

Morb
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1. Abstract

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2. Introduction

The discovery of penicillin shortly before the Second World War has revolutionized human medicine in the western world by saving the lives of millions of soldiers and civilians [11]. It also made major advances in surgery possible [51]. Ever since then antibiotics play a very important role in our well established health system and we are depending on those drugs in order to fight bacterial infections.

Because of the positive experiences people made with penicillin they quickly started to use this compound in an irresponsible way. This led to a penicillin resistance within 32 years after it's discovery [51]. Ever since then it was necessary to repetitively introduce new antibiotics to the market because bacteria evolved resistance against previously developed drugs. Therefor bacteria which are no longer susceptible to antibiotics are nothing new, the response of resistance to antibiotics has its roots as deep as the discovery of antibiotics itself.

2.1 Cause and urgency of antibiotic resistance

In the last few decades a gap opened between the increased use of antibiotics and the decreased development of new drugs [52].

Based on this imbalance antibiotic resistance emerged and nowadays causes a huge problem in the health sector. For example 1.6 € billion and 2.5 million additional hospital days were caused by resistant infections just for the European Union per year [40].

Or it has been shown that methicillin-resistant *Staphylococcus aureus* kills more Americans each year than HIV/AIDS, Parkinson's disease, emphysema, and homicide combined [52].

Between 2000 and 2015 defined daily doses of antibiotics increased by 65 % which demonstrates the tremendous increase of antibiotic consumption [27]. This huge increase over the last two decades is terrifying since it's common sense that this is pushing antibiotic resistance even further.

Surprisingly a big share of antibiotic consumption is unnecessary and irresponsible. Mainly two fields of heavy antibiotic consumption exists and waste of antibiotics take place in both of them. One field is the health sector where a questionable amount of prescriptions happen. Alone in the US approximately 50 % of antibiotics are prescribed unnecessarily [45]. This is mainly because of inaccurate identification of pathogens which could be heavily improved by introducing molecular diagnostic techniques such as PCR [52].

The other field is the globally operating food industry. It's estimated that in the early 2000s 25-50 % of all antibiotic consumption has it's source in the food industry [37]. What makes this extremely high use even less understandable is the fact, that

most of the antibiotics are used prophylactic and to stimulate growth and not in order to cure sick animals.

Now that we have seen the perspective of consumption of antibiotics, it's time to look at the development site of antibiotics.

As seen earlier the global demand of antimicrobials is extremely high, which is why it's surprisingly that the development of antibiotics decreased significantly. For example 19 antibiotics were approved in 1980-1984. In 2010-2014 only six drugs were approved [52]. This observation is not biased by the sampling time but is a tendency which is ongoing since the last three decades.

The little interest in the pharmaceutical industry is caused by the cycle of fighting newly formed resistance with novel drugs itself. Newly approved antibiotics are usually held in reserve and only prescribed for infections that more established antibiotics can't treat. This policy helps to delay the emergence of resistant strains, but it also limits the investment in return [17]. Another reason is that other drugs are used to treat chronic ailments, antibiotics on the other hand are only used for a short time making them a lot less profitable [17]. 0.5 billion \$ [34] is the estimated cost of the development for a novel antibiotic. Considering the reasons mentioned above, such development is seen as a very risky investment [17].

Because of the increase of consumption and the lack of development of antibiotics several resistant bacterial pathogens established themselves in recent times. Among gram-positive bacteria resistant *Staphylococcus aureus* currently poses the biggest threat [52] as mentioned above causing a severe number of deaths. The threat coming from gram-negative pathogens is even more frightening because many emerged multi drug resistance, making it almost impossible to treat infections caused by such pathogens. Common representatives of gram-negative resistant bacteria are *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter* [52] and extended spectrum β -lactamase (ESBL) producing *Escherichia coli* [17].

Since those pathogens listed above and others are becoming more frequently resistant to drugs of last resort the WHO published a list of priority pathogens. This list should encourage development of new antibiotics against pathogens published with this list [53].

Understanding how much antibiotics are prescribed unnecessarily and how irresponsible they are used in the food industry, it's quite obvious that one key of managing resistant pathogens is to stop this extensive overuse. In human medicine new guidelines and methodologies have to be developed which help doctors in the decision whether it's necessary or not to prescribe antibiotics. New guidelines also have to be established in the food industry limiting this excessive prophylactic overuse.

Furthermore the development of antibiotics has to be pushed either by governmental funding/rewards or pharmaceutical companies have to come up with new business models, making the development of antibiotics more profitable.

2.2 Antibiotics and mechanisms of resistance

During the 21st century different classes of antibiotics were discovered and developed. All of them have in common, that they intend to harm the bacterial pathogen, without harming the patient. This is possible by targeting bacteria specific target sites. Such targets are often involved in the synthesis of molecules which are essential for the bacterial cell. For example tetracyclines inhibit bacterial protein synthesis by binding to 30S ribosomal subunit [32]. Other mechanisms are inhibiting bacterial transcription by blocking the process of unwinding of the bacterial DNA (quinolones) or by inhibiting the folic acid metabolism which is targeted by sulfonamides [noauthor·fig.·nodate]. The most common target is cell wall synthesis, since the cell wall is very specific for prokaryotes. β -lactams, a class of antibiotics to which also penicillin belongs, target mechanisms in cell wall synthesis. They are the most prescribed antibiotics in human medicine. That's also why a lot of resistant bacterial pathogens are resistant against members of this class of antibiotics [23]. Since with this thesis I'm focusing on resistance against β -lactams with cephalosporins in particular, I only describe the mechanism of action for this class of antibiotics.

2.2.1 Development and mechanism of action of β -lactams

β -lactam antibiotics act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls [7]. This is a very effective target, since peptidoglycan plays a fundamental role in the cell wall structure. A damaged cell wall leads to bursting of the cell caused by the osmotic pressure from the cytoplasm. This implements that gram-positive bacteria are especially vulnerable to β -lactams because the peptidoglycan forms the very outer layer of their cell membrane [22].

In detail the inhibition of peptidoglycan synthesis happens because the β -lactams are analogues of the amino acid D-alanyl-D-alanine [21]. Those two amino acids form the terminal residues of the NAM/NAG-peptides which are subunits of the peptidoglycan layer [21]. Those subunits are crosslinked to peptidoglycan by DD-transpeptidases. Now the β -lactams also bind to the DD-transpeptidases but they heavily inhibit its activity, which means that the NAM/NAG-peptide subunits are no longer crosslinked [21]. Because β -lactams bind to those DD-transpeptidases those enzymes are also called penicillin binding proteins, coming from penicillin being the most famous representative of β -lactams [21].

All of the β -lactams feature the reactive β -lactam ring, which is a highly strained and reactive cyclic amide. In general five groups of β -lactams can be classified. Those being penams, penems, carbapenems, monobactams and the cefems [8]. The classification is done by subgrouping their ring system which is responsible for the antimicrobial action [19]. Cephalosporins belonging to the cephems are very important because they showed to be very potent and well tolerated by patients thus they are widely used antibiotics [14]. Furthermore Cephalosporins can be divided into five generations which is mostly done based on the time-point of development.

First-generation cephalosporins are very active against gram-positive cocci but other than cefazolin which is used for surgical prophylaxis this generation of drugs is not prescribed often anymore [19]. The second generation of cephalosporins are all active against bacteria covered by first-generation drugs, but have extended coverage against gram-negative bacteria [19]. The tendency of enhanced activity against

gram-negative bacteria is further visible in third-generation cephalosporins. A common representative of this generation is ceftazidime [28]. Only two beta-lactams are classified under the fourth-generation of cephalosporins, those being cefepime and cefpirome [19]. They were developed because bacterial pathogens started to gain resistance against third generation cephalosporins. The newest fifth generation of cephalosporins were explicitly developed to target resistant strains of bacteria but unfortunately drugs belonging to this generation are ineffective against enterococci bacteria [19].

Penams are a large group of β -lactams that include penicillins which are characterized by a basic bicyclic structure [8].

Unlike penams, monobactams have a monocyclic β -lactam ring and are only active against gram-negative bacteria [19]. Lastly carbapenems are also belonging to β -lactams and they have good activity against many gram-negative bacteria [8].

2.2.2 Resistance mechanisms against β -lactams

Because the β -lactam ring in β -lactam antibiotics is very reactive several enzymes are expressed in bacteria which are able to hydrolyze the β -lactam ring [9]. This same ring is also involved in binding to the penicillin binding proteins and therefore hydrolyzed β -lactams are no longer bactericide [9]. Those enzymes called β -lactamases were found before treatment of infections with β -lactams, because they occurred naturally in bacteria being exposed to bactericides produced from fungi [20].

β -lactamases

Nowadays mainly plasmid-mediated β -lactamases are important because they can be easily transmitted via horizontal gene-transfer [35]. The first plasmid-mediated β -lactamase in gram-negative bacteria was described in the early 1960s and called β -lactamase TEM-1 [19]. Because its position on a plasmid and being transposon mediated, TEM-1 was found worldwide only few years later after its first isolation [19]. TEM-1 was able to cause resistance against β -lactams of this time. That's why third-generation cephalosporins such as ceftazidime were developed which can't be hydrolyzed by TEM-1 [19]. Those newly developed extended spectrum cephalosporins where thus heavily used exposing evolutionary formed variants of TEM-1 to selection [19]. This caused further resistance against those newly developed extended cephalosporins [19]. Another anciently known β -lactamase is called SHV-1 which was isolated for the first time in 1974 [29]. It's encoded chromosomally in the majority of isolates of *K. pneumoniae* but is also plasmid mediated when present in *Escherichia Coli* [29].

β -lactamases which are able to hydrolyze extended-spectrum cephalosporins are called extended-spectrum β -lactamases or ESBLs.

Extended spectrum β -lactamases (ESBLs)

Next to the TEM and SHV families new β -lactamases were isolated over the time of the last couple decades. One of them is called CTX-M-1 β -lactamase which was clinically isolated for the first time in Germany in 1986. Its name comes from the high affinity to cefotaxime [19]. Because it only has a sequence identity of 40 % compared to TEM or SHV [10] it got categorized as a new β -lactamase family.

Later on many variants were isolated and the CTX protein family was subdivided into five subgroups, with CTX-M-1 being one of them [19]. It's assumed that they evolved from the β -lactamase precursor AmpC from *Klyzvera ascorbata* [10]. Even though the first CTX-M-1 was isolated in Germany, it's mostly popular in eastern Europe, South America and Japan [10].

Another β -lactamase family belonging to the ESBLs is called OXA. The OXA family was originally created as a phenotypic rather than a genotypic group, based on a specific hydrolysis profile [10]. Therefore the sequence identity compared to other members of this family is only about 20 % [10]. Its name comes from the ability to efficiently hydrolyze oxacillin [10].

2.2.3 General resistance mechanisms

By hydrolyzing β -lactams we have seen one strategy of bacterial resistance against antibiotics. There are other more general mechanisms which protect the pathogen from bactericide effects of antibiotics.

Another pathway of resistant bacteria is to decrease the antibiotic penetration and to increase efflux. That makes a lot of sense, because many antibiotics have targets which are located intracellularly [35]. This implies that bacterial pathogens came up with pathways which are making it more difficult for the antibiotic to reach the cytoplasm, or they introduced pathways which actively transport the antibiotic out of the cell. Some compounds such as β -lactams rely on channels in the membrane of the bacteria called porins, because they are hydrophylic and therefore can not just pass the lipophylic membrane by diffusion [35]. This gives the bacteria the opportunity to reduce the numbers of porins or to change their structure [35]. It's noteworthy that changing permeability is an especially effective strategy for gram-negative bacteria to protect themselves from β -lactams. That's because the β -lactams have to cross the outer membrane in order to inhibit the peptidoglycan synthase [35].

It's much more complicated on the other hand to produce efflux pumps, which actively transport the toxic compound out of the cell. One of the earliest described efflux pump systems are the Tet efflux pumps which are using proton exchange as source of energy [35].

Another mechanism is changing of the target site. Some antibiotics such as rifampin binds to a very conserved pocket within the RNA polymerase of the bacteria. Now by chromosomal mutations coding for this RNA polymerase the bacteria changed the structure of this pocket and therefore they were no longer susceptible to rifampin [35].

2.3 An overview of antibiotic resistant pathogens in Switzerland

For Switzerland the rate of antibiotic consumption divided by the Swiss population stayed the same in recent times, but the absolute consumption increased too. This is because more people were getting treated in hospitals [4] but the general population of Switzerland increased too.

The biggest challenge with resistant pathogens in Switzerland is ongoing in hospitals where yearly 300 people die because of infections with antibiotic resistant

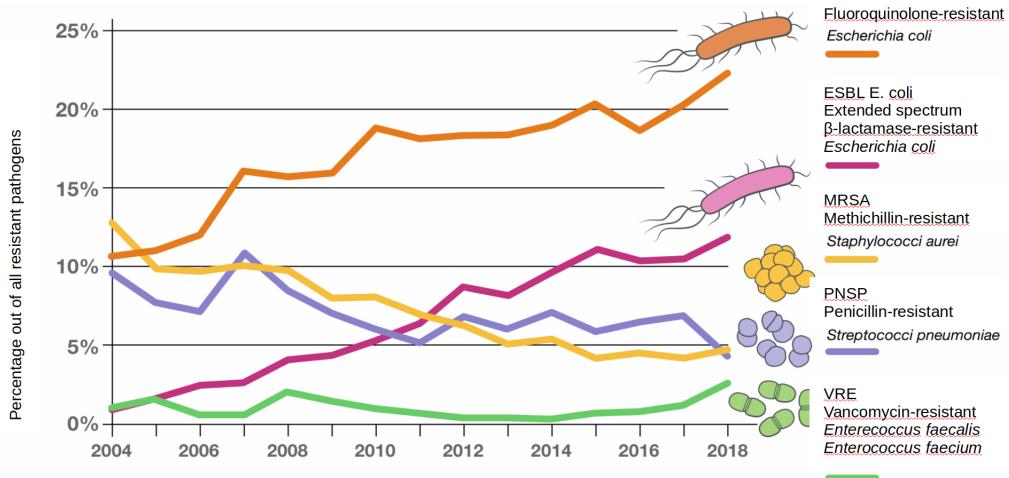


Figure 2.1: Development of popularity of antibiotic resistant pathogens. [3]

bacteria [18]. Hospitals are often hot-spots of resistant bacteria because there is a high density of pathogens, combined with high locally applied antibiotic doses. This generates an increased antibiotic pressure and selection. Unfortunately many patients have a weakened immune system making them a lot more vulnerable to infections with antibiotic resistant bacteria.

Mainly five strains which cause problems in treatment with antibiotics are present in Swiss hospitals. Their increasing or decreasing spread can be seen in Figure 2.1[4].

Staphylococcus aureus is a gram-positive bacterium which is normally present in the upper respiratory tract and on the skin. As a pathogen *Staphylococcus aureus* can cause skin and bone inflammations and is responsible for the most inflammations of surgical wounds [48]. The most common resistance formed by *S. aureus* is resistance against an antibiotic called methicillin, a penicillin like compound. Resistance is achieved by those pathogens by expressing a gene coding for a penicillin binding protein PBP2a which has significantly lower affinity to β -lactams [38]. There are compounds which effectively kill this certain pathogen but resistance against such compounds have been reported already [52]. As seen in Figure 2.1 there is a decrease of this pathogen which could mainly be achieved by aggressive preventive hygiene measures in hospitals and improved screening for this pathogen [52][swiss'hospital].

Streptococcus pneumoniae, a gram-positive bacteria, is normally present in the nose and throat of most individuals [52]. But when *Streptococcus pneumoniae* turns into a pathogen, which mainly happens in elderly patients, it causes severe blood and pulmonary infection causing around 7000 deaths per year [ventola antibiotic'2015e]. Usually resistant *S. pneumoniae* are not susceptible to penicillin like antibiotics by a similar mechanism as described for *S. aureus* (mutations in the penicillin binding proteins). Visible in Figure 2.1, this pathogen is decreasing as well, which is mainly because of the introduction of the PCV7 vaccine. This vaccine provides protection against several pneumococcal strains, which reduces the transmission of resistance [52].

Enterococci such as *Enterococcus faecalis* are responsible for a rather small share of resistant pathogens as visible in Figure 2.1. Unfortunately it's very difficult to treat this gram-positive bacteria because only very few antimicrobial options are

available [52]. This pathogen is so hard to treat because it developed several pathways of mechanism. Modifications in penicillin binding proteins, over-expression of efflux pumps and a cell response that promotes survival in the human host [33].

Escherichia coli belongs to the enterobacteriaceae and is present in every intestine as a gram-negative bacterium. However they are able to cause infections when present in other parts of the body leading mainly to infections of the urinary tract but also in the brains of new borns [3]. Traditionally treatment of *E. coli* was successful by applying doses of third-generation cephalosporines. In the last two decades *E. coli* started to gain resistance against such extended β -lactams by producing extended spectrum β -lactamases (also see section 2.2.2). As seen in Figure 2.1 in 2004 only 1 % of resistant pathogens in Swiss hospitals were ESBL producing *E. coli*. 14 years later they were already responsible for 14 % of infections with resistant pathogens. Luckily ESBL producing *E. coli* are still susceptible to carbapenems but other bacterial strains already developed resistance against carbapenems [50]. That's why carbapenem should only be used as a very last option of treatment of ESBL *E. coli* infections, because chances are quite high, that they are able to evolve resistance against this compound too by for example picking up genes by horizontal gene transfer coding for carbapenemases which are able to hydrolyze carbapenem. [52].

2.4 ESBL *E. coli*

Since by now ESBL *E. coli* can still be treated with antibiotics of last resort such as carbapenems it's extremely important to use those drugs with high awareness. Therefor institutions such as the EUCAST came up with guidelines in order to assist clinical microbiologist in making the right decision in prescribing adequate antibiotics [30]. The EUCAST is also responsible for defining breakpoints. This means they publish data defining which concentration has to be exceeded in order that a certain pathogens is seen as resistant against a specific antibiotic [30].

In the past when it was determined that an infection is caused by *E. coli*, the strain was cultured and susceptibility was tested by determining the MIC. Additionally presence or absence of an ESBL genes was tested by molecular characterisation [26]. Such molecular characterisation was typically done by PCR or microarrays [5]. Now when either the determined MIC was above the breakpoint defined by the EUCAST or the molecular characterisation showed positive results for ESBLs, carbapenemases were described. But it was shown, that sometimes the MIC was below the breakpoint even though ESBLs were detected. Prescribing antibiotics of last resort in such cases was therefor proven to be unnecessary and wrong. As a reaction EUCAST corrected their guidelines and now just susceptibility by MIC determination is considered for making the decision which antibiotic should be prescribed [30]. Unfortunately MIC determination takes about 48 hours which is too long in some cases forcing prescription of carbapenemases even though it's unknown if it's actually necessary. Completely ignoring genomic data seems not ideal, because chances are quite high that whether *E. coli* which have ESBL genes are resistant or not is encoded in the genome. This just means that other characteristics have to be defined, since it's not sufficient to simply look if genes coding for ESBLs are present.

2.5 Studying differences in the genome between extended cephalosporine susceptible and resistant *E. coli*

Some resistant mechanism as for example a change in the target binding site, rely on mutations and therefore imply a change in the genome. Other examples for resistance based on mutations are a different structure of porins making the membrane less permeable for β -lactams, or a change in the structure of efflux pumps which may changes the affinity for a certain substrate. Other mechanisms could rely on up-regulation, which could have its source in a mutation in a promoter region.

As by now it was assumed that the resistance of ESBL *E. coli* is based on the expression of ESBLs, which are able to hydrolyze β -lactams [44]. But as described earlier some *E. coli* which have ESBL coding genes are not resistant. This could mean that extended cephalosporine susceptible *E. coli* which have ESBL genes are not actually expressing such β -lactamases. This could be clarified by studying the ESBL protein levels for susceptible and resistant ESBL *E. coli*. It's also possible, that other resistant mechanisms other than hydrolysis of the compound have an impact on susceptibility.

Either if a change in the expression levels or other mechanisms are responsible for the resistance, it's quite possible that at least a part of the resistance is based on mutations. Those mutations could be identified by comparing the genomes of susceptible and resistant *E. coli*.

Our goal is to identify such mutations which could be involved in the resistance. In order to do so, we pursue two different approaches. One of them is study the genomes of clinical samples of ESBL *E. coli* which are provided by our collaborator who is the head of clinical microbiology at the University hospital of Basel. He took samples of patients who were infected with *E. coli* where ESBLs were detected by PCR. Over the process of treatment he continuously took samples and determined their susceptibility to cefepime and ceftazidime. This lead to a sample collecton of ESBL *E. coli* which were susceptible to extended cephalosporines but evolved resistance, or for some patients resistance was lost over time. By isolating the DNA and deep-sequencing all of the isolates, we identify SNPs in the resistant samples, which are potentially involved in the resistant mechanism.

For the other approach we assemble a system called morbidostat, which allows to automatically culture bacteria. The morbidostat is continiously recording the growth of the cultures and injects appropriate doses of antibiotics. This creates constant inhibition of the growth and forces resistance and selection. Along this pathway we want to take samples and deep-sequence the isolated DNA. Similar to analysis for the clinical samples, we want to identify mutations which accumulated over the time where the bacteria were forced to evolve resistance.

Both approaches rely on next generation sequencing technologies. To get the most accurate genomes we want to use two different next generation sequencing technologies.

Illumina and Nanopore sequencing

Illumina sequencing is a method which produces short reads which are typically 150 base pairs (bp) long. The strong-suit of Illumina sequencing is the low error rate which was determined as 0.24 % per base [39]. A common used Illumina sequencing system is the MiSeq-System which is also used for this project. The downside of this technology is that even though sequencing itself is rather cheap, the instrument costs are high. Furthermore Illumina sequencing has a tendency to generate more reads of GC-enriched DNA fragments [24]. Also it's not possible to assemble structurally correct whole-genomes, because the reads are short.

Oxford Nanopore Technologies allows sequencing with very little laboratory equipment which is also coming with very low instrument costs. This technology produces very long reads which are up to several 100 kbp long. In contrast to Illumina sequencing the error rate per base is 13.6 % [41] which is a lot higher. But because the reads are so long it's possible to assemble structurally correct whole-genomes.

By comparing both technologies, it's pretty obvious that their strong suits are quite contrary. Illumina produces short but accurate reads, Nanopore long and inaccurate reads. Since the costs of both techniques dropped tremendously over the last couple years, it's possible to sequence DNA isolates on both platforms and combine the output in one assembly which is called hybrid assembly. This has the benefit that the advantages of both platforms are combined, leading to assemblies with a low nucleotide error rate but also with high structural correctness.

2.5.1 Principles and experiences with the morbidostat

The morbidostat was initially invented by Toprak et al [1]. Later on Neher et al [16] rebuilt the system in 2015. Based on those two systems we rebuild a morbidostat with small modifications which should improve the handling and mainly make it a lot more compact. This is important since we want to use the morbidostat in a space limiting hypoxi-station.

With the morbidostat 15 cultures can be grown independently in vials. Thereby the growth of each culture is monitored by measuring and saving the optical density (OD). As shown in the left Figure 2.2 measuring of the OD is done by detecting scattered light emitted by LEDs. Based on the growth an appropriate dose of antibiotics is calculated using a control algorithm and injected into the cultures. This leads to an inhibition of the growth without entirely killing every colony forming unit. The appropriate dose of antibiotics is achieved by mixing different drug concentrations using computer controllable pumps which are also injecting the drug into the vials. The morbidostat allows to study drug resistance evolution in real-time, while having an idea of the progress of evolution in resistance.

In the morbidostat, bacteria are grown in a fixed volume. The process of suppressing the growth with antibiotics is divided into cycles which are constantly getting executed. One cycle has following tasks and commands:

- Measuring the OD every several seconds over a defined cycle time Δt , which is typically 10 minutes

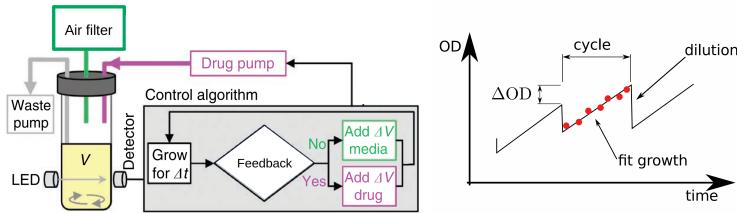


Figure 2.2: Based on the growth a feedback algorithm decides whether and how much drug is getting injected

- Calculating growth rate at the end of the cycle based on the previously collected ODs
- Based on growth, injection of appropriate dose of drug, as calculated by a feedback algorithm
- Remove excess volume (waste)

In the right Figure 2.2 three cycles are shown. Because under certain conditions fluid is getting injected, this leads to a dilution is visible in the OD at the start of the new cycle. ΔOD represents how fast the bacteria are grown. It's the difference of the optical densities from the end of two cycles.

Previous morbidostat experiments and expected outcome

Dösselmann et al [16] used the morbidostat in order to study colistin resistance. With the morbidostat they were able to increase the MIC of colistin for *Pseudomonas aeruginosa* a 100-fold within 20 days [16]. Along the evolutionary pathway they were able to identify a mutation pattern. The mechanism of action for colistin is to displace cations from the phosphate groups of membrane lipids. This leads to disruption of the outer cell membrane causing cell death [13]. Therefor it's not surprise that Bianca et al could identify several mutations coding for proteins involved in the lipopolysaccharide synthesis [16].

The mutations that we find in the analysis form the clinical samples but also from the morbidostat experiment hopefully lead to a better understanding in the resistance mechanism of ESBL *E. coli*. Obviously the data has to be validated by for example synthetically introducing mutations of interest to extended cephalosporine susceptible ESBL *E.*

The mutations can be used by our collaborator who is studying protein levels in ESBL *E. coli*. For example mutations found in promotor region can be compared to protein level expression data in order to tell if they have an impact on the expression.

3. Materials and methods

3.1 Analyzing ESBL *E. coli* isolates from patients of the University hospital Basel

Our collaborator, head of the clinical microbiology from the University hospital Basel, collected 65 isolates of 34 patients who had inflammations caused by ESBL *E. coli*. Over all the sampling period was approximately four and a half years. Sample collection for one patient typically happened within several months.

His group determined the MIC of the extended cephalosporines cefepime and ceftazidime for every collected isolate and short-read sequenced them on a MiSeq Illumina system. Only samples were included where ESBL genes were detected. This resulted in a collection with multiple isolates per patient, where a change of MIC for cefepime and ceftazidime was visible over time caused by gained or lost resistance. Only a few patients showed a significant change in the MIC of cefepime or ceftazidime over time so only those patients of interest could be included in the analysis.

Illumina sequencing

Illumina sequencing was done by our collaborators from the clinical microbiology from the University hospital of Basel. They extracted the DNA from cultured samples 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). The resulting library was sequenced using a MiSeq Illumina platform [2]. The reads produced with Illumina were trimmed with Trim Galore [6].

Nanopore sequencing

Isolates from patients of interest were sequenced with a MinION from Oxford Nanopore. Each library was prepared with a ligation sequencing kit (LSK-108) followed with the native barcoding expansion kit allowing barcoding every sample and loading all of them on a single flow cell (FLO-MIN106D). A detailed protocol of the library preparation is available in the publication of Noll et al [41].

3.1.1 Phylogenetic analysis

Before the genomes of the isolates coming from one patient could be compared phylogenetic analysis had to be done. This was necessary in order to ensure, that one patient was infected with the same ESBL *E. coli* strain over the whole sampling

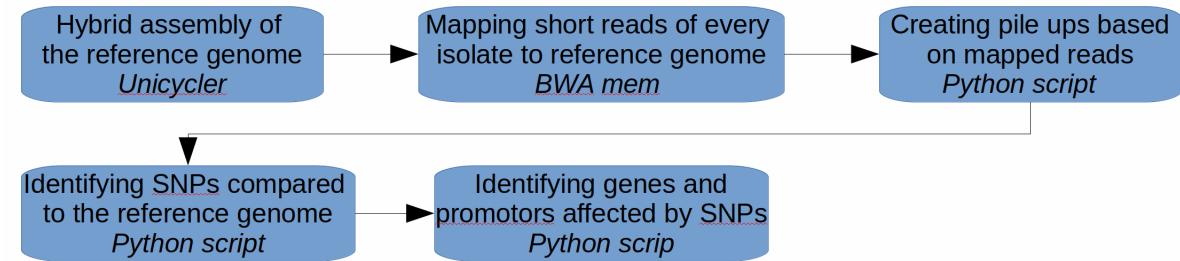


Figure 3.1: Pipe line used for the identification of SNPs and affected genes and promotors

period. This was done by using the panX tool.

PanX is a tool which allows to build a phylogenetic tree, which contains the information about closely related strain are. It's a tool which clusters genes into orthologous groups. From these clusters, panX identifies the core genome which are genes shared by all strains in a group of isolates [15]. Based on those core genomes a strain-level phylogeny can be build, making use of single nucleotide polymorph positions (SNPs). This results in a tree, which visualizes how closely related different isolates are. If isolates have the same common ancestor they map on the same clade of the tree. SNPs in the core-genome then allow to further distinguish the strains and based on the SNPs the isolates are located on different branches of the clade. PanX takes annotated genomes as input files, that's why every sample was short read assembled and annotated.

Short read assembly and genome annotation

Since every isolate was short-read sequenced, all of them were included for the phylogenetic analysis. For assembling spades (v3.12.0) [36] was used. The output of spades was a short-read assembly containing about 100 contigs per assembly. For *E. coli* only a handful of contigs are expected which shows how important long-reads are in order to get structural accurate whole-genome assemblies. But using just short reads was enough because the assembly was only used to identify all genes of every isolate. Identification of the genes was done with prokka (v.1.12) [43]. Prokka first searches a core set of well characterized protein using BLAST+ and then compares reading frames to a database derived from UniProtKB [42]. The results from prokka were stored in a genbank file for every isolate. Based on those genbakn files, the phylogenetic tree was built with panX.

3.1.2 Identification of SNPs

For identifying mutations in form of SNPs a bioinformatical pipeline was established as shown in Figure 3.1 with five steps. Using this pipe line it's possible to identify SNPs which accumulated over time.

Creating a reference genome

As a first step of the pipeline a reference genome was built for every patient of interest. This was done by combining the Illumina and Nanopore sequencing data

of the isolate with the lowest MIC of cefepime and ceftazidime from each patient of interest into a hybrid assembly using Unicycler [54].

Annotation of every build reference genome was done by identification of all genes using prokka [43] (see section 3.1.1). Additionally promotor regions were identified using the promoter prediction tool PePPER [25] and the promoter data base hosted on EcoCyc [46]. PePPER is a tool which takes the assembled sequence as an input and predicts promotor sequences which are returned. Those sequences were mapped against the reference genome using graphmap [47]. Furthermore a promotor data base hosted on EcoCyc was used which contains around 3800 experimentally validated promotors. The sequences from this database was downloaded and every sequence mapped against the reference genome with graphmap. Mapping of PePPER and from the EcoCyc data base was stored in a seperate bam-file for every reference genome.

Mapping of short reads

As a next step all the Illumina short-reads from every isolate of each patient of interest were mapped against the reference genome with BEW mem [31]. This resulted in a bam file for every selected sample

Creating pile ups

For identifying SNPs pile ups are used. Pile ups are count matrices which store how many times which nucleotide is present at a certain position in the genome. Based on those count matrices coverage or base frequency can be calculated, or by comparing pile ups from different isolates of the same patient, it's possible to identify SNPs. Identifying SNPs this way was only possible, because all the short-reads were mapped against the same reference genome.

The pile ups were created for every sample of the selected patients with a custom script pileup.py. The script iterated over every position in the reference genome. Then it checked which base was present in every read which was mapped to this position and stored the nucleotide counts in a matrix. All the pile ups from one patient were stored in a matrix stack. This allowed to easily compare the most abundant base, coverage or base frequency at a certain position over every sample of a patient.

Identification of SNPs

For identification of SNPs a python script analysis_modular.py was used. This script goes though every position in the reference genome. Every pile up for a selected patient is then checked which base is the most abundant. This means if a different base was abundant in a pile up than in the reference genome, a SNP was identified. All the SNPs were stored in a new matrix. Filtering to every found SNP was applied by checking the coverage and base frequency of every position. Only SNPs where the coverage was at least 30 and the base frequency was at least 0. 8 was kept for further analysis.

Identification of genes and promotors affected by SNPs

It was checked if annotation was available for every found SNP. As described in section 3.1.2 genes and promotors were identified for every reference genome.

For checking if a SNP affected a gene, we analyzed the genbank file with [12]. We checked if a SNP is located between a start and an end position of a gene, those positions are stored in the genbank file, where also the information about the gene and the product is available. For checking if a SNP affected a promotor region, we went through the bam-file created based on PePPER and EcoCyc (see 3.1.2). We checked if a SNP is located between a start or end position of a mapped promotor sequence.

3.2 Assembling and handling procedure of the morbidostat

Initially the first morbidostat was build by Toprak et al. [1]. The following system is an adapted version of Topraks built differing mainly in it's pump system, controlling unit and software. Hardware which was not commercially available was built by the in-house mechanic and electronic workshop.

3.2.1 Hardware and setup

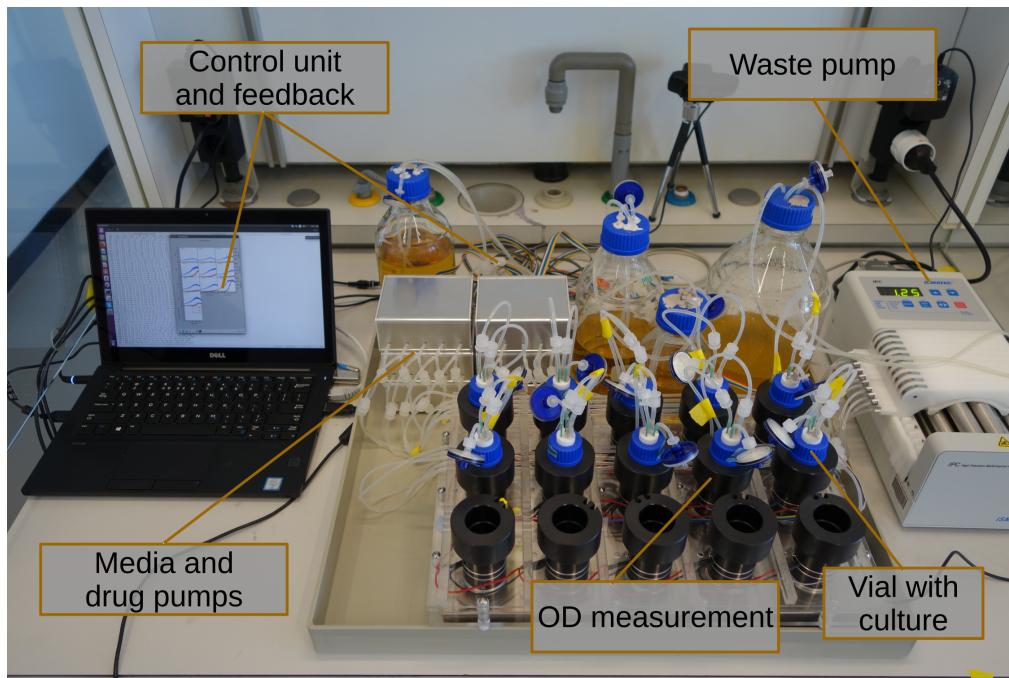


Figure 3.2: Overview of the morbidostat setup. The arduino (controller) is located behind the drug and media pumps.

Figure 3.2 shows the morbidostat setup. The whole setup was placed on a magnetic stirrer which can hold 15 vials. The magnetic stirrer was responsible for a constant mixing of the cultures. On the magnetic stirrer vial holders were placed (black rings

visible in Figure 3.2). In those vial holders the OD measuring unit was integrated. It consisted of an LED and a detector which measured scattered light. The cables leading to the vial holders and connecting the electronics for measuring the OD are visible in Figure 3.2. What can not be seen is that those cables are connected to an arduino which was located behind the pumps. Every vial was connected to four pumps. Three pumps were capable to inject two different drug concentrations and media, the fourth pump was used in order to remove volume which exceeded the culture volume (waste). The connections to the pumps and the bottles were made using silicon tubing and plastic luers.

For controlling the hardware the arduino was used, who processed the OD measurements and turned on/off the pumps injecting fluid to the vials or removing waste. In Figure 3.2 the morbidostat was built up in the open, for later experiments the morbidostat was placed in an hypoxi-station within a biosafety level 2 lab. This was necessary in order for temperature regulation but also for safety reasons (also see section PLACEHOLDER).

OD measurements

For measuring the OD a combination of a light emitting diode (LED) and a phototransistor was chosen. The principle is, that cells cause scattering of a ray of light. By placing an LED at glass wall of the vial and a phototransistor as a detector in a 135 ° angle, the scattering of the light could be measured. More cells caused more scattering which caused more light reaching phototransistor because its angled orientation.

As a LED OPB608A was chosen from TT electronics with a peak wavelength of 890 nm. For detection a PT 333-3C phototransistor was used. One OD measurement is made possible by two independent circuits which are both connected to a 5 V power source and a ground. As shown in figure 3.3 one circuit (colored orange) was powering the LED with a $x \Omega$ resistor connected in serial after the LED. The other circuit (colored blue) was responsible for measuring the scattering of the light with the phototransistor. 15 OD measuring units were split up in three parallel-connected chains representing one row of 5 vials.

Measuring the scattering worked as follows: Light reaching the phototransistor caused an opening in the semiconductor from the phototransistor which led to a current reaching the transistor. The transistor amplified the current. As visible in the blue colored circuit in Figure 3.3 a resistor was connected in serial after the phototransistor. Over this resistor the voltage difference was measured with the arduino. The opening of the semiconductor was proportional to the light which reached it. A bigger opening led to more current which got amplified even more by the transistor. If more current was reaching the resistor over which the voltage was measured, this led to a smaller voltage. In order to get an actual OD, calibration was needed. The voltages were measured which were generated by placing vials with a cell suspension with a certain OD. Because of the linear correlation between the flowing current and the light reaching the phototransistor the correlation between detected voltage and OD could be described in a linear equation. In order that sensitivity of the OD measurement could be changed a potentiometer was added in serial before the voltage measurement with the arduino. It turned out that the system was not as sensitive as thought in advance, so all the potentiometers were opened as much as possible meaning that the highest possible resistor was chosen.

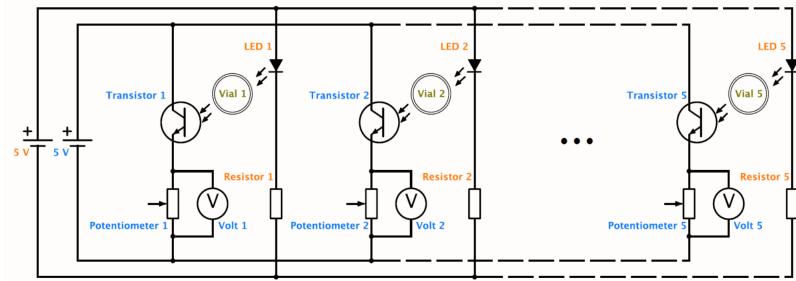


Figure 3.3: Circuit diagram of parallel-connected LED (orange) and phototransistors (blue). This circuit is done independently three times for five vials each. The detector and the LED are orientated in a 135 °angle since this is the best angle for light detection.

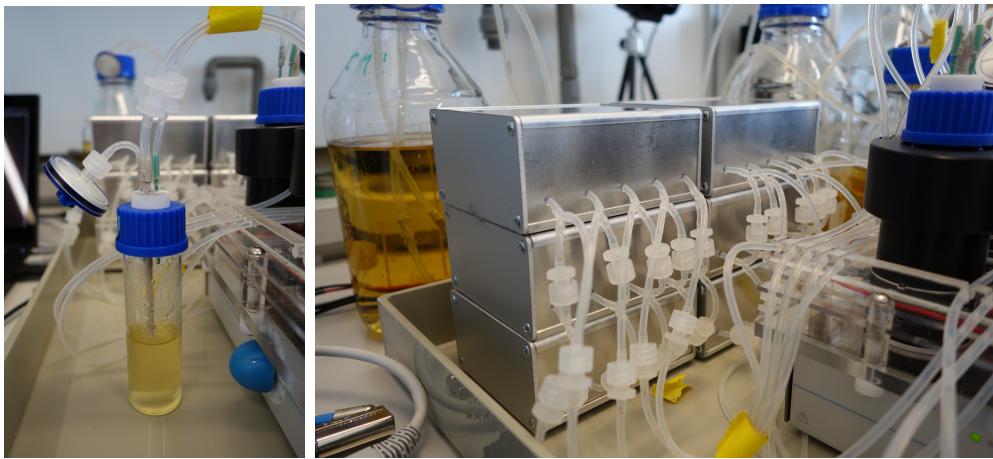


Figure 3.4: Figure left: Vial setup. Figure right: One row of five pumps represents one row of five vials. Since every vial is connected to three pumps, three rows of pumps are stacked on top of each other and connected column by column. This means that one column of three pumps is responsible for injection fluid into one vial. Every outlet from a pump from one column is connected, in order that there is just one tube going to one vial.

Vials and tubing

Every vial was connected to three injecting pumps and one pump which removed the waste. This would have led to four tubes connected to a vial. In order to make the vials more accessible we connected the three tubes from the media and drug pumps together and led the connected tube to the vial which is visible in the right Figure 2.2. Two more inlets per vials were necessary which is also shown in the left figure in 2.2. One inlet set to the height of the desired culture volumewas used to connect the waste pump. The last inlet was connected to an air filter which was necessary to equalize the pressure within the vial.

Pumps

Three connected injecting pumps per vial lead to 45 injecting pumps in total. Mp6 pumps from microComponents were chosen because they have a compact built, having the size of half an USB stick. This is an improvement because the pumps

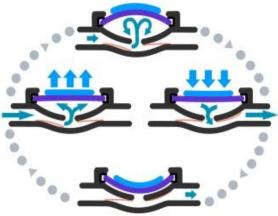


Figure 3.5: Principle of the mp6 pump but two of those piezoelectronics are connected in serial for this pump model [**piezo·pumps**]

used by Toprak et al [49] and Doesselmann [16] were peristaltic pumps which were cubic with a size of approximately 4x4x4 cm.

The functional principle is based on a piezoelectric diaphragm in combination with passive check valves which is shown in figure 3.5. By applying voltage a piezo ceramic mounted on a membrane is deformed resulting in a down stroke. When the voltage decreases again the piezo deform again causing an upstroke of the membrane [**piezo·pumps**]. Because the pump process depends on excitement and relaxation caused by the power, the flow rate generated by the pumps is dependent from the frequency. This also implements that the flow rate is very constant given that the power frequency is also constant. That being the case, the mixing of drug concentration was done by turning on the pumps for a calculated time.

In order to excite and relax the membrane and the piezopumps 230 Volts and a very steady power frequency were needed for this process, making it necessary to control every single pump with a specific mp6-OEM controller. This controller took an input power of 5 V and used about 30 mA of current. The Arduino was not capable of supplying this amount of current for every pump, therefor a separate power adapter was installed to supply the pumps.

The pumps could be controlled by connecting one pin of the controller from the pump to a digital pin of the Arduino. If the pin from the controller was put to ground, the pumps were turned on, if the pin was connected to 5 V the pumps were off. Turning on/off the controller only worked well, when the change of power was very sudden. By adding a pull-down resistor between the digital pins and the ground from the Arduino we could avoid that there was always a very small current flowing through the digital pins. Furthermore it was decided to work with a serial connected inverter, implementing that when the digital pin was set to low, the inverter caused a power of 5 V which meant that the pumps were off. On the other hand when the digital pin was set to high, the inverter produced a power of 0 V which turned the pumps on. As a waste pump a 16-channel peristaltic pump was used which was directly controlled with a digital pin from the Arduino.

3.2.2 Controlling of the morbidostat

The controlling of the morbidostat was divided into two python scripts running on the laptop and one .ino script running on the arduino. Communication of the two hardware devices was possible via an USB cable enabling serial communication. The two python scripts were arduino_interface.py and morbidostat_experiment.py. The arduino_interface.py was responsible for transmitting commands to the arduino via the the serial connection but also for receiving data from the arduino. The

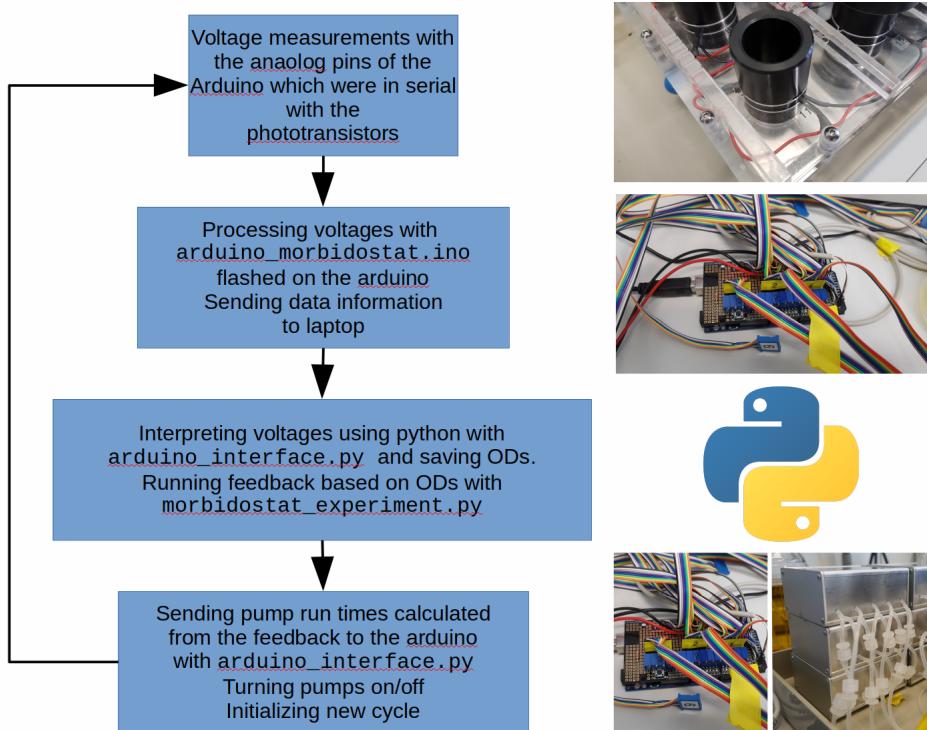


Figure 3.6: Overview of one cycle from the morbidostat.

arduino_morbidostat.ino code interpreted the commands received from the laptop and executed hardware commands. Those commands were measuring voltages of analog pins, needed for the OD measuring, or turning on/off digital pins which turned the pumps on/off (see section 3.2.1). The morbidostat_experiment.py script on the other hand was responsible for saving data, initializing cycles and calculating appropriate drug concentrations. As shown in Figure 3.6 the controlling of the morbidostat was done by constantly executing cycles. One cycle consisted of several steps depending on different scripts.

First the cycle itself was initialized by morbidostat_experiment.py and the ODs were measured and saved for every vial over the defined cycle time (typically 10 minutes).

Measuring of the OD as initialized by morbidostat_experiment.py led to a function call in arduino_interface.py which transmitted a command to the arduino. This command got interpreted and lead to measuring the voltages of the analog pins which were connected to an OD measuring unit as shown in top right of the Figure 3.6. The resulting voltages got transmitted to the arduino_interface.py via the serial communication. This script translated the voltages to optical densities according to a linear equation defined earlier by calibration. Morbidostat_experiment.py was responsible for saving the ODS and averaging those values at the end of the cycle. As a next step a function in morbidostat_experiment.py calculated how much drug should get added to which vial, based on the averaged ODS. The output of the function were runtimes of certain pumps. Those runtimes were transmitted again to the arduino via the arduino_interface.py script and interpreted by the arduino. As shown in the last illustration of Figure 3.6 the arduino switched according digital pins to low for the time as communicated from the laptop.

Feedback

The feedback coded as a function in morbidosta_experiment.py was very important since it was responsible for putting the culture under antibiotic pressure. The outcome of the feedback depended on the growth of the bacteria. Therefor the growth was calculated at the end of every cycle by calculating ΔOD according to the following formula:

$$\Delta OD = (final_OD[x_{cycles_back}] - final_OD[Cycle_{current}])/x$$

The final_OD was calculated at the end of every cycle by averaging every OD measurement gathered over one cycle. As shown in the equation the difference between the final_OD fromt the current cycle and the the final_OD of x cycles back (x being typically 10) was calculated and divided by x. As showin in Figure 3.7 the feedback did several comparisons before calculating an appropriate dose of drug. 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 getting added in this case.

When ΔOD was positive the bacteria were growing. Now it was important to not put the bacteria under selective pressure when the final_OD of the cultures was were small (e.g. 0.03). If drug was added at this stage, this usually led to complete sterilization. That's why a threshold was introduced which was called drug_dilution_threshold. Therefor the next comparison as visible in Figure 3.7 was whether or not the final_OD was higher or smaller than this threshold. If that was not the case the decision was nothing, which means that no fluid was added to the cultures.

However when the final_OD was bigger than the threshold calculation of the appropriate dose was started. This calculation depended on the MIC. Therefore the MIC had to be determined for the choesn bacteria and drug combination before the morbiostat experiment. Calculation of the concentration was divided into two steps consisting of an additive and a multiplicative component. The additive part was mainly important at the beginning of the experiment and was used to approximate a drug concentration in the vials similar to the MIC. Once the concentration in the vials was above the MIC the additive part was ignored. After that the current vial conentration was fed to an multiplicative equation. This equation multiplied the current drug concentration by the ΔOD which resulted in how much the drug concentration in the vial should be increased. This proved to be a good strategy, because fast growth meant stronger inhibition, but when the growth was very small and close to zero, the inhibition was not significantly increased. The multiplicative part also included other variables. For example a target_OD was defined which was used to define which OD should not be exceeded. In general it was the goal to have the OD of the cultures close and steady to the target_OD.

It was chosen to divide the outcome of the multiplicative component by this target_OD. Therefor if the target_OD was set to an high OD, inhibition was smaller than when set to a small OD. Furthermore variables had to be introduced to the multiplicative component which made it possible to make the feedback more aggressive or more sensitive.

Defining the target_OD was a trade off of having a higher probability of mutations for a higher defined target_OD, but keeping the culture at a high OD implemented fewer accuracy of the OD measurement caused by many dead cells.

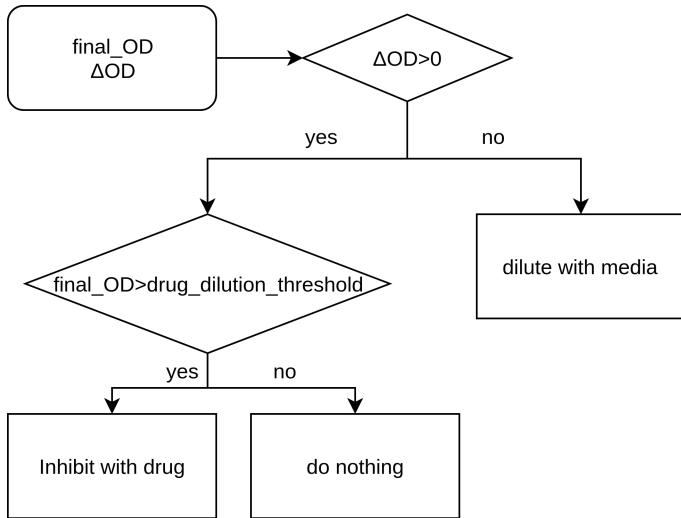


Figure 3.7: Schematic overview of decisions involved in the feedback

3.2.3 Experimental procedure

OD and pump calibration

We chose 1/10 LB media and 9/10 H_2O as a media for all experiments because the bacteria grew too fast by just using medi.. All the culturing of the bacteria was done in a 37 ° incubator. For calibrating the OD measurements an overnight culture with K12 E. coli was inoculated in 5 ml diluted media. The next day the overnight culture was diluted 1/200 in 50 ml diluted media. After a few hours the OD of the culture was measured. Following ODs were prepared with 18 ml diluted media with the appropriate vials for the morbidostat: XXXXXXXXXXXXXXXXXXXXXXX

Then every vial with of one certain OD was placed in every vial holder. With the function calibrate_OD from the morbidostat_experiment.py script, a current measurement was done for every OD and every vial holder. Because the current was measured for different ODs, the relation of current and OD could be described with a linear function.

For calibrating the pumps the function calibrate_pumps from the morbidostat_experiment.py was executed. This function demands the initial weight of every empty vial. After entering every weight, the function turned on every pump for 100 seconds. After that, the vials were weighted again and the values passed to the function which allowed precise calibration of each pump.

MIC determination

Since the feedback was heavily dependent on the MIC, this value had to be determined for every drug which was used to run the morbidostat. Therefor 5 ml of MHB media was inoculated with the bacteria used for the actual experiment and cultured over night. The next day a 1/200 dilution in 20 ml MHB was prepared and cultured for a few hours. In the mean while a 128 fold concentration of the MIC found in the literature for the drug of interest was prepared in MHB. 128 fold was chosen, because the determination was done in a 96 well plate which implements that the MIC found in the literature is in the middle of the plate. The growth of

the diluted culture was constantly monitored by measuring the OD. When the OD of the diluted culture was at 0.08, a 1/100 dilution was done once again. From this final dilution 100 μ l was pipetted in every well from the 96 well expect in the wells from the last column of the plate. Additionally 100 μ l of MHB was added to every well. As a next step 100 μ l from the prepared drug solution was added to the first column of the plate. Then 100 μ l from this column was transferred to next column and mixed. This was repeated until the third last column. This implements that the second last column acts as a control of the cells, since no drug was added. The last column acts as a control for the media. After preparing the well plate it was incubated for 16 hours at 37 ° and after that, the OD of every well was measured using a plate reader. As MIC the column of a certain concentration which inhibited the culture significantly was chosen.

Sterilization of the morbidostat

In order to prevent contamination a solution with sterile water containing 3 % bleach was prepared and pumped through every pump for 5 minutes. After that we let the solution sit in the tubes for about an hour. After that every tube was flushed with sterile water by pumping it through every pump for 5 minutes. All the media, drug bottles and vials with its luer connections were autoclaved before the experiment.

Testing the continuous culture mode from the morbidostat

An overnight culture was set up by inoculating 5 ml of diluted media with K12 XL1-Blue E. coli. The next day a 1/200 dilution in 200 ml was prepared and 18 ml of this suspension was pipetted in 18 sterile vials. A grow curve was determined by starting an experiment with the morbidostat in the GROWTH_RATE_EXPERIMENT modus. The morbidostat was put in the hypoxi-station at 37 ° with an air composition of - % CO_2 and - % nitrogen. The growth rate experiment was done overnight. The next day ideal dilution rates for the continuous experiment were identified, by feeding the growth rates to the morbidostat_simulator.py script which simulates a continuous morbidostat experiment. Also the best parameters for the feedback were determined using the simulator. As a drug amoxicillin was chosen, its MIC was determined as 2 μ /ml according to the procedure in 3.2.3. As a starting concentration 6 μ /ml and 14 μ /ml were chosen for the drug bottles. Tubing, bottles and vials were sterilized according to the section 3.2.3. The continuous morbidostat experiment was started by initializing the experiment with the CONTINUOUS_MORBIDOSTAT modus under the same temperature and air condition as for the grow curve determination. The dilution_factor was set to 0.94. Every other day 200 μ l of the suspension in the vials were transferred into new sterile vials filled with 18 ml diluted media. When a drug bottle was empty, the MIC was changed in the morbidostat_experiment.py according to the concentration which was needed to strongly inhibit the growth. New drug concentrations were chosen based on the newly determined MIC. For the lower concentrated bottle a 3 fold MIC concentration, was chosen. For the higher concentrated drug bottle the concentration was set to 7 fold MIC. At day 4 of the experiment, samples were taken from every vial, by opening the vial in the hypoxi-station and transferring 200 μ l into eppendorf tubes. Those samples were cultured over night in 5 ml diluted media and the next day the MIC was determined. The morbidostat experiment was stopped after 6 days.

3.3 Plasmid construction

Primer design

In total x plasmids were produced with the gibbson assembly cloning procedure. For every β -lactamase found in every sample of interest a plasmid was produced. As target sequences every β -lactamase sequence with it's upstream sequence until the previously located gene plus 50 additional base-pairs was chosen.

4. Results

4.1 Analyzing longitudinally collected isolates from patients of the University hospital Basel

4.1.1 Sample selection based on a pan genome analysis

The phylogenetic tree in 4.1 built with panX shows how closely related the pan genome of the different isolates is. Some isolates from the same patient (eg. Patient21, Patient8) have been isolated which map on different branches. This implements, that some patients got infected with a different strain over time. It's also visible, that some patients are affected by the same outbreak (different patients mapped on the same branch). Since we want to identify changes in the genome caused by evolution of drug resistance, it's important that the patient was infected with the same strain over the entire sample period. Therefor only patients with all the isolates mapped on the same branch in 4.1 could be included in the analysis.

What also had to be considered for selecting patients was that there was a significant change in the MIC over time for the isolates of the same patient. Usually MICs are determined by making a 1:1 dilution row, thus isolates where the MIC changed only by a factor of two, could not be included. Applying those criteria following samples were selected for the analysis:

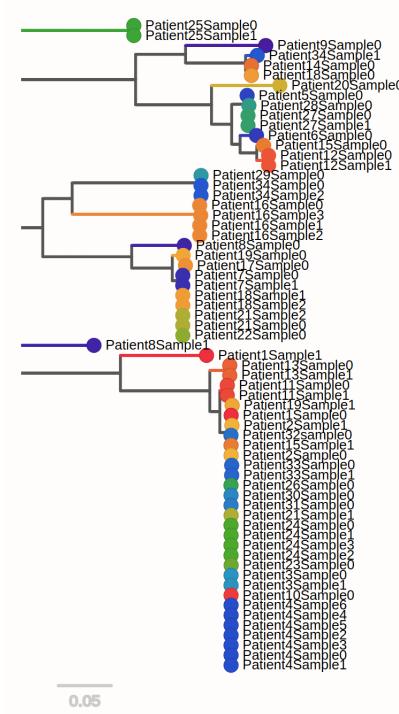


Figure 4.1: Phylogenetic tree build with panX visualizing how closely related the pan genome of isolate is.

Accession	Sample date	MIC Cefepim	MIC Cefepim Sandra	Ceftazidim
Patient12Sample0	9.9.14	4	16	0.75
Patient12Sample1	5.12.14	12	32	2
Patient16Sample0	22.6.12	8	32	2
Patient16Sample1	18.7.13	48	64	8
Patient16Sample2	1.11.13	32	32	12
Patient24Sample0	02.05.11	4	16	1.5
Patient24Sample1	08.15.11	16	32	1.5
Patient24Sample2	11.28.11	3	8	1
Patient25Sample0	15.4.11	64	None	192
Patient25Sample1	22.8.11	6	4	6
Patient33Sample0	26.9.14	24	None	16
Patient33Sample1	29.1.15	1	2	1.5

Table 4.1: Those patients have been selected because they differ significantly in MIC and are mapped on the same branch in panX

5. Discussion

asdfsadfsdfa

6. Declaration

asdfaggj

7. References

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8. Acknowledgements

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