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EFFECTS OF SUBINHIBITORY OFLOXACIN ON SELECTED GENETIC PROCESSES AMONG ENTEROBACTERIA.

BY

Allan W. Abbott B.Sc (Hons)

being a thesis submitted for the Degree of Doctor of Philosophy in the University of Glasgow, Faculty of Medicine, May 1995
The fluorinated 4-quinolone, ofloxacin, interacts with, and inhibits the negative DNA supercolling activity of the bacterial tetrameric enzyme, DNA gyrase. DNA gyrase is one of several topoisomerases that homeostatically regulates the tertiary structure and function of both chromosomal and plasmid DNA. Inhibition of this process is recognised as the precursor to a cascade of cytopathic events that leads to bacterial death.

It is recognised that in stationary phase, changes in gene expression promote diverse mechanisms in bacteria to maintain their 'living state'. Analysis of changes in stationary phase has become increasingly important, since Enterobacteria in many environments are in a constant state of stationary phase primarily through nutrient competition, and that rapid growth is not possible.

The transposition of Tn7 into the IncP plasmid RP4, and the stability and variation of a natural co-integrate plasmid, pOG669, were studied in E. coli K12. The reversion of a spontaneous pleiotropic mutant of Serratia marcescens was investigated in relation to both different growth conditions together with pigmentation and antibiotic resistance. In each of the experimental systems the time course was extended to incorporate possible changes that occurred in stationary phase.

Restriction endonuclease fragmentation patterns (REFP) techniques were used to identify DNA rearrangements in the co-integrate R-plasmid pOG669 and to analyse the insertion of Tn7 into RP4. Subinhibitory ofloxacin lead to DNA rearrangements, that encompassed alteration in the insertion of Tn7, IncX & IncP plasmid maintenance and expression of antibiotic resistance.

Subinhibitory ofloxacin significantly altered the distribution of Tn7 insertion in multiple sites within RP4. Most Tn7 insertions were found to be increased in the 22.2kb KpnI-SmaI fragment and reduced in the 5kb KpnI-SmaI RP4 fragment. Although, fewer insertions were found in the other KpnI-SmaI fragments in RP4, subinhibitory ofloxacin reduced insertions into the 8.5kb and 5.8 fragments. It is proposed this high and low density of Tn7 insertions results from changes in local DNA superhelicity, and led to exposure of insertion sites to Tn7. Preliminary evidence suggests the invA and irfA genes may be contiguous in the RP4 molecule, since no Tn7 insertions
were detected in these regions.

Under different growth conditions, the instability of pOG669 was demonstrated. During logarithmic growth, (repeated subculture) pOG669 was found to be completely eliminated. Subinhibitory ofloxacin increased loss of the co-integrate plasmid pOG669 in non-dividing bacteria (stationary phase) and decreased plasmid loss in bacteria that were in maintenance subculture. pOG669 was found to be unstable in stationary phase and molecular variants were isolated, and included pOG669 variants with DNA rearrangements, kanamycin sensitive variants (KSV). Mixed population variants (MPV) were also isolated, and these comprised of different populations of E. coli K12 J62-1, on the basis of variations in expressed antibiotic resistance of the co-integrate R-plasmid pOG669. MPV1 comprised two populations of E. coli K12 J62-1 with variations in pOG669, and included E. coli K12 J62-1, pOG669 - Ap^KmS & E. coli K12 J62-1, pOG669 - Ap^KmR. MPV2 also comprised two populations of E. coli K12 J62-1 with variations in pOG669, and included E. coli K12 J62-1, pOG669 - Ap^KmS & E. coli K12 J62-1, pOG669 - Ap^KmR. KSV DNA rearrangements extended into the pOG660 component of pOG669, and this region was identified as the source of pOG669 instability. The KSV were derived from a junctional deletion that was adjacent to the Km gene.

S. marcescens GRI 2677-8 is spontaneous non-pigmented resistant mutant and reverted at high frequency to the parental red-pigmented phenotype. Subinhibitory ofloxacin reduced reversion to the pigmented phenotype and data presented shows growth and pigmentation to be closely associated. Spontaneous reversion to the pigmented phenotype of S. marcescens gave rise to loss of resistance to carbenicillin and unrelated antimicrobial agents that included the aminoglycosides, trimethoprim, and chloramphenicol. The evidence suggests that pigmentation and expression of antibiotic resistance are co-regulated by a Regulator of Antibiotic resistance and Pigmentation (RAP). Evidence is also presented that reversion from the non-pigmented phenotype to the pigmented phenotype is coupled with changes in antibiotic resistance, and is linked to functional changes in the RAP regulator(s).
When antimicrobial therapy is optimised for a specific site, subinhibitory concentrations of an antibiotic may occur peripherally to the infection. Similarly, non-compliance and the post-antibiotic effect both generate subinhibitory concentrations of antibiotics. From the results described, exposure of the commensal flora to subinhibitory concentrations of 4-quinolones has the potential to alter the location and mobility of unrelated resistance determinants and effect other evolutionary changes and is therefore likely to be of clinical significance.

This study has demonstrated that changes in antibiotic expression are induced by subinhibitory ofloxacin, as observed in the plasmids pOG669 and RP4. The mobility of these plasmids are also influenced by subinhibitory ofloxacin through their elimination. The insertion and transposition of Tn7 and therefore mobility and transposon resistance determinants have also been influenced by subinhibitory ofloxacin. Therefore, the results presented in this study describe ofloxacin to act on several genetic processes.
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*stationary phase, maintenance, and repeated subculture*

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<tr>
<td><em>att</em>Tn&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Specific Tn&lt;sup&gt;7&lt;/sup&gt; insertion site in the <em>E. coli</em> chromosome</td>
</tr>
<tr>
<td>CFX</td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>C&lt;sup&gt;+&lt;/sup&gt;N</td>
<td>Cinoxacin</td>
</tr>
<tr>
<td>D-ofloxacin</td>
<td>D optical isomer of ofloxacin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR</td>
<td>Directed Repeats</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>FLX</td>
<td>Fleroxacin</td>
</tr>
<tr>
<td>HU</td>
<td>Histone-like protein</td>
</tr>
<tr>
<td>IPP</td>
<td>Intracolonal pigmented papillae</td>
</tr>
<tr>
<td>IR</td>
<td>Inverted Repeats</td>
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<tr>
<td>IZC</td>
<td>Inter-zonal colonies</td>
</tr>
<tr>
<td>KSV</td>
<td>Kanamycin sensitive variant</td>
</tr>
<tr>
<td>Kr</td>
<td>Colonial growth rate (µm hour&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>LFX</td>
<td>Lomefloxacin</td>
</tr>
<tr>
<td>L-ofloxacin</td>
<td>L optical isomer of ofloxacin</td>
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<tr>
<td>MAP</td>
<td>2-methyl-3-amylypyrrole</td>
</tr>
<tr>
<td>MBC</td>
<td>4-methoxy-2,2′-bipyrrrole-5-carboxyaldehyde</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MPV</td>
<td>Mixed Population Variant</td>
</tr>
<tr>
<td>NAL</td>
<td>Nalidixic acid</td>
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<td>NFX (NOR)</td>
<td>Norfloxacin</td>
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<td>OFX</td>
<td>Ofloxacin</td>
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oriT  Origin of transfer
oriV  Origin of vegetative DNA replication
OXO  Oxolinic acid
p+  Bright-red pigmented phenotype
p±  Bright-pink pigmented phenotype
p±  Pale-pink pigmented phenotype
p–  Non-pigmented phenotype
par  partition function
pig  pigmentation gene
PIR  Piromidic acid
PIP  Pipemidic acid
MSOF−  Maintenance subculture without ofloxacin
MSOF+  Maintenance subculture with ofloxacin
pri  DNA primase
RAP  Regulation of Antibiotic resistance and Pigmentation
REFP  Restriction Endonuclease Fragmentation Patterns
RNA  Ribonucleic acid
R Sof−  Repeated Subculture without ofloxacin
R Sof+  Repeated Subculture with ofloxacin
SOF−  Stationary phase without ofloxacin
SOF+  Stationary phase with ofloxacin
SOS  recA-mediated DNA repair system
SPX  Sparfloxacin
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<tr>
<td>SP</td>
<td>Stationary phase</td>
</tr>
<tr>
<td>TES</td>
<td>Solution containing 50mM Tris, 50mM NaCl, and 5mM disodium EDTA (pH 8.0)</td>
</tr>
<tr>
<td>TGE</td>
<td>Buffer solution containing 25mM Tris, 10mM EDTA, 50mM Glucose (pH 8.0)</td>
</tr>
<tr>
<td>tra</td>
<td>region required for conjugal transfer</td>
</tr>
<tr>
<td>trf</td>
<td>transacting replication function</td>
</tr>
<tr>
<td>Tyr-122</td>
<td>tyrosine (amino-terminal) residue at position 122 of the polypeptide sequence of DNA gyrase</td>
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<td>TSX</td>
<td>Tosufloxacin</td>
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I would like to convey my sincerest thanks to my supervisor, Dr. David J. Platt for his advice, encouragement, constructive criticism, patience and understanding throughout the period of this study. I would also acknowledge helpful discussions from Professor Douglas Sleigh and Dr Curtis Gemmell.

I am grateful to Professor Peter Stonier of Hoechst-Roussel for his financial support, without which this study would not have taken place.

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The experimental work and composition are the sole work of the author unless otherwise stated.

22nd May, 1995
CHAPTER 1

INTRODUCTION
1.1. The quinolone antibacterials

1.1.1. Historical development and molecular biochemistry of the 4-quinolones

Although the origins of empirical antimicrobial therapy can be traced back several centuries, the modern era, has been characterised by a wide choice of therapeutic agents since the discovery of sulphonamides in the 1930's. The last six decades have seen an enormous development in available antimicrobial agents tailored to meet the changes in types of infection encountered as well as bacterial susceptibility. These have stimulated an ongoing search for broader spectrum, more active therapeutic alternatives. The last two decades, however, have been dominated by the expansion of the cephalosporins, the development of the \( \beta \)-lactamase inhibitors, and synthetic modification of the 4-quinolone generic group of antimicrobial agents in response to the increasing frequency of bacterial resistance and nosocomial infections.

Nalidixic acid (1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid), is the archetypal member of the first generation quinolones (Figure 1.1), and was originally isolated from a distillate during the synthesis of the antimalarial compound chloroquine (Lesher et al., 1962). It was also the first 4-quinolone introduced into clinical practice for the treatment of Gram-negative urinary tract infections.

Nalidixic acid is more active against Gram-negative than against Gram-positive bacteria, and has proved beneficial in the treatment of urinary tract infections (Stamey et al., 1969), and enteric infections, including dysentery caused by \textit{Shigella sonnei} (Moorhead & Parry, 1965). Although, nalidixic acid is bactericidal, and attains high urinary concentrations of active drug, it is however, considered far from ideal.

The clinical use of nalidixic acid has been limited because of its narrow spectrum of activity, poorer pharmacology, and reputed ease for the rapid emergence of both \textit{in vitro} and \textit{in vivo} resistant organisms (Buchbinder et al., 1962; Barlow, 1963) by mutation (Smith, 1984). In the thirty or so years since the discovery of nalidixic acid, it appears to be exempt from plasmid-mediated resistance (Burman, 1977; Crumplin, 1987). It has also been shown that the mechanism of action of nalidixic acid involves a specific inhibition of chromosome replication (Goss et al.,
Figure 1.1. The first generation quinolone analogues.

**FIRST GENERATION QUINOLONES**

(a) 1,8-naphthyridine

(b) Cinnoline

(c) Pyrido-pyrimidines
Figure 1.1. (Continued).

(d) Quinoline derivatives

![Chemical structures of Oxolinic acid and Flumequine](image)

Oxolinic acid

Flumequine
Such findings have led to the synthesis of novel analogues that would display both improved potency and pharmacology.

The first generation quinolone analogues are shown in Figure 1.1. Nalidixic acid is a naphthyridine, cinoxacin is a cinnoline derivative, pipemidic and piroxicid acids are pyrido-pyrimidines, and flumequine and oxolinic acid are quinoline derivatives. The antibacterial activity of oxolinic acid was found to be approximately 5-10 times that of nalidixic acid (Kaminsky & Meltzer, 1968), and that subsequent development of cinoxacin (Stilwell et al., 1975) differed from oxolinic acid through the use of a cinnoline nucleus rather than a quinoline nucleus. The latter demonstrated that the quinoline nucleus (Figure 1.2) improved antibacterial activity (Stilwell et al., 1975).

Nalidixic acid and its derivatives have now become known generically as the 4-quinolones by virtue of their common molecular skeleton (Smith, 1984), 4-oxo-1, 4-dihydroquinoline (Figure 1.2). The antibacterial activity of these agents was found to increase significantly with the addition of a fluorine residue at position C-6 (Wick et al., 1975). Flumequine (Figure 1.1) was the first fluorinated 4-quinolone. Although, it never reached clinical use, it was a significant discovery in the evolution of the 4-quinolones because of the introduction of a fluorine residue, and that it was comprised of an equimolar mixture of enantiomers. Subsequent attempts were made to derive an agent that possessed a broader spectrum of antibacterial activity. The first of these, was the development of the pyrido-pyrimidine, pipemidic acid (Figure 1.1). Pipemidic acid contains a piperazine substitution at position 7 (Figure 1.1), and this not only enhanced the activity against the Enterobacteria (Matsumoto & Minami, 1975), but there was also significant activity against Pseudomonas aeruginosa (Shimizu et al., 1975), which is intrinsically resistant to nalidixic acid. Despite its extended spectrum of activity, pipemidic acid has only been used clinically in a limited number of countries. Although, pipemidic acid, was the first 4-quinolone with a piperazine substitute, it has nevertheless played a significant role in their evolution.

The first generation 4-quinolones were essentially the prototypes for more recent developments. The knowledge gained from the chemistry of these compounds and the abundance of laboratory data obtained from the investigation of the mechanism of action of nalidixic acid (Goss et al., 1964 &
Figure 1.2. The quinoline nucleus, 4-oxo-1, 4-dihydroquinoline, is the common molecular skeleton of the 4-quinolones. The side-chains (R) provide structure-activity relationships. R₁ (3-6 C-ring or linear branch) controls potency and some effect on pharmacokinetics, R₂ (H-atom) is involved with the gyrase binding site, R₅ (H-atom or NH₂) controls potency and adds Gram-positive activity, R₇ (aminopyrrolidine or piperazine) controls potency, spectrum, and pharmacokinetics. Adapted from Domagala (1994).
1965; Deitz et al, 1966; Crumplin & Smith, 1975 & 1976; Gellert et al, 1976), have led to the discovery of DNA gyrase, a unique enzyme that regulates and controls the tertiary structure of DNA (Menzel & Gellert, 1983). Further to this, several important molecular and bacteriological studies have demonstrated that these agents influence many biological processes in the bacterial cell which involve DNA.

This was the starting point for the development of the newer 4-quinolones. The work of the last decade has concentrated exclusively on the production of 4-quinolone analogues that were both fluorine substituted at C-6 and contained either an aminopyrrolidine or piperazine base-substituent at R-7 (Figure 1.2).

The second generation quinolones as shown in Figure 1.3a, were developed and synthesised for greater antibacterial activity, broader spectrum of activity, improved pharmacological and pharmacokinetic properties, and introduced for the treatment of many bacterial infections (Hooper & Wolfson, 1985; Hooper & Wolfson, 1991). These newer 4-quinolones greatly exceed the antibacterial activity of nalidixic acid, and have brought many bacteria, previously refractory to nalidixic acid, within the range of their therapeutic range (Smith, 1986). The C-6 fluorine substitution contributes to enhanced potency with at least a 10-fold increase in gyrase inhibition and a 5-100-fold decrease in MIC (Domagala, 1994). The R-7 aminopyrrolidine base of tosufloxacin, and the piperazine base of ciprofloxacin, lomefloxacin, ofloxacin, and sparfloxacin (Figure 1.3a) improve antibacterial activity, spectrum, and pharmacokinetics. The pyrrolidines offer higher activity against Gram-positive bacteria, whereas the piperazines display higher activity against Gram-negative bacteria (Sanchez et al, 1988). Alkyl substitution of the R-7 base, as in sparfloxacin, has been shown to further enhance activity against Gram-positive bacteria (Sanchez et al, 1988; Domagala, 1994). Increasing the number of fluorine residues through mono-, di-, and tri-, halogenation of the quinolone nucleus (Figure 1.3a) has been shown to increase the antibacterial activity against Gram-positive bacteria and obligate anaerobes (Mitscher et al, 1990), which are normally both considered intrinsically resistant to the first generation 4-quinolones.

Ofloxacin is currently the only available fluorinated 4-quinolone with optical activity (Figure
and exists as one of two optical stereoisomers or enantiomers in a 1:1 racemic mixture. These stereoisomers are known as D-ofloxacin, which is currently available, and L-ofloxacin (levofloxacin) is advancing towards clinical introduction in 1996. The L-enantiomer is more active against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* with an 8-to 250-fold decrease in MIC (Atarashi *et al.*, 1987; Mitscher *et al.*, 1987).

Figure 1.4 illustrates 4-quinolone analogues currently under investigation. They have been termed the third generation quinolones, and from which the 4-quinolones of the near future may well emerge.
Figure 1.3a. The second generation 4-quinolones or fluorinated 4-quinolones.

SECOND GENERATION QUINOLONES

Fleroxacin

Ciprofloxacin

Norfloxacin

Tosufloxacin

Lomefloxacin

Sparfloxacin
Figure 1.36. Ofloxacin (D & L forms).

D - (+) - ofloxacin

L - (-) - ofloxacin
THIRD GENERATION QUINOLONES

This group includes some examples of prominent quinolone analogues currently under investigation.

- AM - 1091
- AT - 4140
- QA - 241
- WIN - 57273
1.1.2. The *in vitro* activity of the 4-quinolones

A. Gram-negative genera

With the exception of nalidixic acid, the 4-quinolones are highly active against most genera that belong to the enterobacteria, including *E. coli*, *Klebsiella species*, *Serratia species*, *Proteus species*, and those strains resistant to nalidixic acid (Phillips *et al.*, 1988). The newer 4-quinolone agents are also active against *Neisseria species*, *Moraxella catarrhalis*, and *Haemophilus influenzae*, including those strains that produce beta-lactamase, *Legionella pneumophila*, and *P. aeruginosa* (Neu, 1989; Phillips *et al.*, 1988; Wolfson & Hooper, 1989).

B. Gram-positive species

The 4-quinolones are less active against both the *Staphylococci* and *Streptococci* (Eliopoulos & Eliopoulos, 1989, Neu, 1990), than against the aerobic Gram-negative rods. Ofloxacin is the most active against *Staphylococcus aureus* and coagulase-negative *Staphylococcus* species (Lewin & Smith, 1988). Although, ciprofloxacin and ofloxacin are more active than nalidixic acid and norfloxacin against *Streptococcus* species, including *S. faecalis* and *S. pneumoniae*, there is however increasing resistance. The activity of the 4-quinolones is also quite variable against the Gram-positive rods.

**Obligate anaerobes**

Nalidixic acid generally possesses very poor activity against the anaerobes. Although, the activity of the newer generation 4-quinolones against the anaerobes has been greatly enhanced, the *Clostridia* demonstrate varying susceptibility to the 4-quinolones, with the highest degree of activity observed against *C. perfringens* and *C. butyricum*. However, other species including *C. ramosum*, *C. difficile*, and *C. clostridiforme* are less susceptible (Phillips *et al.*, 1988). The 4-quinolones are also active against some members of the *Bacteroides* species including *B. ureolyticus* and *B. fragilis*. *Mobiluncus*, the *Peptostreptococci*, the *Peptococci*, and the *Fusobacteria* have also been shown to be susceptible.

**Miscellaneous bacteria**

The newer 4-quinolones have been shown to exhibit activity against *Mycoplasma pneumoniae*
but less so against *M. hominis* and *Ureaplasma urealyticum*. Both ofloxacin and ciprofloxacin are active against *Chlamydia trachomatis* and members of the *Mycobacterium* species including *M. tuberculosis*.
1.1.3. DNA Structure, Topology, and Topoisomerases

DNA Structure and its intracellular organisation

Since the first proposal of the Watson-Crick model of DNA structure, our understanding of how DNA structure encodes genetic information, allows mutation and recombination, and serves as a template for semiconservative replication and transcription (Watson & Crick, 1953; Watson et al., 1987) have become well established.

Bacterial chromosomal DNA is a large linear covalently closed circular, highly organised molecule (Cairns, 1963). Subsequently, it was found the E. coli chromosome is a circular DNA molecule, approximately 1,300μm long (Cairns, 1963). An essential and ubiquitous property of bacterial DNA is its negative supercoiled state (Stonington & Pettijohn, 1971; Worcel & Burgi, 1972; Wang, 1974; Sinden et al., 1980) with a density of about 1 supercoil per 200 base pairs (Worcel & Burgi, 1972). The homeostatic control and high dynamic state of cellular DNA are now thought to determine the degree of negative supercoiling of DNA (Menzel & Gellert, 1983).

The supercoiled state of double-stranded DNA is required for DNA replication, transcription, recombination, and gene expression (Drlica, 1984). The in vivo configuration of DNA in the bacterial chromosome is torsionally strained and organised into segregated supercoiled domains (Worcel & Burgi, 1972; Sinden & Pettijohn, 1981) (Figure 1.5). Worcel (1974) described how the 1,300μm E. coli chromosome is subdivided into approximately 65 domains of supercoiling (Figure 1.5). Each domain of double-stranded DNA is 20μm long and attached to an RNA core and the size of each domain is reduced by being negatively supercoiled (Wang, 1974) around a cylindrical core to form nucleosome-like structures (Griffith, 1976; Lydersen & Pettijohn, 1977; Eickbush & Moudrianakis, 1978; Drlica & Rouviere-Yaniv, 1987) called compactosomes (Watson et al., 1987). These structures are adhered to a central RNA core containing primarily RNA polymerase Figure 1.5). Compactosomes are complexed with the histone-like protein, HU, and are thought to be associated with bacterial chromatin (Kellenberger, 1987; Rouviere-Yaniv et al., 1992). It has also been shown that HU protein participates in the condensation and compaction of DNA with one dimer of HU per 200 base pairs (Rouviere-Yaniv et al., 1977). HU protein is found in association with RNA polymerase and topoisomerase I in the RNA core (Figure 1.5).
The 1.300μm *E. coli* chromosome is comprised of 65 DNA domains condensed with HU protein and bound to a central RNA polymerase core. This complex is compacted by the negative supercoiling activity of DNA gyrase to form a network of negative supertwisted or supercoiled domains of DNA bound to RNA polymerase. The 4-quinolones inhibit the supercoiling action of DNA gyrase and the compacted DNA-HU-RNA-RNA polymerase complex tends to a more relaxed state. *Adapted from Smith (1986) with permission.*
RNA core
HU protein
Topoisomerases
RNA

Domains lacking Supercoiling

Gyrase
4-Quinolones

Supercoiled domains
This location of HU suggested a role in translation and transcription prior to site-specific recombination and replication (Drlica & Rouviere-Yaniv, 1987; Kellenberger, 1987). One model suggested that the action of HU involved its binding to both DNA (Rouviere-Yaniv, 1977) and the native 30S ribosomal subunit (Suryanarayana & Subramanian, 1978).

Thus, the physical organisation of prokaryotic intracellular DNA occurs at multiple levels (Schmid, 1988) and a major consequence of DNA domains is that chromosomal regions can possess different levels of negative superhelicity (Drlica, 1984). This provides a mechanism that involves the spatial regulation of genes within a chromosomal domain (Schmid, 1988).

**DNA topology and topoisomerases**

**DNA topology**

A fundamental topological problem arises in bacteria because the chromosome is approximately 1300µm in length in *E. coli*, and yet the average size of a bacterium is 1 to 2µm long. For the chromosome to be accommodated into such a smaller cell, the circular DNA molecule must be condensed at least 1,000-fold. Furthermore, in this highly condensed state, the DNA molecule must be able to replicate, segregate (into daughter chromosomes), and allow transcription of individual genes.

Double-helical DNA is comprised of two single anti-parallel strands intertwined around each other (Watson & Crick, 1953). A consequence of this intertwining is that the two strands become linked and in the *E. coli* chromosome, which contains approximately 4 million bp, the strands are intertwined or linked about 400,000 times. This generates a linking number of 400,000 and measures the number of times one strand pierces the plane of the other. Thus, for each cycle of semi-conservative replication, the two DNA strands must unwind 400,000 times through a swivel mechanism which untwists the DNA double helix during strand separation (Cairns, 1963). The topological state of covalently closed circular DNA is dependant on the twist or number of helical turns (the linking number) in the molecule in its native conformation, and the writhe or geometric contortion of the helical axis in space, of the interwound single DNA strands (Fuller, 1971; Wang, 1974; Gellert, 1981). Negative supercoils result from writhe having a negative twist and
intracellular DNA exists in a slightly underwound state with one helical turn, on average every 10.4bp (Sinden et al, 1980; Gellert, 1981). For prokaryotic DNA, the problems of entanglement, strand unwinding, and supercoiling are solved, at least in part, by the DNA topoisomerases (Drlica, 1984; Gellert, 1981; Wang, 1985 & 1987).

DNA topoisomerases

The topological state of closed-circular DNA, and the level of bacterial DNA supercoiling is controlled by the DNA topoisomerases (Cozzarelli, 1980; Gellert, 1981; Wang, 1985). In prokaryotes, the maintenance of duplex DNA in a negatively supercoiled state is important for replication, transcription, and recombination. It is therefore likely that DNA supercoiling is an important aspect of chromosome structure (Wang, 1985; Bjornsti et al, 1986). The topoisomerase enzymes are now known to catalyse changes in the tertiary structure of DNA (Cozzarelli, 1980; Gellert, 1981; Lui & Wang, 1987; Wang, 1985, 1987; Maxwell & Gellert, 1986).

Four bacterial DNA topoisomerases have been isolated from E. coli (Hooper & Wolfson, 1993). Type I topoisomerases include topoisomerases I and III, type II topoisomerases include DNA gyrase and topoisomerase IV, and the special topoisomerases include enzymes that catalyse transposition or integration into and excision of bacteriophage DNA from the bacterial chromosome. Type I and type II topoisomerases differ in their structure and function. The first activity of a topoisomerase to be described was the relaxation of negatively supercoiled closed circular DNA by prokaryotic topoisomerase I (Wang, 1971). The prokaryotic type II topoisomerase, DNA gyrase, is the only topoisomerase able to introduce negative supercoils into DNA (Gellert et al, 1976).

Type I topoisomerases make a transient single strand break in the double helix, followed by passage of the other strand through the break, while type II topoisomerases transiently cleave both strands of the double helix and pass another double helical segment through this break (Morrison & Cozzarelli, 1979; Cozzarelli, 1980; Gellert, 1981). Type I and Type II topoisomerases have been isolated from many species of bacteria, and eukaryotes, including human cells.
Type I topoisomerases

Bacterial DNA topoisomerase I

Nearly twenty-five years ago, it was first identified that extracts of *E. coli* could relax negatively supercoiled DNA (Wang, 1971). *E. coli* type I DNA topoisomerase was found to be responsible for this relaxing activity through unit change in the linking number of the intertwined strands of DNA (Brown & Cozzarelli, 1979). This enzyme is a monomeric protein with a molecular weight of 110kDa and is the product of the *topA* gene, which is located at 28 minutes on the genetic map of the *E. coli* chromosome (Stemglanz *et al.*, 1981; Trucksis & Depew, 1981).

Type I DNA topoisomerases from both prokaryotic and eukaryotic sources relaxes negatively supercoiled DNA through catalytic removal of negative supercoils from DNA, and highly negatively supercoiled DNA is initially relaxed with high efficiency, but the rate slows as the linking number of fully relaxed DNA is approached. It is also known that positively supercoiled DNA is unaffected by this enzyme (Wang, 1971).

The relaxation of negative supercoiled DNA involves a change in the linking number (or number of helical turns) through both the cleavage and rejoining of the DNA chain (Gellert, 1981). Cleavage studies have shown topoisomerase I to possess a high affinity for segments of DNA composed of both a single- and double-stranded region, and that the cleavage site is located within the single-stranded region. Topoisomerase I can both cleave and rejoin DNA repeatedly, in an energy conserving process that involves conservation of the free energy state of the phosphodiester bond in DNA (Depew *et al.*, 1978).

Eukaryotic topoisomerase I is found in association with nuclear chromatin and is of a smaller molecular weight at, 60-75kDa (Gellert, 1981). The eukaryotic enzyme differs from the prokaryotic enzyme in several ways. It is active in the absence of Mg^{2+} ions and in the presence of chelators (Gellert, 1981; Gootz & Osheroff, 1993). Unlike prokaryotic topoisomerase I, eukaryotic topoisomerase I can relax both positively and negatively supercoiled DNA (Champoux & Dulbecco, 1972).
Bacterial DNA topoisomerase III

A second type I topoisomerase, topoisomerase III, been identified in *E. coli*. As with topoisomerase I, topoisomerase III is able to remove negative superhelical twists without the requirement for ATP (Srivenuopal *et al.*, 1984). In addition to its binding with DNA, topoisomerase III binds to RNA, and can cleave both nucleic acids at identical nucleotide sequences (DiGate & Marians, 1992).

Topoisomerase III is the product of the *topB* gene located at 39 minutes on the genetic map of the *E. coli* chromosome (DiGate & Marians, 1989). There is evidence that topoisomerase III may have a role in DNA recombination (Wang *et al.*, 1990).

Type II topoisomerases

Bacterial DNA gyrase

DNA gyrase (EC 5.99.1.3) was the first type II topoisomerase to be isolated from *E. coli* (Gellert *et al.*, 1976), and plays a central role in the structure and function of the bacterial chromosome. DNA gyrase is a large ATP-requiring multisubunit enzyme responsible for the catalytic conversion of relaxed double-stranded DNA into negatively supercoiled DNA (Gellert *et al.*, 1976). The discovery of DNA gyrase was the culmination of work on two classes of DNA synthesis inhibitors, the 4-quinolones, nalidixic and oxolinic acids (Goss *et al.*, 1964) and the coumarins, novobiocin and coumermycin (Gellert *et al.*, 1976; Ryan, 1976).

DNA gyrase is a tetramer composed of two A subunits (GyrA), and two B subunits (GyrB) (Sugino *et al.*, 1977). The A and B subunits were identified on the basis of their selective inhibition with either temperature-sensitive mutations (Gellert *et al.*, 1979; Kreuzer & Cozzarelli, 1979) or specific subunit antibiotics (Gellert *et al.*, 1976 & 1977; Sugino *et al.*, 1977; Orr *et al.*, 1979). The GyrA subunit was identified with the 4-quinolones nalidixic and oxolinic acids (Gellert *et al.*, 1977; Sugino *et al.*, 1977) and the GyrB subunit was identified using novobiocin and coumermycin (Gellert *et al.*, 1976; Orr *et al.*, 1979). The supercoiling reaction is inhibited by both these classes of antibiotic, with the 4-quinolones blocking both the cleavage and re-union processes (Gellert, 1981), and the coumarins inhibit the intrinsic ATP activity of the energy-coupling reaction (Mizuuchi *et al.*, 1982).
1978; Sugino et al, 1978; Sugino et al, 1980). The GyrA subunits, which are encoded by the gyra gene (locus at 48 minutes on the E. coli chromosome), each contain 874 amino acids with a mass of 97kDa, and the GyrB subunits, are encoded by the gyrB gene (locus at 82 minutes on the E. coli chromosome), and each contain 804 amino acids with a mass of 90kDa (Mizuuchi et al, 1978). The tetrameric structure of E. coli DNA gyrase has been confirmed by small-angle neutron scattering, and this yields an estimated molecular mass of 353kDa, which is consistent with the calculated mass of the holoenzyme (A2B2) at 374 kDa (Kneeger et al, 1990). DNA gyrase binds to double-stranded DNA and the DNA supercoiling reaction is driven by a magnesium-dependant hydrolysis of ATP (Gellert et al, 1976; Sugino & Cozzarelli, 1980; Tse et al, 1980). GyrA mediates DNA strand breakage and reunion with the tyrosine residue at position 122 (Tyr-122), forming a transient covalent linkage with the broken 5'-end of the DNA strand (Horowitz & Wang, 1987). GyrB mediates the ATPase activity of DNA gyrase (Lui & Wang, 1987; Morrison & Cozzarelli, 1979), and the N-terminal domain of GyrB (amino acids 2 through 220) is now known to contain the ATP-binding site (Wigley et al, 1991).

When bacteria are exposed to DNA gyrase inhibitors there are two well recognised effects on DNA supercoiling. (1) The specific inactivation of either subunit of DNA gyrase blocks the introduction of titrable supercoils (Gellert et al, 1976; Gellert et al, 1977), and (2) inhibition of either subunit leads to a loss of titrable supercoils from both bacterial chromosomal DNA (Drlca & Snyder, 1978; Manes et al, 1983) and plasmid DNA (Pettijohn & Pfenninger, 1980; Kano et al, 1981; Lockshon & Morris, 1983).

Human cells possess a topoisomerase II. It also cleaves and rejoins double-stranded DNA by introducing two cuts staggered by 4 bp with the 5'-end protruding (Miller et al, 1981; Lui et al, 1983). There is increasing evidence that the mammalian enzyme generates DNA supercoils in those regions of chromatin where genes are actively being transcribed (Smith, 1988), and it is also thought that this enzyme is one of the major structural components of the chromosomal domain that may be sites for active gene expression in eukaryotes (North, 1985; Weintraub, 1985). However, unlike bacterial DNA gyrase, the mammalian enzyme is composed of only two subunits, each with a
molecular weight of 172kDa (Smith, 1984), and can catenate, relax, and unknot DNA by a duplex strand passage mechanism (Sutcliffe et al, 1989).

Mechanism of action of DNA gyrase

The mechanism of DNA supercoiling by DNA gyrase involves the catalytic conversion of relaxed DNA into negatively supercoiled DNA. The process is initiated with the transient cleavage and re-union of double-stranded DNA at specific gyrase binding sites. These specific recognition sites, of 140bp, are found in segments of chromosomal DNA that are in contact with DNA gyrase (Synder & Drlica, 1979; Kleven & Wang, 1980). Within the DNA - DNA gyrase complex, GyrA subunits cleave both duplex strands (Gellert et al, 1977) that are staggered 4bp apart, and the 5'-ends of duplex DNA (with Tyr-122 residues exposed) protrude (Morrison & Cozzarelli, 1979). A single turn of 120- to 150-bp of DNA segment is then wrapped around DNA gyrase, with DNA entry and exit points in close proximity (Kirchhausen et al, 1985; Rau et al, 1987). Kirchhausen et al (1985) have suggested that the gyrase holoenzyme is a "heart-shaped structure" with DNA wrapped between the upper "lobes".

Hydrolysis of ATP induces a structural change within the complex where it is thought increased wrapping of DNA around gyrase occurs (Rau et al, 1987). The DNA is then negatively supercoiled by the GyrB subunits in a reaction that utilises one molecule of ATP for each supercoil formed (Gellert et al, 1977). The number of supercoils for each domain of DNA is approximately 400 (Smith, 1984). The supercoiling process is completed when the negative supercoils are sealed into each chromosomal domain by the GyrA subunits (Gellert et al, 1977). DNA gyrase is distributed along DNA at 100kbp intervals, which corresponds to approximately 1 gyrase molecule per domain of chromosomal DNA (Synder & Drlica, 1979).

In vitro DNA gyrase is also involved in the formation and resolution (catenation and decatenation) of covalently closed circular DNA molecules that are interlocked, like links in a chain (Kreuzer & Cozzarelli, 1980; Mizuuchi et al, 1980; Krasnow & Cozzarelli, 1982; Marians, 1987), and formation and removal of knots within the DNA double-helix (Mizuuchi et al, 1980). DNA gyrase can also catalytically remove positive supercoils (Gellert et al, 1981). Both DNA gyrase and topoisomerase I are regulated at transcriptional level in response to the negative supercoiled state of
DNA (Menzel & Gellert, 1983). Reduction in the level of DNA supercoiling stimulates gyrase expression through increased transcription of the gyrA and gyrB genes (Menzel & Gellert, 1983 & 1987), and suppresses transcription of the topA gene (Tse-Dinh, 1985). These findings suggest that the supercoiled state of DNA is homeostatically regulated (Menzel & Gellert, 1983 & 1987). DNA gyrase plays an essential role in DNA replication and is involved in the initiation (Marians et al, 1977), of elongation (Gellert et al, 1976), and termination (DiNardo et al, 1982). The use of DNA gyrase inhibitors has shown, at least indirectly, that DNA gyrase is also involved in transcription, because an increase in negative supercoiling can increase the expression of the maltose, lactose, and galactose operons (Senzey, 1979; Smith et al, 1978; Smith, 1981; Yang et al, 1979). DNA gyrase may also be involved in DNA recombination (Mizuuchi et al, 1978; Ohtani et al, 1982; Von Wright & Bridges, 1981; Wu et al, 1983;), and DNA repair (Hays & Boehmer, 1978; Phillips et al, 1987). The transposition of Tn5 may also be dependent on the degree of DNA supercoiling of recipient DNA, and is affected by a reduction in gyrase activity by both coumarin inhibitors or gyrA mutations (Isberg & Syvanen, 1982).

**Bacterial topoisomerase IV**

Topoisomerase IV is a membrane-associated enzyme (comprised of the 145kDa combined protein products of parC and parE), that catalyses ATP-dependent relaxation of negatively and positively supercoiled DNAs, and unknotting of unnicked duplex DNA, and is thought to be involved in chromosome partitioning and in removal of DNA supercoils (Kato et al, 1990 & 1992). Unlike DNA gyrase, topoisomerase IV has not shown any DNA supercoiling activity (Hooper & Wolfson, 1993).
1.1.4. The mechanism of action of the 4-quinolones

The quinolone antibacterials are now known to have distinct and remarkable effects on both DNA gyrase and the bacterial cell. Most of the effects of exposure to quinolones were first determined from earlier studies with nalidixic acid and oxolinic acid prior to the discovery of DNA gyrase. Nalidixic acid (NAL) was found to selectively inhibit DNA synthesis, produce DNA degradation, and induce cellular filamentation (Goss et al., 1964 & 1965, Deitz et al., 1966; Cook et al., 1966). NAL is rapidly bactericidal (Goss et al., 1965), and it was found that both chloramphenicol, which inhibits protein synthesis, and dinitrophenol, which uncouples oxidative phosphorylation, reduced its bactericidal activity on *E. coli* (Deitz et al., 1966). These findings demonstrated that competent protein and RNA synthesis were necessary for the bactericidal activity of NAL (Cook et al., 1966; Deitz et al., 1966; Crumplin & Smith, 1975). It has also been shown that although NAL inhibits overall DNA synthesis (Crumplin & Smith, 1976), the major effect of NAL was to produce unsealed nicks in newly synthesised DNA which led to the irreversible degradation of DNA through induced exonuclease action (Crumplin & Smith, 1975 & 1976; Hill & Fangman, 1973) and ultimately cell death.

Currently, the overall mechanisms of bacterial killing by the 4-quinolones are at present not fully understood and further studies will be necessary to determine precisely which mechanisms are responsible for the bactericidal activity of these drugs.

**DNA gyrase - the 4-quinolone target**

Since the discovery that DNA gyrase is the type II topoisomerase responsible for the introduction of DNA supercoiling (Gellert et al., 1976), it has become established through genetic and biochemical studies, the *E. coli* GyrA (*nalA*) subunit is the primary target for NAL and oxolinic acid (Gellert et al., 1977; Sugino et al., 1977). Recently, similar studies have shown the GyrA subunit in other bacterial species, including *Bacillus subtilis* (Sugino & Bott, 1980), *Enterococcus faecalis* (Nakanishi et al., 1991), *Staphylococcus aureus* (Okada et al., 1991), *Campylobacter jejuni* (Gootz & Martin, 1991), *Serratia marcescens* (Masecar & Robillard, 1991), *Pseudomonas*
aeruginosa (Inoue et al, 1987; Masecar et al, 1990), *Haemophilus influenzae* (Setlow et al, 1985), and *Citrobacter freundii* (Aoyama et al, 1988) to be the target of 4-quinolone action. It has also been shown the bactericidal activities of the newer 4-quinolones including ciprofloxacin (Hooper et al, 1987; Robillard & Scarpa, 1988), norfloxacin (Crumplin et al, 1984; Hirai et al, 1986a & 1987; Hooper et al, 1986), and ofloxacin (Sato et al, 1986a & 1986b; Wolfson et al, 1987) against DNA gyrase, are reduced by gyrA mutants. The GyrB (cou) subunit has also been shown to be a target of the 4-quinolones (Inoue et al, 1978; Nakamura et al, 1989; Smith, 1984; Yamagisha et al, 1981 & 1986). It has also been suggested that, to a lesser degree than DNA gyrase, topoisomerase IV is susceptible to the *in vitro* and *in vivo* action of the 4-quinolones (Kato et al, 1992). Ng et al (1992) have demonstrated that in the region of the *parC* and *parE* genes, a new 4-quinolone resistance locus nfxD exists, requiring the presence of the *gyrA* mutation. Studies on purified DNA gyrase have shown NAL, oxolinic acid, and other 4-quinolones to inhibit the activity of DNA gyrase, including the introduction of negative DNA supercoiling, formation and resolution (catenation and decatenation) of cccDNA molecules, formation and removal of knotting within the duplex DNA molecule, and DNA dependent ATP hydrolysis (Drlica, 1984; Gellert, 1981; Wang, 1985 & 1987).

Thus, the bactericidal action of the 4-quinolones action are thought to involve a cascade of cytopathic events (Crumplin et al, 1984; Drlica, 1984; Piddock & Wise, 1987; Reece & Maxwell, 1991), which comprise (a) a primary component that specifically inhibits bacterial DNA gyrase and leads to both the inhibition of chromosomal DNA synthesis (Crumplin & Smith, 1975 & 1976) and an imbalance of bacterial metabolism (Crumplin et al, 1984), and (b) a secondary component resulting from changes in the tertiary structure of chromosomal DNA, through gyrase-induced DNA cleavage, is thought to lead to the inducible production of the *recB* and *recC* exonucleases, and ultimately degradation of bacterial DNA (Crumplin & Smith, 1976; Crumplin et al, 1984; Menzel & Gellert, 1983). Together, both these components constitute the major disruptive bactericidal effects of the 4-quinolones (Smith, 1984 & 1988). Shen et al (1989a) have proposed that, rather
than acting directly on DNA gyrase, the 4-quinolones are thought to bind to saturable sites on supercoiled DNA that involves a quinolone-DNA cooperative binding process. This binding has been suggested to be facilitated by DNA gyrase. Evidence also suggests that DNA gyrase induces a drug binding site on relaxed DNA in the presence of ATP to form a DNA gate (Shen et al., 1989a). The GyrA subunit cleaves the duplex strands that are 4bp staggered apart (Morrison & Cozarelli, 1979), and forms covalent bonds between Tyr-122 and the 5'-ends of the DNA chain (Horowitz & Wang, 1987). These exposed protruding 4bp staggered cuts form a DNA gate, which leads to the formation of a locally denatured DNA bubble that acts as the 4-quinolone binding site (Figure 1.6). The 4-quinolone molecules acquire high binding affinity through a cooperative binding mechanism with the separated 5'-ends of cleaved DNA (Shen et al., 1989b). It is thought, the 4-quinolones self-associate cooperatively into a bioactive tetraplex in the DNA gate and bind with unpaired bases via hydrogen bonding (Shen et al., 1989b). The cooperative 4-quinolone-DNA binding model (Figure 1.6) is in general agreement with the structure-activity relationships of the 4-quinolones (Chu & Fernandes, 1989; Domgala et al., 1986; Mitscher et al., 1989 & 1990; Rosen, 1990; Wentland, 1990).

Bacterial DNA gyrase and eukaryotic topoisomerase II possess remarkable similarities in biochemical mechanisms and amino acid sequences. Therefore, it would be reasonable to question whether 4-quinolone antibacterial agents demonstrate inhibitory activity against eukaryotic topoisomerase II. However, it is generally accepted that the eukaryotic enzyme is not sensitive to inhibition by the 4-quinolones (Bergen, 1988; Hussy et al., 1986; Miller et al., 1981). This is probably due to the structural and functional differences between the bacterial and eukaryotic enzymes and would seem to provide an explanation of why the 4-quinolones are toxic to bacteria but not to humans.

The following are the well established cellular effects of the 4-quinolones on viable bacteria, including effects on bacterial metabolism, bacterial cell surface, and bacterial plasmids.

**Bacterial metabolism**

DNA synthesis is terminated rapidly during exposure to the 4-quinolones. At near growth
Figure 1.6. 4-quinolone-DNA binding model for inhibition of DNA gyrase.

The 4-quinolone molecules form a tetraplex with $\pi-\pi$ stacking of the 4-quinolone rings bound to the DNA gate at the 5'-ends of cleaved DNA. The 4-quinolone high binding affinity occurs via hydrogen bonding (shown by dotted lines) with the exposed unpaired bases of DNA. Adapted from Shen et al (1989b) with permission.
inhibitory concentrations of fleroxacin, large nucleoids in filamentous *E. coli* developed. This suggests chromosome decatennation is inhibited after a round of DNA replication (Georgopapadakou & Bertasso, 1991). High quinolone concentrations inhibit protein and RNA synthesis which leads to cell filamentation (Crumplin et al, 1984; Diver & Wise, 1986; Dougherty & Saukkonen, 1985; Elliott et al, 1987; Smith, 1984) possibly through inhibition of DNA synthesis and induction of the *E. coli* recA (SOS) DNA repair system (Donachie & Robinson, 1987; Phillips et al, 1987; Piddock & Wise, 1987; Walker, 1984). There is also evidence that the quinolone concentration required to inhibit the *in vitro* or *in vivo* level of supercoiling by gyrase with that required to inhibit cell growth demonstrates the *in vitro* level to exceed the *in vivo* by a factor of between 10 to 100 (Gellert et al, 1977; Domagala et al, 1986; Zweerink & Edison, 1985). This is paradoxical since it would be expected that the purified gyrase enzyme would be more and not less sensitive to the 4-quinolones. This has been explained in part that the action of 4-quinolones on bacteria may involve additional mechanisms beyond inhibition of DNA gyrase (Kreuzer & Cozzarelli, 1979). This has lead to the hypothesis that an irreversible 4-quinolone-DNA gyrase complex forms a DNA lesion that acts as a cellular poison (Kreuzer & Cozzarelli, 1979) and therefore initiates the cytopathic cascade of events that lead to cell death.

Many of the quinolones are structurally similar (Figures 1.1-1.4), and therefore share similar cellular effects. However, marked variation in antibacterial potencies have been well documented. These variations parallel differences in DNA gyrase inhibitory concentrations, as has been demonstrated for *E. coli* (Domagala et al, 1986; Hooper et al, 1987) and *Micrococcus luteus* (Zweerink & Edison, 1986).

**Bacterial cell surface**

To reach the intracellular DNA gyrase target in the bacterial cell cytoplasm, the 4-quinolone molecule must access, and traverse through both the outer and inner membranes (Bryan & Bedard, 1991; Nikaido & Vaara, 1985; Hirai et al, 1986a). It has been proposed that the 4-quinolones penetrate the *E. coli* outer membrane by diffusion through OmpF and OmpC porin channels (Cohen et al, 1988; Hirai et al, 1986b). The hydrophilic 4-quinolones, ciprofloxacin, norfloxacain, and
Ofl oxacin enter the bacterial cell via the porin channels, and the hydrophobic 4-quinolone, NAL, penetrates not only through the porin channels but also through the phospholipid regions of the outer membrane. Lipopolysaccharide in the outer membrane has been shown to be a barrier to more hydrophobic 4-quinolones since mutants of *S. typhimurium* with LPS defects show increased susceptibility (Chapman & Georgopapadakou, 1988; Hirai *et al.*, 1986b; Timmers & Sternglanz, 1978).

Exposure to subinhibitory concentrations of quinolones may alter the bacterial cell surface. It has been shown growth of an encapsulated strain of *K. pneumoniae* at subinhibitory concentrations of ciprofloxacin resulted in cell surface changes including, increased complement binding, changes to the outer membrane antigens, and heterogeneous capsular distribution in filamenting cells (Williams, 1987). Growth in the presence of subinhibitory concentrations of quinolones has resulted in reduced adherence of uropathogenic strains to uroepithelial cells (Desnottes *et al.*, 1990). Nal-induced morphological changes have also been observed and attributed to loss of membrane integrity leading to leakage of cytoplasmic constituents through plasmolysis (Dougherty & Saukkonen, 1985; Hooper *et al.*, 1986).

**Bacterial plasmids**

Some plasmids have been shown to be eliminated from their hosts in the presence of subinhibitory quinolone concentrations (Michel-Briand *et al.*, 1986; Platt & Black, 1987; Weisser & Wiedemann, 1985 & 1986). However, the effect on plasmid elimination is highly dependent on the concentration of the quinolone used, the plasmid, and the bacterial host. Coumermycin and novobiocin have been shown to eliminate plasmids through GyrB subunit antagonism (Wolfson *et al.*, 1982; Hooper *et al.*, 1984). The elimination of susceptible plasmids to curing indicates that at subinhibitory concentrations, plasmid DNA replication, partition, or copy number may be more sensitive to the effects of 4-quinolone action than replication of chromosomal DNA (Wolfson *et al.*, 1982; Weisser & Wiedemann, 1986). There is also evidence that certain R-plasmids, including RP4, increase the 4-quinolone susceptibility of their host (Crumplin & Smith, 1981). The 4-quinolones have been shown to inhibit both the conjugative transfer of plasmids (Barbour, 1967; Burman, 1977; Gill &
Iver, 1982; Hirai et al., 1984; Hooper et al., 1989; Michel-Briand et al., 1983; Nakamura et al., 1976; Weisser & Wiedemann, 1987), and the Hfr-mediated transfer of the bacterial chromosome Barbour, 1967; Bouck & Adelberg, 1970; Fenwick & Curtiss, 1973; Hane, 1971; Holom & Pritchard, 1965; Hooper et al., 1989). Hooper et al. (1989) also suggested that disruption of the transfer replication fork was the site for quinolone action. Transfer inhibition is not a unique effect of the quinolones since other antibiotics including both rifampicin and chloramphenicol have also demonstrated similar effects (Fenwick & Curtiss, 1973). It has also been suggested the in vivo lethal effect of the quinolones far exceeds plasmid elimination and transfer inhibition (Weisser & Wiedemann, 1987).

Illegitimate recombination has been reported between pBR322 and bacteriophage λ in the presence of oxolinic acid (Ikeda et al., 1982). Recombination was eliminated by coumermycin, and reduced by oxolinic acid when quinolone resistant gyrA mutants were used (Ikeda et al., 1984). It has also been suggested the recombination sites are similar to gyrase cleavage sites (Ikeda et al., 1984) and that GyrA-DNA cleaved sites mediate recombination (Ikeda & Shiozaki, 1984).

E. coli recA (SOS) DNA repair systems

Damage to bacterial DNA has been shown to occur through quinolone induction of the recA DNA repair system in E. coli (Courtright et al., 1988; Gudas & Pardee, 1976; Phillips et al., 1987; Piddock & Wise, 1987). Induction of the recA DNA repair system by 4-quinolones requires an active DNA replication fork (Gudas & Pardee, 1976) and the functional helicase (unwinding) activity of exonuclease V, the product of the recBC genes (Chaudhury & Smith, 1985). It has been suggested that the RecA protein is activated by DNA damage (Little & Mount, 1982). The most bactericidal concentrations of 4-quinolones have been found to be similar to concentrations that maximally induce the SOS system (Phillips et al., 1987). This suggests the SOS system may be involved in bacterial killing (Chow et al., 1988; Diver & Wise, 1986; Phillips et al., 1987). However, recent studies have suggested the SOS system may not be a mediator of bacterial killing by the 4-quinolones (Lewin et al., 1989; Lewin & Smith, 1990). Therefore, the 4-quinolones may either act on DNA gyrase or saturable sites at the replication fork to produce a
lethal lesion which is converted by exonuclease V to an activating signal for RecA, and binding of multiple RecA molecules may promote error-prone DNA repair (Little & Mount, 1982; Sassanfar & Roberts, 1990).

**Kinetics of 4-quinolone bactericidal activity**

Although, the interaction with DNA gyrase appears to be a necessary prerequisite for the bactericidal activity of the 4-quinolones, our understanding of the molecular events leading to bacterial death remains incomplete. However, common characteristics of bacterial killing by the 4-quinolones have emerged for *E. coli* (Crumplin et al, 1984; Smith, 1984), *S. marcescens* (Lewin et al, 1990), *S. aureus* and *S. warneri* (Lewin & Smith, 1988). Bacterial death is initially rapid but slows when the viable count is approximately 10⁴-fold lower than the initial inoculum (Cook et al, 1966; Courtright et al, 1988; Crumplin et al, 1984; Deitz et al, 1966; Diver & Wise, 1986; Elliott et al, 1987; Goss et al, 1984; Smith, 1984; Wolfson et al, 1989). These kinetics would suggest two components to bacterial killing. The less rapidly killed bacterial cells may represent persisters and do not appear to be resistant or less effectively killed mutants, since neither show resistance to growth inhibition or reduced killing on 4-quinolone re-exposure (Wolfson et al, 1989). The persistence mechanism to the 4-quinolones is as yet unknown. However, it has been suggested this subpopulation may be refractory to the lethal effects of the 4-quinolones, as has been documented for persistant cells exposed to β-lactams (Hoffmann et al, 1972; Holzhoffer et al, 1985).

The viability of *E. coli* KL.16 (ca 10⁷ bacteria/ml) when exposed to a range of concentrations of ciprofloxacin (CFX), nalidixic acid (NAL), norfloxacin (NOR), and ofloxacin (OFX), a demonstrable biphasic response (Figure 1.7) is always observed (Diver & Wise, 1986; Smith, 1984; Winshell & Rosenkranz, 1970). The rate and magnitude of bacterial death is progressively more bactericidal with increasing quinolone concentrations and this phase is due to the progressive inhibition of DNA synthesis (rate of inhibition for CIP>OFX>NOR>NAL). When the most bactericidal concentration is exceeded there is a progressive reduction in bactericidal activity for all 4-quinolones, although, the reduction in bactericidal activity is much more pronounced for nalidixic
acid and norfloxacin. Most bactericidal concentrations of 4-quinolones compare with those concentrations that inhibit bacterial DNA synthesis (Chow et al., 1988), but inhibition of DNA synthesis appears insufficient to affect bacterial death. This progressive reduction in bactericidal activity is through an antagonistic inhibition of RNA synthesis (Crumplin & Smith, 1975). The newer 4-quinolones ciprofloxacin and ofloxacin are less susceptible than nalidixic acid and norfloxacin to antagonism by rifampicin (Crumplin et al., 1984; Deitz et al., 1966; Smith, 1984; Zeiler, 1985; Zeiler & Grohe, 1984). Similarly, it has also been shown that chloramphenicol produces a much less marked antagonism by protein synthesis inhibition (Crumplin et al., 1984; Deitz et al., 1966; Smith, 1984; Zeiler, 1985; Zeiler & Grohe, 1984). Smith (1984) described this phenomenon and suggested the newer 4-quinolones ciprofloxacin and ofloxacin possess a second mechanism of killing in addition to a common mechanism possessed by all 4-quinolones. A third bactericidal mechanism has been described for norfloxacin against non-dividing E. coli under conditions of starvation (Ratcliffe & Smith, 1985).

Antagonism of both RNA synthesis and protein synthesis reduce the 4-quinolone bactericidal effect, but neither affect quinolone inhibition of DNA synthesis (Deitz et al., 1966; Piddock et al., 1990; Winshell & Rosenkranz, 1970). Both antagonisms inhibit protein synthesis, and the lethal effects of the 4-quinolones may involve the synthesis of a protein required for lethality, possibly the RecA protein (Little & Mount, 1982; Smith, 1984). Both competent RNA and protein synthesis are therefore required for the bactericidal activity of the 4-quinolones.
Figure 1.7. Biphasic response of *E. coli* KL16 to different concentrations of the 4-quinolones CFX, NAL, NOR, and OFX. Reprinted partly from Smith (1984) with permission.
1.1.5. Bacterial resistance to the 4-quinolones

With the ever expanding worldwide clinical use of the 4-quinolones, resistance to these agents have been reported (Wolfson & Hooper, 1989), and the mechanisms of resistance has been the subject of many studies. Bacterial resistance to antimicrobial agents are usually mediated either via plasmid-encoded resistance or chromosomal mutation.

Currently, the 4-quinolones have been shown to be exempt from transferrable R-plasmid mediated resistance (Burman, 1977; Crumplin, 1987; Lewin et al, 1990; Smith 1984; Crumplin, 1987). There have been two reports of plasmid-mediated nalidixic acid resistance in Shigella dysenteriae (Panhotra et al, 1985; Munshi et al, 1987). However, both have been questioned on the basis that nalidixic acid resistance could not be transferred and that it was therefore unknown if the generation of nalidixic acid mutants was plasmid-mediated (Crumplin, 1987). It has been suggested that these plasmids do not carry a nal^ gene, but are mutator plasmids increasing the frequency of chromosomal mutations to nalidixic acid resistance (Levy et al, 1988; Lewin et al, 1990). There have been reports however, of a plasmid-mediated mechanism of resistance that may involve drug accumulation. The encoded 50 kDa NorA protein product from the norA gene in S. aureus, has been identified to confer high-level resistance to hydrophilic fluoroquinolones through a process that appears to be membrane associated (Ubukata et al, 1989; Yoshida et al, 1990). When the norA gene was cloned into pBR322, and transformed into E. coli HB101, it conferred resistance to the 4-quinolones. The MICs for the hydrophilic 4-quinolones, ciprofloxacin, enoxacin, norfloxacin, and ofloxacin increased by 8-64 fold, and decreased 2-fold for the hydrophobic 4-quinolones nalidixic acid and sparfloxacin (Ubukata et al, 1989; Yoshida et al, 1990). Although this represents a mechanism whereby a plasmid can confer 4-quinolone resistance, it has yet to be established in clinical isolates (Amyes & Gemmell, 1992).

Chromosomally-mediated resistance to the 4-quinolones

Chromosomal resistance has been shown to occur through two mechanisms - either alteration in the target enzyme, DNA gyrase, or a permeability mutation that reduces drug accumulation (Hooper & Wolfson, 1987; Smith 1986).
Alterations of the GyrA protein subunit in 4-quinolone resistance has been determined in a large number of species, and mutations in the gyrA have been shown to confer high-level resistance to all 4-quinolones. These have been directly identified by genetic mapping or DNA sequencing in several bacteria that include *E. coli* (Cullen *et al.*, 1989; Gellert *et al.*, 1977; Hooper *et al.*, 1986 & 1987; Sugino *et al.*, 1977; Wolfson *et al.*, 1987), *P. aeruginosa* (Hirai *et al.*, 1987; Masecar *et al.*, 1990; Robillard & Scarpa, 1988), *S. marcescens* (Fujimaki *et al.*, 1989; Masecar & Robillard, 1991), and *Klebsiella pneumoniae* (Heisig & Wiedemann, 1991).

Most information on changes in the GyrA protein structure have been acquired from nucleotide sequencing of 4-quinolone resistant gyrA mutants of *E. coli*. Nucleotide sequence analysis has demonstrated most 4-quinolone resistance to be conferred by single changes in amino acid sequence (point mutations) within a small region between amino acids 67 and 100 near the N-terminus of the A subunit (Cambau *et al.*, 1992; Cullen *et al.*, 1989; Hallet & Maxwell, 1991; Yoshida *et al.*, 1990). All of these mutations are clustered in the amino-terminal region of the polypeptide sequence near Tyr-122 of the GyrA subunit (Yoshida *et al.*, 1990; Yoshida *et al.*, 1988). Tyr-122 is also adjacent to the active site of DNA gyrase (Shen *et al.*, 1989b). The 5'-GyrA-Tyr-122-end is covalently bound to DNA when DNA gyrase breaks the phosphodiester bonds of DNA, and therefore mutations close to Tyr-122 lead to changes in the secondary and tertiary structure of GyrA. Resistance to the 4-quinolones is accompanied by changes in the affinities of the 4-quinolone for the DNA gyrase-DNA complex (Amy *et al.* & Gemmell, 1992). Shen *et al.* (1989b) have further suggested resistant DNA gyrase blocks the access of the 4-quinolones to the high affinity binding site. Most alterations in amino acid sequences involved changes in Ser-83 to Leu or Trp, resulting in a 128-fold increase in resistance to NAL, and 32-fold increase in resistance to the newer 4-quinolones (Cullen *et al.*, 1989; Hallet & Maxwell, 1991; Oram & Fisher, 1991; Yoshida *et al.*, 1990). Mutations in the GyrB subunit have been shown to confer changes in 4-quinolone susceptibility in *E. coli* resistance loci *nalC* and *nalD* (Inoue *et al.*, 1987; Yamagishi *et al.*, 1981; Yamagishi *et al.*, 1986). Both loci also encode point mutations, with *nalC* (Lys-447 to Gln) leading to hypersusceptibility to 4-quinolones with a piperazine substituent, and *nalD* (Asp-425...
There have been recent reports of *E. coli* with reduced susceptibility to the 4-quinolones, β-lactams, tetracycline, and chloramphenicol (Hooper *et al.*, 1986; Hirai *et al.*, 1986b). This pleiotropic mutation has also been shown to be due to reduced *ompF* expression, and to be an allele of *marA* (mapping at 34 min on *E. coli* chromosome), which encodes intrinsic multiple antibiotic resistance (George & Levy, 1983). Reduced *ompF* expression has been observed in *marA*, *cfxB*, *norB*, *cfxB* mutants, and they are thought to be closely linked (Hooper & Wolfson, 1993). This resistance mechanism has as yet still to be defined.
to Asn) leading to small increases in 4-quinolone resistance (Yamagishi et al, 1986). The
4-quinolone hypersusceptibility mutation results from an increase in negative charge of the GyrB
subunit, increasing its attraction for the positively charged piperazine group (Yoshida et al, 1991).

The highest levels of 4-quinolone clinical resistance have been observed in gyrA mutants, and E. coli
mutants selected with NAL (at 4xMIC) demonstrated equal numbers of gyrA and gyrB mutations
(Nakamura et al, 1989). 4-quinolone resistance resulting from changes in E. coli topoisomerases I,
III, and IV have not been shown.

Mutations that effect 4-quinolone permeability have been identified in several bacterial species
including, E. coli, Salmonella, Pseudomonas, Klebsiella, and Serratia (Lewin et al, 1990; Piddock
et al, 1989). This mechanism appears to be associated with alterations in the outer membrane
porin OmpF and OmpC, proteins encoded by the ompF and ompC genes. E. coli ompF mutants
have showed a 2-fold increase in resistance to many of the hydrophilic quinolones (Chapman et al,
1989; Chapman & Georgopapadakou, 1988; Cohen et al, 1988; Hirai et al, 1986b; Hooper et al,
1986; Hooper et al, 1987). The small increase in resistance probably results from a compensatory
increase in 4-quinolone efflux through the OmpC porin (Cohen et al, 1989). In contrast, ompC
mutants have practically no detectable change in 4-quinolone resistance (Chapman et al, 1989;
Chapman & Georgopapadakou, 1988; Hirai et al, 1986b; Hooper et al, 1987). It has also been
shown that both inactivation of the ompF gene and mutation in regulatory genes controlling ompF
expression, conferred resistance to 4-quinolones and unrelated antibiotics that include tetracycline,

The clinical frequency and expression of mutational resistance to the 4-quinolones is still
relatively rare, although there is evidence of increased frequency in P. aeruginosa and the
multi-resistant staphylococci (Amyes & Gemmell, 1992). However, in some respects resistant
mutants have been found to be slower growing, temperature sensitive, and auxotrophic (Crumplin &

The mutational resistance to the 4-quinolones for Gram-positive bacteria occurs at a higher
frequency than Gram-negative bacteria both in vivo (Humphreys & Mulivill, 1985) and in vitro
(Reeves et al, 1984; Limb et al, 1987). Gram-positive bacteria are less susceptible to
4-quinolones than Gram-negative bacteria, and no impermeability mutants have been identified in Gram-positive bacteria. As Gram-positive bacteria lack an outer membrane, this would therefore contribute to reduced susceptibility and lack of impermeability mutants (Smith, 1990).
1.1.6. The subinhibitory action of antibacterials

In the clinical environment two aspects of subinhibitory concentrations play an important role in the treatment of infection. Firstly, non-compliance generates subinhibitory concentrations within the human body, and secondly, the post antibiotic effect may be of clinical importance between doses of an antibiotic when the drug concentration may fall below the MIC for an infecting organism. Cessation of antibiotic therapy will also generate a post antibiotic effect and is a characteristic of almost all antimicrobial agents. In this instance the post antibiotic effect is the phenomenon of persisting suppression of bacterial growth as a result of prior antimicrobial exposure. It is well established 4-quinolone therapy suppresses enterobacterial counts to undetectable levels within two to three days (Brumfitt et al., 1984; Reeves, 1986), and after therapy the re-emergence of the Enterobacteriaceae may take a few days (Brumfitt, 1986). The relevance of subinhibitory concentrations of antibiotics is clearly dependent on the in vivo reproduction of in vitro effects. To study the effects of how antibiotics influence cell functions, such as plasmid replication, plasmid elimination, plasmid mutation, and the transfer of transposons from chromosomal DNA to plasmid DNA, and pigmentation reversion in S. marcescens, the concentration of the antibiotic or the time during which the bacterial cell is exposed to it, must be less than the amount required to inhibit bacterial growth. Otherwise, levels approaching MIC and beyond would have bactericidal effects. The 4-quinolones are known to act on DNA replication, the stable maintenance of plasmids, transcription, and transposition (Isberg & Svyanyen, 1985; Reece & Maxwell, 1991), and therefore antibiotics that act at the DNA level are more important at subinhibitory concentrations because they have the potential to alter genetic processes.
1.2. Genetic change in bacteria

Genetic change in bacteria can result from a number of processes including, plasmids, mutation, recombination, transposable genetic elements

1.2.1. Bacterial plasmids

Plasmids are extrachromosomal, intracellular self-replicating covalently closed circular double-stranded DNA replicons. They are ubiquitous within the bacterial kingdom (Sherratt et al., 1979) and by definition are characterised as being dispensable to their host cells except in specialised environments where plasmid-encoded genes including those for antibiotic resistance or degradative metabolic pathways confer a selective advantage. Plasmids are major vehicles for the transfer of genes between bacteria and this transfer plays a vital role in the evolution of bacterial populations.

Plasmids range in size from several to greater than 400 kilobase pairs. The replication time for plasmids is short compared to the bacterial generation time, and plasmid copy number is controlled by the frequency of initiation of replication, which is generally under both chromosomal and plasmid control (Cohen et al., 1985; Kolter & Helinski, 1979; Timmis et al., 1986). Plasmids are categorised as either low copy plasmids (plasmids under stringent control) including R1, RP4 and F, with approximately 1-6 plasmid copies per chromosome, or multicopy plasmids (plasmids under relaxed control) including R6K and ColE1, with approximately 10-60 plasmid copies per chromosome. Although, natural plasmids may have either a high or low copy number, it now appears that for low copy plasmids, their copy number is under cellular control (Sherratt, 1982).

An important property of many plasmids is their ability to be transferred between donor and recipient bacterial cells through conjugal transfer. Self-transmissible plasmids encode genes that promote their transfer (tra) among Gram-negative bacteria (Sherratt, 1986). Conjugation is a complex process specified only by large plasmids. Small non-conjugative plasmids cannot promote their own conjugal transfer, but they can be effectively mobilised by utilising the conjugation apparatus of a co-resident conjugative plasmid. Mobilisation is dependent on the small plasmid possessing both an origin of replication (oriT) and the means by which it can adopt the conjugation
apparatus (Sherratt, 1982; Timmis et al, 1986). Conjugal transfer in Gram-negative bacteria appears to be the major means for dissemination of plasmids, whereas in Gram-positive bacteria, both conjugation and transduction have both been described (Clewell, 1981; Sherratt, 1982). Moreover, alternative plasmid transfer mechanisms have been reported including transformation, and protoplast fusion (Timmis et al, 1986), and conjugative transposons (Thompson, 1986).

**Bacterial phenotypes encoded by plasmid genes**

Plasmids provide a wealth of phenotypic characters for their bacterial hosts (Table 1.1).

**Bacterial pathogenesis**

It is known that plasmid determinants enable their bacterial hosts to both colonise and thrive in changing environments (Timmis et al, 1986). Bacterial pathogenesis is enhanced when plasmid-encoded cell surface adhesins permit enterotoxigenic strains of *E. coli* to adhere to and colonise specific target epithelial cells of the small bowel (Van der Waaij, 1992). Plasmids are also known to encode both bacterial enteroinvasion and multiplication in epithelial cells which lead to epithelial death and mucosal ulceration (Sansonetti, 1992).

**Plasmids and bacterial evolution**

Plasmids are important components in the growth and survival of many bacterial populations (Bennet & Linton, 1986). Plasmid transmissibility, whether by conjugation or transduction, alter bacterial evolution by increasing the gene pool, and such genetic information benefits bacteria which may provide advantages over their competitors.

The evolutionary potential of plasmids are determined by their transmissibility (Anderson, 1968), their recombination properties (Richmond & Wiedemann, 1974), and their ability to incorporate bacterial transposons (Hedges & Jacob, 1974). It is also known R-plasmids may arise from transposition, from either another plasmid or from the chromosome, to a native cryptic plasmid (Roberts et al, 1977). Insertion of transposable elements, involving site-specific-illegitimate
Table 1.1. Examples of bacterial functions encoded by plasmids

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<tr>
<th>Bacterial function</th>
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<tr>
<td><strong>Resistance to antibiotics</strong></td>
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<td>Ap, Cm, Km, and Sm</td>
<td>Sharp <em>et al</em> (1973), McHugh <em>et al</em> (1975)</td>
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<td><strong>Heavy metal resistance</strong></td>
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<td>Silver</td>
<td>McHugh <em>et al</em> (1975)</td>
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<tr>
<td>Antimony III &amp; Arsenate/Arsenite</td>
<td>Silvers <em>et al</em> (1981)</td>
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<tr>
<td>Tellurium</td>
<td>Summers &amp; Silvers (1978)</td>
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<td><strong>Colicin biosyntheses</strong></td>
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<td>Bacquero <em>et al</em> (1978), Hopwood (1978),</td>
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<td>Sidikaro &amp; Nomura (1975)</td>
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<td><strong>Sugar fermentation</strong></td>
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<td><strong>Phage restriction</strong></td>
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<td>Taylor &amp; Grant (1970)</td>
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<td><strong>Virulence factors</strong></td>
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</table>
recombination, into either a plasmid recipient or the chromosome generate DNA rearrangements, and thereby facilitate both plasmid and bacterial evolution. The evolution of plasmids is thought to involve two different types of process (Cohen et al, 1978). Macro-evolution is an evolutionary process that results in the insertion, deletion, or rearrangement of large segments of plasmid DNA. An example of this is the co-integrate R-plasmids. Micro-evolution is an evolutionary process that leads to the insertion, deletion, or substitution of very short segments of DNA. Both these changes can be detected through changes in restriction endonuclease fragmentation patterns of plasmid DNA. These two processes suggest that plasmids evolve both mechanisms. Consequently, natural plasmids have been subjected to both these processes over a long period of time during which they have experienced structural changes to reach their stable state (Cohen et al, 1978).

**Plasmid stability**

One of the most important survival characteristics of naturally occurring plasmids is their ability to ensure that both daughter progeny at cell division contain at least one copy of the plasmid (Roberts et al, 1990). Naturally occurring plasmids are usually stably inherited under nonselective conditions, and the existence of a replication control mechanism ensures a constant number of plasmid copies per chromosome are available. This provides a pool of plasmids for segregation to each daughter cell (Thomas, 1988). Efficient control of plasmid replication is an important prerequisite for plasmid stability. Plasmids that are present in only a few copies per cell must inherit functions for active partitioning (par) or stability (stb). par and stb loci have been identified in several plasmids including R1 (Gerdes & Molin, 1986), F (Mori et al, 1986), and NR1 (Tabuchi et al, 1988). Both stability functions maintain the correct distribution of plasmid copies to daughter progeny (Gerlitz et al, 1990) and the multimer resolution system (mrs) maximises the number of segregating plasmid copies at cell division (Williams & Thomas, 1992).
1.2.2. Mutation

Mutations are produced by several types of changes in the base sequence of DNA. Gene mutations can arise spontaneously or they can be induced. Spontaneous mutations occur in all cells, and induced mutations are produced when an organism is treated with a mutagen (Cairns et al., 1988; Cairns et al., 1990; Davis, 1989; Drake & Baltz, 1976).

Spontaneous mutations arise through DNA replication errors: transition, transversion, frameshift mutations, deletions, lesions, depurination and deamination. Induced mutagenesis can increase the frequency of mutations through base changes, incorporation of base analogues, specific base mispairing, and replication bypass after induction of recA SOS system (Ames, 1979; Hanawalt et al., 1979; Hunter et al., 1986; Lindahl, 1982; Lindahl et al., 1977; Sancar & Rupp, 1983; Sinha, 1987; Singer & Kusmierczuk, 1982). Spontaneous mutations in E. coli and S. marcescens are thought to be the result of illegitimate recombination (Sheratt, 1981; Platt et al., 1983).

Spontaneous mutation rates of E. coli is of the order $10^{-10}$ per base per replication, and spontaneous mutations that lead to gene inactivation occur at about $10^{-5}$ to $10^{-6}$ per gene per cell generation (Drake, 1969; Moses & Summers, 1988). Mutations in various genes involved in SOS repair can reduce the rates of both spontaneous and DNA damage induced mutagenesis (Miller & Kokjohn, 1990; Simonson et al., 1990; Smith & Eisenstadt, 1989; Walker, 1984). Replication errors and spontaneous lesions are known to generate most of the base substitution and frame-shift mutations. Resistance to the newer 4-quinolones as result of spontaneous single-step mutation have been shown to occur less frequently than nalidixic acid, at $10^{-7}$ to $10^{-11}$ (Chin & Neu, 1980 & 1984; Cullmann et al., 1985; Sanders et al., 1984).

Recombination

Homologous and site-specific recombination produce new gene combinations and several E. coli enzymes including those encoded by the recABC genes have been implicated (Bryant et al., 1985; Cunningham et al., 1979; Kowalczykowski, 1987; Nies et al., 1986). It has also been described that homologous, illegitimate recombination (see section 1.2.3) play an important role in plasmid
1.2.3. Transposition

Prokaryotic transposable genetic elements include both transposons (Tn) and insertion sequences (IS). They are discrete mobile DNA elements capable of translocation, at low frequency, into multiple target sites within recipient DNA (Kleckner, 1981; Berg, 1989), without extensive DNA homology (Berg & Berg, 1983; Shapiro, 1983), and in the absence of the recA gene product (Kopecko & Cohen, 1975).

Transposable elements introduce foreign DNA into bacteria and the genetic rearrangements produced have biological importance both in terms of evolution and development. IS elements and transposons have contributed to the evolution of bacteria and their plasmids (Hartl & Sawyer, 1988).

The central property of transposable DNA elements is their ability to insert into recipient DNA sequences. Integration is thought to occur through a recA-independent non-homologous recombination process by an element-encoded transposase (Kopecko & Cohen, 1975). This process is distinct from recA-mediated homologous recombination (Hedges & Jacob, 1974). Although, transposons are known to insert at multiple sites within recipient DNA including bacteriophage DNA, chromosomal and extrachromosomal DNA (Calos & Miller, 1980), their insertion is not entirely random (Cohen & Shapiro, 1980). It is also known that certain regions of recipient DNA are 'hot spots' prone to transposon insertion (Tu & Cohen, 1980). These authors further found Tn3 could be inserted preferentially into recA-independent sites demonstrating sequence homology within inverted repeat (IR) termini. This suggests recognition of homologous DNA sequences may in some way pre-determine both frequency and site-specificity of transposon-associated recombination.

Most prokaryotic transposons promote transposition and DNA rearrangements at frequencies of $10^{-4}$ to $10^{-7}$ per generation (Kleckner, 1981) and are normal constituents of many bacterial genomes, plasmids, and bacteriophages. However, the frequency of transposition of Tn7 to the chromosomal attTn7 sites occurs at $10^{-2}$ to 1.00, and 100-fold lower to other sites (Craig, 1989; Kubo & Craig, 1990). Transposition frequency in a specific bacterium is determined by the
number of target sites and the efficiency of transposition. Transposons can also effect change in both the organisation and the expression of prokaryotic genomes at frequencies comparable to spontaneous mutation rates (Kleckner, 1989). These changes can result through several different pathways including, a direct effect of transposon-mediated insertion or promoted rearrangement, general recombination between two copies of an element present at different locations, and DNA sequences may act as facilitators where illegitimate recombination and mutation processes can produce new regulatory signals and/or new structural gene information.

Transposable elements have three structural features in common (Shapiro, 1983):

(a) inverted repeats (IRs) of 10-40 bps at their ends which flank,
(b) a central core that contains genes required for transposition, and
(c) bounded by short 4-11 bp directed repeats (DRs) of target DNA found on both sides the inserted element.

Transposons encode additional genes in the central core that specify a wide variety of traits that include resistance to antibiotics (Hedge & Jacob, 1974; Kopecko & Cohen, 1975), heavy metal compounds, toxins (virulence factors), and the capacity to ferment sugars or metabolise hydrocarbons (Cohen & Shapiro, 1980).

Insertion sequences (IS elements)

Most of the IS elements (Figure 1.8a) range in size between 700 and 1800 bp, and their termini carry inverted repeats (IRs) of size 10-40 bp (Iida et al., 1983). Inverted repeats (IRs) serve as recognition sequences for transposases that catalyse the fusions of their termini with target DNA (Schmitt, 1986). IS elements contain no detectable genes unrelated to insertion functions, and are compactly organised, and contain one or more structural genes, regulatory information, and transposition determinants (Kleckner, 1981). Examples of IS elements include IS1, IS10, and IS59, which differ in size, the transposition proteins they encode, the sequences at their termini, and the structures they form by transposition (Iida et al., 1983).
Class I (compound) transposons

Class I transposons (Figure 1.8b) were previously described as IS-like elements or composite elements (Berg et al., 1981; Foster et al., 1981; Isberg & Syvanen, 1981) and carry resistance determinants flanked, on either side, with an IS element (Schmitt, 1986). The constituent IS elements can be either direct repeats (DRs) or inverted repeats (IRs) and these elements possess cognate transposases for transposition function (Berg et al., 1981; Grindley & Joyce, 1981; Schmitt, 1986). Class I elements generate both simple conservative transpositions and cointegrate structures, with simple transpositions fivefold more frequent (Berg et al., 1981; Iida et al., 1983; Berg et al., 1988). Examples of Class I transposons include Tn5 and Tn10.

Class II (complex) transposons

Class II transposons (Figure 1.8c) are typified by the Tn3 family which are so similar in structure and function that a common evolutionary origin has been proposed (Casadaban et al., 1981; Heffron et al., 1979). Most Class II transposons are flanked by 30-40bp inverted repeats (IRs) rather than IS elements. The genes for transposition are encoded within the central sequence of the element. Class II transposons are usually 4-5kb in length or more, and most encode antibiotic resistance determinants. The most studied in this group is the β-lactamase (bla)-producing-Tn3. There are two subgroups in this family, determined by interchangeable transposition functions. Tn3, Tn1, Tn2, and Tn1000 form one group, and the other group contains more than ten subgroup members, including Tn21, Tn501, and Tn1721 (Calos & Miller, 1980; Grinsted et al, 1990). Class II elements give rise to cointegrate intermediates during their replicative transposition (Ohtsubo et al., 1981).

Other classes of transposable elements

Class III (transposing bacteriophage) transposons

Members include the two related phages Mu and D108 (Faelen et al., 1978; Hull et al., 1978; Kamp & Kahmann, 1981; Reisbois et al., 1981; Toussaint & Reisbois, 1983). Both use replicative transposition as their mechanism of vegetative replication of the phage genome during
lytic growth (Hull et al., 1978; Kamp & Kahmann, 1981). Two Mu genes, A and B have been shown to directly affect Mu-mediated transposition, and both are required for physical association between a Mu-containing plasmid and the target bacterial chromosome following Mu induction (Chaconas et al., 1981; Coelho et al., 1981). Phage Mu transposes repeatedly during lytic growth, and analyses of intracellular Mu DNAs revealed cointegrates with directed repeats (DRs) of Mu sequences (Chaconas et al., 1981). Transposition of Mu is thought to involve both replicative cointegrate transposition and non-replicative conservative (simple) insertion (Craigie et al., 1988; Mizuuchi & Craigie, 1986).

Class IV (unclassified elements) transposons

There are a few transposable elements that do not fit into any of the above classes. In Gram-negative bacteria, only transposon Tn7 has significantly different properties from any of the other elements. Tn7 transposes at high frequency to a specific chromosomal site, and with a lower frequency to many different sites in plasmids (Barth & Dutta, 1977; Lichtenstein & Brenner, 1981 & 1982). It has also been shown that a relatively large region of Tn7 is occupied for the transposition genes (Craig, 1989). Members include Tn4 and Tn6 (Berg et al., 1975; Datta et al., 1981; Kopecko, 1980; Kopecko et al., 1976).
Figure 1.8. General structure for (a) IS elements, (b) Class I transposons, and (c) Class II transposons.
(a) IS elements, examples include IS1 (768bp), IS10 (1329bp, and IS50 (1543bp).

(b) Class I transposons, examples include Tn5 (5700bp) and Tn/0 (9300bp).

(c) Class II transposons, examples include Tn3 (4957bp).
Transposons or pairs of insertion sequences can provide a selective advantage through the transposition of DNA which may encode important genes that can provide resistance to antibiotics and heavy metals (Berg et al., 1989). These genetic elements can also act as biological switches (Cohen & Shapiro, 1980) by insertion mutation which may cause genes to become dysfunctional (Cohen & Shapiro, 1980; Kleckner, 1981 & 1989), activation of cryptic genes (Parker & Hall, 1990), chromosomal rearrangements by recombination between elements (Sayre & Miller, 1991), and deletions by imprecise or non-specific excision (Erlich, 1989). Transposons, after transposition to plasmids and subsequent conjugative transfer, also achieve greater mobility (Berg, 1977; Cohen & Shapiro, 1980).

Factors influencing the mobility of transposable elements

Several factors have been established as important for the regulation of range and frequency of transposon mobility within bacterial DNA and includes target site-specificity between different transposons. Tn7 recognizes a specific target DNA sequence or 'hot spot' located within the enterobacterial chromosome (Barth et al., 1976) at the attTn7 site, about 84 minutes for E. coli, between the pho8 and glnS genes (Gay et al., 1986; Lichtenstein & Brenner, 1981 & 1982; McKown et al., 1988) as the preferred target site for transposition. The target site preference for the singular attachment site within the chromosome of E. coli, P. aeruginosa, and K. pneumoniae (Lichtenstein & Brenner, 1982; Smith & Jones, 1984) is so specific that when this site is available, virtually all transposition events (transposition rate $10^{-2}$ - $10^{-4}$ to attTn7) are directed there (Lichtenstein & Brenner, 1981). However, if this site is not available, transposition occurs at a 100-fold lower frequency to other secondary sites (Barth & Grinker, 1977; Barth et al., 1978; Craig, 1989; Kubo & Craig, 1990). The transposition of other transposons, for example, Tn5 (Berg, 1989; Lupski et al., 1984) and Tn10 (Halling & Kleckner, 1982; Kleckner et al., 1977 & 1982), also occur at preferred target sites (transposition rate for Tn5 is $10^{-6}$, and for Tn10 is $10^{-7}$ respectively). However, insertion is more random and is not as specific as the Tn7 insertion into the chromosomal 'hot spot' (Halling & Kleckner, 1982).
Current genetic and biochemical evidence suggests three transposition mechanisms notably conservative or simple transposition, replicative transposition, and a combination of both (Berg et al., 1988; Craigie & Mizuuchi, 1985; Pato, 1989).

In conservative transposition, Class I transposons (Tn5 & Tn10) are excised and move from the donor site to the recipient target site. The transposon is not duplicated, and the ends of the donor DNA excision site (the fate of which is not precisely known) are exposed and not rejoined and may act as a signal for exonucleolytic degradation (Berg et al., 1988). Loss of the donor DNA is prevented when cells contain duplicates of the donor sequence. Alternatively, the exposed ends may be repaired in some imprecise manner by non-specific processes unrelated to transposition (Szostak et al., 1983). Further evidence suggests that reconstruction of the donor replicon, known as precise excision, is not dependent on transposon-encoded functions, but is promoted by host functions (Craigie & Kleckner, 1987). The result of conservative transposition is, although one copy is consumed, the other survives, and the resulting cell lineage contains one element at the original site and a second at a new site (Berg et al., 1988).

In replicative transposition, Class II transposons (Tn7) are duplicated. One copy remains within the original donor replicon, whereas a second copy is transferred to the new recipient target site via a co-integrate structure that results from fusion between donor and target sites (Shapiro, 1979). The co-integrate structure becomes resolved by reciprocal site-specific recombination between the directly repeated transposon copies to produce (a) a target replicon with the donor sequence that is genetically identical to the original donor molecule, and (b) a recipient replicon which contains a single copy of the transposable element (Shapiro, 1979; Berg et al., 1988).

The number of transposon copies for many transposons within a bacterial cell is regulated. For example, it is known that two different control mechanisms regulate Tn10 copy number (Kleckner, 1989). The amount of transposase protein is regulated through repressors, and methylation of IS10R by dam methylase (Sayre & Miller, 1991). This involves negative feedback mechanisms, which reduce Tn10 transposition (Simons & Kleckner, 1983). An additional mechanism
controlling transposition of some transposons including, Tn3 and Tn7, is transposition immunity, which is thought to be regulated by the number of transposon copies already present in the host (Sayre & Miller, 1991).

The molecular size of transposable elements can influence transposition rates. Transposition rates for chromosomal insertion, eg. Tn10 and Tn9-like elements, decrease exponentially with increasing transposon length (Morisato et al., 1983; Way & Kleckner, 1985).
1.2.4. DNA rearrangements

A wide variety of DNA rearrangements arise through gene mutation, transposition, and recombination, all of which can lead to changes in chromosome structure. Gene mutation as previously described is caused by several types of change in the base sequence of DNA, including base substitution and deletion. However, it has been recognised that many spontaneous mutations are due to DNA rearrangements that exclude single base changes (Cullum & Saedler, 1981). For example, insertion sequences (Reif & Saedler, 1977) and transposons generate a high frequency of deletions, inversions, and duplications in adjacent DNA sequences (Berg et al, 1989). Transposons can also give rise to deletions in which, part of the element is deleted together with varying lengths of surrounding DNA (Grindley & Reed, 1985). Therefore, such imprecise excision events are now recognised as deletions or inversions that emanate from the internal ends of the IR segments of the transposon (Grindley & Reed, 1985).

Two novel types of DNA elements termed integrons and shufflons have been identified as promoting DNA rearrangements (Hall & Stokes, 1993; Komano et al, 1986 & 1987; Kubo et al, 1988). The IncI plasmid R64 shufflon consists of 4 DNA segments and is a clustered inversion region and the 4 DNA segments invert either independently or in groups (Komano et al, 1990). Movement of this shufflon has been shown to mediate DNA rearrangements (Komano et al, 1990). Integrons represent a family of DNA elements that have evolved by site-specific integration of discrete genes, and have been shown to confer antibiotic resistance (Bissonnette et al, 1991).

Recombination has also been shown to promote DNA rearrangements. DNA rearrangements have also been shown to occur when co-integrate plasmids form through fusion and recombination of dual replicons (Bennett et al, 1986) which may be IS-element- or Tn-element-mediated, or mediated via a recA-independent site-specific recombination (Clerget, 1984; O'Connor & Malamy, 1984).

Many rearrangements in both plasmid and chromosomal DNA can be detected by REFP analysis, and provides a means for the assessment of DNA plasticity in both laboratory and epidemiological investigation (Browning et al, 1995; Rankin et al, 1995; Platt et al, 1988).
1.3. The biology of bacteria in stationary growth phase

Under conditions of nutritional deprivation bacterial cells spend most of their existence in a state where rapid growth is not possible. This state closely resembles the stationary phase of growth in a culture (Roszak & Colwell, 1987), and it has been recognised that changes in gene expression occur on depletion of essential nutrients (Groat et al, 1986). Bacterial cells in stationary phase use several mechanisms to maintain their 'living state' to survive prolonged periods of starvation (Matin et al, 1989; Tormo et al, 1990). It is also known that metabolically active, yet non-dividing cells are maintained by many bacteria (Roszak & Colwell, 1987).

Several studies suggest the development and unusual appearance of mutations during stationary phase in E. coli (Cairns et al, 1988; Hall, 1988; Ryan et al, 1963; Shapiro, 1984). When E. coli enters stationary phase, changes in gene expression are regulated at the level of transcription (Bohannon et al, 1991). Such cells undergo both morphological and physiological changes as well as the ability to induce transcription of specific genes upon the onset of stationary phase (Bohannon et al, 1991; Siegele & Kolter, 1992). A major regulator of the starvation response has been identified in E. coli and is KatF, the product of the katF gene (Lange & Hengge-Aronis, 1991; McCann et al, 1991). It is required for the synthesis of many of the proteins induced by carbon starvation (Siegele & Kolter, 1992). Growth phase-dependent synthesis of acid phosphatase (Dassa et al, 1982; Touati et al, 1986), catalase (Loewen et al, 1985), exonuclease III (Sak et al, 1989) and microcins B17 and C7 (Diaz-Guerra et al, 1989; Hernandez-Chico et al, 1986) increased during nutritional deprivation (Groat et al, 1986). In E. coli 30-50 starvation-induced proteins have been identified (Siegele & Kolter, 1992). It is thought the increase in their synthesis may involve a specific activation of transcription at the onset of stationary phase. At present, their individual roles in the maintenance of the viability remain uncertain. Thus, the activity of certain genes, not essential for growth in log phase but required for survival in the absence of growth, suggests the presence of essential processes unique to stationary phase (Tormo et al, 1990).

Changes in the negative superhelical density of chromosomal DNA has been reported in starved
E. coli cells (Balke & Gralla, 1987), and RNA stability has been shown to decrease. Moreover, 20-40% of total RNA is lost during the first several hours of starvation (Mandelstam, 1960). Both these changes are associated with an increase in spontaneous mutation rate (Cairns et al., 1988; Hall, 1990 & 1991; Tormo et al., 1990). It is also noteworthy that while bacterial cells are in stationary phase they may be sporadically replicating or repairing their DNA, because in a nutritionally deprived state the normal SOS repair enzymes are slow to function, and errors may persist in newly synthesised DNA (Foster, 1992; Ninio, 1991). In fact it has been shown that upon transcription and translation, the cell replicates its DNA and such mutations can become permanent (Rebeck & Samson, 1991). Currently, we know very little about how mutational processes influence the physiology of bacterial cells in different nutritional states (Foster, 1992).

This study is therefore intended to focus primarily on the subinhibitory effects of ofloxacin on changes in genotypic and phenotypic characters involved in the processes of plasmid stability, mutation and transposition, of several members of the Enterobacteriaceae under different growth conditions (and in particular stationary phase cells).
CHAPTER 2

GENERAL MATERIALS AND METHODS
2.1 General bacterial strains and plasmids

The bacterial strains and plasmids used are shown in Table 2.1, which indicates the source and roles of individual strains.

2.2 Maintenance and storage of organisms

Initially all strains (Table 2.1) were revived from -70°C and subcultured onto CLED (Oxoid) agar to isolate pure single colonies and confirm lac phenotype. Antibiotic sensitivity testing (see below) was performed to establish the presence of appropriate resistance determinants. With the exception of S. marcescens GRI 2677-8, all other strains (Table 2.1) were subcultured on a weekly basis and stored at 4°C. S. marcescens GRI 2677-8, upon revival, is pigmented and reverts to the non-pigmented phenotype when subcultured on a daily basis. However, with storage at 4°C GRI 2677-8 reverts to the pigmented phenotype.

2.3 Antibacterial agents and disc diffusion sensitivity testing

Antibacterial agents

Ofloxacin (100% potent powder) was kindly provided by Hoechst Roussel. A stock solution of 1280μg/ml was prepared by dissolving 12.8mg ofloxacin in 0.5mls of 0.1M NaOH, diluted further with sterile distilled water and added to solid or liquid media as appropriate.

The following antibacterial agents were obtained from Sigma and incorporated into CLED agar (Mast) to select for E. coli K12 transconjugants at the stated concentrations: kanamycin (30μg/ml), rifampicin (50μg/ml), tetracycline (30μg/ml), and trimethoprim (150μg/ml).

Sensitivity testing

Antibiotic disc diffusion sensitivity testing was performed using both standard methods (National Committee for Clinical Laboratory Standards, 1990a & 1990b) and the following Oxoid antibiotic discs: amikacin (10μg), ampicillin (10μg), carbenicillin (100μg), cefamandole (30μg), cephazolin (30μg), chloramphenicol (10μg), colistin sulphate (25μg), gentamicin (10μg), kanamycin (30μg), nalidixic acid (30μg), ofloxacin (1μg), streptomycin (10μg), sulphmethoxazole (25μg), tetracycline (10μg), tobramycin (10μg), and trimethoprim (1.25μg).
Table 2.1. General bacterial strains and plasmids

<table>
<thead>
<tr>
<th>(a) Strains &amp; plasmids</th>
<th>Plasmid Profile (mol wt. in kb)</th>
<th>Source/Reference</th>
<th>Phenotype</th>
<th>Experimental Role</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> K12 J62::Tn7</td>
<td>-</td>
<td>D.C.</td>
<td>lac'Tp'pSm'R</td>
<td>R⁺ recipient</td>
</tr>
<tr>
<td><em>E. coli</em> K12 J52.RP4</td>
<td>54</td>
<td>D.C.</td>
<td>lac¹Ap¹Km¹Te¹</td>
<td>R⁺ donor</td>
</tr>
<tr>
<td><em>E. coli</em> K12 J53-2</td>
<td>-</td>
<td>Meynell &amp; Datta¹</td>
<td>lac¹rif¹</td>
<td>R⁺ recipient</td>
</tr>
<tr>
<td><em>E. coli</em> K12 J62-1</td>
<td>-</td>
<td>Meynell &amp; Datta¹</td>
<td>lac¹mal¹</td>
<td>R⁺ recipient</td>
</tr>
<tr>
<td><em>E. coli</em> K12 J62-2</td>
<td>-</td>
<td>Meynell &amp; Datta¹</td>
<td>lac¹mal²</td>
<td>R⁺ recipient</td>
</tr>
<tr>
<td><em>S. marcescens</em> GRI 2677</td>
<td>-</td>
<td>Platt et al (1983)</td>
<td>Ap¹Cz¹Te¹Ch¹</td>
<td>pigmented parent</td>
</tr>
<tr>
<td><em>S. marcescens</em> GRI 2677-8</td>
<td>-</td>
<td>Platt et al (1983)</td>
<td>Ap¹Cz¹Te¹Ch¹</td>
<td>non-pigmented variant</td>
</tr>
<tr>
<td><em>S. typhimurium</em> NCTC73</td>
<td>87</td>
<td>Platt et al (1988)</td>
<td>-</td>
<td>REFP reference²³</td>
</tr>
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(b) Plasmids

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Size (kb)</th>
<th>Properties</th>
<th>Phenotype</th>
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<tbody>
<tr>
<td>RP4</td>
<td>54</td>
<td>IncP R-plasmid</td>
<td>Ap¹Km¹Te¹</td>
</tr>
<tr>
<td>pOG660</td>
<td>87</td>
<td>D.C.</td>
<td>-</td>
</tr>
<tr>
<td>pOG669</td>
<td>139</td>
<td>Co-integrate R-plasmid⁴</td>
<td>Ap¹Km¹</td>
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<tr>
<td>pOG670</td>
<td>52</td>
<td>IncX R-plasmid⁵</td>
<td>Ap¹Km¹</td>
</tr>
<tr>
<td>pSLT</td>
<td>87</td>
<td>SSP⁶</td>
<td>-</td>
</tr>
</tbody>
</table>

¹(1966), ²indigenous plasmid provided reference REFPs, ³pSLT indistinguishable from pOG660 on the basis of PstI, SalI, and AvaII fragmentation patterns, ⁴139kb co-integrate R-plasmid from bovine strain (1985) comprised of ⁵52kb IncX R-plasmid pOG670 and ⁶serotype-specific plasmid (SSP) of *Salmonella typhimurium* LT2, Department Collection, D.C.
2.4. General media

Oxoid Isosensitest Broth and Agar

Isosensitest broth was used to culture organisms overnight and Isosensitest agar was used for disc diffusion sensitivity testing.

Nutrient agar (Oxoid)

Nutrient agar was used for growth of organisms for plasmid DNA extraction in the determination of plasmid profiles using crude lysates.

Brain Heart Infusion Broth (BHI, Oxoid)

Brain Heart Infusion broth was used for growth of organisms for DNA extraction and purification for REP analysis, and for conjugation experiments.

CLED agar (Mast)

CLED agar (Mast) was used for growth, selection of pure colonies, and maintenance of organisms.

CLED agar (Oxoid)

CLED agar (Oxoid) was used for all investigations that involved S. marcescens GRI 2677-8.

2.5. R-plasmid transfer by conjugation

Modification of the broth method of Kraft et al (1983) was used to establish R-plasmid transfer and characterise potential transconjugants. Donor and recipient strains were grown in BHI at 37°C overnight, mixed in the ratio of 1:5 and an equal volume of fresh BHI broth added. After incubation at 37°C for various periods from 30 minutes to 24 hours potential transconjugants were harvested from various combination antibiotic selection CLED agar plates previously inoculated with 10 μl mating mix volumes. Uridine (10 μg/ml) was added to media that contained trimethoprim (Amyes & Smith, 1978) to inhibit trimethoprim antagonists (as found in CLED agar (Oxoid)). The selective medium was designed to inhibit the growth of both donor and recipient organisms but permit the growth of transconjugants. Either nalidixic acid or rifampicin were used as appropriate to inhibit the donor and the antibiotic to which resistance was being transferred, was
incorporated to inhibit the recipient. Transconjugants were purified and tested for homogeneity of
resistance pattern by disc diffusion sensitivity tests. Plasmid transfer was confirmed by
demonstration of plasmid DNA in the transconjugants by agarose gel electrophoresis.

2.6. Molecular Methods

Detection of plasmid DNA

Agarose gel electrophoresis was used for the detection of plasmids (2-200kb in size), in crude
lysates of E. coli.

Plasmid DNA was extracted and separated by agarose gel electrophoresis and the molecular
weight determined using a modification of the method of Platt & Sommerville (1981). From an
overnight culture on Nutrient agar (Oxoid), of 12 - 20 colonies were suspended in 300µl of tris-
borate buffer (89mM tris, 89mM boric acid, 1.25mM disodium EDTA, pH 8.2) in 1.5ml Eppendorf
tubes. 200µl of sodium dodecyl sulphate (SDS) (10% w/v in tris-borate buffer) was added and the
tubes were mixed gently by inversion. After heating at 50°C for 5 minutes the crude lysates (clear
and viscous in appearance) were centrifuged for 15 minutes (9980g) in an Eppendorf micro-
centrifuge to remove cellular debris. A volume of 100µl from the supernatant was loaded on to a
0.7% vertical agarose (Sigma, type II in tris-borate buffer) gel (15x10x0.5cm) together with 5µl
bromophenol blue tracking dye (sucrose 25%, 8mM sodium acetate, 3.5mM SDS, and 0.7mM
bromophenol blue). The gels were then sealed with molten agarose. Electrophoresis was carried
out for 1 hour at constant voltage (100V) followed by 4 hours at a constant voltage of 200V. The
gels were then stained in TES buffer (50mM Tris, 50mM sodium chloride, 5mM sodium EDTA, pH
8.0) that contain 0.165µM (equivalent 6.5ug/ml) ethidium bromide for 15 minutes. Plasmid DNA
was viewed with a ultraviolet transilluminator (365nm) and photographed on Polaroid type 665 film
using a Polaroid MP4 land camera through Kodak filters 25, 12, and 2E. The molecular weights of
plasmids was determined by comparison with control plasmids of known molecular weight:
strain 39R861 (154kb; 67kb; 37kb; 7kb), pOG669 (139kb), RP4 (54kb), pOG670 (52kb).
Restriction Endonuclease Fragmentation Pattern (REFP) analysis of plasmid DNA

Digestion of plasmid DNA with appropriate restriction endonuclease generated fragmentation patterns that enabled the characterisation and general degree of relatedness of plasmid DNA to be determined. In this study, a simple REFP strategy (Platt et al, 1986) was employed to investigate similarities and differences between plasmids and their change(s) through variation.

Restriction endonuclease digestion of purified plasmid DNA was performed using the method of Platt et al (1986). The cells from a 10ml overnight BHI broth culture were harvested through centrifugation at 3000 rpm for 10 minutes and resuspended in 400µl TGE buffer (25mM Tris, 1mM EDTA, 50mM glucose, pH 8.0). The suspension was centrifuged in an Eppendorf micro-centrifuge for 20 seconds, the supernate discarded, and 200µl TGE buffer containing 5mg/ml (freshly prepared) lysozyme (Sigma; E.C. 3.2.1.17) was added. After vortex mixing to evenly suspend the cells, the tubes were incubated on ice for 5 minutes. Cells were lysed by the addition of 400µl alkaline/SDS solution (freshly prepared; 1ml 2 M NaOH, 1ml 10% SDS, and 8mls sterile water) was added to the tubes, mixed gently by inversion until the suspension was clear and viscous. The tubes were incubated on ice for 5 minutes and inverted on two further occasions. 300µl 3M sodium acetate was added and the tubes were mixed gently by inversion until a white precipitate formed, then mixed vigorously, and incubated on ice for 5 minutes. After further mixing the tubes were micro-centrifuged for 2 minutes, the supernate transferred to a fresh tube and 0.5ml phenol/Chloroform mixture (250g phenol, 250mls chloroform, and 50mls TGE buffer) was added, mixed, and centrifuged for 2 minutes. The upper aqueous layer was transferred to a fresh tube and 0.5mls isopropanol was added. The tubes were mixed and incubated at room temperature for 1 hour. The tubes were then micro-centrifuged for 5 minutes, the pellet resuspended in 100µl TE buffer (10mM Tris, 1mM EDTA, pH 8.0), and the incubated at room temperature for 10 minutes. 100µl 7.5M ammonium acetate was added followed by 600µl ice cold 100% ethanol, and incubated at -20°C overnight. After a 2 minute micro-centrifugation, the pellet was resuspended in 160µl TE buffer and 18µl heat inactivated ribonuclease A (Sigma, E.C. 3.1.27.5) was added, mixed, and the tubes incubated at 37°C for 30 minutes. 20µl 2.5 M sodium chloride was added, mixed, and the phenol/chloroform extraction, isopropanol and ice cold ethanol precipitation, and -20°C overnight incubation steps were repeated. The purified DNA pellet was resuspended in 45µl TE buffer and
15μl samples were taken for restriction endonuclease digestion. The following restriction endonucleases were used: AvaiI, EcoRI, HindIII, PstI, and SmaI (Gibco BRL). The restriction digestion mixture contained 15μl plasmid DNA (control contained 2μl phage lambda DNA), 2μl endonuclease (equivalent 20units), 5μl x10 buffer (specific to endonuclease used), and sterile distilled water to a final volume of 50μl. After incubation of 1 to 4 hours at 37°C (30°C for SmaI) 5μl bromophenol blue tracking dye was added to each tube and the total contents loaded on to a 0.8% horizontal agarose gel (15x15x0.3cm) composed of tris-borate buffer containing 0.3μg/ml ethidium bromide. Following overnight electrophoresis with constant 18mA current (in tris-borate buffer containing 0.3μg/ml ethidium bromide), the gel was photographed as described in the previous section. Lane 1 of the gel contained a restriction digest of lambda phage DNA with PstI which provided calibration for determination of molecular weight of plasmid DNA restriction fragments.

The following strategy was employed to characterise plasmids through quantitative assessment of their fragmentation pattern(s). A minimum of 12 restriction fragments from a combination of 2 restriction endonucleases enables the characterisation of plasmid DNA (Platt et al., 1986). PstI was the primary endonuclease used, and SmaI was the secondary endonuclease used if PstI produced 5-20 restriction fragments. If less than 6 restriction fragments were produced using PstI, then the secondary endonuclease was AvaiI. Alternatively, if PstI produced more than 20 restriction fragments, then the secondary endonuclease was HindIII (EcoRI was used on a few occasions). The use of a second endonuclease refined the description of molecular relatedness.

2.7. Statistical analyses

Throughout this study non-parametric method significance tests were employed as these tests critically test the null hypothesis where data that may not be normally distributed. Calculation of standard deviation, standard error, chi-squared (χ²) test for two independent samples and proportions (Siegel, 1956; Wardlaw 1992). Yates' continuity correction was incorporated in contingency tables with df>1 to improve the fit between observed and expected values. Goodness of fit Poisson distributions were determined where;
\[ P(x) = [(u^x e^{-u})]/[x!]^{-1} \]

where, \( P \) = the probability of occurrence of numbers of colonies with IPPs as a function of number of number of IPPs per colony, \( u \) = average number of IPPs per colony, \( x! = 0, 1, 2, 3, \ldots \) etc, \( e \) = base of natural logarithm, and

\[
\chi^2 = \sum_{i=1}^{r} \sum_{j=1}^{k} \frac{(Observed - Predicted)^2}{Predicted}, \text{ with df} = (r-1)(k-1). \]
CHAPTER 3

THE EFFECTS OF SUBINHIBITORY OFLOXACIN ON THE TRANSPOSITION OF Tn7 FROM A CHROMOSOMAL SITE IN E. COLI K12 TO THE INCP PLASMID RP4.
3.1. INTRODUCTION

3.1.1. The transposon Tn7

Tn7 belongs to the Class IV family of unclassified transposable elements (Kleckner, 1981). It is a 14kb element which encodes resistance to trimethoprim and the aminoglycosides streptomycin and spectinomycin (Barth et al., 1976). Tn7 differs from many transposable elements in that it can insert at high frequency into the E. coli chromosomal attTn7 site, and insertion has been shown to be both orientation and site specific (Barth et al., 1976; Lichtenstein & Brenner, 1981 & 1982). When the attTn7 site is not available, Tn7 transposes at a lower frequency to many different sites within plasmids (Barth & Grinter, 1977; Barth et al., 1978; Craig, 1989). Tn7 transposition in vitro requires ATP and Tn7-encoded proteins (Bainton et al., 1991 & 1993; Waddell & Craig, 1988), and it is thought Tn7 transposes via a cut and paste mechanism with Tn7 excised from donor DNA through staggered double-stranded breaks, and is then inserted into attTn7 by the joining of the 3'-Tn7 transposon ends to the 5'-target DNA ends (Bainton et al., 1991; Hagemann & Craig, 1993). Tn7 confers transposition immunity analogous to the Tn3 family, possibly through a mechanism that senses the state of the carrier replicon (Hauer & Shapiro, 1984). Tn7 has terminal IRs, but does not appear to promote co-integrate formation, and there is no evidence to indicate that it encodes a resolvase (Craigie et al., 1985; Grindley & Reed, 1985; Shapiro, 1983)). Tn7 encodes five transposition genes (Figure 3.1), msABCDE (Rogers et al., 1986; Waddell & Craig, 1988). msABC provide functions common to all Tn7 transposition events, and genes msD and msE are thought to determine the target site (Craig, 1991; Flores et al., 1992; Orle & Craig, 1990; Rogers et al., 1986; Tang et al., 1991; Waddell & Craig, 1988). It has been suggested that the gene products of msD (TnsD) and msE (TnsE) are target DNA-binding proteins (Rogers et al., 1986; Waddell & Craig, 1988). TnsD is a specific DNA-binding protein that recognises both attTn7 and pseudo-attTn7 sites and is also associated with high frequency transposition, and TnE is a non-specific DNA-binding protein that promotes low frequency transposition (Craig, 1989; Kubo & Craig, 1990). It has also been speculated that TnsC plays a central role in the selection of target.
Figure 3.1. Physical and genetic map of the 14kb transposon Tn7.

The map is calibrated in kilobases. *tnyABCDE* are the transposition genes, *dhfr*, encodes trimethoprim-resistant dihydröolate reductase (*Tp^r*), *aadA*, encodes adenyln-transferase which inactivates streptomycin (*Sm^r*) and spectinomycin. The numbers give the kb coordinates of the recognised restriction sites. The coordinates for the *KpnI* and *PstI* internal sites are 10.2kb and 13.5kb. RE & LE denote right and left ends respectively. *Adapted from Gosti-Testu et al, 1983; Hauer & Shapiro, 1984; Kubo & Craig, 1990; Lichtenstein & Brenner, 1981 & 1982*.
DNA during Tn7 transposition (Gamas & Craig, 1992).

3.1.2. The effect of 4-quinolones on transposition

Although the primary activity of DNA gyrase inhibitors leads to inhibition of DNA synthesis, secondary effects include inhibition of the transposition of several transposons.

The homeostatic control of negative supercoiled DNA by the DNA topoisomerases are known to play a central role in bacterial metabolism through their regulation of the tertiary structure of DNA (Menzel & Gellert, 1983). This is essential transcription, replication, recombination (Reece & Maxwell, 1991; Wang, 1985), and transposition. Several lines of evidence have shown that the topoisomerases are involved in transposition. topA mutations exert pleiotropic effects on the frequency of Tn5 transposition (Stemglanz et al., 1981). Although, these authors have further suggested that DNA topoisomerase I may be directly involved in Tn5 transposition, they have also demonstrated the frequency of Tn5 transposition is not topA dependent. Similarly, Isberg & Syvanen (1982) have shown in vivo transposition of Tn5 requires topoisomerase activity. They found the DNA gyrase inhibitor, coumermycin, inhibited Tn5 transposition. Both these observations support the hypothesis that the degree of negative DNA superhelicity is important in the mechanism of transposition, since both these topoisomerases have diametrically opposite effects on the supercoiling of covalently-closed-circular- double-stranded DNA. The capacity of the quinolones to influence and modify genetic processes including transposition, implies a potential to influence resistance to unrelated antimicrobial agents (Mamber et al., 1992). Where antimicrobial therapy is optimised for a specific infected site, subinhibitory concentrations of the agent may occur elsewhere in the body (Lorian & De Freitas, 1979; Lorian, 1993). Non-compliance similarly generates subinhibitory concentrations. Subinhibitory concentrations are known to both generate and potentiate the post-antibiotic effect (PAE), which occurs following limited exposure to an antimicrobial agent (MacKenzie & Gould, 1993), and both have been indicated to have clinical significance (Tanaka et al., 1989). It is also known the PAE produced following exposure to
4-quinolones, is dependent on the time required for 4-quinolone-disassociation from receptor binding sites, and the subsequent diffusion out from the bacterial cell (Fuursted, 1987).

Exposure of the commensal flora to subinhibitory concentrations of 4-quinolones may potentiate alteration in the location and mobility of unrelated resistance determinants (MacKenzie & Gould, 1993). The aim of this study was therefore to determine whether exposure to subinhibitory ofloxacin influenced the transposition of Tn7, from a chromosomal site in E. coli K12 to the 60kb conjugative IncF plasmid RP4. Previous work (Barth et al, 1976; Barth & Grinter, 1977) has demonstrated the transposition of Tn7 to multiple sites in RP4 and this system was therefore chosen as a model system in which to investigate the effect of subinhibitory ofloxacin on the transposition of Tn7.
3.2. MATERIALS AND METHODS

3.2.1. Bacterial strains and plasmids

The bacterial strains used are listed in Table 3.1 and indicates the individual strains used in conjugative crosses.

3.2.2. Antibacterial Agents & MIC determination

The MIC of ofloxacin for E. coli J53-2, RP4 was determined using serial dilutions of ofloxacin in isosensitest agar (Oxoid) (Michel-Briand, et al., 1986; Weisser & Wiedemann, 1986). A single subinhibitory concentration of ofloxacin (0.25MIC) was studied.

The following antimicrobial agents were incorporated into CLED (Oxoid) agar. 50μg/ml rifampicin with ascorbic acid 5μg/ml was used to counterselect in conjugative transfer of RP4 to E. coli K12 J62::Tn7. 150μg/ml trimethoprim (Tp) was used to select transconjugants containing Tn7. 10μg/ml uridine (Sigma) was added to CLED (Oxoid) agar containing trimethoprim to inhibit antagonists. 30μg/ml kanamycin (Km) or 30μg/ml tetracycline (Tc) were used separately to select for RP4 transfer respectively.

3.2.3. Rationale of the study

RP4 was transferred to E. coli K12 J62::Tn7 that encoded a chromosomal copy of the transposon Tn7. A single transconjugant colony was grown in Brain Heart Infusion (BHI) broth, divided, and one half exposed to subinhibitory ofloxacin (0.25MIC) (Test population), and the unexposed population (Control) treated in parallel. At intervals up to 120 hours, samples were taken and used as a plasmid donor population in crosses with either Rif & Tp to select for plasmids that had acquired a copy of Tn7 (RP4::Tn7) by transposition from the E. coli K12 J62::Tn7 or Rif & Tc to determine the incidence of RP4 transfer. The incidence of transposition was calculated as a function of RP4::Tn7 mobility relative to RP4 mobility. Tn7 insertion was confirmed by an increase in the molecular weight of RP4 through plasmid profiles. The resultant RP4::Tn7 transconjugants were analysed as described below.
Table 3.1.  Bacterial strains and plasmids for transposition

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Phenotype</th>
<th>Experimental Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli K12 J62::Tn7</td>
<td>lac' pSm' Tc'</td>
<td>Recipient for RP4 and chromosomal Tn7 source</td>
</tr>
<tr>
<td>E. coli K12 J53,RP4</td>
<td>lac' Ap' Km' Tc'</td>
<td>RP4 Donor to E. coli K12 J62::Tn7</td>
</tr>
<tr>
<td>E. coli K12 J53-2</td>
<td>lac' Rif'</td>
<td>Recipient for RP4::Tn7</td>
</tr>
<tr>
<td>RP4</td>
<td>Ap' Km' Tc'</td>
<td>Target plasmid for Tn7</td>
</tr>
</tbody>
</table>

p, partial resistance. Ap, ampicillin. Km, kanamycin. Rif, rifampicin. Sm, streptomycin. Tc, tetracycline. and Tp, trimethoprim. E. coli K12 J62::Tn7 was kindly provided by Hillary Richards.
The above design relied on the conjugative ability of RP4::Tn7. Therefore, transposition events that deleted transfer functions (trc') were selected against, and thereby passed undetected. Moreover, insertion of Tn7 into the trf A & B genes (Figurski et al, 1982) would render the resultant host non-viable, and were similarly excluded.

3.2.4. Conjugation

RP4 was transferred from E. coli K12 J53,RP4 to E. coli K12 J62::Tn7 using Tp & Km selection. 1ml samples at times 0, 4, 24, 48, 96, and 120 hours were taken from the donor culture E. coli K12 J62::Tn7,RP4 in the presence and absence of 0.25MIC ofloxacin. Ofloxacin from the test group was removed by washing the cells three times using sterile BHI broth. RP4::Tn7 was then transferred to the Rif resistant strain E. coli K12 J53-2. Using Rif & Tp selection, the lac' strain E. coli K12 J53-2,RP4::Tn7 was isolated. After mixing, both the donor and recipient populations were centrifuged together for 5 minutes at 1500rpm to enhance contact between donor and recipient cells. A mating time of 90 minutes at 37°C was used to minimise retransfer, and Rif & Tp CLED (Oxoid) agar selection plates were inoculated with 0.1ml volumes of BHI broth culture samples from the mating mixtures prepared at times 0, 4, 24, 48, 96, and 120 hours. Comparison of Ap', Km', Tc', or Tp' transconjugants estimated the incidence of Tn7 transposition to RP4 as RP4::Tn7 derivatives per RP4 transconjugant during the 120 hour exposure to subinhibitory ofloxacin.

3.2.5. Restriction Endonuclease Fragmentation Patterns (REFP) of RP4 DNA

The modified REFP method of Kraft et al (1986) was used, and RP4 DNA was extracted and digested with restriction endonucleases. REFP analysis enabled the location of Tn7 insertion in RP4 to be determined using KpnI & SmaI and KpnI & PstI double digests of purified plasmid RP4 DNA.

3.2.6. Interpretation of REFPs

Tn7 has been shown to possess several internal restriction sites (Figure 3.2), and include KpnI (10.2kb) and PstI (13.5kb), and the physical, genetic, and restriction map of RP4 is well established (Guiney & Lanka, 1989). Therefore, Tn7 insertion sites were determined by
comparison of KpnI & Smal (Tn7 does not possess an internal Smal restriction site) and KpnI & PstI REFPs with wild-type RP4 DNA. Insertion sites were deduced on the basis that any wild-type RP4 fragment deleted indicated Tn7 insertion, and similarly for each insertion the KpnI/Smal double digest showed two new fragments, and each KpnI/PstI double digest showed 3 (inclusive of the 3.3kb internal Tn7 fragment).
3.3. RESULTS

The MIC of ofloxacin for *E. coli* K12 J53-2, RP4::Tn7 was found to be 0.128ug/ml. A total of 312 *E. coli* K12 J53-2, RP4::Tn7 transconjugants that acquired resistance to Tp (142 test group and 170 control group) were examined.

3.3.1. Changes in transposition of Tn7 with time

In the early phase of the experiment (4-48 hours) the number of RP4::Tn7 derivatives increased in both test and control groups (Table 3.2). However, the increase in colony count was lower in the subinhibitory ofloxacin group, with a lower number of RP4::Tn7 derivatives isolated.

In both test and control groups, the number of RP4::Tn7 derivatives decreased during the 48-96 hour phase (Table 3.2) when compared to the 4-48 hour phase. The decrease in the test group was greater when compared to the control group.

3.3.2. RP4 transfer

The number of RP4 transconjugants increased in both test and control groups during the 4-48 hour phase (Table 3.2), although the initial numbers of RP4 transconjugants were lower in the test group. In addition, although the test group demonstrated an increase, this increase when compared to the control group was lower and indicates that subinhibitory ofloxacin reduced RP4 transfer.

The number of RP4 transconjugants decreased in both test and control groups during the 48-96 hour phase (Table 3.2). The number of RP4 transconjugants in both groups at 72 hours were similar, and at 96 hours the number of RP4 transconjugants in the test group was lower compared to the control group.

With one exception, all transconjugants acquired a single copy of Tn7. Tn7 insertion into RP4 in this single transconjugant was absent, and indicates insertion into the chromosome, since Tp resistance was still present. No insertions were observed in either the ampicillin or kanamycin resistance genes. Two control transconjugants were isolated with insertions in the tetracycline resistance gene and this represents less than 1% of those examined.
Table 3.2. The incidence of transposition of Tn7 from a chromosomal site to RP4 after exposure to subinhibitory ofloxacin (0.25MIC) for 4 and 96 hours.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>No. of RP4::Tn7 derivatives</th>
<th>No. of RP4 transconjugants</th>
<th>Incidence of transposition</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 4</td>
<td>83</td>
<td>65,000</td>
<td>1.28 x 10^-3</td>
</tr>
<tr>
<td>T 4</td>
<td>159</td>
<td>25,000</td>
<td>6.36 x 10^-3</td>
</tr>
<tr>
<td>C 24</td>
<td>557</td>
<td>250,000</td>
<td>2.23 x 10^-3</td>
</tr>
<tr>
<td>T 24</td>
<td>222</td>
<td>125,000</td>
<td>1.78 x 10^-3</td>
</tr>
<tr>
<td>C 48</td>
<td>1027</td>
<td>480,000</td>
<td>2.14 x 10^-3</td>
</tr>
<tr>
<td>T 48</td>
<td>473</td>
<td>340,000</td>
<td>1.39 x 10^-3</td>
</tr>
<tr>
<td>C 72</td>
<td>340</td>
<td>116,000</td>
<td>2.93 x 10^-3</td>
</tr>
<tr>
<td>T 72</td>
<td>40</td>
<td>100,000</td>
<td>4.00 x 10^-4</td>
</tr>
<tr>
<td>C 96</td>
<td>48</td>
<td>100,000</td>
<td>4.80 x 10^-4</td>
</tr>
<tr>
<td>T 96</td>
<td>14</td>
<td>50,000</td>
<td>2.80 x 10^-4</td>
</tr>
</tbody>
</table>

C denotes absence of subinhibitory ofloxacin, and T denotes exposure to subinhibitory ofloxacin (0.25MIC).

This is a representative sample of two separate experiments.
3.3.3. The incidence of transposition of Tn7 from a chromosomal site, and determination of location of Tn7 insertion sites in RP4 by REFP analysis

The initial incidence of transposition in the test group was $6.36 \times 10^{-3}$, and $1.27 \times 10^{-3}$ in the control group. Over the 4 - 48 hour period, the incidence of transposition in the test group decreased from $6.36 \times 10^{-3}$ to $1.39 \times 10^{-3}$, and increased in the control group from $1.27 \times 10^{-3}$ to $2.14 \times 10^{-3}$. At 48 hours, the incidence of transposition in the control group was greater than the test group. The incidence of transposition, in the control group at 72 hours was observed to increase further, and in the test group there was a one log unit decrease.

Cumulatively, over the period 4 - 72 hours, the incidence of transposition, increased in the control group and decreased in the test group. At 72 - 96 hours, the incidence of transposition in the test group remained at approximately $10^{-4}$, and in the control group a one log unit decrease was observed. At 96 hours the incidence of transposition was 1.7 times greater in the control group.

Digestion of wild-type RP4 DNA with KpnI & SmaI generated 7 restriction fragments of 22.2kb, 8.8kb, 8.5kb, 7.2kb, 5.8kb, 5kb, and 2kb, and KpnI & PstI generated 5 restriction fragments 27.2kb, 14.2kb, 6.8kb, 6.2kb, 2.8kb (Figure 3.2). Although, there are two 1kb KpnI-SmaI fragments and four 1kb KpnI-PstI fragments, they were not always observed since the REFP gels were run a little longer to maximise resolution of larger fragments.

**KpnI-SmaI RP4 fragments**

RP4::Tn7 DNA from 312 E. coli K12 J53-2,RP4::Tn7 transconjugants was digested with the restriction endonuclease combination KpnI & SmaI. Although insertion sites, for both groups, were widely distributed among these fragments (Table 3.3a & 3.3b), most insertions occurred within the 22.2kb KpnI-SmaI fragment, with fewer than expected insertions within the 8.5kb, 7.2kb, 5.8kb, and 5kb KpnI-SmaI fragments (Table 3.3). There were no observed insertions in the 8.8kb, 2kb, or in any of the 1kb KpnI-SmaI fragments. The incidence of Tn7 insertion within all KpnI-SmaI fragments varied considerably with time, and differed between test and control (Table 3.3).
Figure 3.2. Physical map of RP4 with kb coordinates for \textit{KpnI}, \textit{PstI}, and \textit{SmaI} restriction sites.

The REFP map also demonstrates the sizes of \textit{KpnI-KpnI}, \textit{KpnI-PstI}, \textit{KpnI-SmaI}, \textit{PstI-PstI}, and \textit{SmaI-SmaI} RP4 fragments respectively. Adapted from Guiney \& Lanka (1989).
Table 3.3. The effect of subinhibitory ofloxacin (0.25MIC) on the insertion of Tn7 in Kpn1-SmaI RP4 fragments.

(a) *E. coli* K12 J62::Tn7,RP4 culture not exposed to subinhibitory ofloxacin (control group) prior to transfer to *E. coli* K12 J53-2, and 170 *E. coli* K12 J53-2,RP4::Tn7 transconjugants analysed for changes in Tn7 insertion, and

(b) *E. coli* K12 J62::Tn7,RP4 culture exposed to subinhibitory ofloxacin (test group) prior to transfer to *E. coli* K12 J53-2, and 142 *E. coli* K12 J53-2,RP4::Tn7 transconjugants analysed for changes in Tn7 insertion.
(a) **NO EXPOSURE TO SUBINHIBITORY OFLOXACIN (0.25MIC)**

Variation in the number of Tn7 insertions in each *KpnI-SmaI* RP4 fragment over 120 hours

<table>
<thead>
<tr>
<th><em>KpnI-SmaI</em> Fragment (kb)</th>
<th>4</th>
<th>24</th>
<th>48</th>
<th>96</th>
<th>120</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.2</td>
<td>15</td>
<td>16</td>
<td>13</td>
<td>14</td>
<td>16</td>
<td>74</td>
</tr>
<tr>
<td>8.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8.5</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>10</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>7.2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>5.8</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>9</td>
<td>25</td>
</tr>
<tr>
<td>5.0</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>11</td>
<td>5</td>
<td>43</td>
</tr>
<tr>
<td>2.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

| n                         | 28  | 39  | 27  | 40  | 36  | 170 |

*n*, denotes the total number of transconjugants tested
(b) EXPOSURE TO SUBINHIBITORY OFLOXACIN (0.25 MIC)

Variation in the number of Tn7 insertions in each *KpnI-Smal* RP4 fragment over 120 hours

<table>
<thead>
<tr>
<th>TIME (HOURS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><em>KpnI-Smal</em> fragment (kb)</th>
<th>22.2</th>
<th>8.8</th>
<th>8.5</th>
<th>7.2</th>
<th>5.8</th>
<th>5.0</th>
<th>2.0</th>
<th>1.0</th>
<th>111</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>11</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>24</td>
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<td>1</td>
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<td>106</td>
<td>106</td>
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</tr>
</tbody>
</table>

n, denotes the total number of transconjugants tested
Distribution of Tn7 insertions in RP4

Tables 3.3a & 3.3b show changes in the distribution of Tn7 insertion in each RP4 KpnI/SmaI fragment with time. With the exception of the observed change in Tn7 insertion between 4-24 hours in the 22.2kb fragment in Table 3.3b, the overall insertion of Tn7 remained relatively constant between 4-120 hours for each fragment. This indicates that no new transposition events occurred in stationary phase and subinhibitory ofloxacin was observed not to affect the overall transposition of Tn7. However, subinhibitory ofloxacin was observed to increase new Tn7 transposition events in the 22.2kb fragment between 4-24 hours (Table 3.3b). It was also observed that subinhibitory ofloxacin altered the number and sites of Tn7 insertion in RP4 (as described below).

22.2kb KpnI-SmaI RP4 fragment

Table 3.4 shows that subinhibitory ofloxacin significantly increased (p<0.001) the insertion of Tn7 ($\chi^2=39.9$ with df=1 for two independent proportions) in the 22.2kb KpnI-SmaI RP4 fragment (exposed test group of 111/142 transconjugant colonies, and unexposed control group of 74/170 transconjugant colonies).

8.8kb KpnI/SmaI RP4 fragment

No Tn7 insertions were observed in this fragment (Tables 3.3a & 3.3b).

8.5kb KpnI-SmaI RP4 fragment

Tables 3.4 shows that subinhibitory ofloxacin significantly reduced (p<0.05) the insertion of Tn7 ($\chi^2=4.7$ with df=1 for two independent proportions) in the 8.5kb KpnI-SmaI RP4 fragment (exposed test group of 9/142 transconjugant colonies, and unexposed control group of 25/170 transconjugant colonies).

7.2kb KpnI-SmaI RP4 fragment

A low incidence of Tn7 insertions was observed in both groups (test group of 1/142 transconjugant colonies, and control group of 3/170 transconjugant colonies) Tables 3.3a & 3.3b).
5.8kb KpnI-Smal RP4 fragment

Table 3.4 shows that subinhibitory ofloxacin significantly reduced (p<0.05) the insertion of Tn7 ($\chi^2=3.8$ with df=1 for two independent proportions) in the 5.8kb KpnI-Smal fragment (exposed test group of 10/142 transconjugant colonies, and unexposed control group of 25/170 transconjugant colonies).

5kb KpnI-Smal RP4 fragment

Tables 3.4 shows that subinhibitory ofloxacin significantly reduced (p<0.01) the insertion of Tn7 ($\chi^2=20.5$ with df=1 for two independent proportions) in the 5kb KpnI-Smal fragment (exposed test group of 8/142 transconjugant colonies, and unexposed control group of 43/170 transconjugant colonies).

1kb & 2kb KpnI-Smal RP4 fragments

No Tn7 insertions in either fragment was observed (Tables 3.3a & 3.3b).

Therefore, subinhibitory ofloxacin was found to significantly alter the insertion of Tn7 in the 22.2kb, 8.5kb, 5.8kb, and 5kb KpnI-Smal RP4 fragments.

Tn7 transposition events in the 22.2kb KpnI-Smal RP4 fragment

In stationary phase, subinhibitory ofloxacin (0.25MIC) significantly increased (p<0.05) the insertion of Tn7 ($\chi^2=3.94$ for two independent samples) during the first 24 hours in the 22.2kb KpnI-Smal RP4 fragment (Table 3.3b). It was also observed that no new Tn7 transposition event were observed in this fragment in the unexposed control group (Table 3.3a).

KpnI-PstI RP4 fragments

RP4::Tn7 DNA from 60 E. coli K12 J53-2,RP4::Tn7 transconjugants at 96 hours was digested with the restriction endonuclease combination KpnI & PstI. Most insertions occurred within the 14.2kb KpnI-PstI RP4 fragment (19/30 in the unexposed control group and 14/30 in the exposed test group), and the 27.2kb KpnI-PstI RP4 fragment (7/30 in the unexposed control group and 12/30 in the exposed test group) (Table 3.5). There was a lower incidence of Tn7 insertion in the other KpnI-PstI RP4 fragments. Overall, subinhibitory ofloxacin (0.25MIC) significantly
Table 3.4. Tn7 insertion in the 22.2kb, 8.5kb, 5.8kb, and 5.0kb KpnI-SmaI RP4 fragments.

Comparison of Tn7 insertion in the presence (test group) and absence (control group) of subinhibitory ofloxacin (0.25MIC) using a chi-squared test for independent proportions.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>No. of Tn7 insertions in fragment</th>
<th>No. of insertions not in fragment</th>
<th>TOTAL</th>
<th>STATISTICS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\chi^2$</td>
</tr>
<tr>
<td>22.2kb KpnI-SmaI RP4 fragment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>74</td>
<td>96</td>
<td>170</td>
<td>-</td>
</tr>
<tr>
<td>Test</td>
<td>111</td>
<td>31</td>
<td>142</td>
<td>39.9</td>
</tr>
<tr>
<td>8.5kb KpnI-Sma RP4 fragment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>145</td>
<td>170</td>
<td>-</td>
</tr>
<tr>
<td>Test</td>
<td>9</td>
<td>133</td>
<td>142</td>
<td>4.7</td>
</tr>
<tr>
<td>5.8kb KpnI-SmaI RP4 fragment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Test</td>
<td>10</td>
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<td>142</td>
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</tr>
<tr>
<td>5.0kb KpnI-SmaI RP4 fragment</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>43</td>
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</tr>
<tr>
<td>Test</td>
<td>8</td>
<td>134</td>
<td>142</td>
<td>20.5</td>
</tr>
</tbody>
</table>

$^a$denotes (Total number of insertions in all fragments - Number of insertions in fragment)
Table 3.5. The effect of subinhibitory ofloxacin (0.25MIC) on the fragments from a KpnI-PstI digest at 96 hours.

(a) *E. coli* K12 J62::Tn7,RP4 culture not exposed to subinhibitory ofloxacin (control group) prior to transfer to *E. coli* K12 J53-2, and 30 *E. coli* K12 J53-2,RP4::Tn7 transconjugants analysed for changes in Tn7 insertion, and

(b) *E. coli* K12 J62::Tn7,RP4 exposed to subinhibitory ofloxacin (test group) prior to transfer to *E. coli* K12 J53-2, and 30 *E. coli* K12 J53-2,RP4::Tn7 transconjugants analysed for changes in Tn7 insertion.

**NO EXPOSURE AND EXPOSURE TO SUBINHIBITORY OFLOXACIN (0.25MIC)**

Variation in the number of Tn7 insertions in each *KpnI-PstI* RP4 fragments at 96 hours

<table>
<thead>
<tr>
<th><em>KpnI-PstI</em> Fragment (kb)</th>
<th>(a) Control Group (No exposure to ofloxacin)</th>
<th>(b) Test Group (Exposure to ofloxacin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27.2</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>14.2</td>
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<td>14</td>
</tr>
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<td>6.8</td>
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<td>6.2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2.8</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

(n) | 30 | 30

n, denotes the total number of transconjugants tested
increased (p<0.05) the number of Tn7 insertions ($\chi^2=2.8$ with df=1 for two independent proportions) in the 27.2kb Kpnl-PstI RP4 fragment (Table 3.5), and insertion of Tn7 in the other Kpnl-PstI RP4 fragments were not found to be significantly different from the unexposed control group.

Cumulatively, most Tn7 insertions (a) occurred overall within the 22.2kb & 5kb Kpnl-Smal RP4 fragments, and subinhibitory ofloxacin (0.25MIC) increased insertion in the 22.2kb fragment and decreased insertion in the 5kb fragment, and (b) most insertions occurred overall at 96 hours within the 27.2kb & 14.2kb Kpnl-Smal RP4 fragments, and subinhibitory ofloxacin (0.25MIC) increased insertion in the 27.2kb fragment. Insertion into the 14.2kb Kpnl-Smal-RP4 fragment was observed to decrease in the presence of subinhibitory ofloxacin (0.25MIC), but this was not found to be statistically significant. Comparison of Tn7 insertion into the Kpnl-Smal & Kpnl-PstI RP4 fragments at 96 hours demonstrated that the 22.2kb Kpnl-Smal fragment is found within the 27.2kb Kpnl-PstI fragment, and the 5kb Kpnl-Smal fragment was also found within the 14.2kb Kpnl-Smal fragment.

3.3.4. REFP analysis of Tn7 insertion

Kpnl-Smal RP4 fragments

The combination of Kpnl & Smal, coupled with the prior knowledge that Tn7 possessed an internal Kpnl site, and that certain regions of RP4 were excluded on the basis that Tn7 insertion into the tra and trf genes would render the plasmid non-conjugative or the host non-viable respectively (Figure 3.3), proved useful in establishing which Kpnl & Smal fragments contained potential Tn7 insertion sites.

The Kpnl & Smal combination demonstrated the location of Tn7 insertion could be identified in any of the Kpnl & Smal fragments. Figure 3.4 shows REFPs of Kpnl-Smal RP4 DNA digests with Tn7 insertion at 24 hours in (a) RP4::Tn7 derivatives 1-10 not exposed to subinhibitory ofloxacin, and (b) RP4::Tn7 derivatives 11-20 exposed to subinhibitory ofloxacin (0.25MIC).
Figure 3.3. The genetic structure of RP4 (calibrated in kb).

Abbreviations: oriV (origin of vegetative replication, trfA and B (transacting replication functions), tra 1, 2 & 3 (regions required for conjugal transfer), oriT (origin of transfer), pri (DNA primase), dfr (dihydrofolate reductase, TpF), bla (β-lactamase, ApF), tetA (tetracycline resistance determinant, TcF), tetR (Repressor gene), aphA (aminoglycoside 3'-phospho-transferase, KmF). The dotted lines signify the size of the tra1&2 genes are as yet unknown.

Adapted from Guiney & Lanka (1989).
Most lanes exhibit Tn7 insertion into the 14.2kb KpnI-PstI and 27.2kb KpnI-PstI fragments.

(a) Figure 3.4a [unexposed E. coli K12 J53-2,RP4::Tn7 derivative group (24 hours)]

Lanes 5, 6, 8, 10, 11, 13, and 14 exhibit insertion of Tn7 into the 22.2kb KpnI-SmaI RP4 fragment. Lanes 5, 6, 8, and 13 are similar with a reduction in the size of the 22.2kb fragment. Lane 11 shows the 22.2kb fragment also to be reduced with the resultant 18kb fragment as a doublet, and lane 14 shows the 22.2kb fragment to increase in size, and a smaller 7kb Tn7-KpnI-SmaI fragment results, and lane 10 also demonstrates an increase in size of the 22.2kb fragment, with a resultant double 5kb fragment. Lane 7 demonstrates Tn7 insertion into the 8.5kb KpnI-SmaI fragment with resultant 17.9kb and 4.6kb Tn7-KpnI-SmaI RP4 fragments, lane 9 demonstrates Tn7 insertion into the 5kb KpnI-SmaI fragment with resultant 13.5kb and 5.5kb Tn7-KpnI-SmaI fragments, lane 12 demonstrates Tn7 insertion into the 5.8kb KpnI-SmaI fragment with resultant 10.8kb and 9.6kb Tn7-KpnI-SmaI fragments.

(b) Figure 3.4b [exposed E. coli K12 J53-2,RP4::Tn7 derivative group (24 hours)]

Lanes 15, 16, 18, and 19, are similar and exhibit Tn7 insertion into the 22.2kb KpnI-SmaI fragment. Lanes 17, 20, 24 also demonstrate Tn7 insertion into the 22.2kb fragment, and represent a decrease in the size of the 22.2kb fragment with resultant 18kb doublet fragment. Lane 28 also demonstrates an increase in the size of the 22.2kb with resultant 5.5kb Tn7-KpnI-SmaI fragment. Lane 21 demonstrates Tn7 insertion into 5kb fragment with resultant 12.5kb and 6.0kb Tn7-KpnI-SmaI fragments, and Lane 22 so demonstrates Tn7 insertion into the 5.0kb fragment with resultant Tn7-KpnI-SmaI fragments of 13.5kb and 5.2kb respectively.

KpnI-PstI RP4 fragments

Tn7 insertion was investigated in the KpnI-PstI RP4 fragments in the 96 hour samples. Figure 3.5 shows REFPs of KpnI-PstI RP4 DNA digests with Tn7 insertion at 96 hours with (a) RP4::Tn7 derivatives 21-30 not exposed to subinhibitory ofloxacin, and (b) RP4::Tn7 derivatives 31-40 exposed to subinhibitory ofloxacin (0.25MIC).

(a) Figure 3.5a [unexposed E. coli K12 J53-2,RP4::Tn7 derivative group (96 hours)]

Most lanes exhibit Tn7 insertion into the 14.2kb KpnI-PstI and 27.2kb KpnI-PstI fragments.
Figure 3.4. REFPs of KpnI-SmaI RP4 fragments with Tn7 insertion at 24 hours.

(a) RP4::Tn7 derivatives 1-10 not exposed to subinhibitory ofloxacin (0.25MIC), and
(b) RP4::Tn7 derivatives 11-20 exposed to subinhibitory ofloxacin (0.25MIC).

Lanes 1 & 11 show bacteriophage λ DNA digested with PstI.

Lanes 2, 3, 4, 16, 17, and 18 show wild-type RP4 DNA digested with KpnI, SmaI, and both KpnI & SmaI respectively.

Lanes 5 to 14 show RP4::Tn7 derivatives 1-10, and Lanes 15 to 24 show RP4::Tn7 derivatives 11-20.
3.4a RP4::Tn7 derivatives (1-10, not exposed to subinhibitory ofloxacin)
3.4b RP4::Tn7 derivatives (11-20, exposed to subinhibitory ofloxacin)
Lanes 4, 7, and 9 all show Tn7 insertion into the 27.2kb Kpnl-Pstl fragment. Lanes 5, 6, 8, 10, 11, 12, 13 all show Tn7 insertion into the 14.2kb Kpnl-Pstl with resultant 13.5kb and 11kb (both expressed as a doublet fragment) Tn7-Kpnl-Pstl fragments, and a 3.6kb Tn7-Kpnl-Pstl fragment. The latter new fragment contains the internal Tn7 3.3kb Kpnl-Pstl fragment. Lane 5 demonstrates loss of the 14.2kb Tn7-Kpnl-Pstl fragment with resultant 21.9kb, 3.6kb and 2.8kb doublet Tn7-Kpnl-Pstl fragments. The latter fragments have been derived from the two small Tn7 fragments generated from the internal Kpnl and Pstl sites (3.3kb and 0.5kb respectively). The remaining 11.1kb fragment (resultant from the 14.2kb Kpnl-Pstl fragment) has been inserted with the 10.2kb Tn7 fragment to form the larger 21.3kb Tn7-Kpnl-Pstl fragment.

(b) Figure 3.5b [Test, exposed E. coli K12 J53-2, RP4::Tn7 derivative group (96 hours)]

Most lanes 17-26 exhibit Tn7 insertion into the 14.2kb Kpnl-Kpnl fragment. Lanes 17, 19, 20, 21, 22, 25, and 26 all show the same restriction patterns with resultant new 14.5kb and 3.6kb Tn7-Kpnl-Pstl fragments. Lane 18 is likely to demonstrate Tn7 insertion into the 6.8kb Kpnl-Pstl fragment (evidence of low DNA content and partial digestion) with resultant 13.1kb fragment (in 14.2kb fragment as a doublet), 4.1kb, and 3.6kb Tn7-Kpnl-Pstl fragments. Lane 23 demonstrates Tn7 insertion into the 6.2kb Kpnl-Pstl fragment with resultant 14kb (possible double fragment) and 4.7kb Tn7-Kpnl-Pstl fragments respectively. Lane 24 demonstrates DNA of insufficient concentration to be digested.

Since no new transposition events were observed between 24-96 hours, Figures 3.4 and 3.5 were compared, and Tn7 insertion sites were further discriminated. In particular, the Tn7 insertion sites in the 5kb Kpnl-Smal fragment were found to overlap with the 14.2kb Kpnl-Kpnl fragment, and similarly the 22.2kb Kpnl-Smal fragment overlaps with the 27.2kb Kpnl-Pstl fragment (Figures 3.2 & 3.3). These overlap insertions were confirmed through comparison with Kpnl-Smal & Kpnl-Pstl REFPs for Tn7 insertions at 96 hours (Figures 3.5 & 3.6). The 14.2kb Kpnl-Kpnl fragment is the site of both the tra3 gene and the trfA operon (Figures 3.2 & 3.3), and insertion into these sites would render RP4 non-conjugative and the E. coli K12 J53-2 host non-viable. These sites also encompass approximately half the 14.2kb Kpnl-Kpnl fragment (Figure 3.3). This large fragment
also possesses oriV (1000bp), the origin of vegetative replication and is approximately 5kb upstream from the trf operon. Similarly, this fragment also contains the Tn^5 gene and very few insertions into this resistance determinant were observed. The 22.7kb KpnI-PstI and 22.2kb KpnI-Smal fragments both contain the tral gene and the trfB operon (Figures 3.2 & 3.3), and similarly insertion into these sites would render RP4 non-conjugative and the E. coli K12 J53-2 host non-viable.

Therefore, the fact that the 22.2kb & 5kb KpnI-Smal fragments overlap the 27.2kb & 14.2kb KpnI-PstI fragments, and most Tn7 insertions occurred in these fragments, indicates these regions to be important sites for Tn7 insertion. Figure 3.6 demonstrates Tn7 insertion sites and potential Tn7 insertion sites within the overlap regions of both the 14.2kb KpnI-KpnI and 5kb KpnI-Smal fragments respectively.
Figure 3.5. REFPs of \textit{KpnI-PstI} RP4 fragments with Tn7 insertion at 96 hours.

(a) RP4::Tn7 derivatives 21-30 not exposed to subinhibitory ofloxacin (0.25MIC), and (b) RP4::Tn7 derivatives 31-40 exposed to subinhibitory ofloxacin (0.25MIC).

*Lanes* 1 & 15 show bacteriophage \(\lambda\) digested with \textit{PstI}.

*Lanes* 3 and 16 show wild-type RP4 DNA digested with \textit{KpnI} & \textit{PstI}.

*Lanes* 4 to 13 show RP4::Tn7 derivatives 21-30, and *Lanes* 17 to 26 show RP4::Tn7 derivatives 31-40.

All RP4::Tn7 tracks show the internal Tn7 \textit{KpnI-PstI} fragment.
3.5a RP4::Tn7 derivatives (21-30, not exposed to subinhibitory ofloxacin)
3.5b RP4::Tn7 derivatives (31-40, exposed to subinhibitory ofloxacin)
Figure 3.6. Predicted overlap region for insertion of Tn7 in the 14.2kb *KpnI-KpnI* fragment (red-line) and the 5.0kb *KpnI-SmaI* fragment (green-line).

Each fragment is bounded by the appropriate restriction sites. Abbreviations: *oriV* (origin of vegetative replication), *Tc* (tetracycline resistance gene), *tra3* (transacting replication gene of unknown bp size), *tra3* (third conjugal transfer gene of unknown size), Tn7\(^a\) insertion into *tra2* gene at 21kb (Guiney & Lanka, 1989), *tra2* (second conjugal transfer gene of approximately 7kb extending beyond the 24kb site). The dotted lines signify potential boundaries for *tra3* and *tra2* and designate genes of unknown size. The curved arrows labelled 1 highlight the proposed 3.5kb region for Tn7 insertion sites. The curved arrow labelled 2 demonstrates other potential Tn7 insertion sites within the 14.2kb *KpnI-PstI* fragment between the *oriV* and *tet* genes.
3.4. DISCUSSION

Tn7 differs from other transposons in that it transposes at a higher frequency to the specific attTn7 site within the E. coli chromosome (Lichtenstein & Brenner, 1981 & 1982), and at a lower frequency to multiple sites in plasmids (Barth & Grinter, 1977; Barth et al., 1978). REFP analysis showed significant Tn7 transposition events into the 22.2kb, 8.5kb, 5.8kb, and 5kb KpnI-Smal RP4 fragments (Table 3.3). Tn7 insertions were also observed in the 27.2kb and 14.2kb KpnI-KpnI fragments, which also contain the 22.2kb and 5kb KpnI-Smal fragments.

The transposition of Tn7 to RP4 occurred between 4 and 24 hours in the control group and continued to increase, albeit marginally, up to and including 72 hours, after which diminution was observed (Table 3.2). This assumes conjugation frequency of RP4::Tn7 transfer is the same as RP4 transfer and suggests that the overall number of transposable events was either maintained against a background of logarithmic growth (4-48 hours) as the number of RP4 transconjugants continued to increase, or the replication of E. coli J62::Tn7,RP4 cells matched those cells that died. In other words a net equilibrium had occurred. Subsequently, this was followed by a decrease in stationary phase (72-96 hours) with a constant decrease in the number of RP4 transconjugants.

The overall transposition of Tn7 decreased in the presence of subinhibitory ofloxacin (0.25MIC) during the 120 hour period, and decreased throughout relative to the transferable plasmid. The number of RP4::Tn7 derivatives and RP4 transconjugants, and therefore Tn7 transposition and RP4 transfer were at a lower level when compared to the unexposed control group. This suggests subinhibitory ofloxacin reduced the conjugal transfer of RP4, as observed in Table 3.2. The former supports previous findings (Gill & Iyer, 1981; Weisser & Wiedemann, 1987), that subinhibitory 4-quinolones reduce R-plasmid transfer in E. coli, and for RP4::Tn7 derivatives to be less transferable suggests Tn7 insertion into the pri, tra, and tft genes reduces isolation. In addition to a reduced RP4 transfer, there was also a corresponding reduction in the incidence of Tn7 transposition in the presence of subinhibitory ofloxacin. The fact that reduction in Tn7 transposition mirrors the reduction in RP4 transfer also supports conjugation is reduced. The incidence of Tn7 transposition remained at approximately $10^{-3}$ to $10^{-4}$ during both the logarithmic and stationary phases of growth (Table 3.2).
Ofloxacin significantly altered the insertion of Tn7 into several regions of RP4, notably the 22.2kb, 8.5kb, 5.8kb, and 5kb Kpnl-Smal-RP4 DNA fragments (Tables 3.3 & 3.4). Tn7 was not found to randomly insert into RP4 and therefore produced a diverse population of molecular variants of RP4::Tn7. Subinhibitory ofloxacin increased Tn7 insertions within the 22.2kb Kpnl-Smal RP4 fragment, and these may reside within one of three potential regions. The first of these is within a 2-3kb region between the trfB operon and the oriT site mapping at approximately 33-37 minutes in the RP4 molecule. The second potential region for insertion is between tral and prt genes, mapping at approximately 44-47 minutes. The third potential region for insertion resides between prt and Km^R (aphA) genes mapping at 39.5-40.5 minutes (Figure 3.3). Ofloxacin was also observed to significantly reduce Tn7 insertion in the 8.5kb, 5.8kb, and 5kb Kpnl-Smal fragments, mapping at 1-8.5, 31-37, and 8.5-13.5 minutes respectively.

The 4-quinolones are known to influence transcription through their inhibition of DNA gyrase activity (Lui & Wang, 1987; Sanzey, 1979; Maxwell, 1992), which interferes with the homeostatic mechanism of negative supercoiling of E. coli DNA (Menzel & Gellert, 1983). It is also known the degree of DNA supercoiling affects the regulation of gene transcription (Sanzey, 1979) and inhibition of DNA gyrase changes the tertiary structure of DNA (Menzel & Gellert 1983). This would also suggest that changes in the tertiary structure of DNA, may result through reduction in DNA supercoiling which may lead to relaxation of plasmid DNA and separation of loops of DNA with different superhelical density (Reece & Maxwell, 1991). This may be consistent with the insertion data presented here, since several Kpnl-Smal and Kpnl-PstI fragments have been shown to contain specific regions for Tn7 insertion which, through changes in their superhelical density may unfold the tertiary structure of such local regions of RP4. Therefore, any change in RP4 DNA tertiary structure may either alter or expose insertion sites for Tn7. Certainly, subinhibitory ofloxacin increased the number of Tn7 insertions in the 22.2kb, and reduced Tn7 insertions in the 8.5kb, 5.8kb, and 5kb Kpnl-Smal RP4 fragments. It would be tempting to speculate that these changes in insertion may result from alteration in RP4 DNA superhelical density which may lead to an increase or decrease in exposed Tn7 insertion sites. This would suggest the topology of target RP4 DNA is important for transposition (Berg et al, 1988)
Most Tn7 insertions were observed in the 22.2kb KpnI-SmaI RP4 fragment, and subinhibitory ofloxacin significantly increased Tn7 insertion into this region. This region may therefore be more susceptible to change in DNA superhelical density, and perhaps this region was in a higher negative supercoiled state prior to treatment when compared to the remainder of the RP4 molecule. Subsequently, subinhibitory ofloxacin may have decreased the negative supercoiled state of this region thereby exposing the insertion sites to Tn7. The fact that Tn7 insertion into the other KpnI-SmaI fragments were lower suggests the degree of negative supercoiled state may be different throughout the RP4 molecule. The lack of Tn7 insertion into the 8.8kb KpnI-SmaI RP4 fragment was expected since both the tra3 and trfA genes reside in this fragment (Figure 3.3). Insertion into this fragment would kill the host through expression of the kil-kor regulon and prevent replication and stable maintenance of RP4. The fact that no Tn7 insertions were observed in this fragment would also suggest the tra3 and trfA genes may be contiguous. Similarly, insertion into the pri gene in the 22.2kb KpnI-SmaI fragment would be fatal since pri is thought to co-regulate the trfB-korB operon which may play an important role in the stable inheritance of IncP plasmids including RP4 (Figurski et al, 1982). Therefore, any Tn7 insertion into either of these genes would prevent RP4::Tn7 containing isolates. The lower incidence of Tn7 insertion into the 7.2kb KpnI-SmaI RP4 fragment could also be explained by the fact a large component of this fragment is taken up by the tra2 gene, and any insertion would also be fatal. No insertions were observed in either the 2kb or 1kb KpnI-SmaI, SmaI-SmaI, KpnI-PstI, and PstI-PstI fragments respectively. However, two transconjugants lost their Tc^ which suggests insertion into the 1kb SmaI-SmaI fragment (Figure 3.2). Subinhibitory ofloxacin has altered the insertion of Tn7 in several RP4 regions and this may be consistent with previously observed reduced frequencies of Tn5 transposition in strains of E. coli that lack DNA gyrase (Ishberg & Syvanen, 1982; Sternglanz et al, 1982). This would suggest that DNA gyrase is involved in the transposition mechanism of Tn7, and subinhibitory ofloxacin position altered both location and mobility of unrelated resistance. Therefore, these findings may provide a potential role for 4-quinolones in the evolution of plasmids through changes in the multiple insertion sites for Tn7, and subsequent effects on expression of antimicrobial resistance.
CHAPTER 4

STABILITY AND MOLECULAR VARIATION WITHIN THE CO-INTEGRATE R-PLASMID pOG669.
4.1. INTRODUCTION

4.1.1. Stable maintenance of plasmids

Naturally occurring plasmids are typically categorised as either low copy plasmids (under *stringent* control) including R1, RP4, F, and pOG669 with approximately 1-6 plasmid copies per chromosome, or multicopy plasmids (under *relaxed* control) including R6K and CoIE1 with approximately 10-40 plasmid copies per chromosome.

Recent molecular studies have shown several co-integrate *R*-plasmids to form through replicon fusion (Bennett *et al.*, 1986; Platt *et al.*, 1988). These co-integrate plasmids carry two origins of replication (Crosa *et al.*, 1975), and both are likely to function independently of each other. Similarly, these plasmids may also express dual incompatibility, and if both plasmids are self-transmissible then the host range would be the sum of the range of each individual plasmid.

Three mechanisms that lead to replicon fusion through illegitimate recombination have been proposed and include: (a) IS element-mediated replicon fusion, (b) transposon-mediated replicon fusions, and (c) recA-independent site-specific recombination (Bennett *et al.*, 1986).

Studies of the maintenance of naturally occurring plasmids within bacteria have provided a much greater understanding of the molecular mechanisms involved in plasmid replication (Kolter & Helenski, 1979; Nordstrom, 1985; Uhlin & Nordstrom, 1975). However, less is understood concerning the partition process of low copy number plasmids. Although, the existence of *par* genes has been identified in several low copy plasmids including R1 and RP4 (Gerdes *et al.*, 1985 & 1986), other low copy plasmids have evolved different partition systems to ensure their stable inheritance including the *ccd, sop,* and *stb* loci of plasmid F (Ogura & Hiraga, 1983a & 1983b).

Several stabilisation mechanisms include: the regulation of plasmid initiation and replication which affects the number of plasmid copies for random distribution (Kolter & Helinski, 1979; Molin *et al.*, 1979; Roberts *et al.*, 1990; Thomas, 1988). Partition ensures the physical separation, and distribution of plasmid copies to daughter cells at division (Austin, 1988; Boe *et al.*, 1987;
Molin et al., 1979; Nordström & Austin, 1989; Williams & Thomas, 1992), and monomerisation of plasmid multimers maximises the number of plasmid copies available for segregation (Cohen et al., 1985; Gerlitz et al., 1990; Nordström, 1985; Roberts et al., 1990; Williams & Thomas, 1992). Although, plasmid replication is known to depend upon host replication functions, there is evidence that regulation of plasmid replication, partitioning, and monomerisation are dependent on plasmid genes as in R1, RK2, RP4 and CoIE1 (Gerdes et al., 1986; Gerdes & Molin, 1986; Gerlitz et al., 1990; Ogura & Hiraga, 1983a & 1983b).

The par genes have been identified as a site for DNA gyrase binding in pSC101, and appear to be required for its stable inheritance (Meacock & Cohen, 1980; Wahle & Kornberg, 1988). The level of DNA supercoiling also appears to be involved with partition (Gustafsson et al., 1983; Miller et al., 1990), and it has been suggested that the degree of negative supercoiling of plasmid DNA leads to the interaction of replicated plasmids with bacterially encoded proteins and/or sites, presumably located on cell membranes. This is thought to assist the subsequent distribution of replicated plasmids to daughter progeny at cell division (Miller et al., 1990) and thereby maintain minimal variation in plasmid copy number (Biek & Cohen, 1989; Manen et al., 1990; Stenzel et al., 1987).

Several different stabilisation systems seem to have evolved for low copy number plasmids to ensure their stable inheritance within a population of cells, exemplified by the sop and ccd loci of plasmid F (Ogura & Hiraga, 1983a & 1983b), the parA and parB loci of plasmid R1 (Gerdes et al., 1986; Gerdes & Molin, 1986), and the par loci of plasmids pSC101 (Meacock & Cohen, 1980; Miller et al., 1983) and P1 (Austin & Abeles, 1983).

Plasmid F has been shown to possess two stabilisation mechanisms (encoded by the sopA, -B, -C region and ccdA, -B genes) that operate to ensure effective segregation of plasmid molecules (Ogura & Hiraga, 1983a & 1983b). The first of these, the sopA, -B, -C region is analogous to the partition function although a parB region near oriT has also been identified (Boe et al., 1987), and secondly, the ccdA, -B genes rescue a single copy plasmid molecule in the cell at the point of cell division (Ogura & Hiraga, 1983a). Cell division is thought to be blocked until plasmid replication
ensures segregation of 2 daughter replicons can take place (Ogura & Hiraga, 1983a & 1983b; Gerdes et al, 1985). The parA locus has been shown to be the site of partition in R1 (Gerdes & Molin, 1986), and the parB locus of R1 has also been shown to further stabilise plasmids (Gerdes et al, 1986). parB is also associated with a plasmid-encoded host cell killing mechanism where post-segregational killing of plasmid-free cells, results in the predominance of plasmid-containing cells (Gerdes et al, 1986; Jaffe et al, 1985; Roberts et al, 1994). The parB locus secures the maintenance of segregationally unstable plasmids through a mechanism that involves killing of cells that have become plasmid-free which have arisen from a plasmid-bearing line (Williams & Thomas, 1992). This selective killing of plasmid-free hosts provides an important survival mechanism for naturally occurring plasmids and ensures that each daughter progeny contains at least 1 copy of the plasmid (Roberts et al, 1990). parB has also been shown to influence cell growth in conditions when the copy number approaches 1 (Gerdes et al, 1986). The parB killing mechanism has been demonstrated in R1 (Gerdes et al, 1986; Gerdes, 1988; Gerdes et al, 1990a & 1990b), and the genes hok (host killing) and the unstable antisense sok (suppressor of killing) are required for the stabilising activity of parB. Under normal circumstances the hok lethal product and sok, the inhibitor of hok, are finely balanced. However, overexpression of the lethal hok product has been shown to lead to the rapid death of hosts when the plasmid is lost (and therefore cannot provide the inhibitor). Similarly, overexpression of the sok product inhibits the hok mediated killing and therefore protects plasmid-containing hosts (Gerdes et al, 1986; Roberts et al, 1990). A homologous system in plasmid F has been identified as fim or stm (Golub & Panzer, 1988; Loh et al, 1988). The ccd system of plasmid F also operates similarly (Bernard & Couturier, 1991; Hiraga et al, 1986; Tam & Kline, 1989), as does the pem killer system of R100 (Tsuchimoto et al, 1988). The parB locus mediates the killing of plasmid-free cells at cell division, and the killing factor, the hok toxic product, is a small polypeptide which is not expressed in the plasmid-containing cell, due to an antisense RNA-mediated translational control loop. Rapid degradation of this control RNA induces translation of the lethal product in the plasmid-free cell (Gerdes et al,
1990a & 1990b).

4.1.2. Molecular variation within plasmids

Selection pressure acts on bacterial populations to favour the emergence of genetic variants and the effect of the genetic change may or may not be evident in the observable phenotype. Plasmids play an important role in bacterial reproduction and evolution (Helling et al., 1981) and any change through genetic variation may be the result of rearrangement through transposition, deletion, and insertion of foreign DNA and contribute to molecular variation and subsequent evolution (Cohen et al., 1978). Furthermore, exposure to different selection pressures may increase the diversity of plasmids through changes in, plasmid mobility (Platt et al., 1987; Platt & Black, 1987), and molecular divergence, which may lead to DNA rearrangements (Cullum & Saedler, 1981), and plasmid elimination (Platt & Black, 1987). DNA rearrangements contribute to the molecular variation in plasmids and molecular variants of the serotype-specific plasmid (SSP) of *Salmonella typhimurium*, pOG660, have been demonstrated by REFP analysis (Platt et al., 1988). One such variant, the R-plasmid pOG669, was first recognised during a veterinary outbreak of salmonellosis, and was also found to be a natural co-integrate of pOG660 and the R-plasmid pOG670, both from two different *S. typhimurium* isolates (Platt et al., 1988).

Previous characterisation of pOG669 as a co-integrate derived from analysis of REFPs after digestion with *PstI* and *SmaI*, and consistent with co-integrate formation, was the recognition of new restriction fragments that represented the co-integrate junctions. For *PstI* these fragments were 4.7kb and 2.1kb, and for *SmaI* these fragments were 7kb and 2.2kb respectively.

Previous observations suggested that the pOG669 in an *E. coli* K12 *nalF* host underwent deletion on storage (D. J. Platt, personal communication). Therefore, the aim of this part of the study was to extend these observations and to investigate the subinhibitory effects of ofloxacin on the (1) stability of the co-integrate R-plasmid pOG669, and (2) on molecular variation, and (3) to investigate the role of deletion on both molecular variation and expression of antibiotic resistance of pOG669.
4.2. MATERIALS AND METHODS

4.2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used are listed in Table 4.1 together with a summary of their characteristics.

1. The *E. coli* K12 J62-1 [lac⁺, pro⁻, his⁻, trp⁻, nalF] was used as the recipient strain for the R-plasmid pOG669.

2. *Salmonella typhimurium* NCTC 73, pOG660 was obtained from the National Collection of Type Cultures, and was used to provide reference restriction endonuclease fragment patterns (Platt et al, 1988).

3. *E. coli* K12 J53-2 [pro⁻, met⁻, rif∫] was used as the recipient strain for the R-plasmid pOG670 that specified Ap & Km resistance.

4.2.2. Revival of stored organisms

Bacterial strains (Table 4.1) were revived from -70°C, subcultured onto CLED agar (Oxoid) and the appropriate phenotypes were confirmed together with antibiotic sensitivity patterns by standard methods.

4.2.3. Rationale of the study

The stability of the natural co-integrate R-plasmid pOG669 was studied in *E. coli* K12 J62-1 under different growth conditions either in the presence or absence of subinhibitory ofloxacin (0.25MIC).

A single *E. coli* K12 J62-1, pOG669 colony was used to inoculate 10mls of isosensitest broth (Oxoid) and incubated at 37°C overnight for 18 hours. From this *E. coli* K12 J62-1, pOG669 culture, 100μls was individually added to each of six universal bottles containing 10mls fresh isosensitest broth with or without 0.25MIC ofloxacin, and labelled cultures A-F.

Culture A (designated SOF*: stationary phase culture with 0.25MIC ofloxacin)

10mls of isosensitest broth containing 0.5μg/ml ofloxacin (equivalent to 0.25MIC) was maintained
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<tr>
<th>Host Organism</th>
<th>Plasmid Profile (mol. wt in kb)</th>
<th>Plasmid Designation</th>
<th>Resistance Determinants</th>
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<td>pOG660(^{c})</td>
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<td>Serotype specific plasmid (SSP)</td>
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</table>

\(^{a}\)isolated from bovine strains in 1985, \(^{b}\)IncX R-plasmid isolated from bovine strains in 1985, \(^{c}\)indigenous plasmid pOG660.
in stationary phase at 37°C for 8 weeks.

Culture B (designated SOF²: stationary phase culture without ofloxacin)

10mls of isosensitest broth was maintained in stationary phase at 37°C for 8 weeks.

Culture C (designated MSOF+: maintenance subculture with 0.25MIC ofloxacin)

10mls of isosensitest broth containing 0.5μg/ml ofloxacin (equivalent to 0.25MIC) was maintained at 37°C through serial passage. After 48 hours the 10ml culture was centrifuged at 3K rpm for 5 minutes, the cells were resuspended in 10mls fresh isosensitest broth containing 0.5μg/ml ofloxacin, and re-incubated at 37°C. This procedure was repeated every 48 hours for the duration of the 8 week study.

Culture D (designated MSOF*: maintenance subculture without ofloxacin)

10mls of isosensitest broth was maintained at 37°C through serial passage. After 48 hours the 10ml culture was centrifuged at 3K rpm for 5 minutes, the cells were resuspended in 10mls fresh isosensitest broth, and re-incubated at 37°C. This procedure was repeated daily for the duration of the 8 week study.

Culture E (designated RSOF+: repeated subculture with 0.25MIC ofloxacin)

10mls of isosensitest broth containing 0.5μg/ml ofloxacin (equivalent to 0.25MIC) was incubated at 37°C for 24 hours. This culture was repeatedly subcultured daily for the first week with 20μls taken and used to inoculate 10mls of fresh isosensitest broth and incubated at 37°C.

Culture F (designated RSOF*: repeated subculture without ofloxacin)

10mls of isosensitest broth was incubated at 37°C for 24 hours. This culture was repeatedly subcultured daily for the first week with 20μls taken and used to inoculate 10mls of fresh isosensitest broth and incubated at 37°C.

4.2.4. Sampling

Samples were taken daily from the RSOF+ and RSOF- cultures, every second day from the
Purified pOG669 DNA was initially digested with PstI, and the number of fragments generated determined which additional restriction enzymes were likely to provide optimal fingerprints. Avall was the second enzyme used to further characterise DNA rearrangements. The production of a wide range of restriction fragments enabled adequate specificity.

4.2.5. Plasmid profile analysis

When variation in either Ap & Km sensitivity was observed, the colony was subjected to plasmid profile analysis using a modified method of Platt & Sommerville (1981). pOG669 DNA was obtained from crude lysates, separated by agarose gel electrophoresis, and the molecular weight determined through comparison with plasmids of known molecular weight. This method was used to identify any gross change in plasmid size and/or any evidence of plasmid elimination.

4.2.6. pOG669 DNA restriction endonuclease fragment patterns

All variants in Ap & Km sensitivities were subjected to DNA restriction endonuclease fragmentation patterns (REFP), as previously described (Platt et al, 1986). This technique enabled detection of DNA rearrangement(s). The restriction endonucleases used were PstI and Avall.

Purified pOG669 DNA was initially digested with PstI, and the number of fragments generated determined which additional restriction enzymes were likely to provide optimal fingerprints. Avall was the second enzyme used to further characterise DNA rearrangements. The production of a wide range of restriction fragments enabled adequate specificity.

4.2.7. Interpretation of REFPs

pOG669 possesses two co-integrate junctions (Platt et al, 1988), and the use of PstI and Avall provided evidence of DNA rearrangements that occurred within pOG669. Furthermore, any rearrangements that occurred within or adjacent to the co-integrate junctions were readily identified. SvaI, HindIII and EcoRI refined DNA discrimination further. DNA rearrangements were determined with comparison of restriction fragment patterns observed with wild-type pOG669.
DNA. DNA rearrangements observed within pOG669 were also assessed in comparison with pOG660 and pOG670 REFPs.
4.3. RESULTS

4.3.1. Variation of Ap & Km phenotype

A total of 2200 *E. coli* K12 J62-1, pOG669 colonies isolated from cultures A-F were tested for susceptibility to Ap & Km. The parental Ap\(^{\text{r}}\)Km\(^{\text{r}}\) phenotype was shown in 1063 colonies. Three variant phenotypes were observed and included Ap\(^{\text{s}}\)Km\(^{\text{s}}\) (421 colonies), Ap\(^{\text{s}}\)Km\(^{\text{s}}\) [(36 colonies), kanamycin sensitive variant (KSV)]. The third variant was more complex. When disc sensitivity tests were carried out on individual colonies, evidence of a mixed population was identified. This was observed where the region around the Ap & Km discs showed a more sparse growth of colonies than the semiconfluent growth on the remainder of the plate. This is shown in Figures 4.1 & 4.2 and is referred to as the mixed population variant (MPV), and these phenotypes were seen in 480 colonies, and is described in more detail in 4.3.1(c).

Figure 4.3 shows phenotypic changes in all populations with time. The parental Ap\(^{\text{r}}\)Km\(^{\text{r}}\) phenotype decreased from 1800 plasmid-containing colonies to just over 1063 colonies (the 400 repeated subculture colonies were not included because they would increase the total number of plasmid-free colonies before week 2 when elimination occurred in the stationary phase and maintenance subculture). The number of plasmid-free colonies progressively increased from 0 to 421, and the number of colonies that exhibited the MPV phenotype also progressively increased from 0 to 480. The number of KSV colonies increased, but at a much lower level (0 to 36 colonies). The distribution of each phenotype within cultures A-F are described below.

(a) Ap\(^{\text{s}}\)Km\(^{\text{s}}\) phenotype

Overall, 421 *E. coli* K12 J62-1 colonies (wild-type parent contained pOG669) demonstrated sensitivity to both Ap & Km. Plasmid profiles confirmed in all instances that loss of both Ap & Km resistance was a result of loss of pOG669 (Table 4.2). This represented a frequency for plasmid loss of 421 in 2200. Plasmid loss was significantly greater (p<0.001) in the SOF\(^{\text{+}}\) group (121/450 colonies) compared to the SOF\(^{-}\) group (94/450 colonies) ($\chi^2=20.7$ with df=3 for two independent...
Figure 4.1. Mixed population with *E. coli* K12 J62-1, pOG669-Ap^r^Km^r^ and *E. coli* K12 J62-1, pOG669-Ap^s^Km^s^.

This mixed population variant was designated MPV1.
Figure 4.2. Mixed population with *E. coli* K12 J62-1, pOG669-\(\text{Ap}^\text{r}\text{Km}^\text{s}\) and *E. coli* K12 J62-1, pOG669-\(\text{Ap}^\text{s}\text{Km}^\text{s}\).

This mixed population variant was designated MPV2.
Figure 4.3. Population phenotype changes with time.

Total ApF Km\(^r\) (R\(^+\)), Ap\(^8\) Km\(^s\) (R\(^-\)), MPV, and KSV colonies were counted.
Population phenotype changes with time

![Graph showing population phenotype changes over time with different markers for MPV, R+, and KSV.]
Table 4.2. The distribution of pOG669 elimination from *E. coli* K12 J62-1 colonies in stationary phase, and during maintenance, and repeated subculture over 8 weeks in the presence and absence of subinhibitory ofloxacin (0.25MIC).

50 colonies from each culture per week were tested for loss of Ap & Km resistance and plasmid loss confirmed by plasmid profile. Number of isolated *E. coli* K12 J62-1 colonies that showed pOG669 elimination.

This is a representative sample of two separate experiments.
Number of isolated *E. coli* K12 J62-1 colonies that showed pOG669 elimination

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</table>

*d, days, nt, not tested, "RSOF", remainder 10 colonies were Ap⁺Km⁻ (KSV).*
samples) (Figure 4.4a). In contrast, plasmid loss was significantly greater (p<0.01) in the MSOF\(^{-}\) group (71/450 colonies) compared to the MSOF\(^{+}\) group (45/450 colonies) (\(\chi^2=11.2\) with df=3 for two independent samples) (Figure 4.4b). Although, plasmid elimination increased with time for both stationary phase and maintenance subcultures, the number of colonies that exhibited plasmid loss was however greater in the stationary phase. Table 4.2 and Figures 4.5a, 4.5b, and 4.5c also show differences in the elimination of pOG669.

1. **Stationary phase and maintenance subculture groups** (Figures 4.5a & 4.5b)

(a) Cultures in stationary phase, in the presence and absence of subinhibitory ofloxacin, showed a progressive increase in plasmid elimination (Figure 4.4a). The increase between week 2 and week 4 was higher in the SOF\(^{-}\) group, when compared to the SOF\(^{+}\) group. However, after week 4 the increase in plasmid elimination in the SOF\(^{+}\) group was greater than in the SOF\(^{-}\) group. The rate of plasmid elimination in the SOF\(^{+}\) group continued to increase, whereas in the SOF\(^{-}\) group plasmid loss continued at a constant rate. Thus, in stationary phase cultures, subinhibitory ofloxacin not only increased overall plasmid elimination, but also increased the rate of plasmid elimination.

(b) Cultures in maintenance subculture in the presence and absence of subinhibitory ofloxacin, the pattern of plasmid elimination was different. There was an overall increase in plasmid elimination in both groups (Figure 4.4b). In the MSOF\(^{+}\) group, plasmid elimination was not observed to occur until week 4, and had increased by week 8, whereas in the MSOF\(^{-}\) group, plasmid elimination was observed to occur at week 2, and increased more by week 8 than that observed in the MSOF\(^{+}\) group. The rate of plasmid elimination in the MSOF\(^{-}\) group continued to increase, whereas in the MSOF\(^{+}\) group plasmid loss continued at a constant. Thus, in maintenance subcultures, subinhibitory ofloxacin reduced the rate of plasmid elimination.

Plasmid elimination was greater in stationary phase in the presence of subinhibitory ofloxacin (SOF\(^{+}\) group) with 80% of the overall colonies tested having lost pOG669, and lower in the maintenance phase (MSOF\(^{-}\) group) with 28% of the overall colonies tested having lost pOG669.
Figure 4.4. Plasmid elimination from *E. coli* K12 J62-1 colonies in stationary phase, maintenance, and repeated subculture.

The effects of subinhibitory ofloxacin (0.25MIC) was investigated in each phase. 50 colonies for each culture group per week were tested for Ap & Km susceptibility, and those colonies that showed Ap^5Km^5, were tested for plasmid profile.

(a) *Stationary phase*: 121 SOF^+ colonies and 94 SOF^- colonies showed pOG669 elimination, and

(b) *Maintenance subculture*: 45 MSOF^+ colonies and 71 MSOF^- colonies showed pOG669 elimination.

(c) *Repeated subculture*: 50 RSOF^+ colonies and 40 RSOF^- colonies showed pOG669 elimination.

- ■ - ■ with subinhibitory ofloxacin (0.25MIC)
- □ - □ without subinhibitory ofloxacin
(a) The effect of 0.25MIC OFX on pOG669 elimination in stationary phase
(b) The effect of 0.25MIC OFX on pOG669 elimination in maintenance subculture
(c) The effect of O.25MIC OFX on pOG669 elimination in repeated subculture

- Time (days)
- JS2-1 colonies with pOG669 loss
Although, plasmid elimination in the absence of ofloxacin (SOF and MSOF groups) demonstrated certain similarities in that end-stage elimination at week 8 was very similar. However, in maintenance subculture the effect of subinhibitory ofloxacin produced the opposite effect compared to that in stationary phase, by a reduction in plasmid elimination.

II. Repeated subculture (RSOF+ and RSOF−) groups (Figure 4.4c)

In both groups, elimination of pOG669 was observed (Table 4.2), with 100% elimination in the 50 RSOF+ colonies after week 1, and 80% elimination in the 50 RSOF− colonies after week 2, with the remaining 20% (equivalent to 10 colonies) showed Ap^Km^ (on days 3 and 5, 50 additional colonies tested for Ap & Km susceptibility, showed approximate overall 50% plasmid elimination in repeated subculture for these days). The growth of these cultures was stopped after 7 days on the basis that almost complete elimination of pOG669 was observed. In a replicate experiment, complete elimination of pOG669 was observed from all colonies in both groups after the 7 days.

(b) Ap^Km^ phenotype (KSV)

36 kanamycin sensitive variants were isolated. 26 KSV colonies were isolated in stationary phase between weeks 2 and 8 (Table 4.3). 10 KSV colonies were isolated from the RSOF group. Plasmid profile analysis showed all KSV isolates to harbour between 10 and 15kb reduction in size compared to wild-type pOG669. Thus, this phenotype was not a result of transposition of ampicillin resistance to the chromosome with the subsequent elimination of pOG669.

Overall, in stationary phase, subinhibitory ofloxacin increased the incidence of KSV (SOF+ group 16 of 36, SOF− group 10 of 36). However, this was found not to be statistically significant. 10 of 36 KSV were isolated from the RSOF group, and represents a frequency for KSV in the RSOF group as 10 in 200). No KSV were isolated from the RSOF+ group.

(c) Mixed population variant (MPV)

MPV colonies were first identified on Ap & Km susceptibility tests on isosensitest agar (Oxoid). On isosensitest agar plates there was evidence of a mixed population around both Ap and Km discs
Table 4.3. The distribution of $\text{Ap}^\text{R}\text{Km}^\text{S}$ variants (KSV) isolated from stationary phase and repeated subculture in the presence or absence of subinhibitory ofloxacin (0.25MIC).

<table>
<thead>
<tr>
<th>Culture Group</th>
<th>Week isolated</th>
<th>Number of KSV colonies isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stationary phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOF$^+$</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>SOF$^+$</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>SOF$^-$</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>SOF$^+$</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>SOF$^+$</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>SOF$^-$</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td><strong>Repeated subculture phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSOF$^-$</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>
(Figures 4.1 & 4.2). Figure 4.1 shows a mixed population with *E. coli* K12 J62-1, pOG669-
Ap²Km⁵ and *E. coli* K12, pOG669-Ap⁵Km⁸, and Figure 4.2 shows a mixed population with *E. coli*
K12 J62-1, pOG669-Ap²Km⁵ and *E. coli* K12 J62-1, pOG669-Ap⁵Km⁸. The development of
mixed plasmid phenotypes, either as Ap²Km⁵ and Ap⁵Km⁸, or Ap²Km⁵ and Ap⁵Km⁸ produced two
*E. coli* K12 J62-1 variant populations, MPV1 and MPV2 respectively. Overall, it was observed
that for MPV, 50% were plasmid-containing and 50% were plasmid-free.

This anomaly was an unexpected phenomenon since the original wild-type *E. coli* K12 J62-1
contained the plasmid pOG669. Both MPV1 and MPV2 populations could only have developed on
CLED purity plates, since they were observed at the same time when changes in antibiotic resistance
became apparent at week 2 (Figure 4.3). It is likely that cells at week 2, when sampled from the
stationary phase cultures A & B, and maintenance phase cultures C & D onto CLED purity plates,
were in an unusual state in that single colonies on CLED agar (Oxoid) were composed of half the
cells plasmid-containing, and the other half were plasmid-free. These cells then actively grew from
a state of nutrient deprivation to a state of active growth on fresh nutrients (CLED agar), and
thereby generate 2 cell types in each colony. This represents either a change in genotypic or
phenotypic character of both host and/or plasmid, and for the plasmid-free state to occur, pOG669
must not have partitioned. 50 SOF⁺ and SOF⁻ inter-zonal colonies (IZC) (Figure 4.5) from week 5
were further tested for Ap & Km susceptibility, and the population was curiously found to contain
all three phenotypes (Table 4.4). Notably, the generation of MPV from IZC colonies were de novo.

Table 4.4 also shows that for SOF⁺, the prevalence of Ap⁸Km⁸>Ap⁵Km⁵>MPV>KSV, and for
SOF⁻, the prevalence of Ap²Km⁵>Ap⁸Km⁸>MPV (no KSV colonies were detected). These results
show that in stationary phase subinhibitory ofloxacin reduced the number of resistant colonies,
increased the number of plasmid-free colonies, and increased both MPV and KSV colonies.

Confirmation of whether a genotypic or phenotypic change accounted for the generation of MPV
colonies was determined with conjugative transfer of (a) transitional pOG669 (MPV1 donor with
resident pOG669) into wild-type *E. coli* K12 J53-2 recipient, and re-transfer into transitional
Figure 4.5. Inter-zonal *E. coli* K12 J62-1 colonies (IZC) within ampicillin (Ap) zone.

A mixed population of either MPV1 or MPV2 was prevalent outside zone (A & B). Inside box, 1 of 50 colonies was taken for further Ap & Km susceptibility (X).
Table 4.4. The effect of subinhibitory ofloxacin (0.25MIC) on the prevalence of Ap\(^r\)Km\(^r\), Ap\(^s\)Km\(^s\), MPV, and KSV colonies from single IZC in stationary phase.

PHENOTYPES (No. colonies tested)

<table>
<thead>
<tr>
<th>Culture Group(^1)</th>
<th>Ap(^r)Km(^r)</th>
<th>Ap(^s)Km(^s)</th>
<th>MPV</th>
<th>KSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOF(^+)</td>
<td>17</td>
<td>21</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>SOF(^-)</td>
<td>29</td>
<td>17</td>
<td>4</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^1\)Isolates from week 5 (SOF\(^+\) and SOF\(^-\) groups). ND, none detected. MSOF colonies not tested.
*E. coli* K12 J62-1 recipient (pOG669 previously lost in plasmid elimination experiment as described below), and (b) wild-type pOG669 (*E. coli* K12 J53-2 donor) into transitional *E. coli* K12 J62-1 recipient (pOG669 previously lost in plasmid elimination experiment as described below). No MPV colonies were detected in any of the transconjugants (on basis of no mixed population growth with Ap & Km susceptibility). This indicates the generation of MPV colonies is a phenotypic character.

480 MPV colonies were isolated. Their distribution over the sampling period is shown in Table 4.5) and illustrated in Figures 4.6a & 4.6b. MPV were isolated more significantly from maintenance subcultures (294/450 colonies) as compared to stationary phase cultures (186/450 colonies) ($\chi^2=53.0$ with df=1 for two independent proportions). This indicates that in dividing cells (MSOF groups) MPV are more prevalent and/or in non-dividing cells MPV are less prevalent. These differences therefore indicate preferential is involved with the generation of MPVs. Table 4.5 and Figures 4.6a & 4.6b also show differences in MPV occurrence. In both groups the effect of subinhibitory ofloxacin on the generation of MPV was not statistically significant ($p>0.1$) (Table 4.5). No MPV colonies were detected in either of the RSOF groups.

Although not statistically significant the overall pattern of MPV colony production was different between the SOF+ and SOF- groups (Table 4.5 and Figure 4.6a). Figure 4.6a also shows that for both groups the incidence of MPV colonies increased with time. At week 2 the number of MPV colonies in the SOF- group was just over three-fold lower when compared to the SOF+ group. From weeks 2 to 4 the number of MPV colonies increased six-fold in the SOF- group and marginally decreased in the SOF+ group. Thus, in stationary phase cultures, subinhibitory ofloxacin reduced the overall incidence of MPV production. In maintenance subcultures the number of MPV colonies increased in parallel in both groups with time (Figure 4.6b).
Table 4.5. The distribution of mixed population variant (MPV) prevalence in *E. coli* K12 J62-1 colonies isolated from stationary phase and maintenance subculture in the presence or absence of subinhibitory ofloxacin (0.25MIC).

<table>
<thead>
<tr>
<th>Culture group</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>n</th>
<th>X² (df)</th>
<th>'p'</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stationary phase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOF⁺</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>10</td>
<td>8</td>
<td>10</td>
<td>14</td>
<td>16</td>
<td>16</td>
<td>85</td>
<td>4.1 (3)</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>SOF⁻</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>9</td>
<td>17</td>
<td>17</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>101</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Maintenance subculture</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSOF⁺</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>16</td>
<td>21</td>
<td>23</td>
<td>25</td>
<td>26</td>
<td>27</td>
<td>139</td>
<td>1.8 (3)</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>MSOF⁻</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>16</td>
<td>25</td>
<td>27</td>
<td>28</td>
<td>28</td>
<td>29</td>
<td>155</td>
<td></td>
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</tr>
</tbody>
</table>
Figure 4.6. Incidence of mixed population variants (MPV) in *E. coli* K12 J62-1 colonies from *stationary phase* and *maintenance subculture*.

The effects of subinhibitory ofloxacin (0.25MIC) was investigated in both groups. Each week 50 colonies were tested for Ap & Km susceptibility, and any colony that exhibited mixed population with pOG669 expressing Ap^Km^ & Ap^Km^, or Ap^Km^ & Ap^Km^, were designated MPV1 and MPV2 respectively.

(a) *Stationary phase*: 85 SOF^+ and 101 SOF^- colonies showed occurrence of MPV, and

(b) *Maintenance phase*: 139 POF^+ and 155 POF^- colonies showed occurrence of MPV.

- ■ with subinhibitory ofloxacin (0.25MIC)

- □ without subinhibitory ofloxacin
(a) The effect of 0.25MIC OFX on the incidence of MPV in stationary phase

![Graph showing the effect of 0.25MIC OFX on the incidence of MPV in stationary phase. The x-axis represents time in weeks, ranging from 0 to 8, and the y-axis represents the number of J69-1 colonies exhibiting MPV, ranging from 0 to 20. Two lines are plotted, one indicated by squares and the other by circles, showing the trend over time.]
(b) The effect of 0.25MIC of X on the incidence of MPV in maintenance subculture
4.3.2. Co-integrate junctional DNA rearrangements and characterisation of kanamycin sensitive variants (KSV) by REFP analysis

Figure 4.7 shows *PstI*, *Avall*, and *SmaI* fragmentation patterns of the co-integrate plasmid pOG669, together with those of pOG670 and pSLT, from which it is derived. The co-integrate junctions observed through comparison of each plasmid with each endonuclease are marked by arrows. pOG669 possesses 2 *PstI* junctional fragments of 4.7kb and 2.1kb, 2 *SmaI* junctional fragments of 7kb and 2.2kb, and to date, only 1 *Avall* junctional fragment of 3.9kb has been resolved. The fragmentation patterns in Figure 4.7 provided reference fragmentation patterns for pOG669, pOG670, and pOG660. This enabled identification of DNA rearrangements that occur within or close to co-junctional fragments, and in which component of pOG669 (either pOG670 and/or pSLT).

The plasmids in the KSVs were characterised further using REFP analysis and are shown in Figures 4.8-4.9 for *PstI*, and *Avall* respectively. pOG669 DNA from *E. coli* K12 J62-1, [pOG669 - Ap^F^Km^S^ variants (KSV)] was extracted, purified, and digested with the previously described restriction endonucleases, and changes in fragmentation pattern were observed when compared to wild-type pOG669 DNA.

1. *PstI* fragmentation patterns (Figures 4.8 & 4.10)
   
   (a) 4 fragment deletions of 4.7kb, 4.1kb, 2.3kb, and 1.8kb were deleted in all of the KSV colonies.
   
   (b) 3 additional deletions were observed in KSV colonies 1, 3, 5, 7, and 8 respectively.
      
      (i) deletion of a 6.8kb fragment, which was observed as a doublet fragment in wild-type pOG669 DNA, and
      
      (ii) deletions of 1.6kb and 1.4kb fragments was also observed in these variants.

   (iii) KSV 3 also shows deletion in the 9.5kb fragment.

2. *Avall* fragmentation patterns (Figures 4.9 & 4.10)
   
   (a) 2 fragments of 4.1kb and 3.6kb were deleted in all of the KSV colonies.
Figure 4.7. pOG669, pOG670, and pOG660 digested with PstI, SmaI, and AvaII.

Tracks 1 and 2, Bacteriophage λ digested with PstI and SmaI, Tracks 3, 6, and 9, show pOG670 digested with PstI, SmaI, and AvaII. Tracks 4, 7, and 10, show pOG669 digested with PstI, SmaI, and AvaII respectively. Tracks 5, 8, and 11, show pOG660 digested with PstI, SmaI, and AvaII respectively. The arrows illustrate the junctional fragments with PstI (4.7kb and 2.1kb), SmaI (7kb and 2.2kb), and AvaII (3.9kb).
Figure 4.8. Kanamycin sensitive variants (KSV) 1 to 7 digested with PstI.

Track 1, Bacteriophage λ digested with PstI, Track 2, shows wild-type pOG669 digested with PstI, Tracks 3 to 9, show KSV 1 to 7 digested with PstI. KSV 8 digested with PstI is shown in Figure 4.9 Track 12.
Figure 4.9. Kanamycin sensitive variants (KSV) 1 to 8 digested with AvaII.

Tracks 1 and 2, Bacteriophage λ digested with PstI and AvaII, Track 3, shows wild-type pOG669 digested with AvaII, Tracks 4 to 11, show KSV 1-8 digested with AvaII. Track 12, shows KSV 8 digested with PstI.
Figure 4.10. Computer generated REFP library for E. coli K12 J62-1, pOG669 kanamycin sensitive variants (KSV) 1 to 36 using PstI, and AvaII.
(b) 2 additional fragment deletions were observed in KSV 1, 3, 5, 7, and 8 respectively.

(i) deletion of a 3.1 kb fragment, which is the largest fragment of three grouped fragments as observed in wild-type pOG669 DNA, and

(ii) deletion of the 2.8 kb fragment was also observed in these variants.

KSV colonies 9-36

The PstI and Avall fragmentation patterns observed for this group of KSV colonies are shown in Figure 4.10. KSV 9-27 were isolated from stationary phase cultures, and KSV 28-36 were isolated from repeated subcultures. All were similar to fragmentation patterns of KSV colonies 1-8 (Figure 4.8), in that they possessed the PstI and Avall common deletions, and several of the lower fragment deletions (Table 4.6 & Figure 4.10). Table 4.6 summarises the DNA rearrangements observed for all KSVs and 3 patterns (I-III) of DNA rearrangements have occurred. The 4.7 kb and 1.8 kb PstI fragments deleted in all KSVs correspond to the junctional fragments of the co-integrate. Since both junctions (separated by at least 50 kb) were deleted in all KSVs, the generation of this variant is associated with at least two genetic events. Furthermore, since no variant was isolated with a single junction deleted, it must be the second event that leads to loss of kanamycin resistance. The fact two common junctional deletions were observed, and that the other two common deletions exist within the pOG660 component (as compared with the pOG660 REF in Figure 4.7), provides direct evidence that generation of kanamycin susceptibility resides adjacent to the junctional fragment within the pOG670 component. The additional fragment deletions of 4.1 kb, 2.3 kb, and 1.6 kb all occurred within the pOG660 component of pOG669. The 6.8 kb deletion, observed in 18 of the 36 KSV (50%) colonies, was also found to reside in the pOG660 component, and the 1.6 kb and 1.4 kb deletions were observed in 11 of the 36 KSV (30%) colonies. Avall fragmentation patterns from both Figures 4.7 and 4.9 show that the only common fragment deletion, as observed in all KSV, occurred within the junctional fragment. The second common 4.1 kb fragment deletion was found to reside in the R-plasmid pOG670 component of pOG669, and the remaining observed 3.1 kb and 2.8 kb fragment deletions were also found to reside in the
Table 4.6. Patterns (I-III) of DNA rearrangements for kanamycin sensitive variants (KSV).

<table>
<thead>
<tr>
<th>Pattern</th>
<th>KSVs</th>
<th>PstI deletions (kb)</th>
<th>AvaII deletions (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1,5,7,8,11,12,15,17, 19,22,24,25,29,32,36</td>
<td>6.8;4.7;4.1;2.3;1.8; 1.6;1.4</td>
<td>4.1;3.6</td>
</tr>
<tr>
<td>II</td>
<td>2,4,6,9,10,13,14,16, 18,20,21,23,26,27,28, 30,31,33,34,35</td>
<td>4.7;4.1;2.3;1.8</td>
<td>4.1;3.6</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>9.5;4.7;4.1;2.3;1.8; 1.6;1.4</td>
<td>4.1;3.6</td>
</tr>
</tbody>
</table>
pOG660 component (Figures 4.7 & 4.9). The 3.1kb deletion was found in 22 of the 36 KSV, and the 2.8kb deletion was also found in 22 of the 36 KSV.

4.3.3. Characterisation of resistant *E. coli* K12 J62-1 mixed population variants (MPV) by REFP analysis

As previously described, mixed population variants were derived as three subpopulations descendant from the wild-type parent strain. Figures 4.1 and 4.2 clearly demonstrate the existence of three populations of *E. coli* K12 J62-1, pOG669 - Ap^r^Km^f^, *E. coli* K12 J62-1, pOG669 - Ap^KmS^, and *E. coli* K12 J62-1 - Ap^s^Km^s^. Figure 4.11 shows MPV *PstI* and *Avall* REFPs to be identical to wild-type pOG669. Therefore, this indicates the generation of MPV does not appear to involve DNA rearrangements.
4.11. Computer generated REFP for mixed population variants (MPV) 1-20 using PstI and AvaII.
4.4. DISCUSSION

Relatively little is understood about the molecular mechanisms involved in plasmid stability, and even less is understood about the mechanisms that contribute to plasmid instability. However, it is established that many naturally occurring plasmids are susceptible to both spontaneous fragmentation and recombination (Clowes, 1972; Cohen et al., 1976; Nisioka et al., 1970). These processes have led to bacteria containing a wide range of derivative plasmids (Richmond & Wiedeman, 1974) and may also be responsible for the development of natural co-integrate plasmids.

Maximal loss of pOG669 was observed in repeated subcultures. Subinhibitory ofloxacin eliminated the R-plasmid pOG669 from cultures in stationary phase, and reduced the elimination of pOG669 in maintenance subculture. The effect in stationary phase (nutrient deprivation) may be consistent with host cells being in a highly stressed state and that non-dividing cells may therefore be more susceptible to the elimination effects of ofloxacin under these conditions. Conversely, the reduced elimination observed in maintenance subculture suggests host cells may be in a less stressed state and therefore less susceptible.

It is known the stable maintenance of naturally occurring plasmids within a bacterial population is dependent upon several mechanisms including replication (rep), partitioning (par), and adequate selection pressures (Gerdes et al., 1986). There is also evidence that both rep and par depend on the level of negative DNA supercoiling (Miller et al., 1990), and that inhibition of both DNA replication (rep) and distribution of plasmid copies (par) into viable progeny, will destabilise any existing plasmid population (Austin & Nordstrom, 1990). Although, plasmid loss was observed in cells that were either in stationary phase or in maintenance subculture, nevertheless subinhibitory ofloxacin affected both groups differently. In stationary phase, subinhibitory ofloxacin increased plasmid loss, and in maintenance subculture, subinhibitory ofloxacin reduced plasmid loss. 421 out of 2200 cells (19%) exhibited plasmid loss, and 480 out of 2200 cells (21%) exhibited generation of MPV, and 36 out of 2200 cells (2%) exhibited generation of KSV. The remaining
1263 out of 2200 cells (48%) were fully resistant and therefore still plasmid-containing. Therefore, this higher level of plasmid-containing cells may be associated with the nal^ mediated induction of the recA system in E. coli which is known to block cell division and thereby prevent plasmid elimination (Drlica, 1984; Hooper et al., 1984). The results presented here are consistent with these, since E. coli K12 J62-1 is nal^, and thereby induce the recA system followed by inhibition of DNA gyrase mediated cleavage. There is also evidence that nal^ reduces the ability of bacteria to maintain R-plasmids (Smith, 1984 & 1986).

An explanation for the higher and lower incidence of plasmid elimination, as observed in both stationary phase (SOF^+) and maintenance subculture (MSOF^+), may either involve (a) changes in partition par (Miller et al., 1990) thereby effecting the number of plasmid copies available for distribution into daughter progeny, and/or (b) a threshold level for stable maintenance of plasmid DNA load (Platt & Black, 1987; Platt & Chesham, 1986).

(a) Partition (par)

As the par locus of pSC101 has been shown to contain a strong E. coli DNA gyrase binding site (Wahle & Komberg, 1988), there is evidence that this binding site overlaps par (Tucker et al., 1984). Furthermore, similar par loci have been shown to operate in several plasmids including F (Austin & Wierzbicki, 1983; Ogura & Hiraga, 1983a), R1 and RP4 (Gerdes et al., 1985 & 1986; Gerdes & Molin, 1986), and NR1 (Tabuchi et al., 1988). Therefore, it is possible the par loci in most plasmids may contain this strong DNA gyrase binding site, and this could be a candidate for the site of action for subinhibitory ofloxacin on pOG669 in stationary phase where the number of pOG669 copies may be reduced. There is also recent evidence that the products of parC (ParC) and parE (ParE), which partially derive the recently identified bacterial topoisomerase IV (Kato et al., 1988, 1990 & 1992), are involved with partition in association with the bacterial cell membrane (Kato et al., 1992). Both of which are now thought to have a role in negative DNA supercoiling (Hooper & Wolfson, 1993). These lines of evidence would suggest that the observed effects of subinhibitory ofloxacin on plasmid elimination may result from an effect on plasmid copy partition.
at the bacterial membrane, and on plasmid replication, possibly through changes in plasmid DNA supercoiling. However, in maintenance subculture, the number of pOG669 copies would not be expected to be reduced, and therefore, in these conditions subinhibitory ofloxacin may have reduced the elimination of pOG669 on the basis that not all copies of pOG669 are eliminated.

(b) Threshold level for the stable maintenance of plasmid DNA load

A high frequency of DNA rearrangement leading to plasmid instability has been shown to alter the upper threshold limit to the number of plasmids a strain can stably maintain (Platt & Chesham, 1986; Platt & Black, 1987). Thus, a threshold level may be available to the cell for the stable maintenance of acquired plasmids. However, as pOG669 is a low copy plasmid and as several copies contribute to the plasmid DNA load of the cell, then this threshold may become reduced in the presence of subinhibitory ofloxacin, leading to DNA rearrangements, a reduction in plasmid copy number, and subsequent plasmid elimination. In addition, the stable maintenance of pOG669 in stationary phase may become reduced through the direct reduction of the threshold level thereby reducing plasmid copy number. In maintenance phase the plasmid copy number is higher, and therefore the reduction in plasmid elimination is lower. In repeated subculture virtual 100% elimination was observed, and this may be attributed to the instability mechanisms of pOG669 being accelerated during active logarithmic growth. This would be consistent with a notion that lethal DNA rearrangements lead to plasmid elimination. Recent evidence has suggested that bacteria in stationary phase produce starvation-induced proteins (Siegele & Kolter, 1992), some of which include the exonucleases (Sak et al, 1989). Therefore, the starvation response in stationary phase may activate an error-prone SOS DNA repair process (Foster, 1992; Ninio, 1991) which leads to exonuclease-induced-DNA-rearrangements. These rearrangements may be responsible for spontaneous mutation (Hall, 1990 & 1991; Tormo et al, 1990), which may lead to pOG669 instability and its subsequent reduction in plasmid copy number.

The generation of MPV was an unexpected phenomenon since the original wild-type E. coli K12
J62-1, pOG669 strain was fully plasmid-containing, and during the experiment there was clear evidence that mixed populations had occurred. This change in mixed plasmid status, either $\text{Ap}^\text{R}\text{Km}^\text{R}$/Ap$^\text{R}$Km$^\text{S}$, or Ap$^\text{R}$Km$^\text{S}$/Ap$^\text{S}$Km$^\text{R}$ resulted in the development of the two mixed population variants MPV1 and MPV2 which could only have developed after the cultures were sampled directly onto CLED agar for purity and selection. Perhaps the cells became even more stressed during the transition from stationary phase and maintenance subculture to an active phase on new nutrients. It is conceivable that during plasmid replication, and in particular the partition of daughter progeny, only 1 molecule of pOG669 was transferred to every second daughter cell. In other words, for every two daughter cells, one was plasmid-containing and the other plasmid-free. Notably the incidence of MPV was significantly greater in the maintenance subculture compared to stationary phase, and in both cases when subinhibitory ofloxacin was present, there was a marginal reduction in the number of MPV colonies. This suggests ofloxacin may not be involved in the generation of MPV. The increase in MPV generation was mirrored by an increase in plasmid elimination and this was observed in both stationary phase and maintenance subculture.

This suggests that the stability of pOG669 had changed and perhaps both these observations are linked. Common molecular rearrangements in KSV was also demonstrated, and this lead to loss of kanamycin resistance. Therefore, both these findings would suggest that two different mechanisms are in operation, with one leading to a highly unstable pOG669 (MPV), and the other a derivative of pOG669 (KSV).

Miller et al (1990) demonstrated that plasmids with minimal par region deletions were converted into unstable plasmids, and this may be consistent in the MPV,pOG669 colonies. Subinhibitory ofloxacin through reduction of DNA supercoiling, may therefore increase the rate of their loss. Furthermore, since spontaneous mutations have been shown to contribute to plasmid loss (Heller et al, 1981), and as plasmid loss was observed in MPV in both stationary phase and maintenance subculture, may reflect the absolute instability of pOG669.
REFP analysis demonstrated several different molecular rearrangements in the form of fragment deletions pOG669 DNA in the KSV and several common junctional fragment deletions including two *PstI* deletions, and one *AvaII* deletion were observed. Therefore, one or more of these deletions must be associated with the concomitant loss of kanamycin resistance as observed in all KSV. This was found to occur within the pOG670 component of pOG669 and this is consistent, since the pOG670 component carries the Km resistance determinant. Of the KSV that were isolated, most were from the SOF* group, and most of the overall KSV molecular rearrangements (85% of total) resided within the pOG660 component of pOG669. Therefore, this would suggest the pOG660 component is responsible for the instability mechanism(s) of pOG669.

The generation of both KSV and MPV are likely to have occurred as a result of at least two mutational mechanisms. It is tempting to speculate that these deletions generate, MPV with defective *par*, and KSV through junctional deletions. In addition to this, preliminary studies have demonstrated pOG669 to be stable in *E. coli* K12 J53-2, and similarly, evidence has shown pOG669 to be unstable in *E. coli* K12 J62-1. These findings would suggest that the *nal* phenotype of *E. coli* K12 J62-1 may play an additional role to *par*. This is consistent since inhibition of the *nal* mediated *recA* system would generate plasmid-free cells (Drlica, 1984; Hooper et al, 1984).

It is unknown if other naturally occurring co-integrate plasmids behave in a similar fashion, although it would be tempting to speculate this, since the very nature of the co-integration mechanisms have been established (Bennet et al, 1986). Similarly, constructed co-integrates as a result of illegitimate recombination would be expected to mimic those constructed in the natural environment (Bennet et al, 1986; Platt et al, 1988).

This part of the study would suggest instability of pOG669 leads to the generation of molecular variants through molecular rearrangements, and may therefore influence the evolutionary potential of low copy plasmids. The generation of both KSV and MPV would signify this. However, the fact that the pOG670 component was mostly conserved would further suggest that only small
regions of plasmid DNA are subject to change. Subinhibitory ofloxacin increased pOG669 elimination and the number of KSV, and both would add to a hypothesis that 4-quinolones influence plasmid evolution.
CHAPTER 5

THE SERRATIA MARCESCENS MUTANT GRI 2677-8
5.1. INTRODUCTION

The Gram-negative bacillus, *Serratia marcescens*, is a member of the family *Enterobacteriaceae*, and belongs to the *Klebsiella-Enterobacter-Serratia* tribe (Ewing et al, 1959; Ewing et al, 1962). *S. marcescens* is an organism of modest pathogenicity (Grimont & Grimont, 1978), often associated with infection in the compromised host, after recent surgery and/or previous antimicrobial therapy (Platt & Sommerville, 1981). *Serratia* species are typical opportunistic pathogens and have gained increasing nosocomial significance, further complicated by the emergence of multiple drug resistant strains.

They are intrinsically resistant to some antimicrobial agents including ampicillin and tetracycline, and additional resistance may be either plasmid-mediated (Hedges, 1980; Platt & Sommerville, 1981) or the result of chromosomal mutation (Traub & Kleber, 1977; Traub & Fukushima, 1979).

Pigmented strains are occasionally isolated from patients (Wilkowski et al, 1970), although the majority of clinical isolates are non-pigmented (Blazevic, 1980; Clayton & von Graevenitz, 1966). Historically, strains of *S. marcescens* have been recognised for their blood red-pigment appearance (Bizio, 1823), and production of the blood red-pigment, prodigiosin, is a common characteristic of many strains of *S. marcescens*.

5.1.1. Biosynthesis of prodigiosin

Strains of *S. marcescens* produce prodigiosin via a bifurcated pathway in which two precursors, 2-methyl-3-aminopyrrole (MAP) and 4-methoxy-2,2'-bipyrrrole-5-carboxaldehyde (MBC) are enzymatically condensed to form the tripyrrole prodigiosin (Morrison, 1966; Williams & Hearn, 1967). *S. marcescens* mutants 933 and WF have contributed to the elucidation of the biosynthetic pathway. Each mutant had alternative branches of the pathway blocked and therefore produced a single precursor. When both mutants were grown on the same plate bi-directional cross-feeding of precursors occurred, and prodigiosin was formed at the interface of the both mutants. Identification of the bipyrrrole intermediate structure, MBC, produced by mutant 933 was an important step in determination of the structure of prodigiosin (Santer & Vogel, 1956). Wasserman et al (1960) identified the intermediate structure to be MBC, and Rapoport & Holden (1960 & 1962) confirmed
the structure by synthesis. The final step in the pathway was the identification of MAP which was established through the use of gas chromatography, mass spectroscopy, and nuclear magnetic resonance (Deol et al., 1972).

The genetic control of prodigiosin synthesis is complex with at least nine enzymes required for its biosynthesis (Williams, 1973), and it has been suggested that genes controlling prodigiosin biosynthesis may be clustered (Williams & Qadri, 1980). However, it is not known, either what size the gene cluster is, or whether the pigmentation genes are contiguous (Dauenhauer et al., 1984).

5.1.2. Intracellular location and physiological role of prodigiosin

Synthesis of prodigiosin occurs in the late stages of growth and is therefore thought of as a secondary metabolite. It is also thought to be complexed with the sugar-containing outer membrane glycoproteins (Cruz-Camarillo & Sanchez-Zuniga, 1968) in close association with the cell envelope (Williams & Qadri, 1980). More recently it has been proposed that prodigiosin is present as protein-pigment complexes within the inner cell membrane (Kobayashi & Ichikawa, 1989) and accumulates as periplasmic prodigiosin (Kobayashi & Ichikawa, 1991). Furthermore, the accumulation of prodigiosin increases during active bacterial growth (Geron et al., 1988). The enzyme responsible for the last step in the formation of prodigiosin is also thought to be periplasmic (Matsuyama et al., 1986) since two extracellular enzymes are thought to be released from pigmented Serratia via two different mechanisms (Kobayashi & Ichikawa, 1991). The physiological role of prodigiosin is poorly understood and even now the precise function of prodigiosin remains unknown.

The pigment is thought not to be essential, since white mutants of *S. marcescens* have been shown to grow *in vitro* as well as pigmented strains (Williams, 1973). Nevertheless there is evidence that prodigiosin possesses antimicrobial activity (Gerber, 1975; Williams & Hearn, 1967; Williams & Qadri, 1980) which may enhance survival in nature.

Many pigmented strains of *S. marcescens* spontaneously mutate to non-pigmented variants when subcultured (Bunting 1940 & 1946), and several of these variants revert to the pigmented phenotype
with high frequency. Spontaneous mutation also generates cross-resistance among some aminoglycosides (Traub & Fukushima, 1979), and unrelated agents that include nalidixic acid, trimethoprim, and chloramphenicol (Traub & Kleber, 1977).

*S. marcescens* GRI 2677 is a pigmented plasmid-free clinical isolate, and *S. marcescens* GRI 2677-8 is a spontaneously non-pigmented nutritionally defective (but not auxotrophic) antibiotic resistant mutant. In contrast to the parent strain (GRI 2677), it is resistant to carbenicillin and aminoglycosides. GRI 2677-8 reverts at high frequency to the parental phenotype and it has been suggested this may involve DNA rearrangement(s) by illegitimate recombination (Platt et al., 1983). Revertants accumulate in cultures stored at both room temperature and 4°C, and subcultures of an overnight broth culture contain a high proportion (10 - 20%) of pigmented colonies.

After extended incubation individual non-pigmented colonies develop discrete intracolonial pigmented papillae (IPP) (Platt et al., 1983). These IPPs become progressively evident with increasing colony age, up to 6 days. The number detected per colony ranges from 0 - 8 on CLED agar (Oxoid), but increases on media enriched with additional peptone (Platt & Brown, unpublished observations). Washed suspensions of GRI 2677-8 plated at intervals over a 21 day period show progressive reversion of the population (Platt et al., 1983).

The aim of this part of the study was to extend earlier observations, and to determine the effects of subinhibitory ofloxacin firstly on the reversion of a starved population, and secondly on IPP formation in relation to colonial growth rate.
5.2. MATERIALS AND METHODS

5.2.1. Bacterial strains and media

*S. marcescens* GI 2677-8 was stored in glycerol-peptone at -70°C, and revived by plating onto CLED agar (Oxoid), and incubated at 30°C. CLED agar (Oxoid) was used for (a) growth and maintenance of cultures and (b) to distinguish the non-pigmented phenotype from the parental pigmented phenotype.

To purge initially revived cultures of pigmented revertants, daily subcultures of non-pigmented variants for three consecutive days were performed. CLED agar was also used for the assessment of pigmented variants during population reversion. These variants were classified, as previously described by Platt *et al.* (1983), into groups of decreasing pigmentation: \( p^+ \) (pigmented parent strain), \( p^\pm \), and \( p^- \) (non-pigmented).

Isosensitest broth (Oxoid) was used to culture GI 2677-8 during population reversion. Isosensitest agar (Oxoid) was used for disc diffusion sensitivity testing, and the following antibiotic-impregnated discs (Oxoid): ampicillin (10µg), carbenicillin (100µg), cephazolin (30µg), cefamandole (30µg), colistin sulphate (25µg), tetracycline (10µg), gentamicin (10µg), tobramycin (10µg), amikacin (10µg), streptomycin (10µg), kanamycin (30µg), trimethoprim (1.25µg), sulphmethoxazole (25µg), chloramphenicol (10µg), nalidixic acid (30µg), and ofloxacin (1µg) were used.

5.2.2. Colony reversion assay and determination of the mean colony radial growth rate of GI 2677-8 colonies

The colony reversion assay was carried out in bioassay plates 243 mm² (Gibco/NUNC) that contained 300mls CLED agar (Oxoid). Subinhibitory (0.1MIC and 0.25MIC) ofloxacin was incorporated into the medium. This was inoculated with 100µl of an 8 hour culture of GI 2677-8 diluted to \( 10^{-4} \) to give approximately fifty colonies per plate each separated by at least 1cm. This criterion eliminated competition for nutrients as a source of size variation in resultant colonies. The plates were incubated for 3 days at 30°C followed by a further 2 days at room temperature. For
each colony, the number of intracolonial pigmented papillae of each colony was determined. As IPP formation may be growth rate dependent and it was therefore not be possible to assess the effect of subinhibitory ofloxacin on a single medium. To overcome this disparity, the standard formulation of 5g/l peptone in CLED agar (Oxoid) was supplemented to 12.5g/l and 15g/l to reduce the net inhibitory effect of ofloxacin on growth rate. This provided not only a means to achieve matched growth rate for the comparison of IPP production in the presence of either 0.1MIC or 0.25MIC ofloxacin, but also to observe real effects of subinhibitory ofloxacin on both the reversion process and mean colony radial growth rate. The mean colony radius was determined by measuring the diameter (mm) of fifty GRI 2677-8 colonies at intervals 15, 20, 25, 30, 35, 40, 50, and 60 hours using a Leebrook microbiological zone reader (Leebrook Scientific Instrument Company Limited) giving a linear magnification of 7.5:1 (Platt & Sommerville, 1984).

5.2.3. Rational of population reversion study

In previous studies the population reversion effect was first observed in stationary broth cultures. However, such a result could have been accounted for by either cryptic growth or the higher growth rate of the revertant as compared with GRI 2677-8. To exclude these possibilities the population reversion assay also carried out using washed cell suspensions in distilled water (Platt et al, 1983). Since, 4-quinolones have been shown to act on stationary as well as growing populations (Ratcliffe & Smith, 1984; Zeiler & Grohe, 1984; Zeiler, 1985), the subinhibitory effects were studied under both conditions. Initially, 65mls of isosensitest broth (Oxoid) was inoculated with one pure GRI 2677-8 colony and incubated at 30°C for 8 hours. An eight hour incubation was deemed sufficient to allow the GRI 2677-8 culture to enter the logarithmic phase of growth, and at this stage contain a minimum number of revertants. The appearance of the culture was turbid, indicating sufficient cell numbers were present (as observed in previous experiments with dilution plate analysis). From this 65ml GRI 2677-8 culture, 10mls were individually aliquoted into one of four separate universal bottles labelled culture A to D.
Culture A (starved stationary phase population)

10mls of the original culture was centrifuged at 3000 rpm and the cells were washed, and resuspended in 10mls sterile distilled water.

Culture B (starved stationary phase population with 0.25MIC ofloxacin)

10mls of original culture was centrifuged at 3000 rpm and the cells were washed, and resuspended in 10mls sterile distilled water that contained 0.125μg/ml ofloxacin (equivalent to 0.25MIC).

Culture C (stationary phase population)

10mls of original culture.

Culture D (stationary phase population with 0.25MIC ofloxacin)

10mls of original culture containing 0.125μg/ml ofloxacin (equivalent to 0.25MIC).

Each culture was incubated at 30°C for 25 days and the total count, number of non-pigmented (p⁻) variants, and the total number of pigmented (p⁺) revertants (including p⁺, p±, and p⁻ revertants) were determined by dilution plate viable counts at 5 day intervals. Antibiotic sensitivity patterns for 30 single colonies of non-pigmented (p⁻) variants and pigmented (p⁺, p±, p⁻) revertants were carried out.
5.3. RESULTS

The red-pigmented appearance of GRI 2677 was enhanced on CLED agar (Oxoid) and this provided reproducible discrimination of pigmented variants. Figure 5.1 shows IPPs demonstrating reversion of non-pigmented GRI 2677-8 to the pigmented phenotype after three to five days incubation at 30°C. On revival of GRI 2677-8 from storage, the initial ratio of occurrence of pigmented (p+) and non-pigmented (p-) phenotypes was observed as 4:1. This ratio decreased to less than 1:1000 by daily subculture for 3 days.

5.3.1. The effect of subinhibitory ofloxacin (0.1MIC & 0.25MIC) on colony radial growth rate, numbers, and distribution of IPPs in GRI 2677-8 colonies

The radial growth of colonies was linear between 15 and 60 hours and Figures 5.2-5.4 also show that both 0.1 and 0.25MIC ofloxacin reduced growth rate. Table 5.1 and Figures 5.2-5.4 show the radial growth of GRI 2677-8 colonies to increase with increasing peptone concentration. Table 5.2 and Figure 5.5 show similar colonial and radial growth rates. When the peptone concentration was increased, the colony radial growth rate (Kr) increased (Table 5.2), but inhibition by either 0.1MIC or 0.25MIC subinhibitory ofloxacin was also evident. However, when standard peptone concentration of 5 g/l was compared with a peptone concentration of 12.5 g/l that contained 0.1MIC ofloxacin, and a peptone concentration of 15 g/l that contained 0.25MIC ofloxacin, the colony radial growth rate (Kr) was closely matched (Figure 5.5). Therefore, these concentrations of peptone allowed the effect of subinhibitory ofloxacin on IPP formation to be determined independent of growth rate.

The radial growth rate of GRI 2677-8 on CLED with standard formulation of 5g/l peptone was very similar when compared to either CLED containing 12.5 g/l peptone and 0.1MIC ofloxacin or CLED containing 15g/l peptone and 0.25MIC ofloxacin (Figure 5.5 and Table 5.2). The population distributions as shown in Table 5.3 and Figure 5.6 demonstrate subinhibitory ofloxacin effected a reduction of IPPs per colony. 0.1MIC ofloxacin significantly reduced (p<0.05) the reversion process through reduction of the number IPPs per colony ($\chi^2=8.27$ with df=3 for two independent samples, Table 5.4). 0.25MIC ofloxacin was also shown to significantly decrease (p<0.001) the reversion process through reduction of the number of IPPs per colony ($\chi^2=279.3$ with
Figure 5.1. Colonies of *S. marcescens* GRI 2677-8 that demonstrate intracolonial pigmented papillae (IPP).
Table 5.1. The effect of variation in peptone concentration in the presence and absence of subinhibitory ofloxacin (0.25MIC) on mean colony radius (mm) of GRI 2677-8 colonies.

(a) [peptone 5g/l], (b) [peptone 12.5g/l with 0.1MIC ofloxacin], and (c) [peptone 15g/l with 0.25MIC ofloxacin].

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>[peptone 5g/l]</th>
<th>[peptone 12.5g/l]</th>
<th>[peptone 15g/l]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td>(c)</td>
</tr>
<tr>
<td>15</td>
<td>0.13</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>20</td>
<td>0.42</td>
<td>0.33</td>
<td>0.32</td>
</tr>
<tr>
<td>25</td>
<td>0.65</td>
<td>0.52</td>
<td>0.43</td>
</tr>
<tr>
<td>30</td>
<td>0.92</td>
<td>0.72</td>
<td>0.62</td>
</tr>
<tr>
<td>35</td>
<td>1.13</td>
<td>0.95</td>
<td>0.80</td>
</tr>
<tr>
<td>40</td>
<td>1.38</td>
<td>1.13</td>
<td>0.95</td>
</tr>
<tr>
<td>50</td>
<td>1.90</td>
<td>1.53</td>
<td>1.33</td>
</tr>
<tr>
<td>60</td>
<td>2.33</td>
<td>1.90</td>
<td>1.70</td>
</tr>
</tbody>
</table>

Each mean colony radius was determined from measurements of fifty colonies. The standard error for each measurement was <2%.
Figure 5.2. Radial growth of *S. marcescens* GRI 2677-8 colonies on CLED agar (Oxoid) with peptone concentration of 5g/l.

- [peptone 5g/l]; ▼, ■ [peptone 5g/l] with 0.1MIC, and 0.25MIC ofloxacin respectively.
Figure 5.3. Radial growth of *S. marcescens* GRI 2677-8 colonies on CLED agar (Oxoid) with peptone concentration of 12.5g/l.

- [peptone 12.5g/l]; ▲, ■ [peptone 12.5g/l] with 0.1MIC, and 0.25MIC ofloxacin respectively.
Figure 5.4. Radial growth of *S. marcescens* GRI 2677-8 colonies on CLED agar (Oxoid) with peptone concentration of 15g/l.

- • [peptone 15g/l]; ▼, ■ [peptone 15g/l] with 0.1MIC, and 0.25MIC ofloxacin respectively.
Table 5.2. The effect of variation in peptone concentration in the presence and absence of subinhibitory ofloxacin (0.25MIC) on colonial growth rate (Kr) of GRI 2677-8 colonies.

(a) [peptone 5g/l], (b) [peptone 12.5g/l with 0.1MIC ofloxacin], and (c) [peptone 15g/l with 0.25MIC ofloxacin].

<table>
<thead>
<tr>
<th>[peptone g/l]</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>48.9</td>
<td>40</td>
<td>36</td>
</tr>
<tr>
<td>12.5</td>
<td>53</td>
<td>51</td>
<td>42</td>
</tr>
<tr>
<td>15.0</td>
<td>58</td>
<td>54</td>
<td>48.4</td>
</tr>
</tbody>
</table>
Figure 5.5. Comparison of radial growth of GRI 2677-8 colonies on CLED agar (Oxoid) with variation in peptone concentration.

- [peptone 5g/l]; • [peptone 12.5g/l] with 0.1MIC ofloxacin, and ▲ [peptone 15g/l] with 0.25MIC ofloxacin respectively.
Table 5.3. Comparison of observed number of *S. marcescens* GRI 2677-8 colonies with IPP per colony, with the number of IPP per colony with values predicted from a Poisson Distribution. (a) 5g/l peptone group, 12.5g/l peptone group, and (c) 15g/l group, with and without subinhibitory ofloxacin.

For statistical purposes the numbers of IPP per colony were separated into cells. Statistical Cells I, II, III, and IV represented the number of colonies with IPP as 0-1, 2, 3, and 4-9 IPP per colony respectively (see Table 5.4).
<table>
<thead>
<tr>
<th>CLED agar with [Peptone]</th>
<th>Number of colonies</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Average number of pigmented papillae per colony</th>
<th>$\chi^2$</th>
<th>df</th>
<th>p'</th>
</tr>
</thead>
<tbody>
<tr>
<td>5g/L</td>
<td>80 (O)</td>
<td>0</td>
<td>4</td>
<td>18</td>
<td>44</td>
<td>10</td>
<td>4</td>
<td>2.9</td>
<td>50.8</td>
<td>5</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>80 (P)</td>
<td>4.4</td>
<td>12.77</td>
<td>18.5</td>
<td>17.9</td>
<td>12.98</td>
<td>7.52</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5g/L + 0.1 MIC&lt;sub&gt;min&lt;/sub&gt;</td>
<td>80 (O)</td>
<td>0</td>
<td>24</td>
<td>28</td>
<td>18</td>
<td>4</td>
<td>6</td>
<td>2.25</td>
<td>15.8</td>
<td>5</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>80 (P)</td>
<td>8.43</td>
<td>18.97</td>
<td>21</td>
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<td>9</td>
<td>8.43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5g/L + 0.25 MIC&lt;sub&gt;min&lt;/sub&gt;</td>
<td>80 (O)</td>
<td>16</td>
<td>44</td>
<td>14</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>1.15</td>
<td>12.8</td>
<td>5</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>80 (P)</td>
<td>25.33</td>
<td>29.12</td>
<td>16.75</td>
<td>6.42</td>
<td>1.85</td>
<td>0.42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLED agar with [Peptide]</td>
<td>Number of colonies</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>Average number of pigmented papillae per colony</td>
<td>$\chi^2$</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------------------</td>
<td>---</td>
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<td>--------------------------------</td>
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</tr>
<tr>
<td>12.5g/L.</td>
<td>80 (O)</td>
<td>0</td>
<td>6</td>
<td>14</td>
<td>22</td>
<td>12</td>
<td>16</td>
<td>8</td>
<td>2</td>
<td></td>
<td>3.675</td>
</tr>
<tr>
<td></td>
<td>(P) 2</td>
<td>7.5</td>
<td>13.7</td>
<td>16.8</td>
<td>15</td>
<td>11.3</td>
<td>7</td>
<td>3.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5g/L. + 0.1MIC&lt;sub&gt;min&lt;/sub&gt;</td>
<td>80 (O)</td>
<td>2</td>
<td>6</td>
<td>24</td>
<td>34</td>
<td>14</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td></td>
<td>2.65</td>
</tr>
<tr>
<td></td>
<td>(P) 5.7</td>
<td>15</td>
<td>19.9</td>
<td>17.5</td>
<td>11.6</td>
<td>5.7</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5g/L. + 0.25MIC&lt;sub&gt;min&lt;/sub&gt;</td>
<td>80 (O)</td>
<td>10</td>
<td>20</td>
<td>22</td>
<td>24</td>
<td>4</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td></td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>(P) 12</td>
<td>22.7</td>
<td>21.6</td>
<td>13.7</td>
<td>6.5</td>
<td>2.47</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLED agar with [Peptone]</td>
<td>Number of colonies</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
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<td>---</td>
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<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>15g/L</td>
<td>80 (O)</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>20</td>
<td>8</td>
<td>14</td>
<td>12</td>
<td>10</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>(P) 0.75</td>
<td>3.5</td>
<td>16.3</td>
<td>12.7</td>
<td>14.9</td>
<td>13.9</td>
<td>108</td>
<td>7.2</td>
<td>4.2</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>15g/L + 0.1 MIC&lt;sub&gt;af&lt;/sub&gt;</td>
<td>80 (O)</td>
<td>0</td>
<td>4</td>
<td>14</td>
<td>22</td>
<td>30</td>
<td>10</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(P) 2.8</td>
<td>9.4</td>
<td>15.75</td>
<td>17.6</td>
<td>14.73</td>
<td>9.9</td>
<td>5.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>15g/L + 0.25 MIC&lt;sub&gt;af&lt;/sub&gt;</td>
<td>80 (O)</td>
<td>12</td>
<td>24</td>
<td>22</td>
<td>16</td>
<td>6</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(P) 13.9</td>
<td>24.3</td>
<td>21.3</td>
<td>12.42</td>
<td>5.4</td>
<td>1.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.6. Variation in population distribution of numbers of *S. marcescens* GRI 2677-8 colonies with IPP per colony with the numbers of IPPs per GRI 2677-8 colony.

The peptone concentration was also varied with (a) [peptone 5g/l], (b) [peptone 12.5g/l] with 0.1MIC ofloxacin, and (c) [peptone 15g/l] with 0.25MIC ofloxacin.
Population Distribution of numbers of GRI 2677-8 colonies with pigmented papillae

(a) 
(b) 45-
(c) 45-

Numbers of colonies with pigmented papillae per colony
5.3.2. Characterisation of intracolonic reversion

Figure 5.6 shows the distribution of the number of GRI 2677-8 colonies with pigmented papillae as a function of the number of pigmented papillae per colony. When the distribution for GRI 2677-8 colonies on CLED with standard formulation of 5g/l peptone was compared with colonies grown on CLED with either 12.5g/l peptone that contained 0.1MIC ofloxacin or 15g/l peptone that contained 0.25MIC ofloxacin, there was an observed shift to colonies with decreased reversion (Figure 5.6 and Table 5.3). Furthermore, when similar growth is taken into account, as observed in Figure 5.5, subinhibitory ofloxacin (0.1MIC) significantly reduced reversion, and an increase in subinhibitory ofloxacin to 0.25MIC reduced reversion further with a shift to colonies with fewer IPPs. The results from Table 5.3 indicate the frequency distributions for colonies (grown on CLED with peptone concentrations 5g/l, 12.5g/l that contained 0.1MIC ofloxacin, and 15g/l that contained 0.25MIC ofloxacin) with different numbers of IPPs are represented by 'goodness-of-fit' Poisson Distributions (Table 5.3). Of the 9 distributions tested using 'goodness-of-fit' Poisson, 6 differed significantly (Table 5.2), and 3 did not. This indicates that the reversion process is not necessarily random, and therefore not a typical mutational process.
Table 5.4. The effect of subinhibitory ofloxacin (0.25MIC) on the reversion of the non-pigmented (p−) variant to the parental pigmented (p+) revertant, independent of growth rate.

Comparison of normal peptone concentration of 5g/l in CLED agar (Oxoid) with peptone concentrations of 12.5g/l with 0.1MIC ofloxacin, and 15g/l with 0.25MIC ofloxacin respectively, using a Chi-squared test.

<table>
<thead>
<tr>
<th>STATISTICAL CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>[peptone g/l]</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>12.5 + 0.1MIC</td>
</tr>
<tr>
<td>15.0 + 0.25MIC</td>
</tr>
</tbody>
</table>
5.3.3. Observed variation in parental pigmented phenotypes and antibiotic sensitivity patterns of GRI 2677-8

Although GRI 2677-8 normally reverted to the parental pigmentation phenotype, several pigmented variants were isolated during the population reversion study. Table 5.5 shows two variant sub-populations, pale-pink (p↓) and bright-pink (p↑) phenotypes, of the parental red-pigmented phenotype (p+) were isolated. Table 5.5 also shows that a greater number of bright-pink pigmented variants occurred in stationary phase (nutritionally undeprived), with or without 0.25MIC ofloxacin. Cumulatively, the number of pale-pink variants occurred more frequently with time. Furthermore, by day 25, there were more pale-pink variants in the culture without ofloxacin.

Antibiotic sensitivity patterns for the non-pigmented variant and pigmented revertant of GRI 2677-8 were different (Table 5.6). The critical zones (Table 5.6) were taken from GRI 2677 p+, and any value less than the respective antibiotic zone is still significantly resistant when compared to the usual revertant. Therefore, reversion to the parental pigmented phenotype was associated with loss of resistance to carbenicillin and cefamandole, increased sensitivity to sulphmethoxazole, the aminoglycosides, trimethoprim, and chloramphenicol. There was no change in the sensitivity to nalidixic acid, ofloxacin, rifampicin, and colistin sulphate.

In contrast to Table 5.6, Table 5.7 shows there are further differences in antibiotic patterns for both p↓ and p↑ pigmented variants. The reversion of pigmentation involved four pigmented phenotypes, non-pigmented (p−), pale-pink (p↓), bright-pink (p↑), and parental red-pigmented (p+).
Table 5.5. The number of parental pigmented variant colonies isolated from GRT 2677-8 colonies during population reversion in stationary phase (SP) and starved stationary phase (SS) culture conditions in the presence and absence of subinhibitory ofloxacin (0.25MIC) respectively.

<table>
<thead>
<tr>
<th>Days</th>
<th>SP</th>
<th>SS</th>
<th>SP (0.25MIC)</th>
<th>SS (0.25MIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5p±/362</td>
<td>1p± &amp; 1p±/505</td>
<td>3p±/405</td>
<td>4p± &amp; 1p±/367</td>
</tr>
<tr>
<td>5</td>
<td>0/750</td>
<td>4p±/240</td>
<td>2p±/112</td>
<td>8p±/622</td>
</tr>
<tr>
<td>10</td>
<td>0/420</td>
<td>5p±/680</td>
<td>1p±/950</td>
<td>12p±/194</td>
</tr>
<tr>
<td>15</td>
<td>0/260</td>
<td>18p± &amp; 3p±/300</td>
<td>0/430</td>
<td>21p±/850</td>
</tr>
<tr>
<td>20</td>
<td>0/90</td>
<td>29p±/100</td>
<td>0/35</td>
<td>17p±/52</td>
</tr>
<tr>
<td>25</td>
<td>0/58</td>
<td>32p±/80</td>
<td>0/31</td>
<td>2p± &amp; 7p±/46</td>
</tr>
</tbody>
</table>
Table 5.6. Antibiotic sensitivity patterns (mm) for the non-pigmented \((p^-)\) variant and the parental pigmented \((p^+)^\) revertant of GRI 2677-8.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>((p^-)) variant</th>
<th>((p^+)) revertant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>R</td>
<td>15 (13.00)</td>
</tr>
<tr>
<td>Cephazolin</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>R</td>
<td>11 (9.50)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>15 (13.50)</td>
<td>15 (13.25)</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>15 (13.00)</td>
<td>14 (12.75)</td>
</tr>
<tr>
<td>Colistin sulphate</td>
<td>10 (8.00)</td>
<td>10 (8.00)</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>5 (6.00)</td>
<td>6 (4.50)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Sulphmethoxazole</td>
<td>&lt;5 (7.00)</td>
<td>9 (7.25)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>6 (7.50)</td>
<td>14 (11.75)</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>7 (8.25)</td>
<td>11 (9.25)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>&lt;5 (7.00)</td>
<td>14 (12.00)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>&lt;5 (7.00)</td>
<td>13 (11.00)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>&lt;5 (7.00)</td>
<td>14 (12.00)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>6 (7.25)</td>
<td>17 (15.75)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>6 (7.50)</td>
<td>15 (13.75)</td>
</tr>
</tbody>
</table>

( ) critical zone size shown for those agents to which parent and revertant differed in sensitivity: calculated as \((x - 3SD: revertant)\) and \((x + SD: variant)\)
Table 5.7. Antibiotic sensitivity patterns (mm) for the p± and p± pigmented partial revertants of GRI 2677-8.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>(p±) variant (bright-pink)</th>
<th>(p±) variant (pale-pink)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>11 (I)</td>
<td>12 (I)</td>
</tr>
<tr>
<td>Cephazolin</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>R</td>
<td>(NR) R</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Colistin sulphate</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Sulphmethoxazole</td>
<td>&lt;5 (NR)</td>
<td>&lt;5 (NR)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>7 (NR)</td>
<td>6 (NR)</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>8 (NR)</td>
<td>8 (NR)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>11 (I)</td>
<td>11 (I)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>9 (I)</td>
<td>10 (I)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>11 (I)</td>
<td>13 (R)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10 (I)</td>
<td>12 (I)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>12 (I)</td>
<td>13 (R)</td>
</tr>
</tbody>
</table>

R = no zone. I = intermediate sensitivity (partial reversion) and p+ phenotypes, and NR = no reversion of resistance ie. retention of the variant phenotype (see critical zones in Table 5.6)
Those intermediate in pigmentation also showed partial reversion with respect to \( \beta \)-lactam and aminoglycoside resistance (Tables 5.6 & 5.7). However, the partial resistance of the \( p^- \) variant to sulphmethoxazole, trimethoprim, and chloramphenicol was maintained in both the pale-pink and bright-pink intermediate phenotypes, and indicates that partial reversion did not affect all components of the combined phenotype equally. These results indicate the mechanism of reversion is complex and that the generation of intermediate revertants appeared to be influenced by subinhibitory ofloxacin (0.25MIC).

5.3.4. The effects of subinhibitory ofloxacin (0.25MIC) on the growth kinetics of *S. marcescens* GRI 2677-8 during population reversion under different growth conditions

(a) Culture A (*starved stationary phase population without 0.25MIC ofloxacin*)

When a *starved stationary phase* culture of GRI 2677-8 was incubated at 30°C for 25 days, the following changes in total count, number of non-pigmented variants, and number of pigmented revertants were observed (Figure 5.1a). For purpose of analysis, Figure 5.1a was divided into three separate time periods: 0-15 days, 15-20 days, and 20-25 days respectively. During the first 15 days the total count and the number of non-pigmented variants both decreased by 1 log unit. However, the number of pigmented revertants increased by 0.45 log units. This period shows the decrease in total count is mirrored by the decrease in the number of non-pigmented variants and is attributed to a combination of loss of viability and reversion of \( p^- \) variants to the \( p^+ \) parental phenotype. Between days 15 & 20 there was a decrease of 0.1 log units in the total count, and a decrease of 1 log unit in the number of non-pigmented variants, and a decrease of 0.15 log units in the number of pigmented revertants. The total number of non-pigmented variants decreased below the total number of pigmented revertants. Furthermore, the decrease in the number of non-pigmented cells indicates the reversion rate reached a maximum during this period, together with an increase in cell death. The total count, the number of non-pigmented variants, and the number of
Figure 5.7. Viability counts of population reversion of starved stationary phase culture of S. marcescens GRI 2677-8 incubated at 30°C for 25 days in distilled water in the presence and absence of subinhibitory ofloxacin (0.25MIC).

(a) without 0.25MIC ofloxacin, and (b) with 0.25MIC ofloxacin. • Total count; □ non-pigmented (p*) resistant variant; ▼ pigmented (p+) sensitive revertant.
(a) WITHOUT SUBINHIBITORY OFLOXACIN (0.25MIC)
(b) WITH SUBINHIBITORY OFLOXACIN (0.25MIC)
pigmented revertants decreased at similar rates between days 20 & 25. However, the decrease in the number of non-pigmented variants appeared to reach a plateau where reversion to the parental pigmented phenotype had slowed.

 Cumulatively, the total count decreased by less than 2 log units, the number of variants decreased by more than 2 log units, and the number of revertants increased by 0.75 log units over 25 days.

(b) Culture B (starved stationary phase population with 0.25 MIC ofloxacin)

When starved stationary phase culture of GRI 2677-8 was incubated at 30°C for 25 days in the presence of subinhibitory ofloxacin (0.25MIC), the following changes in total count, number of non-pigmented variants, and the pigmented revertants were observed (Figure 5.7b). During the first 15 days the total count and the number of non-pigmented variants both decreased by 1 log unit. The number of non-pigmented variants decreased more sharply during the first 5 days. However, the number of pigmented revertants increased by 0.8 log units during the first 5 days, followed by a slow decrease of 0.2 log units during the next 5 days, and further followed by a 1 log unit decrease. This period was demonstrated by an overall decrease in reversion. Between days 15 & 20 the total count and the number of non-pigmented variants both marginally decreased, and the number of pigmented revertants decreased further by 0.9 log units. This was consistent by a further reduction in reversion. The total count and the number of non-pigmented variants decreased by 1 log unit, and the number of pigmented revertants reached a plateau between days 20 to 25.

 Cumulatively, the total count decreased by 2 log units, the number of variants decreased by 3 log units, and the number of revertants initially increased by 0.8 log units and then decreased by 2 log units.

The effect of 0.25MIC ofloxacin on a starved stationary phase culture of GRI 2677-8

Comparison of cultures A and B show subinhibitory ofloxacin to influence the reversion process by an initial increase, followed by a 2 log unit decrease in the number of pigmented revertants. Although the reversion process was accelerated, the attained peaks of the number of pigmented
revertants were similar, and thereby demonstrated little effect. Therefore, in starved stationary phase subinhibitory ofloxacin clearly reduced reversion. However, under starvation conditions subinhibitory ofloxacin (0.25MIC) appeared to accelerate the death of revertants.

(c) Culture C (stationary phase population without 0.25MIC ofloxacin)

When a stationary phase culture of GRI 2677-8 was incubated at 30°C for 25 days, the following changes in total count, number of non-pigmented variants, and number of pigmented revertants were observed (Figure 5.8a). For the purpose of analysis, Figure 5.8a was divided into two separate time periods: 0-5 days and 5-25 days respectively. During the first 5 days, the total count increased by 0.7 log units, the number of non-pigmented variants decreased by 0.1 log units, and the number of pigmented revertants increased by 2.7 log units. Stationary phase (in this stationary phase, nutrients deprivation was not initially present as it was a broth culture) enhanced pigmentation which generated the increase in reversion from the non-pigmented to the respective parental pigmented phenotype. A noticeable feature of Figure 5.8a was at day 5 there were more pigmented revertants than non-pigmented variants, where the former contributed to the increase in total count. Between days 5 & 25, the total count decreased by 1.4 log units, the number of non-pigmented variants decreased by 1 log unit, and the number of pigmented revertants decreased by 1.7 log units. Throughout this period there were more cells with the parental pigmented phenotype. The pattern of decrease for total count, number of non-pigmented and pigmented cells were similar. The decline indicates the culture had entered the death phase through lack of nutrients.

Cumulatively, the total count initially increased by 0.7 log units and then decreased by 1.4 log units, the number of variants decreased by 1 log unit, and the number of revertants initially increased by 2.7 log units and then decreased by 1.7 log units.

(d) Culture D (stationary phase culture with 0.25MIC ofloxacin)

When a stationary phase culture of GRI 2677-8 was incubated at 30°C for 25 days in the presence of subinhibitory ofloxacin (0.25MIC), the following changes in total count, number of non-pigmented variants, and the number of pigmented revertants were observed (Figure 5.8b). During
Figure 5.8. Viability counts of population reversion of *stationary phase* culture of *S. marcescens* GRI 2677-8 incubated at 30°C for 25 days in isosensitest broth (Oxoid) in the presence and absence of subinhibitory ofloxacin (0.25MIC).

(a) without 0.25MIC ofloxacin, and (b) with 0.25MIC ofloxacin. • Total count; □ non-pigmented (p−) resistant variant; ▼ pigmented (p+) sensitive revertant.
(a) WITHOUT SUBINHIBITORY OF OXACIN (0.25MIC)

Days

cfu/ml

$10^5$ $10^6$ $10^7$ $10^8$ $10^9$ $10^{10}$
(b) WITH SUBINHIBITORY OFLOXACIN (0.25 MIC)
the first 5 days the total count increased by 0.3 log units, the number of non-pigmented variants
decreased by 0.1 log units, and the number of pigmented revertants increased by 1.7 log units.

Between days 5 & 25 the total count decreased by 1.2 log units, the number of non-pigmented
variants decreased by 1 log unit, and the number of pigmented revertants decreased by 1.5 log units.

Comparison with Figure 5.8a, demonstrates the total number of revertants at day 5 was 1 log unit
lower, which is consistent with ofloxacin inhibiting ofloxacin in a stationary phase culture. The
rate of decline for all groups were similar and the culture had entered the death phase through lack of
nutrients. Cumulatively, the total count initially increased by 0.5 log units and then decreased by
1.75 log units, the number of variants initially increased by 0.5 log units and then decreased by 1.75
log units, and the number of revertants initially increased by 1.5 log units and then decreased by 1
log unit.

The effect of 0.25MIC ofloxacin on a stationary phase culture of GRI 2677-8

Comparison of cultures C and D show that subinhibitory ofloxacin reduced reversion by
approximately 1 log unit. Ofloxacin also inhibited peak reversion, and this demonstrated its effect
on reversion in stationary phase.

Comparison of the effects of 0.25MIC ofloxacin under starved stationary phase and stationary
phase conditions

Although, both starved stationary phase and stationary phase show initial increases in
reversion, the increase in stationary phase was however approximately twice that observed in
starved stationary phase. This was followed by a decrease in reversion in both cultures.
However, in starved stationary phase, the decrease in reversion was twice that observed in
stationary phase. These results indicate that the reversion mechanism in starved non-dividing S.
marscens GRI 2677-8 cells is more susceptible to the effects of subinhibitory ofloxacin.
5.4. DISCUSSION

The spontaneous carbenicillin-resistant non-pigmented (p\(^{-}\)) variant of *S. marcescens* GRI 2677-8 reverted with high frequency to the carbenicillin-sensitive pigmented phenotype. Spontaneous reversion produced a diversity of pigmented variants, and included the red-pigmented (p\(^{+}\)), bright-pink (p\(\pm\)), pale-pink (p\(\mp\)), and non-pigmented (p\(^{-}\)) phenotypes, and this accords with previous findings (Eisenberg, 1914; Bunting, 1946; Labrum & Bunting, 1953; Platt et al., 1983).

Spontaneous reversion to the parental pigmented phenotype gave rise to a loss of resistance to the \(\beta\)-lactams carbenicillin and cefamandole, sulphonmethoxazole, the aminoglycosides, and unrelated antimicrobial agents including trimethoprim and chloramphenicol (Table 5.6). These antimicrobial agents are all structurally unrelated and possess different mechanisms of action. Resistance to these agents is thought to occur via decreased uptake through changes within the cell envelope (Traub & Fukushima, 1979). Both pigmentation and production of a chromosomally-mediated inducible \(\beta\)-lactamase have also been shown to be associated with the cell envelope (Platt et al., 1983).

Population reversion to the parental pigmented phenotype generated the pigmented variants pale-pink (p\(\pm\)) and bright-pink (p\(\mp\)), and comparison of their antibiotic patterns (Tables 5.6 & 5.7) show the pale-pink and bright-pink to be intermediate-phenotypes between the non-pigmented and pigmented phenotypes. The change in resistance to both carbenicillin and the aminoglycosides was observed to occur between the non-pigmented and pale-pink stage, and changes in resistance to sulphonmethoxazole occurred between the bright-pink and red-pigmented stage. Reversion to cefamandole sensitivity also occurred between the bright-pink and parental pigmented stage. Therefore, the 4 stages in the pigmentation process would suggest complex multistate mutational mechanisms may be involved. The first mutation involves changes in resistance to both carbenicillin and the aminoglycosides, and is coupled to a change in pigmentation (non-pigmented to pale-pink). The second mutation involved loss in resistance to sulphonmethoxazole, and further loss
of resistance to chloramphenicol and trimethoprim, and is also coupled to a change in pigmentation (change from bright-pink to red-pigmented). The third change involved loss of cefamandole resistance and is also coupled to a change in pigmentation (non-pigmented to parental red-pigmented). Loss in carbenicillin resistance was progressive with increased sensitivity from the p-phenotype, through the intermediate phenotypes, to the p+ phenotype. Similarly, the increase in aminoglycoside sensitivity was also progressive for these changes in pigmented phenotype. These observations are therefore consistent with both antibiotic resistance and pigmentation being co-regulated, since pigmentation resulted in loss of carbenicillin resistance, increased sensitivity to all the aminoglycosides, and unrelated agents that included chloramphenicol, sulphmethoxazole, and trimethoprim. It is noteworthy that *S. marcescens* is known to possess intrinsic permeability barriers to the β-lactams, and changes in the permeability barrier would account for these changes in resistance patterns. Therefore, the observed change in both pigmentation and antibiotic resistance during reversion would therefore suggest that this spontaneous mutation may be associated with structural changes in the cell envelope. This spontaneous mutation may occur in a regulator of the pigmentation enzymes, which themselves may be membrane-bound. One of the pigmentation enzymes that may be involved is the periplasmic condensing enzyme, which combines the MAP and MBC precursors to form prodigiosin. This is consistent since 2 mutations may account for the two intermediate changes in pigmentation. Thus, the observed changes in both pigmentation and antibiotic resistance presented here are in accordance with previous studies, since pigmentation and β-lactamase have been shown to be associated with the cell envelope (Platt *et al.*, 1983; Sykes & Matthew, 1976; Traub & Fukushima, 1979; Williams & Qadri, 1980).

The genetics and molecular basis of reversion is thought to involve a back-mutational event (Platt & Sommerville, 1981; Platt *et al.*, 1983), and as the pigment, prodigiosin, is bound to a glycoprotein complex within the cell envelope, then a structural mutation in either the pigmentation (*pig*) genes or their regulation may be involved. It is therefore proposed that the likely candidate is a Regulator of Antibiotic resistance and Pigmentation (*RAP*). This structural mutation also changes the
Changes in β-lactam and chloramphenicol resistance may be explained by a mutation in the *mar* locus. Similarly, the resultant changes in membrane permeability (through reduced *ompF* expression) may also influence periplasmic pigmentation. Further work will be required to investigate these possibilities.
permeability of the β-lactams, aminoglycosides, and other unrelated antibiotics through the cell envelope, and is therefore implicated with the changes in pigmentation, and β-lactam, aminoglycoside, and other antibiotic resistance.

The population distributions of numbers of IPP per colony varied between Poisson & Non-Poisson distributions. In growth matched GRI 2677-8 colonies, the increase in peptone concentration in the 5g/l and 12.5g/l + 0.1MIC subinhibitory ofloxacin demonstrated no significant difference from a Poisson distribution in the frequency of colonies with IPP. However, the 15g/l + 0.25MIC subinhibitory ofloxacin group differed significantly from a Poisson distribution. This suggests the Poisson distributions accord with probabilities for random events following a mutation, and that Non-Poisson are non-random. Similarly, an increase in peptone concentration, independent of the effects of subinhibitory ofloxacin, demonstrated Poisson at standardised peptone and Non-Poisson with increased peptone. These findings suggest either two RAP regulators RAPA & RAPB, or a single RAP regulator with two levels of control is involved with the Poisson and Non-Poisson distributions of colonies with IPP.

DNA rearrangement by illegitimate recombination has also been described as a possible mechanism for the occurrence of spontaneous reversion mutation (Platt et al, 1983). This high degree of genetic instability suggests DNA rearrangements result from a change in DNA structure through either transposons, inversions, or deletions of DNA involving an illegitimate recombination events (Shapiro, 1983). DNA inversion as observed with transposition may result from the inversion of a piece of DNA which alternates the position, and thus expression of both the pigmentation and resistance. However, the generation of intermediate phenotypes would contradict this eventuality, since two mutational mechanisms are strongly suggested. Platt et al (1983) demonstrated the reversion process during growth involves a back-mutation, and since the reversion process (with the exception of the [peptone 15g/l + 0.25MIC ofloxacin] generally fits a Poisson distribution, the findings presented here would further suggest the spontaneous reversion occurs via a back-mutation. The reversion rate appears to be more susceptible to the effects of subinhibitory ofloxacin in starved
stationary phase, and this would suggest that in starved GRI 2677-8 cells, the reversion mechanism is different to that of cells in stationary phase.

Viability counts during spontaneous reversion of starved stationary phase and stationary phase cultures of *S. marcescens* GRI 2677-8 (in the presence and absence of 0.25MIC ofloxacin) showed population reversion in a starved stationary phase culture accords with previous findings (Platt et al, 1983). In starved stationary phase, subinhibitory ofloxacin (0.25MIC) produced a step-wise change in reversion, with an initial increase in reversion followed by a significant decrease (Figure 5.7b). Population reversion in a stationary phase culture similarly demonstrated a step-wise change in reversion (Figure 5.8b). However, the increase and subsequent decrease was much more pronounced in the starved stationary phase culture. Both these results suggest that subinhibitory ofloxacin 0.25MIC) affects the reversion process by two mechanisms (a) a initial potentiation of reversion followed by (b) suppression of reversion. The pronounced suppression of reversion in starved non-dividing cells provides further evidence that synthesis of prodigiosin occurs in the late stages of growth. This further suggests that both growth and reversion are closely associated. The results presented here substantiate this, since subinhibitory ofloxacin significantly reduced (p<0.001) the number of IPP per colony and therefore intracolonial reversion in growth matched colonies. In addition, since the growth conditions were adjusted to obtain independence of reversion from growth rate, IPPs in the presence of subinhibitory ofloxacin must result from a direct effect on the reversion mechanism. Furthermore, since the initial mutation is pleotropic, this would also suggest the RAP regulators for antibiotic resistance and pigmentation are the most plausible candidates. The mutagenic potential of 4-quinolones have been described (Hirai et al, 1986a; Phillips, 1987; Hooper et al, 1992; Mamber et al, 1993). Quinolones interact with DNA gyrase and DNA in a manner that results in the induction of the recA SOS DNA repair system. This repair system is error prone and may therefore increase the frequency of bacterial mutations (Phillips, 1987). Although, the potential for mutagenicity is low, nevertheless there is evidence that links 4-quinolones as weak mutagens (Phillips, 1987). It has been reported that the bacterial DNA-
reactive and mutagenic properties of subinhibitory concentrations of quinolones promote increased resistance to several structurally unrelated antimicrobial agents (Mamber et al, 1993). In this context, ofloxacin could be classified as a weak mutagen on the basis of its interference with pigmentation, thereby suggesting a direct genetic effect.
This study focused on the effects of subinhibitory ofloxacin on three different genetic processes among the Enterobacteria. These included (1) the insertion of Tn7 into the IncP R-plasmid RP4, (2) the stability of the R-plasmid pOG669, and (3) the spontaneous reversion mutation of *Serratia marcescens* GRI 2677-8. The overall conclusion was that subinhibitory ofloxacin altered each of these genetic processes. In each of the systems studied the genetic events took place in the absence of ofloxacin, and this allowed the effects of ofloxacin to be evaluated statistically. This approach highlighted quantitative differences in the effects of ofloxacin. Nevertheless, a number of results obtained in one system appear anomalous when compared to another. For example, subinhibitory ofloxacin suppressed reversion in *Serratia marcescens* GRI 2677-8, increased instability of pOG669, and altered the site of Tn7 insertion. It might be argued that ofloxacin indirectly alters the degree of negative supercoiling throughout, but that the systems of transposition mutation and rearrangement have evolved in an environment where DNA supercoiling is constant or at least host-controlled. Alteration of this results in different native cellular processes responding in different ways. Perhaps ofloxacin is equivalent to a chaotropic agent? One of the benefits of examining 3 different processes is that it gives an overview. Thus anomalies not recognised in a more focussed approach become evident and allow the formulation of more precise experimental questions for future investigation.

Subinhibitory ofloxacin significantly altered the pattern of Tn7 insertion into multiple sites in the IncP R-plasmid RP4, and regions of RP4 were therefore found to be either more or less susceptible to insertion of Tn7. However, in *stationary phase*, no new Tn7 transposition events occurred and subinhibitory ofloxacin had no effect. Similarly, the transposition immunity of Tn7 was not affected. This suggested the mechanism of transposition for Tn7 required active chromosomal and RP4 DNA replication during active growth.

Previous studies have shown that topoisomerase I and DNA gyrase are involved in the mechanism of transposition of Tn5 (Stemglanz *et al*, 1981; Isberg & Syvanen, 1982), and that both these enzymes homeostatically control DNA supercoiling (Menzel & Gellert, 1983). Therefore, the
degree of negative DNA supercoiling may be important for the mechanism of transposition. Similarly, the level of DNA superhelicity may be required in stable maintenance of plasmids, and gene expression of pigmentation and antibiotic resistance in *S. marcescens*.

The insertion of Tn7 occurred in several regions of the RP4 molecule, and subinhibitory ofloxacin altered insertion into these regions. Most notable was the increase in insertion into the 22.2kb *KpnI-Smal* fragment, and the decrease in insertion into the 5kb *KpnI-Smal* fragments. For subinhibitory ofloxacin to have exerted changes in insertion, a structural change in the tertiary structure of RP4 would explain these regions to be of different superhelical density. Therefore, alteration in the degree of supercoiling would affect the number of available insertion sites within regions of RP4 DNA. The increase in insertion of Tn7 in the 22.2kb fragment suggested subinhibitory ofloxacin to increase the exposure of Tn7 insertion sites to Tn7, and the decrease in insertion in the 5kb fragment further suggested a reduction in the number of available sites. Ofloxacin specifically inhibits DNA gyrase, and a result of this inhibition, would be to alter the homeostatic balance of negative supercoiling. It is plausible that these regions may contain DNA gyrase binding sites, and their inhibition would contribute to a change in supercoiling, and therefore alter the exposure of Tn7 insertion sites through structural changes within RP4 DNA. This would suggest the topology of target DNA is an important prerequisite for Tn7 insertion. Further investigation would be required to examine this possibility.

Subinhibitory ofloxacin significantly eliminated the co-integrate R-plasmid pOG669 in *stationary phase*, and significantly reduced elimination in *maintenance subculture*, in *E. coli K12 J62-1*. Complete pOG669 elimination occurred in *repeated subculture*. These three different environments altered the stable maintenance of pOG669 with elimination increasing from RSOF>SOF>MSOF. In *maintenance subculture* the higher level of pOG669 stability may be associated with naf^-mediated induction of the *recA* system which reduces plasmid elimination (Drlica, 1984; Hooper *et al.*, 1984). The elimination of pOG669 in both *stationary phase* and *repeated subculture* suggests that an error in plasmid partition may be responsible. In repeated
subculture the rate pOG669 elimination was much greater and this further suggests the rapid loss of pOG669 occurs through an abrupt change in plasmid stability. However, in stationary phase subinhibitory ofloxacin increased both elimination and molecular variation of pOG669. This clearly confirms ofloxacin to have a mechanism of action on non-dividing E. coli. Further evidence of pOG669 instability was demonstrated with the production of two variants. Kanamycin sensitive variants (KSV) resulted from a junctional deletion that produced loss of Km^6, and mixed population variants (MPV1 and MPV2) resulted in marked plasmid instability that produced two mixed populations. MPV must have occurred through a error-prone process that resulted in reduced partition of pOG669 into the two populations. Most KSVs were isolated in stationary phase and several DNA rearrangements were observed, which apart from loss of kanamycin resistance (pOG670) were mostly associated with the pOG660 component of the co-integrate.

pOG669 was clearly unstable in stationary phase with both elimination and the generation of variants occurring, and pOG669 elimination occurred most in repeated subculture. Subinhibitory ofloxacin increased the elimination of pOG669 through a recognised mechanism when the GyrA subunits of DNA gyrase are inhibited, and the resultant unsealed breaks act as signals for the induction of exonucleases leading to rearrangements of pOG669 DNA (Crumplin & Smith, 1975). Therefore, an explanation for this instability may involve the production of starvation-induced proteins and include exonucleases, which are activated through the SOS DNA repair system. This error-prone repair system may lead to either the loss of pOG669 or the generation MPV and KSV. The generation of both MPV and KSV were independent of subinhibitory ofloxacin effects, and they themselves arise through junctional instability in non-dividing cells.

Spontaneous reversion in stationary phase and starved-stationary phase (non-dividing cells) from the non-pigmented variant to the parental pigmented revertant was reduced in the presence of subinhibitory ofloxacin. The reversion process involved the reversion of the non-pigmented phenotype, with the formation of several intermediate phenotypes, to the parental pigmented phenotype. This was coupled to change in antibiotic resistance. Both pigmentation and antibiotic
transposon-mediated resistance. The spontaneous reversion involves a structural back-mutation in the cell envelope (Platt et al., 1983), which may involve a change in the superhelicity of DNA. A change in the degree of negative supercoiling may alter gene expression as observed with both changes in antibiotic resistance and pigmentation. In all three processes, subinhibitory ofloxacin was found to alter the expression, location, and mobility of unrelated resistance determinants. In the clinical environment these findings are important since a drug at subinhibitory concentrations that alters several genetic processes including antibiotic resistance may have important implications. It is well recognised that the 4-quinolones eliminate susceptible plasmids (Wiesser & Wiedemann, 1986) and in this respect pOG669 is susceptible. pOG669 is both ampicillin and kanamycin resistant, and when both are lost through plasmid elimination, the plasmid pool for antibiotic resistance is reduced. An interesting feature of this elimination is that pOG669 is a natural co-integrate R-plasmid that has fused an R-plasmid (pOG670) with pOG660, that had originated in Salmonella typhimurium. pOG660 encodes for virulence, and pOG670 encodes Ap & Km resistances. Therefore, when pOG669 is eliminated, not one, but two plasmids are lost, both of which code for potential problems in the clinical environment. Tn7 also encodes antibiotic resistances (Tp & Sm) and subinhibitory ofloxacin altered both the location and mobility of these resistances. Therefore, here we have a subinhibitory antibiotic that has the potential to alter the transmission of plasmid-mediated and transposon-mediated resistance.

However, there is the possibility of changing the pattern of an infection through sub-MIC effects on the normal commensal flora. In such a situation the balance between pathogenic and non-pathogenic may change. For example, during antibiotic therapy, sites in the body that are peripheral to the site of infection, maybe exposed to sub-MIC levels through, for example, different tissue kinetics. Commensals in these sites may either develop resistance or undergo one of the genetic changes described in this study. Therefore, it may be appropriate for patients on quinolone therapy to be monitored for any changing patterns of resistance to unrelated antibiotics.
The generation of molecular variation through DNA rearrangements were commonly observed in this study. RP4::Tn7 derivatives after Tn7 insertion were isolated, junctional deletions lead to the loss of kanamycin resistance in KSVs, and pigmented variants in S. marcescens were isolated. These findings were also similar in one respect, they all occurred in stationary phase. It is proposed a possible 2 common mechanisms are involved. The first is activation of katF, and the second is alteration in the superhelical density of DNA. It is known that certain proteins are induced during the onset of stationary phase (Groat et al, 1986) and these enable bacteria to adapt to conditions of nutritional deprivation. The katF has been shown to regulate several activities that appear in stationary phase (Bohannon et al, 1991). One of which is the production of exonuclease III (Sak et al, 1989) which may be responsible in part for the observed DNA rearrangements. These DNA rearrangements lead to the generation of KSV. Subinhibitory ofloxacin also altered the availability of Tn7 insertion sites in RP4, and reduced the spontaneous reversion mechanism in S. marcescens. Both these processes are involved with how the regulation of negative supercoiled DNA is controlled. Inhibition of DNA gyrase binding sites alters the insertion of Tn7, and gene expression in S. marcescens leading to variants in pigmentation and antibiotic resistance. DNA supercoiling is thought to interact with transcription and it has been suggested that supercoiling enhances expression from some genes (Rouviere-Yaniv et al, 1991). It would therefore seem plausible that subinhibitory ofloxacin alters the optimal levels of supercoiling for transcription and this may vary from one gene to another (Brahms et al, 1985). This may then lead to gene expression that may be increased, decreased, or unchanged. Such changes in gene expression may lead to alteration, in sites available for Tn7 insertion in RP4, and the regulation of pigmentation and antibiotic resistance in S. marcescens.

The overall results and conclusions of this study are of benefit and concern, as subinhibitory ofloxacin has altered the the stable maintenance of plasmids in terms of their elimination, which is of benefit to medical practice, since a reduction in the number of R-plasmids in an infecting bacterial population will reduce the transmission of antibiotic resistance. Of concern, is the mutational
action of subinhibitory ofloxacin, which although, alters the the location and mobility of Tn7, it nevertheless did not prevent transposition.

**Avenues for future investigations**

The data presented here is for Tn7, and it would therefore seem appropriate to investigate the effects of several subinhibitory concentrations of ofloxacin on other transposons, particularly one from each class. Further investigations would also establish if the insertion site changes observed, is a phenomenon unique to IncP plasmids, or is this a general mechanism of action for ofloxacin on the insertion of transposons into their target site DNA. This would encompass investigation of other plasmid incompatibility groups. Of further interest would be to investigate if subinhibitory ofloxacin influences the insertion of Tn7 to the specific attTn7 site using vector that contains Tn7.

It is well established that 4-quinolones eliminate susceptible plasmids from their bacterial hosts. It would be of interest to investigate the effect of further subinhibitory concentrations of ofloxacin which could be used to determination plasmid elimination kinetics. What is not known, with so many deletions recognised in the pOG660 component, is the effect on the virulence region? If deletions have occurred in this region, then a reduction in plasmid encoded virulence would play a significant role in the bacterial pathogenesis. Similar experiments that would utilise the nal^S^ strain *E. coli* K12 J62-2 could establish whether nal^S^ is indeed important in activation of the rec system. In addition, the stability of pOG669 could also be investigated in a nal^F^ recA^- strain. In fact, preliminary studies have shown pOG669 to be generally stable in this strain, although detailed investigations have not been carried out. Given that junctional fragment were involved it would be possible to sequence and clone these fragments into a range of appropriate vectors to study the specific effect of the junctional sequence in a more defined system.

The system used, relied on a change in sensitivity at the outset. However, from the data obtained it might be possible to select a particular time and study unselected colonies. This would be essential to determine whether smaller deletions across the junctions without effect on Km^F^.
The demonstration that subinhibitory ofloxacin produced a significant decrease in colonial reversion as shown by pronounced left shift in the distribution of IPPs indicates that the system could be developed to provide an assay to assess the mutagenic activity of antimicrobial agents under development. Whereas the Ames test (Ames et al., 1975) uses a back-mutation of single base changes, this reversion is clearly part of a more complex system and may therefore provide additional information. However, at present no attempt has been made to clarify the mechanism in detail. Although this study has extended the information available on GRI 2677-8, the next step would be to investigate the mechanism. The results indicated that it is clearly not a simple back-mutation because of the range of intermediate revertants observed and by inference the primary event (generation of GRI 2677-8 from GRI 2677) must have been a complex rearrangement. Systematic analysis of the molecular differences between parent and variant would provide not only a more solid foundation for the development of the proposed assay system, but may also provide a useful model for the study of genomic evolution. In the latter regard the visibility of pigmentation variation without the requirement for artificial selection such as antibiotics is of considerable benefit.

The first step might be comparison of restriction fragmentation patterns between parent and variant followed by cloning, sequencing and Southern Hybridization of fragments that differ between the two. Further work on the genetic processes that lead to changes in the regulation of pigmentation and antibiotic resistance should incorporate analysis of the pig genes and RAP regulator(s), which would provide a better understanding of the physiological basis of the control of prodigiosin formation. This would involve establishing those physiological factors that would interact with the expression of both genes.

It is not known if S. marcescens GRI 2677-8 has a functional mar gene, and the use of Polymerase Chain Reaction (PCR) and DNA sequencing techniques would establish this one way or the other. If GRI 2677-8 is a marA mutant, then this would also explain the observed changes in β-lactam and chloramphenicol resistance.


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