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Master Thesis Molecular Biology

**Experimental evolution and
genotypic characterization of
ESBL *Escherichia coli***

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Abstract

In Swiss hospitals infections with extended-spectrum cephalosporin resistant *Escherichia coli* (*E. coli*) are emerging [1]. Enzymes called extended-spectrum β -lactamases (ESBL) are able to hydrolyze extended-spectrum cephalosporins. If expressed in *E. coli*, the susceptibility to extended-spectrum cephalosporins is reduced and the strains are called ESBL *E. coli*. Research focused on studying ESBL mediated extended-spectrum cephalosporin resistance, even though other resistance mechanisms possibly affect the resistance level. We studied the evolution of cefepime (an extended-spectrum cephalosporin) resistance in ESBL *E. coli* strains in order to identify new genotypes possibly associated with resistance mechanisms. In order to do so, we studied whole-genome sequencing data from ESBL *E. coli* isolates from patients of the University Hospital of Basel. These isolates evolved resistance to cefepime in patients. A bioinformatic pipeline was developed for analyzing the sequencing data, revealing several single nucleotide polymorphisms (SNPs) in genomes of isolates which evolved resistance. Additionally, we assembled a device called morbidostat, which we used to experimentally evolve resistance to cefepime in ESBL *E. coli* strains. The morbidostat is an automated culturing device continuously applying high antibiotic pressure to bacterial cultures resulting in resistance evolution. We cultured three ESBL *E. coli* strains in the morbidostat with cefepime which highly increased the resistance level of the strains. Samples of strains taken during morbidostat experiments were deep-sequenced and analyzed with the same bioinformatic pipeline which was applied to patient isolates. Compared to the genomes of the strains before culturing with the morbidostat, several SNPs were identified in the genomes of the strains which evolved resistance. Interestingly, we found SNPs in the same genes in patients isolates and morbidostat samples, strongly suggesting the importance of the SNPs for the resistance mechanisms. In particular, we identified SNPs in genes coding for the porins *ompC*, *ompF* and their regulatory system in patient isolates and morbidostat samples. Furthermore, SNPs in genes coding for the DNA-directed RNA polymerase subunit β and the RNA polymerase sigma factor in patient isolates and morbidostat samples affected the transcription machinery.

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Chapter 1

Introduction

The discovery of penicillin in 1928 by Alexander Fleming shortly before the Second World War revolutionized human medicine by saving the lives of thousands of soldiers and civilians suffering from bacterial infections [2]. Fleming received the Nobel Prize for the discovery of penicillin [3]. In his award speech he warned people from irresponsible and excessive penicillin consumption which could lead to penicillin resistance:

“The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant [3]”

Unfortunately, Flemings prediction came true and penicillin resistance emerged shortly after its discovery [4]. Since then it has been necessary to repetitively introduce new antibiotics to the market, because bacteria sooner or later tend to evolve resistance to antibiotics in use.

1.1 Emergence of antibiotic resistance

Antibiotic resistance is emerging worldwide [5]. An increase in antibiotic consumption and a decrease in the development of new antibiotics are the main reasons for the emergence of antibiotic resistance [5]. As shown in Figure 1.1 A and B, consumption mainly increased for countries where a huge population is associated with middle and lower income. Countries with high average income show a rather stable consumption over the recorded years. However, the overall consumption is much higher compared to the countries with middle and low income [6]. This also suggests that the access to antibiotics is uneven.

The number of antibiotics which were approved by the Food and Drug Administration (FDA) decreased in the last four decades as shown in Figure 1.1 C [5]. Whereas between 1980 and 1984 19 antibiotics were approved by the FDA, only six antibiotics were approved between 2010 and 2014 [5]. The decrease in development in new antibiotics mainly has economical reasons. Recently approved antibiotics are usually held in reserve and only prescribed for infections that already established antibiotics cannot treat. This limits the investment of return [7]. Additionally, antibiotics are only used for a short period of time. This makes antibiotics a lot less profitable compared to drugs used for treating chronic diseases or cancer [7]. The investment of return is further limited by the high costs of developing and approving

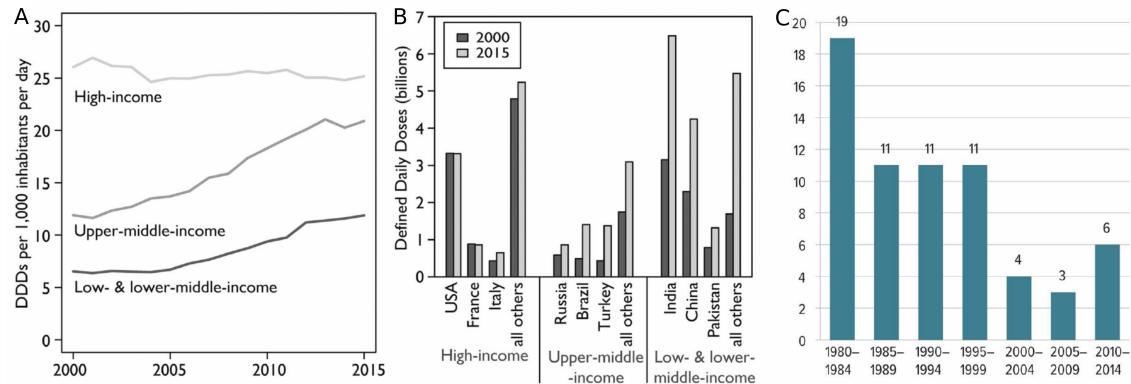


Figure 1.1: A: Worldwide consumption of defined daily antibiotic doses per 1000 inhabitants per day, grouped by income [6]. B: Billions of defined daily doses for selected countries representing different income groups [6]. C: Decrease of FDA approved drugs between 1980 and 2014 [5].

a new antibiotic which is estimated to be 0.5 billion US-Dollar [8].

Antibiotics are used in two main areas: The health sector and intensive animal farming. Unfortunately, in the health sector many antibiotics are prescribed unnecessarily. For instance, it was shown that in 2010 47 millions prescriptions of antibiotics in the USA were redundant [9]. Most of them were prescribed for respiratory conditions most commonly caused by viruses [9]. It is assumed that in the early 2000s 25-50% of all antibiotic consumption took place in intensive animal farming [10]. Most of the antibiotics in intensive animal farming are used as prophylaxis and for stimulating growth, not for curing animals suffering from infections [10][11]. The combination of increased usage and decreased development of antibiotics has caused resistance in all major bacterial pathogens. In Swiss hospitals mainly four resistant pathogens have been reported over the last 15 years [1]: *Escherichia coli* (*E. coli*), *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Klebsiella pneumoniae* [1]. The proportion of resistant *Staphylococcus aureus* decreased because of improved detection and rapid treatment [1]. The proportion of resistant *Streptococcus pneumoniae* declined as well, thanks to the introduction of the PCV7 vaccine which is also effective against resistant *Streptococcus pneumoniae* [1]. However, the proportion of resistant *E. coli* infections increased tremendously over the last 15 years [1], being responsible for 30% of all infections with resistant pathogens in 2018 [1]. *E. coli* evolved resistance to two different classes of antibiotics, those being the fluoroquinolones and the extended-spectrum cephalosporins belonging to the β -lactams [1]. Extended-spectrum cephalosporins comprise some of the latest generations of antibiotics, therefore, the emerging resistance against those antibiotics are causing concerns worldwide.

Different classes of antibiotics were developed during the 20th century and classified by their mechanism of action. The most common classes of antibiotics are shown in Figure 1.2. Mainly three targets of antibiotics exist: Protein synthesis, DNA replication, and the cell wall synthesis [12]. Antibiotics inhibiting protein synthesis target subunits of the 70S ribosome. A common target for antibiotics inhibiting DNA replication is the DNA gyrase which is an enzyme unwinding the DNA, a process necessary for transcription [12]. Lastly, the cell wall synthesis is a very im-

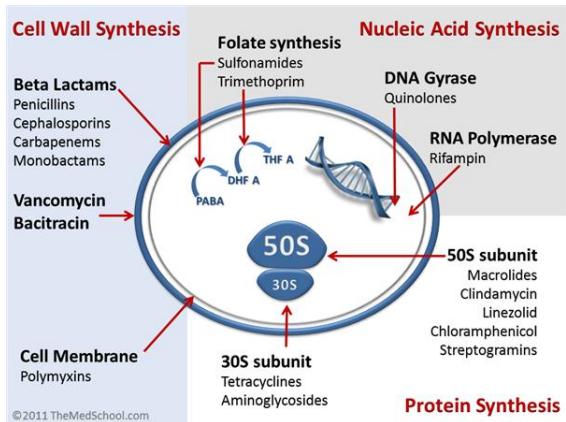


Figure 1.2: Most popular classes of antibiotics and their targets in the bacterial cell [12].

portant target, because it is inhibited by one of the biggest classes of antibiotics the β -lactams [13].

1.2 β -lactams

All β -lactams contain a highly strained and reactive cyclic amide which is responsible for the antimicrobial activity of β -lactams by inhibiting the cell wall synthesis [13]. Interestingly, the cell wall is different in gram-positive and gram-negative bacteria. Gram-positive bacteria consist of a cytoplasmic membrane, surrounded by a cell wall [12]. The gram-negative bacterial cell is surrounded by an additional membrane called outer membrane, which decreases the permeability for substance such as β -lactams [12]. However, β -lactams can reach their target located in the cytoplasm by diffusing through channels called porins [12]. Peptidoglycan consists of long sugar polymers, its structure is shown in Figure 1.3. The polymers themselves consist of the N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) which are alternately linked [13]. The tough, mesh-like characteristics of peptidoglycan are achieved by the bacterial cell by cross linking the long sugar polymers. In particular NAM saccharides have peptide residues often ending with the amino acids D-alanyl-D-alanine [13]. For cross-linking the long sugar polymers, the last D-alanyl amino acid is removed and the resulting acyl species is transferred (cross-linked) to a neighboring peptide chain of a sugar polymer [13]. Cross-linking is mediated by enzymes called DD-transpeptidases [13]. It has been hypothesized that the β -lactam ring shared by all β -lactam antibiotics mimics the D-alanyl-D-alanine and binds to the active site of DD-transpeptidases, causing irreversible inhibition of their activity [13]. A disruption of the peptidoglycan layer induced by inhibited cell wall synthesis leads to lysis of the bacterium [12]. Even though all β -lactams consist of the β -lactam ring, different groups of β -lactams exist. Those being penams, penems, carbapenems, monobactams and cephems [14]. Cephalosporins, belonging to cephems, are very important because they are very potent, well tolerated by patients and thus widely used [15].

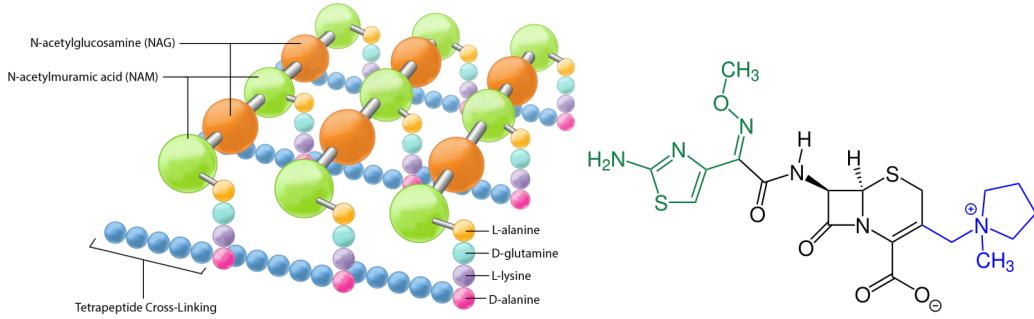


Figure 1.3: Left: Structure of peptidoglycan [16]. Cross-linked peptides attached to the polysaccharides cause a mesh-like structure of the peptidoglycan. Right: Structure of cefepime, a fourth-generation cephalosporin [17]. The structure in black stands for the core structure shared by every cephalosporin. Structures colored in green and blue are the residues of cefepime.

1.2.1 Cephalosporins

All cephalosporins share an identical core structure which is shown in black in Figure 1.3. Different residues attached to the core structure are determining the specific cephalosporin. Five generations of cephalosporins exist. First-generation cephalosporins are very active against gram-positive cocci but are not very potent against gram-negative bacteria [18]. The second-generation cephalosporins are all active against bacteria covered by the first-generation drugs, but have extended activity against gram-negative bacteria [18]. The enhanced activity against gram-negative bacteria is further visible in third-generation cephalosporins. A representative of this generation is ceftazidime [19]. Only two β -lactams are classified as fourth-generation cephalosporins: Cefepime and cefpirome [18]. The residues of cefepime are shown in green and blue in Figure 1.3. The newest fifth-generation of cephalosporins ceftobiprole and ceftaroline were explicitly developed to target multi resistant *Staphylococcus aureus* [18]. Cephalosporins belonging to second or later generations are also referred as extended-spectrum cephalosporins.

1.3 Resistance mechanisms

Generally, there are three different strategies for bacteria to reduce susceptibility against antibiotics which have targets within the cell such as cephalosporins. One strategy is to reduce the uptake of antibiotics through porins [20]. Bacterial cells can limit the uptake by reducing the expression level of porins, or by changing their size [21]. The second strategy is to reduce the affinity or to protect the target site of the antibiotic [22]. Reducing the affinity of the target sites is achieved by point mutations in the gene coding for the target, or by enzymatic alteration of the target site (*e.g.* addition of methyl groups) [22]. The last strategy is to reduce the antibiotic concentration in the cell [22]. Reduction of the antibiotic concentration is achieved by active transport or by degradation. Active transport involves efflux pumps. Those pumps transport the compound through energy-dependent membrane proteins out of the cell [21]. Degradation of antibiotics, usually by hydrolysis, is an enzymatic process and plays an important role in resistance against cephalosporins [23].

1.3.1 Resistance to β -lactams

Resistance to β -lactams is mainly associated with decreased antibiotic concentration in the cell caused by hydrolysis of β -lactams. Hydrolysis of β -lactams is mediated by enzymes called β -lactamases [23]. For some bacterial strains target mutations in the DD-transpeptidases have been reported which were also associated with β -lactam resistance [23]. Additionally, porins have been reported to play an important role in the resistance mechanism against β -lactams, because they effectively can reduce the uptake of β -lactams[23].

β -lactamases

Plasmid-mediated β -lactamases are important for resistance because they can be transmitted via horizontal gene-transfer [22]. The first plasmid-mediated β -lactamase in gram-negative bacteria was described in the early 1960s and called β -lactamase TEM-1 [18]. TEM-1 was found worldwide only a few years later after its first isolation [18]. TEM-1 caused resistance against β -lactams of this time. This is why extended-spectrum cephalosporins such as ceftazidime which could not be hydrolyzed by TEM-1 were developed [18]. Another known β -lactamase is called SHV-1, which was isolated for the first time in 1974 [24]. It is encoded chromosomally in the majority of isolates of *K. pneumoniae*, but is also transmitted via plasmids in *E. coli* [24].

Extended-spectrum β -lactamase (ESBL)

Over time β -lactamases evolved and were able to hydrolyze extended-spectrum cephalosporins. Those novel β -lactamases were called extended-spectrum- β -lactamases (ESBL). CTX-M-1 is an ESBL, which was clinically isolated for the first time in Germany in 1986 [25]. Many variants of CTX-M-1 were isolated and they were divided in five groups [18]. It is assumed that they evolved from the β -lactamase precursor AmpC from *Kluyvera ascorbata* [25]. OXA is another common ESBL family. Originally the OXA family was created as a phenotypic rather than a genotypic group, based on a specific hydrolysis profile [25]. Its name comes from the ability to efficiently hydrolyze oxacillin [25].

1.4 ESBL *E. coli*

The most common ESBL-producing bacteria are *E. coli* and *Klebsiella pneumoniae*. *E. coli* is a gram-negative bacteria and is commonly present in the human intestine [26]. If present in other parts of the body *E. coli* can cause infections [26]. Infections with resistant ESBL *E. coli* increased rapidly over the last 15 years [1].

1.4.1 Identification of ESBL *E. coli* and treatment

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) is an organization advising clinicians on which actions to take in response to results of antimicrobial susceptibility testing (AST) [27]. For *E. coli* where clinicians suspect

the presence of ESBLs, the EUCAST recommends to confirm ESBLs by a standardized ESBL disc test assay [28]. For this assay, *E. coli* are grown with a fixed density on a plate [29]. On this plate discs with cefotaxime, ceftazidime and cefepime alone, but also in combination with the ESBL inhibitor clavulanic acid, are placed [29]. After an incubation time of 18-24 hours the diameter of the inhibition zone on the plates is measured [29]. If the diameter of the inhibition zones, caused by the antibiotic and clavulanic acid combination, is larger than 5 mm, the tested *E. coli* strain is declared as ESBL producing [29]. In those cases clinicians are recommended to use carbapenems for treatment [30]. This procedure, where ESBL detection is only based on AST was introduced in 2013. Before, the EUCAST advised to detect ESBLs with molecular techniques (*e.g.* PCR, microarrays) [31]. Carbapenems were prescribed, if genes coding for ESBLs were found. Interestingly, many *E. coli* strains carrying ESBL genes were susceptible to common cephalosporins [28]. Hombach *et al.* showed that 32.5% of an ESBL *E. coli* isolate collection which all carried the gene coding for CTX-M-1 were susceptible to cefepime [28]. Therefore, carbapenem prescription based on ESBL gene identification was unnecessary in many cases, progressing the carbapenem resistance evolution. This is why the EUCAST changed the guidelines in 2013 and now only AST is considered. In principal it would be helpful for clinicians to include genomic data again, because AST takes very long (about 48 hours) and is not very accurate [29][28].

1.5 Identifying mutations predicting cefepime resistance of ESBL *E. coli*

ESBL genes alone do not determine whether ESBL *E. coli* are resistant to extended-spectrum cephalosporins or not [28]. Instead it is possible that mutations, which have not been identified yet, play an important role in the resistance mechanisms. Mutations could affect promoters and genes responsible for various mechanisms. For example mutations in the promoter of ESBL genes could affect the expression level of ESBLs. Enhanced ESBL expression would likely increase hydrolysis of extended-spectrum cephalosporins. Since extended-cephalosporin concentrations in the bacteria is very dependent on transport through porins, it is quite possible that mutations are affecting genes or promoters of porins. Those mutation could affect the expression level of porins but also their structure, both leading to increased impermeability. Another possibility for targets of mutations are efflux pumps. Mutated efflux pumps could specifically transport cephalosporins out of the cell, decreasing the cephalosporin concentration in the cell.

Mutations can be identified by analyzing genomic data of ESBL *E. coli* strains gaining resistance by evolution. To get access to such genomic data, the same strain of ESBL *E. coli* can be sequenced before and after resistance evolved. This requires access to a clinical isolate collection where susceptibility changed over time. It is also possible to analyze genomic data obtained from ESBL *E. coli* strains which experimentally evolved resistance *in vitro*. Studying resistance with experimental evolution is possible with a device called morbidostat.

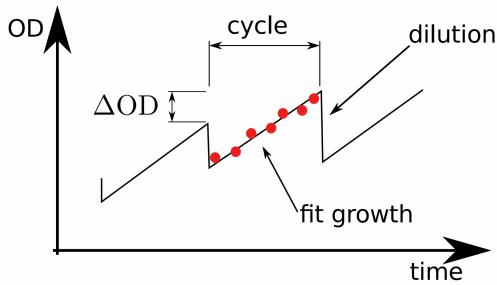


Figure 1.4: Expected outcome for three cycles of culturing with the morbidostat. Individual OD measurements of a cycle are shown as red dots. A fit is calculated approximating every OD measurement. This fit is used for calculating growth rates determining over injected dose of antibiotics. Antibiotics are injected at the end of a cycle, next to inhibition also causing dilution which is visible in the OD measurements of the next cycle.

1.5.1 Principles of the morbidostat

The morbidostat, originally developed by Toprak *et al.*, is an automated culture device [32]. It forces bacteria to gain resistance by applying a constant high antibiotic pressure. Bacteria are grown in a fixed culture volume in vials. The growth in every vial is constantly monitored. Depending on how fast the bacteria grow an appropriate dose of antibiotics is injected into the vials. Culturing multiple days leads to evolution of resistance as phenotypes with increased resistance are constantly selected.

Several tasks are grouped in cycles and repetitively executed by the morbidostat. Over the defined cycle time the optical densities (ODs) of the cultures are constantly measured which is represented as red dots in Figure 1.4. At the end of the cycle a fit is calculated approximating all the OD measurements from one cycle which is shown in Figure 1.4 as a black line going through the red dots. From that fit the growth rate is calculated. A feedback algorithm decides if and how much drug is injected in which vial. The calculated antibiotic concentration is injected with computer controllable pumps. When antibiotics are injected, the cultures are diluted as well, which is visible in the first OD measurement of the next cycle. As a last step of the cycle, volume which exceeds the culture volume is removed. As resistance emerges, samples of the cultures grown in the morbidostat can be taken and sequenced.

1.5.2 Previous morbidostat experiments and expected outcome

Dösselmann *et al.* rebuild the system in 2015 [33] and used it for studying mutations associated with colistin resistance. They were able to increase the minimal inhibitory concentration (MIC) of colistin against *Pseudomonas aeruginosa* 100-fold within 20 days with the morbidostat [33]. The mechanism of action of colistin is to displace cations from the phosphate groups of membrane lipids. This leads to the disruption of the outer cell membrane causing cell death [34]. Dösselmann *et al.* could identify several mutations in genes coding for proteins involved in the lipopolysaccharide

synthesis [33].

1.5.3 Illumina and Nanopore sequencing

Studying changes in the genome relies on accurate sequencing data. Illumina sequencing is a method that produces short reads which are typically 150 base pairs (bp) long. The advantage of Illumina sequencing is the low error rate which was determined as 0.24 % per base [35]. A commonly used Illumina sequencing system is the MiSeq-System. As the reads are only 150 bases long, it is computationally difficult to assemble whole-genomes because overlapping regions are small. Oxford Nanopore Technologies (ONT) produces very long reads which are up to several 100 kbp long [36]. In contrast to Illumina sequencing, the error rate per base is 13.6 % which is a lot higher compared to Illumina sequencing [36]. Indel errors make up for most of the error rate, caused by repetitive regions in the genome [36]. Combining sequencing data from Illumina and ONT in a hybrid-assembly allows to assemble highly accurate whole-genomes. Hybrid-assembling uses the long reads from ONT for scaffolding and the Illumina reads for single-nucleotide accuracy. [36].

1.6 Aim of this thesis

The aim of this Master thesis was to investigate the resistance evolution of ESBL *E. coli* to the extended-spectrum cephalosporin cefepime. The focus was on identifying SNPs in the genome of ESBL *E. coli* which evolved cefepime resistance. In order to do so, we wanted to study genomic data from ESBL *E. coli*, which showed a change in susceptibility against cefepime over the sampling period. Additionally, our aim was to assemble a morbidostat to experimentally evolve cefepime resistance in ESBL *E. coli* and follow the evolution of resistance by studying genomic data. The specific aims were:

- DNA sequencing with Oxford Nanopore Technologies
- *De novo* assembling of ESBL *E. coli* whole-genomes combining Illumina and ONT sequencing data
- Development of a bioinformatic analysis pipeline to identify SNPs in genomes of strains which evolved resistance to cefepime
- Assembling and troubleshooting the morbidostat, use it to experimentally evolve resistance to cefepime in ESBL *E. coli*
- Identification of SNPs in cefepime resistant ESBL *E. coli* patient isolates
- Identification of SNPs in cefepime resistant ESBL *E. coli* where resistance evolved experimentally with the morbidostat

Chapter 2

Materials and methods

This chapter summarizes the material and methods used in this work. ESBL *E. coli* isolates which gained resistance to cefepime and ceftazidime in patients of the University Hospital of Basel were analyzed with a bioinformatic pipeline. Furthermore a morbidostat was assembled which we used to experimentally evolve resistance to cefepime in ESBL *E. coli*. SNPs as a product of resistance evolution were identified following the same bioinformatic pipeline as for the isolates obtained from patients. Code written for running the morbidostat or for identifying SNPs is available on <https://github.com/nahanoo/ESBL-project>.

2.1 ESBL *E. coli* isolates sampled from patients at the University Hospital of Basel

Our collaborators from the clinical microbiology of the University Hospital of Basel provided an isolate collection of 65 ESBL *E. coli* isolates sampled from 34 patients resulting in multiple isolates per patient. Isolates from the same patient are referred to as an isolate series. They determined the isolates as ESBL producing by carrying out combination disk diffusion tests. Furthermore, they determined the minimal inhibitory concentration (MIC) of cefepime and ceftazidime for every isolate. We selected isolate series, where the susceptibility of the isolates changed over the sampling period and where phylogenetic analysis ensured that every isolate of a series was the same strain. Of every selected isolate series we identified SNPs which were potentially caused by resistance evolution.

2.1.1 Disk diffusion tests

As recommended by the EUCAST our collaborators from the clinical microbiology at the University Hospital of Basel determined the *E. coli* isolates as ESBL producing by carrying out standardized disk diffusion tests.

They prepared a cell suspension of each isolate equal to 0.5 McFarland standard. Those suspensions were spread over the entire area of Mueller Hinton agar plates. On those plates disks with either 30 µg ceftazidime, 30 µg ceftazidime + 10 µg clavulanic acid, 30 µg cefepime and 30 µg cefepime + 10 µg clavulanic acid were applied. All plates were incubated for 20 hours. The inhibition zones caused by the disks were measured, if the inhibition diameters were larger than 5 mm the isolates

were determined as ESBL producing. All of the isolates from the collection turned out to be ESBL producing.

2.1.2 MIC determination

The MICs of every isolate were determined by our collaborators from the clinical microbiology at the University Hospital of Basel for the antibiotics ceftazidime and cefepime. This allowed us to select patients with isolates changing their susceptibility over time.

The MICs were determined by performing E tests. From an agar plate they picked a few colonies and diluted them, to 0.5 McFarland with physiological NaCl-solution. The suspension was plated on an agar plate. A reagent strip with either a ceftazidime or cefepime gradient was placed in the middle of the plate. After 20 hours the resulting minimal inhibitory concentration (MIC) was checked.

2.1.3 Selection of isolates suitable for our analysis

We only analyzed isolate series which consisted of more than one isolate, showed a significant change of the MICs and were all the same ESBL *E. coli* strain. To ensure that a isolate series consisted of the same strain, we performed a phylogenetic analysis with every isolate. This analysis was based on Illumina sequences of the isolates provided by our collaborator. For the phylogenetic analysis we used a tool called panX [37].

Illumina sequencing

The DNA from the isolates was extracted using the EZ1 DNA tissue kit on an EZ1 Advanced XL robotic system (Qiagen). The library for the sequencing was prepared using the Nextera XT library preparation kit (Illumina) and the resulting library was sequenced on a MiSeq Illumina platform [38]. The reads produced with Illumina were trimmed with Trim Galore [39].

PanX

PanX is a tool which clusters genes into orthologous clusters [37]. From those clusters, panX identifies the core genome which are genes shared by all isolates in the cluster. Based on those core genomes a strain-level phylogeny is built, making use of single nucleotide polymorph positions (SNPs) within the core genomes [37]. This resulted in a phylogenetic tree with the information how closely related the isolates were.

PanX uses annotated whole-genomes as input files, this is why every isolate was short-read assembled and annotated. For short-read assembling we used spades and for annotating the resulting assemblies we used prokka [40] [41]. Prokka first searches a core set of well characterized proteins using BLAST+ and then compares reading frames to a database derived from UniProtKB [41]. The results from prokka were stored in a genbank file for every isolate. The panX analysis was performed, based on those genbank files.

2.1.4 Identification of SNPs

Considering the phylogenetic analysis and the MICs we selected five patients with at least two isolates per patient which we analyzed. Because the pipeline for identifying SNPs was based on Illumina ONT sequencing data, we sequenced every selected isolate with ONT. Afterwards the selected isolate series were analyzed with the bioinformatic pipeline described in Section 2.2.

ONT sequencing

For ONT sequencing the library with the isolates was prepared with a ligation sequencing kit (LSK-108) followed by the native barcoding expansion kit. This allowed barcoding of multiple isolates and loading all of them on a single flow cell (FLO-MIN106D). As a sequencing device we used the MinION from ONT.

As a first step each DNA isolate was diluted to a concentration of 20 ng/ μ L in 50 μ L nuclease free water (NFW). For end-repairing the DNA 7 μ L NEBnext Ultra II Endrepair/dA-tailing enzyme mix was added to each isolate and incubated at 20°C for 5 min and at 65°C for 5 min. After this step every isolate was cleaned up by adding 60 μ L of AMPure XP beads. The beads were incubated with the isolates for 5 minutes on a rotator and then removed on a magnetic rack. Every isolate was washed with 200 μ L 70% ethanol which was repeated once. The ethanol was removed and every isolate was suspended in 25 μ L NFW. 2.5 μ L of each barcode plus 25 μ L of Blunt/TA ligase was added to each isolate and incubated for 10 minutes. All the isolates were pooled and 500 μ L of AMPure XP beads were added. After incubating the pooled isolate for 5 minutes on the rotator the isolate was washed again twice with 70 % ethanol. All the DNA was eluted in 51 μ L NFW. The final isolate was diluted to a concentration of 35 ng/ μ L. Then for adapter ligation 20 μ L BAM, 30 μ L Ultra II ligation master mix and 1 μ L enhancer were added. After 10 minutes of incubation, 40 μ L AMPure XP beads were added and incubated on the rotator for 5 minutes. The supernatant was removed on the magnetic rack and the DNA was washed twice with ABB. The DNA was eluted and incubated for 10 minutes in 15 μ L ELB. Finally the library was prepared by mixing 15 μ L of eluted DNA in ELB with 25.5 μ L LLB and 35 μ L RBF. The resulting library was loaded on the flow cell, which was primed before with a mixture of 480 μ L RBF and 520 μ L of NFW. The sequencing run was started and simultaneously base-called with Albacore.

2.1.5 Studying copy numbers of ESBL genes

We were also interested to see if the copy numbers of ESBL genes changed while resistance evolved. Possibly the copy numbers affect the ESBL protein level which could explain extended resistance. Therefore, we hybrid-assembled every selected isolate with Unicycler [42] and annotated the assembly with prokka [41]. Afterwards we checked how many ESBL genes were present considering the annotation provided by prokka.

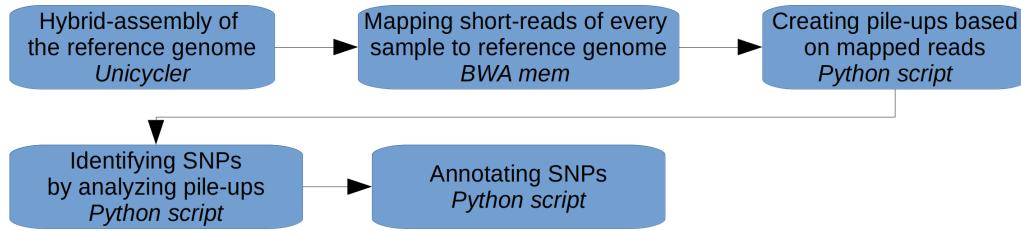


Figure 2.1: Bioinformatic pipeline used for the identification of SNPs and affected genes/promoters.

2.2 Bioinformatic pipeline for identifying SNPs in resistant ESBL *E. coli*

The following pipeline was used to identify SNPs in isolate series which evolved resistance. This pipeline was based on Illumina and ONT sequencing data coming from multiple isolates which were taken while resistance evolved. In principle a hybrid-assembly of the genome was performed for the isolate with the lowest MIC. This genome was used as a reference in order to identify SNPs accumulated in the other isolates where resistance evolved. We also tried to provide annotation for the SNPs.

2.2.1 Creating a reference genome with annotation

As a first step we hybrid-assembled the genome of the isolate with the lowest ceftazidime MIC of an isolate series with Unicycler [42]. Every assembly was annotated with prokka which produced a genbank file for every reference genome [41]. Additionally promoter regions were identified using the promoter prediction tool PePPER [43]. PePPER is a tool which takes whole-genomes as an input and predicts promoter sequences. Those sequences were mapped against the reference genome using graphmap [44]. Furthermore, a promoter database hosted on EcoCyc was used. This database contains around 3800 experimentally validated promoters for *E. coli* [45]. The sequences from this database were downloaded and mapped against the reference genome with graphmap as well [44].

2.2.2 Mapping Illumina sequencing data of the isolate series to the reference genome

As a second step of the pipeline the short-read Illumina sequencing data of every isolate was mapped against the reference genome of the isolate series. Mapping to the reference genome was done with BWA mem [46]. This resulted in a bam file for every isolate of the series. Mapping all the Illumina reads provided us the information which base was present in every Illumina read mapped to a certain position to the reference genome. If the most abundant base of all Illumina reads at a certain position was different than in the reference genome a SNP was present. To make this information more accessible we calculated pile-ups as a third step of our analysis.

2.2.3 Calculating pile-ups

Pile-ups are count matrices storing which base is present how many times at a certain position considering every mapped Illumina read. In order to produce those pile-ups we used a script called `pileup.py`. This script extracted the base counts by going through every position of the sorted bam-file from an isolate. Pile-ups were calculated for every isolate of a series and all the pile-ups stored in a matrix stack.

2.2.4 Identification of SNPs

We identified SNPs by comparing the most abundant base at every position of the matrix-stack with a script called `analysis_modular.py`. If the most abundant base varied between the isolate a SNP was identified. For the rest of the pipeline only SNPs were included where the coverage was at least 30 and the base frequency at least 0.8.

2.2.5 Identifying genes and promoters affected by SNPs

As a last step of the pipeline we checked if annotation was available for the SNPs. For checking if a SNP affected a gene, we analyzed the genbank file with biopython [47]. To check whether a SNP affected a promoter region we analyzed the bam-files which were created with the promoter sequences from PePPER and EcoCyc. We checked if a SNP was located between a start or end position of a mapped promoter sequence.

2.3 Assembling the morbidostat

We experimentally evolved resistance to cefepime with ESBL *E. coli* strains using a morbidostat. We assembled an adapted version of Topraks build differing mainly in its pump system, controlling unit and software[32]. Hardware which was not commercially available was built by the in-house mechanic and electronic workshop.

Figure 2.2 shows the morbidostat setup. As a base for our build we used a magnetic stirrer with 15 slots. On that stirrer we placed three layers of acrylic glass through which we inserted black plastic rings which acted as our vial holders and OD measuring units. The vials placed in the vial holders had three inlets as shown in Figure 2.2. One inlet was used to inject media or antibiotics, one for removing volume exceeding the culture volume and the last one to mount an air filter for pressure equilibration.

To test the hardware, we built the morbidostat in the open as shown in Figure 2.2. For the actual experiments we placed the morbidostat inside a hypoxi-station in a bio safety lab 2. This allowed us to culture the bacteria at 37 °C but also to increase the safety.

2.3.1 OD measuring units

For measuring the ODs we sent a ray of light through the cultures. This ray was scattered by the cells meaning that the ray was thrown off by the cells in multiple directions. With the help of a phototransistor we could measure the scattering with

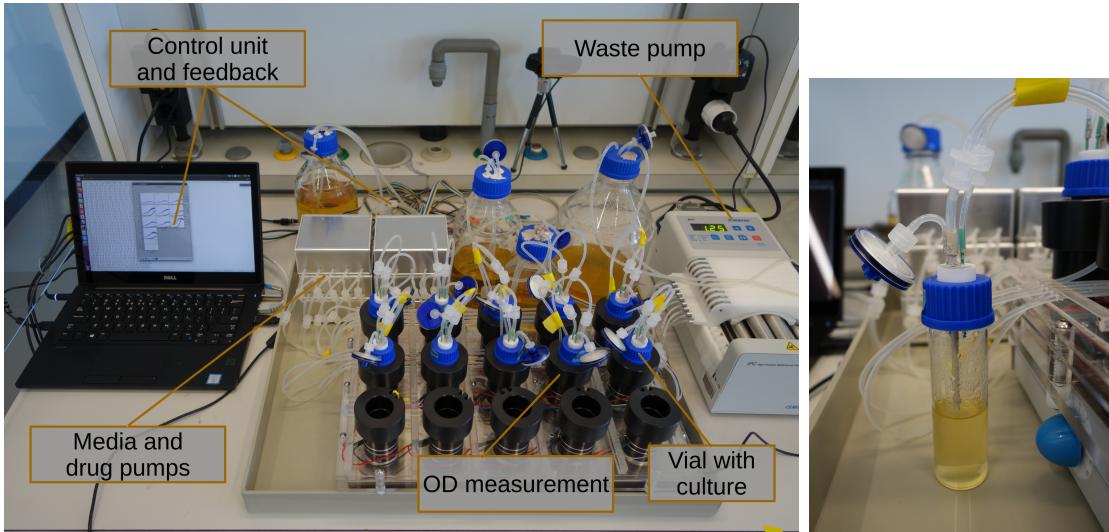


Figure 2.2: Left: Overview of the morbidostat setup. Vials were placed on magnetic stirrer and connected to three drug injecting pumps and one waste removal pump. A microcontroller was connected in serial to a PC, which allows to computer control drug injections and OD measurements. Right: One vial with all the inlets.

analog pins of the microcontroller. Because the scattering was proportional to the cell density, we could translate the scattering into an OD by calibration.

One OD measuring unit integrated in the vial holder is shown in Figure 2.3. Every unit consisted of a vertical-cavity surface-emitting laser (VCSEL) and a phototransistor. We chose OPB608V as a VCSEL with a peak wavelength of 890 nm and PT 333-3C as a phototransistor, both from TT electronics. For each unit the VCSEL and the phototransistor were placed in a 135 ° angle inside the vial holder. Both components were in direct contact with the glass vial. The 15 OD measuring units were divided into three groups of five units which corresponded to one row of vials of the morbidostat. For every group the VCSELs and phototransistors were both connected to independent 5 V circuits. The circuits of one group are illustrated in the left Figure 2.3, where the VCSEL circuit is shown orange and the phototransistor circuit in blue.

The VCSELs were constantly on sending rays of light through the cultures. When an OD measurement was initialized the scattering was detected with the phototransistors. Light reaching the phototransistor caused an opening in the semiconductor from the phototransistor which led to an amplification of the current. This current reached a potentiometer connected in serial over which we measured the voltage with an analog pin of the microcontroller. The opening of the semiconductor altering the measured voltage correlated with how much light reached it. More cells caused more scattering and more light reaching the semiconductor. Therefore, there was a linear correlation between the measured voltage and the cell density in the suspension.

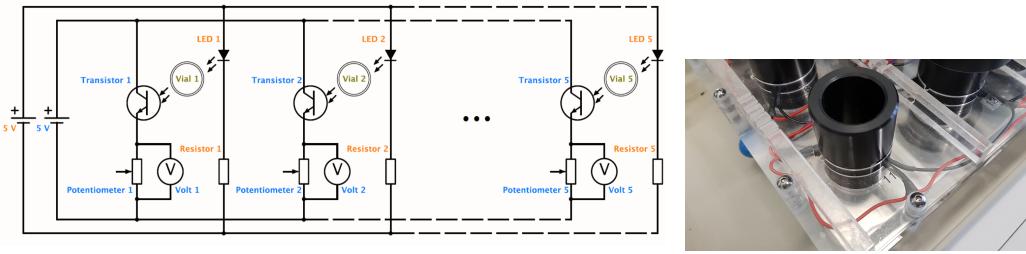


Figure 2.3: Left: Circuit for parallel connected VCSELs (orange). Each VCSEL is connected in serial with a $220\ \Omega$ resistor. The circuit of the phototransistors is shown in blue. The phototransistors are connected in parallel. Each phototransistor is connected to a potentiometer in serial over which the voltage is measured with an analog pin of the microcontroller.



Figure 2.4: Left: Principle of the piezo pumps. Top state: Piezo ceramic (purple) mounted on a membrane (blue) is relaxed. Left valve is open (orange), right valve is closed, liquid enters. Right and bottom state: Voltage is applied to the piezo ceramic deforming the membrane resulting in a down stroke. Left valve is closed, right valve is open. Liquid exits to the right. Voltage decreases again and the piezo ceramic enters its relaxed state again [48]. Middle: Top view of a circuit board with five pumps and five controllers. Right: Bottom view of a circuit board.

2.3.2 Pumps and tubing

Every vial was connected to three injecting pumps. One pump was responsible for injecting media, one for injecting a low-concentrated antibiotic and one for injecting a high-concentrated antibiotic. Mixing of desired antibiotic concentrations was possible by controlling the run times of the pumps. We chose mp6 pumps from microComponents because of their very compact build. The functional principle of the pumps is shown in the left Figure 2.4. We achieved a steady flow rate of the pumps with the mp6-OEM controller from microComponents. On one circuit board five pumps with five mp6-OEM controllers were mounted which is shown in Figure 2.4. Each mp6-OEM controller was connected to one pump, a 5 V power supply, a ground and a digital pin of the microcontroller which allowed computer controlling the run times of every pump. The circuit boards were mounted in a metal box and three of those boxes stacked on top of each other which is shown in Figure 2.5. By packing five pumps in a box and stacking three boxes on top of each other we were able to connect one row of vials to the whole range of antibiotic concentrations with one stack. Pumps of the lowest box were connected to media, pumps of the

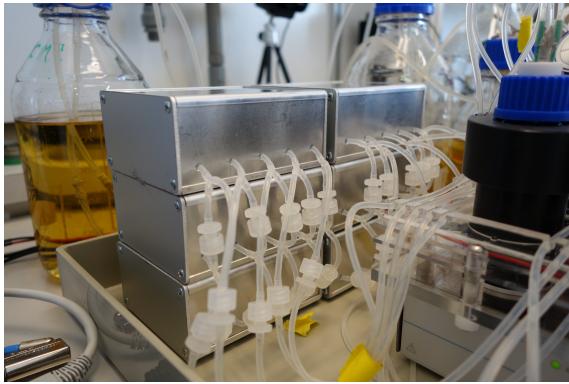


Figure 2.5: Three pump boxes with five pumps and controllers stacked on top of each other. The outlets of the pumps were connected column by column and led to the vials.

middle box to a low-concentrated antibiotic and the pumps of the top box to a high-concentrated antibiotic. We connected the outgoing tubes of the pumps from one stack column by column resulting in one outgoing tube per column as shown in Figure 2.5. Those tubes were led to the vials. This way we connected one vial with three pumps providing all concentrations of antibiotic and media with just one tube leading to the vial. Every vial was also connected to a 16-channel peristaltic pump which removed volume exceeding the culture volume through the gray tube shown in the right Figure 2.2.

Computer controlling the pumps

In order to control the run times of the pumps a pin of every mp6-OEM controller was connected with a digital pin of the microcontroller. If the pin of the mp6-OEM controller received a voltage of 5 V the pumps were off, if the pin was set to ground the pumps were on.

When we set the digital pins of the microcontroller to ground there was still a low current flowing, resulting in a small voltage. The mp6-OEM controllers reacted very sensitive to this small voltage leading to weird behavior of the pumps. We solved this by inserting a pull-down resistor between the digital pins of the microcontroller and the ground which is shown in Figure 2.4. Additionally an inverter was connected in serial to the digital pins. To turn on a pump a digital pin of the microcontroller was set to 5 V. The inverter connected in serial set the signal at the pin of the mp6-OEM to ground and the pump was on. Run time control was possible by setting the digital pins to 5 V for the desired time. The 16-channel peristaltic pump was also computer controllable because the pump was also connected to a digital pin of the microcontroller.

2.3.3 Controlling the morbidostat

As a microcontroller we used an Arduino mega 2560 flashed with an arduino-script called arduino_morbidostat.ino, which allowed us to change the state of digital pins or measuring voltages of analog pins. The microcontroller itself was controlled by a laptop where two python scripts were running. The python script morbidostat_experiment.py decided which analog pins were measured and which digital pins

were set to high for how long. Those tasks were grouped in cycles and repetitively executed. Additionally this script was responsible for storing ODs and injected antibiotic concentrations. We used a second python script called arduino_interface.py to enable the communication between the laptop and the microcontroller. Commands for the microcontroller initialized by morbidostat_experiment.py were encoded in a string by arduino_interface.py which was transmitted to the microcontroller via a serial USB connection. The microcontroller interpreted the string and executed the encoded commands.

We implemented three modes for the morbidostat in morbidostat_experiment.py. Those being the continuous mode which we used for continuous inhibition of the cultures, a growth rate mode where growth rates with no injections were recorded and a fixed OD mode where the OD of a culture was fixed to a certain OD by dilution with media.

Tasks of a continuous morbidostat cycle

As for every mode the continuous mode consisted of several tasks grouped in one cycle. Shown in Figure 2.6 as a first step the microcontroller measured the voltages of the analog pins connected to OD measuring units for a defined cycle time (typically being 10 minutes). Those voltages were constantly sent to the laptop where they were translated to ODs which were saved. After the cycle time the program fit a line to the OD measurements of a cycle for every vial. Using this fit the growth of every vial was calculated and stored. Additionally the measured ODs of a cycle were averaged and stored as well. Then a feedback algorithm calculated how much drug to inject into which vial. Shown in Figure 2.6 the last step of a cycle was to translate the calculated antibiotic concentration into run times of the three pumps connected to a vial. The run times were sent to the microcontroller. The microcontroller turned on the pumps for the calculated time and after removing volume exceeding the culture volume with the peristaltic pump one cycle was finished. The injected volume always remained the same, which was controlled with the dilution factor. The commandflow of the growth rate mode was very similar just that the feedback function was not executed and therefore no fluids were injected into the vials. For the fixed OD mode another function instead of the feedback calculated how much media to inject to keep the OD at a constant value.

Feedback of the continuous mode

The feedback determined how strongly the cultures were inhibited with antibiotics. This feedback was based on the relative difference between the averaged ODs of the current cycle and the averaged ODs of a past cycle. We called the average ODs of a cycle final_OD and the relative difference between two final_ODs ΔOD .

$$\Delta OD = (final_OD_{cycles_back} - final_OD_{current_cycle})/x \quad (I)$$

As shown in Figure 2.7 the feedback did several comparisons before calculating an appropriate dose of antibiotics. As a first step it checked if ΔOD was positive or negative. A negative ΔOD implied that the bacteria were dying. In order to prevent complete sterilization, media was injected in this case, effectively diluting the antibiotic concentration in the vial.

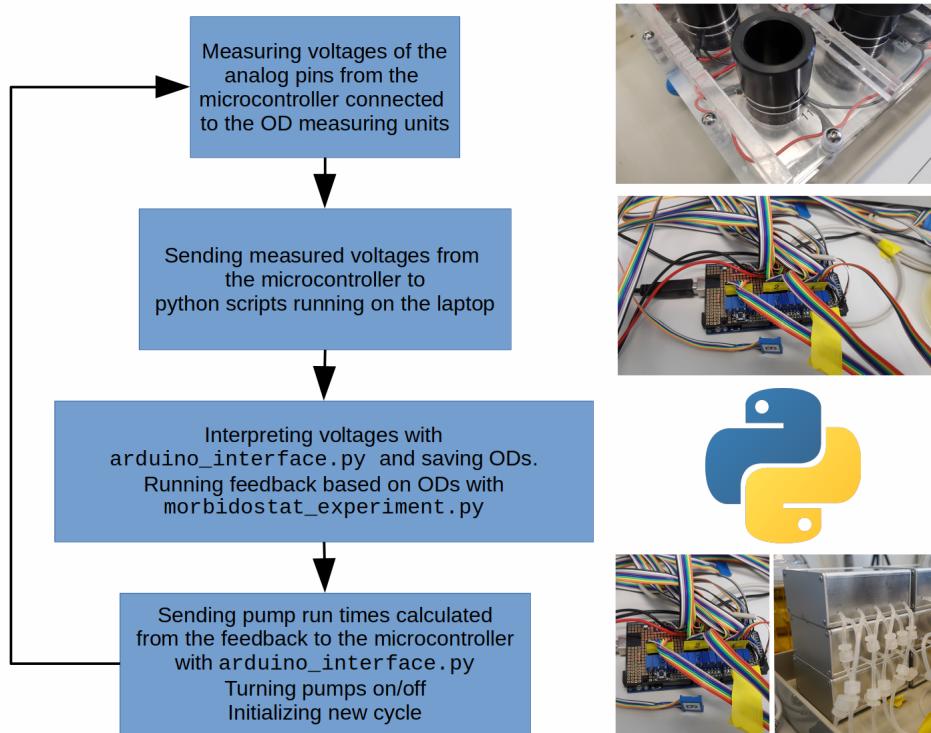


Figure 2.6: Overview of one cycle from the continuous mode of the morbidostat.

When ΔOD was positive the bacteria were growing. Antibiotics were only injected if the bacteria reached a certain OD called drug_dilution_threshold. Therefore, the next comparison as visible in Figure 2.7, was whether or not the final_OD was bigger or smaller than this threshold. If the final_OD was smaller no fluids were injected. However when final_OD was bigger than the threshold, calculation of the appropriate dose was initialized. The calculation itself was split up into two equations.

$$increase_vial_conc = vial_conc + mic_fraction * mic \quad (\text{II})$$

$$increase_vial_conc = vial_conc * c * \Delta OD / target_OD \quad (\text{III})$$

Equation II, mainly important at the beginning of the morbidostat experiments, was used to approximate the MIC of the strains in the vials. This was done by simply adding a fraction of the MIC (mic_fraction) to the current vial concentration (vial_conc). After the MIC was reached this equation was ignored and from now on equation III determined the injected concentration. This equation multiplied the current drug concentration in the vials (vial_conc) by the ΔOD which resulted in how much the drug concentration in the vial was increased (increase_vial_conc). The goal of the feedback was that a certain OD called target_OD was approximated in every vial. In order to do so we divided the second equation by this target_OD. Now if a small target_OD was chosen the increased concentration was divided by a small value causing a higher injected concentration. If target_OD was set to a high value the divided outcome was smaller. As a last step a constant (c) was introduced, which allowed to fine-tune the aggressiveness of the feedback.

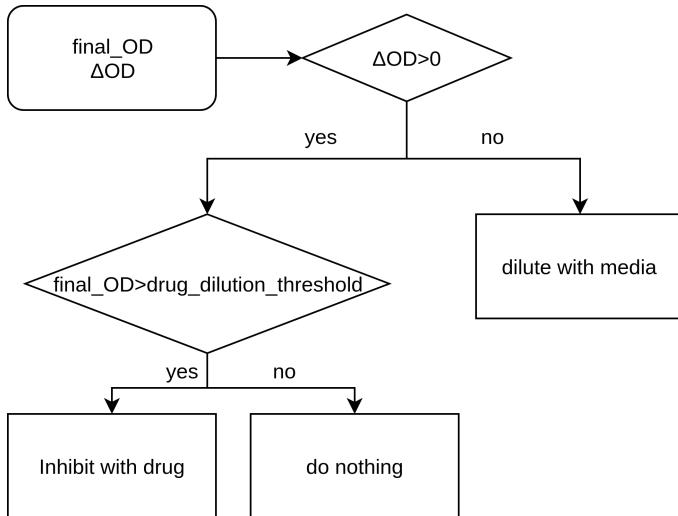


Figure 2.7: Schematic overview of decisions involved in the feedback.

2.3.4 Hardware calibration

OD and pump calibration

The OD measuring units were calibrated by measuring several cultures with a known OD. An overnight culture with K12 XL1 blue *E. coli* was inoculated in 5 ml 9/10 H_2O and 1/10 LB media (also referred to as diluted media). The next day the overnight culture was diluted 1/200 in 50 ml diluted media. After a few hours of day culturing following OD standards were prepared with 18 ml diluted media in the vials for the morbidostat: 0.01 0.021 0.042 0.107 0.192 0.278

Then every vial with a certain OD was placed in every vial holder. With the function calibrate_OD from the morbidostat_experiment.py, a voltage measurement was done for every OD standard and every OD measuring unit. The result of this function was a linear equation which translated the voltages to ODs.

To determine the flow rate of the pumps the function calibrate_pumps from themorbidostat_experiment.py was executed. The weight of every empty vial had to be entered and the function turned on every specified pump for 100 seconds. Afterwards the weight of the vials was entered again and the function calculated the flow rate for the specified pumps.

2.4 Experimentally evolving resistance with the morbidostat

For evolving resistance with the morbidostat Beatrice Claudi from Dirk Bumanns group produced three ESBL *E. coli* strains, by assembling ESBL producing plasmids with Gibson cloning which were transformed into *E. coli* K-12 MG1655 [49]. Working with ESBL *E. coli* K-12 MG1655 increased the safety of the handling, because *E. coli* K-12 MG1655 does not colonize the human intestine.

We cultured the ESBL *E. coli* strains with the plasmid produced with gibson cloning with the morbidostat in the continuous mode leading to increased resistance to ceftazidime within a few days. To confirm emerged resistance the MIC of the strains was

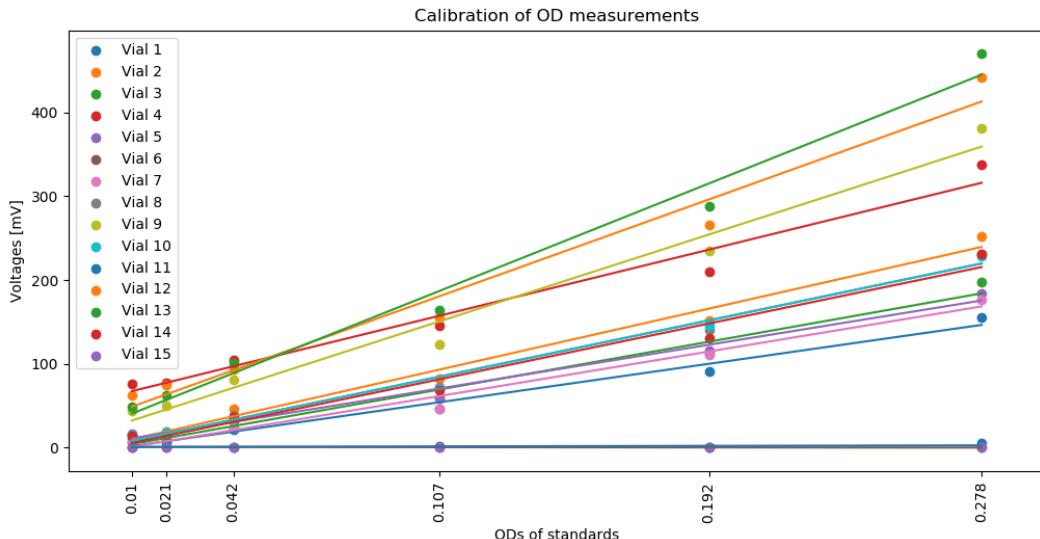


Figure 2.8: Calibration of OD measuring units, with the OD standards on the x-axis and the measured voltages on the y-axis. The line represents the calculated linear equation. The units of vial 5 and 15 were not working.

compared before and after culturing with the morbidostat. Samples taken while culturing with the morbidostat were Illumina and ONT sequenced. The resulting sequencing data was analyzed using the bioinformatic pipeline described in Section 2.2.

2.4.1 Gibson cloning

We extracted three ESBL gene sequences and their upstream sequence up to the previous gene (regulatory sequence) from two isolates of the isolate collection from the University Hospital of Basel (see Section 2.1). An identifier was created for every extracted gene with its regulatory sequence. The extracted sequences are shown in the supplementary in Section 6.2.1. Primers for PCR amplification were designed, based on the extracted sequences.

ID	Product	Source	Fragment size in bp
pEU22	β -lactamase CTX-M-1	Patient 25 isolate 1	1614
pEU23	β -lactamase OXA-1	Patient 25 isolate 1	1789
pEU26	β -lactamase OXA-1	Patient 33 isolate 1	1789

Table 2.1: Designed insert for gibson cloning. The ESBL genes were amplified including its regulatory sequence.

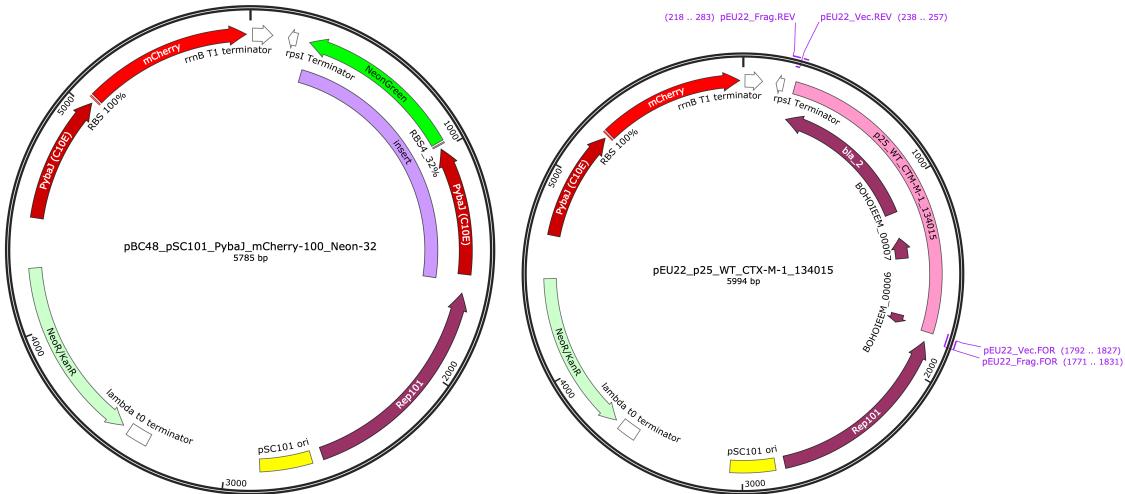


Figure 2.9: Left: Vector used for Gibson cloning. Right: Resulting pEU22 plasmid. The insert, consisting of an ESBL gene and its regulatory sequence amplified with template DNA from patient isolates, replaced NeonGreen which allowed selection for clones with the insert.

ID	Direction	Primer sequence
pEU22	Reverse	cgaaggcagaaccccaTCATCCGAGAGCTTGCATGCC TGCAttacaaacgcgtcggtgacgattttag
pEU22	Forward	GGCTCTTGTATCTATCAGTGAAGCATCAAGACTAAC AAATgcccacagaatgtatgtacgc
pEU23	Reverse	cgaaggcagaaccccaTCATCCGAGAGCTTGCATGCC TGCAttataaatttagtgtgttagaatggatcgatcgatttt
pEU23	Forward	GGCTCTTGTATCTATCAGTGAAGCATCAAGACTAAC AAATgtgtatcccctggcgaaatg
pEU26	Reverse	cgaaggcagaaccccaTCATCCGAGAGCTTGCATGCC TGCAttataaatttagtgtgttagaatggatcgatcgatttt
pEU26	Forward	GGCTCTTGTATCTATCAGTGAAGCATCAAGACTAAC AAATgtgtatcccctggcgaaatg
Vector	Forward	GGCTCTTGTATCTATCAGTGAAGCATCAAGACTAAC AAATgtgtatcccctggcgaaatg
Vector	Reverse	GGCTCTTGTATCTATCAGTGAAGCATCAAGACTAAC AAATgtgtatcccctggcgaaatg

Table 2.2: Primers used for the PCR amplification of the ESBL genes with their regulatory sequence and for controlling the insertion into the vector.

The fragments were amplified using the primer sequences shown in Table 2.2 and the KOD-Hot-start-DNA polymerase with template DNA from the according isolate. The vector shown in Figure 2.9 was amplified by PCR as well using the iProof fidelity polymerase, its sequence is shown in the supplementary (see Section 6.2.1). All PCR reactions were loaded on a 1% agarose gel. Since only bands with the expected lengths were present, the bands were cut out and purified using the NucleoSPin Gel and PCR Clean-up Kit. The DNA was eluted in 30 µL nuclease-free

water. The following Gibson reaction was prepared in order to insert the fragments into the vector:

ID	Fragment	Conc. [ng/ μ L]	Volume [μ L]		
			DNA	GIBSON MIX	H_2O
pEU22	Insert	268	0.22	5	2.75
	Vector	25.66	2.03		
pEU23	Insert	342.9	0.19	5	2.78
	Vector	25.66	2.03		
pEU26	Insert	285.2	0.22	5	2.74
	Vector	25.66	2.03		

Table 2.3: Gibson reaction with the inserts, the vector and the GIBSON mix.

The reactions were incubated for 20 min at 50 °C. From the reactions 2 μ L was added to 100 μ L electrocompetent cells of the strain *E. coli* K-12 MG1655. The mixture was electroporated with a Bio-Rad electroporator, pelleted and resuspended in 1 mL super optimal broth. The cells were shaken for 2 hours at 180 rpm and 37 °C. The electroporated strains were plated on LB plates containing kanamycin and grown over night. Because our vector includes a kanamycin resistance gene, all colonies which grew on the plates had the plasmid. Additionally, the vector had two genes coding for fluorescence proteins, mCherry (red) and NeonGreen (green). As seen in Figure 2.9 the insert replaced the gene coding for NeonGreen. Therefore the Gibson reaction was only successful for clones which were not green. Only red clones were picked and grown over night in LB containing kanamycin. Based on those cultures the clones were checked with a PCR. As a forward primer 'TCT-CAATGGTTCGTTCTCATGG' was used, as a reverse primer 'GGAACAGTAC-GAACGCGCCGA' was used. The PCR control showed positive results and the strains were stocked in LB containing 20% glycerol. The ID created for the plasmids was adopted for the strains.

2.4.2 Culturing ESBL *E. coli* strains with the morbidostat

Before culturing with the morbidostat, we determined the MIC of the ESBL *E. coli* strains and sterilized the device.

MIC determination

We inoculated 5 ml of MHB media with the ESBL *E. coli* strains and cultured the suspension over night at 37 °C. The next day a 1/100 dilution in 20 ml MHB was prepared for every suspension and cultured for a few hours at 37 °C. The growth of the day cultures was constantly monitored by measuring the OD. When the OD of the day culture was 0.08, a 1/100 dilution of every day culture was prepared.

We added 100 μ L of MHB to every well of a 96 well plate. Except for the last column of the plate, we added 100 μ L of the diluted day culture to every well. 100 μ L of a cefepime solution with a concentration of 2.048 mg/mL was added to the first column of the plate. Starting from the first column a 1:1 dilution series was prepared with the the first seven columns. We incubated the plates for 16 hours at 37 ° on a shaker. To get an idea how many cells were used for the MIC determination

10^{-3} and 10^{-5} dilutions of the 1/100 diluted cell cultures were plated on LB plates. After 16 hours the OD of every well was measured using a plate reader. The smallest concentration which inhibited the growth was determined as the MIC.

Sterilization of the morbidostat

We sterilized the morbidostat using two different disinfectants in order to prevent biofilm formation and contamination of our vials. Bottles, vials, tubing and luer connections were autoclaved. Sterile vials were connected to the tubes and the computer controllable pumps were connected to 1 L of 3% citric acid stored in a sterile bottle with sterile tubing. All the tubing going to the vials was flushed with the disinfectant over one hour. The waste pump was turned on leading to disinfection of the tubes connected to the sterile waste bottle. After that the tubing was flushed with 1 L of sterile water. We repeated this procedure but this time using 3% of bleach as disinfectant.

Culturing with the morbidostat

We ran two morbidostat experiments with the ID 01 and 02 with the ESBL *E. coli* strains and *E. coli* K-12 MG1655 as a control. For every experiment we prepared over night cultures of the ESBL *E. coli* strains in 3 ml diluted media with 1% L kanamycin. *E. coli* K-12 MG1655 was cultured over night in 3 mL diluted media. The over night cultures were diluted 200 fold the next day and cultured for a few hours. From those cultures, 200 μ L were transferred into sterile vials containing 18 mL diluted media. We prepared two different antibiotic concentrations which were connected to the pumps. We prepared a concentration of 9 μ g/ml cefepime in 1 L diluted media as a low-concentrated antibiotic solution. For the high-concentrated solution we prepared a cefepime concentration of 21 μ g/mL in 1 L diluted media. The morbidostat experiments were initialized with a target_OD of 0.12, and a dilution factor of 0.91. Every ESBL *E. coli* strain was cultured at least three times with the continuous mode. Additionally we cultured every ESBL *E. coli* strain with the fixed_OD mode. The temperature inside the hypoxi-station was set to 37 °C. The O_2 level was fixed to 10 % and CO_2 fixed to 5 %.

As resistance evolved the antibiotic concentrations of the bottles were changed. We checked which concentration was necessary to strongly inhibit the cultures. New cefepime concentrations which were 3 fold and 7 fold higher than the newly determined inhibiting concentration were prepared and connected. We changed the antibiotic concentrations of the bottles approximately every third day. If the suspension in the vials was extremely milk caused by dead cells we transferred 200 μ L of the suspension to a new sterile vial containing 18 ml diluted media.

During both experiments we took daily samples by opening the vial in the hypoxi-station and transferring 1 mL to an eppendorf tube. The collected samples were centrifuged at 13'000 rpm for 10 minutes and resuspended in 200 μ L LB containing 20 % glycerol. Those suspensions were frozen at -80 °C.

2.4.3 Analysis of the morbidostat samples

Illumina and Nanopore sequencing

We handed the stocks of the cloned ESBL *E. coli* strains and K-12 MG1655 to our collaborators of the University Hospital of Basel where they were sequenced on a MiSeq-Illumina system (see Section 2.1.3). Furthermore, we handed them the stocks for Illumina sequencing of the last sample day of every vial from the 01 experiment. From the 02 experiment we selected the stocks from vial 3,4,5,7 and 8 from the third sample day and the stocks from every vial of the last sample day for Illumina sequencing. Every stock that we handed over for Illumina sequencing was streaked out. We sequenced the ESBL *E. coli* stocks and K-12 MG1655 with ONT. For DNA extraction we inoculated 3 mL LB containing 1% kanamycin with the ESBL *E. coli* stocks. K-12 MG1655 was inoculated in 3 mL LB. The suspensions were cultured over night. The DNA of the resulting over night cultures was extracted following the protocol of the DNeasy Blood & Tissue Kit [50]. The extracted DNA was sequenced with a MinION as described in Section 2.1.4.

Contamination analysis

On the plates where we streaked out every stock that we handed over for Illumina sequencing we saw colonies which had a different shape than *E. coli*. Therefore, we had the suspicion that some stocks were contaminated. Because we had Illumina sequencing data for every stock we could identify the contamination by blasting a few reads [50]. This revealed that the contamination was *Bacillus cereus*. To identify which samples were contaminated, all the Illumina short-reads from every stock were mapped to a *Bacillus cereus* reference genome obtained from NCBI [51] and the ESBL *E. coli* reference genome produced with hybrid-assembling.

Identifying mutations in morbidostat samples

The sample series from the morbidostat experiments were analyzed following the bioinformatic pipeline described in Section 2.2.

Chapter 3

Results

This chapter presents the results of this work. SNPs found in isolate series sampled from patients at the University Hospital of Basel are presented with available annotation. Furthermore, we demonstrate that the assembled morbidostat is very suitable for experimentally evolving resistance to cefepime with ESBL *E. coli*. We also show SNPs in ESBL *E. coli* strains potentially resulting from culturing with high antibiotic pressure.

3.1 Identifying SNPs in ESBL *E. coli* isolates series with changing cefepime susceptibility

First, the phylogenetic tree will be presented that was used to ensure that all isolates of a series were the same strain and to show which isolates were selected for our analysis. Next will be shown which isolates of a series were chosen as a reference and as a last step the identified SNPs compared to the reference are shown.

3.1.1 Selection of isolate series suitable for our analysis

We only included isolate series in our analysis with a significant change of the MICs over the sampling period and where all the isolates from a series were the same ESBL *E. coli*. To ensure strain identity within a series we performed phylogenetic analysis with all isolates with panX [37]. The phylogenetic tree of all isolates is shown in Figure 3.1. This tree revealed that many patients were infected with different ESBL *E. coli* strains over time, suggesting reinfection rather than in-patient evolution. This is visible in the tree where isolates of one patient mapped on different branches. Interestingly some isolates from different patients mapped on the same branch which suggests that those patients were infected from the same outbreak.

We determined a change of the MICs as significant, if the MIC for cefepime increased at least by a factor of three. Applying the criteria of strain identity and significant change of MIC over the sampling period, five isolate series were left over. Those isolates and their MICs are shown in Table 3.1.

3.1.2 Identification of SNPs in patient isolates

After selecting for isolate series suitable for our analysis we determined one isolate per series as a reference. We picked the isolate with the lowest MIC of cefepime as

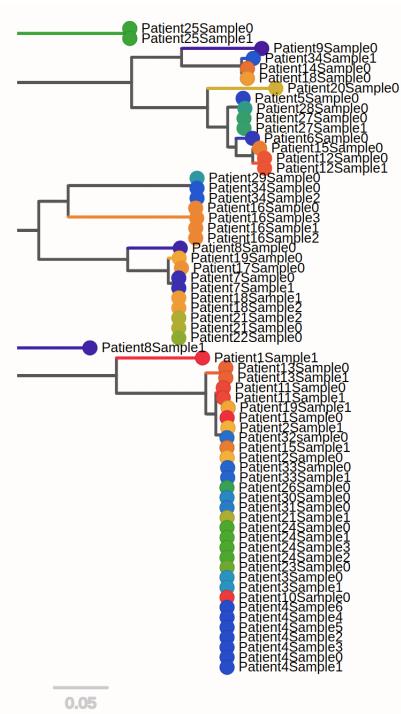


Figure 3.1: Phylogenetic tree built for every ESBL *E. coli* isolate.

Patient	Isolate	Isolate date	MIC cefepime [µg/mL]	MIC ceftazidime [µg/mL]
12	0	09/09/14	4	0.75
	1	05/12/14	12	2
16	0	22/06/12	8	2
	1	18/07/13	48	8
	2	01/11/13	32	12
24	0	02/05/11	4	1.5
	1	08/15/11	16	1.5
	2	11/28/11	3	1
25	0	15/04/11	64	192
	1	22/08/11	6	6
33	0	26/09/14	6	6
	1	29/01/15	1	1.5

Table 3.1: Selected ESBL *E. coli* isolate series and their MIC of cefepime and ceftazidime. Isolates highlighted in orange were chosen as reference for the SNP analysis.

#SNP	Contig	Position	Nucleotide in isolate:	
			0	1
1	0	880249	C	A
2	0	4256761	A	T

Table 3.2: SNPs in the isolates of patient 12

reference. If two isolates of a series had very similar MICs of cefepime (*e.g.* isolate 0 and 2 from patient 24), we picked the isolate with the earlier sample date. The selected references of the series are stained orange in Table 3.1. For the isolate series of patient 25 and 33 resistance decreased over the sample period. Therefore, the reference is isolate 1, and not isolate 0 like for the other isolate series. From the selected references we hybrid-assembled their genome based on Illumina and ONT sequencing data.

We identified SNPs in the selected isolate series by comparing Illumina sequencing data of the isolates with emerged resistance to the genome of the reference of the series. We applied filtering of the SNPs by defining a minimal Illumina coverage of 30 at the position of the SNP. Additionally, only SNPs where the nucleotide in the isolates with emerged resistance changed in 80% of the Illumina reads at this position compared to the reference will be presented.

Isolate series of patient 12

The isolate series of patient 12 consisted of two isolates sampled over three months. The EUCAST classifies strains with cefepime MICs bigger than 4 µg/mL as resistant [52]. The MIC of cefepime of isolate 0 was 4 µg/mL and slightly below the breakpoint, but still very high. For this isolate series we identified two SNPs with no annotation. The SNPs are shown in Table 3.2.

Isolate series of patient 16

From patient 16 three isolates were sampled over five months. The MICs of cefepime from every isolate exceeded the breakpoint. Therefore, all isolates were classified as resistant. Resistance advanced over the sample period visible in the MIC of cefepime of isolate 1 which is 48 µg/mL. We identified over 20 SNPs in the isolates of patient 16, but for only six genes annotation was available. Interestingly one SNP affected a promoter. The SNPs of the isolate series of patient 16 are shown in Table 3.3. The genes affected by the SNPs are shown in Table 3.5 and the affected promoter in Table 3.4.

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#SNP	Contig	Position	Nucleotide in Isolate:		
			0	1	2
1	0	109928	C	A	C
2	0	1895312	A	C	A
3	0	2101128	G	-	-
		2101129	C	-	-
		2101130	A	-	-
4	0	2375277	C	T	C
5	0	3797984	A	G	A
6	0	4200032	T	C	T
7	0	4353560	G	C	G
8	0	5112127	G	T	G
9	0	55597	A	G	G
10	0	922702	G	A	G
11	0	1133762	C	T	T
12	0	1549518	T	G	G
13	0	2016331	A	C	A
14	0	2101129	C	-	-
		2101130	A	-	-
15	0	3920934	A	G	A
16	0	4333944	C	T	T
17	0	4459680	C	-	C
		4459681	C	-	C
18	0	4459684	G	-	G
		4459685	A	-	A
		4459686	A	-	A
		4459687	G	-	G
		4459688	A	-	A
		4459689	G	-	G
19	0	4459692	A	-	A
		4459693	G	-	G
20	0	4459695	G	-	G
21	0	4459697	T	-	T

Table 3.3: SNPs in the isolates of patient 16.

#SNP	Transcription unit	Next upstream gene
16	fes-ybdZ-entF-fepE	<i>fepA</i>

Table 3.4: Identified promoter affected by a SNP in the isolates of patient 16.

The *fepA* promoter sequence affected by a SNP is shown in Figure 3.2. The polymorph position, marked with a red box, was found in the binding site of the σ 70 transcription factor.

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#SNP	Gene	Product	Type and position in gene	Amino acid in isolate:		
				0	1	2
1	<i>ortT</i>	Orphan toxin OrtT	Missense, 44	P	T	P
2	<i>scrY</i>	Sucrose Porin	Missense, 104	L	V	L
3	<i>cpdA</i>	Phosphodiesterase CpdA	In-frame deletion, 162	L	-	-
4	<i>rpoB</i>	DNA-directed RNA polymerase subunit beta	Missense, 113	V	I	V
5	<i>ftsQ</i>	Cell division protein FtsQ	Missense, 207	K	R	K
4	<i>recR</i>	Recombination protein RecR	Missense, 40	M	T	M
5	<i>hcxA</i>	Hydoxycarboxylate dehydrogenase A	Missense, 332	R	G	R
6	<i>ribA</i>	GRP cyclohydrolase-2	Missense, 68	F	L	L

Table 3.5: Genes affected by SNPs found in the isolates of patient 16.



Figure 3.2: This alignment shows the SNP in the fepA promoter. Positions marked with dashed lines are the recognition sites for $\sigma70$ factor.

#SNP	Contig	Position	Nucleotide in isolate:		
			0	1	2
1	0	50032	G	A	G
2	0	418564	C	T	C
3	0	2048961	A	C	C
4	0	2319554	T	A	A
5	0	3444478	T	C	T
6	0	4063226	G	T	T

Table 3.6: SNPs in the isolates of patient 24.

Isolate series of patient 24

The isolate series of patient 24 consisted of three isolates. The MIC of cefepime from isolate 1 exceeded the breakpoint and was classified as resistant. For isolate 2 the resistance decreased again, and dropped below the breakpoint. Identified SNPs in the isolates of patient 24 are show in Table 3.6, the affected genes are shown in Table 3.7.

Isolate series of patient 25

Two isolates were sampled from patient 25. Isolate 0 shows the highest cefepime MIC, which was 64 µg/mL, of all selected isolates. In the case of patient 25 the resistance decreased over the sample period. Isolate 1, with a cefepime MIC of 6 µg/mL was still classified as resistant. The identified SNPs between the isolates of patient 25 are shown in Table 3.8. The genes affected by those SNPs are shown in Table 3.9.

Isolate series of patient 33

As for the series of patient 25, the resistance deceased in the isolates of patient 33. Isolate 0 was classified as resistant, while isolate 1 was classified as cefepime susceptible. Three SNPs were found in the isolates of patient 33, they are shown in Table 3.10 with their annotation in Table 3.11.

3.1.3 Copy numbers of ESBL genes

We checked if the copy numbers of ESBL genes correlated with emerged resistance. Therefore, we analyzed the annotation of the hybrid-assembly of every isolate. Copy numbers of three ESBL genes of the selected isolates are shown in Table 3.1.3. The copy number remained very similar for four out of five patients. Sometimes the ESBL gene was replaced by a different ESBL gene. Only the isolate series of patient 33 showed an increased copy number of ESBL genes while resistance was high, with the copy number decreasing as the resistance decreased. Isolate 0 of patient 33 had 9 copies of CTX-M-1 while isolate 1 only had one.

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#SNP	Gene	Product	Type and position in gene	Amino acid in isolate:		
				0	1	2
1	<i>atl</i>	DNA base-flipping protein	Missense, 87	V	M	V
2	<i>imm_2</i>	Colicin-E7 immunity protein	Missense, 69	K	E	K
3	<i>lldR_2</i>	Lactate Dehydrogenase regulatory protein	Missense, 44	I	S	S
4	<i>dctM_2</i>	TRAP transporter large permease protein	Missense, 112	T	S	S
5	<i>dltA</i>	D-alanine-D-alanyl carrier protein ligase subunit 1	Missense, 692	E	G	E
6	<i>fnr</i>	Fumarate and nitrate reduction regulatory protein	Missense, 31	C	F	F

Table 3.7: Genes affected by the SNPs found in the isolates of patient 24.

#SNP	Contig	Position	Nucleotide in isolates:	
			0	1
1	0	396325	G	A
2	0	396846	G	A
3	0	1996537	-	C
		1996538	-	C
		1996539	-	G
		1996540	-	T
		1996541	-	A
		1996542	-	C
		1996543	-	C
		1996544	-	A
		1996545	-	G
		1996546	-	C
		1996547	-	T
		1996548	-	G
4	0	3743644	A	G
5	0	4785433	G	A
6	0	4785439	G	A

Table 3.8: SNPs in the isolates of patient 25.

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#SNP	Gene	Product	Type and position	Amino acid isolate:	
				0	1
1	<i>ompR</i>	Transcriptional regulatory protein OmpR	Missense, 146	R	H
2	<i>envZ</i>	Osmolarity sensor protein EnvZ	Missense, 84	G	E
3	<i>rfbD</i>	dTDP-4-dehydrorhamnose reductase	Frameshift deletion, 148-295		

Table 3.9: Genes affected by the SNPs found in the isolates of patient 25.

#SNP	Contig	Position	Nucleotide in isolate:		
				0	1
1	0	4008745	A	T	
2	0	4675092	T	A	
3	0	1996537	A	C	

Table 3.10: SNPs in the isolates of patient 33.

#SNP	Gene	Product	Type and position	Amino acid isolate:	
				0	1
1	<i>cydD</i>	ATP-binding/permease protein CydD	Missense, 368	Q	L
2	<i>vnfA</i>	Nitrogen fixation protein VnfA	Missense, 169	N	I

Table 3.11: Genes affected by the SNPs found in the isolates of patient 33.

Patient	Isolate	Copies of genes coding for β -lactamase		
		CTX-M-1	OXA-1	TEM
12	0	1	0	0
12	1	1	0	0
16	0	2	0	1
16	1	2	0	1
16	2	1	0	1
24	0	1	0	2
24	1	1	0	2
24	2	0	0	3
25	0	1	1	0
25	1	1	1	0
33	0	9	0	1
33	0	1	1	0

Table 3.12: Copy number of ESBL genes per isolate.

Vial	Strain ID	ESBL	Mode
1	pEU26	OXA-1	Continuous
2	pEU26	OXA-1	Continuous
3	pEU23	OXA-1	Continuous
4	pEU23	OXA-1	Continuous
5	pEU23	OXA-1	Continuous
9	pEU26	OXA-1	Continuous
10	pEU23	OXA-1	Fixed OD

Vial	Strain ID	ESBL	Mode
1	pEU26	OXA-1	Continuous
2	pEU23	OXA-1	Continuous
3	pEU22	CTX-M-1	Continuous
4	pEU22	CTX-M-1	Continuous
5	pEU22	CTX-M-1	Continuous
7	K12 MG1655	None	Continuous
8	pEU22	CTX-M-1	Fixed OD
9	pEU26	OXA-1	Fixed OD
10	pEU26	OXA-1	Fixed OD

Table 3.13: Left table: Used culturing mode and strains for experiment 01. Right table: Used culturing mode and strains for experiment 02.

3.2 Morbidostat experiments

The experimental evolution of cefepime resistance with ESBL *E. coli* strains was successfully achieved by culturing with the morbidostat. Proof that resistance increased was obtained by comparing the MIC of cefepime from the strains before and after culturing with the morbidostat.

We performed two morbidostat experiments, experiment 01 and experiment 02. By culturing with the fixed_OD with some vials we included controls where no antibiotic-pressure was applied to the strains. Table 3.13 shows which ESBL *E. coli* strain were cultured in which vial, with which mode.

3.2.1 Contamination analysis

For many samples we morphologically determined contaminations. Because we had Illumina sequencing data of every sample, we could determine the contamination as *Bacillus cereus*. To ensure that the stocks of the strains were not contaminated, we mapped the Illumina data of the stocks to the hybrid-assembled *E. coli* reference genome and *Bacillus cereus* genome obtained from NCBI [51]. The percentage of how many reads mapped to *E. coli* and *Bacillus cereus* is shown in Table 3.14. Around 0.44 % of all Illumina reads mapped to *Bacillus cereus* ATCC 14579. In Figure 3.3 one region of an alignment of the Illumina reads to the *Bacillus cereus* genome is shown. It can be seen that the Illumina reads only mapped to very certain regions of the genome of *Bacillus cereus* without overlapping and with a variation of a few bases. Because the reads were not overlapping and they did not map with an identity of 100%, we concluded that the stocks were not contaminated. Also the hybrid-assembly of the reference genome did not show any contigs with *Bacillus cereus* sequences.

We mapped the Illumina reads from the morbidostat samples to the hybrid-assembled *E. coli* genome and the *Bacillus cereus* genome in order to identify contaminated samples. The outcome is shown in Table 3.15. Looking at the percentage of how many reads mapped to *Bacillus cereus* showed that two groups exist. Either 60 % or more mapped to *Bacillus cereus*, implying that the samples were contaminated, or only 0.48% mapped implying that those samples were not contaminated. From all morbidostat samples 12 samples had fewer reads than 0.48% and were not

contaminated.

Stocked strain	Total reads	Reads mapped to <i>Bacillus cereus</i>	Reads mapped to <i>E. coli</i>	% of reads mapped to <i>Bacillus cereus ATCC 14579</i>
pEU22_CTX	3810442	16496	3803789	0.43
pEU23_OXA	4329090	18499	4322708	0.43
pEU26_OXA	4040571	17629	4036167	0.44
K-12 MG1655	4939319	21902	4931214	0.44

Table 3.14: Illumina reads from every stock were mapped to a *Bacillus cereus* genome from NCBI and the *E.coli* reference genome produced with hybrid-assembling [51].

Experiment	Vial	Sample	Total reads	Reads mapped to <i>Bacillus cereus</i>	Reads mapped to <i>E. coli</i>	% of reads mapped to <i>Bacillus cereus</i>
01	1	1	6490750	4967611	272289	76.53
01	2	1	7601839	6001106	418578	78.94
01	3	1	2940556	13976	2934902	0.48
01	4	1	8167777	6150829	281584	75.31
01	5	1	5606842	4089104	178462	72.93
01	9	1	5636939	4466280	268813	79.23
01	10	1	4935808	22368	4928816	0.45
02	1	1	6152378	26815	6143228	0.44
02	2	1	6926506	5180940	291927	74.80
02	3	1	5142535	24909	5136761	0.48
02	3	2	6196309	4694898	279679	75.77
02	4	1	4923087	22342	4915639	0.45
02	4	2	4589212	20628	4582915	0.45
02	5	1	4298753	17870	4292083	0.42
02	5	2	5189054	22772	5181923	0.44
02	7	1	5167596	22583	5159028	0.44
02	7	2	4976635	3015316	107767	60.59
02	8	1	4356280	19067	4350598	0.44
02	8	2	4186125	18526	4181356	0.44
02	9	1	4182681	18487	4176477	0.44
02	10	1	3809536	2576863	136340	67.64

Table 3.15: Illumina reads from every morbidostat sample mapped to a *Bacillus cereus* from NCBI and the *E.coli* reference genome produced with hybrid-assembling [51].

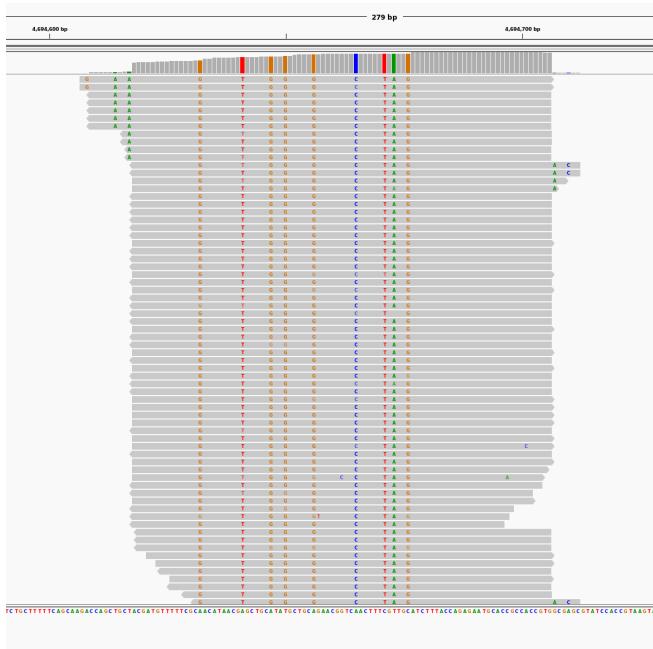


Figure 3.3: Alignment of short reads from pEU23_OXA to the *Bacillus cereus*. The reads mapped with a few SNPs which are colored. The reads also did not overlap.

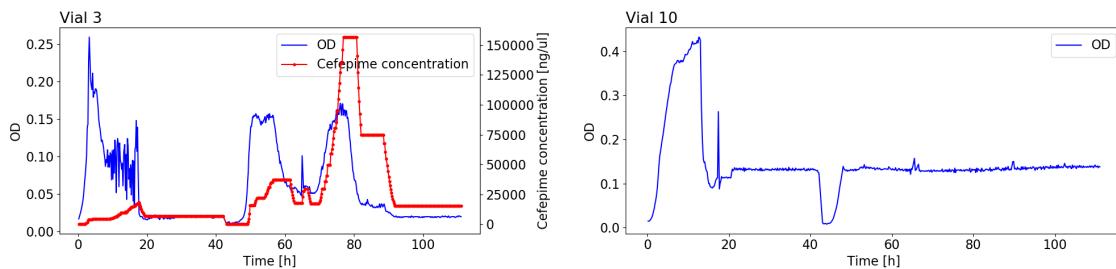


Figure 3.4: Growth curves and cefepime concentration in the vials which were not contaminated from experiment 01.

3.2.2 Growth curves and injected concentrations

We recorded the growth curves and stored the cefepime concentrations of every vial during the entire experiment time of both experiments. This allows us to evaluate how well the implemented feedback algorithm reacted to the change of growth. Figure 3.4 and Figure 3.5 show the recorded ODs and the cefepime concentration in the vials from the morbidostat experiment 01 (Figure 3.4) and 02 (Figure 3.5). From both experiments only the vials with no contamination are shown. After 45 hours 200 μ L of the cell suspensions were transferred into a new vial with 18 mL diluted media. This is causing the abrupt drop of ODs. In vial 3 from the 01 experiment the OD measurements were very noisy at the beginning which was caused by an air cone coming from high stirring with the magnetic stirrer. After 20 hours the stirring was reduced which eliminated the noise. For vial 10 the mode was set to record growth rate instead of fixed OD. The mode was changed to fixed OD after 15 hours. In general the feedback worked really well. The left Figure 3.6 shows the first 45 hours for vial 3 from experiment 02. Drug injection took place after

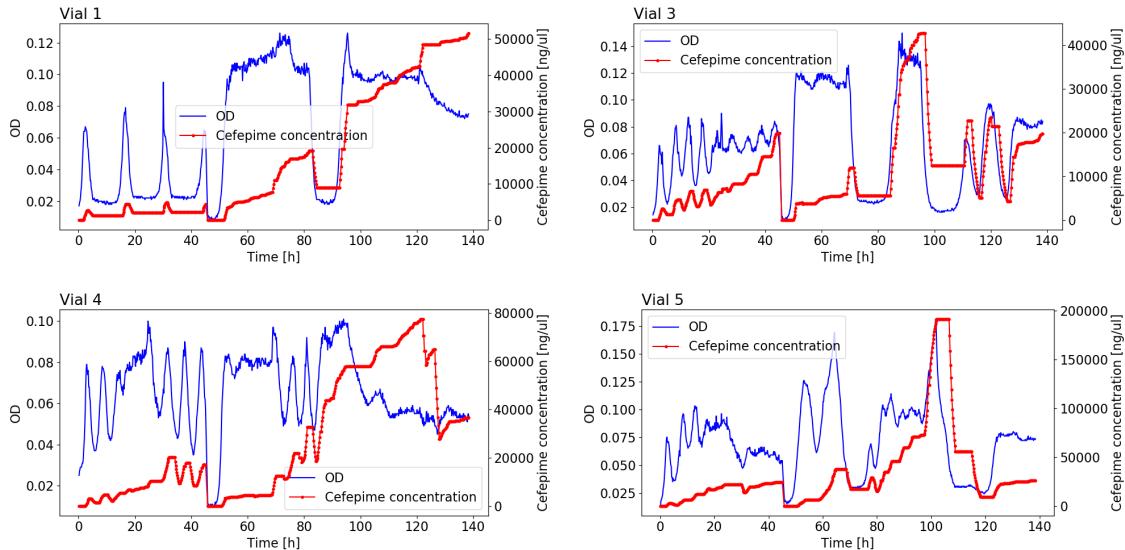


Figure 3.5: Growth curves and cefepime concentration in the vials which were not contaminated from experiment 02.

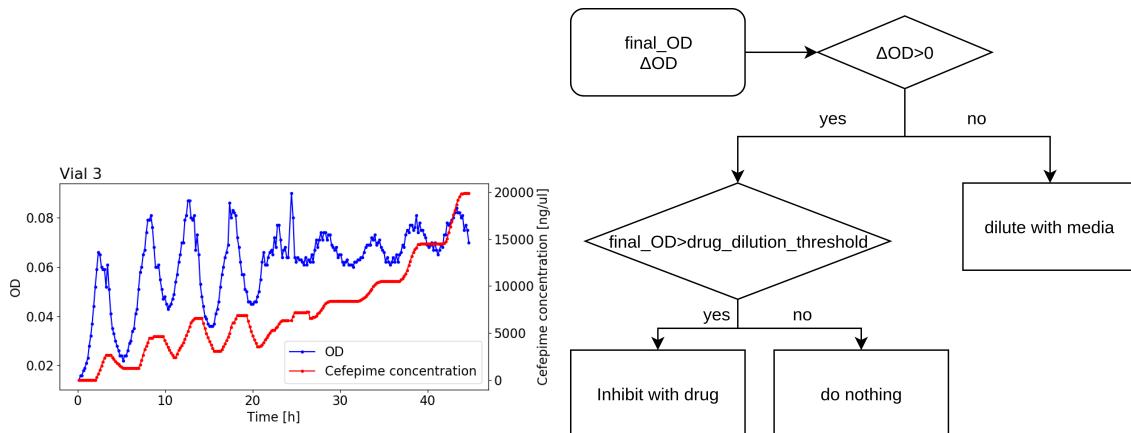


Figure 3.6: Left: First 45 hours from vial 3 of experiment 02. Right: Feedback algorithm deciding over drug injection.

the drug_dilution_threshold was reached. After that the cefepime concentration was continuously increased until the cells started to die. The cefepime concentration which was necessary to kill the majority of the cells was stored. Then the feedback diluted the cultures with media until half of the deadly concentration was reached. After that no injections took place until the culture started to grow again. As a result of the feedback the OD was steady but the cefepime concentration was continuously increased. The deadly cefepime concentration after 40 hours of culturing was already four fold higher than at the beginning.

3.2.3 MIC of the strains used for culturing with the morbidostat

To confirm emerged resistance we determined the MIC of cefepime of the morbidostat samples and the stocks of the strains. The MICs of the strains before culturing

Strain	MIC of cefepime [µg/mL]	Experiment	Vial	MIC of cefepime [µg/mL]
pEU22	8	01	3	512
pEU23	4	01	10	4
pEU26	4	02	1	128
		02	3	512
		02	4	512
		02	5	256

Table 3.16: Left Table: MICs of the strains used for the experiment 01 and 02. Right Table: MICs of the samples from the last sample day of the experiment 01 and 02.

#SNP	Contig	Position	Nucleotide in Sample:	
			0	1
1	0	3403729	G	C

Table 3.17: SNPs in the samples of vial 10 experiment 01.

with the morbidostat are shown in Table 3.16. In Table 3.16 the MIC are shown for the samples of the last sample day of the experiment 01 and 02. It is very clear that culturing with the morbidostat increased the resistance. In three cases the increase was over 100 fold. The sample of vial 10 from experiment 01 shows the same MIC as before culturing with the morbidostat. This is as expected, since this vial was cultured with the fixed_OD mode and no antibiotic pressure was applied.

3.2.4 SNPs of experiment 01

After analyzing the contamination and proving the increase of resistance after culturing with the morbidostat, we present the SNPs which we identified in the samples of the morbidostat. Sample 0 is always the stocked strain before culturing with which the morbidostat was started. The genome of sample 0 is always used as a reference. With this reference we identified SNPs in the samples with evolved resistance applying the same criteria as for the isolate series sampled from patients. We only show vials and samples with no contamination.

Vial 3

Even though the MCI of the sample from vial 3 increased over 100 fold, compared to the MIC determined before culturing with the morbidostat, no SNPs were found.

Vial 10

Vial 10 was cultured with the fixed OD mode. The MIC remained the same before and after culturing with the morbidostat. We were still able to identify two SNPs which are shown in Table 3.17 with their annotation in Table 3.18.

#SNP	Gene	Product	Type and position	Amino acid Sample:	
				0	1
1	<i>tomB_1</i>	Hha toxicity modulator TomB	Missense, 43	I	V

Table 3.18: Genes affected by the SNPs found in the samples of vial 10 experiment 02.

#SNP	Contig	Position	Nucleotide in Sample:		
				0	1
1	1	4290	G		T

Table 3.19: Positions of SNPs in the samples of vial 3 experiment 02.

3.2.5 SNPs of experiment 02

Vial 1

No SNPs were found in the sample of vial 1, even though the vial was cultured in the continuous mode and the MIC increased over 100 fold.

Vial 3

One SNP was found in the samples of vial 3 which is shown in Table 3.19. Interestingly this mutation affects the kanamycin resistance gene of the vector. The change of amino acid caused by the SNP is shown in Table 3.20.

Vial 4

Two SNPs were found for the samples of vial 4 shown in Table 3.21 and the affected genes in Table 3.22.

Vial 5

Three SNPs were found for the samples of vial 5. SNPs are shown Table 3.23, annotations in Table 3.24.

#SNP	Gene	Product	Type and position	Amino acid Sample:	
				0	1
1	<i>neoR/kanR</i>	Kanamycin resistance	Missense, 204	G	V

Table 3.20: Genes affected by the SNPs found in the samples of vial 3 experiment 02.

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#SNP	Contig	Position	Nucleotide in Sample:		
			0	1	2
1	1	2526	T	T	A
2	0	347328	C	C	T

Table 3.21: Positions of SNPs in the samples of vial 4 experiment 02.

#SNP	Gene	Product	Type and position in gene	Amino acid in Sample:		
				0	1	2
1	<i>bla</i>	Beta-lactamase CTX-M-1	Missense, 289	V	V	D
2	<i>ompR</i>	Transcriptional regulatory protein OmpR	Nonsense, 67	R	R	*

Table 3.22: Genes affected by the SNPs found in the samples of vial 4 experiment 02. The star in sample 2 stands for a stop codon.

#SNP	Contig	Position	Nucleotide in Sample:		
			0	1	2
1	0	1570984	C	C	T
2	0	668881	A	T	A
3	0	2898640	G	G	-

Table 3.23: Positions of SNPs in the samples of vial 5 experiment 02.

#SNP	Gene	Product	Type and position in gene	Amino acid in Sample:		
				0	1	2
1	<i>ompC_1</i>	Outer membrane protein C	Silent, 8	L	L	L
2	<i>rpoD</i>	RNA polymerase sigma factor RpoD	Missense, 594	L	Q	L
3	<i>ompF</i>	Outer membrane protein F	Frameshift, 319	G	G	A

Table 3.24: Genes affected by the SNPs found in the samples of vial 5 experiment 02.

Chapter 4

Discussion

In this thesis the evolution of cefepime resistance in ESBL *E. coli* was studied. A bioinformatic pipeline was established, which we used to identify SNPs in the genome while resistance to cefepime evolved in ESBL *E. coli*. This pipeline was applied to next generation sequencing data of ESBL isolates sampled from patients at the University Hospital of Basel. Furthermore, we assembled a morbidostat which we used to experimentally evolve cefepime resistance in ESBL *E. coli*. Samples taken from the morbidostat were analyzed with the established bioinformatic pipeline as well. Technical and experimental challenges are discussed in the following as well as the potential relevance of the identified SNPs and their annotation for cefepime resistance.

4.1 Technical and experimental challenges with the morbidostat

4.1.1 Hardware issues

We faced problems with the computer controllable pumps that we used for injecting antibiotics and media. The controllers and the piezo pumps were very sensitive to small currents flowing through the digital pins of the microcontroller. This caused malfunctions of the pumps. We were able to fix this issue by connecting pull-down resistors to the digital pins of the microcontroller and the ground. Furthermore, we connected inverters in serial to the digital pins, which increased the reliability of the pumps.

We observed in the morbidostat experiment 01 that stirring created air cones which affected the OD measurements. This is why we had to reduce the stirring to a minimum. Generally, strong stirring could be beneficial because it would guarantee equal antibiotic concentrations in the vials. Stirring could be increased if the OD measuring units would be lowered.

We used vials with a polytetrafluoroethylene (Teflon) insert with holes through which we inserted plastic tubes acting as vial inserts. Even though the holes for the plastic tubes were drilled as small as possible, the plastic tubes started to loosen up over time, which we tried to fix by adding silicone. Because of the physical properties of polytetrafluoroethylene silicone did not stick and the plastic tubes could not be sealed properly. Therefore, some vials may have not been completely sealed, potentially increasing the risk of contamination. Furthermore, the chosen

vial design was not ideal for sampling, because we had to unscrew the lid of the vials in the hypoxi-station, which increased the risk of introducing contaminations.

4.1.2 Software issues

Our microcontroller could only execute one task after the other. This means that we had to ensure that the python script was sending the commands one after another, instead of sending multiple commands at once. Therefore, we had to thread the commands. Even though we threaded the commands, we encountered a problem with the microcontroller, caused by receiving multiple commands at once. The OD measurements potentially interfered with changing the state of the digital pins and the microcontroller crashed. The pumps did not turn off and the vials were overflowing. Therefore, we added a reset function which recognized when the microcontroller crashes and automatically hard resets the microcontroller.

4.1.3 *Bacillus cereus* contamination of the morbidostat experiments

The majority of our vials were contaminated with *Bacillus cereus*. Since all the stocked strains which we used for starting morbidostat experiments were not contaminated, the contamination must have entered the system during the experiment. Alternatively, sterilization was not successful. For sterilizing we used 1 L of 3% citric acid and 1 L of 3% bleach. Both solutions were pumped through all the tubing over one hour. Additionally, every piece of hardware in contact with fluids, except the pumps, were autoclaved. *Bacillus cereus* is a gram-positive, endospore-forming bacteria [53]. It is possible that *Bacillus cereus* endospores overcame the sterilization protocol. A study reported that after exposing *Bacillus cereus* endospores to 10% bleach for 15 minutes, they were still able to produce colonies [54]. Alternatively, the *Bacillus cereus* contamination entered the system while taking samples or exchanging the media and drug bottles. As already discussed, the design of the vials was not ideal and contaminations could have entered the device through gaps in the vial inserts. It is also possible that some tube connections were not completely sealed allowing contaminations to enter the device.

Cefepime is supposed to be especially active against gram-positive bacteria [55]. Therefore, it is generally surprising, that *Bacillus cereus* survived culturing with high doses of cefepime. *Bacillus cereus* might have taken up the ESBL plasmid from the ESBL *E. coli* strains by natural competence, which decreased their susceptibility. Possibly, *Bacillus cereus* was killed by the high cefepime concentration, but the antibiotic pressure induced the formation of endospores. For sequencing we inoculated LB containing kanamycin with the sample stocks and cultured them overnight. During overnight culturing the endospores might have switched to the vegetative cycle, causing the contamination in the stocks to be passed for sequencing.

4.2 Bioinformatic challenges

Generally, we identified more mutations in patient isolates than in morbidostat samples. For example the isolates of patient 16 revealed 21 SNPs. One source for this

observation could be that some patients got reinfected with different ESBL *E. coli* strains, even though we tried to ensure strain identity by phylogenetic analysis. Potentially, the phylogenetic tree was not fully resolved and some strains, assumed to be identical, but were actually different. The environment during morbidostat experiments can be fully controlled, because we cultured in the hypoxi-station. The nutrient concentration remained the same, because we cultured with diluted LB media throughout the whole experiment. Therefore, the antibiotic pressure was the main stress causing evolution. On the other hand, in patient isolates the strains had to adapt to many more environmental factors which could further explain why more SNPs were identified in patient isolates. Likely, many patients were treated with other drugs than antibiotics as well, which could also cause evolution of the ESBL *E. coli* strains.

For the isolate series of patient 25 and patient 33 the resistance decreased over the sampling period, which is the opposite compared to the other patient isolate series and the sample series from the morbidostat. For those two resistance did not evolve over the sampling period. Therefore, we defined the most recent sample with the lowest resistance level as reference and identified SNPs in the earliest samples with high resistance levels. We still consider the identified SNPs as relevant, because it is still likely that they can be associated with the corresponding high resistance level. We would have preferred to analyze more isolate series where resistance evolved over the sampling period, but we were restricted by the isolate collection at hand.

The bioinformatic pipeline for identifying SNPs proved to be solid. *De novo* whole-genome assembling with ONT and Illumina sequencing data using the assembler Unicycler resulted in accurate whole-genomes, positively affected by the high coverage that we achieved with ONT sequencing [42]. The rest of the pipeline consisted of established bioinformatic steps such as mapping Illumina reads to reference genomes and identifying SNPs with coverage and base frequency filtering. The weakness of the bioinformatic pipeline was the annotation of the SNPs. For some SNPs no annotation was found by prokka, the tool we used for annotating our reference genome, but blasting of the affected sequences revealed annotations for some additional SNPs [41]. However, we only presented annotations found by prokka [41].

4.3 Culturing with the morbidostat

The assembled morbidostat and the developed feedback algorithm worked very well. The designed OD measuring units proved to be rather sensitive, with the ability to detect ODs starting from 0.01. After optimizing the control of the injecting pumps, they showed to be very reliable and their compact design allowed easy handling of the morbidostat within the space-limited hypoxi-station. The feedback reacted very well to the cultures by diluting with media when the bacteria were dying or by increasing antibiotic concentration if they grew very fast. The developed feedback algorithm might have allowed resistance to evolve faster than expected. Dösselmann *et al.* reported that they cultured *Pseudomonas aeruginosa* with a morbidostat and colistin for 20 days to achieve a 100-fold increase in MIC [33]. We cultured ESBL *E. coli* for only 5 days with our morbidostat and cefepime to achieve a similar increase of the MIC. Since the organism and the antibiotic are not identical, it is difficult to compare how fast resistance evolved. The performed morbidostat experiments proved that our version of the morbidostat is very capable to evolve resistance over

a short period of time.

4.4 Potential resistance mechanisms considering mutated operons

The identified SNPs in ESBL *E. coli* patient isolates and morbidostat samples mainly suggested two targets, which could play an important role in cefepime resistance. One of the targets were porins, in particular the outer membrane protein F (OmpF), the outer membrane protein C (OmpC) and their shared regulatory system. The other target was the RNA polymerase and the sigma factor.

4.4.1 SNPs in porin operons

There are two major outer membrane proteins *E. coli*, OmpF and OmpC [56]. Both are porins in the outer membrane through which cephalosporins pass [57]. A lack of those porins likely heavily reduces the cefepime uptake. Becerio A *et. al* reported that two cefepime resistant ESBL *E. coli* isolates lacked the OmpC and OmpF porins showing the importance of those porins for cefepime resistance [58]. Depending on the osmolarity of the environment *ompF* or *ompC* is preferably expressed. This is regulated by an osmolarity sensor protein EnvZ. EnvZ is a transmembrane sensor which detects the osmotic pressure and phosphorylates the transcriptional regulatory protein OmpR depending on the detected osmolarity [56]. At low osmolarity, EnvZ modulates low levels of phosphorylated OmpR, at high osmolarity EnvZ mediates high levels of phosphorylated OmpR [56]. OmpR is a DNA-binding protein which binds specific regions depending on its phosphorylation status and it is essential for the expression of *ompC* and *ompF* [56]. Phosphorylated OmpR binds to the promoter of *ompC*, if OmpR is unphosphorylated it binds to the promoter of *ompF* [56]. Therefore, phosphorylated OmpR increases the expression of the *ompC* gene while repressing the expression of the *ompF* gene [56]. On the other hand, OmpR which is not phosphorylated increases the expression of *ompF* and reduces the expression of *ompC*.

We found several SNPs in genes of the expressions system of *ompF* and *ompC* in patient isolates and morbidostat samples. One SNP in *envZ* in a patient isolate, in *ompR* one SNP was found in a patient isolate as well as in a morbidostat sample. In the patient isolate the SNP caused a missense mutation in OmpR, whereas in the morbidostat samples the SNP caused a nonsense mutation in OmpR. A mutated OmpR is potentially not able to bind to the the promoters of *ompC* and *ompF*, which could heavily reduce the expression of both outer membrane proteins. This seems especially plausible for the mutated OmpR in the morbidostat sample, because the nonsense mutation heavily impacts the functionality of the protein. The mutated *envZ* found in a patient isolate potentially also affects the expression levels of *ompC* and *ompF*. In *ompC* and *ompF* itself we also found SNPs in morbidostat samples. While *ompC* was affected by a SNP causing a silent mutation in OmpC, the mutation in *ompF* caused a missense mutation in OmpF. The missense mutation in OmpF could cause a change of the structure of the porin, which potentially affects the permeability of the outer membrane for cefepime.

We found another SNP in the promoter of *fepA*. FepA is a gated porin transporting

unspecific hydrophilic substances [59]. The mutation in the promoter potentially repressed the expression of *fepA* which may further decrease the membrane permeability for cefepime.

4.4.2 SNPs in the transcription machinery operon

SNPs were identified targeting the transcription machinery in patient isolates and morbidostat samples. In one patient isolate we found that a mutation in *rpoB* coding for the DNA-directed RNA polymerase subunit β . In a morbidostat sample we found a SNP targeting *rpoD* coding for the RNA polymerase sigma factor. This strongly suggests that some process in the RNA transcription is affecting the cefepime susceptibility. In a preprint Samantha Palace *et al.* reported that they found a SNP in *rpoB* in three ceftriaxone (a third-generation cephalosporin) resistant *Neisseria gonorrhoeae* isolates [60]. They were able to recreate resistance by integrating the mutation in a ceftriaxone susceptible *Neisseria gonorrhoeae* strain, proving the importance of the mutation for ceftriaxone resistance [60]. The mutation in *rpoB* that they identified is not the same as the one that we found. They also reported a mutation in *rpoD* in two *Neisseria gonorrhoeae* isolates and were able to recreate resistance for this mutation [60]. Interestingly, it is not understood yet, how mutations in RpoB or RpoD are impacting cephalosporin resistance.

4.4.3 SNPs affecting other operons

We also identified SNPs in genes for which the evidence was less clear that they potentially impact cefepime susceptibility. For example, one SNP was found in *ortT*. OrtT is a toxin causing membrane damage resulting in reduced growth and increased persistence during stress related to amino acid and DNA synthesis [61]. Cells in the persistent state are generally thought to be more tolerant to antibiotics [61]. While it is difficult to hypothesize why a mutated OrtT was beneficial for the strain, it makes sense that the persistent state is beneficial if antibiotic pressure is applied.

One SNP affected the ESBL gene coding for β -lactamase CTX-M-1 in a morbidostat sample. This caused a missense mutation in the ESBL. The resulting variant is not known in the literature, therefore, it is not possible to estimate whether it is potentially degrading β -lactams more effectively compared to the wild-type.

4.5 Conclusion

We studied cefepime resistance evolution in ESBL *E. coli* patient isolates sampled at the University Hospital of Basel and assembled a morbidostat, which we used to experimentally evolve cefepime resistance in ESBL *E. coli* strains. The assembled morbidostat showed great functionality, increasing the MIC over 100-fold compared to the MIC at the beginning. The bioinformatic analysis of the patient isolates and samples from morbidostat experiments revealed several SNPs in the genomes of ESBL *E. coli* which evolved resistance. Interestingly, some SNPs were found in the same gene in patient isolates and morbidostat samples, strongly suggesting their relevance for cefepime resistance. Two systems were targeted independently by SNPs. One system were the porins, in particular the porins OmpD, OmpF and

their regulatory proteins OmpR and EnvZ. The SNPs found in the porin operons could imply, that the expression level of the porins is reduced, potentially increasing the impermeability of the outer membrane for cefepime. For the other system which was targeted by SNPs, the DNA-directed RNA polymerase subunit β and its RNA polymerase sigma factor, the potential connection to cefepime resistance is unknown. Since the system was targeted by SNPs in patient isolates and morbidostat samples, it is still likely that the identified genotypes are affecting cefepime susceptibility. With our genomic study of cefepime resistance evolution we could identify new genotypes which may impact cefepime susceptibility. Our findings support the relevance of other resistance mechanisms than cefepime degradation by β -lactamases which was the main focus of cefepime resistance investigations so far.

4.6 Outlook

Our goal for future morbidostat experiments is to eliminate contaminations and repeat the resistance evolution experiments with the ESBL *E. coli* strains that we used for this thesis. In order to eliminate the contaminations we will take several additional precautions. We will change the design of the vials and replace the polytetrafluoroethylene inserts with the plastic tubes by septa and syringes. This will guarantee sealed vials and will also improve sampling, because the septa allow sampling with a syringe without opening the vial, reducing the risk of contaminations. We also realized that the injecting pumps were autoclavable, which was the only piece of hardware in contact with fluids, which we had not autoclaved before the morbidostat experiments. In the future we will autoclave the pumps which eliminates further potential sources of contaminations. When we will repeat the morbidostat experiments without contaminations we expect to identify SNPs in the same genes confirming our current findings.

We will also try to extend our patient isolate collection sampled at the University of Basel and we will analyze the additional isolates with the existing bioinformatic pipeline.

To finally confirm the importance of the identified SNPs, we could try to insert some identified mutations in cefepime susceptible *E. coli* strains and check if the created genotypes show changed cefepime susceptibility. If so, this would suggest that the identified SNP is associated with cefepime resistance evolution. Once the morbidostat experiments have been repeated, we could also use the morbidostat for studying resistance evolution of other bacteria and antibiotics.

Chapter 5

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Chapter 6

Supplementary

6.1 Manual for the software control of the morbidostat

This section describes how to switch on specific pumps which is important for disinfecting the morbidostat and how to start morbidostat experiments. First we need to understand the layout of the pumps and how they are assigned. As shown in Figure 6.1 nine pump boxes with five pumps are responsible for injecting antibiotics and media in 15 vials. Every pump box is connected with a numbered connector to the microcontroller. The first layer of pump boxes (connector one, two, three) is named pump1 and connected to media. The second layer (connector four, five, six) is named pump2 and connected to a low-concentrated antibiotic. The top layer (connector seven, eight, nine) is named pump3 and connected to a high-concentrated antibiotic. Within a pump box, every pump has a number as well. The designed layout implies that within one layer of pumps, the individual pumps were numbered from zero to 14. Because the pumps were connected column by column, the same pump number of every box is responsible for injecting fluids in the according vial. Because the pump layout was implemented with zero indexing, the pump number is shifted by one compared to the vial number. *E.g.* P_0 from pump1, P_0 from pump2 and P_0 from pump3 are responsible for injecting media and antibiotics into vial 1.

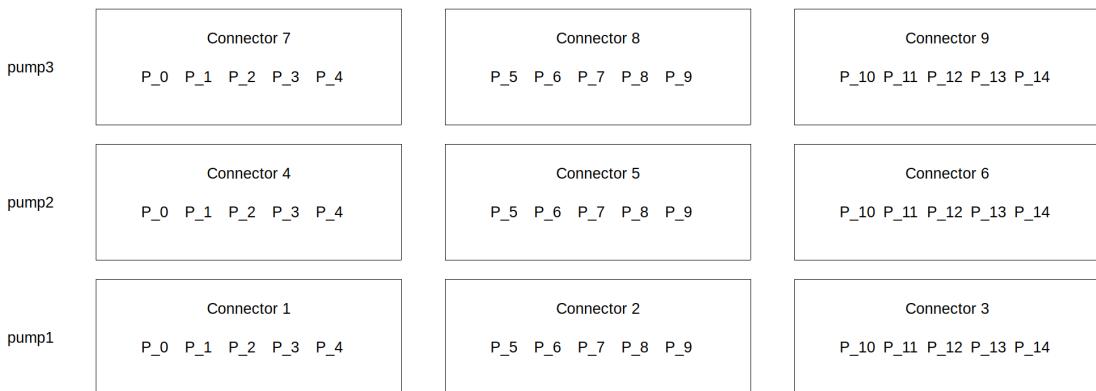


Figure 6.1: Pump layout of the pump stacks. P_N stands for the pump number.

6.1.1 Manually turning on pumps of the morbidostat

Manual control of the morbidostat is possible by running morbidostat_experiment.py in the interactive IPython mode. Therefore, open the terminal and navigate to the location of the morbidostat_experiment.py file.

```
cd /home/python_morbidostat/python_src
```

Run morbidostat_experiment.py in the interactive python mode and initialize the morbidostat. For initializing the morbidostat we need to pass the vial numbers that we want to use to the morbidostat as a list. Indexing starts at zero again. In this example we initialized the morbidostat with the first ten vials.

```
ipython morbidostat_experiment.py --i
morb = morbidostat(vials=[0,1,2,3,4,5,6,7,8,9,10])
```

Within IPython we can call various functions. The following code shows examples of functions for turning on pumps. Text followed by # are comments and not relevant for the functions.

```
morb.run_all_pumps('pump1',30) # Run all pumps of the first
layer pump1 for 30 seconds
morb.morb.run_pump('pump1',0,30) # Run P_0 from pump1 for 30 seconds
morb.morb.inject_volume('pump1',0,10) # Inject 10 mL in P_0 from pump1
morb.morb.run_waste_pump(30) # Run waste pump for 30 seconds
morb.morb.remove_waste(10) # Remove 10 mL of waste from all the vials
```

6.1.2 Starting morbidostat experiments

For starting morbidostat experiments we created a template in a csv file. In this csv file we can specify which vials we want to use for our experiments in which mode. Additionally, we need to specify a few values, mainly important for the continuous mode. We can define which concentrations of the antibiotics we use, which target_OD we would like to approximate, the dilution factor and the MIC of the strains used for the experiment. To edit the template, open the csv file /home/morbidostat/python_morbidostat/setup.csv

strain	K12_OXA				
#times	unit [s,m,h,d]				
cycle	600s				
OD	30s				
experiment	14d				
#parameters					
target OD	0.12				
dilution	0.92				
#drugs	unit	<u>mic</u>			
cefepime	ng/ml	3000			
#bottles					
A	0				
B	9000				
C	30000				
#vials	feedback type (M, OD, G, C)	bottle1	bottle2	bottle3	feedback_drug
1C	A			C	cefepime
2C	A			C	cefepime
3C	A			C	cefepime
4C	A			C	cefepime
5C	A			C	cefepime

Figure 6.2: Template used to start morbidostat experiments. In the column named vials you can enter which vials you want to use for the experiment. Indexing starts at 1. Bottle1 is mapped to pump1, bottle2 to pump2, and bottle3 to pump3.

After you edited the template you must save it. In the same directory a bash script called experiment_from_table.sh is located. We can use this script to start experiments based on our template. Execute the script by typing:

```
./experiment_from_table.sh setup.csv
```

Now the morbidostat experiment is running. You can always change parameters defined in the template while the morbidostat is running. Here are some useful commands while the morbidostat is running.

```
morb.dilution_factor = x # Change the dilution factor
morb.target_OD = x # Change the target_OD
morb.cycle_dt = # # Change the cycle time
morb.interrupt_experiment() # Interrupt the experiment. Necessary if you
want to change the bottles with media or antibiotics
morb.resume_experiment() # Resume the experiment
morb.stop_experiment() # Stops the experiment
morb.drug_concentrations[2][0] = x # Change the drug concentration of bottle2
morb.drug_concentrations[1][0] = x # Change the drug concentration of bottle3
morb.mics[0] = x # Change the mic of the strain
morb.reset_concentrations() # Sets drug concentration in the vials to
0, important if you transfer into a new vial
```

6.2 Gibson cloning

6.2.1 Sequences of fragments

ID: pEU22

```

1 GCCCACAGAA TGATGTCACG CTGAAAATGC CGGCCTTTGA ATGGGTTCAT
51 GTGCAGCTCC ATCAGCAAAA GGGGATGATA AGTTTATCAC CACCGACTAT
101 TTGCAACAGT GCCCTGAAAA CTATATCAA GAAGCCAAT ACGACATGGC
151 GGTGGGTCACT CTCTTGCTAA AGTCATTG GGCATGAA GCCGTGTTTC
201 AAATGATGAT GCTTCATAT AACCTATTT TGTTGTTCAA GTTGATTCC
251 TTGGACTCTT CAGAATACAG ACAGCAAATA AAGACCTTTC GTTTGAAGTA
301 TGTATTTCTT GCAGCAAAAA TAATCAAAAC CGCAAGATAT GTAATCATGA
351 AGTTGTGGAA AAACATATCCG TACAAGGGAG TGTATGAAAA ATGTCTGGTA
401 TAATAAGAAT ATCATCAATA AAATTGAGTG TTGCTCTGTG GATAACTTGC
451 AGAGTTTATT AAGTATCATT GCAGCAAAGA TGAAATCAAT GATTATCAA
501 AAATGATTGA AAGGTGGTTG TAAATAATGT TACAATGTGT GAGAACAGT
551 CTAAATTCTT CGTGAATAG TGATTTTG AGCTAATAAA AAACACACGT
601 GGAATTTAGG GACTATTCAT GTTGTGTTA TTTCGTATCT TCCAGAATAA
651 GGAATCCCCAT GGTAAAAAA TCACTGGCC AGTTCACGCT GATGGCGACG
701 GCAACCGTCA CGCTGTGTT AGGAACTGTG CCGTGTATG CCCAAACGGC
751 GGACGTACAG CAAAAACTTG CCGAATTAGA GCGGCAGTCG GGAGGGAGAC
801 TGGGTGTGGC ATTGATTAAAC ACAGCAGATA ATTCCGAAAAAT ACTTTATCGT
851 GCTGATGAGC GCTTTGCGAT GTGCAGCACC AGTAAAGTGA TGGCCGGGGC
901 CCCGGTGCTG AAGAAAAGTG AAAGCCAACC GAATCTGTTA AATCAGCGG
951 TTGAGATCAA AAAATCTGAC CTTGTTAACT ATAATCCGAT TGGCCAAAAC
1001 CACGTCAATG GGACCGATGTC ACTGGCTGAG CTTACCGCGG CCGGCGCTAC
1051 GTACAGCGAT AACGTGGCGA TGAATAAAGC GATTGGCTCAC GTTGGCGGGC
1101 CGGCTAGCGT CACCCGGTTC GCCCGACACC TGGGAGACG AACGTTCCCGT
1151 CTCGACCGTA CCGAGCCGA GTTAAACAC GCCATTCCGG GCGATCCGGC
1201 TGATACCACT TCACCTCGGG CAATGGCGCA AACTCTCGGG AATCTGACGC
1251 TGGGTAAAGC ATTGGCCGA AGCCAAACGG CCCAGCTGGT GACATGGATG
1301 AAAGCCAATA CCACCCGGTC ACCGACCATT CAGGCTGGA TCCCTGCTT
1351 CTGGGTTGTG GGGGAAAAA CCGGCAGCGG TGGCTATGGC ACCACCAACG
1401 ATATCGGGT GATCTGGCCA AAAGATCGTG CGCCCCCTGAT TCTGTCAT
1451 TACTTCACCC AGCCTCAACO TAAGGCAGA AGCCGTCGGG ATGTATTAGC
1501 GTCGGCCGC AAATCGTCA CCGACCGTTT G

```

Sequence coding for β -lactamase CTX-M-1 marked in red. The sequence was extracted from patient 25 sample 1.

ID: pEU23:

```

1 GTGATCCCC GGGCAAATG CGCCTTGTA GCAGAGTTT TGAAATGTA
51 GGCCTTTGA TAAGACAAAA GGCTGCCTC TCGCTAACT TGCAACAGT
101 CCTTTAAGCG TGCCTAATAAA GCCCTACA AATTGGGAGT TAGACATCA
151 GAGCAACGC AAAACAAAGT TAGGCATCA AAAGTACAG ATCGTGACA
201 ACAGCAACGA TTCCGTCA CA TGCGCCTC TGACTGAGCA TGACTTGCG
251 ATGCTCTAG AGTGGCTAA TCGATCTC ATCGTCGAGT GGTGGGGCG
301 AGAAGAAGC CGCCCACA TTGCTGACGT ACAGGAACAG TACTTGCAA
351 GCGTTTAG GCAAAGAGTC GTCACTCCA ATATGCAT GCTGATGGA
401 GAGCCGATT GGTATGCCA GTCGTACGT GCTTTGGA GCGGGGACG

```

451 ACGGTGGAA GAAGAAACCG ATCCAGGAGT ACGCGGAATA GACCAGTTAC
 501 TGGCGAATGC ATCACAACGT GGCAAAGGCT TGGGAACCAA GCTGGTTCGA
 551 GCTCTGGTTG AGTTGCTGTT CAATGATCCC GAGGTCACCA AGATCCAAC
 601 GGACCCGTG CCGAGCAACT TGCGAGCGAT CCGATGCTAC GAGAAAGCGG
 651 GGTTTGAGAG GCAAGGTACC GTAACCACCC CATATGGTCC AGCCGTGTAC
 701 ATGGTTCAAA CACGCCAGGC ATTGAGCGA ACACGCAGTG ATGCCTAAC
 751 CTTCCATCGA GGGGGACGTC CAAGGGCTGG CGCCCTTGGC CGCCCTCAT
 801 GTCAAACGTT GGGCGAACCC GGAGCCTCAT TAATTGTTAG CCGTTAAAAT
 851 TAAGCCCTT ACCAAACCAA TACTTATTAT GAAAAACACA ATACATATCA
 901 ACTTCGCTAT TTTTTAATA ATTGCAAATA TTATCTACAG CAGCGCAGT
 951 GCATCAACAG ATATCTCTAC TGTTGCATCT CCATTATTG AAGGAACGTGA
 1001 AGGTTGTTTT TTACTTTACG ATGCATCCAC AAACGCTGAA ATTGCTCAAT
 1051 TCAATAAAGC AAAGTGTGCA ACGCAAATGG CACCAAGATT AACTTTCAAG
 1101 ATCGCATTAT CACTTATCGC ATTTGATGCC GAAATAATAG ATCAGAAAAC
 1151 CATATTCAAA TGGGATAAAA CCCCCAAAGG AATGGAGATC TGGAACAGCA
 1201 ATCATAACACC AAAGACGTGG ATGCAATTTC CTGTTGTTTG GGTTTCGCAA
 1251 GAAATAACCC AAAAATTGG ATTAAATAAA ATCAAGAATT ATCTCAAAGA
 1301 TTTTGATTAT GGAAATCAAG ACTTCTCTGG AGATAAAAGAA AGAAAACAACG
 1351 GATTAACAGA AGCATGGCTC GAAAGTAGCT TAAAAAATTG ACCAGAAGAA
 1401 CAAATTCAAT TCCTGGTAA AATTATTAAT CACAATCTCC CAGTTAAAAA
 1451 CTCAGCCATA GAAAACACCA TAGAGAACAT GTATCTACAA GATCTGGATA
 1501 ATAGTACAAA ACTGTATGCC AAAACTGGTG CAGGATTACAC AGCAAATAGA
 1551 ACCTTACAAA ACGGATGGTT TGAAGGGTTT ATTATAAGCA AATCAGGACA
 1601 TAAATATGTT TTTGTGTCCC CACTTACAGG AAACATTGGGG TCGAATTAA
 1651 CATCAAGGCAT AAAAGCCAAG AAAATGCCGA TCACCAATTCTT AAACACACTA
 1701 AATTTATA

Sequence coding for β -lactamase OXA-1 marked in red. Source: The sequence was extracted from patient 25 sample 1.

ID: pEU26

1 GTGATCCCCT GGGCGAAATG CGCCTGGTAA GCAGAGTTTT TGAAATGTAA
 51 GGCCTTGAA TAAGACAAAA GGCTGCCTCA TCGCTAACTT TGCAACAGTG
 101 CCTTTAAGCG TGCATAATAA GCCCTACACA AATTGGGAGT TAGACATCAT
 151 GAGCAACGCA AAAACAAAGT TAGGCATCAC AAAGTACAGC ATCGTGACCA
 201 ACAGCAACGA TTCCGTCACA CTGCGCCTCA TGACTGAGCA TGACCTTGCG
 251 ATGCTCTATG AGTGGCTAAA TCGATCTCAT ATCGTCGAGT GGTGGGGCGG
 301 AGAAGAAGCA CGCCCGACAC TTGCTGACGT ACAGGAACAG TACTTGCCAA
 351 GCGTTTAGC GCAAGAGTCC GTCACTCCAT ACATTGCAAT GCTGAATGGA
 401 GAGCCGATTG GGTATGCCA GTCGTACGTT GCTCTTGGAA CGGGGACGG
 451 ACGGTGGAA GAAGAAACCG ATCCAGGAGT ACGCGGAATA GACCAGTTAC
 501 TGGCGAATGC ATCACAACGT GGCAAAGGCT TGGGAACCAA GCTGGTTCGA
 551 GCTCTGGTTG AGTTGCTGTT CAATGATCCC GAGGTCACCA AGATCCAAC
 601 GGACCCGTG CCGAGCAACT TGCGAGCGAT CCGATGCTAC GAGAAAGCGG
 651 GGTTTGAGAG GCAAGGTACC GTAACCACCC CATATGGTCC AGCCGTGTAC
 701 ATGGTTCAAA CACGCCAGGC ATTGAGCGA ACACGCAGTG ATGCCTAAC
 751 CTTCCATCGA GGGGGACGTC CAAGGGCTGG CGCCCTTGGC CGCCCTCAT
 801 GTCAAACGTT GGGCGAACCC GGAGCCTCAT TAATTGTTAG CCGTTAAAAT
 851 TAAGCCCTT ACCAAACCAA TACTTATTAT GAAAAACACA ATACATATCA

901 ACTTCGCTAT TTTTTAATA ATTGCAAATA TTATCTACAG CAGGCCAGT
951 GCATCAACAG ATATCTCTAC TGTTGCATCT CCATTATTG AAGGAACTGA
1001 AGGTTGTTT TTACTTTACG ATGCATCCAC AAACGCTGAA ATTGCTCAAT
1051 TCAATAAAGC AAAGTGTGCA ACGCAAATGG CACAGATTG AACTTTCAAG
1101 ATCGCATTAT CACTT~~ATGGC~~ ~~ATTTGATGCG~~ ~~GAAATAATAG~~ ~~ATCAGAAAAC~~
1151 ~~CATATTCAAA~~ ~~TGGCATAAAA~~ ~~CCCCCAAAGG~~ ~~AATGCAGATC~~ ~~TGGAACAGCA~~
1201 ~~ATCATACACC~~ ~~AAAGACGTGG~~ ~~ATGCAATT~~ ~~CTGTTGTTG~~ ~~GGTTTCGCAA~~
1251 ~~GAAATAACCC~~ ~~AAAAAATTGG~~ ~~ATTAAT~~ ~~ATCAAGAATT~~ ~~ATCTCAAAGA~~
1301 ~~TTTTGATTAT~~ ~~GGAAATCAAG~~ ~~ACTTCTCTGG~~ ~~AGATAAGAA~~ ~~AGAAACAACG~~
1351 ~~GATTAACACA~~ ~~AGCATGGCTC~~ ~~GAAACTAGCT~~ ~~TAAAAATTTC~~ ~~ACCACAAGAA~~
1401 ~~CAAATTCAAT~~ ~~TCCCTCCGTAA~~ ~~AATTATTAAT~~ ~~CACAATCTCC~~ ~~CAGTTAAAAAA~~
1451 ~~CTCAGCCATA~~ ~~GAAAACACCA~~ ~~TAGAGAACAT~~ ~~GTATCTACAA~~ ~~GATCTGGATA~~
1501 ~~ATAGTACAAA~~ ~~ACTGTATGGG~~ ~~AAAACTGGTG~~ ~~CAGGATTCAC~~ ~~AGCAAATAGA~~
1551 ~~ACCTTACAAA~~ ~~ACGGATGGTT~~ ~~TGAACGGTTT~~ ~~ATTATAAGCA~~ ~~AATCAGGACA~~
1601 ~~TAAATATGTT~~ ~~TTTGTGTCCG~~ ~~CACTTACAGG~~ ~~AAACTGGGG~~ ~~TGGAATTAA~~
1651 ~~CATCAAGCAT~~ ~~AAAAGCCAAG~~ ~~AAAAATGCCA~~ ~~TCACCATTCT~~ ~~AAACACACTA~~
1701 ~~AATTTATA~~

Sequence coding for β -lactamase OXA-1 marked in red. Source: The sequence was extracted from patient 33 sample 1.

ID: pSC101_vector

1 CTAGAGGCAT CAAATAAAAC GAAAGGCTCA GTCGAAAGAC TGGCCCTTTC
51 GTTTTATCTG TTGTTGTCG GTGAACGCTC TCCTGAGTAG GACAAATCCG
101 CCGGGAGCGG ATTTGAACGT TCGGAAGTGC TAAAGATAAGG GAGGAAAAGA
151 GACTGGATTC GGACTTTAGC CATAAAAAAA CCCGCCGAAG CGGGTTTTTT
201 CGCTAACAGT TGCTCTGCCGA AGCAGAACCC CATCATCCGA GAGCTTGCAT
251 GCCTGCATAT TTGTTAGTCT TGATGCTTCA CTGATAGATA CAAGAGCCAT
301 AAGAACCTCA GATCCTTCCG TATTTAGCCA GTATGTTCTC TAGTGTGGTT
351 CGTTGTTTTT GCGTGAGCCA TGAGAACGAA CCATTGAGAT CATGCTTACT
401 TTGCATGTCA CTCAAAAATT TTGCTCAAA ACTGGTGAGC TGAATTTTG
451 CAGTTAAAGC ATCGTGTAGT GTTTTCTTA GTCCGTTACG TAGGTAGGAA
501 TCTGATGTAA TGGTTGTTGG TATTTGTCA CCATTCAATT TTATCTGGTT
551 GTTCTCAAGT TCGGTTACGA GATCCATTG TCTATCTAGT TCAACTTGGGA
601 AAATCAACGT ATCAGTCGGG CGGCCTCGCT TATCAACCAC CAATTCATA
651 TTGCTGTAAG TGTAAATC TTTACTTATT GGTTCAAAA CCCATTGGTT
701 AAGCCTTTA AACTCATGGT AGTTATTTC AAGCATTAAAC ATGAACCTAA
751 ATTCACTCAAG GCTAATCTCT ATATTCGCT TGTGAGTTT CTTTGTGTT
801 AGTTCTTTA ATAACCACCTC ATAAATCCTC ATAGAGTATT TGTTTCAAA
851 AGACTTAACA TGTTCCAGAT TATATTTC TGAATTTTTT AACTGGAAAAA
901 GATAAGGCAA TATCTCTTCA CTAAAAACTA ATTCTAATT TTGCTTGTGAG
951 AACTTGGCAT AGTTGTCCA CTGGAAAATC TCAAAGCCTT TAACCAAAGG
1001 ATTCCCTGATT TCCACAGTTC TCGTCATCAG CTCTCTGGTT GCTTTAGCTA
1051 ATACACCATA AGCATTTCCTA CTACTGATGT TCATCATCTG AGCGTATTGG
1101 TTATAAGTGA ACGATACCGT CCGTTCTTC CTTGTAGGGT TTTCAATCGT
1151 GGGGTTGAGT AGTGCACAC AGCATAAAAT TAGCTGGTT TCATGCTCCG
1201 TTAAGTCATA GCGACTAATC GCTAGTCAT TTGCTTTGAA AACAACTAAT
1251 TCAGACATAC ATCTCAATTG GTCTAGGTGA TTTAATCAC TATACCAATT
1301 GAGATGGGCT AGTCAATGAT AATTACTAGT CCTTTCCCT TGAGTTGTGG

1351 GTATCTGTAA ATTCTGCTAG ACCTTGCTG GAAAACTTGT AAATTCTGCT
1401 AGACCCCTCTG TAAATTCCGC TAGACCTTGT TGTTGTTTTT TTGTTTATAT
1451 TCAAGTGGTT ATAATTATA GAATAAAGAA AGAATAAAAA AAGATAAAAA
1501 GAATAGATCC CAGCCCTGTG TATAACTCAC TACTTTAGTC AGTTCCGCAG
1551 TATTACAAAA GGATGTCGCA AACGCTGTT GCTCCTCTAC AAAACAGACC
1601 TTAAAACCCT AAAGGCTTAA GTAGCACCCT CGCAAGCTCG GGCAAATCGC
1651 TGAATATTCC TTTTGTCTCC GACCATCAGG CACCTGAGTC GCTGTCTTT
1701 TCGTGACATT CAGTCGCTG CGCTCACGGC TCTGGCAGTG AATGGGGTA
1751 AATGGCACTA CAGGCCCTT TTATGGATTG ATGCAAGGAA ACTACCCATA
1801 ATACAAGAAA AGCCCGTCAC GGGCTTCTCA GGGCGTTTA TGGCGGGTCT
1851 GCTATGTGGT GCTATCTGAC TTTTGCTGT TCAGCAGTTC CTGCCCTCTG
1901 ATTTCCAGT CTGACCACTT CGGATTATCC CGTGACAGGT CATTCAAGCT
1951 GGCTAATGCA CCCAGTAAGG CAGCGGTATC ATCAACAGGC TTACCCGTCT
2001 TACTGTCCCT AGTGCTTGGG TTCTCACCAA TAAAAAACGC CCGGCGCAA
2051 CCGAGCGTTC TGAACAAATC CAGATGGAGT TCTGAGGTCA TTACTGGATC
2101 TATCAACAGG AGTCCAAGCG AGCTCTCGAA CCCCAGAGTC CCGCTCAGAA
2151 GAACTCGTCA AGAAGGCGAT AGAAGGCGAT GCGCTCGA TCAGGAGCGG
2201 CGATACCGTA AAGCACGAGG AAGCGGTCA CGCATTGCCCC GCCTAAGCTCT
2251 TCAGCAATAT CACGGTAGC CAACGCTATG TCCTGATAGC GGTCCGCCAC
2301 ACCCAGCCGG CCACAGTCGA TGAATCCAGA AAAGCGGCCA TTTTCCACCA
2351 TGATATTCCG CAAGCAGGCA TCGCCATGGG TCACGACGAG ATCCTGCCG
2401 TCGGGCATGC GCGCCTTGAG CCTGGCGAAC AGTCGGCTG GCGCGAGCCC
2451 CTGATGCTCT TCGTCCAGAT CATCCTGATC GACAAGACCG GCTTCCATCC
2501 GAGTACGTGC TCGCTCGATG CGATGTTTCG CTTGGTGGTC GAATGGCAG
2551 GTAGCCGGAT CAAGCGTATG CAGCCGCCGC ATTGCATCAG CCATGATGGA
2601 TACTTCTCG GCAGGAGCAA GGTGAGATGA CAGGAGATCC TGCCCCGGCA
2651 CTTGCCCAA TAGCAGCCAG TCCCTTCCCG CTTCACTGAC AACGTGAGC
2701 ACAGCTCGC AAGGAACGCC CGTCGTGGCC AGCCACGATA GCCGCGCTGC
2751 CTCGTCTGC AGTTCAATTCA GGGCACCGGA CAGGTCGGTC TTGACAAAAA
2801 GAAACGGGCG CCCCTGCGCT GACAGCCGGA ACACGGCGGC ATCAGAGCAG
2851 CCGATTGTCT GTTGTGCCCA GTCATAGCCG AATAGCCTCT CCACCCAAGC
2901 GGCGGAGAA CCTGCGTGCA ATCCATCTTG TTCAATCATG CGAAACGATC
2951 CTCATCCTGT CTCTTGATC GATCTTGATC CCCTGCGCCA TCAGATCCTT
3001 GGCGGCAAGA AAGCCATCCA GTTTACTTTG CAGGGCTTCC CAACCTTACC
3051 AGAGGGCGCC CCAGCTGGCA ATTCCGACGT CTAAGAAACC ATTATTATCA
3101 TGACATTAAC CTATAAAAAT AGGCGTATCA CGAGGCCCTT TCGTCTTCAC
3151 CCGTATAATC CTTACTCGT TAAAGGCTTT TCTTAGGAAA GTTGGCCATT
3201 TCTTAATTCA GCCATTAATT AAGAAATATT AAGAATATTCT CTGGCTATTT
3251 TCTCCTGTCA GAGTCTATTG TTTTAGCCTG AAAAGCTAAA AAACGTTAAC
3301 CCAATGATTA CACAAACAAT AAAACTGGTT CCTTTTAGG CGACCGACGA
3351 TCACTGTTAA AATTGAAAAA AGTATGGCAA CACGCGGCTT TCACGCAATT
3401 GTAATTTTA GTAATATGAC GATGAAAAGT TTTTTAGAGT AGATTATAGT
3451 TAAATCATAA GGTGACGTGG GAAGTACCAAG GTTAGTTAGT TGTATCCATC
3501 CGAAGGTGT TCGGTTAGTT TAAGCCACTA AAAAGGGGAT GCATTATGGG
3551 CGAATACTCA CCCAAAAGAC ATGATATAGC ACAGCTAAA TTTCTTGGC
3601 AAACCCCTGTA TCATGACTGT CTTGCAAACC TTGAAGAAG CAACCATGGC
3651 TGGGTTAATG ACCCGACCTC AGCAGTCAAC CTGCAAGCTCA ATGAGCTGAT
3701 AGAGATCCTC TAGATTTAAG AAGGAGATAT ACATATGGTG AGCAAGGGCG

3751 AGGAGGATAA CATGGCCATC ATCAAGGAGT TCATGCGCTT CAAGGTGCAC
3801 ATGGAGGGCT CCGTGAACGG CCACGAGTTC GAGATCGAGG GCGAGGGCGA
3851 GGGCCGCCCG TACGAGGGCA CCCAGACCGC CAAGCTGAAG GTGACCAAGG
3901 GTGGCCCCCT GCCCTTCGCC TGGGACATCC TGTCCCCTCA GTTCATGTAC
3951 GGCTCCAAGG CCTACGTGAA GCACCCGCC GACATCCCC ACTACTTGAA
4001 GCTGTCCTTC CCCGAGGGCT TCAAGTGGGA GCGCGTGATG AACTTCGAGG
4051 ACGGCGGCGT GGTGACCGTG ACCCAGGACT CCTCCCTGCA GGACGGCGAG
4101 TTCATCTACA AGGTGAAGCT GCGCGGCACC AACTTCCCCT CCGACGGCCC
4151 CGTAATGCAG AAGAAGACCA TGGGCTGGGA GGCCTCCTCC GAGCGGATGT
4201 ACCCCGAGGA CGGCGCCCTG AAGGGCGAGA TCAAGCAGAG GCTGAAGCTG
4251 AAGGACGGCG GCCACTACGA CGCTGAGGTC AAGACCACCT ACAAGGCCAA
4301 GAAGCCCGTG CAGCTGCCCG GCGCCTACAA CGTCAACATC AAGTTGGACA
4351 TCACCTCCA CAACGAGGAC TACACCATCG TGGAACAGTA CGAACGGGCC
4401 GAGGGCCGCC ACTCCACCGG CGGCATGGAC GAGCTGTACA AGTAATGCAG
4451 GCATGCAAGC T



Erklärung zur wissenschaftlichen Redlichkeit

(beinhaltet Erklärung zu Plagiat und Betrug)

Masterarbeit

Titel der Arbeit (*Druckschrift*):

Experimental evolution and genotypic characterization of ESBL Escherichia Coli

Name, Vorname (*Druckschrift*):

Eric Ulrich

Matrikelnummer:

13-109-814

Mit meiner Unterschrift erkläre ich, dass mir bei der Abfassung dieser Arbeit nur die darin angegebene Hilfe zuteil wurde und dass ich sie nur mit den in der Arbeit angegebenen Hilfsmitteln verfasst habe.

Ich habe sämtliche verwendeten Quellen erwähnt und gemäss anerkannten wissenschaftlichen Regeln zitiert.

Diese Erklärung wird ergänzt durch eine separat abgeschlossene Vereinbarung bezüglich der Veröffentlichung oder öffentlichen Zugänglichkeit dieser Arbeit.

ja nein

Ort, Datum:

Basel, 9.8.2019

Unterschrift:

Dieses Blatt ist in die Bachelor-, resp. Masterarbeit einzufügen.