Resistance to Fluoroquinolones in *Escherichia coli*: Prevention, Genetics and Fitness Costs

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Abstract

Antibiotic-resistant bacteria are increasingly a major healthcare problem but very few new classes of antibiotics have been discovered or launched in recent decades. Approaches to dealing with the problem include learning how bacteria evolve to resistance and improving dosing regimens with current antibiotics so as to reduce the selection of resistant bacteria.

This thesis presents studies examining whether antibiotic dosing at high levels can prevent the selection of fluoroquinolone-resistant mutants in Escherichia coli. It also addresses the genetics of fluoroquinolone resistance in E. coli in relation to fitness costs for the resistant bacteria, and the evolution of E. coli to reduce the costs of resistance.

The mutant prevention concentration (MPC) of ciprofloxacin was measured for a set of clinical urinary tract infection E. coli strains showing that MPC could not be predicted from the minimum inhibitory concentration (MIC). Results from an in vitro kinetic model showed that an AUC/MPC >22 for ciprofloxacin was the single best pharmacodynamic index that predicted prevention of resistance emergence in the wild-type. Simulating currently approved dosing regimens for three different fluoroquinolones it was found that only a few were effective in preventing the selection of a small sub-population of pre-existing mutants.

Step-wise selection of fluoroquinolone resistance showed that the accumulation of mutations usually reduced bacterial fitness in vitro and in vivo. Systematic construction of isogenic resistant strains confirmed this result and revealed that some combinations of resistance mutations mutually compensate and increase both resistance and fitness. It was discovered that mutations altering RNA polymerase could ameliorate the fitness costs of fluoroquinolone resistance. Thus, the major fitness cost of fluoroquinolone resistance is due to defective transcription.

The finding that fluoroquinolone resistance mutations can increase resistance while mutually compensating their fitness costs, shows that resistance to fluoroquinolones can continue to evolve in the absence of antibiotic selection.

Keywords: Fluoroquinolone, Escherichia coli, Resistance, Gyrase, Topoisomerase IV, MPC, Fitness compensation, Urinary Tract Infection

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urn:nbn:se:uu:diva-7851 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7851)
Det Okända, de otrampade stigarna, sökandet efter kunskap
lockar oss vidare mot nya upptäckter och aha-upplevelser.
Om vi finge svar på allt, vore mycket så mycket mindre,
-ja, för vissa vore livet kanske till och med outhärdligt!
Per Aspera Ad Astra

Till Minne av Farfar Marcus
List of papers

This thesis is based on following papers, which will be referred to in the text by their Roman numerals:

Mutant prevention concentrations of ciprofloxacin for urinary tract infection isolates of Escherichia coli.
*Journal of Antimicrobial Chemotherapy* 2005; 55: 938-943

II) Olofsson S.K, Marcusson L.L., Komp Lindgren P, Hughes D, Cars O.
Selection of ciprofloxacin resistance in *Escherichia coli* in an *in vitro* kinetic model: relation between drug exposure and mutant prevention concentration.
*Journal of Antimicrobial Chemotherapy* 2006; 57: 1116-1121

III) Olofsson S.K, Marcusson L.L., Strömbäck A, Hughes D, Cars O.
Dose-related selection of fluoroquinolone-resistant *Escherichia coli*. Submitted

IV) Komp Lindgren P, Marcusson L.L., Sandvang D, Frimodt-Møller N, Hughes D
Biological cost of single and multiple norfloxacin resistance mutations in *Escherichia coli* implicated in urinary tract infections.
*Antimicrobial Agents and Chemotherapy* 2005; 49:6 2343-2351

V) Marcusson L.L., Hughes D.
Increased bacterial fitness selected with antimicrobial drug resistance.
Submitted

VI) Marcusson L.L., Hughes D
Fitness compensation in fluoroquinolone resistant *Escherichia coli* by mutant RNA polymerase.
Manuscript

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>Area under the serum concentration-time curve</td>
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<tr>
<td>Cfu</td>
<td>Colony forming unit</td>
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<tr>
<td>$C_{\text{max}}$</td>
<td>Maximal serum concentration</td>
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<tr>
<td>DDD</td>
<td>Defined daily doses</td>
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<tr>
<td>dTMP</td>
<td>Deoxythymidine 5’-phosphate</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EARSS</td>
<td>European Antimicrobial Resistance Surveillance System</td>
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<tr>
<td>FQ</td>
<td>Fluoroquinolones</td>
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<tr>
<td>HGT</td>
<td>Horizontal gene transfer</td>
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<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<tr>
<td>MPC</td>
<td>Mutant prevention concentration</td>
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<tr>
<td>MSW</td>
<td>Mutant selective window</td>
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<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards Institute (previously NCCLS)</td>
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<tr>
<td>OMP</td>
<td>Outer membrane porin</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PMF</td>
<td>Proton motive force</td>
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<tr>
<td>PD</td>
<td>Pharmacodynamics</td>
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<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAP</td>
<td>Ribonucleic acid polymerase</td>
</tr>
<tr>
<td>SRGA</td>
<td>Swedish reference group for antibiotics</td>
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<tr>
<td>$T_{1/2}$</td>
<td>Elimination half-life</td>
</tr>
<tr>
<td>$T&gt;MIC$</td>
<td>Time over MIC</td>
</tr>
<tr>
<td>$T&gt;MPC$</td>
<td>Time over MPC</td>
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<tr>
<td>QRDR</td>
<td>Quinolone resistance determining region</td>
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INTRODUCTION

The story of microorganisms and the discovery of antibiotics

As its name indicates, contagion is an infection that passes from one thing to another. The infection is precisely similar in both the carrier and the receiver of the contagion; we say the contagion has occurred when a certain similar taint has affected them both.

So, when a person die of drinking poison, we say perhaps that they were infected, but not that they suffered contagion; and in the case of things that naturally go bad when exposed to the air, such as milk, meat, etc., we say that they have become corrupt, but not that they have suffered contagion, unless indeed the air itself has also become corrupt in a precisely similar way.

Girolamo Fracastoro, 1546

These thoughts and observations might have been the starting point of the understanding of infectious bacteria (the contagion) and their relation to disease. The observation of small ‘animalcules’ by Anton van Leeuwenhoek almost 200 years later also gave a glimpse into the invisible world of microorganisms and encouraged the development of microbiology. The pioneers who followed, during the golden age of microbiology, included among others, Pasteur and Koch. Their work was incredibly important in leading to our understanding of microbiology and its relationship to infections. Pasteur’s proposal of the germ theory of disease provided a satisfying explanation for the origin of many human illnesses, and Koch’s postulates provided a rigorous analytical framework for future investigations into the causes of infectious disease.

In 1909 a new era began with the discovery in Paul Ehrlich’s lab of the sulfa drug. This was probably one of the most important medical discoveries of the period and encouraged the search for other anti-infective agents paving the way for the discovery of the chemotherapeutic agents and antibiotics we use today. The discovery made by Alexander Fleming two decades later that Penicillium notatum had an inhibitory effect on staphylococci would come to save the lives of many millions of people, but not until given impetus by the outbreak of World War II. The success of penicillin influenced many other scientists and pharmaceutical companies to discover and develop new classes of antibiotics over the following years (Brock, 1999).
Antibiotics and their targets

Antibiotics can be divided into different classes according to their mechanism of action (Figure 1). Antibiotics target vital mechanisms within the bacterial cell including cell wall formation, folic acid metabolism, DNA replication, RNA transcription, and protein synthesis.

β-lactam antibiotics are by volume of use the single most important group and inhibit cell wall formation by blocking the cross-linking of carbohydrates in the peptidoglycan layer (Waxman and Strominger, 1983). The trimethoprim and sulfonamides were the first antimicrobials drugs developed and they target the folic acid metabolism pathway which is involved in production of the essential coenzyme tetrahydrofolate and the synthesis of dTMP and purines. DNA replication is targeted by the fluoroquinolones, a group of synthetic antimicrobial drugs which interfere with DNA gyrase and topoisomerase IV. These enzymes are required for the unwinding of DNA during chromosome replication and transcription by the introduction of negative supercoils (Marians and Hiasa, 1997). RNA polymerase which transcribes RNA from DNA is inhibited by the binding of rifampicin to the polymerase (Hartmann et al., 1967). After transcription, messenger RNA’s are translated into proteins by the translation machinery which includes the ribosome and its many interacting factors. A great many antibiotics target the protein synthesis machinery. Among these, the macrolides, like erythromycin, binds to the peptide exit tunnel of the 50S ribosomal subunit, inhibiting further protein synthesis. Aminoglycosides have their mode of action by irreversibly attaching to the 30S subunit of the ribosome, thereby inhibiting protein synthesis (Waksman, 1965). Tetracyclines also interfere with the translation machinery by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site in the 30S subunit of the ribosome.

Figure 1. Some commonly used antibiotics and their mode of action.
Antibiotic resistance development

Evolution of resistance

Resistance towards antibiotics is not a new phenomenon; it has existed in nature a long time before the word was coined as a problematic concept in our era. Antibiotics are natural compounds and microorganisms in nature produce by themselves almost all of the different antibiotic classes known (Datta and Hughes, 1983). Even though the resistance phenomenon towards these “natural” antibiotics is not new per se, the increased prevalence of resistance among infectious bacteria towards antibiotics used in human medicine during the last couple of decades is alarming. Analysis of the “Murray collection” of bacterial strains supports the conclusion that resistance development has increased drastically because of antibiotic use and misuse over the last sixty years. Thus, clinical strains collected between 1917 and 1952 did not have any significant resistance towards antibiotics used today (Datta and Hughes, 1983). The forces that drive and select resistance evolution can be divided into four main categories; (i) spontaneous mutations and mutator strains within the bacterial population that generate new variants, (ii) horizontal gene transfer between different strains that spread resistance genes, (iii) phenotypic tolerance of some bacteria to antibiotics that prevents eradication, and (iv) antibiotic selective pressure that drives the selection of resistant variants in the population.

Spontaneous mutations and mutator bacteria

Mutations play an important role in the evolution process because they generate new variants of existing genes. Mutations are also a potential threat to the organism’s viability because they may reduce fitness. All organisms are under periodic environmental pressure to change and adapt, and new mutations can provide solutions to these pressures. But making the wrong changes can be lethal and to maintain genetic stability there are many repair and accuracy systems to limit the accumulation of mutations. There is a fine balance between the accumulation of adaptive changes within the genome and the risk of loss of fitness associated with new mutations. The average spontaneous mutation rate in bacteria is $10^{-9}$-$10^{-10}$ mutations per base pair per generation (Andersson and Hughes, 1996; Drake et al., 1998).

Mutator strains are genetic variants within the population that exhibit a higher mutation rate than normal cells, usually because they lack one or more of the systems that support the high fidelity of DNA replication, or the repair of mismatch of mutations in the double helix. Mutator bacteria are always present at a low frequency in bacterial populations due to spontaneous mutagenesis affecting repair and fidelity genes. Mutators can vary in their mutation rate dependent on their mutator genotype (Baquero et al.,
One of the strongest mutator phenotypes involves knockout mutations affecting the methyl-directed mismatch repair system. Mutations within the *mutHLS*, *uvrD* and *dam* genes that are a part of this system result in up to a $10^3$-fold increase in mutation rate compared to the wild-type (Komp Lindgren *et al.*, 2003; LeClerc *et al.*, 1996; Matic *et al.*, 1997; Modrich, 1989, 1991). When bacteria are exposed to different environments e.g. antibiotic selective conditions, a strain with a mutator phenotype can have a selective advantage over non-mutator strains and become enriched during selection (Mao *et al.*, 1997; Miller *et al.*, 2004). Since mutators are more likely to generate adaptive mutations that will be beneficial in a selective environment. They will reshape the population and hitchhike with the fitter phenotype which outcompetes the pre-existing population. As expected, mutators accelerate antibiotic resistance development in vitro (Schaaff *et al.*, 2002), and this was also seen in vivo when patients were treated with high levels of antibiotics (Gustafsson *et al.*, 2003b). In a recent study when a collection of fluoroquinolone-resistant *Escherichia coli* (E. coli) urinary tract infection (UTI) strains were investigated, a positive correlation was found between the number of mutations conferring resistance to fluoroquinolones and the relative increase in mutation rate (Komp Lindgren *et al.*, 2003). It has also been showed that even weak mutators can drive the evolution of fluoroquinolone resistance in *E. coli* (Örlen and Hughes, 2006).

Extra chromosomal transfer of resistance

Horizontal gene transfer (HGT) plays an important role in the evolution of bacteria. It has been shown that bacterial genomes are genetic mosaics due to the acquisition of exogenous DNA and its incorporation into the chromosome (Blattner *et al.*, 1997). Resistance mechanisms can be transferred by HGT between microorganisms by transformation or via vectors such as plasmids, transposons and bacteriophages. Plasmids provide a versatile and efficient means for HGT of resistance genes and many plasmids carry systems that promote their transmission to other bacterial cells. Plasmids can replicate and maintain themselves in a host cell, they can integrate into the bacterial chromosome, and sometimes when they excise they take chromosomal genes with them (Bryan, 1988). Plasmids often carry integrons which are assemblies of multiple resistance genes which are transferred between cells together with the plasmid, but can also be inserted into the chromosome by recombination (Recchia and Hall, 1997). Transposons can also contain a variety of resistance genes, but in contrast to integrons they are responsible for their own transfer and integration into the bacterial chromosome or plasmid genome.
Antibiotic tolerance

There are genetic factors within the bacterial genome that by themselves do not increase resistance to antibiotics but can ensure that a certain proportion of the bacterial population is resistant. For example, the tolerance phenomenon enables a small fraction of cells to survive despite the presence of antibiotics, e.g. the cell wall alterations facilitating β-lactam resistance (Novak and Tuomanen, 1999; Tuomanen et al., 1991). Accumulating evidence suggests that antibiotic tolerance, or ‘persistence’, results from heterogeneity in bacterial populations and the ability of some bacteria to enter into a dormant (non-dividing) state (Dhar and McKinney, 2007; Lewis, 2007).

Antibiotic selective pressure

When antibiotics are used in treatment of pathogenic strains there will be a selection pressure for antibiotic resistance both in the bacterial population within the person being treated and in the bigger human population consuming the antibiotic. Quantitative models and correlations between antibiotic usage and resistance support the conclusion that the volume of drug use in a society is an important factor in resistance development (Austin et al., 1999). Other factors include the inappropriate use of antibiotics, for example, antibiotic dosing at concentrations that select for the growth of resistant bacteria. In addition, the co-selection of different resistance determinants due to genetic linkage or cross-resistance will be factors driving resistance development. Under gradual antibiotic pressure bacteria will adapt by genetic changes that enable its survival in specific environments. For example, when antibiotics are used in therapy, concentrations can vary in different body compartments and may not eradicate the complete bacterial population; concentration variations like this will rather create an environment suitable for the selection of a stepwise increase in resistance (Austin et al., 1998; Baquero et al., 1993, 1997). Low-level resistant bacteria may then survive and continue to evolve and become high-level resistant.
Mechanisms of resistance

Bacteria become resistant to antibiotic drugs by decreasing the effective drug concentration at the drug target. This can be achieved in three different ways: by reducing the interaction between the drug and the drug target (by target modification or target protection); by reducing the concentration of drug in the cell (by decreased influx or increased efflux); or by modification or inactivation of the drug (Figure 2).

Reduced interaction by target modification and protection

Antibiotics are usually developed to bind to and inhibit a specific target. This target can become directly altered by mutations that reduce the affinity of the antibiotic to its binding site. The consequence is thereby a decrease in the effective concentration of the drug at the target. An alternative mechanism to reduce the interaction between the drug and the target is the production by the bacteria of a protein product that protects the target from the antibiotic. Recently a plasmid-encoded resistance protein QnrA, was shown to protect DNA gyrase and topoisomerase IV against fluoroquinolone binding (Tran and Jacoby, 2002; Tran et al., 2005). Resistance associated with chromosomal mutations is usually inherited vertically whereas plasmid-borne (or transposon-associated) resistance determinants such as QnrA can more easily be transmitted horizontally between bacteria.

Reduced accumulation of antibiotics by decreased influx or increased efflux

Bacterial outer membrane proteins (OMPs) play a major role in controlling the diffusion of ions and different small molecules into and out of the bacterial cell (Benz and Bauer, 1988). Mutations or deletion of OMPs could create an obstacle for the entrance of certain antibiotics. It has been shown that some porin–deficient strains are more resistant towards tetracyclines, chloramphenicol and certain β-lactam antibiotics than porin-sufficient strains (Harder et al., 1981).

Antibiotics can be exported from the cell by efflux pumps which are driven either by ATP hydrolysis or by the proton motive force (PMF). There are five different classes of bacterial efflux pumps, The first class, called the ATP-binding cassette (ABC) superfamily, includes both specific and multidrug-transporters (MDR). The remaining four classes are included in the PMF dependent family. These are the Small Multidrug Resistance Family (SMR), the Major Facilitator Superfamily (MFS), the Multidrug and Toxic Compound Extrusion Family (MATE), and the Resistance/Nodulation/Cell Division Family (RND). One of the best characterized efflux pumps in E.coli belongs to the RND family and is made of the multiprotein transmembrane complex AcrAB-TolC. The AcrAB-TolC efflux pump is active in effluxing
many different drugs from *E. coli*, including fluoroquinolones, tetracyclines and chloramphenicol.

*Modification and inactivation of the drug*

Chemical modification of an antibiotic by enzyme activity can result in its inactivation. Antibiotic resistance by enzymatic modification is usually specific for a single drug class, e.g. β-lactamases act on β-lactam antibiotics, chloramphenicol acetyltransferases act on chloramphenicol, and aminoglycoside acetyltransferase act on aminoglycosides. The β-lactamases work by opening the β-lactam ring thereby inactivating the antibiotic before it reaches its target (Livermore, 1995). An enzyme that modifies fluoroquinolones has recently been identified in gram-negative bacteria (Robicsek *et al.*, 2006). This is a variant of an aminoglycoside-modifying enzyme (*aac (6')-Ib*) that has acquired the ability to acetylate and reduce the activity of certain fluoroquinolones.

*Figure 2. Different resistance mechanisms of the bacterial cell.*
Spread of antibiotics in society and the increasing resistance problem

Antimicrobial drug resistance presents an ever-increasing global public health threat that involves all major microbial pathogens and antimicrobial drugs. This is, at least in part, a consequence of the widespread misuse of antibiotics within the community over the last couple of decades. It has been estimated that at least 40% of all antibiotics prescribed in the industrialized countries of the West seem to have an incorrect basis; being, for example, prescriptions for viral infections (Wise et al., 1998). The usage of antibiotics in many countries without any prescription is also a problem that increases the rate of resistance development (Cars and Nordberg, 2004).

Even countries with very strict controls in place have a problem with increased antibiotic selective pressure. For example, in the five year period between 2000-2005, the use of antibiotics increased by 13% measured in defined daily doses (DDD)/1000 inhabitants within hospitals in Sweden. The most common antibiotics used were cephalosporins, penicillins, tetracyclines and fluoroquinolones (Cars and Olsson Liljequist, 2005). In Europe as a whole, the number of countries with a frequency of fluoroquinolone resistant E.coli above 25% doubled from 2004-2005, and now comprises six countries. Only four countries among the 28 surveyed maintained fluoroquinolone resistance levels at or below 5% (EARSS).
*Escherichia coli* and Urinary Tract Infections

The work of this thesis has been performed with *Escherichia coli* as the model organism (*Figure 3*). The gram-negative rod-shaped bacteria *E. coli* belongs to the *Enterobacteriaceae* family. In addition to being the causative agent of several diseases, *E. coli* is also one of the best characterized microorganisms.

*E. coli* is a member of our intestinal commensal flora. However, some strains of *E. coli* are pathogenic and cause diarrhea, gastroenteritis, sepsis and (UTI) (Kaper *et al.*, 2004). *E. coli* is responsible for 80% or more of uncomplicated UTI. UTI are usually classified according to their syndromes: (i) asymptomatic bacteriuria; where there is growth of bacteria in the urine but without any general symptoms, (ii) cystitis; where the infection is restricted to the bladder, (iii) acute pyelonephritis; refers to the inflammation of the renal pelvis and the kidneys. Pathogenic strains that are able to colonize the small intestine and urethra contain specific adherence factors that are very important for their virulence, e.g. type 1 fimbriae that are especially important in the colonization of the bladder (Johnson, 1991). Several toxins, such as haemolysin, cytotoxic necrotizing factors and proteases are also produced in different amounts varying between different uropathogenic *E. coli* (Johnson and Stell, 2000).

*Figure 3. Electron scanning micrograph of* Escherichia coli.*

(Kindly provided by Leif Ljung)
Fluoroquinolones and their targets

Fluoroquinolones

This group of antibiotics are analogs of nalidixic acid which was initially isolated as a bi-product of anti-malarial research (Lesher et al., 1962; Neu, 1987). The chemical development of the fluoroquinolones (FQ) has been very active, with both second (e.g. norfloxacin, ciprofloxacin) and third (e.g. levofloxacin, gatifloxacin and moxifloxacin) generations of the drugs being used clinically (Figure 4). These antimicrobials have broad spectrum activity and very good pharmacokinetic properties such as relatively long half-lives due to slow elimination, good distribution into body compartments and fluids as well as good penetration into human cells.

Figure 4. Chemical structures for three fluoroquinolones

Gyrase and topoisomerase

DNA gyrase and topoisomerase IV are bacterial type II topoisomerases. Type II topoisomerases alter DNA supercoiling in a reaction that involves making a double-strand break in the DNA, in contrast to type I topoisomerases (e.g. topoisomerase I) that make single-stranded cuts in DNA. DNA gyrase and topoisomerase IV are essential enzymes in the bacterial cell. DNA gyrase introduces negative supercoils in DNA using the energy of ATP hydrolysis, whereas topoisomerase IV counters this activity by relaxing negative supercoils. Two other activities of topoisomerase IV are unknotted DNA and decatenation, for example at the end of chromosome replication (Figure 5). DNA gyrase is a tetramer composed of two GyrA and two GyrB subunits encoded by gyrA and gyrB, respectively. GyrA is involved in the double-strand breakage and reunion reaction, whereas GyrB is responsible for the ATPase activity of the enzyme. The introduction of negative supercoiling by DNA gyrase is important for the binding of DnaA initiator protein to the replication origin of E. coli, and also for facilitating the bi-directional progression of the replication forks around the chromosome (Postow et al., 2001). Topoisomerase IV has about 40% amino acid homology with DNA
gyrase and is also a tetramer with two ParC and two ParE subunits. Both enzymes have also been reported to function in the initiation and progression of RNA polymerase by modulating the superhelicity of the DNA and relieving the positive tension that builds up in front of the transcription machinery (Figure 5) (Liu and Wang, 1987; Nöllmann et al., 2007; Peter et al., 2004; Wu et al., 1988).

Figure 5. Illustration of DNA gyrase and topoisomerase IV involvement in DNA replication and transcription.

Drug and target interaction
Fluoroquinolones act by making a ternary complex of the drug, topoisomerase and DNA, that remains bound on the cut DNA, thereby inhibiting further replication and bacterial growth. The cleavage reaction is initiated by gyrase first wrapping the DNA around itself and then making a double cut of the two strands. The DNA binds at two recognition helixes of the GyrA dimer whereupon the drug then gets bound, one drug molecule at each recognition helix, since there are probably two quinolones per complex created. It has been suggested that the gyrase, quinolone, and DNA complex forms before cleavage of the DNA occurs, and that it is stabilized even more after cleavage (Kampranis and Maxwell, 1998). In gram-negative bacteria the preferred target for nalidixic acid and ciprofloxacin binding is the DNA gyrase before topoisomerase IV. In gram-positives e.g. Staphylococcus aureus and Streptococcus pneumoniae the primary target has been shown to be topoisomerase IV (Munoz and De La Campa, 1996; Ng et al., 1996; Pan et al., 1996; Pan and Fisher, 1996, 1999) In contrast, when sparfloxacin was tested against S. pneumoniae, mutations in gyrA appeared as first-step mutations
while second-step mutations appeared in the grlA gene (parC) (Pan and Fisher, 1997). This suggests that structural differences between fluoroquinolones may be responsible for the preferred target binding of either DNA gyrase or topoisomerase IV.

**Effects on both DNA replication and RNA transcription**

DNA synthesis is inhibited within minutes after formation of the DNA:gyrase:drug complex (Fournier *et al.*, 2000; Snyder and Drlica, 1979), since the complex forms immediately in front of the replication fork (Drlica *et al.*, 1980). Ternary complex formation is a reversible event (Goss *et al.*, 1965), whereas the release of broken DNA from the complex is irreversible (Krasin and Hutchinson, 1977). Bacteriostatic concentrations of oxolinic acid trap gyrase and block DNA synthesis without releasing broken DNA from gyrase-DNA complexes. Release of DNA occurs only at higher bactericidal oxolinic acid concentrations (Chen *et al.*, 1996; Snyder and Drlica, 1979).

RNA transcription is also affected by formation of the DNA:gyrase:drug complex. Purified gyrase in complex with ciprofloxacin on DNA has been shown to block the movement of RNA polymerase, but at a slower rate than inhibition of DNA synthesis (Goss *et al.*, 1965; Willmott *et al.*, 1994). Termination of transcription seems to happen 20 bp upstream of the complex in *E. coli* (Willmott *et al.*, 1994). Quinolone-mediated inhibiton of transcription seems to be bacteriostatic (Drlica and Malik, 2003).

There is a clear distinction between the bacteriostatic and lethal effects of the fluoroquinolones since the formation of the DNA:gyrase:drug complex only inhibits the growth of bacteria whereas it is the release of DNA breaks from the complex that results in cell death (Chen *et al.*, 1996). Different fluoroquinolones seems to have different bacteriostatic or bactericidal effects. Compounds that contains a C8-methoxyl group has been shown to be particularly lethal, and incubation of wild-type cultures on agar containing C8-methoxyl fluoroquinolones produced no resistant mutants (Zhao *et al.*, 1997). Even though bacterial strains might give similar MIC values, different compounds could be different in their lethal ability (Goldman *et al.*, 1996; Morrissey and George, 2000). Some compounds will trap single-strand breaks or cause release of pairs of single-strand breaks easier than others (Chen *et al.*, 1996; Krasin and Hutchinson, 1977; Snyder and Drlica, 1979).
Fluoroquinolone resistance

Resistance toward fluoroquinolones in gram-negative bacteria is the result of a multistep selection event. Lowered susceptibility towards fluoroquinolones is associated with (i) alterations within the topoisomerases, (ii) protection of the target from drug attack, (iii) changes in proteins responsible for intracellular drug concentration, and (iv) drug modification.

Target mutations

Resistance towards fluoroquinolones seems to primarily involve alterations within the genes of the drug targets, DNA gyrase and topoisomerase IV, being gyrA, gyrB and parC, parE respectively. It has been shown that DNA gyrase is the primary target for fluoroquinolones in E.coli and that mutations within its genes reduce the susceptibility towards the drug (Drlica et al., 1980; Heaton et al., 2000). GyrA resistance mutations map in a region called the quinolone resistance-determining region (QRDR) which encodes amino acids 51 through 106 (Friedman et al., 2001; Yoshida et al., 1990). Mutations in parC and parE have no effect on resistance in E. coli by themselves but rather in combination with mutations in gyrA and gyrB (Breines et al., 1997; Khodursky et al., 1995) and (Paper V).

Target protection

Until recently fluoroquinolone resistance was thought only to be acquired by chromosomal alterations. Plasmid mediated resistance towards nalidixic acid in Shigella dysenteriae had been reported, but not confirmed (Munshi et al., 1987). However, in 1998, Martinez-Martinez and co-workers found a multiresistance plasmid, pMG252, in a clinical isolate from Klebsiella pneumoniae which could be transferred between isolates and which when transferred to E.coli conferred a low level of resistance and led to the generation of high-level fluoroquinolone resistant mutants at 100 times the frequency of a plasmid-free strain. The gene responsible for the increase in resistance towards fluoroquinolones was designated qnr (Martinez-Martinez et al., 1998).

Qnr is a member of the pentapeptide repeat family and protects DNA gyrase and topoisomerase IV from fluoroquinolone binding (Tran and Jacoby, 2002; Tran et al., 2005). Jacoby et al. recently found a new qnr gene (qnrB) that seems to have the same protective action, but has less than 40% amino acid identity to the original qnrA gene. Another qnr gene, qnrS, has also recently been found in a plasmid from a Shigella flexneri isolate (Hata et al., 2005). Yet another member of this pentapeptide repeat family has been shown to have a DNA-like structure in Mycobacterium tuberculosis and it apparently mimics DNA as a substrate for DNA gyrase (Hegde et al., 2005).
**Influx and Efflux mediated resistance**

The *mar* (multiple antibiotic resistance) locus (*Figure 6*) is part of a regulon that has been shown to be involved in controlling the level of fluoroquinolone resistance because when its repressors are mutated resistance levels to fluoroquinolones and many other drugs increase (Cohen *et al.*, 1988a; Cohen *et al.*, 1988b; Cohen *et al.*, 1989; Okusu *et al.*, 1996; Piddock *et al.*, 1991). The *mar* regulon also controls genes that are involved in cellular metabolism, physiology and virulence (Barbosa and Levy, 2000; Pomposiello *et al.*, 2001).

The *marRAB* locus consists of an operator, from which two different transcripts can be made, namely; *marC* and *marRAB* (*Figure 6*). MarR act as a negative regulator of the operon, whereas MarA is a positive transcriptional activator, and when overexpressed it will result in multiple antibiotic resistance involving the expression of at least 60 different genes in *E.coli* (Barbosa and Levy, 2000; Pomposiello *et al.*, 2001). This upregulation includes the transcription of *acrA* and *tolC*, which are components of the AcrAB-TolC efflux pump. MarA is also involved in the downregulation of the gene for the outer membrane protein, OmpF, through which hydrophilic substances enter the cell (Cohen *et al.*, 1988b; Cohen *et al.*, 1989).

Fluoroquinolone resistant clinical isolates have often been associated with a multiple antibiotic resistance phenotype. This is due to the decreased intracellular drug concentrations achieved by the combined effects of decreased expression of the OmpF porin and overexpression of the AcrAB-TolC efflux pump (Cohen *et al.*, 1988b; Okusu *et al.*, 1996). The overexpression of this efflux pump is also associated with organic solvent tolerance (OST) in *E.coli* (Wang *et al.*, 2001). Data has shown that highly fluoroquinolone resistant *E.coli* exhibit OST up to 30% more than sensitive *E.coli* strains (Kern *et al.*, 2000; Oethinger *et al.*, 1998). There was a good correlation between the OST phenotype and the presence of mutations within the *marOR* and *acrR* genes in a set of clinical UTI *E.coli* that were fluoroquinolone resistant (Komp Lindgren *et al.*, 2003).
Drug modification
Enzymes that degrade fluoroquinolones have been found only in fungal systems (Martens et al., 1996; Wetzstein et al., 1997; Wetzstein et al., 1999). However, recently it has been shown that a variant of the aminoglycoside acetyltransferase; (aac (6')-Ib) called aac (6')-Ib-cr modifies not only aminoglycosides but also fluoroquinolones, resulting in reduced fluoroquinolone activity (Robicsek et al., 2006).
Persistant bacteria

When a bacterial population is growing exponentially and is then exposed to bactericidal concentrations of antibiotic, the expectation is that the population should decrease until all bacteria are dead. However, it has been shown that a small fraction of the population often remains alive. This phenomenon has been called phenotypic tolerance (Balaban et al., 2004; Miller et al., 2004; Wiuff et al., 2005). The phenomenon shows that a bacterial population that is genetically homogeneous can be physiologically heterogeneous in relation to its antibiotic susceptibility. Persister cells of *Staphylococcus aureus* were isolated already in the 1940’s when exposed to penicillin (Bigger, 1944). When *E.coli* was exposed to increasing levels of ciprofloxacin over time a surviving population of $10^{-7}$-$10^{-8}$ was isolated and the persisters were not altered in their susceptibility to the drug (Marcusson et al., 2005; Olofsson et al., 2006). There are many different suggestions about what underlies this tolerance phenotypes. An interesting observation is that there is cell-to-cell variation in growth rate within a genetically homogenous population, and that the cells that have the slower growth rate have an increased resistance towards ampicillin exposure (Balaban et al., 2004). Induction of the SOS-response has also been shown to be involved in the disturbance of cell wall synthesis and growth, thereby protecting *E.coli* from ampicillin (Miller et al., 2004). Mutations within the *hipA* and *hipB* genes have been associated with the enhanced probability of survival of *E.coli* in the presence of β-lactams and fluoroquinolones (Moyed and Bertrand, 1983; Scherrer and Moyed, 1988; Wolfson et al., 1990). The toxin-anti toxin systems including HipAB and RelE can be upregulated and overexpressed giving a high toxin concentration in cell, leading to the shutdown of multiple drug targets, thereby preventing the drug from inactivating the target molecules (Keren et al., 2004). A majority of infections are associated with bacterial biofilms where the survival of persisters has been shown to play a significant roll (Lewis, 2005). The importance of persisters in clinical infections has been mathematically simulated in relation to dosing regimens, showing that phenotypic tolerance could prevent clearance of the infection by the antibiotic (Wiuff et al., 2005).
The biological cost of being resistant

Antibiotic resistance is frequently associated with a biological fitness cost associated with the resistance mutations acquired. Since antibiotics usually target essential genes and functions within the cell, mutations within these genes, will most often negatively affect bacterial metabolism. Biological costs reduce the growth fitness of bacteria, a parameter that can be measured by competition experiments between clonally or isogenic related susceptible and resistant strains in vitro or in vivo.

There are many examples of antibiotic resistance mutations reducing the fitness of bacteria. Fusidic-acid resistant *Staphylococcus aureus* containing mutations in EF-G which is involved in translation have reduced fitness both in vitro and in vivo (Nagaev et al., 2001). This is also true for fusidic acid resistant and streptomycin resistant *Salmonella typhimurium* measured in vitro and in vivo (Björkman et al., 2000). Similarly, rpoB mutations conferring resistance to rifampicin in *E.coli*, and rpsL mutations conferring resistance to streptomycin in *E.coli* and *S. typhimurium* reduce bacterial fitness both in vitro and in vivo (Björkman et al., 2000; Levin et al., 2000; Reynolds, 2000). In a competition experiment made on human skin, fusidic acid resistant *Staphylococcus epidermis* with fusA mutations had reduced fitness relative to an isogenic antibiotic-susceptible strain (Gustafsson et al., 2003a).

However, there are also some antibiotic resistance mutations that seem to have relatively low fitness costs for the bacteria. For example, certain mutations in rpsL in *E.coli*, *S. typhimurium* and *Mycobacterium tuberculosis*, some gyrA and parC mutations in *Streptococcus pneumoniae*, and some mutations within katG in *M. tuberculosis* have each been shown to cause no measurable reduction in growth rate (Björkman et al., 1998; Björkman et al., 1999; Gillespie et al., 2002; Pym et al., 2002; Sander et al., 2002).

There are very few experimental studies concerning the biological fitness effects on *E.coli* of fluoroquinolone resistance. However, some recent studies showed that the accumulation of fluoroquinolone resistance mutations in *E.coli* was associated with a large reduction in biological fitness both in vitro and in vivo (Gualco et al., 2007; Komp Lindgren et al., 2005).
Fitness compensation

An antibiotic-resistant mutant has a beneficial advantage in an antibiotic-containing environment, and the mutant genotype will prevail and increase in frequency in the population, even though it might have a decreased fitness compared to the antibiotic-susceptible parent in an antibiotic-free environment. However, if the antibiotic selection is removed from the environment this mutant genotype would be at a disadvantage in competition with the susceptible wild-type strain, and might be driven to extinction.

There are two evolutionary pathways that could lead to the survival of the resistant strain: (i) reversion to its ancestral genotype, i.e. reverting to antibiotic susceptibility (ii) the acquisition of compensatory mutations that would ameliorate the fitness loss associated with resistance while maintaining the resistance phenotype (Björkman et al., 1998; Reynolds, 2000). The first pathway has a low probability, since a reversion would usually require a single specific nucleotide substitution to return to its original antibiotic-sensitive state. The second evolutionary route is more likely in most cases since the genetic target for a compensatory mutation that would give an increased fitness is likely to be larger. How frequent and restorable the fitness compensation might be depends on the environmental factors.

Compensatory evolution has been observed both in vitro (Björkman et al., 1998; Björkman et al., 2000; Johanson et al., 1996; Johnsen et al., 2002; Levin et al., 2000; Maisnier-Patin et al., 2002; Nagaev et al., 2001; Reynolds, 2000; Schrag et al., 1997), in vivo (Björkman et al., 2000; Johnsen et al., 2002; Nagaev et al., 2001) and in clinical situations (Björkholt et al., 2001; Nagaev et al., 2001). Streptomycin resistant rpsL mutants in S. typhimurium were shown to be compensated by different mutations in ribosomal proteins S4, S5 and L19 (Björkman et al., 1999; Maisnier-Patin et al., 2002). Studies both in S. typhimurium and S. aureus showed that compensated mutants became both partly and fully restored in their fitness without effecting their high level of resistance (Björkman et al., 1998; Björkman et al., 2000; Nagaev et al., 2001). The same compensatory pattern was seen with rifampicin resistant E.coli and in clinical isoniazid resistant M.tuberculosis (Reynolds, 2000; Sherman et al., 1996). Compensatory evolution will tend to stabilize antibiotic resistance in a bacterial population (Björkman et al., 2000; Levin et al., 2000).
Susceptibility versus resistance

Antibiotic susceptibility is defined and usually measured by the antibiotic concentration that it required to have an effect on the specific organism. The bacterium is considered susceptible if the agent restricts bacterial growth. In contrast, if the agent does not restrict bacterial growth, the bacteria can be considered to be resistant. Bacteria may be inherently resistant to certain antibiotics, for example because the drug fails to enter the bacterial cell, or they may acquire resistance, for example by mutation or HGT of resistance genes.

Dosing regimens: pharmacokinetics and pharmacodynamics

Pharmacological parameters of antibiotics are most important to consider in respect to their in vivo activity and their administration to clear infections (Figure 7). Dosing regimens are usually based on the pharmacokinetics and the pharmacodynamics of the antibiotic. Pharmacokinetics (PK) involves the absorption, distribution and elimination of the drug. Pharmacodynamics (PD) shows the relationship between serum concentrations of the drug and the pharmacological and toxicological effects of the drug. After a drug has been administrated it will initially reach a maximum serum concentration (C_max) within the body. This concentration gradually falls as the drug is metabolised or excreted. The area under the serum concentration-time curve (AUC) represents the total amount of drug exposure in the body for a particular dosing regimen.

The Minimum inhibitory concentration - MIC

The aim of antibiotic therapy is to eradicate or inhibit growth of the infecting bacteria. The concentration of drug needed to inhibit the growth of bacteria in vitro is called the Minimum Inhibitory Concentration (MIC). The definition is set as the lowest concentration needed to inhibit visible growth in liquid media or on solid media under standardized conditions. These conditions are set by national or international organizations such as the CLSI in the USA, and the SRGA in Sweden. Another parameter that can correlate with success in antibiotic treatment is the amount of time the antibiotic concentration remains over the MIC so as to eradicate the infection (T>MIC). It is important to maintain antibiotic serum concentrations above the MIC value to eliminate re-growth of the bacteria (Craig, 1998).
Mutant prevention concentration – MPC
Mutant Prevention Concentration (MPC) dosing is a relatively new concept that has been introduced to prevent the selection or enrichment of drug resistant mutants during therapy. The MPC is the lowest drug concentration that inhibits growth of the least-susceptible single-step mutant in a large \((10^{10} \text{ cfu})\) bacterial population.

**AUC/MIC**
Fluoroquinolones exhibit concentration-dependent killing kinetics and the AUC/MIC ratio is a PK/PD parameter that shows a good correlation with bactericidal efficacy (Craig, 1998). The relationship between the AUC/MIC and the eradication of the infection has been calculated for different antibiotics. Studies have shown that an AUC/MIC value of \(\geq 125\) for intravenous ciprofloxacin was associated with a satisfactory outcome for seriously ill patients (Forrest et al., 1993). In contrast, levofloxacin seemed more effective requiring a lower AUC/MIC value of \(~100\) to clear the infection (Craig, 1998).

**AUC/MPC**
The mutant prevention concentration has also been included in these formulas since it is important to restrict the growth of resistant mutants during therapy. There have been efforts made to measure the AUC/MPC and the time within the so called Mutant Selective Window (MSW) which is the concentration range where mutants are selected (Blondeau et al., 2001; Dong et al., 1999; Drlica, 2003; Firsov et al., 2003; Zinner et al., 2003). An in vitro study showed that an AUC/MPC of \(\geq 22\) was needed to restrict mutant outgrowth of wild-type *Escherichia coli* using ciprofloxacin (Olofsson et al., 2006). Similar values were found in an in vivo study where rabbits were treated with levofloxacin against a *Staphylococcus aureus* infection, where an AUC/MPC > 25 was needed to restrict the acquisition of resistance (Cui et al., 2006).

**Breakpoints**
Breakpoints are used as a tool to translate the actual MIC values of bacteria in relation to specific drugs into a simple categorization as susceptible, intermediate or resistant. Breakpoints are defined based on the pharmacokinetic data on the relationship between the drug and specific species, as well as on the basis of a population analysis of the range of drug susceptibility values in the natural bacterial population. Breakpoints are used clinically as a warning system in relation to the suitability of a particular drug treatment, and to follow the evolution of resistance patterns in the population.
Figure 7. Pharmacodynamic and pharmacokinetic parameters important for effective bacterial elimination and mutant prevention.
Taking on the resistance problem - Restricting development of mutants

*Increasing resistance towards fluoroquinolones*

Fluoroquinolones are a very important class of antibiotics with 18% of total market share (Kresse et al., 2007). According to the EARSS database 25 of 28 European countries showed a significant increase in resistance towards fluoroquinolones between the years 2001 and 2005. The northern countries still remain at a low level of resistance whereas the southern countries, including Bulgaria, Cyprus, Spain, Italy, Malta and Portugal, have doubled their resistance levels between 2004 and 2005 and each country report fluoroquinolone resistant *E.coli* levels above 25% (EARSS).

*How do we prevent the resistance development?*

As fluoroquinolone resistance develops in a step-wise manner it can be hard to distinguish when bacterial strains has passed the point of no return. Dealing effectively with the resistance problem will probably require a series of different approaches including (i) the development of new antimicrobial agents, (ii) efforts to reduce selection pressure for resistance by reducing the consumption of antibiotics and (iii) the optimisation of dosing regimens so as to minimize the selection of resistant mutants. The following section will discuss the importance of an anti-mutant dosing strategy.

*Mutant prevention concentration*

The mutant prevention concentration is defined as the drug concentration that will prevent the least susceptible, single-step mutant in a large bacterial population (>10^10) from growing (Baquero, 1990; Baquero and Negri, 1997b; Dong et al., 1999; Drlica, 2003). Thus, the bacteria would need to acquire two or more resistance mutations to be able to grow at the MPC. This is unlikely to happen since the mutation frequency for resistance is ~10^-7, and would thus require a bacterial population of ~10^14 for a double mutant, which is not common in most infections (Low, 2001).

The MPC concept was coined after the discovery made in studies with *Mycobacterium* where the recovery of resistant mutants showed two distinct drops in response to treatment with increasing concentrations of drug. Thus, when cells were treated with increasing concentrations of fluoroquinolones, the number of cfu first dropped at a concentration corresponding to the level that inhibited the susceptible cells within the population, corresponding to the MIC of the population. The plateau of surviving cfu at higher drug concentrations above MIC was due to the presence of resistant mutant popula-
tions that were present at low frequencies ($10^{-7}$). As the antibiotic concentration was increased further there was an additional drop in cfu due to the inhibition of the least susceptible, single-step mutants in the population. This higher drug concentration was referred to as the MPC (Dong et al., 1999; Sindelar et al., 2000; Zhou et al., 2000).

The method used to determine the MPC level is by applying $>10^{10}$ cells onto a series of drug containing agar plates and incubating for a minimum of 48 hours. The MPC is the lowest drug concentration at which no resistant colonies grow (Dong et al., 1999). There has been several studies that have measured MPC for a range of different pathogens and a variety of different antibiotic compounds (Hansen and Blondeau, 2005; Hansen et al., 2006; Hermsen et al., 2005; Li et al., 2004; Linde and Lehn, 2004; Marcusson et al., 2005; Metzler et al., 2004; Randall et al., 2004; Rodriguez et al., 2004a; Rodriguez et al., 2004b; Rodriguez et al., 2005; Smith et al., 2004; Wetzstein, 2005).

**Mutant selective window**

When antibiotics are used to treat bacterial infections, antibiotic gradients are formed within the human body (Baquero and Negri, 1997a; Negri et al., 1994). Baquero introduced the notion that there existed a dangerous drug concentration range were drug-resistant mutants were selected (Baquero and Negri, 1997b). The mutants were said to “hill climb” by achieving additional mutations to be able to survive at higher drug concentrations (Figure 8). These concentration ranges will be achieved within the human body in a series of compartments dependent on diffusion rates as well as elimination rates. These antibiotic concentration ranges will be an optimal environment for the selection of bacterial populations harbouring antibiotic resistance determinants, while at the same time suppressing or slowing growth of the susceptible population (Baquero, 1990; Baquero and Negri, 1997b).

The MSW hypothesis suggests that mutants are most likely to be selected within the drug concentration window between the MIC and the MPC (Zhao and Drlica, 2001, 2002). Below MIC there is no drug selection effect. Within the MSW susceptible cells are inhibited while resistant cells are selected and grow. Above the MSW (above MPC) even resistant cells are inhibited. It has been shown that mutants having a low level of resistance towards a drug will in a stepwise way be selected for, and will become even less susceptible towards the drug. (Dong et al., 1999; Zhao and Drlica, 2001, 2002; Zhou et al., 2000). Concentration-dependent selection towards ceftotaxime, giving low-level antibiotic-resistant genetic variants, has been demonstrated both in vitro and in vivo (Negri et al., 2000). In a study with *S. aureus* exposed to
fluoroquinolone concentrations that were below, within, and above the MSW, mutant enrichment was only observed when the concentration range fell within the MSW, for at least 20% of the time (Firsov et al., 2003). These results suggest that the drug concentration in therapy should never be within the MSW under any circumstances. The aim should be to develop dosing regimens that would restrict the selection of resistance; by maintaining drug concentrations above the MPC throughout therapy (Drlica and Malik, 2003).

**Figure 8.** Mutants will “Hill climb” within the Mutant Selective Window.

**Combination-therapy**

By using only one antibiotic in therapy there will always be a risk of selecting mutants since there is only one target for the antibiotic. This risk is greater if the antibiotic concentrations during therapy are not kept above the MPC. In cases where MPC treatment is not desirable (e.g. for reasons of toxicity) then combination therapy, using two antibiotic simultaneously, would probably restrict resistance development. To be able to use dual compounds in therapy requires that the antibiotics target different intracellular mechanisms and may also require that they have similar pharmacokinetic properties. It is important that neither of the drug concentrations fall below its MIC during the period of therapy, as that would be equivalent to mono-therapy for part of the treatment period (Drlica, 2001; Zhao and Drlica, 2001).
PRESENT INVESTIGATIONS

Understanding how antibiotic resistance develops is fundamental knowledge required in the battle to reduce the emergence and spread of resistance. The aims of this research investigation were the following:

- To measure for a set of clinical UTI *E. coli* isolates *in vitro* their MPC values for ciprofloxacin and ask whether these could be predicted from their MIC values.

- To apply an *in vitro* kinetic model to investigate the MPC-related pharmacodynamic indices of ciprofloxacin that predict the prevention of resistance.

- To investigate the relationship between different approved fluoroquinolone dosing regimens and the selection of pre-existing resistant mutants.

- To make a step-wise selection for fluoroquinolone resistance and measure the associated fitness costs both *in vitro* and *in vivo*.

- To investigate different combinations of mutations associated with fluoroquinolone resistance and measure their effects on resistance and bacterial fitness.

- To identify fitness-compensating mutations in highly fluoroquinolone-resistant *E. coli*. 
Prevention of resistance development and alternative dosing regimens

Study I) Mutant prevention concentrations of ciprofloxacin for urinary tract infection isolates of Escherichia coli.

The mutant prevention concentration (MPC) is defined as the drug concentration needed to prevent the least susceptible resistant mutant in a large bacterial population from growing. The fluoroquinolones are an important group of antimicrobials used to treat various different infections, in particular those caused by Gram-negative pathogens (Kresse et al., 2007).

The study included to measure the MPC level of ciprofloxacin for a set of UTI E. coli isolates with different levels of susceptibility and determine whether MPC could be predicted from MIC. In our test panel we had isolates that had MIC values ranging from completely susceptible up to those that exceeded the CLSI (NCCLS) breakpoint for ciprofloxacin (4 µg/ml). These latter strains had MPCs greater than 32 µg/ml, and was not further evaluated since they would need very high dosing (≥32 µg/ml) during monotherapy which would not be possible with approved dosing procedures (Boy et al., 2004; Talan et al., 2004). 22 of the isolates that had MICs below the breakpoint for ciprofloxacin susceptibility were shown to have MPC values that ranged between 0.1 µg/ml and 5 µg/ml (Figure 9). MIC was not a good predictor of MPC, and correlated badly with a linear regression value of $R^2 = 0.58$. For most isolates the ratio MPC/MIC was ~16. The MSW however varied greatly between strains. Four strains with low MICs (0.016 – 0.023 µg/ml) had unusually high MPCs of 1 – 2.5 µg/ml, giving an MPC/MIC >100. This shows that the MPC cannot be accurately predicted from MIC. There are presumably other genetic factors causing the high MPC in these strains which are not reflected in the MIC. For most of the susceptible strains it should be possible to dose above MPC using monotherapy with currently approved dosing procedures (Boy et al., 2004; Talan et al., 2004), thus reducing the chance of selecting resistance.
One concentration step below the MPC level of each strain tested, resistant mutants were selected and tested for their MIC values. These mutants frequently had MICs equal to, or greater than, the MPC for that strain. A potential consequence of sub-optimal therapy that selects for resistance is illustrated by these mutants. This implies that they are not the product of single-step mutations. A logical explanation for this is that within the MSW some mutants that survive grow poorly, and are subject to selection for second-step mutations that enhance growth associated with increasing the level of resistance (see also Paper V). Multi-step selection for colony growth has been well documented in the phenomenon of adaptive mutation (Foster, 2004; Rosenberg and Hastings, 2004; Roth and Andersson, 2004). At MPC, in contrast, the growth of first-step mutants is completely inhibited and a multi-step evolution process is prevented.

At the end of the MPC experiment, i.e after 96 h, the MPC plates were screened for potential survivors. Surviving cfu was isolated after 96 h incubation at MPC at a frequency of $\sim 10^{-8}$ from the strains C47, C50 and Nu14, and at $\sim 10^{-7}$ for C97. A strain with a heritable enhanced survival frequency (C97-1) relative to its parent C97, was also isolated. Survival at MPC was not associated with an increase in MIC, nor was it associated with mutations in the hipA or hipB genes where a mutation that enhances survival in the presence of $\beta$-lactams and fluoroquinolones has previously been identified (Moyed and Bertrand, 1983; Scherrer and Moyed, 1988; Wolfson et al., 1990). These results rule out mutations in hipA or hipB as the cause of enhanced survival at MPC in these isolates. Thus, although some cells survive

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**Figure 9.** 22 clinical strains and their MIC and MPC values.
prolonged exposure to MPC, there were no resistant mutants among the survivors, showing that the MPC concept works as expected. Whether persisters are important for re-infection of UTI are not known and needs further investigation.

This study has shown that variable genetic factors in clinical isolates determine the drug concentration needed to prevent accumulation of mutants. The MPC cannot be accurately predicted by determining the MIC for the strain.

In the *in vitro* assay of MPC described here, constant antibiotic concentrations were used throughout the experiments. As has been pointed out many times (Allen *et al.*, 2003; Blondeau *et al.*, 2001; Dong *et al.*, 1999; Drlica and Malik, 2003; Firsov *et al.*, 2003; Firsov *et al.*, 2004; Zinner *et al.*, 2003) to be clinically useful, the MPC concept should be tested with consideration for the drugs pharmacokinetic properties, i.e. the critical magnitude of the drug exposure required to prevent emergence of resistant mutants in relation to the MPC needs to be determined.
Study II) Selection of ciprofloxacin resistance in *Escherichia coli* in an *in vitro* kinetic model: relation between drug exposure and mutant prevention concentration.

To be able to evaluate the significance and reliability of MPC for clinical application it is important to measure this under conditions that closely mimic the clinical environment. Previous studies done with fluoroquinolones integrating PK/PD implied that the area under the concentration-time curve AUC/MIC and the maximum concentration of the drug in serum C\text{max}/MIC are the most important pharmacodynamics indices that predict the efficacy of bacterial killing (Blondeau *et al.*, 2004; Zinner and Firsov, 1999; Zinner *et al.*, 2004).

In this study the aim was to investigate and evaluate the MPC of ciprofloxacin for two susceptible wild-type strains (Nu14, CCUG17620) and one strain carrying a single resistance mutation in the *gyrA* gene (Nu118) by using an *in vitro* kinetic model. MPC was determined by using a constant infusion of ciprofloxacin and then related to the PK/PD of the drug. The latter was assessed by the prevention of growth of resistant mutants in relation to different dosing profiles.

The study was started by measuring the effects of static ciprofloxacin concentrations for the three strains (Nu14, CCUG17620 and Nu118), by exposing them to the drug for 24 h. For both the susceptible strains an MPC of 0.128 mg/L ciprofloxacin was needed to prevent any mutant formation. This corresponds to a MPC/MIC of ~16, which is in full agreement with the previous *in vitro* study on agar (Paper I). The MPC level determined for the *gyrA* mutant gave a lower MPC/MIC index of ~4, giving no mutants at 0.188 mg/L. All strains gave resistant mutants under the MPC i.e. within the MSW, as expected.

Next, the T > MPC\text{PR} was measured in a pharmacokinetic model. In the first round of experiments Nu14 was exposed to a static concentration at its MPC (128 mg/L) for 2-18 h before initiation of the dilution phase with a T\text{1/2} of 4 h. The results showed that a T > MPC of 18 h was sufficient to prevent mutant growth (*Figure 10, left panel*). In the second trial the C\text{max} was varied for each strains drug dilution with a T\text{1/2} of 4 h that was begun at time zero. It was found that for both susceptible strains resistance development was prevented when a C\text{max} of 64xMIC, corresponding to a T>MPC of 8 h (33% of the dosing interval) was achieved (*Figure 10, right panel*). For the mutant Nu118 a C\text{max} of only 8xMIC prevented resistance development, corresponding to a T>MPC of 4 h (17% of the dosing interval). Results in this study
suggest that these parameters by themselves do not predict the prevention of resistance development.

**Figure 10.** Ciprofloxacin concentration and time needed to get the mutant prevention concentration for wild-type Nu14. Solid lines indicates concentrations that selected resistant mutants; dashed lines indicates the concentration that prevented mutant formation.

As in the previous study (Paper I) there was a surviving population at the end of the experiments. These persisting cells were unchanged in their MIC compared to their original MIC values. Persistance of bacterial sub-populations in the presence of antibiotics is not a new phenomenon (Lewis, 2005). The number of persisters found at the highest ciprofloxacin concentrations tested here were: for Nu14, $10^4$ cfu/mL (1024 x MIC); CCUG17620, $10^6$ cfu/mL (128 x MIC); and Nu118, $10^7$ cfu/mL (32 x MIC).

The PK/PD parameters were measured and suggest that the index that correlated best with the prevention of mutant development was AUC/MPC. For both the susceptible strains the AUC/MPC index gave a value of $\geq 22$ that was associated with the prevention of resistance development. AUC/MIC is the parameter that has been associated with efficient bacterial killing and is used for determining current dosing regimens (Craig, 1998). The results in this study suggest that AUC/MPC might be a more predictive parameter to consider in dosing for the prevention of mutant development.
Study III) Dose-related selection of fluoroquinolone-resistant Escherichia coli.

Fluoroquinolone doses used in treatment vary according to the suspected pathogen and the localization and severity of the infection. By using an *in vitro* kinetic model the aim was to study the effectiveness of six different approved dosing regimens of commonly used fluoroquinolones and to evaluate their potential to select pre-existing mutants.

The plan was to investigate the effects of clinically used doses of norfloxacin, ciprofloxacin and moxifloxacin on survival and selection in *E. coli* populations with pre-existing fluoroquinolone-resistant mutants. Cultures of susceptible wild-type (*araBΔ*) and isogenic single- (*gyrA S83L*) or double (*gyrA S83L, marRΔ*) fluoroquinolone-resistant mutants were mixed, and then exposed to fluoroquinolones for 24 h in an *in vitro* kinetic model. The antibiotic concentrations used corresponded to approved clinical doses of norfloxacin, 200 mg administered twice daily; ciprofloxacin, 100 – 750 mg administered twice daily; and moxifloxacin, 400 mg administered once daily. Resistance development was monitored together with MIC determinations of survivors and sequencing of known resistance genes.

All tested doses were sufficient to eradicate the susceptible wild-type strain. Norfloxacin 200 mg administered twice daily selected for both the single and double resistant mutants. Dosing with ciprofloxacin 250 mg administered twice daily eradicated the *gyrA* S83L single mutant, but 750 mg administered twice daily was required to eradicate the double-mutant population. Moxifloxacin administrated as 400 mg once daily was sufficient to eliminate the wild-type and the single mutant but did not completely remove the double mutant.

Five mutants that had an increased MIC were isolated at the end point of the ciprofloxacin experiments and the genes which are most associated with fluoroquinolone resistance were sequenced. In one double- mutant exposed to a ciprofloxacin dose of 500 mg administered twice daily, a novel *parC* mutation was identified: *parC* G171C. The mutant had a ciprofloxacin MIC of 1.5 µg/ml, corresponding to a two-step increase on the Etest scale compared to the parental strain. The remaining four mutant strains did not reveal any new mutations within *gyrA, gyrB, parC, parE, marOR, or acrR*. This is consistent with published findings that many mutations causing small increases in fluoroquinolone resistance are not found in the classical resistance-associated genes (Paper IV).
The pharmacodynamic parameter AUC/MIC is known to correlate with efficacy of the fluoroquinolones. Clinical and experimental studies have shown that an AUC/MIC value of about 100-125 is sufficient to predict clinical and microbiological success (Forrest et al., 1993; Odenholt and Cars, 2006; Wright et al., 2000). For the wild-type population, AUC/MIC values were above 100 for all the tested fluoroquinolone doses, which was sufficient for full eradication in all cases. In the present study an AUC/MPC\textsubscript{wild-type} value of 35 prevented selection of the single mutant when present initially as 1% of the total population. These results suggest that AUC/MPC is a useful predictor of outcome. Among antibiotic-resistant mutants isolated from patients there is a great variation in both MIC and MPC (Komp Lindgren et al., 2003) and (Paper I) and pharmacodynamic indices such as AUC/MIC and AUC/MPC could vary significantly depending on these values.

This study shows that several of the currently recommended doses of norfloxacin, ciprofloxacin and moxifloxacin are not sufficiently effective to prevent the selection of fluoroquinolone resistant mutants in \textit{E. coli} when serum concentrations are simulated. The pharmacodynamic exposure needed to prevent selection of resistance found in this study may well be obtained in the urine where the drug is concentrated. However, some bacteria may reside in the bladder epithelium (Kau et al., 2005) and selection may also take place in the commensal flora. Ciprofloxacin 750 mg administered twice daily was the only tested clinical dose that eradicated all \textit{E. coli} bacteria, including single and double mutants. Thus, focusing on the target pathogen in a simplified system, ciprofloxacin 750 mg administered twice daily would be the preferred choice for treatment. Moxifloxacin 400 mg administered once daily also showed high efficacy. It is of the highest importance that, during drug development, antibiotics as well as their dosing regimes should be carefully selected based on the pharmacokinetic and pharmacodynamic properties that support prevention of antibiotic resistance.
The genetics of resistance and bacterial fitness

IV) Biological cost of single and multiple norfloxacin resistance mutations in *Escherichia coli* implicated in urinary tract infections.

Resistance to fluoroquinolones in UTI *E. coli* is associated with multiple mutations, typically altering DNA gyrase, DNA topoisomerase IV, and regulation of AcrAB-TolC-mediated efflux. This investigation asked whether there was a fitness cost associated with the accumulation of multiple resistance-associated mutations.

The susceptible *E. coli* UTI isolate Nu14 were used to select resistant mutants through 3-5 successive steps with norfloxacin. Each selection step was made at twice the MIC of the selected strain. After each selection the MIC of the selected mutants were measured and regions of *gyrA*, *gyrB*, *parC*, and *parE*, previously associated with resistance mutation, and all of *marOR* and *acrR* were sequenced. The first selection step yielded mutations in *gyrA*, *gyrB*, and *marOR*. 2 mutants of each class were used as the starting point for subsequent selection steps which yielded mutations in *gyrA*, *parE*, and *marOR*, but not in *gyrB* or *parC* or *acrR*. Resistance-associated mutations were identified among these six genes in almost all isolates after selection steps 1 and 2, but in less than 50% of isolates after subsequent selection steps.

Selected strains were competed *in vitro*, in urine, and in a mouse UTI infection model, against the starting strain, Nu14. First step mutations were not associated with significant fitness costs, having fitness values very close to those of the parental Nu14 strain. Among the nine second-step mutants recovered, six were fit within ±3% of the Nu14 value, close to the standard error of these measurements, while three were significantly less fit, with growth rates 13 – 16% slower than Nu14. The accumulation of three or more resistance-associated mutations was strongly associated with a large reduction in biological fitness with generation times increased by between 6% and 55% with a mean increase in generation time of 31%. The reduced fitness was observed both *in vitro* and *in vivo*. Two fifth-step mutants tested had generation times increased by 23% and 43% respectively, relative to Nu14.
Interestingly, in some lineages a partial restoration of fitness was associated with the accumulation of additional mutations in late selection steps. In one of the lineages specific resistance-associated mutations were identified for each of the selection steps. Each of the strains from two different lineages (2a and 2b) was competed with Nu14 to measure their relative fitness both in vitro and in vivo in a mouse model (Figure 11). In lineage 2a the in vitro results showed a progressive decline in fitness with each of the four selection steps increasing the resistance. In contrast, the in vivo results for the same lineage showed a similar pattern with decreased fitness in the first three selection steps, but increased fitness associated with acquisition of the fourth mutation. In the second lineage (2b) the in vitro and in vivo results followed each other. A small fitness loss was associated with the first mutation, followed by a greater loss associated with the second mutation, and finally a partial restoration of fitness associated with the third mutation.

Figure 11. The fitness cost in vitro and in vivo for two different pathways of accumulating resistance mutations.

This study show that the accumulation of resistance-associated mutations is associated with a loss of bacterial fitness, but also suggest that particular combinations of these resistance mutations can partially compensate for these fitness losses.

The reversal of fitness costs associated with the accumulation of mutations is interesting in terms of the clinical development of fluoroquinolone resistance. Thus, if the multiple mutations required for resistance impose a severe fitness cost, resistant bacteria will be under selection pressure to avoid, minimize, or compensate for these costs. There could also be a parallel accumulation of fitness-compensating mutations either within or outside of the genes associated with resistance. In the clinical setting these fitness-compensating mutations might be acquired in the time-interval between an-
tibiotic treatment (selection) which has shown to be the case in vitro in the absence of antibiotics (Björkman et al., 1998; Nagaev et al., 2001). The suggestion made is that the relative biological costs of multiple mutations may influence the evolution of \textit{E. coli} strains developing resistance to fluoroquinolones.
V) Increased bacterial fitness selected with antimicrobial drug resistance

It is known that fluoroquinolone resistance mutations accumulate in a step-wise manner and that this accumulation of mutations is associated with growth rate reduction. This study investigated in a systematic way the consequences of specific combinations of mutations on resistance and fitness. A set of isogenic strains carrying combinations of resistance mutations found commonly in clinical isolates were constructed and their associated levels of resistance and fitness were measured.

A typical highly resistant *E. coli* UTI isolate, C1186, was the basis for the mutations used in these constructions. The clinical strain is highly-resistant to fluoroquinolones with a MIC for ciprofloxacin of 32 µg/ml. It carries resistance mutations altering topoisomerases (*gyrA* S83L, D87N; *parC* S80I), and up-regulating drug efflux (*marOR* small deletion, and amino acid substitution; *acrR* IS1 insertion). C1186 has a growth advantage of 3% per generation in competition against a laboratory wild-type. A total of 28 isogenic derivatives of wild-type MG1655 were constructed each mimicking in part the complex resistance genotype of C1186.

MIC for ciprofloxacin was measured for each of the 28 strains. Single mutations in *gyrA*, S83L and D87N, increased MIC 24-fold and 15-fold respectively. Knockout mutations in *marR* and *acrR* each increased MIC only 2 - 3-fold, while the substitution S80I in *parC* had no effect on MIC. Double mutation combinations had MIC’s 8 – 63-fold wild-type level, with the combination ΔmarR + ΔacrR having the smallest increase. Triple mutation combinations had MIC’s in the range 0.38 – 32 µg/ml, with 5/9 above the breakpoint that defines clinical resistance in Europe (1 µg/ml) (EUCAST, 2006), and 2/9 above the breakpoint currently used in the USA (4 µg/ml).

The 28 strains were also tested in growth competitions against wild-type to measure their fitness. Some single mutations *gyrA* S83L, *gyrA* D87N and *parC* S80I were near neutral (having a fitness index of 1.0), whereas ΔmarR and ΔacrR reduced fitness significantly having fitness values of 0.83 and 0.92, respectively. The mean fitness of single resistance mutations was 0.95, ranging from 0.83 – 1.01. With two mutations the fitness decreased to a mean value of 0.89, ranging between 0.79 – 0.99, whilst with three mutations it was reduced even further to 0.80 ranging between 0.54 – 0.98. However, the addition of a fourth resistance mutation reversed this downward trend and increased mean fitness to 0.87 with a range of 0.81 – 0.92. The ultimate strain, LM705 which contained all five mutations mimicking the resistance genotype of C1186, had a fitness of 0.90.
Six strains were of special interest since they showed that an additional resistance mutation increased both resistance and fitness at the same time. In five of the six strains the additional mutation that increasing resistance was gyrA S83L. In four cases this mutation increased the resistance level to 32 µg/ml. When analyzing the fitness of these strains the average increase in fitness associated with the acquisition of an extra mutation was 10% ranging between 5-24%. From the data it can be concluded that for certain mutation combinations, fluoroquinolone resistance correlates positively with fitness.

An important clinical implication of this correlation between resistance mutations and fitness is that once a strain has acquired the first few resistance mutations (and still lies well under the breakpoint defining clinical resistance) it can continue to evolve high-level fluoroquinolone resistance, even in the absence of further antibiotic selective pressure.

\[\text{Figure 12. Illustration of increasing resistance to ciprofloxacin due to accumulation of mutations (top panel). Loss in fitness by accumulation of up to three resistance mutations but an increase in fitness by the addition of the two later resistance mutations (bottom panel).}\]
A novel evolutionary strategy that reduces the fitness costs for bacteria of mutations conferring antimicrobial drug resistance has been identified. This study shows how bacteria can reduce mutational fitness costs while simultaneously increasing their level of drug resistance. The details of how the evolution of fluoroquinolone resistance plays out in nature will depend on the particular selective environment. Bacteria that progress down an evolutionary path with reduced fitness relative to a competing population may continue to extinction, or may, given the opportunity by mutation, acquire a change that increases their relative fitness thus enhancing their chances of survival (Figure 13).

Figure 13. Illustration of different possible evolutionary pathways dependent on bacterial fitness and resistance accumulation. (A) Resistance mutations can be accumulated that have low or no fitness burden on the bacterial population. (B) Resistance mutations that have a fitness cost can become fitness compensated without changing the resistance level. (C) Resistance mutations that lower the fitness of a bacterial population can lead to extinction. (D) Fitness can be compensated and simultaneously co-select for increased resistance.
VI) Fitness compensation in fluoroquinolone resistant *Escherichia coli* by mutant RNA polymerase

Knowledge from much published previous work is that the evolution of susceptible bacteria to drug resistance, whether by mutation or by horizontal gene acquisition usually is associated with a loss of bacterial fitness expressed as reduced growth rate *in vitro* and reduced virulence *in vivo*. *E. coli* lineages were previously evolved (Paper IV) in the laboratory to high-level fluoroquinolone resistance which resulted in reduced fitness compared to their parental strain. There are several strategies bacteria can exploit to reduce or avoid these resistance-associated fitness costs while still maintaining drug resistance, (i) they could accumulate only cost-free or low-cost resistance mutations, (ii) but it is also possible to acquire so-called fitness compensating mutations that ameliorate the fitness costs of resistance without losing drug resistance.

Antibiotic-susceptible *E. coli* were evolved step-wise to high level resistance to the fluoroquinolone ciprofloxacin. This resulted in lineages that were shown by DNA sequencing to contain multiple mutations altering genes coding for DNA gyrase, DNA topoisomerase IV, and genes controlling the regulation of drug efflux. Two highly-resistant mutants were named LM597 and LM642, respectively. The growth rates of these mutants were measured and showed that they grew at less than half the wild-type rate. These strains were then cycled in drug-free rich medium to select for bacteria with new mutations ameliorating the fitness costs of resistance. Two fast-growing clones with improved growth rates were chosen for further analysis. LM668 was derived from LM597, while LM683 was derived from LM642. The MIC of ciprofloxacin in the fast-growing mutants was reduced relative to the parental strains but in each case remained well over the breakpoint for clinical resistance.

To identify compensatory mutations responsible for the improved growth rate in the two strains a transposome, <R6Kori/KAN-2>, was introduced separately into cultures of LM668 and LM683, with selection for kanamycin resistance at 25 µg/ml. This resulted in populations of cells with the transposome inserted into the chromosome at random positions. Kanamycin-resistant transformants of each strain were pooled separately and bacteriophage P1 was grown on each pool. The P1 pool for each of the fast growing strains was then used to transduce its slow growing parental strain selecting for the transposome and screening for fast-growing colonies. Fast growing transposome-linked transductants were retained from both the slow growing strain LM597 and LM642. Chromosomal DNA was prepared from each of the candidate strains, cut and ligated into a circular plasmid form and trans-
formed by electroporation in a pir\(^{+}\) E. coli selecting for kanamycin resistance. Plasmid DNA prepared from the transformants was sequenced to identify the site of transposome insertion into the chromosome. Sequencing revealed that the transposome insertions linked to fast growth in the derivative of LM668 were inserted at two different sites; within frwD and in between ppc and argE. Both of these insertion sites are in the same region of the chromosome (Figure 14a), and the mutation responsible for fast growth in LM668 was provisionally designated fast-1. The mutation that compensated growth in LM683 was linked to a transposome inserted within gspD (Figure 14b) in its derivative, and the mutation responsible for fast growth in this strain was provisionally designated fast-2.

To identify the mutations fast-1 and fast-2 three-point mapping was performed. The transposome-linked fast-1 strain was subjected to transduction with btuB::Tn10. This gave the gene order fast-1 – Tn5 – btuB::Tn10. We also used spontaneous rifampicin-resistant rpoB mutations in a second mapping experiment of the same region and fortuitously discovered that these rpoB mutations conferred the fast-growth phenotype. These mutations in rpoB were designated fast-3 and fast-4 (Figure 14a). A similar mapping experiment was made for a derivative of the fast-2 strain that had been transduced with a strain containing zhd-126::Tn10, rpsE100. Fast-2 maps on the distal side of rpsE in a region carrying many ribosomal protein genes and the RNA polymerase α-subunit gene rpoA (Figure 14b).

![Figure 14](image)

**Figure 14.** Location of the compensatory mutations, fast-1, fast-3, fast-4 and fast-2, in the E. coli genome.
DNA sequence analysis identified fast-2 as the mutation Arg195Pro in rpoA. The mutations fast-3 and fast-4 are His526Tyr and Leu533Pro in rpoB. The fast-1 mutation has yet to be identified. These are the first mutations shown to compensate the fitness defects associated with fluoroquinolone resistance.

This study has shown that several individual mutations altering RNA polymerase (RNAP) can substantially improve the growth fitness of multiply-mutant fluoroquinolone resistant strains. Mutations were identified in the α- and the β-subunits of RNA polymerase. It seems that mutations that alter RNA polymerase can significantly improve the growth rate of fluoroquinolone resistant strains with reduced fitness.

The suggested model to explain the data is the following: (i) Normal growth rate is strongly dependent on the rate of gene transcription; (ii) The rate of transcription is dependent on type II topoisomerase activity to modulate the superhelicity of DNA; (iii) Fluoroquinolone-resistant mutant topoisomerases are less efficient at modulating superhelicity and this reduces the rate of transcription and thus growth rate; and (iv) Mutant RNA polymerase is less sensitive to local DNA topology and thus less dependent on topoisomerase activity for transcription. These phenotypes could be mediated at the level of transcription initiation or possibly at the level of Rho-dependent transcription termination.

One of the implications of this model is that some fluoroquinolone resistant clinical isolates may be expected to carry mutations in RNAP or its associated regulatory factors, that can promote transcription efficiently, independent of the actions of topoisomerases in re-modelling the structure of DNA.
Is there a solution of the antibiotic resistance problem and perspectives for the future

So we are facing a real problem with the increasing resistance development towards the antibiotics used to treat for small infections as well as life threatening diseases. Because there has been a misuse of antibiotics since their introduction we now see many antibiotic classes lose their activity towards important bacterial pathogens.

The work presented in this thesis has involved studies to increase our understanding of the genetic basis for the development of resistance to fluoroquinolones, and also how we can use new concepts in dosing that may help prevent the selection of mutants during treatment.

Fluoroquinolone resistance involves accumulating multiple mutations in the target genes for the drug and in genes regulating drug efflux. Resistance is associated with a loss in growth rate both \textit{in vitro} and \textit{in vivo} as shown in one of the studies. By constructing different combinations containing common fluoroquinolone resistance mutations it was possible to analyse how resistance evolution can occur more in detail. The data presented here shows that some resistant strains that had acquired resistance mutations and decreased fitness, could, by acquiring an additional resistance mutation increase their fitness as well as their resistance level. By these results a novel evolutionary strategy has been discovered: that reducing the fitness costs of resistance for bacteria can correlate with increased antimicrobial drug resistance. There have been discussions about whether it would be possible to prevent resistance development by decreasing the use of antibiotics. In this context the findings in these studies are very disturbing since they imply that bacteria could, without being subject to antibiotic selection, increase their resistance level by selection for increased growth fitness. The last study investigated the nature of fitness-compensatory mutations and the discovery made was that fitness loss in highly fluoroquinolone resistant \textit{E. coli}, is most likely due a reduced efficiency of transcription, and that this can be compensated by mutations altering RNA polymerase. It will be interesting to determine whether fitness-compensatory mutations within RNA polymerase can explain the high fitness found in resistant clinical strains.
The mutant prevention concentration measured for a set of clinical UTI strains suggested that they had complex genetic backgrounds resulting in a great variety in their MPC values. There was a low correlation between the MPC values and the corresponding MIC values. These results suggest that MPC should be included among the PK/PD parameters used to determine the optimal dosing regimens for fluoroquinolones since this concentration and not MIC will minimize the selection of mutants. To be able to use the MPC concept in clinical therapy one needs to investigate it in relation to other PK/PD parameters. The results from Paper II showed that $T>MPC$ and $AUC/MPC$ were the single PK/PD parameters that best predicted the prevention of resistance development. Efforts were made to test the ability of different approved fluoroquinolone doses that are used to treat various infections to prevent the selection of resistant mutants. It was found that only some approved dosing regimens eradicated or prevented the selection of resistant mutants, while other approved doses were less effective and selected pre-existing resistant mutants.

As we face a serious global resistance problem we need to adopt a series of measures to address the problem. These include (i) decreasing antibiotic use to reduce selection pressure for resistance, (ii) improving our knowledge of how bacteria become resistant and how they ameliorate the resulting fitness costs, (iii) improving dosing regimens with the aim of reducing the emergence of resistance and preventing the selection of resistant mutants.
Swedish Summary


Efter att ha selekterat ökad fluorokinolonresistens i en vildtypesstam konstaterades en ökad resistens på grund av flertalet uppkomna mutationer. I de flesta fall erhölls även en minskad tillväxthastighet både in vitro och in vivo. I vissa fall sågs även en tillväxtökning tillsammans med ökad selekterad resistens men några mutationer kunde inte identifiera vare sig i gyrA, gyrB, parC, parE, marR eller acrR som var förknippade med denna tillväxtökning. En av studierna syftade till att undersöka olika evolutionära vägar som kan leda fram till höggradig resistens mot fluorokinoloner, därför konstruerades stammar innehållande olika kombinationer av mutationer som vanligen finns i kliniskt högresistanta stammar och sedan mätte tillväxthastigheten samt resistensnivån hos dessa stammar. En starkkorrelation mellan antal mutatio- ner och reducerad tillväxthastighet, men vissa kombinationer av mutationer
resulterade i bättre tillväxt än andra. Det som var mest intressant var att en enda mutation hos vissa stammar kunde resultera i ökad resistens och samtidigt ökad tillväxt. Dessa data indikerar en helt ny resistensmekanism där en mutation som leder till ökad antibiotikaresistens samtidigt leder till ökad tillväxt.

En av studierna visar hur så kallade kompenserande mutationer hos högreresista-
tanta bakterier kan återställa tillväxthastigheten. Sekvenseringsresultat visa-
de en sådan mutation i *rpoA*, som kodar för en subenhet i RNA-polymeras.
Resistens mot fluorokinoloner påverkar replikationen, men även transkriptionen, genom att topoisomerasaktiviteten blir mindre effektiv. Hypotesen som framkommit av detta arbete är att en mutation i RNA-polymeras kan överkomma transkriptionsproblemet och därmed öka tillväxten.

Dagens antibiotikadoser baseras på den minsta hämmande koncentrationen ("minimal inhibitory concentration", MIC) som hämmar tillväxten av bakterien. Ett nytt koncept innebär att man i stället använder högre koncentrationer av antibiotika, så kallad mutationshindrande koncentration ("mutation prevention concentration", MPC). Syftet med en så hög koncentration är att man därmed skulle kunna motverka att mutanter selekteras under behandling. I en av studierna undersöcktes hur hög denna MPC var i kliniska urinvägspatogena stammar av *E. coli*, slutsatsen var att koncentrationen som behövdes för att hindra uppkomst av ytterligare resistensmutationer var olika i olika stammar. Detta gällde även för stammar som man tror är väldigt känns-
liga för antibiotika. MPC-värdet kunde inte förutsägas ur MIC-värdet.

I en av studierna testades även detta i en farmakodynamisk modell som visa-
de att T>MPC (tiden då antibiotikakoncentrationen i serum är på MPC-nivå) samt AUC/MPC (AUC = "area under curve", d v s den totala antibiotikamängden i serum under den tidsperiod under vilken mätningar gjorts) var viktiga parametrar för att motverka selektion av resista-
tanta mutanter. Några vanligt förekommande fluorokinolondoser som används vid klinisk behandling av olika infektioner testades även och resultaten visade att vissa var bättre än andra på att motverka resistensutveckling beroende på ursprungspopulationen av bakterier. Fler studier behövs dock för att särskilt veta om det är i urinvägarna eller någon annan stans i kroppen selektionen sker och vilken dos som skulle vara bäst för att elimineras bakterier på ett specifikt infek-
tionsställe utan att selektera mutanter.

Det finns ett stort behov av att utveckla nya antibiotika eftersom bakterier snabbt utvecklar multiresistens mot tillgängliga antibiotika. För att kunna göra det på rätt sätt måste man förstå hur bakterierna anpassas för att överle-
va antibiotikabehandling samt ligga ett steg före dess evolution.
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