Quantitative relationship between antibiotic exposure and the acquisition and transmission of resistance in bacteria in the laboratory

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The cover of this thesis was designed by N. Händel and illustrates bacterial colony growth in a petri dish in close vicinity to two antibiotic discs. It exemplifies two major characteristics of bacteria that have been studied and compared extensively in this thesis: susceptibility (left) and resistance (right) to antibiotics. The author also included two chemical molecules in the background scatter. The carbon atom forms the basis of all known life on Earth and is shown in the form of the simplest organic molecule structure – a hydrocarbon CH₄. The second molecule is a simplified depiction of benzene that is characterized by 6 carbon atoms joined in a ring. Benzene is often found in chemical structures of antibiotics.

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Table of contents

**Chapter 1:** General introduction.................................................. 7

**Chapter 2:** Compensation of the metabolic costs of antibiotic resistance by physiological adaptation in *Escherichia coli*................................................................. 29

**Chapter 3:** Interaction between mutations and regulation of gene expression during development of *de novo* antibiotic resistance............................................................... 57

**Chapter 4:** Effects of stress, ROS and the SOS response during the *de novo* acquisition of antibiotic resistance in *Escherichia coli*................................................................. 79

**Chapter 5:** Factors that affect the transfer of a β-lactam resistance conferring plasmid................................................................. 99

**Chapter 6:** General discussion.......................................................... 119

**References** .......................................................................................... 129

**Summary/Samenvatting**........................................................................ 149

**List of Publications**................................................................................ 159

**Acknowledgements**................................................................................ 161
Chapter 1

General introduction
Introduction

Microbial organisms are ubiquitous, essential components of all ecosystems and have evolved in over more than 3.8 billion years to their present variety [1]. Although largely invisible to the eye, prokaryotes and lower eukaryotes are an essential component to the earth’s ecological system. The complex and diverse metabolism of microbes performs indispensable transformations in the biogeochemical cycles of the biosphere and the production of important components of the earth’s atmosphere [2]. Bacteria and fungi have major roles in food production and nutrient recycling and microbial communities reside on our skin, and in our mouth, esophagus, stomach, colon, and vagina [3].

Microbes have the ability to survive using a diverse set of strategies to dominate, co-exist, or compete for nutrients. Both bacteria and fungi do not live in isolation, they exist in large communities. Complex multicellular structures observed during the formation of biofilms demonstrate the possibility of single cells to interact and communicate with each other through the production and excretion of signaling molecules. Signaling has been shown to occur on an intra- and interspecies level and can present both cooperative or selfish behavior [4]. Signaling molecules are mostly produced and used by microbes for increased nutrient acquisition, survival in specific environmental niches, protection or as weapon against competitors [5]. Components belonging to the functional class of antibiotics are able to kill or inhibit microbial growth of potential competitors. The main action of antibiotics was thought for a long time to be solely harmful to other organisms. Since the beginning of the 21st century more and more evidence exits that antibiotics are not only weapons against competitors. Antibiotics have been shown to trigger altered gene expression in a concentration dependent manner, whereby every component induces a very specific microbial response and can be toxic at high concentrations [6]. In the thesis we will focus on prokaryotes (bacteria) and their interactions with antibiotics.

The discovery and introduction of manmade antibiotics in the 1930s initiated a period of drug innovation and utilization in the clinical and agricultural sector. But soon after the introduction, antibiotic resistant bacteria appeared in hospitals, where initially most antibiotics were being used [7]. Antibiotics are commonly used for the treatment of infectious diseases. Such
antimicrobials are also applied as prophylaxes for reducing the risk of infection resulting from complex medical interventions, such as organ transplants and cancer chemotherapy [8].

In addition, antibiotics were introduced in the agricultural sector for non-therapeutic purposes as growth promoters to improve food conversion [8]. With the dramatic increase of antibiotic use in the past 80 years, microbes have evolved strategies to become and remain drug resistant. Bacteria can get resistant through phenotypic adaptation, mutation or the transfer of mobile elements in order to protect or substitute the drug target, detoxify the cell through increased efflux or prevent the drug accumulation inside the cell (table 1). For instance, $\beta$-lactam antibiotics impede proper biosynthesis of the cell wall. Some bacterial species are able to induce overproduction of enzymes, so called $\beta$-lactamases, that can hydrolyze this drug. Other organisms can profit from the transfer and expression of mobile elements that encode for $\beta$-lactamases. Resistance to the antibiotic class of quinolones is mainly brought about by alterations of the drug target. As quinolones bind to the enzyme gyrase which is involved in DNA replication, resistance can evolve by mutations that change the quinolone binding pocket and thus prevent inhibition of the gyrase activity. In addition, decreased expression of porins or increased expression of multidrug efflux pumps results in a reduced accumulation of the drug inside the cell.

Nowadays, resistance has become a threat to human health as only very few last resort antibiotics are available that still show antimicrobial activity against resistant strains from common pathogens. As innovative strategies or new antimicrobial components are lacking, international organizations, such as the World Health Organization (WHO), and researchers raise alarms about the increasing number of drug resistant microbes [9, 10]. The European Commission estimated in 2011 that within the EU antibiotic resistance caused 25,000 human deaths annually and extra healthcare costs and productivity losses of at least € 1.5 billion [8].
Table 1. Modes of action and resistance of commonly used antibiotics adapted from reference Morar and Wright (2010) [11].

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>Example</th>
<th>Target</th>
<th>Mode(s) of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactam</td>
<td>Penicillins (ampicillin, amoxicillin); Cephalosporins (cephamycin)</td>
<td>Peptidoglycan biosynthesis</td>
<td>Hydrolysis, efflux, altered target</td>
</tr>
<tr>
<td>Quinolone</td>
<td>Enrofloxacin, Ciprofloxacin</td>
<td>DNA replication</td>
<td>Acetylation, efflux, altered target</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Minocycline, Tigecycline</td>
<td>Translation</td>
<td>Monooxygenation, efflux, altered target</td>
</tr>
<tr>
<td>Aminoglycoside</td>
<td>Gentamicin, Streptomycin</td>
<td>Translation</td>
<td>Phosphorylation, acetylation, nucleotidylaction, efflux, altered target</td>
</tr>
<tr>
<td>Glycopeptide</td>
<td>Vancomycin, Teicoplanin</td>
<td>Peptidoglycan biosynthesis</td>
<td>Reprogramming peptidoglycan biosynthesis</td>
</tr>
<tr>
<td>Macrolide</td>
<td>Erythromycin, Azithromycin</td>
<td>Translation</td>
<td>Hydrolysis, glycosylation, phosphorylation, efflux, drug target</td>
</tr>
</tbody>
</table>

Drug resistance is not exclusively a recent phenomenon. The metagenomic analysis of ancient DNA isolated from 30,000-year-old Beringian permafrost sediments demonstrated the existence of a highly diverse collection of genes encoding resistance to β-lactam, tetracycline and glycopeptide antibiotics [12]. The dramatic overuse of antibiotics in the past 80 years created a selective pressure for antibiotic resistance genes that have since then spread among a variety of different bacterial species and over long distances. The tetracycline conferring resistance gene tetM, for example, was found in staphylococci, streptococci, enterococci, clostridia, listeria and enteric bacteria [13]. A broad host range plasmids, like the RP1 that encodes resistance to ampicillin, tetracycline and kanamycin and that originated in a species of Pseudomonas, enables the transfer of (multiple) resistance determinants to most, if not all Gram-negative bacteria [14].
To understand why we face an epidemic of antibiotic resistant bacteria the following sections will be used to highlight (1) factors that increase the antibiotic concentration in the environment and (2) genetic and physiological effects of antibiotics that drive the acquisition of resistance.

1. Factors that increase the antibiotic concentration in the environment

1.1. Overuse and misuse in hospitals

Human health care faces increasing emergence and spread of antibiotic resistant bacteria worldwide. In many cases, only few antimicrobials remain for effective treatment. In particular methicillin-resistant and vancomycin-tolerant *Staphylococcus aureus* and gram-negative bacteria producing extended spectrum β-lactamases (ESBLs) with resistance to multiple other antibiotics pose a threat to human health [15]. Because of their ability to cure infections, antibiotics have become indispensable. At the same time there is a substantial unnecessary use of antimicrobial compounds, effectively selecting for resistant microbes [16]. Inadequate treatment can be avoided by clinicians who ensure the selection of the appropriate antimicrobial with activity against the identified pathogen. A clinicians duty must be that antibiotic administration follows requirements such as adequate dosing, proper interval administrations and avoidance of unwanted drug interactions and patients should adhere strictly to the instructions they receive from their prescribing physicians [15]. Misuse of antimicrobial compounds could be avoided by educating staff and by focusing on improving adherence to guidelines for the use of antimicrobial agents.

Unfortunately, misuse of drugs is still a widespread and common phenomenon. For example, Werner et al. (2011) showed that 39% of all requested therapeutic fluoroquinolones were inappropriate among hospitalized patients in the MetroHealth Medical Center (Ohio, US), whereby the most frequent errors were deviation from standard use for viral or non-infectious diseases and inappropriate length of treatment [17]. Despite the intention to cure patients, 27% of treatment showed even adverse effects including gastrointestinal problems, colonization by resistant pathogens, or *Clostridium difficile* infection. The misuse of antibiotics does not leave the host unaffected, even though the individual might seem healthy. The effect of ciprofloxacin treatment of a usual 5 days course on 3 healthy individuals showed a dramatic
and immediate antibiotic induced decrease in the phylogenetic diversity of the microbial gut ecosystem. Abundance of about a third of the bacterial taxa was affected [18]. The bacterial community was reassembled after approximately 4 weeks, but several taxa did not recover within 6 month.

The widespread use of antibiotics has most likely already induced changes in the human microbiome. In 2009, Sommer et al. investigated the microbial community of 2 unrelated healthy individuals who had not been treated with antibiotics for at least one year [19]. Half of the identified resistance genes in aerobic cultured isolates were identical to the ones found in major pathogens, even though this fraction represents only a small subset of the gut community. The pure existence of these resistance genes in healthy individuals can result in rapid enrichment, selection and further spread of resistance upon antibiotic exposure.

The reduction of excessive antibiotic use in hospitals is beneficial in the battle against the emergence of antibiotic resistance. Restricting fluoroquinolone usage to its minimum in an intensive care unit for 6 months resulted in a 75.8% reduced prescription rate of this antibiotic class [20]. Subsequently, the frequency of resistant *P. aeruginosa* dropped from 71.3 to 52.4%. Several studies showed similar trends upon the limitation of antibiotic usage indicating that restricting is an intervention of prime importance [21-23].

After their use in man, antimicrobials enter the sewer system. Depending on their polarity, water solubility and persistence, antibiotics are released into rivers or accumulate with sewage sludge [24]. Transfer of resistance conferring plasmids has been demonstrated to occur in aquatic environments, such as seawater [25]. Individual drug concentrations in the aquatic environment are found to be rather low [26, 27], but the additive effect of antibiotics sharing the same mode of action could present a stressful environment for sensitive bacteria, inducing cellular protection mechanisms or bacterial mutagenesis (see section 2.2) and in turn select for resistance.

### 1.2. Overuse in agriculture

Antibiotics are commonly administered to food-animals for prophylactic and therapeutic use. Subtherapeutic quantities of antibiotics are also administered in many countries to healthy food-animals as it enhances the growth rate and feed-
to-weight ratio for poultry, swine, and beef cattle [28, 29]. Soon after the introduction of antibiotics in livestock in the 1950s a warning against evolving resistance was issued in response to the discovery of transferable oxytetracycline resistance in *Salmonella enterica* Serovar Typhimurium [30]. Another wakeup call in the early 1990s was the link between growth promoting avoparin usage and glycopeptide-resistant enterococci isolated in humans and this has led to a reduction of antibiotics in livestock all over Europe [31, 32]. Resistance genes can be transferred to the microbiota of farm workers that can pass them on to family members [33, 34].

Reduction in antibiotic usage for food-animals has been shown to reduce resistance level in microbes. In Denmark, for example, 73% of *Enterococcus faecium* isolates in broiler chickens were vancomycin-resistant before growth promoting antibiotics were prohibited. Following a ban in 1997, the resistance level dropped to 6% in 2000 [35]. This and similar outcomes resulted in the ban for the use of all antibiotics as growth promoting agents in 2006 all over Europe. In the Netherlands the total sales of antibiotics for veterinary use dropped from 519 tons in 2009 to 244 tons in 2012, corresponding to an overall decrease of 53% that has been observed across all livestock sectors [36].

Unfortunately, the drastic reduction of antibiotic usage for food-animals does not solve the worldwide threat of antimicrobial resistance overnight. If there is no reservoir for susceptible bacteria available to recolonize the host, elimination of resistance determinants occurs very slowly, if at all. Frei et al. (2001), for example, demonstrated that gram-positive bacteria isolated from intestines of broilers were resistant to bacitracin, even though only their parental generation was treated with this drug. This study illustrates the possibility of the transfer of resistance determinants to descendants without any antibiotic pressure and shows the difficulty of eliminating resistance genes from the environment [37].

Evaluations about antibiotic usage in food-animals vary widely, whereby estimates indicate that 50 - 80% of the total antibiotic production is still consumed in livestock [38]. After the ban on using antibiotics as growth promoters in the EU, more therapeutic antimicrobials had to be used in some countries in order to control animal diseases. In Denmark, for example, it was reported that after an initial halving of antibiotic usage in food animals, the
amount of therapeutic antimicrobials increased again by more than 10% over
the last ten years [39]. Once administered, veterinary antibiotics are excreted
through animal urine and feces and are often not entirely removed during
sewage treatment [40], causing accumulation of resistance genes in the soil. In
the Netherlands, for example, the abundance of 18 different major antibiotic
resistance gene classes found in the soil increased in the last 70 years [41].
Especially tetracyclines were found to be at least 15 times more abundant than
in 1970 demonstrating the clear link between overuse of antibiotics and the
accumulation of resistance determinants in the environment. Hamscher et al.
(2002) studied the distribution and persistence of antibiotics in a field fertilized
with liquid manure and detected average concentrations of tetracycline of 172
µg/kg soil at a depth of 30 cm one month after manure application [42]. The fate
of antibiotics in the soil or groundwater strongly depends on their physical and
chemical properties. Many substances, such as tetracyclines, are polar and
barley soluble in water [43]. Thus, antibiotic contamination in aquatic
environments due to manure application may be restricted to only some
antibiotic classes. Nevertheless, existence of only small amounts of antibiotics
in soil can result in the selection of resistant bacteria.

Food animals can harbor antibiotic resistant bacteria that may spread to soil,
ground and surface water through the use of manure on agricultural fields [44-46]. Even though these bacteria might not persist in the environment for a long
time, resistance conferring genes may be transferred to resident soil
microorganisms through conjugation. As only less than 1% of the soil bacteria
are cultivable [47], quantification of horizontal transfer events occurring in soil
is difficult. Studies using cultivatable organisms or phylogenetic analysis,
however, suggest that the soil presents a huge reservoir for the transfer of
resistance genes between bacteria [48, 49]. In addition, many food-borne
pathogens can end up on meat products during slaughter. Thus, consumers of
meat are threatened through mishandling of raw meat or insufficient cooking.
The consumption of raw vegetables grown on manure treated fields turns out to
be a route of human exposure to antibiotic-resistant bacteria as well [50-52].
However, Abriouel et al. (2008) showed that enterococcal isolates from fruits
and vegetables were much less antibiotic resistant compared to clinical isolates
[53]. The cycle of treating animals with antibiotics and applying untreated
manure on croplands results in spread of resistance genes to indigenous soil bacteria through horizontal gene transfer, a dissemination route that could transfer resistance back to humans as illustrated in figure 1. The use of similar antibiotics in the agricultural and medical sector increases the possibility of selection and transfer of resistance genes between animals and man even more. Mobile resistance elements, virulence genes and the genomic backbone identified in both sectors show significant genetic similarities [54, 55].

**Figure 1.** Environmental and social factors driving the acquisition of resistance to antibiotics in bacteria.

### 1.3. Social issues and public misuse

The increase in internet access enables humans around the globe to order and obtain medicines anonymously without consultations with clinicians. Drugs can be advertised through the internet and the medical guidance offered is usually of low quality. The resulting increase in self-medication selects for resistant microbes. Usage in countries with less restrictions regarding drug prescriptions and lower costs counters the effect of adherence to strict guidelines elsewhere.
Morgan et al. (2011) demonstrated that self-medication and non-prescribed antimicrobials are associated with the choice of an inappropriate drug itself, but also incorrect dosing [56]. Mostly, inappropriate behavior can be attributed to the lack of awareness of the potential consequences [57]. Studies focusing on the link between awareness and behavior in European countries showed that awareness of antibiotic resistance was lowest in countries facing the highest rates of antibiotic resistant microbes. In 2011 the north-south gradient of E. coli isolates resistant to third-generation cephalosporins ranged from 3% in Sweden to 36% in Cyprus [58].

Even if people are aware of the problem, international travel contributes to the spread of resistant microbes around the globe [59]. A very clear example of how international travel can contribute to the spread of resistant pathogens in globally distant areas is illustrated by the New Delhi metallo-β-lactamase 1 (NDM-1). In 2008, NDM-1 was first characterized in isolates of a K. pneumoniae and E. coli recovered from a Swedish patient who has been hospitalized earlier in New Delhi, India [60]. NDM-1 was produced both by a K. pneumoniae isolate from urine and a faecal E. coli isolate from the same patient indicating the occurrence of in vivo transfer. Acquisition of the NDM-1 lactamase compromises the efficacy of almost all β-lactams, including carababenemes that are known as the last resort β-lactams [61]. The epidemiological link between NDM-1 and the Indian subcontinent was further strengthened by additional isolation of NDM-1 producers from patients in India, Pakistan and the UK, whereby many of the UK hospitalized patients had been travelling to India or Pakistan within the past year [62]. Up to now, NDM-1 positive isolates have been reported in 40 countries covering all continents except Antarctica, illustrating that the threat of antibiotic resistance transcends national borders [63]. Controlling this epidemic spread of NDM-1 and other resistance genes requires cooperation between health authorities and elaborate strategies to slow down evolution and spread of bacterial resistance.
2. Genetic and physiological effects of antibiotics that drive the acquisition of resistance

2.1. Phenotypic heterogeneity

Antibiotic exposure induces phenotypic and genotypic heterogeneity across an isogenic culture [64]. High drug concentrations kill the vast majority of bacteria within a bacterial population. A small portion of dormant and refractory sensitive bacteria, so called persister cells, remain [64-67]. In contrast to resistant bacteria, persisters do not show genetic changes related to resistance, whereby tolerance to high drug concentrations is supposed to predominantly arise from physiological processes [68]. Regrowth of persisters and subsequent drug exposure results in a killing curve identical to that of the original bacterial population indicating that the trait is not inherited by the progeny of persisters [69]. The phenomenon of transient and non-inherited drug tolerance was mainly overlooked, as microbial residues can be eliminated by the host immune system in the majority of cases. However, if the antibiotic fails to effectively reach at least some cells in vivo or the drug level drops, one single surviving bacterium can restart a new cycle of infection and cause a chronic or relapsing disease [66]. Because of the low frequency of $10^{-5}$ surviving tolerant cells in populations of E. coli or P. aeruginosa and the metastable phenotypic tolerance, studying persister cells is still challenging [66]. Even though the phenomenon of surviving Staphylococcus colonies upon penicillin treatment was first recorded in the early 1940’s [67, 70], breakthrough in persister research was reported only 40 years later. Moyed and Bertrand (1983) succeeded in isolating persister mutants known as hip that exhibit an increased abundance of cells in the persister state compared to its E. coli K-12 parental strain [71]. Analysis and mapping of this gene locus revealed the contribution of the hipAB operon in the formation of persisters. Overexpression of the protein kinase HipA triggers growth arrest due to the synthesis of ppGpp by the enzyme RelA. As a consequence macromolecular synthesis is inhibited in E. coli, causing growth arrest, and enabling resistance to antibiotics which only kill during growth, such as β-lactams [72]. The phenomenon of persistence, as well as the underlying molecular mechanisms and its relevance within a culture is still under discussion and far from being fully understood. An alternative model suggests that persistence results from distinct and varying cellular physiologies or
processes occurring in every bacterial population [68, 73]. Stochastic fluctuations within a culture that is facing high drug dosage could display a strategy of cells evading antibiotic lethality by occupying different mechanisms and associated phenotypes. In general, heterogeneity of growth rates within an isogenic culture and especially after prolonged antibiotic treatment might have an important impact on the evolution of antibiotic resistance as well.

2.2. Mutator strains and antibiotic induced mutagenesis

Genetic changes introduced by spontaneous mutations are a major driver of bacterial evolution and adaptation. Success and persistence of mutant variants within a bacterial population strongly depends on the cost and benefit of the particular mutation on the overall fitness of the variant. *E. coli* K-12 has a rate of deleterious mutations per genome per replication of $2 \times 10^{-4}$ [74]. The rate of beneficial mutations however was found to be only $4 \times 10^{-9}$ [75]. Because most mutations are neither beneficial nor lethal, bacteria prevent excessive accumulation of genetic changes by using complex DNA repair mechanisms, such as the mismatch repair system (MMR), lowering the replication error rate to $10^{-9}$ to $10^{-11}$ per base replication [76, 77]. Common infections can contain $10^{10}$ organisms and therefore hundreds of resistant cells can be already present prior to administration of the antibiotic [78]. Deactivating genes involved in the MMR results in $10^{2}$- to $10^{4}$-fold increased mutation rates [79, 80]. Changes in the genome by mutations are not exclusively linked to actively dividing cells as it was assumed in early studies of Luria and Delbrück [81]. It now has been shown that mutations may occur also in non-dividing cells and are then commonly known as adaptive mutations or stress induced mutagenesis [82, 83]. Like phenotypic heterogeneity, increased genetic variability under stressful conditions could display another strategy of cells evading antibiotic lethality. Modulating mutation rates within a population can increase the probability of generation of successful adaptive mutations. Highly variable mutation rates result in the so-called mutator phenotype that has been observed at an unexpectedly high frequency of at least 1% in pathogenic strains of *E. coli* and *S. enterica* [84]. As mentioned earlier, most of the introduced mutations are not beneficial. Selection should therefore drive the occurrence of mutator strains to its lower limit [85].
However, Blazquez et al. (2003) reported a prevalence of mutator mutants up to 20% in pathogenic *Pseudomonas* isolated from the lungs of patients undergoing antibiotic therapy [86]. This suggests that natural isolates are exposed to situations where mutator phenotypes confer a selective advantage [87]. Prolonged antibiotic therapy may select for mutator alleles and can be the cause for the emergence of antibiotic resistant bacteria that exhibit mutations in multiple gene loci.

Mutation rates can be modulated on the population level by selecting mutator bacteria over non-mutators or by induction of the bacterial stress response [87]. Many kinds of stress can result in genetic changes, including Single Nucleotide Polymorphisms (SNP), deletions and insertions [88, 89], variations in copy number [90], chromosomal rearrangements [91] and movement of mobile elements [92]. Within the last 20 years multiple studies proved that bacteria are not only passively waiting for the optimal set of mutations to appear, but instead are able to induce transient mutagenesis mechanisms in response to stress. One of these mechanism is the SOS response that has recently been reviewed by Baharoglu and Mazel (2014) [93]. Briefly, the SOS response is activated by the formation of single-stranded DNA (ssDNA) which can be caused by multiple events, such as DNA damage or stalled replication. After the RecA coprotease has bound to the ssDNA in the damaged region, the produced filament catalyzes the auto-proteolysis of the repressor LexA. Derepression of the SOS regulon, comprised of about 40 genes in *E. coli* [94], initiates expression of the error-prone DNA polymerases Pol-II, DinB (Pol-IV) and UmuCD (Pol-V), which are responsible for increased mutation rates [95].

Another mechanism of induced mutagenesis depends on the accumulation of RpoS, a specialized sigma factor, and is reviewed in [96]. RpoS-dependent gene expression upon stress, nutrient deprivation or entering the stationary phase increases resistance not only to the encountered stress, but also to other stressful treatments. RpoS interacts with the RNA polymerase and regulates directly or indirectly about 500 genes in *E. coli*, which correlates to approximately 10% of the whole genome [97].
2.3. Biological cost of mutations conferring antibiotic resistance

The success of mutations among a population does not only depend on the benefits, but also on the biological cost. Experimental studies of the cost of resistance have focused on comparing growth rates, survival and the competitive performance of susceptible and resistant cells [98]. A number of studies reported fitness costs due to chromosomal mutations or plasmid carriage [99-101], but the existence of resistance with low or no biological cost [100] indicates that the fitness burden of resistance can vary depending on external conditions.

The epidemic of antibiotic resistance in bacteria observed nowadays would not occur if every additional mutation that contributes to resistance further reduced the strain’s fitness. Moreover, success of the paired genetic changes is strongly affected by the degree and strength of the genetic interaction itself, so called epistasis [102]. Interaction between underlying phenotypes causing epistasis can be positive in the case of beneficial mutations or negative caused by deleterious mutations [103]. A vast majority of allelic combinations conferring resistance to different classes of drugs were found to exhibit positive epistasis [104, 105]. Thus, the cost of double mutations was smaller than the expected cost of each individual and independent mutation. Fitness benefits of mutations in the absence of antibiotic pressure [105] preserve mutants even when antibiotic usage is reduced. The occurrence of positive epistasis can prolong the time expected for elimination of (multi-)drug resistant bacteria in a culture. For example, Trindade et al. (2009) demonstrated that some double resistances entail no measurable fitness cost, in contrast to the individual resistance [104]. Hence reducing solely the antibiotic usage is not likely to result in a proportional lowering of resistance levels. Moreover, underlying epistatic mechanisms that stabilize the resistant population and restore bacterial fitness can result in increased persistence of multi-resistant bacteria in the environment, even if the antibiotic pressure is inexistent.

2.4. Random drift and clonal interference

The accumulation of beneficial mutations is the major driver of adaptation and evolutionary processes towards antibiotic resistance. Whether a mutation gets fixed in a population is influenced by several factors. First, random genetic drift
can affect the diversity of the outgrowing population. Most mutations are lost if they occur only at low frequency [106]. In large populations random drift is generally considered weak compared to selection [106]. However, random drift is believed to play an important role in populations adapting to novel environments starting with a small population size.

Muller (1964) postulated in the context of sexual reproduction that when a beneficial mutation reaches a frequency that is high enough, selection dynamics become more dominant [107]. If two or more mutations arise in the same population, sexually reproducing organisms can recombine to form a fitter double mutant. In asexual populations, however, individuals with the highest fitness will spread most in the population. Another important fact is the cost of each mutation in one individual of the population. Thus, even mutations with a large effect on resistance could be outcompeted unless they appear in a genetic background supported by compensatory mutations. This so-called ‘clonal interference’ between beneficial mutations has several consequences in asexual populations for the dynamics of evolution towards antibiotic resistance [106, 108]. With increasing mutation rate or population size, the chance of fixation of the beneficial mutation declines, therefore to be successful, substitutions should entail larger fitness gains [109, 110]. However, small effect mutations are more common than mutations resulting in larger fitness gains [75]. Thus, double mutants have a higher probability to get fixed in a population and large asexual populations routinely harbor triple or quadruple mutations [111]. Whether and when a single mutation or multiple mutations become dominant in the population mainly depends on mutation rate and population size [112]. The effect of clonal interference can be studied under laboratory conditions by continuous culturing or serial passaging of cells under defined conditions. Lang et al. (2013) illuminated the process of adaptation by using whole-genome and whole-population sequencing in 40 replicate cultures of \textit{Saccharomyces cerevisiae} [89]. This study showed that individual evolving sub-populations harboring multiple genetic changes move synchronously through the population and compete with each other. Overall, clonal interference seems to represent an optimized adaptive strategy of asexual populations to deal with and rapidly react to changing and fluctuating environments.
2.5. Protection of a population’s sub-fraction by altruism

The coexistence of sensitive, intermediate-resistant and highly resistant cells growing within a clonal population is well documented [113-115]. Gaining resistance conferring mutations at the cost of fitness by a few individuals can support the overall adaptation and resistance of the whole population when these produce and share the metabolite indole that is involved in stress signaling in *E. coli* [115]. In response to this signal, more sensitive cells overproduced drug efflux pumps and induced oxidative stress-protective mechanisms. Thus, more sensitive cells were able to survive high drug dosages that would have killed them without the support of the more resistant ones (figure 2a). Enhancing the survival capacity of more sensitive cells increases the diversity within a population. This evolutionary strategy preserves the potential for the population to rapidly return to its genetic origin upon drug removal.

Bacterial growth within a self-produced polymer matrix can enhance antibiotic tolerance dramatically due to the exclusion of biocides from the bacterial community. This way a so-called biofilm is built-up consisting of polysaccharides, DNA and proteins and persistence of e. g. staphylococcal infections [116, 117]. A main characteristic of a biofilms structure is the heterogeneity within a population’s metabolic activity caused by the gradients of nutrients and oxygen within this biofilm [118-120]. Oxygen levels for example are high at the outer layers of the biofilm, but low in the center, affecting growth, metabolic activity and protein synthesis of bacteria.

Biofilms offer protection against antibiotics (figure 2b). For example β-lactam antibiotics act most effectively on rapidly dividing cells and hence are not suited to eradicate infections of *Staphylococcus* or *Pseudomonas* that are characterized by slow growth in biofilm structures [121, 122]. In addition, chromosomally encoded β-lactamases expelled into the biofilm matrix can degrade penicillin and similar antibiotics before they reach sensitive cells located in the biofilm center [123, 124]. Thus, multiple resistance mechanisms interact with each other to increase survival in biofilm structures upon drug exposure, whereby the outer layer of the biofilm protects the inner center.
**2.6. Dynamics of the selection for antibiotic resistance**

Increasing environmental antibiotic concentration, overuse and abuse enhance the selection of resistant mutants and the rate of mutation. If the bacterial population is not killed immediately by a given drug, cells are exposed to a stressful environment and the mutation rate increases. In 1990, Baquero described a dangerous range of drug concentrations in which resistant mutants were selected with highest frequencies [125]. The correlation of the drug concentration and the rise of resistant clones has been characterized extensively [78, 126]. When exposing bacteria to antibiotics at concentrations below the minimum inhibitory concentration (MIC) outgrowth might be slowed down, but the vast majority of the population is not killed and could build up resistance. Close to the MIC, the highest propensity for the evolution of resistant clones is observed [126, 127]. Further increase of the drug dosage results in a sharp drop of viable colonies. The steep decrease in mutant frequency occurs because concentrations above the MIC select against the wild-type and consequently the number of cells that can evolve into resistant mutants is lowered. At even higher drug concentrations intermediate resistant clones are challenged as well. If no resistant mutant can be detected, the drug concentration is high enough to
prevent resistant mutants from emerging (MPC, mutant prevention concentration). The drug dosage between the MIC and MPC is known as mutant selection window (MSW) and resistance is thought to primarily develop in this range of concentrations [78, 128, 129]. Antibiotic concentrations below the MIC induce *de novo* acquired resistance as well [127, 130-133]. For example, exposing cells to stepwise increasing and sublethal concentrations of three different antibiotics separately showed a rapid induction of *de novo* acquired resistance [130]. Within 15 days *E. coli* got highly resistant to amoxicillin, enrofloxacin and tetracycline. Upon removal of the antibiotic amoxicillin and enrofloxacin resistance was found to be permanent, indicating underlying genetic changes. Feng *et al.* (2014) showed in a similar approach that cells with reduced susceptibility to amoxicillin outcompeted the ancestor *in vitro* whenever antibiotics were present, even at relatively low concentrations [134]. Gullberg *et al.* (2011) reported that the mutant selection concentration (MSC) is dependent on the drug-bug combination and on the particular resistance mutation. In fact, a reduction of the drug concentration to 230-fold lower than the MIC caused enrichment of a particular ciprofloxacin resistant *E. coli* mutant [127]. In agreement with *in vitro* obtained data, emergence of resistance clones *in vivo* was shown to occur already at very low (2.5% of the therapeutic dose) drug dosages in the microbiota of chicken guts [133].

Sublethal drug concentrations are not only selecting for *de novo* acquired resistance mutations, but also for transfer of resistance conferring genes through conjugation. For example, highest conjugation rates of a tetracycline resistance conferring plasmid were found to occur at moderate selection pressures [135]. Thus, the occurrence of intermediate or fully resistant sub-populations should be prevented by applying short and intensive drug treatments to avoid emergence and outgrowth of resistant clones.

The research reported in this thesis was designed to identify factors driving the evolution and spread of resistance in bacteria. As reviewed above, the knowledge about the epidemiology on the one hand and the molecular mechanisms on the other hand is considerable. Less effort has been devoted to the kind of questions that would allow for the understanding of the epidemiology in the framework of the physiology and molecular biology of the
organisms involved. The first step towards this goal is to reveal the quantitative relationship between the antibiotic use and the acquisition and transmission of resistance. Subsequently, development of resistance to different antibiotic classes and overall molecular and physiological changes of the cell upon acquisition of resistance have to be addressed. Factors affecting the rate of transfer of plasmids carrying resistance genes must be identified as well. The combination of the different type of results provides a first step towards understanding the epidemiology of antibiotic resistance as a result of the ecology and physiology of the cell (figure 3).

**Figure 3.** Schematic overview of cellular routes in order to circumvent antibiotic mediated cell death. This thesis focuses on the quantitative relationship between antibiotic usage and development and dissemination of antibiotic resistance in bacteria (framed box).
3. Thesis outline

Development and spread of antibiotic resistance is a major health threat. The quantitative relationship between the usage of antibiotics and the development and transmission of resistance can identify driving forces of the evolution and spread of drug resistance. This insight may in turn provide starting points for improved treatment strategies. Chapter 1 reviews the widespread use of antibiotics and the occurrence of resistance genes in the environment focusing on the correlation between exposure to antibiotics and the rise of antibiotic resistance in the past 70 years. In addition, physiological and genomic factors are discussed that influence and drive the development and spread of resistance in bacteria.

Acquisition of antibiotic resistance in bacteria is often associated with a metabolic burden, resulting in a decreased fitness compared to their susceptible counterparts in the absence of the antibiotic. Chapter 2 provides new insights on the potential of the model organism *E. coli* MG1655 to compensate for metabolic costs of antibiotic resistance. Changes in transcriptomic profiles of cells that were made resistant by stepwise increasing the antibiotic concentration are analyzed in the framework of physiological parameters. The overall outcome indicates that the effect of the acquisition of resistance consists not so much of an extra energy requirement, but more a reduced ecological range.

Chapter 3 concentrates on the interrelationship between changes in gene expression and DNA mutations during the acquisition of resistance to amoxicillin, enrofloxacin and tetracycline. The response of sensitive cells to sub-lethal drug concentrations is compared to that of resistant cells. Mutations in resistance conferring regions are monitored during the buildup of amoxicillin and enrofloxacin resistance in order to study the evolutionary pathway leading to resistance. Additionally, the effect of subsequent drug removal was documented in enrofloxacin adapted bacteria by culturing resistant cells further without antibiotics and analyzing the transcriptomic, genomic and resistance profile.

Based on the transcriptomic comparison discussed in chapter 2 and 3, selected gene candidates were studied in chapter 4 regarding their potential to influence the adaptation in response to increasing concentrations of amoxicillin, enrofloxacin and tetracycline. This study focusses on the role of the SOS
response, the transcriptional activator \textit{gadE}, the outer membrane porin \textit{ompF} and the transcriptional regulator involved in superoxide removal \textit{soxS} in the adaptation process. In addition, the potential of \textit{E. coli} to adapt to antibiotics in a non-optimal growth environment, such as a lowered pH and increased salt concentrations, is investigated.

\textbf{Chapter 5} concentrates on factors that influence the transfer rate of an antibiotic resistance conferring plasmid. As transfer of resistance genes from the agricultural sector to the human health care is a major health hazard, an ESBL-carrying \textit{E. coli} isolate from chicken meat is used as plasmid donor to study the influence of parameters, such as cell density, growth rate or antibiotic pressure on conjugation rates. Through the use of the Ion Proton next generation sequencing technology the resistance conferring plasmid is identified, as well as mutations in transconjugant cells that may compensate for plasmid carriage.

\textbf{Chapter 6} will summarize and discuss the outcomes obtained in this thesis project. Similarities and differences in the buildup of resistance to antibiotics belonging to different classes are discussed, as well as the role of adjusting central regulatory networks and cellular consequences of becoming resistant.
Chapter 2

Compensation of the metabolic costs of antibiotic resistance by physiological adaptation in *Escherichia coli*

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This chapter has been published

Abstract

Antibiotic resistance is often associated with metabolic costs. To investigate metabolic consequences of antibiotic resistance, the genomic and transcriptomic profile was compared between an amoxicillin resistant *E. coli* strain and the wild-type it was derived from. 125 amino acid substitutions and 7 mutations that were located <1000 bp upstream of differentially expressed genes were found in resistant cells. However, a broad induction and suppression of genes was observed when comparing the expression profile of resistant to wild-type cells. Expression of genes involved in the cell wall maintenance, DNA metabolic processes, cellular stress response and respiration was most affected in resistant cells regardless of the absence or presence of amoxicillin. The SOS response was downregulated in resistant cells. The physiological effect of the acquisition of amoxicillin resistance in cells grown in chemostat cultures consisted of an initial increase in glucose consumption that was followed by an adaptation process. Furthermore, no difference in maintenance energy was observed between resistant and sensitive cells. In accordance with the transcriptomic profile, exposure of resistant cells to amoxicillin resulted in a reduced salt and pH tolerance. Taken together, the results demonstrate that the acquisition of antibiotic resistance in *E. coli* is accompanied by specifically reorganized metabolic networks in order to circumvent metabolic costs. The overall effect of the acquisition of resistance consists not so much of an extra energy requirement, but more of a reduced ecological range.
Introduction

Antibiotic resistance in bacteria is often associated with a metabolic burden resulting in decreased fitness compared to their susceptible counterparts in the absence of the antibiotic [98, 136-138]. Bacteria can become resistant to antibiotics by genetic mutation, transfer and expression of resistance genes from resistant to susceptible organisms or phenotypic adaptation. These changes can modify and unbalance bacterial metabolism, thereby impairing the physiological efficiency [98]. However, bacteria have a remarkable capacity to compensate for and reduce these physiological costs [138, 139]. Decreasing the metabolic burden of drug resistance by compensatory adaptation can stabilize resistant bacterial populations [140].

Changes in the outer membrane porins, penicillin binding proteins or efflux pumps can result in an increased resistance of *E. coli* to β-lactams [141]. In addition, resistance to β-lactams can occur due to production of β-lactamase, either chromosomally encoded or plasmid mediated [142]. Bacteria exposed to antibiotics utilize complex protection mechanisms, such as the SOS response that triggers transcription of genes involved in repairing DNA damage [143, 144], or the ROS system of reactive oxygen inducible genes [145]. It is to be expected that these adaptations require an energy investment.

Gene expression can be regulated in response to drug exposure in a manner that strikes a balance between efficient energy metabolism and adjustment to changing environments [146]. The acquisition of antibiotic resistance seems to have a specific effect on bacterial physiology rather than a general burden by overexpression of genes conferring resistance [147-149]. For example, the overexpression of the multidrug efflux pump MexEF-OprN in antibiotic resistant *Pseudomonas aeruginosa* cells did not result in a decreased fitness [147]. The downregulation of several quorum-sensing regulated genes indicates that acquisition of resistance can be accompanied by modifications attuned to specific ecosystems.

The general assumption is that long-term adaptation is mainly caused by either genetic mutations or by horizontal gene transfer, for example by the acquisition of plasmids. In contrast, short-term adaptation is believed to be purely phenotypic, whereby the inherent susceptibility to drugs in a population does not change [150]. Therefore, changes at the gene expression level are
supposed to be either rapidly induced in response of the drug or permanently altered by genetic modifications in the promoter region of either the resistance conferring gene or a respective regulator [150, 151].

Resistance to amoxicillin could be induced in *E. coli* by growth in the presence of stepwise increasing antibiotic concentrations, resulting in a 100-fold increase in the minimal inhibitory concentration (MIC) [130]. During the process of adaptation the cells initially increased specific glucose consumption, indicating a metabolic cost of resistance. After several cycles of growth in the presence of the antibiotic the metabolic cost decreased [130]. This suggests a dependence of the metabolic cost on the length of exposure to the antibiotic, indicating that the organism undergoes complex metabolic adaptations that compensate for the cost of resistance. Since the resistance persists during growth in the absence of the antibiotic, it seems logical to assume that it is caused by mutations rather than regulation of gene expression. This remains to be proven.

The radical based theory [145, 152, 153] proposes that oxygen radicals play a central role in cell death upon exposure to bactericidal antibiotics. If this is indeed the case, then sub-lethal levels should induce defense mechanisms that can in turn be detected by induction of the relevant genes. Furthermore the physiological effects on the cell must be detectable as well.

It is unlikely that the enduring metabolic adaptations that compensate for the initial metabolic costs of resistance have no other physiological consequences. Therefore these metabolic consequences need to be documented in more detail and potential negative effects of acquisition of resistance investigated. To this aim the genomic and transcriptomic profile of drug sensitive and amoxicillin resistant *E. coli* cells were compared. The large set of genes that were significantly up- and downregulated suggested several physiological consequences. One of the most prominent turned out to be a reduced ecological range, due to a diminished ability to control intracellular parameters upon changing environmental conditions.
**Material and Methods**

*Bacterial strains, growth media and culture conditions*

The drug sensitive wild-type *E. coli* MG1655 and an amoxicillin resistant *E. coli* strain with a MIC of 512 µg/ml were used throughout. The latter was derived from the first by stepwise increasing the amoxicillin concentration with every transfer cycle when almost normal growth occurred [130]. After approximately 100 generations the MIC of *E. coli* for amoxicillin increased from 4 to 512 µg/ml. Batch cultures of *E. coli* were grown at 37°C in a phosphate buffered (100 mM Na₂H₂PO₄) defined minimal medium containing 55 mM glucose with a pH of 6.9 [154]. For continuous cultivations of *E. coli* the glucose (5 mM) and Na₂H₂PO₄ (10 mM) concentrations were reduced and the pH was controlled at 6.9 with NaOH. Media were autoclaved for 20 min at 120°C, with the exception of glucose (10 min, 110°C). Four different *Staphylococcus aureus* strains were donated by Drs J. Wagenaar and W.J.B. van Wamel. These strains were isolated from two veal calf farms, MRSA0026 and MRSA0027 from one and MRSA0028 and MRSA0029 from another. For the cultivation of the *S. aureus* strains a chemically defined medium (CDMPC) [155] was used. The MRSA strains were assigned to the sequence type (ST) 398 and had the following characteristics [156]: MRSA0026: mecA+, spa t011, MIC methicillin 4 µg/ml; MRSA0027: mecA-, spa t011, MIC methicillin 1 µg/ml; MRSA0028: mecA+, spa t034, MIC methicillin 64 µg/ml; MRSA0029: mecA-, spa t034, MIC methicillin 1 µg/ml. *Enterococcus faecium* E1039 (ampicillin (MIC 0.0625 µg/ml) and vancomycin sensitive), E1162 (ampicillin resistant (MIC 4 µg/ml), vancomycin sensitive), E155 (ampicillin (MIC 8 µg/ml) and vancomycin resistant) were donated by Drs R.J.L. Willems and W. van Schaik and grown in MRS bouillon (VWR International, Darmstadt, Germany). The *E. faecium* strains were characterized by multilocus sequence typing (MLST) [157, 158]. E1039 belongs to the ST42 and differs in 4 out of 7 MLST genes from E1162 and E155. The E1162 and E155 (referred to as VS2) strains were assigned to ST17 and are clonally related [157].

Pre-cultures for inoculation of 96-well plates, batch and continuous cultures were grown in shake-flasks overnight shaken at 200 rpm at 37°C. For cultivation of sensitive and resistant cells under different conditions (sodium
chloride, pH) 96-well plates were used. Growth was followed over time for 23 h in a microtiter plate reader, measuring OD$_{600}$ every 10 min, with shaking in between. Results were obtained by calculating $\mu_{\max}$ based on averaged OD$_{600}$ values of 2 independent replicates, each performed as 2 technical replicates. The significance ($P \leq 0.05$) of differences between $\mu_{\max}$ values was determined by a Student’s t test.

Continuous cultivations were performed in a Sixfors (Infors AG, Bottmingen, Switzerland) fermenter vessel with a working volume of 250 ml, stirring at 250 rpm and a constant temperature of 37°C. The pH was controlled by automatically pumping sterile 2N NaOH into the vessel. Continuous cultivations were started as batch cultivations for 24 hours and switched to chemostat mode by activating feed and waste pumps. Growth conditions were maintained and culture parameters (pH, temperature, stirrer) recorded by the controller in the Sixfors fermentation unit. If the culture parameters, including the OD, remained constant for 5-7 volume changes samples were taken and if necessary new parameters were set. Growth in chemostat cultures was followed by measuring OD and dry weight. The dry weight was determined by filtering 5 ml of the culture volume on a pre-weighted filter and measurement of the filter weight after drying over night at 110°C.

**Determination of the glucose concentration**

At steady state culture liquid was harvested, immediately filtered (0.2 µm pore size) and stored at -80°C. Glucose concentration of thawed samples was determined enzymatically according to Bergmeyer [159].

**2',7'-Dichlorofluorescein oxidation**

The level of intracellular reactive oxygen species (ROS) was measured by using the fluorescence dye 2',7'- dichlorofluorescein diacetate (H$_2$DCFDA) according to an adapted protocol of Jakubowski and coworkers [160]. Briefly, bacteria were grown for 3 hours as described above to an OD$_{600}$ of approximately 0.3. A sample of 10 ml was incubated 1 hour with 10 µM H$_2$O$_2$ or different concentrations of amoxicillin (1 or 4 µg/ml for sensitive cells; 150 or 512 µg/ml for resistant cells). The H$_2$DCFDA (dissolved in DMSO, 100 mM) was added to a final concentration of 100 µM. After incubating the bacteria 30 min in the
dark at 37°C and 200 rpm, cells were pelleted, washed, suspended in 1 ml buffer and disrupted by bead beating. Cell extracts were clarified by centrifugation. Fluorescence of the cell lysate was read on a Fluostar Optima spectrofluorometer (BMG Labtech) with an excitation wavelength of 470 nm and an emission wavelength of 529 nm. Results were obtained by calculating the mean fluorescence of 2 independent replicates, each performed as 2 technical replicates. Mean fluorescence was normalized to the protein concentration determined with the Thermo Scientific Pierce Micro BCA Protein Assay Kit.

**MIC readings and antibiotics**

MIC values were determined as previously described [161]. Briefly: duplicate serial dilutions of a factor of 2, ranging from 1 to 2.048 µg/ml of amoxicillin, were made in 96-well plates. Additionally, two wells were used to analyze growth without antibiotics. Amoxicillin stock solutions contained a concentration of 10 mg/ml, were 0.2-mm filter-sterilized and stored at 4°C prior to use. For MIC readings bacterial cells were inoculated into each well to a starting OD$_{600}$ of 0.05. Growth was followed over time for 23 h in a microtiter plate reader, measuring OD$_{600}$ every 10 min, with shaking in between. A Thermo Scientific Multiskan FC with SkanIt software was used for the 96-well measurements. The MIC was defined as the lowest concentration of antibiotic that reduced the growth to an OD of 0.2 or less after 23 hours.

**β-lactamase assay**

The β-lactamase activity was determined by using the chromogenic substrate nitrocefin according to an adapted protocol of O'Callaghan and coworkers [162]. Cells were grown until OD$_{600}$ 1.0 and harvested by washing 1 ml with 100 mM sodium phosphate buffer (pH 7.0). After disrupting the cells by sonification, cell extracts were clarified by centrifugation. β-lactamase activity was determined by measuring the rate of nitrocefin hydrolysis (final concentration 100 µM) at 390 nm at 30°C in 100 mM sodium phosphate buffer pH 7.0. Enzyme activity was normalized to the protein concentration of the supernatant that was determined with the Thermo Scientific Pierce Micro BCA
Protein Assay Kit. Specific β-lactamase activities are presented as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.

**Microarray**

RNA isolation, yield and quality

In total 3 biological replicates of each experimental condition were grown in batch cultures. Cells were cultured overnight, inoculated in fresh medium to an OD of 0.2 with or without amoxicillin and harvested at an OD_{600} of 1.0. The pellet was flash-frozen in liquid nitrogen and stored at -80°C. The total RNA was extracted by adding 500 µl of RNeasy lysis buffer containing 1% mercaptoethanol and incubation at room temperature for 5 min. The lysed cells were extracted twice with acid phenol, followed by two chloroform extractions. Subsequently the total RNA was precipitated with isopropanol and incubated overnight at -80°C. After centrifugation for 30 min at 4°C, the pellet was washed with ice-cold 75% ethanol. Subsequently, the RNA was re-dissolved in 100 µl RNase free water. Samples were purified with the RNeasy Kit (Qiagen). The amount of RNA was measured on the NanoDrop ND-1000 (Thermo Scientific). The integrity of the RNA samples was investigated with the BioAnalyzer (Agilent Technologies) using the RNA nano 6000 kit (Agilent Technologies). Labeling, microarray hybridization, scanning and data processing was performed at the MicroArray Department of the University of Amsterdam.

Labeling protocol

Per test sample, 5 µg total RNA combined with 1 µg random octamers (Biolegio) in 4.5 µl H₂O was heated to 65°C for 10 min to denature the RNA and was allowed to cool in an ice-waterbath for 10 min. This 4.5 µl was made to 10 µl with a first strand mastermix containing final concentrations of 50 mM Tris-Cl (pH 8.3), 3 mM MgCl₂, 75 mM KCl, 200 mM Raffinose (Sigma-Aldrich), 0.015% Triton X-100, 30 ng Actinomycin-D (Sigma-Aldrich), 0.01M DTT, 0.5 mM dGAC, 0.35 mM dUTP, 0.15 mM dUTP-Cy3 (test) or dUTP-Cy5 (common reference) (GE Healthcare) and 200U SuperScript-II (Life Technologies). This mixture was incubated for 2 min at 25°C, 120 min at 42°C and 15 min at 70°C. Finally, 1.5 µl of 2.5M NaOH was added to hydrolyze the
remaining RNA by heating for 10 min at 70°C. 8.5 µl 2M MOPS was added for neutralization and the labeled cDNA was purified with the E.Z.N.A. MicroElute RNA Clean-up Kit (Omega Biotek). Dye incorporation and cDNA yield was measured on the NanoDrop ND-1000 (Thermo Scientific) yielding 2-2.5 µg per sample and a frequency of incorporation > 10 pmol/µg. The common reference was made by an equimolar pool of all test samples (5 µg per sample) and subsequently labeled as the test samples with Cy5 incorporation.

Microarray hybridization, scanning & data processing
Each hybridization mixture was made up from 750 ng Test (Cy3) and 750 ng Reference (Cy5) sample. Samples were dried and 1.98 µl of water was added. The hybridization cocktail was made according to the manufacturer’s instructions (Nimblegen Arrays User’s Guide – Gene Expression Arrays Version 5.0, Roche Nimblegen). Hybridization samples were loaded onto 12x135k custom designed microarrays against *E. coli* (OID 38205, Design 120315). Microarrays were hybridized for 18 hours at 42°C with the NimbleGen Hybridization System 4 (Roche Nimblegen). Afterwards, the slides were washed according to the Nimblegen Arrays User’s Guide – Gene Expression Arrays Version 5.0 and scanned in an ozone-free room with a Agilent DNA microarray scanner G2565CA (Agilent Technologies). Feature extraction was performed with NimbleScan v2.6 (Roche Nimblegen). Data for biological replicates were normalized, averaged and analyzed. Genes were considered to be differentially expressed when they had a ≥ 2-fold increase or decrease in transcript and demonstrated a significant change in levels of expression (P ≤ 0.05) as determined by a Student’s t test.

Genome sequencing and assembly
Bacterial genome Roche/454 Titanium shotgun sequencing was performed according to the manufacturer's instructions. The sequence reads were assembled using the Roche GS De Novo Assembler (Newbler) v 2.3 and the Roche GS Reference Mapper v 2.0.0.12. Alignment of the contigs was done with MAUVE [163] using the progressive MAUVE algorithm in its default settings (http://asap.ahabs.wisc.edu/mauve/), using the reference database sequence for *E. coli* strain MG1655 from NCBI (Refseq: NC_000913,
Genbank: U00096). The genomes were annotated using the Annotation Engine at the Institute for Genome Sciences of the University of Maryland School of Medicine (http://ae.igs.umaryland.edu/cgi). Alignment of the Roche 454 reads was done with a tool developed in house, RoVar (http://trac.nbic.nl/rovar), which uses BLAT, version 34 [164]. The following criteria were chosen for selecting structural variations: (i) the region of the structural variation should be present only once in the reference sequence; (ii) no perfect match reads should align; and (iii) at least 3 reads should unambiguously support each structural variation. This approach was utilized to ascertain that the identified differences are almost certainly not the effect of sequencing errors.

Amplification and sequencing of the ampC promoter
The E. coli ampC promoter region was amplified and sequenced using 5’-GGGATCTTTTGTTGCTCT-3’ as forward and 5’-CTTCATTGGTCGCGATT-3’ as reverse primers. PCR conditions for amplification reactions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 35 s at 95°C, 55 s at 49°C, and 90 s at 72°C, and a final 90 s extension at 72°C. Reactions were performed in 50 µl volumes with Taq DNA polymerase (Thermo Scientific). PCR products were purified with the MSB® Spin PCRapace kit (Invitek) and sequenced by Macrogen Europe.

Results
Regulation of gene expression
The physiological consequences of exposure to antibiotics are reflected on the transcriptome of E. coli after adaptation to and subsequent additional exposure to amoxicillin (figure 1). The expression of a total number of 4237 genes was assessed. Growth at 1 µg/ml amoxicillin did not induce differential gene expression in wild-type cells when only genes showing a statistically significant minimally 2-fold change are considered to be differentially expressed. The strain made permanently resistant by growing it at increasing levels of amoxicillin [130] had after growth in medium without antibiotic 32 upregulated and 79 downregulated genes compared to the wild-type it originated from. This suggests that the changes in gene expression that accompany the acquisition of
Reduction of metabolic costs of antibiotic resistance

resistance are enduring. Growth of this resistant strain in the presence of 150 μg/ml amoxicillin resulted in the upregulation of 109 and downregulation of 133 genes compared to the wild-type. When the resistant strain grown with amoxicillin is compared to the same strain in the absence of the antibiotic, 4 genes were downregulated and 8 upregulated. Overall, in resistant cells more genes were downregulated than upregulated compared to the wild-type and exposure to the antibiotic increased both numbers.

The transcriptomics of selected functionally related groups of genes based on standard GO terms found by the DAVID bioinformatics tool [165] are summarized in table 1. Genes belonging to the functional group of the membrane or transport were both down- and upregulated in resistant cells compared to the wild-type. In the functional groups of cation binding, cellular respiration and electron transport chain only upregulation of genes occurred. The overlap of genes that are categorized to different groups, such as the

![Figure 1](image.png)

**Figure 1.** Total number of up- and downregulated genes in amoxicillin resistant cells compared to the wild-type in the absence (-AMX) or presence of amoxicillin (+AMX, wild-type: 1 μg/ml, resistant cells: 150 μg/ml). Selected differentially expressed genes of each condition examined are presented in the figure with the fold change in brackets. Genes are considered to be differentially expressed when the expression ratio exceeds a factor of two and shows a significant different (95% confidence) log expression ratio \( \geq \pm 0.5 \).
cellular respiration, transport and membrane group, is illustrated in figure 2. Differentially up and downregulated genes can be associated with multiple groups. Hence, the number of differentially regulated genes does not match with the total number of genes categorized into groups.

**Figure 2.** Overlap of upregulated genes categorized into the cellular respiration, transport and membrane group of amoxicillin resistant *E. coli* cells in the presence of amoxicillin compared to the wild-type (0.25xMIC; wild-type: 1 μg/ml, resistant cells: 150 μg/ml amoxicillin, respectively). Genes are considered to be differentially expressed when the expression ratio exceeds a factor of two and shows a significant different (95% confidence) log expression ratio greater than or equal to ± 0.5.
Table 1. Summary of gene numbers showing differentially regulation in selected functional groups of *E. coli* wild-type (WT) and antibiotic resistant cells (AR) with or without amoxicillin found with DAVID Bioinformatics Resources 6.7 (32).

<table>
<thead>
<tr>
<th>Functional group</th>
<th>total number</th>
<th>AR vs WT</th>
<th>ARamox vs WTamox</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>upregulated</td>
<td>downregulated</td>
</tr>
<tr>
<td>Whole genome</td>
<td>4237</td>
<td>32</td>
<td>79</td>
</tr>
<tr>
<td>Membrane</td>
<td>1093</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Transport</td>
<td>820</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Cation binding</td>
<td>637</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Iron ion binding</td>
<td>78</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrate catabolic process</td>
<td>114</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>DNA metabolic process</td>
<td>183</td>
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<tr>
<td>DNA repair</td>
<td>75</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>DNA replication</td>
<td>59</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Transposition, DNA mediated</td>
<td>18</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>Cellular response to stress</td>
<td>158</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td>SOS response</td>
<td>23</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>Response to antibiotic</td>
<td>84</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Cellular respiration</td>
<td>85</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Electron transport chain</td>
<td>112</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

* Only those genes that were significantly differentially expressed (P > 0.95 confidence) and at least 2-fold altered gene expression are listed. AR versus WT: comparison of unexposed cells; ARamox vs WTamox: comparison of amoxicillin exposed cells (0.25xMIC; 1 and 150 µg/ml amoxicillin for WT and AR, respectively)
Genes involved in the SOS response were suppressed in resistant cells compared to the wild-type both in the absence and presence of amoxicillin (table 2). No significant upregulation of any SOS response gene in drug resistant cells was found. Upon comparable exposure to amoxicillin (0.25xMIC) both strains should be affected to similar extent, but only in resistant cells further downregulation of the SOS response genes was observed. The most reduced expression in drug resistant cells within this functional group was found for umuD, that is part of the error prone repair of DNA. Other important genes involved in regulating the SOS response, recA, dinI, umuC, recX and lexA, were also suppressed.

The GO term “response to antibiotics” (GO:0046677), includes any process that results in a change in state or activity of a cell or an organism as a result of an antibiotic stimulus such as movement, secretion, enzyme production, gene expression, etc. [166]. In resistant cells grown in the presence of amoxicillin 4 out of 84 genes of this group were upregulated (table 1). The strongest upregulation regardless of the presence of amoxicillin, approximately 100-fold, occurred in ampC which encodes for a β-lactamase. To examine the effect of this upregulation on the phenotype, the specific β-lactamase activity was determined (table 3). Whereas no β-lactamase activity could be detected in wild-type cells, resistant cells showed a specific activity of 310.7 ± 57.9 U/mg. The presence of 150 μg/ml amoxicillin during growth did not result in a further increased β-lactamase activity. The β-lactam resistance protein Blr that increases resistance to a number of antibiotics which inhibit peptidoglycan synthesis [167] had a 2.2-fold higher expression in resistant cells exposed to amoxicillin. The highest increase in expression (16.2-fold) of a drug efflux related gene was observed for sugE which belongs to the small multidrug resistance family [168]. Two multidrug efflux transporter, mdtM and mdtK were more than 2-fold up regulated in the resistant strain, but solely in the presence of amoxicillin. However, other well-known genes belonging to the GO term “response to antibiotics”, such as the mar and acr operon, showed no significant differential expression.
Table 2. Fold change of selected up- and downregulated genes in amoxicillin resistant *E. coli* cells compared to the wild-type without (-AMX) or with amoxicillin (+AMX, 0.25xMIC).\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Upregulated</th>
<th></th>
<th>Downregulated</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- AMX</td>
<td>+ AMX</td>
<td>- AMX</td>
<td>+ AMX</td>
</tr>
<tr>
<td><strong>Membrane</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ompW</em></td>
<td>1.3(^b)</td>
<td>2.1</td>
<td>-4.7</td>
<td>-5.5</td>
</tr>
<tr>
<td><em>copA</em></td>
<td>1.4(^b)</td>
<td>2.3</td>
<td>-1.6(^b)</td>
<td>-2.5</td>
</tr>
<tr>
<td><em>cusC</em></td>
<td>4.9</td>
<td>23.5</td>
<td>-1.5(^b)</td>
<td>-1.5(^b)</td>
</tr>
<tr>
<td><em>ble</em></td>
<td>11.0</td>
<td>12.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Transport</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>dcuC</em></td>
<td>2.0</td>
<td>3.0</td>
<td>-1.5(^b)</td>
<td>-2.9</td>
</tr>
<tr>
<td><em>sugE</em></td>
<td>13.8</td>
<td>16.2</td>
<td>-1.5(^b)</td>
<td>-2.9</td>
</tr>
<tr>
<td><em>frdA</em></td>
<td>28.7</td>
<td>37.6</td>
<td>-2.8</td>
<td>-3.3</td>
</tr>
<tr>
<td><em>frdB</em></td>
<td>31.3</td>
<td>60.4</td>
<td>-3.3</td>
<td>-2.4</td>
</tr>
<tr>
<td><em>frdD</em></td>
<td>25.3</td>
<td>44.7</td>
<td>-3.7</td>
<td>-2.5</td>
</tr>
<tr>
<td><strong>Response to antibiotic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>mdtM</em></td>
<td>1.1(^b)</td>
<td>2.4</td>
<td>-3.2</td>
<td>-3.0</td>
</tr>
<tr>
<td><em>mdtK</em></td>
<td>1.9(^b)</td>
<td>2.9</td>
<td>-3.3</td>
<td>-2.8</td>
</tr>
<tr>
<td><em>blr</em></td>
<td>1.7</td>
<td>2.2</td>
<td>-3.8</td>
<td>-3.1</td>
</tr>
<tr>
<td><em>ampC</em></td>
<td>97.1</td>
<td>106.2</td>
<td>-4.7</td>
<td>-3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-5.5</td>
<td>-4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-6.7</td>
<td>-7.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-9.1</td>
<td>-7.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-9.2</td>
<td>-7.4</td>
</tr>
</tbody>
</table>

\(^a\) Genes are considered to be differentially expressed if they showed significantly different (95% confidence) log expression ratio greater than or equal to ± 0.5.

\(^b\) Not significantly differentially expressed (p > 0.05).
Chapter 2

Table 3. Specific β-lactamase activity of wild-type (WT) and amoxicillin resistant (AR) cells cultured with or without sub-inhibitory amoxicillin (AMX) concentrations.

<table>
<thead>
<tr>
<th>Cells and drug</th>
<th>β-lactamase activity(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0</td>
</tr>
<tr>
<td>WT + 1 μg/ml AMX</td>
<td>0</td>
</tr>
<tr>
<td>AR</td>
<td>310.7 ± 57.9</td>
</tr>
<tr>
<td>AR + 150 μg/ml AMX</td>
<td>300.0 ± 57.5</td>
</tr>
</tbody>
</table>

\(^a\) Specific activity reported in nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. Results are presented as the means and standard deviations from 3 independent measurements.

Genes categorized in the functional group of the membrane were both up- and downregulated compared to the wild-type (table 1). Resistant cells had an approximately 12-fold increased expression of \(bhc\) that is assumed to be involved in transport or lipid storage necessary for membrane repair or maintenance [169]. When resistant cells were exposed to amoxicillin upregulation was observed of \(cusR\) (2.3-fold), a known regulator of genes related to copper efflux [170], of \(cusF\) coding for the copper efflux/system (3.7-fold), of \(cusC\) (23.5-fold), of the multicopper oxidase \(cueO\) (2.5-fold) and of the \(Cu^+\) efflux ATPase \(copA\) (2.3-fold). Altered expression of porins or restriction of their functions due to point mutations have been linked to antibiotic resistance [171]. However, amoxicillin resistant cells showed only a 2.1-fold increased expression of \(ompW\) in response to the antibiotic. The expression of \(dinF\), a member of the multidrug and toxic compound extrusion (MATE) group [172], was approximately 5-fold suppressed in resistant cells upon addition of amoxicillin.

The functional group of transport, which includes metabolic enzymes, had 13 up- and 13 downregulated genes in resistant cells compared to the wild-type. The numbers of differentially expressed genes increased to 34 up- and 20 downregulated genes, when these cells were exposed to amoxicillin (table 1). Expression of genes coding for proteins involved in electron transport and carbon metabolism was clearly affected. In the absence of amoxicillin 5 genes involved in cellular respiration (\(narg, frdABCD\) operon) were upregulated in resistant cells compared to wild-type. In resistant cells exposed to amoxicillin
24 genes involved in cellular respiration were upregulated, including the \textit{frd} operon. The \textit{frd} operon encodes for the enzyme fumarate reductase and allows for the utilization of fumarate as a terminal electron acceptor instead of oxygen for anaerobic oxidative phosphorylation in \textit{E. coli} [173]. The genes \textit{frdB} and \textit{frdA} which encode the enzyme fumarate reductase were upregulated (table 2). All genes encoding succinate dehydrogenase that catalyzes the physiologically opposite reaction were downregulated. Two enzymes, succinate dehydrogenase and fumarate reductase catalyze the interconversion of succinate to fumarate. \textit{SdhA}, \textit{sdhC} and \textit{sdhD} are 1.5-, 3.3- and 2.9-fold downregulated upon amoxicillin exposure, respectively. This corroborates a partial substitution of succinate dehydrogenase by fumarate reductase [174]. The expression of the transporter \textit{dcuC} that is responsible for the uptake of C4-dicarboxylates such as fumarate under anaerobic conditions, increased 3-fold upon amoxicillin exposure. Genes involved in acid and salt resistance (\textit{gadABC}, \textit{gadE}, \textit{gadWX}, \textit{hdeAB}, \textit{evgAS}) [175, 176] were less expressed in resistant cells compared to the wild-type (table 2).

\textit{Mutations found in amoxicillin resistant cells}  
Sequencing of the entire genome revealed a total of 789 mutations in the genome of amoxicillin resistant cells compared to the wild-type. These genomic alterations include 250 in-frame mutations, whereas 125 genetic alterations resulted in amino acid substitutions. In the out-of-frame regions 589 mutations were found. Only few genes that had mutations in the upstream noncoding region (<1000 bp) or coding region showed a significantly differentially expression in the presence and/or absence of amoxicillin (table 4). In \textit{E. coli} the promoter region of \textit{ampC} is embedded within the coding sequence of the upstream located \textit{frd} operon [177].

Depending on the selection criteria, amoxicillin resistant cells had or did not have an adenine insertion at the nucleotide position 351 in the \textit{frdD} gene (-13 insertion in the \textit{ampC} promoter). To decide this point five independent cultures of the wild-type were made resistant by growth at steadily increasing levels of amoxicillin and the region was sequenced following PCR. An insertion at nucleotide position 348 was observed in all five strains thus obtained. This insertion is located in the interspace between the -10 and -35 regions of the
upstream located Pribnow box of \( \text{ampC} \). In resistant cells addition of amoxicillin barely influenced the expression of \( \text{ampC} \), indicating that the mutation in the promoter region was crucial for the 100-fold upregulation compared to wild-type cells.

No mutations were found in the upstream or coding regions of penicillin binding proteins. Several genes encoding for transporters (e.g. 19 bp upstream of \( \text{aroP} \)) showed mutations in the upstream region (<1000 bp), but expression was not significantly altered. Amino acid alterations were found in the coding region of 2 genes encoding for multidrug efflux transporters: \( \text{YeeO} \) (W2L), \( \text{MdtF} \) (F897V).

**Physiological consequences of the acquisition of antibiotic resistance**

The metabolic costs of exposure to amoxicillin were investigated using continuous cultures. The sudden addition of sub-lethal amoxicillin concentrations of 1 (wild-type) and 150 (resistant) µg/ml to cultures growing at steady state rates of D 0.2 and 0.4 h\(^{-1}\) resulted in a jump in the specific glucose consumption (\( q_{\text{gluc}} \)), except in the case of slow growing resistant cells (figure 3). The wild-type cells returned to the original \( q_{\text{gluc}} \) after 48 hours (D=0.2 h\(^{-1}\)), or 24 hours (D=0.4 h\(^{-1}\)). The time frame of the recovery indicates that approximately 10 cell divisions were needed for the metabolic adjustments that restored the \( q_{\text{gluc}} \) to be completed. In resistant cells growing at D=0.4 h\(^{-1}\) the increased glucose consumption upon addition of amoxicillin remained elevated, indicating no further adjustments of the carbon metabolism took place.
Table 4. Mutations of selected genes in out-of-frame, in-frame or promoter regions of amoxicillin resistant cells (AR).a

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position of mutation</th>
<th>-AMX</th>
<th>+AMX</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Outframe</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pdhR</td>
<td>522 bp upstream, deletion</td>
<td>-1.9b</td>
<td>-2.9</td>
<td>Transcriptional regulator of pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>cusF</td>
<td>58 bp upstream, T → C</td>
<td>2.3</td>
<td>3.7</td>
<td>Putative periplasmic copper-binding protein</td>
</tr>
<tr>
<td>insL</td>
<td>61 bp &amp; 59bp upstream, T → C</td>
<td>-2.9</td>
<td>-1.6</td>
<td>IS186 hypothetical protein</td>
</tr>
<tr>
<td>dcuC</td>
<td>343 bp upstream, deletion</td>
<td>2.0</td>
<td>3.0</td>
<td>Dicarboxylate transport protein</td>
</tr>
<tr>
<td>iraM</td>
<td>528 bp upstream, insertion</td>
<td>-2.2</td>
<td>-1.6</td>
<td>Inhibitor of σS proteolysis, contributes to acid resistance</td>
</tr>
<tr>
<td>narG</td>
<td>460 bp upstream, insertion</td>
<td>2.6</td>
<td>5.1</td>
<td>Nitrate reductase 1, alpha subunit</td>
</tr>
<tr>
<td>waaA</td>
<td>121 bp upstream, insertion</td>
<td>1.6</td>
<td>2.0</td>
<td>3-deoxy-D-manno-octulosonic-acid transferase</td>
</tr>
<tr>
<td><strong>Promoter region</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ampC</td>
<td>-12 insertion of A</td>
<td>97.1</td>
<td>106.2</td>
<td>β-lactamase; penicillin resistance</td>
</tr>
</tbody>
</table>
### Table continued from previous page

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position of mutation</th>
<th>-AMX</th>
<th>+AMX</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inframe</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>insB</em>-4</td>
<td>F81L; R65S; Y128H</td>
<td>2.3</td>
<td>2.5</td>
<td>IS1 protein InsB</td>
</tr>
<tr>
<td><em>gadB</em></td>
<td>D233H; T214P</td>
<td>-5.3</td>
<td>-22.9</td>
<td>Glutamate decarboxylase isozyme</td>
</tr>
<tr>
<td><em>evgS</em></td>
<td>N57I</td>
<td>-1.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-2.8</td>
<td>EvgS sensory histidine kinase, regulates multidrug resistance</td>
</tr>
<tr>
<td><em>nirC</em></td>
<td>W28L</td>
<td>1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5</td>
<td>nitrite reductase activity</td>
</tr>
<tr>
<td><em>gadA</em></td>
<td>H465Y; Q459K</td>
<td>-5.4</td>
<td>-4.4</td>
<td>Glutamate decarboxylase A</td>
</tr>
<tr>
<td><em>frdD</em></td>
<td>V117V</td>
<td>25.3</td>
<td>44.7</td>
<td>Fumarate reductase subunit D/ampC promoter region</td>
</tr>
<tr>
<td><em>narG</em></td>
<td>T236T; A242A; Y245Y; S255S; V257V; T260T</td>
<td>2.6</td>
<td>5.1</td>
<td>Nitrate reductase 1, alpha subunit</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mutations in the outframe (<1,000 bp upstream of differentially expressed genes) or inframe regions of genes are presented when expression was significantly changed at the same time in the presence (+AMX) and/or absence (-AMX) of amoxicillin. Genes were considered to be differentially expressed when a minimally 2-fold change is significantly different (95% confidence).

<sup>b</sup> Not significantly (p > 0.05) differentially expressed.

<sup>c</sup> Differs from selection criteria (ii) described in methods section. Perfect match reads and structural variations were identified.
Figure 3. Specific glucose consumption ($q_{gluc}$) of *E. coli* wild-type (WT) and amoxicillin resistant *E. coli* strain (AR) in continuous culture at a dilution rate of (a) 0.2 h$^{-1}$ and (b) 0.4 h$^{-1}$. After cells reached steady state ($t=0$) *E. coli* wild-type (WT) and the drug resistant (AR) strain were exposed to 1 and 150 µg/ml amoxicillin, respectively.

The metabolic costs of the acquisition of resistance were estimated by measuring and comparing the maintenance energy, defined as all energy not devoted to growth [178, 179], in the *E. coli* strains of this study, 4 methicillin sensitive or resistant *Staphylococcus aureus* and 3 *Enterococcus faecium* strains. The extrapolation of $q_{gluc}$ to $D=0$ h$^{-1}$ revealed no difference in maintenance energy between wild-type and *in vitro* adapted resistant cells of *E. coli* (figure 4a). Similarly, no difference in maintenance energy was observed between methicillin sensitive and resistant *S. aureus* strains (figure 4b). In contrast, clinical isolates of *E. faecium* with different resistance patterns for ampicillin and vancomycin had different maintenance energy requirements. The ampicillin and vancomycin sensitive *E. faecium* 1039 and ampicillin resistant but vancomycin sensitive *E. faecium* 1162 showed the lowest maintenance energy (figure 4c). The ampicillin and vancomycin resistant *E. faecium* E155 had a higher maintenance energy compared to *E. faecium* 1039 and the genetically related *E. faecium* E1162. This suggests that, at least in this single case, ampicillin resistance did not require extra energy, but vancomycin resistance did.
Chapter 2

Figure 4. Maintenance energies of (a) *E. coli* wild-type (WT) and amoxicillin resistant *E. coli* (AR) (b) methicillin sensitive *S. aureus* (MSSA0027 and MSSA0029) and methicillin resistant *S. aureus* (MRSA0026 and MRSA0029) and (c) *E. faecium* E1039 (ampicillin and vancomycin sensitive), E1162 (ampicillin resistant, vancomycin sensitive), E155 (ampicillin and vancomycin resistant). By measuring the specific glucose consumption rate ($q_{\text{gluc}}$) as a function of the dilution rate ($D$) in steady-state chemostat cultures and extrapolating by linear regression to a $D$ of 0 h$^{-1}$ the maintenance energy was estimated.

The downregulation of genes involved in salt and pH control suggested that the costs of resistance might consist of a reduced ecological range rather than metabolic efficiency. To examine whether resistance goes at the expense of decreased salt and pH tolerance, the $\mu_{\text{max}}$ of the drug sensitive and resistant strain was compared at different sodium chloride concentrations with and without sublethal amoxicillin levels (figure 5). The amoxicillin concentrations used resulted in approximately 25% reduction of growth (final OD) and are equivalent to $\frac{1}{2}$ MIC value. The $\mu_{\text{max}}$ of the wild-type and the drug resistant
strain did not differ from each other in a pH range of 6 to 7. The addition of sublethal concentrations of amoxicillin lowered the $\mu_{max}$ of resistant cells in the range of pH 6 to 6.5 more than that of sensitive cells. This suggests that the ability to resist amoxicillin comes at the expense of reduced pH control.

Figure 5. Maximal specific growth rate ($\mu_{max}$) of *E. coli* wild-type (WT) and the amoxicillin resistant *E. coli* (AR) strain with left: varying pH values in a range from 6 to 7.5; and right: increasing sodium chloride concentrations with or without sub-inhibitory concentrations of amoxicillin of 2 and 256 µg/ml, respectively. Results were obtained by calculating $\mu_{max}$ based on averaged OD$_{600}$ values of 2 independent replicates. The significance ($P \leq 0.05$) of the difference in $\mu_{max}$ was determined by a Student’s $t$ test.

In the presence of amoxicillin increased salt concentrations had a stronger effect on growth rate of resistant cells compared to the wild-type. The drug sensitive strain is able to grow with 2% sodium chloride and 2 µg/ml amoxicillin. In contrast, exposure to 2% sodium chloride and sublethal drug concentrations resulted in complete growth inhibition of resistant cells. The $\mu_{max}$ of the resistant strain exposed to sublethal levels of amoxicillin was by far most affected by 1% salt. It is noteworthy that only in the presence of amoxicillin the ecological range of resistant cells was affected.

Reactive oxygen species (ROS) are thought to have an essential role in the mechanism by which antibiotics destroy cells [145, 153]. ROS production did not differ significantly between wild-type and amoxicillin resistant cells (figure 6). Furthermore, increased intracellular ROS levels resulting from growth in the presence of different amoxicillin concentrations were not observed.
Antibiotic resistance in bacteria is often associated with a decreased fitness compared to their susceptible counterparts [98, 136, 180]. However, no difference in maintenance energy was observed in our study between an amoxicillin resistant *E. coli* strain and the wild-type it was derived from. Of the *E. faecium* and *S. aureus* strains tested, only one strain, which was also resistant to vancomycin, had an increased maintenance energy. The approximately 10 doubling times needed for reduction of the initial metabolic costs of dealing with sublethal levels of amoxicillin suggest that more modifications occurred than just the induction of enzymes. The dynamics of the carbon metabolism observed in this study are in agreement with the notion that the physiological response depends on the length of exposure to the antibiotic [130]. Overall, it seems that the initial physiological burden is followed by a long-term adaptation process resulting in the reduction of metabolic costs.

**Figure 6.** Intracellular level of reactive oxygen species in wild-type (WT) and amoxicillin resistant (AR) *E. coli* cells as measured with 100 μM H₂DCFDA. Bacteria grown for 3 hours to an OD₆₀₀ of approximately 0.3 were incubated for 1 hour with or without amoxicillin (untreated) or 10 μM H₂O₂ (pos. control). Sub-MIC: 1 and 150 μg/ml amoxicillin for WT and AR, respectively. MIC: 4 and 512 μg/ml amoxicillin for WT and AR. Results are presented as the means and standard deviations from 2 independent measurements.
In this view, the observed enduring changes in the transcriptomic profile could be part of an energy saving mechanism. Changes in expression level were most significant in four main groups: cell wall maintenance, DNA metabolic processes, cellular stress response and respiration and the electron transport chain. Exposure of resistant cells to amoxicillin resulted in additional physiological adjustments that increased the number of differentially expressed genes to 242. Almost all of these additional genes belonged to the same four groups. Persistent suppression of the SOS defense mechanism in resistant cells could contribute to a reduction of metabolic costs counterbalancing the increased expression of genes conferring resistance (e.g. *ampC*, *blr*).

A well-known phenomenon is the acquisition of compensatory mutations to reduce the metabolic burden in antibiotic resistant cells and thus to restore bacterial fitness [181]. It is not obvious by which molecular mechanism the long term changes in expression level observed in the present study are achieved. Mutations in promoter regions cannot account for all, as in resistant cells only 7 mutations were found <1000 bp upstream of genes that were differentially expressed (table 4). The long-term nature of the changes in expression level was shown by the high number of differentially regulated genes of resistant cells compared to the wild-type in the absence of the antibiotic (table 1). The small number of mutations in the upstream region of differentially expressed genes in resistant cells suggests that the enduring impact of amoxicillin exposure on the overall transcriptomic profile is attained by other mechanisms as well.

The substantially higher expression of a set of genes involved in energy metabolism in resistant cells upon exposure to non-lethal levels of amoxicillin indicates a switch in metabolism. In *E. coli* the *frd* operon is repressed by oxygen and nitrite [182]. The downregulation of succinate dehydrogenase and upregulation of fumarate reductase indicates a partial switch in metabolism from aerobic to anaerobic in drug exposed resistant cells. This is supported by the induction of the anaerobic dimethyl sulfoxide reductase and nitrate reductase A.

The observed switch to a partial anaerobic metabolism is in agreement with the radical-based theory [152, 153, 183]. In this line of thinking, the induction of the *frd* operon might benefit resistant cells by avoiding ROS-mediated cell death resulting from β-lactam action in *E. coli*. The ROS defense mechanism was repeatedly induced in sensitive cells by exposure to β-lactams [183, 184].
Conceivably, reducing the superoxide production in drug adapted cells could contribute to the high level antibiotic resistance. In resistant cells in the present study neither the ROS nor the SOS response genes were upregulated regardless of the presence or absence of amoxicillin (table 2) and ROS production in amoxicillin exposed wild-type and resistant cells did not differ significantly. These observations are more in agreement with those that have suggested cell death from antibiotics without the involvement of ROS [185, 186]. The altered expression pattern of genes involved in the tricarboxylic acid cycle found in this study corresponds well with the surge in NADH upon exposure to ampicillin and norfloxacin [153]. The upregulation of the \( \textit{frd} \) operon in resistant cells, possibly inducing fumarate respiration and thus resulting in NADH depletion, may counter the elevated NADH dependent superoxide production via the electron transport chain [153]. The role of ROS that is crucial according to the radical-based theory might also be secondary, as cells may produce ROS when the metabolism is disturbed by antibiotics. Our results cannot distinguish without a doubt between these two possibilities, but are more supportive of a secondary role of ROS.

Cycles of copper oxidation and reduction can produce ROS [187]. The 24-fold increased expression of \( \textit{cusC} \) in resistant cells exposed to amoxicillin suggests a contribution of the CusCFBA copper/silver efflux system to resistance. Upregulation of the \( \text{Cu}^+ \) efflux ATPase \( \textit{copA} \) corroborates the impact of copper homeostasis in resistant cells exposed to amoxicillin. The most important functions of copper in \( \textit{E. coli} \) are in the cytochrome c oxidase and related enzymes that are oxygen-dependent terminal oxidases in the respiratory chain. The bactericidal action of Cu(II) mainly results from the direct interactions between copper species, such as Cu(II) or Cu(I), and cell components [188]. The induction of specific copper efflux systems in amoxicillin resistant cells suggests copper induced damage upon drug exposure.

The simultaneous up regulation of both \( \textit{ampC} \) and \( \textit{frd} \) found in this study is not necessarily the effect of co-regulation, but could also be caused by the switch to a metabolism resembling that found under anaerobic conditions. Even though the \( \textit{ampC} \) promoter is embedded in the \( \textit{frd} \) operon, an independent expression of the closely located \( \textit{ampC} \) and \( \textit{frdD} \) genes was demonstrated [189, 190]. The \( \textit{frd} \) operon is induced by fumarate and anaerobic conditions [174].
The constitutively low-level \textit{ampC} expression in wild-type \textit{E. coli} cells is not inducible, but regulated in a growth rate dependent attenuator mechanism [190]. The weak \textit{ampC} expression in wild-type cells may be caused by a change in the nucleotide sequence of the conserved Pribnow box and an interbox distance of only 16 bp [190]. High-level expression of \textit{ampC} depends on an optimal sequence of bases (17 bp) between the -10 and -25 regions in the Pribnow box [191, 192]. The insertion of one adenine nucleotide in amoxicillin resistant cells in the interspace between the -10 and -35 box created an optimal distance in the Pribnow box and thus resulted in a 100-fold upregulation. This strong upregulation of \textit{ampC} in resistant cells correlates well with the enhanced specific \textit{β}-lactamase activity of \(310.7 \pm 57.5\) U/mg. A comparable activity of \(700\) U/mg was measured in \textit{E. coli} ATCC 35218 harboring the TEM-1 plasmid [193].

The physiological cost of adaptation is compensated by trimming down other cellular regulatory mechanisms such as the pH or salt control. The consistently lower expression of genes involved in acid resistance (\textit{gadABC, gadE, gadWX, hdeAB, evgAS, nhaA}) [175, 176] corresponds to the reduced pH range in the presence of amoxicillin. Similarly, resistant cells were less salt-tolerant, possibly due to downregulation of \textit{gadXW} which could have caused a reduced \(\text{Na}^+\) dependent regulation of \textit{gadE, gadA} and \textit{gadBC}, resulting in this lower resistance to increased sodium chloride concentrations [194]. The downregulation of the sodium-ion transporter \textit{nhaA} in resistant cells further lowers \(\text{Na}^+\) tolerance of the resistant strain.

The main mechanisms of resistant \textit{E. coli} to prevent amoxicillin induced cell damage are summarized in figure 8. Overall, the specific up- and downregulation of genes that accompanies the acquisition of resistance seems instrumental in reducing the metabolic costs. The enduring nature of adaptation at the expression level shows that permanent changes can restore fitness by a very specific process of up- and downregulation of metabolic and regulatory networks. The costs of resistance in the case of the induced ability to withstand amoxicillin investigated in this study seem to consist of a reduced ecological range, rather than increased energy metabolism. Given the more or less constant conditions in the host, this seems understandable from an evolutionary point of view.
Figure 8. Schematic model summarizing the main metabolic consequences of amoxicillin resistance in *E. coli*. In drug exposed resistant cells gene expression of alternative electron acceptors (*frdABCD*, *narGHJI*, *dmsABC*) is induced indicating a partial switch in metabolism from aerobic to anaerobic. Depletion of NADH may counter the elevated NADH dependent superoxide production via the electron transport chain that was proposed by Kohanski and coworkers as a common mechanism of cell death induced by bactericidal antibiotics [153]. Metabolic changes in amoxicillin resistant cells include a suppressed SOS response compared to sensitive cells regardless of the presence or absence of amoxicillin. Resistance is further enhanced by a mutation in the promoter region of *ampC* resulting in increased expression of the β-lactamase.

Acknowledgements

The authors thank Drs R.J.L. Willems and W. van Schaik for donation of the *Enterococcus faecium* strains and stimulating discussions and Drs J. Wagenaar and W.J.B. van Wamel for the *Staphylococcus aureus* strains. Drs J. Piet and S.A.F.T. van Hijum assisted with the genome analysis.
Chapter 3

Interaction between mutations and regulation of gene expression during development of de novo antibiotic resistance

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Abstract

Bacteria can become resistant not only by horizontal gene transfer or other forms of exchange of genetic information, but also *de novo* by adaptation at the gene expression level and through DNA mutations. The interrelationship between changes in gene expression and DNA mutations during acquisition of resistance is not well documented. In addition, it is not known whether the DNA mutations leading to resistance always occur in the same order and whether the final result is always identical. The expression of over 4000 genes in *Escherichia coli* was compared upon adaptation to amoxicillin, tetracycline and enrofloxacin. During adaptation known resistance genes were sequenced for mutations that cause resistance. The order of mutations varied within two sets of strains adapted in parallel to amoxicillin and enrofloxacin, respectively, whereas the buildup of resistance was very similar. No specific mutations could be related to the rather modest increase in tetracycline resistance. Ribosome sensed induction and efflux pump activation initially protected the cell through induction of expression and allowed it to survive low levels of antibiotics. Subsequently mutations were promoted by the stress-induced SOS response that stimulated modulation of genetic instability and these mutations resulted in resistance to even higher antibiotic concentrations. The initial adaptation at the expression level enabled a subsequent trial and error search for the optimal mutations. The quantitative adjustment of cellular processes at different levels accelerated the acquisition of antibiotic resistance.
Introduction

The *de novo* acquisition of resistance against antibiotics is known to be accompanied by certain mutations and differential expression of specific genes [131, 140, 146, 148, 151]. The “radical-based” theory [152, 153] proposes that bactericidal antibiotics cause cell death by a single mechanism, driven by the accumulation of oxygen radicals in the cells. In that case the cellular response to sub-lethal concentrations of antibiotics should be similar even for compounds belonging to different classes of bactericidal drugs, such as β-lactams or fluoroquinolones. The outcome might differ for bacteriostatic drugs, for example tetracycline. The radical-based theory, however, is the subject of debate [195]. The revelation of a common denominator on the adaptation processes to different antibiotics could illuminate the question of a single mechanism from a different angle.

Resistance can easily be induced in *E. coli* by exposure to step-wise increasing sub-lethal concentrations [130]. The effects of the acquisition of resistance to amoxicillin on the overall physiology is a complex set of adaptations at the gene expression level, preventing metabolic costs at the expense of the ecological range [196]. After the initial stage, the prolonged exposure to antibiotics modulates the SOS-response, leading in turn to mutations that cause resistance [197]. The mutations generate the more permanent resistance, that remains long after the antibiotic pressure has been removed [148]. Resistance to amoxicillin is primarily caused by overexpression of *ampC* due to mutations in the promoter region [198]. The effectiveness of fluoroquinolones is greatly reduced by mutations in *gyrA* and *parC* [199]. If these mutations are induced by a single mechanism, a common pattern in the incidence may reveal itself.

The questions therefore poses itself whether the response of the cell upon exposure to different antibiotics is very similar, as common mechanism of action and induction of resistance would suggest, or that it differs for each class of antibiotics. In addition the delicate interplay between changes at the expression level and the induction of mutations has not been documented as well. To investigate differences in cellular response of *E. coli* upon exposure to
various antibiotics, the effects were documented of adaptation to amoxicillin, enrofloxacin and tetracycline, each belonging to a different class of compounds.

Material and Methods

Bacterial strains, growth media and culture conditions and MIC measurement

The drug sensitive wild-type *E. coli* MG1655 was used throughout. Amoxicillin, tetracycline and enrofloxacin resistance was induced by stepwise increasing the drug concentration with every transfer cycle when almost normal growth occurred [130]. Batch cultures of *E. coli* were grown at 37°C in a phosphate buffered (100 mM total NaH₂PO₄ and Na₂HPO₄) and defined minimal medium containing 55 mM glucose with a pH of 6.9 [200]. The MIC values were measured by following growth in 96 well plates as described previously [161], using duplicate serial dilutions of a factor of 2, ranging from 0 to 1024 μg/ml of the antibiotic. The MIC was defined as the lowest concentration of antibiotic that reduced the growth to an OD₅₉₅ of 0.2 or less after 23 hours.

Amplification and sequencing of resistance conferring gene loci

Amplification was performed in 50-µl working volumes with *Taq* DNA polymerase (Thermo scientific), using the following parameters: denaturation at 95°C for 5 min, followed by 35 cycles of 35 s at 95°C, 55s at 49°C and 90s at 72°C, and finally 90s extension at 72°C. The PCR products were purified with the MSB Spin PCRapace kit (Invitek) and sequenced by Macrogen Europe. Oligonucleotide primers used throughout this study are listed in table 1.
Table 1. Oligonucleotide primers used in this study for the detection of alterations in the \textit{ampC} promoter region of amoxicillin resistant and \textit{gyrA}, \textit{gyrB} or \textit{parC} gene loci for enrofloxacin resistant \textit{E. coli} cells.

<table>
<thead>
<tr>
<th>Gene range (no. of nucleotides)</th>
<th>Oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{ampC} promoter</td>
<td>Forward 5<code> GGGATCTTTTGGTGTCTCT 3</code></td>
</tr>
<tr>
<td></td>
<td>Reverse 5<code> CTTCATTGGTCGCCGTATT 3</code></td>
</tr>
<tr>
<td>\textit{gyrA}</td>
<td>Forward 5<code> AGGTTAGGAAATTTTGGGTTGG 3</code></td>
</tr>
<tr>
<td></td>
<td>Reverse 5<code> GTAGAGGGATAGCGGTAGTAG 3</code></td>
</tr>
<tr>
<td>\textit{gyrB}</td>
<td>Forward 5<code> ACGATAGAAGAAGGTCAACA 3</code></td>
</tr>
<tr>
<td></td>
<td>Reverse 5<code> CTCCAGACCAAGACAAAA 3</code></td>
</tr>
<tr>
<td>\textit{parC}</td>
<td>Forward 5<code> TATGCGTGGAATATCGGT 3</code></td>
</tr>
<tr>
<td></td>
<td>Reverse 5<code> GAAGGCTGGCGGAATAAGT 3</code></td>
</tr>
</tbody>
</table>

**Microarray RNA analysis**

Three biological replicates were inoculated to an \textit{OD}_{600} of 0.2 in fresh medium with or without antibiotics and harvested at an \textit{OD}_{600} of 1.0. Antibiotics were added at 0.25xMIC: 1 (WT) and 150 \(\mu g/ml\) (adapted) for amoxicillin; 0.125 and 128 \(\mu g/ml\) for enrofloxacin and 0.25 and 16 \(\mu g/ml\) for tetracycline. The pellet was flash-frozen in liquid nitrogen and stored at -80\(^\circ\)C. The total RNA was extracted by adding 500 \(\mu l\) of RNeasy lysis buffer containing 1% mercaptoethanol and incubated at room temperature for 5 min. The lysed cells were extracted twice with acid phenol, followed by two chloroform extractions. Total RNA was precipitated with isopropanol, incubated overnight at -80\(^\circ\)C and centrifuged for 30 min at 4\(^\circ\)C. The pellet was washed with ice-cold 75% ethanol and re-dissolved in 100 \(\mu l\) RNAse free water. The RNA samples were purified with the RNeasy Kit (Qiagen). The amount of RNA was measured on the NanoDrop ND-1000 (Thermo Scientific). The integrity of the RNA samples was investigated with the BioAnalyzer (Agilent Technologies) using the RNA nano 6000 kit (Agilent Technologies). Labeling, microarray hybridization, scanning and data processing was performed at “The MicroArray Department” of the University of Amsterdam as described previously [196].

**Genome sequencing and assembly**

Whole genome sequencing was performed described previously on a Roche 454 platform [196, 201]. The following criteria were utilized to rule out sequencing errors: (i) the region of the structural variation is unique in the reference
sequence; (ii) no perfect match reads can be aligned; and (iii) at least 3 or more reads should unambiguously support each SNP.

**Results**

*Expression profiles*

Expression profiles of strains made permanently resistant by growth at stepwise increasing concentrations [130] were compared to those of the wild-type to establish the role of differential expression in the *de novo* acquisition of resistance (figure 1). The final MIC’s were between 256 and 1024 μg/ml for amoxicillin, 512 - 1024 μg/ml for enrofloxacin and 64 μg/ml for tetracycline. Replicates were analyzed of a single adapted strain for each antibiotic. Possibly using different strains varying results might have been obtained, but physiological parameters, such as growth rate, enzyme activities or stress tolerance turned out to be very similar on other studies using more strains [130, 196]. This indicates that the outcome of the regulatory processes was similar as well.

Overall, roughly twice as many genes were significantly (factor > 2 at 95% confidence, N=3) downregulated as upregulated upon adaptation to amoxicillin, but for enrofloxacin and tetracycline this was the opposite (figure 1a). Expression of over 200 genes varied by a factor between 2 and 3, either up or down, and just 127 between 3 and 10 fold (figure 1b). Only very few genes were higher than 20-fold differentially expressed in resistant cells compared to the wild-type. The acid stress chaperone *hdeB* [202] was suppressed in amoxicillin and tetracycline resistant cells 200- and 82-fold respectively. Enrofloxacin resistant cells showed a 65-fold increased expression of the superoxide response regulon *soxS*. Amoxicillin resistant cells showed a strong induction of the *frd* operon, of the putative amino acid transporter *yjeM* and a 97-fold induction of *ampC* expression.
Mutations and expression in de novo resistance

Figure 1. Expression profiles of cells adapted to antibiotics and grown at 0.25xMIC, compared to the wild-type in the absence of antibiotics. (a) Number of differentially up- and downregulated genes in amoxicillin (Amx; MIC = 512 μg/ml), tetracycline (Tetra; MIC = 64 μg/ml) or enrofloxacin (Enro; MIC = 512 μg/ml) adapted E. coli cells compared to the wild-type. Genes are listed when expression is significantly (95% confidence) changed by a factor exceeding 2. (b) Number of genes that are up- or downregulated, grouped according to the factor of the differential expression in E. coli cells resistant to enrofloxacin (Enro), tetracycline (Tetra) and amoxicillin (Amx).

A consistent pattern does not present itself when analyzing and comparing expression patterns, but some aspects deserve notice. Adaptation to tetracycline required by far the largest number of differentially expressed genes, but the adaptation to this antibiotic was less successful than to the others. Only 4 genes, hdeA and gadABC, were differentially regulated in all three resistant cell types. These genes are part of the acid resistance systems [203]. Expression of a pH inducible protein involved in stress response (inaA), the major oxygen-insensitive nitroreductase (nfsAB) and the multidrug efflux transporters (acrAB, mdtG) [204] were significantly induced in tetracycline and enrofloxacin resistant cells. The regulator of acrAB, acrR, was upregulated 5.9 times,
resulting in a very comparable upregulation of *acrA* by 7.9 and *acrB* by a factor of 5.8. In the same cells pilus and membrane associated genes (*fimACDI*, *ompF*) were suppressed.

The diverging character of the expression profile is also seen when comparing differentially regulated genes according to their function (table 2). For example, only in tetracycline resistant cells 16 and 11 genes showing significant differential expression were clustered into the functional groups of transcription and ATP binding, respectively. However, upon induction of resistance to each of the three antibiotics, functional groups of cell wall and membrane, iron ion binding or cellular and anaerobic respiration were affected, but not identical genes. Overall, for all three antibiotics, the acquired resistance was accompanied by a large set of differentially expressed genes, both up and down, but the physiological roles are not always obvious.

**Mutations**

The results of the whole genome sequencing on several of the strains yielded no other SNP’s or other mutations that correlated consistently with increasing resistance than those described below, which are known from the literature. Genes that are both up- or downregulated and mutated are discussed below.

As *de novo* resistance against amoxicillin in *E. coli* involves primarily mutations in the Pribnow box promoter region of the *ampC* gene coding for a β-lactamase [177, 191, 198, 205], this region was sequenced in 7 parallel cultures during the building–up of a more than 500-fold increased resistance to amoxicillin (figure 2 and 3). In total 12 such mutations were observed of which only 5 were present in the final cultures. An insertion of a T at the -15 position in the Pribnow box was found in the final samples of 5 out of 7 strains, twice accompanied by an apparently not essential G to T mutation at position +33. The two other strains had a T to A mutation at position -32, also in the Pribnow box, always complemented by another mutation. This succession of events suggest that with increasing antibiotic concentrations some mutations were fixed in the culture, while other disappeared, even though these originally may have contributed to the increase of resistance [198]. The final outcome can be described as the result of a set of events that do not always occur in the same
order, concurring with some of the existing hypotheses on successful adaptation as a result of mutations [89, 206, 207].

Table 2. Summary of differential regulation in selected functional groups of tetracycline (Tetra), enrofloxacin (Enro) and amoxicillin (Amx) resistant *E. coli* compared to the wild-type found with DAVID Bioinformatics Resources 6.7 [165].

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Tetra</th>
<th>Enro</th>
<th>Amx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>up</td>
<td>down</td>
<td>up</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>101</td>
<td>49</td>
<td>83</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>22</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>Cell wall</td>
<td>16</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>Iron ion binding</td>
<td>13</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Response to drug</td>
<td>5</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Nucleotide binding</td>
<td>14</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Transcription</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP binding</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metal ion binding</td>
<td>20</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>Cellular respiration</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Anaerobic respiration</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

*a* Genes were analyzed showing a minimally 2-fold significantly different expression (95% confidence).
Resistance to quinolones, inhibitors of DNA replication that bind to DNA gyrase [199], is generally associated with mutations in \textit{gyrA}, though mutations in \textit{parC}, coding for a topoisomerase [208] and \textit{gyrB} [209] can further increase levels of resistance. The more than 1000-fold increase of the MIC for enrofloxacin was accompanied by 3 to 5 mutations (figure 4). The initial mutations always occurred in the \textit{gyrA} gene and were rarely lost or reversed. In 4 out of 7 times it was the well-documented S83L mutation [210, 211]. For the highest levels of resistance at least one of the several observed mutations in \textit{parC} was necessary as well. Mutations in \textit{gyrA} and \textit{parC} were not reversed during 30 days of continued growth in either absence or presence of the antibiotic (figure 5). Two strains had (a) mutation(s) in \textit{gyrB} that, with one exception, only occurred at the very end of the experiments and could not be correlated to additional resistance because saturation levels had already been reached. Therefore it seems that mutations in \textit{gyrB} did not directly influence the level of resistance, but an indirect relationship by stabilizing the quinolone binding pocket [209], cannot be excluded. Compared to \textit{ampC} in the case of amoxicillin, the path to resistance is therefore more focused for \textit{gyrA}, but just as variable for \textit{parC} and \textit{gyrB}. Conceivably, in the case of \textit{de novo} enrofloxacin resistance, the initial adaptation at the expression level makes the subsequent
occurrence of mutations possible by protecting the cell against the antibiotic immediately after exposure when mutations have not yet taken place.

**Figure 3.** Genetic modifications in the *ampC* promoter region of *E. coli* MG1655 during the acquisition of amoxicillin resistance. The measured MIC as a function of the amoxicillin concentration in the culture and the mutations found in the *ampC* promoter sequence over the course of stepwise increasing amoxicillin concentrations for 7 replicate cultures of *E. coli* MG1655. For every concentration PCR products of 2 clones were sequenced. The * symbol indicate mutations that were found only in one colony. Green: mutations in the *ampC* attenuator region, yellow: -10 box, blue: interbox distance, red: -35 box.
Figure 4. Genetic modifications in resistance conferring genes of *E. coli* MG1655 during the adaptation to enrofloxacin. Top: plot of the measured MIC as a function of the enrofloxacin concentration in the medium. Below: mutations in *gyrA*, *parC*, *gyrB* as a function of the MIC during growth at stepwise increasing enrofloxacin concentrations in 7 replicate cultures of *E. coli* MG1655. For every concentration 2 clones were sequenced. The * symbol indicates mutations found only in one colony. blue: mutations in *gyrA*, red: *parC*, grey: *gyrB*. 
Despite the large number of differentially expressed genes, no consistent mutations could be discerned by whole genome sequencing during the development of tetracycline resistance, which was limited to a factor of 16, or 4 steps of 2 (data not shown and [130]). Hence the moderate resistance that was developed against tetracycline must be primarily due to adaptations at the gene expression level. Alternatively, the mutations in seemingly unrelated genes may have contributed to the rather modest resistance that was built-up against tetracycline. More likely, the lack of effective mutations could explain why tetracycline adapted cells had most differentially expressed genes (figure 6a).

**Figure 5.** Mutations found in resistance conferring regions of **gyrA**, **parC**, **gyrB** in 2 enrofloxacin resistant *E. coli* replicates cultured for 30 days in the presence or absence of the antibiotic. Blue: mutations in **gyrA**, red: **parC**, grey: **gyrB**. The * symbol identifies mutations found only in one of the two colonies that were sequenced for each data point.
Regulatory processes

Names and roles of genes that are both mutated and differentially expressed are presented in table 3. These genes were considered of special interest as they are affected by both mechanisms that can confer resistance in the absence of exchange of genetic information. Most of these doubly affected genes are involved in transport or metabolism and only very few, notably ampC [192], are known resistance genes. If simple mutational pathways would be assumed in the development of de novo resistance, then mutations could be directly linked to expression. However, the data suggest that development of de novo resistance requires cellular adjustments regulated in a far more complex manner. Only very few genes were mutated and differentially regulated at the same time, despite the huge number of differentially expressed genes and mutations identified. This indicates that a complex set of adjustments operates within the regulatory network of the cell during adaptation (table 3).

While different genes are mutated upon induction of resistance to different antibiotics, it is not obvious that the same applies to differential regulation. Conceivably a distinction must be made between genes involved in the primary processes, such as efflux pump, and a set of secondary genes that are differentially regulated to compensate and adjust for the effects of the primary effectors. If the same mechanisms are involved in countering the effects of each antibiotic, a similar response at the expression level, up- and downregulating at least a similar set of secondary genes, can be expected. The overlap of genes involved in acquired resistance against the three antibiotics through differential expression was surprisingly small (figure 6a and b). Only strains made resistant to enrofloxacin and tetracycline shared a considerable number of co-regulated genes. The widely varying sets of differentially regulated genes suggest that different mechanisms are involved in adapting to each antibiotic.
Table 3. Genes that were simultaneously mutated in the in-frame and/or out-of-frame (> 1,000 bp upstream of differentially expressed genes) and significantly (minimally 2-fold at 95% confidence) differentially expressed, even after the antibiotic was removed from the medium.

<table>
<thead>
<tr>
<th>Antibiotic, position and gene</th>
<th>Position of mutation</th>
<th>Without indicated antibiotic (fold change)</th>
<th>With indicated antibiotic (fold change)</th>
<th>Gene Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enrofloxacin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outframe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yiep</td>
<td>684 bp upstream G to T, 688 T to A, 706 A to G, 753 T to A</td>
<td>2.5</td>
<td>2.3</td>
<td>predicted transcriptional regulator</td>
</tr>
<tr>
<td>pptA</td>
<td>95 bp upstream G to T</td>
<td>2.3</td>
<td>2.1</td>
<td>probable 4-oxalocrotonate tautomerase</td>
</tr>
<tr>
<td>stpA</td>
<td>35 bp upstream, deletion</td>
<td>-3.3</td>
<td>-3.4a</td>
<td>H-NS-like DNA-binding protein with RNA chaperone activity</td>
</tr>
<tr>
<td>fimA</td>
<td>452 bp upstream, deletion</td>
<td>-7.9</td>
<td>-8.5</td>
<td>major type 1 subunit fimbrin</td>
</tr>
<tr>
<td>Inframe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dgt</td>
<td>G77G</td>
<td>3.1</td>
<td>2.9</td>
<td>dGTPase, binds single-stranded DNA</td>
</tr>
<tr>
<td>fimD</td>
<td>T331Y</td>
<td>-3.8</td>
<td>-3.9</td>
<td>outer membrane protein; export and assembly of type 1 fimbriae glutamate decarboxylase A, confers resistance to extreme acid conditions</td>
</tr>
<tr>
<td>gadA</td>
<td>S24P; R31; D39A; F43L</td>
<td>3.2</td>
<td>3.2</td>
<td>predicted multidrug transporter subunit of ABC superfamily: ATP-binding component</td>
</tr>
<tr>
<td>mldA</td>
<td>N234T</td>
<td>2.5</td>
<td>2.4</td>
<td>Transporter: outer membrane porin F</td>
</tr>
<tr>
<td>ompF</td>
<td>I336I; I336N</td>
<td>10.1</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td><strong>Amoxicillin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outframe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>insL</td>
<td>61 bp &amp; 59bp upstream, T to C</td>
<td>-2.9</td>
<td>-1.6</td>
<td>IS186 hypothetical protein</td>
</tr>
<tr>
<td>iraM</td>
<td>528 bp upstream, insertion</td>
<td>-2.2</td>
<td>-1.6</td>
<td>Inhibitor of σ₅ proteolysis, contributes to acid resistance Nitrate reductase 1, alpha subunit</td>
</tr>
<tr>
<td>narG</td>
<td>460 bp upstream, insertion</td>
<td>2.6</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>Promoter region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ampCb</td>
<td>-12 insertion of A</td>
<td>97.1</td>
<td>106.2</td>
<td>β-lactamase; penicillin resistance</td>
</tr>
<tr>
<td>Inframe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>insB-4</td>
<td>F81L; R65S; Y128H</td>
<td>2.3</td>
<td>2.5</td>
<td>IS1 protein InsB</td>
</tr>
<tr>
<td>gadB</td>
<td>D233H; T214P</td>
<td>-5.3</td>
<td>-22.9</td>
<td>Glutamate decarboxylase isozyme</td>
</tr>
<tr>
<td>gadA</td>
<td>H465Y; Q459K</td>
<td>-5.4</td>
<td>-4.4</td>
<td>Glutamate decarboxylase A</td>
</tr>
</tbody>
</table>
### Chapter 3

Table continued from previous page

<table>
<thead>
<tr>
<th>Antibiotic, position and gene</th>
<th>Position of mutation</th>
<th>Without indicated antibiotic (fold change)</th>
<th>With indicated antibiotic (fold change)</th>
<th>Gene Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tetracycline</strong>&lt;br&gt;Outframe</td>
<td>&lt;br&gt;<strong>fimA</strong>&lt;br&gt;62 bp upstream, deletion&lt;br&gt;198 bp upstream, T to G&lt;br&gt;198 bp upstream, deletion</td>
<td>8.3</td>
<td>9.1</td>
<td>major type 1 subunit fimbrin (pilin)</td>
</tr>
<tr>
<td><strong>higB</strong>&lt;br&gt;199 bp upstream, G to A&lt;br&gt;200 bp upstream, C to A&lt;br&gt;212 bp upstream, C to A&lt;br&gt;392 bp upstream, insertion</td>
<td>3.6</td>
<td>12.6</td>
<td>translation-dependent mRNA interferase, toxin of the HigB-HigA toxin-antitoxin system</td>
<td></td>
</tr>
<tr>
<td><strong>ompF</strong>&lt;br&gt;89 bp upstream, deletion</td>
<td>-10.7</td>
<td>-1.9</td>
<td>outer membrane porin F</td>
<td></td>
</tr>
<tr>
<td><strong>Inframe</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>cpxP</strong>&lt;br&gt;S152V</td>
<td>2.9</td>
<td>1.1</td>
<td>regulator of the Cpx response and possible chaperone involved in resistance to extracytoplasmic stress</td>
<td></td>
</tr>
<tr>
<td><strong>gadA</strong>&lt;br&gt;H465Y; Q459K; Y393Y; D233Y; T214S</td>
<td>-2.7</td>
<td>-41.5</td>
<td>glutamate decarboxylase A, part of the glutamate-dependent acid resistance system 2</td>
<td></td>
</tr>
<tr>
<td><strong>gadB</strong>&lt;br&gt;H465H; Q459E; N81Y&lt;br&gt;I336I; I336N; Y332H; K299N; T298A; T298I; T298T; D288H</td>
<td>-2.7</td>
<td>-40.7</td>
<td>glutamate decarboxylase B</td>
<td></td>
</tr>
<tr>
<td><strong>ompF</strong>&lt;br&gt;-10.7</td>
<td>-1.9</td>
<td>Transporter: outer membrane porin F</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ybfD</strong>&lt;br&gt;A194A; F206F; P218P; K219E</td>
<td>2.0</td>
<td>1.2</td>
<td>putative DNA ligase</td>
<td></td>
</tr>
<tr>
<td><strong>yliE</strong>&lt;br&gt;M89N</td>
<td>-2.2</td>
<td>-1.1</td>
<td>predicted c-di-GMP-specific phosphodiesterase</td>
<td></td>
</tr>
</tbody>
</table>

*Not significantly (p > 0.05) differentially expressed

*Differs from selection criteria (ii) described in methods section. Perfect match reads as well as structural variations were identified.
A second question regarding regulation at the expression level is how many genes are rapidly induced upon initial exposure to an antibiotic. To answer this question, the effect of the antibiotic on the wild-type was studied by exposing the cells to 0.25×MIC, allowing growth, but assuring effect of the antibiotic (figure 7a). Exposure of cells to amoxicillin had the smallest effect on the transcriptomic profile. While wild-type cells showed no significant change in gene expression when exposed to 0.25×MIC, amoxicillin resistant cells showed 4 suppressed and 8 induced genes. In contrast, the use of 0.125 μg/ml
enrofloxacin (0.25×MIC) for the wild-type showed a massive effect on the transcriptomic profile with 303 suppressed and 206 induced genes. When the wild-type was made permanently resistant to enrofloxacin, the global transcriptomic effect of enrofloxacin exposure was reversed and only a single gene was differentially expressed by growth at 0.25×MIC (128 μg/ml).

![Figure 7](image)

**Figure 7.** Change in transcriptomic profile of wild-type and antibiotic resistant *E. coli* cells in response to short term (< 10 generations) drug exposure and long term adaptation (> 100 generations). (a) Number of up and down regulated genes after antibiotic exposure (0.25×MIC: 1 μg/ml amoxicillin; 0.25 μg/ml tetracycline and 0.125 μg/ml enrofloxacin) in wild-type (WT) and antibiotic resistant (AR) cells compared to wild-type. (b) Number of up- or downregulated genes compared to wild-type for the wild-type exposed to 0.25×MIC enrofloxacin (WT(exposed)), enrofloxacin adapted cells (Enro), enrofloxacin adapted exposed to 0.25×MIC (Enro(exposed)) and enrofloxacin adapted and cultured for 30 days without the antibiotic (Enro 30).

To study the opposite effect of rapid induction, growth of adapted cells was followed in the absence of the antibiotic. The number of differentially regulated genes in enrofloxacin resistant cells reduced from 120 differentially regulated genes after the adaptation phase to 23 genes after growth for 30 days without antibiotic (figure 6) while the MIC remained above 1024 μg/ml. Only one gene, the NADH:quinone oxidoreductase *azoR*, had a 2.7-, 6.25- and 2.2-fold suppressed expression in wild-type exposed to enrofloxacin, cells after adaptation and adapted cells grown in the absence of enrofloxacin, respectively (figure 6b). No mutation was found in the coding or upstream region (>1000 bp) of *azoR*. 
Interaction between adaptation of expression and mutations

The effects of short term exposure on expression levels differ strongly from those of long term adaptation. To elucidate these differences the induction of resistance by adaptation was compared to the immediate regulation of gene expression upon initial exposure of the wild-type to antibiotics. This is illustrated by the expression levels of the wild-type in response to short term exposure and adapted cells in the presence or absence of the antibiotic (figures 7a and b). When the role of mutations is large, as for amoxicillin resistance (figure 7a) and high levels of enrofloxacin resistance (figure 7b), the role of expression is limited. When mutations have no obvious role, as in the cases of tetracycline resistance and the initial reaction to enrofloxacin exposure, a large number of genes is differentially regulated. These observations suggest that the cell initially reacts to the exposure to antibiotics by differentially expressing genes, but when subsequently successful mutations occur, the role of differential expression is reduced. Thus the role of mutations in the acquisition of resistance is exactly mirrored by that of differential expression. As an exception, the constitutive upregulation of ampC as a consequence of a mutation in the Pribnow box regulating its expression, is a remarkable combination of both. We expected to find physiological differences, such as lower growth rates, upon acquisition of resistance. In reality only cells exposed to tetracycline had lower growth rates (data not shown and [130]).

Discussion

Taking into consideration all information on the response at the molecular level of E. coli to exposure to antibiotics of this and other studies [115, 131, 152, 196, 207, 212-214], a picture emerges of the cell exploring all possible escape routes both at the transcriptional and at the mutational level. The overall result is an intricate set of interactions between mutations and quantitative adaptations at the enzymatic level that result in enhanced resistance of the cell to the antibiotic it encounters. For each of the three antibiotics applied in this study, the outcome was different, as well as the strategy, as far as that term is valid in this context, since intent cannot be implied. The example of tetracycline is one extreme, as
no mutations seem to be involved consistently and many genes are differentially expressed. Enrofloxacin resistance is the other extreme since it is caused by mutations, with a very limited role for regulation of expression once the crucial mutations have occurred. The middle path is taken towards amoxicillin resistance, as the consistent mutations affect the expression of $ampC$, a $\beta$-lactamase that deactivates amoxicillin effectively [196].

The mutations induced in this study by stepwise increasing exposure to amoxicillin are identical to those found in clinical AmpC hyperproducing $E.\ coli$ isolates [198, 215] and seem to have the same effect. Acquisition of enrofloxacin resistance utilized different evolutionary pathways that lead to identical phenotypic outcomes. In 36 clinical $E.\ coli$ isolates, the most abundant change in $gyrA$ was found to be an amino acid change of serine to leucine at position 83 [210], in accordance to our findings. This suggests that while initially some variation occurred in the mutations, the successful final set is limited. This implies a “gambling for existence” search procedure for the optimal mutations, maintaining the most beneficial ones, as shown for long-term adaptation and evolution [216]. The different strategies possibly reflect the different operating mechanisms of the antibiotics, amoxicillin blocking cell-wall synthesis, while tetracycline inhibits protein synthesis and enrofloxacin DNA synthesis.

Interestingly, the essential mutations were not reversed when the antibiotic was no longer present, suggesting that the cell pays a limited metabolic price for the mutations, if at all. This in turn raises the question what the advantage is of the wildtype allele. The implication for patient treatment is considerable, as even low levels of induced amoxicillin resistance caused the susceptible population to be outcompeted by cells made moderately resistant due to short term exposure to non-lethal concentrations of antibiotics [217]. The remarkable capability of $E.\ coli$ to get highly resistant to amoxicillin and enrofloxacin within 100 generations and without observed fitness costs is reflected in the abundant observations of tremendous spread of antibiotic resistance in the environment.

In conclusion, de novo resistance to antibiotics is brought about by a complex interaction of cellular processes, involving both adaptation of expression levels and mutations. Ribosome sensed induction and efflux pump
activation are examples of mechanisms that initially protect the cell through induction of expression [218] and allow it to survive low levels of antibiotics. Subsequently mutations are promoted by the SOS response that stimulates the horizontal exchange of resistance genes [197], or stress-induced modulation of genetic instability [219] and result in resistance to higher concentrations. The quantitative adjustment of cellular processes at different levels [220] facilitate the rapid evolution observed during the acquisition of antibiotic resistance.

Acknowledgements

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Chapter 4

Effects of stress, ROS and the SOS response during the de novo acquisition of antibiotic resistance in *Escherichia coli*

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This chapter has been submitted
Abstract

The worldwide increase of antimicrobial resistance threatens human health as it causes therapy failure and increased costs for control of infectious diseases. Hence, strategies to slow down the evolution of resistance in bacteria are urgently needed. De novo acquisition of resistance due to mutations and/or phenotypic adaptation has previously been shown to occur rapidly as a result of interactions of gene expression and mutations [221]. Based on that study, deletion mutants were selected to investigate the contribution of several individual genes in the de novo acquisition of antibiotic resistance in E. coli. In addition, the potential of bacteria to become resistant to antibiotics under non-optimized conditions, such as a lowered pH or increased salt concentration, was documented. The results indicate that the SOS response and global transcriptional activators, such as gadE, play a crucial role in to the development of antibiotic resistance. Deletion of the transcriptional regulator soxS that is involved in superoxide removal slowed down the acquisition of resistance, but only to bactericidal antibiotics. Acquisition of resistance in the presence of a second stressor resulted in a lower adaptation rate and could therefore present a useful strategy to combat evolution of resistance. The outcome suggests that a central cellular mechanism is crucial for development of resistance. In this system genes involved in regulation of transcription that were not identified by changes in expression level upon exposure to antibiotics also play a role.
Introduction

The worldwide emergence of antibiotic resistant bacteria poses a serious threat to human health, as costs of treatment increase and the outcome is negatively affected. Bacteria can become resistant de novo by genetic or phenotypic changes, but also through acquisition of resistance conferring plasmids. Induction of resistance to antibiotics was shown to occur very rapidly as a result of exposure to stepwise increasing sub-lethal drug concentrations [130]. Within 15 daily transfers, corresponding to only 90 generations, bacterial cells developed genetic and permanent transcriptional changes [221]. These cellular modifications allow the population on the one hand to grow in the presence of high antibiotic pressure, but on the other hand they may decrease fitness or cause a metabolic burden [130, 151, 221]. For example, the adaptation of E. coli to amoxicillin was accompanied by a reduced ecological range, corroborating the notion of physiological costs of resistance [196].

In order to be able to develop countermeasures that avoid or slow down the acquisition of antibiotic resistance, it is essential to understand how bacteria respond to drug exposure. Many studies investigated adaptation under optimized culture conditions and revealed underlying molecular mechanisms in response to a single stress agent, namely the antibiotic itself [88, 153, 221, 222]. But how bacteria adapt to antibiotics under non-optimized conditions and thus in the presence of two or more stressors is still poorly understood and generally not well known. One of the aims of this study is to investigate the potential of E. coli to adapt to antibiotics in a non-optimal growth environment, in this case a lowered pH and increased salt concentrations.

The genetic background is likely to affect the ability to adapt and become resistant. Genes that were permanently differentially regulated in E. coli cells resistant to amoxicillin, enrofloxacin or tetracycline compared to their sensitive ancestor [221] are interesting candidates for such research. The current study focusses on the role in adaptation to antibiotics of the transcriptional activator gadE, the outer membrane porin ompF and the transcriptional regulator soxS that is involved in superoxide removal. ΔgadE, ΔsoxS, ΔompF mutants were exposed to stepwise increasing antibiotic concentrations. The data shows a divergent adaptation rate compared to the wild-type. Furthermore, the role of
the SOS response was examined by exposing $\Delta recA$ cells to increasing concentrations of drugs belonging to the different antibiotic classes.

**Material and Methods**

**Bacterial strains, growth media, antibiotics and MIC measurement**

The drug sensitive wild-type *E. coli* MG1655 was used as control throughout this study. Single knockout strains ($\Delta recA$, $\Delta gadE$, $\Delta soxS$, $\Delta ompF$) were purchased from a BW25113 deletion library [223]. Cells were grown in 100 ml flasks at 37°C in a phosphate buffered (100 mM Na$_2$H$_2$PO$_4$) and defined minimal medium containing 55 mM glucose and shaken at 200 rpm [200]. If not stated otherwise, the pH was set to 6.9 with 4 N NaOH. In experiments performed with varying environmental conditions, the pH was set to 6.0 or an additional 2% NaCl was added to the medium. To study the development of resistance under non-optimal conditions, wild-type cells were adapted during one week by daily transferring cells into fresh medium at an OD of 0.1 for each condition tested: (1) pH 6.9, 0% additional NaCl; (2) pH 6.0, 0% additional NaCl, (3) pH 6.9, 2% additional NaCl. Amoxicillin, tetracycline and enrofloxacin stock solutions (10 mg/ml) were 0.2-µm filtersterilized and stored at 4°C. Light sensitive tetracycline stock solutions were wrapped in tinfoil to prevent exposure to light. Wild-type and deletion mutants were grown with 1.25 µg/ml amoxicillin, 0.5 µg/ml tetracycline or 0.125 µg/ml enrofloxacin to induce the buildup of resistance. The starting enrofloxacin concentration for the $\Delta recA$ deletion mutant was reduced to 0.03125 µg/ml, as cells showed no growth with higher concentrations of this antibiotic. Resistance to antibiotics was induced by stepwise increasing the drug concentration with every transfer cycle when almost normal growth occurred [130]. The MIC values were measured by following growth in 96 well plates as described previously [161], using duplicate serial dilutions of a factor of 2, ranging from 0.5 to 1024 µg/ml of the antibiotic. Additionally, two wells were used to follow growth without any antibiotic. The MIC was defined as the lowest concentration of antibiotic that reduced the growth to an OD$_{595}$ of 0.2 or less after 23 hours.
**Amplification and sequencing of resistance conferring gene loci**

Amplification was performed in 50-µl working volumes with Taq DNA polymerase (Thermo scientific), using the following parameters: denaturation at 95°C for 5 min, followed by 35 cycles of 35 s at 95°C, 55 s at 49°C and 90 s at 72°C, and finally 90 s extension at 72°C. The MSB Spin PCRAPace kit (Invitek) was used to purify the amplified PCR products. Oligonucleotide primers used for amplification of resistance conferring regions are identical to those used previously [221]. PCR products were sequenced by Macrogen Europe.

**RNA isolation and quantitative real-time PCR**

*E. coli* pre-cultures were grown overnight without antibiotics in defined minimal medium under different conditions. Cells were inoculated to an OD of 0.2 in fresh medium with or without 0.125 µg/ml enrofloxacin. When cultures reached an OD of 1.0, cells were harvested directly into RNAlater RNA Stabilization Reagent (Qiagen). After centrifuging 3 min at 4000 rpm and 4°C, pellets were flash-frozen in liquid nitrogen and stored at -80°C. The total RNA was extracted by adding 500 µl of RNeasy lysis buffer containing 1% mercaptoethanol and incubated at room temperature for 5 min. The lysed cells were extracted twice with acid phenol, followed by two chloroform extractions. Total RNA was precipitated with isopropanol, incubated overnight at -80°C and centrifuged for 30 min at 4°C. The pellet was washed with ice-cold 75% ethanol and re-dissolved in 100 µl RNAse free water. The RNA samples were purified with the RNeasy Kit (Qiagen) and RNA quality was verified on a 1% agarose gel to assure absence of RNA degradation. The amount of RNA was measured on the NanoDrop ND-1000 (Thermo Scientific). DNA residues were removed with the help of Ambion® TURBO DNA-free™ DNase Treatment and Removal Reagents according to the manufacturer’s instructions. First strand cDNA was synthesized from RNA (1 µg) using the RevertAid First Strand cDNA Synthesis kits (Fermentas). The final concentrations of cDNA and primers in a total volume of 50 µl were 20 ng and 50 nM, respectively. The optimal primer concentration was verified beforehand for each primer pair. Primers for quantitative real-time PCR (qRT-PCR) used in this study are shown in table 1. qRT-PCR was performed with the 7300 real-time PCR system (Applied Biosystems) and universal cycling conditions (2 min at 50°C, 10 min
at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C) using the Power SYBR® Green PCR Master Mix (Life Technologies). Cycle threshold (Ct) values were determined by automated threshold analysis with ABI Prism version 1.0 software. The relative abundance was calculated and normalized with the ΔΔC_T method, using idnT as the reference gene according to Zhou et al. [224]. Stable expression of the reference gene was verified with expression data obtained in a microarray study comparing antibiotic exposed sensitive and antibiotic resistant E. coli cells to wild-type expression levels [221]. qRT-PCR performance under the above mentioned conditions was validated according to the manufacturer’s instructions using serial dilutions of template cDNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>idnT</td>
<td>Forward 5’ CGCCACTACGCTGATTGCT 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ TCACGTACCGCCCATTTGCA 3’</td>
</tr>
<tr>
<td>rpoS</td>
<td>Forward 5’ TCGCCCGCCGATGA 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ GCGGGGCAATTTTTAACA 3’</td>
</tr>
<tr>
<td>soxS</td>
<td>Forward 5’ GGCCGGCGGTGGATGTTG 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ GTCCATTGCGATATCCAAAT 3’</td>
</tr>
<tr>
<td>umuD</td>
<td>Forward 5’ TGATATTGTCATCGCTTGATTG 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’ CGGGCGTAGTTGCAATTTT 3’</td>
</tr>
</tbody>
</table>

Table 1. Primer used in this study for qRT-PCR.

Results

Effect of selected gene deletions on the acquisition of antibiotic resistance

Single deletion of promising gene candidates (gadE, soxS, ompF) that could affect the buildup of resistance to 3 different antibiotics had only a marginal effect on the sensitivity to amoxicillin and tetracycline compared to the wild-type (table 2). Only when measuring the MIC for enrofloxacin in the ΔsoxS and ΔgadE strain an 8- and 4-fold reduction of the MIC was observed.
Table 2. MIC for wild-type (WT) and deletion mutants grown in defined mineral or LB medium for amoxicillin, enrofloxacin and tetracycline.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ΔsoxS</th>
<th>ΔgadE</th>
<th>ΔompF</th>
<th>WT in LB</th>
<th>ΔrecA in LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC for amoxicillin</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>[µg/ml]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIC for enrofloxacin</td>
<td>0.5</td>
<td>0.0625</td>
<td>0.125</td>
<td>1</td>
<td>0.25</td>
<td>0.0313</td>
</tr>
<tr>
<td>[µg/ml]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIC for tetracycline</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>[µg/ml]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Induction of resistance to amoxicillin and enrofloxacin was affected in different ways by these gene deletions. During the acquisition of resistance to amoxicillin only ΔsoxS cells showed a reduced adaptation rate, and then again only at high amoxicillin concentration above 128 µg/ml (figure 1). Whereas the wild-type reached the highest amoxicillin concentration of 1024 µg/ml after 18 days, ΔsoxS cells were only able to grow with 256 µg/ml after 26 days. Development of enrofloxacin resistance was more effected by selected deletions. Both the ΔgadE and ΔsoxS mutant adapted slower to increased enrofloxacin concentrations compared to the wild-type (figure 2). The deletion of the outer membrane protein F in the ΔompF strain seemed to be beneficial in the beginning compared to the wild-type, but cells stopped growing at day 7. Re-inoculated ΔompF cells from the -80°C glycerol stock, grown at 1 µg/ml enrofloxacin, showed similar adaptation rates to increasing enrofloxacin concentration as the wild-type. Overall, only deletion of soxS affected development of resistance to the two bactericidal antibiotics enrofloxacin and amoxicillin. To test whether the lack of soxS hampers the acquisition of resistance to a bacteriostatic antibiotic as well, ΔsoxS cells were made resistant to tetracycline (data not shown). However, no difference in the buildup of resistance to tetracycline in the ΔsoxS mutant was observed compared to the wild-type strain.
Figure 1. Acquisition of resistance to amoxicillin in *E. coli* wild-type and ΔsoxS, ΔompF and ΔgadE knockout strains in mineral medium. Cells were adapted by stepwise increasing the drug concentration by a factor of 2 when growth was comparable to wild-type cells.

Figure 2. Acquisition of resistance to enrofloxacin in *E. coli* wild-type and ΔsoxS, ΔompF and ΔgadE knockout strains in mineral medium. Cells were adapted by stepwise increasing the drug concentration by a factor of 2 when growth was comparable to wild-type cells.
Acquisition of antibiotic resistance with inefficient SOS-response in LB medium

To study the development of antibiotic resistance in cells with an inefficient SOS response, complex LB medium had to be used, as the ΔrecA strain was not able to grow in mineral medium. The time needed to adapt to enrofloxacin and tetracycline in wild-type cells and complex medium was similar to rates obtained in mineral medium (figure 2 and 3). However, adaptation of wild-type cells to amoxicillin seemed to benefit from complex medium as growth in the presence of 1280 µg/ml was already observed after 10 days.

Surprisingly, no mutation in the promoter region of ampC was found when wild-type cells were grown at 320 and 1280 µg/ml (table 3). In contrast, adaptation of wild-type cells in mineral medium to amoxicillin concentrations higher than 20 µg/ml resulted in genetic modifications of the ampC promoter region as shown in this and previous studies. Therefore results were verified twice by sequencing relevant PCR products, but no mutation could be detected.

The MIC for amoxicillin and tetracycline was only marginally affected by the lack of recA (table 2). Adaptation in response to increasing amoxicillin and tetracycline concentrations was generally slower in recA deficient mutants, but ΔrecA cells were able to adapt to both antibiotics (figure 3A and 3C). Adaptation to amoxicillin in ΔrecA cells was characterized by long plateaus at 4 and 8 µg/ml, corresponding to the range of concentration in which resistance conferring mutations occur in the ampC promoter region [221]. In contrast, the MIC value for enrofloxacin in the ΔrecA strain showed an 8-fold reduction compared to the wild-type (table 2). As a consequence, the starting sub-MIC concentration had to be lowered for ΔrecA cells from 0.125 µg/ml used for wild-type cells to 0.03125 µg/ml. After 6 days of growth only in LB medium and passaging a fraction every day into fresh medium containing 0.03125 µg/ml enrofloxacin, sufficient growth was obtained to continue the process of adaptation (figure 3B). After 20 days ΔrecA cells were only adapted to a concentration of 0.25 µg/ml, corresponding to the MIC value of the wild-type in LB. In contrast to the wild-type that showed a first resistance conferring mutation in gyrA with 0.125 µg/ml, the first mutation in gyrA of ΔrecA cells was only identified at a concentration of 0.25 µg/ml (table 5).
Figure 3. Acquisition of resistance to amoxicillin (a), enrofloxacin (b) and tetracycline (c) in *E. coli* wild-type and ∆recA cells in complex LB medium.
Role of stress, ROS and SOS response in de novo acquired resistance

Table 3. Mutations found in the ampC promoter region of wild-type (WT) and deletion mutant cells during the acquisition of resistance to amoxicillin (AMX).

<table>
<thead>
<tr>
<th>Concentration AMX (µg/ml)</th>
<th>WT</th>
<th>ΔsoxS</th>
<th>ΔompF</th>
<th>ΔgadE</th>
<th>WT in LB</th>
<th>ΔrecA in LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.5</td>
<td>interbox*</td>
<td>-</td>
<td>-</td>
<td>-35 box*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-10 box; -35 box</td>
<td>-10 box*</td>
<td>-35 box</td>
<td>-35 box</td>
<td>-10 box</td>
<td>-10 box</td>
</tr>
<tr>
<td>20</td>
<td>-10 box; -35 box</td>
<td>terminator</td>
<td>-35 box</td>
<td>-35 box</td>
<td>interbox</td>
<td>-10 box; interbox</td>
</tr>
<tr>
<td>320</td>
<td>-10 box; -35 box</td>
<td>terminator, interbox</td>
<td>-35 box*</td>
<td>-35 box</td>
<td>-</td>
<td>-10 box; interbox</td>
</tr>
<tr>
<td>1280</td>
<td>-10 box; -35 box; interbox</td>
<td>terminator, interbox</td>
<td>-35 box*</td>
<td>-35 box</td>
<td>-</td>
<td>-10 box; interbox</td>
</tr>
</tbody>
</table>

*mutation only found in one of two sequenced replicates

Acquisition of antibiotic resistance in varying environments
Development of resistance under non-optimized conditions, such as increased salt concentration or reduced pH, deviated from adaptation achieved in optimized mineral medium (pH 6.9, 0% additional NaCl) (figure 4 and 5). Below amoxicillin and enrofloxacin concentration of 8 to 16 µg/ml adaptation rates were similar for both antibiotics and all conditions studied (figure 4 and 5). Only above this threshold adaptation rates of cells grown under non-optimal conditions slowed down. Sequencing of resistance conferring regions in gyrA revealed different patterns in accumulation of genetic variations (figure 5, table 6). With reduced pH or increased salt concentration no mutation was found in gyrA when cells were grown in the presence of 0.125 µg/ml enrofloxacin. In contrast, wild-type cells cultured in LB or mineral medium under optimized
conditions showed mutations with the lowest enrofloxacin concentration used (table 4 and 5).

**Table 4.** Mutations found in resistance conferring regions of *gyrA* and *parC* in the wild-type (WT) and deletion mutants during the acquisition of resistance to enrofloxacin (enro).

<table>
<thead>
<tr>
<th>Concentration enro (µg/ml)</th>
<th>WT <em>gyrA</em></th>
<th>ΔsoxS <em>gyrA</em></th>
<th>ΔompF <em>gyrA</em></th>
<th>ΔagadE <em>gyrA</em></th>
<th>WT <em>parC</em></th>
<th>ΔsoxS <em>parC</em></th>
<th>ΔompF <em>parC</em></th>
<th>ΔagadE <em>parC</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.125</td>
<td>S83L</td>
<td>-</td>
<td>D87G</td>
<td>D87G</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.25</td>
<td>S83L</td>
<td>D87Y</td>
<td>D87G</td>
<td>G81C; R38R*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>S83L</td>
<td>G81D</td>
<td>D87G; R38R*</td>
<td>G81C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>D87G; S83L</td>
<td>G81D</td>
<td>D87G; S83L</td>
<td>G81D</td>
<td>S80I</td>
<td>S80I</td>
<td>-</td>
<td>S80R</td>
</tr>
<tr>
<td>1024</td>
<td>D87G; S83L</td>
<td>G81D</td>
<td>D87G; S83L</td>
<td>G81D</td>
<td>S80I</td>
<td>D79A</td>
<td>E84G</td>
<td>S80R</td>
</tr>
</tbody>
</table>

*mutation only found in one of two sequenced replicates
**Table 5.** Mutations found in resistance conferring regions of \( \textit{gyrA} \) and \( \textit{parC} \) in the wild-type and \( \Delta \text{recA} \) deletion mutant during the acquisition of resistance to enrofloxacin in LB medium.

<table>
<thead>
<tr>
<th>Concentration enrofloxacin (µg/ml)</th>
<th>WT ( \textit{gyrA} )</th>
<th>( \Delta \text{recA} ) ( \textit{gyrA} )</th>
<th>WT ( \textit{parC} )</th>
<th>( \Delta \text{recA} ) ( \textit{parC} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.125</td>
<td>G81D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.25</td>
<td>S83L</td>
<td>D87Y</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>S83L</td>
<td>D87Y</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>S83W</td>
<td>ND</td>
<td>G78D</td>
<td>ND</td>
</tr>
<tr>
<td>1024</td>
<td>S83L</td>
<td>ND</td>
<td>S80R</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Table 6.** Mutations found in resistance conferring regions of \( \textit{gyrA} \) and \( \textit{parC} \) in wild-type cells adapted to enrofloxacin (enro) in mineral medium with different conditions (pH 6 or 2% salt).

<table>
<thead>
<tr>
<th>Concentration enro (µg/ml)</th>
<th>Control ( \textit{gyrA} )</th>
<th>pH 6 ( \textit{gyrA} )</th>
<th>2% salt ( \textit{gyrA} )</th>
<th>Control ( \textit{parC} )</th>
<th>pH 6 ( \textit{parC} )</th>
<th>2% salt ( \textit{parC} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.125</td>
<td>S83W;D87Y*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.25</td>
<td>S83W</td>
<td>D87G</td>
<td>D87T</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>S83W</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>S83W</td>
<td>S83W</td>
<td>S83L</td>
<td>G78D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1024</td>
<td>S83W; A84P</td>
<td>G81D</td>
<td>S83L;D87G</td>
<td>G78D</td>
<td>-</td>
<td>E84G</td>
</tr>
</tbody>
</table>

*mutation only found in one of two sequenced replicates
Figure 4. Acquisition of resistance to amoxicillin under varying conditions (pH 6 or 2% salt) in *E. coli* wild-type and cells in mineral medium. Cells were adapted to different conditions by daily passaging to fresh medium without any antibiotic for seven days.

Figure 5. Acquisition of resistance to enrofloxacin under varying conditions (pH 6 or 2% salt) in *E. coli* wild-type. Cells were adapted to different conditions by daily passaging to fresh medium without any antibiotic for seven days.
Role of stress, ROS and SOS response in de novo acquired resistance

Once cells were able to grow with 16 µg/ml enrofloxacin in LB or under optimized conditions in mineral medium the well-known G78D mutation was observed in \( \text{parC} \). But again, under non-optimal conditions mutations in \( \text{parC} \) were only found at higher enrofloxacin concentrations. \( \text{E. coli} \) cells made resistant to 1024 µg/ml enrofloxacin medium with a lowered pH of 6 showed no mutation in \( \text{parC} \) at all. Resistance to enrofloxacin strongly depends on mutations in \( \text{gyrA} \) and \( \text{parC} \). Several studies proved that bacteria are not only passively waiting for the optimal set of mutations to appear and that prokaryotes are able to induce transient mutagenesis mechanism in response to stress [225]. To test whether a changed general stress (\( \text{rpoS} \)), oxidative (\( \text{soxS} \)) or SOS response (\( \text{umuD} \)) account for a later accumulation of mutations and protects the cells from antibiotics under non-optimal conditions, expression levels of \( \text{rpoS} \), \( \text{soxS} \) and \( \text{umuD} \) were compared to the wild-type with qRT-PCR (figure 6). In agreement with a previous microarray study, an enrofloxacin resistant strain (Enro) was used as control showing a strong induction for \( \text{soxS} \), but equal expression levels of \( \text{rpoS} \) and \( \text{umuD} \) compared to the wild-type in the absence of antibiotics [221]. Non-optimized conditions and exposure to 0.125 µg/ml enrofloxacin did not alter the expression of \( \text{soxS} \). However, \( \text{umuD} \) was at least 3-fold down regulated under all deviating growth conditions tested with and without enrofloxacin exposure. This general master stress regulator showed a 4 fold induction in cells upon culturing them in 2% NaCl when exposing them to 0.125 µg/ml enrofloxacin.
Discussion

Acquisition of antibiotic resistance is influenced by many intertwined factors, such as regulatory processes in response to DNA or oxidative damage, but also external culture conditions. Complex LB medium promotes the buildup of amoxicillin resistance in *E. coli* wild-type cells compared to minimum mineral medium. Cells grown on minimal glucose containing medium might be partially starved for amino acids that could potentially be a stressful situation by itself, challenging growth and possibly evolution of antibiotic resistance [226]. No mutation was found in wild-type cells grown at high concentrations of amoxicillin. In contrast to previous findings using mineral medium [196, 221], no *ampC* promoter mutation was found when cells were grown in complex medium at amoxicillin concentrations higher than 20 µg/ml. A considerable number of studies have already observed the coexistence of sensitive, intermediate-resistant and highly resistant cells growing within a clonal
Role of stress, ROS and SOS response in de novo acquired resistance

population [113, 115]. In biofilm matrices, for example, overproduction of chromosomal β-lactamases degraded penicillin like antibiotics before they even reached the sensitive cells located in the biofilm center [123, 124]. Thus, complex medium could increase variations within a clonal population and protect a population’s sub-fraction by altruism.

Comparing the expression and mutational profiles between wild-type and antibiotic resistant cells does not represent the full picture of changes a cell has to undergo during the acquisition of resistance. Resistance to fluoroquinolones is generally associated with mutations affecting the DNA gyrase [221, 227, 228]. Furthermore, up-regulation of efflux mechanisms has been shown to increase the MIC at least 4-fold [227]. Previously, we compared the transcriptomic profile of wild-type with that of cells made resistant to enrofloxacin. A strong down regulation (10-fold) of ompF and induction of soxS (65-fold) was observed in resistant cells [221]. The single ompF deletion mutant, however, showed no advantage in response to increasing enrofloxacin concentrations, even though ΔompF was associated with increased fluoroquinolone resistance in several previous studies [227, 229, 230]. However, fluoroquinolones can enter cells via multiple porins (OmpA, OmpC and OmpF) as well as non-porin pathways directly passing through the lipid bilayer [230]. Based on that, Chapman and Georgopapadakou proposed that the total uptake of fluoroquinolones is not limited to porins, but influenced by the hydrophobicity of the quinolone [230]. Thus, deletion of only one porin does not entirely protect the cell and hence does not change the adaptation rate, but might play an important but not yet identified role in the concerted response of adaptation to antibiotics.

In contrast to the ΔompF mutant, deletion of soxS and gadE slowed down the adaptation process, even though gadE was not found to be differentially expressed in enrofloxacin adapted cells. Deletion of soxS, however, did alter the process of adaptation to amoxicillin and enrofloxacin, although soxS was not differentially expressed in amoxicillin resistant cells. Overall, the process of adaptation to antibiotics seems far more complex and dependent on the combined response of regulatory networks than changing expression of single genes encoding for porins or multidrug efflux pumps.
The production of reactive oxygen species (ROS) has been proposed as an essential step in the killing by bactericidal antibiotics, such as amoxicillin and enrofloxacin [152]. Interestingly, induction of resistance to amoxicillin and enrofloxacin was slowed down in ΔsoxS mutants, indicating that cells were hampered by the increased production of superoxide due to exposure to bactericidal antibiotics. In contrast, acquisition of tetracycline resistance (bacteriostatic) was not affected by the deletion of soxS. Kohanski et al. [153] proposed that different classes of bactericidal antibiotics generate varying levels of ROS regardless of their drug-target interactions. The observations made in this study are in line with the radical based theory and support the concept of ROS production in cells that are exposed to bactericidal antibiotics. Thus, understanding of underlying antibiotic actions and fundamental cellular mechanisms in response to antibiotic exposure is needed for a broader understanding of the evolution of antibiotic resistance.

Resistance was mainly induced under optimized conditions in this and also in previous studies [88, 130, 131, 161, 231]. Applying a second stressor, such as a non-optimal pH or increased NaCl concentration has a strong effect on the adaptation rate. For both antibiotics, amoxicillin and enrofloxacin, adaptation rates clearly slowed down above 16 µg/ml indicating that growth in the presence of two stressors decreases the adaptation rate. Thus, adaptation to the presence of one antibiotic occurs fast and comes at a cost of the ecological range as shown previously [196]. But cells that are challenged by two stressors pay by slowing down the adaptation rate. The source of the second stressor might be important as well. Previously it was shown that E. coli made resistant to amoxicillin showed a decreased ecological range with lower maximum growth rates at a pH of 6 or 2% NaCl compared to the wild-type [196]. The search for combination of stressors that slow down the evolution of resistance could therefore present a useful strategy to combat evolution of resistance.

In addition to differences in adaptation rates under different conditions, genetic alterations deviated from those found under optimal culture conditions. Acquisition of enrofloxacin resistance was accompanied by mutations in gyrA already at the lowest concentration of 0.125 µg/ml in 7 biological replicates [221]. Changing environmental conditions resulted in a delay of the first gyrA and parC mutation (table 6). The same effect was also observed in the recA
deletion mutant (table 5). Acidic conditions are generally associated with the induction of oxidative damage related genes [232]. However, adaptation to acidic conditions for 1 week reduced soxS expression 3-fold. Moreover, the adaptation to acidic and altered salt conditions lowered expression of umuD, which depends on RecA activation, at least 4-fold (figure 6). This could have created a genetic background similar to the recA deletion mutant and therefore caused the delayed genetic change in gyrA and parC. Thus, delayed mutations found in cultures that were adapted to enrofloxacin in non-optimized conditions are in agreement with results obtained in the recA deletion mutant and hence illustrate the importance of the SOS response in the acquisition of antibiotic resistance. Because of the crucial role in modulating the adaptation, mutation [95] and horizontal transfer rate [197], inhibition of the SOS response could prolong the efficacy of current fluoroquinolone antibiotics and provide an alternative to combat the evolution of bacterial resistance to antibiotics.
Chapter 5

Factors that affect the transfer of a β-lactam resistance conferring plasmid

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\textsuperscript{a}University of Amsterdam, Laboratory for Molecular Biology and Microbial Food Safety, Swammerdam Institute of Life Sciences, Amsterdam, The Netherlands; \textsuperscript{b}MicroArray Department and Integrative Bioinformatics Unit, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands; \textsuperscript{c}Netherlands Food and Consumer Product Safety Authority, Office for Risk Assessment, Utrecht, The Netherlands

This chapter has been submitted
Abstract

The spread of antibiotic resistant bacteria worldwide presents a major health threat to human health care that results in therapy failure and increasing costs. The transfer of resistance conferring plasmids by conjugation is a major route by which resistance genes disseminate at the intra- and interspecies level. High similarities between resistance genes identified in foodborne and hospital-acquired pathogens suggest transmission of resistance conferring and transferrable mobile elements through the food chain. To identify factors that drive the spread of resistance genes an extended-spectrum β-lactamase (ESBL)-carrying *E. coli* isolate from chicken meat was selected as plasmid donor for this study. Sequencing analysis of the isolated plasmid revealed the existence of a CTX-M-1 harboring IncI1 plasmid that confers high levels of resistance to β-lactam antibiotics. Transfer experiments with the plasmid carrying donor cells and the β-lactam sensitive *E. coli* MG1655 lab strain demonstrated that increase in cell density, energy availability and growth rate result in elevated plasmid transfer efficiency. Raising the antibiotic concentrations above the minimum inhibitory concentration (MIC) resulted on the one hand in reduced transfer rates, but on the other hand selected for the resistant and plasmid carrying *E. coli* food-isolate. Based on the mutational pattern of transconjugant cells, a common mechanism is proposed which compensates for fitness costs due to plasmid carriage by shutting down unnecessary cell functions. Reducing potential fitness costs due to maintenance and expression of the plasmid could contribute to persistence of resistance genes in the environment even without antibiotic pressure. Taken together, the results discern factors that drive the spread and persistence of resistance conferring plasmids in natural occurring isolates and shows how these contribute to transmission of resistance genes through the food chain.
Introduction

Bacteria can become resistant against antibiotics by phenotypic adaptation, genetic changes or uptake and incorporation of resistance genes. Resistance conferring plasmids (R-plasmid) play an important role in the dissemination of antimicrobial resistance genes enabling the latter mechanism [7, 233, 234]. Transfer of R-plasmids through conjugation dramatically enhances the spread of antibiotic resistant bacteria. This in turn causes a range of problems, such as increased treatment costs and a lack of effective components against multidrug resistant pathogenic bacteria [7]. Molecular comparison between resistant *E. coli* isolates from poultry and humans revealed a high proportion of identical plasmid sequences [235], suggesting transmission of extended-spectrum β-lactamase (ESBL) genes through the food chain. Livestock-associated methicillin-resistant *S. aureus* transferred directly from animals to farm workers or close relatives [236, 237]. Thus, transfer of R-plasmids from agricultural bacteria to human pathogens creates a potential link between selection for resistance at farms and clinical cases involving resistant strains.

Antibiotics belonging to the β-lactam class are among the most important antimicrobials used in veterinary and human medicine. Resistance to β-lactams is caused by the production of antibiotic inactivating enzymes, named β-lactamases [238]. So far, more than 150 β-lactamases have been reported in many different genera of *Enterobacteriaceae* and *P. aeruginosa* and new β-lactamases continue to emerge worldwide [239]. The spread of ESBL enzymes that are also able to hydrolyze cephalosporins, such as ceftazidime and cefotaxime [240, 241] contributes to the overall increase in resistance. Plasmid-mediated ESBLs most commonly belong to the TEM and SHV gene families often found among *Enterobacteriaceae* [242, 243], but since their discovery in 1986 a dramatic increase in CTX-M enzymes, which exhibit a higher β-lactamase activity against cefotaxime, has been reported [244, 245]. At least 109 CTX-M variants have been identified, whereby CTX-M-15 and CTX-M-14 are the most dominant enzymes [246].

Spreading of resistance genes involves two steps: the successful incorporation of the gene and the subsequent selection for resistance. This study focuses on the first step, as less information about it is available. Although the worldwide distribution of various ESBLs and a large variety of other plasmids
is reported, factors that drive the transfer of R-plasmids have rarely been described. It is generally assumed that sub-lethal antibiotic concentrations in the environment increase the transfer of R-plasmids via the induction of the SOS response [197, 247]. In contrast, the occurrence of plasmid transfer events without any antibiotic pressure have also been reported [248, 249]. As transfer of resistance genes from the agricultural sector to human health care is a major hazard, we used an ESBL-carrying *E. coli* isolate from chicken meat as plasmid donor to study factors that drive the transfer of ESBL-plasmids. Analysis of the unknown plasmid revealed homology to a recently sequenced IncI1 plasmid carrying a β-lactamase of the CTX-M-1 type that has been identified in natural isolates of *E. coli* [250]. Cell density, availability of an energy source, growth rate and antibiotic pressure affected transfer rates between donor and acceptor cells. Furthermore, fitness costs of carrying the plasmid and compensatory mutations were studied to evaluate the possibility of persistence in the environment.

**Material and Methods**

*Bacterial strains, growth media and antibiotics*

Throughout this study a β-lactam sensitive, but chloramphenicol resistant (chlorR) *E. coli* MG1655 YFP (kindly provided by MB Elowitz) [251] was used as acceptor and a β-lactam resistant ESBL carrying *E. coli* isolated from chicken meat (ESBL242, kindly provided by B Wit of the Netherlands Food and Consumer Product Safety Authority) functioned as donor. Batch cultures of both strains were grown at 37°C in a phosphate buffered (100 mM total NaH2PO4 and Na2HPO4, pH 6.9) and defined minimal medium containing 55 mM glucose [154]. For continuous cultures, the glucose level was reduced to 5 mM and the Na2H2PO4 concentration to 10 mM. Media were autoclaved for 20 min at 120°C, with the exception of glucose (10 min at 110°C). Precultures were grown in shake flasks overnight and shaken at 200 rpm at 37°C for inoculation of 96-well plates and batch and continuous cultures. Continuous cultures were performed in a Sixfors (Infors AG, Bottmingen, Switzerland) fermenter vessel with a working volume of 250 ml. Temperature and stirrer rate were kept constant at 37°C and 250 rpm, respectively. The pH was controlled.
Transfer of a resistance conferring plasmid

by automatically pumping sterile 2 N NaOH into the vessel. After starting the continuous cultures in batch modus for 24 hours, the culture was switched to continuous mode by activating the feed and waste pumps. Temperature, pH and stirrer rate were recorded by the controller in the Sixfors fermentation unit. Both acceptor and donor strains were grown separately without antibiotics, at the same growth rate. Growth conditions were maintained constant throughout experiments. When culture parameters, including OD and cell counts, remained constant for 5 to 7 volume changes, acceptor and donor cells were mixed in a ratio of 1:1 in a third sterile empty vessel. The dilution rate (D), which at steady state equals µ, specific growth rate, of each individual and the subsequent mixed culture was identical. 5 mL of the culture was sampled periodically for 48 hours. The total numbers and ratio of acceptor, donor and transconjugant cells in the vessel were determined by plating an appropriate dilution of the culture on antibiotic selective agar plates. The threshold for detection of transconjugant cells was 10 per mL in the total incubation.

For transfer of resistance conferring genes in batch cultures, acceptor and donor cells were grown separately in shake flasks. If not stated otherwise, cells were mixed in a ratio of 1:1 to an initial OD$_{600}$ of 0.01. Mating of acceptor and donor cells on LB agar plates can be excluded as no transconjugants were observed when strains were cultured under conditions that did not allow plasmid transfer and subsequently plated on LB plates. Maximum specific growth rates of acceptor, donor and transconjugants were measured by inoculating overnight grown cells in 20 ml fresh Evans media to an OD$_{600}$ of 0.1 and following growth at 37°C and 200 rpm. Growth rates were calculated from data obtained in log phase and are reported as averages of two independent replicates.

**MIC measurement**

The MIC values were measured by following growth in 96 well plates as described previously [161], using duplicate serial dilutions of a factor of 2, ranging from 0.5 to 1024 μg/ml of the antibiotic. Additionally, two wells were used to document growth in the absence of antibiotics. Cells were inoculated to an OD$_{600}$ of 0.05. Growth was followed over time for 23 h in a microtiter plate reader, measuring the optical density at 595 nm every 10 min, with shaking in between. The MIC was defined as the lowest concentration of antibiotic that
reduced the growth to an OD$_{595}$ of 0.2 or less after 23 hours. A Thermo Scientific Multiskan FC with SkanIt software was used for recording and analyzing the 96-well measurements. Amoxicillin, ampicillin and chloramphenicol stock solutions in a concentration of 10 mg/ml were 0.2-mm filter sterilized and stored at 4°C prior to use.

**Measurement of β-lactamase activity**

The β-lactamase activity was determined by using the chromogenic substrate nitrocefin according to an adapted protocol of O’Callaghan and coworkers [162]. Cells of an overnight grown culture were inoculated in fresh media and grown to an OD$_{600}$ of 1.0. After harvesting and washing 1 ml of the culture with 100 mM sodium phosphate buffer (pH 7.0), cells were lysed using the same buffer containing 1% Triton X-100. Cell extracts were clarified by centrifugation for 1 min at 12,000 rpm. Lactamase activity was determined by measuring the rate of nitrocefin hydrolysis (final assay concentration 100 mM) at 390 nm at 30°C in 100 mM sodium phosphate buffer (pH 7.0) using the BMG Fluostar Optima plate reader. Enzyme activity was normalized to the protein concentration determined with the Thermo Scientific Pierce Micro BCA Protein Assay Kit. Specific β-lactamase activities are presented as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.

**Isolation of genomic and plasmid DNA**

Genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen) according to the manufactures instructions for Gram-negative bacteria. 0.5 mL of an overnight grown culture was used as starter material. To avoid co-purification of RNA, 4 µl of RNase (stock 180 mg/ml) was added to the sample after cell lysis. For plasmid isolation the Qiagen Plasmid Maxi Kit was used according to the manufacturer’s instructions. Plasmids with a size of 1 kb and >10 kb were separated by DNA extraction from agarose gels and subsequent purification using the QIAquick Gel Extraction Kit. The amount and quality of DNA was measured on the NanoDrop ND-1000 (Thermo Scientific) and verified on a 1% agarose gel.
Transfer of a resistance conferring plasmid

Next-generation sequencing
gDNA libraries were generated according to the manufacturers’ protocols using the Ion Xpress™ Plus gDNA Fragment Library Preparations (Life Technologies). Shearing of 100 ng gDNA was performed using the Covaris® M220 Focused-ultrasonicator following the 200-bp protocol provided by Life Technologies. Bar-coded libraries were prepared using the Ion Plus fragment library kit (Life Technologies) and the Ion Xpress DNA bar coding kit (Life Technologies) according to the 200-base-read Ion Proton libraries instructions of the manufacturer. The size distribution and yield of the barcoded libraries were assessed using the 2200 Tapestation System with Agilent High Sensitivity D1000 ScreenTapes (Agilent Technologies). Sequencing templates were prepared using the Ion PI Template OT2 200 Kit v3 on an Ion OneTouch 2 system and enriched on an Ion OneTouch ES system (Life Technologies). Sequencing was performed on the Ion Proton system using the Ion PI Chip v2 and the Ion PI Sequencing 200 kit v3 (Life Technologies) according to the manufacturers’ protocols.

Data analysis
The FASTQ files were subjected to a quality control procedure, using in-house software and fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Tmap [252] was used to map all accepted reads in all samples onto the E. coli reference genome (str. K-12 substr. MG1655, complete genome; NC_000913.3). The plasmids were identified by de novo assembly of the reads using CLC Genomics Workbench (http://www.clcbio.com/). The 1 kb plasmid sample contained only a small number of reads (<1%) that mapped on the reference genome, and all reads were used for de-novo assembly of the plasmid. The assembly resulted in several potential plasmid sequences. The reads from the 1 kb plasmid sample were mapped back onto these potential sequences using Tmap, to determine which of these sequences was most likely present in the sample in high abundances. The >10 kb plasmid sample contained a large number of reads (>1%) that mapped on the reference genome, and only those reads not mapping on the genome were used for de novo assembly of the plasmid using CLC Genomics Workbench. The assembly resulted in several potential plasmid sequences with interpretable BLAST hits in the NCBI
databases [253]. The reads from the >10 kb plasmid sample were mapped onto these BLAST hits to determine if these sequences were likely to be present in the sample in high abundances. Finally, single nucleotide variants, insertions and deletions were identified in the experimental *E. coli* samples compared to the reference genome using the Genome Analysis Toolkit (GATK; [254]), with BWA replaced by Tmap.

**Results**

*Identification of plasmids in ESBL242 cells*

A β-lactam resistant *E. coli* strain was used throughout that was isolated from chicken meat and had a MIC for ampicillin >1024 µg/ml (table 1). Isolation and sequencing of the plasmids it contained revealed one small plasmid with a length of 1554 bp. This small element showed 99.7% identity with a previously identified mobile element pJJ1886-1 (Accession number CP006784) which does not confer antibiotic resistance according to ResFinder 1.4 [255, 256]. The second plasmid was identified as pESBL-283 (Accession number CP008736) with a size of 110137 bp. This plasmid belongs to IncI1 family and harbors a CTX-M-1 β-lactamase and plasmid addiction systems [250].

**Table 1.** Minimum inhibitory concentration (MIC), maximum growth rate (µ\text{max}) and β-lactamase activity of acceptor, donor and transconjugant cells.

<table>
<thead>
<tr>
<th></th>
<th><em>E. coli</em> MG1655/YFP</th>
<th>ESBL242</th>
<th>Transconjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC for ampicillin [µg/ml]</td>
<td>2-4</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
</tr>
<tr>
<td>µ\text{max} [h⁻¹]</td>
<td>0.71 ± 0.01</td>
<td>0.82 ± 0.01</td>
<td>0.67 ± 0.03\textsuperscript{a}</td>
</tr>
<tr>
<td>β-lactamase activity\textsuperscript{b}</td>
<td>21.6 ± 2.9</td>
<td>649.1 ± 3.0</td>
<td>704 ± 226.8\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Average µ\text{max} of three transconjugants randomly chosen from three different transfer experiments. All individual transconjugants were grown in two replicates.

\textsuperscript{b}Specific activity is reported in nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. The results are presented as the means and standard deviations of two independent measurements.

\textsuperscript{c}β-lactamase activity was averaged from transconjugants obtained in two individual experiments.
Transfer of the resistance conferring plasmid

Initially the energy dependence of plasmid transfer and the effects of cell density and incubation time were examined. In mineral medium without antibiotics, transfer from the ESBL242 donor (ampR, amoxR) to the *E. coli* MG1655 acceptor (chlorR) occurred at high rates, but only at cell densities higher than $3 \times 10^5$ cell/ml (figure 1). No transfer was observed below a total cell density of $2.85 \times 10^5$ cells/ml, when mixing donor and acceptor cells in a ratio of 1:1 and incubating for 1 hour. Increased cell densities resulted in higher transfer with a maximum of $74.6 \times 10^3$ transconjugants/ml at a total cell density of $7.2 \times 10^7$ cells/ml.

In minimal medium that lacks glucose and antibiotics, no transconjugant cells were detected when donor and acceptor cells were mixed at a ratio of 1:1 in a range of $1 \times 10^5$ cells/ml to $7.0 \times 10^7$ cells/ml. This suggests that the transfer, incorporation and activation of the resistance conferring plasmid strongly depends on the availability of an energy source. Transconjugants obtained in three different experiments were tested regarding the resistance pattern and growth rate. All tested transconjugants showed a high MIC for chloramphenicol.
and ampicillin. The growth rate of transconjugant cells was comparable to that of the acceptor strain. The high β-lactamase activity indicates that carrying and expressing the ESBL-plasmid had no major impact on the fitness of the host (table 1).

To study the effect of antibiotic concentration on plasmid transfer, acceptor and donor cells were mixed in a ratio of 1:1 and growth of each fraction and the emergence of transconjugants were followed over time by plating small samples on antibiotic selective agar. The highest plasmid transfer rate was observed in the absence of any antibiotics (figure 2A-D). The number of transconjugants and transfer rates obtained in the presence of ampicillin and amoxicillin were very similar (figure 3). To examine the ability of transconjugant cells to function as donor of the ESBL-plasmid, acceptor and transconjugant were mixed in a ratio of 1:1. A transfer efficiency of 84 out of 100 cells indicated that the vast majority of acceptor cells had picked up the ESBL-plasmid. It must be kept in mind that transconjugants that emerged early in the experiment may have contributed to the rise in number of transconjugants at the end of the experiment. Exposing the mixed culture of ESBL242 donor and E. coli acceptor cells to 4 µg/ml ampicillin, corresponding to the MIC of the acceptor strain, resulted in a decrease of transconjugants/ml of about 100 fold (figure 2A and 3). The acceptor cell number increased slower during the first 4 hours of co-culturing with 4 µg/ml ampicillin than in the absence of antibiotics, contributing to the observed lower number of transconjugants. With ampicillin concentrations of 50 and 512 µg/ml this effect becomes even more obvious, as can be seen in the strong decrease of evolved transconjugants (figures 2C-D and 3).
**Figure 2.** Transfer of pESBL-283 from ESBL242 (donor, amp$^R$) to *E. coli* MG1655 (acceptor, chlor$^R$) as a function of time during co-culture in a ratio of 1:1 with (a) 0 µg/ml, (b) 4 µg/ml, (c) 32 µg/ml and (d) 1024 µg/ml ampicillin.

**Figure 3.** Number of evolved transconjugants/ml after co-culturing ESBL242 (donor, amp$^R$) and *E. coli* MG1655 (acceptor, chlor$^R$) for 24 hours. Cells were mixed in a ratio of 1:1 with varying antibiotic concentrations.
Continuous cultures with glucose as the rate-limiting substrate were used to simulate the more natural conditions of a sub-maximal growth rate and limited energy levels. Transfer experiments in chemostats at dilution rates (D) of 0.2 and 0.4 h⁻¹, corresponding to approximately 0.3 and 0.6 times the maximum growth rate of *E. coli* respectively, also showed decreasing transconjugants/ml at high antibiotic concentration (figure 4). Increasing the growth rate resulted in a higher number of transconjugants/ml and thus higher transfer efficiencies (figure 5). In agreement with results obtained in batch culture, ESBL-plasmids were picked up by almost every available acceptor cell, regardless of the antibiotic concentration (figure 4). The observed higher biomass yield of the ESBL242 donor strain might have caused the reduction of acceptor and transconjugant cells over a long period of continuous growth (0.066 g dry weight mm⁻¹ glucose for acceptor versus 0.094 g dry weight mm⁻¹ glucose for donor cells at D=0.2 h⁻¹). Therefore the total number of transconjugants/ml after 6 hours seems higher compared to 24 hours (figure 4 and 5).
Figure 4. Transfer of pESBL-283 from ESBL242 (donor, amp<sup>R</sup>) to *E. coli* MG1655 (acceptor, chlor<sup>R</sup>). After donor and acceptor cells reached steady states in separate chemostats, cultures were mixed in a ratio of 1:1 in an empty reactor vessel and immediately supplied with fresh medium at the same dilution rate. Experimental conditions in each of the panels: (a) 0 µg/ml at D=0.2 h<sup>-1</sup>, (b) 512 µg/ml ampicillin at D=0.2 h<sup>-1</sup>, (c) 0 µg/ml at D=0.4 h<sup>-1</sup> and (d) 512 µg/ml ampicillin at D=0.4 h<sup>-1</sup>.

Figure 5. Number of transconjugants during continuous cultivation of ESBL242 (donor) and *E. coli* MG1655 (acceptor) cells at t=6h or 24h. *E. coli* MG1655 and ESBL242 cells were cultivated separately and continuously at a dilution rate of D=0.2 or 0.4 h<sup>-1</sup> and subsequently mixed with a ratio of 1:1 (t=0h).
Because of the global spread of ESBL carrying Enterobacteriaceae, β-lactam antibiotics are used often in combination with β-lactamase inhibitors, such as sulbactam or clavulanic acid, to restore antibiotic sensitivity. Generally, β-lactams and inhibitors are administered in a ratio of 2:1. Clavulanic acid itself had only little microbial effect on the ESBL242 growth, but when grown with amoxicillin and clavulanic acid the MIC was reduced to 16 and 8 μg/ml, respectively. Co-incubation in the presence of half the MIC concentration, 8 μg/ml amoxicillin in combination with 4 μg/ml clavulanic acid immediately yielded transconjugants, and affected outgrowth of both strains during the first 6 hours (figure 6). Based on the high β-lactam activity measured in donor cells, this period of time could correspond to the time needed for antibiotic degradation by non-inhibited β-lactamases. The number of transconjugants/ml determined after 24 h were similar to co-incubations in the absence of antibiotics (figure 3 and 6).

Figure 6. Transfer of pESBL-283 from ESBL242 (donor, ampR) to E. coli MG1655 (acceptor, chlorR) in a ratio of 1:1 with 8 μg/ml amoxicillin and 4 μg/ml clavulanic acid.
**Genetic changes found in selected transconjugants**

Transconjugants, evolved in either batch or continuous cultures were selected and sequenced in order to identify compensatory mutations in response to plasmid carriage (table 2 and 3). As to be expected, the resistance conferring plasmid pESBL-283 was recovered in the ESBL242 strain and all transconjugants. In contrast, the small plasmid with a size of 1554 bp was only found in some transconjugants independent of antibiotic use or culture condition (table 2). This class of small cryptic plasmids was described earlier as selfish DNA that persists simply due to its ability to replicate and to its stability based on high copy number [257].

**Table 2.** Plasmids found in the acceptor, donor and selected transconjugants. Plasmids of ESBL242 were isolated, separated and identified by sequencing. Transconjugants that evolved in different experiments were selected and sequenced.

<table>
<thead>
<tr>
<th>Sample</th>
<th>pJJ1886-1(^a)</th>
<th>pESBL-283(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli MG1655 (chlor(^R))</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ESBL242 (amp(^R))</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>D=0.4h(^{-1}) 0 µg/ml</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>D=0.4h(^{-1}) 0 µg /ml</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>D=0.4h(^{-1}) 50 µg /ml</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>D=0.4h(^{-1}) 512 µg /ml</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>D=0.4h(^{-1}) 512 µg /ml</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Batch 0 µg /ml t=0h</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Batch 0 µg /ml t=24h</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>Batch 50 µg /ml t=24h</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Batch 512 µg /ml t=24h</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Batch Amx/Clav t=24h</td>
<td>-</td>
<td>X</td>
</tr>
</tbody>
</table>

\(^a\) Accession number CP006784  
\(^b\) Accession number CP008736
Table 3. Mutations found in the genome of selected transconjugant cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Variant</th>
<th>Position</th>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>D=0.4h⁻¹</td>
<td>SNP</td>
<td>2 nucleotides downstream of galS C -&gt; A</td>
<td>rpoD</td>
<td>RNA polymerase, sigma 70 (sigma D) factor</td>
</tr>
<tr>
<td>0 µg/ml</td>
<td>SNP</td>
<td>AA 103 R -&gt; C (CGT/TGT)</td>
<td>malT</td>
<td>MalT transcriptional activator</td>
</tr>
<tr>
<td></td>
<td>SNP</td>
<td>AA 243 L -&gt; Q (CTG/CAG)</td>
<td>fliN</td>
<td>flagellar motor switch protein FliN</td>
</tr>
<tr>
<td>DEL</td>
<td>AA 81 CTG/C-G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D=0.4h⁻¹</td>
<td>SNP</td>
<td>2 and 3 nucleotides downstream of galS GC -&gt; AA</td>
<td>rpoD</td>
<td>RNA polymerase, sigma 70 (sigma D) factor</td>
</tr>
<tr>
<td>0 µg/ml</td>
<td>SNP</td>
<td>AA 103 R -&gt; C (CGT/TGT)</td>
<td>malT</td>
<td>MalT transcriptional activator</td>
</tr>
<tr>
<td></td>
<td>SNP</td>
<td>AA 243 L -&gt; Q (CTG/CAG)</td>
<td>fliN</td>
<td>flagellar motor switch protein FliN</td>
</tr>
<tr>
<td>DEL</td>
<td>AA 81 (CTG/C-G)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D=0.4h⁻¹</td>
<td>SNP</td>
<td>23 nucleotides downstream of yfjR</td>
<td>fimH</td>
<td>minor fimbrial subunit, D-mannose specific adhesion</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>SNP</td>
<td>AA 77 V -&gt; A (GTC/GCC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D=0.4h⁻¹</td>
<td>SNP</td>
<td>AA 334 K -&gt; I (AAA/ATA)</td>
<td>rpoC</td>
<td>RNA polymerase, β' subunit</td>
</tr>
<tr>
<td>512 µg/ml</td>
<td>SNP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D=0.4h⁻¹</td>
<td>INS</td>
<td>36 nucleotides after AA 300</td>
<td>rpoC</td>
<td>RNA polymerase, β' subunit</td>
</tr>
<tr>
<td>512 µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
No consistent mutation was found in all transconjugants sequenced (table 3). In total 11 different SNPs, one insertion and 4 deletions were found across all 10 transconjugants that have been analyzed. Two replicates from the same experiment showed similar, but not entirely identical outcomes. For example, one transconjugant evolved in continuous culture at D=0.4 h$^{-1}$ showed downstream of galS only one SNP, whereas the second transconjugant replicate colony had one additional SNP at the neighboring nucleotide. During continuous cultivation the highest number of at least four genetic alterations was found in the absence of antibiotic pressure.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Variant</th>
<th>Position</th>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 0 µg/ml</td>
<td>SNP</td>
<td>AA 90 Q -&gt; Stop (CAG/TAG)</td>
<td>fliC</td>
<td>flagellar biosynthesis; flagellin</td>
</tr>
<tr>
<td>t=0h</td>
<td>DEL</td>
<td>2 nucleotides downstream of galS C -&gt; -</td>
<td>Not annotated</td>
<td>-</td>
</tr>
<tr>
<td>Batch 50 µg/ml</td>
<td>DEL</td>
<td>15kb fragment from fliA to otsA</td>
<td></td>
<td>Fragment includes flhDC operon involved in flagellar biosynthesis and assembly</td>
</tr>
<tr>
<td>t=24h</td>
<td>SNP</td>
<td>AA 77 V -&gt; A (GTC/GCC)</td>
<td>fimH</td>
<td>minor fimbrial subunit, D-mannose specific adhesin</td>
</tr>
<tr>
<td></td>
<td>DEL</td>
<td>85 nucleotides downstream of yfjM (CGCACTATG/-)</td>
<td></td>
<td>CP4-57 prophage, antitoxin of the RnlA-RlnB toxin-antitoxin system</td>
</tr>
<tr>
<td>Batch Amx/Cla</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>v t=24h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In transfer experiments with 512 µg/ml only one mutation was identified in two sequenced colonies in the β' subunit of the RNA polymerase rpoC. The transconjugant that has evolved in batch cultures directly after mixing the cells (t = 0h) in the absence of antibiotics showed no additional mutation, whereas the transconjugant from the same experiment but selected after 24 hours had a SNP in the flagellar biosynthesis protein FliC and a deletion 2 nucleotides downstream of galS that has also been identified in continuous cultures.

**Discussion**

The rapid transfer of resistance genes in the absence of antibiotic pressure reported in this study partly explains the global spread of resistance conferring plasmids seen in the agricultural and medical sector [258, 259]. Despite the fact that the probability of random cell-to-cell contact is higher in suspension, conjugation rates in suspension were similar to rates in biofilms [260]. As found before [135], rates of transfer were highest in the absence or with low antibiotic concentrations and increased at higher growth rates. Transfer rates observed in this study, however, were much higher than measured for a plasmid with the size of 95 kb conferring resistance to tetracycline [135]. Transfer of resistance conferring elements strongly depends on the donor cell and on information encoded on the plasmid itself [261]. Besides the CTX-M-1 β-lactamase, the plasmid used in this study carried genes for conjugational transfer proteins and plasmid addiction systems. Once the acceptor cell obtained the pESBL-283 it replicates and maintains the resistance conferring element. We observed in our experiments that no antibiotic pressure was needed to increase the fraction of plasmid carrying cells.

Energy availability and cell density seem to be important factors influencing the efficiency of transfer events. The latter one corresponds to the chance event of cell-to-cell contact and contact time that has been postulated to stimulate transfer events [260, 262]. Conjugation as an energy demanding process requires the availability of an energy source as shown for *E. coli* (this study) and other bacteria [263]. However, in natural systems, such as seawater, nutrient limitation did not seriously impede conjugation, whereby starved cells were able to transfer plasmids as well [25]. The difference in methodology may explain varying outcomes. This study focused on short-term plasmid transfer events that
can occur within one hour, for example during food processing or in the gastrointestinal tract. Studies using natural habitats that are nutrient limited consisted of experiments that measure transfer efficiencies over 3 days [25].

Plasmids are believed to impose fitness costs on the host cell in the absence of the antibiotic [264]. Acquisition of the ESBL harboring plasmid caused only a marginal reduction of the maximum specific growth rate of 6% in transconjugants compared to recipient cells (table 1). Expressing and maintaining the ESBL-plasmid in transconjugant cells resulted in a comparable resistance pattern against ampicillin and similar β-lactamase activity as determined for donor cells. Over evolutionary time fitness costs are ameliorated not only when resistance emerged de novo [196, 221], but also when cells acquire resistance conferring plasmids by horizontal gene transfer [101, 265-267]. Sequencing of selected transconjugants evolved in different experiments revealed no common compensatory mutation across all cells. However, genes involved in the biosynthesis and assembly of flagellar proteins seem to be hotspots for identified genetic alterations (table 3). The transconjugant evolved in batch culture after 24 hours in the presence of 50 µg/ml ampicillin was characterized by the loss of the FlhDC master operon that regulates the synthesis and assembly of flagella [268]. In addition, FlhD participates in the regulation of 29 operons, including genes involved in anaerobic metabolism and the Entner Doudoroff pathway [269]. Loss of flhD allows E. coli to use a wider range of carbon sources compared to the wild-type [268]. Mutations that enhance the metabolic efficiency confer a colonization advantage as it was observed for an E. coli MG1655 strain with a lack of flhD that evolved in a streptomycin-treated mouse intestine [270]. Motility is a phenotype that can be crucial to fitness in many environments entailing high energy demands [268, 271]. Thus, alterations in motility associated genes can decrease the fitness burden and enhance metabolic efficiency at the same time indicating a common strategy to reduce fitness costs.

Based on the observed high transfer rates without any antibiotic pressure and marginal fitness costs that can be compensated for, a picture emerges of well-adapted and easily spreading resistance plasmids. Microbial environments, such as the digestive tract, seem to be the optimal locus for conjugation by providing nutrients and the chance of cell-to-cell contact, even between
different species [272, 273]. Thus, this study illustrates the importance of limiting the use of antibiotics to its minimum to avoid selection for resistant strains, but also the need of adequate treatment and hygiene procedures to prevent transfer of resistance conferring plasmids to antibiotic sensitive cells.
Chapter 6

General discussion
Discussion

Within this thesis, the de novo buildup of resistance to different classes of antibiotics and subsequent cellular consequences are investigated. The conducted research serves to establish a quantitative relationship between drug usage and the acquisition and transmission of resistance. Results obtained regarding molecular and physiological alterations in resistant cells are used to identify common drivers of the acquisition and transfer of resistance that will be discussed below.

1. De novo buildup of antibiotic resistance through mutations

The de novo buildup of resistance to different classes of antibiotics can occur rapidly within 60 to 100 generations under laboratory conditions and success strongly depends on the resistance conferring mutation that is acquired and established within the culture as shown in chapter 2 and 3. No common evolutionary pathway for the different types of antibiotics could be observed that leads to high levels of resistance. The different mutational outcomes indicate a trial and error search for the most optimal set of mutations. The first mutation linked to resistance appears at antibiotic concentrations that activate the SOS response. Absence of the SOS response results in long adaptation plateaus and thus reduced adaptation rates. In fact, cells with a deficient SOS response are not able to induce resistance against enrofloxacin, corroborating the crucial function of the SOS response as major driver of de novo acquired antibiotic resistance on the genomic level.

Sequencing of more than one colony revealed that one culture does not harbor a homogeneous set of mutants. Moreover, recent studies revealed that multiple mutations move synchronously through the culture [89] and diversifying lineages can coexist for many years within one patient [274]. Recently, Hammer et al. (2014) demonstrated that genetically distinct bacterial populations interact with each other through the exchange of metabolites resulting in increased antimicrobial resistance and virulence properties [275]. Thus, diversity within a bacterial community can contribute to the ability to tolerate fitness-reducing mutations.

A subset of mutations that were identified in chapter 3 and 4 could be ascribed to confer only low level resistance. For example, genetic alterations
that are known from the literature to confer only moderate resistance to antibiotics, such as the -11 C -> T alteration in the *ampC* promoter [198], have only been identified at low concentrations of amoxicillin. The moderate increase in promoter strength most likely resulted in lower fitness burdens compared to other mutations inducing promoter strength to a higher level. The antibiotic concentration applied limits the set of mutations that is selected for and hence the metabolic costs. Taken together, the data suggests that *de novo* acquisition of resistance is mainly brought by antibiotic induced mutagenesis and the cost of resistance conferring mutations determines the mutant(s) that get(s) established within a culture.

The medium composition turned out to be crucial as well. Using complex LB medium instead of mineral medium resulted in a higher adaptation rate for amoxicillin. Thus, nutrient rich environments, such as fertilized soil, human blood or the gastrointestinal tract can present reservoirs that enhance the possibility of the *de novo* buildup of resistance in the presence of sub-lethal antibiotic concentrations.

2. **The costs of *de novo* acquired resistance**

Acquisition of antibiotic resistance through mutation or phenotypic adaptation is believed to impose a fitness cost compared to the ancestor in the absence of the drug [98, 151]. The initial response to sub-lethal concentrations of antibiotics causes a broad alteration of the transcriptomic profile in wild-type cells. To cope with increased antibiotic concentrations, cells need to invest energy in order to overexpress drug efflux pumps or antibiotic degrading enzymes which results in a metabolic burden. Exposing bacteria to increasing sub-lethal concentrations of antimicrobials allows the cell to adapt to new environments by seemingly permanent and very specific changes in gene expression. This change of response on the transcriptomic level starting with a broad alteration and ending in a changed expression of single genes that contribute to resistance is crucial for the current understanding of mechanisms for the compensation of fitness costs. The current notion is that during the process of adaptation, bacterial cells seem to specifically rewire cellular networks in order to compensate for metabolic burdens. In agreement with the outcome of chapter 2 and 3, the build-up of resistance requires the concerted response of elements
and regulatory networks that play a primary role in microbial physiology, namely the electron transport chain, the metabolism of amino acids, fatty acids or nucleotides, motility and the intrinsic stress response [151, 276, 277].

Mostly, studies focus on comparing growth rates to the wild type as indicator for the biological costs of resistance. But results of chapter 2 indicate that additional cellular strategies can be used to compensate for costly mutations. By reducing the ecological range, the organism can grow as well as the ancestor and by decreasing the expression of at that moment unneeded cellular systems, such as the SOS response in resistant cells, energy can be more efficiently directed towards the maintenance of resistance. Alterations in the transcriptomic profile and in the regulatory network structure of resistant pathogens have been linked to fundamental phenotypic differences among microbial species [278, 279], suggesting a variety of strategies for adaptation.

Upon induction of resistance and subsequent removal of the drug, resistance conferring mutations are neither lost nor reversed. Moreover, differential expression of genes was reduced and approached wild-type levels without changing the resistance nor mutational pattern. This suggests that upon reduction of antibiotic usage the fraction of resistant bacteria will decrease depending on the overall biological cost of resistance. As mentioned earlier, diversity within a bacterial community can ensure collective recovery of bacterial fitness comparable to that of the wild-type through the exchange of metabolites resulting in increased antimicrobial resistance and virulence properties [275]. Thus, reduction of antibiotic usage will lower the fraction of resistant bacteria, but they may not be completely eliminated. As soon as antibiotics are applied again, resistant bacteria will immediately spread and overgrow sensitive microbes. Thus, by finding common weak spots of resistant bacteria, such as a reduced ecological range, strategies can be invented to specifically select for the sensitive variant.

3. The role of reactive oxygen species (ROS) in the induction of resistance

As described above, phenotypic adaptation mainly occurs in two steps: first the cell responds to drug exposure and later optimization of cellular networks takes place to decrease the metabolic burden of resistance. Upon antibiotic exposure of sensitive wild-type cells, broad and rather unspecific alterations of the
transcriptomic profile are observed. For example, exposure of drug sensitive cells to sub-lethal concentrations of enrofloxacin induced expression of genes involved in homeostasis of the cellular membrane and respiration as well as the regulation of cellular stress response (chapter 3). Expression of soxS that is involved in the removal of cell damaging superoxide [280, 281] was not significantly changed in enrofloxacin exposed wild-type cells. Upon induction of resistance, however, soxS was 64-fold upregulated illustrating the before mentioned specific and permanent alterations as part of the cellular phenotypic adaption.

The soxS deletion mutant showed a drastically decreased adaptation rate in response to increasing concentrations of enrofloxacin, but also amoxicillin. The so-called “radical based theory” proposes that bactericidal antibiotics, such as amoxicillin and enrofloxacin, produce reactive oxygen species (ROS) and that these fulfil a central role in cell killing [152, 153]. Thus, the role of soxS is in line with this radical based theory and is consistent with the existence of a central mechanism underlying bactericidal drugs belonging to different antibiotic classes.

In line with the above, bactericidal antibiotics alter cellular respiration and induce noxious levels of intracellular hydrogen peroxide triggering a distinct oxidative damage response [282]. Dwyer et al. (2014) also found that bactericidal antibiotics elevate oxygen consumption suggesting alterations to bacterial redox physiology. To counteract elevated ROS formation due to increased cellular respiration, resistant cells may use alternative strategies to convert glucose to energy and biomass. In amoxicillin resistant cells, studied in chapter 2, no induction of genes coding for ROS scavenging enzymes is observed. The increased expression of alternative electron acceptors, however, suggests a partial metabolic switch from aerobic to anaerobic conditions. Amoxicillin resistant cells showed an induction of the fumarate reductase and the nitrate reductase A that are induced under anaerobic conditions, but are involved in the formation of ROS or reactive nitrogen species (RNS) at the same time [283, 284]. Whether cells made permanently resistant to a range of bactericidal antibiotics show indeed decreased levels of ROS formation needs to be proven.
The impact and role of ROS in drug action and the development of antibiotic resistance is still under discussion. Intermediate levels of ROS derived from antibiotic-induced alterations in metabolism may activate the general stress response and promote mutagenesis. In contrast, high ROS levels cause cell death by damaging DNA, lipids and proteins [285, 286]. Therefore it seems plausible that resistant cells use strategies to combat elevated ROS production, such as the overexpression of ROS scavengers that has been observed in enrofloxacin adapted cells. Another strategy could be to simply shut down respiration. Upon exposure to selected antimicrobials, E. coli and other pathogens can adopt a respiration-deficient state known as a small colony variant (SCV) and thereby become frequently resistant to antimicrobials [287]. Taken together, results obtained in this thesis and other studies indicate that resistant bacteria follow two main strategies to combat elevated ROS production. Firstly, upregulation of ROS scavenging genes can decrease the concentration of ROS inside the cell and/or secondly, a metabolic switch towards anaerobic conditions enables the cell to circumvent ROS mediated killing.

4. Factors that drive the transfer of resistance conferring genes

Resistance genes can be transferred from one cell to another by transformation (uptake of naked DNA), transduction through viruses or conjugation in which DNA is transferred through a pore [288]. The β-lactam resistance conferring plasmid pESBL-283 that encodes for a CTM-X-1 β-lactamase very rapidly transferred from an E. coli donor strain that was isolated from chicken meat to a commonly used E. coli MG1655 lab strain (chapter 5). The genetic information encoded on the plasmid itself contributes to rapid transfer and persistence within the mixed culture. Bacteria invest a considerable amount of effort in keeping themselves free of invading DNA. Restriction-modification systems present the primary defense mechanism against foreign DNA. After cleaving foreign DNA, linear fragments become substrates for more extensive nucleolytic degradation by the RecBCD enzyme that is part of the SOS response [289]. The β-lactam resistance conferring plasmid pESBL-283 encoded on the one hand for an anti-restriction protein and on the other for psiB, a plasmid SOS inhibition protein B,
blocking the hosts defense mechanism and contributing to the persistence of the plasmid within the culture [250].

Recently Gullberg et al. (2014) demonstrated that the maintenance of a 220 kbp plasmid depends on the balance between the cost of plasmid carriage and a selective pressure in the presence of sub-lethal concentrations of antibiotics or heavy metals. By shutting down costly and not necessarily needed cell functions, such as motility, *E. coli* cells are able to compensate for metabolic costs [271]. The naturally occurring pESBL-283 was transferred without any known pressure as the plasmid did not encode for heavy metal resistance. No common genetic alteration in the genomic profile of selected transconjugants could be identified and mutations were mostly located in genes involved in biosynthesis or assembly of flagellar proteins.

In urinary tract infections motility and chemotaxis have been suggested to contribute to virulence by enabling the organism to escape host immune responses [290, 291]. In fact, Lane et al. (2005) showed that non-motile mutants were able to colonize the urinary tract during an independent challenge, but each of the mutants was consistently outcompeted by the wild-type strain [292]. The authors suggest that flagella and flagellum-mediated motility and chemotaxis are expressed transiently to aid in colonization and afterwards repressed to avoid activation by the immune response. Underlining the versatility of stress adaptation strategies, the results of chapter 5 indicate that shutting down energy dependent motility systems could be an important driver for the spread and persistence of resistance conferring plasmids in the environment.

5. **Common drivers of antibiotic resistance in bacteria**

5.1. **Cell density**

Results of this project in conjunction with earlier studies reveal common drivers for the acquisition of resistance through mutations, as well as for phenotypic adaptation and horizontal gene transfer. One major driver is the bacterial density of the culture itself, that seems to be crucial for the transfer of resistance conferring genes and determines the probability of a mutation to become fixed in a culture. Only above a certain cell density threshold that enables the opportunity for cell-to-cell contact, transfer of resistance genes is observed. In
addition, de novo acquisition of resistance by mutation or phenotypic adaptation is influenced by the factor cell density in various ways. Large populations can increase the number of competing beneficial mutations, and hence are expected to increase the intensity of clonal interference [112]. Following this line of reasoning, Tan et al. (2012) showed that individual E. coli cells as part of dense populations resist eradication by considerably higher drug concentrations compared to lower densities [293]. This so called “inoculum effect” is based on the amount of antibiotic per cell. This very specific antibiotic load per cell determines whether individual cells grow or die, resulting in density-dependent metastability that can decide the outcome of an antibiotic treatment. Overall, low cell densities inhibit the emergence and transmission of resistance.

5.2. Nutrient availability

The medium composition has an effect on the de novo acquisition of resistance due to mutations and on transfer of resistance through conjugation. In chapter 4 it is shown that the MIC of E. coli wild type cells is increased for amoxicillin and tetracycline when cultured in complex medium instead of mineral medium. High-level resistance to amoxicillin was observed within 60 generations when cells were cultured in complex medium. In contrast, in order to obtain a comparable resistance phenotype in mineral medium the adaptation phase had to be prolonged to at least 90 generation. Reduction of nutrient availability results in growth arrest. Starved cells and/or not dividing bacteria are partially or completely refractory to killing by antibiotics from most, if not all, major antibiotic classes [150]. Thus, the more nutrients are available under stressful conditions the faster bacteria adapt to their environment and become resistant. Any additional stress in the form of a second stressor, be it reduced nutrient availability or a lowered pH, leads to prolonged adaptation rates. The process of conjugation is also dependent on nutrient availability (chapter 5). If acceptor and plasmid donor cells get starved no transfer of resistance conferring genes is observed, illustrating the importance of energy availability and bacterial growth in the development and spread of antibiotic resistance.
5.3. **SOS response**

As mentioned earlier, results of this thesis and other studies demonstrate the importance of the bacterial SOS response in the acquisition of resistance conferring mutations. SOS deficient mutants show decreased adaptation rates in response to increasing amoxicillin or tetracycline concentrations or even lack the possibility to adapt to certain antibiotics, such as enrofloxacin. Several studies have highlighted the role of the SOS response in conjugative DNA transfer as well [184, 197, 294]. Antibiotics that inhibit DNA replication and cell viability activate the SOS response. This in turn induces mutagenesis and promotes high-frequency horizontal transfer events. Taken together, a lack of the SOS response extends the adaptation phase or prevents the transfer of antibiotic resistance genes [197].

5.4. **Compensation of fitness costs results in the persistence of antibiotic resistance**

Acquisition of antibiotic resistance through mutations, phenotypic adaptations or horizontal gene transfer is supposed to carry a fitness cost in the absence of the antibiotic [98, 100]. Single chromosomal mutations that confer antibiotic resistance and that are introduced in an isogenic bacterial background or occur spontaneously, usually result in a decreased growth rate of the mutant strain [100, 295, 296]. Replication and maintenance of accessory elements, such as resistance conferring plasmids, can also lead to a decreased bacterial fitness [297]. In contrast, results of this thesis show that the cell counteracts initial fitness burdens by introducing compensatory mutations, changing cellular networks or decreasing the amount of energy channeled into at that moment less essential cellular processes. In fact, secondary mutations that compensate for the initial cost of resistance are more common than reversion to the sensitive allele [99]. This in turn leads to the establishment of the resistant mutant within the culture even when the drug is removed afterwards. Thus, the terms fitness cost or metabolic burden of resistance does only account for the initial phase of the acquisition of resistance and can be overcome by cellular mechanisms that enable adaptation to new environments within a short period of time.
6. Concluding remarks

Taken together, the outcome of this project illustrates that a reduction in the antibiotic usage might not result in the immediate disappearance of resistant bacteria that are already found ubiquitous in human and environmental reservoirs. Nevertheless, reducing the environmental antibiotic load to a minimum can decrease the chance for de novo acquisition of resistance and selection for resistant bacteria. When antimicrobial compounds need to be applied, well-calculated drug dosage should prevent the buildup and transmission of resistance genes, for example by a short and intense antibiotic therapy with drug concentrations exceeding the mutant selection window. Since resistant microbes are found ubiquitous nowadays on food [298] or even in public transport systems [299], appropriate hygiene becomes highly important in order to prevent health threatening infections with multi-resistant microorganisms.

To minimize the probability of de novo acquisition of resistance during antibiotic therapy, novel approaches are currently under investigation. The rapid evolution of drug resistance and the lack of new antibiotics is spurring attention towards multidrug combination treatments. Importantly, however, certain drug-drug interactions that show increased growth inhibition compared to each individual drug were found to increase relative selection in favor of resistance [300]. On the other hand, recent studies indicate that the use of drug cycling or alternating antibiotic treatments slow down the evolution of resistance [301, 302]. In general, this study showed that application of a second stressor significantly decreases the adaptation rate. Thus, optimizing current treatment strategies still creates enormous potential to slow down de novo acquired resistance and to gain control over the development and spread of antibiotic resistance in microbes.


References


134


134
References


References


References


References


References


References


Summary/Samenvatting
The worldwide emergence and spread of antibiotic resistant bacteria represent a major threat to human health care as the chance of therapy failure and costs for treatment increase. Bacteria can get resistant through three main mechanisms: 1) phenotypic adaptation (e.g. upregulation of drug efflux pumps), 2) mutations that alter the drug target and/or 3) transfer of resistance conferring genes between bacteria through conjugation. To curb the continuous rise of drug resistant bacteria worldwide, new strategies are urgently needed that counteract the development and spread of resistance. Development of innovative countermeasures depends on a deeper understanding of how sensitive and drug resistant bacteria respond to different drug levels, circumvent metabolic costs and the development of knowledge of the factors that can affect the acquisition of drug resistance. Therefore, the main object of my thesis was to characterize and quantify the relationship between antibiotic usage and development and transmission of resistance using laboratory cultures of *E. coli* as a model. The genetic and physiological changes that were shown in this study to accompany the acquisition of resistance indicate that bacteria are able to acquire resistance and compensate for metabolic costs due to various and highly flexible strategies.

**Chapter 1** describes how overuse and misuse of antibiotics in agriculture and medicine in the past 70 years has caused a dramatic increase of microbial resistance worldwide. In addition, this chapter shows that antibiotics are not only harmful to bacteria, but in fact can induce protection mechanisms at sub-lethal as well as at normally lethal drug concentrations. Molecular and physiological changes in resistant cells compared to their ancestors are complemented by the accumulation of compensatory mutations that minimize metabolic costs of resistance and therefore enable the establishment of antibiotic resistant cells in natural reservoirs.

In **chapter 2** the transcriptomic profile and physiological properties of *E. coli* cells made resistant to amoxicillin are characterized. While no difference in the energy consumption was observed, permanent changes in the transcriptomic profile of resistant cells indicated a change in pH and salt tolerance of resistant cells. Comparing the maximum specific growth rate of sensitive and resistant cells at different pH values or increased salt concentrations showed that resistant cells had a lesser ability to endure environmental stress. Hence, the overall
outcome indicates that the effect of the acquisition of resistance consists not so much of an extra energy requirement, but results more in a reduced ecological range.

Chapter 3 compares the cellular response to antibiotics on the transcriptomic level of sensitive E. coli cells and cells made resistant to one of the three antibiotics belonging to different drug classes: amoxicillin (β-lactam, bactericidal), enrofloxacin (fluoroquinolone, bactericidal) and tetracycline (bacteriostatic). Sensitive wild-type cells respond strongly to sub-lethal antibiotic exposure as illustrated by a high number of differentially regulated genes. Resistant cells on the other hand were characterized by a limited set of permanently up- or down regulated genes even without any antibiotic exposure when compared to the wild-type. Beyond that, drug exposure had no major additional effects on the gene expression in antibiotic resistant cells. When drug resistant cells were further on cultivated in the absence of antibiotics the number of differentially regulated genes in resistant cells reduced to a figure close to that observed in wild-type cells without losing resistance. Acquisition of antibiotic resistance follows flexible evolutionary pathways yielding the same phenotypic outcome but with different resistance conferring mutations. Overall, this study demonstrates that de novo resistance to antibiotics is brought about by a complex interaction of cellular processes, involving both adaptation of expression levels and mutations.

Based on the transcriptomic profile of cells made resistant to amoxicillin, tetracycline and enrofloxacin, respectively (chapter 2 and 3), I selected interesting gene candidates and investigated their role in the acquisition of resistance in chapter 4. The results indicate that the SOS response and global transcriptional activators, such as gadE, play a crucial role in the development of antibiotic resistance. Deletion of soxS, which is involved in the removal of superoxide slowed down the acquisition of resistance to amoxicillin and enrofloxacin but not to the bacteriostatic antibiotic tetracycline. These observations provide additional evidence for the so-called “Radical-based theory”, which states that bactericidal antibiotics produce reactive oxygen species (ROS) that contribute to antibiotic mediated cell killing. Furthermore, I tested whether sub-optimal environmental conditions act as second stressors and as such reduce the rate at which cells become drug resistant. Increased salt
concentrations or changed pH decreased the adaptation rate and could therefore present a useful starting point to develop strategies to combat evolution of resistance.

Chapter 5 concentrates on factors that influence the transfer rate of an antibiotic resistance conferring plasmid through conjugation between bacteria. A food isolate of *E. coli* that contained an Extended Spectrum β-Lactamase (ESBL)-plasmid was used as donor throughout. The resistance conferring plasmid was identified as a CTX-M-1 β-lactamase harboring IncI1 plasmid that confers high levels of resistance to β-lactam antibiotics. Transfer experiments with plasmid carrying *E. coli* donor cells and the β-lactam sensitive *E. coli* MG1655 lab strain as acceptor demonstrated that an increase in cell density, energy availability and growth rate resulted in elevated plasmid transfer efficiencies. Raising the antibiotic concentrations above the minimum inhibitory concentration (MIC) reduced transfer rates on the one hand, but on the other hand selected for the resistant and plasmid-carrying *E. coli* food-isolate. Acquisition and maintenance of resistance conferring plasmids are believed to impose fitness costs on the host cell in the absence of the antibiotic. Transconjugant cells had a marginal but significant reduction of the maximum specific growth rate of 6% compared to their ancestor. In order to study genetic modifications in cells that had picked up the resistance plasmid, the whole genome was sequenced of selected transconjugants and compared to the wild-type. The mutational pattern of transconjugant cells revealed common hotspots for genetic changes in motility associated and energy demanding processes. Thus, a common mechanism for decreasing the metabolic burden due to plasmid carriage and maintenance was proposed that could cause the rapid spread and persistence of resistance conferring plasmids in the environment.

Chapter 6 aims at identification and discussion of common drivers of the acquisition and transfer of resistance in bacteria, such as nutrient availability and the cost of resistance. The previous chapters illustrate that the environmental antibiotic levels should be decreased in order to prevent *de novo* acquisition of resistance in bacteria. The data that we obtained corroborate the need to adhere strictly to medical protocols, many of which recommend short but intense antibiotic therapies. New insights obtained in this thesis could be deployed to optimize current and generate innovative antibacterial treatment
strategies based on the combination of antibiotic and environmental stresses. Such developments should help in gaining control over the development and spread of antibiotic resistance in microbes.
De wereldwijde opkomst en verspreiding van antibioticaresistente bacteriën vormen een serieuze bedreiging voor de gezondheidszorg omdat het risico dat een therapie niet aanslaat steeds groter wordt en bovendien de behandelingskosten sterk toenemen. Bacteriën kunnen op drie manieren resistent worden. (1) Door fenotypische aanpassing, bijv. door het aanmaken van meer effluxpompen. (2) Door genetische mutaties, die leiden tot verminderingde binding van het antibioticum aan het doelwitenzyme, en/of (3) door overdracht van resistentiegenen via bacteriële conjugatie. Om de wereldwijde toename van antibioticaresistente bacteriën tegen te gaan, zijn er dringend nieuwe benaderingswijzen nodig. De ontwikkeling van fundamenteel nieuwe tegenmaatregelen staat of valt met een beter begrip van hoe gevoelige en resistente bacteriën reageren op verschillende antibioticaconcentraties en hoe bacteriën er in slagen de bijkomende metabole kosten van resistentieontwikkeling te minimaliseren. Het vereist ook een dieper inzicht in de factoren, die resistentieverwerving beïnvloeden. Ik heb mij daarom in mijn proefschrift vooral ten doel gesteld om onder gecontroleerde omstandigheden en met de darmbacterie *E. coli* als modelorganisme het verband tussen overmatig antibioticagebruik en de ontwikkeling en overdracht van antibioticaresistentie te karakteriseren en te kwantificeren. De met de verwerving van resistentie gepaard gaande genetische en fysiologische veranderingen, die ik in mijn studie heb geïdentificeerd, wijzen er op dat bacteriën op verschillende manieren antibioticaresistentie kunnen verwerven en bovendien compensatiemechanismen kunnen ontwikkelen om de bijbehorende metabole kosten te minimaliseren.

**Hoofdstuk 1** beschrijft hoe gedurende de afgelopen zeventig jaar overmatig gebruik en misbruik van antibiotica in bijv. de veefokkerij en in de geneeskunde hebben geleid tot een dramatische, wereldwijde toename in bacteriële resistentie. Dit hoofdstuk laat verder zien dat ruim gebruik van antibiotica niet alleen gepaard gaat met de ontwikkeling van resistentiemechanismen, die bacteriën in staat stellen subletale antibioticaconcentraties beter te verdragen, maar ook met de ontwikkeling van resistentiemechanismen, die groei mogelijk maken bij aanvankelijk letale concentraties. Naast moleculaire en fysiologische aanpassingen, die rechtstreeks met resistentieontwikkeling te maken hebben, is er ook sprake van ophoping van een andere klasse van mutaties. Deze helpen
bacteriën om de metabole kosten van de resistentieontwikkeling te minimaliseren, waardoor ze kunnen overleven in natuurlijke reservoirs.

In **Hoofdstuk 2** worden het transcriptieprofiel en de fysiologische eigenschappen gekarakteriseerd van *E. coli* cellen, die resistent zijn geworden tegen amoxicilline. Hoewel gevoelige en resisteerte cellen niet verschillen in hun energieverbruik, traden er blijvende veranderingen in het transcriptieprofiel op, die wijzen op veranderingen in pH-gevoeligheid en zouttolerantie. Vergelijking van de maximale specifieke groeisnelheid van gevoelige en resistente cellen bij verschillende zuurgraden of verhoogde zoutconcentraties toonde aan, dat antibacterieresistente cellen gevoeliger waren voor belastende milieuomstandigheden. Samengevat, wijzen de resultaten er op, dat resistentieverwerving niet zo zeer meer metabole energie kost, maar het ecologische spectrum verkleint.

**Hoofdstuk 3** vergelijkt de transcriptionele respons tegen antibiotica tussen gevoelige cellen en cellen, die resistentie hebben verworven tegen respectievelijk drie antibiotica, die elk behoren tot een verschillende klasse van antibioticum. De gebruikte antibiotica zijn amoxicilline (een β-lactam, bacteriedodend), enrofloxacine (een fluoroquinolone, bacteriedodend) en tetracycline (bacteriestatisch). Gevoelige, wildtype cellen reageren sterk op blootstelling aan subletale antibioticaconcentraties, hetgeen zich uit in een groot aantal differentieel gereguleerde genen. Resistente cellen echter werden gekenmerkt door een beperkt aantal blijvend opgereguleerde of omlaaggereguleerde genen, zelfs in de afwezigheid van een antibioticum, terwijl de expressie van de overige genen slechts beperkte veranderingen vertoonde. Wanneer het antibioticum verwijderd werd, nam het aantal differentieel gereguleerde genen in resistente cellen sterk af zonder dat de resistentie verloren ging. Resistentieverwerving volgt wisselende, evolutionaire wegen, die leiden tot hetzelfde fenotype, maar het uiteindelijke fenotype is gebaseerd op verschillende, resistentieverlenende genetische mutaties. Samengenomen, laten de resultaten van deze studie zien dat *de novo* resistentie tegen antibiotica tot stand komt door een complexe interactie van cellulaire processen, die zowel aanpassingen in genexpressie als genetische mutaties inhouden.

In **Hoofdstuk 4** heb de mogelijke bijdrage van een aantal genen in resistentieverwerving onderzocht. Deze heb ik geselecteerd aan de hand van de
transcriptieprofielen verkregen met cellen, die resistentie hadden verworven tegen respectievelijk amoxicilline, enrofloxacine en tetracycline (zie hoofdstuk 2 en 3). De resultaten wijzen er op, dat de zogenaamde SOS respons en globale transcriptie-activatoren zoals *gadE* een sleutelrol spelen in de ontwikkeling van antibioticaresistentie. Deletie van het *soxS* gen, dat betrokken is bij de verwijdering van het zuurstofradicaal superoxide, vertraagde de resistentieverwerving tegen amoxicilline en enrofloxacine, die beide bacteriedodend zijn, maar niet tegen het bacteriestatische antibioticum tetracycline. Deze waarnemingen leveren aanvullende bewijzen voor de zogenaamde “Radicalentheorie” (Radical-based theory), die zegt dat bacteriedodende antibiotica leiden tot de verhoogde vorming van reactieve zuurstofradicalen (ROS, reactive oxygen species), die bijdragen aan antibiotische celdoding. Ik heb verder uitgezocht of suboptimale omgevingsomstandigheden werken als secundaire stressfactoren en als zodanig de snelheid verminderen waarmee cellen antibioticaresistent worden. Verhoogde zoutconcentraties en veranderingen in de zuurgraad vertraagden inderdaad de aanpassingsnelheid en zouden zodoende een waardevol uitgangspunt kunnen bieden voor de ontwikkeling van nieuwe strategieën om resistentieontwikkeling te bestrijden.

**Hoofdstuk 5** richt zich op factoren, die de snelheid beïnvloeden waarmee resistentieverlenende plasmiden via bacteriële conjugatie worden overgedragen. In deze experimenten werd een voedseldolaat van *E. coli* gebruikt, dat een Extended Spectrum β-Lactamase (ESBL)-plasmide als donor bevatte. Het resistentieverlenende plasmide werd geïdentificeerd als een IncI1 plasmide dat een CTX-M-1 β-lactamase gen bevat en zodoende een hoge mate van bescherming tegen β-lactam antibiotica biedt. Overdrachtsexperimenten met plasmidedragende *E. coli* donor cellen en de β-lactamgevoelige *E. coli* MG1655 laboratoriumstam als acceptor toonden aan dat plasmide overdracht toenam bij een toename in celdichtheid, energiebeschikbaarheid en groeisnelheid. Wanneer de antibioticaconcentratie verhoogd werd tot boven de minimale inhiberende concentratie (MIC), nam de overdrachtssnelheid van het plasmide af, maar tegelijkertijd leidde deze stap tot selectie van de uit het voedsel geïsoleerde *E. coli* stam met het resistentieverlenende plasmide. Het mutatiepatroon van verder geëvolueerde transconjugant cellen onthulde gemeenschappelijke,
voorkeursplaatsen voor bepaalde genetische veranderingen, die processen betroffen die te maken hebben met het bewegingsvermogen en met andere energievereisende processen. Ik stel voor dat een dergelijke gang van zaken zou kunnen dienen om de metabole belasting als gevolg van het aanmaken en onderhouden van het plasmide te verminderen en daarmee bij zou dragen aan de snelle verspreiding en persistentie van resistentieverlenende plasmiden in het milieu.

**Hoofdstuk 6** behelst de identificatie en bespreking van gemeenschappelijke, drijvende factoren achter de verwerving en overdracht van antibioticaresistentie, zoals beschikbaarheid van voedingsstoffen en de metabole kosten van resistentie. De voorafgaande hoofdstukken hebben laten zien dat het antibioticapeil in het milieu moet dalen om *de novo* resistentieverwerving in bacteriën te voorkomen. Als antibiotica noodzakelijk zijn, moet streng worden vast gehouden aan medische protocollen, die korte maar intense antibioticatherapiën voorschrijven. De nieuwe inzichten, die in dit proefschrift verkregen zijn, kunnen worden gebruikt om de huidige behandelingsstrategieën te optimaliseren teneinde de ontwikkeling en verspreiding van antibioticaresistentie in bacteriën onder controle te brengen.
List of publications


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