amino-acid residue confers a greater level of resistance. The lack of additional mutations in ribosomal S12 protein for 2nd to 4th step streptomycin selected mutants (Strep MIC=100→1500mg/L) suggested the involvement of alternative targets in streptomycin action, with S12 possibly forming only part of a streptomycin binding pocket. S12 mutations were not demonstrated in four generations of neomycin, kanamycin, tobramycin and gentamicin selected mutants, indicating that this protein is not a target of these antibiotics. Streptomycin mutants lacked cross-resistance to other aminoglycosides again suggesting that S12 is involved exclusively in streptomycin action. Cross-resistance was demonstrated between neomycin, tobramycin, kanamycin and gentamicin resistant mutants, indicating a common target, most probably a facet of their shared structural similarity.

The results from this thesis suggested that streptomycin has a protein synthesis dependent mechanism of cell death, beyond initial target binding. The action of streptomycin as a protein synthesis inhibitor is well documented, however this fails to explain why it is bactericidal to cell, when other common protein synthesis inhibitors do not exert a bactericidal effect. Chloramphenicol and spectinomycin were demonstrated to have an antagonistic effect on streptomycin activity, indicating a protein synthesis requirement in cell death. Streptomycin was shown to inhibit its own activity by acting as a bacteriostatic protein synthesis inhibitor at low concentrations. The action of streptomycin as a protein synthesis inhibitor is therefore not responsible for cell death, with action dependent upon a yet unidentified protein synthesis requiring bactericidal pathway.

An understanding of bacterial targets will allow drugs to be modified which may not select resistance in the same fashion. Knowledge of how resistance emerged in the clinic will allow better drug use and dosing policies. The deduction that drug-target interaction does not explain the consequent bactericidal cell death, suggests that the future of antimicrobial design is dependent upon looking beyond initial target protein interactions to understand complex cellular dynamics, to elucidate the pathways of antimicrobial induced cell death.

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