Optimization of treatment protocols to prevent de novo development of antibiotic resistance in Pseudomonas aeruginosa
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*Yanfang Feng*
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Yanfang Feng

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The cover picture, a chess play between Ms. Yanfang Feng and Mr. *Pseudomonas aeruginosa*, was kindly drawn by QianQian Zhang for this thesis. The idea was adapted from Lei Chen and Yan Liang (http://www.eurekalert.org/multimedia/pub/84598.php). This drawing was used to illustrate the resourceful *Pseudomonas aeruginosa* could rapidly come up with the novel solution to survive whenever a new treatment strategy was applied by human being, just like what was shown by the author in this thesis.

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Chapter 1

General introduction
**Chapter 1**

**Antibiotic resistance increases the risks of infectious diseases**

Antibiotics are antimicrobials that either kill or inhibit the growth of bacteria by interacting with specific targets. Since penicillin was discovered by Alexander Fleming in 1928, a large number of antibiotics has been discovered or synthesized (1, 2). They are characterized by various modes of action, including inhibition of cell wall synthesis, interference with DNA replication or RNA synthesis, as well as disruption of protein synthesis (3). The introduction of antibiotics has revolutionized medicine and saved millions of lives by making previously lethal infectious diseases curable. Since 1930s, millions of tons of antibiotics have been produced and applied in clinical practice. These antibiotics play crucial roles in almost all aspects of modern medicine. Without antibiotics, doctors are unable to perform basic surgeries, cure cancers efficiently, or prevent common infections from becoming life-threatening (1).

The unmitigated success of antibiotics, however, did not last long as bacteria, owing to their remarkable adaptability, are able to acquire resistance towards the therapeutic drug in response to drug exposure (4, 5). This can be well illustrated by the fact that the history of antibiotic discovery is concomitant with the development and spread of resistance in pathogens. Penicillinase, being isolated a few years after penicillin was discovered, was soon prevalent in bacteria that previously did not or rarely produce it (6). Sulfonamide resistance emerged in the strains of *Streptococcus pyogenes* in hospitals at the same time that this antibiotic was used in clinics (7). More and more *Mycobacterium tuberculosis* isolates resisted the therapeutic effects of streptomycin soon after this drug was introduced to treat tuberculosis (8). Similarly, the corresponding resistance has closely followed application of other drugs in clinical practice (4).

Antibiotic resistance is defined as the inability of an antibiotic to effectively kill bacteria or control their growth. The level of resistance of a bacterium to a certain drug is quantified by the minimal inhibitory concentration (MIC) of this drug that prevents visible growth. By comparing the measured MIC with clinical susceptibility breakpoints, the suitability of a specific antibiotic considered for treatment of a pathogen can be predicted. The prevalence of antibiotic resistance has been reported in almost all clinically significant pathogenic species, including *Acinetobacter baumannii*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and species of *Enterobacteria*, *Shigella*, and *Salmonella*, to
a different extend (7, 9-18). With world globalization, resistant strains travel from locations where resistance is acquired to faraway places which were never before confronted with such infections, making the problem a worldwide issue (19). An excellent example is the ESBL Klebsiella pneumoniae that translocated from India to the United Kingdom and Europe in less than 24 hours (20).

The emergence of multidrug resistant (MDR) strains makes the situation even worse. It generally renders the infections more refractory and costly to treat. In the worst case, patients may lose their lives because of the MDR organisms since none of the available antibiotics can clear the infection. The globally notable MDR strains in hospitals and community are Acinetobacter baumannii, Enterococcus faecium, Enterobacter cloacae, Klebsiella pneumoniae, Mycobacterium tuberculosis, Pseudomonas aeruginosa and Staphylococcus aureus (21-28). The MDR bacteria especially found in developing countries include MDR Salmonella enteritidis, Shigella flexneri as well as Vibrio cholera (29).

The economic burden caused by antibiotic resistance is alarming. According to the Centers for Disease Control and Prevention (CDC), antibiotic resistance costs an estimated $55 billion a year and more than 8 million additional days of hospital stay in United States (30). Earlier, the EU commission reported 25,000 deaths and an extra cost of at least 1.5 billion annually as consequence of antibiotic resistance within EU (31). Unlike these economic parameters, the long-term effects of antibiotic resistance to patients and their surroundings are hard to estimate but potentially more harmful (32). The real unaffordable cost, however, is a so called post-antibiotic era when no effective drugs are available for common infectious pathogens (33). To curb the prevalence of antibiotic resistance, novel drugs, especially those targeting MDR organisms, are urgently needed. However, the pipelines for development of new antibiotics have dried up (34). Therefore, preserving the currently available antibiotics for as long as possible by prudent administration is of paramount importance. In order to do so, thoroughly understanding the mechanisms by which the bacteria become resistant towards the therapeutic drugs has been on the top of the list of current scientific research.

Molecular mechanisms of antibiotic resistance

Bacteria may acquire antibiotic resistance either by de novo evolution or by acquiring resistant genes by horizontal gene transfer from another bacterium (35, 36). The de novo development of resistance can be achieved through multiple
ways including single nucleotide substitution, deletion, and insertion, as well as duplication of resistance-conferring genes. These genomic changes result in drug resistance, as shown in Figure 1, through alteration of the cell target where the drug attacks, over-activation of the production of the enzyme that hydrolyzes or inactivates antibiotics, decrease of the uptake of the antimicrobial molecules, or induction of the efflux pumps to expel antibiotics out of the cell (37, 38).

In addition to de novo evolution, bacteria can also become resistant by genetic exchange with other bacteria. Antibiotic resistance genes are able to transfer from one bacterium to the other with the aid of mobile genetic elements such as plasmids, bacteriophages and transposons (39, 40). Plasmid-mediated antibiotic resistance is commonly reported in pathogenic bacteria. It involves almost all classes of antibiotics currently used in clinics, especially the frequently applied beta-lactams, aminoglycosides, cephalosporins and fluoroquinolones (41-43). There is no doubt about the association of phages with the formation of mobile resistance elements because of the occurrence of resistant genes flanked by the phage “fingerprints” (44). Transposons also play an important role in transmission and spread of resistant genes, as exemplified by the transposons accommodating tet(M) tetracycline resistance genes (45).

Although resistance has originated from the biological process of evolution, the de novo development of drug resistance during a specific treatment of a patient is not yet well documented, as opposed to plasmid-conferred antibiotic resistance. The difficulties to accomplish this work in vivo are several. First, it is hard to acquire in vivo samples from the location inside the body (organ) where the infection occurs. Likewise, measurement of the drug concentration reaching the target pathogens in real time is also fraught with technical difficulties. Second, the previous drug exposure as well as the concurrent treatments for other symptoms irrelevant to bacterial infections, are expected to interfere with the resistance development towards the antibiotics. Third, the co-existence of multiple bacterial species in vivo complicates the analysis. Fourth, the sole role of de novo evolution in resistance development can be hardly assessed as simultaneous genetic transmission cannot be excluded for infections in a hospital environment. These difficulties can be overcome by the application of in vitro continuous culture cultivation models, e.g. chemostat, morbidostat, hollow fiber infection model, combined with the Pharmacokinetic/Pharmacodynamic (PK/PD) data obtained from the patient population (46-48). Within in vitro models, bacterial growth conditions can be strictly manipulated and factors of therapeutic regimens can be
General introduction

populations, the molecular mechanisms behind the occurrence and development of resistance can be also assessed over time (49).

Besides the evolutionary trajectory towards drug resistance, the destiny of acquired resistance after treatment has also aroused significant concerns because it determines the persistence and dissemination of resistance. It was predicted that bacteria may lose or reverse a resistant phenotype when the selective pressure exhibited by antibiotic treatments is halted, because antibiotic resistance may be accompanied by fitness cost (50, 51). Such fitness cost, in the form of reduction of growth rate, minimization of ecological range or variation of virulence, has been documented in resistant bacteria. However, absence of fitness cost might also be observed, as shown by both in vitro and in vivo data (52, 53). This is explained by suggesting that either the resistance mutations barely compromise

Figure 1. Examples of mechanisms of antibiotic resistance in a bacterial cell, being adapted from reference 37 & 38.
bacterial fitness, or that fitness-compensating mutations occurred concomitantly. Nevertheless, long-term tracking of the fate of the resistance mutations after the removal of antibiotics across the whole genome is currently still lacking.

**Current guidelines aiming to reduce antibiotic resistance**

Making a rational drug choice is essential for preventing antibiotic resistance. There is consensus that the antibiotic should be chosen specifically according to the infection-causing pathogen since some pathogens are inherently resistant to certain antibiotics (54, 55). Among the drugs with activity against the target bacteria, the ones with narrow coverage are preferred over those having broad spectrums, with the intention to avoid selection of resistance among other microorganisms irrelevant to the infection (56). The local epidemiology of antibiotic resistance patterns and the drug consumption history of the patients would assist determination of the empirical treatment (57). If time is allowed, prescription of drugs based on the result of the drug susceptibility test is more ideal not only in terms of therapeutic effects but also from the perspective of the prevention of resistance development (58). Unfortunately, this is hard to adopt in the clinic, especially at the starting of the therapy, partly because the current methods for determining drug susceptibility are not so rapid yet.

Apart from the aforementioned principles, there are other proposed tactics on how to avoid resistance by adjusting application of the drugs during treatment. One of such tactics is alternating different drugs in a single therapy, which was proposed as the use of one drug may be capable to select against the resistance built up during the application of the other drug (59-62). This strategy, however, has not been introduced to the clinic. One of the main reasons is that the evidence supporting the potential collateral sensitivity in clinical pathogenic isolates remains equivocal compared to the cross resistance (63). Besides drug rotation, combination of multiple drugs is also expected to be effective in decreasing the development of resistance. This idea is based on the assumption that the chance of the occurrence of mutation conferring resistance towards all the drugs applied concurrently is very rare (62, 64). However, evidence in support of combination treatment is still scarce and inconclusive (65, 66).

Besides choice of antibiotic, dosage is a well known element directly influencing development of resistance in bacteria (67, 68). Based on this observation, the concept of mutation prevention concentration (MPC) has been proposed (69, 70).
General introduction

This concept, as its name indicates, is used to define a dosing level that is able to block the emergence of resistant mutants. Beside the MPC, the Pharmacokinetic (PK)/Pharmacodynamic (PD) parameters such as $C_{\text{min}}$ (the minimal concentration of an antibiotic)/MIC ratio and AUC (area under the curve, indicating the total exposure of an antibiotic to an organism)/MIC ratio, are also evaluated with respect to their effects in curbing the occurrence of resistance (71, 72). However, the application of these parameters in practice is very challenging since the actual drug concentration reaching the infected compartment or location is dynamic over time and varies in individuals. In some cases, the drug exposure can become intermittent owing to the poor compliance of patients or improper dose intervals. How these dosing patterns encountered in patients are relevant to the development of resistance in bacteria is still not understood.

Treatment duration is also important. As it has been often suggested, the therapeutic course has to be as short as possible to prevent emergence of drug resistance (56, 73, 74). This seems reasonable as the shorter the bacteria interact with the antibiotics, the less they would develop resistance. However, a realistic treatment lasts normally more than a few days in order to avoid a relapse of the infection (75, 76). In the case of some infections like tuberculosis, the treatment might last several months or even years (77). Considering the remarkable genetic capabilities of bacteria, the occurrence of resistance development is very likely already during a single treatment. However, no clear cut-off of treatment period has been recommended for specific drugs aiming to prevent the pathogens from becoming resistant.

Research questions and thesis outline

This thesis attempts to answer the following questions: First, whether the therapeutic levels of drugs expected in patients are able to cause the de novo development of resistance? Second, how the resistance evolved in the initial treatment influences the therapeutic effects of the subsequent treatments? In the third place, how the genome of bacteria dynamically changes during and after the treatment with different types of antibiotics? Finally, what is the optimal way to administrate antibiotics to guarantee both control of infections and prevention of resistance?

This thesis consists of six chapters. Chapter 1 serves as a general introduction. It starts with pointing out the severity of antibiotic resistance problem in
Chapter 1

clinical pathogens and its heavy burden for the whole society. After that, the mechanisms by which the clinical pathogens acquire resistance are briefly described. At the same time, the lack of information about de novo acquisition of antibiotic resistance during the patient therapy, as well as the necessity of tracking the genome-wide mutations during and after the antibiotic therapy are discussed. Finally, the current recommendations for administering antibiotics in order to avoid development of antibiotic resistance are summarized. The lack or incompleteness of evidence supporting the specific recommendations is noticed as well.

Chapter 2 concentrates on examination of development of antibiotic resistance during simulated antibiotic treatment of *Pseudomonas aeruginosa* (*P. aeruginosa*) infections *in vitro*. Two first-line anti-*P. aeruginosa* antibiotics, ceftazidime and meropenem, are studied. Their concentration-time profiles resulting from clinically used treatment regimens are simulated by computer modeling. Among the profiles, the 5\(^{th}\) percentile, called “low”, 50\(^{th}\), dubbed “medium” and “high” (95\(^{th}\) percentile) profiles are mimicked in chemostats. During the simulation of treatment, drug susceptibility, cellular morphology and the number of surviving cells are followed every day. What is also included in the daily assessment is the proportion of different levels of resistant subpopulations, the mutations in the resistance-relevant genes and the fitness cost defined as reduction of growth rate.

Chapter 3 focuses on elucidating how therapy failure of an initial antibiotic treatment may influence the effects of subsequent treatments. In this study, initially is tested whether resistance would occur during the treatment with sublethal concentrations of amoxicillin. The influence of the amoxicillin-resistant mutants acquired in the first treatment on the subsequent amoxicillin therapy is assessed by performing the growth competition experiments with the original wild type *Escherichia coli* (*E. coli*) strain and the mildly or highly amoxicillin-resistant *E. coli* strain evolved from it. The influence is also examined when the second treatment is switched to a third generation cephalosporin by comparing the evolutionary speed towards cefotaxime resistance of the wild type *E. coli* strain and the *E. coli* strain made mildly amoxicillin-resistant.

Chapter 4 highlights the linkage between the increase of antibiotic resistance levels and the dynamics of mutations across the whole genome of bacteria. For this purpose, *P. aeruginosa* is exposed to stepwise increasing concentrations of five
General introduction

medically relevant antibiotics: ceftazidime, meropenem, piperillin/tazobactum, ciprofloxacin and tobramycin. Whole genome sequencing was applied to identify the genomic changes at multiple time points during the resistance development process. The reversibility of the acquired resistance after the treatment and its corresponding genomic variations are also explored. Since resistance towards beta-lactam antibiotics is often related to overexpression of beta-lactamases, the relationship between beta-lactamase activity and resistance levels of P. aeruginosa towards beta-lactam drugs is documented. The fitness, as demonstrated by the maximum growth rate, the pH and salt tolerance, and the maintenance energy of P. aeruginosa before and after the acquisition of resistance, is also investigated.

Chapter 5 presents the comparison of a series of treatment strategies with respect to both bacteria killing efficacy and resistance preventive potential, aiming to provide better evidence for recommendations of antimicrobial administration guidelines. The tested administration protocols, including continuous infusion, fluctuating infusion, intermittent treatment, drug alteration as well as drug combination, are mimicked in chemostats. All the drug concentrations applied are those expected to occur in patients. To gain insights into the differences of evolutionary trajectories among the assessed treatment regimes, the mutation profiles of the oprD gene are followed during each mimicked treatment due to the fact that the meropenem resistance is closely related to oprD mutations in P. aeruginosa.

Chapter 6 summarizes the results achieved in this thesis. In addition, we elaborate on the discussion of several issues concluded from chapter 2 to chapter 5, including de novo development of antibiotic resistance during treatment and its consequences, the complexity of evolutionary trajectory towards antibiotic resistance, completion and improvement of the guidelines to antibiotic administration.
Chapter 2

Development of antibiotic resistance during simulated treatment of *Pseudomonas aeruginosa* in chemostats

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Chapter 2

Abstract

During treatment of infections with antibiotics in critically ill patients in the intensive care resistance often develops. This study aims to establish whether under those conditions this resistance can develop de novo or that genetic exchange between bacteria is by necessity involved. Chemostat cultures of Pseudomonas aeruginosa (P. aeruginosa) were exposed to treatment regimes with ceftazidime and meropenem that simulated conditions expected in patient plasma. Development of antibiotic resistance was monitored and mutations in resistance genes were searched for by sequencing PCR products. Even at the highest concentrations that can be expected in patients, sufficient bacteria survived in clumps of filamentous cells to recover and grow out after 3 to 5 days. At the end of a 7 days simulated treatment, the minimal inhibitory concentration (MIC) had increased by a factor between 10 and 10,000 depending on the antibiotic and the treatment protocol. The fitness costs of resistance were minimal. In the resistant strains, only three mutations were observed in genes associated with beta-lactam resistance. The development of resistance often observed during patient treatment can be explained by de novo acquisition of resistance and genetic exchange of resistance genes is not by necessity involved. As far as conclusions based on an in vitro study using P. aeruginosa and only two antibiotics can be generalized, it seems that development of resistance can be minimized by treating with antibiotics in the highest concentration the patient can endure for the shortest time needed to eliminate the infection.
Introduction

Antimicrobial treatment of critically ill patients in the intensive care is often complicated by antimicrobial resistance (AMR) development of the targeted microorganisms, even though these were susceptible at the start of treatment (78). The acquired resistance complicates any further antimicrobial treatment that might be needed and can endanger the health of the patient in the case of recurring infections and should therefore be prevented as much as possible. Conversely, timely and appropriate antibiotic therapy significantly reduces mortality in septic patients on ICU’s (79-81) and resistance decreases the chance of appropriate empirical therapy.

Rational design of strategies to prevent development of resistance require a thorough understanding of the processes causing it. Antibiotic resistance can be acquired by transfer of genetic information between bacteria at the infection site, or it can develop de novo through genetic mutations, as a result of exposure to the drug (82, 83). Alternatively, it is conceivable that the resistant variants of the target pathogen were present before start of the treatment, but due to extremely low prevalence did not influence the measured minimal inhibitory concentration (MIC). For optimization of treatment protocols aimed at eliminating an infection successfully without causing emergence of resistance it is important to distinguish between these scenarios. Due to the complexity of all potential and actual interactions between microbes at an infection site, it is not possible to ascertain with certainty the occurrence of de novo development of resistance in patients during treatment. Simulation of the treatment in chemostats, however, can provide the necessary controlled and reproducible conditions.

A generally accepted principle for treatment protocols to prevent development of resistance is to have the antibiotic concentration exceed the mutant prevention concentration (MPC) for as much of the treatment period as possible (69). Treatment principles are based on the average susceptibility of the targeted microbes. However, there is considerable variability between individual bacterial cells and microbes possess effective strategies to rapidly increase their resistance against antibiotics (84, 85). As a result, treatment principles that are based on the functional average cannot exclude the possibility of a few cells surviving and becoming resistant. Though some general guidelines for antibiotic stewardship have been formulated (86),
the actual influence of different treatment protocols on the likelihood that resistant cells will emerge is poorly understood (68). This study addresses the question whether under conditions mimicking those at an infection site during antibiotic treatment, pathogens can survive and emerge resistant to the antibiotic that was applied. *Pseudomonas aeruginosa* was chosen as model organism because it is a major nosocomial pathogen associated with high mortality rates among critically-ill patients (87). Two commonly used drugs to treat *P. aeruginosa* infections, ceftazidime and meropenem, were selected to represent the third-generation cephalosporins with anti-*Pseudomonas* activity, and the carbapenem class of antibiotics, respectively.

**Materials and Methods**

**Selection of drug level exposure to ceftazidime and meropenem**

Ceftazidime and meropenem concentration-time curves were computer-simulated following clinically used dosing regimens for ceftazidime (a 1000 mg iv loading dose followed by a continuous infusion of 3000 mg over 24 hours) and meropenem (a 1000 mg iv bolus infusion administered over 30 minutes three times per day). The simulations were performed on the basis of published population pharmacokinetic models for ceftazidime and meropenem in critically ill patients. These models also account for inter-individual variability in drug exposure (88, 89). One thousand simulations per drug were performed, resulting in a range of concentration-time profiles representative for the critically ill patient population treated according to the applied dosing regimens. From this range, the 5, 50 and 95 percentile of the concentration-time profiles of ceftazidime and meropenem were selected to be mimicked in the chemostat. The 50 percentile was chosen to represent the exposure in a typical critically ill patient, while the 5 and 95 percentile were chosen to illustrate the inter-individual variability in drug exposure within the population. Simulations were performed with nonlinear mixed effects modeling (NONMEM) software package (version 7.2, ICON plc, Dublin, Ireland).

**Bacterial strain, antibiotics, growth medium and culture conditions**

The strain used throughout the study was the antibiotic-susceptible wild type strain *Pseudomonas aeruginosa* ATCC27853. Cultures were grown at 37°C in cation-adjusted Mueller Hinton Broth (Sigma-Aldrich), autoclaved at 115°C.
Resistance development during the simulated treatment for 10 minutes. Continuous cultures were performed in Sixfors fermenter vessels (Infors AG, Bottingen, Switzerland) with a working volume of 250 mL, air flow 0.1 l/min, at 37°C and stirred at 250 rpm. In the absence of antibiotics cell density was approximately $10^9$ cells/ml. The pH was regulated at 7.0 by automatically adding sterile 2 N NaOH. Samples were taken at exactly 24 hours intervals for a variety of parameters, such as optical density (OD$_{595}$), MIC and maximal growth rate ($\mu_{\text{max}}$) measurement, bacterial cell morphological observation, bacterial cell counts and sequencing of resistance genes. Steady state was assumed when after a minimum of 5 volume changes all culture parameters had reached constant values. A dilution rate of $0.3^{-1}$h was chosen to mimic the submaximal growth rate that pathogens can be expected to have at an infection site. To determine maximum growth rates ($\mu_{\text{max}}$) the growth of batch cultures was followed for 23 hours by measuring the optical density at 595 nm. The $\mu_{\text{max}}$ was calculated based on the averaged growth rates during exponential phase of 4 independent replicates.

The correct antibiotic concentration-time profiles in the chemostat were maintained by computer controlled continuous infusion of ceftazidime and programmed interval pumping of meropenem. Stock solutions of ceftazidime (Fresenius Kabi) and meropenem (Fresenius Kabi) were prepared freshly for every experiment by dissolving the drugs in water and filter sterilizing (0.2 $\mu$m). To allow for continuous infusion in the culture, ceftazidime was added to the culture medium stock bottle which was kept on ice throughout experiments, and changed once in two days. The stability of ceftazidime in culture medium kept on ice was tested and no significant degradation was seen over a period of three days. Meropenem is unstable even at low temperature (90, 91). Therefore, the experimental drug concentration was maintained by computer controlled pumping of stock solutions kept on ice for a maximum of 8 hours.

**Minimum inhibitory concentration (MIC) and mutant prevention concentration (MPC)**

MIC was measured by following growth in 96-well plates as described previously (92). The antibiotic concentrations ranged from 0.06 mg/l to 1,024 mg/l. All measurements were performed in duplicates which had identical results in all measurements. The MIC was defined as the minimal concentration of antibiotic that limited growth to an OD$_{595}$ of 0.2 or less after
Chapter 2

23 hours. The starting OD was 0.05.

MPC was determined by inoculating $>10^{10}$ cells on antibiotic containing Mueller-Hinton agar plates (Sigma-Aldrich). The reported MPC is the lowest concentration that showed no growth after 48 hours at 37°C. All tests were performed in four replicates.

**Cell density, cell counting and morphological observation**

The cell density was measured spectrophotometrically at a wave length of 595 nm. Cell morphology was observed microscopically using a light microscope at 400X. The total cell number of intact bacteria was determined by colony counts of bacteria grown on antibiotic-free agar plates after appropriate dilutions. To establish the resistant fraction, equal volumes were spread on LB agar plates containing antibiotics. The drug concentrations were 8 mg/l, 24 mg/l, 48 mg/l for ceftazidime, representing the 5, 50 and 95 percentile steady-state concentrations resulting from the computer simulation. For meropenem the 5, 50 and 95 percentile trough concentrations were 0.6 mg/l, 5 mg/l and 15 mg/l respectively. The number of colonies observed after overnight incubation on plates containing a specific antibiotic concentration was used as measure for the number of cells being able to survive at this concentration. This number was divided by total cell count on plates without antibiotics to determine the resistant fraction at that concentration.

**Amplification and sequencing of resistance genes**

Mutational changes that are likely to contribute to resistance were identified in a separate study by whole genome sequencing of cultures that had been made resistant by exposure to stepwise increasing concentrations of ceftazidime or meropenem (manuscript in preparation). Such mutations were found in the following genes in ceftazidime exposed cells: $ampD$, $dacB$, $hfq$, and $yerD$ and in cells adapted to meropenem: $oprD$, $mexR$ and $mexB$. PCR products of four colonies were sequenced to detect the presence of mutations in these genes related to meropenem resistance, but fourteen in the case of ceftazidime, because these sometimes occurred in low frequency. The primers used for PCR to amplify the relevant regions are given in Table 1.
Resistance development during the simulated treatment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ampD</td>
<td>Forward 5<code> GTAGACCACCACCAGAA 3</code></td>
</tr>
<tr>
<td></td>
<td>Reverse 5<code> AATACCTTCTCCTGCAGC 3</code></td>
</tr>
<tr>
<td>docB</td>
<td>Forward 5<code> ATCGGGCTTGAGAAT 3</code></td>
</tr>
<tr>
<td></td>
<td>Reverse 5<code> TTGCGTGATGTCCC 3</code></td>
</tr>
<tr>
<td>hfq</td>
<td>Forward 5<code> CCCCAGATGCACCA 3</code></td>
</tr>
<tr>
<td></td>
<td>Reverse 5<code> TTGCGCGTCTGTTTCG 3</code></td>
</tr>
<tr>
<td>yerD</td>
<td>Forward 5<code> GACATGAAAGCCGGAG 3</code></td>
</tr>
<tr>
<td></td>
<td>Reverse 5<code> CGAAGAAGGTGACTCCA 3</code></td>
</tr>
<tr>
<td>oprD</td>
<td>Forward 5<code> CTGCCTGCTATAAGTTAG 3</code></td>
</tr>
<tr>
<td></td>
<td>Reverse 5<code> CTAGGCCCTCTTTTATA 3</code></td>
</tr>
<tr>
<td>mexR</td>
<td>Forward 5<code> AAGCGGATACCTGAAAACG 3</code></td>
</tr>
<tr>
<td></td>
<td>Reverse 5<code> AAGCCTCGGTTGAAAAACA 3</code></td>
</tr>
<tr>
<td>mexB</td>
<td>Forward 5<code> TCGAGTTAAGACGCTG 3</code></td>
</tr>
<tr>
<td></td>
<td>Reverse 5<code> TGGAAGTCGCGGATCA 3</code></td>
</tr>
</tbody>
</table>

Table 1. Primers used for PCR reactions to amplify the relevant regions of the indicated genes.

Statistical analysis

In all experiments duplicate measurements were performed for most parameters, with the notable exception of cell counts, as this parameter showed noticeable variation between replicates and was therefore carried out in fourfold. Still, only rarely the difference between the highest and lowest value exceeded 10%. To ascertain reproducibility all experiments were repeated, with invariably an almost identical outcome. Of all experiments only the first version is reported, as averaging values would not be correct, since each data point depends on the preceding and therefore replicates are not independent measurements. Including both experiments in the graphs would result in an uninterpretable figure. As differences in values of minimally a factor of 10, but up to 10,000, were measured with a precision of a factor of 2, P values always were less than 0.001, making statistical analysis irrelevant.

Results

The 5, 50, and 95 percentile concentration-time profiles resulting from the computer-simulations, which are representative for the expected concentration-time profiles in critically ill patients, were mimicked for each antibiotic in the chemostat. The acquired concentration-time profiles
in the chemostat matched with the simulated ceftazidime and meropenem concentration-time curves as intended (88, 89) (Figure 1). There was considerable inter-individual variation in the concentrations calculated for patients, which is illustrated by the large difference between the 5 and 95 percentile values for both antibiotics. Both antibiotics showed a rapid drop in concentration after the initial loading dose, which could not be completely mimicked in the chemostat. Otherwise the simulated and the chemostat antibiotic concentrations were equivalent. The measured mutation prevention concentrations (MPC) were 48±18.5 mg/l for ceftazidime and 16±9.2 mg/l for meropenem, which are approximately the 95 percentile concentrations of these two drugs.

The bacterial cell density of the culture decreased by 2 to 4 factors of ten within the first 1-5 days (Figure 2). Decrease and recovery depended on the antibiotic concentrations applied. In all cases, the cultures almost completely returned to the initial density within maximally 7 days. The fastest recovery happened at the 5 percentile concentration of ceftazidime and the slowest
Resistance development during the simulated treatment

upon exposure to the 95 percentile concentration of meropenem. In none of the experiments a wash-out occurred, as would be indicated by a complete disappearance of all bacterial cells. Instead, already after 24 hours the initial rapid decline slowed and some growth took place, though initially not always enough to completely counterbalance the dilution rate of $0.3 \text{ h}^{-1}$.

Cell morphology was observed under the microscope at several stages during initial exposure to antibiotics and the subsequent recovery. In Figure 3, the morphology of a culture grown in the absence of ceftazidime is compared

Figure 2. Density of a culture of *P. aeruginosa* exposed to ceftazidime and meropenem in the chemostat ($D = 0.3 \text{ h}^{-1}$) at concentrations simulating the 5, 50 and 95 percentile of the concentration-time profiles as they are expected in critically ill patients. Cultures were in steady state in the absence of antibiotics before day 0.

Figure 3. Comparison of cell morphology of regular growing culture and a culture growing at the 50 percentile concentration of ceftazidime during the initial exposure. Exposure to other concentrations of ceftazidime and all experimental concentrations of meropenem yielded a similar morphology.
to that of a culture exposed to ceftazidime at the time when the cell density was lowest. Long filaments were seen when the culture was stressed upon initial exposure to ceftazidime. After the cultures recovered, the morphology was identical to that of the starting culture. The results at different concentrations were very similar and so was the morphology upon exposure to meropenem.

The MIC increased by between 4 and 7 two-fold increasing steps during the length of the simulated treatment (Figure 4). For both antibiotics, the most rapid increase occurred at the median concentration. The 5 percentile concentration yielded a smaller and slower increase in MIC. The cells exposed to the highest drug level (95 percentile) were the last to show an increased MIC, however the final MIC values were the highest. After a continuously increasing phase, the MICs remained constant for all concentrations. The adaptation to meropenem seemed to require more time than to ceftazidime.

When resistance develops during antibiotic treatment of an infection, initially only a minute fraction of the total number of cells will be resistant. Still, the size and existence of this fraction is highly relevant as it forms the source for outgrowth of the resistant pathogens later on (Figure 5). The fraction of cells resistant to the 5, 50 and 95 percentile concentrations was determined by plating on agar plates containing these levels of each antibiotic. The largest fraction resistant to ceftazidime arose in the culture exposed to

![Figure 4. Minimum inhibitory concentrations (MIC) of P. aeruginosa culture as a function of time (days) during exposure to the 5, 50, and 95 percentile of concentration-time profiles as they are to be expected in critically ill patients of ceftazidime (left panel) and meropenem (right panel) in chemostats (D = 0.3 h⁻¹).]
Resistance development during the simulated treatment

Figure 5. Fraction of cells from chemostat cultures that can grow on plates containing the 5, 50, 95 percentile of the ceftazidime steady-state concentration and the meropenem trough concentration (8 hour after drug administration) as these are to be expected in critically ill patients, as a function of time (days) during growth at the indicated antibiotic levels.

The maximum growth rate ($\mu_{\text{max}}$) is often considered a measure for the relative fitness of bacteria. To examine whether the acquisition of resistance was accompanied by a loss of relative fitness, the $\mu_{\text{max}}$ in the absence of antibiotics was determined on samples taken from the chemostat cultures.
exposed to the highest level (95 percentile) of ceftazidime and meropenem. In response to growth at these drug levels, the $\mu_{\text{max}}$ decreased slightly in cells that became resistant to ceftazidime and decreased for only one day upon exposure to meropenem (Figure 6). The small difference suggests that relative cell fitness was barely affected during the evolution of drug resistance.

Contrary to the expectations based on whole genome sequencing of strains made resistant to very high concentrations of antibiotics by step-wise increasing exposure, the number of mutations detected after growth in the presence of ceftazidime and meropenem was very limited. Exposure to ceftazidime yielded two point mutations in $ampD$ gene (Stop86E and V155G) (Table 2). In the case of meropenem several mutations were detected in $oprD$: Two point mutations (E107D, G166S), an insertion of C between position 389 and 390 and a deletion of A at position 296. In only one condition, exposure to the 95 percentile for 7 days, an insertion of G occurred between position 296 and 297 of $mexR$. Mutations occurred either relatively shortly after the start of the exposure, or only at the very end and only at the highest concentration, in the case of $mexR$ and meropenem.

![Figure 6. Maximum growth rate ($\mu_{\text{max}}$) in the absence of antibiotics of cells taken from chemostat cultures exposed to the 95 percentile of concentration-time profiles as they are to be expected in critically ill patients of ceftazidime or meropenem as a function of time (days). For each time point, the growth rate of four independent samples was measured.](image-url)
Discussion

The whole range of simulated antibiotic concentrations could induce the development of antibiotic resistance in slow-growing cultures of *P. aeruginosa* within 7 days, even at the highest drug levels. These results nicely dovetail with an earlier study that used shorter duration of exposure and less varied conditions. Given the experimental conditions, these observations indicate that horizontal gene transfer is not required for development of resistance within a short time span during treatment. This seemingly contradicts, at least in the case of *P. aeruginosa*, the theory that high dosing is sufficient to avoid resistance development as the drug kills pathogenic bacteria before the bacteria have the chance to evolve drug resistance (68, 69). Apparently bacterial cells can survive and recover under conditions that do not allow normal growth. In any event, these observations may explain the occurrence of resistant infections shortly after high dose treatment was applied in the clinical setting (78).

The *de novo* development of antibiotic resistance is a complex interplay of initial changes in expression levels of a large number of genes and subsequent

<table>
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Table 2. Mutations detected in *P. aeruginosa* cultures during exposure to the 5, 50, and 95 percentile of concentration-time profiles as they are to be expected in critically ill patients of ceftazidime (ampD, dacB, hfq, yerD) and meropenem (oprD, mexR, mexT). Four and 14 randomly selected colonies were tested for meropenem and ceftazidime culture, respectively. No mutations were found in dacB, hfq, yerD and mexT.
Chapter 2

mutations in a few specific genes (82, 84, 85, 94). Some forms of resistance induce almost no fitness costs, hence a resistant mutant can often maintain itself once it emerges in a bacterial population (95). Simulation of empirical treatment of infections by primary care physicians by exposing *Escherichia coli* to therapeutic concentrations of antibiotics during short time periods, resulted in resistance levels which could complicate subsequent treatment should this be necessary (96). Protocols for treatment in the intensive care are explicitly designed to prevent this emergence of resistance (97). In this study, however, exposure of *P. aeruginosa* to concentrations of ceftazidime and meropenem equal to those in the plasma of ICU-patients for the usual treatment duration did allow resistant cells to develop and grow out and to finally dominate the culture. Combining the considerations discussed above, it seems that from the viewpoint of prevention of emergence of resistance, treatment with the highest dose the patient can endure for the shortest time that eliminates the infection, may be optimal.

The survival of 0.001%-1% of cells at drug concentrations of 1 to 15 times the MIC observed in this study could possibly be attributed to drug resistance heterogeneity (98), collective resistance (99, 100), the production of filaments (101, 102), or a combination of these. According to the concept of resistance heterogeneity, a few cells among a population could temporarily tolerate higher drug concentrations by modifying cellular functions relevant to drug resistance, e.g. drug efflux, drug degrading enzymes, metabolic dormancy, etc. (84, 103). If the drug pressure is continued, these phenotypically resistant cells could acquire genetic mutations to achieve long lasting resistance (85). The filamentation observed in this study at moments that the cells are under severe stress resulting from exposure to the antibiotics, is fully in line with the concepts described above.

The in some cases observed absence of cells able to grow at antibiotic concentrations encountered in the growth medium can be explained by assuming that the proportion of phenotypic resistant cells in the culture is below the detection limit of 10 cells/ml. According to the collective resistance theory (99, 100), a small fraction of cells can escape the killing by lethal drug concentrations through co-operation among the susceptible cells by e.g. cooperative antibiotic inactivation, formation of biofilms, clustering, etc. Absence of resistant cells at the beginning of the treatment corresponds well with this explanation. The morphological changes seen in this study indicate
that filamentation may be an essential step bacteria use to establish antibiotic resistance, in agreement with other studies (99, 101, 102). Essential for this concept are the multiple chromosomes encompassed in a single filament. Recombination among these chromosomes on the one hand helps repairing DNA damage caused by antibiotic exposure, on the other hand it accelerates drug resistance evolution by increasing the mutagenesis rate (101). Once the mutant chromosome is generated, it would be separated from the filament and propagate normal sized, resistant progeny.

The initial cell density in the chemostats of approximately $10^9$ CFU/ml was much larger than that in bacteremia ($10^{4-6}$ CFU/ml), but comparable to what is found outside of the systemic circulation (104, 105). The cell number can increase to as much as $10^{8-9}$ CFU/mL in *Pseudomonas* biofilms, e.g. in sputum samples of cystic fibrosis patients (104, 105). Population size is one of the key factors influencing the evolution of antibiotic resistance (106). Resistant subpopulations are present in low frequencies (i.e. $10^{-6-10^{-8}}$), so large population sizes will boost the rate at which bacteria can evolve (69). In addition, collective resistance is expected to occur more readily at high cell density. The dependence of the evolutionary path on the selective power (107, 108), may explain the observation that the time required for emergence of resistance increases and that the fraction of resistant cells decreases with increasing antibiotic concentration. This notion is further supported by the observation that at some concentrations cells resistant to different antibiotic levels were co-selected and their ratio maintained to the end.

Even at the highest tested concentrations as they are expected in patients, drug resistance will emerge and develop if treatments last long enough, underscoring the importance of limiting the length of treatments. The importance of short treatments is further stressed by the observation that the decrease of maximal growth rate of resistant cells is of short duration. Once cells have become resistant, the growth rate rapidly restores to rates comparable to those of susceptible cells. Hence an infection can be hardly cleared in subsequent treatments (109). Once drugs kill the majority of the pathogenic bacteria, the immune system usually normally clears the left-over cells (110). Therefore the survival of a small number of cells and the following development of resistance under adequate drug exposure will less likely happen in patients with competent immunity. Consequently, attention should be given to the design of the dosing regimen for patients with compromised
immunity.

The overall conclusion of this study is that even the highest antibiotic concentrations measured in patients, although considerably exceeding the MIC, can still induce development of antibiotic resistance in a realistic treatment time. Induction of resistance occurs more rapidly at the lower concentrations than at the higher levels. The consequences are not only development of high levels of resistance but also recovery of fitness of resistant variants within the time frame of the treatment. This in turn might severely hamper a subsequent treatment, should this be necessary. Therefore, to prevent the occurrence of antibiotic resistance, strategies of high doses combined with the shortest treatment courses that is sufficiently effective, seem most effective.

Acknowledgments

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Chapter 3

Experimental simulation of the effects of an initial antibiotic treatment on a subsequent treatment after initial therapy failure

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Abstract

Therapy failure of empirical antibiotic treatments prescribed by primary care physicians occurs commonly. The effect of such a treatment on the susceptibility to second line antimicrobial drugs is unknown. Resistance to amoxicillin was rapidly induced or selected in *Escherichia coli* (*E. coli*) at concentrations expected in the patient’s body. Strains with reduced susceptibility outcompeted the wild-type whenever antibiotics were present, even in low concentrations that did not affect the growth rates of both strains. Exposure of *E. coli* to amoxicillin caused moderate resistance to cefotaxime. The combined evidence suggests that initial treatment by amoxicillin has a negative effect on subsequent therapy with beta-lactam antibiotics.
Effects of the developed resistance on the subsequent therapy

Introduction

Bacterial infections are often initially treated empirically with first-line antibiotics, such as amoxicillin. Because of the global prevalence of antibiotic resistance, the failure of the first therapy has become a frequent event (111). Subsequent treatment is required in such cases to eradicate the enduring infection. It is not clear, however, how the initial antibiotic therapy influences the follow-up treatment. Clinical observations suggest that there is an effect of the first on the later treatments, but the microbial physiology that can explain this effect is not understood at present.

Within the patient, it is not possible to distinguish between certain cells becoming resistant and an already existing resistant subpopulation becoming dominant at the infection site during antimicrobial therapy. Therefore, the initial question to be addressed is whether under conditions that can be expected to occur in a patient during treatment using a given dosing schedule, pathogens that are initially susceptible to the antibiotic applied can become resistant. The ensuing question is whether a strain that has developed resistance can hamper the follow-up treatment, due to simultaneously acquired reduced susceptibility to the next antimicrobial drug, in particular when a more potent drug of the same class is chosen.

The de novo emergence of resistance as a result of adaptation and mutations due to exposure to antibiotics is well-documented (82, 83, 112, 113). When resistance is not acquired through horizontal gene transfer, amoxicillin resistance in *E. coli* is mostly caused by the induction of AmpC beta-lactamase (84, 114, 115). Exposure to non-lethal levels of antibiotics induces a complex series of adaptations at the expression and cellular level affecting metabolism, regulation, virulence, DNA repair and stress response (84, 116, 117). Those changes might result in cross-resistance to other antibiotics that are eligible for subsequent treatments, especially if they have similar mechanisms (4). Even if the increase of resistance may appear limited, at the infection site, such effects could determine the difference between the elimination and survival of pathogens.

When antibiotic treatment is applied, another possible development in an infection site is the selection of a subpopulation that is already moderately to highly resistant. In fact, the survival of cells of a less susceptible subpopulation is a digital event: either it happens or it does not. According to the mutant
selection window hypothesis, selection of resistant cells only occurs when the drug concentration exceeds the MIC (Minimal Inhibitory Concentration) of the susceptible cells ($\text{MIC}_{\text{susc}}$), but is below that of the resistant variants (MPC) (Mutant Prevention Concentration) (69, 118). However, concentrations lower than $\text{MIC}_{\text{susc}}$ can also effectively select for resistant strains, as long as the fitness cost of resistance does not exceed the metabolic advantages (67, 109). That selection for resistance at low antibiotic levels occurs in an in vivo model was shown in rabbits infected with Staphylococcus aureus (119).

Levels of the antibiotic are not constant within the patient during a therapy. Only when the medicine is delivered intravenously can constant blood levels be expected. Typically, oral therapy involves the patient taking a dose at more or less regular intervals for some time, while the kidneys or liver remove the antibiotic after it has reached the bloodstream. In addition, the antibiotic may not penetrate well to the infection site, reducing the exposure of the pathogens even further (120, 121). Concentrations at which the selection for resistance takes place may therefore be encountered under a variety of conditions. Pre-existing mutations can be selected at high concentrations, but de novo mutations and adaptation at the expression level occur mostly when levels are low between the administrations of the doses (122). These conditions can be simulated in the laboratory and the insights thus obtained used to improve treatment strategies.

The aim of this study is to simulate in vitro the outcome of a situation in which an initial amoxicillin treatment fails to cure an E. coli infection, since the drug concentration attained at the infection site is sub-lethal. By documenting the development of resistance as an effect of this event and the selection of strains that have become moderately resistant by this simulated treatment, the effects of a failed initial treatment on subsequent antibiotic therapy can be envisaged. The outcome of this in vitro study using E. coli as a model organism suggests that an initial amoxicillin treatment of an amoxicillin susceptible strain can negatively influence the outcome of continued amoxicillin treatment or a follow-up therapy with the third-generation cephalosporin cefotaxime. Even though cefotaxime is a beta-lactam antibiotic just as amoxicillin, it is still commonly prescribed in The Netherlands for follow-up treatment, as it is considered more potent, and while more than 40% of the isolates from general practice are resistant to amoxicillin, only 3%–6% are resistant to cefotaxime. The research presented below puts the effectiveness of this practice into doubt.
Effects of the developed resistance on the subsequent therapy

Materials and methods

Bacterial strains, growth medium and culture conditions

All tested strains were derived from the antibiotic-susceptible wild-type E. coli MG1655 strain (WT). The strain, denoted as WT-YFP, which contains the YFP (yellow fluorescent protein) gene and is resistant to chloramphenicol (123), was kindly provided by M. Elowitz. The strains named M8 and M8-YFP were created by growing the WT and the WT-YFP, respectively, at 2 μg/mL amoxicillin for 5 days. These strains became moderately resistant against amoxicillin as a result (MIC 16–32 μg/mL). The strain indicated as M256 was grown at increasing levels of amoxicillin for 2 weeks [5] and had a permanent MIC of 512 μg/mL afterwards. The WT-YFP had the same amoxicillin MIC (4 μg/mL) as the WT, but had an MIC to chloramphenicol of 128 μg/mL.

Batch cultures were grown at 37°C in the defined minimal mineral Evans medium containing 100 mM Na$_2$HPO$_4$ buffer and 55 mM glucose with a pH of 6.9 (124). For the cultivation of continuous cultures, the concentrations of glucose and Na$_2$HPO$_4$ were decreased to 5 mM and 10 mM, respectively. The pH was maintained at 6.9 by pumping 2 N NaOH. The media were autoclaved for 20 minutes at 121°C, with the exception of glucose, which was autoclaved for 10 minutes at 110°C and added afterwards. Amoxicillin stock solutions of 10 mg/mL were 0.2 mm filter-sterilized and preserved in 4°C prior to use.

Precultures for the inoculation of 96-well plates, batch cultures and continuous cultures were grown overnight in 100 mL flasks shaken at 200 rpm at 37°C. The precultures of susceptible strains (WT, WT-YFP) were grown without antibiotics, while the M8 and M8-YFP precultures were cultivated in medium containing 2 μg/mL amoxicillin and 256 μg/mL for the M256 strain. The experimental concentrations used for simulating suboptimal amoxicillin treatment were 1 or 2 μg/mL. For daily transfers, fresh medium and amoxicillin stocks were used.

Continuous cultures were carried out in Sixfors fermenter vessels (Infors AG, Bottmingen, Switzerland) consisting of 6 vessels with a working volume of 250 mL, at 37°C and stirred at 250 rpm constantly. The pH of the cultures was regulated at 6.9 by automatically adding the sterile 2 N NaOH. The culture's parameters, such as pH, temperature and the stirring, were monitored by the controller system of the Sixfors fermenter unit. Amoxicillin treatment was initiated after all the parameters, including the culture's OD, reached steady state at a dilution rate (D)
of 0.3 h\(^{-1}\). To mimic the exposure in infection sites as a result of three oral doses per day, amoxicillin was pumped in for an hour, reaching a maximum concentration in the vessel of 2 \(\mu\)g/mL. It was diluted out to approximately 0.3 \(\mu\)g/mL during the subsequent 7 hours. The treatment regimen was simulated by repeating these 8-hours cycles over 5 days. Samples were taken at exactly 24-hours intervals for MIC measurement and sequencing of the promoter of the \(ampC\) lactamase gene during the entire treatment simulation.

**MIC measurement and antibiotics**

The MIC values were measured in 96-well plates, as described previously (92). The highest amoxicillin concentration was 1,024 \(\mu\)g/mL with serial dilutions by a factor of 2 until 0.5 \(\mu\)g/mL. The test culture was inoculated to a starting OD\(_{600}\) of 0.05 in the wells. Growth was followed for 23 hours by reading OD\(_{595}\) every 10 minutes with shaking in between and analyzed by the SkanIt software of the Thermo Scientific Multiskan FC (Filter-based Microplate Photometer). All measurements were performed as two technical replicates. The MIC was defined as the minimal concentration of antibiotic that limited growth to an OD of 0.2 or less after 23 hours.

**Competition experiment among susceptible and resistant Strains**

A total of three sets of competition experiments were carried out in this study: WT-YFP and M256, WT-YFP and M8 and M8-YFP and M256. For each experiment, the overnight cultures of the two strains were mixed together at ratios of 1:1, 1:100 and 1:10,000 (resistant:susceptible). The mixed culture was grown in shake flasks containing Evans medium either without antibiotics or with 2 \(\mu\)g/mL amoxicillin for 24 hours. During the cultivation, the ratio of resistant to susceptible was tracked at t=0, t=3, t=6 and t=24 hour by counting colonies from samples on LB (Luria broth) agar plates containing amoxicillin or chloramphenicol. Plates containing 34 \(\mu\)g/mL chloramphenicol were used to distinguish WT-YFP and M8-YFP strains from their competitors; the plates containing 10 \(\mu\)g/mL or 50 \(\mu\)g/mL amoxicillin were used to select the M8 strain or M256 strain from the mixed cultures; the plates without any antibiotics were used to count the cell numbers of the whole population. Controls showed no growth of susceptible strains on the antibiotic containing plates.
Effects of the developed resistance on the subsequent therapy

Amplification and sequencing of the \textit{ampC} promoter

The promoter region of the \textit{ampC} gene was amplified by PCR and sequenced using 5'-GGGATCTTTTGTTGCTCT-3' as the forward primer and 5'-CTTCATTGCTGCTATT-3' as the reverse primer. Amplification was performed in 50-μL working volumes with Taq DNA polymerase (Thermo Scientific), using the following parameters: denaturation at 95°C for 5 minutes, followed by 35 cycles of 35 seconds at 95°C, 55 seconds at 49°C and 90 seconds at 72°C; and finally, 90-seconds extension at 72°C. The PCR products were purified with the MSB (Minimal Salt Binding) Spin PCRapace kit (Invitek) and sequenced by Macrogen Europe.

Adaptation of \textit{E. coli} to cefotaxime

Three strains were adapted to cefotaxime: WT, M8 and M256. The preculture was initially adapted to the sublethal concentrations: 0.06 μg/mL for WT and M8, 0.5 μg/mL for M256. Whenever normal or approximately normal growth (OD$_{600}$ > 75% of OD$_{600}$ for normal growth) occurred, an aliquot of the culture, resulting in an OD$_{600}$ of 0.1, was used to start two more incubations: one at the same concentration of cefotaxime, the other at double concentration. The stepwise increasing exposure to cefotaxime was continued for 15 days at most. The MIC value was determined every day, and the \beta-lactamase activity was measured before and after the adaptation.

Determination of \beta-lactamase activity

To measure the activity of \beta-lactamase, an assay based on the chromogenic substrate, nitrocefin, was applied (125, 126). Briefly, 1 mL of a culture grown to OD$_{600}$ of 1.0 was harvested by washing in sodium phosphate buffer (100 mM, pH 7.0). The cells were lysed in sodium phosphate buffer containing 1% Triton X-100, and the cell extracts were centrifuged (15,000 rpm, 5 min, 4°C). \beta-lactamase activity was determined by measuring the amount of nitrocefin (final concentration: 100 μM) hydrolyzed by 8 μL of the testing sample per minute at 390 nm at 30 °C within 82 μL of sodium phosphate buffer. The final enzyme activity was normalized to the protein concentration of the samples, which was measured using the Thermo Scientific Pierce Micro BCA (Bicinchoninic acid) Protein Assay Kit.
Chapter 3

Results

To illustrate the effect of amoxicillin on the growth of *E. coli* MG1655 wild-type, cultures were compared at 0, 2 and 4 μg/mL of this antibiotic (Figure 1). Growth was almost completely inhibited at 4 μg/mL. At 2 μg/mL, the initial growth rate equaled to that of the control, but the culture started to collapse after approximately 5 hours. This suggests that most cells died as the cell wall disintegrated. It seems that afterwards, some of the cells that remained alive grew out, and after 23 hours, a density was reached nearly identical to that of the control. This two-stage growth curve indicates that the rapid adaptation that allows cultures of *E. coli* to withstand amoxicillin levels close to the MIC is caused in part by the survival of a small subpopulation when the majority of the cells succumb. In a similar manner, growth was followed for the same strain that was
made resistant by exposure to 2 μg/mL amoxicillin for five days (indicated as M8), or by exposure to step-wise increasing concentrations of amoxicillin (83) (indicated as M256) at the highest concentration that allowed growth, 256 μg/mL. At this concentration of amoxicillin, this highly-resistant strain had a lower growth rate, but reached a final density of about 75% of the control. At 2 μg/mL amoxicillin, the two adapted strains grew at the same rate as they did in the absence of antibiotics, indicating that no residual effects remained.

To assess whether amoxicillin-susceptible E. coli could become resistant due to exposure to levels of amoxicillin below the MIC, the increase of the MIC was measured during growth at 2 μg/mL amoxicillin (Figure 2). In the presence of 2 μg/mL amoxicillin, the MICs of the wild-type (WT) and WT-YFP strains rapidly increased from 4 μg/mL to 16 or 32 μg/mL within 24-hour exposure, and eventually reached 32 or 64 μg/mL after another 4 days of culture. The WT-YFP (yellow fluorescent protein) strain became resistant slightly faster. This acquired resistance remained or decreased by only a factor of two during growth in the absence of amoxicillin for the following five days.

In order to mimic carbon and energy limited growth and exposure to fluctuating drug concentrations, as might take place in the blood of a patient, a chemostat culture of WT was grown at a specific growth rate (D) of 0.3 h⁻¹ and maximally 2

![Figure 2. The increase of the MIC during growth of E. coli in the presence of 2 μg/mL amoxicillin for five days and subsequently in absence for another five days. WT, E. coli MG 1655. WT-YFP, WT with genes coding for chloramphenicol resistance and a yellow fluorescent protein. In the chemostat (D = 0.3 h⁻¹), a pulse of amoxicillin reaching maximally 2 μg/mL in the culture vessel was given every 8 h.](image-url)
μg/mL amoxicillin. To simulate the usual treatment regimen of three oral doses per day over five days, the antibiotic was pumped in for one hour, reaching the maximum concentration, followed by 7 hours, during which the drug was steadily diluted. Under these conditions, E. coli builds up resistance gradually (Figure 2). The MIC was elevated by a factor of two after every 1-2 days. Still, the final MIC of 32 μg/mL is considered clinically resistant according to the EUCAST (The European Committee on Antimicrobial Susceptibility Testing) system (127).

The irreversible nature of the increase in MIC suggests that a mutation is involved, not only adaptation at the expression level. To verify this, the promoter of the ampC lactamase gene, which is known to be involved in amoxicillin resistance (84), was sequenced to detect relevant mutations for all daily samples (Table 1). The WT flask culture first developed a mutation weakening its attenuator on the third day, followed by a mutation in the Pribnow box that optimizes the promoter function (115) on the following day. The WT-YFP flask culture only acquired the

<table>
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Table 1. Mutations in the promoter region of the E. coli ampC lactamase gene. Mutations in the promoter region of the ampC lactamase gene of WT and WT-YFP strains cultivated in flasks and WT-YFP cultivated in a chemostat vessel (see Figure 2). Each data point represents two sequenced PCR reactions from separate colonies from a plated-out stablile sampled at the indicated day. Only in one case, Day 5 of the WT in a shaking flask, did the outcome of the two colonies differ. The strains obtained after five days are used for further experimentation and subsequently indicated as M8 and M8-YFP.
Effects of the developed resistance on the subsequent therapy

same Pribnow box mutation, but did so already on the second day of exposure. The other mutation was not observed. In the chemostat, however, there was no mutation detected throughout the exposure period, suggesting that adaptations at the expression level were sufficient to induce considerable resistance. The observations described above suggest that a typical five-day treatment of amoxicillin can induce resistance to a level that hampers further treatment, should this be necessary.

In order to explore whether *E. coli* cells that acquired resistance can cause treatment failure in follow-up therapy, competition experiments in a co-culture were carried out between wild-type (WT-YFP) and cells made moderately resistant (M8) or highly resistant (M256), as described above. Two sets of growth competition experiments were performed. In the first, the ratios between fully susceptible WT-YFP and the highly amoxicillin-resistant strain, M256, during growth at different amoxicillin levels were followed (Figure 3a). The second focused on the WT-YFP and the mildly resistant M8 strain (Figure 3b). The resistant and susceptible strains were mixed together in starting ratios of approximately 1:1, 1:100 or 1:1,000 and grown in medium containing 2 μg/mL amoxicillin or the same medium without antibiotics. Each line of Figure 3 represents a separate competition experiment in which changes in the ratio were monitored at 0, 3, 6 and 24 hour. In the absence of antibiotics, the ratios between the different strains remained basically unchanged, while the culture density increased by approximately a factor of 1,000. In the presence of amoxicillin, the resistant strains overgrew the susceptible. The change in the ratio was independent of the initial ratio. Changes were more drastic when M8 was competing with WT-YFP than in the case of WT-YFP and M256, indicating that at these low levels of amoxicillin, the moderately resistant strain had more advantage than the highly resistant one. Very similar results were obtained at 1 μg/mL amoxicillin (data not shown).

Since the moderately resistant M8 strain was more effective than the highly resistant M256 in outcompeting the sensitive strain, the ability of M8-YFP, made moderately resistant against amoxicillin in the same way as the M8 strain, to outgrow M256 was explored. The ratios in the co-culture were basically constant during growth. In the absence of antibiotics, the M8-YFP strains seemed to have initially a marginal advantage, if at all. The disadvantage at 4 μg/mL amoxicillin was also minute or absent. Given that the most drastic change in the ratio was a factor of 10, compared to up to 10^10 in the other experiments, the expected outcompeting of M256 by M8-YFP was effectively not observed (Figure 4).
Figure 3. Competition experiments between M8 (a) or M256 (b) and WT·YFP with different initial ratios (1:1, 1:100 and 1:10,000) in the absence or presence of 2 μg/mL amoxicillin.
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Figure 4. Competition experiment between M256 and M8 (see Figure 1) in an initial ratio of 1:1 and exposed to zero or 4 μg/mL amoxicillin.

To evaluate the effects of previous amoxicillin exposure on the efficacy of subsequent cefotaxime treatment, the potential of adaptation to cefotaxime was compared among WT, M8 and M256. The M256’s initial MIC for cefotaxime exceeded that of the WT by a factor of four; that of M8, by a factor of two. The cultures of the two biological duplicates of the M256 strain both collapsed during the adaptation, one on the eighth day, the other on the thirteenth day (Figure 5). However, the adjustment

Figure 5. The MICs as a function of time during the adaptation of E. coli strains to stepwise increasing concentrations of cefotaxime, starting at 0.06 μg/mL for WT (MIC = 1 μg/mL) and WT 5a (see Figure 1; MIC = 2 μg/mL) and at 0.5 μg/mL for the two duplicate strains of M256, indicated as M256-1 and M256-2.
MICs for cefotaxime of all tested strains were increased by three two-fold steps during adaptation. To assess whether the overexpressed beta-lactamase of the M256 strain is further induced during the development of cefotaxime resistance, the beta-lactamase activity was measured before and after adaptation to cefotaxime (Table 2). Before adaptation, the M8 and WT strain presented almost the same level of beta-lactamase activity, while M256 exhibited an activity more than 200 times higher than WT and M8. After adaptation, the beta-lactamase activities of M8 and WT remained on the same level as prior to adaptation, but the enzyme activity of M256 was negligible, indicating that E. coli’s development of resistance to cefotaxime is less likely to be caused by overexpression of beta-lactamase and furthermore suggesting that cefotaxime does not induce an increase of beta-lactamase activity. As a result, the amoxicillin resistant M256 strain started out somewhat more resistant to cefotaxime, as well, and reached higher levels of cefotaxime resistance than the amoxicillin sensitive strains before collapsing when the beta-lactamase activity decreased.

### Discussion

It is not uncommon in clinical practice that different antibiotics might be employed successively to cure a single infection after initial therapy failure (111). In case amoxicillin is not sufficiently effective, cefotaxime, a third generation cephalosporin, is sometimes applied for the follow-up treatment. Therefore, the effect of the initial amoxicillin treatment on the effectiveness of cefotaxime was

<table>
<thead>
<tr>
<th>Strains</th>
<th>Beta-lactamase activity</th>
<th>Before adaptation</th>
<th>After adaptation</th>
</tr>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
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<td>0</td>
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<tr>
<td>WT</td>
<td>21.6 ± 2.9</td>
<td>23.3 ± 4.5</td>
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<tr>
<td>M8</td>
<td>21.4 ± 3.3</td>
<td>17.2 ± 1.3</td>
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</tr>
<tr>
<td>M256</td>
<td>491.3 ± 3.6</td>
<td>3.6 ± 1.8</td>
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</tr>
</tbody>
</table>

Table 2. Specific activities of the beta-lactamase of the WT, M8 and M256 strains (see Figure 1), before and after adaptation to cefotaxime (see Figure 5). The results are presented as the means and standard deviations of three biological duplicates. For each biological replicate, two independent measurements were performed.
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addressed. In The Netherlands, resistance against cefotaxime is far less common than resistance against amoxicillin. As cefotaxime is in addition considered more potent, it is often used for follow-up treatment after amoxicillin failed to cure the infection, even though both belong to the beta-lactam class. In establishing this practice, the effects of the first treatment on the effectiveness of the subsequent one were not considered. We have explored in vitro how preceding exposure to amoxicillin influences the susceptibility of a culture to amoxicillin itself or cefotaxime as an example of another beta-lactam antibiotic. Extrapolated to medical practice, this implicates that an initial amoxicillin therapy may negatively influence the clinical outcome of subsequent treatments with the same or another beta-lactam antibiotic.

Failure of an initial amoxicillin treatment might be encountered clinically in those cases that the drug concentration reaching the infection site is lower than the optimal level (128). In an infection site, the bacterial population is likely to be heterogeneous and consisting of cells and strains possessing a range of MICs. The actual antibiotic concentration at the infection site might be lethal for some strains, while others survive, even if they are not defined as clinically resistant (128). This notion can be illustrated by the result that most wild-type cells died initially when exposed to 2 μg/mL amoxicillin, while some cells grew out after 18 h (Figure 1). These surviving cells could rapidly develop moderate, but long-lasting, resistance as the exposure to amoxicillin continued (Figure 2), in agreement with the large number of physiological and genetic changes that are induced by sublethal levels of antibiotics (82, 84). One example of these changes is the point mutation enhancing the promoter of the ampC beta-lactamase gene (Table 1). However, this is not the case for the chemostat culture, where no mutations appeared in the same region, implying that adaptation not involving AmpC beta-lactamase can also result in lasting amoxicillin resistance (84).

From a clinical perspective, prolonged or repeated treatment with a single antimicrobial drug may appear to be a poor practice, but this may be common during self-medication, particularly if antimicrobials are easily accessible, as is the case in many countries where antimicrobial drugs are available over the counter (129). From a scientific point of view, it is also useful to document the effects of repeating or prolonged treatments with the same antibiotic, even if this is clinically less relevant. The outcome of the competition experiments between wild-type cells and the same strain made moderately resistant by a simulated treatment with amoxicillin suggests that the effect of such an initial treatment on a subsequent
course is quite dramatic (Figure 3). These findings correspond well with earlier studies (67, 130) on the effects of low concentrations of antibiotics on resistance development. When both strains can grow well in separate cultures at the level of antibiotics applied, the mildly resistant strain will take over completely, even if at the start, it is only present as 0.01% of the population. This effect cannot be explained by a difference in growth rates only, and the mechanism behind this rapid take-over is presently not understood. The effect is that the lower boundary of the mutant selection window extends to a level far below the MICs of the susceptible strains, as suggested before on other grounds (69, 131). The effect of exposure to low concentrations of antibiotics is further illustrated by Figure 2, showing that a sub-MIC level of amoxicillin caused the development of resistance by a factor of 16, as the MIC jumped in two days from four to 64 μg/mL. Therefore, it seems that low levels of antibiotics may very well cause great risks of developing resistance.

The supposed fitness costs of antibiotic resistance (109) are not reflected in the competition experiments between the different strains in the absence of antibiotics, as the ratio did not change while the cell density increased by three orders of magnitude. This is in line with the conclusion of a physiological comparison of resistant and susceptible strains, that the price for resistance is not so much metabolic, but rather, a reduced ecological range (84). It also explains the effect of low concentrations of antibiotic, as a small reduction of the initial growth rate of the sensitive strain (Figure 1) is enough to have a strong influence on the ratio in the co-culture. The observation that the moderately resistant M8 strain outcompetes the sensitive strain more effectively than the highly resistant M256 strain (Figure 3) most likely is not accounted for by metabolic differences between the strains, but by the high beta-lactamase activity of the latter (Table 2). By relatively rapidly clearing the medium from amoxicillin, the M256 strain in fact removes this hurdle for the wild-type cells. Similarly, the equal growth rates of the moderately and highly resistant strains in co-culture can be understood by the elimination of the antibiotic by the beta-lactamase of the M256 strain. A comparable effect might occur in an infection site with a mixture of pathogens with different sensitivities to antibiotics, where at least one pathogen is capable of producing enzymes that lyse antibiotics efficiently.

Both moderate- and high-level resistance to amoxicillin raised the MIC for cefotaxime in the E. coli MG1655 variants. Part of this effect may be caused by the induction of high levels of AmpC beta-lactamase, due to mutations in the promoter region in the highly resistant variant (84, 115), even though the affinity of
this enzyme for cefotaxime is comparatively weak (132). The effect remains strong enough to lift the MIC in the range of clinically resistant, for the length of a standard antibiotic treatment. After some time, cefotaxime does become effective again, as this antibiotic does not induce AmpC (132) and did not maintain the induction of AmpC (Table 2), but for the outcome of the treatment, this might no longer be relevant. The observed cross-resistance of moderately amoxicillin resistant strains for cefotaxime was not caused by elevated AmpC levels (Table 2), indicating that other cellular processes are involved, as well.

The overall conclusion of this study is that exposure of pathogens to concentrations of antibiotics that fall within the mutant selection window should be avoided as much as possible. Such concentrations can be encountered not only as the result of poor medical practice, but also due to undesirable procedures in agriculture, such as giving antibiotics as a growth-promoter. Both in human medicine and in veterinary practice, considerable thought must be given to which antibiotics are used after treatment failure of the first drug. Using a second antibiotic that has the same or a similar mechanism seems imprudent.

Conclusions

The overall conclusion from the combined considerations discussed above is that the unsuccessful treatment with one antibiotic can severely hamper a follow-up treatment not only with the same, but also with another, antibiotic of the same class. The exposure to concentrations that simulate those during antibiotic therapy for a similar amount of time led to a consistent and lasting increase in the MIC, severely altering the susceptibility of the strain. A similar conclusion was reached in an in vivo situation when studying flock treatment of chickens (133). Obviously, the best way to prevent these course of events is to render the initial treatment fail-proof by increasing the concentration to a level that ascertains the death of all pathogens. Practical restrictions, such as the tolerance of the patient for the drug, may limit the maximal concentration below the optimal level.

Acknowledgments

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Chapter 4

Dynamics of mutations during development of resistance by *Pseudomonas aeruginosa* against five antibiotics

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Abstract

Pseudomonas aeruginosa (P. aeruginosa) is an opportunistic pathogen that causes considerable morbidity and mortality, specifically during intensive care. Antibiotic-resistant variants of this organism are more difficult to treat and cause substantial extra costs compared to susceptible strains. In the laboratory, P. aeruginosa rapidly developed resistance to five medically relevant antibiotics upon exposure to stepwise increasing concentrations. At several time points during the acquisition of resistance, samples were taken for whole-genome sequencing. The increase in the MIC of ciprofloxacin was linked to specific mutations in gyrA, parC, and gyrB, appearing sequentially. In the case of tobramycin, mutations in fusA, HP02880, rplB, and capD were induced. The MICs of the beta-lactam compounds meropenem and ceftazidime and the combination of piperacillin and tazobactam correlated linearly with beta-lactamase activity but not always with individual mutations. The genes that were mutated during the development of beta-lactam resistance differed for each antibiotic. A quantitative relationship between the frequency of mutations and the increase in resistance could not be established for any of the antibiotics. When the adapted strains are grown in the absence of the antibiotic, some mutations remained and others were reversed, but this reversal did not necessarily lower the MIC. The increased MIC came at the cost of moderately reduced cellular functions or a somewhat lower growth rate. In all cases except ciprofloxacin, the increase in resistance seems to be the result of complex interactions among several cellular systems rather than individual mutations.
Introduction

The medical consequences of antibiotic resistance, such as fewer options for and increased costs of treating infectious diseases, are well recognized. The pathway to resistance consists of sequential mutations or acquisition of resistance genes driven by the selective pressure caused by antibiotic exposure (82). Once resistance has been acquired, the cell rarely reverses to become sensitive again, compensating for the metabolic costs instead (50, 134). The increased level of resistance caused by an antibiotic treatment typically prescribed by primary care physicians is very noticeable when subsequent further treatment is necessary (96). Hence, in order to limit the development of resistance when antibiotics have to be used, treatment protocols need to be devised to prevent this side effect. Rational design of such protocols requires knowledge of the molecular mechanisms that cause resistance. One of the central questions is whether similar mechanisms are operational for all drugs or whether resistance to each drug is induced in a distinct manner. Other basic questions center on evolutionary pathways to clinically significant resistance and the persistence of molecular changes after treatment.

Molecular changes that cause the development and persistence of drug resistance can be identified by combining experimental evolution and whole-genome sequencing (WGS), provided that the proper controls are used (135, 136). This study used the pathogen *Pseudomonas aeruginosa* as a model to achieve this goal, as it is an important opportunistic pathogen, for example, in patients suffering from cystic fibrosis (137). Several antibiotics are used as the treatment of choice for intensive care patients infected with *P. aeruginosa*. The bacteria were adapted to the following most-often-used drugs: the fluoroquinolone ciprofloxacin, the aminoglycoside tobramycin, and the beta-lactam antibiotics ceftazidime, meropenem, and piperacillin in combination with the beta-lactamase inhibitor tazobactam. This experimental design allows the comparison of three classes of antibiotics and has three members of a single class to document the variability within a class as a biological control for the interclass comparison. The outcome suggests that the *de novo* buildup of resistance cannot be attributed only to DNA mutations but rather develops as a result of intricate interactions between cellular adaptation and mutations.
Chapter 4

Materials and Methods

Bacterial strains, growth media, and culture conditions

The antibiotic-susceptible wild-type strain *P. aeruginosa* ATCC 27853 was used as the ancestor strain in all resistance evolution experiments. Batch cultures were grown in either rich or defined minimal medium to assess the influence of the growth environment on the development of resistance. The rich medium was cation-adjusted Mueller-Hinton broth (Sigma-Aldrich) autoclaved at 115°C for 10 minutes. The minimal medium was Evans medium containing 55 mM glucose at pH 6.9 (124). Evans medium was autoclaved for 20 minutes at 121°C, with the exception of glucose, which was autoclaved for 10 minutes at 110°C and added afterward. Continuous cultures were performed only with Evans medium with the concentrations of glucose and Na₂HPO₄ lowered to 5 and 10 mM, respectively.

Precultures for the inoculation of 96-well plates, batch cultures, and continuous cultures were grown overnight in 100 ml flasks shaken at 200 rpm at 37°C. Continuous cultures were carried out in Sixfors fermenter vessels (Infors AG, Bottmingen, Switzerland) consisting of six vessels with a working volume of 250 ml at 37°C and constant stirring at 250 rpm. The pH was maintained at 6.9 by automatically adding sterilized 2 N NaOH. Culture parameters such as pH, temperature, and the stirring rate were monitored continuously. The continuous culture was assumed to have reached a steady state when all of the parameters measured, including cell density and optical density at 600 nm (OD₆₀₀), remained constant after five to seven volume changes. Samples were taken at every steady state to determine the dry weight and number of cells and the glucose concentration of the culture medium.

Evolution experiments

For experiments documenting the development of resistance, cultures were initially grown at the maximum antibiotic concentration that allowed growth. Whenever normal or approximately normal growth (OD₆₀₀ at >75% of the OD₆₀₀ for normal growth) occurred, a small aliquot of the culture was used to start two more incubations, one at the same concentration and the other at double the concentration (83). The stepwise increasing exposure to an antibiotic was stopped when the saturation level of the antibiotic was reached or continued for 30 days at most. After the adaptation, cultures were grown in fresh medium.
without drugs for 15 days to observe the sustainability of the acquired resistance after treatment. Independent duplicates were performed with each antibiotic. The MIC was determined every day for both duplicates. Daily samples were preserved at -80°C for further tests, including WGS, beta-lactamase activity measurement, and fitness evaluation. These tests were done with samples revived at the concentration of antibiotic to which they were adapted.

**Antibiotics and MIC measurement**

Five antibiotics were tested in this study, i.e., three beta-lactam antibiotics, ceftazidime, meropenem, and piperacillin, combined with the beta-lactamase inhibitor tazobactam; the aminoglycoside tobramycin; and the fluoroquinolone ciprofloxacin. The 10 mg/ml stock solutions of ceftazidime (Fresenius Kabi), meropenem (Fresenius Kabi), piperacillin-tazobactam (Fresenius Kabi), and ciprofloxacin (Fluka) were filter sterilized (0.2 µm) and preserved in a freezer at -20°C. Each stock solution of these drugs was used only once and remade freshly every week. The tobramycin was purchased in a solution of 80 mg/2 ml (Obracin), stored according to the manufacturer’s instructions, and used before the expiration date.

MICs were measured by monitoring the growth of cells exposed to antibiotic concentrations increasing by factors of 2 in 96-well plates as described previously (92). The ranges of antibiotic concentrations were adjusted according to the expected resistance level of the sample tested. All measurements were performed in duplicate. The starting \( \text{OD}_{600} \) was 0.05. The MIC was defined as the minimal concentration of antibiotic that limited growth to an \( \text{OD}_{600} \) of ≤ 0.2 after 23 hours.

**De novo sequencing and annotation of the reference strain**

The culture’s genome was isolated with the DNeasy blood and tissue kit (Qiagen). De novo sequencing of the reference strain was performed by using the Illumina and PacBio platforms at BaseClear B.V. (Leiden, The Netherlands). For Illumina sequencing, high-molecular-weight genomic DNA (gDNA) was used as the input for library preparation with the Illumina Nextera XT library preparation kit (Illumina). Briefly, the gDNA was fragmented by random transposon integration, DNA adapters with sample-specific bar codes were added, and the library was amplified by PCR. The resulting Illumina library was checked on a Bioanalyzer (Agilent) and quantified. The library was multiplexed, clustered, and sequenced
on an Illumina HiSeq 2500 by a paired-end 125-cycle protocol. For PacBio sequencing, high-molecular-weight gDNA was sheared to about 10-kb lengths with gTUBES (Covaris) and further processed into a PacBio sequencing library by standard protocols (Pacific Biosciences). The resulting PacBio library was checked on a Bioanalyzer (Agilent), quantified, and sequenced on a PacBio RSII.

The quality of the Illumina FASTQ sequences was enhanced by trimming off low-quality bases with the program bbduk, which is part of the BBMap suite, version 34.46. The quality-filtered sequence reads were assembled into a number of contig sequences with ABYSS version 1.5.1 (138). These contigs were then linked and placed into superscaffolds based on the alignment of the PacBio CLR reads with BLASR (139). From the alignment, the orientation, order, and distance between the contigs were estimated. This analysis was performed with the SSPACE-LongRead scaffold, version 1.0 (140). The gapped regions within the superscaffolds were (partially) closed in an automated manner with GapFiller, version 1.10 (141). The complete sequence was reached in one scaffold with a total size of 6,827,737 bp.

Genome annotation of the assembled contig or scaffold sequences was performed with the BaseClear (BaseClear B.V., Leiden, The Netherlands) annotation pipeline, which is based on the Prokka Prokaryotic Genome Annotation System (Victorian Bioinformatics Consortium, Melbourne, Australia).

WGS and data analysis

Whole genome sequencing (WGS) was applied at those points where the MIC significantly increased in response to drug exposure at the end of the drug exposure period and after 15 days of continued growth in the absence of the drug. Strains grown in either Mueller-Hinton broth or mineral medium for 30 days served as controls for mutations occurring during growth in the absence of antibiotics. DNA was also collected with the DNeasy blood and tissue kit (Qiagen). gDNA libraries were generated according to the manufacturer’s protocols with the Ion Xpress Plus gDNA Fragment Library Preparations (Life Technologies). Shearing of 100 ng of gDNA was performed with the Covaris M220 Focused-ultrasonicator in accordance with the 200-bp protocol provided by Life Technologies. Barcoded libraries were prepared with the Ion Plus fragment library kit (Life Technologies) and the Ion Xpress DNA bar-coding kit (Life Technologies) according to the instructions of the manufacturer of the 200-base-read Ion Proton libraries. The
Dynamics of mutations during development of resistance

size distribution and yield of the bar-coded libraries were assessed with the 2200 Tapestation System by using Agilent High Sensitivity D1000 ScreenTapes (Agilent Technologies). Sequencing templates were prepared with 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 with the Ion Proton system with the Ion PI Chip v2 and the Ion PI Sequencing 200 kit v3 (Life Technologies) according to the manufacturer’s protocols.

The FASTQ files were subjected to quality control procedures. The quality of individual samples was assessed with fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). In addition, several quality metrics (sequencing depths, read length distributions, read quality distributions, mean read quality along the read, base frequencies at each read position) were compared across the samples, in relation to the experimental factors, with in-house software based on samtools (142) and R statistical software (https://www.r-project.org/). Tmap (143) was used to map all accepted reads from all samples to the P. aeruginosa ATCC 27853 reference genome. The Ion Proton system generates sequencing reads of variable lengths, and Tmap combines a short-read algorithm (144) and long-read algorithms (145, 146) in a multistage mapping approach. The average sequencing depth was 220, with a range of 185 to 253. Deviations from the reference genome, such as single-nucleotide variants, insertions, and deletions, were identified with the standalone Torrent Variant Caller, v4.2. (Thermo Fisher Scientific, Waltham, MA). The abundance of the mutations is presented as a mutation frequency calculated as the ratio of the number of reads containing a genetic variation to the overall read number.

Possible gene duplication was searched for by aligning reads of experimental samples with the reference genome and calculating the number of reads that map for every gene. Both forward and reverse complemented reads were counted in nonoverlapping “windows” of 100 nucleotides. Counts were normalized and copy numbers were estimated with haplocln.mops, which is especially designed for haploid organisms (http://www.bioinf.jku.at/research/ehec/ehec.html) (147). Pairwise comparisons of the control and experimental samples for every gene were performed in the R programming language to calculate differences as follows: $\text{diff} = \log_2 \text{sample count} - \log_2 \text{control count}$. Genes were plotted in the order of the genome and assessed for individual genes or cluster of genes that gained a copy or was deleted. A gene was considered to have been duplicated if the ratio exceeded 1, representing a single duplication, and it occurred in more
than one experimental sample. Applying these standards, no gene was found to have been duplicated.

To distinguish mutations caused by sequencing errors or selected for by the growth medium, the detected genetic variations were excluded in the final analysis when one of the following conditions applied: (i) the Phred quality score was <20, (ii) the depth was <100, (iii) mutations appeared only once and at a frequency of <10%, or (iv) mutations also occurred in cells growing in the absence of drugs for 30 days. Mutations with Phred quality scores between 10 and 20 and/or sequencing depths between 40 and 100 were included when they were found more than once.

**Reproducibility of mutations detected**

WGS of one of the duplicate strains was performed, and PCR was used to ascertain the presence of these mutations in the other replicate at the same time points. The primers used are shown in Table 1. The PCR products of six colonies for each combination of antibiotic and time point were purified with the MSB Spin PCRapace kit (Invitek) and sequenced by Macrogen Europe by Sanger sequencing with an ABI 3730XL DNA analyzer.

**Beta-lactamase assay**

Beta-lactamase activity was measured with an assay based on the chromogenic substrate nitrocefin (125). Briefly, 1 ml of culture was harvested and washed in buffer (pH 7.0). The cells were lysed in 1% Triton X-100, and the lysates were centrifuged. Beta-lactamase activity was determined as the rate of nitrocefin hydrolysis with protein as the normalization factor.

**Assessment of fitness cost**

To determine maximum growth rates ($\mu_{\text{max}}$) the growth of batch cultures was monitored for 23 hours by measuring $\text{OD}_{600}$. The $\mu_{\text{max}}$ was calculated as the average growth rate of four independent replicates during exponential growth. Dry weight was calculated as the added weight on a preweighed filter dried overnight at 110°C. Cell number was quantified by counting colonies on antibiotic-free agar plates after dilution. Glucose concentrations were determined enzymatically.
Dynamics of mutations during development of resistance

Results

To explore the role of mutations in the de novo development of resistance by *P. aeruginosa*, cells were made resistant to medically relevant antibiotics and WGS was performed at several time points during this process. Resistance to three beta-lactam antibiotics, ceftazidime, meropenem, and piperacillin, in combination with the beta-lactamase inhibitor tazobactam; to the aminoglycoside tobramycin; and to the fluoroquinolone ciprofloxacin was induced. Identification of the mutations that accompany the acquisition of resistance allowed a comparison of the potential differences within the beta-lactam class of antibiotics and the expected differences between the representatives of separate classes. Since preliminary experiments showed that the acquisition of resistance differs when *P. aeruginosa* is grown in mineral or rich medium, both media were used.

None or barely any resistance to the beta-lactam antibiotics developed during growth and exposure in mineral medium (Figure 1). There was no meaningful difference in the development of resistance to tobramycin and ciprofloxacin in mineral or rich medium (compare Figure 1 and 2). In rich medium, the MIC of a drug for *P. aeruginosa* increased within 20 to 30 days by 7 to 11 2-fold steps, depending on the drug (Figure 2). Once the drug was removed and growth continued in its absence, the MIC decreased slightly or not at all. The difference between the replicates in the acquisition of resistance was meaningful only in the case of the 8-fold lower MIC of piperacillin-tazobactam for one replicate than for

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Table 1. Sequences of the primers used for PCR of the areas of interest within the genes indicated

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<th>Gene</th>
<th>Primer sequence (5'-3')</th>
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<tr>
<td><em>ptrB</em></td>
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<tr>
<td><em>pyrD</em></td>
<td>GAGCAGAAAAGCCCAGAG / CGAGGAAAGGTSACTACA</td>
</tr>
<tr>
<td><em>gya</em></td>
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<tr>
<td><em>pykB</em></td>
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<tr>
<td><em>PMP356</em></td>
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<td><em>UN22880</em></td>
<td>CGCGGCTGTTT / GCGTTRIGCG</td>
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<tr>
<td><em>rubB</em></td>
<td>GGGCCAGCGG3TNA / CTTGGG3EGTST</td>
</tr>
<tr>
<td><em>cppB</em></td>
<td>MTTGCGGAGGCAA / AAA GCA GTC GCT TC</td>
</tr>
</tbody>
</table>

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59
the other. The MIC of ceftazidime decreased for one replicate after exposure was ended but not in the other.

At 4 or 5 time points (indicated with red arrows in Figure 2), WGS of one of the replicates was performed with a sequencing depth of roughly 200 times. The hypothesis was that mutations would appear at a low frequency in the beginning and over time become dominant. The actual outcome differed from that expected to various degrees (Figure 2). In the figures, mutations are omitted that appeared only once and at a frequency of <10%. The presence of the same mutations in the replicate cultures was checked by sequencing PCR products of the DNA in the region around the mutation. Unless specifically mentioned, the first and second replicates had the same mutations. The theoretical possibility that additional mutations had occurred in the replicate strain was not verified experimentally.

The accumulation of mutations was comparatively straightforward when cells were exposed to ciprofloxacin. After five transfers, the usual T83I gyrA mutation for fluoroquinolones (85) appeared, accompanied by a mutation in CPA2 (Figure 2A). After another seven transfers, the CPA2 mutation disappeared entirely and two parC mutations emerged in less than half of the population. Analysis of the individual reads, averaging 200 bases, suggests that the neighboring parC mutations never occurred in a single chromosome. In the next stage, when the highest MIC had been reached, only one of the two parC mutations, P85L in the

![Figure 1. Antibiotic resistance development by P. aeruginosa expressed as 2-fold increases (Incr.) in MICs as a function of time (days) in minimal medium with stepwise increasing concentrations of the antibiotics indicated.](image-url)
Dynamics of mutations during development of resistance

Figure 2. Increase (Incr.) in the MIC by factors of 2 and frequencies of mutations observed in the replicates on which WGS was performed as a function of time (days) in the presence of the antibiotic indicated and after its removal from the growth medium. All mutations found at frequencies exceeding 0.5 were also discovered in the second replicate by PCR, except for the parC mutations in response to ciprofloxacin, where P85L in the first was replaced by S87N in the second. Piper, piperacillin; Tazo, tazobactam.

first replicate and S87N in the second, remained and a gyrB mutation appeared in the whole population. Even though these mutations remained, the MIC was
slightly reduced during growth in the absence of ciprofloxacin. Exposure to tobramycin immediately yielded a mutation in *fusA* in the whole population and a low frequency of mutation in *HP02280* (Figure 2B). Both were found at a frequency of one a few transfers later, accompanied by a mutation in *rplB*. When the highest MIC was reached, a new *capD* mutation attained a frequency of almost one as well. After 15 transfers in drug-free medium, the *HP02280* mutation disappeared and the MIC was considerably reduced.

The picture for the three beta-lactam antibiotics was considerably more complex. No persistent mutations were observed in the initial stages, when considerable resistance had already developed. When cells were already almost completely resistant to the combination of piperacillin and tazobactam, two mutations were observed, the usual one in *ampC* and an unknown single-nucleotide polymorphism in a noncoding region (Figure 2C). Both remained when the antibiotic pressure was removed, while the MIC remained unchanged. Growth in the presence of meropenem initially resulted in one synonymous mutation with a frequency of one in a hypothetical protein (Figure 2D). This mutation was no longer observed afterward. Instead, a low-frequency mutation appeared in *oprD*, which codes for a membrane channel known to be involved in meropenem resistance (148). In the next stage, at an intermediate MIC, the frequency of none of the six observed mutations exceeded 0.5. The mutations occurred three times in *oprD*, twice in *mexR*, and once in a noncoding region. None of the different

![Image](image.png)

Figure 3. (A) 13-Lactamase activities measured in cells adapted to the beta-lactam antibiotics indicated and after subsequent growth for 15 days in the absence of the drugs. The unit of 13-lactamase activity is nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. (B) Increase (Incr.) in the MIC as a function of 13-lactamase activity. The correlation between 13-lactamase activity and MIC is statistically significant for the two antibiotics indicated with asterisks. Piper, piperacillin; tazo, tazobactam.
Dynamics of mutations during development of resistance

mutations detected in a specific gene occurred together on the chromosome of a single cell. In cells with the highest MIC, mutations in oprD, mexR, mexB, and a noncoding region had taken over the entire population and attained a frequency of one. After removal of the drug, the MIC remained unchanged at high levels, but two new mutations were detected, while the earlier oprD mutation decreased to a very low frequency. The exact same synonymous mutation that was observed at the start of the meropenem experiment also appeared upon exposure to ceftazidime (Figure 2E). While the MIC increased steadily, low-frequency mutations appeared and disappeared. Only at the very end, when the MIC had decreased because of removal of the drug, was a mutation in prkC, which codes for a serine/threonine-protein kinase, detected in the entire population.

The experimental samples were searched for evidence of gene duplication or deletion, but none was found, indicating that under these experimental conditions, only point mutations occurred.

Strains adapted to ceftazidime maintained their beta-lactamase activity when grown in the absence of a drug, but meropenem- and piperacillin-tazobactam-adapted cells did not (Figure 3A). Since the MICs of the latter two antibiotics did not decrease during growth in their absence, beta-lactamase activity cannot be the sole factor determining the MICs. Still, there is a considerable correlation between the measured beta-lactamase activity and the increase in the MICs of the three beta-lactam antibiotics (ceftazidime, \( R^2 = 0.926 \); piperacillin-tazobactam, \( R^2 = 0.921 \); meropenem, \( R^2 = 0.635 \) ) (Figure 3B). This correlation does not necessarily mean that beta-lactamase activity determines the MIC of this class of antibiotics. The positive cross-resistance of only two to four 2-fold steps (Table 2) suggests that a common mechanism is merely part of the total beta-lactam resistance system. Cross-resistance with the antibiotics of the other classes is either absent

<table>
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<th>Ceftazidime</th>
<th>Meropenem</th>
<th>Piperacillin-tazobactam</th>
<th>Tobramycin</th>
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<td>-2/+3</td>
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Table 2 Cross-resistance after drug exposure/removal*  *Shown are the increases (+) or decreases (-) in the MICs of the antibiotics at the top upon acquisition of resistance to the antibiotics on the left. After P. aeruginosa became resistant to tobramycin, it was more sensitive to the other antibiotics. *3 – no change in the MIC.
or negative. The negative cross-resistance of tobramycin-adapted cells indicates that becoming resistant to this antibiotic increases sensitivity to the others.

The costs of resistance for the cell were measured as a decrease in $\mu_{\text{max}}$ (Figure 4A) and as maintenance energy, defined as energy metabolism devoted to purposes other than growth (Figure 4B). Resistance to all antibiotics caused some decrease in $\mu_{\text{max}}$. This decrease was statistically significant in cells adapted to ceftazidime, meropenem, and tobramycin. Ceftazidime-adapted cells had the lowest $\mu_{\text{max}}$ of 0.85 h$^{-1}$, compared to 1.15 for the wild type. Maintenance energy could only be

![Figure 4](image_url)

**Figure 4.** $\mu_{\text{max}}$ and maintenance energy of strains adapted to the antibiotics indicated immediately after growth in the presence of the drugs (after exposure) and after 14 days of subsequent growth in their absence (after removal). An asterisk indicates that the growth rate was significantly lower than that of the wild type, while double asterisks indicate that the growth rates before and after growth in the absence of the antibiotic differed significantly. Panels: A, $\mu_{\text{max}}$ in duplications (Piper, piperacillin; tazo, tazobactam); B, estimation of the maintenance energy by extrapolation to $D$ (specific growth rate in duplication per h) = 0 of the specific glucose (gluc) consumption measured at several dilution rates; C, $\mu_{\text{max}}$ of wild-type *P. aeruginosa* and cells adapted to tobramycin (TBM) in the presence or absence of the drug as a function of pH.
measured in cells adapted to ciprofloxacin and tobramycin, because only these grew in mineral medium. In rich medium, too much glucose and other potential carbon and energy sources are available to use the carbon and energy source as a growth rate-limiting factor. The results indicate an increase in the maintenance energy by up to 0.3 mM of glucose/10^{10} cells/h when cells become resistant to ciprofloxacin and roughly half of that amount in the case of tobramycin. Taken together, these observations indicate that \textit{P. aeruginosa} pays a modest metabolic price for becoming resistant to the antibiotics tested.

Induced resistance in \textit{Escherichia coli} came at the price of a reduced ability to grow under less-than-optimal conditions (84). Therefore, growth at nonoptimal pHs and salt concentrations was measured in \textit{P. aeruginosa} as well. Only cells adapted to tobramycin had a reduced pH range (Figure 4C), indicating that regulation of the internal pH interfered with counteraction of the drug. In all other cases, growth rates at different pHs and salt concentrations were similar to those of the wild type (data not shown).

\textbf{Discussion}

The outcome of this study suggests that \textit{P. aeruginosa} acquires resistance to the five antibiotics tested by different genetic mechanisms. WGS did not reveal mutations common to all five types of resistance, not even when limited to the three beta-lactam antibiotics. Some of the genes that were mutated were identified as resistance genes in \textit{P. aeruginosa} before (149, 150). There was no clear correlation between the frequency of specific mutations and the increase in the MICs of any of the antibiotics tested, though for fluoroquinolone resistance, the \textit{gyrA} mutation is always required. Below, we will argue that all of the data combined indicate that, as shown for \textit{E. coli} (84, 85), mutations alone cannot explain the development of resistance but that an intricate interaction between the adaptation of cellular systems and the DNA mutation level leads to higher levels of resistance.

Intrinsic resistance of \textit{P. aeruginosa} to fluoroquinolones is due to four RND-type drug efflux pumps of the Mex-Opr families (151), which were not affected during \textit{de novo} development of ciprofloxacin resistance in this study. In a hospital setting, fluoroquinolone use did not correlate with the occurrence of the \textit{gyrA} and \textit{parC} mutations found in this study (152). Therefore, it seems that several distinct mechanisms can neutralize the effects of fluoroquinolones on \textit{P. aeruginosa}. 
The role of the initial CPA2 mutation is unclear, but mutations often co-occur without an obvious functional relationship during the adaptation to changing environmental conditions in P. aeruginosa (153).

Cell elongation and clustering were shown to be essential steps during the initial development of resistance in P. aeruginosa (154). Therefore, the role of fusA, which codes for an elongation factor, in the initial protection against tobramycin can be understood in terms of its function in biofilm formation by Pseudomonas chlororaphis (155). The rplB gene codes for a 50S ribosome-associated L2 protein that is known to be involved in bactobolin resistance (156). The effect of the rplB mutation may be exerted through interaction with the 30S target protein of aminoglycosides (151). The CapD protein has a function in type 1 capsular polysaccharide biosynthesis (157) and therefore can have an indirect effect on the cellular access of aminoglycosides.

The cefotolozane-tazobactam combination caused several mutations during the development of resistance in P. aeruginosa (158), but the ampC mutation was the only functional one observed when piperacillin-tazobactam was used in this study. Since this mutation appeared at the very last MIC increase stages, its contribution may not have been very important. Possibly, the induction of increased beta-lactamase activity did not involve DNA mutations but was the result of increased expression that remained at higher levels even after many generations, as found in E. coli (84). In contrast, the highest levels of meropenem resistance correlated with mutations in the well-known resistance genes of the oprD and mex families (159). There are, however, several other genes involved in resistance to beta-lactam antibiotics (160, 161) that did not show mutations in this study. The dacB mutations observed at low frequencies during the development of resistance to ceftazidime influence the expression of AmpC beta-lactamases (162). The ampD mutations observed after the growth of cells made resistant to ceftazidime in antibiotic-free medium fulfill a role in peptidoglycan synthesis (163). The mechanism by which the two mutations in yerD, which codes for the large subunit of glutamate synthase, interact with ceftazidime resistance is unclear. It is possible that genetic hitchhiking is the cause of these mutations, rather than a functional relationship (164).

While development of resistance to tobramycin and ciprofloxacin seems to be a more or less linear process with two unsuccessful mutations in the initial stages in the case of ciprofloxacin, the process is less straightforward in the case of beta-
lactam antibiotics. In the latter case, the diverse-community model (165, 166) seems more applicable, in particular in the case of ceftazidime. In the early stages of development of resistance to meropenem and ceftazidime, multiple mutations in different genes are observed at frequencies of less than one and sometimes even at very low frequencies. Either the mutated cells also provide neighboring cells with protection against the antibiotic (99) or it represents evolution by trial and error. Alternatively, the well-documented regulation at the level of cellular processes (84, 103) may be the origin of the initial increase in the MIC observed in a similar manner in the case of all five antibiotics tested.

The fitness costs of resistance can come as a reduced growth rate, lower pathogenicity (50, 109), or a reduced ecological range (84). The $\mu_{\text{max}}$ of *P. aeruginosa* was affected less than its maintenance energy, indicating that the operation of, for example, efflux pumps requires additional energy (167). The increased maintenance energy was observed in the absence of antibiotics, indicating a more or less permanent change in metabolism. The moderate effect on $\mu_{\text{max}}$ is in agreement with observations on clinical strains (52). Whether the acquisition of resistance in vitro also influences pathogenicity, as in these clinical strains, is unclear. No mutations were observed in known pathogenicity genes, but expression levels may have been affected.

The overall conclusion from the comparison of the development of resistance to five antibiotics by *P. aeruginosa* is that no common mutations or mechanisms can be discerned. Only resistance to ciprofloxacin is built up in a straightforward manner by mutations in the genes coding for the target proteins. The genes affected are different for each of the three beta-lactam antibiotics. While this does not exclude the possibility of a common mechanism regulating the acquisition of resistance, as would be expected, assuming a common killing mechanism for bactericidal antibiotics (3, 168), it does not support that notion either. The lack of correlation between the persistence of mutations and the MIC after removal of the antibiotic from the growth medium indicates that these mutations are not the sole factor determining the MIC.

**Acknowledgements**

We thank C. Schultz, C. J. Hodiamont, and R. M. Van Hest for stimulating discussions. This study was financed by the Netherlands Food and Consumer Product Safety Authority.
Chapter 5

Optimization of therapy against *Pseudomonas aeruginosa* with ceftazidime and meropenem using chemostats as model

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This chapter has been submitted
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Abstract

*Pseudomonas aeruginosa* is an opportunistic pathogen that can cause life-threatening infections in patients admitted to intensive care units. Resistance rapidly develops against the drugs of choice, ceftazidime and meropenem. Several therapeutic protocols were compared, to test which protocol leads to the optimal combination of reduction in cell density and limiting resistance. The insights gained can contribute to the design of optimal treatment protocols. Chemostat cultures were exposed to ceftazidime and meropenem concentrations measured in the blood of patients at low (5th percentile), medium (50th percentile) or high (95th percentile) levels in a variety of therapy protocols to simulate treatment of infections. Cultures exposed to ceftazidime recovered after 1 day at low, 2 days at medium and 3 days at high concentrations and developed corresponding levels of resistance. Patterns were very similar for meropenem except that recovery started at day 1, 3 and 5 with the low, medium and high concentrations respectively. Fluctuating levels and intermittent treatment achieved similar reduction of cell numbers, but caused less resistance. Treatment alternating ceftazidime and meropenem reduced cell numbers more than monotherapy, while strongly limiting development of resistance. Combination therapy was even more effective in both respects. Therapeutic goals are best reached with least risk for development of resistance when ceftazidime and meropenem are used in combination or alternating, at the highest concentrations the patient can endure. Monotherapy should also apply the highest concentration that is safe for the shortest time that achieves the treatment objective.
Introduction

Antimicrobial resistance (AMR) renders previously life-saving drugs less and less effective (19, 169) and few new antibiotics are expected to enter the market in the near future. Even if new antibiotics were to be introduced, development of resistance is always a risk to be mitigated as much as possible (4). Multidrug-resistant Gram-negative bacteria cause additional suffering and increased treatment costs compared to their susceptible counterparts (170). Optimizing treatment strategies in such a manner that the therapeutic goal is achieved while minimizing development of AMR, is one way to prolong the useful life of existing antibiotics (171, 172). Optimal treatment regimens in terms of the height of the dose and the duration of the treatment course have long been recognized as crucial to preventing the emergence of AMR strains (67, 68). Multi-drug resistant (MDR) Pseudomonas aeruginosa is a major problem during treatment of critically ill patients in intensive care units and therefore P. aeruginosa was chosen as model organism for this study on optimization of treatment protocols.

Genetic mutations underlie de novo acquisition of resistance during antibiotic treatment (173), hence prevention of DNA mutations will forestall development of AMR. According to this line of thought, the concept of the mutant prevention concentration (MPC) was introduced by Drlica and Zhao (69, 70). The MPC can be derived from pharmacokinetic/pharmacodynamic (PK/PD) parameters including minimal inhibitory concentration (MIC) and area under the time-concentration curve (AUC) (174). Treatment strategies to prevent build-up of AMR have been proposed based on these principles (72, 175). Simulation of patient treatment in chemostat cultures using actual patient PK/PD data showed that the development of resistance by P. aeruginosa observed in intensive care units (ICU) can be explained by de novo acquisition of AMR (154).

One of the strategies suggested for the prevention of the development of AMR is the use of alternating or combination treatments with antibiotics belonging to different classes (176, 177). The rationale behind this idea is that adaptation to one drug will impede acquisition to another one from a different class. Since the adaptation of P. aeruginosa to two beta-lactam antibiotics, ceftazidime and meropenem, involves very different mutations (173, 178), alternating between these two drugs might also be beneficial. Just as in reaction to exposure to antibiotics from different classes, collateral sensitivity might develop (60). Both in hospitals and in an experimental setting, such a strategy to clear infections while
limiting AMR, has shown promise (176, 177). Data actually demonstrating that
drug-cycling can be successful in preventing AMR development during antibiotic
treatment are scarce (179, 180). Some evidence suggests that combining two
or more drugs at the same time prevents increase of resistance (62, 64, 73, 74),
while other lines of analysis reach the opposite conclusion (65, 66).

One of the main challenges in comparing different studies is the wide variety
of organisms, antibiotics, concentrations and modes of exposure that have been
applied. Therefore, a comparison of different treatment strategies within a fixed
experimental setting, using the same target organism and antibiotics throughout,
is likely to provide useful insights. We studied ceftazidime and meropenem
treatment protocols, two first-line antibiotics for treatment of *P. aeruginosa*
infections in intensive care units, in chemostat cultures of *P. aeruginosa*, with the
aim to determine the optimal therapeutic efficacy (reduction in cell density) while
limiting development of resistance. As such, we aimed to provide experimental
evidence to support proposals for changes in patient treatment protocols. The
comparison of the different treatment protocols suggests that applying the highest
exposure that is safe for the patient for the shortest time needed to achieve the
therapeutic objective, is the best way to prevent build-up of AMR. Combining or
alternating two antibiotics, even if they belong to the same class, provides an
additional barrier for development of AMR.

**Material and Methods**

**Bacterial strain, growth medium and culture conditions**

The antibiotic-susceptible wild type strain *Pseudomonas aeruginosa* ATCC27853
was used for all experiments. Cultures were grown on cation-adjusted Mueller
Hinton Broth (Sigma-Aldrich) autoclaved at 115 °C for 10 minutes. Continuous
cultures to simulate growth of *P. aeruginosa* in patients were performed in Sixfors
fermenter vessels (Infors AG, Bottingen, Switzerland) with a working volume of
250ml, an airflow of 0.10 liter/min and a stirring rate of 200 rpm, operating at 37
°C. The pH was maintained at 7.0 by automatically introducing sterile 2 N NaOH.
The dilution rate was set at 0.3 h to mimic the growth rate of pathogens expected
at infection sites. The culture was assumed to have reached steady state when
after at least 5 volume changes optical density (OD) remained stable. It was
experimentally ascertained that neither glucose nor oxygen was a growth rate
limiting factor. During antibiotic treatment samples were taken every 24 hours to
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follow cellular parameters and AMR level.

Antibiotic treatments

Two first line anti-P. aeruginosa antibiotics, ceftazidime (Fresenius Kabi) and meropenem (Fresenius Kabi), were chosen for in vitro simulation of treatments. As both drugs are reported to be chemically unstable, fresh solutions were prepared whenever needed by dissolution in water followed by filter (0.2 µm) sterilization. Three concentration levels, labelled “low”, “medium” and “high” for brevity were tested, representing the 5 (8 mg/l for ceftazidime, 0.6 mg/l for meropenem), 50 (24 mg/l for ceftazidime, 5 mg/l for meropenem) and 95 percentile of drug concentration ranges (48 mg/l for ceftazidime, 15 mg/l for meropenem), observed in blood of ICU patients (154) after a standard clinical dosing regimen (1000 mg iv loading dose followed by a continuous infusion of 3000 mg over 24 hours for ceftazidime and a 1000 mg iv bolus infusion administered over 30 minutes three times per day for meropenem). The drug was injected directly in the culture during pulse treatment, or added to the culture medium stock bottle when continuous infusion was required. The medium stock solution was used maximally for 3 days if the antibiotic was introduced by injection. The stability of ceftazidime in Mueller Hinton Broth was tested at room temperature and no significant decrease of antimicrobial activity was observed over three days. Decay of meropenem was not observed over a 3 days period as antimicrobial activity was equal for newly made solutions and one kept at room temperature during that time. Switches between different concentrations or drugs during treatment were realized by programmed timers which were connected to the medium pumps. Each experiment testing a treatment strategy was performed in duplicate to detect biological variation.

Cell density and minimum inhibitory concentration (MIC)

Therapeutic effects of specific treatments were evaluated by following cell density. Cell density was assessed as OD$_{595}$ counting under the microscope and counting colonies on antibiotic-free agar plates after dilution. At least two duplicates were applied for cell counting of each sample.

MIC was measured by following growth of cells exposed to 2-fold serial diluted concentrations of antibiotic in 96-well plates as described before (92). The maximum concentration of antibiotic was chosen according to the expected susceptibility of the test culture. At the start of the measurements OD$_{595}$ was 0.05,
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equivalent to $10^5$ cells. The MIC value was defined as the minimal concentration that limited growth to an OD$_{595}$ of 0.2 or less after 23 hours. All measurements were performed in duplicate and reported as the mean value.

**Amplification and sequencing of oprD gene**

Samples for PCR were taken from randomly selected colonies growing on cell count plates inoculated at different time points, to detect the mutations within the $oprD$ gene. The gene was amplified by PCR and sequenced with 5-CTGGTGCTTTAGTTAG-3 as forward primer and 5-CTACGCCCTTCCCTTTATA-3 as reverse primer. PCR reactions were performed in 50-μl working volumes using Taq DNA polymerase 4 (Thermo scientific). The procedure was: denaturation at 95°C for 5 minutes, followed by 30 cycles of 35 seconds at 95°C, 55 seconds at 56°C and 90 seconds at 72°C, and finally 90-seconds extension at 72°C. The PCR products were purified by MSB Spin PCRapace kit (Invitek) and sequenced by Macrogen Europe.

**Results**

Treatment of *Pseudomonas aeruginosa* was simulated in chemostats with the aim to mimic conditions at infection sites more realistically than batch cultures would do, because cells growing at high rates are more sensitive to antibiotics than those growing slowly.

**Ceftazidime**

Cultures exposed to “low” concentrations of ceftazidime, corresponding to the lowest 5 percentile encountered in blood of ICU patients, recovered rapidly after an initial 100 fold decline in cell numbers (Figure 1). At medium (50 percentile) and high (95 percentile) levels the recovery started after 48 and 72 hours respectively. In all cases cultures fully recovered in less than the week a treatment in the ICU minimally lasts. By then, the MIC of the culture exposed to the low treatment had increased 32-fold and the MIC of those encountering the other two regimens more than 100-fold.

Plasma concentrations of antibiotics are unlikely to be constant for long. Therefore changes in blood levels were imitated by alternating low and high concentrations in 12 hour cycles. When the initial exposure is high, the recovery of the culture occurs slightly later than when treatment is started with the low level (Figure 1). The final level of resistance is the same and equal to the MIC.
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Figure 1. Time-kill kinetic and resistance development in *P. aeruginosa* upon exposure to constant, fluctuating and intermittent treatment of ceftazidime. L, M and H indicate the low (5%), median (50%) and high (95%) concentrations expected in ICU patients respectively when the clinically used dosing regime (1000 mg IV loading dose followed by a continuous infusion of 3000 mg over 24 h) is administered. Results of constant concentrations are shown in section A. In section B, 12-hour H and 12-hour L were cycled. Intermittent treatment is given in C where a high concentration pulse was given every 12 hours or a 2-days constant treatment was followed by a 1-day absence of antibiotics. The results below were obtained by calculating the average value of two independent replicate experiments with each measurement as a technical duplicate. The error bars indicate the standard error of the 4 measurements.

of cells after the constant low level exposure. Variations in treatment protocols that might be needed because of the situation of a specific patient were imitated by pulse treatment with 12 hour intervals and by constant levels for 48 hours followed by 24 hours without antibiotics. Cells exposed to intermittent levels of ceftazidime overcame the treatment after one day and became equally rapidly resistant. The cells treated with an antibiotic free interval never reached the control density completely and became resistant only after 7 days (Figure 1).
Figure 2. Killing kinetics and resistance development in *P. aeruginosa* upon constant, fluctuating or intermittent exposure to meropenem. All symbols are defined as in Figure 1. The clinical dosing regime simulated is a 1000 mg iv bolus infusion administered over 30 minutes three times per day. * indicates that this data point the culture recovered in one experimental run to cell density around $10^8$ CFU/ml but remained at $10^6-7$ CFU/ml in the other.

**Meropenem**

The exact same experiments as were performed exposing *P. aeruginosa* to ceftazidime were also carried out with meropenem (Figure 2). Exposure to constant concentrations of meropenem yielded almost the same results as for ceftazidime, except that recovery started later and took longer to complete. Recovery started only after 5 days at the high level, compared to after three in the case of ceftazidime. In the case of meropenem, exposure to high (95%) concentrations also resulted in more than 100-fold of resistance. When the concentrations fluctuated, starting with low concentrations delayed development.
of resistance by about one day. Intermittent treatment led to almost immediate recovery and considerable resistance, but the interrupted treatment did not allow complete recovery and caused only 8-fold increase in resistance, against more than 100-fold after constant treatment for 7 days.

Development of resistance to meropenem in *P. aeruginosa* is known to be accompanied by DNA mutations in the *oprD* gene that appear at some point in time after exposure to meropenem (173). To examine whether the same *oprD* mutations

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</tbody>
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Table 1. Mutations in *oprD* gene of *P. aeruginosa* upon exposure to different meropenem treatment strategies simulated in chemostat. Three randomly selected colonies were tested for each of the two independent populations. The results for the second replicate are shown in italics with the color grey. The number of the colonies in which the mutations were found is indicated in superscript. Symbol “-” indicates that no mutations were found. No mutations were identified in *oprD* while meropenem was alternated or combined with ceftazidime.
appeared regardless of treatment regimen, PCR products of this gene were sequenced every day for every treatment to follow appearance of the mutations (Table 1). Many different mutations appeared and disappeared. Some of these were unique to a single day and condition, while others appeared at several time points in more than one condition. Part of this variation may be due to the fact that three colonies were picked for each measurement, but largely it reflects the trial and error mutagenic process also found in other experimental systems. Figure 3 presents an overview of the number and nature of mutations in the oprD gene. Stop codons are remarkably often introduced, 16 times. Deletions, insertions and substitutions causing amino acid changes occur in comparable numbers (8, 11 and 12 times respectively). By far the highest number of mutations (19) occurred after constant exposure to low concentrations. The treatment regimens based on high levels caused 3 mutations each, but different ones (Figure 3; Table 1).

**Ceftazidime-meropenem combination**

Treating patients with two antibiotics can be achieved by giving both simultaneously or by alternating between them. Cells exposed to low levels of

![Figure 3](image-url)
Optimization of antibiotic therapy

Figure 4. Killing kinetics and resistance development in *P. aeruginosa* upon exposure to the combination of ceftazidime and meropenem. Symbols as in figure 1. Only low and medium concentrations levels were assessed, as the high concentrations eliminated the culture from the chemostat. The average values are presented of two replicate experiments analyzed in duplicate. The error bars indicate standard errors.

both drugs started recovering after two days, compared to one day when only one of each was applied (Figure 4). Not much resistance was developed against either antibiotic, except for a 50-fold increase of MIC against ceftazidime in cells treated with the low concentrations. Medium levels of both drugs kept the cell number down by a factor of between 100 and 10,000 for the entire 7-day period. After exposure to medium levels of both drugs, the resistance was far lower than after constant exposure to medium levels of the single drugs.

Starting alternating treatments with ceftazidime was more effective than starting with meropenem in the sense that recovery started later (Figure 5). Most resistance against ceftazidime, just as much as after constant exposure to high concentrations of ceftazidime only, developed after cycling the two drugs at low concentrations. This was unexpected in the light of all other experiments where low level exposure caused low level resistance. Relatively little resistance built up against meropenem. Starting with meropenem allowed slightly faster recovery of the culture, but in the case of medium concentrations, not a complete one in the 7 days of the experiment (Figure 5). Cycling low levels led to an 8- to 50-fold increase in ceftazidime resistance, independent of the initial antibiotic. Meropenem resistance at the end of the 7-day period was intermediate.

**Discussion**

*De novo* development of antimicrobial resistance by *P. aeruginosa* occurs as result of simulated antibiotic treatment, applying concentrations of antibiotics as they are expected in blood of ICU patients (154). Fast growing cells are more sensitive to antibiotics than slow growing cells (181). Therefore chemostat cultures make
a better model for infections than batch cultures, because less than maximal growth rates expected in infection sites can be achieved. Overall, the controlled conditions and limited growth rates in chemostats imitate the conditions in patients best. It must be kept in mind though that the chemostat has its limitations as model, because the influence of the immune system on the overall outcome cannot be incorporated. The present study compared several treatment regimens with the aim of proposing potential treatment protocols that achieve the therapeutic goal of optimal reduction in cell density at minimal development of resistance.

Remarkably, the worst outcome is that of the standard therapeutic protocol applied during the 7-day period usual in the ICU for ventilator-associated pneumonia, in the sense that the bacteria are not optimally eliminated and formidable development of resistance occurred. If treatment at the concentration corresponding to the high level (95 percentile of expected drug concentrations) could be stopped after 3 days, the best outcome achievable with constant concentrations would be reached, while the increase of the MIC is limited to a
factor of approximately 10. Over the entire 7-day period, the therapeutic result of the high level can be obtained by alternating high and low levels, but the final resistance is lower in this case (Figure 1 and 2). Initial high concentrations seem optimal to prevent mutations (173). This corresponds with the notion that not only the MIC of susceptible cells is relevant to predict treatment outcome, but that the elimination of potential persisters requires an exposure exceeding the MIC (68, 73, 74). The known mutant prevention concentration (MPC) (154) was exceeded here, but still cells survived and became resistant. This implies that dosing above the MPC, which is determined using 2-days assays, does not prevent resistance development during longer exposure, and hence previously proposed strategies based on MPC (69, 70, 72, 175) may not always be effective, unless persister cells are eliminated by the immune system. The early appearance of mutations during constant exposure in this study provides supporting evidence for this conclusion.

The effectiveness of the combination and alternating treatments is demonstrated by the complete elimination of all cells when high (95%) concentrations were applied, a result not attained with either meropenem or ceftazidime alone. Alternating two drugs is known to impede the evolution of resistance towards either or both of the drugs, especially those exhibiting collateral sensitivity (60, 61, 177). Even though the two drugs used here were from the same class, both the interaction with the cell and the mutations that accompany resistance against the antibiotic (173) were sufficiently different for the two drugs to have synergistic effects. A comparable synergism between two antibiotics of the same class applied in the framework of a single treatment was also shown for aminoglycosides (182). These conclusions are in agreement with other lines of evidence (62, 64, 74). The efficacy of the application of two drugs alternating or combined is further illustrated by strongly reduced final resistance, which reached levels comparable to those of monotherapy only in the case of ceftazidime at low levels of exposure. However, even when dual therapy is applied the higher concentrations are far more effective, both in reducing bacterial cell numbers and in preventing resistance.

Development of resistance against meropenem is associated with mutations in oprD (173). In line with the notion that low concentrations select most for resistance, the highest number of mutations was found in cells exposed to low concentrations. The prevention of any mutations by combination and alternating treatments and the low number of mutations observed in the fluctuating and intermitted treatments plead for further investigations to enable their
incorporation in therapeutic protocols. This suggestion is supported by similar comparisons of single and multiple drug regimens (61, 62). The variety in conditions in this study resulted in a far larger number of mutations than found after a step-wise increase of the meropenem concentration. This dependence of the evolutionary pathway on the external conditions has been observed on other systems as well (107, 108). Loss of function mutations far dominated the total number of mutations encountered (28 out of 34), indicating that disabling the outer membrane pore oprD makes the cells resistant to meropenem by not allowing the compound to enter the cell.

Conclusions

Combining all available information, and with the disclaimer that chemostat data may not always be applicable to the patient, a number of conclusions can be drawn:

• In monotherapy with ceftazidime or meropenem the highest safe exposure for the patient should be applied for the shortest time that achieves the therapeutic goal.

• Application of fluctuating concentrations of both ceftazidime and meropenem may accomplish the same reduction in cell density as constant concentrations, while causing less resistance.

• An intermittent treatment regimen causes less resistance than would be expected based on general knowledge of antibiotic treatment.

• Therapy with alternating ceftazidime and meropenem may be more successful than single drug therapy at the same concentrations, while also reducing development of resistance.

• When possible, combination therapy with ceftazidime and meropenem may be superior to treatment with each of the antibiotics alone, both in clearing the infection and in limiting development of resistance. Again, the highest dosing possible should be applied.

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Optimization of antibiotic therapy

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Chapter 6

General discussion
Chapter 6

De novo development of antibiotic resistance during treatments and its consequences

1. Evolution of antibiotic resistance during the treatment

The role of patient-to-patient transmission in spreading antibiotic resistance in hospitals and clinics has been well documented in a large number of studies using molecular epidemiology as main instrument (183-188). In contrast, the contribution of de novo development caused by antibiotic therapy is rarely reported, possibly because of the complexity of translating in vivo conditions to an experimental setting. The experimental simulation of patient conditions is the core of this project. In chapter 2, 3 and 5, it is demonstrated that the build-up of resistance observed in clinical pathogens can also be solely caused by de novo mutations emerging and accumulating in response to antibiotic concentrations expected in patients. Resistant isolates have been discovered in hospitals that are very similar to those recovered from the experimental situation (189-194). The statement that bacteria can rarely acquire resistance by de novo during a single treatment lasting only a few days is therefore not supported by this observation, once more indicating the necessity of prudent use of antibiotics.

2. Consequences of de novo evolved resistance

It has been generally noticed that when an initial antibiotic therapy is not concluded satisfactorily, the subsequent treatment is also less likely to succeed since resistance may have evolved. A simple explanation is that continuation of the treatment without awareness of the emerged of drug resistance, as shown in chapter 3 and other laboratory studies, will select for existing resistant cells among the bacterial population (67, 130, 195). Moreover, the cells that are more resistant evolve further even when the selection pressure is sustained at a low concentration, as exemplified by the data of chapter 2 to 5 as well as other in vitro studies of resistance evolution (112, 131, 196). Clinically, the positive correlation between the prevalence of antibiotic resistance and the increase of the antibiotic consumption has been proven more than once, confirming the aggravating effects of frequent application of a single drug on the problems of drug resistance (197-202).

Treatment failure in patients resulting from the emergence of drug resistance necessitates deployment of more effective drugs afterwards. However, as
clarified in chapter 3, a negative effect of the initial treatment failure is to be expected on the success of the subsequent treatment, even when a structurally and functionally improved drug is chosen. The well known reason for this is that treatment with one drug can produce resistance to several other drugs (203-205). For example, exposure to imipenem can make Acinetobacter baumannii resistant towards several cephalosporin and carbapenem drugs through over-expression of carbapenemase, efflux pumps or/and AmpC β-lactamase expression (206). The other negative influence predicted is that adaptation to one drug may accelerate development of resistance of bacteria towards other drugs. This suggestion is based on the theory that for all bactericidal drugs, a common mechanism is operational in killing bacteria (117, 168, 207, 208).

The extra expenditure, the extension of hospital stay as well as the elevation of mortality rate due to antibiotic resistance all manifest how difficult it is to treat the infectious diseases caused by drug-resistant pathogens (209-214). This difficulty is expected to be infinitely exacerbated considering that the evolved resistance is able to spread within the ecosystem, especially among the vulnerable people, such as those in the intensive care units of the hospital. Accordingly, it is crucial to prevent the acquisition of drug resistance in clinical pathogens in the first place when initial treatment is given. This holds regardless of whether enrichment of existing resistance or de novo development of resistance during the previous treatment is obstructing the efficacy of subsequent therapy.

Complexity of the evolutionary paths towards antibiotic resistance

1. Determinism and stochasticity

As shown in chapter 4, almost all genetic mutations that appeared during adaptation of P. aeruginosa to increasing concentrations of meropenem, ciprofloxacin and piperclillin/tazobactam can be repeatedly detected in independent biological duplicates. This remarkable parallelism of evolution has also been noticed by many other researchers investigating different microorganisms (82, 85, 153, 215-218). The deterministic strength for such parallel evolution is considered to be the interplay between different mutations: Positive epistatic effects of two resistant mutations could lead significantly higher resistance levels than the sum of resistance caused by these two mutations separately; Though some mutations do not directly bring about resistance, they
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favor development of resistance to antimicrobial agents by alleviating fitness cost of other essential mutations conferring drug resistance (219-222). The sequential emergence and fixation of specific mutations seen along the gradual increase of resistance in chapter 4 are in accordance to this theory.

The concept of evolutionary parallelism entails that it may be possible to predict, diagnose and address the problem of antibiotic resistance by targeting only a handful of genes (204, 216, 223). However, this may in fact not completely be correct. The reason is that evolution is also a stochastic process, as illustrated by the data of chapter 4 where genetic variation in genes relevant to ceftazidime resistance were found predominantly in one population but not in the other. The randomness of these mutations can be explained by genetic hitchhiking and clonal interference: Because of genetic hitchhiking, mutations without connection to acquisition of resistance can also be fixed in the population. Nevertheless, the most fit mutation might be outcompeted by a less fit one on account of clonal interference (164). Besides genetic hitchhiking and clonal interference, the drug regime and the growth environment are also essential elements changing the bacteria’s evolutionary routes towards antibiotic resistance: Data of chapters 4 and 5 show that continuous application of lethal concentrations of meropenem gives rise to the random occurrence of multiple loss-of-function mutations in the oprD gene, rather than the stepwise occurrence and selection of a few mutations detected when the concentration of the antibiotic was increased gradually. Similar findings were also reported in other studies (107, 108, 224). As for the influences of growth medium, the failure of adaptation of P. aeruginosa to three beta lactam drugs in minimal medium in chapter 4 demonstrates that certain environments may not permit evolution of resistance to take place, at least not within short time periods, even though growth in the absence of antibiotics is not compromised in this condition. The cause of this failure is not clear, but several studies report the influence of growth conditions on the evolution of drug resistance (153, 218, 225). Except the factors tested in this thesis, the evolutionary path has been also shown by others to be influenced by the presence of additional types of stress, the co-existence of heterogeneous organisms, the genetic background and the population size of pathogenic bacteria (149, 165, 226-230).

2. Genomic and non-genomic interaction

The key message of chapter 4 is that the increase of resistance seems to be the result of a complex interaction between several cellular systems, rather than
individual mutations, in agreement with earlier results (85). The survival of bacterial cells without resistance mutations, especially in the initial period of resistance development, is most likely to be attributed to adaptive changes on the transcriptional level. This form of adaptation can e.g. be realized through overexpression of efflux pumps, activation of drug-degrading enzymes, expressional suppression of outer membrane proteins, or changes in other cellular process indirectly related with antibiotic tolerance (103). Except for these solutions, bacteria can also transiently protect themselves from the lethality of antibiotics if slow growth, or, in the extreme case, a dormant state is sustained (231). Because of heterogeneity, subpopulations of a clonal bacterial population might be characterized with distinctively slow growth, thus being able to survive persistently in response to high exposure of antibiotics, so called persisters (232-234). Formation of certain physical structures such as multinucleated filaments and biofilms, were also shown to be beneficial for bacteria that need to survive in the presence of antibiotics (101, 104, 235). Another non-genetic strategy that bacteria can rely on to become tolerant to antibiotic treatment is extending the lag phase (236).

Resistance conferred not by genetic mutations, but by changes in expression levels plays an essential role in acquisition of resistance, because development of resistance does not always correlate with specific mutations (chapter 4). However, how these non-genetic mechanisms interact with genetic mutations has rarely been studied. Based on these findings, we proposed that the initial regulation of gene expression prepares the way for subsequent mutational events. Transcriptional adaptation allows bacteria to survive at lower drug stress, and thus gain time to search for optimal mutations by trial and error. Persistence, as an independent biological process, has also recently been concluded to be complementary to molecular resistance during the bacterial adaptation to stress (237). More research is needed to interpret the complex interactions between molecular evolution and non-genetic adaptations (238, 239).

3. Individualism and collectivity

After growth in the presence of an antibiotic for some generations, one would expect that all the individual cells of a bacterial population possess the corresponding resistance mutations in order to survive exposure to the antibiotic. The mutation frequency data in cultures exposed to ciprofloxacin, tobramycin, piperclillin/tazobactam or meropenem, highlight the randomness of resistance
development. During resistance development, there are moments where the sum of the mutation frequencies in a specific gene is less than 1. In other words, part of the population has no mutations, as exemplified by the evolution of ceftazidime resistance described in chapter 4. The survival of this subpopulation might be due to the appearance of non-genomic adaptations discussed above, but should possibly also be credited to a phenomenon named “collective resistance”. Under the right conditions, a small portion of resistant cells protects the other more susceptible cells and ultimately allows the survival of the overall population in an environment with antibiotics (99, 100, 240, 241). A good example of such collective resistance would be that resistant cells overproduce extracellular drug degrading enzymes and thus rescue the vulnerable cells in their vicinity. This is most likely the case in our study of ceftazidime resistance as the small number of mutations consists of those activating expression of beta lactamase, and foreseeably led to collective resistance of the whole population. Signaling pathways can also be utilized by sensitive cells to intercept stress signals from their resistant neighbors and thereby start cellular adaptation processes to enhance their own drug tolerance. However, whether this also plays a role in the experiments of this thesis is not clear. It should be pointed out that collective resistance not only occurs within the same bacterial species but also can happen among different species (242).

4. Persistence and reversibility

It was generally assumed that the genetic and phenotypic modifications that result in antibiotic resistance also lead to concomitant reductions in bacterial fitness, virulence and ecological niches. As a consequence resistance is expected to be reversible after treatment is halted (84, 95, 243-246). However, more and more evidence, including the data in chapter 4 of this thesis, is not in agreement with this notion (50, 52, 53). The evolved resistance is more likely to be persistent, rather than reversed, when drug exposure is ended for several reasons. First, reverse mutations occur less often than compensatory ones, and therefore readily go into stochastic extinction during genetic drift (247, 248). Second, the epistatic interactions between resistant and compensatory mutations constrain the occurrence of reverse mutations since fitness cost would be incurred by reverse mutations. Finally, mutations that partly or fully compensate the fitness cost of resistance in bacteria are expected to delay, or outcompete the co-existing reverse mutations (249, 250).
Despite the rarity of reversal of resistance mutations, a few mutations are shown in chapter 4 to disappear after the culture was grown for two weeks in the absence of drugs. This suggests, in agreement with the modest reduction of growth rate and the significant elevation of maintenance energy measured after the drug exposure in some of the resistant strains, that fitness costs are indeed incurred by development of resistance. Besides loss of evolved mutations, acquisition of new mutations was also seen when the resistant culture was transferred to drug-free medium, implying systematic re-adaptation of the resistant culture to the new drug-free environment. The resistant phenotype after treatment is an integrated result of the persistence of resistance mutations acquired during the drug exposure and the emergence of newly adaptive variations incurred after drug withdrawal.

**Completion and optimization of current guidelines for antibiotic administration**

1. Reduction of unnecessary usage is always desirable

Because of the incredible evolutionary ability of bacteria towards antibiotic resistance once selection stress is present, as shown in chapters 2 to 5 of this thesis as well as other studies, reduction of inappropriate usage of antibacterial agents is under all circumstances recommended to combat antibiotic resistance (251-255). Campaigns carried out to reduce inappropriate antibiotic use such as the Get Smart: Know When Antibiotics Work campaign have been developed both nationally and locally in the United States and Europe (256, 257). Through these campaigns, inappropriate prescribing practices have been significantly decreased (258, 259).

Measures advocated so far include restriction of prescription of antibiotics to treat non-bacterial infections, curtailing the treatment course if antibiotic usage is necessary and limitation of the usage of antibiotics outside the clinic. The overprescription of antibiotics for treating infections is enormous. According to estimations from the United States, over half of all antibiotics prescribed to cure acute respiratory tract infections, including upper respiratory tract infections, otitis media, sinusitis, pharyngitis, and bronchitis, in outpatient setting, are not necessary because these infections are not caused by bacteria (260-262). Such inappropriate use of antibiotics in China, India and other countries with less strict regulation of antibiotic usage is expected to be even more common (263-265).
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Besides elimination of usage of antibiotic in infections not caused by bacteria, shortening treatment length may be the most clinically applicable way to reduce antibiotic consumption. In most clinical scenarios, the recommended duration of therapy in published guidelines is based on tradition and expert opinions. However, the available randomized controlled trials (RCTs) prove that the short-term and long-term treatments of many infectious diseases are not significantly different in terms of clinical success, relapse of infection and adverse events (266-275). Thus, it is suggested to apply the RCTs to optimize antibiotic therapy duration and to use biomarkers, such as procalcitonin, in conjunction with signs of clinical recovery to discontinue antibiotic therapy when this can be safely done (276-280). In addition, reducing usage of antibiotics in agriculture, for food animals and for other purposes rather than clinical therapy is also recommended (281-283). To deploy these measures effectively, thorough knowledge of antibiotic resistance, rapid diagnostic methods, validated antimicrobial stewardship as well as supporting policies are all indispensable.

2. Concerns about “Hit hard and hit fast”

“Hit hard and hit fast” is a strategy a German scientist, even years before the discovery of penicillin proposed to combat bacterial infections. The aim of this strategy was to kill all bacteria inside a person. However, this aim was soon realized to be unpractical because bacteria evolve. Despite of failure in wiping out all pathogens, “hit hard and hit fast” has become the principle for current antibiotic administration from the perspective of resistance prevention (121, 284-286). Its accuracy was proven by the prevention of resistance when dosing is persistently high, and at the same time, treatment duration is short enough. However, the mutation prevention concentration acquired in vitro may not work in patients, given that in vivo growth conditions, population size, duration of drug exposure and efficiency of immune system are different from those in vitro. More importantly, it is hard to keep the concentration during the whole treatment course over the MPC because of the highly dynamic drug concentration in patients (287-289). Without certainty of the exact value for MPC for a specific condition in patients, blind introduction of the highest dose that is acceptable for patients would lead to the emergence of the highest resistance if any pathogens survive, as demonstrated in chapter 5. Besides the dosage, treatment duration is also essential in order to prevent occurrence of resistance, since resistance development may be initiated when bacteria are continuously exposed to antibiotics. Taken together, to make the approach of “hit hard and hit fast” work practically, both the antibiotic
dosage and the treatment course need to be elaborately devised taking careful consideration of the specific drug, pathogen and the condition of the patient.

### 3. Monotherapy, alternating or multi-therapy?

In the simulations of chapter 5, cycling different drugs in one treatment performed better in both infection control and limiting development of resistance, compared to the single-drug strategy using the same concentrations. This observation strongly supports the proposal of applying alternating drugs to prevent de novo development of antibiotic resistance. To make this proposal suitable for implementation in the clinic, searches have been carried out for the optimized pair of drugs that can be cycled during one therapy. The outcome was that resistance to either of the rotated drugs would happen if two drugs are collaterally sensitive, such as most of aminoglycoside antibiotics with beta lactam drugs (59-62). However, our results in chapter 5 showed that cycling two drugs with different targets and resistance mechanisms, but without collateral sensitivity also has a positive influence in both enhancing bacterial killing and postponing resistance development. In addition to the choice of drugs, the order of the drugs in the alteration is also a success factor for the alternating therapy, as discussed in chapter 5 and elsewhere (290). The latter study suggested that choosing the drug with larger fitness costs to initiate therapy is helpful in reducing resistance, but more evidence is needed to confirm this presumption.

Treatment of infections with multiple drugs at one time has long been suggested to be a solution to combat the problem of antibiotic resistance (56, 291-293). Our results described in chapter 5 are in agreement with this suggestion. However, the benefits of multi-treatments have not been decisively demonstrated in clinic trails (294, 295). This uncertainty may be the result of several factors: Firstly, the effects of the drug combination are dependent on the concentration of each constituent drug, as shown in chapter 5. However, because of the pharmacokinetic properties, there are inevitably periods where drug concentrations in patients cannot be high enough to prevent mutations. What's more important is that most drugs tested have synergistic effects in bacterial killing. The pairing of these drugs has been shown to stimulate resistance evolution more in comparison to drugs with antagonistic relationships (296, 297).

The common factor essential for prevention of mutations in all treatment strategies is drug concentration. As seen in chapter 5, alternating and combining different drugs lowers mutation prevention concentrations, but can still permit
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resistance to develop if the concentration is too low. Thus, whilst exact practices may vary, the principle of "hit hard" should be strictly obeyed when new treatment tactics are considered to be translated into medical practice.
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Summary/Samenvatting
Summary/Samenvatting

The ever-increasing rate of drug resistant bacteria has been one of the most challenging problem worldwide. Because of the indispensable role of antibiotics in our modern medicine, curbing the development of antibiotic resistance is a goal as essential as controlling the deterioration of disease in patient treatments. Therefore, the main subject of this thesis was to research the evolution of resistance in bacteria, exampled mostly by a clinically leading pathogen \textit{P. aeruginosa}, towards clinically relevant antibiotics. The ultimate purpose is to explore the optimal way to administrate drugs in order to cure the infection, and at the same time maximally reduce the occurrence of antibiotic resistance.

\textbf{Chapter 1} introduces the background where the research questions of this thesis are raised. It begins with the description of the urgency the antibiotic resistance problem. Afterwards, the current knowledge gaps about whether, when and how the bacterial population is able to evolve resistance \textit{de novo} during the therapeutic course of antibiotics are proposed. What is also indicated is the limitation of the information in the destiny of the evolved mutations after the treatment is halted. Finally, it is pointed out that the evidences for the currently used recommendations for antibiotic administration are rare and elusive.

\textbf{Chapter 2} documents whether the resistance can develop \textit{de novo} in patient by mimicking the treatment of \textit{P. aeruginosa} infection \textit{in vitro} with concentrations reached in patients. It is found that even at the highest concentration sufficient bacteria survive in clumps of filamentous cells, and then recover and grow out after 3 to 5 days. Within one week, the MIC has increased by a factor between 10 and 10,000 depending on the antibiotic and the treatment regimes. Hence, the outcome suggests that the occurrence of resistance often observed during patient treatment can be explained by \textit{de novo} acquisition of resistance and genetic exchange of resistance genes is not by necessity involved.

\textbf{Chapter 3} illustrates the influences of the therapy failure of an initial antibiotic treatment on the effects of subsequent treatments. It was shown that the amoxicillin resistance could be rapidly induced in \textit{E. coli} at concentrations expected in the patients. These resistant population was rapidly enriched when amoxicillin was applied for the second time. If the other structurally and functionally advanced beta lactam such as cefotaxime was chosen for the subsequent therapy, the effects was not expected to be optimistic owing to the appearance of cross resistance. To conclude, the initial treatment by amoxicillin has a negative effect on subsequent therapy with beta-lactam antibiotics.
Chapter 4 explores how the whole genome varies while the drug resistance is rising as results of the stepwise increasing of antibiotic exposure in bacteria exampled as *P. aeruginosa*. The increase in the MIC of ciprofloxacin and tobramycin was found to be linked to sequentially appeared mutations. However, this is not the case in the three beta-lactam drugs. The resistance-related mutations differed for each specific antibiotic. The reversal of evolved mutations after the treatment may not necessarily cause the reduction of resistance. The cellular functions or the growth rate seemed compromised because of the acquisition of antibiotic resistance. Overall, the results suggest that the evolution of antibiotic resistance is likely to be the result of complex interactions among several cellular systems rather than individual mutations.

Chapter 5 assesses a large variety of therapeutic protocols for *P. aeruginosa* infections simulated with chemostat. The results enlighten that therapeutic objective is best reached with the least amount of resistance induced when drugs are used in combination or alternating protocols at the highest concentrations the patient can endure. If monotherapy is the choice, the optimal administration strategy would be the highest dosage applied with the shortest course.

Chapter 6 discusses the results achieved in this thesis. Firstly, the de novo development of antibiotic resistance during treatments and its consequences are stressed based on the findings in chapter 2 and 3. According mainly to the contents of chapter 4, the complexity of the evolutionary paths towards antibiotic resistance is then explicated from the perspective of its determinism and stochasticity, genomic and non-genomic interaction, individualism and collectivity, persistence and reversibility. At the last, suggestions about completion and optimization of current guidelines for antibiotic administration are presented with reference to the findings of chapter 5.
Summary/Samenvatting

De globale toename van antibioticaresistentie in bacteriën is een probleem bij de bestrijding van infectieziekten. Vanwege de onmisbare rol van antibiotica in de moderne medische wereld is het tegengaan van antibioticaresistentie een belangrijke overweging bij het behandelen en genezen van individuele patiënten. De focus van dit proefschrift ligt daarom op het onderzoeken van de evolutie van resistentie tegen klinisch relevante antibiotica. Als modelorganisme is de opportunistisch pathogene bacterie *P. aeruginosa* gebruikt. Het uiteindelijke doel is het vinden van een optimale behandelingssstrategie waarbij complete genezing gecombineerd wordt met minimale ontwikkeling van antibioticaresistentie.

**Hoofdstuk 1** introduceert het onderwerp en schetst het kader waarin de onderzoeks vraag is geformuleerd. Allereerst wordt beschreven hoe urgent het huidige resistentieprobleem is. Gebaseerd hierop volgen een aantal vragen over het hoe, wat, waar, en wanneer de bacteriële populatie *de novo* resistentie ontwikkelt wanneer patiënten behandeld worden met antibiotica. Ook wordt benadrukt hoe beperkt de huidige informatie is over het effect van het staken van een behandeling met antibiotica op resistentie bacteriën. Als laatste wordt er vastgesteld dat er op dit moment niet genoeg bekend is over het effect van de huidige behandelprotocolen op de ontwikkeling van antibioticaresistentie.

In **Hoofdstuk 2** wordt door het *in vitro* nabootsen van huidige behandelingssregimes de ontwikkeling van de *de novo* resistentie in *P. aeruginosa* beschreven. Zelfs bij de hoogste concentraties waaraan patiënten tijdens behandeling blootgesteld worden, slagen bacteriën erin te overleven door in filamentieuze vorm samen te klonteren. Na drie tot vijf dagen herstellen deze bacteriën en vormen ze een nieuwe, resistente, populatie. Afhankelijk van het type antibioticum en de gekozen behandelingssstrategie stijgt de MIC binnen één week met een factor 10 tot 10.000. Deze data laten zien dat de antibiotica resistentie die op dit moment tijdens behandeling van patiënten ontstaat, verklaard kan worden door het *de novo* verwerven van resistentie. Het uitwisselen van resistentiesgappen is hierbij niet noodzakelijk.

**Hoofdstuk 3** illustreert hoe therapiefalen de daaropvolgende behandelingen kan beïnvloeden. Het blootstellen van *E. coli* aan klinisch relevante concentraties van het bètalactam antibioticum amoxicilline leidt tot snelle ontwikkeling van resistentie. Een latere herhaling van de behandeling met amoxicilline resulteert in een snellere toename en groei van deze resistente populatie. Bovendien heeft het ontstaan van kruisresistentie als gevolg dat andere bètalactam antibiotica
ook niet altijd meer gebruikt kunnen worden voor behandeling. Een voorbeeld hiervan is cefotaxim, een derde-generatie cefalosporine. Uit deze data kan geconcludeerd worden dat de initiële behandeling met amoxicilline een negatief effect heeft op toekomstige behandelingen met andere bètalactam antibiotica.

In hoofdstuk 4 wordt de genetische variatie van P. aeruginosa tijdens stapsgewijze blootstelling aan toenemende concentraties antibiotica onderzocht. Voor ciprofloxacine en tobramycine kan de toename in MIC gelinkt worden aan specifieke mutaties die sequentieel na blootstelling aan antibiotica verschijnen. Dit is in tegenstelling tot resistentie die ontwikkelt na blootstelling aan drie verschillende bètalactam antibiotica. Voor elk antibioticum kunnen verschillende mutaties geïdentificeerd worden die leiden tot resistentie. Het kwijtraken van de verkregen mutaties leidt echter niet altijd tot een afname in resistentie. Daarnaast heeft het ontwikkelen van resistentie een negatieve invloed op de groeisnelheid van de bacterie of het functioneren van de cel. Samengevat laten deze resultaten zien dat de ontwikkeling van antibiotica resistentie niet het gevolg is van enkele, losse mutaties, maar eerder voortvloeit uit de complexe interactie van verschillende cellulaire systemen.

Hoofdstuk 5 beschrijft hoe antibioticaresistentie ontstaat als verschillende therapiemodellen worden nagebootst in een chemostaat. Het behandelen met een combinatie van verschillende antibiotica of een afwisselend regime met de hoogst mogelijke concentraties zorgt ervoor dat resistentie zo beperkt mogelijk blijft. Als er toch voor therapie met een enkel antibioticum gekozen wordt, is een zo kort mogelijke behandeling met een zo hoog mogelijke concentratie de meest optimale keuze.

In hoofdstuk 6 worden de resultaten uit dit proefschrift besproken en in de juiste context geplaatst. Als eerste wordt de evolutie van antibiotica resistantie besproken. Aan de hand van data uit de hoofdstukken 2 en 3 worden de mogelijke gevolgen hiervan benadrukt. De resultaten uit hoofdstuk 4 worden gebruikt om de complexiteit van de evolutie van antibioticaresistentie te bespreken. Een aantal verschillende aspecten, waaronder determinisme versus stochastiek, de interactie tussen het genoom en de rest van de cel, individualisme versus collectiviteit, en persisten eneversversibilidaditeit worden hierbij in overweging genomen. Tenslotte worden er, gebaseerd op de resultaten uit hoofdstuk 5, enkele aanbevelingen gegeven om de huidige behandelprotocol len te optimaliseren en aan te vullen.
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Acknowledgements
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