Molecular Interactions Between DNA gyrase and the Quinolone Antibacterials

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Abstract

DNA gyrase is an essential bacterial topoisomerase with a central role in many cellular processes. Its lack of homology to eukaryotic topoisomerases has been the basis of its role as an important drug target. The quinolone antibacterials have previously been shown to "poison" DNA gyrase of *Escherichia coli*, resulting in rapid bacterial cell death by a pathway which has been a subject of controversy. Following on from the finding that the complex of quinolone and gyrase on DNA results in truncated mRNAs *in vitro*, the results presented in this thesis show that *in vivo*, the addition of quinolone drugs results in the production of truncated mRNAs which are presumably translated into truncated proteins. This is expected to cause total deregulation of cellular processes and result in cell death.

It has also been shown that the newer more potent quinolone drugs such as ciprofloxacin and ofloxacin have an additional effect on the bacterial cells in that the chromosome is broken down into fragments of DNA, some of which are estimated to be as small as 4kb in length. This process appears similar to the degradation of chromosomal DNA that occurs during apoptosis of some eukaryotic cells.

The acquisition of quinolone-resistance in *E. coli* and *Salmonella* has also been investigated. Firstly, part of *gyrA* of *S.typhimurium* NCTC5710 was sequenced and found to be 94% homologous at the nucleotide level to the corresponding sequence of *E.coli*. Following on from this, clinical isolates were screened by DNA sequencing and amino acid mutations in the "quinolone resistance determining region" (QRDR) of *gyrA* identified. Mutations in *gyrA* such as

serine-83 to leucine, serine-83 to phenylalanine, aspartate-87 to tyrosine, aspartate-87 to asparagine and aspartate-87 to glycine were found to have arisen in the QRDR of *gyrA* in common with other quinolone resistance mutations previously found.

It has been shown that high levels of *gyrA* are toxic to *E. coli*, probably as a result of loss of homeostatis of the supercoiling of the bacterial chromosome when large levels of DNA gyrase are present. This result was firstly inferred by the ability to clone *gyrA* into a low copy number plasmid but not into a high copy number plasmid. Cloning of *gyrA* into a vector in which the copy number could be altered confirmed that high level expression of *gyrA* is detrimental to the cell.

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Declaration

The research described in this thesis is the sole work of the undersigned author unless otherwise stated.

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Publications to date

J.C.Brown, P.M.A.Shanahan, M.V. Jesudason, C.J.Thomson and S.G.B.Amyes (1996) Mutations responsible for reduced susceptibility to 4-quinolones in clinical isolates of multi-resistant *Salmonella typhi* in India. *Journal of Antimicrobial Chemotherapy* **37** p891-900.

J.C.Brown, C.J.Thomson and S.G.B. Amyes (1996) Mutations in the *gyrA* gene of clinical isolates of *Salmonella typhimurium* and three other *Salmonella* spp. leading to decreased susceptibilities to 4-quinolone drugs. *Journal of Antimicrobial Chemotherapy* **37** p351-356.

J.C.Brown and S.G.B. Amyes (1996) (In Press) Molecular Detection of Quinolone Resistance in Methods in Molecular Medicine Eds. N. Woodford and A. Johnson

Presentations

J.C.Brown, C.J.Thomson and S.G.B.Amyes (1995) Poster presentation at the 3rd Tripartite meeting, Scottish Microbiology Association, Kilkenny, Eire. Mutations in the *gyrA* gene of *Salmonella typhi* associated with 4-quinolone resistance

J.C.Brown, C.J.Thomson and S.G.B.Amyes (1995) Poster presentation at the 7th European Congress of Clinical Microbiology and Infectious Diseases,Vienna, Austria. Mutations in the *gyrA* gene of *Salmonella typhi* associated with 4-quinolone resistance

J.C.Brown, P.M.A.Shanahan, M.V.Jesudason C.J.Thomson and S.G.B.Amyes. Slide presentation at the 35th Interscience Congress of Antimicrobial Agents and Chemotherapy, San Francisco 1995. Mutations in *gyrA* responsible for quinolone resistance in multi-resistant *Salmonella typhi*: an emerging therapeutic problem?

J.C.Brown, C.J.Thomson and S.G.B.Amyes. Slide presentation at the 35th Interscience Congress of Antimicrobial Agents and Chemotherapy, San Francisco 1995. False positive results in identification of quinolone-resistant mutations in the *gyrA* gene of *Escherichia coli* by *Hinf*I restriction

J.C.Brown, C.J.Thomson and S.G.B.Amyes. Poster presentation at the 96th American Society of Microbiology General Meeting, New Orleans 1996. The relationship between expression of *gyrA* from a plasmid and the minimum inhibitory concentration of quinolone drugs in quinolone-resistant *Escherichia coli*.

1 Introduction

1.1 An overview

In 1963, with the advent of electron microscopy, Cairns (1963) showed that the *Escherichia coli* chromosome is circular. From this microscopic data, it was inferred that a DNA "swivelling" mechanism had to be present in bacterial cells to separate the two strands of DNA prior to replication. Since then enzymes with this kind of activity, called topoisomerases, have been identified and extensively studied and found to be essential components of all known cells. Bacterial DNA topoisomerases allow DNA replication to proceed by unwinding the DNA double helix and also these enzymes have implicit roles in DNA recombination, decatenation, unknotting, transposition and transcription and hence gene expression (Reece and Maxwell, 1991b). Current interest in DNA topoisomerases stems from the fact that they play a critical role in DNA maintenance and are the primary cellular targets for some of the most widely prescribed antibiotics and anticancer drugs used in the treatment of human diseases (Bodley and Liu, 1988).

On studying the integrative recombination of bacteriophage λ in an *E.coli* derived cell free system, Gellert *et al* (1976) demonstrated that a negatively supercoiled DNA substrate was required for phage integration. It was also found that this negatively supercoiled DNA could only be exchanged for a relaxed DNA substrate if an *E.coli* cell fraction was added along with ATP (adenosine triphosphate) to the cell free system (Gellert *et al*, 1976). The energy dependent enzyme present in this cell fraction with a negative supercoiling activity was named DNA gyrase, and it became the prototype of a new class of

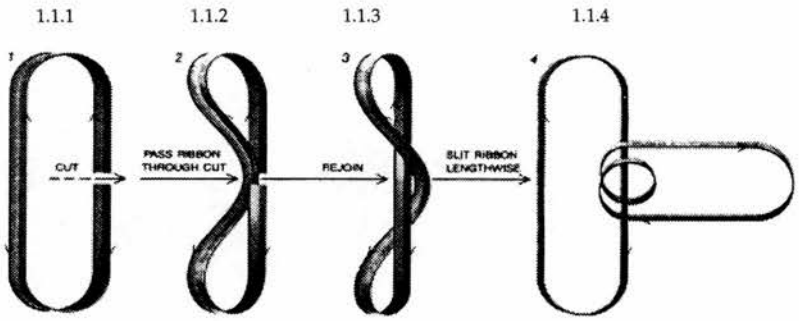
topoisomerase enzymes that possessed the unique ability to catalyse the introduction of negative supercoil turns into closed circular double stranded DNA. Although all known DNA topoisomerases in eukaryotes and prokaryotes are able to relax negatively supercoiled DNA, only bacterial DNA gyrase can introduce negative supercoils into DNA by an energy dependent process.

1.2 DNA Structure, Supercoiling, and Topology

To appreciate fully the roles of topoisomerases, it is necessary to understand their mechanism of action at a molecular level (for a review see Wang, 1982). Topoisomerases change the linking number between two intertwined single-stranded DNA rings, that is, the number of times the two strands of a DNA duplex circle are interwound.

The mechanism by which the linking number of a DNA duplex is changed is illustrated in Figure 1.1, with the double-stranded DNA represented as a circular ribbon and the two anti-parallel strands shown by the two edges of the ribbon. In the first diagram 1.1.1, the two strands are unlinked, therefore the linking number (Lk) is zero. The action of topoisomerases is demonstrated in 1.1.2 with the introduction of a temporary double-stranded break in the duplex which is then resealed after the passage of another double-stranded segment through it. In this nick-resealing mode of action, the linking number changes from 0 to +2. The linking number of a duplex is a topological invariant up until the point that the rings are no longer intact. The name given to identical rings differing only in their linking numbers is topoisomers and these structures can be distinguished by gel electrophoresis. The linking number of a DNA duplex at its most stable conformation is known as Lk_0, however unlike Lk, Lk_0 is not a topological invariant as its value is only fixed if the conditions specifying the most stable state are unchanged.

Figure 1.1: The mechanism by which the linking number of a DNA duplex is changed (Taken from Wang, 1982).



If the linking number deviates increasingly from Lk_0, the duplex becomes more strained, accumulating deformations and distortions of the DNA to create a topological form of DNA known as supercoiled DNA. Supercoiled DNA with a linking number greater than Lk_0 is known as positively supercoiled whereas supercoiled DNA with a linking number less than Lk_0 is negatively supercoiled. Some of the ways that negatively supercoiled DNA can become distorted and an electron micrograph of relaxed and supercoiled DNA are shown in Figure 1.2 and Figure 1.3 respectively.

Figure 1.2: Some of the ways that negatively supercoiled DNA can become distorted (Taken from Wang, 1982).

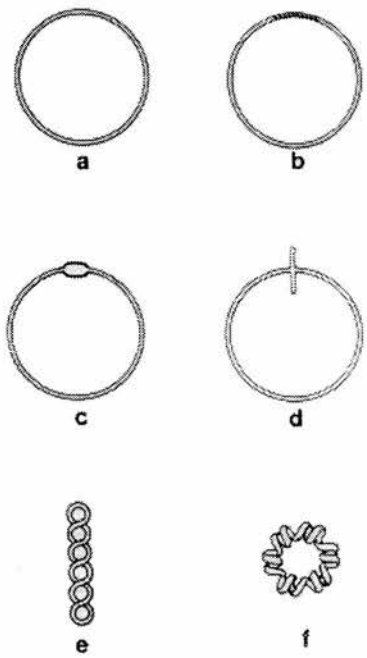
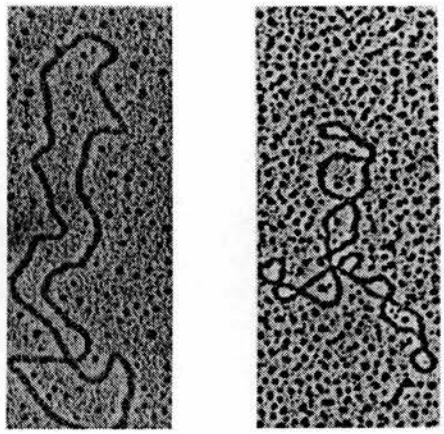


Figure 1.3: Electron micrograph of relaxed and supercoiled DNA (Taken from Wang, 1982).



1.3 Type II DNA topoisomerases

Type II DNA topoisomerases characteristically change the DNA topology by introducing a break in both DNA strands and changing the linking number of DNA in steps of two, thus differing from the action of type I topoisomerases which change the linking number in steps of one. Type II topoisomerases are essential cellular proteins and temperature-sensitive mutations in their genes have been shown to result in cell death at the restrictive temperature (Kato *et al*, 1990; Kato *et al*, 1988) whereas type I topoisomerases are dispensable and their loss is compensated for by mutations in the genes for the type II topoisomerases causing a decrease in supercoiling efficiency (DiNardo *et al*, 1982). All topoisomerases are by definition able to change the topology of DNA, through breakage and reunion of DNA strands, however each enzyme has a different activity. The two type I topoisomerases present in *E.coli*, topoisomerase I and topoisomerase III relax and decatenate DNA respectively in the absence of ATP (Wang, 1971; DiGate and Marians, 1988) whereas the two type II topoisomerases in *E.coli*, gyrase and topoisomerase IV preferentially supercoil and decatenate the DNA respectively (Adams *et al*, 1992) in an energy dependent fashion.

Type II topoisomerases are ATP-dependent, and function as dyadic molecules. In bacteria, two or more polypeptide chains form each half of the topoisomerase whereas in eukaryotes, identical polypeptide chains, ranging from 160 to 180kDa form the two halves of the enzyme. In *E.coli*, two distinct type II topoisomerases exist: DNA gyrase and DNA topoisomerase IV. These two enzymes have clear and defined functions in the cell: whilst gyrase removes positive supercoils and introduces negative ones, it can also unlink knots and catenanes generated by recombination. However, gyrase does not play a major role in unlinking catenanes formed during replication, and this function is predominantly the responsibility of topoisomerase IV (Adams *et al*, 1992). Considering that there is noticeable structural homology between gyrase and topoisomerase IV (see 1.3.4), it is interesting that the two enzymes have clearly defined roles in the cell with minimal functional overlap.

1.3.1 Biochemistry of DNA gyrase

Biochemical investigations have shown that DNA gyrase is formed from two different subunits called GyrA and GyrB (Klevan and Wang, 1980). Sugino *et al* (1977) demonstrated that the ratio of these two subunits was equimolar by mixing different amounts of the two gyrase subunits with DNA before separating the DNA-protein complex from free protein by gel filtration. These gyrase-DNA complexes were found to consist of two homodimers of the A and B proteins of size 97kDa and 90kDa respectively (Swanberg and Wang, 1987; Yamagishi *et al*, 1986). A brief summary of some of the properties of GyrA and GyrB of *E.coli* is given in Table 1.1.

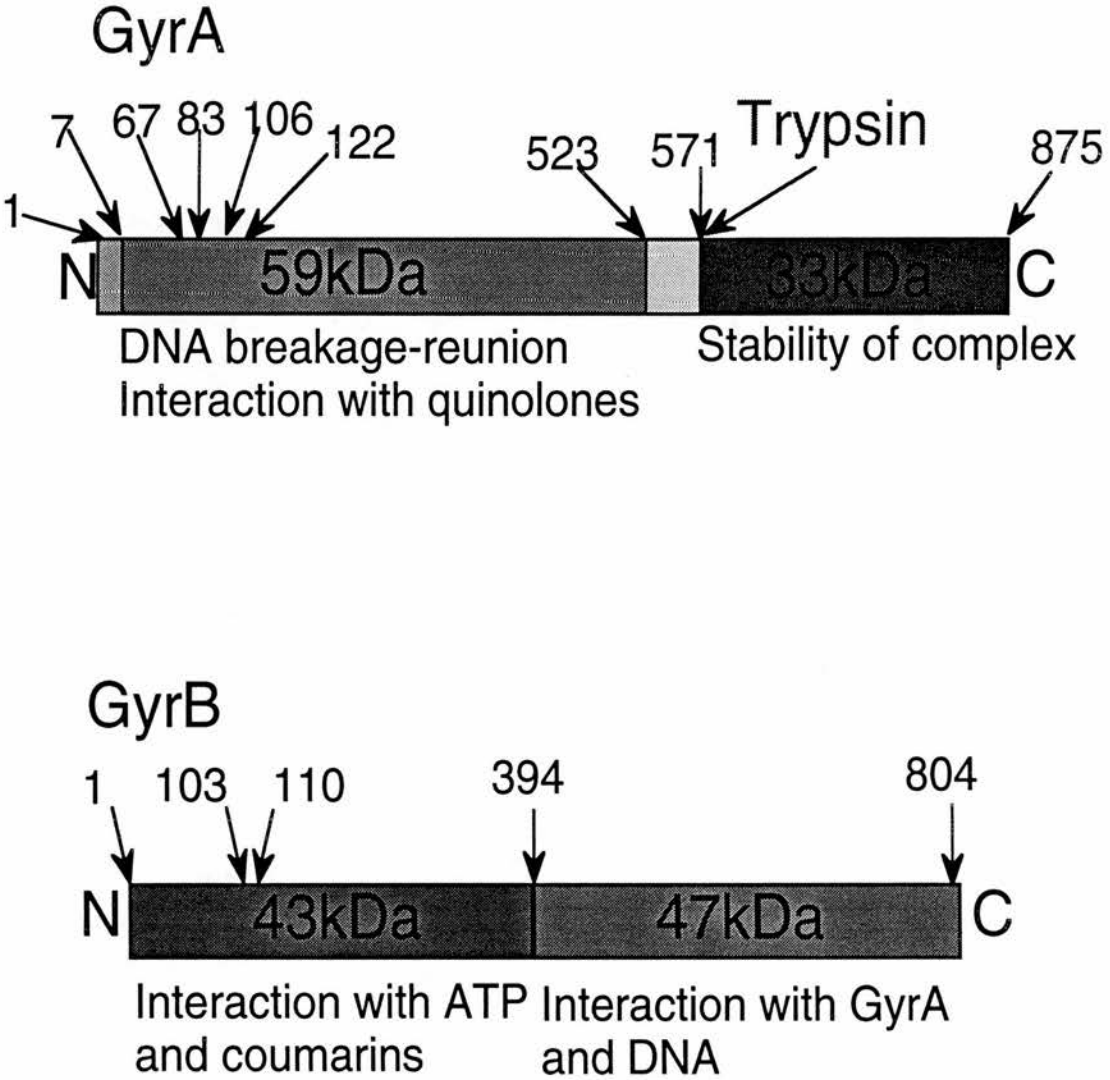
Table 1.1: Properties of *E.coli* GyrA and GyrB

	GyrA	**GyrB**
Gene	*gyrA*	*gyrB*
Length of gene	2625bp	2412bp
Molecular weight	96887	89893
Amino acids	875	804
Function	Breakage and reunion of DNA	ATPase activity
Drug interactions	Quinolone drugs	Coumarin drugs

The GyrA subunit has been shown to comprise of two discrete domains with different functional and structural features. Treatment of the A subunit of *E.coli* DNA gyrase with trypsin yields two stable fragments, one of 64kDa and one of 33kDa (Reece and Maxwell, 1989; Reece and Maxwell, 1991a; Reece and Maxwell, 1991c) as shown in Figure 1.4. These two domains have been cloned and their products investigated biochemically. The supercoiling reaction of DNA gyrase requires both subunits, studies of partial reactions, protein structure

and the effects of inhibitors have shown that specific functions can be assigned to the two subunits as shown below.

Figure 1.4: Domain model showing the two subunits of *E.coli* DNA gyrase. Amino acid positions are numbered and the various domains represented. Adapted from Reece and Maxwell, 1991b.



GyrA

The 64kDa N-terminal fragment of GyrA was found to be necessary for DNA supercoiling, covalent attachment to DNA (Horowitz and Wang, 1987) and interfacing with GyrB and was thus concluded to be the DNA breakage-reunion domain of the A subunit (Reece and Maxwell, 1989). The quinolone drugs which interfere with DNA strand passage act upon this region of GyrA (Yoshida *et al*, 1988; Cullen *et al*, 1989; Hallett and Maxwell, 1991). In *in vivo* assays, the 64kDa N-terminal fragment was found to be able to carry out DNA cleavage and religation (Reece and Maxwell, 1989), however DNA supercoiling was found to proceed more efficiently in the presence of the 33kDa fragment (Reece and Maxwell, 1991c). Truncated versions of this polypeptide were constructed by introducing stop codons into the DNA sequence of the cloned fragment (Reece and Maxwell, 1991a). It was thus found that the first six amino acids of the N-terminal fragment, amino acids 1 to 6, can be deleted with no apparent deleterious effect on the breakage-reunion reaction with DNA (Reece and Maxwell, 1991a). However the breakage-reunion reaction is completely abolished by removing the first 69 amino acids of the GyrA subunit (Reece and Maxwell, 1991a). Experiments have shown that the smallest GyrA section capable of breakage and reunion is the region encoded by amino acids 7 to 523 (Reece and Maxwell, 1991a). All the catalytic activity of GyrA is believed to be contained in this 64kDa fragment.

Investigations into the 33kDa C-terminal fragment indicated no enzymatic activity (Reece and Maxwell, 1991c). Instead this region of GyrA has been shown to possess a DNA-binding activity and is thus thought to stabilise the gyrase-DNA complex (Reece and Maxwell, 1989). By generating a clone which overproduced the 33kDa subunit, it was found that this polypeptide could form

a complex with the 64kDa protein and also the GyrB subunit (Reece and Maxwell, 1991c). It was also shown that the 33kDa protein is involved in the wrapping of DNA around gyrase and this stabilises the complex by acting as a non-specific DNA-binding protein (Reece and Maxwell, 1991c).

GyrB

On purification of GyrB, a truncated version of the protein named topoII' was commonly found and further investigations identified it as the C-terminal half of the GyrB subunit (from amino acid 394 to the end) which probably resulted from proteolytic cleavage during cell lysis (Brown *et al*, 1979; Gellert *et al*, 1979; Adachi *et al*, 1987). This fragment was found to be able to relax and cleave DNA in conjunction with GyrA but could not negatively supercoil DNA (Brown *et al*, 1979; Gellert *et al*, 1979) and further studies showed that the C-terminal GyrB fragment interacted with GyrA (Brown *et al*, 1979). The rest of the subunit (amino acids 1 to 393) was studied by inserting premature stop codons into the middle of the gene resulting in a protein that had ATPase activity as well as containing a binding site for ATP (Jackson *et al*, 1991; Ali *et al*, 1993). These results confirmed the findings of Adachi *et al* (1987) who showed that the N-terminal domain of GyrB included the site of ATP hydrolysis and is the region where coumarin drugs such as novobiocin and coumermycin A1 interact (Ali *et al*, 1993). In conclusion, it is the GyrB N-terminal fragment that confers the ability of the enzyme to negatively supercoil DNA.

A summary of reactions carried out by GyrA and GyrB is displayed in Table 1.2.

Table 1.2: Reactions of DNA gyrase

	Subunits required	ATP required	Inhibited by quinolones	Inhibited by coumarins
Supercoiling	A +B	Yes	Yes	Yes
Relaxation of:				
a)Negative supercoils	A+B	No	Yes	No
b)Positive supercoils	A +B	Yes	Yes	-
Catenation	A+B	Yes	Yes	Yes
Decatenation	A+B	Yes	Yes	-
Unknotting	A+B	Yes	Yes	Yes
DNA cleavage	A+B	No	No	No
ATPase	B	Yes	No	Yes

1.3.2 Crystallographic investigations into DNA gyrase

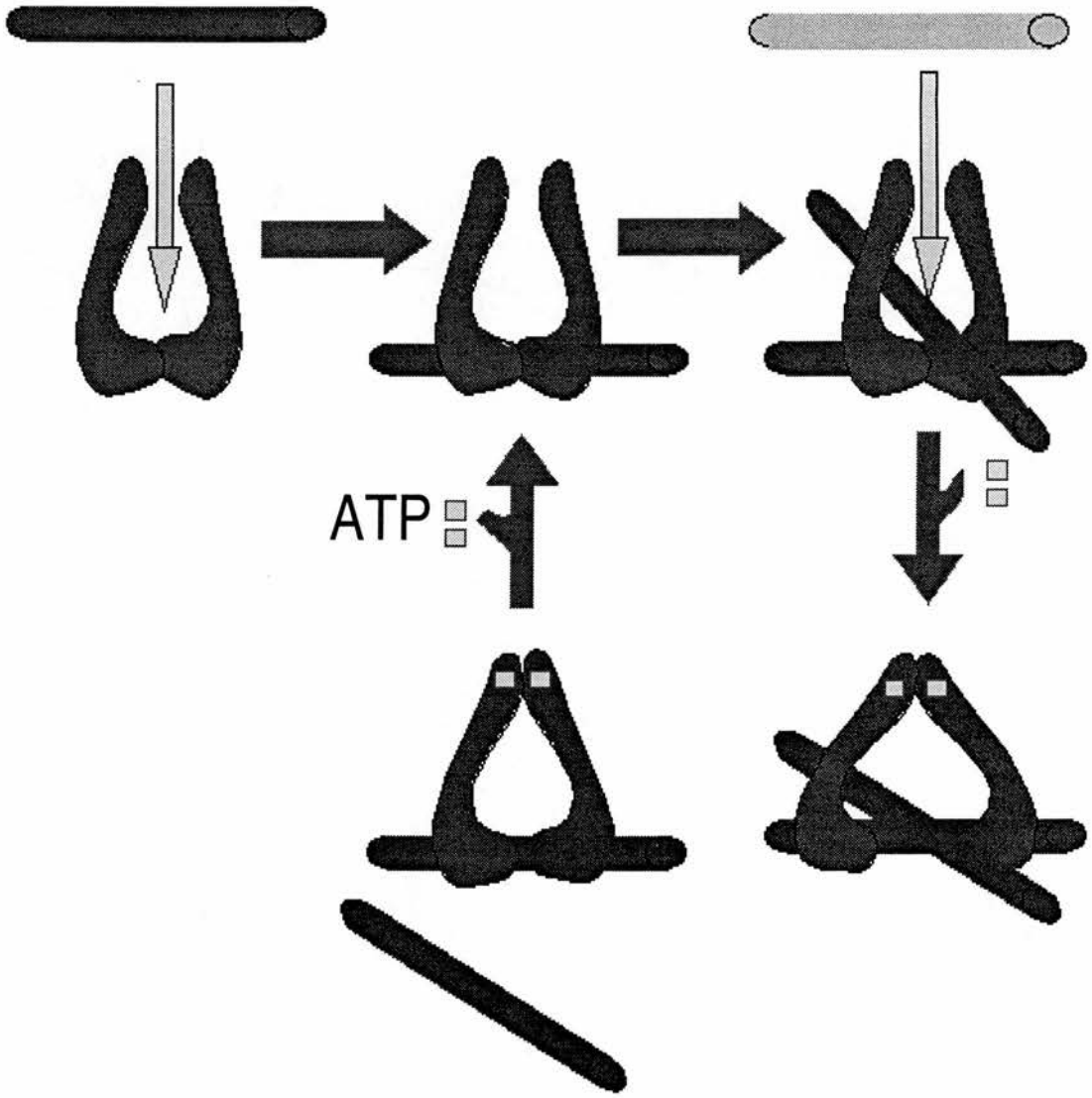
The N-terminal domain of GyrB has been co-crystallised with 5'ADPNP, (a non-hydrolysable analogue of ATP) and its structure determined at 2.5Å (Wigley *et al*, 1991). The crystal structure information showed two distinct domains, both with novel protein folds. Dimerisation of the fragment was also found to occur in the presence of 5'ADPNP. Whilst the N-terminal domains bind and hydrolyse ATP, the C-terminal domains form the sides of a 20Å hole through the dimer. It is this hole that is believed to be important in DNA strand passage during the supercoiling reaction. The diameter of the hole is the same as the diameter of the DNA helix, suggesting that this hole is the gateway through which the DNA passes in the supercoiling reaction. This hole running through the protein was also shown to be lined by positively charged arginine residues which is thought to form some kind of DNA-binding surface .

1.3.3 Molecular mechanism of DNA gyrase

The critical residue in the active site of the *E.coli* DNA gyrase enzyme responsible for DNA cleavage has been mapped to tyrosine-122 on the A subunit (Horowitz and Wang, 1987). This residue is believed to become covalently bound to a 5′ group of DNA through an O^4-phosphotyrosine bond (Horowitz and Wang, 1987) when the enzyme cleaves the phosphodiester bonds of DNA. The 5′ O^4-phosphotyrosine bond formed conserves the energy of the broken phosphodiester bond, allowing the DNA to be ligated without an additional energy source.

DNA topoisomerases catalyse the interconversion of topological isomers of DNA, for example the integration of λ DNA into the bacterial chromosome. This process involves the breakage of at least four strands of duplex DNA and rejoining the ends to different partners without altering the linking number. An outline of the mechanism by which this occurs is displayed in Figure 1.5.

Figure 1.5: Molecular mechanism of DNA gyrase (Taken from Orphanides and Maxwell, 1994).



DNA gyrase transports one double stranded DNA segment through an enzyme-mediated double-stranded break in another (Roca, 1995). This is accomplished by creating a temporary "gate" in one DNA helix which opens to let the other helix be transported through. The break in the DNA is then resealed. A nucleophilic transesterification reaction occurs between a pair of tyrosyl residues

(located on each half of the gyrase holoenzyme) and two DNA phosphodiester bonds four base pairs apart. Mechanistically this occurs by the covalent linkage of the phenolic oxygens of the tyrosines to the phosphoryl groups at the 5′ ends of the transiently broken DNA. This leaves a pair of hydroxyl groups on the recessed 3′ ends. The DNA strands then separate around this area and the pair of tyrosine-linked 5′ ends move away from each other, opening a "gate" after the binding of ATP. This enzyme-mediated gate is then closed once the second DNA segment has been transported. The covalent bonds between gyrase and DNA are broken by a transesterification reaction occurring between the pair of 3′ hydroxyl groups and the phosphotyrosyl linkages. This causes the religation of the two cleaved DNA strands into a continuous segment. Hydrolysis of ATP resets the enzyme. The hole generated is believed to be as large as 35 to 40Å.

1.3.4 DNA topoisomerase IV

In *E.coli*, DNA topoisomerase IV is encoded by the *parC* and *parE* genes (Kato *et al*, 1990). This enzyme is essential for the partitioning of newly replicated chromosomes and plasmids (Adams *et al*, 1992) and this has been shown by the use of temperature-sensitive mutants of topoisomerase IV which accumulate double-stranded nucleoids at the restrictive temperature (Kato *et al*, 1988). Knocking out topoisomerase IV from cells results in a phenotype where catenated plasmids fail to separate (Adams *et al*, 1992). It has also been shown to have a relaxation activity (Peng and Marians, 1993) although *in vivo*, its decatenating activity is more important than its relaxation activity (Kato *et al*, 1992). ParC and ParE have been found to have a high degree of similarity to GyrA and GyrB, respectively. ParC is 730 amino acids long and 36% identical to GyrA, whereas ParE is 601 amino acids long and 40% identical to GyrB (Kato

et al, 1990). Although topoisomerase IV has no supercoiling activity, it has ATP-dependent DNA relaxation and decatenation activities (Kato *et al*, 1992).

1.4 Cellular Action of DNA gyrase

1.4.1 Intracellular localisation of GyrA and GyrB

Immunogold labelling of GyrA and GyrB showed that these two proteins were found mainly in the cytoplasm and only less than 10% of the total GyrA and GyrB content was located in the nucleoid (Thornton *et al*, 1994). It is estimated that at least 1000 to 3000 molecules of each GyrA and GyrB is present in each cell. Simultaneous labelling with both anti-GyrA and anti-GyrB revealed that there was no obvious association between GyrA and GyrB in the cell, instead the subunits seemed to exist as monomers instead of forming the DNA gyrase tetramer. This study concluded that only a small fraction of GyrA and GyrB is actually associated with the nucleoid and loops of peripheral DNA with the majority remaining free in the cytoplasm (Thornton *et al*, 1994).

1.4.2 The importance of supercoiling in bacterial cells

Bacterial DNA is negatively supercoiled as demonstrated by psoralen binding studies (Sinden *et al*, 1980). Psoralen binds to negatively supercoiled DNA so if bacteria are treated with coumermycin A1, psoralen is shown to bind with a lower affinity since there is a decreased superhelical tension in the cell. Supercoiling of DNA in bacterial cells has an implicit role in determining cellular functions. The maintenance of the correct level of supercoiling on specific sites of the bacterial chromosome dictates the probability of processes such as DNA replication, transcription and recombination of occurring. In *E.coli* the optimum range of supercoiling for a certain section of the chromosome is maintained by a balance of DNA gyrase and topoisomerase I activities, as well as by the effects of transcription (see 1.4.6).

1.4.3 The role of gyrase in the maintenance of fixed supercoiling levels in *E.coli*

The levels of supercoiling on the bacterial chromosome are strictly regulated by the action of the topoisomerases. It has been shown that gyrase inhibitors, particularly the coumarin family of drugs, result in a decrease in the superhelicity of both the bacterial chromosome and plasmids (Drlica and Snyder, 1978; Kano *et al*, 1981; Manes *et al*, 1983). It is thought that maintaining the balance of supercoiling levels in the cell is the role of topoisomerase I and gyrase with neither topoisomerase III nor topoisomerase IV having a significant role. Gyrase and topoisomerase I have different substrate affinities: where gyrase is more active on relaxed templates (Sugino and Cozzarelli, 1980), topoisomerase I has a greater specificity for highly negatively supercoiled DNA *in vitro* (Wang, 1971). However, once DNA supercoiling drops below "normal" levels, topoisomerase I is not found to be a major source of DNA relaxation *in vivo*. Perturbations of supercoiling are also believed to be corrected by the changes of gene expression in the genes encoding gyrase and topoisomerase I (see 1.4.5). While gyrase increases the superhelical tension of DNA by decreasing the DNA linking number, topoisomerase I does the opposite. Cells that have mutations in *topA*, the gene encoding topoisomerase I, commonly have compensatory mutations in *gyrA* and *gyrB*, resulting in a decreased superhelical tension so the supercoiling does not increase to excessively high levels (Pruss *et al*, 1982). While higher than optimum levels of negative supercoiling resulting from an absence of topoisomerase I is deleterious to the cell, a reduction in gyrase activity is not (DiNardo *et al*, 1982). Sequence analysis has shown that in an *E.coli topA* mutant, the *gyrA* gene is modified in such a way that Ala-569 and Thr-586 are interchanged (Oram and Fisher, 1992). The apparent importance of these changes is that they flank Arg-571, the site of trypsin cleavage that divides the

GyrA subunit into two domains (see Figure 1.4). This suggests there is selective pressure to maintain the net supercoiling activity within a certain range.

1.4.4 The effect of supercoiling on transcription

Many promoters are sensitive to supercoiling and the expression of certain bacterial operons is sensitive to inhibitors of gyrase, presumably because of a change in superhelicity of the template (reviewed by Drlica, 1984). Cloned random fragments of *E.coli* DNA (Menzel and Gellert, 1987a) and randomly inserted Mud*lac* fusions in *S.typhimurium* (Jovanovich and Lebowitz, 1987) have been used to demonstrate that up to 70% of promoters respond in a significant manner to gyrase inhibition by coumermycin A1. This emphasises the relationship between DNA supercoiling and transcription. For example, negatively supercoiled φX174 DNA has been found to be a better template for transcription *in vitro* than relaxed DNA (Hayashi and Hayashi, 1971). The binding of *E.coli* RNA polymerase to DNA causes the double helix to unwind (Saucier and Wang, 1972), as a result, negative supercoiling should promote RNA polymerase binding. Therefore an increased rate of initiation as a result of facilitated RNA polymerase binding would be expected to result in an increase in the overall transcription rate and number of transcription complexes (Richardson, 1974; Richardson, 1975). However the situation is more complex given that some promoters are stimulated by relaxation of DNA whilst some are unaffected by either supercoiling or relaxation.

On examining a range of promoters it can be concluded that different promoters require different amounts of DNA supercoiling to facilitate structural transitions needed for the initiation of transcription. The variation of the optimum levels of supercoiling for the expression of different genes was demonstrated *in vitro*

by the use of abortive initiation assays (Borowiec and Gralla, 1987). Results showed that the rate of open complex formation in three *lac* promoter variants peaked at different superhelical densities where these variants had single base pair changes in the spacer and -35 regions as well as in the -10 region where DNA unwinding occurs. From this it was concluded that supercoiling induced deformation of the entire *lac* promoter region may be needed for proper recognition by RNA polymerase. An alternative explanation is that changes in the sequence in one section of the promoter may determine the ease of a transition such as melting in another part through the topological coupling of changes in the conformation of the supercoiled DNA. Therefore in some cases, the supercoiling optimum is a characteristic of the interaction between RNA polymerase and the promoter. Experiments carried out studying the gene expression of λ under different superhelical tensions *in vitro* showed that an increase in supercoiling results in an increased amount of RNA synthesis but a decreased amount of early gene transcription (Botchan *et al*, 1973) by raising the transcriptional abilities of promoters not used on linear DNA (Botchan, 1976). Therefore different genes show different responses to changes in supercoiling and in this way, differential effects on gene expression can be achieved. Supercoiling can increase the amount of transcription of a particular gene both by increasing the rate of transcription from existing initiation sites and making new transcription sites available, this effect being promoter specific.

1.4.5 Relaxation-stimulated transcription

In contrast to the view that negatively supercoiled DNA stimulates transcription, some promoters have been found to be stimulated when in a relaxed conformation. Relaxation-stimulated transcription (RST) has been found to occur in the promoters of *gyrA* and *gyrB* (Menzel and Gellert, 1983). On the

addition of coumermycin or novobiocin, the rate of synthesis of the *gyrA* and *gyrB* gene products increase 10 to 20 fold (Menzel and Gellert, 1983). Also in experiments with a temperature-sensitive gyrase mutant strain, on raising the temperature to the restrictive temperature, the rate of synthesis of GyrA and GyrB was again shown to rise (Menzel and Gellert, 1983). Therefore in the case of the promoters of *gyrA* and *gyrB*, a decrease in DNA negative supercoiling increases promoter activity. Experiments using an *in vitro* transcription-translation system showed that expression of *gyrA* and *gyrB* increased 100-fold if the template was relaxed and an inhibitor of gyrase, such as coumermycin, was added to keep the template in a relaxed form (Menzel and Gellert, 1983). However inhibiting gyrase by nalidixic acid has been shown to display a much smaller increase in the expression of gyrase. Menzel and Gellert (1987b) went on to show that this regulation of gyrase expression occurs at the level of transcription since regions of *gyrA* and *gyrB* fused to *galK* makes *galK* activity inducible *in vivo*, to coumermycin. On the other hand, the expression of topoisomerase I responds to changes in supercoiling in the opposite way, that is it is inhibited by treatments which relax DNA supercoils and it is stimulated by treatments that increase the levels of negative supercoiling (Tse-Dinh and Beran, 1988).

Since this response is analogous to feedback inhibition, it can be concluded that supercoiling is likely to be a normal regulator of these genes. One can see how RST has a homeostatic role in the cell so that a decrease in DNA supercoiling stimulates the *gyrA* and *gyrB* promoters to synthesise DNA gyrase to increase supercoiling to its normal level and an increase in negative supercoiling stimulates the production of topoisomerase I.

1.4.6 The effect of transcription on supercoiling

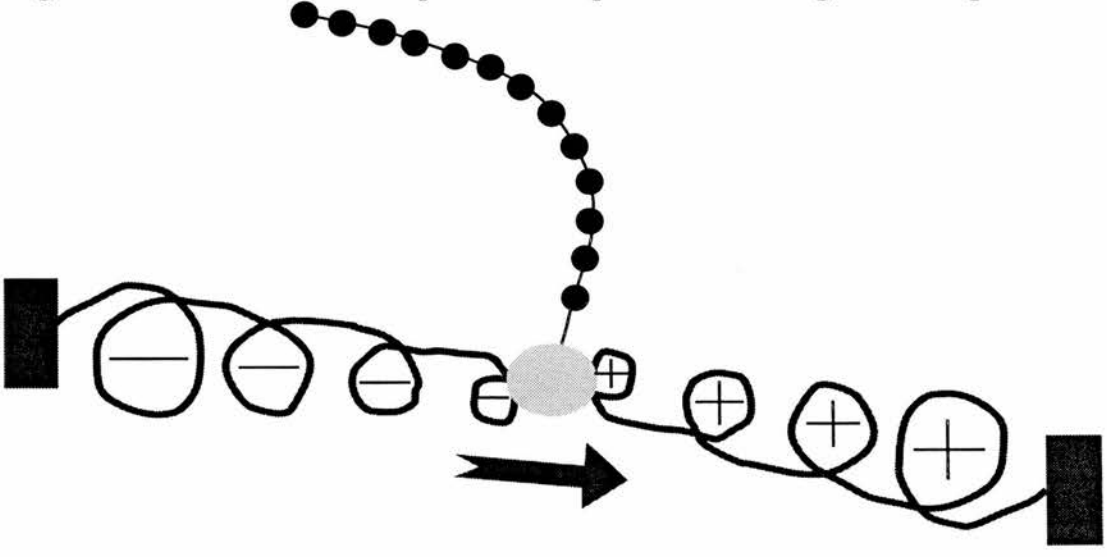
Transcription has a major effect on the local and global supercoiling state of DNA. As transcription has been shown to have a profound effect on supercoiling (Pruss and Drlica, 1986), localised variations in supercoiling can be achieved. Supercoiling provides a way for the transcription of one gene to affect adjacent genes via DNA topology. It can be imagined that depending on local transcriptional activities in the bacterial chromosome, supercoiling in localised regions of the chromosome could become quite different from the global level. It has been found that different plasmids from the same strain can have very different levels of supercoiling. Increased negative supercoiling results from transcription from a strong promoter rather than a weak one. Both divergent transcription and the membrane attachment of one of the transcription units are required for transcription to profoundly alter DNA supercoiling. In wild type bacteria, gyrase and topoisomerase I have the capacity to keep pace with transcription-generated topological problems and are therefore major determinants of DNA supercoiling in cells (Cook *et al*, 1992).

1.4.7 Function of DNA gyrase in the replication of the *E.coli* chromosome

DNA gyrase is involved in the initiation, chain elongation and termination of DNA replication (Baker and Kornberg, 1988; Baker *et al*, 1986). At the origin of replication, gyrase is required for the unwinding of the double helix (Baker and Kornberg, 1988). DNA gyrase is one of five essential proteins involved in the elongation stage of replication of the *E.coli* chromosome. Unwinding the DNA duplex causes a "strain" to build up in front of the replication machinery

as a result of the generation of positive supercoils as displayed in Figure 1.6. The function of gyrase is to remove this topological strain. DNA gyrase reduces the linking number of the parental strands prior to them becoming unwound by the DnaB helicase (Baker *et al*, 1986). The remaining proteins required for elongation of the nascent DNA strand are DnaB helicase which generates single-stranded DNA by unwinding the DNA duplex, DnaG primase which is needed to "prime" DNA synthesis by forming oligonucleotide primers, DNA polymerase III holoenzyme which replicates the single-stranded DNA templates by extending the primers formed by primase and the single-stranded DNA binding protein (SSB) which binds to the unwound DNA strands and melts any secondary structure so the replication machinery can run smoothly. Unsurprisingly, this elongation stage of DNA replication is inhibited by gyrase inhibitors such as novobiocin and nalidixic acid (Drlica, 1984). Once the bacterial chromosome has been completely replicated, the two catenanes must be unlinked and moved to opposite ends of the cell before cell division. This process, as mentioned before, is principally carried out by topoisomerase IV.

Figure 1.6: Generation of positive supercoils during DNA replication.



1.4.8 Segregation of bacterial chromosomes

Catenanes are the structures in which bacterial chromosome are found during the later stages of DNA replication when the replication forks approach the terminus region. The two linked daughter chromosomes must be unlinked before partitioning occurs into daughter cells. Although both gyrase and topoisomerase IV can decatenate *in vitro*, topoisomerase IV is the principle decatenating enzyme in bacterial cells. Gyrase can however substitute in some ways for topoisomerase IV, however it has to be present at high concentrations to do this (Kato *et al*, 1992). It has been suggested that the specificity of topoisomerase IV rather than gyrase for the terminus region may result from topoisomerase IV and the other terminus-acting proteins being sequestered in a "termisome complex" (Rothfield, 1994). One of the two subunits of topoisomerase IV, ParC has been found by Kato *et al* (1992) to be attached to the cytoplasmic membrane. Of further interest is the fact that ParC can be released from the membrane subsequent to DNase I treatment. Therefore it seems that the segregation machinery is likely to be located at the inner face of the membrane (Kato *et al*, 1992).

1.4.9 The role of DNA gyrase in recombination

In recombination, the supercoiling induced by gyrase is essential. Some types of general recombination are sensitive to inhibition of gyrase *in vivo* (Hays and Boehmer, 1978) and transpositional recombination of Mu needs a supercoiled DNA substrate (Mizuuchi and Craigie, 1986). The involvement of gyrase in illegitimate recombination has also been demonstrated at least *in vitro*, possibly with the interchange between gyrase molecules bound to different sites on the DNA fragment (Naito *et al*, 1984).

1.5 DNA gyrase as a drug target

As DNA gyrase is unique to prokaryotes, it is an ideal target for antibiotics. Antibacterial drugs which specifically target DNA gyrase will damage the bacterial pathogens in a way that will be described later while leaving the host unaffected. There are two main classes of antibacterial drugs which specifically target DNA gyrase: the quinolones and the coumarins. DNA topoisomerase-targeting drugs can be divided into these two classes based on their mode of action:

Class I includes the quinolones which target DNA gyrase. Drugs in class I are "topoisomerase poisons", which act by stabilising the covalent topoisomerase-DNA complex as will be described in section 1.6.2. Two naturally occurring polypeptides target DNA gyrase with a similar mode of action. These are microcin B17 (Vizan *et al*, 1991) and the CcdB gene encoded toxin carried on the F plasmid (Miki *et al*, 1992), both of which will be discussed in 1.5.6-1.5.7.

Class II includes the coumarin family of antibiotics which interact with the DNA gyrase ATPase domain (Contreras and Maxwell, 1992). Drugs in class II are distinguished from those in class I in that they interfere with the catalytic functions of DNA topoisomerases without trapping the covalent intermediate and are thus "topoisomerase inhibitors" rather than "topoisomerase poisons".

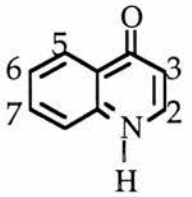
1.5.1. Quinolone antibacterial agents: an overview

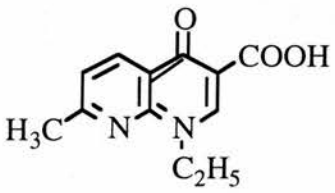
The quinolone drugs are synthetic compounds which do not resemble any compounds found in living organisms. The first quinolone to be synthesised was nalidixic acid which was introduced into the field of antimicrobial

chemotherapy in 1962 (Lesher *et al*, 1962). This antibacterial agent was used extensively for the treatment of urinary tract and enteric infections, particularly against members of the *Enterobacteriaceae*, to which it has a high efficacy. Despite extensive clinical use, resistance to nalidixic acid was found to remain relatively low. A few years later in the 1970s, several other antibacterial agents that were chemically related to nalidixic acid, oxolinic acid and cinoxacin, were introduced. However, the most significant discovery in the development of quinolone antibacterial agents came later when it was discovered that the insertion of a fluorine at position 6 in the basic nucleus enhanced and broadened the antimicrobial activity. This resulted in the development of newer quinolone drugs with antibacterial activities approximately a thousand times that of nalidixic acid (Smith and Lewin, 1988). Newer quinolones such as norfloxacin, enoxacin, ofloxacin and ciprofloxacin are active against both Gram positive and Gram negative bacteria (Wolfson and Hooper, 1985).

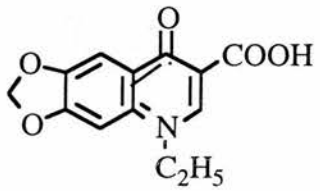
The quinolone antibacterial agents can be divided into four general groups: naphthyridines, cinnolines, pyridopyrimidines and quinolones. The chemical structure of the basic nucleus and its derivatives are shown in Figure 1.7. The common 4-quinolone skeleton (4-oxo-1,4-dihydroquinolone) is produced by inserting an oxygen at position 4 in the basic nucleus. Nalidixic acid and enoxacin are naphthyridines and have an additional nitrogen atom at position 8, thus they are 8-aza-4-quinolones. Quinolone antibacterial agents that derive from the quinolone nucleus are simply known as 4-quinolones or quinolones.

Figure 1.7: The 4-quinolone skeleton and structures of drugs derived from it

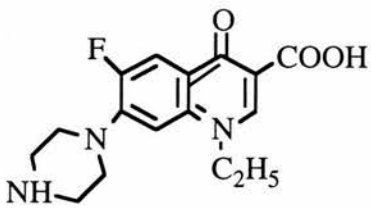




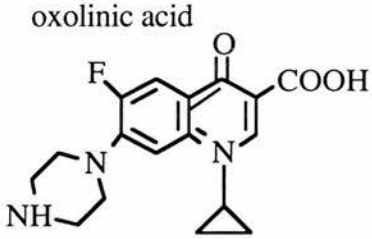
nalidixic acid



oxolinic acid



norfloxacin



ciprofloxacin

It has been shown that for greatest activities, quinolone drugs have a carboxyl group at position 3, a keto group at position 4 and a fluorine at position 6 and a piperazinyl group or a methyl-substituted piperazinyl group at position 7 (Chu and Fernandes, 1989).

1.5.2 Binding of quinolones to DNA gyrase/ DNA

Before binding studies had been carried out, it was believed that quinolone drugs bound directly to GyrA (Gellert *et al*, 1977; Sugino *et al*, 1977), since quinolone resistance mutations occurred in the *gyrA* gene. However, the occurrence of quinolone resistance mutations in *gyrB* suggested that the quinolone binding site might encompass both subunits (Yamagishi *et al*, 1986).

Binding studies carried out by Shen and Pernet (1985) suggested that quinolones bound to DNA but not to gyrase, nor its individual subunits and it was separately observed that the binding of quinolones to DNA causes the DNA to unwind to a small extent (Tornaletti and Pedrini, 1988). It has been suggested that quinolone drugs bind preferentially and cooperatively to single-stranded DNA (Shen and Pernet, 1985; Shen *et al*, 1989a) which led to the idea that drugs interact with a single stranded pocket of DNA formed during the strand passage reaction catalysed by gyrase. This hypothesis was modified slightly when it became apparent that DNA gyrase was able to stimulate the binding of quinolones to double stranded DNA (Shen *et al*, 1989b). Shen *et al* (1989c) later proposed a cooperative drug-DNA binding model for the binding of quinolones to the gyrase-DNA complex. It was suggested that the enzyme cuts double-stranded DNA, producing a four base pair stagger and the resulting exposed single stranded regions form the quinolone binding site. The quinolone drugs are thought to interact with the bases through hydrogen bonds which occur between the 3-carboxy and 4-oxo groups which almost all quinolones possess. At least four molecules are believed to bind per binding site and ring stacking and hydrophobic interactions contribute to this co-operativity. Shen *et al* (1989c) postulated that gyrase interacts via the group at the C7 position of the drug (Figure 1.7), however Maxwell later pointed out that the C7 group is rather variable (Maxwell, 1992). However, a new angle has recently been added to this drug binding hypothesis with the discovery that mutations in *gyrA* which have tyrosine-122 replaced with either phenylalanine or serine (and thus cannot cleave DNA) still can bind quinolone drugs (Critchlow and Maxwell, 1996) suggesting that DNA cleavage by gyrase is not a prerequisite for drug binding. Critchlow and Maxwell (1996) proposed that a pre-cleavage complex between gyrase, quinolone and DNA exists which is converted to a, perhaps more stable,

complex following cleavage. This could occur if quinolones interact with a gyrase-DNA complex in a similar way to the interaction of eukaryotic topoisomerase II inhibitors with topoisomerase and DNA (Freudenreich and Kreuzer, 1993). Intercalation of the planar ring of the quinolone drug into the internucleotide space next to the cleaved phosphodiester bond could occur where it can interact with residues of gyrase. This would occur either in the presence or absence of DNA cleavage but in the presence of quinolones would likely favour DNA cleavage (Critchlow and Maxwell, 1996).

1.5.3 Resistance to the quinolone drugs

Increasingly, the occurrence of *E.coli* and *S.typhimurium* isolates highly resistant to fluoroquinolones has been reported recently (Heisig *et al*, 1993; Heisig, 1993; Heisig *et al*, 1994; Griggs *et al*, 1994; Heisig *et al*, 1995). Bacterial resistance to the quinolone drugs has been frequently shown to occur concurrently with a mutation in either *gyrA*, *gyrB* or both (Reece and Maxwell, 1991b).

1.5.4 The Quinolone Resistance Determining Region of *gyrA*

It appears that the major target of quinolone drugs is GyrA (Yoshida *et al*, 1988). Point mutations arising in the DNA sequence of *gyrA* associated with quinolone resistance seem to map in a specific region of the protein called the quinolone resistance determining region (QRDR) (Yoshida *et al*, 1990). The QRDR spans the area of the protein between alanine-67 and glutamine-106 as shown previously in Figure 1.4. Structurally, the importance of the region is believed to result from its proximity to tyrosine-122. Residues which have been found to change correlating with an increase in the minimum inhibitory concentration (MIC) of quinolone drug are as follows: alanine-67, glycine-81, serine-83,

alanine-84, aspartate-87 and glutamine-106 (Reece and Maxwell, 1991b), however serine-83 and aspartate-87 are thought to be the most critical (Yoshida *et al*, 1990; Oram and Fisher, 1991; Hallett and Maxwell, 1991).

Table 1.3: Mutations in the quinolone resistance determining region of the *gyrA* gene of *E.coli*

Position in GyrA	Amino acid change	Change in MIC of ciprofloxacin (mg/L)[a]	Reference
67	Ala→Ser	4	Yoshida *et al*, 1990
81	Gly→Cys	8	Yoshida *et al*, 1990
81	Gly→Asp	ND	Cambau *et al*, 1993
83	Ser→Ala	4	Hallett and Maxwell, 1991
83	Ser→Leu	32	Yoshida *et al*, 1990
83	Ser→Trp	32	Yoshida *et al*, 1990
84	Ala→Pro	8	Yoshida *et al*, 1990
87	Asp→Asn	16	Yoshida *et al*, 1990
87	Asp→Val	5	Yoshida *et al*, 1990
87	Asp→Tyr	8	Heisig *et al*, 1993
106	Gln→Arg	4	Hallett and Maxwell, 1991
106	Gln→His	4	Yoshida *et al*, 1990

[a] The change in MIC is given as the factor by which the MIC has changed compared to the original parent strain

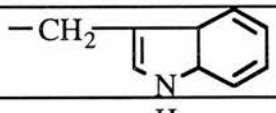
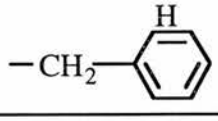
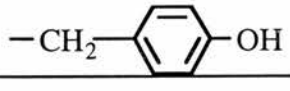
Given that the amino acid changes leading to the highest levels of ciprofloxacin resistance are serine-83 to leucine and serine-83 to tryptophan as highlighted in Table 1.3, it was suggested that only such bulky hydrophobic residues at this position would be able to confer the quinolone resistant phenotype (Yoshida *et al*, 1990). However, site-directed mutagenesis (SDM) carried out by Hallett and Maxwell (1991) showed that changing serine-83 to alanine in the GyrA subunit of *E.coli* resulted in a gyrase protein conferring resistance to ciprofloxacin in an *in vitro* assay, suggesting that it is the loss of the hydroxyl group that is responsible for the resistance phenotype. Interestingly, the MIC_{50} of ciprofloxacin of strains carrying the serine-83 to alanine mutation is lower than that seen with strains with serine-83 to leucine and serine-83 to tryptophan mutations (Cullen *et al*, 1989; Yoshida *et al*, 1990; Yoshida *et al*, 1988). It has been speculated that the bulkiness of the leucine and tryptophan residues contributes further to the level of quinolone resistance, presumably by destabilising the gyrase-quinolone interaction more, perhaps by preventing quinolone entry into the "quinolone pocket", the region of the complex in which the drug is thought to bind.

SDM was also carried out on glutamine-106 of GyrA. Glutamine-106 to histidine is the only quinolone resistance mutation reported to occur naturally at this position (Yoshida *et al*, 1988; Yoshida *et al*, 1990). The MIC of ciprofloxacin for glutamine-106 to histidine was only found to be four times more than the wild type value, consistent with the proposal by Yoshida *et al* (1990) that this change leads to low level resistance (Yoshida *et al*, 1990). However Hallett and Maxwell (1991) found that changing glutamine-106 to arginine resulted in a level of quinolone resistance comparable to that induced by the mutation serine-83 to alanine, suggesting that the level of resistance is determined by the specific

amino acid that is substituted. Interestingly, both histidine and arginine are positively charged residues whereas glutamine is polar so both these changes could cause destabilisation of the gyrase-quinolone interaction. It has also been proposed that residues serine-83, aspartate-87 and glutamine-106, being hydrophilic, may participate in hydrogen-binding interactions with the drug molecules (Hallett and Maxwell, 1991). However, in the model of quinolone binding postulated by Shen *et al (1989c),* the hydrogen-bonding interactions are thought to occur between the drug and hydrogen-bond donors on the DNA while enzyme-drug interactions are believed to occur between the C7 group of the quinolone and DNA gyrase. The mutations serine-83 to alanine and glutamine-106 to arginine do not affect the cleavage reaction *per se* but instead cause the interaction of the quinolone with the enzyme to become destabilised.

Extensive site-directed mutagenesis has been carried out on serine-83 (Yonezawa *et al*, 1995a). Artificial mutants of *E.coli* GyrA were generated and the effect of the mutations on the level of quinolone resistance studied. It was found that converting serine-83 to tryptophan, leucine, phenylalanine, tyrosine, alanine, valine and isoleucine resulted in bacterial resistance to the quinolones. Changing serine-83 to glycine, asparagine, lysine, arginine and aspartate was found not to confer resistance. Table 1.4 describes the measured MIC of nalidixic acid after changing serine-83 by SDM to an amino acid of different chemical structure.

Table 1.4: The MIC of nalidixic acid after changing serine-83 of GyrA of *E.coli* to an amino acid of a different chemical structure.

83rd amino acid	MIC of nalidixic acid (mg/L)	Amino acid side-group
Serine	6.25	$-CH_2-OH$
Glycine	6.25	$-H$
Lysine	6.25	$-CH_2-CH_2-CH_2-CH_2-NH_3^+$
Arginine	6.25	$-CH_2-CH_2-CH_2-NH-C(=NH_2^+)-NH_2$
Asparagine	12.5	$-CH_2-C(=O)-NH_2$
Aspartate	12.5	$-CH_2-C(=O)-O^-$
Alanine	50	$-CH_3$
Valine	50	$-CH(CH_3)-CH_3$
Isoleucine	100	$-CH(CH_3)-CH_2-CH_3$
Tryptophan	100	
Phenylalanine	100	
Tyrosine	400	
Leucine	400	$-CH_2-CH(CH_3)-CH_3$

On interpreting these results, it was concluded that, for quinolone resistance to occur, the amino acid at position 83 has to be hydrophobic and the hydroxyl group of serine was not important in the quinolone-gyrase interaction. It was also concluded that serine-83 did not directly interact with other amino acids. These findings modify the previous ideas of Hallett and Maxwell (1991) as Yonezawa *et al* (1995a) found that replacing serine-83 with tyrosine confers quinolone resistance, thus it cannot be the loss of the hydroxyl group that results in quinolone resistance, since tyrosine also has a hydroxyl group although the latter amino acid has a far more bulky structure than does serine (see Table 1.4). Another group have proposed that the hydroxyl group of serine might be part of an important hydrogen bonding or metal-liganding interaction essential for the action of quinolones (Oram and Fisher, 1991). The results of the work carried out by Yonezawa *et al* (1995a) dispute the theory that the hydroxyl group plays an important role in the quinolone-gyrase interaction and strengthen the hypothesis that the 83rd amino acid must be hydrophobic to express the quinolone resistant phenotype. It was further proposed that serine-83 is at the entrance to the quinolone pocket.

1.5.5 The Quinolone Resistance Determining Region of *gyrB*

The *gyrB* gene has also been described as having a quinolone resistance determining region (Yoshida *et al*, 1991) however mutations in *gyrB* seem to be of minor importance compared to mutations in *gyrA*. This region is around the centre of the gene and the important residues seem to be aspartate-426 and lysine-447 (Yoshida *et al*, 1991). Changing aspartate-426 to asparagine and lysine-447 to glutamate has the effect of raising the MICs of some quinolone drugs (Yamagishi *et al*, 1986). A small increase in the MICs of acidic quinolones such as nalidixic acid, oxolinic acid and cinoxacin is detectable for both

mutations, however only the change aspartate-426 to asparagine conferred resistance to fluorinated quinolones also. It was also found that changing lysine-447 to glutamate results in an increased sensitivity to fluoroquinolones (Yamagishi *et al*, 1986). It has been postulated that either nalidixic acid could become adjacent to the quinolone resistance determining region of *gyrB* when bound or that the mutations in the quinolone resistance determining region of *gyrB* may result in changed resistance levels as a result of different contacts with GyrA being formed which may then change the ease of binding of nalidixic acid to the GyrA protein (Yamagishi *et al*, 1986).

1.5.6 The CcdB/ CcdA system of *E.coli*

The *ccdA* and *ccdB* genes of *E.coli* are present on the F plasmid and function to preserve stable maintenance of the plasmid by forming a poison-antidote mechanism (Bernard and Couturier, 1992). Production of CcdB results in an inhibition of DNA partitioning and cell division which is lethal to the cell, however under normal circumstances, it is neutralised by CcdA (Miki *et al*, 1992). The half life of CcdA is shorter than that of CcdB so if the F plasmid is lost, decay of CcdA leads to activation of CcdB and subsequent cell death by filamentation (Miki *et al*, 1992). It has been shown that CcdB forms a complex with GyrA in a manner very similar to that of the quinolone drugs (Bernard *et al*, 1993) whilst *E.coli* mutants with resistance to the cytotoxic activity of CcdB have been shown to have a mutation in *gyrA* converting arginine-462 to cysteine (Bernard and Couturier, 1992). The CcdB protein has been shown to be responsible for gyrase-mediated double-stranded DNA breakage (Bernard *et al*, 1993), resulting in the stimulation of the SOS response (Bernard and Couturier, 1992). However in the *gyrA462* mutant, the SOS response is not activated indicating that the *gyrA462* mutant protein does not damage the DNA, unlike

the wild-type (Bernard and Couturier, 1992). This evidence suggests that CcdB, like the quinolone drugs is a DNA gyrase poison (Bernard and Couturier, 1992). CcdB has indeed been found to trap gyrase into a cleavable complex and this reaction can be reversed by adding CcdA (Bernard *et al*, 1993). The mechanism of DNA strand cleavage by CcdB shows close parallels to the action of the quinolones. However unlike the quinolones, which carry out the DNA cleavage reaction in the presence or absence of ATP, CcdB cleavage of DNA requires ATP (Bernard *et al*, 1993). It is interesting that quinolone resistant bacteria are sensitive to CcdB, thus possibilities are open for the development of new gyrase-targeting drugs based on CcdB (Bernard *et al*, 1993).

1.5.7 Microcin B17

Microcin B17 is a low molecular weight peptide antibiotic produced by certain *E.coli* strains (Herrero and Moreno, 1986). The mechanism of action of microcin B17 has been shown to involve DNA replication arrest and a consequent induction of the SOS response through inhibition of DNA gyrase (Herrero and Moreno, 1986). Cells treated with microcin B17 have been found to induce production of SOS-induced proteins such as RecA, SulA and UmuC, indeed *recA*$^-$ and *recBC*$^-$ strains are hypersensitive to this antibiotic. Microcin B17 is mutagenic, however this effect is either thought to be a result of poorly repaired DNA lesions or an increase in UmuCD expression (Herrero and Moreno, 1986) whilst induction of the SOS response is RecBCD dependent as with the quinolones (see 1.6.8). *E.coli* mutants resistant to microcin B17 have been isolated and shown to map in *gyrB* (Vizan *et al*, 1991) resulting from a tryptophan-751 to arginine conversion in the GyrB protein. The mode of action of the antibiotic is to cause double-stranded cleavage of chromosomal DNA so that its

mechanism is similar to that of the quinolones.

1.5.8 DNA topoisomerase IV as a drug target

The two proteins constituting DNA topoisomerase IV, ParC and ParE have been purified and studied in respect to them forming a putative antimicrobial target (Hoshino *et al*, 1994). The specific activity of topoisomerase IV decatenation was found to be five times greater than topoisomerase IV relaxation suggesting that the decatenation activity of topoisomerase IV seems the most relevant for the study of drug inhibition. Topoisomerase IV was found to be less sensitive to quinolones than was gyrase and the 50% inhibitory concentrations for decatenation were found to be significantly lower than this for type I topoisomerases. A positive correlation was found between the inhibitory activity against topoisomerase IV decatenation and that for gyrase thus topoisomerase IV certainly is a candidate target for quinolone drugs in intact bacteria. It is imagined that when quinolone drugs are present in the cell in high concentrations, these drugs would be likely to inhibit the decatenation activities of topoisomerase IV as well as the supercoiling activities of gyrase. *In vitro*, only gyrase and topoisomerase IV segregate intact catenated DNA in the presence of ATP (Adams *et al*, 1992) but recent work has suggested that DNA gyrase may not decatenate DNA in bacterial cells (Adams *et al*, 1992; Hiasa *et al*, 1994). Formation of catenanes by DNA gyrase tends only to happen under conditions of aggregated DNA, for example in the presence of high spermidine concentrations (Kreuzer and Cozzarelli,1980; Mizuuchi *et al*, 1980). The IC_{50} for nalidixic acid against the decatenation reaction of topoisomerase IV was found to be eight times higher than the IC_{50} for the supercoiling reaction of gyrase (Hoshino *et al*, 1994).

Residues corresponding to the quinolone resistance determining region and coumarin resistance region are conserved in topoisomerase IV, suggesting that quinolone and coumarin drugs may also inactivate topoisomerase IV. It has been found that although topoisomerase IV is inhibited by quinolone and coumarin drugs, it is not as sensitive to these drugs as is gyrase (Peng and Marians, 1993). Sensitivity to novobiocin and oxolinic acid has been observed for the *E.coli* DNA topoisomerase IV relaxation reaction (Kato *et al*, 1992) but the decatenation reaction seems to be much less sensitive. If topoisomerase IV is indeed membrane bound, it is possible that *in vivo* the enzyme does not come into contact with drugs such as novobiocin and oxolinic acid.

1.6 Effect of DNA gyrase inhibition at a cellular level

1.6.1 Effect of quinolones on the bacterial chromosome

Experiments carried out investigating the effect of quinolones on the bacterial chromosome were undertaken in the late 1970s and used oxolinic acid as a representative quinolone (Snyder and Drlica, 1979). It was found that the incubation of *E.coli* cells with oxolinic acid resulted in the cleavage of DNA after addition of sodium dodecyl sulphate (SDS). It was estimated that there are about 45 breaks in the chromosome, a value similar to the number of domains of supercoiling found in the isolated chromosome. Since the *E.coli* chromosome is about 4700kb, this corresponds to approximately one gyrase site per 100kbp (Drlica *et al*, 1980). This information is interesting given that the number of active DNA gyrase sites has been estimated as approximately 50 per cell (Snyder and Drlica, 1979). The number of supercoiled domains per cell has also been estimated as being 50 (Worcel and Burgi, 1972), suggesting that one gyrase complex may be associated with each domain of supercoiling (Snyder and Drlica, 1979). By using low oxolinic acid concentrations, it was realised that each gyrase cleavage site consists of two independent drug targets, one on each strand (Snyder and Drlica, 1979). This conclusion was drawn since single-stranded sites were found to predominate at low oxolinic acid concentrations and the number of cleaved single stranded sites were found to parallel the inhibition of DNA synthesis. A further conclusion of Snyder and Drlica (1979) was that each gyrase site was contacted by two nicking-sealing subunits, one on each strand, and for DNA synthesis to continue, both subunits would have to be functional. The effect of oxolinic acid on the cell is rapid, with DNA synthesis decreasing rapidly within a few minutes, even when low drug

concentrations are used and few cleavable complexes are formed (Snyder and Drlica, 1979).

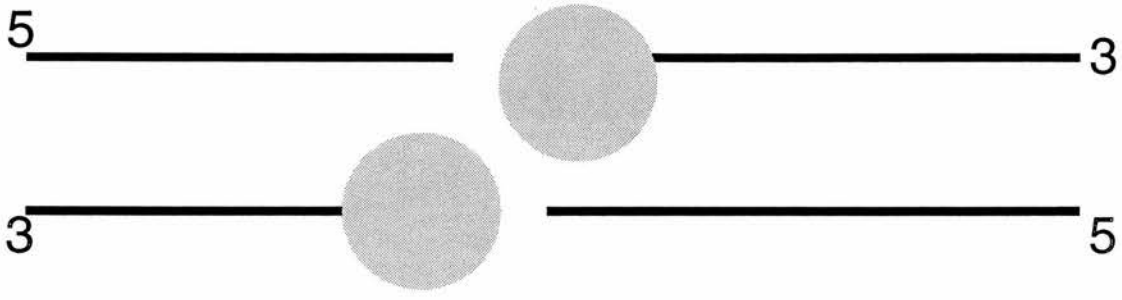
Drlica *et al* (1980) postulated that this rapid inhibition of DNA synthesis resulted from oxolinic acid combining with gyrase and forming a barrier to the movement of replication forks. Inactivation of gyrase in replicating regions of the chromosome would understandably inhibit DNA synthesis quickly. Kinetic measurements carried out by Drlica *et al* (1980) showed that inactivating gyrase at the replication forks was the major cause of inhibition of DNA synthesis and gyrase sites elsewhere on the chromosome did not contribute to this inhibition to any major extent.

1.6.2 Implications of "Cleavable Complex" Formation

The bactericidal effect of quinolones is complex and presently poorly understood. Quinolone-mediated cell death does not occur by the inhibition of DNA supercoiling which is demonstrated by the fact that the IC_{50} value (the concentration of drug at which 50% inhibition of supercoiling occurs) for a particular quinolone drug exceeds the MIC by a factor of 10 to 100 (Domagala *et al*, 1986; Gellert *et al*, 1977; Zweerink and Edison, 1986). It was postulated by Kreuzer and Cozzarelli (1979) that quinolone drugs act as "topoisomerase poisons" by blocking all activities of DNA gyrase, with these drugs trapping the gyrase enzyme at a certain stage in the reaction cycle, thus inhibiting the supercoiling of DNA and arresting the movement of polymerases. Cell death would be expected to occur when only a small fraction of intracellular gyrase molecules had quinolone bound to them, that is at a concentration of drug that would not affect supercoiling appreciably. On the addition of SDS to a reaction mixture of chromosomal DNA, gyrase and oxolinic acid, the DNA is found to

be cleaved on both strands with a 4bp stagger and protruding 3′ ends (Gellert *et al*, 1977; Sugino *et al*, 1977; Morrison and Cozzarelli, 1979). These 3′ ends provide a template primer for DNA polymerase (Morrison and Cozzarelli, 1979), however the cleaved DNA is resistant to labelling with T4 polynucleotide kinase even after treatment with proteinase K suggesting that the denatured enzyme remains attached to the cleaved DNA and is covalently bonded to both 5′ terminal extensions as illustrated in Figure 1.8.

Figure 1.8: Diagrammatic representation of the gyrase-DNA "cleavable complex" (represented by the yellow circle) on double stranded DNA.

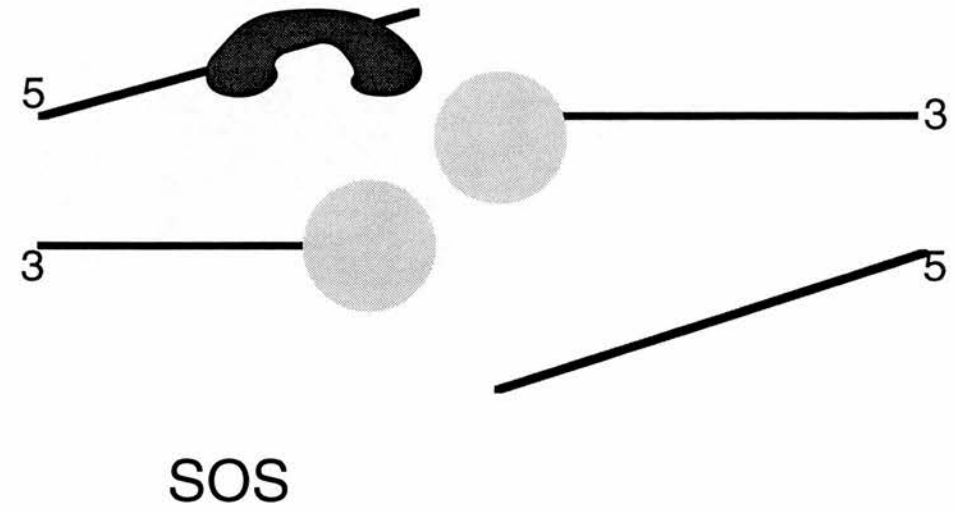


1.6.3 The effect of quinolone drugs on transcription

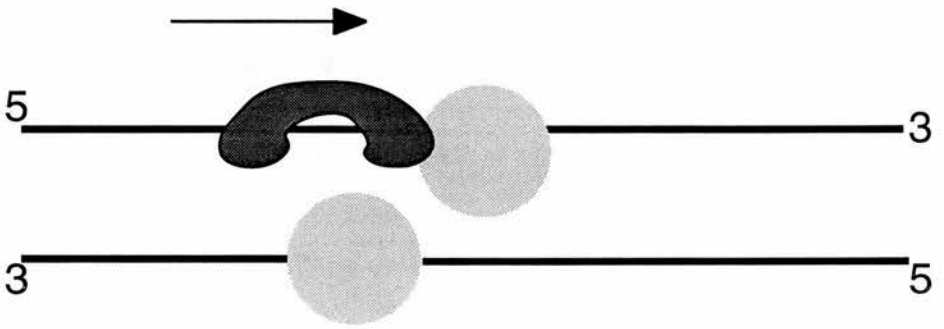
Experiments carried out by Willmott *et al* (1994) have shed some light on the mode of action of quinolone drugs. By the use of an *in vitro* transcription system, the effect of quinolones on transcription was studied. It was found that the complexes of gyrase and quinolone with DNA result in transcriptional arrest by both *E.coli* and T7 RNA polymerases, seen by the production of short transcripts. However, when DNA gyrase carrying a point mutation conferring resistance to quinolones was introduced into the system, it was found that transcriptional arrest only occurred at much higher concentrations of the quinolone drug. It was therefore found that the amount of ciprofloxacin needed

to stall transcription significantly is of a comparable level with the amount required to promote DNA cleavage by gyrase (Hallett and Maxwell, 1991). Changing the active site tyrosine-122 to serine results in the lifting of this transcriptional block *in vitro*, suggesting that DNA cleavage is required for polymerase blocking (Critchlow and Maxwell, 1996). Interestingly, DNA synthesis by DNA polymerase I is inhibited by gyrase and quinolone drug together but not by either component alone Willmott *et al* (1994). These observations confirm those of Chow *et al* (1988) who showed that the addition of ciprofloxacin, norfloxacin and difloxacin to a logarithmic culture of *E.coli* resulted in a rapid decrease in DNA synthesis. Even low levels of oxolinic acid have been found to form cleavable complexes on DNA, thus inhibiting DNA synthesis and RNA synthesis in proportion to the number of complexes formed (Snyder and Drlica, 1979). Manes *et al* (1983) found that oxolinic acid inhibits RNA synthesis at concentrations at which DNA supercoiling is unimpeded. Therefore, DNA synthesis and transcription are much more sensitive to quinolone action than is DNA supercoiling and it is probably the inhibition of the former that is the lethal event. Camptothecin, a eukaryotic type I topoisomerase inhibitor which is also a "topoisomerase poison" has been shown to cause replication fork and transcriptional arrest (Bendixen *et al*, 1990; Hsiang *et al*, 1989). Formation of the cleavable complex consisting of quinolone-gyrase and DNA seems to stimulate various activities as described in Figure 1.9. Briefly, the SOS response can be induced by either the formation of free ends after collision of the cleavable complex with the transcriptional machinery (Possibility1) or the induction of DNA repair (Possibility 2).

Figure 1.9: Diagrammatic representation of quinolone-mediated transcription arrest and its implications.



Possibility 1: The cleavable complex is broken by the movement of the transcriptional machinery for example



Possibility 2: The distortions in the DNA duplex are detected by repair enzymes, for example, Uvr endonuclease, which sets off the SOS response

1.6.4 Formation of Double-Stranded Breaks (DSBs) by the Quinolones

After the formation of the cleavable complex, two potential double-stranded DNA break (DSB)-forming scenarios can be imagined (Figure 1.9). It is probably the case that when the replication or transcription machinery collides with the "cleavable complex" on the DNA, it stops and reinitiates some distance away, leaving a gap in the DNA to which RecA can bind. Another possibility is that the impact of the replication or transcription complex on the "cleavable complex" rips it apart, resulting in a double-stranded break possibly with the 5′ end attached to the GyrA subunit and the 3′ end free (Morrison and Cozzarelli, 1979). Also distortions in the DNA helix are detected by repair enzymes such as Uvr endonuclease which initiates DNA repair mechanisms (Kuemmerle and Masker, 1980; Sancar and Rupp, 1983). Whether or not the action of quinolones results in free DNA ends in bacterial cells has been a debatable point. Work carried out by Snyder and Drlica(1979) showed that treating *E.coli* cells with oxolinic acid at concentrations sufficient to completely block the synthesis of DNA did not result in much DNA fragmentation as mostly intact nucleoids containing negative supercoiled DNA were isolated after sedimentation into ethidium-containing sucrose gradients (Snyder and Drlica, 1979). However if the isolated nucleoids were then treated with SDS to denature the proteins, fragments of DNA were detected. Therefore the gyrase enzyme holds the DNA together in bacterial cells and the lesion can be thought of as a complex of quinolone-gyrase-DNA containing a double-stranded break. These lesions would act as barriers to the passage of the replication fork and transcriptional machinery and would be prone to breakage in an actively replicating/ transcribing cell. The formation of DSBs in cells is potentially lethal and to

protect itself, the cell mounts a DNA repair response. Single-stranded DNA ends produced by DSB formation are bound by RecA which protects them against further damage and induces binding of RecBCD. It is not surprising that lack of these important recombination and repair enzymes in *recA*$^-$, *recB*$^-$ and / or *recC*$^-$ cells causes hypersensitivity to quinolone drugs (Lewin *et al*, 1989a). The hypersensitivity of *recA13* and *recB21* mutants is thought to be as a result of their lack of recombinational repair (Lewin *et al*, 1989a). A major determinant of the cytotoxicity of Class I drugs seems to be the interconversion of dormant single or double stranded breaks in a inhibitor-topoisomerase-DNA ternary complex into an irreversible double-stranded DNA break. The fact that yeast RAD52 mutants (RAD52 is a repair enzyme which reseals DNA double stranded breaks) are very sensitive to Class I drugs (Nitiss and Wang, 1988) gives weight to this hypothesis.

1.6.5 Repair of quinolone-induced DNA lesions

Genetic studies which made use of cells with mutations in several of the DNA recombination and repair genes showed that it is mainly such genes that affect the survival of the bacterial cell after treatment with quinolones (Lewin *et al*, 1989a). RecA has a central role in the protection of free DNA ends before they are repaired, ensuring that DNA is sufficiently intact before cell division can proceed. Another important enzyme is RecBCD (Exonuclease V) which is comprised of three subunits encoded by the *recB*, *recC* and *recD* genes (Smith, 1988). RecBCD has DNA helicase, single-strand DNA nuclease and double-strand DNA nuclease activities which combine to expose single-stranded DNA with a free end which acts as a substrate for RecA. RecBCD can only unwind DNA which has a free duplex end so once it has attached itself to the DNA it moves along, unwinding and rewinding DNA. The recombination activity of

RecBCD is stimulated by a sequence in DNA called Chi (5′ GCTGGTGG 3′). When RecBCD encounters a Chi sequence, the nucleolytic activity of RecBCD causes cleavage of the exposed single-stranded region near Chi so rewinding of the DNA cannot occur. As a result, a gap in the DNA and a single-stranded tail are produced, both of which RecA can bind. This structure is thus competent for DNA strand exchange with a homologous sequence.

1.6.6 The endo-exonuclease activity of RecBCD

Endo-exonucleases are thought to be present in all living organisms enhancing the survival of organisms by promoting recombination and repair of DNA. This leads to diversification of the genome, an advantageous ability in a changing environment. However if genomic DNA becomes damaged to such an extent that it cannot be repaired, it is the function of the endo-exonucleases to degrade it thus, in this way the population is relieved of cells containing detrimental mutations. Endo-exonucleases have both endonuclease and exonuclease activities. RecBCD in *E.coli* is an endo-exonuclease, possessing single-strand specific endonuclease and single-strand and double-strand exonuclease activities (Fraser, 1994). The breaks generated by the endonuclease activity have 3′ OH and 5′ P termini and the exonuclease activity on either linear single-stranded or double-stranded DNA results in the generation of small oligonucleotides with 5′ P ends. RecBCD is highly processive on double-stranded linear DNA, yielding single strands of DNA which are thousands of nucleotides long (Fraser, 1994).

1.6.7 Induction of the SOS response by the quinolones

The quinolone drugs have been shown to induce the SOS response in *E.coli*

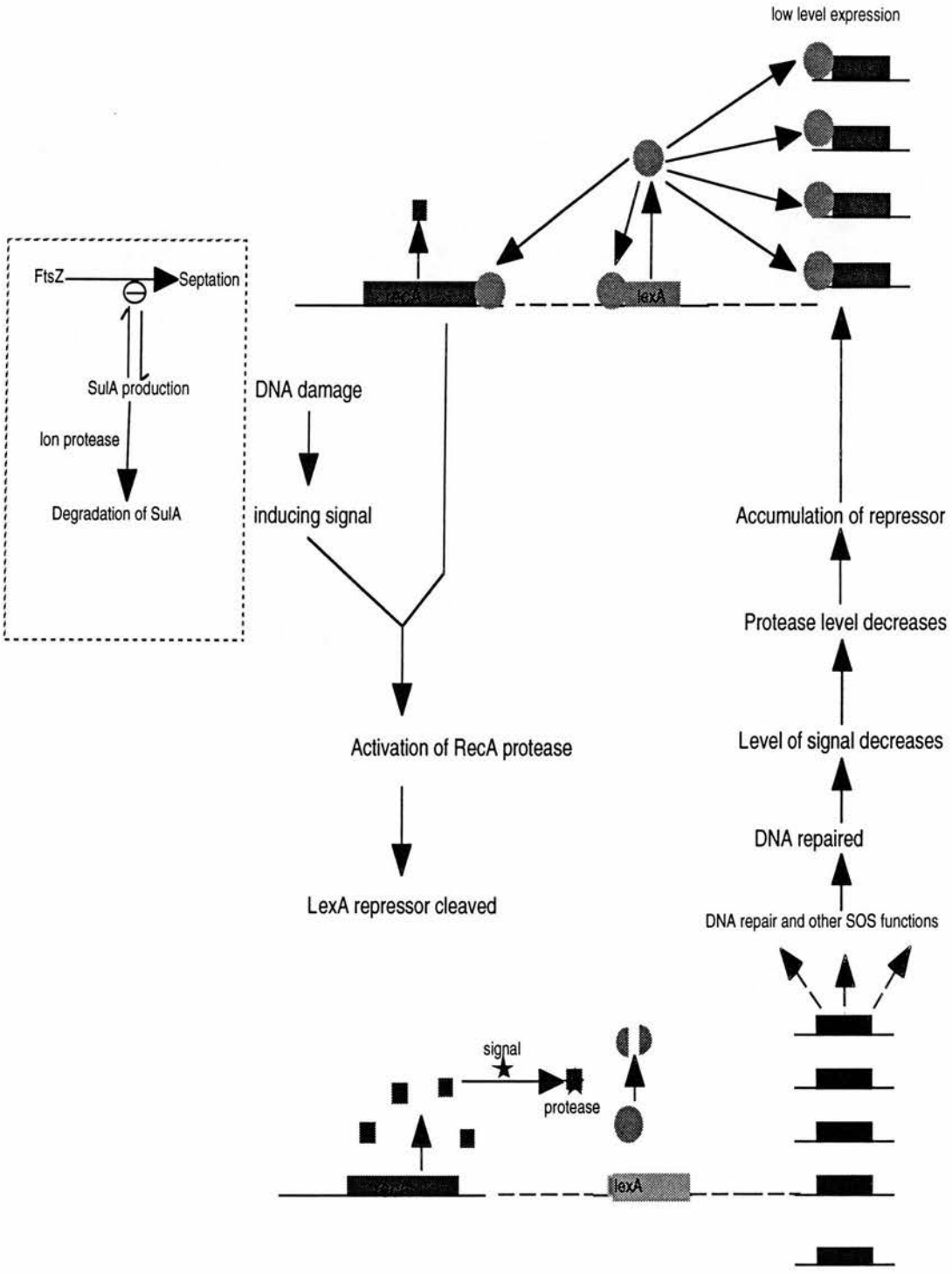
(Phillips *et al*, 1987). The maximal induction of the SOS response was found to occur at the OBC, suggesting that maximal induction of the SOS response might be lethal (Phillips *et al*, 1987). Concentrations of nalidixic acid greater than the OBC have been found to result in less DNA damage and less bactericidal activity (Crumplin and Smith, 1975). Also, agents that induce the SOS response are known to increase the mutation rate in *E.coli* (Quillardet *et al* 1985). This mutagenic effect can only occur in cells that are sensitive to quinolones and can mount an SOS response. Interestingly, the SOS response is also switched on in response to gyrase inhibition by coumermycin, albeit slowly at over two hours after addition of the drug (Menzel and Gellert, 1983). This in itself emphasises the essential nature of correct supercoiling levels in bacterial cells.

1.6.8 Induction of the SOS response is mediated by RecA

DNA damage in bacterial cells is detected by RecA, a protein central to recombination (Smith, 1988). As previously mentioned, RecA binds to single-stranded regions of DNA, coating it so it is protected from nuclease action (Smith, 1988). Binding to DNA in such a manner stimulates a catalytic ability of RecA to be able to cleave certain cellular repressors. This protease activity of RecA specifically cleaves the LexA repressor into two fragments. This results in the induction of the "SOS response", a response which is stimulated after treatment with DNA damaging agents which give rise to regions of single-stranded DNA, either by excision damage or because the damage is encountered by a replication complex (Goss *et al*, 1965; Crumplin *et al*, 1984; Benbrook and Miller, 1986). The action of RecBCD on DNA damaged by nalidixic acid is perhaps the signal for induction of the SOS response (Walker, 1984; Bailone *et al*, 1985). Each gene which is part of the SOS response has an operator recognised by the LexA repressor (Gottesman, 1984). Once RecA has cleaved the LexA

repressor, transcription of the SOS genes proceeds. The *recA* and *lexA* genes are also repressed by LexA. When DNA is damaged, the rate of synthesis of both RecA and LexA increases. More RecA is required for the high rate of DNA repair by recombination whereas more LexA is needed for the time that the DNA is repaired and repression of the SOS genes can again occur. Until this time, LexA is destroyed at a steady rate by RecA (Gottesman, 1984). Meanwhile, production of the SOS-induced SulA protein results in inhibition of septation. SulA binds to FtsZ and thereby prevents its action in the initiation of cell division. As long as SulA is produced, the cell cannot divide and instead is seen to form long filaments. This allows the cell to be able to recover from DNA damage before division resumes when SulA is degraded by the Lon protease. The stimulation of the SOS response by DNA damage is illustrated in Figure 1.10.

Figure 1.10: The stimulation of the SOS response by DNA damage



1.6.9 The Involvement of the SOS Response in Repairing Quinolone-damaged DNA

It has been suggested previously that either the SOS response or recombinational repair systems of *E.coli* are responsible for repairing DNA damage caused by quinolone drugs (Drlica, 1984). The effect of quinolones on the bacterial cell does not seem to be mediated only through the SOS response (Lewin *et al*, 1989a). Lewin *et al* (1989a) used a cell with a *lexA3* mutation, the LexA protein of which was resistant to proteolytic cleavage by RecA and the SOS response was not induced. However this strain was as sensitive to nalidixic acid as the wild type, at a number of concentrations (Lewin *et al*, 1989a). It would be expected that either a decreased or increased sensitivity to nalidixic acid would result depending on the action of the SOS response to either repair the damaged DNA or to kill the cell. It is also interesting that a *recA13recB21* double mutant was found to have an increased sensitivity to nalidixic acid than either single mutant (Lewin *et al*, 1989a). The fact that two mutations blocking the same pathway have an additive effect in this situation, disputes that the induction of the SOS response is solely responsible for repair. It therefore seems that it is the recombination activities of RecA and RecBCD that seem to be important in repair of quinolone-induced DNA lesions and the SOS response only seems to have a minor role.

1.6.10 Quinolone Cell Death Mechanisms

There are obviously many unanswered questions as far as cell death goes. Another confusing issue is the fact that the newer quinolones have been reported not to need protein synthesis to die but the older quinolones such as nalidixic acid do (Lewin *et al*, 1991). Experiments carried out have shown that the newer quinolones can kill nongrowing cells, cells in stationary phase and cells treated

with protein- and RNA-synthesis inhibitors (Zeiler, 1985; Lewin *et al*, 1990; Eng *et al*, 1991). Conclusions drawn separately by Diver and Wise (1986) and Smith (1984) suggested that in ciprofloxacin-challenged *E.coli*, there are at least two modes of cell death. Firstly, more than 90% of cell death is thought to occur by a mechanism not involving additional protein synthesis. This is the mechanism that Smith (1984) referred to as Mechanism B and was found only to occur with potent fluorinated quinolones such as ciprofloxacin or ofloxacin. Secondly, a minor cell death mechanism exists which does require protein synthesis and leads to cell filamentation and vacuolation. This mechanism was named Mechanism A by Smith (1984) and was found to occur with all quinolones. A third mechanism, Mechanism C, has also been identified which apparently works with ciprofloxacin, ofloxacin and norfloxacin (Ratcliffe and Smith, 1985). RNA and protein synthesis but not cell division are thought to be required. Howard *et al* (1993) proposed that Mechanism C is mediated via a drug interaction with the GyrB subunit. Experiments carried out by Crumplin and Smith (1976) demonstrated that single-stranded DNA precursors accumulated in nalidixic acid challenged cells. Smith(1984) proposed that this nicked DNA would form a substrate for cellular exonucleases which would cause a certain amount of DNA degradation and would then induce the SOS response. Whether or not the SOS response would be able to rescue the cell to any extent would presumably depend on the extent of gyrase inhibition and therefore inhibition of proteins needed to repair the DNA. Evidence for the SOS response would be observed by signs of filamentation and /or vacuolation of the cell which would themselves contribute as a secondary mechanism of cell death.

1.6.11 Topoisomerase IV and cytotoxicity

The inhibition of topoisomerase IV does not seem to lead to cytotoxicity when

inhibited by quinolones thus differing from the inhibition of gyrase. Since the cytotoxic effect of quinolones on gyrase is believed to result from cellular enzymes, for example the DNA replication complex, tracking along the chromosome and colliding with the cleavable complex, splitting it and leaving double strand breaks which then cannot be resealed, it can be concluded that for the inhibition of topoisomerase IV to be cytotoxic, this enzyme would also have to be present in front of the replication complex. As discussed in 1.5.8, the roles of gyrase and topoisomerase IV seem to be specific and it is likely that topoisomerase IV is localised to the terminus region of the chromosome and could even be membrane-bound (Rothfield, 1994). The fact that topoisomerase IV is unlikely to be present in front of the replication complexes on the chromosome explains the lack of cleavable complexes formed between topoisomerase IV, quinolone and DNA. Even though quinolones have been shown to inhibit topoisomerase IV, cytotoxicity will not result because of the lack of cleavable complex formation by the enzyme. Levels of quinolones completely inhibiting topoisomerase IV have been shown to be over one hundred times the normal MICs of potent quinolones (Nitiss, 1994). Although some quinolone-resistant *E.coli* have been found to have mutations in topoisomerase IV which results in an increased resistance to quinolones (Heisig, 1996; Vila *et al*, 1996), topoisomerase IV is only a minor target of quinolones in *E.coli* compared to gyrase and a decreased susceptibility to quinolones cannot occur without a mutation in DNA gyrase. Therefore while gyrase inhibition is potentially bactericidal, topoisomerase IV inhibition is largely bacteriostatic. This is thought to be as a result of its location in the cell membrane and/or behind the replication fork (Khodursky *et al*, 1995).

1.7 Aims of this thesis

1. To sequence the QRDR of *gyrA* of *S.typhimurium* and *S. typhi.*

2. To identify mutations arising in the QRDR of *gyrA* of *S.typhimurium* and *S.typhi* in response to a decreased susceptibility of quinolone drugs.

3. To study the difference in amino acid substitutions in the QRDR of *gyrA* between *E.coli* and *Salmonella* and to relate them to differential codon usage.

4. To clone *gyrA* from *E.coli* into a suitable cloning vector and study its effect on the cell when expressed at high levels.

5. To investigate the molecular mechanisms of quinolone-mediated cell death by attempting to correlate viability of the bacterial culture to integrity of the chromosomal DNA when electrophoresed on both pulsed field and conventional agarose gels.

2 Materials and Methods

2.1 Reagents

Chemicals and reagents were all supplied by Sigma Chemicals (Poole, Dorset) unless otherwise indicated. All antimicrobial agents were freshly prepared with sterile distilled water. Manufacturers of the antibiotics along with the solvents used to dissolve the powders are listed in Table 2.1.

Table 2.1 Antimicrobial Agents

Antimicrobial Agent	Abbreviation	Solvent	Manufacturer
Ampicillin	Amp	Water	Sigma
Chloramphenicol	Cm	Ethanol	Sigma
Ciprofloxacin	Cip	Water	Bayer
Nalidixic acid	Nal	Water	Sigma
Norfloxacin	Nfx	0.5M NaOH	Mercke, Sharp and Dohme
Ofloxacin	Ofx	0.5MNaOH	Roussel Laboratories Ltd
Oxolinic acid	Oxl	0.5M NaOH	Sigma
Rifampicin	Rif	Water	Ciba Laboratories
Streptomycin	Sm	Water	Sigma
Tetracyclin	Tet	Water	Sigma
Trimethoprim	Trim	Lactic acid	Wellcome Foundation

2.2 Bacterial and Phage Maintenance

Bacterial strains used in this study are listed in Table 2.2. Bacteria were either maintained on Luria-Bertani (LB) agar plates at room temperature or in Frozen Storage Buffer at -70°C for longer term storage. To prepare bacteria for frozen storage, a fresh 5ml overnight culture was prepared with antibiotic selection if required. A volume of 0.9ml of this culture was added to 0.1ml Frozen Storage Buffer [50% (v/v) glycerol] in sterile cryovials (Alpha Laboratories, Eastleigh, Hants), briefly vortexed and stored at -70°C. Strains were sub-cultured directly from stock onto solid media, for verification of phenotype and purity prior to use but were never passaged. Phage lysates were stored at 4°C as suspensions to which a few drops of chloroform were added to prevent microbial growth.

Table 2.2 Bacterial Strains

Strain	Genotype	Reference
E.coli K12 TG1	*thi, Δ(lac–proAB)*, F'[lacI^{q}lacZΔM15]	Anon (1991)
E.coli DH5α	*thi, Δlac*U169 (Φ80*lacZ*ΔM15)	Hanahan (1983)
E.coli KNK453	*gyrA*^{ts}	Kreuzer and Cozzarelli, 1979
E.coli XL-1 BLUE MRF'	Δ(*mcrA*)183, D(*mcrCB-hsdSMR-mrr*) *endA1, supE44, thi01, recA1, gyrA96, relA1, lac*[*F'proAB, lacIqZDM15, Tn10 (tet^R)*]	Stratagene
E.coli SOLR	e14$^-$(*mcrA*), Δ(*mcrCB-hsdSMR-mrr*)171, *sbcC, recB, recJ, umuC::Tn5(kanR), uvrC, lac, gyrA96, relA1, thi-1, endA1, lR,* [F' *proAB, lacIqZDM15*] Su-	Stratagene

The clinical strains which were investigated in Chapter 3 were received as

follows. The *S.typhimurium* isolates from Vellore, India were collected by Dr Hilary-Kay Young (University of Dundee) in 1984. The *S.typhi* isolates from Vellore, India were collected by Dr Mary Jesudason (Christian Medical College and Hospital, Vellore, India) between 1992 and 1994. The clinical isolates of *E.coli* and *S.typhimurium* collected from a variety of British hospitals were received from a culture collection documented by Dr Robert Paton (University of Edinburgh).

Plasmids used and constructed in the course of this study are listed in Table 2.3.

Table 2.3 Plasmids

Plasmid	Size	Markers	Description	Reference
pBR322	4.3kb	Amp^R Tet^R	Cloning vector	Balbas *et al*, 1986
pUC18	2.7kb	Amp^R*lacZ*	Cloning vector	Norrander *et al*, 1983
pKIL119	3kb	Kan^R	Encodes *ccdB*	Bernard, 1995
pNotI	-	Amp^R	Encodes *Not*I	New England Biolabs
pOU61	9kb	Amp^R	Cloning vector	Larsen *et al*, 1984
pBluescript	2.9kb	Amp^R *lacZ*	Cloning vector	Stratagene
pPH3	8kb	Amp^R	Encodes *gyrA*	Hallett *et al*, 1990
pNJR3-2	28kb	Tet^R	Encodes *gyrA*	Robillard, 1990
pBRgyrA	6.8kb	Amp^R	Encodes *gyrA*	This work
pOUgyrA	11.8kb	Amp^R	Encodes *gyrA*	This work

2.3 Growth Media and Buffers

Luria-Bertani (LB) broth [1%w/v Bactotryptone (Difco), 0.5% (w/v) Bacto yeast extract (Difco), 1% (w/v) sodium chloride] and Luria-Bertani (LB) agar [Luria-Bertani broth +1.5% bacteriological agar number 1 (Oxoid)] were used routinely for all bacterial manipulations except where stated. For work with phage λ, the media were supplemented with 10mM $MgSO_4$ and 0.2% maltose to maximise expression of the λ receptor protein.

2.4 Growth of Bacteria

Bacteria were routinely grown as liquid overnight cultures at 37°C. A single colony from a freshly streaked out strain was inoculated aseptically into broth and shaken overnight in a temperature-controlled orbital shaker (Gallenkamp). If the strain, or a plasmid harboured by it, contained antibiotic resistance markers, the appropriate antibiotic was added to the culture for selection purposes.

2.5 Determination of the Minimum Inhibitory Concentration of a particular antibiotic

Minimum Inhibitory Concentrations (MICs) were performed on Isosensitest (IST) agar following the British Society for Antimicrobial Chemotherapy (BSAC) guidelines for susceptibility testing where applicable (Phillips *et al*, 1991). MICs were determined by agar double dilution of the antimicrobial agent. Agar, containing the appropriate concentrations of antimicrobial agent, was inoculated with a 2μl spot of each organism, delivered from a multipoint inoculator (Denley, Billinghurst, Surrey). Bacterial strains to be tested were inoculated into Luria

Bertani broth and incubated for 16 hours at 37°C before dilution in 0.7% sterile saline to give an inoculum of 10^4 colony forming units (cfu/spot) in each case. Inoculated agar plates were incubated overnight at 37°C.

2.6 Determination of the Optimum Bactericidal Concentration of a particular antibiotic

Determination of the optimum bactericidal concentration (OBC) of a particular antibiotic was performed on a log phase culture of the bacteria to be tested. Various concentrations of antibiotics were added to a specified volume of culture and allowed to grow for a specified length of time. Aliquots of culture (neat) and 10^{-2} and 10^{-4} dilutions (in 0.7% sterile saline) of the aliquots were plated out on McConkey agar (Oxoid), incubated overnight and the viable counts determined. The concentration of antibiotic corresponding to the most cell death was termed the OBC.

2.7 Preparation of Plugs for Pulsed Field Electrophoresis

Pulsed field electrophoresis of chromosomal DNA was carried out by embedding a specified number of cells in low melting point agarose (Biorad) to minimise shearing of DNA. The bacterial culture was centrifuged (at 3000rpm for 15 minutes) in a bench top centrifuge, drained and the pellet resuspended in 100µl SE buffer [10mM NaCl, 25mM EDTA pH7.5] and the OD_{590} adjusted to 1. An equal amount of bacterial culture in SE buffer was added to 1% low melting point agarose (Biorad) in a sterile Eppendorf tube and mixed thoroughly. A 80µl volume of this mixture was inserted into a well of the plug mould (Biorad) and allowed to set at 4°C. The set plugs were transferred to a 2ml sterile bijoux containing 2ml lysis buffer [1%w/v N-lauroylsarcosine, 50mM EDTA pH9.5

with 0.5mg/ml proteinase K freshly prepared]. The plugs were deproteinised overnight and the following day, washed three times thoroughly with 2ml TE buffer [10mM Tris, 1mM EDTA pH8]. A 1% agarose gel was prepared by the addition of 1g Pulsed Field grade agarose (Biorad) to 100ml 0.5xTBE [10xTBE is 0.89M Tris-borate pH8.3, 20mM EDTA]. The plugs were inserted into the gel and sealed by pipetting 5ml of molten agarose over them.

2.8 Pulsed Field Electrophoresis

A pulsed field gel electrophoresis tank (Chef DR-II, Biorad, UK) was used to separate the DNA fragments. The initial pulse time was 1 second, the final pulse time was 60 seconds, the voltage was 200V and the temperature kept constant at 14°C for 16 hours. To size the DNA fragments, λ concatemer markers (Biorad,UK) were used. After 16 hours, the gel was stained in 50μg/l ethidium bromide and photographed over UV light from a transilluminator (UV Products, Cambridge).

2.9 Preparation of Competent Cells

To prepare competent *E.coli* cells, the method of Chung and Miller (1988) was used. A single colony of the appropriate strain of bacteria was inoculated into 5ml of LB broth containing a suitable antibiotic and shaken overnight at 37°C. This was then diluted 1 in 100 into a flask containing LB broth and grown until an OD_{600} of between 0.3 and 0.4. The cells were subsequently placed on ice and transferred to a sterile thick-walled glass universal bottle and pelleted at 3000rpm in a benchtop centrifuge for 10 minutes. The broth was poured off and after allowing the pellet to drain briefly by inversion, the cells were again placed on ice. Ice-cold TSS buffer [1% (w/v) Bactotryptone (Difco), 0.5% (w/v)

Yeast extract (Difco), 1% (w/v) NaCl, 10% (w/v) PEG3350, 20mM $MgSO_4$, 10mM PIPES pH6.5] was added in a volume equivalent to 0.1x original volume of cells and the cells resuspended by vortexing. A volume of DMSO equivalent to 0.05X original volume of cells was added after which the cells were ready for transformation.

2.10 Transformation of competent cells with plasmid DNA

Transformation of competent cells (prepared as described in Section 2.9), was carried out by adding 1-100ng of plasmid DNA (in a volume of <10µl) to 100µl of cells. The tubes were left on ice for a period of 30 minutes, after which 400µl of LBG (LB broth + 20mM glucose) was added to each, mixed and the cells incubated at 37°C for one hour to allow expression of plasmid antibiotic resistance genes. Aliquots of 200µl of the incubated cells were then spread on appropriate antibiotic-containing plates and incubated overnight at the appropriate temperature. During the cloning experiments, inserts that had been ligated into *lacZ* of the cloning vector could be identified by plating the transformants out on agar plates which contained 20µg/ml X-gal and 0.1mM IPTG. Plasmids which carried inserts which interrupted expression of *lacZ* appeared white, whereas those which had no insert remained blue.

2.11 Small Scale Plasmid Preparation

Routine preparations of plasmid DNA were performed by a modification of the alkaline lysis method of Birnboim and Doly (1979). A 5ml volume of LB broth (plus suitable antibiotic selection) was inoculated with a single colony of the plasmid bearing strain, and incubated with continuous shaking at the appropriate temperature (typically 37°C) overnight. The culture was then

centrifuged at 3000rpm in a benchtop centrifuge. The supernatant was discarded, and the bacterial pellet resuspended in 0.1ml of Solution I [1% glucose, 10mM EDTA and 25mM Tris-HCl (pH8)]. To this cell suspension, 0.2ml of Solution II [0.2M NaOH, 1% SDS] was added, mixed by gentle inversion of the tube and incubated on ice for 5 minutes to allow lysis to proceed. A volume of 0.15ml of Solution III [3M sodium acetate (pH5.0)] was then added and the contents of the tube mixed vigorously by extensive vortexing (vortexing was only carried out when the plasmids to be extracted were less than 10kb, plasmids larger than this would be likely to shear by this step). After leaving the prep on ice for 10 minutes to allow precipitation of chromosomal DNA and insoluble cellular debris, the mixture was then spun in a microfuge for 10 minutes. The resulting supernatant (about 0.5ml) was transferred to a fresh Eppendorf tube and 0.5ml of phenol/ chloroform/ isoamyl alcohol (ratio 25:24:1) was added, mixed by vortexing and centrifuged in a microfuge for 2 minutes. The upper aqueous phase was transferred to a fresh tube and the plasmid DNA could then be recovered from solution by ethanol or isopropanol precipitation. Typically, the final pellet of nucleic acid was resuspended in TE buffer (10mM Tris, 1mM EDTA pH8) containing RNase A (20µg/ml). It was usually observed that 5ml of overnight culture yielded approximately 3-5µg of plasmid DNA.

2.12 Large Scale Plasmid Preparation

To prepare large scale quantities of plasmid DNA, a Qiagen midiprep kit (Qiagen, Chatsworth, California) was used. A 25ml volume of culture (high copy number plasmids) or 100ml volume of culture (low copy number plasmids) routinely yielded 50µg to 100µg DNA.

2.13 Preparation of chromosomal DNA

Chromosomal DNA was prepared by a slight modification of the protocol of Zyskind and Bernstein (Zyskind & Bernstein, 1992). A single colony was inoculated into 5ml of nutrient broth and grown overnight in a 37°C incubator. A 1.2ml volume of this culture was spun in a microcentrifuge (Microcentaur MSE) for a period of 15 seconds. The broth was discarded and the cell pellet resuspended in a volume of 0.31ml HTE buffer [50mM Tris-HCl, pH 8.0, 20mM EDTA] by briefly vortexing to which 0.35ml of a 2% solution sarcosyl in HTE was added with brief inversion of the tube, followed by 5µl RNase [10mg/ml in TE buffer]. A volume of 35µl of pronase [10mg/ml in 10mM Tris-HCl pH8.0, 10mM NaCl, 0.1mM EDTA buffer] was added and the tubes incubated in a 50°C waterbath until lysis had occurred. Three rounds of phenol/chloroform extraction were carried out and the resulting lysate treated with a 10% volume of 3M sodium acetate and a 100% volume of isopropanol at -20°C. DNA precipitation was achieved after placing the tubes at -70°C for 15 minutes followed by centrifugation at 13,000rpm in a microcentrifuge for 20 minutes. The resulting pellet was briefly washed by the addition of 70% ethanol and then dried in a vacuum desiccator. Resuspension of the pellet in 100µl of TE gave a solution of DNA suitable for amplification by the Polymerase Chain Reaction.

2.14 Amplification of DNA by the Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) amplification of a specified fragment of DNA was carried out in a total volume of 100µl prepared in a 0.5ml polypropylene microcentrifuge tube (Alpha Laboratories) with a Techne PHC-2 Dri-Block Cycler (Cambridge, Cambs.). PCR amplifications consisted of 1xPCR buffer

(Gibco, BRL), 2.5mM $MgCl_2$ (Gibco, BRL), 200-400μM each dNTP (Boehringer Mannheim), 10pmoles of each primer (Oswel, Department of Oswel, Department of Chemistry, University of Edinburgh), 10ng DNA template and 1 unit of *Taq* polymerase (Gibco, BRL). Reaction mixtures were overlaid with one or two drops of mineral oil to reduce evaporation, and the tips of the tubes were smeared with a thin film of silicone grease to improve thermal contact with the heating block. After completion of the PCR reaction, samples were placed on ice to quench any further reaction.

Two sets of primers, designed on inspection of the *gyrA* sequence of *Escherichia coli* (Yoshida *et al*, 1988), were used during the course of this work, one set amplified the first 620bps of *gyrA* including the QRDR and the other set amplified the whole gene including promoter and terminator sequences.

Primer sequence	Amplification Conditions
Region amplified: +1 to +620bps	
5 ' ATGAGCGACCTTGCGAGAGAAATTACACCG 3 '	96°C for 15s
5 ' TTCCATCAGCCCTTCAATGCTGATGTCTTC 3 '*	96°C for 15s
* indicates that a biotin tag was added to the end	50°C for 30s
	70°C for 90s
	70°C for 5 mins
Region amplified: -70 to +2840bp	
5 ' CCGCGGATCCGAATAAAGCGTATAGGTTTA 3 '	94°C for 3 mins
5 ' TCGAATTCGCCCAGACTTTGCAGCCTGG 3 '	94°C for 3 mins
	56°C for 3 mins
	74°C for 3 mins
	74°C for 5 mins

2.15 Restriction of DNA

Endonuclease cutting of DNA was typically performed in volumes of between 20 and 100µl. These contained the requisite amount of DNA (usually 1-10µg) and the appropriate Promega restriction buffer (Promega, Madison, WI, USA) at 1x concentration. The restriction enzyme (Promega, Madison, WI, USA) was usually present in a 2-5 fold excess, i.e. 2-5 units per microgram of DNA. The digests were made up to their final volume with distilled water. The complete restriction digests were incubated at the recommended temperature (usually 37°C) for 1-3 hours. The products of the reaction were either directly analysed by agarose gel electrophoresis, or phenol extracted, ethanol precipitated and dissolved in a suitable volume of TE buffer [10mM Tris, 1mM EDTA pH8] for future manipulations.

2.16 Conventional Agarose Gel Electrophoresis

Agarose gel electrophoretic analysis of DNA was either performed with TAE [10X TAE is 40mM Tris-acetate pH7.6, 1mM EDTA] or TBE [10X TBE is 0.89M Tris-borate pH8.3, 20mM EDTA] buffer. The gels were made up by melting the appropriate amount of agarose (Gibco BRL) in either 1x TAE or 1x TBE in a microwave oven. Gels were cast in an appropriate casting tray and once set, placed in a gel electrophoresis tank (Bethesda Research Laboratories Horizon 20.25 gel tank, Life Technologies, Petersburg, Florida or a Pharmacia GNA-100 minitank) and immersed in the same buffer (either 1xTAE or 1xTBE) used in the preparation of the gel. The DNA samples containing 1x tracking dye [30%w/v glycerol, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol] were loaded

into the wells at one end of the tray and then electrophoresed at between 50V and 100V for a time, the length of which depended on the size of the DNA fragments concerned. After completion of electrophoresis, gels were stained in water containing 50µg/l ethidium bromide for about an hour. The gels could be photographed with Polaroid film and UV transillumination (UV Products, Cambridge).

For RFLP analysis, Metaphor agarose (FMC Bioproducts, supplied by Flowgen, UK) was used on its merits of discriminating between small fragments of DNA.

2.17 Size Fractionation of DNA

DNA of a certain size (or range of sizes) was isolated from agarose gels by a Geneclean kit (Bio 101 Inc, La Jolla, California, USA). The appropriate DNA band (or range of bands) was located in an ethidium bromide stained gel under UV transillumination and cut out with a clean razor blade in as small a volume of agarose as possible. Gel slices were transferred to Eppendorf tubes, the weight of the slice determined and 3 volumes of saturated sodium iodide solution added. These were incubated at 50°C until the gel slice had dissolved. A 5µl volume of glassmilk was then added and the suspension mixed well and left on ice for 5 minutes. Tubes were spun in a microfuge for 5 seconds and the supernatant poured off. The pellets were washed three times in 0.5ml of Geneclean New Wash Solution (supplied with the kit), centrifuging and resuspending the pellets each time. After the final washing, all traces of the wash solution were removed with a Pasteur pipette and the pellets were then suspended in 5µl distilled water. These were incubated at 50°C for 2-3 minutes, centrifuged for 30 seconds and the DNA containing supernatant transferred to a fresh tube. A further 5µl of distilled water was added to the glassmilk pellets

and the procedure was repeated to give a final DNA containing solution with a volume of 10µl. This DNA solution could be directly used for further manipulations.

2.18 Ligation of DNA

Ligations of DNA were typically performed in a final volume of 10µl, or 5µl when ligating into λZAPII (Stratagene, La Jolla, California). The constituents of the ligation reactions were an appropriate amount of vector and insert (both extracted with phenol/ chloroform and ethanol precipitated), ligation buffer and 0.1 unit of T4 DNA ligase (Gibco BRL) made up to the correct volume with distilled water. The ligation reactions were incubated for 16 hours at 12°C.

2.19 Creation of a chromosomal library in the vector λZAPII

The vector, λZAPII pre-digested with *EcoR*I and calf intestinal alkaline phosphatase (Stratagene, La Jolla, California, USA) in a volume of 1µl (1µg) was added to 3µl *EcoR*I -digested *E.coli* chromosomal DNA, 0.5µl 10X T4 DNA ligase buffer (Gibco,BRL) and 0.5µl T4 DNA ligase (Gibco, BRL). This ligation mixture was incubated at 12°C overnight.

2.20 Packaging of λZAPII

After ligation of DNA into this vector, packaging of the phage was necessary prior to infection into the plating cells. To the 5µl ligation mixture (see above), the two components of the Gigapack II Gold Packaging extract (Stratagene, La Jolla, California, USA) were added, following the manufacturer's protocol. Incubation at room temperature for 2 hours proceeded, after which 500µl of

SM buffer [0.5% (w/v) NaCl, 0.2% (w/v) $MgSO_4$, 5% 1M Tris-HCl pH7.5, 0.01% gelatin] was added along with 20µl chloroform. This aliquot of packaged phage was stored at 4°C until it was used to infect the prepared plating cells (see section 2.21).

2.21 Preparation of λ Plate Lysates

A single colony of *E.coli* XL-1 BLUE MRF' was inoculated into 50ml LB broth supplemented with 20mM $MgSO_4$ and 20% maltose for 4-6 hours at 37°C. The culture was then spun down in a benchtop centrifuge at 3000rpm for 10 minutes, after which the broth was discarded and the pellet resuspended in 10mM $MgSO_4$. The OD_{600} of the cells was measured and the cells titered with 10mM $MgSO_4$ to an OD_{600} value of 0.5. These plating cells in a volume of 200µl were added to 1µl of packaged phage particles (see section 2.20). This was allowed to incubate at 37°C for 15 minutes, after which 3ml of Top Agar [LB broth + 0.7% agarose] at 55°C was added and the mixture immediately poured onto fresh LB agar plates. After 10 minutes, the plates were transferred to a 37°C incubator overnight or until visible lysis had occurred. Since the λZAPII vector contains *lacZ*, estimation of the proportion of plaques formed from phages containing inserts to those which did not, could be accomplished by the addition of 50µl 250mg/l X-gal and 15µl 10mM IPTG to the top agar. After lysis had occurred, those plaques formed from phage containing inserts appeared clear, whereas those which had formed by the re-ligation of the two arms of the vector, appeared blue.

2.22 Titering the phage library

A single colony of *E.coli* XL-1 BLUE MRF' was inoculated into 50ml LB broth supplemented with 20mM $MgSO_4$ and 20% maltose for 4-6 hours at 37°C. The

culture was then spun down in a benchtop centrifuge at 3000rpm for 10 minutes, after which the broth was discarded and the pellet resuspended in 10mM $MgSO_4$. The OD_{600} of the cells was measured and the cells titered with 10mM $MgSO_4$ to an OD_{600} value of 0.5. These plating cells in a volume of 200µl were added to various dilutions of packaged phage particles (see 2.20). This was allowed to incubate at 37°C for 15 minutes, after which 3ml of Top Agar [LB broth + 0.7% agarose] at 55°C was added and the mixture immediately poured onto fresh LB agar plates. After 10 minutes, the plates were transferred to a 37°C incubator overnight or until visible lysis had occurred. The number of plaques were counted and the pfu/ml of the library was determined.

2.23 Preparation of Single Stranded DNA prior to DNA Sequencing

Sequencing was carried out directly from the PCR fragment with Dynabeads M-280 (Dynal A.S., N-0212, Oslo, Norway) to separate the strands. A 40µl volume of PCR-amplified DNA was added to 40µl of washed Dynabeads according to the manufacturer's protocol and the strands subsequently separated.

2.24 DNA Sequencing

A 5µl volume of the single-stranded DNA template attached to the Dynabeads prepared as above was used routinely in the sequencing reaction which was carried out with a Sequenase 2.0 kit according to the manufacturer's protocol with [^{35}S]-dATP (Amersham Life Sciences, UK) radiolabel.

2.25 DNA Sequencing Gel Electrophoresis

DNA sequencing was performed with a Nucleic Acid Sequencing Cell (Bio-

Rad, Watford, Herts). The glass sequencing gel plates were thoroughly cleaned with ethanol and distilled water and assembled together with 0.2mm spacers separating the two plates. The top plate was siliconized with Gel Slick (AT Biochem, Malvern, Pennsylvania), in order to prevent the gel sticking to both the plates when the apparatus was disassembled. The gel was prepared as described by Ausubel *et al* (1995). For the plug, 20ml of the gel solution was polymerised by the addition of a 100µl of a 25% ammonium persulphate solution and 100µl TEMED. Immediately, this was poured into the gel casting tray to form a plug. For each ml of remaining gel solution, 1µl of 25% ammonium persulphate solution and 1µl of TEMED was added and the solution poured carefully between the two plates of the gel apparatus. The flat edge of a shark toothed comb was pushed between the plates to layer the top of the gel. The gel was set aside for at least one hour to allow polymerisation. Once set, the comb was removed and the top of the gel was thoroughly flushed with distilled water. The sharktooth comb was then replaced with the points downwards just touching the surface of the gel. The gel was clamped into the sequencing apparatus and 0.6x TBE solution poured into the top and bottom reservoirs. The gel was then pre-run at 50W for 1 hour. The gel was ready to be loaded with the sequencing reactions. The samples were loaded in the order G, A, T and C immediately after denaturing the DNA by heating the samples to 80°C for 2 minutes to denature the DNA. Immediately after this incubation, 2-3µl of each sample were loaded onto the gel. The gel was then electrophoresed at 50W for 2-6 hours, depending on which part of the sequence was to be read. Once electrophoresis was complete, the glass plates were removed from the apparatus and the top plate very carefully removed. The bottom plate with the gel attached was then placed in a fixing bath containing 15% methanol and 5% acetic acid in water for 30 minutes. The plate and gel were then removed and a

sheet of Whatman 3MM paper placed on top of the gel. Even pressure was then applied and the paper peeled off taking the gel with it. The gel attached to the paper was covered in Saran Wrap and dried in a vacuum gel drier for 1-2 hours at 80°C. When dry, the gel was placed in an autoradiography cassette (Amersham Life Sciences, UK) and exposed to Kodak Biomax MR-1 film at room temperature for 24 hours after which the film was developed at the X-ray department, Royal Infirmary of Edinburgh.

2.26 Extraction of RNA

Total RNA was extracted by the use of a RNeasy kit (Qiagen, Chatsworth, California). Potentially contaminating RNases were removed from the working area by applying RNase ZAP (Ambion, Austin, Texas, USA) to pipettes and bench surfaces.

2.27 Electrophoresis of RNA

RNA was electrophoresed in a formaldehyde containing gel. The gel was prepared by melting an appropriate amount of agarose in water, cooling to 60°C and adding 5x gel buffer [0.2M morpholinopropane sulfonic acid pH7, 50mM sodium acetate and 5mM EDTA pH8) and formaldehyde to give 1X and 2.2M final concentrations respectively. The RNA sample in a volume of 9µl was added to 4µl 5x gel buffer, 7µl formaldehyde and 20µl formamide and incubated at 65°C for 15 minutes. After chilling on ice and briefly pulsing in a microcentrifuge, 4µl of sterile-DEPC-treated formaldehyde gel loading buffer [50% glycerol, 1mM EDTA pH8, 0.25% bromophenol blue, 0.25% xylene cyanol FF] was added. Each mixture was divided between two wells on opposite halves of the gel, as by loading in duplicate, one half of the gel could be blotted

(see 2.28) and the other half could be stained with ethidium bromide. The samples were electrophoresed in 1x formaldehyde gel running buffer for 3 to 4 hours at 100V. After electrophoresis, half of the RNA gel was soaked in 0.5M ammonium acetate for 20 minutes in a RNase-free glass dish. This solution was then poured off and replaced with 0.5µg/ml ethidium bromide in 0.5M ammonium acetate and stained for 40 minutes. The gel was destained in 0.5M ammonium acetate for one hour and the RNA fragments visualised on a UV transilluminator.

2.28 Preparation of Northern Blots

The half the gel that was not stained with ethidium bromide was thoroughly washed in DEPC-treated water before soaking in 5mM sodium hydroxide for 30 minutes. This solution was replaced with 20xSSC [3M sodium chloride, 0.3M trisodium citrate adjusted to pH7 with 1M HCl] and soaked for 45 minutes. The gel was then transferred to a RNase free glass baking dish and any unused areas of the gel trimmed away with a razor blade. The procedure then that followed that described in Ausubel *et al*, (1995).

2.29 Plaque Lifts

The *E.coli* genomic library prepared in λZAP was screened by carrying out plaque lifts. Plating cells were prepared as described before and infected with an appropriate volume of phage. The phage lysates were plated out on 24.3cm by 24.3cm Petri dishes and incubated overnight. The plates were then chilled for two hours at 4°C to prevent top agar from sticking to Hybond N+ filter (Amersham Life Sciences, USA). The filter was applied to the surface of the Petri dish and the transfer allowed to proceed for two minutes. The filter was

then denatured after lifting by submerging in denaturing solution [1.5M NaCl, 0.5M NaOH] for 2 minutes, neutralised for 5 minutes in neutralising solution [1.5M NaCl, 0.5M Tris-HCl pH8], rinsed for 30 seconds in rinsing solution [0.2M Tris-HCl pH7.5, 2X SSC] before blotting briefly on Whatman 3MM filter paper. After baking the filter for 2 hours at 80°C, it was hybridised as explained in 2.30.

2.30 Hybridisation of Plaque Lift Filters

Hybridisation was carried out by the use of a ECL 3' oligo labelling kit (Amersham, Bucks) following the manufacturer's protocol. The probe used was a oligonucleotide specific to *gyrA* (described in Table 2.4) that had been previously labelled with fluorescein.

2.31 Hybridisation of Northern blot filters

Hybridisation of Northern blot filters was carried out following the protocol detailed in Ausubel *et al*, (1995). The probe used was either a fluorescein-labelled oligonucleotide specific to gyrA (as described in 2.30) or a 620bp PCR-amplified fragment of *gyrA* (described in Table 2.4) into which ^{32}P-dCTP (Amersham Life Sciences, UK) had been incorporated by random prime labelling. 20ng of purified DNA was made up to 24µl in distilled water and boiled for 3 minutes. The mixture was placed at 37°C and added to 10µl OLB buffer [OLB buffer was made from the following components: Solution O (1.25M Tris-HCl pH7.5, 0.125M $MgCl_2$), Solution A (1ml Solution O, 18µl β-mercaptoethanol), Solution B (2M Hepes, pH 6.6) and Solution C (Hexadeoxyribonucleotides (Pharmacia) at 4.5mg/ml). Solutions A, B and C were mixed at a ratio of 100: 250: 150 and the OLB mix was stored at -20°C]. To this, 2µl BSA (10mg/ml), 1µl of each of

dATP, dGTP and dTTP (stock solutions of 10mM), 10µl ^{32}P-dCTP and 1ml Klenow polymerase (Gibco BRL) was added. The reaction was placed at 19°C for 30 minutes then at 30°C for 30 minutes before termination by the addition of 150µl OLB stop buffer [25mM NaCl, 25mM Tris-HCl pH7.5, 5mM EDTA, 1% SDS). The DNA was purified through a Sephadex G-50 spun column, the 200µl solution collected being immediately useable as a probe. The blot was pre-hybridised in 25ml of pre-hybridisation buffer [5XSSC, 0.5% SDS, 100mg/ml denatured, fragmented salmon sperm DNA (Genebloc, MBI Fermantas), 50X Denhardt's reagent (5g Ficoll, 5g polyvinylpyrrolidone, 5g BSA and water to 500ml)] for 2 hours at 68°C after which this solution was replaced with 25ml hybridisation buffer [pre-hybridisation buffer + 9% dextran sulphate]. The ^{32}P-labelled probe (prepared as described in 2.31) was denatured by heating the probe at 96°C for 5 minutes before rapidly chilling on ice and adding to the hybridisation mix. This was incubated overnight at 68°C. The filter was washed of excess probe and non-specific hybrids by 3 washes of 30 minutes at 55°C in RNA wash buffer [0.1% SDS, 5mM Tris-HCl pH7.5, 25mM NaCl and 1mM EDTA]. The filter was then air dried for 3 minutes and sealed into a thin plastic bag ready for autoradiography. Filters were placed between two Ilford "plus-X" intensifying screens in an autoradiographic cassette with a sheet of X-ray film and stored at -70°C until development.

3 Detection of mutations in the QRDR of *gyrA* in quinolone-resistant bacteria

3.1 Introduction

Since the introduction of quinolone drugs such as ciprofloxacin, ofloxacin and nalidixic acid for the treatment of *Enterobacteriaceae* infections, there have been numerous reports of clinical isolates showing a reduced susceptibility to these drugs (Wiedemann and Heisig, 1994). By far the most heavily reported type of mutation causing this decreased susceptibility is a change in the *gyrA* gene (Reece and Maxwell, 1991b), occurring in the quinolone-resistance determining region (QRDR). Such mutations can be identified by various molecular techniques and subsequently correlated to a change in the minimum inhibitory concentration (MIC) of the cell for a particular drug compared to a sensitive isolate.

Mini-surveys were carried out on four separate groups of clinical *Enterobacteriaceae* isolates and DNA sequencing was performed to detect mutations that had arisen in response to challenge with the quinolone drugs. The sequencing results were confirmed by showing that a change in the restriction pattern of this region of the QRDR had also occurred. The four groups of clinical isolates studied were as follows:

- *Salmonella typhimurium* isolates from Vellore, India, collected in 1984
- *Salmonella typhi* isolates from Vellore, India, collected during the period 1992 to 1994
- *Salmonella* species (various) from a variety of British hospitals collected during the period 1989 to 1992
- *Escherichia coli* isolates from a variety of British hospitals collected during the period 1989 to 1992

3.2 DNA Sequencing of the QRDR of *gyrA* of *S.typhimurium* NCTC 5710

At the onset of this work, a published DNA sequence of any part of the *gyrA* gene of *S.typhimurium* was unavailable. Consequently, part of the *gyrA* gene of the standard sensitive isolate *S.typhimurium* NCTC5710 was sequenced so that any point mutations found in the DNA of the clinical isolates could be compared to the sensitive NCTC isolate. This NCTC strain was sensitive to ciprofloxacin, ofloxacin and nalidixic acid; the MICs of each of these antibiotics were 0.008mg/l, 0.064mg/l and 2mg/l respectively. A 620bp fragment incorporating the QRDR of the enzyme was amplified by the polymerase chain reaction from chromosomal DNA and both DNA strands consequently sequenced. A DNA sequence 486 base pairs long was achieved and is shown in Figure 3.1 along with the predicted amino acid sequence.

Figure 3.1: The DNA and predicted amino acid sequence of *S. typhimurium gyrA* (nucleotides 72 to 557, amino acids 24 to 185). The nucleotide sequence of the *gyrA* gene is presented from the 5' (left) to 3' (right) end. The deduced amino acid sequence is given below the DNA sequence. Amino acid positions are numbered, starting at codon 24. Deviations from the nucleotide sequence of *E.coli* (Yoshida *et al*, 1988) are highlighted in bold.

```
TAT GCG ATG TCG GTC ATT GTT GGC CGT GCG CTG CCA GAT GTC CGA GAT GGC CTG
Tyr Ala Met Ser Val Ile Val Gly Arg Ala Leu Pro Asp Val Arg Asp Gly Leu
    25                  30                  35                  40

AAG CCG GTA CAC CGT CGC GTA CTT TAC GCC ATG AAC GTA TTG GGC AAT GAC TGG
Lys Pro Val His Arg Arg Val Leu Tyr Ala Met Asn Val Leu Gly Asn Asp Trp
            45                  50                  55

AAC AAA GCC TAT AAA AAA TCT GCC CGT GTC GTT GGT GAC GTA ATC GGT AAA TAC
Asn Lys Ala Tyr Lys Lys Ser Ala Arg Val Val Gly Asp Val Ile Gly Lys Tyr
60                  65                  70                  75

CAT CCC CAC GGC GAT TCC GCA GTG TAT GAC ACC ATC GTT CGT ATG GCG CAG CCA
His Pro His Gly Asp Ser Ala Val Tyr Asp Thr Ile Val Arg Met Ala Gln Pro
        80                  85                  90                  95

TTC TCG CTG CGT TAC ATG CTG GTG GAT GGT CAG GGT AAC TTC GGT TCT ATT GAC
Phe Ser Leu Arg Tyr Met Leu Val Asp Gly Gln Gly Asn Phe Gly Ser Ile Asp
                100                 105                 110

GGC GAC TCC GCG GCA GCA ATG CGT TAT ACG GAG ATC CGT CTT GCG AAA ATC GCC
Gly Asp Ser Ala Ala Ala Met Arg Tyr Thr Glu Ile Arg Leu Ala Lys Ile Ala
    115                 120                 125                 130

CAC GAA CTG ATG GCC GAT CTC GAA AAA GAG ACG GTC GAT TTC GTG GAT AAC TAT
His Glu Leu Met Ala Asp Leu Glu Lys Glu Thr Val Asp Phe Val Asp Asn Tyr
            135                 140                 145

GAC GGT ACG GAA AAA ATT CCG GAC GTC ATG CCG ACC AAA ATT CCG AAT CTG CTG
Asp Gly Thr Glu Lys Ile Pro Asp Val Met Pro Thr Lys Ile Pro Asn Leu Leu
150                 155                 160                 165

GTG AAC GGT TCT TCC GGT ATC GCA GTA GGT ATG GCA ACG AAT ATC CCG CCG CAC
Val Asn Gly Ser Ser Gly Ile Ala Val Gly Met Ala Thr Asn Ile Pro Pro His
        170                 175                 180                 185
```

The nucleotide sequence of this region was found to bear an overall amino acid identity of 100% with the corresponding region of *E. coli* GyrA (Yoshida *et al,* 1988). The homology of *S. typhimurium* with *E. coli* at the nucleotide level was found to be 94%. In this region of 486 nucleotides, 27 changes were found compared to *E.coli,* all of which occur in the third position of the codon and are synonymous. The amino acid sequence of this region of *gyrA of S. typhimurium* was compared to that of *E. coli, Staphlylococcus aureus and Bacillus subtilus* as shown in Figure 3.2.

Figure 3. 2: Alignment of the amino acid sequences derived from the region of the nucleotide sequence between bases 72 and 557 of the *gyrA* genes (amino acids 24 to 185) of *Salmonella typhimurium, Escherichia coli* (Yoshida *et al,* 1988), *Staphylococcus aureus* (Sreedharan *et al,* 1990) and *Bacillus subtilis* (Moriya, Ogasawara & Yoshikawa, 1985). Codon homology is highlighted in bold.

S.typhimurium **YAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDWNKAYK**
E.coli **YAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDWNKAYK**
S.aureus **YAMSVIVARALPDVRDGLKPVHRRILYGLNEQGMTPDKSYK**
B.subtilis **YAMSVIVSRALPDVRDGLKPVHRRILYAMNDLGMTSDKPYK**

KSARV**VG**D**VIGKYHPHGDS**AVYDTI**VRMAQ**P**F**S**L**R**Y**M**L**VDG**Q**GNFGS**I**DGDSAAAMR**
KSARV**VG**D**VIGKYHPHGDS**AVYDTI**VRMAQ**P**F**S**L**R**Y**M**L**VDG**Q**GNFGS**I**DGDSAAAMR**
KSARI**VG**D**VM**GKYHPHGDS**SIYEAM**VRMAQ**D**F**SY**R**Y**P**L**VDG**Q**GNFGS**M**DGDGAAAMR**
KSARI**VGEVIGKYHPHGDS**AVYESM**VRMAQ**D**F**NY**R**Y**M**L**VDG**H**GNFGS**V**DGDSAAAMR**

YTEI**R**L**AKI**AH**E**LMA**D**LE**K**ETV**D**F**V**DNYDG**T**E**K**I**P**D**V**M**P**TKI**PNLL**V**NG**SS**GIAVGM**
YTEI**R**L**AKI**AH**E**LMA**D**LE**K**ETV**D**F**V**DNYDG**T**E**K**I**P**D**V**M**P**TKI**PNLL**V**NG**SS**GIAVGM**
YTEARMT**KI**TL**E**LLR**D**IN**K**D**T**I**D**F**I**DNYDG**N**E**R**E**P**S**V**L**P**ARF**PNLLA**NG**AS**GIAVGM**
YTEARMS**KI**SM**E**ILR**D**IT**K**D**T**I**D**YQ**DNYDG**S**E**R**E**P**V**V**M**P**SRF**PNLL**V**NG**AA**GIAVGM**

ATNIPPH
ATNIPPH
ATNIPPH
ATNIPPH

3.3 The Effect of Mutating *S.typhimurium* NCTC5710 to a Higher Level of Ciprofloxacin Resistance on the Sequence of *gyrA*

This ciprofloxacin-sensitive strain was then selected for ciprofloxacin resistance by daily inoculation into broth containing a sub-inhibitory concentration of ciprofloxacin until the MIC reached 0.512mg/l, a 64-fold increase. Although ofloxacin and nalidixic acid were not used in this selection process, the MICs of these antibiotics increased to 2mg/1 and 256mg/1 respectively.

S. typhimurium NCTC5710, mutated to a 64-fold higher level of ciprofloxacin resistance was found to have undergone a G to T transition at the first position of codon number 87, resulting in the substitution of a tyrosine residue for the original aspartic acid. This is in keeping with the recent proposal of Yonezawa *et al* (1995b) that a quinolone resistant phenotype is exhibited on the conversion of aspartate at position 87 to a non-acidic amino acid such as tyrosine. This is only the second report of the substitution of aspartate-87 by tyrosine in response to a higher level of ciprofloxacin resistance, the first was been reported in a ciprofloxacin resistant clinical isolate of *Pseudomonas aeruginosa* (Kureishi *et al*, 1994). Similar changes of aspartate-88 to tyrosine in *Haemophilus influenzae* (Georgiou *et al*, 1996)and aspartate-91 to tyrosine in *Helicobacter pylori* (Moore *et al*, 1996) have also been reported.

In the same way, the *gyrA* DNA sequences of ten of the Indian *S. typhimurium* isolates were investigated.

3.4 Investigating the QRDR of *gyrA* of the Multi-Resistant Clinical Isolates of *S. typhimurium* from Vellore, India

One hundred and one clinical isolates of *S. typhimurium* were collected from Vellore, India in 1987. The MICs of these strains were measured and a number of those showing either a relatively higher resistance or in one case, a higher sensitivity to ciprofloxacin, were investigated further. The average MIC of ciprofloxacin, ofloxacin and nalidixic acid for these 101 strains were 0.016mgl, 0.064mg/1 and 4.0mg/1 respectively. Ten strains were investigated further as described in sections 3.4.1 -3.4.2.

3.4.1 DNA Sequencing of the QRDR of the *gyrA* gene of the *S. typhimurium* strains isolated from Vellore, India

A 620bp fragment incorporating the QRDR of the enzyme was amplified by the polymerase chain reaction from chromosomal DNA and both DNA strands consequently sequenced. The nucleotide changes in the QRDR of *gyrA* found as a consequence of DNA sequencing are shown in Table 3.1.

Table 3.1: Minimum Inhibitory Concentrations (MIC) of the investigated *Salmonella typhimurium* strains from Vellore, India along with any base pair changes found as a consequence of DNA sequencing. The base change resulting in an amino acid substitution is highlighted in bold and underlined.

STRAIN	MICs (mg/L)			Nucleotide	Amino acid
	Ciprofloxacin	Ofloxacin	Nalidixic acid	change*	change
VI13	0.128	0.512	256	GAC→G**G**C	Asp-87→Gly
VI16	0.128	0.512	128	GAC→**A**AC	Asp-87→Asn
VI253	0.256	0.512	256	TCC→T**T**C	Ser-83 →Phe
				GAC→**A**AC	Asp-87→Asn
VI258	0.064	0.064	2	NO CHANGE	NO CHANGE
VI264	0.128	0.512	256	GAC→G**G**C	Asp-87→Gly
VI325	0.128	0.512	128	GAC→G**G**C	Asp-87→Gly
VI343	0.128	0.512	128	GAC→**A**AC	Asp-87→Asn
VI358	0.008	0.064	4	NO CHANGE	NO CHANGE
VI367	0.128	0.256	128	GAC→G**G**C	Asp-87→Gly
VI385	0.064	0.256	128	GAC→G**G**C	Asp-87→Gly

* Note that the first codon for each pair is the codon sequence of the NCTC and clinical sensitive isolates and not the sequence of the codon before mutation to quinolone resistance. It is possible that the pre-mutation sequence of codon 83 of the parent strain is different to that above.

Various mutations were found in the QRDR of those strains possessing higher MICs. Mutations such as aspartate-87 to glycine, aspartate-87 to asparagine and serine-83 to phenylalanine in the QRDR seemed to be, at least, partly responsible for the decreased susceptibilities for ciprofloxacin, ofloxacin and nalidixic acid. In strains VI13, VI264, VI325, VI367 and VI385, the acidic aspartate residue was substituted by a neutral non-polar glycine residue and in strains VI16, VI253 and VI343 the same aspartate residue became asparagine, a neutral polar amino acid. Again, this correlates with the hypothesis proposed by Yonezawa *et al* (1995b) that the substitution of an acidic amino acid at position 87 with a non-acidic amino acid results in a decreased susceptibility to quinolone drugs. In strain VI253, serine-83, a neutral polar amino acid was substituted by phenylalanine, a neutral non-polar hydrophobic amino acid. Again this agrees with the hypothesis of Yonezawa *et al* (1995a) that the 83rd amino acid has to be hydrophobic to express the quinolone-resistance phenotype. The fact that strain VI258 (MIC of ciprofloxacin 0.064mg/l) has no change in its nucleotide sequence despite requiring eight times as much ciprofloxacin to inhibit its growth compared to the NCTC strain (MIC of ciprofloxacin 0.008mg/l) suggests that the decreased susceptibility of VI258 must be as a result of a change occurring somewhere other than in *gyrA*. This could possibly be a change in outer membrane proteins, efflux mechanisms or in *gyrB* or *parC* (see later). It is interesting that strain VI385 (MIC of ciprofloxacin 0.064mg/l) was found to have a change in the nucleotide sequence of *gyrA* resulting in aspartate-87 being

replaced with glycine. Although VI258 and VI385 have similar susceptibility levels to ciprofloxacin, it is apparent that their resistance mechanism must differ since the measured MICs of ofloxacin and nalidixic acid are very different between the two strains. It is possible that the level of resistance conferred upon the *S. typhimurium* strains by a change in *gyrA* modifying aspartate-87 to glycine is very similar to that resulting from a change in *gyrA* modifying aspartate-87 to asparagine. This conclusion was reached on the basis that all the strains but one that had either a mutation in *gyrA* consistently had a MIC of ciprofloxacin of 0.128mg/l. The one strain that had a MIC above 0.128mg/l, strain VI253, was found to have not one but two mutations: aspartate-87 to asparagine and serine-83 to phenylalanine. Therefore the combination of a mutation at serine-83 and aspartate-87 results in the MIC of the cell raising above 0.128mg/l.

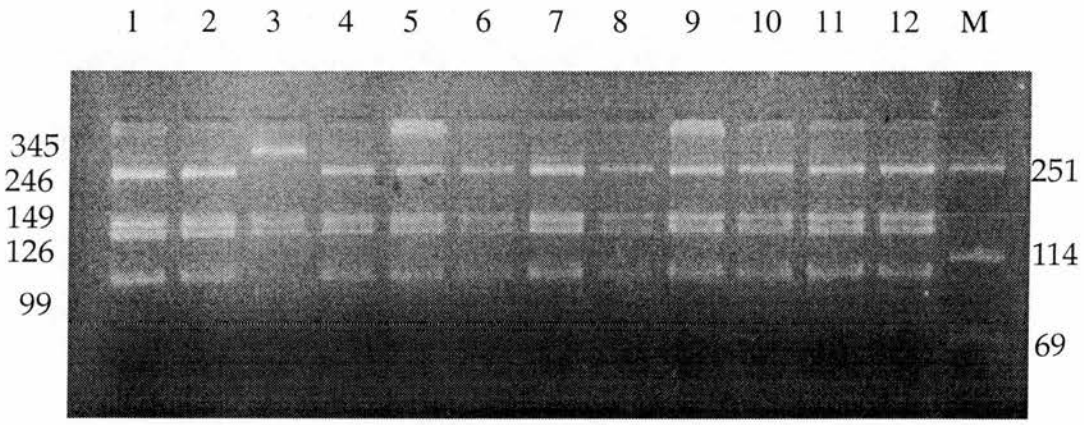
3.4.2 Restriction Fragment Length Polymorphisms (RFLPS) of the QRDR of *gyrA* of the *Salmonella typhimurium* Clinical Isolates

Restriction Fragment Length Polymorphisms (RFLPS) were carried out on the QRDR of the *Salmonella typhimurium* clinical isolates with the restriction endonuclease *Hinf*I. As a result of certain common *gyrA* mutations, for example some found in *E. coli* (Fisher *et al*, 1989), often causing the abolition of a *Hinf*I restriction site in *gyrA* occurring within a region of the enzyme centred around serine-83, *Hinf*I restriction was used as a screen for amino acid substitutions in the QRDR. The sequence recognised and cleaved by this enzyme is G/ANTC, thus a *gyrA* PCR fragment of *S. typhimurium* amplified from a strain with no amino acid substitution at serine-83 (nucleotide sequence encoding amino acids 82 and 83 being GATTCC) would be cleaved by the enzyme whereas a *gyrA* PCR fragment amplified from a quinolone-resistant strain with a mutation at

serine-83 (replaced by, for example, phenylalanine) would not be cut since this would change the nucleotide sequence at this area to GATTTC. In quinolone-sensitive *S. typhimurium*, the DNA sequence encoding these two amino acids is GATTCC.

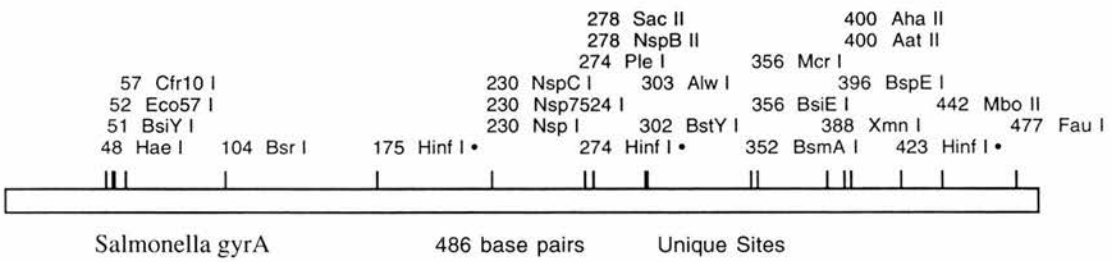
As can be seen in Figure 3.3, the *gyrA* PCR fragment amplified from strain VI253 shows a different restriction pattern from all the other strains by virtue of its point mutation at serine-83 which abolishes the *Hinf*I site. The enzyme *Hinf*I cuts the amplified 620bp fragment of the wild-type *S. typhimurium gyrA* three times as shown in Figure 3.4. This results in the digestion of the fragment into four sections of lengths 246bp, 99bp, 149bp and 126bp. Since the PCR fragments from VI13, VI16, VI258, VI264, VI325, VI343, VI358, VI367 and VI385 do not have a mutation at serine-83, they followed the pattern of the restriction of the wild-type fragment. However, the presence of a serine-83 to phenylalanine substitution in the *gyrA* gene of *S. typhimurium* VI253 removed the *Hinf*I site in the codons for amino acids 82 and 83, resulting in the generation of only three fragments of lengths 345bp, 149bp and 126bp.

Figure 3.3: Restriction fragment length polymorphisms in the amplified 620bp section *of gyrA* in *S. typhimurium* strains sensitive or with a decreased susceptibility to ciprofloxacin. Lanes 1 to 12 inclusive: PCR fragments have been restricted with *Hinf*I.



Lanes: 1, VI13; 2, VI16; 3, VI253; 4, VI258; 5, VI264; 6, VI325; 7, VI343; 8, VI358; 9, VI367; 10, VI385; 11, *S. typhimurium* NCTC5710 (ciprofloxacin sensitive); 12, *S. typhimurium* NCTC5710 (mutated to MIC of 0.512mg/l, see section 3.3); M, Molecular weight markers of sizes 25lbp, 114bp and 69bp as shown to the right of the gel. Fragment sizes of the restricted DNA are indicated in base pairs to the left of the gel.

Figure 3.4: Diagrammatic representation of the *Hinf*I restriction sites in the sequenced region of *gyrA* of *S. typhimurium*. The restriction map is the same for *S.typhi* (see 3.5.2).



3.5 Investigating the QRDR of *gyrA* of the Multi-Resistant Clinical Isolates of *S. typhi* from Vellore, India

3.5.1 Levels of Resistance to the Quinolone Drugs

Fifteen *S. typhi* strains isolated in the Christian Medical Centre, Vellore, India were examined and compared throughout with sensitive *Salmonella* spp. control strains. The MICs of ciprofloxacin, ofloxacin and nalidixic acid were determined as shown in Table 3.2. Twelve out of 15 of the Indian isolates were found to have MICs of ciprofloxacin of 0.256mg/l, a level considerably higher than expected. The three *S. typhi* strains which were relatively sensitive to ciprofloxacin were also sensitive to ofloxacin and nalidixic acid. Similarly the isolates showing decreased susceptibilities to ciprofloxacin had high levels of resistance to these two other drugs. It is interesting that the three remaining *S. typhi* strains sensitive to ciprofloxacin were isolated from patients in 1992 and 1993 whereas 11 of the 12 isolates which were markedly more resistant to this drug were isolated in 1994. Only one of the isolates (ST5), which was isolated in 1992 had a decreased susceptibility to quinolone drugs and investigating the cause of this revealed that the acquisition of quinolone resistance must result from a totally different mutation compared to the other resistant isolates (see later).

3.5.2 DNA Sequencing of the QRDR of the *gyrA* gene of *S. typhi* strains isolated from Vellore, India

A 620 bp fragment was amplified from the *gyrA* gene of each of the *S. typhi* strains as before and the double-stranded DNA separated into single strands by the use of Dynabeads. The nucleotide sequences of the three ciprofloxacin-sensitive *S. typhi* strains (ST3, ST7 and ST12) and ciprofloxacin-sensitive *S. typhimurium* were found to be identical. A ciprofloxacin-sensitive strain of *S. arizoniae* was also used as a sensitive control strain for sequence comparisons since a NCTC *S. typhi* could not be obtained. The nucleotide sequence of ciprofloxacin-sensitive *S. typhi* along with the predicted amino acid sequence is identical to that of *S.typhimurium* as shown in Figure 3.1. Deviations from the amino acid sequence of both ciprofloxacin-sensitive *S. typhi and S. typhimurium* were found in all of the twelve isolates with decreased susceptibilities to ciprofloxacin as shown in Table 3.2.

Table 3.2 : Minimum Inhibitory Concentrations (MIC) of the investigated *S.typhi* strains from Vellore, India along with any base pair changes found as a consequence of DNA sequencing. The base change resulting in an amino acid substitution is highlighted in bold and underlined.

ISOLATE	MICs(mg/L)			Nucleotide*	Amino acid
	Ciprofloxacin	Ofloxacin	Nalidixic acid	changes	change
ST1	0.256	0.512	256	TCC →T**T**C	Ser-83→ Phe
ST2	0.256	0.512	256	TCC →T**T**C	Ser-83→ Phe
ST3	0.016	0.064	4	NO CHANGE	NO CHANGE
ST4	0.256	0.512	256	TCC →T**T**C	Ser-83 →Phe
ST5	0.256	0.512	256	NO CHANGE	NO CHANGE
ST6	0.256	0.512	256	TCC →T**T**C	Ser-83 →Phe
				GAC→ **T**AC	Asp-87 →Tyr
ST7	0.016	0.032	4	NO CHANGE	NO CHANGE
ST8	0.256	0.512	256	TCC →T**T**C	Ser-83 →Phe
ST9	0.256	0.512	256	TCC →T**T**C	Ser-83 →Phe
ST10	0.256	0.512	256	GAC →**T**AC	Asp-87 →Tyr
ST11	0.256	0.512	256	TCC →T**T**C	Ser-83→Phe
ST12	0.016	0.032	4	NO CHANGE	NO CHANGE
ST13	0.256	0.512	>256	TCC →T**T**C	Ser-83 →Phe
ST14	0.256	0.512	>256	TCC →T**T**C	Ser-83 →Phe
ST15	0.256	0.512	>256	TCC →T**T**C	Ser-83 →Phe

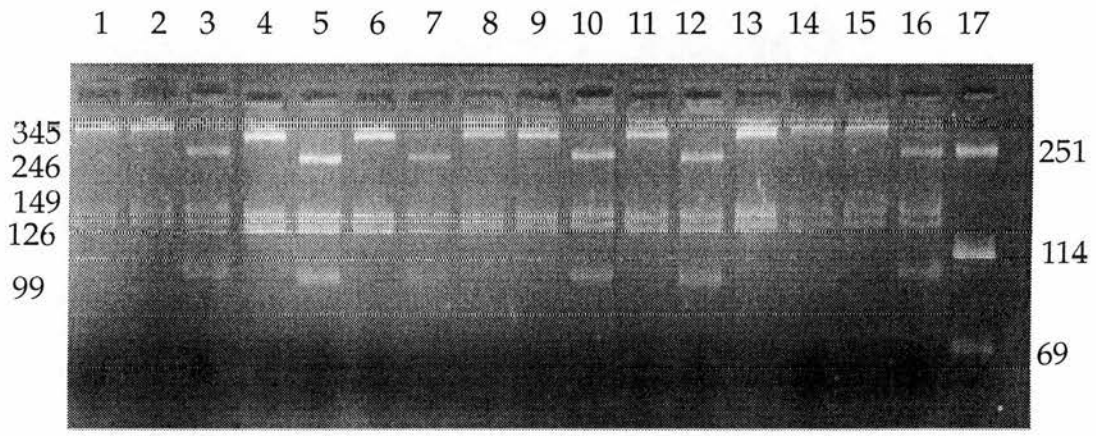
* Note that the first codon for each pair is the codon sequence of the NCTC and clinical sensitive isolates and not the sequence of the codon before mutation to quinolone resistance. It is possible that the pre-mutation sequence of codon 83 of the parent strain is different to that above.

In strains ST1, ST2, ST4, ST8, ST9, ST11, ST13, ST14, ST15 a single substitution of serine at position 83 to phenylalanine was found to have occurred. Again this correlates with the hypothesis that changing serine-83 to a hydrophobic amino acid results in the expression of a quinolone-resistant phenotype (Yonezawa *et al*, 1995a). Strains ST6 and ST10 were found to carry different mutations, namely a double mutation of serine-83 to phenylalanine and aspartate-87 to tyrosine in ST6 while ST10 showed a single mutation of aspartate-87 to tyrosine. However a different type of quinolone-resistance mutation was concluded to be the cause of the high quinolone MICs in strain ST5. On sequencing the QRDR of this strain, no amino acid substitutions were found. Quinolone-resistance in ST5 is thus likely to be attributed to either an outer membrane protein mutation or a mutation affecting efflux or perhaps a mutation in *gyrB* or *parC*. It is interesting that ST5 was isolated in 1992 whereas all the other quinolone-resistant isolates with mutations in *gyrA* were isolated two years later in 1994.

3.5.3 Restriction Fragment Length Polymorphisms (RFLPS) of the QRDR of *gyrA* of the *Salmonella typhi* Clinical Isolates

The amplified PCR-products were restricted with *Hinf*I as described in 3.4.2 in order to confirm the point mutations around serine-83. As can be seen in Figure 3.5, the *gyrA* PCR fragments amplified from strains ST3, ST5, ST7, ST10, ST12 and ST16 show a different restriction pattern from all the other strains since there is no point mutation at serine-83 which leaves the *Hinf*I site. The enzyme *Hinf*I cuts the amplified 620bp fragment of the wild-type *S. typhi gyrA* three times as shown in Figure 3.4. This results in the digestion of the fragment into four sections of lengths 246bp, 99bp, 149bp and 126bp. Since the PCR fragments from ST1, ST2, ST4, ST6, ST8, ST9, ST11, ST 13, ST14 and ST15 do have a mutation at serine-83, thus abolishing the *Hinf*I site, the PCR product is only cut into three fragments of lengths 345bp, 149bp and 126bp.

Figure 3.5: Restriction fragment length polymorphisms in the amplified 620bp section of *gyrA* in *S. typhi* strains sensitive or with a decreased susceptibility to ciprofloxacin. Lanes 1 to 17 inclusive: PCR fragments have been restricted with *Hinf*I.



Lanes: 1, ST 1; 2, ST2; 3, ST3; 4, ST4; 5, ST5; 6, ST6; 7, ST7; 8, ST8; 9, ST9; 10, ST10; 11, ST11; 12, ST12; 13, ST13; 14, ST14; 15, ST15; 16, Ciprofloxacin-sensitive *Salmonella typhimurium* NCTC5710;. 17, Molecular weight markers of sizes 251bp, 114bp and 69bp as shown to the right of the gel. Fragment sizes of the restricted DNA are indicated in base pairs to the left of the gel.

3.6 Investigating the QRDR-containing region of *gyrA* of multi-resistant clinical isolates of *Salmonella* from British hospitals

Ten clinical isolates of various *Salmonella* species were received from a variety of British hospitals during the period 1989 to 1992. The MICs of ciprofloxacin, ofloxacin and nalidixic acid for the strains were measured and are displayed in Table 3.3.

The strains were investigated further by sequencing the QRDR of the *gyrA* gene and confirming these results by *Hinf*I digestion as before as described in sections 3.6.1 -3.6.2.

3.6.1 DNA Sequencing of the QRDR of the *gyrA* gene of *Salmonella strains* isolated from British hospitals

Part of the *gyrA* gene of each was sequenced as before and any point mutations that were detected in the QRDR are shown alongside the MIC results in Table 3.3.

Table 3.3: Minimum Inhibitory Concentrations (MIC) of the investigated *Salmonella* strains along with any base pair changes found as a consequence of DNA sequencing. The base change resulting in an amino acid substitution is highlighted in bold and underlined.

STRAIN	MICs mg/l			Nucleotide*	Amino Acid
	Ciprofloxacin	Ofloxacin	Nalidixic Acid	Change	Change
CIP179	0.064	0.512	4	NO CHANGE	NO CHANGE
CIP182	0.004	0.032	4	NO CHANGE	NO CHANGE
CIP184	1	2	>256	TCC→T**T**C	Ser-83→Phe
CIP185	1	2	>256	TCC→T**T**C	Ser-83→Phe
CIP247	0.064	0.128	8	NO CHANGE	NO CHANGE
CIP248	0.256	0.512	>256	TCC→T**T**C	Ser-83→Phe

* Note that the first codon for each pair is the codon sequence of the NCTC and clinical sensitive isolates and not the sequence of the codon before mutation to quinolone resistance. It is possible that the pre-mutation sequence of codon 83 of the parent strain is different to that above.

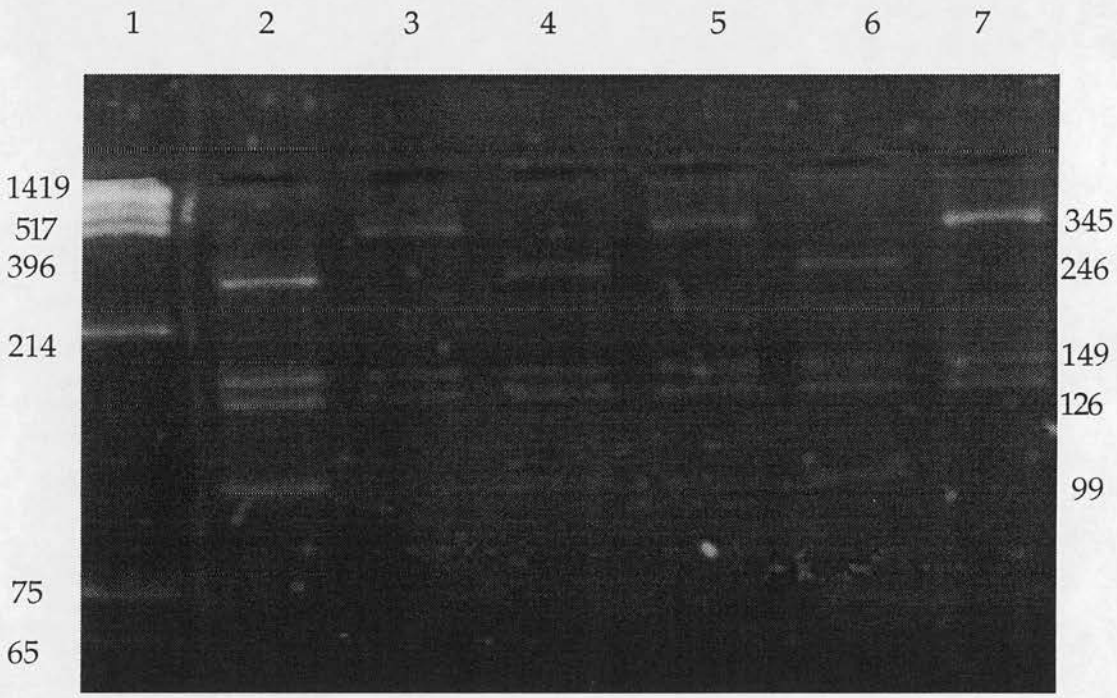
Isolates CIP179 and CIP184, CIP182 and CIP185, CIP247 and CIP248 are paired strains with CIP179, CIP182 and CIP247 being isolated pre-therapy and CIP184,

CIP185 and CIP248 being isolated post-therapy. It is interesting that during therapy each of the three isolates became less susceptible to the action of ciprofloxacin, ofloxacin and nalidixic acid which could be correlated to a mutation at serine-83 of *gyrA* changing serine-83 to phenylalanine.

3.6.2 Restriction Fragment Length Polymorphisms (RFLPS) of the QRDR of *gyrA* of the *Salmonella* Clinical Isolates

The amplified PCR-products were restricted with *HinfI* as before in order to confirm the detected changes around serine-83. As can be seen in Figure 3.6, the *gyrA* PCR fragments amplified from strains CIP179, CIP182 and CIP247 show a different restriction pattern from all their post-therapy partners since there is no point mutation at serine-83 which leaves the *HinfI* site. As a result the PCR fragment is digested into four sections of lengths 246bp, 99bp, 149bp and 126bp. Since the PCR fragments from the post-therapy isolates CIP184, CIP185 and CIP248 do have a mutation at serine-83, thus abolishing the *HinfI* site, the PCR product is only cut into three fragments of lengths 345bp, 149bp and 126bp.

Figure 3.6: Restriction fragment length polymorphisms in the amplified 620bp section of *gyrA* in the clinical *Salmonella* strains sensitive or with a decreased susceptibility to ciprofloxacin. Lanes 2 to 7 inclusive: PCR fragments have been restricted with *Hinf*I.



Lanes:1, pUC18 plasmid DNA digested with *EcoR*I and *Hinf*I (sizes of fragments 1419bp, 517bp, 396bp, 214bp, 75bp and 65bp as shown to the left of the gel); 2, CIP179; 3, CIP182; 4, CIP184; 5, CIP185; 6, CIP247; 7, CIP248. Fragment sizes of the restricted DNA are indicated in base pairs to the right of the gel.

3.7 Investigating the QRDR-containing region of *gyrA* of multi-resistant clinical isolates of *E. coli*

Ten clinical isolates of *Escherichia coli* were received from a variety of British hospitals during the period 1989 to 1992. The MICs of ciprofloxacin, ofloxacin and nalidixic acid for the *E. coli* isolates were measured and are displayed in Table 3.4. Part of the *gyrA* gene of each was sequenced as before and any point mutations that were detected in the QRDR are shown alongside the MIC results in Table 3.4.

The strains were investigated further by sequencing the QRDR of *gyrA* and confirming the results by digestion of the PCR product as described in sections 3.7.1 -3.7.2.

3.7.1 DNA Sequencing of the QRDR of the clinical isolates of *E. coli*

Table 3.4: Minimum Inhibitory Concentrations (MIC) of the investigated *E. coli* strains along with any base pair changes found as a consequence of DNA sequencing. The base change resulting in an amino acid substitution is highlighted in bold and underlined.

STRAIN	MICs (mg/l)			Nucleotide*	Amino acid
	Ciprofloxacin	Ofloxacin	Nalidixic acid	Change	Change
CIP108	4	8	256	TCG →T**T**G	Ser83 →Leu
				GAC→ **T**AC	Asp87 →Tyr
CIP109	8	8	256	TCG →T**T**G	Ser83 →Leu
				GAC →**A**AC	Asp87→Asn
CIP262	0.256	0.512	256	TCG →T**T**G	Ser83 →Leu
CIP65	1	1	256	TCG →T**T**G	Ser83 →Leu
CIP113	2	4	256	TCG →T**T**G	Ser83 →Leu
CIP223	2	4	256	TCG →T**T**G	Ser83→ Leu
CIP218	4	4	256	TCG →T**T**G	Ser83 →Leu
CIP261	16	16	256	TCG →T**TC**	Ser83 →Phe
CIP66	0.008	0.032	2	NO CHANGE	NO CHANGE
CIP110	0.032	0.032	8	NO CHANGE	NO CHANGE

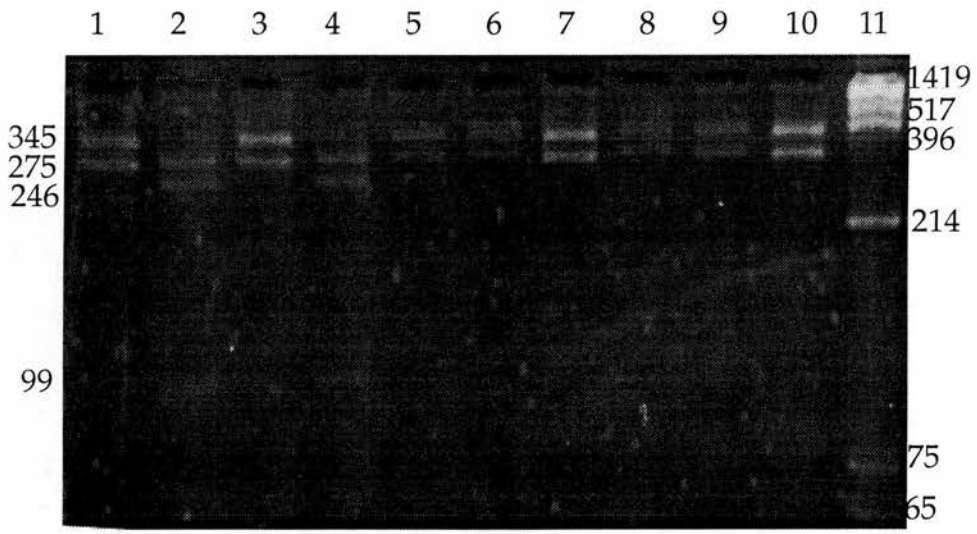
* Note that the first codon for each pair is the codon sequence of the NCTC and clinical sensitive isolates and not the sequence of the codon before mutation to quinolone resistance. It is possible that the pre-mutation sequence of codon 83 of the parent strain is different to that above.

Compared to the quinolone-sensitive isolates CIP66 and CIP110, the quinolone-resistant isolates showed a variety of mutations in the *gyrA* gene such as serine-83 to leucine in isolates CIP262, CIP65, CIP113, CIP223 and CIP218, and serine-83 to phenylalanine in isolate CIP261. High level quinolone resistance in isolates CIP108 and CIP109 (MICs of ciprofloxacin 4mg/1 and 8mg/1 respectively) was found to be as a result of two mutations in *gyrA* as shown in Table 3.4 although there is the possibility that additional mutations could also be present.

3.7.2 Restriction Fragment Length Polymorphisms (RFLPS) of the QRDR of *gyrA* of the *E. coli* Clinical Isolates

The amplified PCR products were restricted with *Hinf*I as before to confirm the sequencing results.

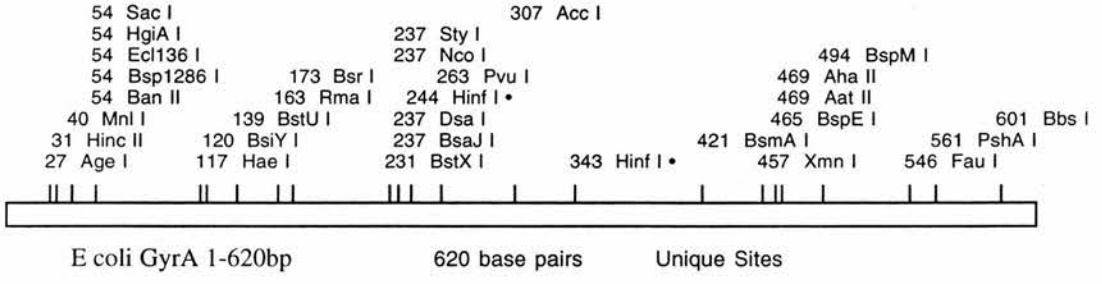
Figure 3.7: Restriction fragment length polymorphisms in the amplified 620bp section of *gyrA* in the clinical *E.coli* strains sensitive or with a decreased susceptibility to ciprofloxacin. Lanes 1 to 10 inclusive: PCR fragments have been restricted with *Hinf*I.



Lanes: 1, CIP65; 2, CIP66; 3, CIP109; 4, CIP110; 5, CIP108; 6, CIP113; 7, CIP218; 8, CIP223; 9, CIP261; 10,CIP262; 11, pUC18 plasmid DNA restricted with *Eco*RI and *Hinf*I (sizes of fragments 1419bp, 517bp, 396bp, 214bp, 75bp and 65bp as shown to the left of the gel). Fragment sizes of the restricted DNA are indicated in base pairs to the right of the gel.

A *Hinf*I restriction map of this amplified region is shown in Figure 3.8. Correlating with the fact that isolates CIP108, CIP109, CIP262, CIP65, CIP113, CIP223, CIP218 and CIP261 showed a change from serine-83 at the QRDR of *gyrA*, the PCR products amplified from these strains were cut by *Hinf*I once resulting in the production of two fragments of lengths 345bp and 275bp as shown in Figure 3.7. Likewise the fact that strains 66 and 110 showed no change at serine-83 was confirmed by the generation of three fragments on *Hinf*I digestion.

Figure 3.8: Diagrammatic representation of the *Hinf*I restriction sites in the amplified region of *gyrA* of *E.coli*



3.8 Discussion

All the strains investigated, except one, which showed a decreased susceptibility to quinolone drugs was shown to have incorporated an amino acid mutation at either serine-83 and/or aspartate-87 of the QRDR of *gyrA*. The importance of these two amino acids of the QRDR has been investigated previously. The conversion of serine-83 to a hydrophobic amino acid has the effect of making the cell less susceptible to the action of quinolone drugs (Yonezawa *et al*, 1995a), presumably by weakening the interaction between DNA gyrase and the quinolone-DNA complex (Oram and Fisher, 1991). Likewise, changing aspartate-87 to a non-negatively charged amino acid results in a decreased susceptibility to quinolone drugs. Therefore, serine-83 and aspartate-87 must be key residues involved in the interaction between DNA gyrase, DNA and the quinolone drug . Indeed, this has been speculated previously (Hallett and Maxwell, 1991). It has been postulated that this region of the protein is involved in the action of quinolone drugs with its close contact to the site of DNA attachment at tyrosine-122. All of the changes above could modify the strength of binding between the quinolone and gyrase either by the presence of differently charged and sized residues making this site of DNA breakage and reunion less accessible, or through loss of the hydrogen bonding capabilities of serine-83. Clinical resistance to the quinolone drugs resulting from a mutated GyrA protein seems to almost universally arise from a mutation in this stretch of the enzyme known as the quinolone resistance determining region (QRDR) (Yoshida *et al*, 1990).

Quinolone-resistant clinical isolates of *E. coli* and *S. typhimurium* with similar mutations in the QRDR have been recently widely reported (Heisig, 1994; Heisig *et al*, 1995; Ruiz *et al*, 1995; Truong *et al*, 1995; Griggs *et al*, 1996; Bazile-Pham-

Khac *et al*, 1996). While quinolone resistant *E. coli* isolates commonly show a serine-83 to leucine (Heisig *et al*, 1993; Yoshida *et al*, 1988; Oram and Fisher, 1991) or serine-83 to tryptophan (Cullen *et al*, 1989; Yoshida *et al*, 1988; Oram and Fisher, 1991) change, quinolone-resistant *Salmonella* seem to be more inclined to a serine-83 to phenylalanine change (this work, also Griggs *et al*, 1996; Reyna *et al*, 1995). The triplet encoding serine-83 of GyrA in *Salmonella* was found to be TCC compared to TCG in *E. coli*. Therefore a change from TCC (serine) to TTC (phenylalanine) which only involves one nucleotide change is understandable. However, in *E. coli*, the triplet encoding serine-83 is TCG which has been previously found to change to TGG (tryptophan) (Oram and Fisher, 1991), or TTG (leucine) (this work) in response to quinolone challenge. A mutation in the triplet involving two nucleotide changes was found to occur in this study. A clinical isolate of *E. coli*, CIP261, was found to have a serine-83 to phenylalanine mutation as a result of a change in the DNA sequence from TCG to TTC. This change is novel in *E. coli* and possibly results from the differential codon usage within *E. coli* species. Since *E. coli* and *S. typhimurium* can be considered to be diverging from a common ancestral genome, it would be expected that somewhere between the classic "definition" of a *E. coli* bacterium and a *S. typhimurium* bacterium, there is some "middle ground", where the codon usage is perhaps different. It would have been interesting to examine the parent strain of CIP261, in order to ascertain what the nucleotide of codon 83 was before the isolate had mutated to quinolone resistance. As the occurrence of two mutations adjacent to each other is probably quite a rare event, it is possible that the original codon sequence was TCC as in *Salmonella* species. To reconfirm the clinical records that it was *E.coli* rather than *Salmonella*, features of the DNA sequence were examined further, revealing that the *gyrA* sequence of CIP261 was more similar to *E.coli* than *Salmonella*.

Although the patterns of codon usage in *E. coli* and *S. typhimurium* are similar (Sharp and Li, 1986; Ikemura, 1985), there are certainly some differences in silent-base composition, occurring as the result of a mutation-selection balance. The average divergence of *E. coli* genes compared to *S. typhimurium* has been shown to be 15.6% (Sharp, 1991), however since proteins mutate at different rates, this is only a very average value and for highly evolved proteins such as DNA gyrase, the degree of change of the amino acid sequence would be expected to be minimal. However, since codon preferences vary from one organism to another depending on the abundance and anticodon sequence of the various tRNAs in the cell (Ikemura, 1981), a mutation occurring in one position of the triplet could easily change the same amino acid in two species to two different amino acids, as shown to happen with the isolates investigated in this chapter. As the GC contents of *E.coli* and *S.typhimurium* are vary slightly, it is understandable that the two species are predisposed to different amino acid substitutions at the same codon.

From a more clinical perspective, the results show the occurrence of a decreased susceptibility to quinolone drugs in the isolated bacteria. The ease with which mutations in *gyrA* are acquired has been demonstrated in 3.3 where a NCTC strain of *S.typhimurium* was inoculated daily into broth containing increasing levels of ciprofloxacin. A mutation in *gyrA* converting aspartate-87 to tyrosine was readily acquired and resulted in the MIC of ciprofloxacin of the cell increasing 64-fold from 0.008mg/l to 0.512mg/l. At this point, the experiment was stopped, however, further mutation of the strain by increasing amounts of ciprofloxacin may have resulted in the occurrence of further mutations, perhaps changing serine-83 to a hydrophobic amino acid. It is significant that of the

twelve isolates of *S.typhi* with decreased susceptibility to quinolone drugs (see 3.5), only ST10 did not respond to ciprofloxacin therapy when this drug was administered to a patient (M. Jesudason, personal communication). The eleven other isolates examined had the same MIC for ciprofloxacin (0.256mg/l), thus there is a possibility that these isolates could also become more resistant to quinolone drugs actually during the course of therapy, if, for example, a sub-therapeutic dose of the drug was given. However, for the purpose of this study, the amount of quinolone drug administered to patients on a clinical basis was found to be sufficient to inhibit the growth of these isolates.

Clinical isolates which did not respond to quinolone therapy have previously been described and shown to have much greater MICs of quinolone drugs (Piddock *et al*, 1993). However, even although the isolates examined in this study still have MICs below the recommended breakpoint concentration for the quinolone drugs, it is possible that under conditions of prolonged quinolone therapy, further mutations in *gyrA*, *gyrB* or another locus may arise, resulting in a highly quinolone-resistant isolate not amenable to quinolone therapy, such as that described by Heisig (1993).

Although it is well-accepted that the occurrence of mutations at serine-83 and/or aspartate-87 of *gyrA* of the *Enterobacteriaceae* contribute to quinolone resistance, the lack of correlation between MIC and point mutation in *gyrA* for the above isolates can possibly be attributed to the occurrence of additional mutations in other loci. Changes in outer membrane protein mutations, changes affecting efflux mutations in *gyrB*, changes in *par*C and/or *parE* have all been shown previously to contribute to quinolone resistance. For example, two isolates of *Salmonella*, CIP185 and CIP248 with the same amino acid mutation

at serine-83 resulting in the replacement of serine-83 by phenylalanine were shown to have MICs of ciprofloxacin of 1mg/l and 0.256mg/l respectively. This is in keeping with the ideas discussed before that high level quinolone resistance in clinical bacteria is rarely the result of a single mutation, instead it arises from a sequential build-up of alterations in permeability and in the structural genes mainly of *gyrA* and *parC*.

Recent evidence has demonstrated the contribution of mutations in *parC* in the development of high-level quinolone resistance in *E.coli* (Heisig, 1996). This underlines the role of topoisomerase IV as a secondary, less sensitive target for quinolone action in *E.coli*. Despite *parC* mutations being detected in quinolone resistant *E.coli* with mutations in the QRDR of *gyrA*, bacteria harbouring mutations in *parC* alone have not been identified. It is likely that on further investigation of the clinical isolates examined in this chapter, further mutations in the outer membrane proteins, genes affecting the efflux mechanisms, *parC* and *parE* would be revealed. Armed with all this information, it would then be possible to assign different numerical levels of quinolone resistance to different resistance mechanisms. Genetic strain construction experiments *in vivo* could then be used to confirm such results.

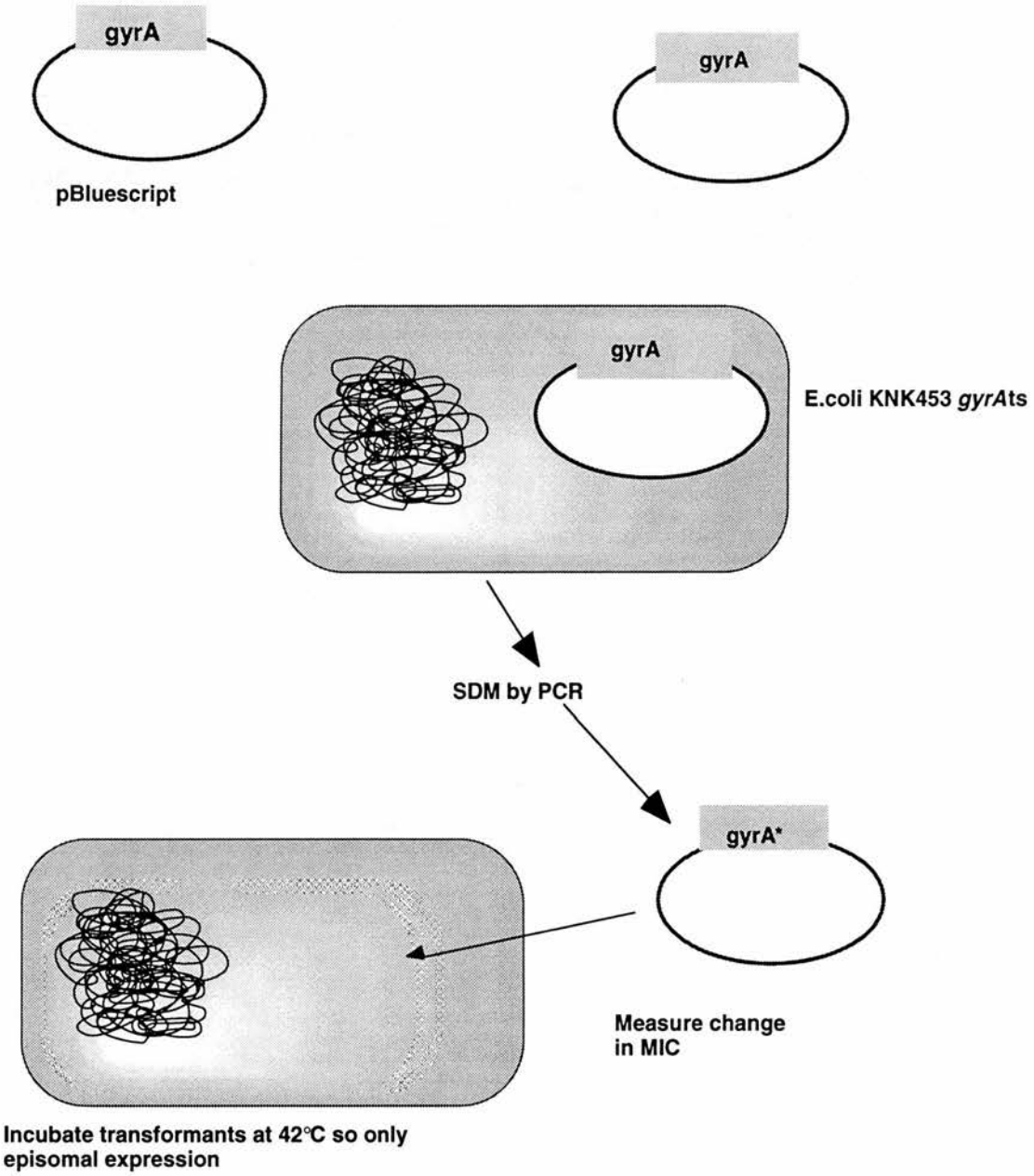
Therefore, in conclusion, the quinolone resistant clinical isolates examined in this study were all found to possess mutations in the QRDR of *gyrA* at amino acid positions that had been previously proposed to be critical for the affinity of binding to the DNA-quinolone complex (Hallett and Maxwell, 1991). Only one isolate, *S.typhi* ST5, was found not to have a changed DNA sequence of *gyrA* despite showing a decreased susceptibility to the quinolone drugs. This and the fact that there is only a vague correlation between MIC and mutations in *gyrA* points towards the presence of mutations in other loci.

4 Cloning of *gyrA* and investigating its high copy expression

4.1 Introduction

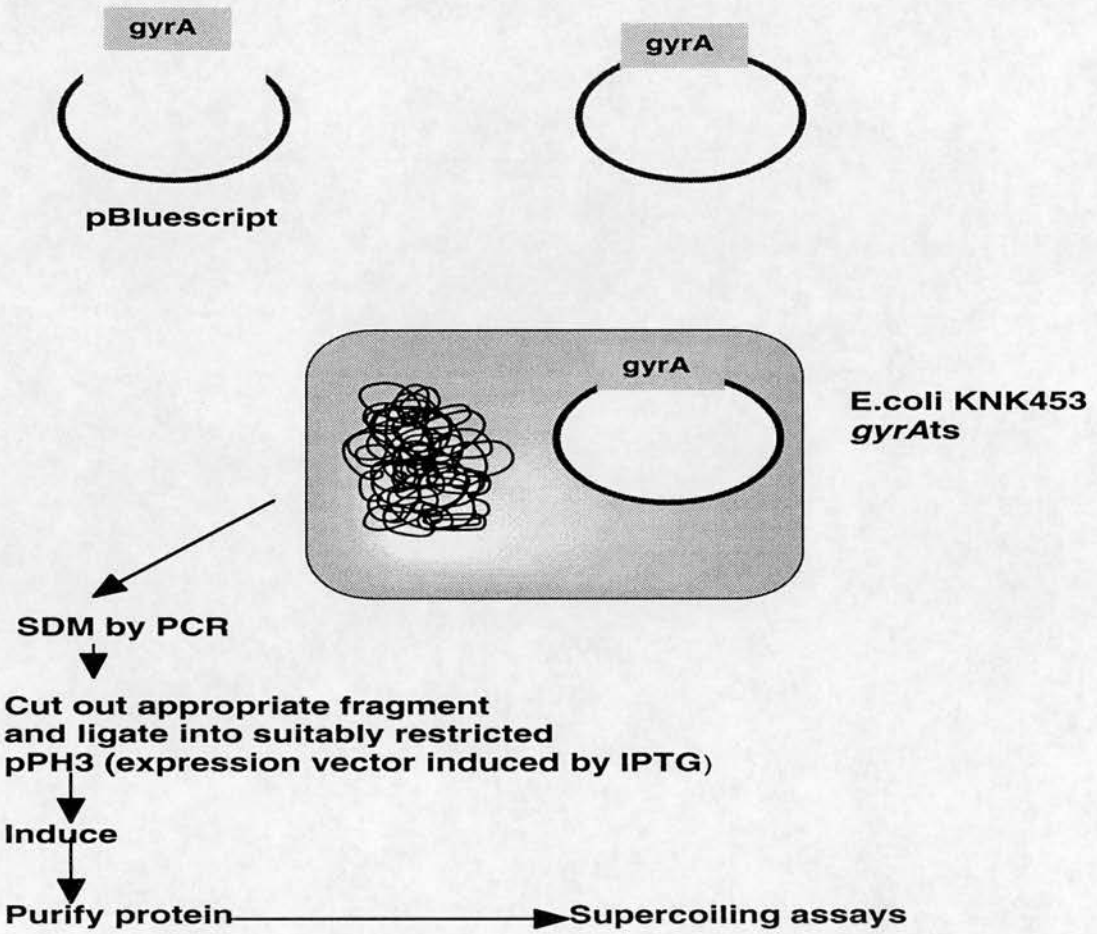
At the onset of this work, it was aimed to examine the effect of mutations arising in the QRDR of *gyrA* in a neutral genetic background where any contribution to the quinolone-resistance phenotype by other mutations such as outer membrane protein mutations and mutations affecting efflux which were probably present in the previously investigated clinical strains could be eliminated. It was planned to clone *gyrA* with its promoter into a high copy number plasmid from which single-stranded DNA could be extracted, then engineering point mutations in the DNA of the QRDR of *gyrA* artificially by site-directed mutagenesis, so the effect of the changed amino acid on the MIC of the cell could easily be ascertained. The *gyrA* temperature-sensitive ($gyrA^{ts}$) strain *E.coli* KNK453 (Kreuzer and Cozzarelli, 1979) was to be used as the host for the various manipulations by virtue of its ability to grow at 30°C but not at 42°C. The vector pBluescript was favoured for the cloning of *gyrA* since, being a phagemid vector, single-stranded DNA could easily be obtained on infection with helper phage. Figure 4.1 shows the various stages that had to be carried out in this investigation.

Figure 4.1: Plan of action to investigate changing serine-83 and aspartate-87 of GyrA of *E.coli* on the Minimum Inhibitory Concentration of ciprofloxacin the cell by inserting point mutations in these positions to create amino acid changes



However, during the course of the initial stages of this work, these results were published by another group (Yonezawa *et al*, 1995a, 1995b). Therefore the aim was altered to study the effect of changing the important tyrosine-122 residue in GyrA again by site-directed mutagenesis to chemically similar and dissimilar amino acids and assess how this affected the efficiency of the supercoiling reaction. A scheme for this is presented below (Figure 4.2).

Figure 4.2: Plan of action to investigate changing tyrosine-122 of GyrA of *E.coli* on the supercoiling reaction by inserting point mutations in this position to create amino acid changes



During the initial course of this work, these results were published by Critchlow and Maxwell, (1996). The highly competitive nature of these studies confirmed their importance and indeed, the construction of *gyrA*-containing plasmids generated some interesting results which will be discussed below.

4.2 Construction of pBluescript carrying *gyrA*

4.2.1 Strategy 1: Cloning of *gyrA* from a genomic library of *E.coli*

Cloning

Chromosomal DNA extracted from *E.coli* TG1 was fully digested with the restriction enzyme *EcoR*I and the restriction product run out on a 1% agarose gel. DNA fragments in the size range 4kb to 11kb were cut out, purified and ligated into the vector λZAPII which had been predigested with *EcoR*I and dephosphorylated with calf intestinal alkaline phosphatase (CIAP). The products of the ligation were packaged into Gigapack II Gold packaging extract and the efficiency of the packaging verified by titering the phage library in *E.coli* XL-1 BLUE and plating out on LB agar overlaying with top agar containing IPTG and X-gal. The number of clones containing inserts was found to be approximately 100 times greater than those not containing inserts.

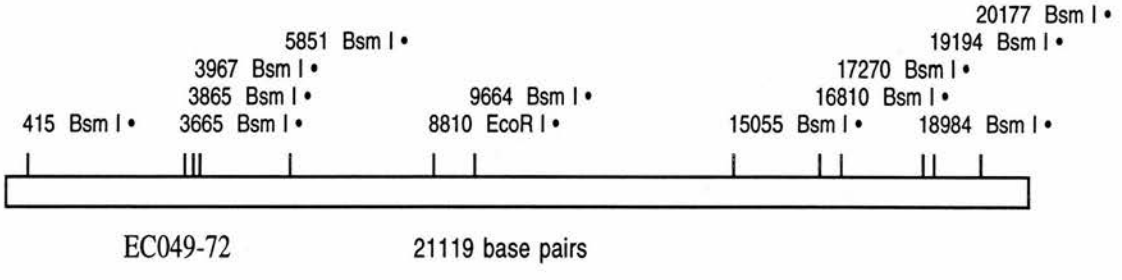
Screening

The phage library was plated out on large Petri dishes and plaque lifts performed. After baking the membrane, hybridisation was carried out by the use of the plasmid pPH3 which contains a copy of the *E. coli gyrA* gene (excluding the upstream promoter sequences). After developing the film corresponding to the blot, 3 putative positively hybridising plaques were revealed. The corresponding plaques were cored out of the master plate and stored in SM buffer at 4°C with a few drops of chloroform. On carrying out a secondary screen, it was found that the three putative plaques were actually false positives.

Conclusions

It was decided to discontinue with this strategy since a restriction map of this region of *E.coli* chromosomal DNA downloaded from Genbank showed that *gyrA* was present on a *EcoR*I-*EcoR*I fragment which was at least 8810bp long. It was therefore speculated that the presence of excessive upstream and downstream regions also present in the potential construct was deemed unnecessary and it was feared that the overproduction by pBluescript of other genes present in the construct might lead to misleading results. It was thereafter aimed to achieve a much neater construct, only containing *gyrA* to eliminate this possibility.

Figure 4.3: A simplified restriction map of the area surrounding and including *gyrA* of *E. coli*, present on contigs ECD049.72 (21119bp) showing the *Bsm*I and *EcoR*I sites.



The *gyrA* gene is present on the 3250bp *Bsm*I-*Bsm*I fragment between nucleotides 415 and 3665 on this contig.

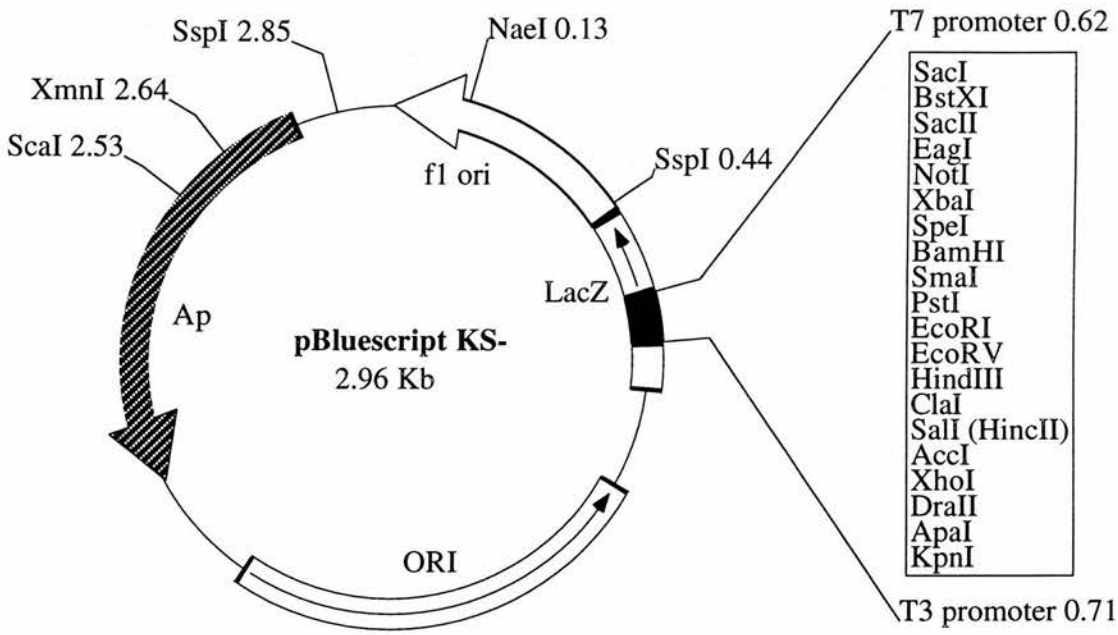
4.2.2 Strategy 2: Cloning of *gyrA* from a cosmid

Cloning

The *gyrA* gene of *E. coli* was excised from the cosmid vector pNJR3-2 on a 3.3kb fragment, by the restriction enzyme *Bsm*I. This *Bsm*I fragment had 3' overhangs

on each side, therefore the enzyme T4 DNA polymerase was used to produce blunt-ended fragments. This DNA fragment was ligated into pBluescript which had been digested with *Sma*I to create blunt ends.

Figure 4.4: Diagram of pBluescript showing the relevant restriction sites



The ligation mixture was transformed into *E.coli* XL1-BLUE and the cells plated out onto LB agar plates containing ampicillin, IPTG and X-gal. After overnight incubation at 37°C, three white colonies resulted which were subsequently analysed.

Screening

Plasmid preps of the three white colonies were separately restricted with *Eco*RI and *BamH*I and compared to pBluescript with no insert. It was expected that the presence of the 3.3kb inserted fragment containing *gyrA* would generate an extra band compared to pBluescript alone. Instead, anomalous restriction patterns were observed. Genetic complementation studies were then carried

out by the use of the *gyrA*^{ts} strain *E.coli* KNK453. The three putative positive colonies were transformed into *E.coli* KNK453 and the cells plated out on LB agar plates containing ampicillin and incubated at either 30°C or 42°C. However it was found that none of the putative plasmids was able to complement *E.coli* KNK453, thus none carried a functional *gyrA* gene.

Conclusions

It was thus concluded that the insert present in the three clones was not the required one. There is a possibility that during the gel extraction of the *Bsm*I-*Bsm*I 3.3kb fragment, the DNA became sheared and smaller fragments carrying the incomplete *gyrA* gene were ligated into pBluescript instead. It was decided that this strategy should be discontinued since extraction of sufficient quantities of DNA from pNJR3-2 was difficult. The cosmid pNJR3-2 is present in a low copy number in the cell and the fact that only a 3.3kb fragment of the total 28kb cosmid is useful, makes the whole extraction procedure very inefficient. Therefore a more specific strategy was adopted.

4.2.3 Strategy 3: Cloning of *gyrA* from a PCR product

Cloning

The *gyrA* gene of *E.coli* was PCR-amplified from a chromosomal prep of *E.coli* TG1. The primers used were as follows:

CCGCGGATCCGAATAAAGCGTATAGCTTTA

TCGAATTCGCCCAGACTTTGCAGCCTGG

As shown by underlining, a *BamH*I restriction site was introduced into the upstream primer and a *Eco*RI restriction site introduced into the downstream primer to facilitate subsequent cloning steps.

The resulting 2910bp PCR fragment was digested with the restriction enzymes *BamHI* and *EcoRI* and the product of restriction run out on a 1% agarose gel. The DNA fragment was cut out, purified and ligated into pBluescript that had been digested also with *BamHI* and *EcoRI*. The ligation mixture was transformed into *E. coli* XL1-BLUE and the cells plated out on LB agar plates containing ampicillin, IPTG and X-gal. Forty-eight white colonies were picked and plasmid DNA extracted. The plasmid DNA from the putative recombinants was restricted with an appropriate restriction enzyme. However, unexpected restriction patterns were again observed.

Screening

To investigate whether the putative plasmids constructed did contain *gyrA*, genetic complementation studies were again carried out. The 48 putative positive colonies were transformed into *E.coli* KNK453 and the cells plated out on LB agar plates containing 50mg/1 ampicillin and incubated at either 30°C or 42°C. The results of the complementation experiments revealed that none of the recombinants actually carried a functional *gyrA* gene.

Conclusions

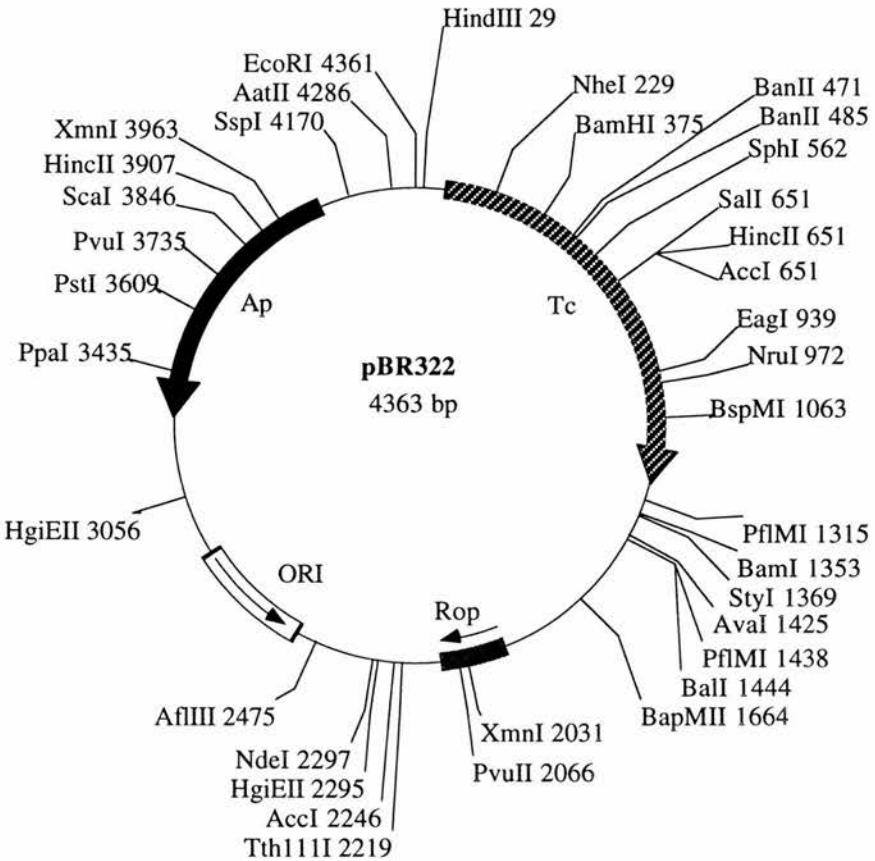
As a result of the inability to clone *gyrA* into pBluescript, it was decided to attempt to clone *gyrA* into a lower copy number plasmid such as pBR322. It was thought that the overexpression of *gyrA* may be deleterious to the cell through imbalancing the homeostatis of supercoiling in the cell. If there was such a deleterious effect on the cell, cloning of *gyrA* into pBR322 (copy number 15 to 20 compared to a copy number of 200 to 1000 with pBluescript), would have the advantage that less GyrA molecules would be produced.

4.3 Construction of pBR322 carrying *gyrA*

4.3.1 Strategy 4: Cloning of gyrA from a PCR product into pBR322

The *gyrA* gene of *E.coli* was PCR-amplified from a chromosomal preparation of *E.coli* TG1 as before and the resulting PCR fragment digested with *EcoR*I and *BamH*I. The product on the restriction was run out on an agarose gel, purified and ligated into pBR322 that had also been digested with *EcoR*I and *BamH*I.

Figure 4.5: Restriction map of pBR322



The ligation mixture was transformed into *E.coli* XL1-BLUE and the cells plated out on LB agar plates containing ampicillin. Eighteen putative positives were picked and plasmid DNA extracted. The resulting plasmids were digested with an appropriate restriction enzyme to confirm the presence of the insert. Again, spurious results were achieved and none of the putatives complemented the *gyrA*^{ts} strain *E.coli* KNK453 at 42°C.

Conclusions

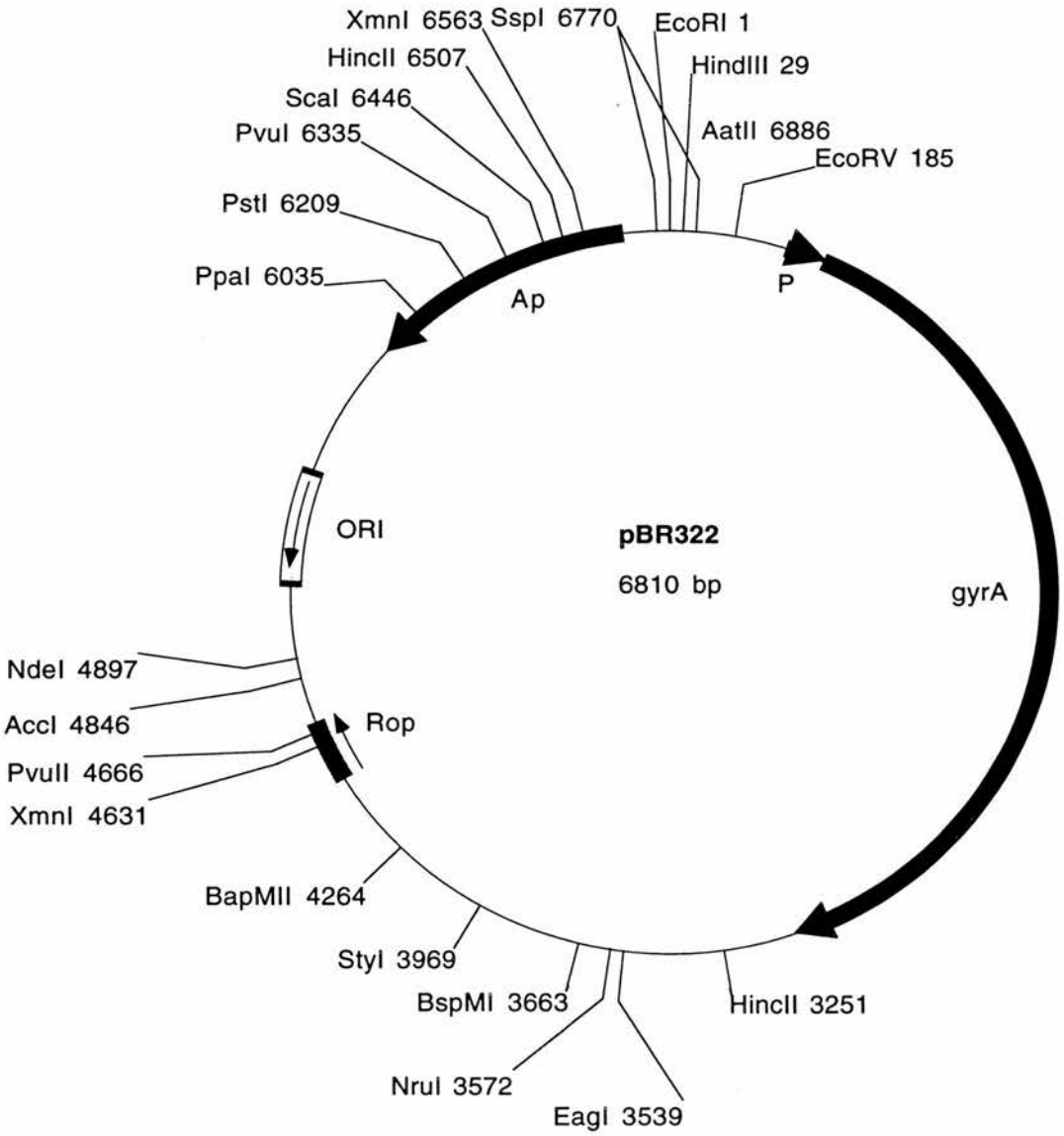
The inability to be able to clone *gyrA* into pBR322 was unexpected, since the cloning of a similar construct had been published before (Swanberg and Wang, 1987). However, it seemed that the exact positioning of *gyrA* in pBR322 was crucial since the *tet* gene of pBR322 is supercoiling sensitive (Jaworski *et al*, 1989). Bearing this in mind, the cloning procedure was slightly modified as described below.

Strategy 4b: Cloning of *gyrA* from a PCR-amplified PCR product into pBR322

Instead of cloning the PCR-amplified *gyrA* *EcoR*I-*BamH*I fragment into the *EcoR*I and *BamH*I sites of pBR322 as before, the fragment was cloned into *BamH*I-digested pBR322, the ends of which had been filled in with T4 DNA polymerase. The ligation mixture was transformed into *E.coli* XL1-BLUE and the cells plated out on LB agar plates containing ampicillin. Many putative positives were generated, twelve were picked from which plasmid DNA was extracted. The resulting plasmids were digested with *Sma*I to confirm the presence of the insert. All of the twelve putatives were shown to produce a 6810bp fragment on digestion with *Sma*I, which was the size of fragment expected from *Sma*I

digestion of the desired construct. To confirm that this was indeed the correct construct, the plasmid was transformed into *gyrA*^{ts} *E.coli* KNK453 and grown at 42°C. The plasmid was found to complement *E.coli* KNK453 at 42°C, therefore a suitable *gyrA*-containing clone had finally been achieved. After re-confirming the status of the plasmid by various restriction enzyme digests (data not shown), this plasmid was named pBRgyrA. A diagram of pBRgyrA with some restriction sites is shown in Figure 4.6.

Figure 4.6: Diagram of pBRgyrA



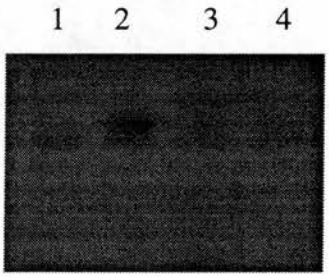
4.4 Is high-level expression of *gyrA* toxic to *E.coli*?

The inability to clone *gyrA* into a high copy number plasmid prompted the relationship between the level of expression of *gyrA* and cell viability to be investigated. It seemed possible that high level expression of *gyrA* affected the levels of supercoiling in the cell and thus affected cell viability. The fact that *gyrA* was cloned without problem into pBR322 suggested that levels of up to 15 to 20 plasmid-encoded copies per cell of *gyrA* could be tolerated.

In order to investigate this further, the PCR-fragment carrying *gyrA* was amplified as before, restricted with *EcoR*I and *BamH*I and ligated into the *EcoR*I and *BamH*I sites of plasmid pOU61 to form construct pOUgyrA. Plasmid pOU61 is a temperature-dependent cloning vector which is present in one copy per chromosome at temperatures below 37°C but at 42°C, shows uncontrolled replication (Larsen *et al*, 1984). By growing *E.coli* TG1 transformed with pOUgyrA at temperatures at and above 37°C and correlating the proportion of *gyrA* mRNA transcripts and amounts of GyrA with cell viability, the possible high level toxicity of GyrA was investigated.

A log culture of *E.coli* TG1 containing pOUgyrA was grown at temperatures 30°C, 35°C, 37°C and 42°C for two hours. RNA was extracted from each culture, electrophoresed, blotted and hybridised to a *gyrA* probe. The results of this Northern blot are displayed in Figure 4.7.

Figure 4.7: Northern blot showing the increase in *gyrA* transcripts produced by strain *E.coli* TG1pOUgyrA at increasing temperatures. Runaway replication of plasmid pOUgyrA is achieved by increasing the incubation temperature (see text for details).



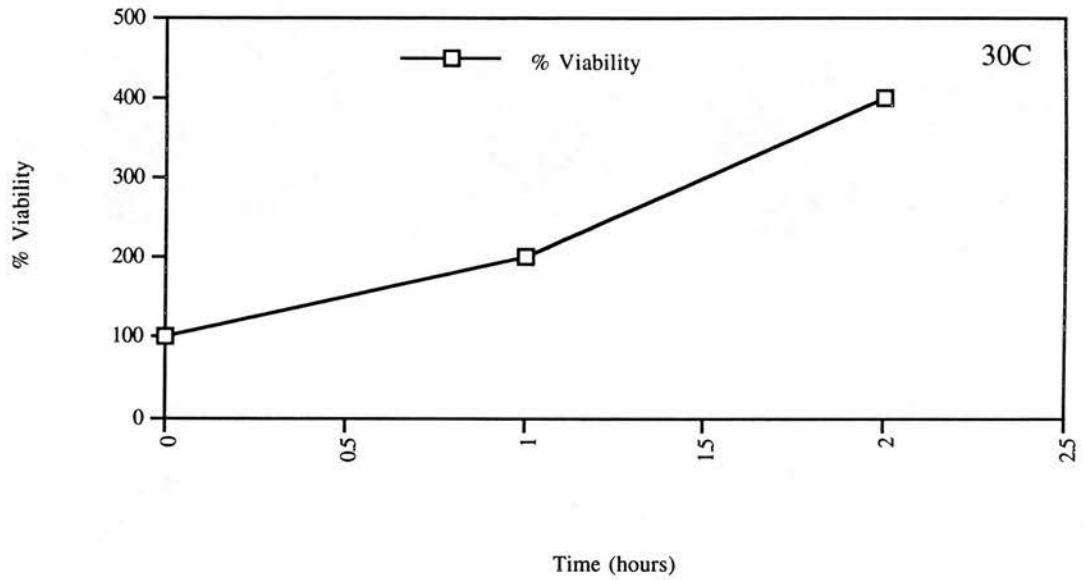
A log culture of *E.coli* TG1 containing pOUgyrA was grown at temperatures 30°C, 35°C, 37°C and 42°C for two hours. RNA was extracted from each culture, electrophoresed, blotted and hybridised to a *gyrA* probe. Lane 1: *E. coli* TG1 pOUgyrA grown for 2 hours at 30°C; Lane 2: *E. coli* TG1 pOUgyrA grown for 2 hours at 35°C; Lane 3: *E. coli* TG1 pOUgyrA grown for 2 hours at 37°C; Lane 4: *E. coli* TG1 pOUgyrA grown for 2 hours at 42°C.

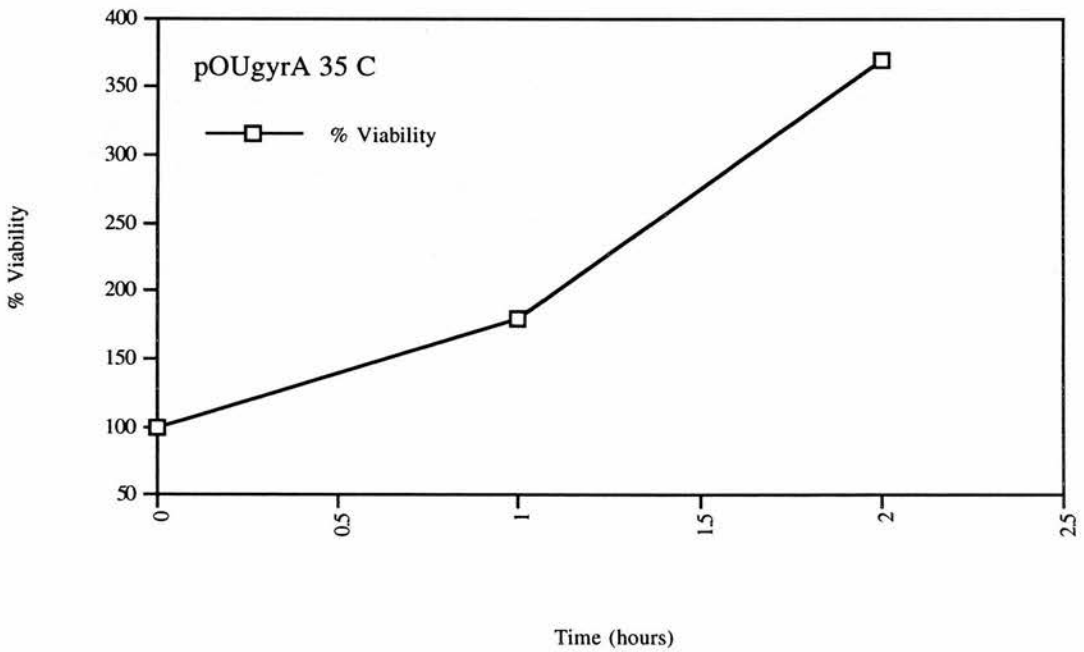
The results of the Northern blot show that between 30°C and 35°C, the levels of *gyrA* mRNA in *E.coli* TG1 pOUgyrA increases (Figure 4.7, Lanes 1 and 2). However, the *E.coli* TG1 pOUgyrA cultures grown at 37°C and 42°C show less *gyrA* transcripts (Figure 4.7, Lanes 3 and 4). This can be explained by the fact that incubation of the cultures at the higher temperatures for 2 hours results in cell death by overexpression of *gyrA* and subsequently less *gyrA* transcripts are detected. It was apparent that more than one band of RNA hybridising to the *gyrA* probe was present at the higher temperatures (Figure 4.7, Lanes 3 and 4). The reason for this higher molecular weight band is unknown.

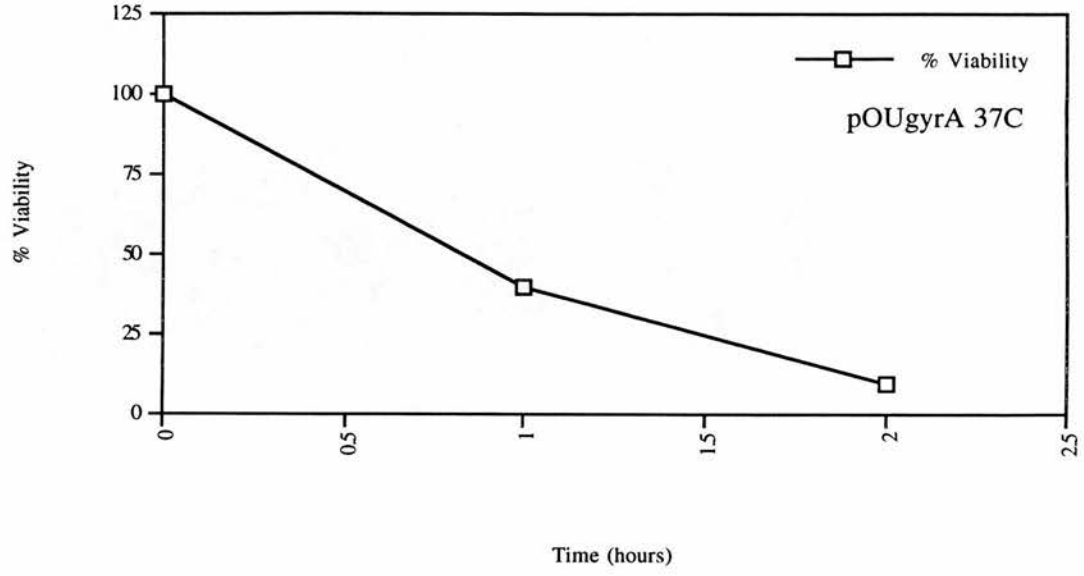
A 100µl volume of each culture was also removed after one hour to measure the cell viability. The cell viability at each temperature measured is plotted in Figure 4.8.

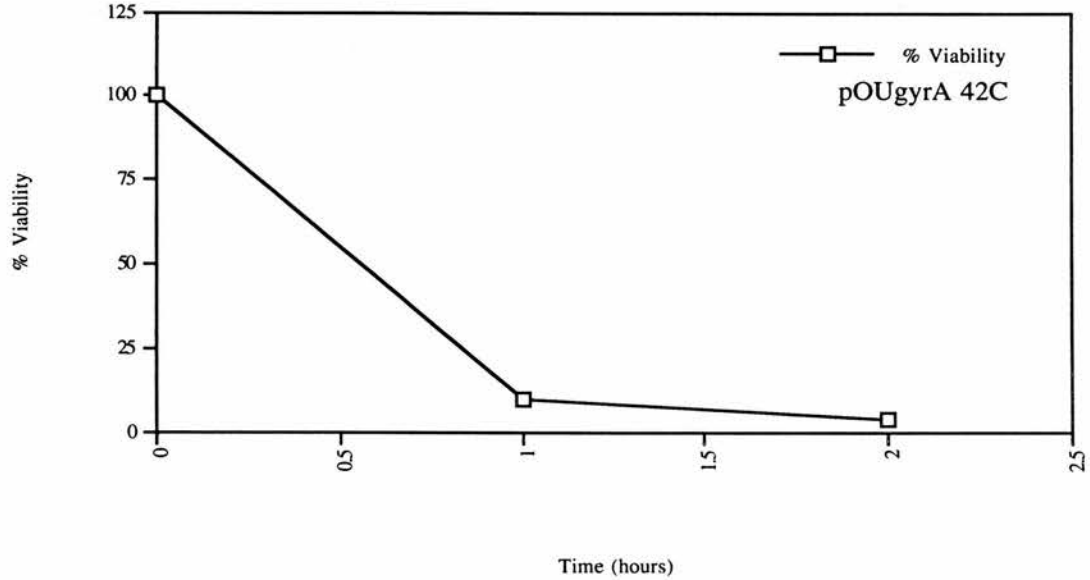
The graphs add weight to the proposal that GyrA is toxic when expressed at high levels in *E.coli*. The percentage viability of four cultures derived from the same parent but grown at different temperatures was calculated at two time points: one hour and two hours. The *E.coli* TG1 pOUgyrA culture that was grown at 30°C shows normal growth kinetics with the culture doubling in size every hour. The pattern of growth of *E.coli* TG1 pOUgyrA grown at 35°C almost is identical to that of the previous culture grown at 30°C, however, the viable count was actually found to be lower. The fact that pOU61 shows partial runaway replication is reflected by the results of the *E.coli* TG1 pOUgyrA culture grown at 37°C which decreases in cell viability with time. After one hour, 40% of the original population were found to be viable and after 2 hours 10% were viable. However, at 42°C, a rapid loss of cell viability was observed (10% of the original population after one hour and 4% of the original population after two hours) suggesting even more so that over-expression of GyrA may be detrimental to the cell.

Figure 4.8: Graphs plotting viability of culture *E.coli* TG1pOUgyrA at increasing temperatures






125
100
75
50
25
0
% Viability
% Viability
pOUgyrA 37C
0
0.5
1
1.5
2
2.5
Time (hours)


125
100
75
50
25
0
% Viability
% Viability
pOUgyrA 42C
0
0.5
1
1.5
2
2.5
Time (hours)

4.5 Discussion

By attempting to clone *gyrA* into a high copy number plasmid, it was found that expression of this gene may be toxic at high levels. This theory was strengthened by cloning *gyrA* into pOU61, a runaway replication vector which is present in one copy per cell at 30°C but shows uncontrollable replication at 42°C (Larsen *et al*, 1984). When *gyrA* was expressed at a high level (confirmed by Northern blotting, Figure 4.7), the viability of the culture was observed to decrease dramatically (Figure 4.8). The fact that more *gyrA* transcripts were detected at 35°C than at 30°C (Figure 4.7, compare Lane 2 to Lane 1) and the viable count of the culture at 35°C was lower confirms this. Although it is likely that the overexpression of GyrA in the cell affects the viability of the culture, it is possible, however, that the sheer density of plasmid DNA has the same effect. On exploring the possibility that *gyrA* was toxic to the cell at high levels (this work; A.Maxwell, personal communication), a search of the literature was carried out to investigate what cloning vectors had been previously used by other groups to clone *gyrA* (Table 4.1)

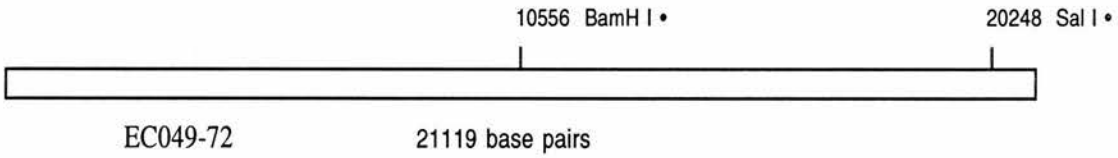
Table 4.1: Descriptions of plasmids into which *gyrA* has been cloned.

Plasmid	Backbone	Uninduced copy number	Reference
pMK90	pBR322	15 to 20	Mizuuchi *et al*, 1984
pTS7	pACYC184	5 to 6	Saiki *et al*, 1994
pPH3	pUC18	Zero	Hallett *et al*, 1990
pBP515	pRSFK	low	Heisig and Wiedemann, 1991
pAW012	pBR322	15 to 20	Nakamura *et al*, 1989
pAW011	pBR322	15 to 20	Yoshida *et al*, 1988
pNJR322	pLA2917	low	Robillard, 1990
pJSW101	pUC19	200	Soussy *et al*, 1993

pSLS447	pBR322	15 to 20	Swanberg and Wang, 1987

All the *gyrA*-encoding vectors but one previously made had been based on low copy numbers such as pBR322. The one *gyrA*-encoding vector that was expressed in high copy number was pJSW101 (Soussy *et al*, 1993). By cloning and expressing *gyrA* in pUC19, GyrA would be expected to be present in at least 200 to 1000 extra copies per cell, more if the gene was readily transcribed. Plasmid pJSW101 is described as a pUC19-based plasmid with a *BamHI-SalI* fragment from *E.coli* KL16 containing *gyrA* cloned into the *BamHI* and *SalI* sites (Soussy *et al*, 1993). However when a restriction map of this area was constructed, the nearest *BamHI* site was found to be at least 6891bp from the end of *gyrA* and a *SalI* site on the other side of the gene could not be found. Therefore the accuracy of the construction of this plasmid may be dubious.

Figure 4.9: Simplified restriction map of the region surrounding *gyrA* showing *BamHI* and *SalI* sites.



The inability to clone *gyrA* into the *EcoRI* and *BamHI* sites of pBR322 possibly was a result of the insert affecting the negative supercoiling of the plasmid. It has been observed previously that some inserts cloned into the *BamHI* site of pBR322 result in the occurrence of deletions (Jaworski *et al*, 1989). These authors suggested that the deletions resulted from genetic recombination because of a change in the negative supercoiling that occurred when transcription of the *tet* gene was disrupted. Although it was concluded that cloning some inserts in

the *BamHI* site would lead to deletions whereas cloning into the *EcoRI* site would not (Jaworski *et al*, 1989), the fact that *gyrA* itself is a supercoiling-specific gene may affect the negative supercoiling of the plasmid in such a way such that inserting the gene into the *EcoRI* and *BamHI* sites is impossible.

The high copy number toxicity of *gyrA* in *E.coli* almost definitely will result from a loss of control of supercoiling levels. Maintaining the correct supercoiling levels in *E.coli* is of upmost importance as transcription of many genes is supercoiling sensitive (see 1.4.2- 1.4.3). There also seems to be a mechanism to keep the amounts of cellular GyrA and GyrB at similar levels. However, studies have shown that overproduction of *gyrA* has no effect on supercoiling, suggesting that GyrA is in excess compared to GyrB, consistent with the results of extraction studies (Staudenbauer and Orr 1981). It has been suggested that it might be the concentration of GyrB rather than that of GyrA which controls the levels of supercoiling. It is possible that very high level expression of GyrA in *E.coli* may affect the cell somehow either by GyrA binding and interfering with a different target other than GyrB. Another explanation is that high level expression of *gyrA* results in the production of inclusion bodies. Overexpression of proteins commonly results in misfolding of the polypeptide so it cannot take up its native aggregated state and instead remains as an aggregated inclusion body (Wetzel, 1994).

In conclusion, overexpression of GyrA in *E.coli* is probably harmful to the cell, either as a result of loss of control of supercoiling, incorrect binding to other targets in the cell or the formation of insoluble aggregates of GyrA in inclusion bodies.

5 The Cellular Response to Quinolone Drugs

Although the quinolones have been shown to target DNA gyrase and poison the enzyme through the formation of the "cleavable complex", the actual cellular process of death has remained elusive. The experiments described in this chapter attempted to explain some of the molecular aspects of quinolone-induced cell death.

5.1 The effect of quinolone drugs on the viable count of *E.coli*

E.coli TG1 was investigated regarding its response to nalidixic acid and ciprofloxacin. Various concentrations of nalidixic acid (Figure 5.1) or ciprofloxacin (Figure 5.3) were added to a log phase culture of *E.coli* TG1 and 2 hours later a sample withdrawn and the viable count calculated. The results are displayed in Figures 5.1 and 5.3 below. From the graphs, the Optimum Bactericidal Concentration (OBC) of nalidixic acid and ciprofloxacin were estimated as being 90mg/l (Figure 5.1) and 0.15mg/l (Figure 5.3) respectively. These values and the shapes of the graphs were identical to previous data published for *E.coli* KL16 (Smith, 1986). As expected, the proportion of bacteria surviving treatment with a range of concentrations of quinolones followed a biphasic response with concentrations less than and more than the OBC resulting in a decreased proportion of bacterial death. The bacteriostatis at concentrations greater than the OBC has been correlated with an increased inhibition of RNA and thus protein synthesis. Therefore at quinolone concentrations higher than the OBC, the proposed bactericidal event responsible for cell death after treatment with nalidixic acid and partly responsible for cell death after treatment with ciprofloxacin is prevented from taking place (Deitz, Cook and Goss, 1966).

The experiment was then modified slightly by the addition of 170mg/l chloramphenicol 1 hour prior to the addition of the OBC of quinolone drug to inhibit protein synthesis. The viable counts are displayed in Figure 5.2 and Figure 5.4. As shown in Figure 5.2, the addition of chloramphenicol resulted in inhibition of cell death by nalidixic acid over the period of 3 hours tested. Instead, bacteriostatis results which agreed with previous findings that *de novo* protein synthesis is required for cell death after treatment with nalidixic acid (Smith, 1984). In contrast, the bactericidal effect of ciprofloxacin was not found to be as susceptible to antagonism by inhibition of protein synthesis as was that of nalidixic acid as shown in Figure 5.4. This again agrees with previous findings that there seem to be two mechanisms of cell death after treatment of *E.coli* with ciprofloxacin: a bactericidal event which requires *de novo* protein synthesis and a bactericidal event that does not (Smith, 1986).

Figure 5.1: Viable count of *E. coli* TG1 challenged with various concentrations of nalidixic acid

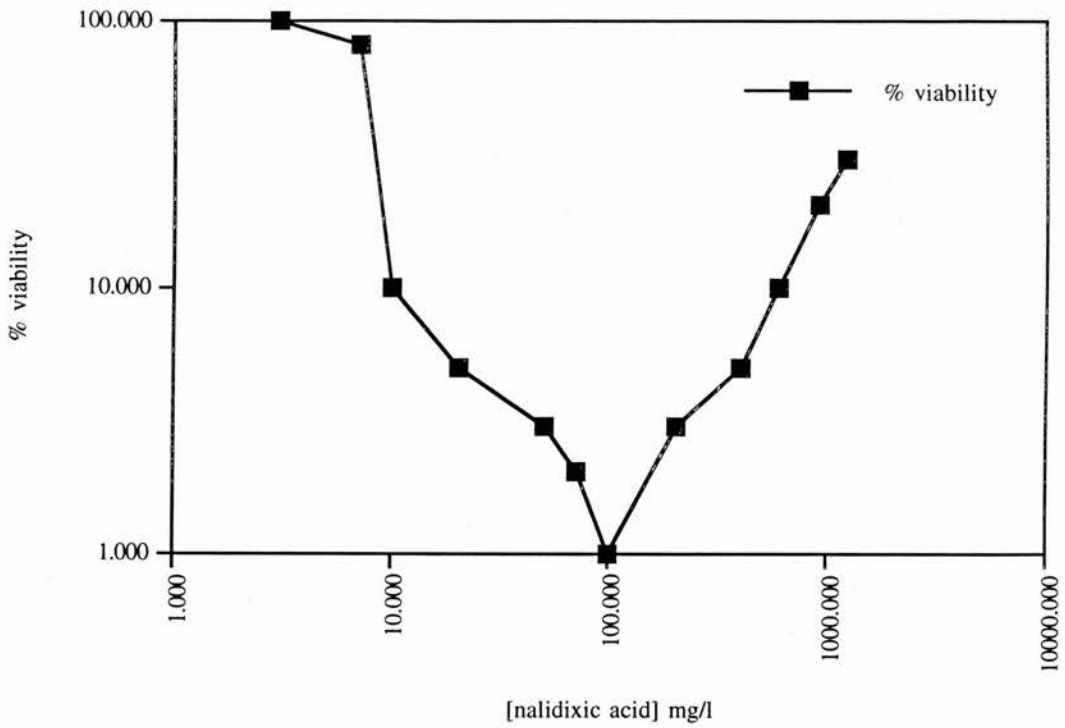


Figure 5.2 Viable count of *E. coli* TG1 challenged with the Optimum Bactericidal Concentration of nalidixic acid in the present of 170mg/l chloramphenicol over 3 hours

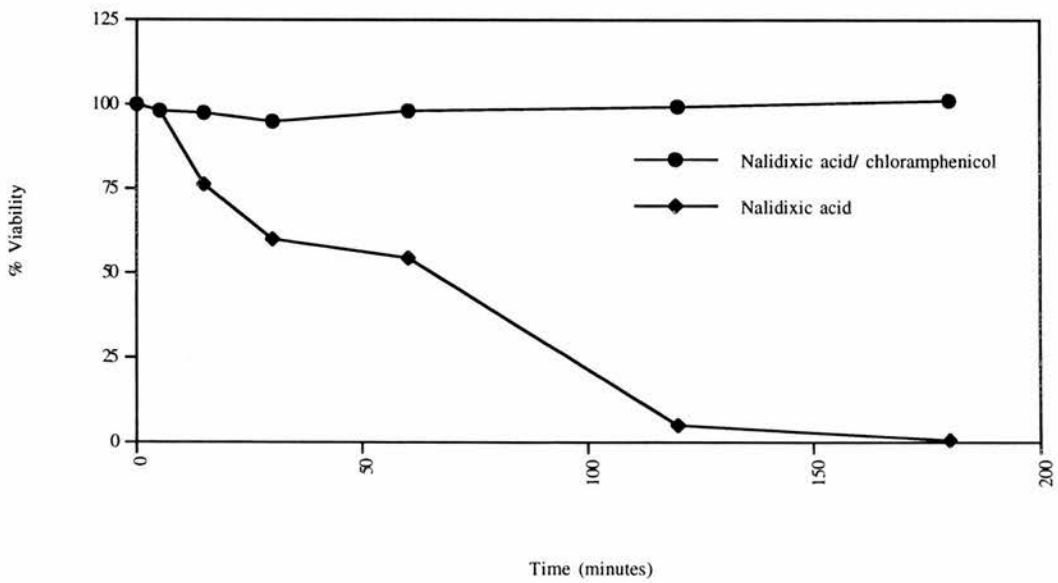


Figure 5.3: Viable count of *E. coli* TG1 challenged with various concentrations of ciprofloxacin

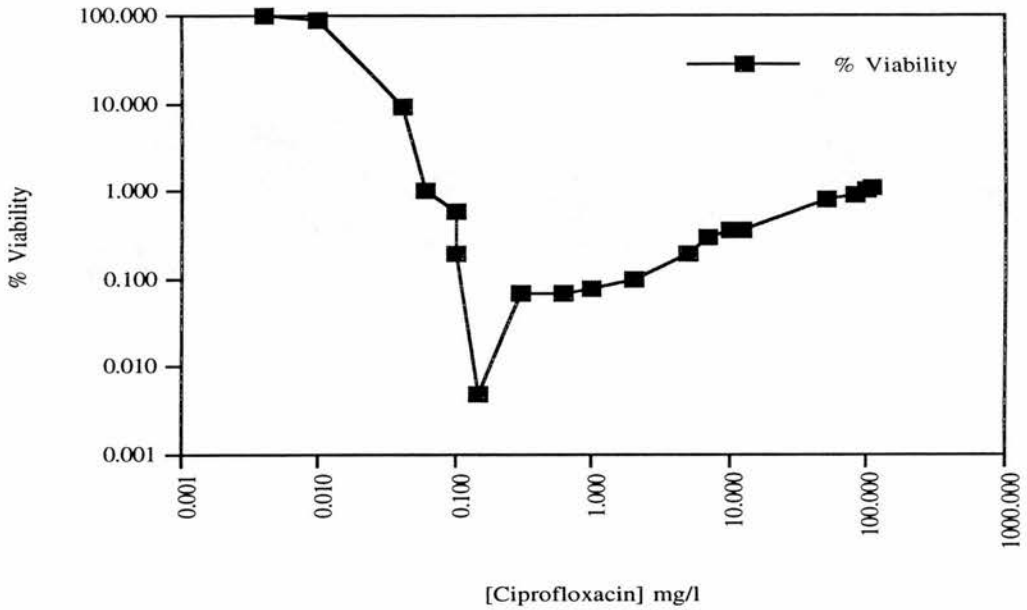
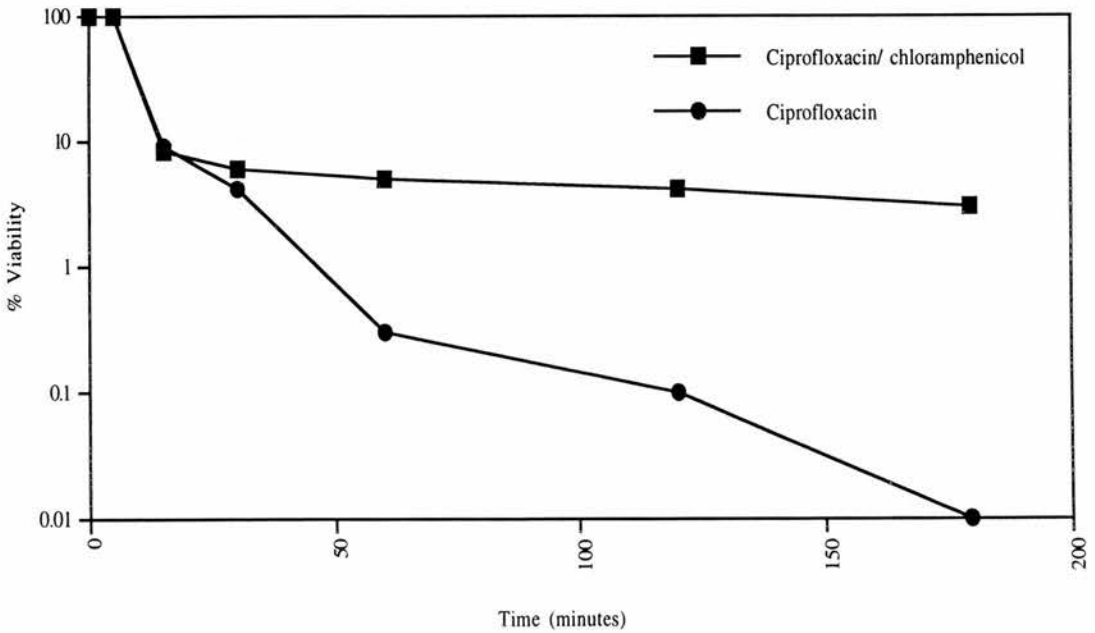


Figure 5.4 Viable count of *E. coli* TG1 challenged with the Optimum Bactericidal Concentration of ciprofloxacin in the presence or absence of 170mg/l chloramphenicol over 3 hours



5.2 The effect of quinolone drugs on the integrity of chromosomal DNA

To investigate the mechanisms of *E.coli* cell death further, the effect of the addition of quinolone drugs on the integrity of the bacterial chromosome was then studied. As a result of cleavable complexes on the chromosome giving rise to double-stranded DNA breaks which are potentially lethal, it was hoped that studying the effects of the quinolone drugs on the chromosome would be informative.

E.coli TG1 was grown up to log phase, the culture split into various aliquots which were then challenged with a specified amount of either nalidixic acid or ciprofloxacin. The cultures were incubated for a further period of time. To prepare the DNA for electrophoresis, different methods were employed as described below.

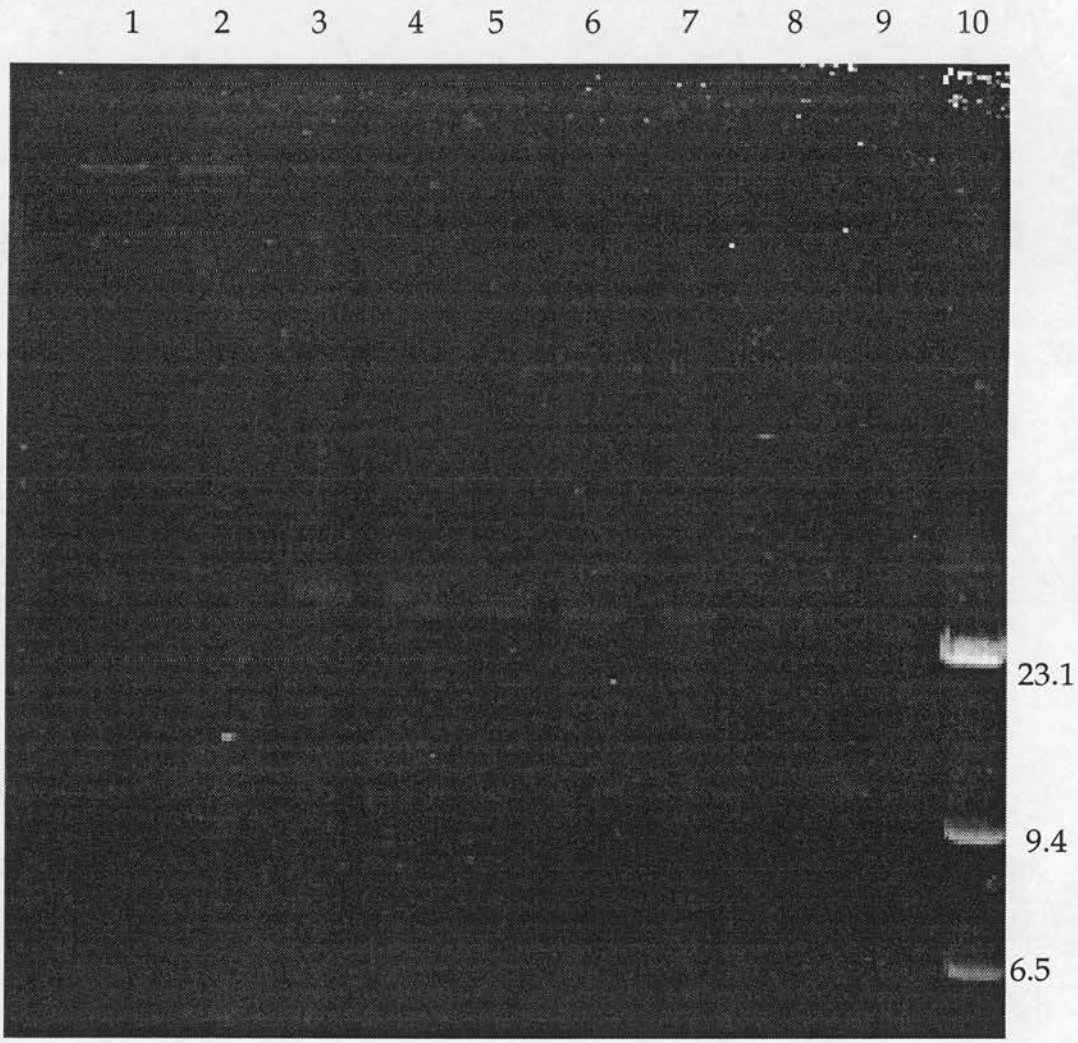
(i) Gentle lysis of bacterial cells with SDS

The method of Walker *et al* (1993) was followed. Briefly an equal volume of 2% (w/v) SDS was added to the bacterial culture. As well as causing cell lysis, any ongoing endogenous DNA cleavage would be suppressed and the chromosomal DNA would be effectively "deproteinized". It was found that the addition of 50mg/ml RNase (60 minutes at 50°C) and 0.5mg/ml proteinase K (60 minutes at 50°C), improved the appearance of the DNA. These lysates were loaded onto a gel and subjected to conventional agarose gel electrophoresis.

As shown in Figure 5.5, the chromosomal DNA extracted from *E.coli* untreated with ciprofloxacin remained intact and did not leave the wells of the agarose

gel as a result of its high molecular weight (Figure 5.5, Lane 1). However with increasing amounts of ciprofloxacin added to the bacterial culture (Figure 5.5, Lanes 2-9), the DNA was observed to have left the wells and form a band just above the top band of the λ *Hind*III markers (23.1kb). This DNA is believed to correspond to a size of 50-300kbp (Walker *et al*, 1993). The results show that increasing the concentration of ciprofloxacin results in the cleavage of the bacterial chromosome into smaller fragments of approximately 50-300kbp.

Figure 5.5: The effect of various concentrations of ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below is a photograph of the conventional agarose gel on which the fragments of DNA were resolved.

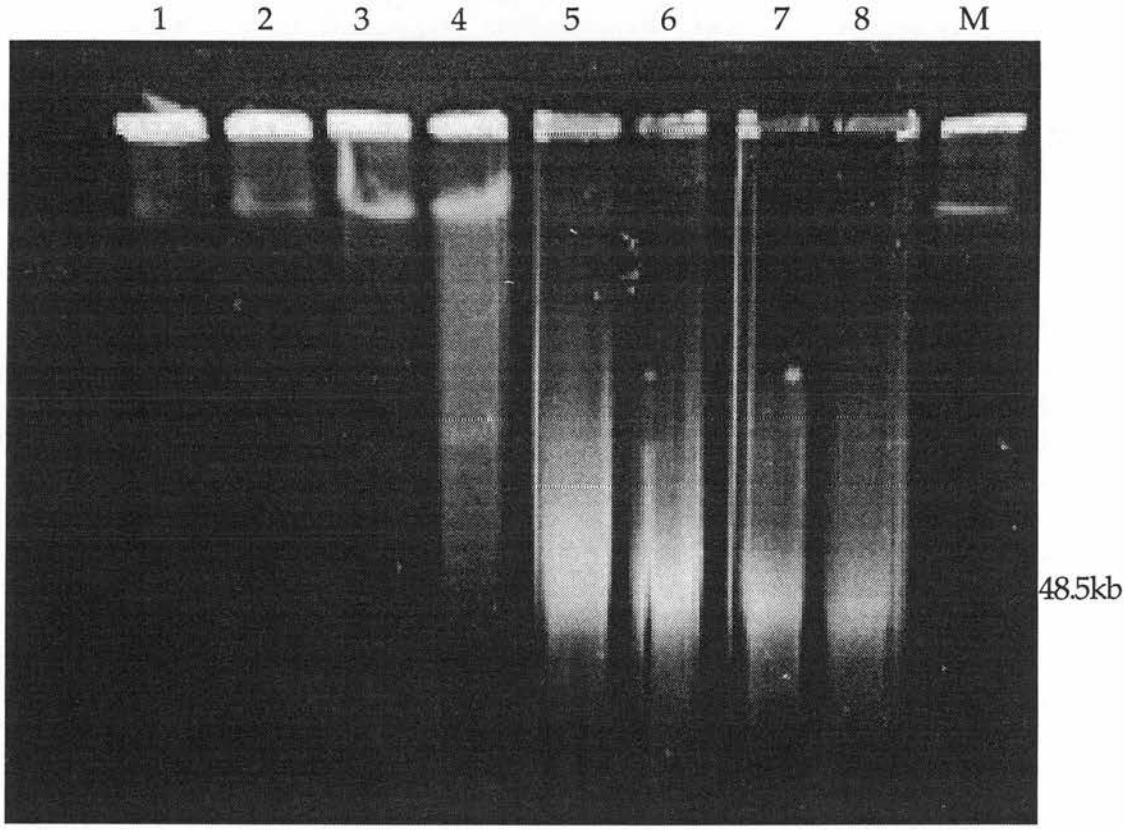


Cells were challenged with various concentrations of ciprofloxacin and an aliquot of cells removed after 10 minutes to assess DNA damage. Lane 1, no ciprofloxacin; Lane 2, 0.004mg/l ciprofloxacin; Lane 3, 0.008mg/l ciprofloxacin; Lane 4, 0.016mg/l ciprofloxacin; Lane 5, 0.032mg/l ciprofloxacin; Lane 6, 0.064mg/l ciprofloxacin; Lane 7, 0.128mg/l ciprofloxacin; Lane 8, 0.256 mg/l ciprofloxacin; Lane 9, 0.512mg/l ciprofloxacin; Lane 10, λ *Hin*dIII markers.

(ii) Lysis of cells in agarose plugs

As before, a logarithmic culture of *E.coli* TG1 was challenged with different concentrations of ciprofloxacin (Figure 5.6) or nalidixic acid (Figure 5.7), incubated for a further 60 minutes and the cells embedded in agarose plugs. Pulsed field gel electrophoresis was then used to separate the DNA fragments.

Figure 5.6: The effect of various concentrations of ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below is a photograph of the pulsed field gel on which the larger fragments of DNA were resolved.

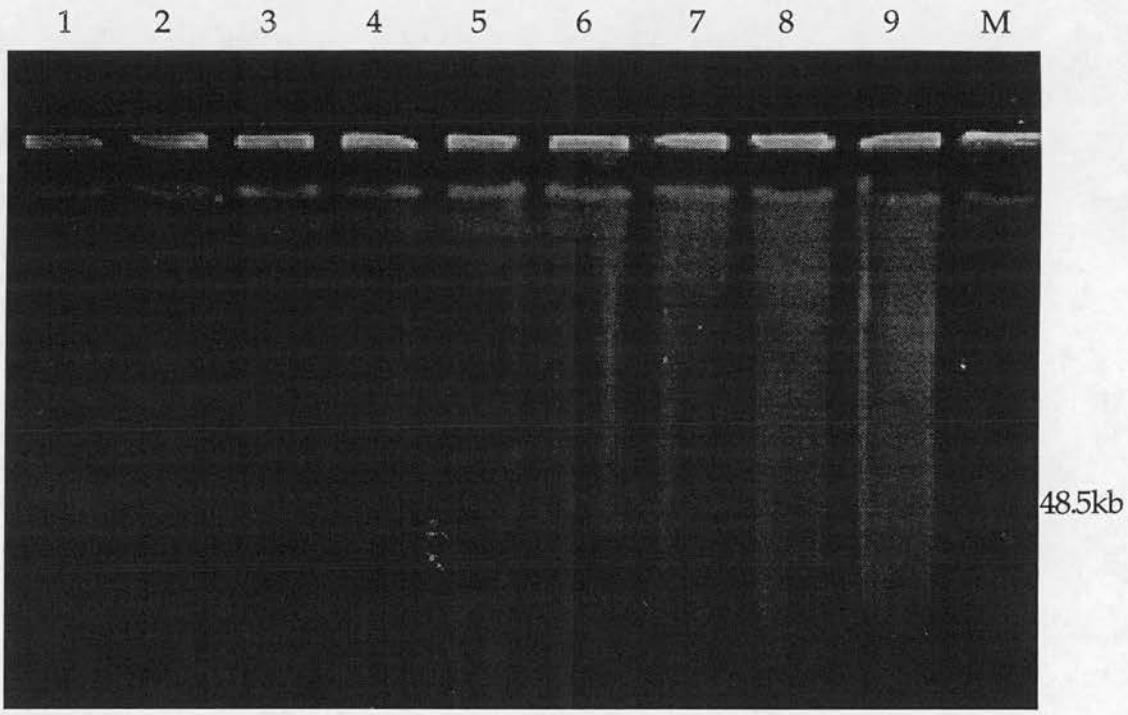


Cells were challenged with various concentrations of ciprofloxacin and an aliquot of cells removed after 60 minutes to assess DNA damage. Lane 1, no ciprofloxacin; Lane 2, 0.001mg/l ciprofloxacin; Lane 3, 0.004mg/l ciprofloxacin; Lane 4, 0.016mg/l ciprofloxacin; Lane 5, 0.064mg/l ciprofloxacin; Lane 6, 0.15mg/l ciprofloxacin; Lane 7, 0.5mg/l ciprofloxacin; Lane 8, 5 mg/l ciprofloxacin ; Lane M, λ concatemer markers.

On analysing the DNA by pulsed field electrophoresis, it appeared that at low ciprofloxacin concentrations (0.001mg/l, Figure 5.6, Lane 2), the chromosomal DNA remained intact. However it seemed that the addition of increasing amounts of ciprofloxacin up to the minimum inhibitory concentration (MIC) (0.004mg/l, Figure 5.6, Lane 3), the chromosomal DNA became increasingly damaged. The addition of a higher concentration of ciprofloxacin than the MIC caused the damage of the chromosomal DNA into even smaller fragments as shown in Figure 5.6, Lane 4 and even more so in Lane 5. In Lanes 6 to 8, a faint chromosomal DNA band could be seen, however the remainder of the DNA seemed to be on average much smaller. Compared to the DNA in the previous lane (Figure 5.6, Lane 5 [ciprofloxacin]=0.064mg/l), the DNA in Lane 6 [ciprofloxacin]=0.15mg/l seemed to lack the very high molecular weight fragments with the majority of DNA fragments appearing as being less than 48.5kb (bottom band of λ concatemer markers). At a concentration of ciprofloxacin above the OBC (0.5mg/l, Figure 5.6, Lane 7), even less DNA in the high molecular weight range was observed with some of the DNA appearing again to be of size less than 48.5kb. However on challenge with the highest tested amount of ciprofloxacin (Figure 5.6, Lane 8, [ciprofloxacin]=5mg/l), it appeared that there was not so much DNA damage at this concentration compared to the last two lower concentrations.

Therefore from these data alone, it seemed that the most ciprofloxacin-induced damage to the bacterial chromosome occurs around the OBC but at ciprofloxacin concentrations above and below this value, the bacterial chromosome is less degraded. The characteristics of the DNA at the various concentrations of ciprofloxacin used seemed to follow the pattern of the viable count of the culture at various concentrations of the drug (Figure 5.3).

Figure 5.7: The effect of various concentrations of nalidixic acid on the integrity of *E. coli* chromosomal DNA. Displayed below is a photograph of the pulsed field gel on which the larger fragments of DNA were resolved.



Cells were challenged with various concentrations of nalidixic acid and an aliquot of cells removed after 60 minutes to assess DNA damage. Lane 1, no nalidixic acid; Lane 2, 1mg/l nalidixic acid; Lane 3, 5mg/l nalidixic acid; Lane 4, 10mg/l nalidixic acid; Lane 5, 50mg/l nalidixic acid; Lane 6, 90mg/l nalidixic acid; Lane 7, 200mg/l nalidixic acid; Lane 8, 500mg/l nalidixic acid; Lane 9, 1000mg/l nalidixic acid; Lane M, λ concatemer markers.

As can be seen in Figure 5.7, minimal, if any, damage to the chromosomal DNA occurs at low nalidixic acid concentrations (Figure 5.7, Lanes 2 to 4, representing [nalidixic acid] 1mg/l to 10mg/l) compared to the drug-free control (Figure 5.7, Lane 1). Even around the MIC of nalidixic acid (3mg/l), minimal double stranded DNA breaks are observed. However on addition of 50mg/l nalidixic acid (Figure 5.7, Lane 5) to the bacterial culture, the chromosomal DNA appears much more damaged. At the concentration of nalidixic acid corresponding to the OBC of nalidixic acid, 90mg/l, (Figure 5.7, Lane 6) and at concentrations above (Lanes 7 to 8), the chromosomal DNA seems increasingly damaged, suggesting that the more nalidixic acid that is added to the culture, the more DNA damage by double-stranded breaks. This is in contrast with the effect of ciprofloxacin on chromosomal DNA (Figure 5.6) since at higher ciprofloxacin concentrations (Figure 5.6, Lane 9), there appeared to be less double-stranded DNA breaks. The other difference between the action of ciprofloxacin and nalidixic acid on the bacterial chromosome was that while with nalidixic acid, the chromosome always seemed to remain comparatively intact despite extensive double stranded DNA breaks, at certain concentrations of ciprofloxacin, the bacterial chromosome appeared less intact and the majority of the DNA seemed to be of much smaller size.

Since these preliminary experiments suggested that the mode of action of ciprofloxacin was different to that of nalidixic acid with regards to the formation of double stranded DNA breaks and smaller DNA fragments at high antibiotic concentrations, it was decided to carry out a more detailed study.

Investigating the extent of DNA damage at different concentrations of ciprofloxacin at different time points

Different amounts of ciprofloxacin, ranging from 0 to 100mg/l were added to a log phase culture and incubated for either 0, 5, 15, 30, 60, 90, 120, 180 or 360 minutes. The cells were treated as described in (ii) above and the DNA fragments separated by pulsed field gel electrophoresis. A small section of the agarose plug was cut off and run in a conventional agarose gel to ascertain the effect of the drugs on the chromosome as regards to the smaller fragments. It was found that this method of preparing DNA for conventional gel electrophoresis was better than that used previously since loading the SDS-treated lysate directly into the wells resulted in the mixture escaping from the wells. Both pulsed field gels and conventional agarose gels showing the effects of different concentrations of ciprofloxacin over different time points are displayed in the following Figures 5.8 to 5.19 along with a graph plotting the viable counts of the different cultures at the specified time points.

Figure 5.8: The effect of 0.001mg/l ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.8A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.8B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.8C)

Figure 5.8A

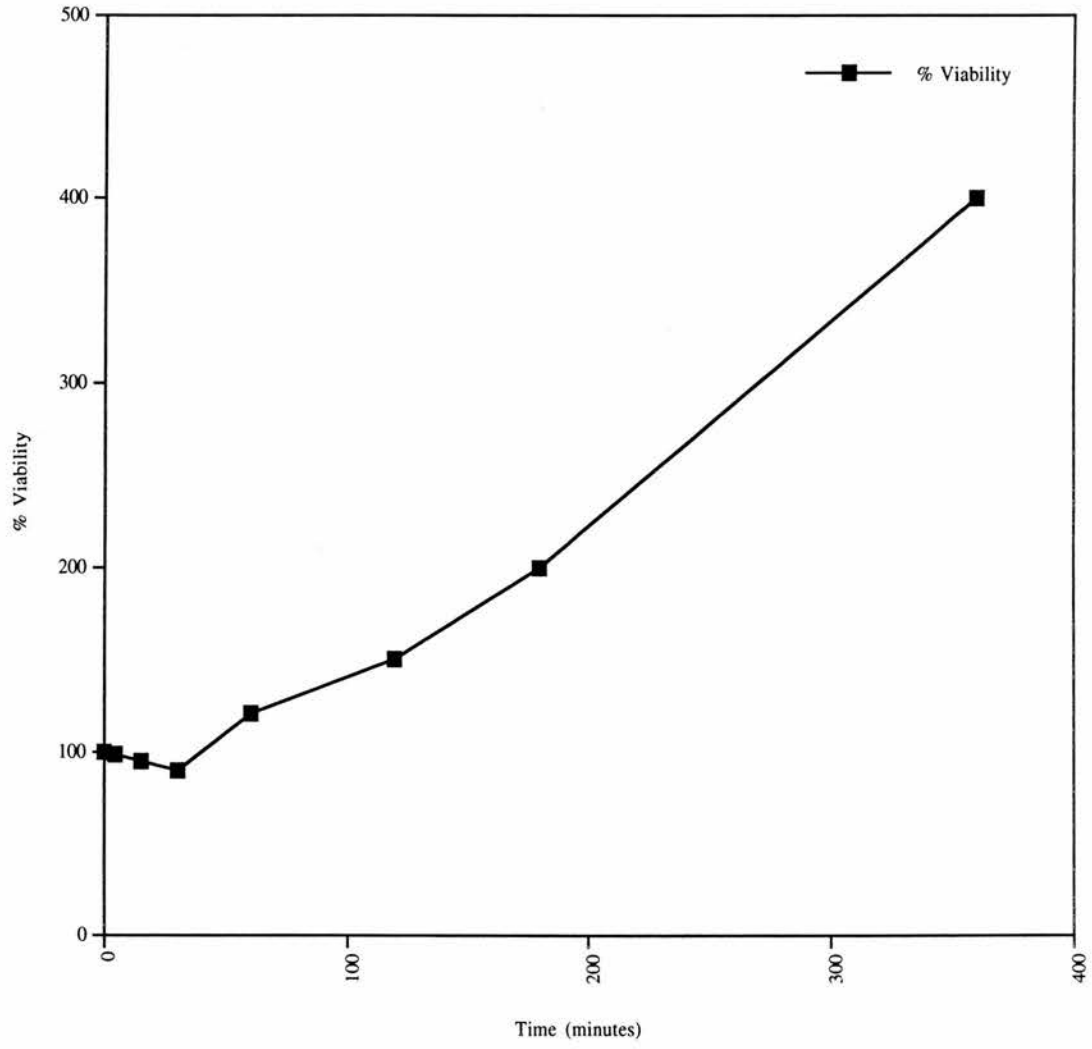


Figure 5.8B

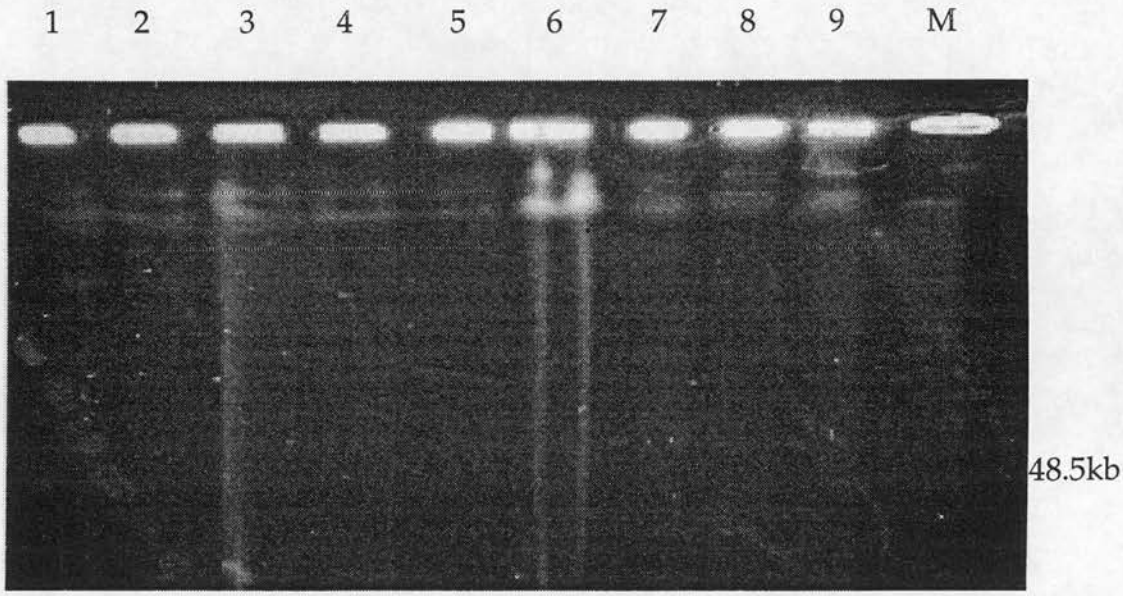
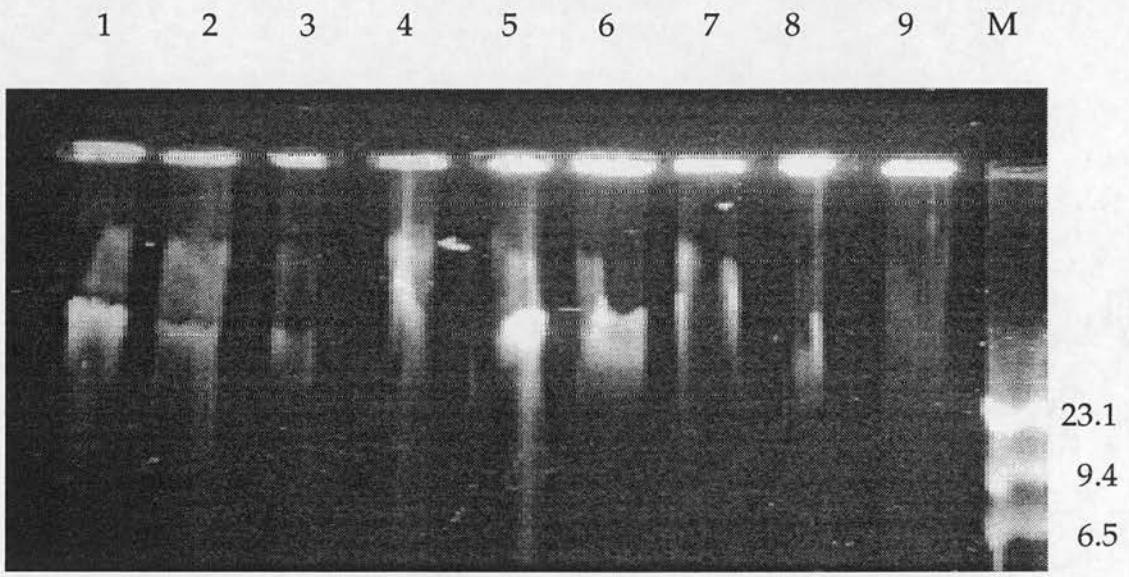


Figure 5.8C



Cells were challenged with 0.001mg/l ciprofloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ *Hind*III markers.

As shown in Figure 5.8B, the addition of 0.001mg/l ciprofloxacin causes minimal degradation of chromosomal DNA. At all the time points tested from t=0 to t=360, the chromosomal DNA appears intact and free from double stranded DNA breaks. The lower molecular weight DNA range is shown in Figure 5.8C. As expected, the DNA resolves as 50kb to 300kb fragments, compared to the λ *Hind*III markers. It appears that at this ciprofloxacin concentration, minimal DNA damage occurs to the chromosome and correspondingly, the viable count of the ciprofloxacin-challenged culture, over the time points tested, despite an initial lag on addition of ciprofloxacin, showed the kinetics expected of a healthy dividing culture (Figure 5.8A).

Figure 5.9: The effect of 0.004mg/l ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.9A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.9B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.9C)

Figure 5.9A

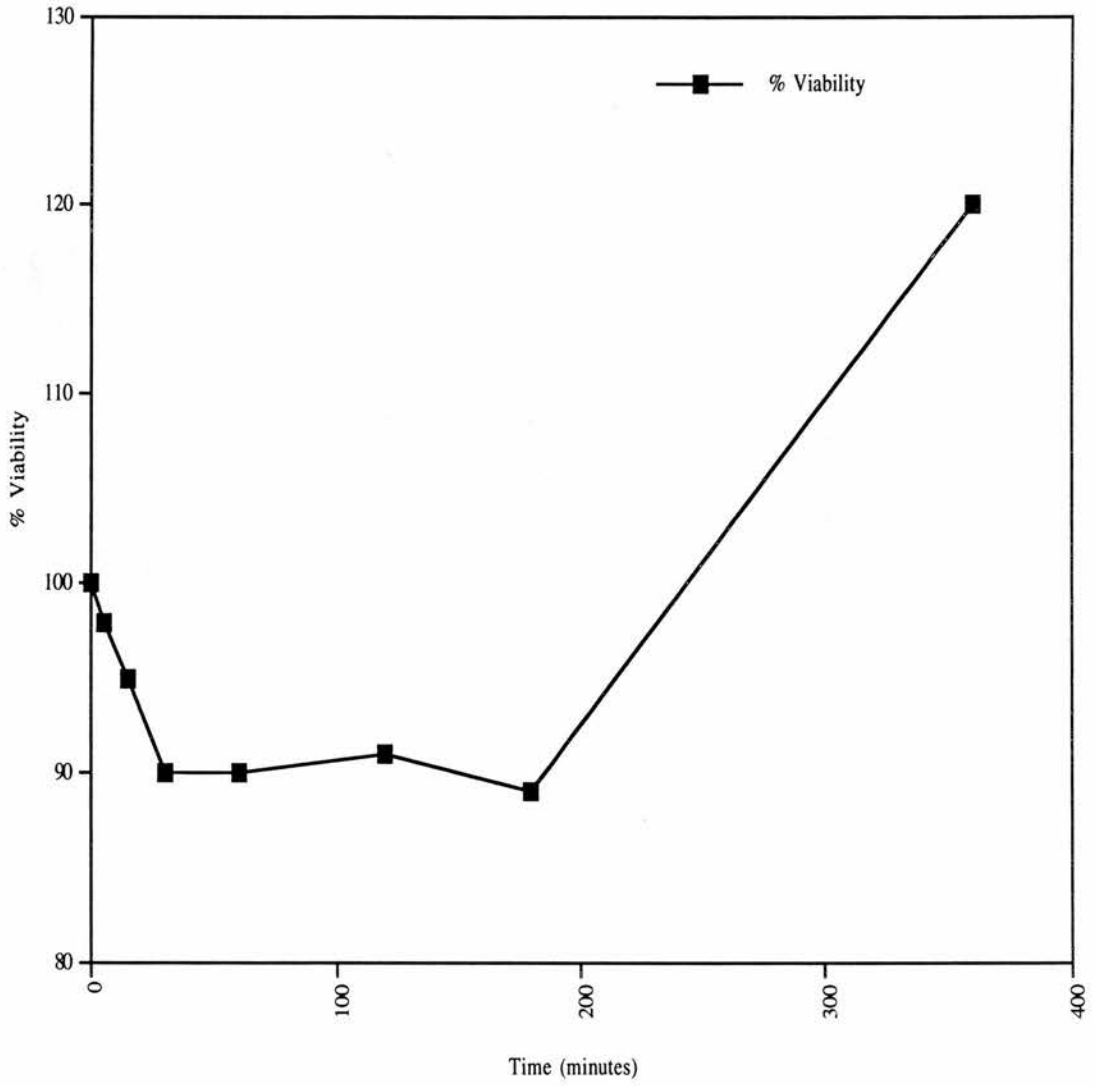


Figure 5.9B

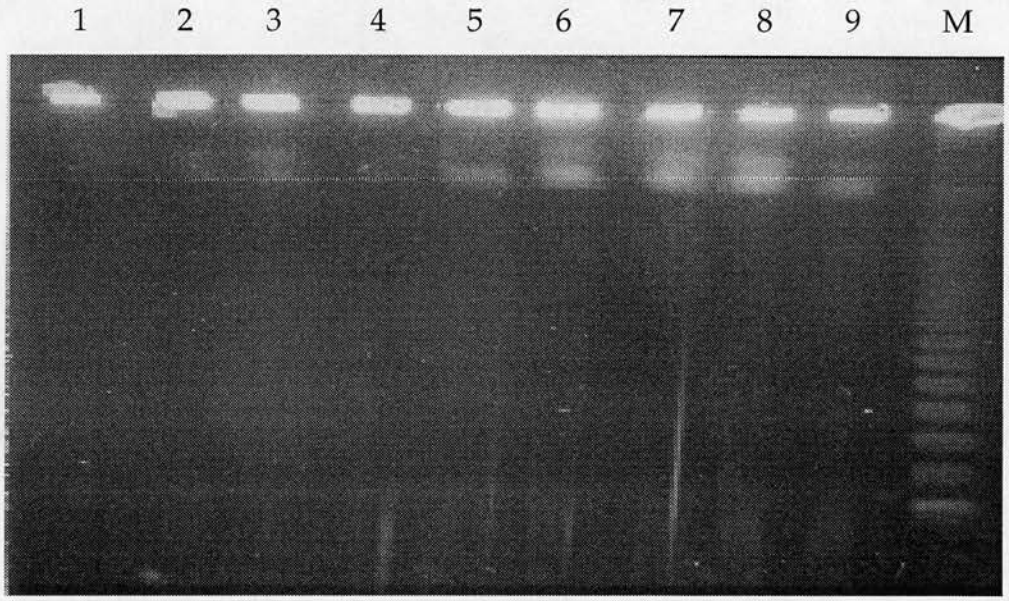
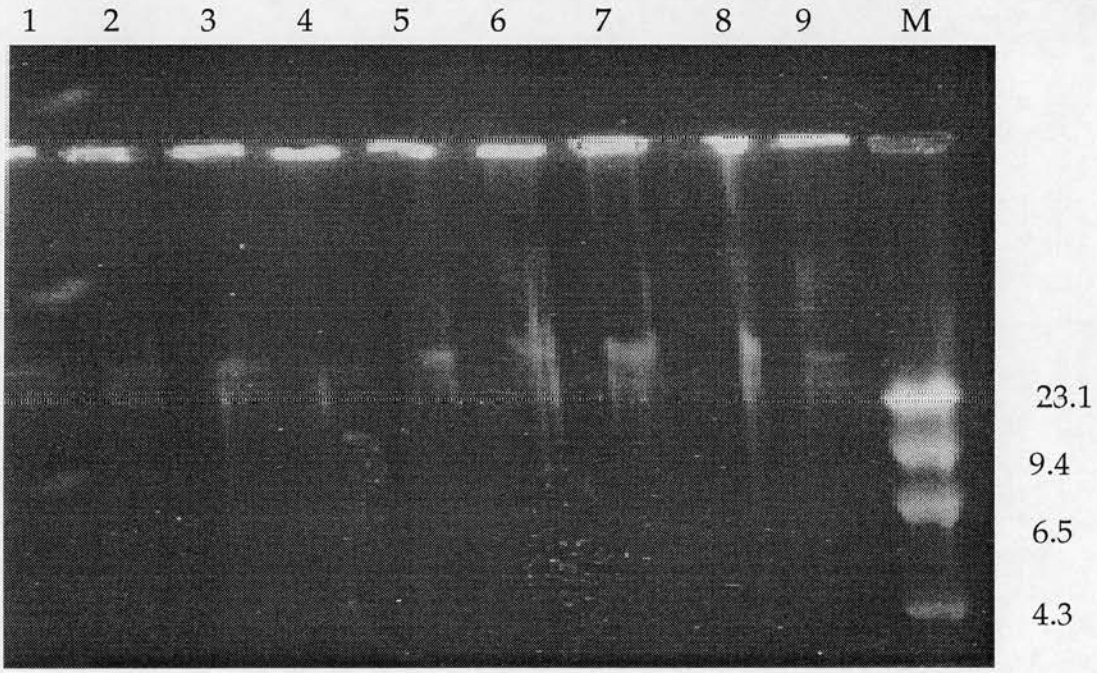


Figure 5.9C



Cells were challenged with 0.004mg/l ciprofloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0 ; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ *Hind*III markers.

As shown in Figure 5.9B, the addition of 0.004mg/l ciprofloxacin (the MIC) causes minimal degradation of chromosomal DNA. At all the time points tested from t=0 to t=360 minutes, the chromosomal DNA appears intact and only contains minimal double stranded DNA breaks. The extent of the double strand DNA breaks actually appears to increase with time up until 60 minutes (Figure 5.9B, Lane 4) and then remain constant up to 360 minutes (Figure 5.9B, Lane 9) and the smallest fragments resolved by the pulsed field gel seem to be slightly smaller than the bottom band of the λ concatamers (48.5kb). To resolve such small fragments, a conventional agarose gel was used as shown in Figure 5.9C. As expected, the DNA resolves as 50kb to 300kb fragments, compared to the λ *Hind*III markers. On addition of 0.004mg/l of ciprofloxacin to the bacterial culture, the viable count was found to decrease slowly to 90% of the original population at 30 minutes, stabilise at this level up until 180 minutes and then increase to 120% of the population at 360 minutes. This suggests that the cells cannot divide for 180 minutes after the addition of the MIC of ciprofloxacin as a result of DNA damage, however once the necessary repairs had been made, cell division can resume once again.

Figure 5.10: The effect of 0.016mg/l ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.10A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.10B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.10C)

Figure 5.10A

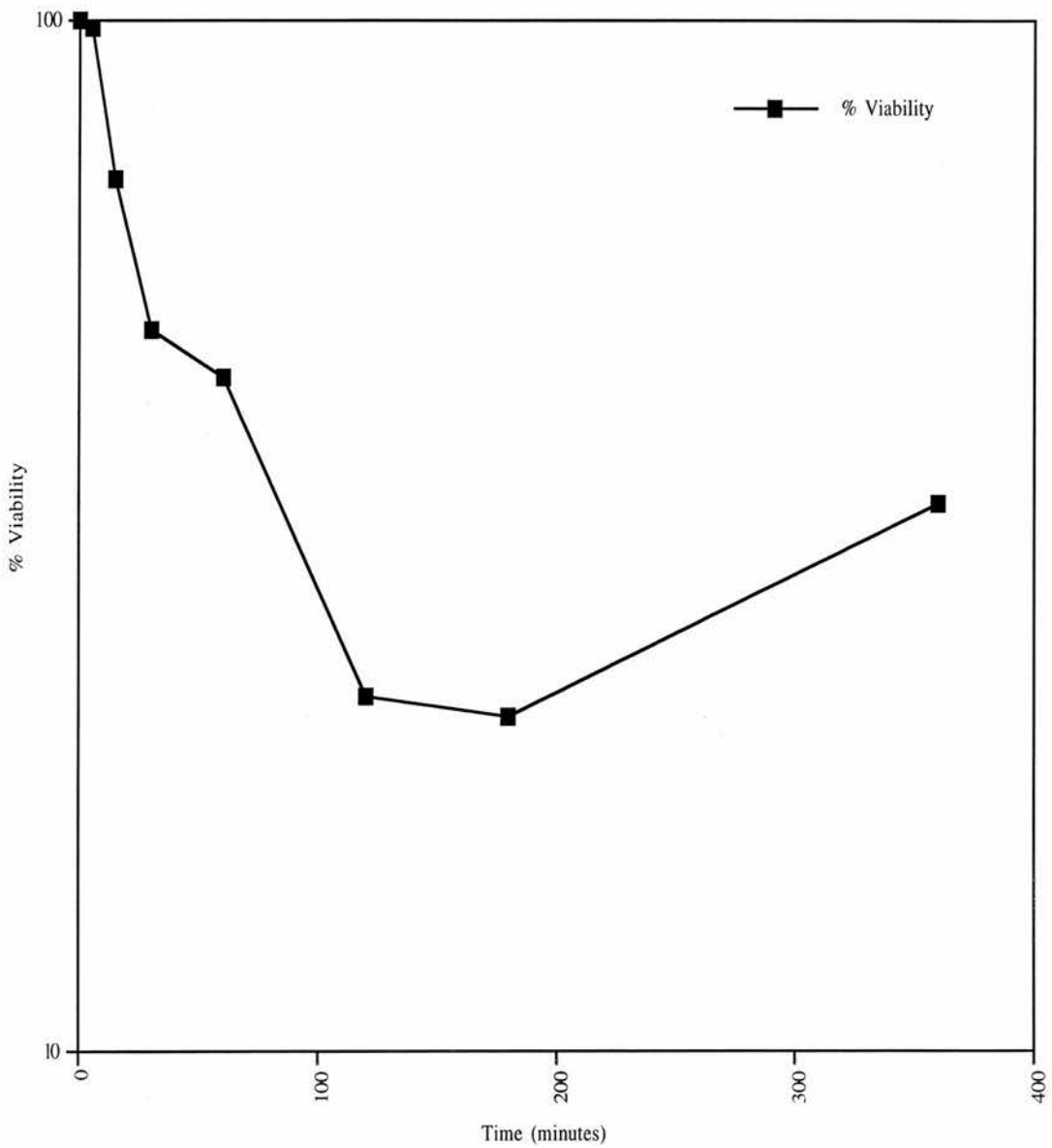


Figure 5.10B

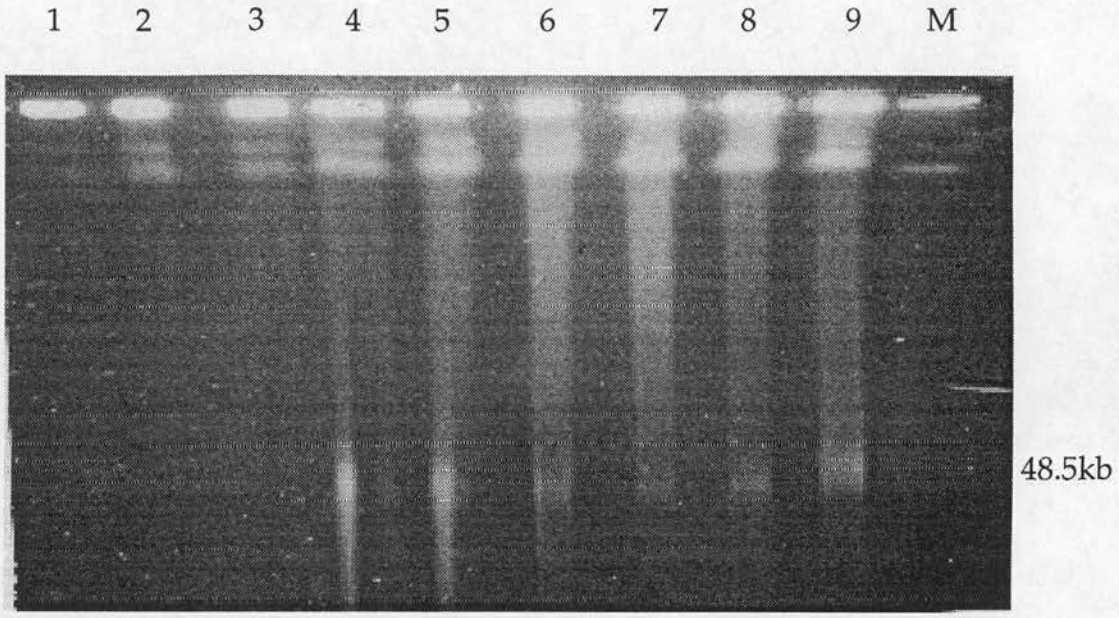
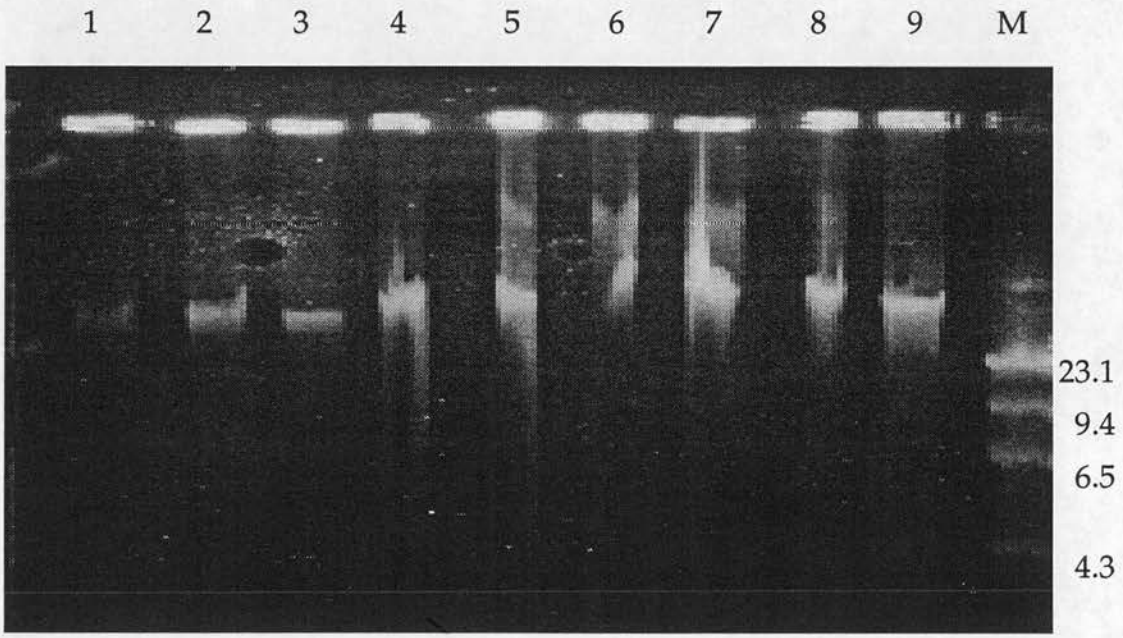


Figure 5.10C



Cells were challenged with 0.016mg/l ciprofloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer /λ *Hind*III markers.

As shown in Figure 5.10B, the addition of more than the MIC of ciprofloxacin (0.016mg/l) causes increasing double stranded DNA breaks. The formation of these double stranded DNA breaks is evident even after 5 minutes (Figure 5.10B, Lane 2) and after 30 minutes (Figure 5.10B, Lane 4) there appears to be increasing amounts of smaller fragments of DNA. Interestingly after 90 minutes, the amount of smaller fragments of DNA seemed to decrease (Figure 5.10B, Lane 6) and this trend continued up until 360 minutes (Figure 5.10B, Lane 9). At the later time points, even though there appeared to be less smaller DNA fragments, there appeared to be less evidence of extensive double stranded DNA breaks with a persisting population of DNA fragments of size approximately 48.5kb. Despite the extensive double stranded breakage, at this concentration of ciprofloxacin, the bacterial chromosome appeared largely intact.

These observations correlate with the results of resolving the smaller fragments on a conventional gel. As shown in Figure 5.10C, the majority of the DNA extracted from the cells at the early time points (t=5 minutes, t=15 minutes, Figure 5.10C, Lanes 2 and 3) is resolved at a position on the gel corresponding to a molecular size of 50kb to 300kb. However incubating the cells for 30 minutes after challenge with 0.016mg/l ciprofloxacin seemed to reveal the production of smaller fragments of sizes as small as 4kb (compared to the λ *Hind*III markers, Lane M). The amount of lower molecular weight DNA appeared to decrease after 60 minutes and from this time point onwards, the DNA again appeared to be 50kb to 300kb. Correspondingly, the viable count of culture decreased after 15 minutes and even more so after 30, 60 and 120 minutes (Figure 5.10A). The viable count of the culture then stabilised for one hour between 120 minutes and 180 minutes during which time DNA repair was probably being carried out. However, after this time point, an increase in cell viability was observed.

These results suggest either the action of DNA repair enzymes in the cell after 120 minutes or that the cells with heavily damaged DNA die and are perhaps removed from the population by cell lysis.

Figure 5.11: The effect of 0.064mg/l ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.11A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.11B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.11C)

Figure 5.11A

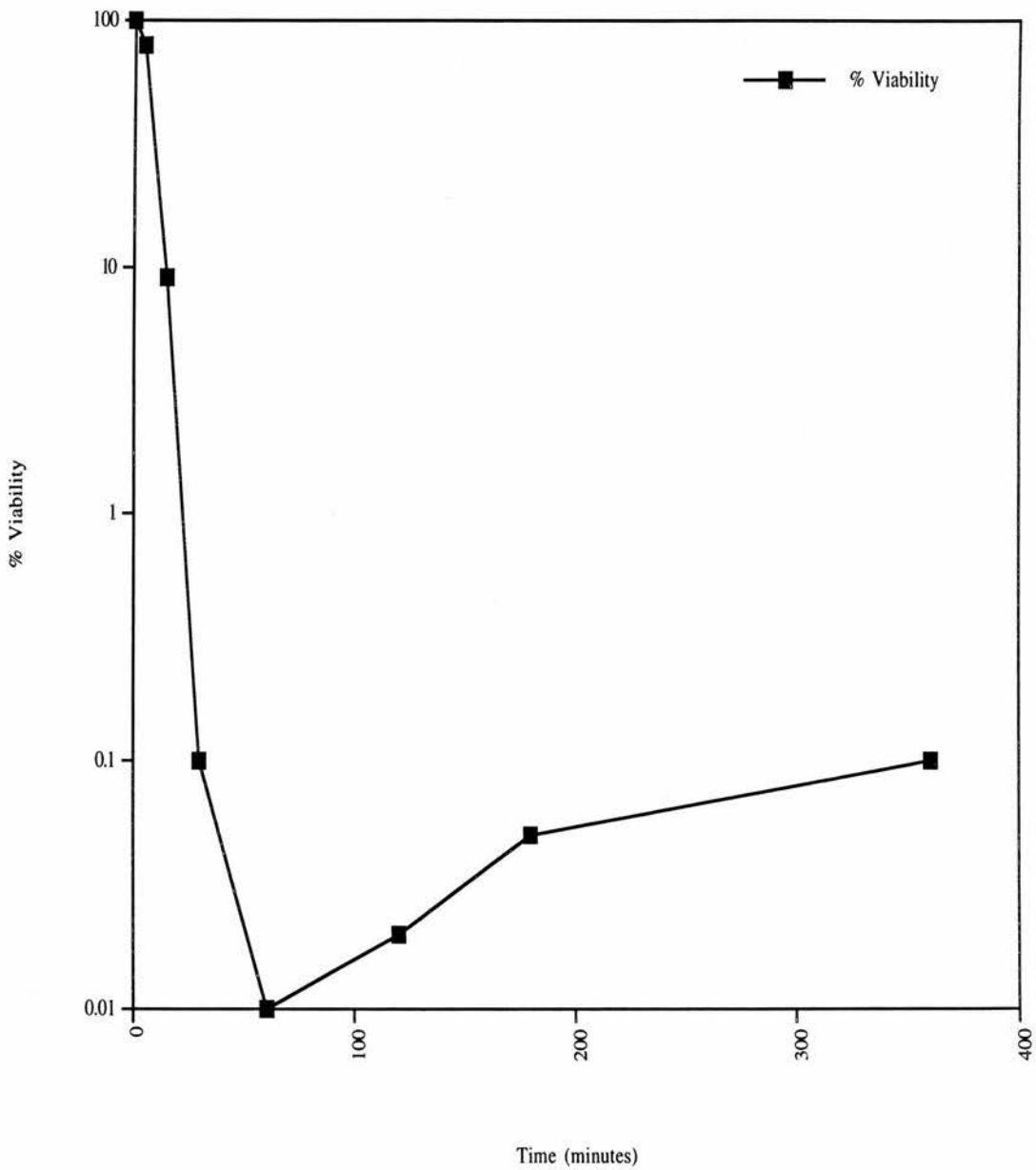


Figure 5.11B

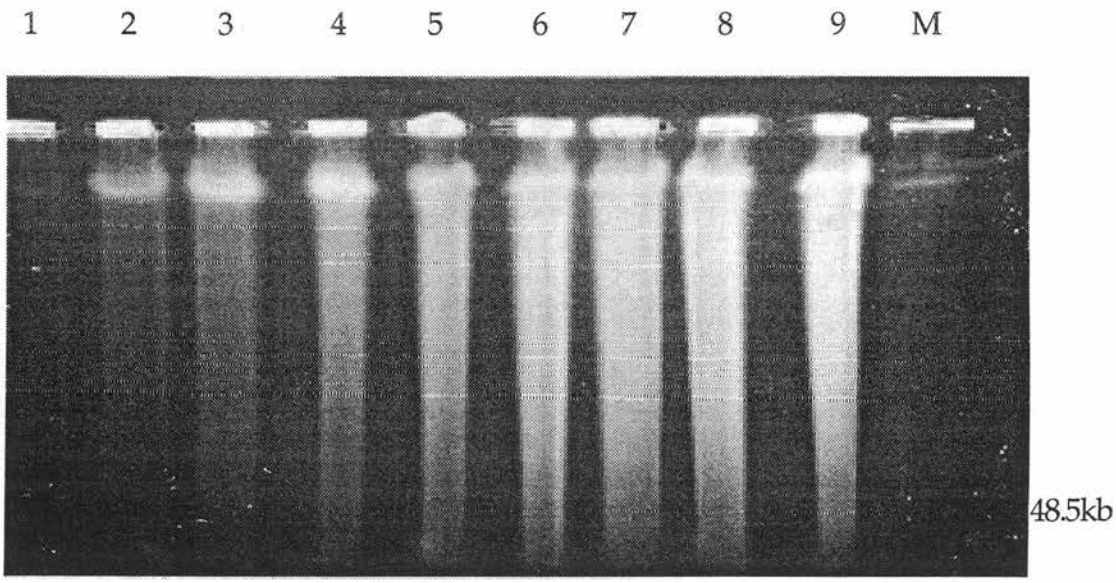
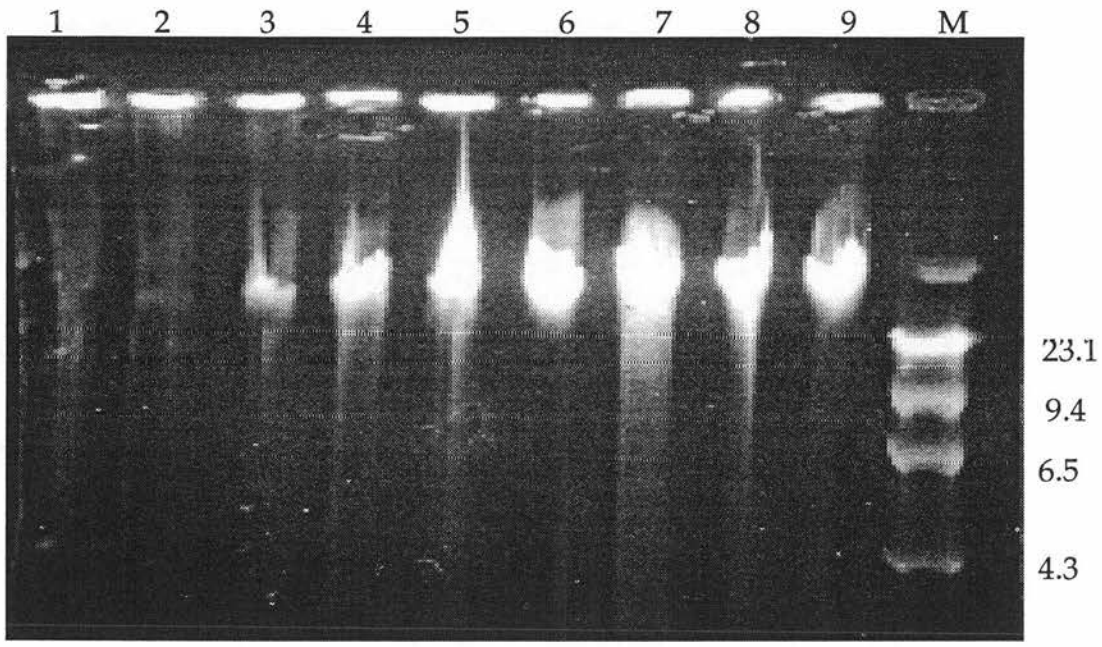


Figure 5.11C



Cells were challenged with 0.064mg/l ciprofloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ *Hind*III markers.

As shown in Figure 5.11B, the addition of 0.064mg/l ciprofloxacin causes even more extensive double stranded DNA damage. Again, the formation of these double stranded DNA breaks is evident even after 5 minutes (Figure 5.11B, Lane 2) and after this time point there seems to be evidence of more smaller fragments of DNA. With increasing time, there appears to be more DNA double stranded breaks, however despite this, at least some chromosomal DNA remains intact. Therefore these results differ from these discussed previously (Figure 5.10B, [ciprofloxacin]=0.016mg/l) in that at this concentration of ciprofloxacin (0.064mg/l) there appears to be no active DNA repair reducing the appearance of the DNA double stranded breaks over time. Bearing this in mind, it is unlikely that the explanation for less lower molecular weight DNA at increasing time points in the previous figure (Figure 5.10) is as a result of bacterial lysis removing the heavily damaged cell from the population. If this were the case, the effect would be much more pronounced in Figure 5.11 as four times more ciprofloxacin was added to the cell. However it is still possible that cell lysis only occurs at lower ciprofloxacin concentrations but this is unlikely. Therefore it appears that, at lower ciprofloxacin concentrations, a more pronounced DNA repair process is responsible for the presence of less smaller DNA fragments at the highest time points. It is likely that at a concentration of 0.064mg/l ciprofloxacin, the DNA repair process is saturated with double stranded DNA breaks and at this concentration, DNA repair is inefficient.

These conclusions correlate with the results of the conventional agarose gel used to separate the lower molecular weight DNA fragments shown in Figure 5.11C. After 15 minutes of ciprofloxacin challenge, the chromosomal DNA was seen to become degraded into smaller fragments, some of which appeared to be less than 4.3kb (compared to λ*Hind*III, Lane M). It is interesting that at the

highest time points (180 minutes and 360 minutes, Lanes 8 and 9), there seemed to be a decrease in the amount of lower molecular weight DNA so an active repair process does indeed seem plausible.

The results of the viable count demonstrated that there is a decreased viability of the bacterial culture after 15 minutes which becomes more pronounced after 30 and 60 minutes. However, cell viability does seem to increase after 120 minutes, perhaps because the cell has had more of a time to be able to repair its DNA and cells that have escaped death by the addition of the drug can commence cell division.

Figure 5.12: The effect of 0.15mg/l ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.12A) and a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.12B).

Figure 5.12A

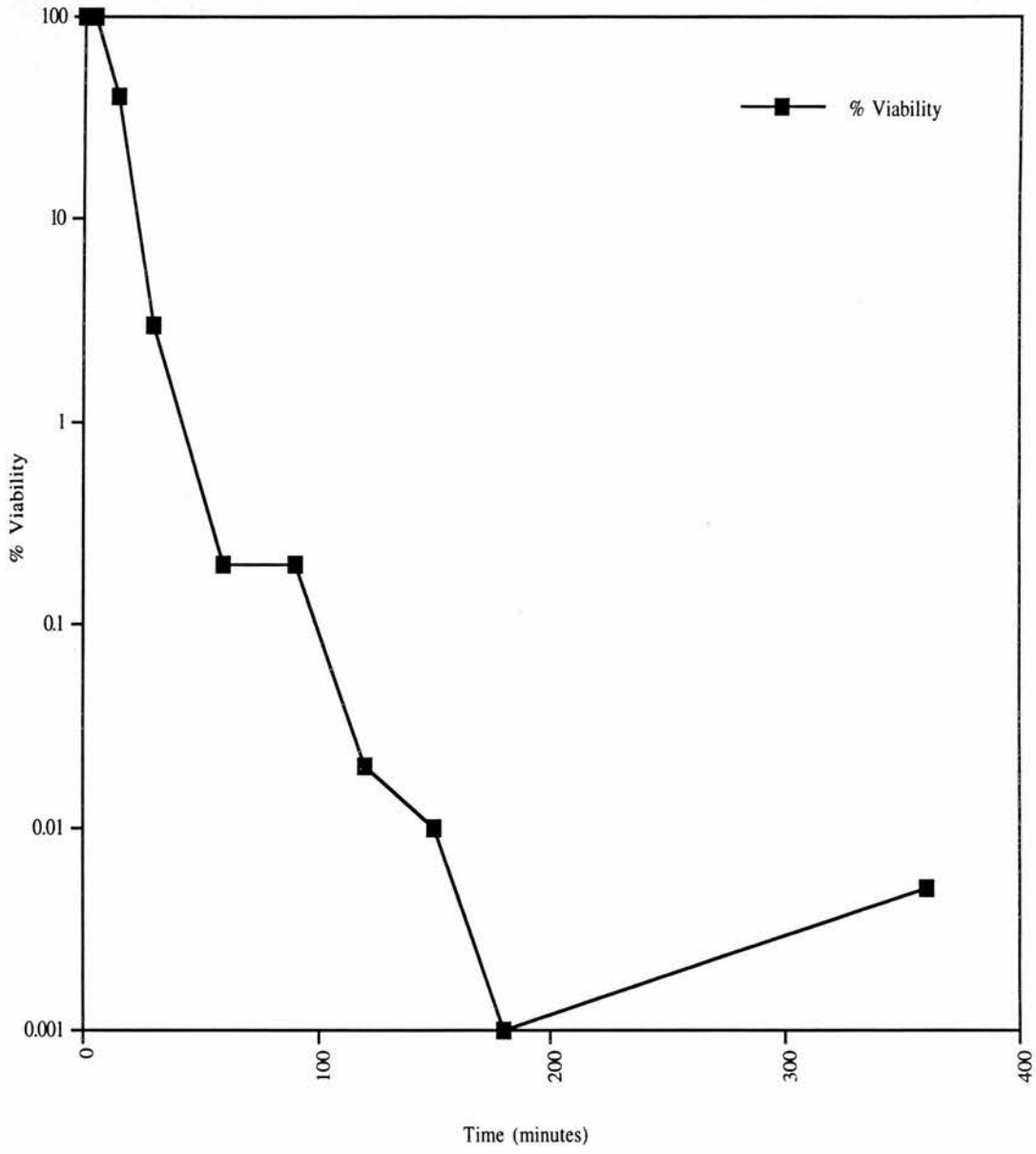
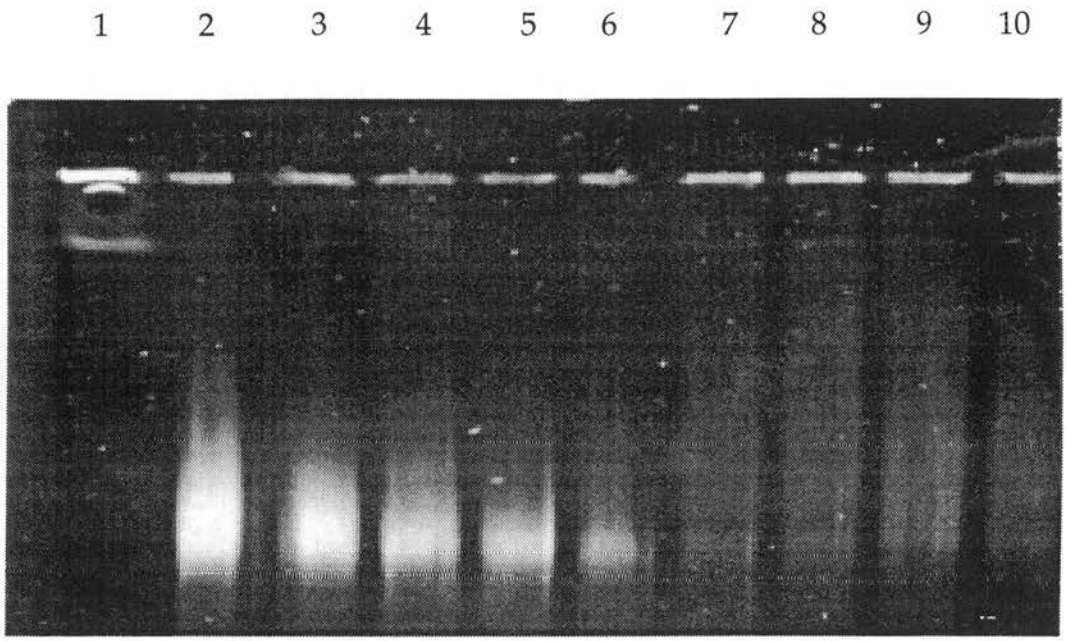


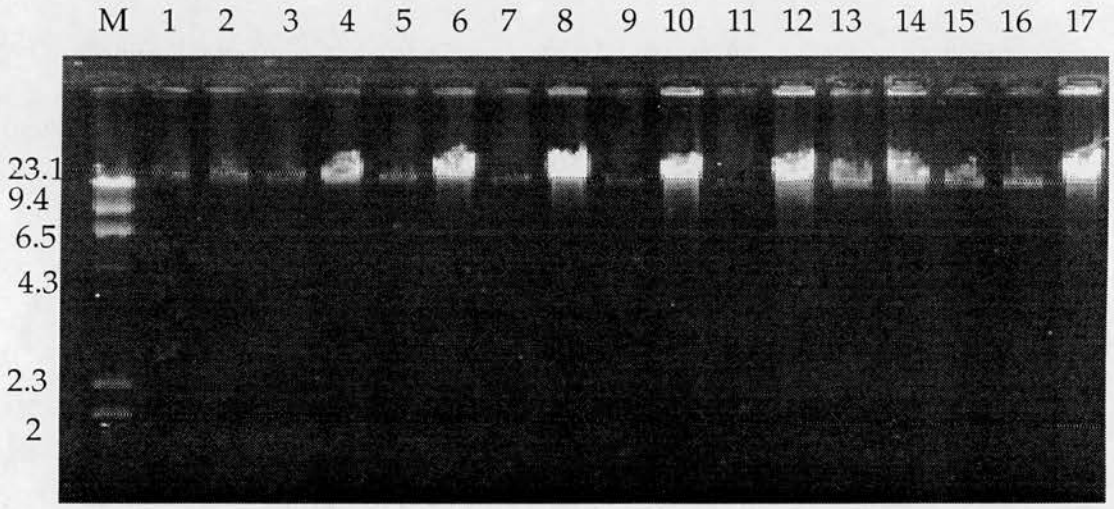
Figure 5.12B



Cells were challenged with 0.15mg/l ciprofloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=150; Lane 8, t=180; Lane 9, t=240; Lane 10, t=300.

The effect of the OBC of ciprofloxacin on the production of smaller fragments of DNA was then studied as before. A log culture of *E.coli* TG1 was grown up, challenged with 0.15mg/l ciprofloxacin and samples withdrawn at various time points. The DNA extracted from these samples was electrophoresed on a conventional agarose gel as shown in Figure 5.13. This was directly compared with a culture challenged with the OBC of nalidixic acid in order to investigate any differences in the sizes of DNA fragments generated by the addition of the two antibiotics.

Figure 5.13: The effect of 0.15mg/l ciprofloxacin or 90mg/l nalidixic acid on the integrity of *E. coli* chromosomal DNA. Displayed below is a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved.



Cells were challenged with 0.15mg/l ciprofloxacin or 90mg/l nalidixic acid and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane M, λ *Hind* III markers; Lane 1, Antibiotic-free culture t=0; Lane 2, ciprofloxacin treated culture t=5; Lane 3, nalidixic acid treated culture t=5; Lane 4, ciprofloxacin treated culture t=15; Lane 5, nalidixic acid treated culture t=15; Lane 6, ciprofloxacin treated culture t=30; Lane 7, nalidixic acid treated culture t=30; Lane 8, ciprofloxacin treated culture t=60; Lane 9, nalidixic acid treated culture t=60; Lane 10, ciprofloxacin treated culture t=120; Lane 11, nalidixic acid treated culture t=120; Lane 12, ciprofloxacin treated culture t=180; Lane 13, nalidixic acid treated culture t=180; Lane 14, ciprofloxacin treated culture t=270; Lane 15, nalidixic acid treated culture t=270; Lane 16, nalidixic acid treated culture t=360; Lane 17, ciprofloxacin treated culture t=360.

On the addition of the OBC of ciprofloxacin (0.15mg/l), it appeared that rapid DNA degradation occurred, even after 5 minutes (Figure 5.12B, Lane 2). With increasing time, a definite trend was seen to emerge which showed that increasing DNA double stranded breaks were evident and the intact chromosomal band always seen at the top of the gel had disappeared. This suggested that the DNA inside the cells was most damaged after 60 minutes (Figure 5.12B, Lane 5). After this point, the DNA appeared to become less damaged and the band at the top of the gel corresponding to intact chromosomal DNA seemed to reappear. Again, the reason for this is unclear, either the DNA becomes more repaired because the antibiotic becomes less active, the DNA is repaired by the action of DNA repair enzymes or the cells badly affected by the antibiotic die and burst and are therefore removed from the population, leaving the cells that have survived.

The results of analysing the lower molecular weight fragments by conventional gel electrophoresis (Figure 5.13) confirms these ideas. DNA degradation was seen to occur as soon as 15 minutes after the addition of ciprofloxacin and increased over time (Figure 5.13, Lane 4). Extensive DNA damage releasing DNA fragments as small as 4kb was observed on this gel. The results of the viable count showed that there was rapid death as a result of ciprofloxacin addition and even after 15 minutes, only 50% viability was seen. After 30 minutes just over 90% of the bacterial population had died and after one hour, more than 99.9% were non-viable. Further decreases in the viability of the culture was observed; however after 180 minutes, it appeared that a very small percentage of the culture was viable and could divide. This agreed with the idea of an active repair process suggested by the results of the pulsed field gel (Figure 5.12B).

Figure 5.14: The effect of 0.5mg/l ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.14A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.14B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.14C)

Figure 5.14A

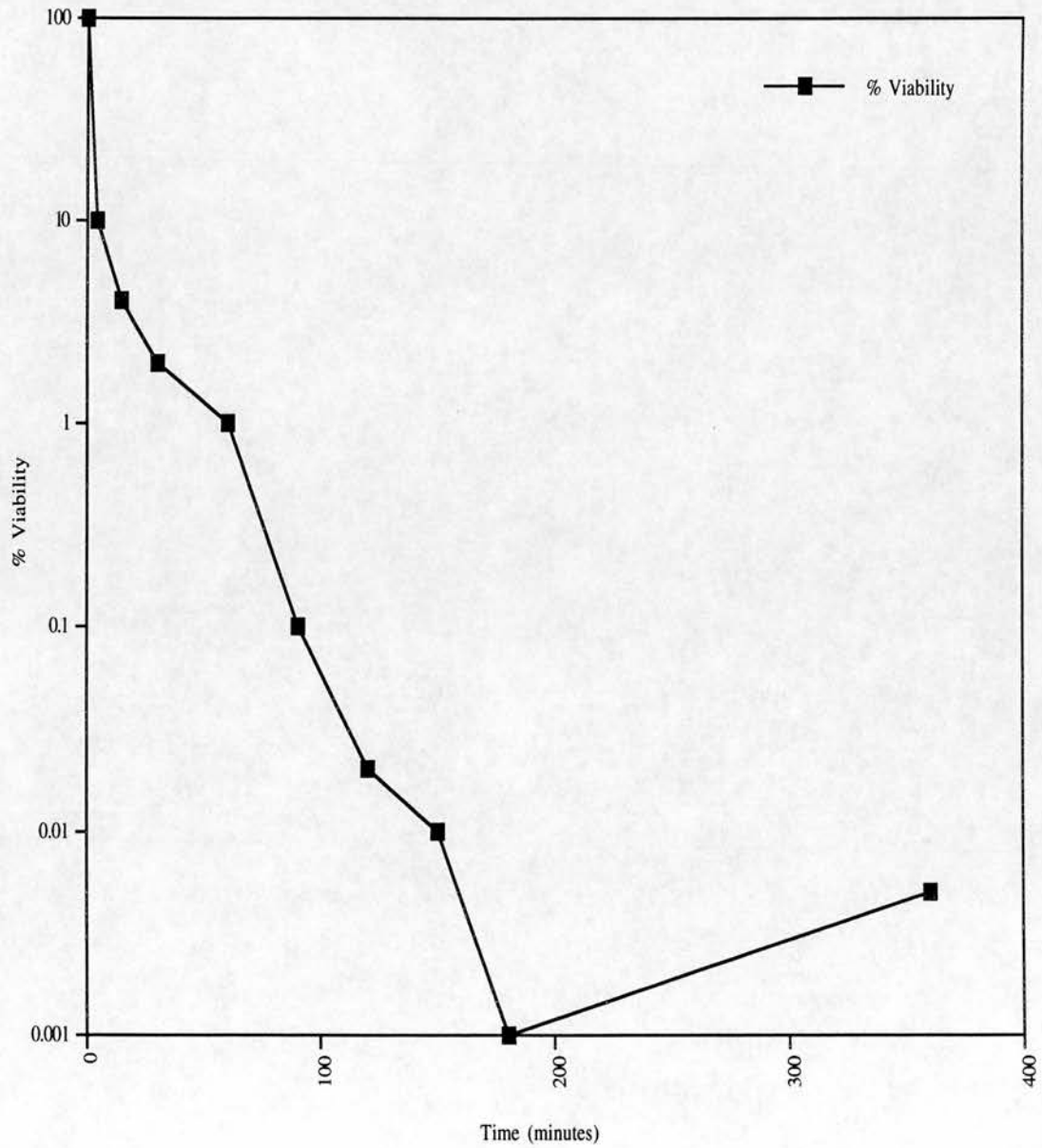


Figure 5.14B

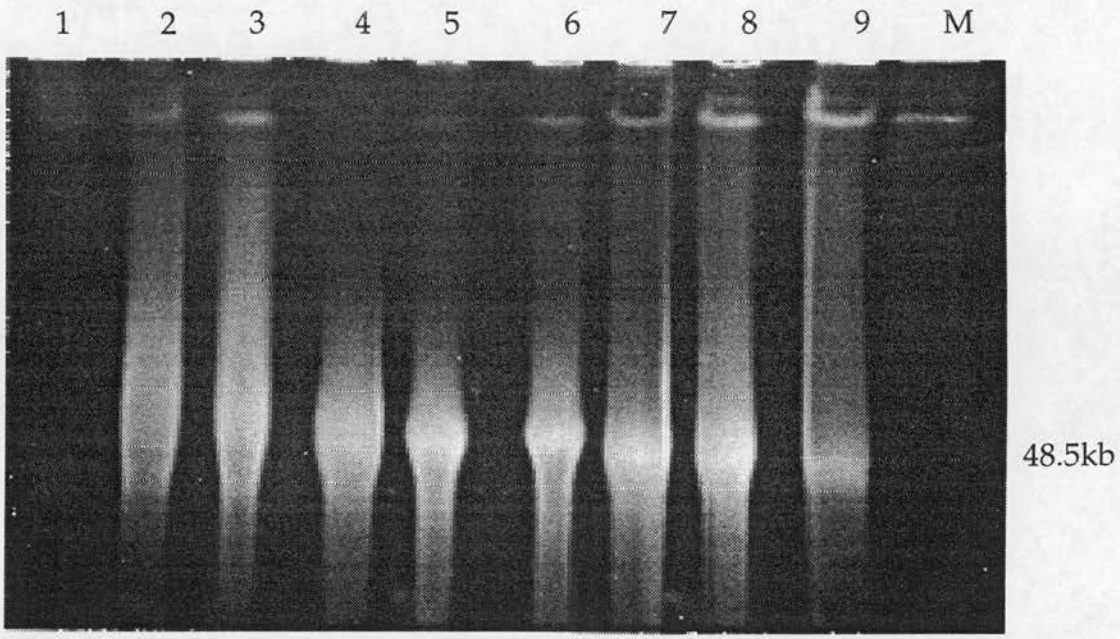
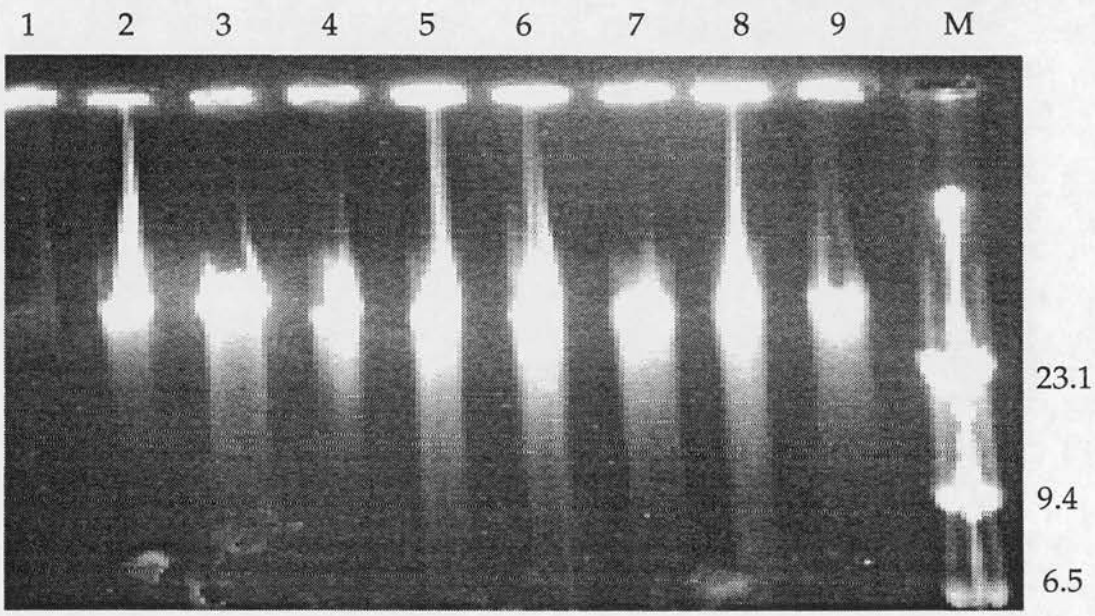


Figure 5.14C



Cells were challenged with 0.5mg/l ciprofloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer/ λ *Hind*III markers.

As shown in Figure 5.14B, the addition of 0.5mg/l ciprofloxacin results in a response following the same pattern as that seen in Figure 5.12. Briefly, the formation of double stranded DNA breaks was evident after 5 minutes as before and after 30 minutes more smaller DNA fragments were observed (Figure 5.14, Lane 4). At this stage, a definite reduction in the proportion of larger DNA fragments was seen although a small trace of the intact chromosomal DNA was evident. With increasing time, more larger DNA fragments were observed, again suggesting the presence of a DNA repair mechanism. After 360 minutes (Figure 5.14, Lane 9), chromosomal DNA appeared to contain approximately the same extent of double stranded DNA breaks as at after 15 minutes (Figure 5.14, Lane 3). The presence of some sort of DNA repair mechanism is reaffirmed by the results displayed in Figure 5.14C. The amount of DNA in the lower molecular weight range (compared to λ*Hind*III markers, Figure 5.14C, Lane M), can be seen to increase to an optimum occurring around 60 minutes (Figure 5.14C, Lane 5) after which the average molecular weight of DNA fragments seems to increase (Figure 5.14, Lanes 6 to 9). This correlates with the results of the pulsed field gel, already discussed above.

The viable count carried out on the culture demonstrated that there was again a rapid decrease in viability even after 5 minutes and more so after 15 minutes and 30 minutes (Figure 5.14A). The optimum amount of cell death was observed to occur at 180 minutes, after which the viable count started to increase up until 360 minutes. This data correlates to the pulsed field and conventional gels, reaffirming the view that there is an active DNA repair mechanism.

Figure 5.15: The effect of 1mg/l ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.15A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.15B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.15C)

Figure 5.15A

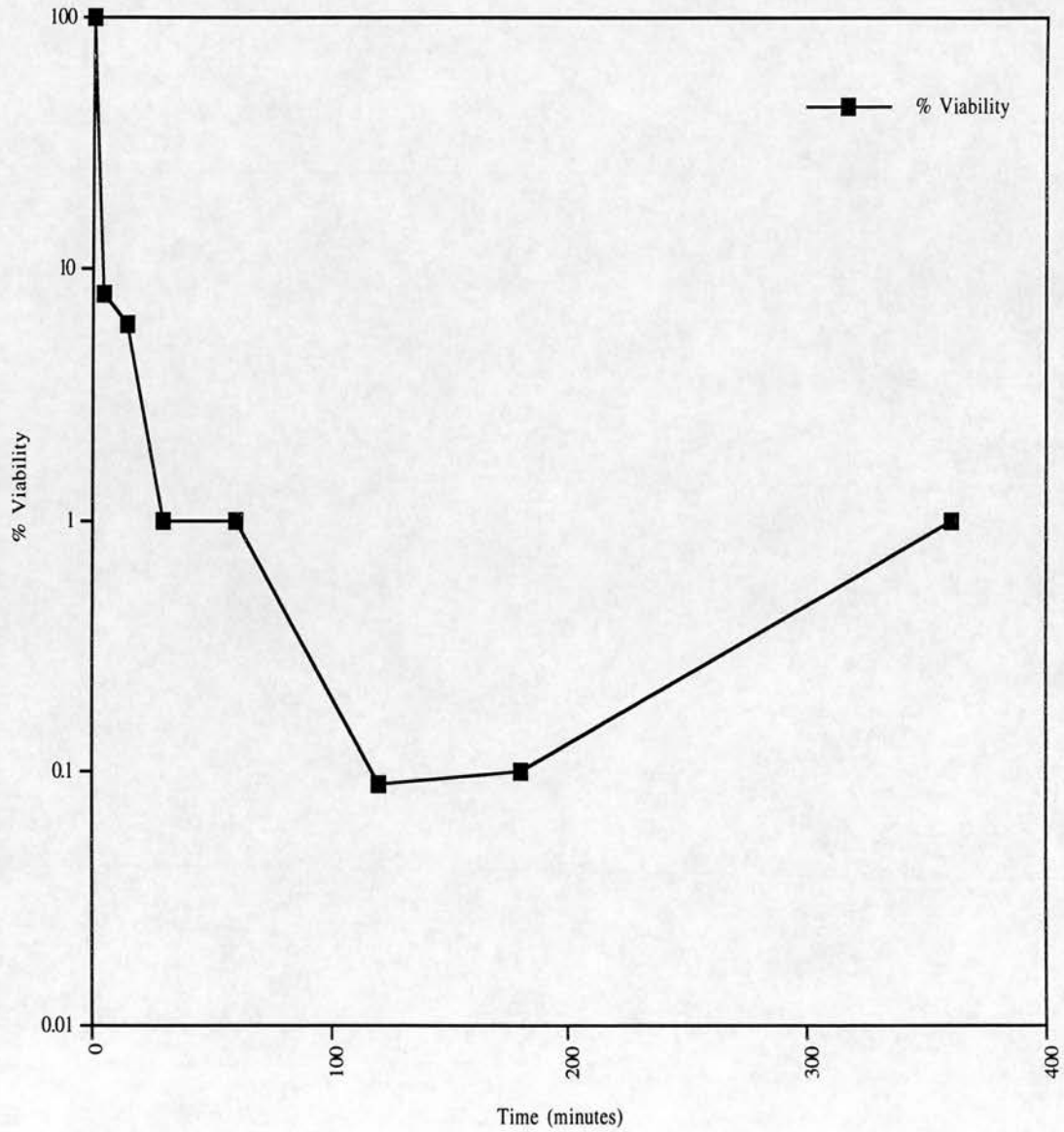


Figure 5.15B

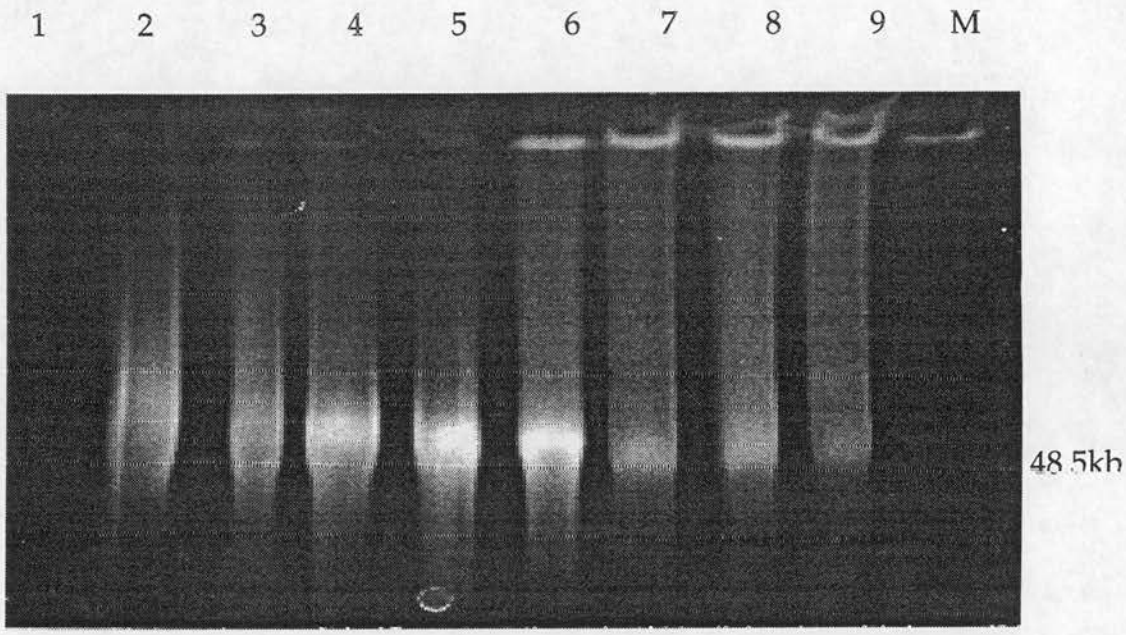
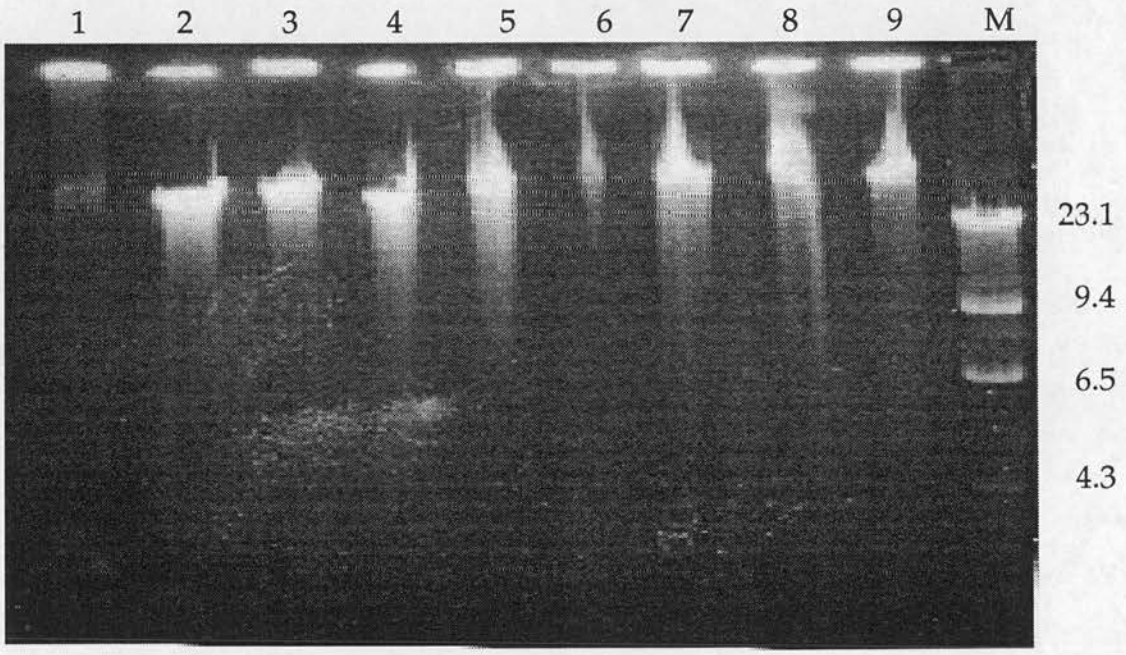


Figure 5.15C



Cells were challenged with 1mg/l ciprofloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ *Hind*III markers.

Strengthening the conclusions drawn before, the results from a similar experiment but this time with 1mg/l ciprofloxacin (Figure 5.15) again suggested the presence of a DNA repair mechanism. As before, the DNA was shown to incur double stranded breaks after 5 minutes (Figure 5.15, Lane 2) and the most damage was observed at 60 minutes (Figure 5.15B, Lane 5). After this point, the DNA appears more intact and a definite decrease in the amount of lower molecular weight fragments was evident (Figure 5.15B, Lanes 6 to 9). These results were again confirmed by the pattern of DNA damage observed by conventional electrophoresis where an increase in the average molecular weight of the DNA fragments was seen to occur with time (Figure 5.15C, Lanes 7 to 9) after 60 minutes (Figure 5.15C, Lane 5) and the results of the viable count (Figure 5.15A) which showed that the bacterial culture increased in size by a factor of one hundred after 180 minutes.

Figure 5.16: The effect of 5mg/l ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.16A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.16B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.16C)

Figure 5.16A

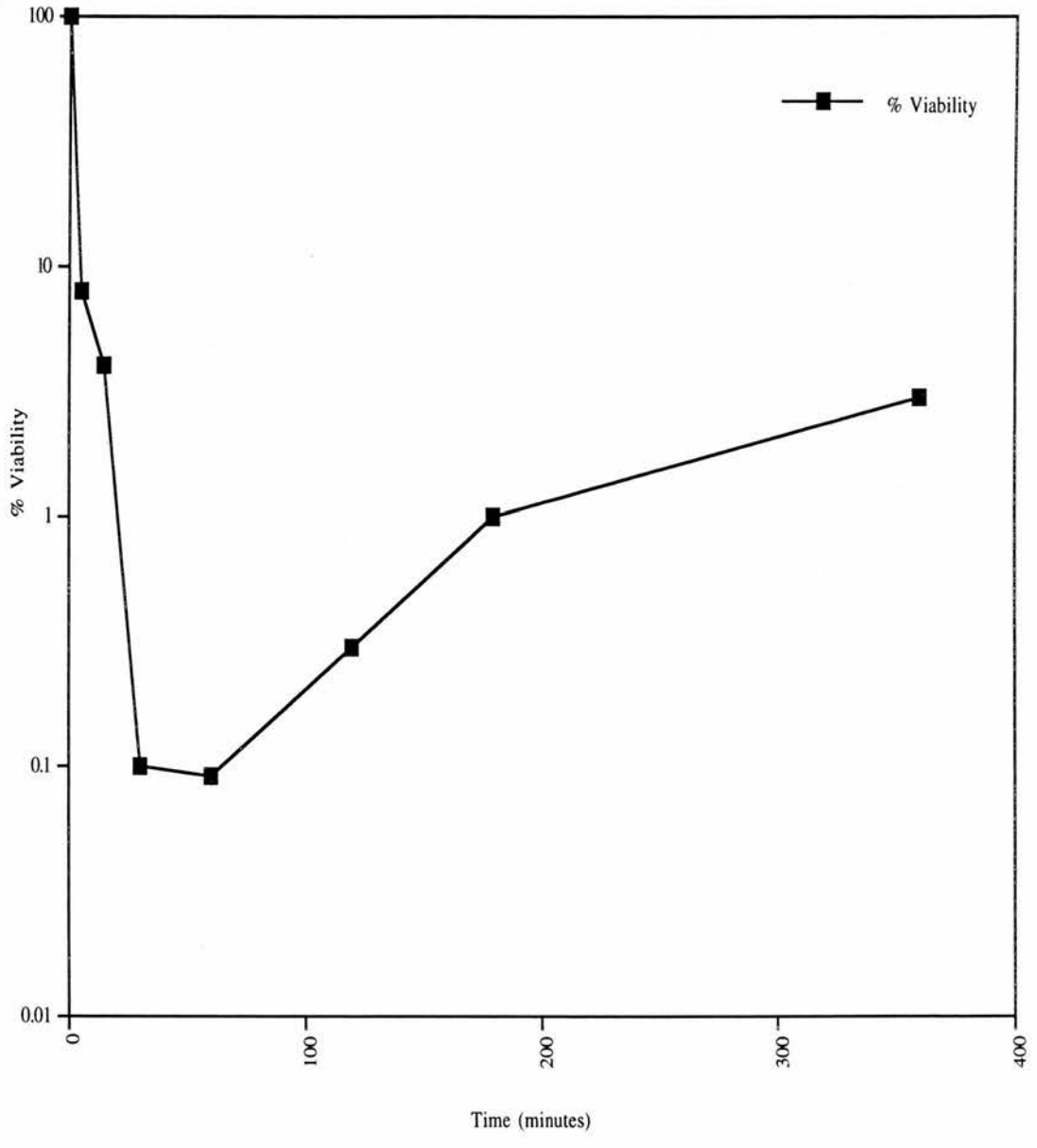


Figure 5.16B

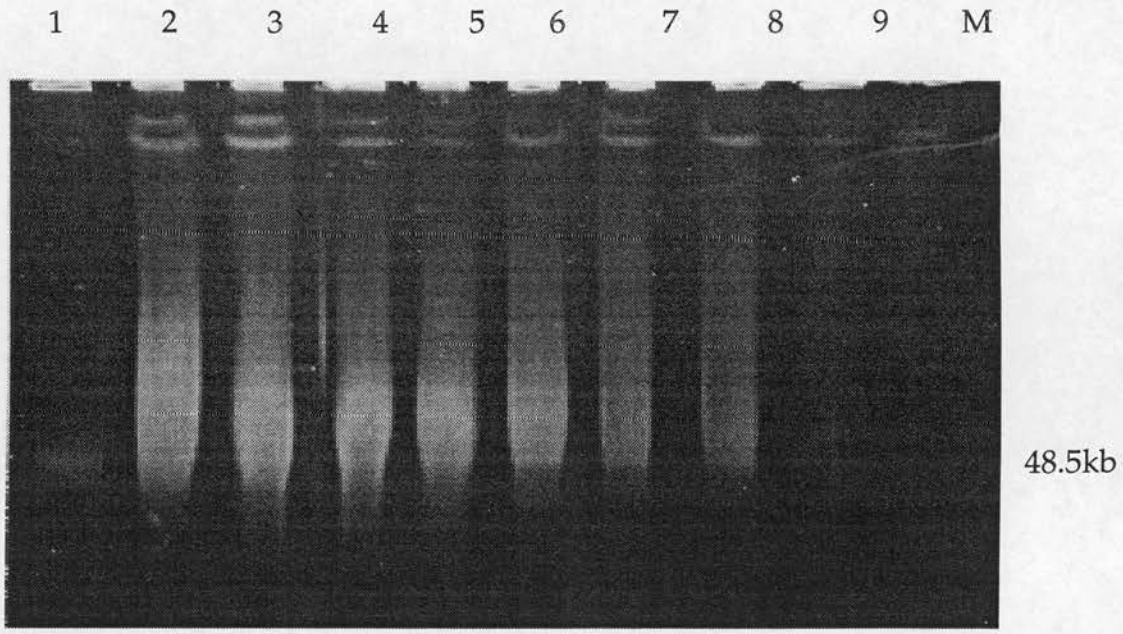
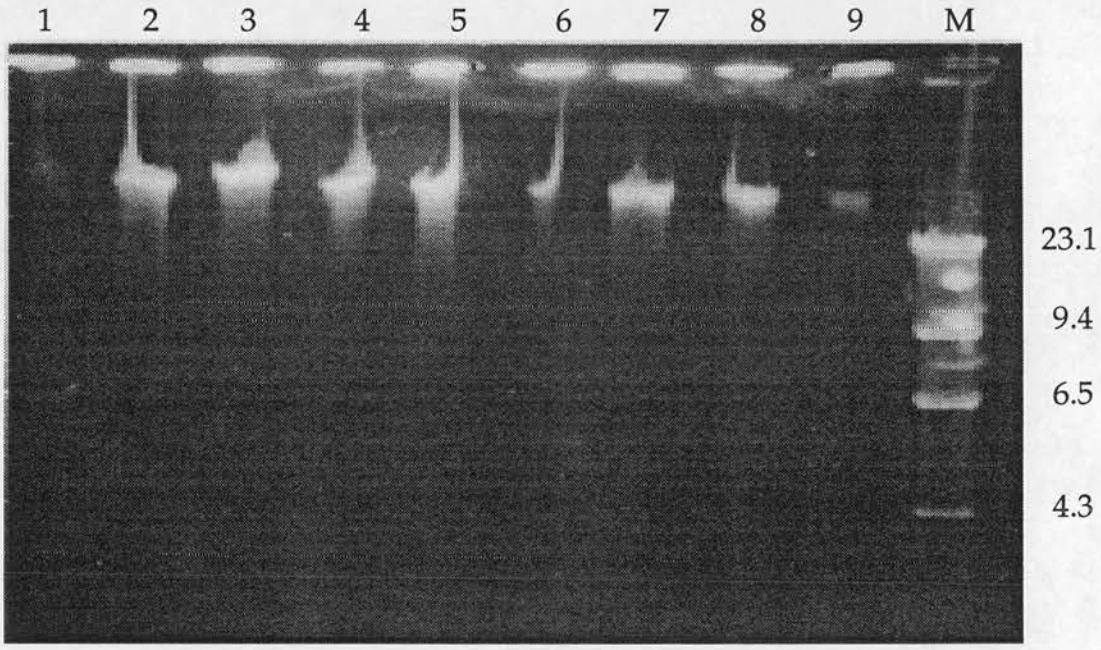


Figure 5.16C



Cells were challenged with 5mg/l ciprofloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer/ λ *Hind*III markers.

The result of challenging the cells with 5mg/l ciprofloxacin followed the same pattern as before, the only difference being that at time points that had before correlated with extensive DNA damage and lack of high molecular weight fragments, that is at 60 minutes (Lane 5, Figures 5.12B and 5.14B) did not appear to give rise to so much DNA damage at this ciprofloxacin concentration. Evidence of at least some larger chromosomal DNA fragments was always present and the average size of fragments seemed to increase gradually after 60 minutes incubation (Figure 5.16B, Lanes 5 to 9). This observation is confirmed by the results of the conventional gel electrophoresis of the smaller DNA fragments where double stranded breaks were seen to become slightly more prominent from 5 minutes incubation to 60 minutes incubation (Figure 5.16C, Lanes 2 to 5) but then became less obvious after 60 minutes (Figure 5.16C, Lanes 6 to 9). However even at the time points at which DNA double stranded breaks were seen, the fragments produced were not as small as seen previously at lower ciprofloxacin concentrations. The results of the viable counts followed the same pattern as before, however, a significant increase in the number of viable colonies from 60 minutes to 360 minutes was observed (Figure 5.16A).

At this higher concentration of ciprofloxacin, it seemed that although double stranded DNA breaks were present as before, the DNA seemed not to be degraded into smaller molecular weight fragments. It even appeared that the proposed DNA repair mechanism was more efficient at higher ciprofloxacin concentrations. Almost identical patterns were seen to occur at even higher concentrations of ciprofloxacin tested as shown in the following three figures: Figure 5.17 corresponding to 10mg/l ciprofloxacin, Figure 5.18 corresponding to 50mg/l ciprofloxacin and Figure 5.19 corresponding to 100mg/l.

Figure 5.17: The effect of 10mg/l ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.17A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.17B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.17C)

Figure 5.17A

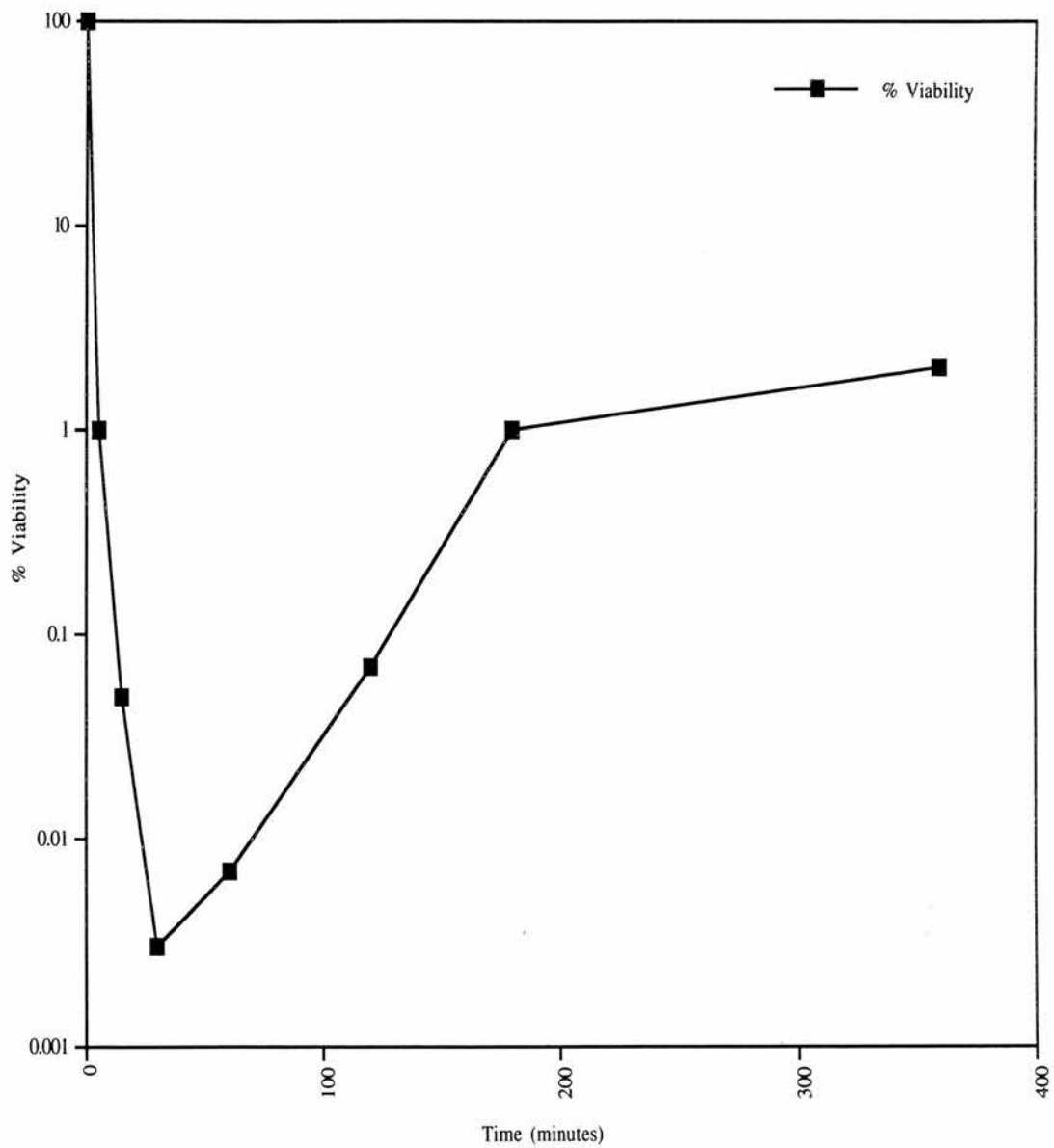


Figure 5.17B

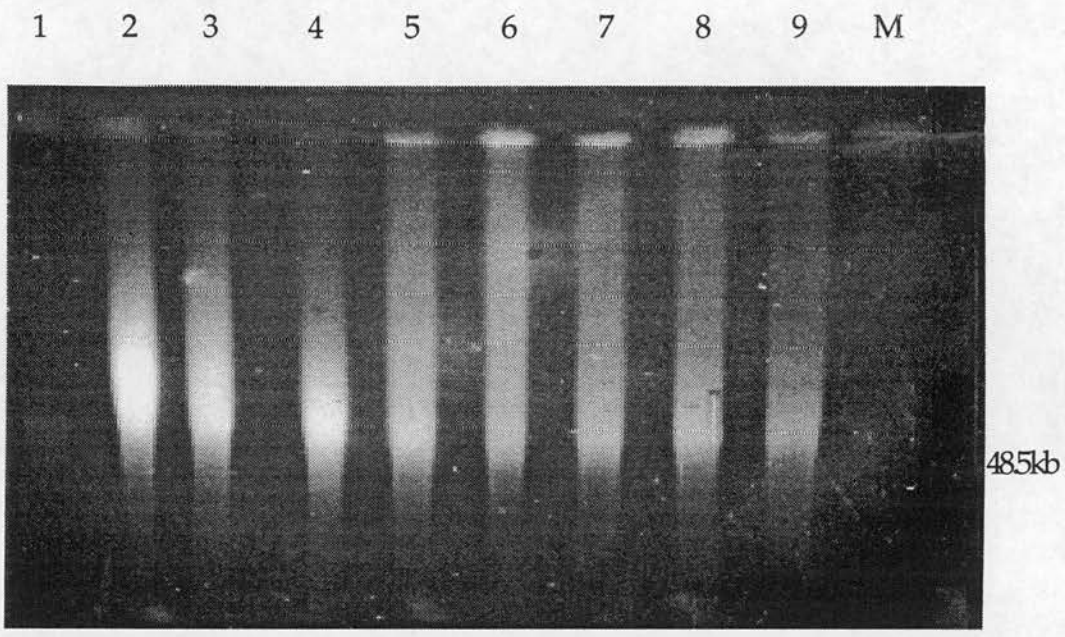
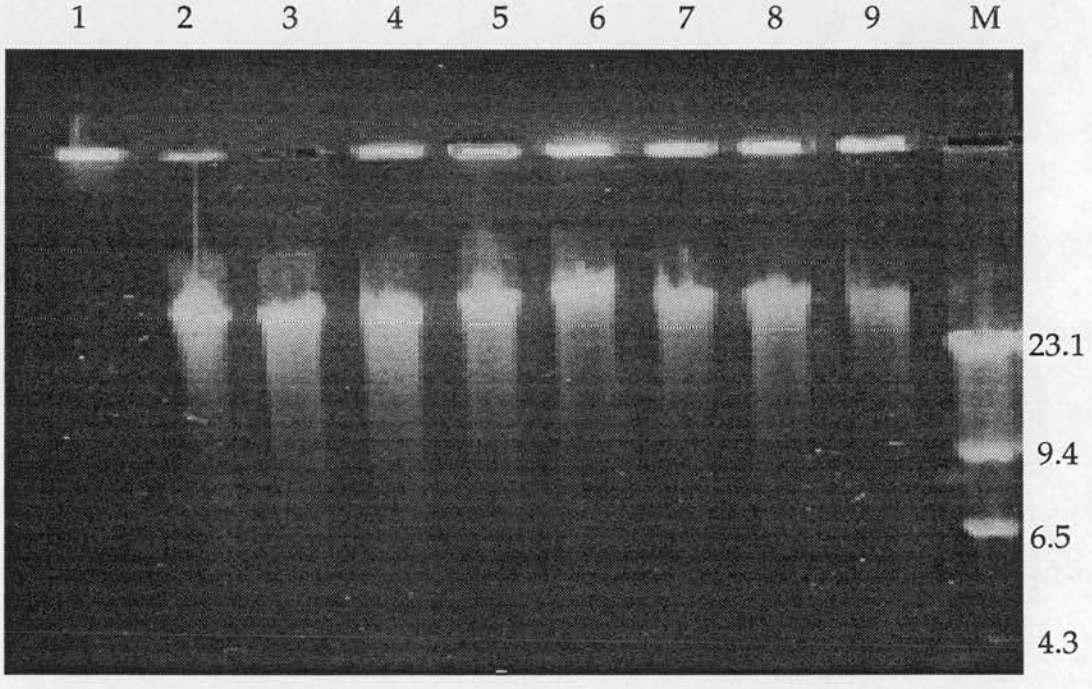


Figure 5.17C



Cells were challenged with 10mg/l ciprofloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ *Hind*III markers.

Figure 5.18: The effect of 50mg/l ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.18A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.18B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.18C)

Figure 5.18A

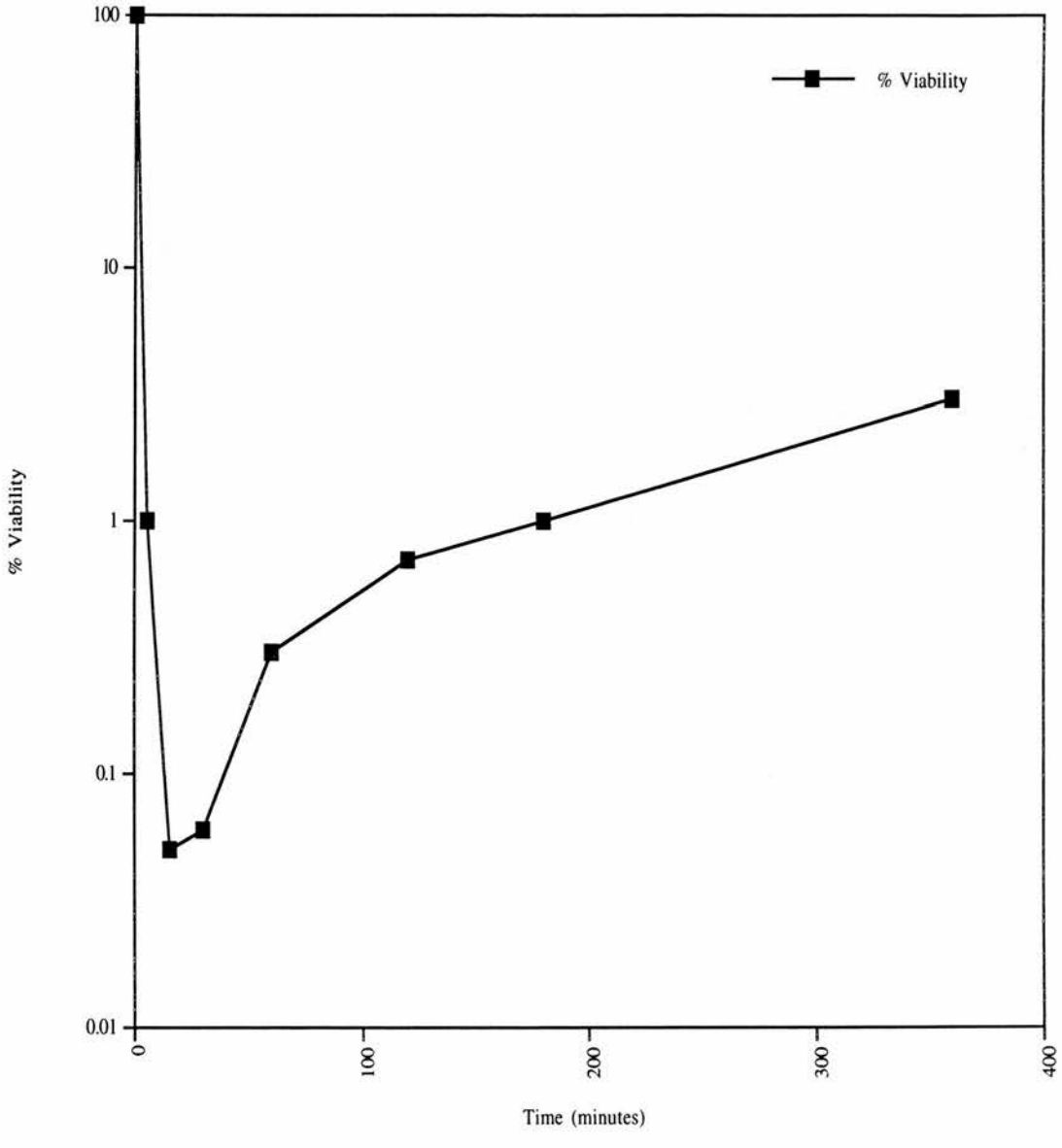


Figure 5.18B

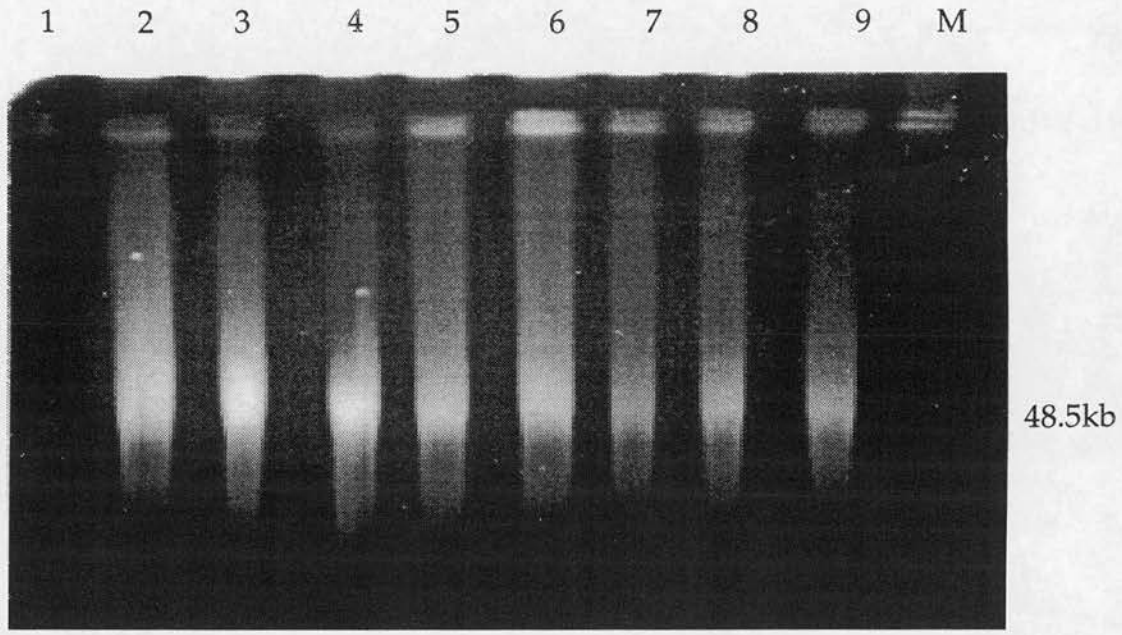
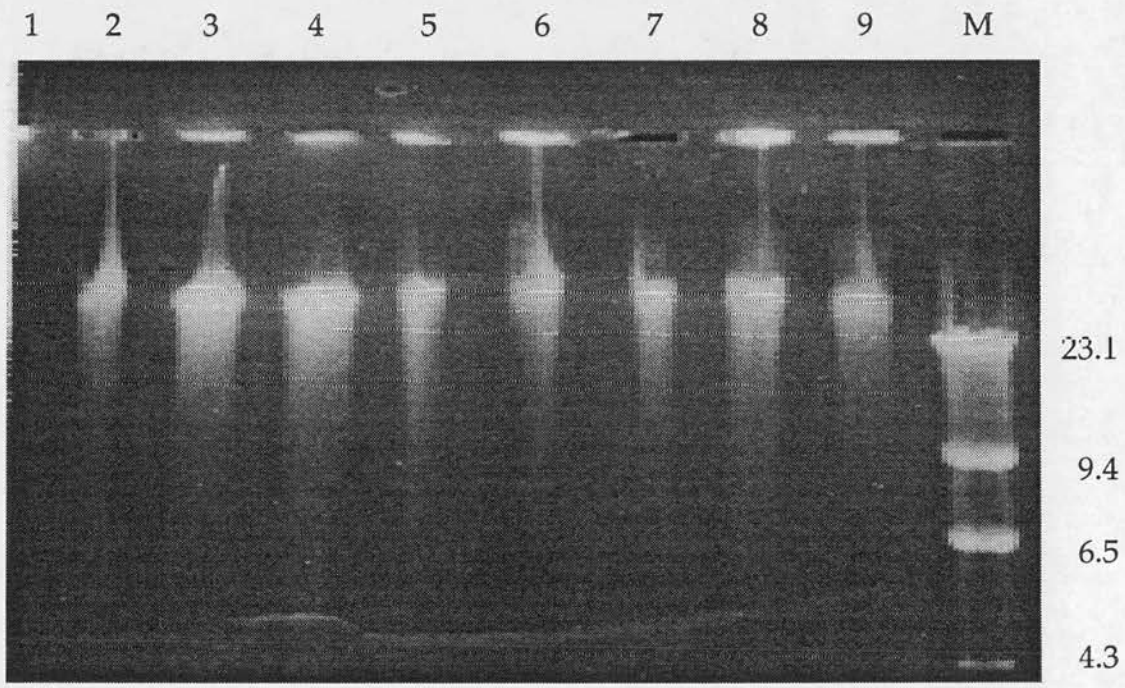


Figure 5.18C



Cells were challenged with 50mg/l ciprofloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer/ λ *Hind*III markers.

Figure 5.19: The effect of 100mg/l ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.19A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.19B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.19C)

Figure 5.19A

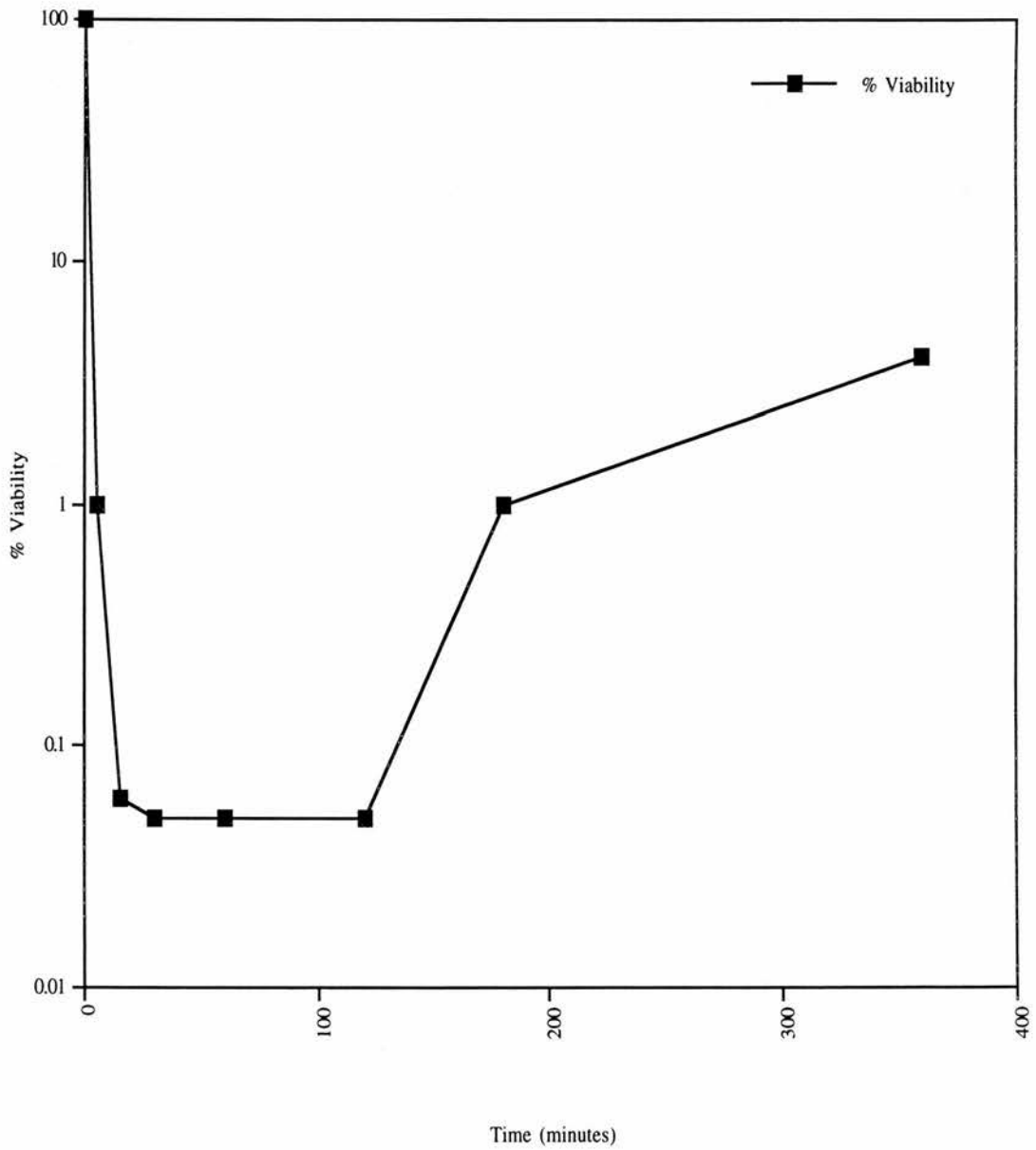


Figure 5.19B

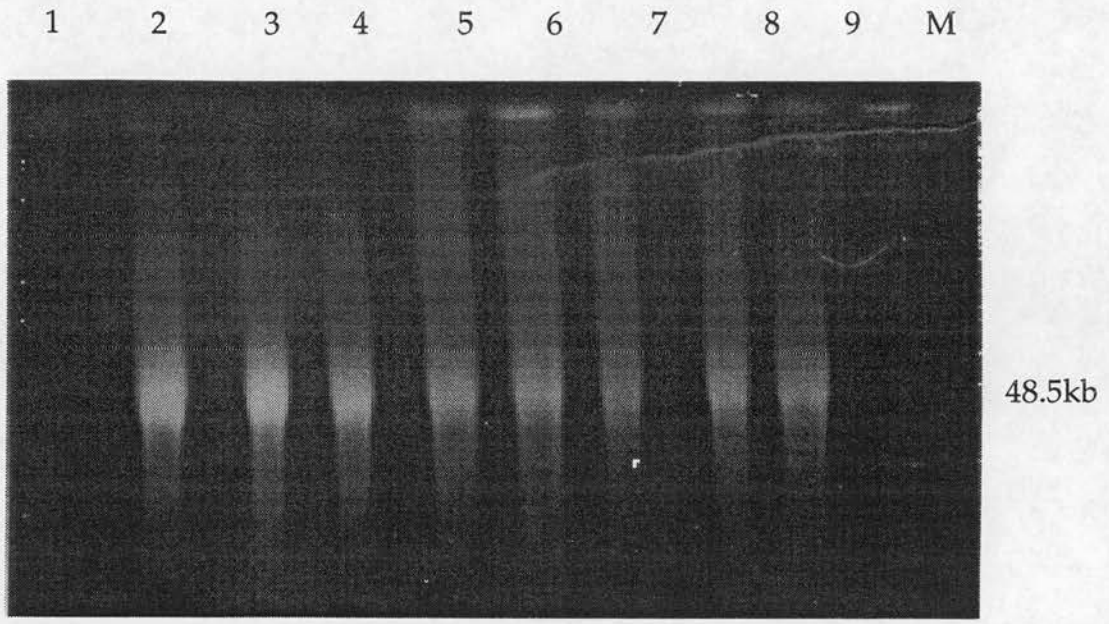
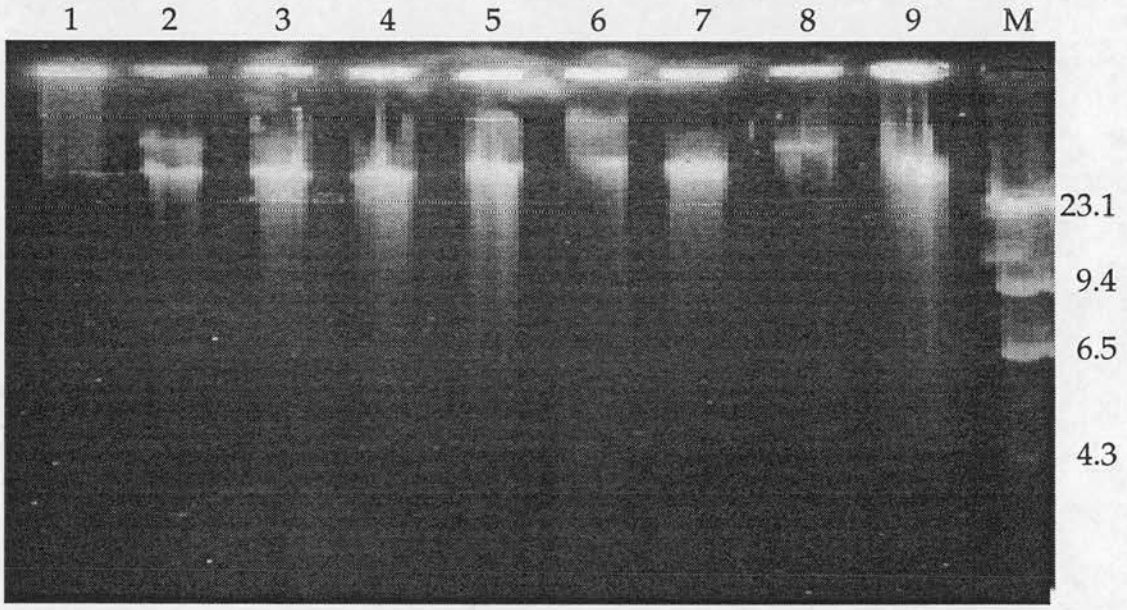


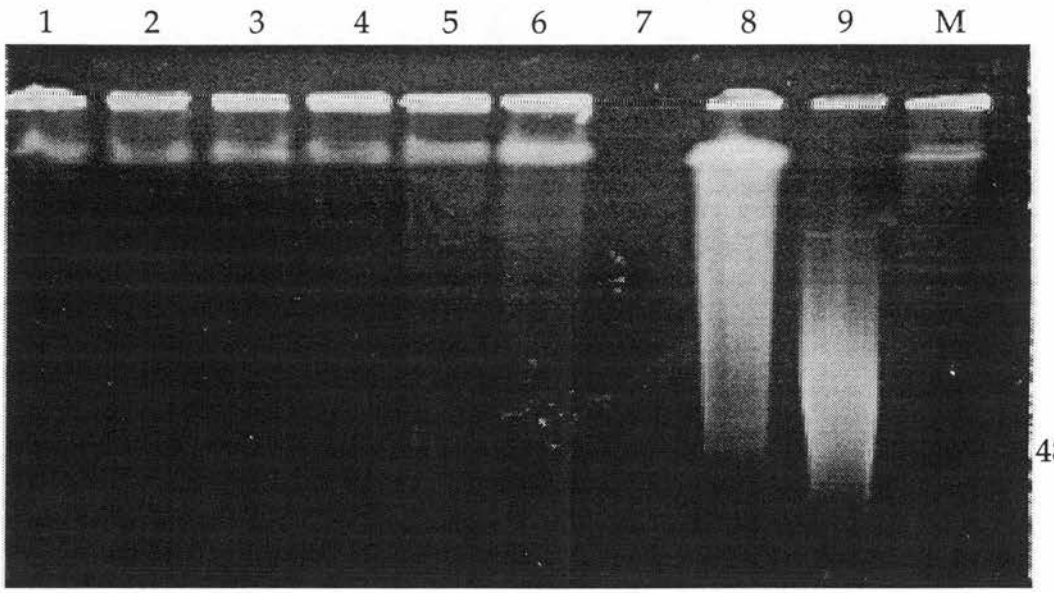
Figure 5.19C



Cells were challenged with 100mg/l ciprofloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ *Hind*III markers.

The effect of ciprofloxacin on a different strain of *E.coli* other than *E.coli* TG1 was then studied. *E.coli* strain CIP223 (described in 3.7) has a MIC of ciprofloxacin of ciprofloxacin of 2mg/l and thus requires higher levels of ciprofloxacin to cause bacterial cell death. A culture of *E.coli* CIP223 was challenged with various concentrations of ciprofloxacin, allowed to grow for one hour and its DNA analysed by pulsed field electrophoresis as before. The results are displayed in Figure 5.20.

Figure 5.20: The effect of various concentrations of ciprofloxacin on the integrity of *E. coli* CIP223 chromosomal DNA. Displayed below is a photograph of the pulsed field gel on which the large fragments of DNA were resolved.



Cells were challenged with various concentrations of ciprofloxacin and an aliquot of cells removed after one hour to assess DNA damage: Lane 1, no ciprofloxacin; Lane 2, 0.00025mg/l ciprofloxacin; Lane 3, 0.001mg/l ciprofloxacin; Lane 4, 0.004mg/l ciprofloxacin; Lane 5, 0.016mg/l ciprofloxacin; Lane 6, 0.064mg/l ciprofloxacin; Lane 7, blank well; Lane 8, 0.5mg/l ciprofloxacin; Lane 9, 5mg/l ciprofloxacin ; Lane M, λ concatemer markers.

The results establish that *E.coli* CIP223 shows the same production of double stranded breaks and DNA degradation as *E.coli* TG1. The difference is that only minimal double stranded breaks are detected at concentrations of ciprofloxacin up until 0.064mg/l (Figure 5.20, Lane 6), after which DNA degradation is apparent after the addition of 5mg/l ciprofloxacin (Figure 5.20, Lane 9). Therefore the addition of ciprofloxacin has the same effect on the chromosomal DNA in *E.coli* CIP223 as *E.coli* TG1, however the pattern of DNA degradation and double strand break formation in E.coli CIP223 occurs at a higher ciprofloxacin concentration.

Since challenge of *E.coli* TG1 with ciprofloxacin seemed to culminate in a different response to nalidixic acid with regards to degradation of DNA at the OBC, further experiments were carried out to investigate this. As mentioned before, microbiological experiments carried out previously demonstrated that the fluoroquinolones ciprofloxacin and ofloxacin possess an additional mechanism of bactericidal action (Mechanism B) compared to the basic quinolone response, shown to occur with nalidixic acid (Mechanism A). The results achieved from the experiments described in this chapter suggested that the additional bactericidal mechanism of action of ciprofloxacin but not of nalidixic acid, was the degradation of DNA into small fragments at the OBC (compare Figure 5.6, Lane 6 to Figure 5.7, Lane 6). After incubating the cells for 360 minutes at the OBC of ciprofloxacin, DNA fragments as small as 4kb are released but at the optimum bactericidal concentration of nalidixic acid, these small fragments are not visible (Figure 5.13).

This destruction of the bacterial chromosome into small fragments is reminiscent of apoptosis in eukaryotic cells. Apoptosis is the name given to the changes through which a cell passes when, for example, it is damaged to such an extent that it is past the point of being repairable. Apoptosis, or physiological cell death occurs when a cell within an organism dies by a mechanism orchestrated by proteins encoded by the host's genome. In this way unwanted cells are killed which is important, for example, in ageing, in development and in defence. It is very interesting that topoisomerase II-inhibiting drugs such as VM26 and VP16 which have an identical mode of action to the quinolone drugs actually elicit apoptosis in thymocyte and lymphocyte cells (Walker *et al*, 1991). The mechanism by which these drugs function, that is formation of the "cleavable complex" and formation of double stranded DNA breaks, results in DNA which is considered by the cell as being too damaged to repair. In eukaryotic cells, the

nucleus shrinks, the nuclear membrane breaks down, the chromatin collapses and nucleases pass from the cytoplasm to the nucleus and destroy the DNA. It has been shown that the interaction with the eukaryotic antineoplastic drugs VM26 and VP16 has two components, firstly the interaction of the drug with topoisomerase II and secondly the activation of endonucleolytic activities associated with apoptosis. Enzymes which have been discussed previously as having a role in this process are endo-exonucleases. For example in human leukemic CEM cells an endo-exonuclease has been connected with the chromatin DNA fragmentation that accompanies apoptosis (Fraser, 1994). Parallels have been drawn between these eukaryotic enzymes which processively degrade regions of DNA in cells that have become too damaged to be repaired and the RecBCD enzyme of *E.coli* (Fraser, 1994). As discussed in 1.6.6, RecBCD has both endonuclease and exonuclease activities and is normally involved in repair of double stranded DNA breaks. However, if the bacterial cell becomes damaged beyond repair, the RecBCD enzyme becomes reckless and degrades the chromosomal DNA to acid-soluble nucleotides (Smith, 1988).

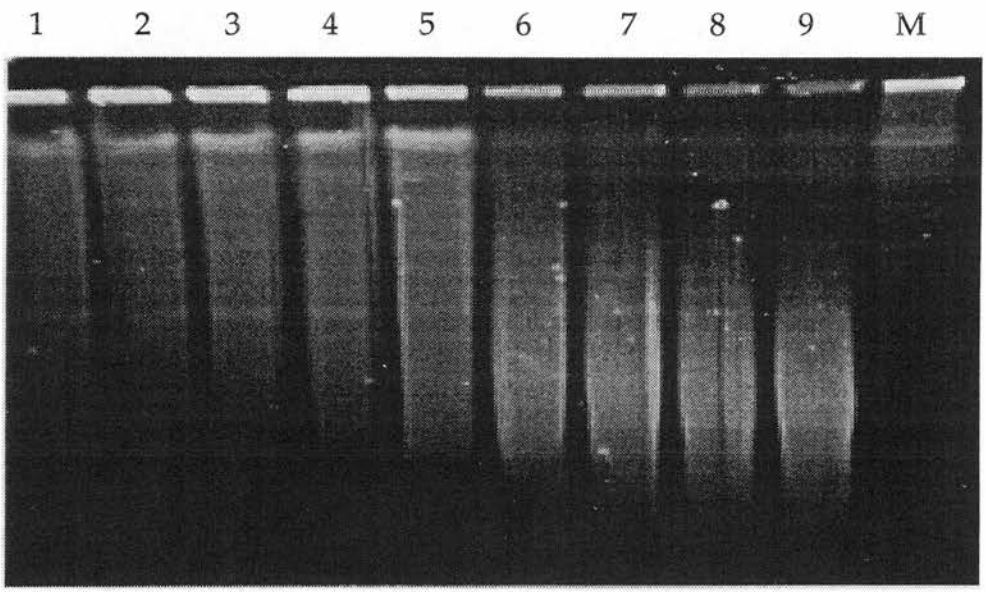
Unlike many enzymes that have been previously discussed to be involved in the response of the cell to quinolones, RecBCD is not part of the SOS regulation, nor is it inducible. Therefore, in order to show that it was indeed RecBCD that was involved in this "apoptosis"-like DNA degradation effect, the presence of DNA degradation in the presence of a protein synthesis inhibitor would have to be shown.

5.3 Is the "apoptosis"-like DNA degradation dependent on protein synthesis?

In the light of the ideas described above, experiments were then carried out to investigate whether the "apoptosis"-like response observed to occur on challenge with ciprofloxacin occurred in the absence of ongoing protein synthesis. If the "apoptosis" -like response was still seen to occur, the production of a DNA damage-inducible nuclease could be ruled out and the DNA damage could be attributed to a constitutive nuclease such as RecBCD.

A logarithmic culture of *E.coli* TG1 was challenged as before with various concentrations of ciprofloxacin, however, one hour prior to the addition of ciprofloxacin, 170mg/l chloramphenicol was added to inhibit all ongoing protein synthesis. Samples of the cultures after one hour were embedded in agarose as before and electrophoresed on pulsed field gels.

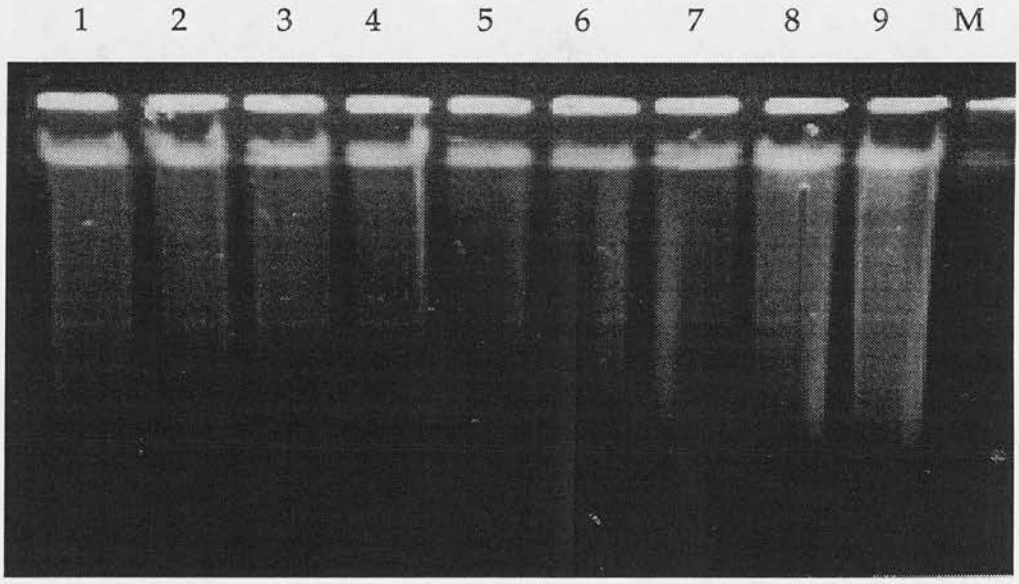
Figure 5.21A: The effect of the inhibition of protein synthesis prior to the addition of various concentrations of ciprofloxacin on the integrity of *E.coli* chromosomal DNA. Displayed below is a photograph of the pulsed field gel on which the large fragments of DNA were resolved.



Protein synthesis was inhibited by the addition of 170mg/l chloramphenicol one hour prior to the addition of various concentrations of ciprofloxacin and an aliquot of cells removed after one hour to assess DNA damage: Lane 1, no ciprofloxacin; Lane 2, 0.00025mg/l ciprofloxacin; Lane 3, 0.001mg/l ciprofloxacin; Lane 4, 0.004mg/l ciprofloxacin; Lane 5, 0.016mg/l ciprofloxacin; Lane 6, 0.064mg/l ciprofloxacin; Lane 7, 0.15mg/l ciprofloxacin ; Lane 8, 0.5mg/l ciprofloxacin; Lane 9, 5mg/l ciprofloxacin ; Lane M, λ concatemer markers.

It was found that at the lowest concentration of ciprofloxacin (Figure 5.21A, Lane 2), or even in the absence of ciprofloxacin (Figure 5.21A, Lane 1), DNA double stranded breaks were seen to occur routinely. This is possibly as a result of protein synthesis inhibition affecting the cellular manufacture of DNA repair enymes which would normally repair any DNA nicks. Although it has been hypothesised that RecBCD, the predominant DNA repair enzyme is constitutively expressed and is responsible for the DNA degradation, it is possible that on addition of chloramphenicol to the cell, the inhibition of other accessory repair enzymes results in more persistent DNA double stranded breaks (Figure 5.21A, Lanes 1 to 5). As shown in Figure 5.21A, Lane 6, on addition of 0.064mg/l ciprofloxacin, DNA degradation starts to proceed, and is increasingly predominant with higher ciprofloxacin concentrations (Figure 5.21A, Lane 9). At the OBC and high ciprofloxacin concentrations, smaller DNA fragments were detected, as had been found to occur also in the presence of ongoing protein synthesis. Therefore it was concluded that the "apoptosis"-like DNA degradation found to occur with ciprofloxacin also proceeded in the absence of protein synthesis. The results of inhibiting protein synthesis on nalidixic acid-induced cell death are shown in Figure 5.21B.

Figure 5.21B: The effect of the inhibition of protein synthesis prior to the addition of various concentrations of nalidixic acid on the integrity of *E. coli* chromosomal DNA. Displayed below is a photograph of the pulsed field gel on which the large fragments of DNA were resolved.



Protein synthesis was inhibited by the addition of 170mg/l chloramphenicol one hour prior to the addition of various concentrations of nalidixic acid and an aliquot of cells removed after one hour to assess DNA damage: Lane 1, no nalidixic acid; Lane 2, 1mg/l nalidixic acid; Lane 3, 5mg/l nalidixic acid; Lane 4, 10mg/l nalidixic acid ; Lane 5, 50mg/l nalidixic acid; Lane 6, 90mg/l nalidixic acid; Lane 7, 200mg/l nalidixic acid; Lane 8, 500mg/l nalidixic acid ; Lane 9, 1000mg/l nalidixic acid ; Lane M, λ concatemer markers.

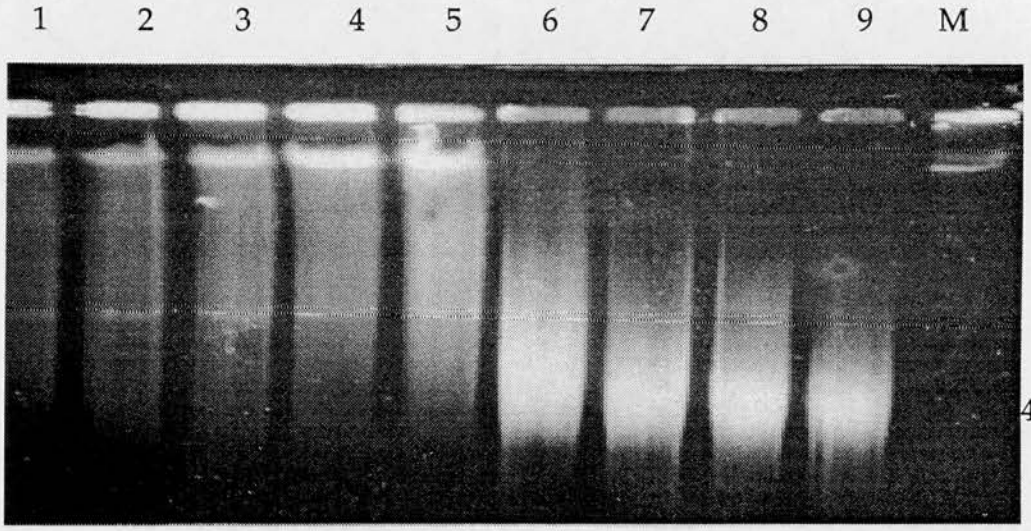
Even when no nalidixic acid was added to the bacterial culture, more DNA damage was observed compared to the addition of nalidixic acid in the absence of chloramphenicol (Figure 5.7). The inhibition of protein synthesis by chloramphenicol is believed to affect the repair of "routine" DNA damage in the cell. Even at high nalidixic acid concentrations, there was still found to be evidence of intact bacterial chromsomal DNA. In conclusion, nalidixic acid, although resulting in double stranded breaks at high concentrations, does not cause degradation of the chromosomal DNA.

It was interesting that the addition of ciprofloxacin in the absence of protein synthesis (Figure 5.21A) resulted in what appeared to be incomplete degradation of the bacterial chromsome. Comparing the effect of various concentrations of ciprofloxacin in the presence and absence of protein synthesis (Figure 5.6 and Figure 5.21A respectively), it is apparent that a band on the pulsed field gel corresponding to intact chromosomal DNA is present with the latter but not the former. Although the addition of chloramphenicol and ciprofloxacin together do show an "apoptosis"-like degradation of DNA and the bulk of the larger DNA fragments seem to be degraded into smaller fragments (Figure 5.21A, Lanes 6 to 9), the bacterial chromosome remains intact to some degree. This can possibly be explained by the fact that the mode of action of chloramphenicol has been shown to cause loops of DNA to migrate from the cytoplasm to the nucleoid mass, thus the bacterial chromosome may become less accessible to the quinolone drugs and the enzymes responsible for the degradation (see later).

5.4 Is the "apoptosis"-like DNA degradation dependent on RNA synthesis?

In the same way, the RNA synthesis dependence of the "apoptosis"-like DNA degradation was investigated. By adding rifampicin to the bacterial culture one hour prior to the addition of various concentrations of ciprofloxacin, the effect of the inhibition of RNA synthesis on this "apoptosis"-like effect was studied. As shown in Figure 5.22, the inhibition of RNA synthesis did not affect the "apoptosis"-like DNA degradation. This reconfirmed that the nuclease responsible for degrading the chromosomal DNA was constitutively expressed. It was found that at the lowest concentration of ciprofloxacin (Figure 5.22, Lane 2), or even in the absence of ciprofloxacin (Figure 5.22, Lane 1), DNA double stranded breaks were seen to occur routinely. This is possibly as a result of RNA synthesis inhibition affecting the cellular manufacture of DNA repair enzymes which would normally be around to mend any DNA nicks. Although it has been hypothesised that RecBCD, the predominant DNA repair enzyme is constitutively expressed and is responsible for the DNA degradation, it is possible that on addition of rifampicin to the cell, the inhibition of other accessory repair enzymes results in more persistent DNA double stranded breaks (Figure 5.22, Lanes 1 to 5). As shown in Figure 5.22B, Lane 6, on addition of 0.064mg/l ciprofloxacin, DNA degradation starts, and is increasingly predominant with higher ciprofloxacin concentrations (Figure 5.22, Lane 9). At the OBC and high ciprofloxacin concentrations, the DNA was found to be in the lower molecular weight range, as had been found to occur also in the presence of ongoing RNA synthesis.

Figure 5.22: The effect of 160mg/l rifampicin added one hour prior to the addition of various concentrations of ciprofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below is a photograph of the pulsed field gel on which the large fragments of DNA were resolved.



RNA synthesis was inhibited by the addition of 160mg/l rifampicin one hour prior to the addition of various concentrations of ciprofloxacin and an aliquot of cells removed after one hour to assess DNA damage: Lane 1, no ciprofloxacin; Lane 2, 0.00025mg/l ciprofloxacin; Lane 3, 0.001mg/l ciprofloxacin; Lane 4, 0.004mg/l ciprofloxacin; Lane 5, 0.016mg/l ciprofloxacin; Lane 6, 0.064mg/l ciprofloxacin; Lane 7, 0.15mg/l ciprofloxacin; Lane 8, 0.5mg/l ciprofloxacin; Lane 9, 5mg/l ciprofloxacin; Lane M, λ concatemer markers.

In order to show that the concentrations of chloramphenicol and rifampicin added did not affect the production of double stranded DNA breaks, different concentrations of chloramphenicol (Figure 5.23) or rifampicin (Figure 5.24) were added to a logarithmic culture of *E.coli* TG1 as before.

As shown in Figures 5.23B, 5.23C, 5.24B and 5.24C the same amount of DNA damage was found to be constant throughout different concentrations of chloramphenicol and rifampicin and the viable count was not seen to differ significantly.

Figure 5.23: The effect of various concentrations of chloramphenicol added on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.23A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.23B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.23C)

Figure 5.23A

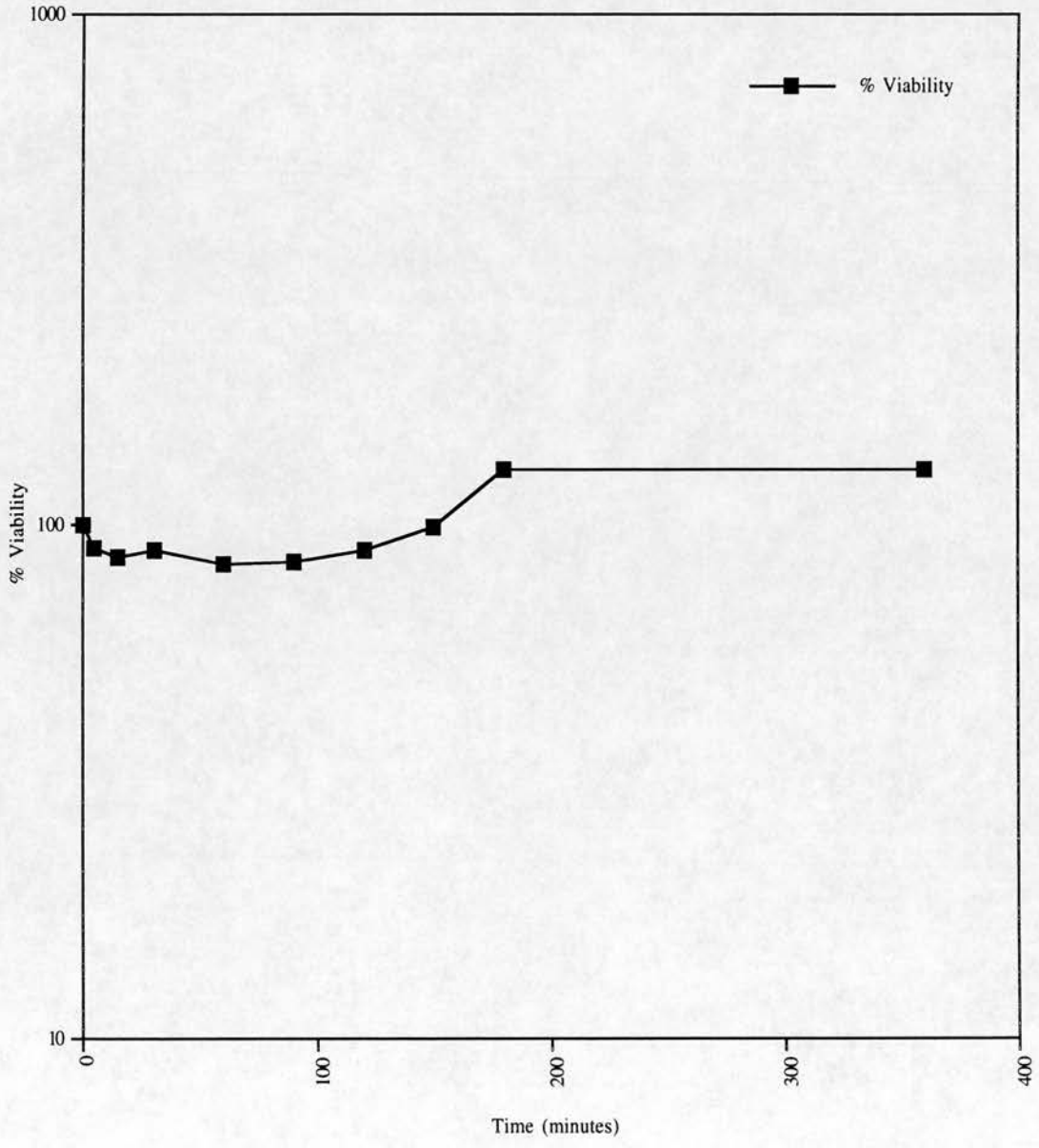


Figure 5.23B

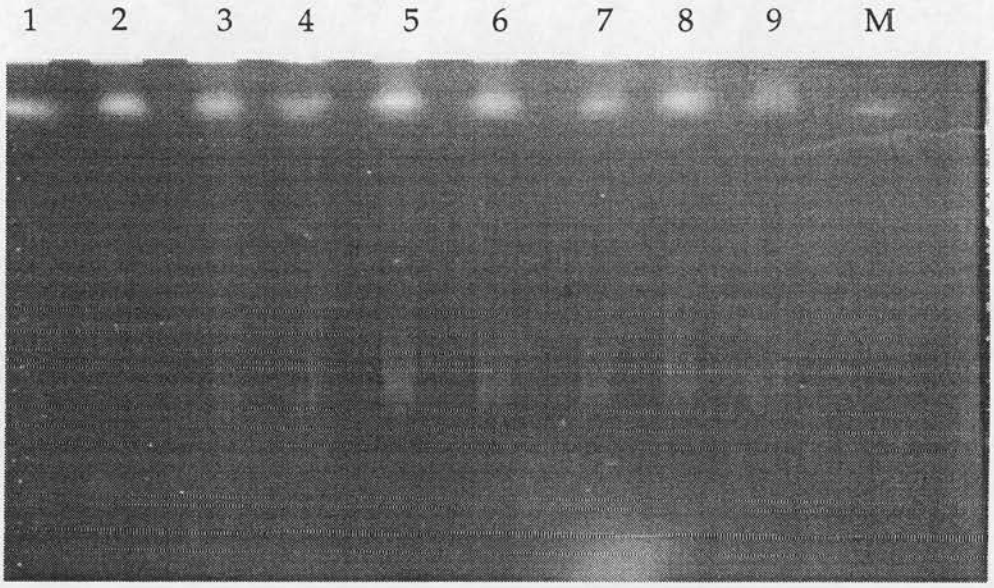
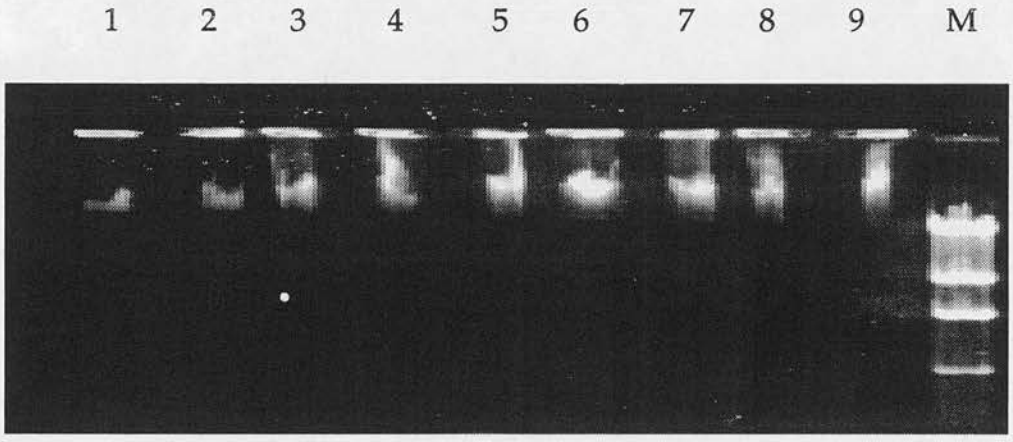


Figure 5.23C



Cells were challenged by the addition of various concentrations of chloramphenicol and an aliquot of cells removed after 60 minutes to assess DNA damage: Lane 1, no chloramphenicol; Lane 2, 20mg/l chloramphenicol; Lane 3, 40mg/l chloramphenicol; Lane 4, 80mg/l chloramphenicol; Lane 5, 120mg/l chloramphenicol; Lane 6, 170mg/l chloramphenicol; Lane 7, 200mg/l chloramphenicol; Lane 8, 240mg/l chloramphenicol; Lane 9, 300mg/l chloramphenicol; Lane M, λ concatemer/ λ *Hind*III markers.

Figure 5.24: The effect of various concentrations of rifampicin added on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.24A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.24B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.24C)

Figure 5.24A

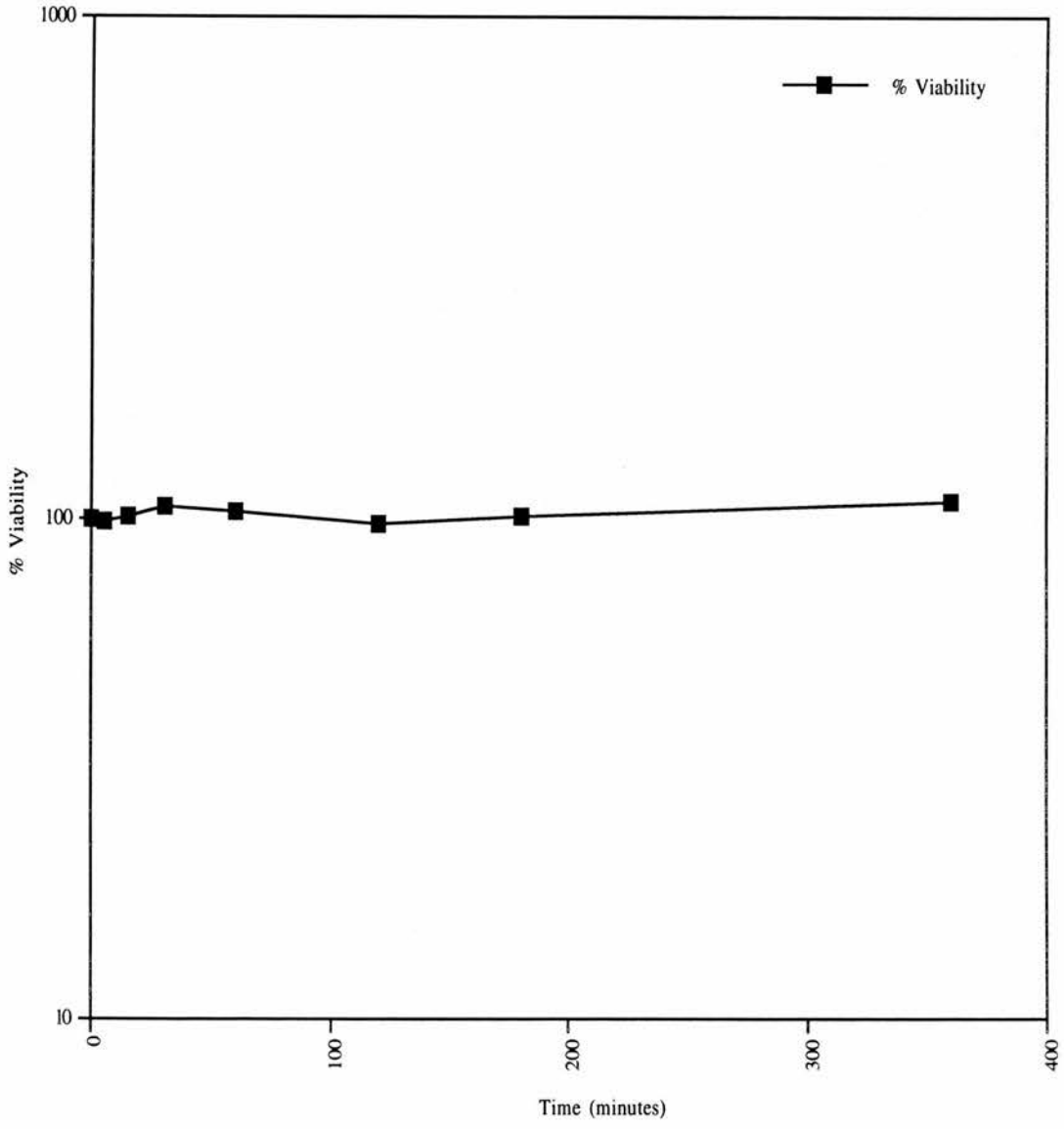


Figure 5.24B

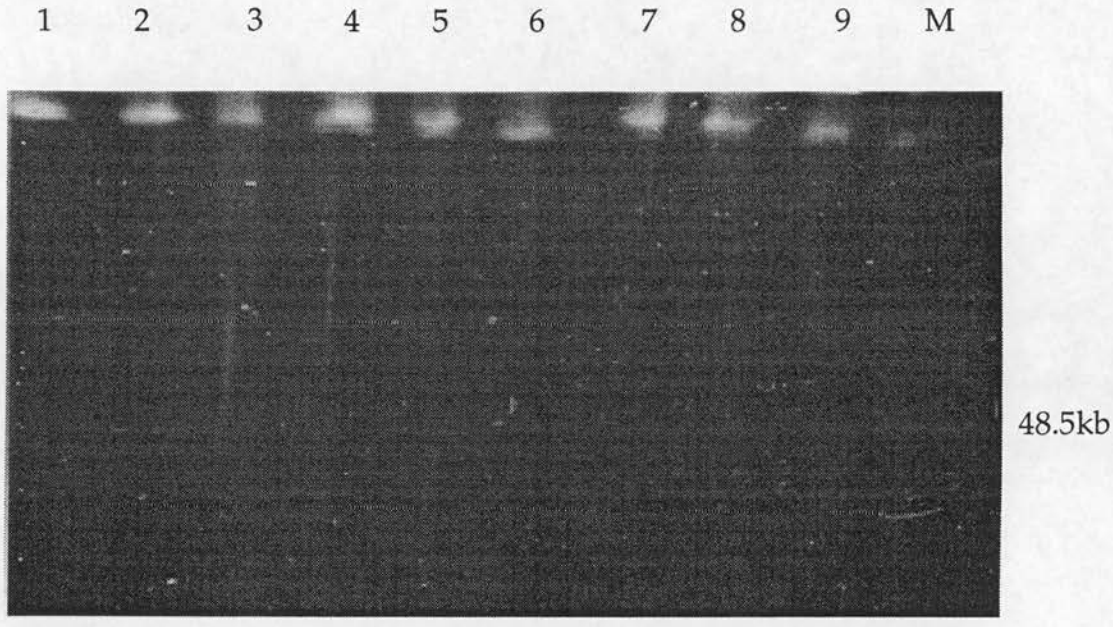
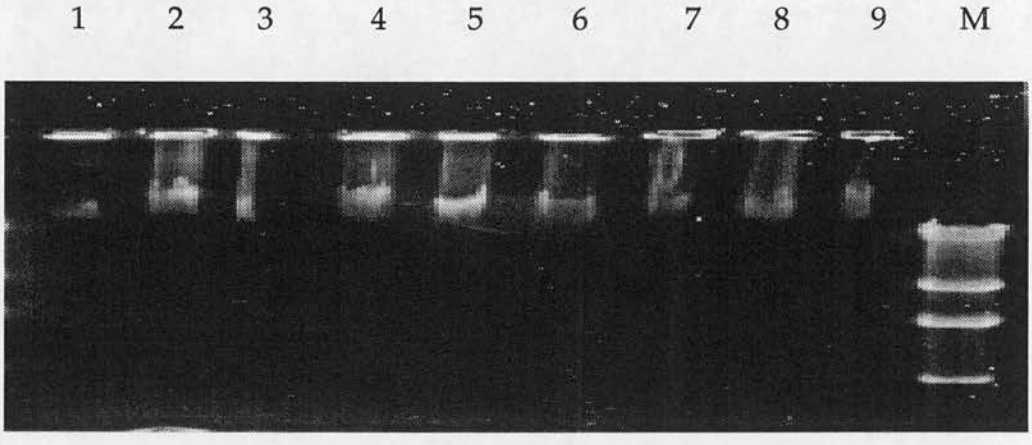


Figure 5.24C



Cells were challenged by the addition of various concentrations of rifampicin and an aliquot of cells removed after 60 minutes to assess DNA damage: Lane 1, no rifampicin ; Lane 2, 20mg/l rifampicin; Lane 3, 40mg/l rifampicin; Lane 4, 80mg/l rifampicin; Lane 5, 120mg/l rifampicin; Lane 6, 160mg/l rifampicin; Lane 7, 200mg/l rifampicin; Lane 8, 240mg/l rifampicin; Lane 9, 300mg/l rifampicin; Lane M, λ concatemer / λ *Hind*III markers.

5.5 Is the "apoptosis"-like DNA degradation occurring after ciprofloxacin challenge Mechanism B?

As mentioned before, the bactericidal activities of nalidixic acid and ciprofloxacin have been differentiated into Mechanism A and Mechanism B (Smith, 1986). Mechanism A is common to all quinolones and is abolished by the inhibition of RNA synthesis. Mechanism B is unique to ofloxacin and ciprofloxacin and does not require RNA or protein synthesis. The results above suggested that the "apoptosis"-like effect was possibly what had been referred to as Mechanism B since it was found to be unique to ciprofloxacin, does not require RNA or protein synthesis and causes rapid cell death. As shown in Figure 5.7, the addition of the OBC of nalidixic acid did not cause this "apoptosis"-like effect, and the addition of chloramphenicol to inhibit protein synthesis did not change the pattern of DNA degradation (Figure 5.21B).

The inhibition of protein synthesis increased the viable count of the nalidixic acid-challenged bacterial culture as shown in Figure 5.2, despite the formation of double stranded DNA breaks which are in this case non-lethal. If this "apoptosis" -like effect was indeed Mechanism B, then as well as occurring in response to ciprofloxacin-challenge, it would also be expected to occur in response to ofloxacin challenge. On the other hand, it would not be expected to occur in response to norfloxacin and oxolinic acid which have been shown only to elicit Mechanism A in the same way as nalidixic acid. This was investigated as described in 5.6 to 5.7.

5.6 Does ofloxacin elicit the "apoptosis"-like response?

In the same way as before, a logarithmic culture of *E.coli* TG1 was challenged with the OBC of ofloxacin (Figure 5.25). Aliquots of the culture were extracted at different time points and embedded in agarose plugs. Photographs of the gels after pulsed field and conventional electrophoresis are displayed below in Figure 5.25.

Figure 5.25: The effect of 0.9mg/l ofloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.25A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.25B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.25C)

Figure 5.25A

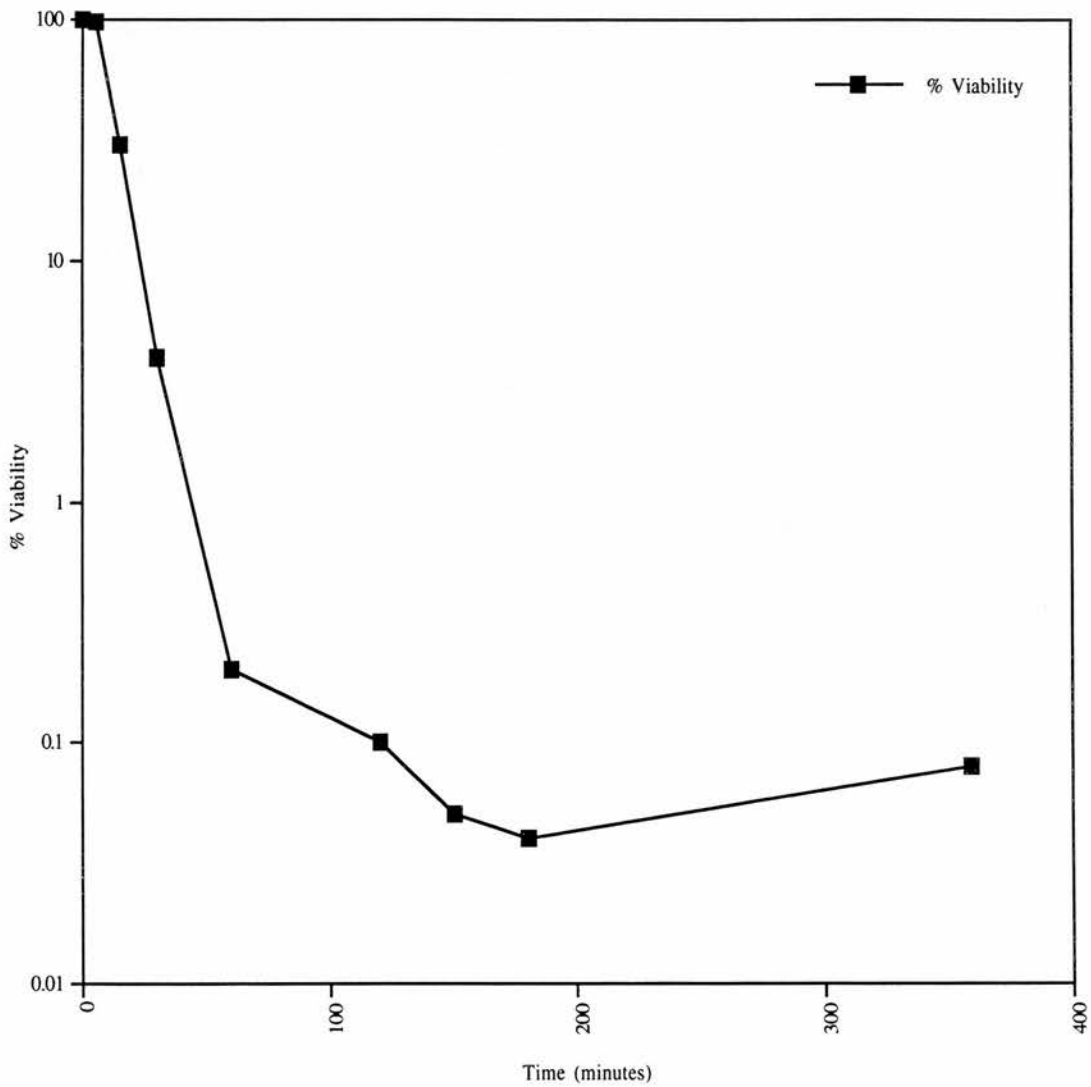


Figure 5.25B

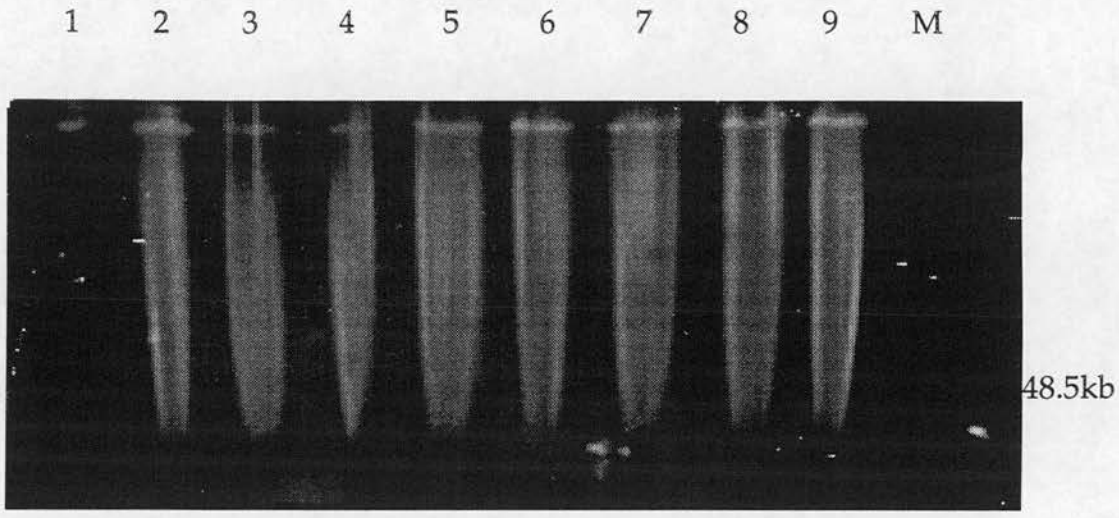
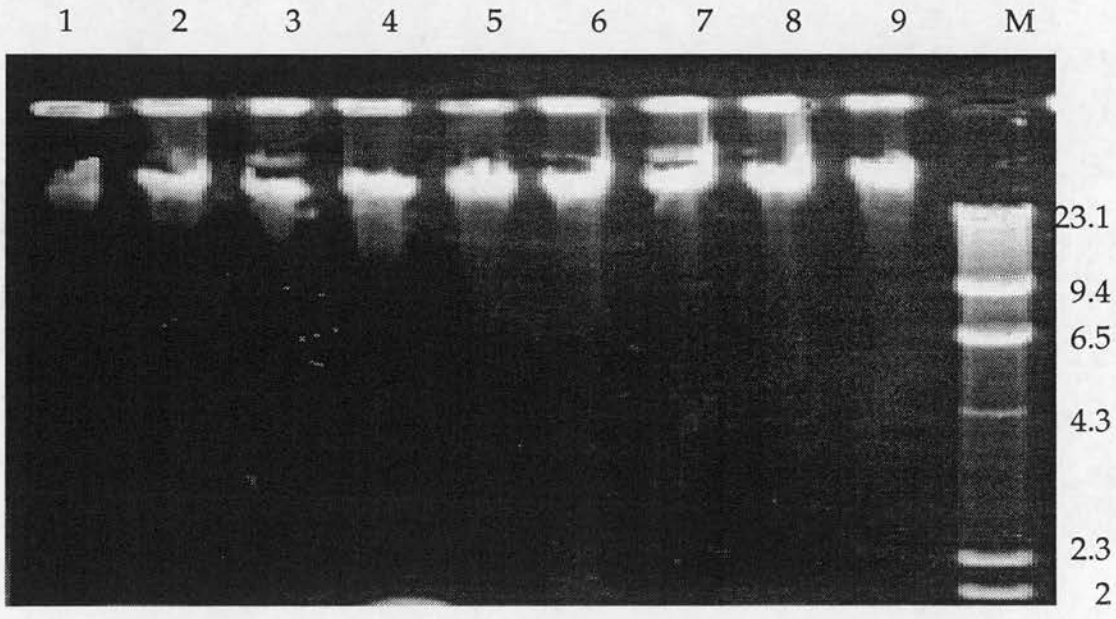


Figure 5.25C



Cells were challenged with 0.9mg/l ofloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ*Hind*III markers.

Addition of ofloxacin at the OBC to a logarithmic culture of *E.coli* TG1 suggested that this antibiotic did elicit the "apoptosis"-like response. Even after 5 minutes, a decrease in the viable count of the culture was observed (Figure 5.25A), corresponding with degradation of the chromosomal DNA and loss of high molecular weight fragments (Figure 5.25B). It appeared that an active repair mechanism was again present as the DNA seemed to become more intact with time. However the extent of the "apoptosis"-like response was much less pronounced with ofloxacin than it was with ciprofloxacin (Figure 5.12B).

5.7 Do norfloxacin and oxolinic acid elicit the "apoptosis"-like response?

In the same way as before, a logarithmic culture of *E.coli* TG1 was challenged with either the OBC of norfloxacin or oxolinic acid (Figures 5.26 and 5.27). Aliquots of the culture were extracted at different time points and embedded in agarose plugs. Photographs of the gels after pulsed field and conventional electrophoresis are displayed below in Figures 5.26 and 5.27.

Figure 5.26: The effect of 1.5mg/l norfloxacin on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.26A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.26B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.26C)

Figure 5.26A

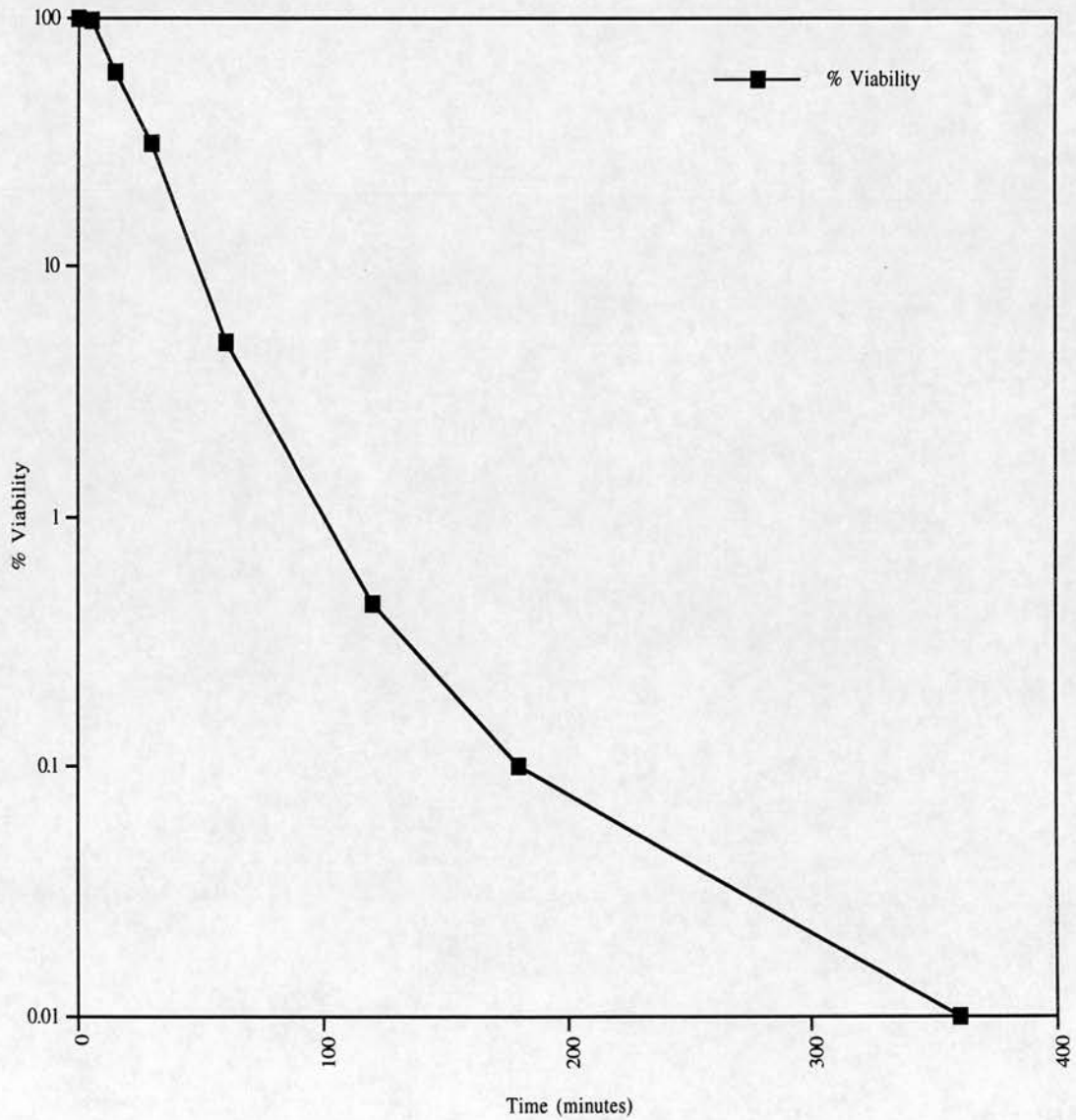


Figure 5.26B

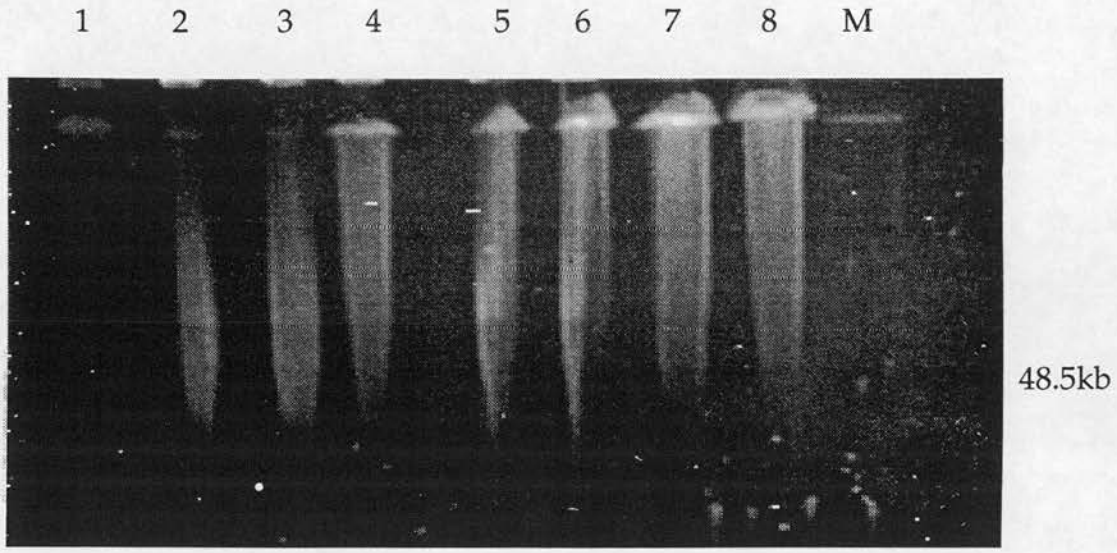
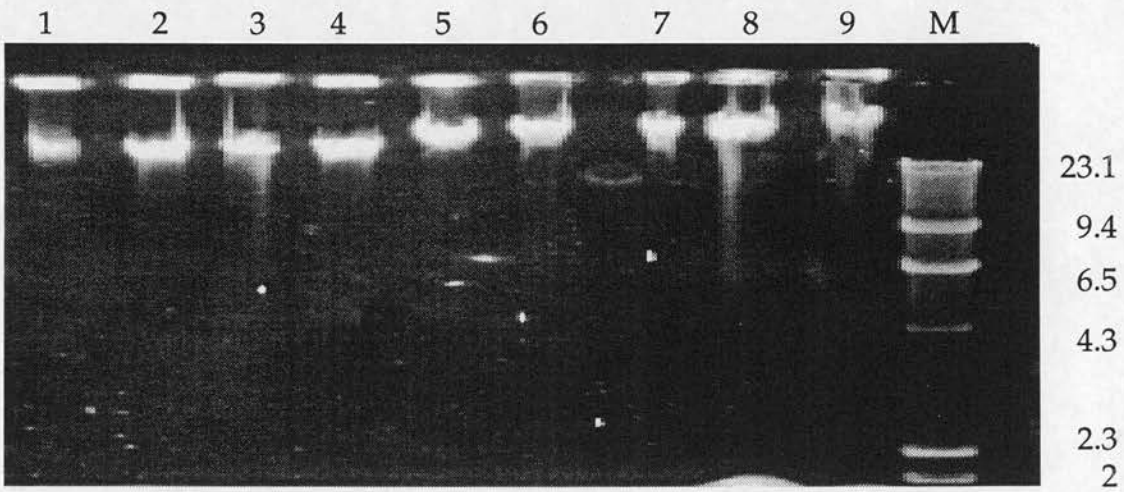


Figure 5.26C



Cells were challenged with 1.5mg/l norfloxacin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ *Hind*III markers.

Figure 5.27: The effect of 9mg/l oxolinic acid on the integrity of *E. coli* chromosomal DNA. Displayed below are a graph plotting the viable counts of the culture at the specified time points (5.27A), a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.27B) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.27C)

Figure 5.27A

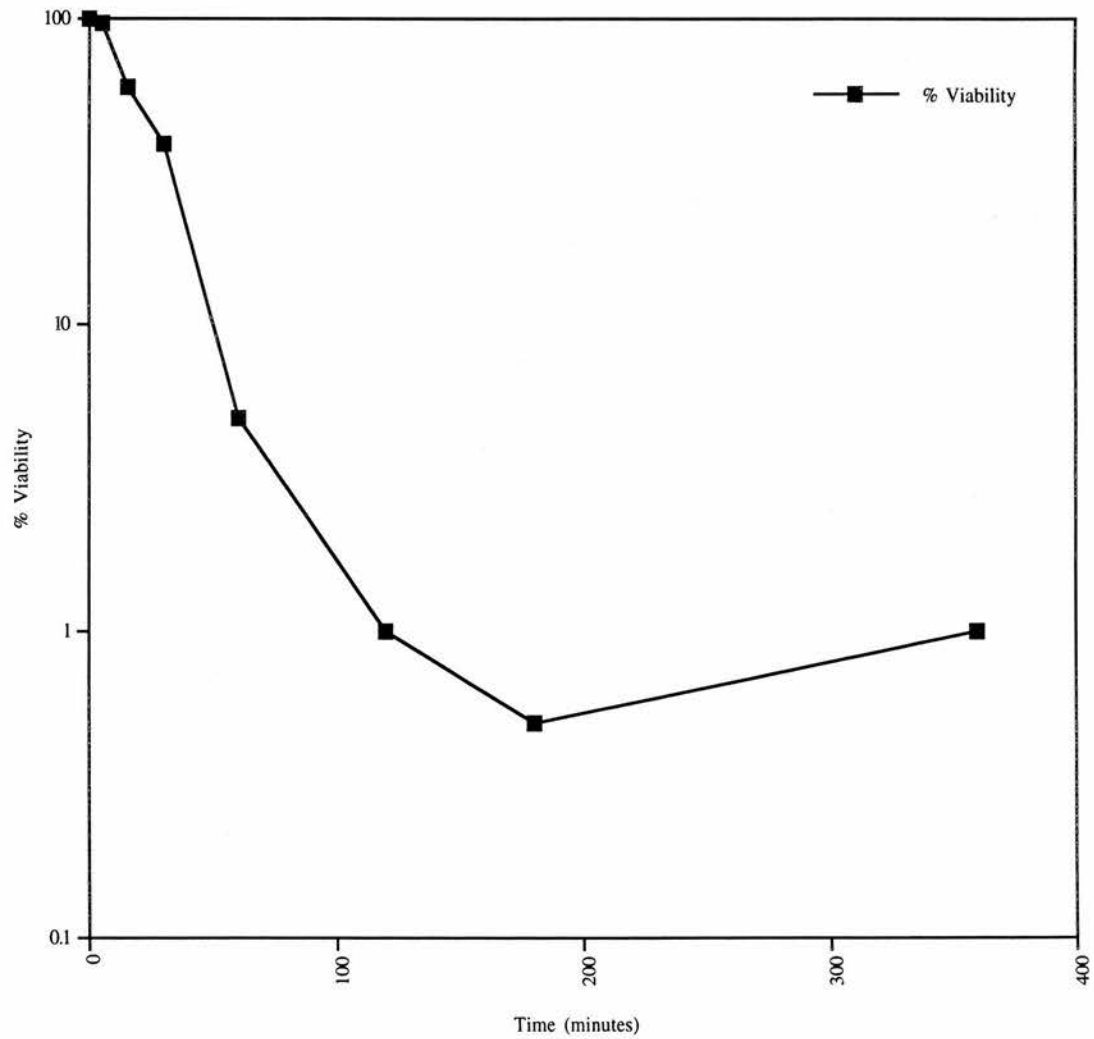


Figure 5.27B

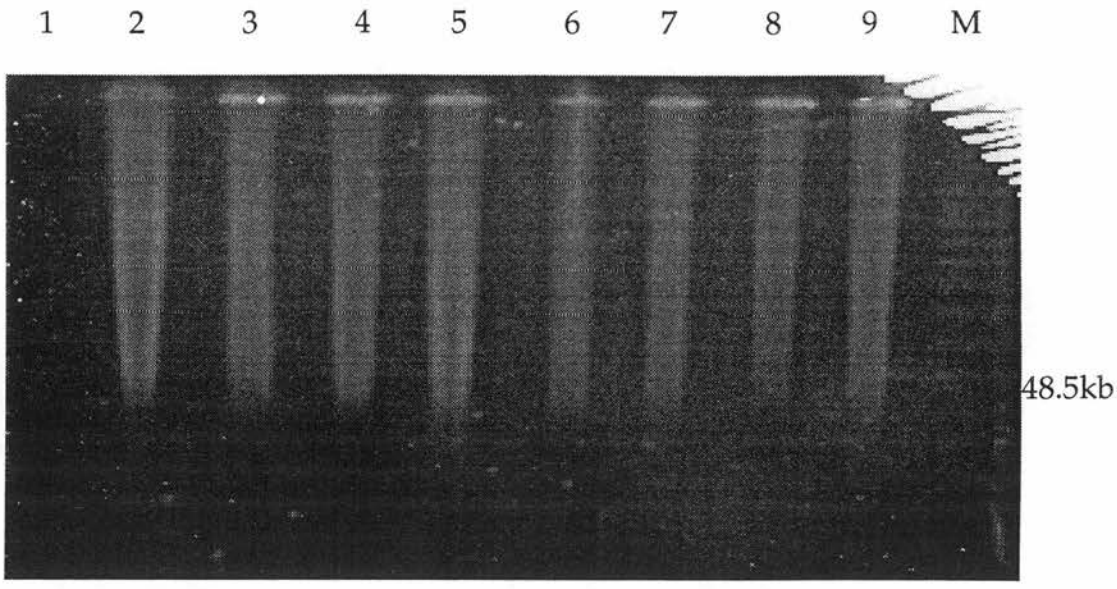
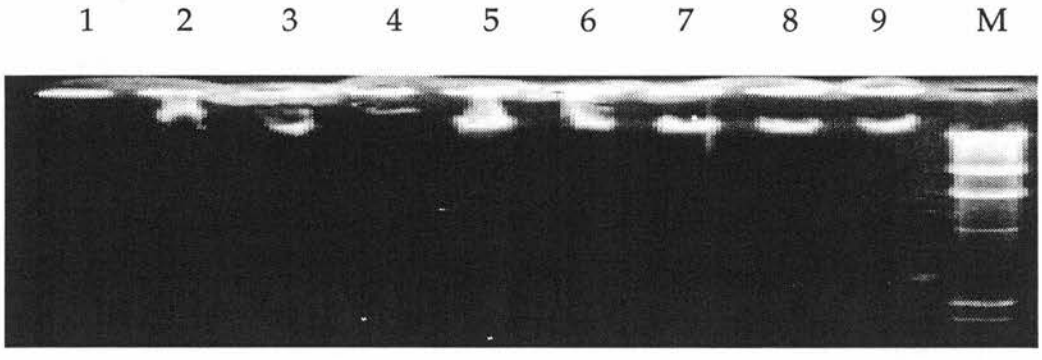


Figure 5.27C



Cells were challenged with 9mg/l oxolinic acid and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ *Hind*III markers.

The "apoptosis"-like response did not seem to occur with oxolinic acid (Figure 5.27). Instead, extensive double stranded breaks were observed but no small DNA fragments (Figure 5.27C). However some loss of the chromosomal DNA was detected five and fifteen minutes after addition of the OBC of norfloxacin to the bacterial culture (Figure 5.26B). This suggested that the "apoptosis"-like effect might be a characteristic of norfloxacin-mediated as well as ofloxacin- and ciprofloxacin-mediated cell death.

It also seemed that the formation of double stranded DNA breaks was lethal in the case of fluoroquinolones such as ciprofloxacin, ofloxacin and norfloxacin but non-lethal in the case of quinolones such as nalidixic acid and oxolinic acid. To confirm whether the formation of double stranded DNA breaks could indeed be the lethal event, two further experiments were carried out.

5.8 Is double stranded break formation the lethal event in fluoroquinolone challenged cells?

The formation of double stranded DNA breaks in fluoroquinolone challenged cells was mimicked by two separate experiments in order to ascertain that it was the formation of double stranded breaks by the fluoroquinolones that was the lethal event and not another property of the antibiotics. Two different plasmids were used to introduce double stranded DNA breaks into the chromosome and the effect on the integrity of the DNA and on the viability of the cells was investigated.

5.8.1 Use of a *ccdB*-carrying plasmid to mimick quinolone double stranded DNA breakage

As mentioned before, the CcdB toxin targets the GyrA subunit of DNA gyrase and poisons it in the same way as do the quinolones (Bernard *et al*, 1993). By doing so, double stranded breaks are formed in the chromosome and the cell dies. It has previously been shown by comparing the viscosity of total DNA extracted from $CcdA^{-}CcdB^{-}$ bacteria and from $CcdA^{+}CcdB^{+}$ bacteria that CcdB fragments the genomic DNA (Bernard and Couturier, 1992). However the appearance of the genomic DNA on a pulsed field gel was not ascertained.

A plasmid carrying the *ccdB* gene under an inducible promoter pKIL119 was transformed into *E.coli* TG1. A logarithmic culture of the resulting strain *E.coli* TG1pKIL119 was grown up and production of CcdB induced by the addition of 0.5mM IPTG. Aliquots of the culture were extracted at specified time points and treated as before.

Figure 5.28: The effect of *ccdB* expression on the integrity of *E. coli* chromosomal DNA. Displayed below is a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.28A) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.28B).

Figure 5.28A

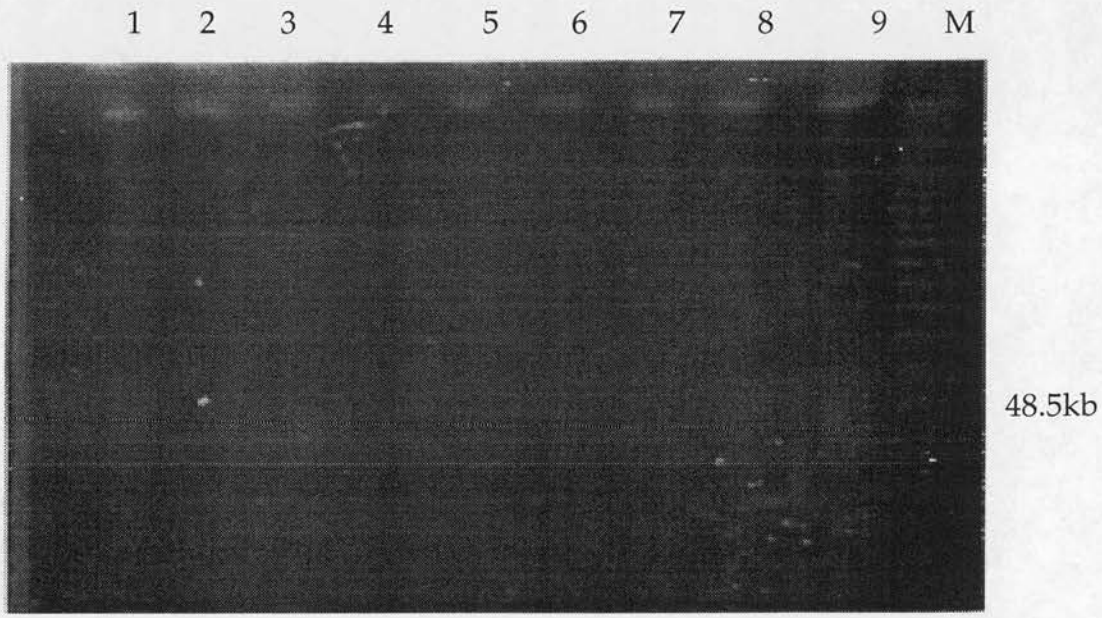
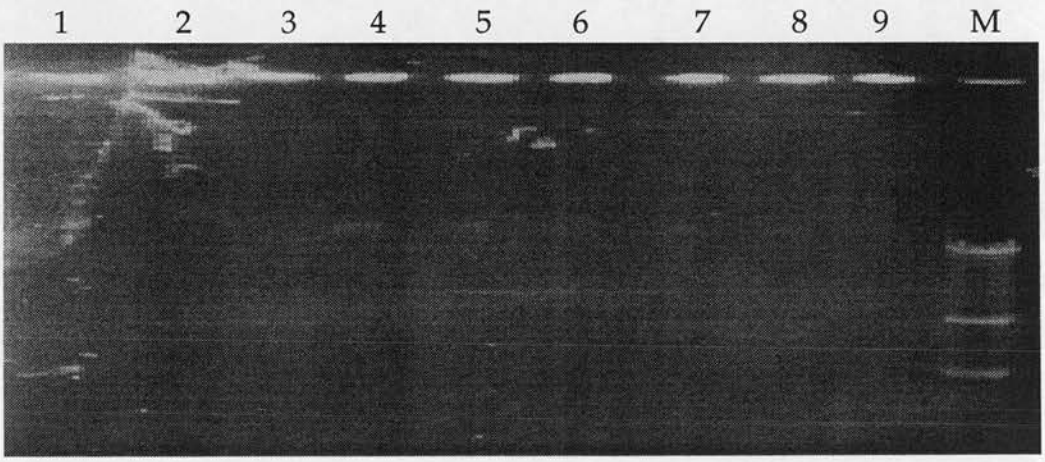


Figure 5.28B



Cells carrying pKIL119 were induced with 0.5mM IPTG and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer/ λ *Hind*III markers.

The results of inducing expression of CcdB in *E.coli* revealed that this topoisomerase poison did not culminate in the same effect as did ciprofloxacin. At all the time points investigated, intact chromosomal DNA was always detected. However a faint band was seen corresponding to a 48.5kb DNA fragment. This band was also detected on the conventional agarose gel (Figure 5.28B). The identity of this band is discussed later. However by no means the cellular response to CcdB appeared as destructive as that to ciprofloxacin. The results of the viable count experiments revealed that a rapid loss of viability occurred on induction of *ccdB* by the addition of IPTG (data not shown). Therefore even the minimal double stranded breaks detected on the gels corresponded to cell death.

5.8.2 Use of a *Not*I-carrying plasmid to mimick quinolone-induced double stranded DNA breakage

The *Not*I restriction endonuclease is known to cleave the *E.coli* chromosome at 22 sites (Condemine and Smith, 1990), releasing fragments of size 20kb to 100kb. Therefore this rare-cutting endonuclease was used to mimick quinolone-induced double stranded breaks.

A plasmid carrying the *Not*I-encoding gene under an inducible promoter was transformed into *E.coli* TG1. A logarithmic culture of the resulting strain *E.coli* TG1pNotI was grown up and production of *Not*I induced by the addition of 0.5mM IPTG. Aliquots of the culture were extracted at specified time points and treated as before.

Figure 5.29: The effect of *Not*I expression on the integrity of *E. coli* chromosomal DNA. Displayed below are a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.29A) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.29B)

Figure 5.29A

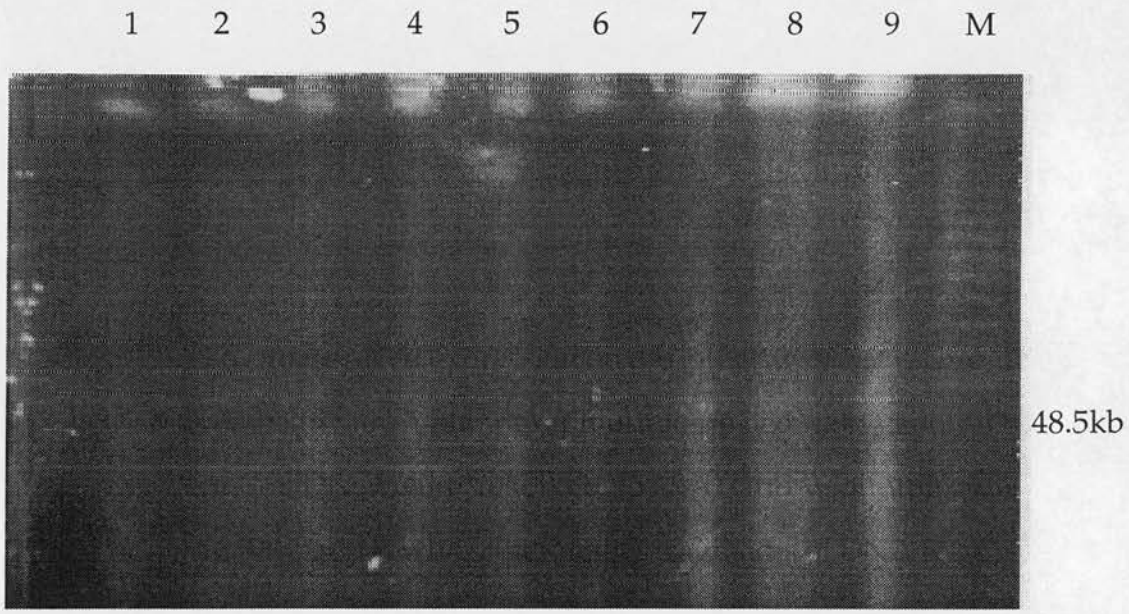
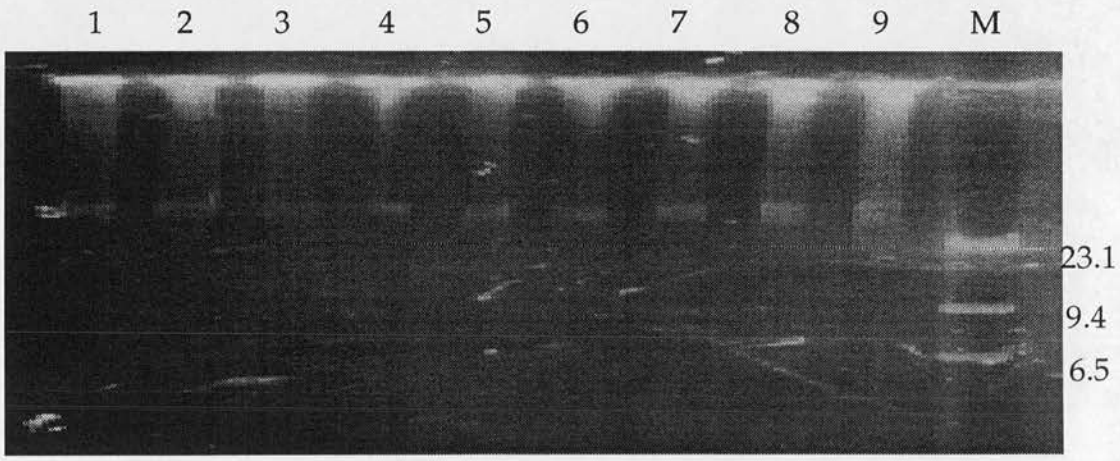


Figure 5.29B



Cells carrying pNotI were induced with 0.5mM IPTG and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60 ; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ *Hind*III markers.

The induction of *Not*I in *E.coli* resulted in the production of a smear of chromosomal DNA fragments on the pulsed field gel (Figure 5.29A). Surprisingly, this effect was also seen prior to the addition of IPTG (Figure 5.29A, Lane 1). It can possibly be concluded that the expression of *Not*I is "leaky" and occurs even when the promoter is not stimulated. The extent of DSBs actually seemed to increase with time (compare Figure 5.29A Lane 2 to Lane 9) and the presence of a band corresponding to a DNA fragment of size 48.5kb (compared to the bottom band of λ concatemers) was detected. This is shown more clearly in Figure 5.29B. The identity of this band is discussed further later. Again, The results of the viable count experiments revealed that a rapid loss of viability occurred on induction of *Not*I by the addition of IPTG (data not shown). Therefore even the minimal double stranded breaks detected on the gels corresponded to cell death.

5.9 Is this "apoptosis"-like effect involved in the bactericidal mechanisms of other antibiotics than the fluoroquinolones?

Having related a bacterial "apoptosis"-like effect to ofloxacin and ciprofloxacin mediated cell death and possibly to norfloxacin mediated cell death, the occurrence of this cell death mechanism in response to other unrelated antibiotics was investigated. The effect of growth-inhibiting concentrations of three antibiotics from three separate classes on the integrity of chromosomal DNA was studied: ampicillin (an inhibitor of cell division), trimethoprim (an inhibitor of dihydrofolate reductase) and streptomycin (an inhibitor of protein synthesis). The results are displayed in the following Figures 5.30 to 5.32.

Figure 5.30: The effect of 1mg/l ampicillin on the integrity of *E. coli* chromosomal DNA. Displayed below are a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.30A) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.30B).

Figure 5.30A

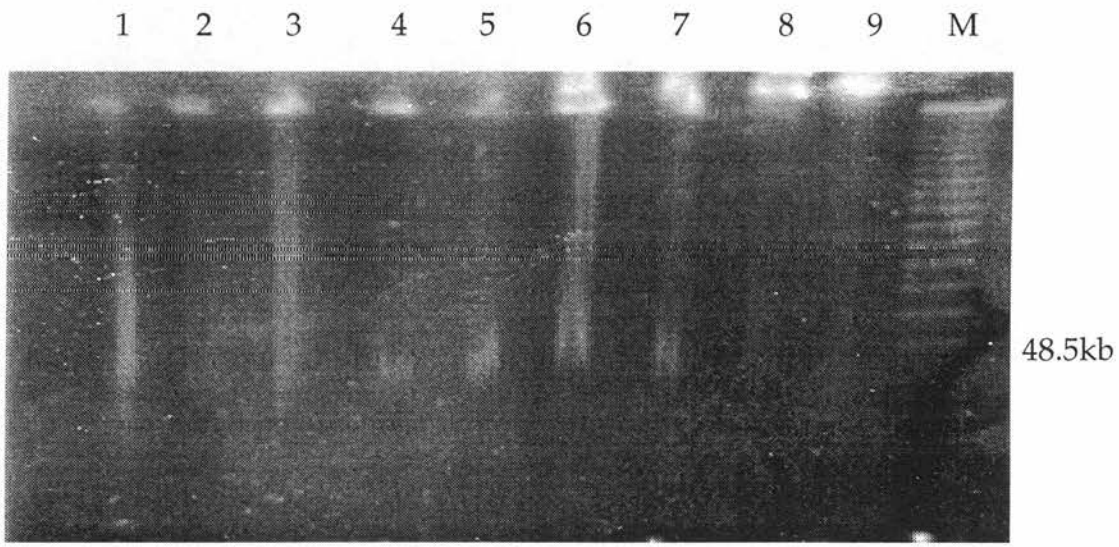
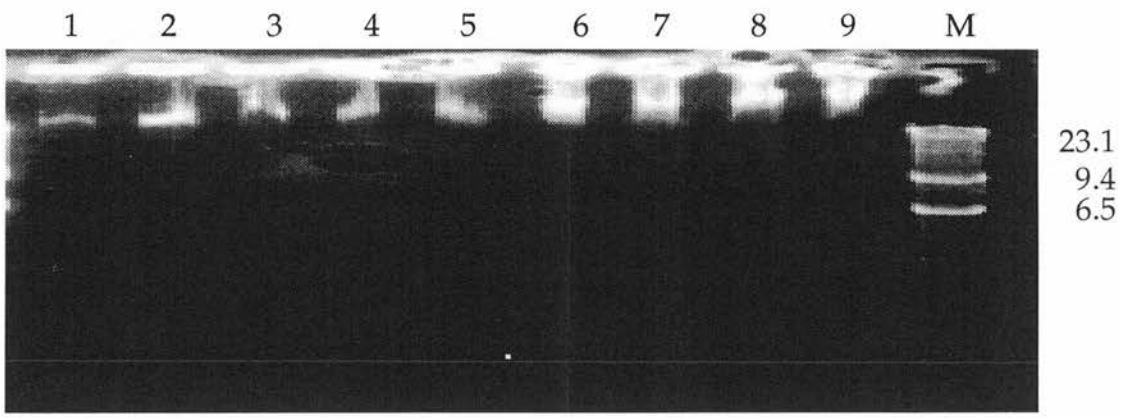


Figure 5.30B



Cells were challenged with 1mg/l ampicillin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ *Hind*III markers.

Figure 5.31: The effect 1mg/l trimethoprim on the integrity of *E. coli* chromosomal DNA. Displayed below are a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.31A) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.31B).

Figure 5.31A

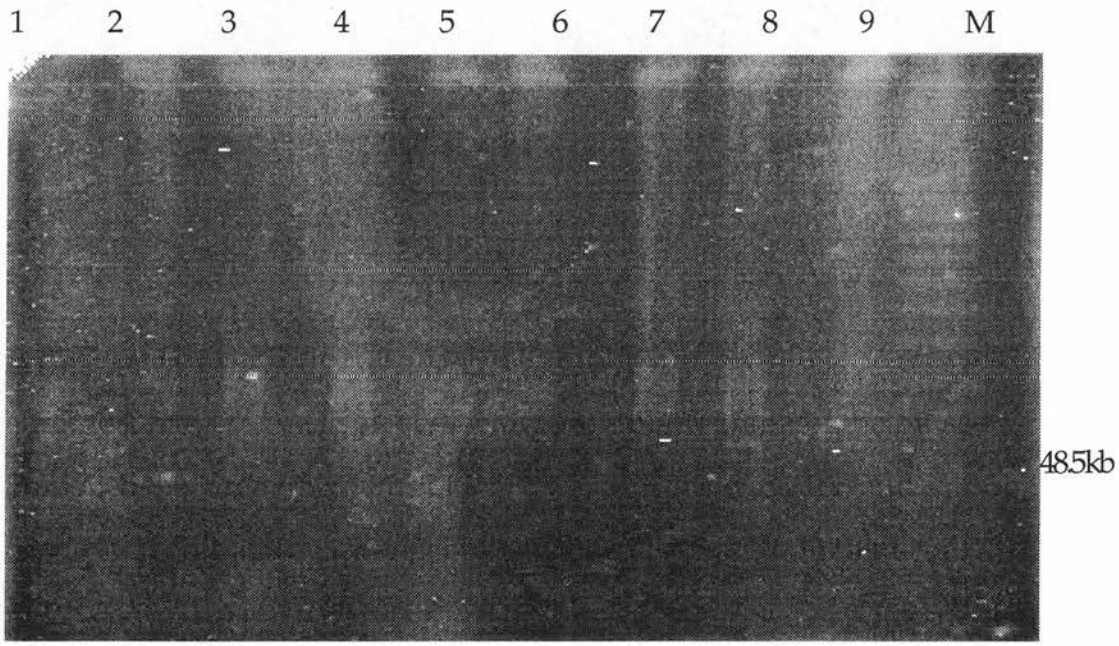
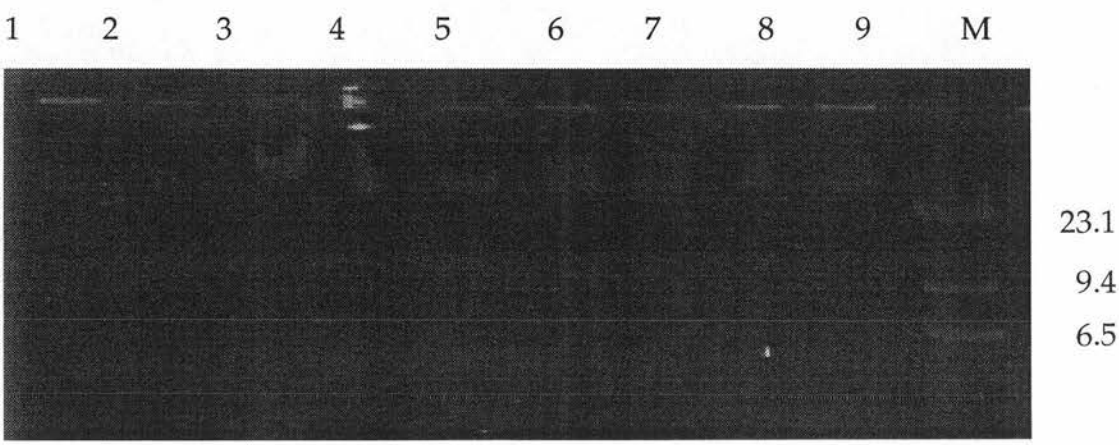


Figure 5.31B



Cells were challenged with 1mg/l trimethoprim and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ *Hind*III markers.

Figure 5.32: The effect of 25mg/l streptomycin on the integrity of *E. coli* chromosomal DNA. Displayed below are a photograph of the pulsed field gel on which the large fragments of DNA were resolved (5.32A) and a photograph of the conventional agarose gel on which the smaller fragments of DNA were resolved (5.32B).

Figure 5.32A

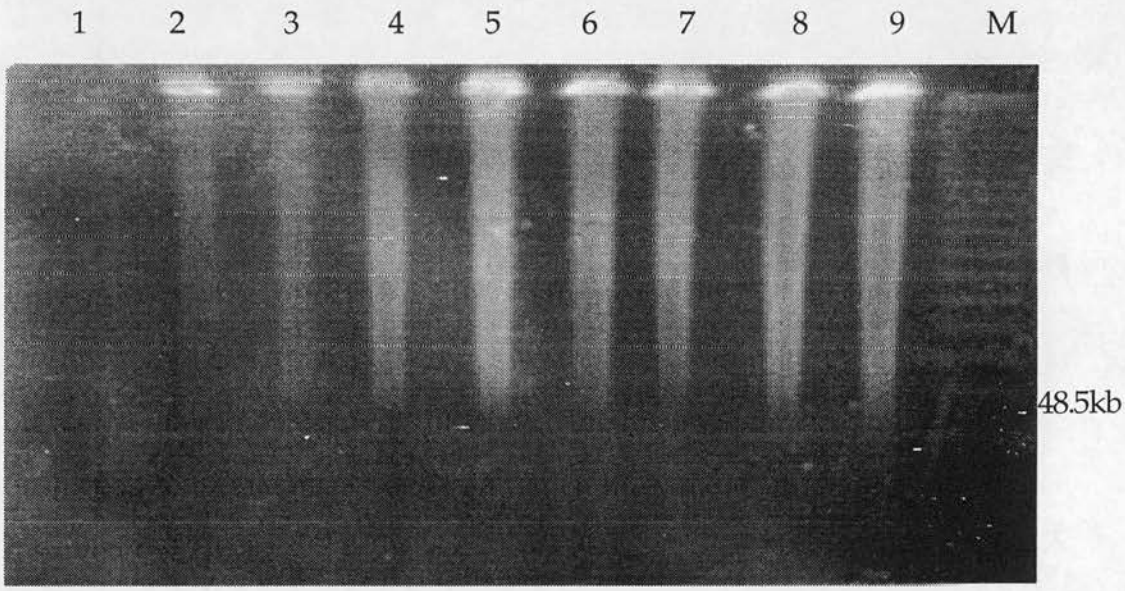
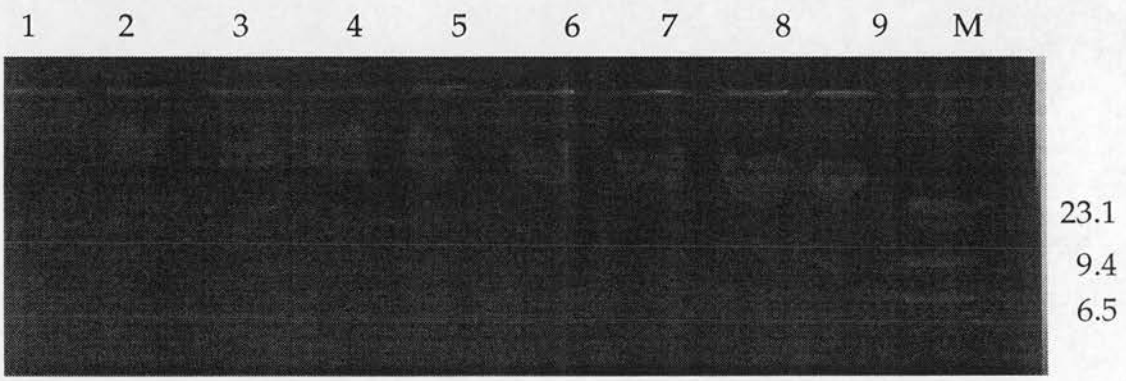


Figure 5.32B



Cells were challenged with 25mg/l streptomycin and an aliquot of cells removed at the following time points (in minutes) to assess DNA damage: Lane 1, t=0; Lane 2, t=5; Lane 3, t=15; Lane 4, t=30; Lane 5, t=60; Lane 6, t=90; Lane 7, t=120; Lane 8, t=180; Lane 9, t=360; Lane M, λ concatemer / λ *Hind*III markers.

The results in Figures 5.30 to 5.32 display some interesting results. While ampicillin and trimethoprim do not appear to affect the integrity of the DNA, the addition of streptomycin was found to induce DSBs in a manner very similar to that of nalidixic acid. The reason for this may be a result of streptomycin's mode of action as an antibiotic affecting the accuracy of protein manufacture. As will be discussed in more detail later, streptomycin results in misreading of the mRNA at the ribosome and subsequently mutant proteins are formed. It can be imagined how such proteins could damage the cell in various ways as well as prevent the correct repair of DNA. Therefore as a result, any DNA nicks that arise in the cell cannot be repaired, as seen in Figure 5.32A.

5.10 What is Mechanism A?

Having investigated the molecular aspects of Mechanism B of the quinolone drugs, attention was turned to the bactericidal pathway named Mechanism A. Mechanism A as described before, was the name given to the cell death occurring with all quinolones which was dependent on protein and RNA synthesis (Smith, 1986). The cause of this bactericidal mechanism has been the source of much debate with suggestions that the production of a toxic protein might be the key event (Courtright *et al*, 1988). These results suggested that it was RNA and protein synthesis *per se* that were lethal to the cell after quinolone challenge. In considering the roles of rifampicin and chloramphenicol in the inhibition of RNA and protein synthesis, it is vital to realise at which point in transcription and translation these compounds function. Rifampicin and chloramphenicol are both bacteriostatic antibiotics, rifampicin works by binding to the β subunit of RNA polymerase and prevents the initiation of transcription, whereas chloramphenicol prevents the initiation of protein synthesis by binding to the 50S ribosomal subunit.

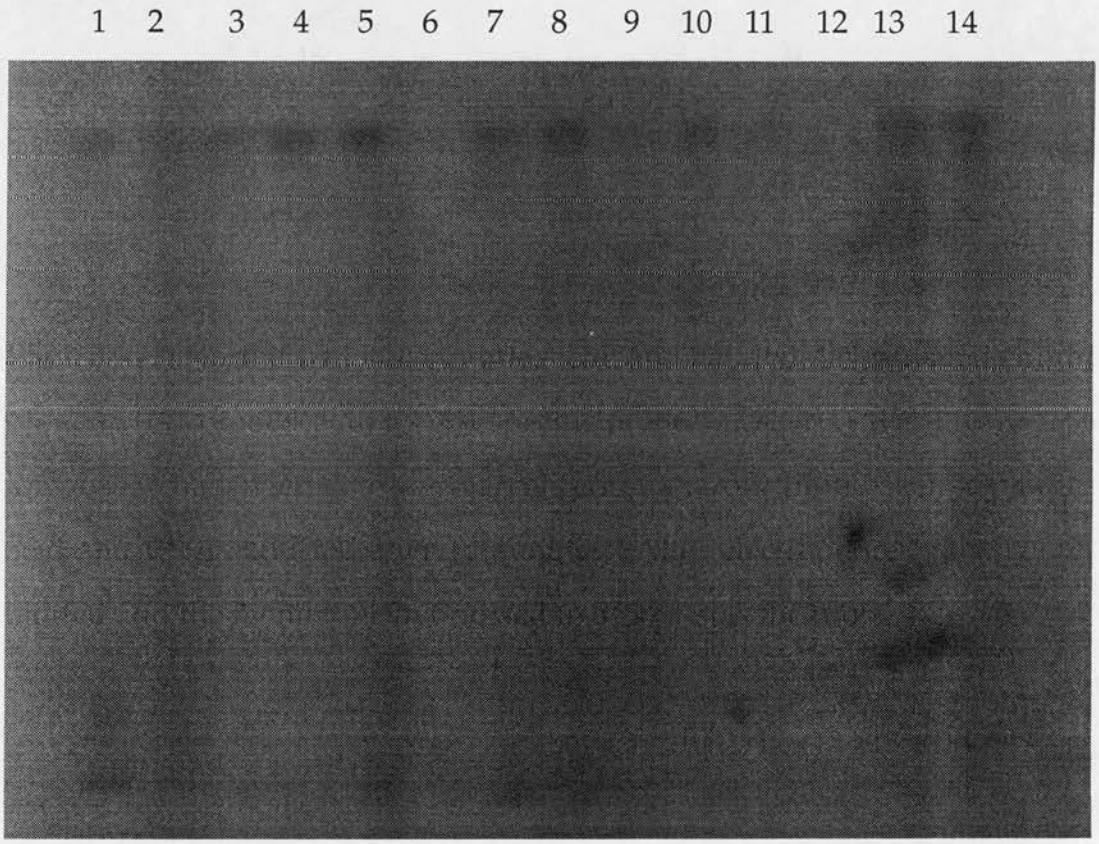
This information, coupled with the findings of Willmott *et al* (1994) which showed that the complex of quinolone, gyrase and DNA *in vitro* results in the production of truncated transcripts by RNA polymerase was the basis for the next set of experiments. Truncated transcripts were believed to be produced since the quinolone-gyrase-DNA poisoned complex acted as a barrier to the movement of RNA polymerase which fell off the template 10 to 20bp before the quinolone-gyrase complex on the DNA, releasing a shortened mRNA. If this also happened *in vivo*, one could expect that the production of truncated mRNAs and hence truncated polypeptides would result in a complete de-regulation of the cell.

By adding either rifampicin or chloramphenicol to inhibit either RNA or protein synthesis respectively, it can be understood how this quinolone-induced lethal event could be avoided. Although a cell deficient in RNA and/or protein synthesis would not be able to grow and divide, once the effect of the RNA and/or protein synthesis inhibitor and the quinolone wore off, the cell would again be functional.

To investigate this quinolone-induced RNA polymerase block that occurred *in vitro* also occurred *in vivo*, a logarithmic culture of *E.coli* TG1 was challenged with the OBC of nalidixic acid for specified amounts of time and RNA extracted. After electrophoresis, the RNA was blotted and the membrane probed with a probe specific to *gyrA*. A *gyrA* oligo probe labelled with fluorescein was initially used to detect the prematurely truncated transcripts. However this non-radioactive system was found not to be sensitive enough, considering that it was aimed to detect single-copy *gyrA* mRNAs of varying length. The use was then made of a ^{32}P-dCTP labelled *gyrA* probe which was hoped to be much more sensitive.

Preliminary results show that a "smear" of *gyrA* mRNA of varying length was detected, correlating with the *in vitro* results as shown in Figure 5.33. The addition of lower concentrations of ciprofloxacin and nalidixic acid correlates with much truncation of *gyrA* (Figure 5.33, Lanes 4,5,10,13 and 14) whereas higher concentrations of ciprofloxacin and nalidixic acid correlate with no detectable *gyrA* transcripts, confirming the theory that at high quinolone concentrations, transcription is inhibited.

Figure 5.33: Northern blot of ciprofloxacin- and nalidixic acid-treated cultures of *E.coli* TG1 probed with a *gyrA*-specific probe. *E.coli* TG1 was grown up to log phase, challenged with various concentrations of ciprofloxacin or nalidixic acid and RNA extracted after 10 minutes. After electrophoresis the gel was blotted and the membrane hybridised to a *gyrA*-specific probe.



Lane 1, 0.15mg/l ciprofloxacin; Lane 2, 0.5mg/l ciprofloxacin; Lane 3, 1mg/l ciprofloxacin; Lane 4, 0.004mg/l ciprofloxacin; Lane 5, 0.008mg/l ciprofloxacin; Lane 6, 5mg/l ciprofloxacin; Lane 7, 0.002mg/l ciprofloxacin; Lane 8, 0.001mg/l ciprofloxacin; Lane 9, no ciprofloxacin; Lane 10, 3mg/l nalidixic acid; Lane 11, no nalidixic acid; Lane 12, 100mg/l nalidixic acid; Lane 13, 5mg/l nalidixic acid; Lane 14, 10mg/l nalidixic acid.

5.11 The Contribution of SOS Mutagenesis to Quinolone-Induced Cell Death

Although the two major pathways of quinolone-induced cell death have been elucidated by this work, it is also likely that some minor cellular pathways contribute. For example, quinolone drugs have previously been shown to cause a loss of membrane integrity and leakage of intracellular material (Dougherty and Saukkonen, 1985). Another cellular response to quinolone drugs is the SOS response, although there has been much debate whether or not the SOS response increases cell viability after quinolone challenge (Lewin *et al*, 1989; Walters *et al*, 1989; Piddock and Wise, 1987). Proteins produced in the response to DNA damage such as RecA, UmuD, UmuC and SulA although offering a protective role to the cell, do not actually seem to affect cell viability. The reason for this could be that so many cellular repair functions rely on the nicking-resealing activity of DNA gyrase. One of the SOS functions induced by quinolone drugs was investigated in the course of this work. SOS mutagenesis in *E.coli* is carried out by RecA, DNA polymerase III, UmuD, UmuD' and UmuC (Murli and Walker, 1993; Peat *et al*, 1996) and the result is that error-prone DNA replication occurs although the mutation rate is unknown. A correlation between *umuC* induction and a *Salmonella* mutagenicity assay for quinolone antimicrobials has previously been ascertained (Power and Phillips, 1993). To study how errors in DNA replication might affect the viability of the cell, 0.002mg/l ciprofloxacin (a level below the MIC so the cells could still divide) was added to a logarithmic culture of *E.coli*, the culture allowed to continue to grow and a chromosomal preparation of DNA carried out. Taking *gyrA* as a representative gene, a part of the *gyrA* gene was sequenced but no mutations were found. This probably suggests that the DNA replication mutations only occurred at a low frequency, the mutations were detrimental and the cell died

or that they were later repaired by the DNA repair machinery.

5.12 Discussion

By relating the viable count of a quinolone challenged *E.coli* culture to double stranded DNA breaks (DSBs) and DNA degradation, the molecular mechanisms involved in quinolone cell death have been investigated. The previously unexplained mechanism "Mechanism B" has been thoroughly studied at a molecular level and shown to correlate with the microbiological observations. Preliminary findings showed that the molecular mechanism of Mechanism A correlates with the *in vitro* investigations (Willmott *et al*, 1994).

5.12.1 The Molecular Basis of Mechanism B

Mechanism B had previously been described as a bactericidal response of the cell as a result of challenge with quinolone drugs which could not be inhibited by RNA synthesis- and protein synthesis-inhibitors. This bactericidal mechanism had been found only to be possessed by ciprofloxacin (Smith and Lewin, 1988), DR-3355 (Lewin and Amyes, 1989), fleroxacin (Lewin and Amyes, 1990), tomefloxacin (Lewin *et al*, 1989b), ofloxacin (Smith and Lewin, 1988) and perfloxacin (Lewin and Amyes, 1990). Both DNA damage and the inhibition of any further DNA replication have been previously discussed in relation to quinolone binding to an actively replicating gyrase-DNA complex and the occurrence of a potentially cytotoxic event (Crumplin and Smith, 1976). Maxwell (1992) speculated that Mechanism B was the result of the formation of a stable ternary complex of quinolone, gyrase and DNA which then forms a barrier to the passage of DNA and RNA polymerases. The lesion that resulted was proposed to be the basis for cell death. Howard *et al* (1993) proposed that Mechanism B resulted from SOS processing of quinolone-damaged DNA and that the GyrA subunit is the site of action of Mechanism B. All these previous

observations were found to fit in with the model proposed by this work.

Differences in the state of the bacterial genomic DNA were observed by electrophoresis depending whether the antibiotic used elicited Mechanism B as well as Mechanism A. Those tested which had been shown before to exhibit Mechanism B, that is ciprofloxacin and ofloxacin, were shown to give rise to DNA degradation after 60 minutes which was likened to the apoptosis induced by eukaryotic topoisomerase targeting drugs (Walker *et al*, 1991). The quinolone antibiotics only possessing Mechanism A that were tested (nalidixic acid and oxolinic acid) did not show this "apoptosis"-like effect even although extensive DSBs were observed. Norfloxacin, which had previously been shown not to elicit Mechanism B, showed a very similar response to that of ofloxacin during the course of this work, suggesting that the action of norfloxacin is somewhat similar to ciprofloxacin and ofloxacin.

5.12.2 The formation of "cleavable complexes"

The similarity of mode of action between the quinolone antibacterials and some anti-cancer drugs on DNA gyrase and topoisomerase II respectively prompted parallels to be drawn as concerns the mechanism of cell death (Maxwell, 1992; Liu, 1989; Hsiang, Libou and Lou, 1989). Topoisomerase II-targeting drugs also form "cleavable complexes" with topoisomerase II and DNA in which the enzyme becomes covalently but reversibly linked to the broken ends of DNA. Three possible outcomes then exist for the cleavable complex:

1. The cleavable complex could be converted to a permanent enzyme-linked strand break by random thermal motion (although this is probably a rare event).

2. The cleavable complex could be dissociated into functional enzyme and intact DNA.

3. The cleavable complex could be processed by either the DNA replication machinery, the transcriptional machinery or the DNA repair machinery into a form of DNA damage that is irreversible.

It is such damage resulting from the breakage of the cleavable complexes that is thought to be cytotoxic. However, the reversible nature of cleavable complexes means that cytotoxicity results from the small fraction that become irreversible. Since DSBs are in fact routinely repaired, only a certain number of DSBs will become cleavable complexes of which only some will accumulate in irreversible DNA damage. Another similarity between cleavable complex formation in prokaryotes and eukaryotes is that the lethal effects of topoisomerase II-targeting drugs also appear to involve the action of the nucleic acid synthesis machinery in the conversion of topoisomerase II cleavable complexes to lethal lesions. It has been found in eukaryotes that the addition of DNA or RNA synthesis inhibitors reduce the lethality of the topoisomerase II drugs (D'Arpa *et al*, 1990; Kaufman *et al*, 1991) presumably by reducing the number of potentially lethal collisions between the nucleic acid synthesis machinery and the cleavable complexes. However a basal level of cell death was detected in the lack of nucleic acid synthesis suggesting either that a small level of DNA synthesis is ongoing or that there is another pathway creating DSBs. In *E.coli*, the inhibition of RNA synthesis has also been shown to lessen the bactericidal effect of quinolone drugs although this may also be explained by a different theory (see later).

5.12.3 Parallels with eukaryotic cells

In eukaryotes, the existence of repair mechanisms for cleavable complex-induced damage is indicated by the hypersensitivity of DNA repair mutants to the lethal effects of topoisomerase-targeting drugs, for example the RAD52 mutants of *Saccharomyces cerevisiae*. These RAD52 mutations are extremely sensitive to the topoisomerase targeting drugs m-AMSA and camptothecin which induce high levels of both gene conversion and reciprocal exchange consistent with the type of damage repaired by the RAD52 pathway (Nitiss and Wang, 1988). The RAD52 gene product has been implicated in DSB repair and it is interesting that X-ray sensitive CHO cells defective in DSB repair are also sensitive to topoisomerase II-targeting drugs (Caldecott *et al*, 1990).

5.12.4 The Involvement of an Endo-exonuclease

In *E.coli*, it has been previously found that DNA repair mutants such as *recB*$^-$ or *recC*$^-$ cells are hypersensitive to the lethal effects of quinolone drugs (Lewin *et al*, 1989). Strengthening the evidence that RecBCD was the endo-exonuclease responsible for this DNA degradation is the fact that parallels have previously been drawn between RecBCD in *E.coli* and RAD52 in *S.cerevisiae* (Fraser, 1994). From this it was concluded that possibly the same type of pathway was responsible for the cytotoxicity of the quinolone drugs in *E.coli* as the cytotoxicity of the topoisomerase II-targeting drugs in eukaryotes. Indeed, the fact that nalidixic acid-treated cells of *E.coli* (Cook *et al*,1966a; Grigg, 1970; Hill and Fangman, 1973) and *B.subtilis* (Cook *et al*, 1966b) have been already shown to degrade their DNA suggested that DNA degradation may be associated with cell death. Smith (1984) proposed that nicked DNA formed by the action of

quinolone-inhibited DNA gyrase acts as a substrate for exonucleases which degrade the DNA. The identity of the nuclease responsible for degrading the chromosomal DNA in eukaryotes is unclear. However, evidence of a DNase I-like enzyme in rat thymocytes (Peitsch *et al*, 1993), a Ca^{2+}-dependent DNase I-like endonuclease in virally transformed murine fibroblasts (Ucker *et al*, 1992) and an inducible lymphocyte nuclear endonuclease associated with apoptosis (Khodarev and Ashwell, 1996) have been published. It has been found that the human leukemic CEM cell endo-exonuclease plays a role in the apoptotic cell death induced in response to cytotoxic doses of various DNA damaging agents. The degradative activity of this eukaryotic endo-exonuclease involved in apoptosis has been likened to RecBCD of *E.coli* (Fraser, 1994). Also the RAD52 mutation in *S.cerevisiae* has been shown to result in a loss of nuclear endo-exonuclease and a deficiency in recombination and recombinational DSB repair. This observation provides a strong parallel between the functions of the eukaryotic and prokaryotic endo-exonucleases. As discussed in 1.6.6, RecBCD has both endonuclease and exonuclease activities and is normally involved in the repair of DSBs and the repair of collapsed replication forks (Kuzminov, 1995a,b). However if the bacterial cell becomes damaged beyond repair, the RecBCD enzyme becomes "reckless" and degrades the chromosomal DNA to acid-soluble nucleotides (Smith, 1988). It has also been proposed that the presence of lesions in template DNA or a protein-mediated block to the progress of replisomes results in the breakage of arrested replication forks, albeit an apparently self-destructive behaviour of the cell (Kuzminov, 1995a,b). Experiments have suggested that replication forks that are broken as a result of treatment with nalidixic acid are repaired by the RecBCD recombinational pathway (Kuzminov, 1995a,b). However the very nature of RecBCD repair suggests that if the DNA was badly damaged, DNA degradation rather than

DNA repair would be the prominent activity. The results of experiments carried out by Lewin and Smith (1990) suggested that RecBCD may be the enzyme responsible for the DNA breakdown that was observed in bacteria challenged with quinolones. Indeed pulse-chase experiments carried out on quinolone-challenged *B.subtilis* indicated that the DNA degradation started from the replication fork and continued sequentially along the chromosome from the "new" to the"old" DNA (Ramareddy and Reiter, 1969). Although Lewin and Smith (1990) found that DNA breakdown did occur with quinolone-challenged cells, they could not correlate it with the bactericidal effect of the drug. If, for example, they had studied the DNA fragment sizes on pulsed field and conventional gels, as described in this chapter, they would have possibly arrived at a correlation. The multiple mutagen sensitivities of *recB*$^-$ and *recC*$^-$ cells reflects a deficiency in post-replication repair processes, especially recombinational DSB repair which attempt to rid the cell of DNA damage not excised by various specific excision-repair enzymes before replication. Therefore as a result of the parallels previously drawn between RecBCD and enzymes involved in apoptosis in eukaryotes, and the fact that *recB*$^-$ and *recC*$^-$ cells were hypersensitive to the quinolones, it would be interesting to investigate the possible role of RecBCD in the apparent "apoptosis"-like effect that had been observed after treatment of *E.coli* cells with ciprofloxacin. The breakage of stalled replication forks has been found to be followed by the RecBCD-dependent degradation of the newly synthesised DNA (Kuzminov, 1995a,b). Interestingly, this degradation has been found not to occur in a *recB*$^-$ cell (Buttin and Wright, 1968). By repeating the ciprofloxacin-challenge experiments in a *recB*$^-$ or *recC*$^-$ *E.coli* strain and studying the DNA on a pulsed field gel, the presence or absence of an "apoptosis"-like effect would confirm participation of this enzyme. The single-strand specificity of RecBCD-mediated DNA degradation is reflected by the observation that if the degradation is allowed to proceed to completion, only half of the newly

incorporation label is made acid-soluble (Buttin and Wright, 1968; Veomert and Kuempel, 1973).

Unlike many enzymes that have been previously discussed to be involved in the response of the cell to quinolones, RecBCD is not part of the SOS regulon, nor is it inducible. Therefore, in order to show that it was indeed RecBCD that was involved in the "apoptosis"-like DNA degradation effect, the presence of DNA degradation in the presence of a protein synthesis inhibitor had to be shown. To investigate this, an amount of chloramphenicol sufficient to inhibit protein synthesis was added one hour prior to the addition of various concentrations of ciprofloxacin. The degradation of chromosomal DNA and the apparent loss of the larger fragments of DNA (Figure 5.21A) suggested that this "apoptosis"-like degradation was protein synthesis independent.

5.12.5 Factors determining the lethality of DSBs

Although DSBs were observed in the chromosomal DNA of the cultures challenged with 0.001mg/l to 0.064mg/l ciprofloxacin, no "apoptosis"-like effect was seen. Also in the nalidixic acid and oxolinic acid challenged cultures, addition of these two antibiotics at their OBCs did not result in the same extent of DNA degradation that was seen with ofloxacin and ciprofloxacin at their OBCs. Even when the bactericidal effect of nalidixic acid was inhibited by the addition of chloramphenicol (Figure 5.21B), DSBs were observed to be present in the chromosomal DNA. This suggested that DSB formation was sometimes non-lethal and possibly reversible. These observations agreed with those of Lewin and Smith (1990) who detected degradation of DNA in bacteria exposed to nalidixic acid even when chloramphenicol or rifampicin was also present, that is when nalidixic acid was not bactericidal. However on the other hand

evidence has previously suggested that DSBs are a type of DNA damage that is highly lethal as it has been shown that repairable single stranded breaks may be converted into DSBs (Boothman and Pardee, 1989). Indeed, single-stranded breaks in the bacterial chromosome (Hill and Fangman, 1973) enlarging to double stranded gaps (Fenwick and Curtis, 1973) have been observed in bacteria treated with nalidixic acid. This suggested that it was the extent of DSB formation that dictated the lethality on the bactericidal cell. Such a scenario can easily be imagined since a cell with only a few DSBs in its chromosomal DNA would possibly have the repair proficiency to mend the breaks whereas a cell with an abundance of DSBs would possibly be unable to carry out sufficient DNA repair and subsequently die. Therefore the binding affinity of a particular quinolone for the gyrase-DNA complex probably determines the likelihood of irreversible DSB formation since a drug that binds tightly to the gyrase-DNA complex and forms a irreversible cleavable complex would be more likely to result in lethality that a drug which bound less tightly and formed a more reversible cleavable complex. This is illustrated in Figure 5.34.

Figure 5.34:

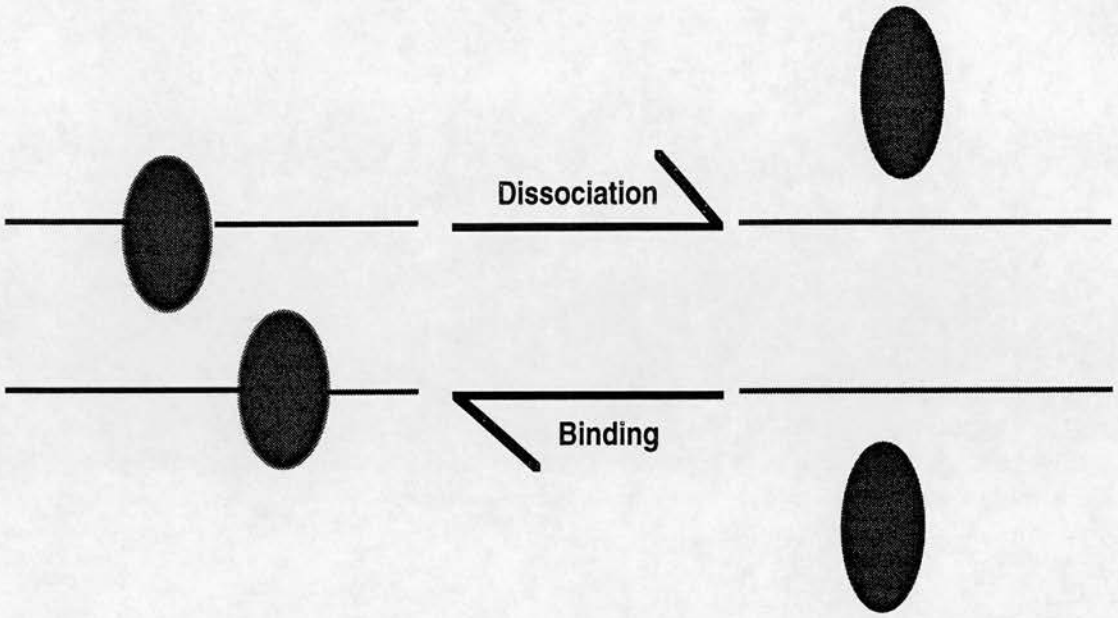


Table 5.1: Potencies of quinolone drugs for cleavage of DNA and inhibition of supercoiling by DNA gyrase

Drug	Gyrase cleavage µg/ml	Gyrase 50% inhibition µg/ml	Antibacterial activity µg/ml	Mechanism B? Yes/No
Nalidixic acid	50	>100	6.3	No
Oxolinic acid	10	25	0.2	No
Norfloxacin	1	5.5	0.1	No/ Yes
Ofloxacin	5	6.3	0.1	Yes
Ciprofloxacin	0.5	5.3	0.025	Yes

Data taken from Domagala *et al* (1986)

From these data, it seemed that the higher the affinity of the quinolone drug for the DNA gyrase cleavage reaction, the more DNA degradation leading to a potential "apoptosis"-like effect occurred. Drugs binding tightly to the gyrase-DNA complex and readily causing DNA cleavage were possibly more likely to

form an irreversible cleavable complex and therefore stimulate the endo-exonuclease function of RecBCD. Although less norfloxacin was required to elicit cleavage of DNA than ofloxacin, it is possible that ofloxacin perhaps has a more marked effect which affects cell death in a different way.

5.12.6 The Relationship between Cell Death and Transcription/ Replication

In all the experiments carried out involving the addition of ciprofloxacin to the bacterial culture, an optimum in DNA degradation was observed to occur at around 60 minutes after which the DNA seemed to become more intact. This is likely to be as a result of DNA repair enzymes which start to function again after the antibiotic begins to become less active. From the viable count experiments, it was found that on addition of a concentration of ciprofloxacin less than or equal to the MIC (Figures 5.8A and 5.9A), the bacterial culture stopped dividing for some time but then reinitiated cell division and resumed normal growth kinetics. At concentrations of ciprofloxacin exceeding the MIC, the culture rapidly died with minimum viability after 15 to 60 minutes but then seemed to start division once again. It was found that at higher ciprofloxacin concentrations (5mg/l, 10mg/l, 50 mg/l, 100mg/l- Figures 5.16A, 5.17A, 5.18A, 5.19A), the extent of cell death was not as severe as with lower ciprofloxacin concentrations (0.15mg/l, 0.5mg/l- Figures 5.12A and 5.14A). At the higher ciprofloxacin concentrations, recovery seemed to be quicker also. The conclusion from this set of viable counts was that at higher ciprofloxacin concentrations, there was greater viability of the culture. This could possibly be attributed to the fact that at high ciprofloxacin concentrations, no transcription or replication fork movement can occur as a result of widespread cleavable complex formation. Since in this case the cleavable complexes will not be broken apart, the rapid

death observed to occur with the lower ciprofloxacin concentrations did not occur. This would explain why on the pulsed field gel there is always evidence of intact chromosomal DNA at higher ciprofloxacin concentrations and the chromosome is not as degraded as seen with the OBC of ciprofloxacin for example. This scenario reflects the effect of topoisomerase II-targeting drugs on topoisomerase II in the presence of nucleic acid synthesis inhibitors. As mentioned previously, low levels of killing occur in quiescent eukaryotic cells suggesting that active DNA replication and transcription contributes to cell death (D'Arpa and Liu, 1989).

Therefore it appeared that an "apoptosis"-like effect was occurring with ofloxacin and ciprofloxacin however the effect of these antibiotics differed from the effect of eukaryotic topoisomerase II-targeting drugs in that the bacterial "apoptosis" seemed to be reversible to some extent whereas apoptosis in eukaryotic cells certainly is not.

5.12.7 The preponderance of DNA migrating as a 50kb fragment

By running both pulsed field and conventional agarose gels, both high molecular weight and low molecular weight fragments can be detected. Only ciprofloxacin (Figure 5.12B and 5.13) and ofloxacin (Figure 5.25B) were expected to be associated with DNA degradation resulting in the production of DNA fragments as small as 4kb (Figure 5.13). However addition of norfloxacin to the bacterial culture yielded surprising results: norfloxacin also showed this "apoptosis"-like effect. On studying the pattern of DNA degradation after ciprofloxacin or ofloxacin challenge by pulsed field gel electrophoresis, it was apparent that there was a preponderance of DNA fragments in the 50kb size region. The production of DNA fragments in the 50kb size range was also seen to some

extent to happen with nalidixic acid, oxolinic acid and norfloxacin (Figures 5.7, 5.27 and 5.26) and has been reported also to occur with enoxacin (Courtright *et al*, 1988). This can be explained by the action of RecBCD and the spacing of the replication/ transcription complexes. The binding of RecBCD to the free ends of DNA after the collapse of replication forks for example, has already been discussed. The double stranded DNA is then unwound and degraded until a Chi sequence is reached. At this point, the affinity of the RecD subunit for the RecBC heterodimer is weakened and the resulting protein, RecBC loses its exonuclease activity. The enzyme then becomes proficient as a recombinogenic helicase. The binding of RecA to the single-stranded DNA, as well as protecting it from further nuclease degradation and stimulating recombination, induces the SOS response. In badly damaged cells, it is likely that single-stranded DNA of variable length will be released which may migrate as a 50kb fragment when electrophoresed. Although it has been estimated that there are 5kb between Chi sequences in *E.coli* (Myers and Stahl, 1994), it is unlikely that all the Chi sequences would be involved at once as this would result in very large scale degradation of the chromosome. Another explanation is that if the replication/ transcription complexes were 50kb apart on the bacterial chromosome, stalling of two by gyrase poisoning would result in degradation of DNA, leaving single-stranded fragments plus a 50kb double-stranded DNA fragment.

If the average fragment size after DNA degradation is around 50kb then it can be estimated that there are about 90 cuts per *E.coli* chromosome. The number of DNA gyrase binding sites on the bacterial chromosome has been a matter of contention, with one estimate of one gyrase binding site per domain giving 45 DNA breaks in the chromosome after oxolinic acid treatment (Snyder and Drlica, 1979), another estimate of as many as 10,000 (Franco and Drlica, 1988),

suggesting that gyrase probably can access most of the chromosome as required for the enzyme to concentrate around replication forks (Drlica *et al*, 1980) and downstream of active promoters (Kato *et al*, 1990). It is the replication-fork associated DNA gyrase molecules (Drlica *et al*, 1980) and the transcription complexes associated DNA gyrase molecules that are rapidly inhibited by the addition of quinolone. *E.coli* growing in a rich medium divides once every 25 minutes; however the time required to duplicate is less than that needed for chromosomal DNA replication which takes about 40 minutes. As a result, multiple replication bubbles in the bacterial chromosome are present. In estimating the numbers of potential cleavable complexes that could be formed after quinolone challenge, the large number of transcriptional complexes on the bacterial chromosome also has to be taken into account. Although DNA degradation occurs at both replication forks, the selectivity shown means that only one of the two strands of newly synthesised DNA is degraded (Veomert and Kuempel, 1973). The preponderance of 50kb fragments may well reflect the spacing of DNA replication forks and transcriptional machinery on the bacterial chromosome.

5.12.8 Is this really bacterial "apoptosis"?

By referring to the DNA degradation observed with ofloxacin and ciprofloxacin as "apoptosis", it must be clarified what exactly is meant by "apoptosis". As mentioned previously, apoptosis is a name given to the cellular processes associated with programmed cell death (PCD) that occur mainly during development of eukaryotes (Vaux and Strasser, 1996). Similar patterns of high molecular weight DNA fragmentation to those observed by this work have been shown to occur in many cell types stimulated to undergo apoptosis (Oberhammer *et al*, 1993). Although the concepts of DNA degradation carried

out by endo-exonucleases in response to heavy DNA damage appear identical in *E.coli* and eukaryotic organisms and the effect of such agents on bacterial DNA has been likened to apoptosis (Fraser, 1994), an actual PCD mechanism would not seem to be a well-evolved process in single-celled organisms. However evidence of PCD-like responses on *E.coli* have been reported. Firstly, some bacterial plasmids can program the death of plasmid-free segregants so that no plasmid-free cells (which would overgrow the plasmid carriers) can survive. An example of this is the previously described CcdB/ CcdA system of *E.coli* (Bernard *et al*, 1993). Secondly some type II methylases and their cognate methylases are plasmid-encoded and work as toxin-antidote pairs with the DNA methylase protecting the chromosomal DNA from endonucleolytic attack by the restriction enzyme. These restriction enzymes cleave chromosomal DNA directly whereas CcdB acts by converting gyrase into a DNA-damaging agent through the formation of DSBs.

The difference between the PCD-like responses described above and the RecBCD-mediated DNA degradation response found to occur after ciprofloxacin and ofloxacin challenge is that while both the CcdB/ CcdA and restriction enzyme /methylase systems are plasmid-mediated and have evolved as a result of the selfishness of the plasmid, RecBCD is chromosomally encoded and any evolution of this enzyme to become selfish would be antagonistic to the evolution of the whole organism. PCD responses have however been reported to occur in *E.coli*, for example if the bacteria has a "feast or famine" way of life in which self-sacrifice is common (Yarmolinsky, 1995). Yarmolinsky (1995) proposed that for bacteria to profit from the death of their own kind, they must be heterogeneous. In a clonal population of bacteria, temporal and positional variation seems to be quite normal. A variety of colonial forms exist as the

result of changes in cell density, nutrient supply, substratum surface, plasmid burden, incident variation, viral infection or the passage of time. In a moribund subpopulation it seems that starvation induces *E.coli* to become hypermutable (Rosenberg *et al*, 1994). These hypermutable individuals may go unnoticed as they may not be colony-formers. Since bacteria grow as clones, it has been suggested that a virally infected cell committing suicide for example is a pacifist strategy as by doing this, the other cells would escape infection (Shub, 1994). Therefore, although it is tempting to speculate that bacteria with DNA so badly damaged might kill themselves in a bid to protect the rest of the population, considering the intrinsic selfishness and competitiveness of bacteria, this proposal is unlikely.

5.12.9 The Molecular Implications of Mechanism A

Mechanism A had previously been identified as the bactericidal pathway common to all quinolone drugs which was somehow associated with RNA and protein synthesis. Although there had been speculation of production of a SOS-encoded toxic protein which killed the cells (Smith, 1984; Dietz *et al*, 1966), with the *in vitro* evidence that the quinolone-gyrase-DNA complex led to the formation of a barrier to RNA polymerase resulting in truncated transcripts (Willmott *et al*, 1994) it seemed more likely that it was the general act of RNA and protein synthesis that was bactericidal. Rifampicin, which inhibits RNA synthesis, and chloramphenicol, which inhibits protein synthesis, have both found to completely block the bactericidal effect of nalidixic acid. The importance of this information is the realisation of where exactly rifampicin and chloramphenicol target their effect. Rifampicin acts on the β subunit of bacterial RNA polymerase by preventing completion of the initiation phase while chloramphenicol binds to the 50S subunit of the bacterial ribosome.

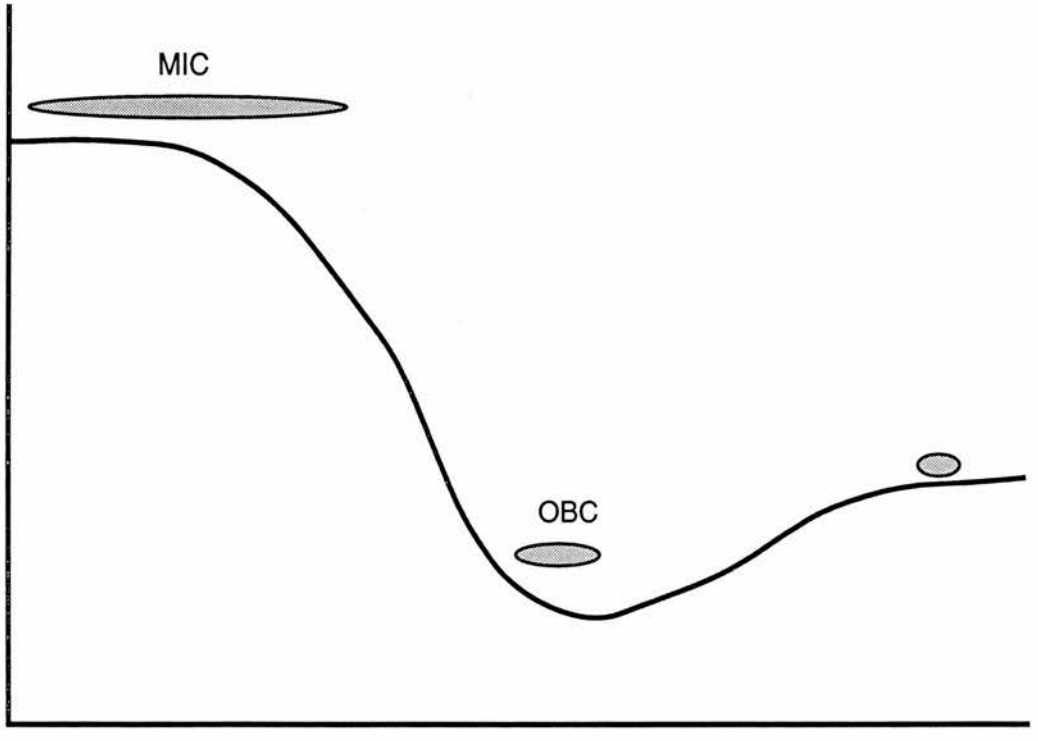
However, inhibiting protein synthesis with streptomycin for example which binds to sites on the 30S subunit of the bacterial ribosome causing an alteration in codon: anticodon recognition, was not found to inhibit the bactericidal effect (Lewin and Smith, 1989). Therefore it was concluded that it was the interruption of RNA synthesis and protein synthesis *per se* that was toxic to the cell. To verify that the truncation of RNA transcripts also occurred *in vivo* as *in vitro*, various concentrations of nalidixic acid and ciprofloxacin were added to a logarithmic culture, RNA extracted after 10 minutes, blotted and hybridisation of the blot carried out with a *gyrA* probe. It was hoped that with increasing quinolone concentration, truncated mRNAs would be more apparent as shown by a smear on the Northern blot. However, after the addition of 90mg/l nalidixic acid and above, it was hoped that no mRNA would be detected, presumably as a result of high quinolone concentrations inhibiting all the transcriptional machinery. Since DNA gyrase is present in front of the transcriptional machinery as well as at the gyrase binding sites, inhibition of the RNA polymerase-associated DNA gyrase at high quinolone concentrations would cause a drastic inhibition of RNA synthesis. However, constraints of time meant that this technique could not be optimised and only a preliminary Northern blot is shown (Figure 5.33). Use of the ECL kit to detect truncated mRNAs was an unfortunate choice as the kit is not sensitive enough to detect single copy mRNAs. By the time that this was realised and a ^{32}P-labelling procedure attempted, this experiment had to be put on hold.

The implications of this are severe for the cell as an abundance of prematurely terminated mRNAs will be translated into incorrectly folded proteins (since transcription and translation in bacteria are tightly coupled). The result would be expected to be a complete deregulation of cellular functions. By inhibiting

RNA synthesis and protein synthesis prior to quinolone challenge, it is apparent how under these conditions, the quinolones only elicit a bacteriostatic effect.

Other important mRNAs that would also be expected to be prematurely terminated are those encoding the messages for SOS-associated and repair proteins. After quinolone challenge, the induction of the SOS response is a major event, resulting in the production of proteins such as UmuC, SulA, UvrA, UvrB, RecA and LexA (Gottesman, 1984). For example, the level of SulA in the cell increases significantly. Therefore in the presence of quinolone drug, it would be expected that some of the *sulA* mRNA transcripts would be truncated especially if there is a gyrase binding site in the *sulA* gene. If this was the case, this would explain the morphology of *E.coli* cells at different concentrations of quinolone (Figure 5.35).

Figure 5.35: Morphology of *E.coli* cells at different concentrations of quinolones



At the MIC of the quinolone drug, extensive filamentation is seen with cells

appearing many times their normal length (Diver and Wise, 1986). After this point, the cells seem to be less filamented and at the OBC, a more mixed culture of some filamented cells and some not so filamented cells is apparent. Filamentation is brought about by the SulA protein which inhibits the initiation of cell division by FtsZ at the septum (Dai *et al*, 1994). The importance of SulA being SOS-induced is that when cells undergo damage, they are inhibited by SulA to divide until the damage has been repaired by the SOS response. At the OBC of quinolone drug, if some of the *sulA* mRNAs were truncated, less active SulA would be expected to be produced. Perhaps incorrectly folded SulA interferes with another protein other than FtsZ and thus causes deregulation of the cell. It is also possible that not as much SulA is produced at the OBC than at the MIC because RNA synthesis is blocked by the transcription-associated gyrase being poisoned. However since cell death at the OBC is an active process seeming to be dependent on at least some transcription and translation, the first explanation is possibly more likely.

It is unknown whether mRNA truncation occurs at certain sites, for example the gyrase binding sites (Willmott *et al*, 1994) or randomly throughout the genome depending on the position of the replication fork. It is possible that some proteins will tend to be truncated more than others depending where they are on the chromosome. Changes in the protein profiles of quinolone-challenged *E.coli* cells have previously been reported (Guan *et al*, 1992), however it is unclear whether the appearance of a quinolone-induced protein band on a SDS-PAGE gel relates to a SOS-induced protein or a constitutively expressed protein which is truncated. Since the production of truncated proteins is probably quite a random event, depending on the position of the cleavable complex, it is possibly rather difficult to detect truncated proteins on a

conventional SDS-PAGE gel. However, electrophoresis of proteins on a native gel would probably more accurately show if the proteins were correctly folded or not.

It is interesting that the proposed molecular basis of Mechanism A appears similar to the molecular basis of the action of the aminoglycoside group of antibiotics. Aminoglycosides such as gentamicin, streptomycin, amikacin, kanamycin, tobramycin and neomycin elicit their antibacterial effect by binding to sites on the 30S subunit of the bacterial ribosome, causing an alteration in codon: anticodon recognition. This results in misreading of the mRNA and hence in the production of defective bacterial proteins which is bactericidal.

Previous experiments carried out investigating the effect of streptomycin on quinolone-mediated cell death (Lewin and Smith, 1989) led to the conclusions that it was the action of streptomycin on the bacterial membrane that resulted in the synergistic effect of these two antibiotics. However, the effect of streptomycin on the bacterial membrane is actually mediated by the formation of membrane channels by misread proteins (Davis *et al*, 1986). Although it is possible that the synergistic effect of quinolones and streptomycin results from streptomycin damaging the membrane rather than the continuation of protein synthesis in general, it is also tempting to speculate that the damage induced by the quinolones (especially nalidixic acid) on the bacterial membrane (Dougherty *et al*, 1985) is also as a result of misformed proteins.

12.10 The Synergistic Effect of Mechanism A and Mechanism B on the Bacterial Cell

Antibiotics which elicit both Mechanism A and Mechanism B on *E.coli* have been observed to result in quicker killing of the cell. For example, the addition of ofloxacin or ciprofloxacin (which work by both Mechanism A and Mechanism B) at the OBC has been found to result in a 90% loss of viability in 19 minutes, compared to 52 minutes with the OBC of norfloxacin, 58 minutes with the OBC of oxolinic acid and 62 minutes with nalidixic acid (Smith, 1984; Smith, 1988). The viability studies carried out during the course of the presently described research suggested that *E.coli* TG1 was killed even quicker with cells appearing non-viable even after 5 minutes. Since nalidixic acid and oxolinic acid have been shown to result in degradation to a small degree (Lewin and Smith, 1990), it is proposed that all the quinolone antibacterials can elicit cell death by both Mechanism A and Mechanism B, however, depending on their ability to cleave and bind to DNA, one mechanism of death is probably overshadowed by the other. Addition of nalidixic acid to the cell possibly results in truncated mRNAs, misfolded proteins and deregulation of the cell by pathways previously discussed such as insertion of faulty proteins into the cell membrane. Although nalidixic acid forms a cleavable complex with gyrase and DNA, its binding to DNA can probably be repaired eventually if the cell does not die before by faulty protein deregulation. On the other hand, ciprofloxacin which is much more adept at DNA cleavage (see Table 5.1) possibly forms a much tighter cleavable complex which cannot be repaired easily, thus enzymes such as RecBCD are stimulated and result in wide scale DNA degradation. Although the formation of truncated mRNAs has been shown to occur with quinolones such as ciprofloxacin and ofloxacin (Willmott *et al*, 1994), this is probably just a

minor mechanism of cell death as the cell will die before from the production of DSBs as shown by the "apoptosis"-like response.

5.12.11 Another recently proposed model for quinolone-induced cell death

The basis for quinolone-induced cell death in *E.coli* proposed in this thesis fits in with previous observations and ideas as discussed. However another explanation for quinolone-induced cell death has recently been proposed (Chen *et al*, 1996). Although the results of the experiments performed by Chen *et al* (1996) add weight to the ideas raised by this thesis, the role of topoisomerase IV as a drug target is more strongly considered. In agreement with the results presented in this thesis, Chen *et al* (1996) propose that the inhibition of DNA synthesis is not by itself the cause of the bactericidal effect of oxolinic acid (which was used as the standard quinolone drug). Also these authors concluded that formation of the quinolone-gyrase-DNA complex as detected by SDS-dependent DNA fragmentation is not alone sufficient to kill cells as even at high concentrations, the nucleoids maintained most of their integrity. However at higher quinolone concentrations, the nucleoid structure was found to change to some degree. All of these observations are compatible with the proposals of this thesis. The formation of cleavable complexes was detected even when chloramphenicol had been added to the culture to inhibit protein synthesis (Chen *et al*, 1996). The lethal effect of oxolinic acid was suggested to result from the intracellular release of DNA ends and not simply the formation of quinolone-gyrase-DNA complexes. Chen *et al* (1996) found that treating the cells with chloramphenicol blocked the release of the free ends associated with the addition of oxolinic acid. These ideas are in keeping with the results of this thesis in that lack of protein synthesis reduces cell death to some extent with quinolones

such as oxolinic acid and nalidixic acid possibly by decreasing the number of potential lethal collisions between a cleavable complex and moving replication forks/ transcriptional machinery. Chen *et al* (1996) do not consider this option and instead propose that a repair factor, independent of the SOS response is responsible for dissociating the quinolone-gyrase-DNA complex and forming a DSB. The speculated repair factor would however have to turn over very rapidly as chloramphenicol inhibits nalidixic acid-induced lethality even when added 2 to 3 hours after the quinolone (Deitz *et al*, 1966). Such a factor has yet to be identified. Chen *et al* (1996) acknowledge the existence of Mechanism B and suggest that this chloramphenicol-insensitive cell death mechanism in *E.coli* results from the dissociation of gyrase. It is perhaps more likely that instead of the DNA gyrase enzyme dissociating, it is broken apart by the movement of DNA replication machinery and/or transcriptional machinery as suggested in this thesis. In agreement with these ideas, Chen *et al* (1996) postulate that ciprofloxacin at low concentrations behaves much like oxolinic acid at high concentrations since chloramphenicol can inhibit the bactericidal action of ciprofloxacin at low concentrations. These authors concluded that the potency or concentration of a quinolone appears to shift its mode of action from a mechanism requiring protein synthesis (Mechanism A) to one that does not (Mechanism B). Chen *et al* (1996) propose that chloramphenicol blocks the lethal effects associated with topoisomerase IV-ciprofloxacin interactions and one or more components of the SOS response eliminate the gap of time between addition of ciprofloxacin and topoisomerase IV-mediated cell death.

A difference between the size of DNA fragments generated by ciprofloxacin treatment and oxolinic acid treatment was observed (Chen *et al*, 1996) in agreement with this thesis. However, Chen *et al* (1996) conclude that this is a

result of ciprofloxacin trapping both gyrase and topoisomerase IV on the chromosome. As discussed previously, topoisomerase IV is thought to occupy different domains of the chromosome than does gyrase. Unlike gyrase, it is not present in front of the replication fork or transcriptional machinery and instead it has been localised to the bacterial membrane and the region of the chromosome opposite the origin of replication. In light of these ideas, it is unlikely that cleavable complexes are formed between topoisomerase IV, quinolone and DNA and instead the reason for the existence of the smaller DNA fragments detected after ciprofloxacin challenge possibly results from ciprofloxacin forming a tighter and therefore more lethal cleavable complex. Topoisomerase IV is believed to be a secondary target for quinolone drugs in *E.coli*, however its inhibition is bacteriostatic rather than bactericidal (Khodursky *et al*, 1995).

5.12.12 Conclusions

In conclusion, this work has revealed that an "apoptosis"-like response seems to correlate with Mechanism B after challenge of *E.coli* TG1 with concentrations of ciprofloxacin and ofloxacin at and above the OBC. This result is interesting as it confirms that the mechanism of action of prokaryotic and eukaryotic topoisomerase II-poisoning drugs is very similar. Addition of eukaryotic topoisomerase II-poisoning drugs such as VP16 and VM26 to eukaryotic cells results in the cell dying and the cell death is indicative of that typified by apoptosis. In *E.coli* as well as eukaryotes, DSBs have been demonstrated to correlate with cell death. The enzyme primarily responsible for this "apoptosis"-like effect in *E.coli* is believed to be RecBCD, however other enzymes could play a minor role. The "apoptosis"-like effect was found not to occur with three differently acting antibiotics: ampicillin, trimethoprim and streptomycin, suggesting that the "apoptosis"-like effect is related to the formation of the

gyrase-DNA-quinolone cleavable complex and furthermore, the strength of quinolone-binding to the complex. It was also shown that the quinolone antibacterials result in premature transcription termination *in vivo* in agreement with the *in vitro* results (Willmott *et al*, 1994) and it is proposed that this formation of truncated mRNA *in vivo* corresponds to Mechanism A. Other minor mechanisms possibly also contribute to quinolone-mediated cell death. Changes in membrane permeability, induction of SOS mutagenesis and death by lethal filamentation all are also believed to add to the quinolone antibacterials repertoire of armoury.

6 Conclusions

Various molecular interactions between DNA gyrase and the quinolone antibacterials have been investigated and described in this thesis. Decreased susceptibilities of clinical *Salmonella* strains have been shown to correlate to point mutations in the quinolone resistance determining region such as serine-83 to leucine, serine-83 to phenylalanine, aspartate-87 to tyrosine, aspartate-87 to asparagine and aspartate-87 to glycine when compared to the *gyrA* sequence of a standard NCTC *Salmonella* control. The results suggest a general trend towards quinolone resistance in clinical strains, particularly those isolated in India.

Experiments showed that expression of GyrA was toxic at a high copy number, possibly by disturbing the supercoiling *status quo* of the bacterial cell. By correlating expression of *gyrA* mRNA to cell viability, high expression of *gyrA* mRNA was shown to result in reduced cell numbers. This observation explained the failure of cloning *gyrA* into a high copy number vector such as pBluescript but not a medium copy number vector such as pBR322.

The effect of various quinolone antibacterials on the bacterial cell was studied at a molecular level. By examining the cellular DNA on pulsed field gels, all the quinolone drugs tested produced double stranded breaks which did not necessarily correlate to loss of viability. Potent fluroquinolones such as ciprofloxacin and ofloxacin resulted in the production of severe double stranded breaks which lead to further degradation of the DNA to fragments which were as small as 4kb. The pattern of degradation on a pulsed field gel was likened to that observed during eukaryotic apoptosis. The similarities and differences of eukaryotic apoptosis and quinolone-induced bacterial cell death were discussed

in depth including the involvement of DNA repair enzymes. However physiologically, eukaryotic apoptosis differs from quinolone-induced cell death in that the former is a controlled process with the aim of destroying the cell and removing it from the populataion, for example during development. Quinolone-induced cell death, on the other hand, is the result of an antibiotic acting on the cell culminating in cell death through double stranded break formation. The formation of severe double stranded breaks, as seen with ciprofloxacin and ofloxacin, was correlated to Mecahnism B and is a result of the high affinity of these antibiotics with DNA gyrase and DNA. The formation of truncated mRNAs and therefore truncated misfolded proteins was correlated to Mechansism A and almost definitely occurs with all quinolone drugs.

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