

Morb
University of Basel

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1. Abstract

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2. Introduction

2.1 Cause and urgency of antibiotic resistance

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. It also made major advances in surgery possible. 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 [20].

Because of the positive experience people made during the Second World War with antibiotics, they quickly started to use them in an irresponsible way. This caused a Penicillin resistance within 32 years after it's discovery [20]. Therefore people realized early on, that extensive use of antibiotics are a threat for the efficiency of those drugs. But the discovery of new antibiotics gave them confidence and the excess of usage of antibiotics went on. Not only were antibiotics continuously prescribed inappropriately they also got introduced into agriculture. Unfortunately the tendency of irresponsible use of antibiotics increased in the last couple decades.

For Switzerland the rate of antibiotic consumption divided by the Swiss population stayed the same in recent times, but the absolute consumption increased. This is because more people were getting treated in hospitals [8] but the general population of Switzerland increased too. Furthermore antibiotic resistance is a global issue. Compared to other countries Switzerland is on the lower end when it comes to antibiotic doses per 1000 people [8]. Additionally other countries also have a very high use of antibiotics in agriculture, exposing a lot of bacteria to a lot of antibiotics, giving the bacteria an ideal environment to evolve and gain resistance. Since traveling started to get a lot more popular with long-distance traveling in particular, there is a globally ongoing exchange of resistant pathogens. This increases the difficulties of managing resistant pathogens because fighting the antibiotic resistance crisis locally is not going to help much because of this global exchange.

In Switzerland the biggest challenge with resistant pathogens is ongoing in hospitals. In such institutions, obviously a lot of people need treatment against bacterial infections, leading to an accumulation of pathogens but also to very locally applied high doses of antibiotics. Therefore Swiss hospitals turned into hot-spots of antibiotic resistant pathogens. Unfortunately a lot of people within the hospitals are weakened by diseases causing a worse functional immune system and therefore such patients are a lot more vulnerable to infections with antibiotic resistant bacteria. This combination of an increasingly number of antibiotic resistant bacteria and very vulnerable patients lead to a very urgent problem in modern medicine causing about 300 deaths, patients having to spend more time in hospitals and high costs [7].

2.1.1 An overview of antibiotic resistant pathogens in Swiss hospitals

Mainly four strains which cause problems in treatment with antibiotics are present in Swiss hospitals:

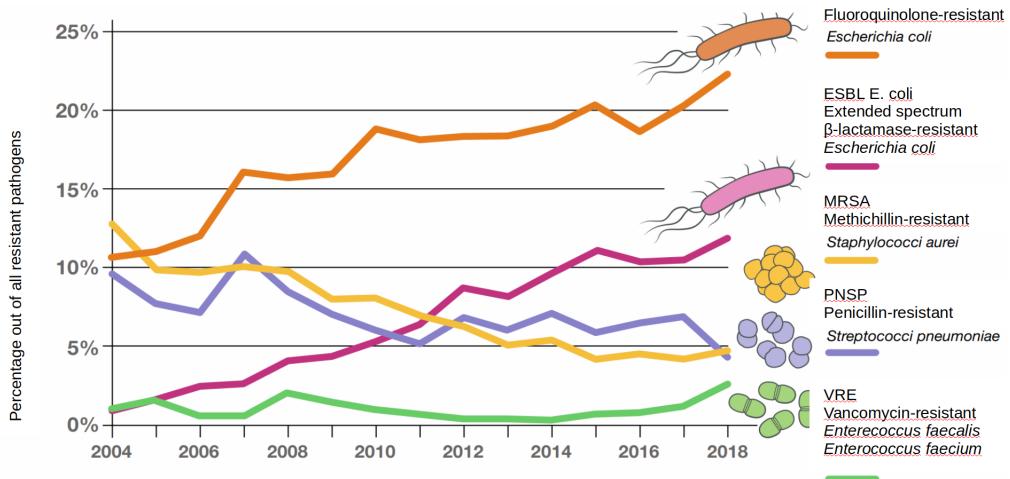


Figure 2.1: Development of popularity of antibiotic resistant pathogens. [9]

Staphylococcus aureus usually doesn't cause any diseases in healthy people. But if someone's immune system is weakened they can cause skin and bone inflammations. *Staphylococcus aureus* is causing the most infections for surgical wounds.

Streptococcus pneumoniae are present normally in the nose and throat of everyone. For weak people this strain can turn into a pathogen causing several thousands blood infections and pulmonary infections per year.

Klebsiella pneumoniae is one of the biggest problems for swiss hospitals. They can cause severe pulmonary infections and blood infections which can be very dangerous for newborns [9].

Escherichia coli (E. coli) belongs to the enterobacteriaceae and is present in every intestine where they are not dangerous. 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 brain of new borns.

Mainly *E. coli* and *Staphylococcus aureus* have been increasingly reported in context of antibiotic resistant infections over the last 15 years as visible in Figure 2.1. On the other hand antibiotic resistant infections with *Streptococci pneumoniae* and *Enterococcus* remained more or less the same or decreased slightly. It is noteworthy how fast the numbers of infections with extended spectrum β -lactamase (ESBL) producing *E. coli* increased within 15 years. In 2004 they only made up for about 1 % of all antibiotic resistant pathogens, 2018 they are already at 12 % with an uprising tendency. This is causing a lot of concerns and problems in hospitals because as the name already implies, those pathogens are able to hydrolyze an extended spectrum of β -lactams forcing doctors to prescribe antibiotics of last resort.

Because of this threat coming from ESBL *E. coli* I dedicate this thesis entirely to this pathogen. Unfortunately it is not completely understood yet, how those enterobacteriaceae are able to produce such a strong resistance against many β -

lactams. My goal is to generally get a better understanding of genes involved in the evolution of resistance and potentially identify new genes which could play an important role in the resistant mechanisms.

2.2 Extended-spectrum -lactamases (ESBL) E. Coli

Extended β -lactamases (ESBLs) are certain β -lactamases which are able to hydrolyze extended-spectrum cephalosporins with an oxyimino side chain. Such cephalosporins are aztronam, cefotxamie or ceftazidime for example [12]. Some ESBLs arose by mutations in genes for common plasmid-mediated β -lactamases such as TEM and SHV enzymes. Those ancestors were able to hydrolyze regular β -lactamases but were susceptible to cephalosporins [3]. There are also new families of ESBLs, such as CTX-M and OXA [10] which evolved independently from the ESBLs which have their origin in the TEM and SHV enzymes.

Those newly formed β -lactamases are increasingly reported as reasons for resistance, in contrast to the other β -lactamases related to TEM and SHV which generally decrease in popularity. CTX-M is a group of β -lactamases with CTX-M-1 being the most popular one. There are many more characterized CTX-M enzymes, most of them differ by just one amino acid. With a sequence identity compared to TEM and SHV of only 40 % [10], it's assumed that they evolved from a different precursor. 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. The OXA family was originally created as a phenotypic rather than a genotypic group, based on a specific hydrolysis profile. Therefore the sequence identity compared to other members of this family is only about 20 %. Its name comes from the ability to efficiently hydrolyze oxacillin [10].

ESBL E. Coli can be detected using isothermal amplification, PCR or microarrays. Since ESBL E. coli are able to hydrolyze cephalosporine, doctors prescribed carbapenems or potent β -lactam- β -lactamase inhibitor (BLBLI) combinations, when ESBLs were detected with the methods above. However it turned out that some E. coli expressing ESBLs are actually susceptible to oxymino-cephalosporins [12]. This showed, that the prescription of antibiotics of last resort because a pathogen tested positive for ESBLs was unnecessary in some cases. Since antibiotics of last resort are incredibly valuable it's in the interest of everyone to only use them if urgently necessary. Irresponsible prescription on the other hand will cause further resistance in a rather short time scale. Since a simple presence/absence check of ESBLs is not giving the necessary information in order to decide whether antibiotics of last resort are needed, those tests are now getting ignored as ordered by the EUCAST [4]. It's recommended to test susceptibility *in vitro* which takes about 48 hours which is a lot longer than genetically testing. For patients in acute stages of inflammation 48 hours is too long, forcing doctors to still prescribe antibiotics of last resort even when it's not known if the pathogen would be susceptible to common cephalosporins. Since some ESBL producing E. coli are genetically resistant but phenotypically susceptible there must be other mechanisms involved in the resistance path next to a simple presence or absence of β -lactamases.

Other ways to protect the pathogen from the drug could be a less permeable

outer membrane of the pathogen. It's also possible that resistant pathogens have a higher efflux activity, leading to an active transport of the drug, out of the cell. In combination this would mean a lower influx and a higher efflux, which would work in favor of the pathogen. Furthermore another option would be that the copy number of genes coding for ESBLs varies within ESBL E. coli, deciding over resistance or susceptibility.

With this thesis I try to get a better understanding on which strategies are involved in resistant ESBL E. coli. I'm doing this by combining two different approaches, one being a bioinformatic analysis with ESBL E. coli isolates from patients, the other being a more experimental approach.

For the first part of this project I try to identify changes in the genome from ESBL E. coli samples isolated from our collaborator Adrian Egli at the University hospital Basel. Those samples made a transformation from being susceptible to β -lactams to resistant, or they lost their ability to hydrolyze cephalosporins.

For the experimental part of this project I assemble a system called morbidostat which allows to continuously monitor E. coli cultures and automatically putting them under antibiotic pressure, by injecting appropriate drug doses. This causes evolution and selection which can be observed in real time. Along this process samples can be taken and analyzed with a similar pipeline as for the clinical isolates.

2.3 Longitudinally collected ESBL E. coli isolates from patients from the University hospital Basel

Our collaborator Adrian Egli, head of the clinical microbiology from the University hospital Basel, collected Isolates of patients who were infected with ESBL E. coli. His group determined the MIC of the cephalosporines cefepime and ceftazidime for every collected isolate and short-read sequenced thme on a MiSeq Illumina system. This resulted in a data 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. The principle of this analysis is, to build a very accurate reference genome for each patient based on the isolate with the lowest MIC for the tested cephalosporines. The isolate with the lowest MIC is also called wild-type. Then the other isolates with a higher MIC can be compared to this reference genome and changes can be identified.

This can be done by mapping the Illumina sequencing data from the resistant isolates to the reference genome from the wild-type. This makes possible to identify single-nucleotide polymorph positions (SNPs) in the genome of the resistant samples. Because tools for annotating genomes are available, it's possible to filter for SNPs which are affecting known genes or promoter regions. Based on this information it should be possible to get more insights in the evolution of resistance.

Since this analysis is depending on very accurately assembled reference genomes we additionally long-read sequenced isolates of interest with Oxford Nanopore Technologies. Illumina returns very accurate short reads (75 bp) but because the reads are so short it's computationally not possible to assemble those reads structurally correct. On the other hand reads produced with Nanopore Technologies are extremely long (up to 200 kbp) but quite inaccurate based on issues with repetitive

nucleotide sequences. However both techniques combined together return an assembly with a nucleotide error rate from Illumina but also with a high accuracy in terms of structural information. This strategy of combining short- and long-reads is also known as hybrid assembly.

2.4 Building a morbidostat

Since we wanted to experimentally force susceptible ESBL E. coli to gain resistance over a rather short time period, we had to come up with a system which allows to constantly put E. coli under antibiotic pressure. Such constant antibiotic pressure forces evolution of resistance and for selection of mutants. Such a system has already been invented by Toprak et al. [1] which he called morbidostat, an automated continuous culture device. Based on the invention of Toprak we rebuild a morbidostat with small modifications which should improve the handling and mainly make it a lot more compact which is important since we want to use the morbidostat in a space limiting hypoxi-station. With our version of 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). Based on the growth an appropriate dose of antibiotics is injected into the culture leading 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. Since the goal is to identify changes in the genome while the strain gains resistance, samples have to be collected every other day, in order that the DNA can be isolated and sequenced.

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
- 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
- Getting rid off volume which exceeds fixed culture volume

2.4.1 Drugs of interest and bacteria of choice for the experiments

Choice of ESBL bacteria for the morbidostat experiments

Initially the idea was to cultivate the wild-type isolates from the sample collection provided from Adrian Egli and which were also used for the bioinformatics analysis. This would have been nice, because it would have allowed to compare evolution taking place under physiological conditions and forced evolution by the morbidostat.

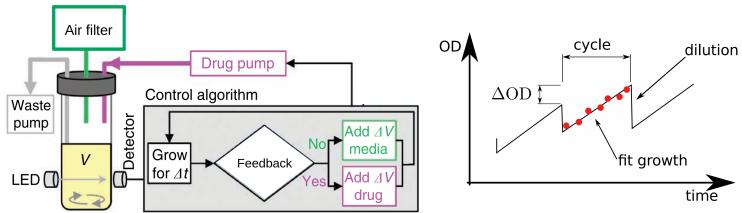


Figure 2.2: Based on the growth a feedback algorithm decides whether and how much drug is getting injected

Even though the morbidostat is run inside an air tight hypoxystation within a bio safety lab 2, it was decided that it's too risky to do the experiments with the ESBL E. coli coming from the hospital. Instead we decided to clone the β -lactamase sequences from the wild type samples into a plasmid transformed into K12 E. coli which is not able to infect humans.

Concerning the drugs, cefepime and ceftazidime were chosen, since those drugs were also used for the treatment of the ESBL E. coli from the sample collection from the hospital.

Both cephalosporines are effectively bactericidal against gram-negative bacteria and act by inhibiting the synthesis of the peptidoglycan layer of gram negative bacterial cell walls. Cefepime is regarded as a 4th-generation cephalosporin and was developed with ceftazidime as a foundation. Resistance against ceftazidime often emerged due to hydrolysis by the AmpC β -lactamase. Cefepime was designed with a change in its 3' side chain, which should protect the drug from hydrolysis [2].

3. Materials and methods

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

3.1.1 Sample collection and selecting patients of interest

Adrian Eglis group collected 65 isolates from 34 patients over a time span of approximately four and a half years. Sample collection from one patient happened within several months. In order to find out that a certain patient was infected with the same strain over the sample period a phylogenetic tree was build with panX which visualizes how closely related the pan genome of different isolates is. This was done by assembling every isolate with its short-read Illumina sequences provided by Adrian Egli using spades (v3.12.0) [16]. Every resulting assembly was annotated using prokka (v.1.12) [18]. The genbank file returned from prokka was used to run panX [11].

3.1.2 Sequencing and assembling of the reference genome

Nanopore sequencing

Every sample of interest (4.1) was 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 description of the protocol is available in Nicholas Noll publication [5].

Illumina sequencing

Illumina sequencing was done by our collaborator Adiran Egli from the University hospital of Basel. His team extracted the DNA from cultured isolates 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 preparatino kit (Illumina). The resulting library was sequenced using a MiSeq Illumina platform [5].

Assembling of the reference gemoe

Before assembling trimming was performed for all the Illumina reads using Trim Galore [19]. Both sequencing data from Illumina and Nanopore were combined into a hybrid assemly using Unicycler [6]. This was done for every isolate with the lowest MICs for every patient and defined as reference genome.

3.1.3 Identifying SNPs

Based on the reference genome built according to the section 3.1.2 pile ups were created with every sample for each patient. This means, that the Illumina reads for every isolate of each patient were mapped to the reference genome which was stored as a bam-file. As a mapper BWA MEM [13] was used. The pile ups were produced with a custom script written by Richard Neher. 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, using the CIGAR-code of the read. This resulted in a count matrix for every position in the reference genome allowing to calculate base frequency and the coverage for every position. The python pileup.py script used to produce the pile ups is available on GitHub.

Pile ups in form of count matrices were calculated for every sample. Since they were produced using the same reference sequence per patient, all the pile ups for each contig from every sample could be stored in one matrix, giving a list of matrices per patient. This was very handy, because this data structure allowed to easily compare positions over different samples.

Using the pile ups every position for every patient was checked for variance with another script called analysis_modular.py. Since many positions showed variance caused by sequencing and mapping errors filtering was applied by setting a threshold for coverage and base frequency. As such thresholds 30 was chosen for the minimum coverage and 0.8 for the minimum base frequency.

Annotating the genomes

In order to possibly find out what in in the genome is affected by the SNPs, the genes were identified using prokka [18]. Additionally promoters were classified using the promoter prediction tool PePPER [14] and the promoter data base hosed on EcoCyc [17]. With this annotation it was possible to identify some genes and promoters altered by SNPs.

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

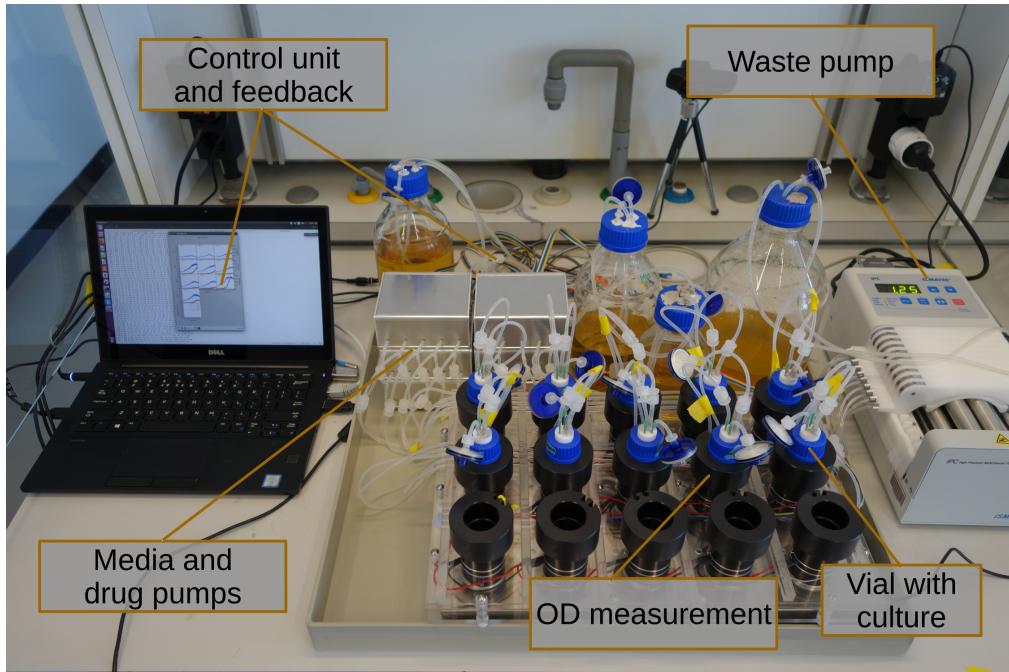


Figure 3.1: Overview of the morbidostat setup. Behind the drug and media pumps the arduino is located.

In Figure 3.1 the morbidostat setup is shown. Only 10 cultures were used but potentially 15 cultures can be monitored and inhibited with this setup. On the magnetic stirrer which caused constant mixing the electronics for measuring the OD was placed integrated in a vial holder. In this holder the vials sit and were connected to a media bottle, two drug bottles with different concentrations, one waste pump and an air filter. The connection was made using silicon tubing and plastic luers. For controlling the hardware an arduino was used, who processed information and executed commands fed from a python program which ran on a laptop. In Figure 3.1 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.

OD measurements

For measuring the OD a combination of a light emitting diode and a phototransistor was chosen. The principle is, that at lower ODs more light could get through the culture which caused a bigger opening in the semiconductor from the phototransistor. This led to more flowing current in the base region, which could be detected with the Arduino. Since a current was detected this implemented, that a calibration was necessary in order that the translation to an OD could be done.

As a light emitting diode OPB608A was chosen from TT electronics with a peak wavelength of 890 nm. For detection a PT 333-3C phototransistor was used. The 15 OD-measuring installations have been split up into three parallel-connected chains representing one row of 5 vials. Each chain was connected to a 5 V power supply and grounded as shown in Figure 3.2. To limit the current a resistor of $?\Omega$ was

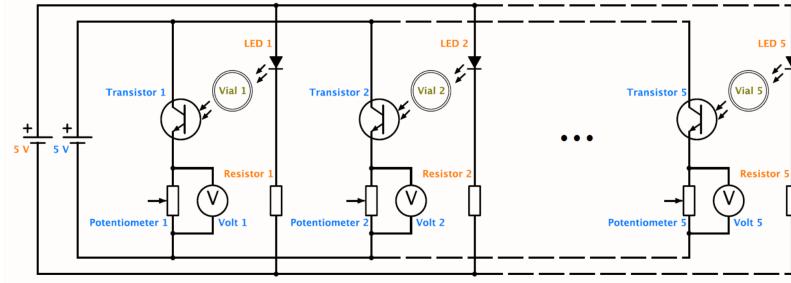


Figure 3.2: Left figure: Circuit diagram of parallel-connected LED (orange) and phototransistors (blue). This circuit is done independently three times for five vials each. The detector and the LED are orientated in a 135 °angle since this is the best angle for light detection. Right figure: vial setup

added for every chain. The current was getting measured by an analog pin, which was connected to a shield mounted on the arduino. In order that sensitivity could be changed a potentiometer was added in serial before the current measurement. It turned out that the system was not as sensitive as thought in advance, so all the potentiometers were opened as much as possible.

Vials and tubing

Every vial was connected to three injecting pumps and one pump which sucked off the waste, meaning every volume which was above the desired culture volume as shown in Figure 3.3. The three injecting pumps were able to inject media or two different drug concentrations. Since it was a lot easier to make one inlet in the vial instead of three, the tubes which are injecting fluid were connected before the vial, leading to only one inlet. Because fluid getting added an air filter had to be mounted, necessary for equalizing air pressure.

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.

The functional principle is based on a piezoelectric diaphragm in combination with passive check valves. By applying voltage the membrane is deformed, when the voltage decreases again the membrane drops down [15]. 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.

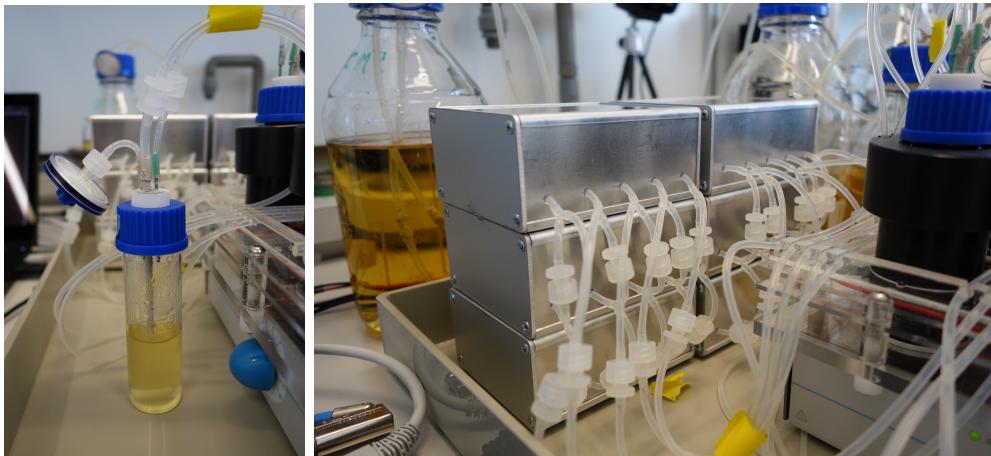


Figure 3.3: Figure left: Vial setup. Figure right: One row of five pumps represents one row of five vials. Since every vial is connected to three pumps, three rows of pumps are stacked on top of each other and connected column by column. This means that one column of three pumps is responsible for injection 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.

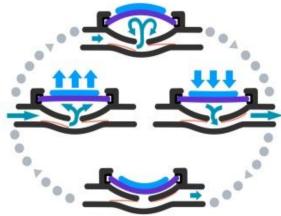


Figure 3.4: Principle of the mp6 pump but two of those piezoelectronics are connected in serial for this pump model [15]

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. Unfortunately there was always a very small current flowing through the digital pins of the Arduino even when the pin was set to low. This caused problems with the controller of the pumps which could be solved by adding a pull-down resistor between the digital pins and the ground from the Arduino. 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 control

The controlling of the morbidostat was set up as an interaction of python programs running on a laptop and the .ino code running on the Arduino. The communication between the different types of code is possible because strings can be sent with the arduino to the laptop which can be interpreted with the serial package from python. On the other hand, strings sent from python can be interpreted with the Arduino. Even though it was necessary to thread the information sent with python in order to not create an overflow of commands on the arduino.

There were mainly several python scripts running on the laptop, one called arduino_interface.py which was handling the communication with the Arduino and another script called morbidostat_experiment.py which was responsible for the feedback, meaning that it calculated the necessary runtimes of the pumps for each vial. This program was also responsible for constantly initiating the cycles. With a third script morbidostat_setup.py the experiment was initialized at the beginning and with morbidostat_monitor.py information produced from the morbidostat such as OD and drug injections could be monitored. Every script is available on GitHub.

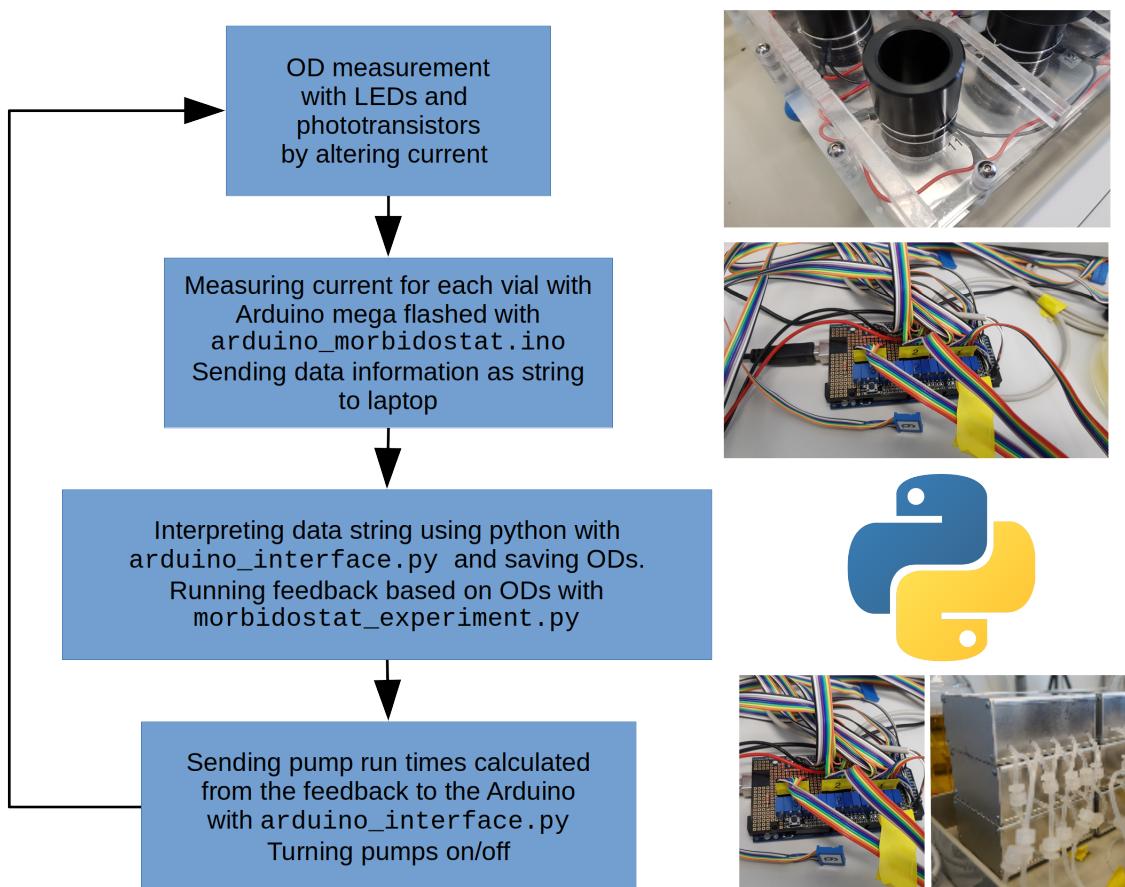


Figure 3.5: Overview of the morbidostat control. The arduino_morbiostat.ino was running in a constant loop() measuring the currents for each vial. Threading had to be set up in the arduino_interface.py in order that the Arduino didn't crash. The ODs and the injections could be monitored with morbidostat_monitor.py. Changes in the control could be done by changing variables made possible by running morbidostat_experiment.py in the interactive ipython mode.

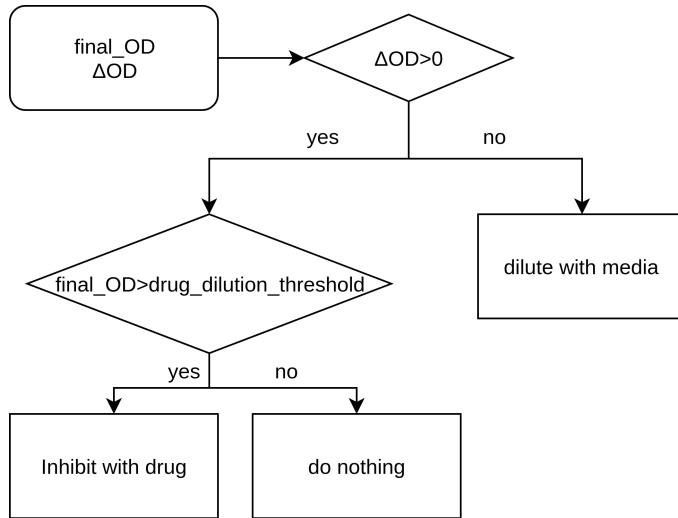


Figure 3.6: If ΔOD is negative, implementing that the cells are dying, media is getting added in order to reduce drug concentration. If it's positive the final_OD is compared to the drug_dilution_threshold, if it's bigger the cells are getting inhibited with drug, if it's smaller no liquid is added.

Feedback

For successfully putting the culture under antibiotic pressure it was a necessity to have a well working feedback which decided whether and how much drug was getting added to the culture. Every OD measurement in one cycle was averaged for every culture and called final OD. Using the final OD the growth of each culture could be described using following formula:

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

It was important that the feedback didn't inject drug when the cultures were at a very low optical density because drug injection would may led to complete sterilization. Therefor a threshold was introduced which was called drug_dilution_therschold, which prevented drug injection if the final_OD was below the OD defined in the drug_dilution_threshold. Furthermore the goal of the feedback was to keep the cultures at defined OD. This desired OD was called target_OD. Defining this 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. Combining described variables above the feedback was implemented in morbidostat_experiment.py as shown in 3.5:

3.2.3 Experimental procedure

OD and pump calibration

As a media for all experiments a mixture of 1/10 LB media and 9/10 H_2O was chosen. All the culturing of the bacteria was done in a 37 ° incubator. For calibrating the OD measurements an overnight culure 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: XXXXXXXXXXXXXXXXXXXXXXXXX

Then every vial with 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 µl was pipetted in every well from the 96 well expect in the wells from the last column of the plate. Additionally 100 µl of MHB was added to every well. As a next step 100 µl from the prepared drug solution was added to the first column of the plate. Then 100 µl 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 te feedback were determined using the simulator. As a drug amoxicillin was chosen, it's 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

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. Therefore 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:

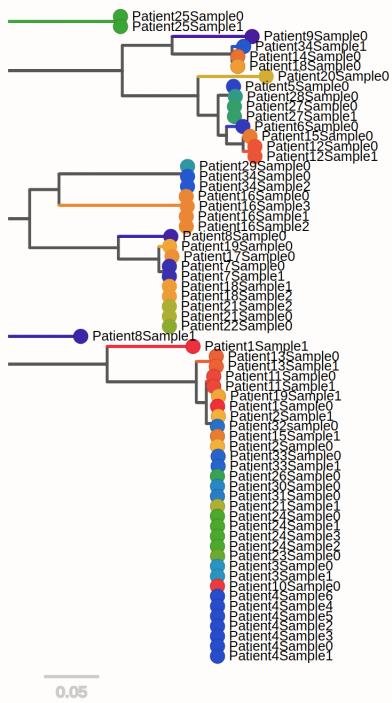


Figure 4.1: Phylogenetic tree build with panX visualizing how closely related the pan genome of isolate is.

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

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6. Declaration

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7. References

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8. Acknowledgements

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