National Technical University of Athens

Technical University of Denmark

Analyzing Collateral Effects of Resistance Adaptation in Nosocomial Pathogens

Diploma Thesis

Konstantinos Asimakopoulos

Supervisors

Professor Evangelos Topakas

NTUA – School of Chemical Engineering

Professor Morten O. A. Sommer

DTU – Systems Biology

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Contents

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Abstract

Pathogenic bacteria are an important cause of high mortality rates and high healthcare costs in developing countries. The emergence of resistant species has made the development of new antimicrobial drugs necessary. A first approach to this issue is to understand how bacteria evolve their resistance mechanisms.

On this basis, we performed adaptive evolution experiments of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baimannii*, *Pseudomonas aeruginosa* and *Enterobacter cloacae*) organisms using a suite of five antibiotics. The results showed that resistance adaptation is a complex phenomenon that affects major phenotypical features such as the growth rate, which is mainly slowed down. Collateral sensitivity and resistance experiments revealed that adaptation resulted in cross-resistance across different classes of antibiotics in the majority of the cases showing that the mechanisms of resistance are not unique for each drug.

The data and the knowledge gathered from the work done in this thesis can be used as a guiding tool during sequence analysis of the adapted strains. We expect that our final findings will shed light on the genetic responses of the ESKAPE pathogens exposed to antimicrobials.

Introduction

History of Bacterial Diseases

Bacteria were discovered in the late 1670s by Antonie van Leeuwenhoek, a Dutch cloth merchant who was an expert at lens grinding so that he could examine the weave of cloth. He observed bacteria for the first time in an unnatural water environment, pepper water[1]. Two more centuries were needed until it was proven that bacteria could cause disease. This proof was obtained from several scientists, but the most crucial evidence came from the work of Louis Pasteur and Robert Koch in the 1860s and the 1870s. Pasteur showed that bacteria could not grow in sealed broth if the broth was boiled first. In 1890 Koch published his four criteria for establishing a relationship between the presence of a bacteria and a disease, after his discovery of anthrax bacillus and tubercle bacillus, the causative agents of anthrax and the tuberculosis disease, respectively[2].

However the deadly power of bacteria was known to humans long before their discovery. There are records of infectious diseases even in B.C.E. In 430 B.C.E the plague of Athens killed 20% of the Athenian troops that were fighting in the Great Peloponnesian War. In 2005 a correlation was made between DNA extracted from dental pulp of three teeth recovered from Kerameikos cemetery in Athens and a known pathogen. The scientists identified nucleotide sequences from a pathogenic bacterium, *Salmonella Enterica Serovar Typhi*, which causes typhoid fever[3]. The Plague of Justinian (541 to 750 A.D), which eliminated one quarter to one half of the human population in the Eastern Mediterranean region, was likely a bubonic plague.

Discovery and Production of Antibacterial Agents

The inference that bacteria cause diseases led many researchers to start searching for ways to confront the problem. The 20th century is the era of the discovery and evolution of antimicrobial compounds. In 1909 Paul Ehrlich, a German physician, discovered the active compound 606, which was toxic against Treponema pallidum, a bacteria that causes syphilis[4]. A landmark in the history of medicine is 1932, when Bayer chemists, Josef Klarer and Fritz Mietzsch, first synthesized sulfoaminochrysoidine as part of a research program designed to find compounds that might act as antibacterial drugs in the body. In the late autumn 1932, Gerhard Domagk tested sulfoaminochrysoidïne in mice and found the drug effective against some important bacterial infections. In 1935 the drug was released in the market under the name Prontosil and Gerhard Domagk received the Nobel Prize in Medicine in 1939[2].

A year later Howard Florey and his team in Oxford devised a method for mass producing penicillin, the great discovery of Alexander Fleming in 1928. Florey and Ernst Boris Chain discovered penicillin's therapeutic action and its chemical composition, which was confirmed by X-Ray Crystallography done by Dorothy Hodgkin. For their research in penicillin Chain, Florey and Fleming received the Nobel Prize in Medicine in 1945[5, 6]. In the following 30 years several screening projects were carried out and a large number of antibacterial agents were introduced into the market (Figure 1).

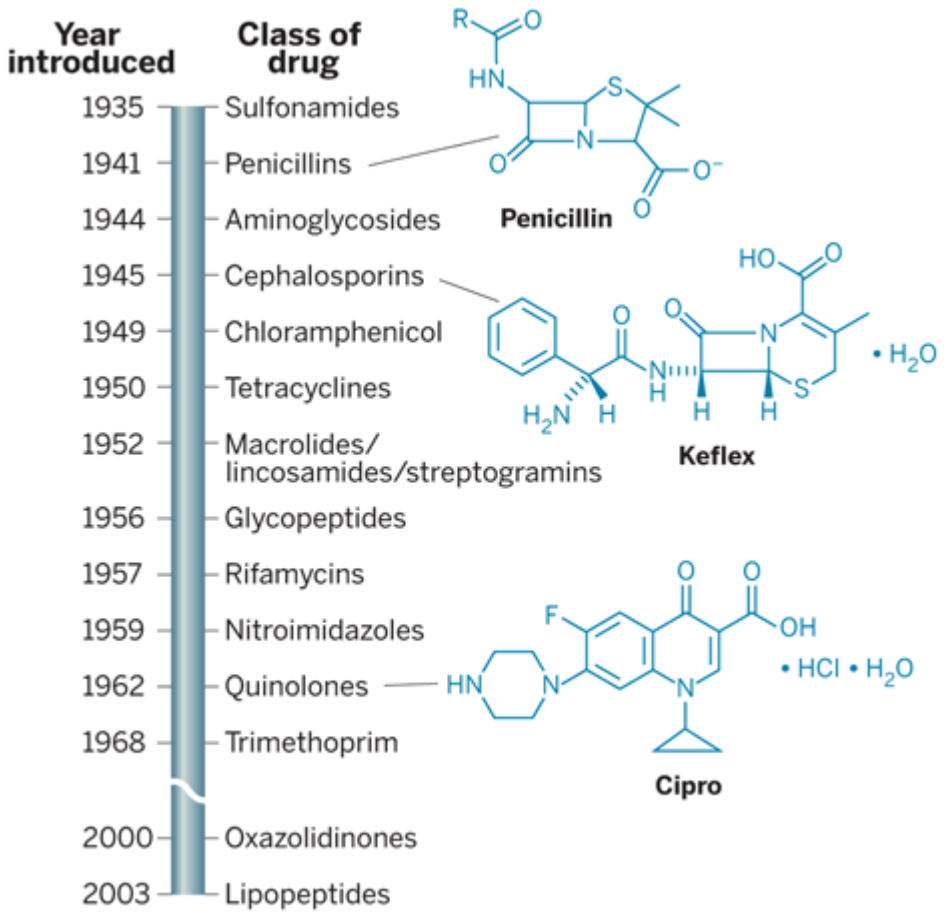

Figure 1: Timeline of classes of antibacterial agents introduced in the markets[7]

Antibiotic Resistance

After the 1950s the global availability of antibiotics increased abruptly. The uncontrolled sales of antibiotics and therefore the widespread use of them caused the emergence and spread of pathogenic bacteria resistant to many antibiotics. In addition to that, only two new classes of antibiotics of novel mechanisms of action (linezolid and daptomycin) have been introduced into the market during the last three decades which led to the continuous use of the same antibiotic classes for the last 40-50 years.

Today infectious diseases still remain the main cause of human deaths. The mortality rates due to multidrug-resistant bacterial infections are high. Each year about 25,000 patients in the European Union die from an infection caused by multidrug resistant bacteria and more than 63,000 patients in the United States die every year from hospital-acquired bacterial infections[8]. More than 1.5 billion euros are spent each year as a result of extra healthcare costs and productivity losses due to infections by multi-drug resistant bacteria[9]. Therefore there is a strong need for introducing new ways of responding to the antibiotic resistance challenge with the discovery and development of new therapeutic agents and a better understanding of antibiotic resistance mechanisms.

ESKAPE Problem

The Infectious Diseases Society of America has chosen a group of multi-drug resistant bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) and has given them the acronym ESKAPE because of their capability to escape bactericidal effects of antibiotics[10].

Enterococcus faecium

Enterococcus faecium is a gram positive bacterium that can be commensal in the human intestine, but can also be pathogenic. It can also be found in the oral cavity and the vaginal tract. It can cause nosocomial bacteremia, surgical wound infection, endocarditis and urinary tract infections[10]. *E. faecium* is capable of surviving for extended periods in soil, sewage and inside hospital on many different kinds of surfaces. It is capable of growing under a wide range of environmental conditions, including temperatures ranging from 10 to 45 degrees Celsius, basic or acidic, hypertonic or isotonic environments[11].

E. faecium can be highly drug resistant and acquires its resistance by plasmids, by conjugative transposons, by chromosomal genes that encode resistance and by sporadic mutations. There are reports that refer to *E. faecium* strains as being resistant to vancomycin[12], penicillin[13], gentamicin[14] and teicoplanin[15]. For instance, high-level penicillin resistance in *E.* faecium is most commonly associated with accumulation of point mutations in the penicillin binding region of PBP5[16] (acquired resistance), in the contrary *E. faecium* is resistant to tobramycin with MICs as high as 1000 μg/ml because of the aminoglycoside 6' acetyltransferase, which is encoded from its genome[17] (intrinsic resistance).

Staphylococcus aureus

S. aureus is a gram positive coccal bacterium which is commensal in the respiratory tract and on the skin[18]. However, it can also be pathogenic and cause skin and soft tissue infections such as abscesses, furuncles and cellulitis. It can also cause much more serious infections such as bloodstream infections, pneumonia, or bone and joint infections[19]. It is one of the five most common causes of hospital acquired infections and is often the cause of postsurgical wound infections. Each year around 500,000 patients in United States' hospitals contract a staphylococcal infection[20].

S. aureus is adept at adapting quickly to antibiotic treatment. The most common mechanisms used by *S. aureus* are the enzymatic inactivation of the antibiotic, the decreasing of the affinity of the target molecule of the antibiotic by altering its structure, and efflux pumps. The genes responsible for the antibiotic resistance are acquired through horizontal gene transfer or by spontaneous mutations[21].

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a major multidrug-resistant (MDR) pathogen since the early 1960s. Unfortunately, many MRSA outbreaks frequently arise with a recent example being intensive care unit (ICU)-associated bacteremia in London[22]. Recent studies from Europe state that the average excess costs per MRSA infected patient range from 5,700 € to 10,000 €[23]. Methicillin, like all penicillins, exerts its action by blocking the penicillin binding proteins (PBPs) which are responsible for the construction and the maintenance of the bacterial cell wall. The MRSA strains acquired a gene called PBP2a which was not blocked by methicillin and could replace the other PBPs. The gene that encodes PBP2a is mecA and its presence means MRSA is not only resistant to methicillin but also to all β-lactam antibiotics including synthetic penicillins, cephalosporins and carbapenems[24].

Klebsiella pneumoniae

Klebsiella pneumoniae is a Gram negative, non-motile, rod shaped bacterium which is found in the nasopharynx and the intestinal tract[25]. However it can also be pathogenic and because of its ability to spread rapidly in the hospital environment, these bacteria tend to cause nosocomial outbreaks. It can cause urinary tract infections, septicemias, pneumonia, soft tissue infections, thrombophlebitis, diarrhea, upper respiratory tract infection and meningitis[25, 26]. It is also an opportunistic pathogen for patients with chronic pulmonary disease, enteral pathogenicity and rhinoscleroma[25]. Due to the extensive spread of antibiotic-resistant strains, especially of extended-spectrum β-lactamase (ESBL)-producing strains, there has been renewed interest in *Klebsiella* infections[27].

Since ESBL production frequently is accompanied by multi-resistance to antibiotics, therapeutic options have become limited. However, ESBL-producing *Klebsiella* strains remain susceptible to carbapenems with a small number of reports referred to imipenem resistant *K. pneumoniae* strains[28]. ESBLs are usually plasmid mediated and easily transmitted among different members of the *Enterobacteriaceae*. Unfortunately, *Klebsiella* strains are accompanied by a relatively high stability of the plasmids encoding ESBLs. Long hospital stays are a serious factor for the acquisition of resistance[25].

Acinetobacter baumannii

Acinetobacter baumannii is a rod shaped Gram negative bacterium which has emerged as one of the most troublesome pathogens for health care institutions globally. It has a remarkable ability to upregulate or acquire resistant determinants, making it one of the most threatening organisms in the current antibiotic era[29]. *A. baumannii* can survive in a hospital environment for prolonged periods, thus potentiating its ability for nosocomial spread[30]. The most common infection caused by this organism is hospital–acquired pneumonia[31] but there are reports also of *A. baumannii* infecting the central nervous system, skin and soft tissue, and bones.

A. baumannii has several mechanisms of acquiring antibiotic resistance. The most prevalent mechanism of β-lactam resistance in *A. baumannii* is enzymatic degradation by β-lactamases. However multiple mechanisms often work in concert to produce the same phenotype[32]. *A. baumannii* is intrinsically resistant to cephalosporins as all strains are chromosomally encoded with AmpC cephalosporiases[33]. As far as it concerns aminoglycosides, it is reported that the presence of genes encoding for

aminoglycoside-modifying enzymes within class 1 integrons is highly prevalent in multidrug resistant *A*. *baumannii* strains[34]. Finally, there are also reports of *A. baumannii* strains with mutations in the gyrA and parC genes which lead in the synthesis of a modified DNA gyrase which is stable against quinolones[30].

Pseudomonas aeruginosa

P. aeruginosa is a Gram negative, motile, rod shaped bacterium which is abundant in various moist environments. This opportunistic pathogen is associated with hospital-acquired infections, most notably in immunocompromised individuals[35]. It can cause urinary tract infections[36], ventilator associated pneumonia[34], surgical site infection[34], ocular infections[37], skin and soft tissue infections and burn sepsis[38]. Patients with AIDS or cystic fibrosis face an increased risk of acquiring an infection and developing complications[35].

What makes *P. aeruginosa* uniquely problematic is its inherent resistance to many drug classes, its ability to acquire resistance via mutations to all relevant treatments, its high and increasing rates of resistance locally and its frequent role in serious infections[39]. Mechanisms underlying antibiotic resistance have been found to include production of antibiotic-degrading or antibiotic-inactivating enzymes, outer membrane proteins to evict the antibiotics and mutations to change antibiotic targets[39].

Enterobacter cloacae

Enterobacter cloacae is part of the normal flora of the gastrointestinal tract of 40 to 80% of people and is widely distributed in the environment[40]. *Enterobacter* infections are increasing in frequency, particularly in intensive care units. This nosocomial pathogen can cause a range of infections such as bacteremia, lower respiratory tract infection, skin and soft tissue infections, urinary tract infections, endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, and ophthalmic infections[41].

The first complete genome sequence of *E. Cloacae* isolated from human cerebrospinal fluid was reported in 2010. It was reported that the organism carries genes for 37 multidrug efflux proteins, 7 antimicrobial peptide resistance proteins, and 11 β-lactamases, suggesting its broad range of antibiotic resistance[42]. A number of agents remain effective for treatment. Among the beta-lactams, the fourth generation cephalosporins and carbapenems are the most attractive options. Aminoglycosides

retain good activity but usually require combination with another agent. Quinolones are highly active against most strains, but emerging resistance is a major concern[43].

Antibiotics used in the experiments

Cefepime

Cefepime is a fourth-generation cephalosporin antibiotic developed in 1994 and marketed under the name Maxipime. It is usually reserved to treat moderate to severe nosocomial pneumonia, infection caused by multiple drug resistant bacteria such as *P. aeruginosa*. Cefepime acts by binding to penicillin-binding proteins and inhibiting the synthesis of the bacterial cell wall[44]. Zwitterionic fourth-generation cephalosporins combine the properties of rapid bacterial outer membrane penetration with high stability to AmpC β-lactamase and with good affinity for the penicillin-binding proteins[45]. Cefepime has in vitro activity against Gram-positive organisms including *S. aureus* and penicillin-sensitive, -intermediate and -resistant *Streptococcus pneumoniae* similar to that of cefotaxime and ceftriaxone$_{44}$. Cefepime also has good activity against Gram-negative organisms, including *P. aeruginosa*, similar to that of ceftazidime. Importantly, cefepime is stable against many of the common plasmid- and chromosome-mediated β-lactamases and is a poor inducer of AmpC β-lactamases[44].

Ciprofloxacin

Ciprofloxacin is a broad spectrum fluoroquinolone antibacterial agent which exerts its antimicrobial effect by preventing energy dependent negative supercoiling of bacterial DNA through gyrase inhibition[46]. Fluoroquinolones are effective agents that target both gram-negative and gram-positive bacteria and are recommended for severe bacterial infections[47]. Ciprofloxacin attains therapeutic concentrations in most tissues and body fluids. The results of clinical trials with ciprofloxacin have confirmed its clinical efficacy and low potential for adverse effects. These include complicated urinary tract infections, sexually transmitted diseases (gonorrhoea and chancroid), skin and bone infections, gastrointestinal infections caused by multi-resistant organisms, lower respiratory tract infections (including those in patients with cystic fibrosis), febrile neutropenia (combined with an agent which possesses good activity against Gram-positive bacteria), intra-abdominal infections (combined with an antianaerobic agent) and malignant external otitis[48].

Meropenem

Meropenem is a carbapenem antibiotic showing a strong antibacterial activity to a wide range of bacteria strains from Gram-positive bacteria to Gram-negative. Carbapenems act as mechanism-based inhibitors of the peptidase domain of PBPs (Penicillin-binding proteins) and can inhibit peptide cross-linking as well as other peptidase reactions. A key factor of the efficacy of carbapenems is their ability to bind to multiple different PBPs[49]. A remarkable feature of meropenem is that its toxicity to the central nerve and the kidney are significantly low, while conventional carbapenem antibiotics are problematic in these toxicities[50].

Tetracycline

Tetracycline is a broad spectrum polyketide antibiotic which exhibits activity against a wide range of microorganisms including Gram-positive and Gram-negative bacteria, chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites[50]. The first tetracycline-resistant bacterium, *Shigella dysenteriae*, was isolated in 1953. Tetracycline resistance now occurs in an increasing number of pathogenic, opportunistic, and commensal bacteria. The presence of tetracycline-resistant pathogens limits the use of this agent in treatment of disease. Tetracycline inhibits protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site[50]. Tetracycline resistance is often due to the acquisition of new genes, which code for energy-dependent efflux of tetracyclines or for a protein that protects bacterial ribosomes from the action of tetracyclines[51].

Gentamicin

Gentamicin is an aminoglycoside antibiotic produced by *Micromonospora purpurea*. It was discovered in 1963 by Weinstein, Wagman *et al*. at Schering Corporation in Bloomfield, N.J. [52]. Gentamicin is a bactericidal antibiotic that works by irreversibly binding the 30S subunit of the bacterial ribosome, interrupting protein synthesis[53]. An important characteristic of this drug is its capability to remain active even after autoclaving[54]. The mechanisms of resistance to gentamicin are: decreased cell permeability, alterations at the ribosomal binding site and aminoglycoside modifying enzymes found on plasmids and transposons[55].

Thesis Investigations

Although we know the ESKAPE pathogens possess a remarkable ability to acquire resistance for a broad range of antibiotics, few adaptive evolution studies have ever been performed on these medically important species. The information we have about the mechanisms of resistance come from highly resistant clinical isolates that have been sequenced. In my thesis work I performed a systematic adaptive evolution of the ESKAPE organisms under uniform growing conditions using the aforementioned suite of antibiotics. Populations were adapted to each one of the five drugs. Drug combinations were not investigated here. Following adaptation, individual isolates of the most resistant populations were investigated for collateral sensitivity and resistance to the four other antimicrobials. The purpose of this research is to shed light on the evolutionary path that leads to resistance acquisition in the ESKAPE organisms.

Overall image of the adaptive evolution experiments

Table 1: Adaptive evolution experiments. S. aureus to cefepime and K. pneumoniae to Meropenem needed to be redone

	Cefepime	Meropenem	Tetracycline	Gentamicin	Ciprofloxacin
E. faecium	✓	✓	✓	✓	✓
S. aureus	✗	✓	✓	✓	✓
K. pneumoniae	✓	✗	✓	✓	✓
A. baumannii	✓	✓	✓	✓	✓
P. aeruginosa	✓	✓	✓	✓	✓
E. cloacae	✓	✓	✓	✓	✓

Table 1 includes the 30 adaptive evolutions that were performed for the project by Marius Faza, former employee of Systems Biology department in DTU. Two of them failed, marked with a cross in table 1, and therefore needed to be redone.

Materials and Methods

Bacteria and Reagents

S. aureus strain Newman and *K. pneumoniae* (DSM 30104) were adapted to cefepime and meropenem, respectively. MICs tests and kinetic experiments were performed to six bacterial organisms: *E. faecium* (DSM 2146), *S. aureus* strain Newman, *K. pneumoniae* (DSM 30104), *A. baumannii* (ATCC 17978), *P. aerugnosa* (PAO1) and *E. cloacae* (ATCC 13047). The drugs used in these experiments were: cefepime, meropenem, tetracycline, gentamicin and ciprofloxacin. All adaptive evolution experiments, MICs and kinetics experiments were performed using Mueller – Hinton Broth (MHB) media with an addition of 0.5% glucose. The drug stocks were refreshed every ten days.

Adaptive Evolution Experiments

Before the beginning of the evolution experiments it was necessary to determine the IC90 of the wild type (WT) strains. Therefore, MIC experiments were established for *S. aureus* to cefepime and *K. pneumoniae* to meropenem. Twenty four well plates were used for the evolution experiments. Both adaptive evolutions were performed in triplicates. Positive and negative controls accompanied every adaptation experimental step. The negative control included media only and was used as an indicator of contamination and as a way to measure the optical density of pure media. The positive control, apart from the media, contained 75 µl of the inoculating strain. The inoculating strains were subjected to increasing drug concentrations in two-fold steps. After an eighteen hour incubation period at 37 ^{o}C, the optical density (OD) at 600 nm wavelength was measured on a BioTek Epoch plate reader. The value of the positive control was used to normalize the evolution data. In order to determine the well, from which the next day's experiment would be inoculated, a maximum limit of 60% bacterial inhibition was chosen. This value was selected based on previous work[56]. The replicate that presented the best growth below and closer to the 60% inhibition cut off (passage value) was used as the inoculating material for the next experiment. For each replicate eleven wells were used, two for the negative and positive controls and nine for the increasing concentrations of which three were below the previous day's passage value, in two fold steps. 50 µl volume of the passaged well was inoculated into 5000 µl of fresh media containing the passage concentration of the drug and the solution was incubated overnight at 37 ^{o}C. A portion of the overnight culture was saved in glycerol stocks. This same process was repeated eighteen times for both evolution experiments.

Collateral Sensitivity and Resistance Experiments

Bacterial isolates from the last evolution step, which had been saved as glycerol stocks, were tested to determine their IC90 fold improvement over the IC90 of the non-adapted WT. Every adapted strain was also tested to the other antibiotics to determine if adaptation to one drug resulted in cross resistance to another. In order to determine the IC90 of the adapted strains we used 96 well micro titer plates and the experiments were performed in quadruplicates. The concentration of the drugs for each experiment followed a two-fold steps mode. Similarly to the adaptive evolution experiments positive and negative controls were used in each test. Adapted strains from the glycerol stocks were streaked into LB petri plates and incubated at 37^{o}C overnight. Four colonies from each plate were picked, inoculated into MHB+0.5% glucose media and incubated at 37^{o}C for 4 – 6 hours. After the growth period the pre-cultures were diluted and used as inoculant for the 96 well micro titer plates. The inoculated plates were incubated at 37^{o}C overnight for at least 16 hours and after the growth period OD600 was read on a BioTek Epoch plate reader.

Kinetics Experiments

Kinetic experiments were performed for all the adapted strains in order to calculate the generation time *G* and the growth rate *k*. All kinetic experiments were performed in 96 well micro titer plates in quadruplicates. Adapted strains from the glycerol stocks were streaked into LB petri plates and incubated at 37 ^{o}C overnight. A single colony was picked, inoculated into MHB+0.5% glucose media and incubated at 37 ^{o}C for 4 – 6 hours. After the growth period this solution of cells was used as inoculant for the 96 well micro titer plates. The inoculated plates were placed in Elx808 BioTek plate reader at 37 ^{o}C, shaking for a certain period of time (All *E. faecium* isolates were incubated for 14 hours without shaking. All other isolates were incubated for 12 hours, shaking) and OD630 was measured every 5 minutes.

Data Analysis

For the analysis of the experimental data Excel and Prism software was used. First, from all the OD600 values of the inoculated wells the average value of the negative controls was subtracted. The resulting values were then divided by the positive control

OD600 value. The result of the division is the fraction of cells able to grow at a particular drug concentration. Inhibition is calculated as: 1-fraction of cell survivors. Inhibition data were plotted in Prism and IC90 was read from the graph.

The OD630 values from the kinetic experiments were plotted in Prism and the time values at 0.1 OD630 and 0.25 OD630 were read from the accrued graph. Generation time is calculated as: $G = \frac{\Delta t}{3.3 \bullet \log(\frac{N0.25}{N0.1})}$. Where Δt = time of 0.25 OD630 – time of 0.1 OD630 and N = concentration of cells. Growth rate is calculated as: $k = \frac{2.303 \bullet \log(\frac{N0.25}{N0.1})}{\Delta t}$

Adaptive Evolution of *S. aureus* to Cefepime

S. aureus strain Newman was isolated in 1952 from a human infection and since then has been used in a variety of experiments[57]. Its genome sequence was published in 2008 (Journal of Bacteriology by Tadashi Baba et al.) and showed that Newman lack antibiotic resistant determinants compared to MRSA[58]. Recently, five genes have been found to confer antibiotic resistance, though none of them are β-lactamases[59].

Determining WT MIC

Prior to the adaptation process the Inhibitory Concentration 90% (IC90) of the WT strain to cefepime needed to be determined. This was done for two reasons. First, all the data gathered for the adapted strains, were compared to the IC90 of the non-adapted strain, secondly, the WT IC90 provided a starting point for our adaptation experiment.

The IC90 experiment was performed in a micro titer 96 well plate, the plan of which is presented in figure 1.

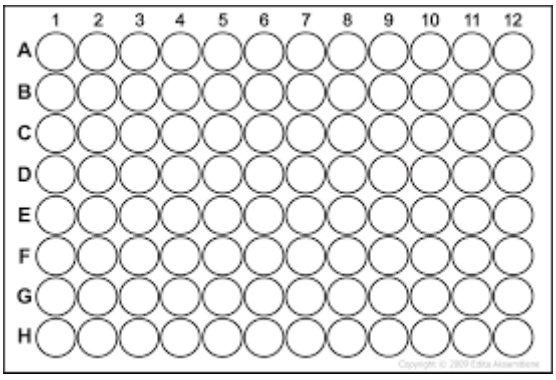

Figure 1: 96 well micro titer plate's plan

The negative control was placed in column 1 and the positive control in column 2. Column 3 to 12 contained increasing concentrations of cefepime. Concentrations increased between columns in two fold steps so that a very broad range of values could be covered. The experiment was performed using four technical replicates. The results of this experiment are presented in figure 3, from which we determined that the IC90 of the WT strain of *S. aureus* to cefepime was 1.45 µg/ml.

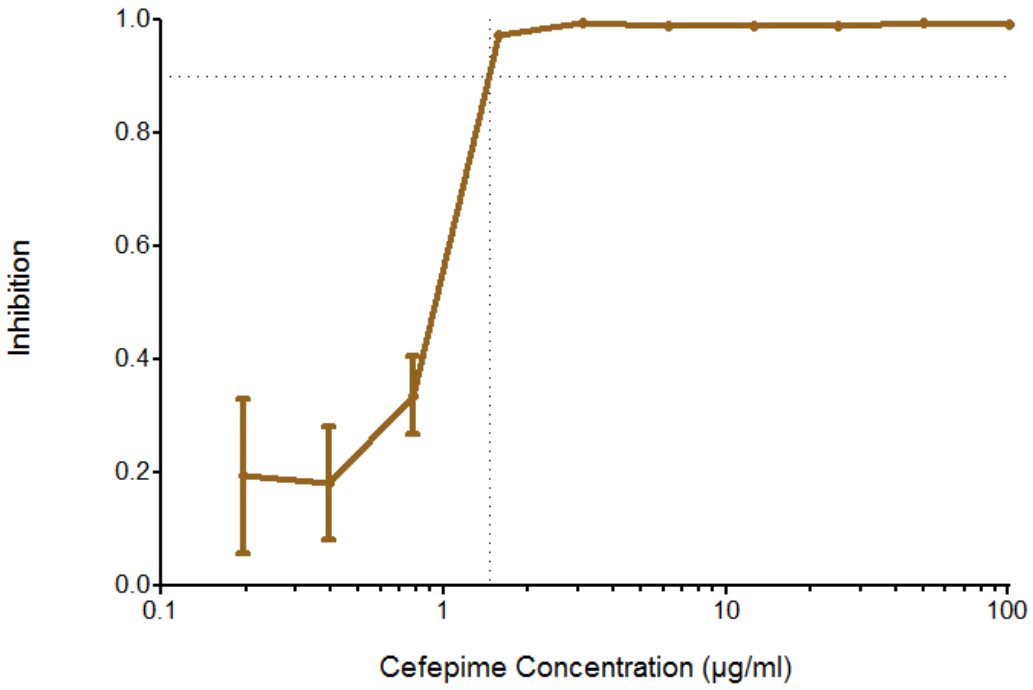

Figure 2: Inhibition of Staphylococcus aureus WT in a gradient of cefepime concentrations. Determination of IC90 at 1.45 µg/ml

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Explanation of the adaptive evolution experiment – Day 1

The adaptive evolution experiments were performed in 24 well plates, the template of which is presented in figure 3.

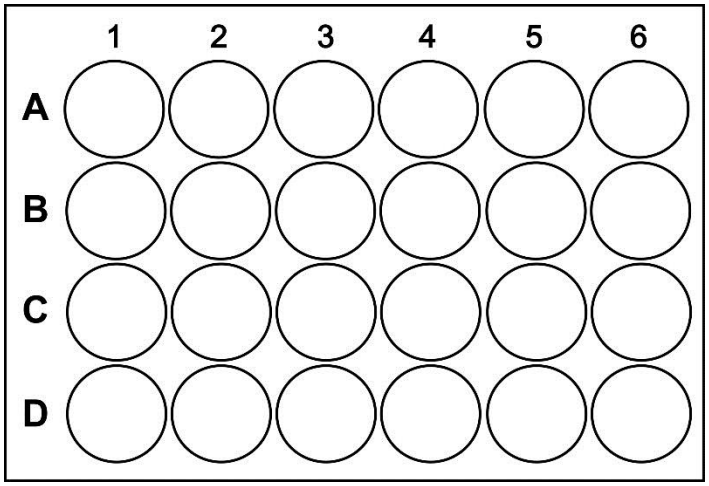

Figure 3: 24 well plate's template

A single *S. aureus* colony was grown up overnight and then used as inoculant in the evolution experiments. The experiment was performed in triplicate and gave rise to 3 lineages: A, B and C. Each evolution step used two 24-well plates. The highest concentration was placed in the first plate in column 6 to allow for a larger concentration range to be tested. For instance, the highest drug concentration of lineage A was placed in well A6, of lineage B in well B6 and of lineage C in well C6. Concentrations then decreased in two-fold steps across plates 1 and 2. In row D on plate 1 the first three wells were used as negative controls and the well D4 was used as positive control for lineage A, well D5 as positive control for lineage B and well D6 as positive control for lineage C. The determined IC90 concentration was used to center the concentration range considered in Day 1 of the evolution experiment. The concentrations chosen for Day 1 are presented in Table 2. Each well contained 1500 µl of the appropriate drug concentration in MHB+0.5% glucose media (except for the negative and positive controls that contained pure media without drug). Apart from the negative controls, the rest wells were inoculated with 75 µl of *S. aureus* WT.

Table 2: Template of the two 24 well plates for Day's 1 experiment. The values in the blue boxes are concentrations of cefepime in µg/ml

PLATE 2

	1	2	3	4	5	6
A	Empty	Empty	Empty	0,06	0,125	0,25
B	Empty	Empty	Empty	0,06	0,125	0,25
C	Empty	Empty	Empty	0,06	0,125	0,25
D	Empty	Empty	Empty	Empty	Empty	Empty

PLATE 1

	1	2	3	4	5	6
A	0,5	1	2	4	8	16
B	0,5	1	2	4	8	16
C	0,5	1	2	4	8	16
D	Negative Control	Negative Control	Negative Control	Positive control of lineage A	Positive control of lineage B	Positive control of lineage C

Following 16 hours of incubation at 37 ^{o}C, we measured the OD600 of the wells and the results of Day 1 are presented in Table 3.

Table 3: Calculation of OD600 from Epoch Plate Reader for Day 1 (Values in white cells). On the top (blue cells) the values refer to the concentration of cefepime in the wells of the same column. D row of Plate 1 contains the negative and positive controls as shown in Table 2.

PLATE 2

				0,06	0,125	0,25
A				0,528	0,514	0,468
B				0,516	0,446	0,428
C				0,555	0,507	0,466
D						

PLATE 1

	0,5	1	2	4	8	16
A	0,449	0,405	0,153	0,095	0,093	0,098
B	0,289	0,301	0,13	0,091	0,088	0,093
C	0,288	0,336	0,147	0,09	0,087	0,097
D	0,077	0,074	0,068	0,573	0,602	0,642

An average of the three negative controls was taken and subtracted from the remaining values. The resulting values were then normalized by their respective positive control, so all values from lineage A were divided by the positive control of lineage A, the values from lineage B by the positive control of lineage B and the values from lineage C by the positive control of lineage C. The results were then subtracted from 1 in order to determine the fraction of the population inhibited by the drug concentration. The results for Day 1 are presented in Table 4.

Table 4: Percentage of inhibition in each well.

PLATE 2

	0,06	0,125	0,25
A	15%	17%	26%
B	17%	30%	33%
C	10%	19%	26%

PLATE 1

	0,5	1	2	4	8	16
A	29%	38%	85%	96%	96%	95%
B	59%	57%	89%	97%	97%	96%
C	60%	51%	86%	97%	97%	95%

We used the following criteria to determine which wells would be used as inoculum in the next evolution experiment: the highest concentration for which no more than 60% inhibition was present. In Day 1 the wells with 0.5 μg/ml cefepime concentration for all three lineages were selected.

Explanation of the adaptive evolution experiment – Day 2 to Day 18

The passaged concentration from Day 1 was placed in column 1 of plate 1. In this case 0.5 μg/ml for all three lineages. The other concentrations follow the two-fold increasing and decreasing method as in Day 1. Each lineage was inoculated using the selected well of the previous day into a new plate. For example, all the wells referred to lineage A (Plate 1: A1 to A6 and D4, Plate 2: A4 to A6) were inoculated from the passaged well of Day 1 (A1 from Plate 1 in our case). The same pattern applied for lineages B and C. The inoculation of Day 2 plates is presented in figure 4. The same process was repeated until Day 18, which was the last adaptation day of this experiment. The passage wells were determined with the 60% criteria after analysis of the data in Microsoft Excel.

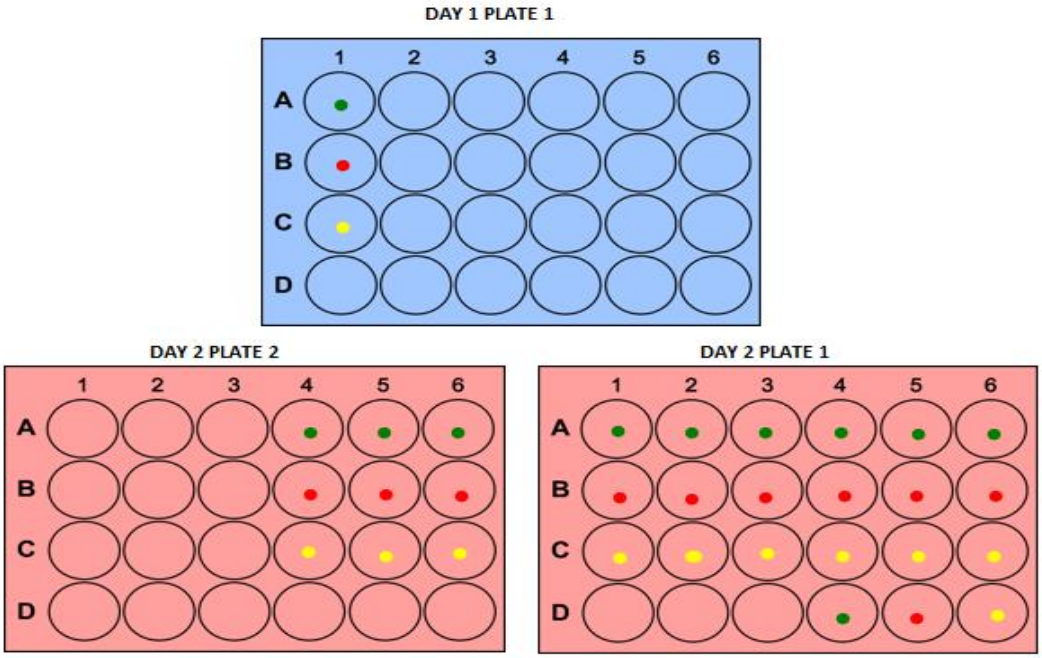

Figure 4: Inoculation of Day 2. Lineage A: GREEN, Lineage B: RED, Lineage C: YELLOW

Results after the completion of the adaptation

The adaptation of *S. aureus* to cefepime lasted 18 days and the results for each lineage are presented in figure 5.

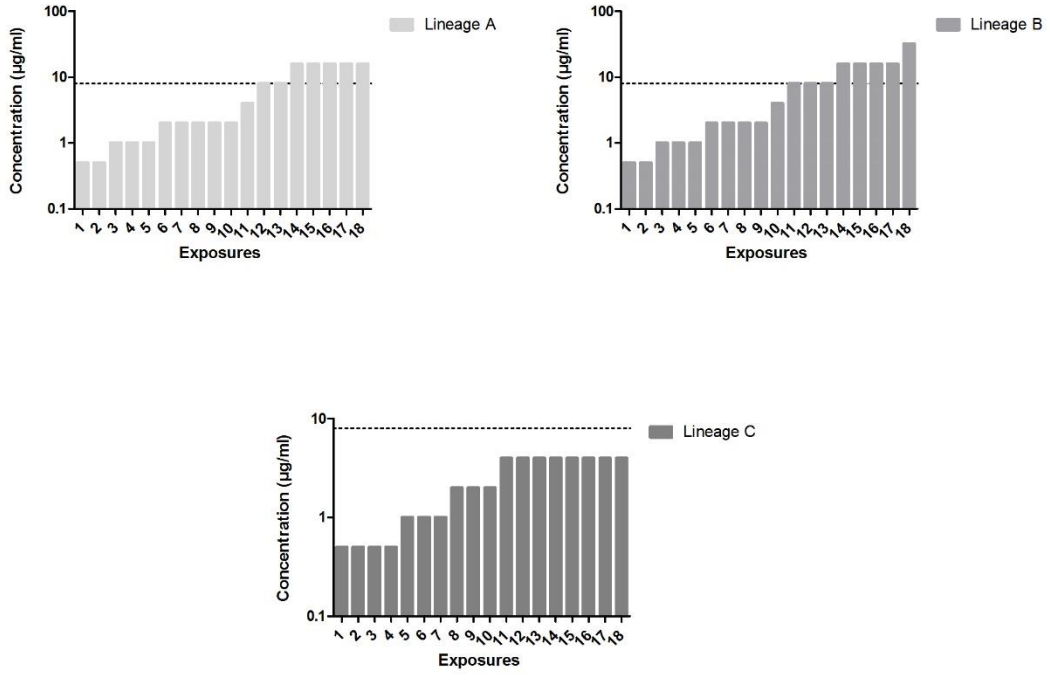

Figure 5: Adaptation of Staphylococcus aureus strain Newman to cefepime. Passage drug concentrations graph. Dash line refers to the ECOFF of Staphylococcus aureus to cefepime which is 8 µg/ml (EUCAST)

We observe that the three lineages differed in their adaptation to cefepime. This is to be expected because *S. aureus* is a living organism with a complicated metabolic network and broad range of genetic mechanisms. A possible explanation for this variation could be that the mutations which occurred in lineage C did not confer high levels of resistance to cefepime where as the mutations that occurred in lineages A and B did. Following 18 days of adaptation lineage A could grow in 16 µg/ml of cefepime, lineage B could grow in 32 µg/ml and lineage C could grow in 4 µg/ml. Following 18 days of exposure, all three lineages reached or surpassed the clinical MIC breakpoint of Staphylococcus aureus to cefepime given by EUCAST (4 µg/ml)[60]. Clinical antimicrobial susceptibility breakpoints are used to predict the clinical outcome of antimicrobial treatment.

Another important parameter concerning antibiotic resistance is the epidemiological cut-off (ECOFF) value, as it is acronymically labelled by EUCAST. ECOFF values are bacteria and drug specific and represent the highest value within a WT MIC distribution. The ECOFF of *S. aureus* to cefepime is 8 µg/ml and the distribution is presented in Figure 6[60]. Lineages A and B surpass the ECOFF value and can be considered clinically resistant.

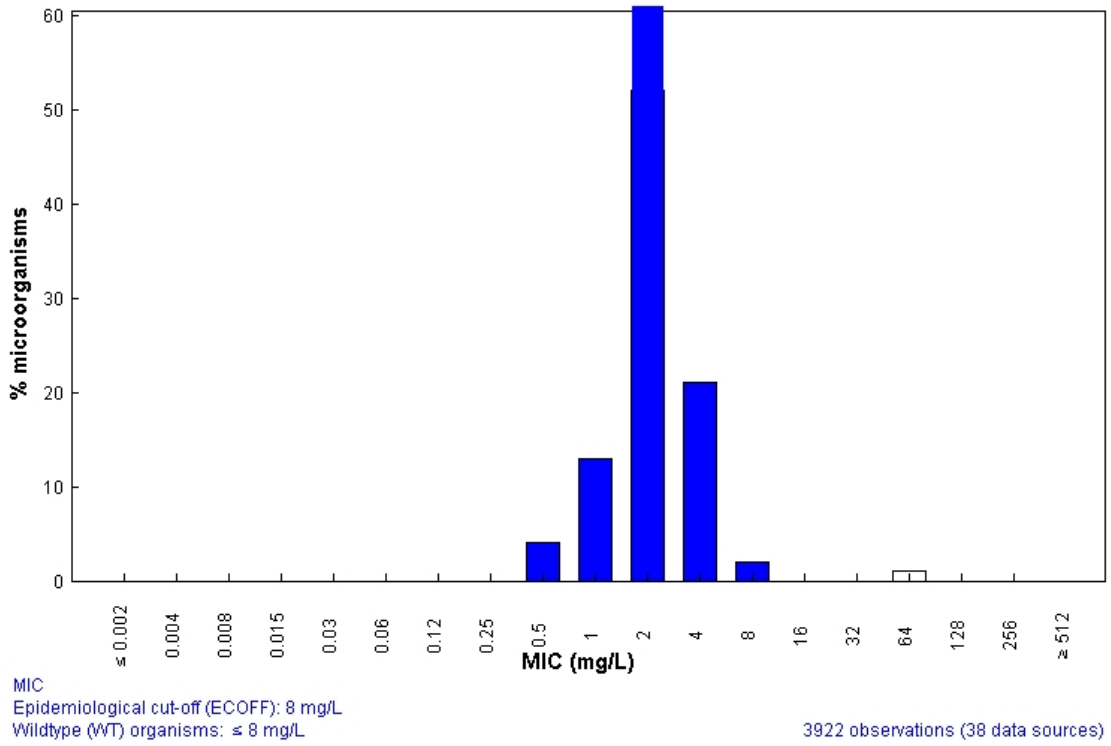

Figure 6: MIC distribution of S. aureus to cefepime from 38 data sources published by EUCAST

Adaptation of *S. aureus* to cefepime was rapid. Lineages A and B exceeded ECOFF value after 13 days, an observation that confirms that this bacteria has the ability to

evolve multi-drug resistant mechanisms. It is likely that this resistance was achieved via mutations affecting the affinity of cefepime's binding to penicillin binding proteins, however, without sequencing data we cannot be sure of this.

MIC of the adapted *S. aureus* strain Newman lineages to cefepime

Following the completion of the adaptation procedure we investigated how the IC90 of the three lineages changed after 18 days of exposure to cefepime. MIC experiments were performed for lineages A, B and C to cefepime. The results of the MIC tests are presented in Figure 7. The IC90 of lineage A was determined to be 85 µg/ml, lineage B was determined to be 65 µg/ml and lineage C was determined to be 20 µg/ml. Lineages A and B adapted to a higher cefepime concentration than lineage C. It is unsurprising that their respective IC90 values would be greater than lineage C. Relative to the WT, the fold improvement of lineage A was 59x WT IC90, of lineage B was 45x WT IC90 and of lineage C was 14x WT IC90.

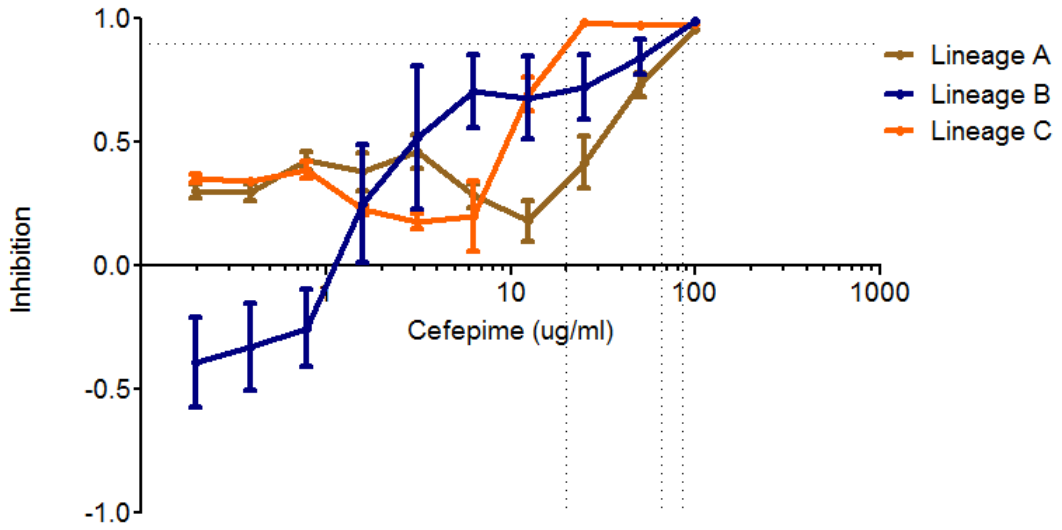

Figure 7: MIC test of Lineages A, B and C to cefepime after 18 days of adaptation

Adaptive Evolution of *K. pneumoniae* to meropenem

Information about *K. pneumoniae* (DSM 30104)

K. pneumoniae (DSM 30104) is a clinical isolate whose genome was published at Journal of Bacteriology in 2012[62]. Fifteen antibiotic resistance genes were identified within its genome, including genes coding for class A beta-lactamase, a multidrug resistant efflux pump and a potassium antiporter. There were also 10 genes related to lactamase function, including gloB and ampC, which have the functions of encoding a metallo-beta-lactamase superfamily hydrolase and beta-lactamase class C, respectively[62].

K. pneumoniae WT MIC test

An MIC test was performed for *K. pneumoniae* to meropenem before beginning of the adaptive evolution experiment. The IC90 was determined at 0.033 μg/ml (Figure 8).

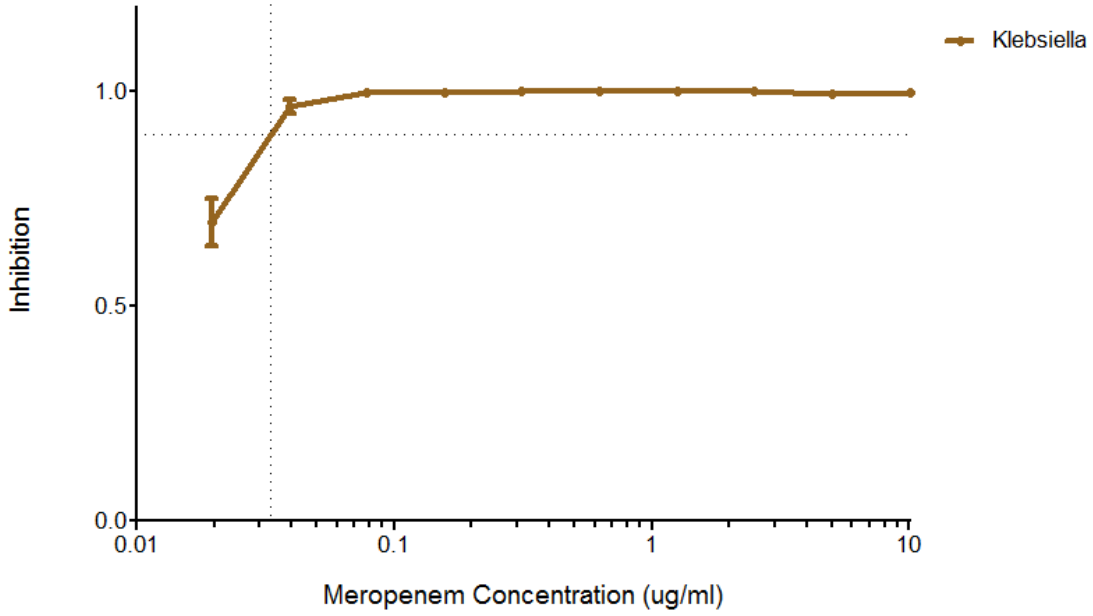

Figure 8: MIC test of Klebsiella pneumoniae WT to meropenem

K. pneumoniae adaptive evolution experiment

The procedure that was followed for the 18 days adaptive evolution of *K. pneumoniae* to meropenem was the same as in the case of *S. aureus* to cefepime. Results of the adaptation are presented in Figure 9.

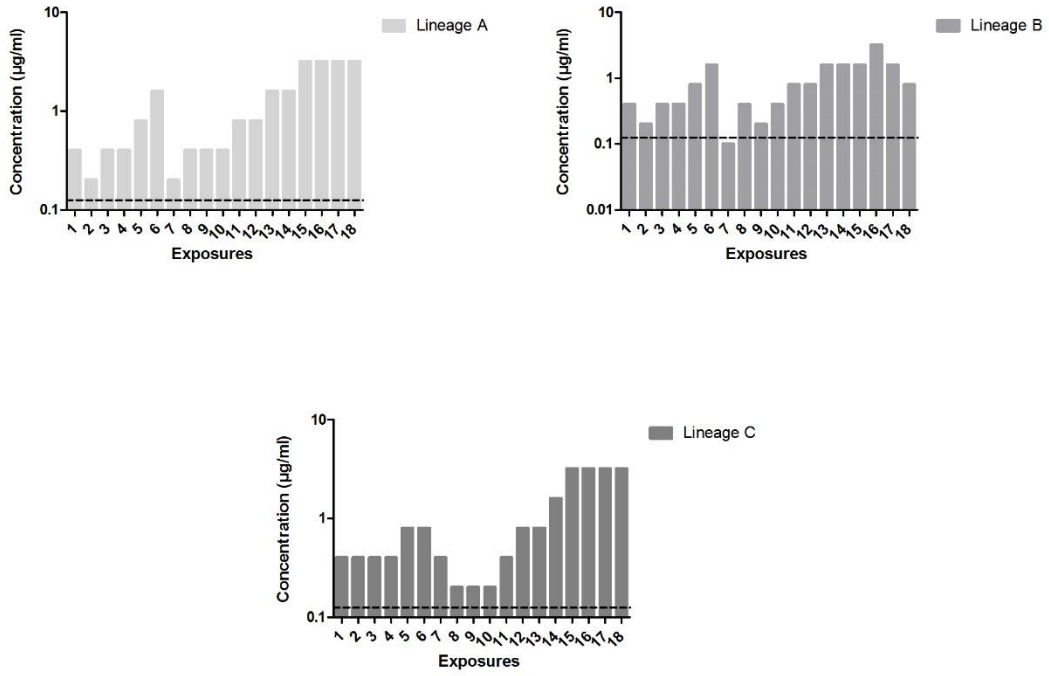

Figure 9: Adaptive evolution of Klebsiella pneumoniae to meropenem. Y axis: Passage concentration (µg/ml), X axis: exposure (days). The dashed line represents the ECOFF value, which is at 0.125 µg/ml

Lineage A adapted quickly to meropenem, and then at Day 7 a big drop in the passage concentration appeared. After this drop or valley, lineage A steadily started to adapt in higher drug concentrations. Lineage B experienced two adaptation valleys, one at Day 7 and one at Day 9. Thereafter, the drug concentration increased up to Day 16, when it started dropping again. Lineage C had one adaption valley starting from Day 7 and continuing until Day 10. Thereafter it adapted steadily to higher drug concentration reaching a maximum value at Day 15.

Every day we selected the strongest population to passage and this choice lead to adaptation bottlenecks. What we observe here is that resistance comes at a cost to the organism and this cost is manifested as slower growth or weaker growth. The adaptation valleys represent this phenomenon.

Following 18 days of adaptation lineages A and C were able to grow in 3.2 µg/ml of cefepime, while Lineage B was able to grow in 0.8 µg/ml.

The epidemiological cut off (ECOFF) for meropenem tested in *Klebsiella pneumoniae* is 0.125 µg/ml given by EUCAST[63].

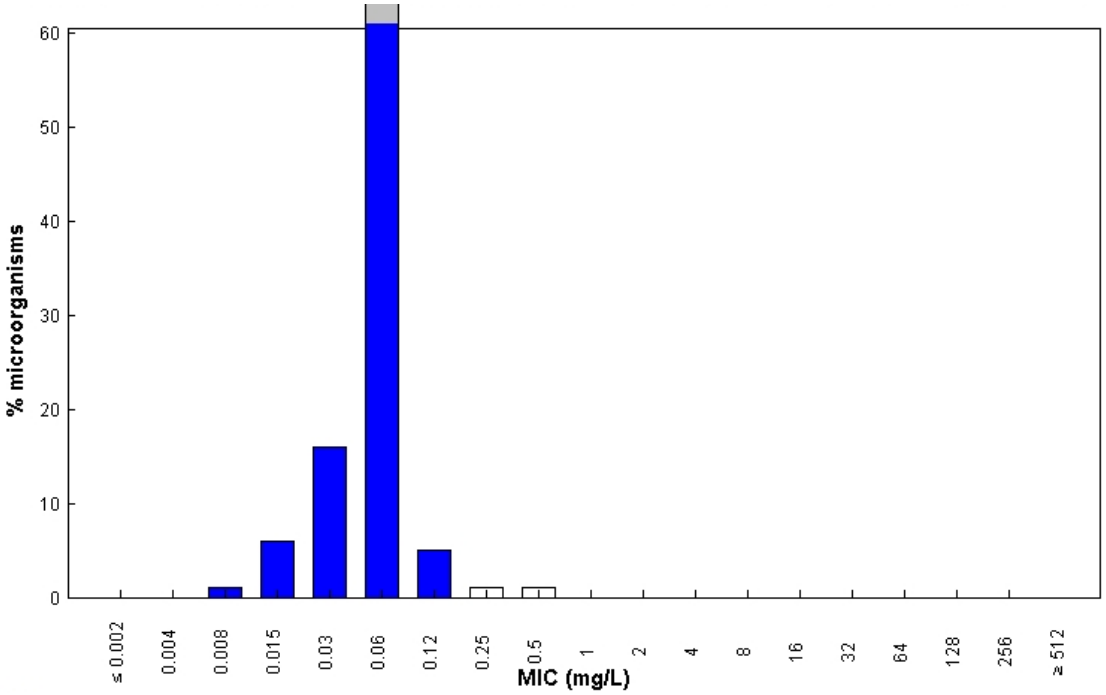

Figure 10: MIC distribution of Klebsiella pneumoniae to meropenem published by EUCAST

In our adaptive evolution experiment all lineages surpassed the ECOFF value. This observation confirms that all our lineages became clinically resistant to meropenem. The main mechanisms of resistance in our case were likely β-lactamases since carbapenems are β-lactam antibiotics and from previous work our strain was known to possess GloB, AmpC and 8 more genes that code for β-lactam resistance[62].

MIC test of the adapted *K. pneumoniae* lineages to meropenem

Following the adaptation process we determined the fold improvement of the IC90 value of all three lineages over the WT IC90 using MIC tests. The results are presented in Figure 11. The IC90 for lineage A was at 4 µg/ml and 2.6 µg/ml for lineages B and C, indicating that adaptation was nearing its end. This corresponds to a fold improvement of 121 times for lineage A and 79 times for lineages B and C. All three lineages became more than 50 times more resistant to meropenem than the non-adapted WT strain.

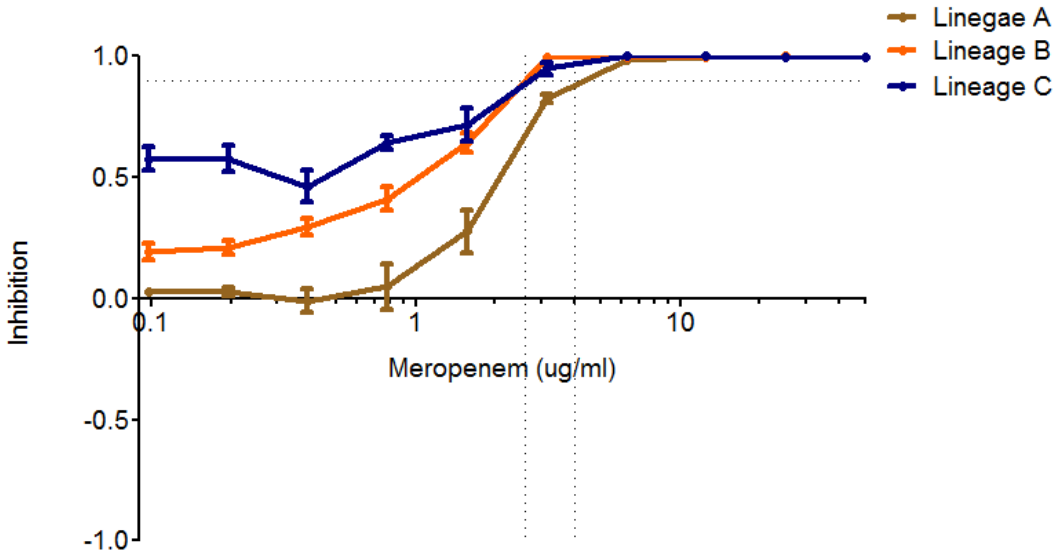

Figure 11: MIC test of K. pneumonia to meropenem lineages A, B, C after 18 days of exposure.

Introduction to Cross Resistance and Sensitivity Experiments

Apart from the two adaptive evolutions that we performed, 28 additional adaptive evolutions had already been done by Marius Faza, a postdoc in the Sommer Lab. Isolates of all adapted populations were tested to the drug they were adapted to and to the four other drugs used in this work. The information derived from these experiments help us to understand how resistance amongst different classes of drugs is related. The bacteria used in these experiments are *E. faecium* (DSM 2146), *Staphylococcus aureus* strain Newman, *K. pneumoniae* (DSM 30104), *A. baumannii* (ATCC 17978), *P. aerugnosa* (PAO1) and *E. cloacae* (ATCC 13047). The drugs used in these experiments were: cefepime (cephalosporin), meropenem (carbapenem), tetracycline (polyketide), gentamicin (aminoglycoside) and ciprofloxacin (fluoroquinolone). From now on in the thesis the drugs will be also referred with their acronyms, Cefepime – FEP, Meropenem – MEM, Tetracycline – TET, Gentamicin – GEN and Ciprofloxacin – CIP. The target of each drug is presented in Table 5. Although they belong to different classes of antibiotics, the targets of FEP and MEM, and GEN and TET were common. Necessary for the following analysis are the WT IC90s of the WT strains, which are presented in Table 6.

Table 5: target of each antibiotic in the bacterial cells

DRUG	CLASS	ABBREVIATION	TARGET
cefepime	cephalosporins	FEP	cell wall
ciprofloxacin	fluoroquinolones	CIP	DNA gyrase
gentamicin	aminoglycosides	GEN	ribosome 30s
meropenem	carbapenems	MEM	cell wall
tetracycline	tetracyclines	TET	ribosome 30s

Table 6: WT IC90s of all species to the suite of drugs. Values are in µg/ml

WT IC90s	MEM	TET	CIP	GEN	FEP
A. baumannii	0,07	0,35	0,05	0,16	1,25
E. cloacae	0,14	1,06	0,03	1,17	0,14
P. aeruginosa	0,60	2,70	0,08	0,29	0,75
K. pneumoniae	0,03	0,57	0,15	0,29	1,95
S. aureus	0,27	0,14	0,08	0,36	1,45
E. faecium	0,75	0,14	1,39	4,60	5,00

Cross Resistance of *Staphylococcus aureus* (Newman)

Results of the collateral sensitivity and resistance tests for all adapted *S. aureus* strains are shown in Figure 12. The color of the cells represent the intensity of the fold susceptibility or the fold resistance over the WT strain. Cells that have shades of blue show that the adapted stain is more susceptible and cells that have shades of brown show that the adapted strain is more resistant. Heat map values represent an average IC90 of four biological replicates divided by the WT IC90 of the selected drug. These values reflect resistance or sensitivity gained as a result of adaptation to a single drug.

HEATMAP

	Fep	Cip	Gen	Mem	Tet
Fep A	58,62	1,07	0,78	14,81	5,00
B	44,83	0,96	0,36	0,89	0,43
C	13,79	0,73	0,83	7,78	17,14
Cip A	0,91	1066,67	3,06	1,19	1,43
B	1,31	2866,67	2,67	0,78	2,07
C	1,62	2066,67	2,67	1,37	2,07
D	0,91	833,33	4,03	1,00	0,93
Gen A	1,59	2,00	158,33	0,93	0,50
B	1,17	1,47	291,67	0,52	0,46
C	1,59	0,87	291,67	1,96	1,43
D	0,72	1,00	158,33	0,52	0,89
Mem A	2,76	0,80	3,61	10,37	0,21
B	1,24	0,80	3,33	10,00	1,29
C	0,66	0,80	1,94	9,26	1,11
D	3,17	7,33	3,89	10,74	1,43
Tet A	1,59	0,93	1,25	0,48	6,07
B	0,46	1,27	2,08	8,15	16,43
C	0,79	0,93	0,83	6,67	13,57
D	1,45	1,27	1,25	0,56	12,14

Figure 12: Cross resistance/susceptibility of S. aureus strain Newman to FEP CIP MEM GEN and TET

Adaptation to FEP confers cross-resistance to MEM and TET (lineages A and C). Adaptation to CIP confers slight cross resistance to GEN. The IC90 of CIP adapted to CIP was more than 10^3 times the WT. Adaptation to GEN does not result in collateral resistance or susceptibility. Adaption to MEM confers slight cross-resistance to GEN. TET adaptation resulted in MEM cross resistance in two out of four lineages. Overall adaptation of *S. aureus* to antimicrobial environments results in limited cross-resistance and in no collateral sensitivity.

Cross Resistance of *Acinetobacter baumannii* (ATCC 17978)

A. baumannii strain ATCC 17978 was isolated from a case of meningitis in 1951. Its sequence was published in 2007[64]. It was reported that *A. baumannii* ATTCC 17978 possessed 74 potential drug-resistant genes, including 32 efflux pumps and 11 permeases of the drug/metabolite transporter (DMT) superfamily. This strain is also reported to be resistant to β-lactams and have weak resistance to tetracycline and aminoglycosides. The cross-resistance of *A. baumannii* to our suite of drugs is presented in Figure 13.

HEATMAP

	Fep	Cip	Gen	Mem	Tet
Fep A	20,00	5,00	39,06	2,86	2,86
B	10,00	4,80	15,63	6,43	2,29
C	11,20	11,00	51,88	2,14	2,86
D	7,60	6,00	15,63	2,86	1,79
Cip A	8,00	8000,00	0,94	7,86	6,57
B	5,60	8000,00	1,56	7,86	5,43
C	6,40	10000,00	23,75	2,14	3,14
D	16,00	10000,00	7,81	4,14	2,86
Gen A	17,60	9,60	8750,00	2,14	0,77
B	15,20	6,20	4812,50	1,10	0,43
C	15,20	9,60	7312,50	1,10	0,43
Mem A	10,64	4,50	3,44	80,00	0,09
B	20,00	3,20	3,44	72,86	0,09
C	13,60	3,20	1,25	314,29	0,18
D	80,00	20,00	44,57	44,57	0,08
Tet A	3,60	3,40	1,00	4,43	114,29
B	3,60	3,40	1,00	4,43	114,29
C	7,52	9,40	0,42	5,71	59,14
D	3,60	3,40	1,00	4,43	29,43

Figure 13: Heatmap: Cross resistance/susceptibility of A. baumannii to FEP CIP MEM GEN and TET

Adaptation of *A. baumannii* to FEP resulted in an increase of WT IC90 by 8 to 20 times, but resulted in collateral cross resistance to GEN (16-39x WT IC90). Additional notable cross-resistance was observed at CIP (>5x WT IC90). IC90 of CIP adapted to CIP is 10^4 times the WT. In addition, adaptation to CIP confers cross-resistance to the remaining drugs. Adaption to GEN confers resistance to FEP and CIP. After the adaptive evolution of *A. baumannii* to GEN the IC90 increased from 4813 times in lineage B up to 8750 times in lineage C. The strains adapted to MEM became more than 10 times more sensitive to TET. Adaption to MEM also confers resistance to FEP, CIP and GEN. Although the evolution of resistance to FEP is explainable because they both are β-lactam antibiotics and therefore share common target, the increased susceptibility in TET needs further research. Adaption to TET confers slight resistance to FEP, CIP and MEM. The presence of several genes in *A. baumannii* may explain the collateral resistance and sensitivity observed here.

Cross Resistance of *Enterococcus faecium* (DSM 2146)

The genome of *E. faecium* DSM 2146 has not been sequenced, so we do not know if it has any resistance genes. The first *E. faecium* whole-genome sequence was published in 2012 (by Margaret M. C. Lam in Journal of Bacteriology)[65]. It was a strain isolated from a patient's bloodstream in Australia reported to have the vancomycin resistant gene VanB. Later in 2012 the complete genome sequence of *E. faecium* TX16 (also referred as DO) was published[66]. This is an isolate from a hospitalized patient with endocarditis. Four tetracycline resistance genes were reported related to tetracycline efflux pumps. The results from our cross resistance experiments in *E. faecium* are presented in figure 14.

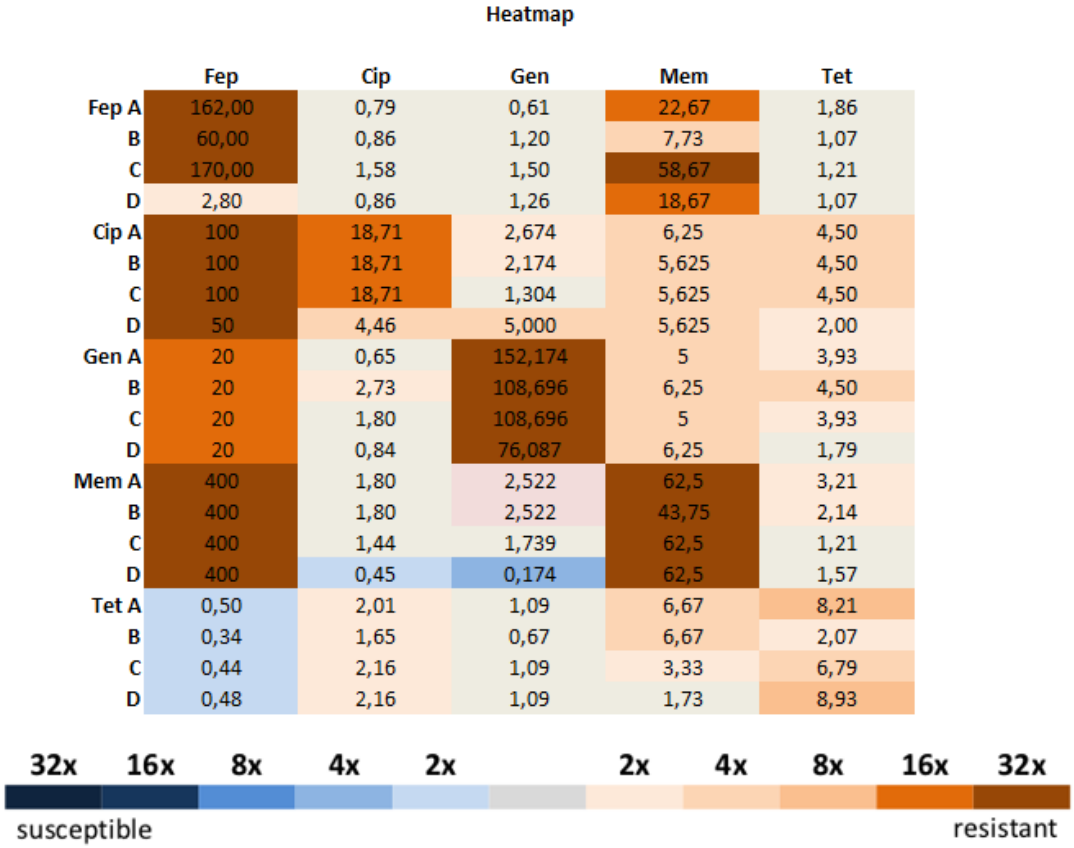

Figure 14: Heatmap: Cross resistance/susceptibility of E. faecium to FEP CIP MEM GEN and TET

Adaptation to FEP resulted in 20 times cross-resistance to MEM. Adaptation to CIP confers very strong cross resistance to FEP (100x WT IC90) and more moderate cross-resistance to MEM (6x WT IC90) and TET (5x WT IC90). Adaptation to GEN resulted in 20x IC90 in FEP and also confers resistance to MEM (WT IC90) and TET (4x WT IC90). Adaptation to MEM confers cross-resistance to FEP. Finally, *E. faecium*

adapted to TET, not only, conferred a slight cross resistance to MEM (2-6x WT IC90) and CIP (2x WT IC90), but also a slight cross sensitivity to FEP (2x WT IC90).
Firstly, notable is that adaptation of *E. faecium* to TET did not result in large improvement in IC90. Secondly, *E. faecium* developed resistance to FEP after adaption to CIP, GEN and MEM. We know that carbapenems and cephalosporins have the same mechanism of action which may explain the cross resistance between FEP and MEM. However, it is difficult to explain, without sequencing data, why resistance to a fluoroquinolone or an aminoglycoside leads to cross-resistance to FEP. In a publication by LB Rice et al., it is stated that *E. faecium* amplifies its resistance against β-lactam antibiotics with combinations of mutations in PBP5 gene[67]. However, there is no evidence that adaptation to CIP or GEN results in mutations to the PBP5 gene.

Cross Resistance of *Pseudomonas aeruginosa* (PAO1)

Strain PAO1 is a wound isolate and the most widely used strain of *P. aeruginosa*. Its genome was published in 2000[68]. There are 34 known resistance genes occurred with the majority being related with multi-drug resistant efflux pumps achieved by outer membrane proteins. The researchers stated that *P. aeruginosa* contains the highest proportion of regulatory genes observed for a bacterial genome. These regulatory genes may play a role in resistance adaptation.
Analysis of the cross resistance of *P. aeruginosa* PAO1 was performed and the data derived from our study are presented in Table 9. Adaptation to FEP limited cross-resistance to other drugs. Out of four lineages only lineage C surpassed more than 10 times the IC90 value of the WT strain. Adaptation to CIP resulted in cross-resistance to TET (4-8x WT IC90) and FEP (8-16x WT IC90). Adaptation to GEN confers slight cross-resistance to the remaining drugs (3-6x WT IC90). Strains adapted to MEM gained cross-resistance to TET (15x WT IC90) and a slight cross resistance to FEP (4-7x WT IC90) and CIP (4x WT IC90). Adaptation to TET confers cross-resistance to all remaining drugs.

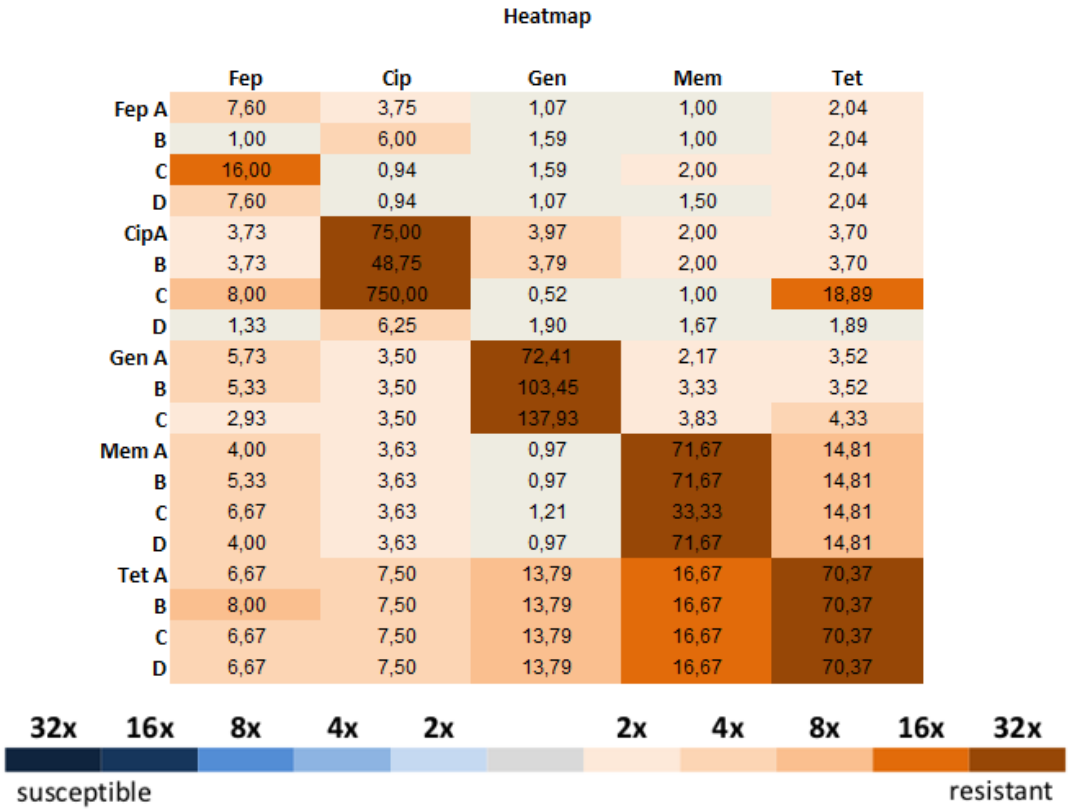

Figure 15: Heatmap: Cross resistance/susceptibility of P. aeruginosa to FEP CIP MEM GEN and TET

P. aeruginosa has difficulty evolving strong antibiotic resistance mechanisms against FEP and it is for this reason that it is commonly used against this specific bacteria. The ECOFF value of *P. aeruginosa* to FEP is 8 µg/ml and apart from lineage C none of the remaining three lineages surpassed it. In research published in the journal of Antimicrobial Chemotherapy in 2002, scientists stated that the pharmacodynamics profile of cefepime predicts acceptable levels of success against *P. aeruginosa*[69]. In a similar study with extended-infusion cefepime used as treatment for *P. aeruginosa* infections, the results showed that cefepime reduces mortality, reduces the mean time of stay in the intensive care unit by 3.5 days and reduces the hospital costs by $ 23,183per person[70].

Cross Resistance of *Enterobacter cloacae* (ATCC 13047)

E. cloacae (ATCC 13047) was isolated from human cerebrospinal fluid by Edwin Oakes Jordan in 1890. The genome of this strain was published in 2010[42]. This strain has a broad range of antibiotic resistance mechanisms including efflux pumps and β-lactamases. *E. cloacae* was challenged to the suite of 6 drugs that we used and the

adapted strains were tested for possible development of collateral sensitivity or resistance.

The data we gathered from the MIC experiments are presented in figure 16.

HEATMAP

	Fep	Cip	Gen	Mem	Tet
Fep A	17,86	1,21	0,38	3,21	1,08
B	17,86	1,21	0,43	1,79	1,08
C	32,86	1,21	1,71	3,57	0,62
D	8,57	1,03	0,20	0,71	0,94
Cip A	2,50	758,62	0,38	1,00	1,60
B	1,79	758,62	0,11	0,93	3,87
C	1,79	1724,14	0,21	0,43	1,42
D	2,14	1379,31	0,21	0,93	1,13
Gen A	4,29	0,66	19,66	1,07	1,04
B	4,29	1,10	19,66	1,57	1,04
C	1,79	0,55	31,62	0,86	0,75
D	3,93	1,10	19,66	1,00	1,04
Mem A	85,71	2,76	0,68	357,14	1,70
B	71,43	2,41	0,47	135,71	1,04
C	42,86	1,24	0,43	285,71	0,54
D	42,86	0,62	0,52	164,29	1,04
Tet A	7,86	37,93	0,26	0,79	160,38
B	15,00	20,69	0,44	1,50	150,94
C	8,57	20,69	0,51	1,14	188,68
D	3,93	9,66	0,85	1,29	37,74

Figure 16: Heatmap: Cross resistance/susceptibility of E. cloacae to FEP CIP MEM GEN and TET

Adaptation to FEP results in a cross sensitivity to GEN (2-5x WT IC90) and cross resistance to MEM (3x WT IC90). CIP adapted strains became 5 to 10 times more sensitive to GEN. Adaptation to GEN confers cross-resistance to FEP (4x WT IC90). We also observed an unequal bidirectional collateral resistance between FEP and MEM, which was stronger for MEM adapted strains (43 – 85x WT IC90). Finally, adaptation to TET resulted in cross-resistance to FEP (4-15x WT IC90) and CIP (10-38x WT IC90).

Compared to MEM, FEP is a more stable and strong β-lactam antibiotic against *E. cloacae*. We observe that the lineages adapted to MEM had greater cross-resistance to FEP than lineages adapted to FEP. This observation suggests that carbapenem resistant *E. cloacae* should not be treated with cephalosporins such as cefepime.

Another observation that we should highlight is the one direction increased resistance of TET adapted lineages in CIP, a fluoroquinolone that targets DNA gyrase and topoisomerase IV. The main mechanism of resistance to quinolones is accumulated

mutations in gyrA, gyrB, parA and parC genes[71]. However, the possibility that adaption to TET leads to accumulated mutations in these genes unlikely. Thus, we can attribute this resistance increase to mutations related with cell wall permeability, such as efflux pumps. Sequencing results will clarify this.

Cross Resistance of *Klebsiella pneumoniae* (DSM 30104)

K. pneumoniae (DSM 30104) is a multi-drug resistant strain as previously stated. Following the adaptive evolution to each one of the drugs, we tested the collateral sensitivity and resistance of each adapted strain. The collected data are presented in Figure 17.

HEATMAP

	Fep	Cip	Gen	Mem	Tet
Fep A	8,00	0,48	0,86	24,24	14,04
B	14,56	0,97	0,93	45,45	21,93
C	3,74	1,72	2,59	10,00	17,54
D	2,00	0,97	0,86	27,27	18,42
Cip A	0,04	39,66	0,22	0,82	2,63
B	0,02	79,31	0,24	1,12	5,26
C	0,05	151,72	0,24	1,09	1,32
D	0,07	35,86	0,31	2,12	2,63
Gen A	0,07	0,76	155,17	1,52	3,68
B	0,28	0,83	275,86	4,24	4,56
C	0,08	0,45	137,93	5,76	2,63
D	0,04	0,24	1,21	2,12	1,40
Mem A	3,08	2,48	0,52	121,21	22,81
B	6,15	6,90	1,00	78,79	26,32
C	5,13	6,90	1,00	78,79	24,56
Tet A	0,07	1,10	0,52	6,67	31,58
B	0,05	0,13	1,00	1,58	17,54
C	0,55	0,97	0,52	1,82	149,12
D	0,29	0,83	0,97	2,33	149,12

Figure 17: Cross resistance/susceptibility of K. pneumoniae to FEP CIP MEM GEN and TET

Strains adapted to FEP develop stronger resistance to MEM (10-45x WT IC90) and TET (14-22x WT IC90) than FEP (2-15x WT IC90). Resistance to CIP and GEN remains at the same levels as the WT. Adaption to CIP results in 16 times cross sensitivity to FEP. Cross-resistance to TET (3-5x WT IC90) and cross sensitivity to GEN (4x WT IC90) were also observed. Adaptation to GEN confers cross resistance to MEM (2-6x WT IC90) and TET (2-5x WT IC90) and cross sensitivity to FEP (4-25x

WT IC90). Adaptation to MEM results in strong cross-resistance to TET (23-26x WT IC90) and cross-resistance to FEP (3-6x WT IC90) and CIP (2-7x WT IC90). Adaptation to TET confers a cross-resistance to MEM (2-7x WT IC90) and strong cross sensitivity to FEP (14x and 20x WT IC90) for two out of four lineages.

The reason for which three out of four adaptations lead to collateral sensitivity to FEP is that the WT strain had an IC90 value, which is 10 times the ECOFF value. This means that DSM 30104 is FEP resistant. In order to understand what exactly happened we need sequencing results. The bidirectional cross-resistance between FEP and MEM has already been explained previously. Other observed instances of cross resistance may be the result of general resistant mechanisms like efflux pumps.

Overall Collateral Sensitivity and Resistance Data Correlation

Apart from the heatmaps that provide an inner species overview and constitute an important source of information for the collateral effects of antimicrobial exposure, we are also interested in how adaptation to a single drug resulted in cross resistance or cross sensitivity to other drugs across all the species. We, therefore, constructed graphs for each antibiotic including the fold IC90 change of all the bacteria in the other four drugs.

Bacteria adapted to FEP

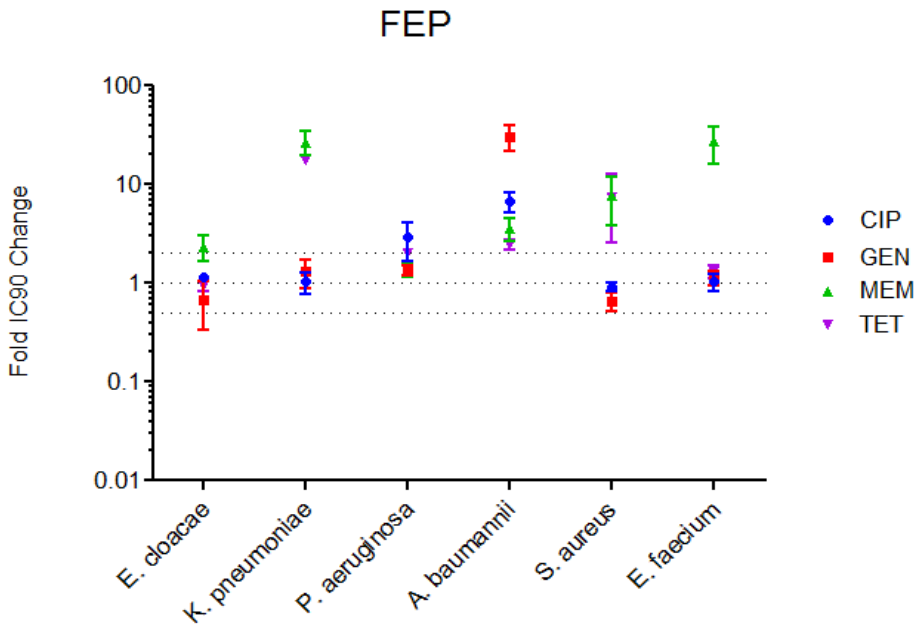

Figure 18: Fold IC90 change of FEP adapted bacteria to the rest of the drugs

Adaptation to FEP has collateral effects to CIP only in the cases of *P. aeruginosa* and *A. baumannii*. *A. baumannii* has the highest fold IC90 change in this case, but still the IC90 value is not 10x higher than the WT's. *A. baumannii* is also the only species to which adaption to FEP resulted in 30 times increased resistance to GEN. The other bacteria remain almost equally resistant to GEN as the WT strain. Another interesting observation that derives from the graph is that adaption to FEP caused 27x fold IC90 change for both *K. pneumoniae* and *E. faecium*. This is may be caused from mutations to some common genes in both bacteria. Sequencing data are needed to explain in depth these findings. In general, adaptation to FEP results in cross-resistance to MEM. Finally, *K. pneumoniae* is the only species that developed more than 10 times increased resistance to TET after adaptation to FEP. Four out of six species gained resistance to TET after adaptation to FEP. Overall adaptation to FEP did not cause collateral sensitivity to any combinations of bacteria and drugs except for *E. cloacae* and *S. aureus* to GEN.

Bacteria adapted to CIP

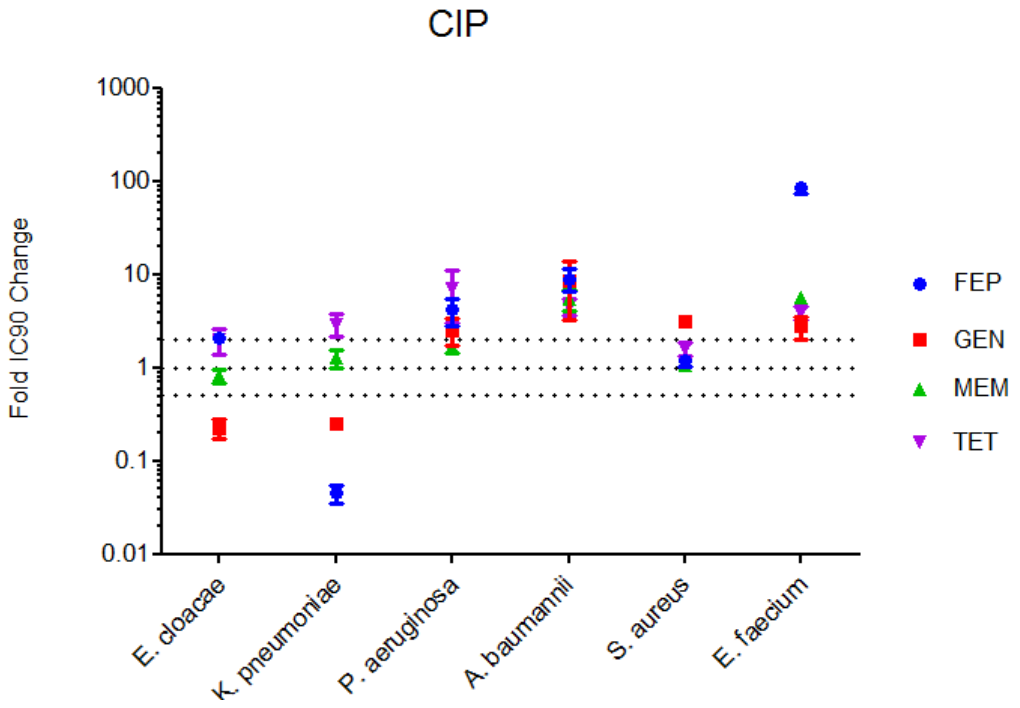

Figure 19: Fold IC90 change of CIP adapted bacteria to the rest of the drugs

Adaptation to CIP resulted in collateral sensitivity to GEN in both *E. cloacae* and *K. pneumoniae* and cross-resistance to the remaining species. *K. pneumoniae* also became sensitive to FEP. However, strong cross-resistance to FEP was observed in *E. faecium* after adaptation to CIP. Equally interesting is that adaption to FEP for both of the aforementioned bacteria resulted in no collateral effects to CIP (figure 18). Cross-resistance to MEM was observed for three out of six species. Finally, adaptation to CIP resulted in cross-resistance to TET in all species. Only three cases of cross sensitivity occur and therefore, overall adaptation to CIP results in collateral resistance in the majority of the cases.

Bacteria adapted to GEN

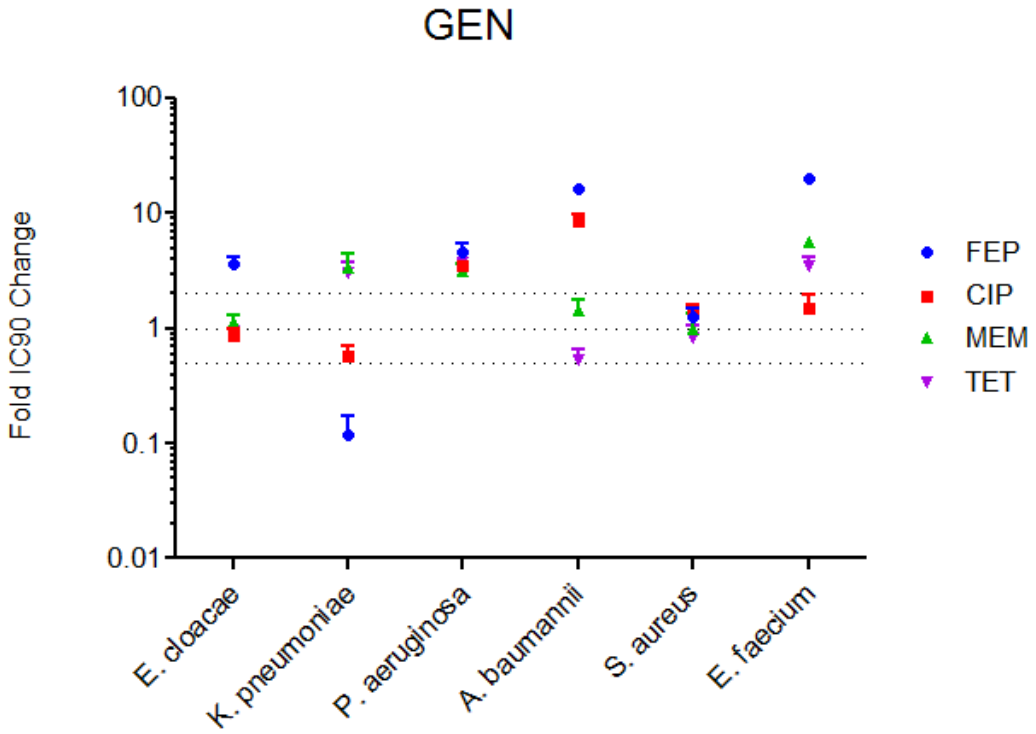

Figure 20: Fold IC90 change of GEN adapted bacteria to the rest of the drugs

Adaptation to GEN resulted in cross-resistance to FEP, CIP and MEM in four out of six species and cross-resistance to TET in three out of six species. Cross sensitivity occurred only in three cases. Overall adaptation to GEN resulted in cross-resistance to the majority of the species and drugs.

Bacteria adapted to MEM

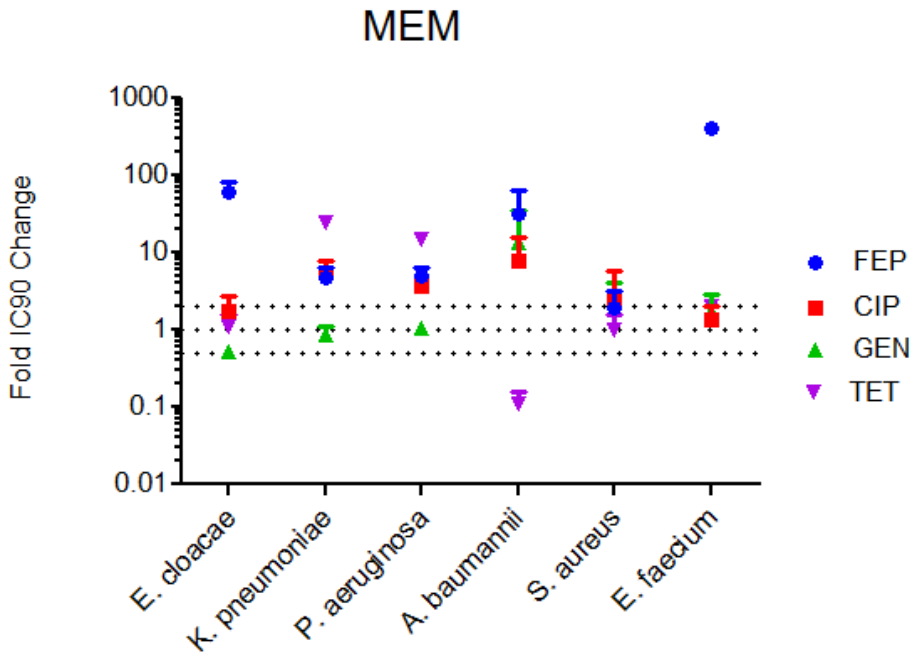

Figure 21: Fold IC90 change of GEN adapted bacteria to the rest of the drugs

The most important observation from Figure 21 is that MEM adaptation results in cross-resistance to FEP across the species, which is very strong in *E. faecium*. Slight cross-resistance across the species appeared also in the case of CIP. No trends occurred for GEN and TET. Overall adaptation to MEM resulted in cross-resistance to the majority of the cases. Cross sensitivity was only observed in a single case.

Bacteria adapted to TET

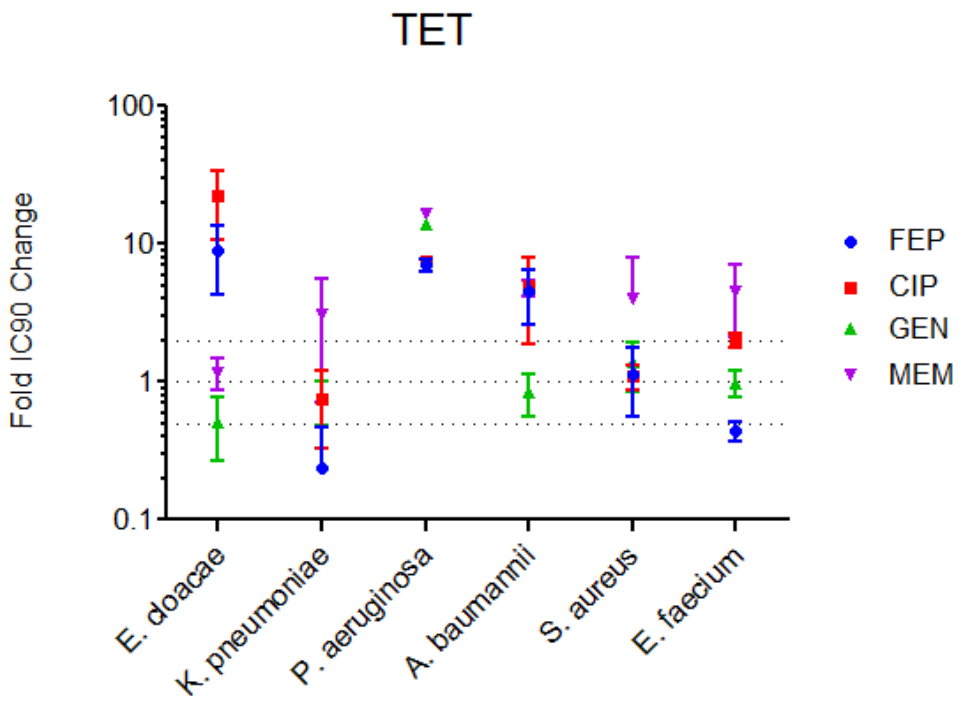

Figure 22: Fold IC90 change of TET adapted bacteria to the rest of the drugs

Adaptation to TET resulted in cross-resistance to MEM across the species (apart from *E. cloacae*). It also caused cross-resistance to CIP in four out of six species. Finally, adaptation to TET did not cause any trends of cross resistance or cross sensitivity to FEP with three values being in the resistance side, two in the susceptibility side and one neutral.

Growth Experiments

The effects of adaptation on growth rate (k) and generation time (G) were investigated. The purpose of these experiments was to locate any adverse effects in the growth kinetics of the strains exposed to antibiotics. If an organism grows very slowly as a result of resistance adaptation then it will likely be outcompeted in an environment containing unadapted species. The experimental method used in order to extract the G and k values is explained in the Materials and Methods section. However, an example will be presented analytically for the better understanding of the procedure.

Staphylococcus aureus strain Newman adapted to FEP growth experiments

S. aureus strain Newman adapted to FEP lineages A, B, C and WT were streaked in LB plates and grown overnight at 37 ^{o}C. A single colony was then picked from each petri plate and inoculated in MHB + 0.5% glucose liquid media and incubated at 37 ^{o}C for 4 to 6 hours. The pre-culture was then diluted to approximately 10^3 cells/μl and 10 μl of this diluted pre-culture was used as an inoculant for a 96 micro titer well plate filled with media. The experiment was performed in quadruplicate technical replicates. The inoculated plate was placed in Elx808 BioTek plate reader shaking at 37 ^{o}C for 12 hours and OD630 was measured every 5 minutes. The data gathered from the plate reader were exported to Prism software and then was used to create the kinetic graphs. The growth curves for *S. aureus* adapted to FEP and *S. aureus* WT are presented in Figure 23.

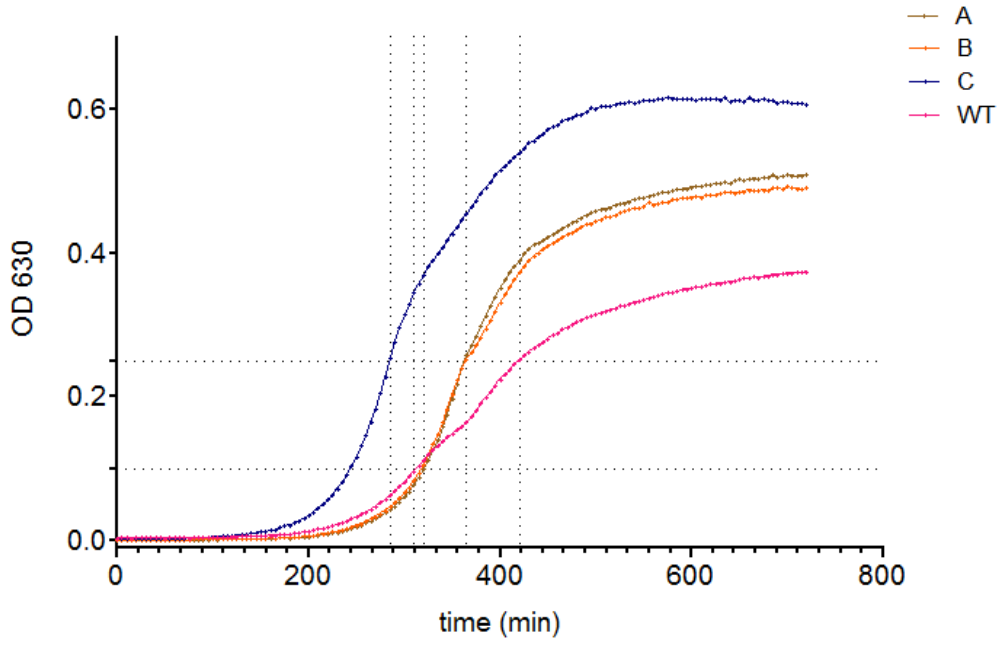

Figure 23: Growth curves for S. aureus strain Newman WT and FEP adapted lineages A, B, C

From the graphs we can observe that the FEP adapted strains grow faster than the WT strain. This is an interesting observation as it shows that resistance adaptation did not have a negative effect to the growth rate of *S. aureus*.

For each curve the 0.1 OD630 and the 0.25 OD630 were determined and then used to calculate the generation time and growth rate (Materials and Methods section).

Table 12: Minutes of growth needed for each strain to reach 0.1 and 0.25 OD630

	OD 630	
	0.1	0.25
A	320 min	365 min
B	320 min	365 min
C	245 min	285 min
WT	310 min	420 min

Using the time values we calculate the generation time and the growth rate. A generation time is the time it takes for one cell to become two. Growth rate k is derived from the first grade differential equation that characterizes the exponential growth period.

Table13: Generation time G and growth rate k for S. aureus strain Newman WT and lineages A, B, C adapted to FEP

	G (min)	k (min-1)
A	34	0,0204
B	34	0,0204
C	30	0,0229
WT	84	0,0083

Large G values and low k values indicate slow growth. From table 13 we confirm our previous observation that the WT strain grows slower than the strains adapted to FEP.

Overall growth experiments

The same procedure, described in the previous section, was performed for all the adapted strains. The calculated k and G values for each strain were used to determine how resistance adaptation affects the kinetics growth of adapted populations. Growth values were used to draw general conclusions about adaptation to a particular drug and about the species overall.

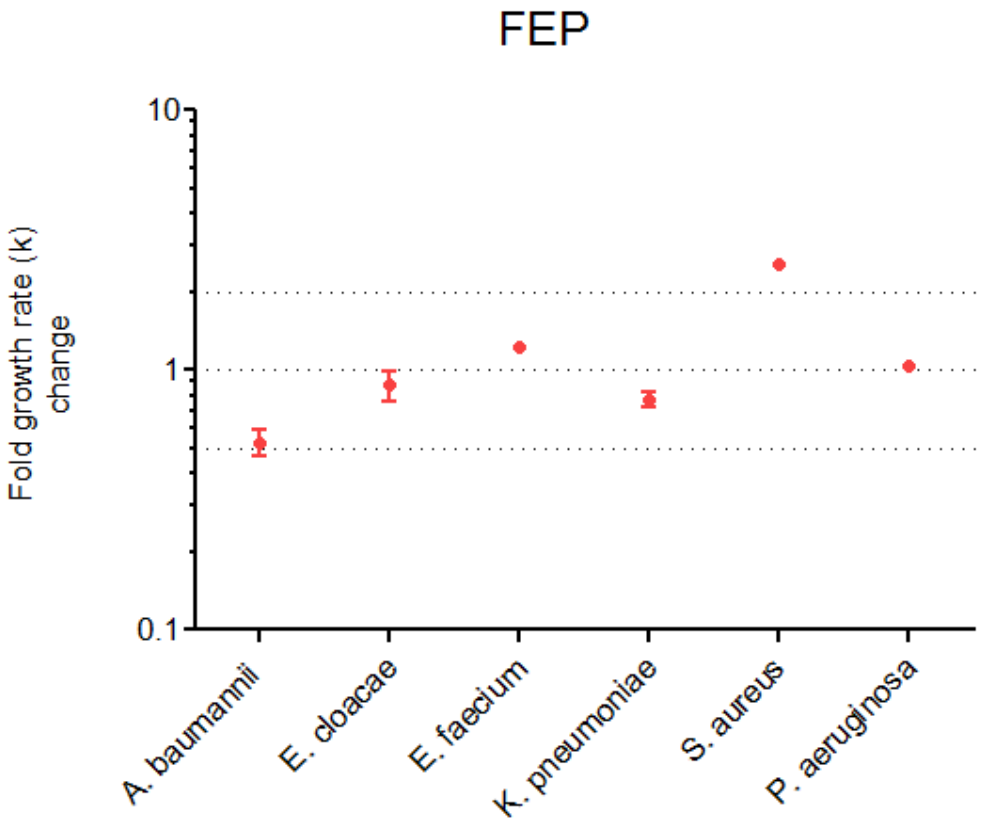

Figure 24: k fold over WT for strains of each bacteria adapted to FEP

From Figure 24 we observe that adaptation to FEP resulted in slower growth for *A. baumannii*, *E. cloacae* and *K. pneumoniae*. On the other hand, the growth rate increased in the cases of *E. faecium* and *S. aureus*. Adaptation to FEP had no effects on the growth rate of *P. aeruginosa*.

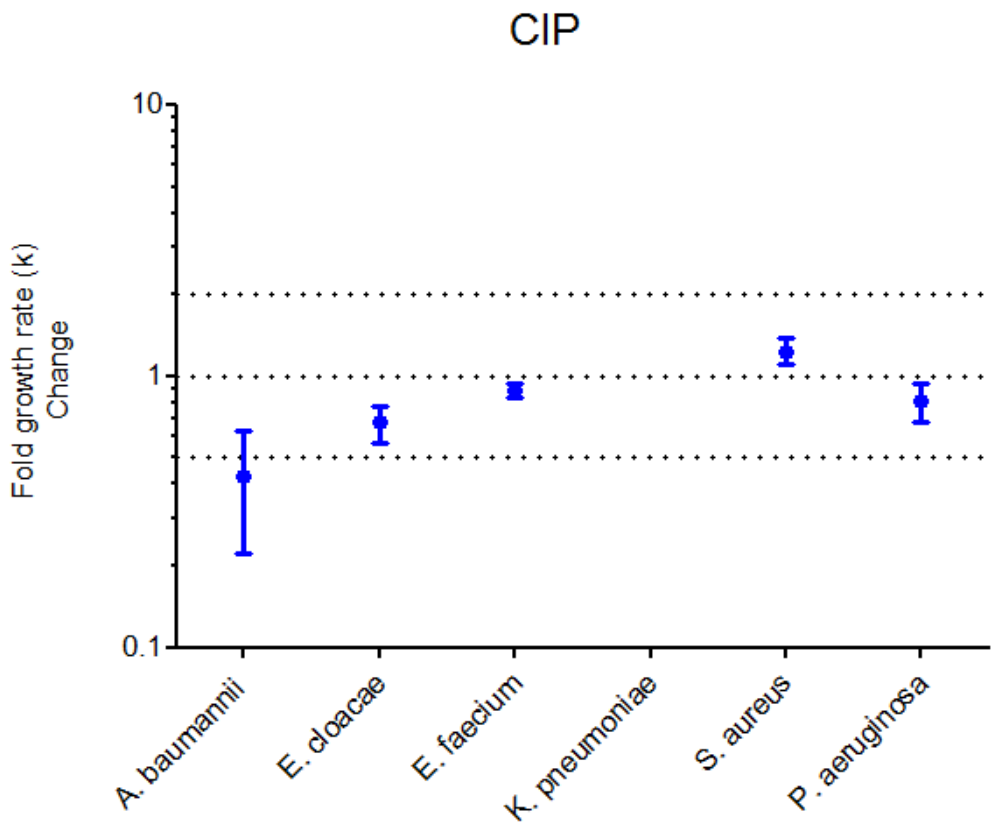

Figure 25: k fold over WT for strains of each bacteria adapted to CIP

Overall CIP adaptation slows growth. The only exception observed is *S. aureus* whose growth rate is increased 1.2x WT. *K. pneumoniae* adapted to CIP was not able to be grown successfully in this experiment, but will be investigated in a later date.

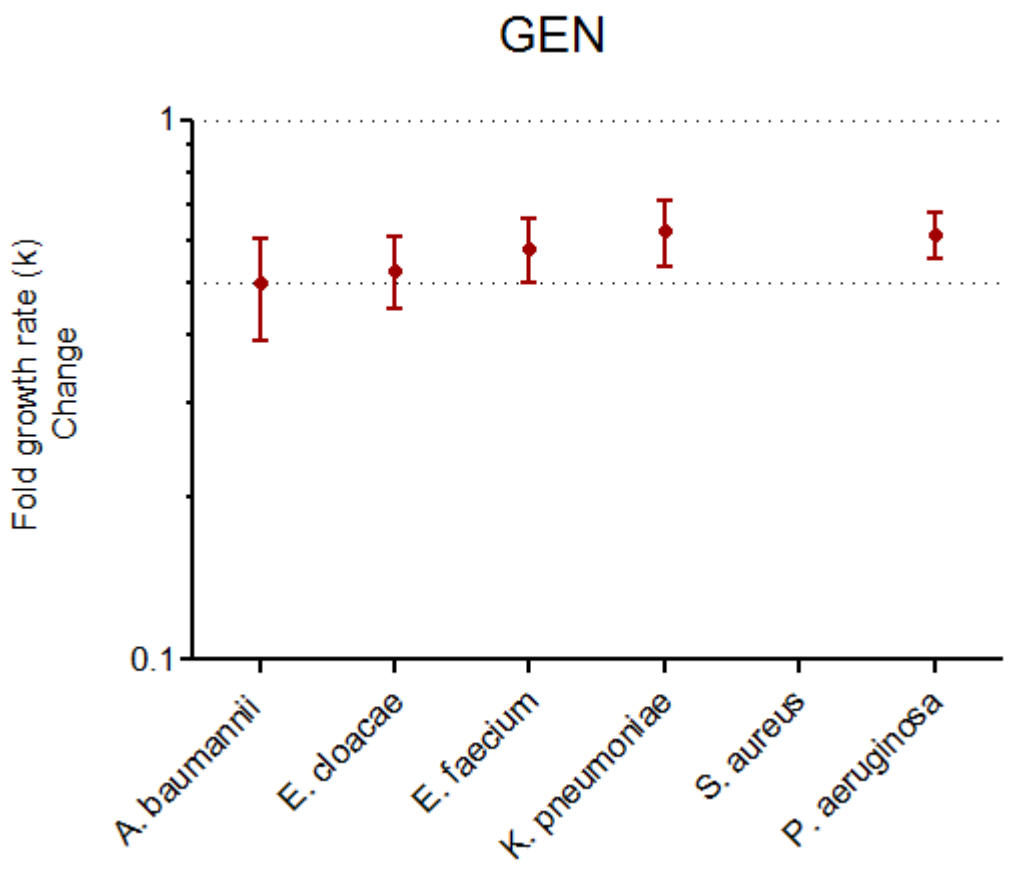

Figure 26: k fold over WT for strains of each bacteria adapted to GEN

An interesting observation from Figure 26 is that adaption to GEN resulted in slower growth for all the bacterial species tested. There are no values for *S. aureus* adapted to GEN strains because these lineages repeatedly did not meet our growth criteria. All cultures went into stationary phase at much lower OD values indicating that adaptation to GEN decreases *S. aureus* ability to grow to large density.

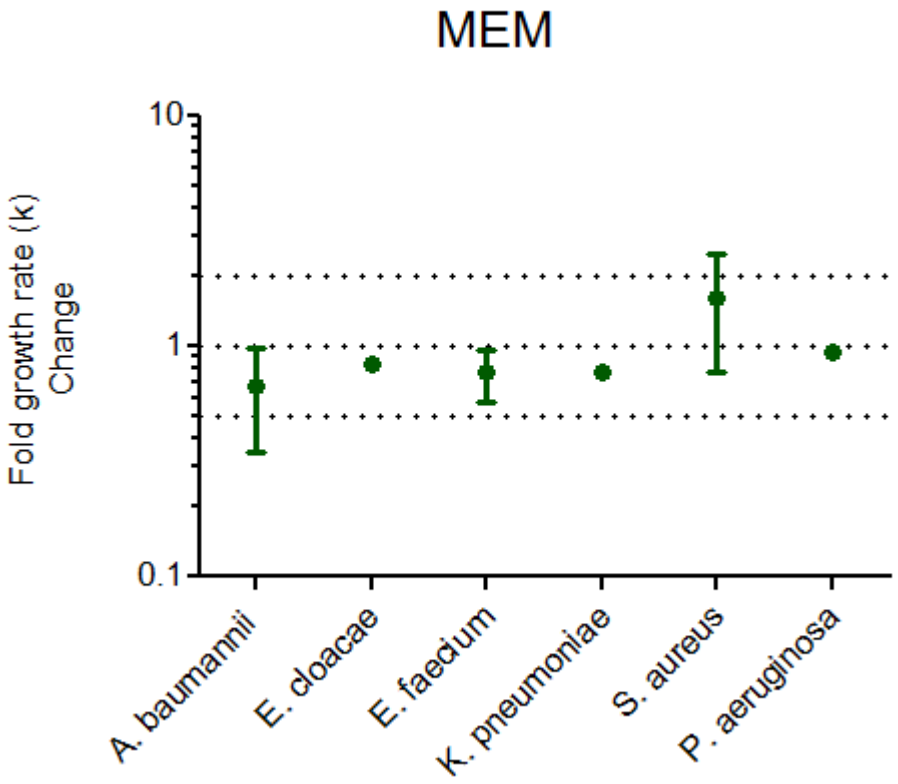

Figure 27: k and G fold over WT for strains of each bacteria adapted to GEN

In *A. baumannii* and *S. aureus* there is a deviation amongst the k fold values with the majority of them depicting a slower growth for *A. baumannii* and a faster growth for *S. aureus* adapted to MEM strains. Overall, adaptation to MEM resulted in slower growth rates for four out of six species. The growth rate of *P. aeruginosa* remained at the same level as the WT's.

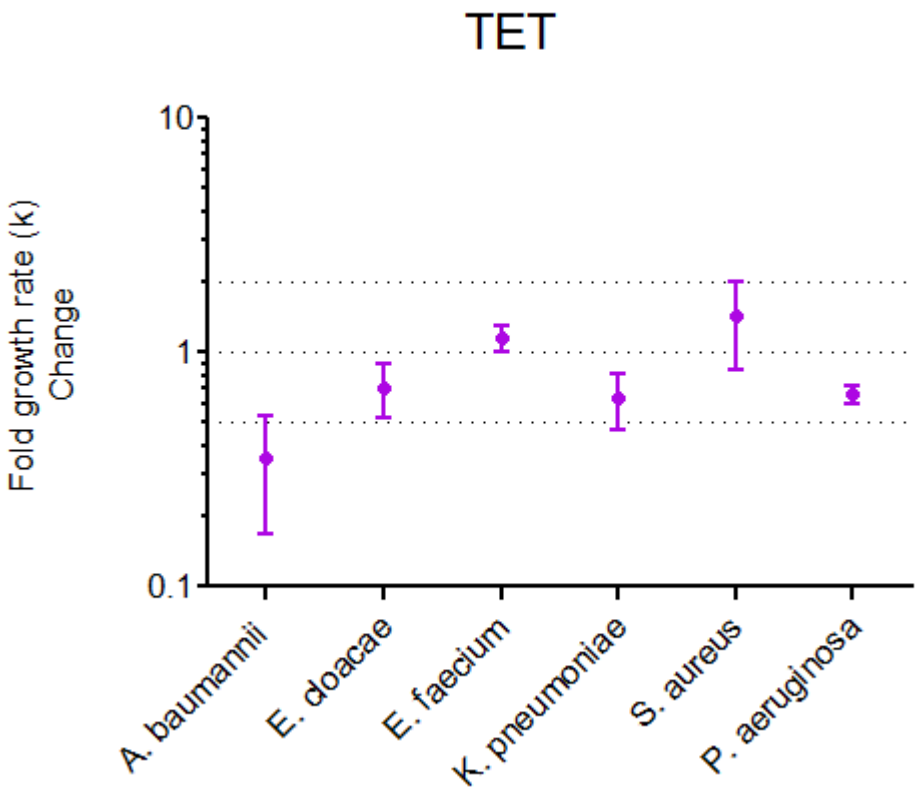

Figure 28: k and G fold over WT for strains of each bacteria adapted to GEN

A. baumannii adapted to TET grew slower than the WT strain with a deviation in the values for each lineage. *E. cloacae* and *P. aeruginosa* TET adapted strains had also a slower growth compared to the WT strains. There is a deviation also in the values for *S. aureus* with the average value showing that the adapted strains have a faster exponential phase than the WT strains.

An important overall observation that we can make is that exposure of *A. baumannii* to antibiotics leads to adverse effects in its growth kinetics, as in all cases the growth rate k value is smaller compared to the WT's. In addition gentamicin is a drug that slows down the growth of all strains.

Conclusions

The objective of this work was to observe and assess the adaptive evolution of ESKAPE pathogens to a suite of different classes of antibiotics and understand how resistance amongst those drugs is related. On this basis we performed adaptation of *S. aureus* and *K. pneumoniae* to cefepime and meropenem respectively, followed by

phenotypic characterization of all adapted ESKAPE organisms. Our results suggest that resistance adaptation results in cross-resistance in all species for the majority of the drugs investigated. Collateral sensitivity may occur, but certainly is a phenomenon that needs to be investigated. The strength of cross resistance or cross susceptibility varies for different bacteria and different drugs. In addition, adaptive evolution of *K. pneumoniae* to meropenem and *S. aureus* to cefepime shows that resistance adaptation is not a linear phenomenon, but a very complex one. In order to shed more light on this complexity we performed growth kinetics experiments. The results showed that resistance adaptation results in slowing down growth in the majority of the cases. However, accelerated growth was observed in *S. aureus* isolates adapted to five of the six drugs.

Many of the observations made across the experiments cannot be understood and evaluated without further investigation. Sequencing of the adapted strains is necessary so as to understand the mechanisms leading to resistance. The data obtained here can be used as a compass to direct analysis of sequencing data. We expect that the results from the sequencing data will explain the various complex phenotypes observed in this work and that they may be applied to a broader range of antibiotics.

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Appendices

Collateral Resistance and Sensitivity Experiments – IC90 Data

Table 14: IC90s of S. aureus adapted strains

IC90 after evolution					
	Fep	**Cip**	**Gen**	**Mem**	**Tet**
Fep A	85,00	0,08	0,28	4,00	0,70
B	65,00	0,07	0,13	0,24	0,06
C	20,00	0,06	0,30	2,10	2,40
Cip A	1,32	80,00	1,10	0,32	0,20
B	1,90	215,00	0,96	0,21	0,29
C	2,35	155,00	0,96	0,37	0,29
D	1,32	62,50	1,45	0,27	0,13
Gen A	2,30	0,15	57,00	0,25	0,07
B	1,70	0,11	105,00	0,14	0,07
C	2,30	0,07	105,00	0,53	0,20
D	1,05	0,08	57,00	0,14	0,13
Mem A	4,00	0,06	1,30	2,80	0,03
B	1,80	0,06	1,20	2,70	0,18
C	0,95	0,06	0,70	2,50	0,16
D	4,60	0,55	1,40	2,90	0,20
Tet A	2,30	0,07	0,45	0,13	0,85
B	0,67	0,10	0,75	2,20	2,30
C	1,15	0,07	0,30	1,80	1,90
D	2,10	0,10	0,45	0,15	1,70

Table 15: IC90s of P. aeruginosa adapted strains

IC90 after evolution					
	Fep	**Cip**	**Gen**	**Mem**	**Tet**
Fep A	5,70	0,30	0,31	0,60	5,50
B	0,75	0,48	0,46	0,60	5,50
C	12,00	0,08	0,46	1,20	5,50
D	5,70	0,08	0,31	0,90	5,50
Cip A	2,80	6,00	1,15	1,20	10,00
B	2,80	3,90	1,10	1,20	10,00
C	6,00	60,00	0,15	0,60	51,00
D	1,00	0,50	0,55	1,00	5,10
Gen A	4,30	0,28	21,00	1,30	9,50
B	4,00	0,28	30,00	2,00	9,50
C	2,20	0,28	40,00	2,30	11,70
Mem A	3,00	0,29	0,28	43,00	40,00
B	4,00	0,29	0,28	43,00	40,00
C	5,00	0,29	0,35	20,00	40,00
D	3,00	0,29	0,28	43,00	40,00
Tet A	5,00	0,60	4,00	10,00	190,00
B	6,00	0,60	4,00	10,00	190,00
C	5,00	0,60	4,00	10,00	190,00
D	5,00	0,60	4,00	10,00	190,00

Table 16: IC90s of K. pneumoniae adapted strains

IC90 after evolution					
	Fep	**Cip**	**Gen**	**Mem**	**Tet**
Fep A	15,60	0,07	0,25	0,80	8,00
B	28,40	0,14	0,27	1,50	12,50
C	7,30	0,25	0,75	0,33	10,00
D	3,90	0,14	0,25	0,90	10,50
Cip A	0,08	5,75	0,07	0,03	1,50
B	0,04	11,50	0,07	0,04	3,00
C	0,10	22,00	0,07	0,04	0,75
D	0,13	5,20	0,09	0,07	1,50
Gen A	0,14	0,11	45,00	0,05	2,10
B	0,54	0,12	80,00	0,14	2,60
C	0,15	0,07	40,00	0,19	1,50
D	0,07	0,04	0,35	0,07	0,80
Mem A	6,00	0,36	0,15	4,00	13,00
B	12,00	1,00	0,29	2,60	15,00
C	10,00	1,00	0,29	2,60	14,00
Tet A	0,14	0,16	0,15	0,22	18,00
B	0,09	0,02	0,29	0,05	10,00
C	1,07	0,14	0,15	0,06	85,00
D	0,56	0,12	0,28	0,08	85,00

Table 17: IC90s of E. faecium adapted strains

IC90 after evolution					
	Fep	**Cip**	**Gen**	**Mem**	**Tet**
Fep A	810,00	1,10	2,80	17,00	0,26
B	300,00	1,20	5,50	5,80	0,15
C	850,00	2,20	6,90	44,00	0,17
D	14,00	1,20	5,80	14,00	0,15
Cip A	500,00	26,00	12,30	50,00	0,63
B	500,00	26,00	10,00	45,00	0,63
C	500,00	26,00	6,00	45,00	0,63
D	250,00	6,20	23,00	45,00	0,28
Gen A	100,00	0,90	700,00	40,00	0,55
B	100,00	3,80	500,00	50,00	0,63
C	100,00	2,50	500,00	40,00	0,55
D	100,00	1,17	350,00	50,00	0,25
Mem A	2000,00	2,50	11,60	500,00	0,45
B	2000,00	2,50	11,60	350,00	0,30
C	2000,00	2,00	8,00	500,00	0,17
D	2000,00	0,62	0,80	500,00	0,22
Tet A	2,50	2,80	5,00	5,00	1,15
B	1,70	2,30	3,10	5,00	0,29
C	2,20	3,00	5,00	2,50	0,95
D	2,38	3,00	5,00	1,30	1,25

Table 18: IC90s of E. cloacae adapted strains

IC90 after evolution					
	Fep	**Cip**	**Gen**	**Mem**	**Tet**
Fep A	2,5	0,035	0,45	0,45	1,15
B	2,5	0,035	0,5	0,25	1,15
C	4,6	0,035	2	0,5	0,66
D	1,2	0,03	0,23	0,1	1
Cip A	0,35	22	0,45	0,14	1,7
B	0,25	22	0,13	0,13	4,1
C	0,25	50	0,25	0,06	1,5
D	0,3	40	0,25	0,13	1,2
Gen A	0,6	0,019	23	0,15	1,1
B	0,6	0,032	23	0,22	1,1
C	0,25	0,016	37	0,12	0,8
D	0,55	0,032	23	0,14	1,1
Mem A	12	0,08	0,8	50	1,8
B	10	0,07	0,55	19	1,1
C	6	0,036	0,5	40	0,57
D	6	0,018	0,61	23	1,1
Tet A	1,1	1,1	0,3	0,11	170
B	2,1	0,6	0,52	0,21	160
C	1,2	0,6	0,6	0,16	200
D	0,55	0,28	1	0,18	40

Table 19: IC90s of A. baumannii adapted strains

IC90 after evolution					
	Fep	**Cip**	**Gen**	**Mem**	**Tet**
Fep A	25,00	0,25	6,25	0,20	1,00
B	12,50	0,24	2,50	0,45	0,80
C	14,00	0,55	8,30	0,15	1,00
D	9,50	0,30	2,50	0,20	0,63
Cip A	10,00	400,00	0,15	0,55	2,30
B	7,00	400,00	0,25	0,55	1,90
C	8,00	500,00	3,80	0,15	1,10
D	20,00	500,00	1,25	0,29	1,00
Gen A	22,00	0,48	1400,00	0,15	0,27
B	19,00	0,31	770,00	0,08	0,15
C	19,00	0,48	1170,00	0,08	0,15
Mem A	13,30	0,23	0,55	5,60	0,03
B	25,00	0,16	0,55	5,10	0,03
C	17,00	0,16	0,20	22,00	0,06
D	100,00	1,00	23,00	3,12	0,03
Tet A	4,50	0,17	0,16	0,31	40,00
B	4,50	0,17	0,16	0,31	40,00
C	9,40	0,47	0,07	0,40	20,70
D	4,50	0,17	0,16	0,31	10,30

ECOFF values

Table 20: ECOFF values of ESKAPE pathogens to the suite of drugs used in the experiments. ND = Not Determined

ECOFF values (µg/ml)

	FEP	**CIP**	**GEN**	**MEM**	**TET**
A. baumannii	ND	1	4	2	ND
E. faecium	ND	4	32	8	4
E. cloacae	0,125	0,125	2	0,125	16
K. pneumoniae	0,125	0,125	2	0,125	8
P. aeruginosa	8	0.5	8	2	ND
S. aureus	8	1	2	0.5	1

Growth Kinetics Experiments

Table 21: S. aureus growth kinetics data

	OD 0.1 (min)	OD 0.25 (min)	delta (min)	G (min)	k (min-1)	k fold over WT	G fold over WT
Fep A	320	365	45	34,27	0,0204	2,444	0,41
B	320	365	45	34,27	0,0204	2,444	0,41
C	245	285	40	30,46	0,0229	2,750	0,36
Cip A	250	350	100	76,15	0,0092	1,100	0,91
B	210	290	80	60,92	0,0115	1,375	0,73
Mem A	260	320	60	45,69	0,0153	1,833	0,55
B	330	470	140	106,61	0,0065	0,786	1,27
C	280	375	95	72,34	0,0096	1,158	0,86
D	290	330	40	30,46	0,0229	2,750	0,36
Tet A	260	320	60	45,69	0,0153	1,833	0,55
B	245	380	135	102,80	0,0068	0,815	1,23
C	275	380	105	79,96	0,0087	1,048	0,95
D	320	375	55	41,88	0,0167	2,000	0,50

Table 22: P. aeruginosa growth kinetics data

	OD 0.1 (min)	OD 0.25 (min)	delta (min)	G (min)	k (min-1)	k fold over WT	G fold over WT
Fep A	275	350	75	57,11	0,0122	1,032	1,00
B	275	350	75	57,11	0,0122	1,032	1,00
C	275	350	75	57,11	0,0122	1,032	1,00
D	275	350	75	57,11	0,0122	1,032	1,00
Cip A	295	415	120	91,38	0,0076	0,645	1,60
B	300	420	120	91,38	0,0076	0,645	1,60
C	305	410	105	79,96	0,0087	0,737	1,40
D	285	350	65	49,50	0,0141	1,191	0,87
Gen A	370	490	120	91,38	0,0076	0,645	1,50
B	395	505	110	83,76	0,0083	0,704	1,38
C	345	500	155	118,03	0,0059	0,499	2,07
Mem A	265	350	85	64,73	0,0108	0,911	1,13
B	275	350	75	57,11	0,0122	1,032	1,00
C	275	360	85	64,73	0,0108	0,911	1,13
D	275	360	85	64,73	0,0108	0,911	1,13
Tet A	295	410	115	87,57	0,0080	0,673	1,53
B	295	400	105	79,96	0,0087	0,737	1,40
C	295	420	125	95,19	0,0073	0,619	1,67
D	285	410	125	95,19	0,0073	0,619	1,67

Table 23: K. pneumoniae growth kinetics data

	OD 0.1 (min)	OD 0.25 (min)	delta (min)	G (min)	k (min-1)	k fold over WT	G fold over WT
Fep A	240	285	45	34,27	0,0204	0,906	1,29
B	250	310	60	45,69	0,0153	0,680	1,71
C	250	310	60	45,69	0,0153	0,680	1,71
D	220	270	50	38,07	0,0183	0,816	1,43
Gen A	260	335	75	57,11	0,0122	0,544	2,14
B	232	290	58	44,17	0,0158	0,703	1,66
C	270	365	95	72,34	0,0096	0,429	2,71
D	225	275	50	38,07	0,0183	0,816	1,43
Mem A	240	292	52	39,60	0,0176	0,784	1,30
B	285	340	55	41,88	0,0167	0,741	1,38
C	292	345	53	40,36	0,0173	0,769	1,33
Tet A	280	335	55	41,88	0,0167	0,741	1,57
B	200	250	50	38,07	0,0183	0,816	1,00
C	310	405	95	72,34	0,0096	0,429	1,90
D	310	380	70	53,30	0,0131	0,583	1,40

Table 24: E. faecium growth kinetics data

	OD 0.1 (min)	OD 0.25 (min)	delta (min)	G (min)	k (min-1)	k fold over WT	G fold over WT
Fep A	300	365	65	49,50	0,0141	1,181	0,93
B	280	340	60	45,69	0,0153	1,280	0,86
C	290	350	60	45,69	0,0153	1,280	0,86
D	240	305	65	49,50	0,0141	1,181	0,93
Cip A	285	390	105	79,96	0,0087	0,731	1,50
B	220	300	80	60,92	0,0115	0,960	1,14
C	225	305	80	60,92	0,0115	0,960	1,14
D	395	480	85	64,73	0,0108	0,903	1,00
Gen A	395	500	105	79,96	0,0087	0,731	1,24
B	385	495	110	83,76	0,0083	0,698	1,29
C	305	475	170	129,45	0,0054	0,452	2,00
D	630	805	175	133,26	0,0052	0,439	2,06
Mem A	360	495	135	102,80	0,0068	0,569	1,59
B	260	335	75	57,11	0,0122	1,024	1,07
C	290	390	100	76,15	0,0092	0,768	1,43
D	260	370	110	83,76	0,0083	0,698	1,57
Tet A	260	320	60	45,69	0,0153	1,280	0,86
B	245	305	60	45,69	0,0153	1,280	0,71
C	240	310	70	53,30	0,0131	1,097	1,00
D	260	340	80	60,92	0,0115	0,960	1,14

Table 25: E. cloacae growth kinetics data

	OD 0.1 (min)	OD 0.25 (min)	delta (min)	G (min)	k (min-1)	k fold over WT	G fold over WT
Fep A	250	305	55	41,88	0,0167	0,770	1,22
B	220	260	40	30,46	0,0229	1,059	0,89
C	290	360	70	53,30	0,0131	0,605	1,56
D	220	260	40	30,46	0,0229	1,059	0,89
Cip A	255	300	45	34,27	0,0204	0,941	1,00
B	310	405	95	72,34	0,0096	0,446	2,11
C	385	450	65	49,50	0,0141	0,652	1,44
D	430	495	65	49,50	0,0141	0,652	1,44
Gen A	290	355	65	49,50	0,0141	0,652	1,44
B	300	370	70	53,30	0,0131	0,605	1,56
C	245	390	145	110,42	0,0063	0,292	3,22
D	295	370	75	57,11	0,0122	0,565	1,67
Mem A	220	270	50	38,07	0,0183	0,847	1,11
B	240	290	50	38,07	0,0183	0,847	1,11
C	220	270	50	38,07	0,0183	0,847	1,11
D	275	330	55	41,88	0,0167	0,770	1,22
Tet A	310	390	80	60,92	0,0115	0,529	1,14
B	340	410	70	53,30	0,0131	0,605	1,56
C	280	335	55	41,88	0,0167	0,770	1,22
D	200	245	45	34,27	0,0204	0,941	1,00

Table 26: A. baumannii growth kinetics data

	OD 0.1 (min)	OD 0.25 (min)	delta (min)	G (min)	k (min-1)	k fold over WT	G fold over WT
Fep A	200	320	120	91,38	0,0076	0,554	2,00
B	260	360	100	76,15	0,0092	0,665	1,25
C	220	350	130	98,99	0,0070	0,512	1,86
D	300	480	180	137,07	0,0051	0,370	2,57
Cip A	220	300	80	60,92	0,0115	0,832	1,33
B	250	560	310	236,06	0,0030	0,215	4,43
C	230	520	290	220,83	0,0032	0,229	4,14
Gen A	280	450	170	129,45	0,0054	0,391	2,13
B	335	445	110	83,76	0,0083	0,605	1,83
Mem A	280	380	100	76,15	0,0092	0,665	1,67
B	220	280	60	45,69	0,0153	1,109	0,75
C	430	600	170	129,45	0,0054	0,391	2,13
D	275	410	135	102,80	0,0068	0,493	2,25
Tet A	185	360	175	133,26	0,0052	0,380	2,19
B	195	520	325	247,49	0,0028	0,205	4,06
C	220	520	300	228,45	0,0031	0,222	3,75
D	185	295	110	83,76	0,0083	0,605	1,38