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Constraints on up-regulation of drug efflux in the evolution of ciprofloxacin resistance

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Abstract

Praski Alzrigat, L. 2017. Constraints on up-regulation of drug efflux in the evolution of ciprofloxacin resistance. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1335. 48 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9923-5.

The crucial role of antibiotics in modern medicine, in curing infections and enabling advanced medical procedures, is being threatened by the increasing frequency of resistant bacteria. Better understanding of the forces selecting resistance mutations could help develop strategies to optimize the use of antibiotics and slow the spread of resistance.

Resistance to ciprofloxacin, a clinically important antibiotic, almost always involves target mutations in DNA gyrase and Topoisomerase IV. Because ciprofloxacin is a substrate of the AcrAB-TolC efflux pump, mutations causing pump up-regulation are also common.

Studying the role of efflux pump-regulatory mutations in the development of ciprofloxacin resistance, we found a strong bias against gene-inactivating mutations in *marR* and *acrR* in clinical isolates. MIC and fitness measurements revealed that amino acid substitutions conferred smaller susceptibility reductions and smaller fitness costs than gene-inactivating mutations, suggesting that resistance mutations in clinical isolates are selected for high fitness rather than high resistance (**Paper I and II**).

We asked whether the high fitness costs of *marR*-inactivating mutations could be ameliorated without affecting the resistance phenotype. Multiple independent lineages were experimentally evolved to select for improved growth fitness. Whole genome sequencing revealed mutations affecting *marA*, *lon* and *arcA* as potential compensatory pathways. For the *marA* and *lon* mutations the improved growth rate was associated with an increased susceptibility (*arcA* is being investigated). (**Paper III**).

An evolution experiment selecting for ciprofloxacin resistance revealed upon whole genome sequencing the expected mutations in drug target and efflux-regulatory genes, but also in genes encoding aminoacyl-tRNA synthetases. We investigated two independently selected *leuS* mutations, and concluded that they contributed to ciprofloxacin resistance by activating the stringent response that in turn caused up-regulation of genes involved in efflux. However, these *leuS* mutations incur a high fitness cost (**Paper IV**).

To summarize, the research findings in this thesis suggest that the potential ciprofloxacin resistome may include more genes than previously thought, but a strong selection for high fitness selectively purifies many resistance mutations from clinical isolates. In conclusion, selection for high relative fitness constrains the spectrum of mutations that survive and get fixed in clinical populations of bacteria.

Keywords: ciprofloxacin, antibiotic resistance, drug efflux, bacterial fitness, marR, acrR, marA, experimental evolution

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To my parents

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Praski Alzrigat, L.**, Huseby, D.L., Brandis, G. and Hughes, D. (2017) Fitness cost constrains the spectrum of *marR* mutations in ciprofloxacin-resistant *Escherichia coli*. *Journal of Antimicrobial Chemotherapy*, revision returned after review.

II **Praski Alzrigat, L.**, Huseby, D.L. and Hughes, D. (2017) A biased spectrum of mutations in *acrR* among ciprofloxacin-resistant isolates of *Escherichia coli.* Submitted.

III **Praski Alzrigat, L.**, Huseby, D.L. and Hughes, D. (2017) Genetics of fitness-compensation in experimentally evolved *marR* mutants of *Escherichia coli*. Manuscript.

IV Garoff, L., Huseby, D.L., **Praski Alzrigat, L.** and Hughes, D. (2017) Functional significance of *leuS* mutations in *Escherichia coli* resistance to ciprofloxacin. Manuscript.

Contents

Abbreviations

ATP	adenosine triphosphate
CNS	central nervous system
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FACS	fluorescence-activated cell sorter
HGT	horizontal gene transfer
HTH	helix-turn-helix
IM	inner membrane
MACS	magnetic-activated cell sorter
MDR	multi-drug resistance
MIC	minimal inhibitory concentration
mRNA	messenger RNA
OM	outer membrane
ORF	open reading frame
PBP	penicillin-binding protein
QRDR	quinolone resistance-determining region
RNA	ribonucleic acid
RND	resistance-nodulation division
SDS	sodium dodecyl
SIR	susceptible, intermediate or resistant
UTI	urinary tract infection

Introduction

Background of antibiotics

Most antibiotics originate from natural compounds produced by environmental microorganisms and plants, possibly for the purpose of being used as weapons against other microorganisms competing in the same microenvironment or as signaling molecules that regulate bacterial behavior in microbial communities (Linares et al. 2006). For example, penicillins and cephalosporins derive from fungi, while streptomycin, erythromycin, tetracycline and vancomycin derive from *Streptomyces* bacteria. Antibiotics can also be designed, either synthetically or by modifying antibiotics of natural origin. Modified variants of natural antibiotics include for example second and third generations of penicillins and cephalosporins. Fluoroquinolones, such as ciprofloxacin, are one of very few purely synthetic classes of antibiotics.

Alexander Fleming's discovery of penicillin (Fleming 2001) and Howard Florey and Ernst Chain's development of penicillin for medical use (Ligon 2004) is one of the most important developments in medical history. Penicillin was the first so-called "wonder drug" in the fight against infectious diseases and sparked the "golden age" of antibiotics during the 1950s and 1960s when most antibiotics known today were discovered. Antibiotics have since their introduction into medical practice saved millions of lives.

When antibiotics first came into use they were very efficient in clearing bacterial infections and the general belief was that infectious diseases were under control and would perhaps even be eradicated. It soon became clear, however, that bacteria had ways to evade antibiotics – they could develop resistance. In fact, bacteria have developed resistance to every antibiotic class within a few years of the introduction of each new class into medical practice (Fig. 1) (Clatworthy et al. 2007).

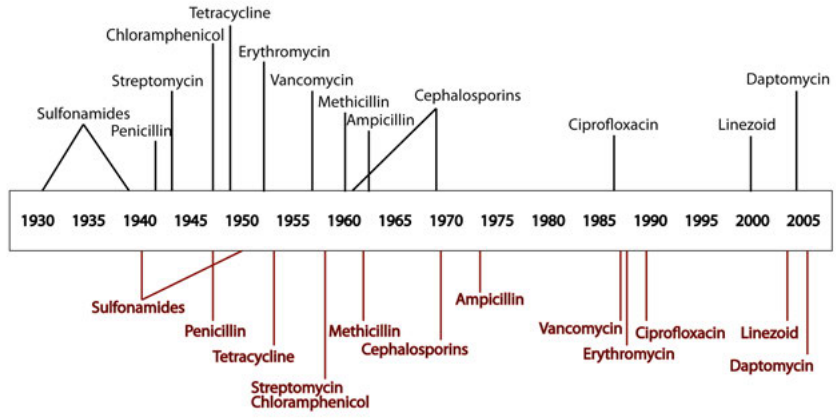


Figure 1. Timeline of the introduction of different antibiotics into medical practice (shown above the axis) and the evolution of resistance of those antibiotics (shown below the axis).

The impact of resistance to antibiotics

The increasing resistance to antibiotics poses a very real threat of a return to the pre-antibiotic era with decreased ability to treat infectious diseases and increased health risks for vulnerable patients (Baquero & Blázquez 1997). Common infections that have easily been treated with antibiotics may once again become deadly. Many medical procedures such as hip replacements, heart transplants, chemotherapy and even catheter insertions require effective antibiotics to avoid bacterial infection. Ineffective antibiotics due to bacterial resistance may render common medical procedures highly risky. The need for longer treatments, longer stays at the hospital and the increasing need to use more toxic antibiotics all contribute to increase patient morbidity. Antibiotic resistance also imposes a financial burden on society due to increased healthcare costs and productivity losses, estimated at EUR 1.5 billion per year in the EU in 2007 (ECDC 2009).

The emergence of bacteria that are resistant to multiple antibiotics (multi-drug resistance, MDR) adds another layer to the already severe crisis of antibiotic resistance (Levy & Marshall 2004; Giamarellos-Bourboulis, Papadimitriou, et al. 2006). There are several clinically important bacterial species where multi-drug resistant variants are already at very high frequencies in human infections, such as *Klebsiella pneumoniae* (Giamarellos-Bourboulis, Tziortzioti, et al. 2006) and *Mycobacterium tuberculosis* (Matteelli et al. 2014), for which there are virtually no antibiotic options left to use.

Classes of antibiotics

Antibiotics usually target essential bacterial pathways that are sufficiently different from or not present in human cells to avoid toxicity. Common targets are cell wall synthesis, folate synthesis, protein synthesis, DNA replication and RNA transcription. Antibiotics clear infections either by inhibiting growth (bacteriostatic), or killing (bacteriocidal), the infecting bacteria. Some antibiotics are active against a wide range of Gram-negative and Gram-positive bacteria (broad-spectrum antibiotics), for example tetracyclines and macrolides. Others, for example aminoglycosides and rifampicin, are active against a more limited range of bacterial species (narrow-spectrum antibiotics).

Cell wall synthesis inhibitors

Penicillins, cephalosporins, monobactams and carbapenems are cell wall synthesis inhibitors. They share a chemical structure (β-lactam ring) able to bind to and inactivate penicillin-binding proteins (PBPs) involved in cross-linking peptidoglycan, a crucial step in cell wall synthesis. The resulting weakened cell wall will eventually burst from the osmotic pressure and kill the cell.

Folate synthesis inhibitors

The folate synthesis pathway is responsible for nucleotide production and is the target of the antibiotic classes sulfonamides and trimethoprim. Sulfonamides inhibit the dihydropteroate synthase, while trimethoprim inhibits dihydrofolate reductase, two different steps in the pathway. Since bacteria are dependent on this pathway to get access to nucleotides in order to grow and replicate, blocking the pathway will kill the cell.

Ribosomal inhibitors

There are many antibiotics that target the ribosome. Some examples are aminoglycosides and tetracyclines that bind to the 30S ribosomal subunit, and macrolides and chloramphenicol that bind to the 50S ribosomal subunit. These antibiotics block different steps in translation by binding to different parts of the ribosome. Inhibiting protein synthesis also inhibits growth and replication.

DNA gyrase inhibitors

DNA gyrase and topoisomerase IV are responsible for regulating supercoiling of the DNA during processes such as DNA replication, gene transcription, and chromosome decatenation. Fluoroquinolone antibiotics bind to the

enzyme-DNA complex thereby arresting replication, transcription progression, or decatenation, eventually leading to cell death.

RNA polymerase inhibitors

The RNA polymerase is targeted by a group of antibiotics called rifamycins. They bind to RpoB, the β-subunit of the RNA polymerase, and thereby sterically block the elongating mRNA at transcription (Wehrli & Staehelin 1971; Campbell et al. 2001). The inhibition of RNA synthesis will eventually lead to cell death. Rifampicin, a rifamycin derivative, is especially clinically important as it is one of the key drugs in anti-tuberculosis therapy (Conde & Lapa E Silva 2011).

Development of resistance to antibiotics

Antibiotics revolutionized medicine as once-deadly infections now could be treated easily. The Achilles heel of antibiotics is, however, development of resistance by the pathogenic bacteria. Antibiotic resistance is when bacteria are able to continue to grow and replicate in the presence of normally lethal concentrations of the antibiotic. Since the first clinical use of penicillin, resistance has been observed already within a few years after introduction into medical use for virtually all antibiotics (Palumbi 2001). Bacteria can acquire resistance by *de novo* mutations or by horizontal gene transfer (HGT), but can also have a degree of intrinsic resistance.

Intrinsic resistance

Intrinsic, or inherent, resistance can be due to naturally occurring genes in the bacterium's chromosome, such as AmpC β-lactamases that inactivate β-lactam antibiotics (eg penicillins) or genes encoding efflux systems that pump drugs out of the cell (Alekshun & Levy 2007). Gram-negatives have a natural insensitivity to many antibiotics due to their outer membrane, which is impermeable to many molecules (Cox & Wright 2013). Bacteria can also be intrinsically resistant because they lack the molecule targeted by the antibiotic. For example, *Enterococci* are intrinsically resistant to cephalosporins since they lack the penicillin-binding proteins that these antibiotics target (Murray 1990).

Acquired resistance

As bacteria are exposed to antibiotics, they need to adapt to the new environmental conditions or they will die. The antibiotic pressure selects for variants with mutations that will help the bacteria survive despite the pres-

ence of antibiotics. The average rate of spontaneous mutations in bacteria is 10^{-9} to 10^{-10} mutations per base pair per generation (Andersson & Hughes 1996). Most random mutations are expected to be deleterious (causing a decrease in fitness ranging from nearly neutral to mildly deleterious to lethal), or neutral (for example, some synonymous amino acid substitutions), with only a very few mutations being beneficial (Drake et al. 1998).

Antibiotic pressure also selects for the development of mutator strains. Mutators are variants within a population that have an elevated mutation rate (Miller 1996). This genetic variant is usually due to mutations in genes coding for proteins that protect and repair DNA (frequently in *mutL and mutS* of the mismatch repair system) (Denamur & Matic 2006). The higher mutation rate increases the probability of beneficial mutations occurring and can therefore accelerate the development of resistance. Bacteria can also become resistant by acquiring foreign DNA via HGT. The mechanisms of HGT involve a number of gene transfer systems, such as transformation (uptake of free DNA from the surroundings), conjugation (transfer of plasmids containing resistance genes from one bacterium to another via a pilus), and transduction (transfer of resistance genes via bacteriophage) (Ochman et al. 2000).

HGT is also associated with co-selection of resistance to more than one antibiotic (Andersson & Hughes 2010). This occurs when resistance genes are closely located on the same genetic element such as a plasmid, transposon or integron. This can lead to an increased resistance to an antibiotic even if the bacteria are not exposed to that antibiotic. Cross-selection of resistance can also happen in cases where resistance to one antibiotic leads to resistance to an entire class of antibiotics due to similarities in their structures.

Definition of bacterial resistance

A very general definition of resistance is the capacity of bacteria to withstand the harmful effects of antibiotics. Resistant bacteria can continue to grow and replicate in the presence of antibiotics in contrast with susceptible bacteria that are killed or growth-inhibited. In a clinical setting it is important to have a standard for defining and measuring resistance to antibiotics in order to optimize treatment. One such standardized measure is the minimal inhibitory concentration (MIC), which defines the minimal concentration of an antibiotic that is able to prevent further growth of the bacterium under an agreed set of conditions. MIC can be measured using broth dilutions with progressively higher concentrations of the antibiotic, or using commercially available E-test strips impregnated with a gradient of antibiotic concentrations. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) sets standardized breakpoints, and interpretive crite-

ria, to be used by clinicians in the definition of bacteria isolated from patients as susceptible, intermediate or resistant (SIR) based on MIC measurements (Turnidge & Paterson 2007). Breakpoints are based on the distribution of MIC values observed in a large number of bacterial isolates. An epidemiological breakpoint refers to the minimum drug concentration that inhibits growth of a wild-type strain (wild-type cut off value) and any isolate with an MIC above the breakpoint is by definition a less susceptible variant. In contrast, the clinical breakpoint is defined so as to distinguish strains based on a prediction of the likely therapeutic outcome of antibiotic use. The clinical breakpoint is predictive of therapeutic success or failure. A strain isolated from a patient with a MIC for a certain antibiotic higher than its clinical breakpoint value is defined as clinically resistant to that antibiotic, and if that antibiotic were used in standard therapy there would be a high likelihood of therapeutic failure.

Resistance mechanisms

Bacteria develop resistance to antibiotics by *de novo* mutations or incorporation of foreign genetic material (HGT). These genetic changes give resistance through the following main mechanisms: i) modification or inactivation of the antibiotic, ii) reduced influx or increased efflux, iii) modification of the target and iv) overproduction of resistance and target genes (Coates et al. 2002).

Modification or inactivation of the antibiotic

There are several examples of HGT-acquired genes that code for enzymes that inactivate antibiotics or modify them in ways that reduce their toxicity. A good example are the β-lactamases that are commonly spread by plasmids, transposons and integrons. Hundreds of these enzymes have been characterized (Jacoby & Munoz-Price 2005). They confer resistance to β-lactam antibiotics such as penicillins and cephalosporins by catalyzing the cleavage and inactivation of their β-lactam ring necessary for anti-bacterial activity. There are also aminoglycoside-modifying enzymes that N-acetylate (acetyltransferases), phosphorylate (phosphotransferases) or adenylate (nucleotidyltransferases) the aminoglycosides resulting in structural changes that impair target binding (Wright 2005; Ramirez & Tolmasky 2010). The genes coding for these aminoglycoside-modifying enzymes are transferred between bacteria as part of integrons, transposons, plasmids or other mobile genetic elements.

Reduced influx or increased efflux

In order for an antibiotic to be effective, it needs to bind to a target with enough saturation to inhibit some essential reaction. By decreasing the intracellular concentration of an antibiotic, either through reduced membrane permeability or through increased expression of efflux pumps, the probability of achieving a level of drug saturation high enough to block the target's normal function is lowered. Mutations that down-regulate the expression of outer membrane porins (eg OmpF in *Escherichia coli*) can reduce the entry of some antibiotics into the bacterial cell. Mutations that up-regulate the expression of efflux pumps (eg the AcrAB-TolC efflux pump, the major efflux pump in *E. coli*) will increase the active removal of some antibiotic out of the cell. In *E. coli*, the OmpF influx and AcrAb-TolC efflux systems are co-regulated by the global transcriptional regulator MarA. Salicylate is known to activate transcription of *marA* (Cohen et al. 1993a; Vila & Soto 2012). MarA then regulates the expression of a number of genes, for example down-regulates porin expression and up-regulates efflux pump expression. Mutations that lead to overexpression of *marA* therefore lead to increased resistance to many antibiotics.

Modification of the target

In order for an antibiotic to be effective against the bacteria it also needs to bind to the target sufficiently tightly. Certain mutations can alter the binding site in the target so that the affinity of the antibiotic to the target is reduced. For example, single amino acid substitutions in *gyrA* are enough to cause reduced binding affinity of fluoroquinolones to DNA gyrase (Hooper 1999). Also in the case of *rpoB* is it sufficient with single amino acid substitutions to reduce the affinity and cause resistance to rifampicin (Telenti et al. 1993).

Overproduction of resistance and target genes

Antibiotic resistance is also associated with gene amplification. Having multiple copies of a gene leads to a higher level of expression of the gene product. This can confer antibiotic resistance through overproduction of for example antibiotic modifying enzymes, efflux pumps and target molecules. An amplification including five genes required for dihydrofolate biosynthesis (*folCEPBK*) was found to confer resistance to sulfonamide in *Streptococcus agalactiae* (Brochet et al. 2008). The resistance was identified to be the result of increased expression of dihydropteroate synthase (*folP*), the target of this antibiotic (Roland et al. 1979). Increased gene copy number also widens the mutational target and increases the chance of beneficial resistance mutations.

Fitness

Fitness can in very basic terms be defined as the ability of an individual or genotype to survive and reproduce in a specific environment. In the process of natural selection, the fittest genotypes will have a greater chance of surviving and reproducing, thereby contributing their genes to the next generation and such genotypes will tend to increase in proportion in the population. In the competition among genotypes in a given environment, it is in other words the fitness of one genotype compared with another (relative fitness) that is interesting. The relative fitness is often calculated as the absolute fitness of each genotype divided by the absolute fitness of the fittest genotype (or any genotype you want to compare with) (Orr 2009).

Bacterial fitness can be determined by comparing relative rates of growth or by pairwise competitions. Growth rates can be measured using for example a Bioscreen C reader (Labsystems), which regularly (eg every four minutes) records the optical density at 600 nm. This gives a measurement of the bacterium's doubling time during exponential growth and can distinguish differences of about 5% or greater in growth rate. Measuring relative fitness by pairwise competitions is a technique with a higher sensitivity. For example, a culture containing a 1:1 mixture of two competing strains is allowed to grow through several successive growth cycles, and is assayed for changes in the relative frequencies of the two competitors after each cycle. Using strains with fitness-neutral fluorescent markers and FACS (fluorescence-activated cell sorter) or MACS (magnetic-activated cell sorter) will allow detection of fitness differences of <0.1% per generation (Brandis & Hughes 2016). Growth rate analysis take into account only the exponential growth phase, whereas competitions measure fitness in the entire growth cycle; lag phase, exponential phase and stationary phase.

Fitness cost of resistance mutations

Development of resistance to antibiotics gives the resistant bacteria an advantage over the susceptible bacteria under conditions where both are exposed to the drug. Antibiotic resistance is, however, often associated with a reduction in bacterial fitness and a reduced competitive ability against susceptible bacteria in the absence of the drug. Bacterial fitness is most often defined in terms of relative growth rate, but can also be defined in terms of colonization, transmissibility or virulence. The negative effect of resistance mutations on growth has been shown for several mutations and species (Andersson & Hughes 2010; Nagaev et al. 2001; Marcusson et al. 2009; Schrag et al. 1997). It can be due to antibiotics binding to essential enzymes with the consequence that mutations in these target enzymes that increase resistance to the drug, will also affect the enzyme's functionality, making the

bacteria grow slower. Another example is resistance mutations leading to overexpression of efflux pumps. This will increase resistance, but only at a cost for the cell, both due to the increased production cost and to the increased risk of pumping out, not only undesired compounds but also nutrients out of the cell as many pumps are non-specific. The magnitude of the fitness cost of being resistant can vary depending on the particular mutation and some resistance mutations might even be cost free (Sander et al. 2002; Marcusson et al. 2009; Sirgel et al. 2012).

Fitness cost of resistance mutations in clinical isolates

Do relative fitness costs matter in the selection of resistance mutations in clinical isolates? Bacteria from clinical isolates have been subjected to the competitive conditions within a patient, including competing for resources and survival in the context of the patient's bacterial flora. Clinical isolates may also have been subjected to a variety of other conditions, both within and without a human host, resulting in very complex history that might be reflected in many differences in genotype between different clinical isolates. It is therefore difficult to make simple direct genome comparisons between isolates to determine what genetic differences can be attributed to what effects. There are, however, studies that support that fitness costs do matter also in clinical isolates. A K42R mutation in the 30S ribosomal protein S12 in *M. tuberculosis* gives streptomycin resistance. It has no measurable fitness cost *in vitro*. *In vitro* selected streptomycin resistant mutants had a variety of K42 amino acid substitutions at approximately equal frequencies, while 89 of 90 resistant clinical isolates carried the no-cost substitution K42R, suggesting that there is in this case a preference of low cost mutations in clinical isolates (Böttger et al. 1998). Also, in our studies of the spectra of *marR* and *acrR* mutations appearing in fluoroquinolone-resistant clinical isolates (**Paper I**, **Paper II**), we found that low cost, low MIC single amino acid substitution mutations were strongly preferred over higher cost, higher MIC inactivating mutations in clinical isolates, whereas in *in vitro*-selected strains subjected to a less competitive environment than in a patient, we found the opposite mutational distribution. This suggests that also in these cases, there is a preference for low cost mutations in clinical isolates.

Fitness compensatory evolution as a response to growth inhibition

Since antibiotic resistance is often associated with a fitness cost, it would be expected that as the antibiotic selective pressure is relieved, slow-growing resistant mutants would be outcompeted by faster-growing susceptible bacte-

ria. This would suggest that limiting the use of antibiotics could limit the frequency of resistant mutants in the population. There are, however, many *in vitro* studies that have reported contradicting observations, including mutants with restored fitness and maintained resistance (Andersson & Hughes 2010; Björkman et al. 2000). One explanation can be that some mutations have no cost, or a very low, associated cost. The decreased fitness in resistant mutants can also be counterbalanced by acquiring additional mutations that reduce the fitness cost without reducing resistance (Maisnier-Patin & Andersson 2004; Björkman et al. 1998). Such compensatory mutations, as well as low-cost mutations, can stabilize resistant bacterial populations, even in the absence of antibiotic pressure. Another alternative for a resistant slow growing mutant to adapt to an environment without the selective pressure of antibiotics is to revert back to the wild-type version of the gene. This is, however, less common than compensatory mutations since there is a higher number of possible compensatory mutations (larger mutational target) than back mutations (usually single nucleotide substitution) (Andersson & Hughes 2010).

There are several types of compensatory mutations. Intragenic compensatory mutations (within the same locus as the resistance mutation) restore an altered structure and function of a protein. An example is an *E. coli gyrB* (one of the two subunits of DNA gyrase) mutation giving coumarin resistance whose fitness cost can be compensated by a second mutation in *gyrB*. The compensatory mutation changes the structure of GyrB, thereby stabilizing DNA gyrase and restoring the activity of the enzyme (Blance et al. 2000). Extragenic compensatory mutations (at other loci) can, partly or completely, restore an altered function of a protein. For example, in **Paper III** we found that *lon* mutations can partly compensate for the fitness cost conferred by an inactivating *marR* mutation. The *marR* mutation results in increased expression of the regulator MarA and efflux pump components AcrAB and TolC, leading to a high fitness cost. Lon is a protease that cleaves MarA, among other proteins. The data suggest that the *lon* mutation results in increased MarA cleavage, leading to less up-regulated expression of AcrAB and TolC and a subsequent reduction in fitness cost. Intergenic (a variety of extragenic) compensatory mutations restore an altered structure and function of multi subunit molecules such as RNA polymerase. In this case, the resistance mutation is in one subunit and the compensatory mutation is in another. An example is that the fitness cost of a resistance conferring mutation in the ribosomal protein S12 has been observed to be compensated by mutations in other ribosomal proteins S4, S5 or L19 (Björkman et al. 1998; Maisnier-Patin et al. 2002; Schrag et al. 1997; Brandis et al. 2012; Brandis & Hughes 2013).

Fitness compensation in clinical isolates

Due to the complex genotypes of clinical isolates, it is difficult to prove that fitness compensation occurs during development of clinical resistance. One example of clinical fitness compensation evolution is, however, found in *M. tuberculosis* development of resistance to kanamycin (Shcherbakov et al. 2010). Among several different point mutations in the gene for 16S rRNA conferring a kanamycin resistance phenotype (A1408G, C1409U, G1491U and C1409A/G1491U) one particular mutation, A1408G, was most frequent (95%) in clinical isolates while each of the other single mutations was found at a low frequency of 1-2 %. Surprisingly the double mutation (C1409A/G1491U) occurred as frequently as the rare single mutations. Each point mutation was introduced into an isogenic wild-type background to study their individual impact on fitness and resistance. The experimental measurements supported the clinical importance of low fitness cost mutations, and the existence of cost-compensatory mutations. Thus, the most frequently occurring mutation A1408G was shown to be a low cost mutation conferring high-level resistance (MIC >1024). The rare single mutations, G1491U and G1409U, had very high fitness costs (12% per generation) and intermediate resistance (MIC 64). The most important result was that the double mutation, C1409A/G1491U, showed an intermediate resistance (MIC 16), but was more or less cost free (0.1% per generation). This suggests that in the clinical isolate C1409A compensates for the fitness cost of G1491U resulting in a genotype conferring drug-resistance at a very low fitness cost.

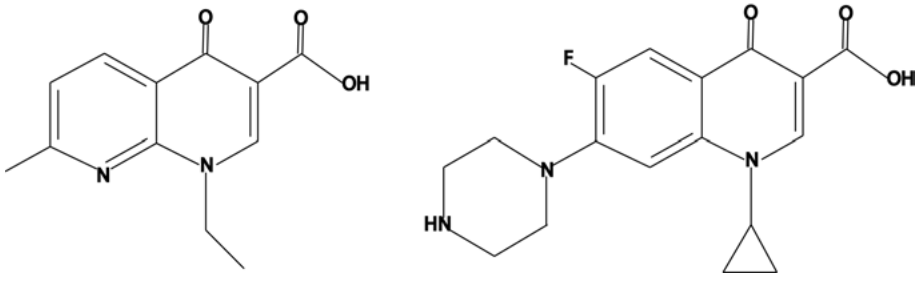
Bacterial need to balance fitness cost with the advantage of increased resistance

The wild-type drug-susceptible genotype is normally the most fit. Mutations conferring antibiotic resistance are beneficial under antibiotic pressure, although they often cause a fitness cost rendering the mutations disadvantageous in the absence of antibiotics. The beneficial effect of resistance and the fitness cost counteract each other. In other words, there is a trade-off between having resistance and growing faster. For example, the balance between being resistant by keeping the antibiotics out of the cell and the cost of overexpressing efflux pumps needs to be fine-tuned for the mutant genotype to survive in the population. Different mutations will have different trade-offs between resistance and fitness cost, which will affect their success in the natural selection in different environments (in the presence/absence of antibiotics, at different concentrations of antibiotics).

Fluoroquinolones

The fluoroquinolones are among the most important and most frequently prescribed antibiotics available today. They are a class of antibiotics of purely synthetic origin. They are valued for their broad-spectrum activity, good pharmacokinetic properties and rapid bacteriocidal activity.

Nalidixic acid was the first quinolone to be developed (Lesher et al. 1962). It was discovered in 1962 as a by-product of chloroquine (an antimalarial agent) synthesis and was found to have antibacterial activity. Nalidixic acid was mainly used to treat urinary tract infections (UTIs) caused by Gram-negative bacteria. It had otherwise limited clinical use due to low serum concentrations, high MIC, toxicity (gastrointestinal tract and central nervous system, CNS) and the rapid emergence of resistance (Emmerson & Jones 2003). Several other quinolones (eg pipemidic acid, oxolinic acid and cinoxacin) were developed through modification of nalidixic acid, but they offered only marginal improvements. In the 1970s, the first fluoroquinolones were developed by adding a fluoro-group to the structure (Fig. 2). This addition increased the target enzyme inhibition more than 10-fold and improved MIC up to 100-fold (Andersson & MacGowan 2003). The fluoroquinolone norfloxacin (developed in 1978) showed improved activity against Gram-negatives and also had some Gram-positive activity. Norfloxacin sparked a rapid development of many fluoroquinolones (eg pefloxacin, fleroxacin, ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin) with improvements in pharmacokinetic parameters and activity spectrum and many of them are still in clinical use today. Novel fluoroquinolones currently in clinical trials include for example, avarofloxacin, delafloxacin, finafloxacin, zabofloxacin and nemonoxacin (reviewed in Kocsis et al. 2016). These new agents show enhanced antibacterial activity, importantly also against strains already exhibiting resistance to existing fluoroquinolones.



Nalidixic acid Ciprofloxacin

Figure 2. Chemical structures for the quinolone nalidixic acid and the fluoroquinolone ciprofloxacin.

Ciprofloxacin

Ciprofloxacin is one of the most successful and widely used fluoroquinolones. It is a second-generation fluoroquinolone and was introduced into clinical use in 1986. It is a broad-spectrum antibiotic with activity against most Gram-negative bacteria (eg *Enterobacteriaceae*, *Haemophilus influenza*, *Neisseria* spp., *Pseudumonas aeruginosa*) and some Gram-positive bacteria (eg *Staphylococcus aureus, Streptococcus pneumonia*). It is used to treat a wide range of bacterial infections including UTIs, gastrointestinal infections, respiratory tract infections, sexually transmitted diseases and skin and bone related infections (Ball 2000). Side effects reported to be associated with the use of ciprofloxacin are, for example, gastrointestinal tract and CNS disturbances, skin rashes and hepatoxicity.

Ciprofloxacin mechanism of action

Ciprofloxacin, as well as the other fluoroquinolones, acts by inhibiting the two essential bacterial enzymes DNA gyrase and DNA topoisomerase IV. DNA gyrase is responsible for regulating the supercoiling of DNA that arises as the transcription and replication complexes move along the DNA. DNA topoisomerase IV is involved in the unlinking of replicated daughter chromosomes after DNA replication. Both of these enzymes are required for growth and replication and inhibition of them will kill the cell.

DNA gyrase

DNA gyrase is a tetramer composed of two subunits of GyrA (encoded by *gyrA*) and two subunits of GyrB (encoded by *gyrB*). The bacterial closed circular chromosome is condensed by supercoiling in order to fit into the cell. Relaxing of the supercoiling is necessary when DNA is to be replicated or genes transcribed. As replication and transcription complexes move along the DNA, helicases separate the two strands of the double helix to expose the DNA. This strand separation generates over-winding of the double stranded DNA in front of the replication and transcription complexes and would, if left untouched, end up inhibiting further strand separation and replication/transcription. DNA gyrase removes the positive supercoils generated by the separation of the strands by moving ahead of the helicase, catalyzing a double stranded break in the supercoiled DNA, passing another part of the supercoiled DNA through the created opening in the DNA and then resealing the DNA. By doing this, the region ahead of the helicase is prevented from getting so tightly wound that the helicase is blocked or the DNA molecule breaks. This process is driven by adenosine triphosphate (ATP) hydrolysis. In the DNA gyrase tetramer, it is the A subunits that bind and cleave the DNA, while the B subunits provide the ATPase activity.

DNA topoisomerase IV

DNA topoisomerase IV is also a tetramer built up by two subunits of ParC (encoded by *parC*) and two subunits of ParE (encoded by *parE*). Just like DNA gyrase, topoisomerase IV is able to catalyze double stranded breaks, pass another region of double stranded DNA through the gap and reseal the broken strands. As a round of replication of a circular DNA molecule is almost completed, the resulting two daughter chromosomes are still interlinked and need to be separated and segregated into separate daughter cells. The main role of topoisomerase IV is this decatenation of replicated daughter chromosomes.

Ciprofloxacin inhibition of DNA gyrase and DNA Topoisomerase IV

Ciprofloxacin binds to DNA gyrase/topoisomerase IV that is in complex with DNA. This binding blocks the DNA-enzyme complexes and prevents the double stranded breaks catalyzed by DNA gyrase/topoisomerase IV from being resealed. Further DNA replication or gene transcription is thereby inhibited. The build up of double stranded DNA breaks eventually results in cell death.

Resistance to ciprofloxacin

The extensive use of ciprofloxacin in both clinical and veterinary medicine has resulted in rapid emergence of resistance. The development of resistance to ciprofloxacin in *E. coli* is a multistep selection process. Individual resistance mutations usually result in only small increases of the MIC and to reach clinically relevant levels of resistance (the European clinical breakpoint is currently 0.5 mg/L (EUCAST 2017)) requires an accumulation of several mutations. There are two main mechanisms of resistance to ciprofloxacin: mutations in the genes coding for the target enzymes DNA gyrase and topoisomerase IV and mutations in genes coding for regulators of drug influx and efflux. There is also plasmid-mediated ciprofloxacin resistance; for example the Qnr protein that is able to protect the target enzymes from interaction with the drug.

Resistance due to altered drug target enzymes

The main mechanism of ciprofloxacin resistance seems to be alteration of the target enzymes DNA gyrase and topoisomerase IV, meaning one or several mutations in the genes *gyrA*, *gyrB*, *parC* or *parE* (Hooper 1999). In *E. coli*, resistance mutations in *gyrA* are more frequent than in *gyrB*. They cluster in a small part of the N-terminal region of *gyrA*, the quinolone resistance-

determining region (QRDR), including amino acids 67 to 106 (Yoshida et al. 1990). It is believed that mutations in QRDR alter the binding site of ciprofloxacin leading to reduced drug binding strength (Willmott & Maxwell 1993). Mutations within *parC* and *parE* alone do not confer resistance in *E. coli*. However, second step mutations in especially *parC* in strains that have already acquired a mutation in *gyrA* can lead to high levels of resistance (Bachoual et al. 1998; Marcusson et al. 2009).

Resistance due to altered intracellular concentrations of the drug

In order to reach its target enzymes, ciprofloxacin needs to cross the outer and inner membranes of *E. coli* (and of other Gram-negative bacteria). The outer membrane contains pore-forming proteins, porins. The major non-specific porin in *E. coli* is OmpF. It allows the diffusion of small molecules, such as essential solutes but also ciprofloxacin, into the cell (Koebnik et al. 2000). The expression of *ompF* is under the regulation of the global transcriptional regulator MarA, which in its turn is regulated by the repressor MarR. Mutations that lead to inactivation of the repressor MarR result in increased expression of MarA. MarA will activate the expression of many genes within its regulon, including *micF*. MicF is an antisense-RNA that binds to OmpF mRNA, thereby prevents its translation. This down-regulation of porin expression on the surface of the bacterial cell and resulting reduced influx of antibiotics can contribute to the resistance phenotype (Fernández & Hancock 2012). Ciprofloxacin can, however, also cross the outer membrane by diffusion through the phospholipid bilayer of the membrane (Chapman & Georgopapadakou 1988).

MarA also regulates the expression of AcrAB-TolC, the major efflux pump in *E. coli*. The AcrAB-TolC efflux pump can transport a wide range of substrates, for example antibiotics (including ciprofloxacin), organic solvents and bile salts out of the cell. Mutations that lead to the inactivation of the repressor MarR and increased expression of MarA will result in increased expression of the components of the efflux pump, AcrA, AcrB and TolC. The expression of AcrA and AcrB is also under the regulation of their operon's local repressor AcrR. Mutations that inactivate AcrR lead to increased expression of AcrA and AcrB. This up-regulation of the efflux pump and resulting increased transportation of antibiotics out of the cell will contribute to resistance. Due to the pump's wide range of substrates, including many antibiotics, its up-regulation leads to the development of a multi-resistance phenotype (Alekshun & Levy 1997).

Resistance due to target enzyme protection acquired through HGT

Genes encoding an efflux pump, *qepA* (Périchon et al. 2007), enzymes that can modify and reduce the activity of ciprofloxacin, *aac(6')-Ib* (Strahilevitz et al. 2009), or proteins that can bind to the target enzymes and thereby inhibit ciprofloxacin binding, *qnrA*, *qnrB*, *qnrC*, and *qnrS* (Martínez-Martínez et al. 1998; Strahilevitz et al. 2009) have been found on plasmids. Thus ciprofloxacin resistance can also be mediated through HGT. The Qnr proteins can bind to and protect both DNA gyrase and topoisomerase IV, and confer a similar level of resistance as caused by a single target mutation in *gyrA*. However, having acquired a plasmid carrying *qnr* and then acquiring another ciprofloxacin resistance mutation in for example *gyrA* will generate a higher level of resistance (Jacoby 2005).

Resistance to ciprofloxacin in clinical isolates

Fluoroquinolones were introduced into medical practice in the late 1980s. Upon their launch it was widely believed that fluoroquinolone resistance would not emerge since they are purely synthetic and the target bacteria had never encountered them before. However, resistance emerged within months and some of these cases led to clinical failure of treatment (Piddock 1990; Piddock et al. 1993). Figure 3 illustrates the increase in the proportion of fluoroquinolone-resistant *E. coli* clinical isolates in Europe from 2005 to 2015. The most frequent mutation observed in ciprofloxacin resistant clinical isolates of *E. coli* is in *gyrA* Ser83, often together with *gyrA* Asp87 (Everett et al. 1996; Oram & Fisher 1991; Komp Lindgren et al. 2003). The presence of a mutation in *parC* is also very frequent. The mutations in *parC* occur most commonly in Ser80, followed by Glu84, usually carried together with one or two *gyrA* mutations (Komp Lindgren et al. 2003; Heisig 1996; Huseby et al. 2017). Ciprofloxacin resistant clinical isolates are sometimes found to have resistance mutations in the target enzymes in combination with mutations affecting efflux. Those are in most cases mutations in *marR* and *acrR* that up-regulate the expression of the AcrAB-TolC efflux pump (Komp Lindgren et al. 2003). The following 5 mutations are typical of highly resistant clinical isolates: *gyrA* Ser83Leu, Asp87Asn, *parC* Ser80Ile, *marR* small deletion and *acrR* IS insertion (Marcusson et al. 2009; Komp Lindgren et al. 2003; Huseby et al. 2017).

Mutations in *gyrB* are rare. Many studies have failed to identify *gyrB* mutations in ciprofloxacin resistant clinical isolates (Komp Lindgren et al. 2003; Bachoual et al. 1998; Fendukly et al. 2003). Also *parE* mutations are uncommon and seem to be less important to ciprofloxacin resistance (Everett et

al. 1996; Ruiz et al. 1997). However, *parE* has been less studied than *gyrA* and *parC* in resistant clinical isolates and might need to be further evaluated.

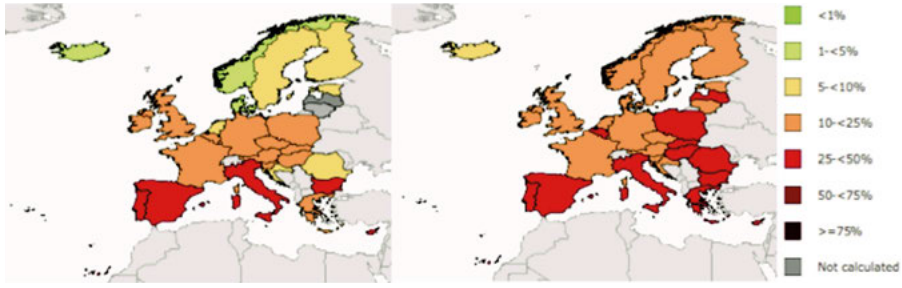


Figure 3. Percentages of clinical isolates of *E. coli* resistant to fluoroquinolones in Europe from 2005 to 2015 illustrating the increase of fluoroquinolone resistance. (ECDC 2017)

Multi-drug efflux pumps

Bacteria have membrane transport systems important for controlling the influx of essential nutrients and the efflux of toxic compounds, including antibiotics, to maintain cellular homeostasis. Efflux pumps play an important role in the development of bacterial multi-drug resistance. The expression of efflux pumps is often under the control of transcriptional regulators, at a local or global level. These regulators often recognize the same range of compounds as the pumps they regulate export out of the cell. In this way the regulators can act as sensors and respond to unfavorable levels of toxic compounds. The level of efflux observed in antibiotic-susceptible wild-type strains of *E. coli* is not enough to give clinically relevant levels of resistance to most antibiotics, including ciprofloxacin (note: in some bacterial species, such as *P. aeruginosa* resistance to tetracycline and chloramphenicol (Li et al. 1994), is intrinsic because of their wild-type level of efflux pump activity). However, under the selective pressure of antibiotics a mutant variant of a susceptible strain that overexpresses one or more efflux pumps can be selected and express a resistant phenotype. This overexpression of efflux pumps is often due to mutations inactivating the transcriptional regulators.

Bacteria often carry genes encoding several different efflux pump systems. Efflux pump systems are divided into five major classes: i) the ATP-binding cassette (ABC) superfamily, ii) the major facilitator (MFS) superfamily, iii) the multi-drug and toxic compound extrusion (MATE) family, iv) the small multi-drug resistance (SMR) family and v) the resistance-nodulation-division (RND) family. The ABC family transporters use ATP hydrolysis to provide energy for the transportation of compounds out of the cell. All other

families are dependent on the transmembrane electrochemical gradient of protons or sodium ions making them proton/compound antiporters (Putman et al. 2000). Some efflux pumps are specific for one substrate, while others are capable of transporting a wide range of structurally dissimilar compounds out of the cell.

AcrAB-TolC is the major efflux pump in *E. coli*

In *E. coli*, and other Gram-negative bacteria, compounds to be secreted out of the cell from the cytosol need to cross the inner membrane, the periplasm and the outer membrane. The RND type AcrAB-TolC efflux pump of *E. coli* is a tripartite system composed of a transporter protein (AcrB) in the inner membrane, a membrane fusion protein (AcrA) in the periplasm and an outer membrane protein (TolC) that together form a continuous channel out of the cell (Fig. 4). This pump is a very non-specific pump capable of transporting a diverse array of compounds with little chemical similarity, among them many antibiotics. Known substrates of the AcrAB-TolC efflux pump includes antibiotics such as fluoroquinolones, chloramphenicol, β-lactams, tetracycline, rifampin, novobiocin, fusidic acid, nalidixic acid, cationic dyes such as ethidium bromide and acriflavin and detergents such as sodium dodecyl sulfate (SDS), triton X-100 and bile salts, as well as short-chain fatty acids (Koronakis et al. 2004). A common feature of the substrates of the AcrAB-TolC efflux pump seems to be the presence of a lipophilic domain (Nikaido 1996).

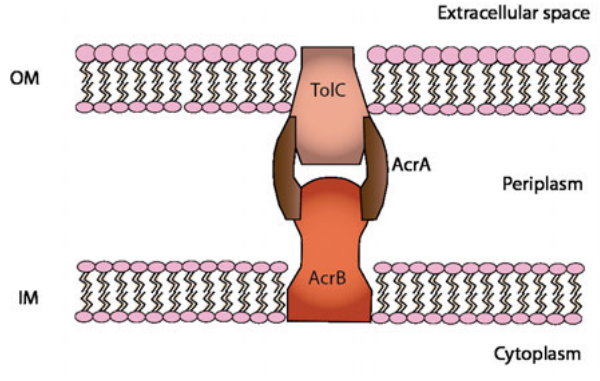


Figure 4. The AcrAB-TolC efflux pump of *E. coli*. AcrB is the inner membrane (IM) transporter responsible for recognizing substrates and providing energy by proton motive force. TolC is the outer membrane (OM) channel. AcrA is the membrane fusion protein linking AcrB and TolC to each other to form a continuous channel out of the bacterial cell.

AcrB – transporter protein

AcrB is a 1049 amino acid protein containing 12 transmembrane α-helices and two large loops located in the periplasm (Murakami et al. 2002). In the AcrAB-TolC structure, AcrB appears as a trimer with a central pore. The large transmembrane domain anchors the trimer in the inner membrane. The large periplasmic part of trimeric AcrB consists of a pore domain and a TolC docking domain. The TolC docking domain is a funnel like structure, which narrows to a pore as it leads into the transmembrane domain where it widens to a large central cavity. AcrB is responsible for substrate recognition/selection and energy transduction. Substrates bind to the wall of the central cavity of the transmembrane domain as well as to a periplasmic binding site (Yu et al. 2005; Aires & Nikaido 2005). AcrB is a proton antiporter and powers efflux by exchanging one H^+ ion for one substrate molecule.

Crystal structures of AcrB reveal that the AcrB monomer can take on three different conformational states; loose (L), tight (T) and open (O) (Pos 2009). Cycling of these conformational states is suggested to drive the transportation of compounds out of the cell. At low levels of substrate, an LLL conformation of the trimer is described. Binding of substrate to one of the three L monomers (low binding affinity) results in its conversion from L to T (strong binding affinity). For this T monomer to change its conformation to O (no substrate binding affinity) and release the substrate to the outside, another substrate molecule has to bind to a different L monomer in the trimer. The conversion from T to O is proposed to be an energy dependent step powered by proton transport from the periplasm to the cytoplasm. Upon the release of the substrate, the O monomer converts back to the L conformation.

TolC – outer membrane exit channel

The TolC monomer is a 493 amino acid protein containing a β-domain, an α-helical domain and a mixed α/β-domain (Koronakis et al. 2000). TolC exists as a trimer in the outer membrane forming a single exit channel out of the cell. The TolC trimer forms a cylinder like structure made up of a β-barrel channel embedded in the outer membrane and an α-helical barrel extending into the periplasm. A mixed α/β-domain forms a belt around the mid-section of the periplasmic domain. The opening in the outer membrane is constitutively open to the outside of the cell, while the periplasmic opening is closed (or rather, too small to allow passage of substrates) until a substrate binds to and is being channeled through AcrB to the periplasmic opening of TolC. The conformational changes in AcrB during substrate translocation (or interactions with the hexameric AcrA) are suggested to trigger a conformational change in TolC enlarging the diameter of the opening and allowing passage of the substrate (Koronakis et al. 2000).

The AcrB trimer is assembled constitutively, that is in absence of substrate and TolC. As substrate binds to AcrB, TolC is recruited and docks onto AcrB in the periplasm, their association being stabilized by the adaptor protein AcrA (Du et al. 2014). The assembly of the AcrAB-TolC machinery is only transient. When the substrate has been transported out of the cell, the complex disassembles again (Thanabalu et al. 1998).

AcrA – adapter protein

AcrA is a 397 amino acid lipoprotein. It assembles as a hexamer in the AcrAB-TolC structure (Du et al. 2014). As described above, AcrA couples the inner membrane transporter (AcrB) and the outer membrane exit channel (TolC). It is believed to be anchored to the inner membrane through a single transmembrane helix and then extends through the periplasm far enough to interact with both AcrB and TolC.

AcrZ – suggested modulator of AcrB activity

Recently, a small 49 amino acid protein, AcrZ (previously uncharacterized protein YbhT) was identified as a binding partner of the transporter AcrB (Hobbs et al. 2012). It folds into a hydrophobic α-helix that fits into a groove of the transmembrane domain of AcrB (Du et al. 2014). Cells lacking *acrZ* were found to be sensitive to some of the antibiotics (tetracycline, puromycin and chloramphenicol) exported by the AcrAB-TolC efflux pump, suggesting that AcrZ may function by affecting the substrate specificity of AcrB (Hobbs et al. 2012), possibly by changing the conformation or the organization of surface residues of the substrate binding pocket in AcrB (Du et al. 2014). The expression of *acrZ* is co-regulated with the expression of the genes encoding the other pump components, *acrA*, *acrB* and *tolC*, by MarA (as well as SoxS and Rob).

Regulation of the expression of the AcrAB-TolC efflux pump

Mutations that lead to overexpression of AcrAB-TolC increase the MIC for ciprofloxacin and many other antibiotics. The expression of AcrAB-TolC is negatively regulated by the local repressor *acrR* (*acrA* and *acrB*) and positively regulated by the global transcriptional regulator *marA*, which in turn is negatively regulated by the repressor *marR*. Mutations in either *acrR* or *marR* can cause increased expression of the AcrAB-TolC efflux pump. Also mutations in the promoter regions to which the regulators bind can affect expression.

AcrR – local repression

AcrA and AcrB are encoded by the *acrAB* operon and their expression is modulated by the local repressor AcrR, a member of the TetR family of transcriptional regulators (Fig. 5). The regulator gene, *acrR*, is located directly upstream of *acrAB*, but is divergently transcribed. AcrR also represses its own expression. A 24 base pair inverted repeat (IR) sequence overlapping the *acrAB* promoter region is suggested to be the target-binding site for AcrR (Su et al. 2007). Two dimers of AcrR cooperatively bind to the IR sequence thereby blocking the RNA polymerase from transcribing the genes (Li et al. 2007). However, AcrR does not bind so strongly and allows a certain level of constitutive expression of AcrAB. This confers intrinsic resistance, though not high enough to reach a clinically relevant level. AcrR can bind a wide variety of structurally different ligands overlapping with the substrates of the AcrB pump. Ligand binding to AcrR triggers conformational changes resulting in the release of the repressor from the target DNA and allowing transcription to occur.

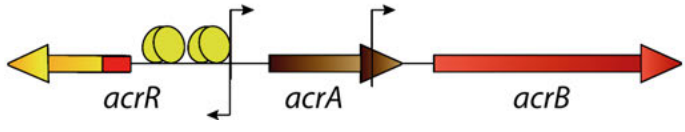


Figure 5. The *acrAB* operon is repressed by the divergently transcribed local dimeric repressor protein AcrR. Two dimers of AcrR cooperatively bind to an inverted repeat sequence in the promoter region of the *acrAB* operon and thereby blocks RNA transcription. The box in red represents a marbox to which the global transcriptional regulator MarA can bind to up-regulate the expression of *acrAB* (see below).

AcrR (Fig. 6) is a 215 amino acid protein folded into nine α-helices and divided into two domains, the N-terminal DNA-binding domain and the C-terminal ligand-binding and dimerization domain (Li et al. 2007). Helices α1 (amino acids 7-27), α2 (34-41), α3 (45-51) and part of α4 (55-62) make up the DNA-binding domain. Helices α2 and α3 form the helix-turn-helix (HTH) DNA binding motif typical of the TetR family and other prokaryotic transcriptional regulators. Aligning the HTH motif sequences of members of the TetR family show that amino acids L34, R45, A47, Y49, W50, H51 and K55 are well conserved indicating that they could be especially important for the DNA binding (Ramos et al. 2005). A speculative model of AcrR bound to DNA suggests that Y49, H51 and K55 make phosphate contacts with the target DNA and that R45 interacts directly with four different bases of the DNA, indicating critical roles of these amino acids for DNA binding (Li et al. 2007).

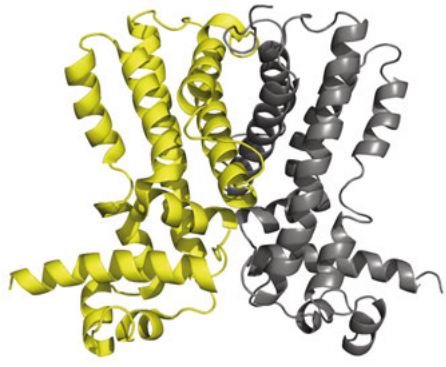


Figure 6. A ribbon diagram of dimeric AcrR showing one monomer in yellow and the other in grey. The figure was made with PyMOL (DeLano 2002) from the crystal structure of AcrR from *E. coli* (Li et al. 2007; pdb-code 2QOP).

Helices α4 (amino acids 63-65 and 69-80), α5 (85-102), α6 (104-115), α7 (122-151), α8 (160-180) and α9 (190-204) form the C-terminal ligand-binding and dimerization domain. Formation of the dimer mainly involves α8 and α9, but also α6 and α7 to a certain extent. Several close contact pairs have been identified from the crystal structure of AcrR, for example F114 and E176', R168 and N177', M201 and M201' and Q200 with C205' and P206' (the prime sign, ', refers to the second subunit) (Li et al. 2007). The helices comprising the C-terminal domain together form a large internal cavity predicted to serve as a ligand-binding pocket. In this mostly hydrophobic binding pocket are W63, I70 and F114 indicated to be especially important for binding ligands (Li et al. 2007). Deeply buried in the pocket is E67, a negatively charged amino acid expected to be important for binding positively charged ligands.

MarA – global regulation

The expression of the AcrAB-TolC efflux pump is also controlled by the global transcriptional regulator MarA and/or by the closely related regulators SoxS and Rob (members of the AraC family of transcriptional regulators). These homologous proteins can each bind to the same 20 base pair DNA sequence, the so-called marbox, located within the promoter region of many genes. The regulons of these three regulator proteins have a high degree of overlap (a common set of about 40 genes (Duval & Lister 2013)), but each is activated by a different signal or signals. MarA is activated by, for example, salicylate, SoxS by oxidative stress and Rob by, for example, bile salts. The overlap in regulons, and their responsiveness to different stimuli, allows for

a co-ordinated response to different extracellular threats. There are thousands of marbox sequences distributed throughout the *E. coli* genome. However, a functional marbox needs to be in a specific orientation and position relative to the -35 and -10 RNA polymerase signal sequences for the binding of a regulator protein to have an effect on transcription (Martin et al. 1999).

MarA is a 127 amino acid protein composed of seven α-helices folding into two domains, each containing a HTH DNA binding motif (Rhee et al. 1998). MarA binds to the marbox as a monomer, but the monomer binds at two sites within the marbox due to the two HTH motifs. The formation of a MarA-DNA-RNA polymerase ternary complex activates transcription. The MarA regulon consists of a diverse collection of genes. Barbosa & Levy (2000) characterized the MarA regulon by using DNA microarrays containing most of the genomic open reading frames (ORFs) of *E. coli* and comparing the expression of the genome in presence and absence of MarA. cDNA was prepared from RNA extracted from *mar*-deleted and *mar*-expressing strains and hybridized to microarrays. The microarrays identified 62 genes whose expression was affected by *marA* expression; 47 genes were up-regulated and 15 genes were down-regulated. These genes are spread throughout the chromosome and are involved in a wide range of cellular functions. It is not yet fully understood how (or whether) all of these genes interact in the development of an MDR phenotype. Among the up-regulated genes are the efflux pump genes *acrA*, *acrB* and *tolC* as well as *micF*, a gene encoding an antisense RNA that decreases the expression of the outer membrane porin OmpF. Increased expression of these four genes together results in a decreased influx and an increased efflux of certain antibiotics and thereby contributes to an MDR resistance phenotype.

MarA does not activate each gene of the regulon to the same extent. This is mainly due to small differences in the marbox sequences that results in different repressor-binding strengths. The binding strength to and thereby transcriptional regulation of the target genes is also dependent on the concentration of the MarA regulator in the cell.

MarR – repressor of the *marRAB* operon

The intracellular level of MarA is controlled by the local repressor MarR. *marA* is part of the *marRAB* operon (Fig. 7). The mar locus consists of two divergent transcriptional units (regulated from different promotors) that flank the operator region *marO*. One operon includes *marC*, a gene of unknown function. The divergent operon comprises *marRAB*, encoding the local transcriptional repressor MarR, the global transcriptional regulator MarA and MarB, a gene reported to repress the expression of the *marRAB* operon by reducing the rate of transcription of *marA* via an unknown indirect mechanism (Vinué et al. 2013).

*Figure 7.*The *mar* locus encodes *marC* (unknown function) and the *marRAB* operon transcribed divergently; *marR* (the local transcriptional repressor), *marA* (the global transcriptional regulator) and *marB* (suggested regulator of *marA* transcription). MarR represses by binding as a dimer to two sites within *marO* (the operator region located in between *marC* and the *marRAB* operon). The -35 and -10 RNA polymerase signal sequences are indicated. As MarR repression is relieved, *marRAB* is transcribed. MarA binds to marbox sequences in the genome to regulate transcription of genes such as the *marRAB* operon, *acrAB*, *tolC* and *micF*, among many others.

MarR represses transcription of *marRAB* by binding as a dimer to each of two direct repeats, sites I and II, within the *mar* operator, preventing binding of the RNA polymerase. The MarR binding sites are located downstream of and do not overlap with the MarA binding site (the marbox). A moderate degree of competition between the two proteins does however exist (Martin et al. 1996). When an inducer of expression binds to MarR, the conformation of MarR changes to a non-DNA-binding conformation. As MarR no longer blocks the transcriptional starting site, RNA polymerase can now bind and start transcribing the *marRAB* operon. Once MarA is expressed, it binds to the marbox upstream of the *marRAB* operon up-regulating its own production. This positive feedback loop results in highly elevated intracellular levels of MarA that can then bind to the marboxes of genes in the MarA regulon to regulate their expression. The different genes in the MarA regulon are activated by different MarA concentrations. Known inducers of the *marRAB* operon are salicylate and other aromatic acids, uncoupling agents such as carbonyl cyanide m-chlorophe- nylhydrazone and redox-cycling compounds such as menadione and plumbagin (Cohen et al. 1993b; Hächler et al. 1991; Alekshun & Levy 1999), while the natural inducer of MarR derepression remains unknown. Since MarR is known to regulate resistance to structurally different antibiotics, it has been suggested that it is unlikely that MarR can bind to such a wide variety of compounds with enough specificity and that it therefore could instead be a cellular product generated at exposure to the compounds that is the actual inducing signal for MarR derepression. Hao and collegues (Hao et al. 2014) reported that copper(II) ions are able to cause dissociation of MarR from its target DNA. The copper(II) ions generate disulfide bonds between two MarR dimers by oxidizing a cysteine residue (Cys80) on MarR and this dimerization of MarR dimers impairs MarR's DNA-binding ability causing dissociation of MarR from its target DNA and derepression of the *marRAB* operon. They showed that presence of salicylate

and the antibiotics Norfloxacin (a fluoroquinolone) and Ampicillin increases the level of intracellular copper and induces MarR derepression, suggesting that MarR is a copper sensor and that copper(II) ions could be the natural inducer of MarR derepression. Increased expression of the *marRAB* operon also results from mutations in *marR* or *marO*.

MarR is a 144 amino acid protein folded into both α-helices and β-sheets and can be divided into a dimerization domain and a DNA-binding domain (Fig. 8) (Alekshun et al. 2001). The N- and C-terminal regions (amino acids 10-21 and 123-144 respectively) form the dimerization domain and intertwine with the corresponding regions in the other subunit of the dimer. The crystal structure reveals amino acids involved in interacting with the second subunit, for example hydrogen bonds between Lys24 and Pro144' and Glu10 and Lys140' and salt bridges between Asp67 and Arg73' and Asp67' and Arg73. The DNA-binding domain is composed by amino acids 55-100, which build up a winged-helix DNA binding domain (α2-β1-α3-α4-β2-W(wing)1-β3). Helix α4 contributes most to DNA recognition.

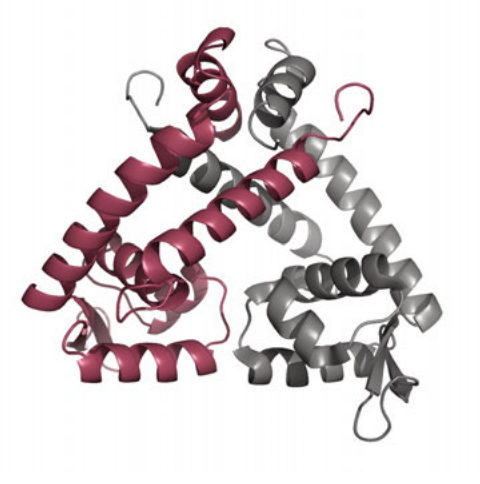


Figure 8. A ribbon diagram of dimeric MarR showing one monomer in winered and the other in grey. The figure was made with PyMOL (DeLano 2002) from the crystal structure of MarR from *E. coli* (Alekshun et al. 2001); pdb-code 1JGS).

Ciprofloxacin resistance due to overexpression of the AcrAB-TolC efflux pump in clinical isolates

Overexpression of the AcrAB-TolC efflux pump alone does not confer a clinical level of ciprofloxacin resistance in *E. coli*. However, in combination with mutations in topoisomerase genes, mainly *gyrA* and *parC*, overproduction of the efflux pump contributes to high levels of resistance. Increased expression of the AcrAB-TolC efflux pump in clinical isolates has been identified to be due to one of four mechanisms; i) mutations in the local repressor *acrR*, ii) mutations in *marR* repressing the global transcriptional regulator MarA, iii) mutations in *marO* to which MarR binds to repress and iv) insertion sequences upstream of *acrAB* and *tolC* (Piddock 2006) that may provide alternative transcriptional initiation sequences. A number of insertion elements, duplications, deletions and single amino acid substitutions inactivating AcrR and MarR and resulting in increased efflux expression and ciprofloxacin resistance have been observed in clinical isolates of *E. coli* (Webber & Piddock 2001; Wang et al. 2001; Komp Lindgren et al. 2003; Linde et al. 2000; Oethinger et al. 1998; **Paper I**; **Paper II**). Some insertion sequences have promoters that can affect the expression of downstream genes. If such insertion sequences are present in front of genes encoding components of the efflux pump, those genes can be overexpressed contributing to resistance. Swick and collegues (Swick et al. 2011) saw a correlation between the number of antibiotics clinical isolates of *E. coli* were resistant to and overexpression of AcrAB. The stronger the MDR phenotype, the higher the probability that the clinical isolate also overexpressed AcrAB. This correlation supports the importance of efflux as a mechanism contributing to antibiotic resistance, in particular MDR.

Present investigations

Background

Ciprofloxacin is a clinically important fluoroquinolone with good activity against a broad range of clinically relevant Gram-negative and Gram-positive bacteria, including *Escherichia coli*, one of the main causes of urinary tract infections and invasive septicemia. However, the rapid development of resistance to ciprofloxacin observed in clinical isolates reduces its therapeutic usefulness. Clinical resistance to ciprofloxacin requires acquisition of several genetic alterations, almost always in drug target genes (*gyrA*/*gyrB*), sometimes in combination with genes involved in up-regulation of drug efflux (*marR*/*acrR*/*soxR*). Since neither *marR* nor *acrR* is essential, and up-regulation of drug efflux resulting in resistance can be caused by inactivating either repressor, the target size for viable mutations is very large and includes both inactivating and single amino acid substitution mutations. Many but not all clinical isolates that express resistance to ciprofloxacin carry mutations in *marR* and/or *acrR*. In contrast, almost all resistant clinical isolates carry single amino acid substitution mutations in the drug target gene *gyrA*. This contrast is surprising because the spontaneous mutation rate to inactivate non-essential repressors is expected to be several orders of magnitude greater than the rate of specific amino acid substitution mutations in an essential gene such as *gyrA*. There is an additional unexpected bias in the mutations that are found among the clinical resistant isolates that putatively up-regulate efflux via mutations in *marR* and *acrR*. Namely, that a selection to inactivate the repressors would be expected to result in a strong bias in the mutational spectrum in favour of gene-inactivating mutations over single amino acid substitutions. However, when the genotypes of ciprofloxacin-resistant clinical isolates of *E. coli* are examined, the spectrum of observed putative mutations in *marR* and *acrR* is not found to be heavily biased in favour of inactivating mutations, but rather in favour of amino acid substitutions. We set out to identify which selective forces constrain the evolution of mutations up-regulating drug efflux in the development of ciprofloxacin resistance.

Aims of the thesis

Given observations of unexpectedly low frequencies of inactivating mutations in the transcriptional regulators *marR* and *acrR* in ciprofloxacin-resistant clinical isolates and based on our hypothesis that this bias in favour of single amino acid substitutions could be due to fitness cost differences, the aims of this thesis were:

1. To identify the mutational distributions (inactivating versus single amino acid substitution mutations) that appear in the efflux regulators MarR and AcrR in clinical isolates and evaluate if they are dominated by inactivating mutations, as would be expected by selection for maximum relief of repression. (**Paper I and II**).

2. To investigate what selective forces shape the distribution of mutations that appears. Do the different types of mutations have different effects on drug susceptibility and, or, on the relative fitness of the mutants? (**Paper I and II**).

3. To examine by experimental evolution whether *marR* mutations conferring a higher level of resistance at a high fitness cost can evolve to acquire mutations that improve fitness and whether this can be achieved without reversing the resistance phenotype. Such a scenario would greatly improve the chances of high resistance genotypes to stabilize in bacterial populations also in environments without the selective pressure of the drug. (**Paper III**).

Results

Paper I - Fitness cost constrains the spectrum of *marR* mutations in ciprofloxacin-resistant *Escherichia coli*.

When studying the spectrum of mutations in *marR* (repressor of the *marRAB* operon) in ciprofloxacin-resistant clinical isolates, we could determine that there is a strong bias against inactivating mutations. We constructed isogenic strains carrying 36 different *marR* mutations, both inactivating and single amino acid substitution mutations, identified in ciprofloxacin-resistant clinical isolates or selected for resistance *in vitro*. We measured the effect on minimal inhibitory concentration (MIC), relative fitness in growth competition assays in the presence and absence of ciprofloxacin, and expression levels of *marA* (transcriptional regulator regulated by *marR*) and *acrA*, *acrB* and *tolC* (drug efflux pump components regulated by *marA*) of each individual *marR* mutation. In general the mutations identified in clinical isolates conferred low levels of resistance combined with low growth fitness costs

suggesting that selection favours mutations that have the lowest fitness costs, even though these confer only modest reductions in drug susceptibility. We also found a strong correlation between the level of expression of *marA* and the level of expression of efflux pump components and MIC, supporting a functional connection between *marR* mutations and reduced susceptibility to ciprofloxacin.

Paper II - A biased spectrum of mutations in *acrR* among ciprofloxacin-resistant isolates of *Escherichia coli.*

In **Paper II** we studied the spectrum of mutations in *acrR* (repressor of the *acrAB* operon) appearing in ciprofloxacin-resistant clinical isolates of *E. coli* and what selective pressures constrain which types of mutations appear. As in the case of *marR* (**Paper I**) we could confirm a strong bias against gene-inactivating mutations in *acrR* among clinical isolates. We constructed isogenic strains carrying 30 different *acrR* mutations, both inactivating and single amino acid substitution mutations, identified in ciprofloxacin-resistant clinical isolates or selected for resistance *in vitro*. For each strain, MIC for ciprofloxacin and relative competitive growth fitness in the presence and absence of ciprofloxacin were measured. qPCR was used to measure the effect of individual *acrR* mutations on the expression levels of efflux pump components *acrA* and *acrB*. In general single amino acid substitution mutations caused lower levels of resistance and incurred lower fitness costs, supporting the hypothesis that mutations with low fitness costs have a selective advantage over mutations conferring higher levels of resistance.

Paper III - Genetics of fitness-compensation in experimentally evolved *marR* mutants of *Escherichia coli*.

We experimentally evolved multiple independent lineages of strains carrying predicted inactivating mutations in *marR* that confer large reductions in susceptibility to ciprofloxacin combined with high fitness costs to select for improved competitive growth fitness (strains studied in **Paper I**) for 500 generations. We found that compensatory evolution had occurred in all evolved lineages. Putative compensatory mutations were identified by whole genome sequencing and they, in particular those identified in strains that had acquired only a single mutation, suggest that a mutation in any one of three different genes, *marA* (the positive regulator of AcrAB), *lon* (a protease that controls levels of MarA protein) or *arcA* (a global transcriptional regulator) is sufficient to compensate for the fitness cost of the *marR* mutation. The data suggest that mutations in *marA* or *lon* act by reducing expression of the AcrAB-TolC efflux pump and restore growth fitness at the cost of losing resistance to ciprofloxacin. In **Paper I** we found that resistance mutations in

clinical isolates are primarily selected for high relative fitness rather than for increased resistance and the data in **Paper III** suggest that fitness-compensatory mutations are subsequently selected to reduce the fitness cost caused by resistance mutations by down-regulating the expression of MarA, the positive regulator of the Mar regulon (including the AcrAB efflux pump). In contrast, the mutations in *arcA* increase growth fitness of the evolved strains without increasing the susceptibility to ciprofloxacin (and without affecting the expression of the AcrAB-TolC pump). The mechanism of action of the *arcA* mutations is currently under investigation.

Paper IV - Functional significance of *leuS* mutations in *Escherichia coli* resistance to ciprofloxacin.

During an evolution experiment selecting for resistance to ciprofloxacin, we discovered that in addition to the occurrence of expected mutations in drug target genes and genes regulating drug efflux, mutations also arose frequently in aminoacyl-tRNA synthetase genes. In **Paper IV** we study the contribution of two independently selected *leuS* mutations to the evolution of ciprofloxacin resistance and the mechanism by which the *leuS* mutations reduce ciprofloxacin susceptibility. We found that introducing a *leuS* mutation into a strain with a drug target or efflux resistance mutation increases MIC and competitive fitness as a function of increasing ciprofloxacin concentration, confirming that *leuS* mutations contribute to decreased drug susceptibility. Furthermore, deletion of *relA* abolished the ability of *leuS* mutations to increase the MIC, suggesting that the mechanism of reduced susceptibility is via induction of the stringent response. This was also supported by RNA sequencing analysis of strains carrying the *leuS* mutations in which 119 genes in another study shown to be up-regulated by the onset of the stringent response were up-regulated in the strains with the *leuS* mutations. The RNA sequencing analysis further showed that transcript levels of efflux genes *mdtK*, *acrZ* and *ydhJK* mRNA were altered in the *leuS* mutants, suggesting that increased efflux is likely to be responsible for the resistance phenotype, which is currently under investigation.

Concluding remarks

1. Our data show that the spectrum of ‘resistance’ mutations accessible among clinical isolates is narrower than previously thought. That is, the majority of mutations that arise spontaneously in the efflux regulators *marR* and *acrR* have associated fitness problems that select against their fixation in bacterial populations. This explains the observation that, although efflux regulator mutations occur at a frequency that is at least 2 orders of magni-

tude greater than that of mutations occurring in drug target genes, such efflux mutations are not universally found in resistant clinical isolates.

2. Our experimental evolution data suggest that the high-frequency low-fitness 'resistance' mutations that occur in *marR* cannot easily evolve to reduce their fitness cost without simultaneously losing their resistance phenotype. We can speculate that the amelioration of the mutational fitness cost is constrained by the complexity of the Mar regulon. Mutations that inactivate the MarR repressor cause a constitutive activation of MarA, positive regulator of the entire Mar regulon. If the fitness costs are caused by misregulation of several components of the Mar regulon then there may be no simple genetic path to amelioration, other than reducing the expression of the global regulator MarA. Such a path reduces the fitness cost but simultaneously also reduces the expression of the AcrAB pump and this reduces resistance to ciprofloxacin. Our current data (we do not yet know how the ArcA mutants function) suggests that the high-cost mutants affecting *marR* cannot be compensated without reducing overexpression of the AcrAB pump.

3. The discovery of mutations in tRNA synthetases as contributors to the evolution of resistance to ciprofloxacin shows that the potential 'resistome' for this drug is much larger than previously realized. We showed that the resistance phenotype of the *leuS* mutations is mediated through increased drug efflux. However, as with the mutations affecting *marR* and *acrR*, the mutations in *leuS* come with a significant fitness cost. This fitness cost could explain why such mutations in *leuS* or any of the other synthetases have not been identified as contributing to resistance in clinical isolates.

In conclusion, the research finding in this thesis suggest that while the potential ciprofloxacin resistome may include many additional genes than previously suspected, that this is strongly counteracted by the selection in clinical isolates for low fitness cost genetic alterations. Ultimately, the selection for bacteria with a high relative fitness severely constrains the genetic alterations that survive and get fixed in clinical populations of bacteria.

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