Virtual Practical "Antibiotics: Targets and resistance mechanisms – the MEGA-plate experiment"

Introduction Antibiotics

Welcome to the virtual practical on the spontaneous evolution of antibiotics-resistant bacteria on a MEGA-plate. Here, we will first briefly discuss antibiotics and their characteristics, then we will have a look at the experimental setup of the MEGA-plate and finally analyse and discuss the results of the experiment.

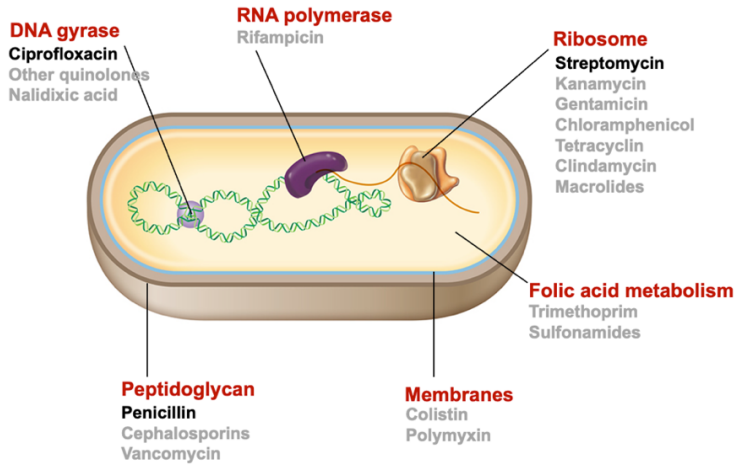
Among the many microorganisms only a small number of organisms are pathogenic to humans, animals or plants. These microorganisms are very important for human and veterinary medicine and in plant pathology. Antibiotics are antimicrobial substances that can be used to fight infectious diseases. Antibiotics are produced by particular microorganisms. A lot of antibiotics are also chemically modified in order to increase their effect or to stabilise them.

Of the thousands of antimicrobial substances that have been identified to date, only few are suitable for therapeutic use. These substances have revolutionised modern medicine as they allow to fight infections caused by bacteria and fungi. Dosage and duration of the therapy must be adapted to the respective pathogen. At the same time, possible side effects towards the host should be minimised.

Q1: Why do you think microorganisms produce antibiotics themselves? Write down a keyword.

Antibiotics exert their antibacterial effects by targeting different essential structures and processes in bacterial cells. For medical applications, it is important that an antibiotic does not interfere with structures or processes in human cells. In the following figure you can see that e.g. the antibiotics **Penicillin** and **Ciprofloxacin** target the biosynthesis of the bacterial cell wall and interfere with DNA gyrase, an enzyme involved in replication of circular DNA, respectively.

Q2: Explain why these antibiotics are not supposed to target human cells.

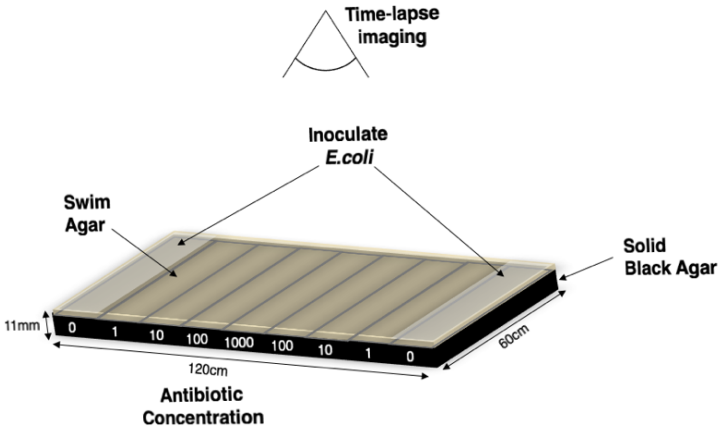


Q3: Let's repeat what we have learned until now. Decide which statement(s) about antibiotics is/are true.

- ☐ *Every antibiotic works against all kinds of bacteria.*
- ☐ *Some bacteria are naturally resistant to some antibiotics.*
- ☐ *Antibiotics are secondary metabolites (natural products) of microorganisms that are directed against other microorganisms.*
- ☐ *Bacteria can obtain antibiotic resistance by mutations or gene transfer.*

The MEGA-plate Experiment

In order to study how bacteria evolve resistance to antibiotics under laboratory conditions, scientists developed a device called the MEGA-Plate (MEGA stands for Microbial Evolution and Growth Arena). The MEGA-Plate is a bacterial growth plate similar to a petridish. It is filled with black-colored nutritious agar overlaid by soft agar allowing bacterial motility.



The MEGA-Plate is divided into sections with different amounts of antibiotics. The sections at either end has no antibiotic. The first sections toward the center has a concentrations of antibiotic to just inhibit growth of the wild-type of the bacterium (indicated with 1x). The next two sections toward the center have an even higher concentration of the antibiotic (10×, 100×). The center section has the highest concentration of antibiotic (1000×), which is 1000 times higher than the 1× sections near the ends. The bacterium *E. coli* is then added into the sections at the ends without antibiotics. Using time-lapse imaging from above one we then observe how the bacteria grow and spread over time - over ten days to be exact.

In the original experiment the researchers used two different antibiotics, each in a separate MEGA-plate experiment: Trimethoprim and Ciprofloxacin.

Q4: How do these two antibiotics act? See the figure on page one for help.

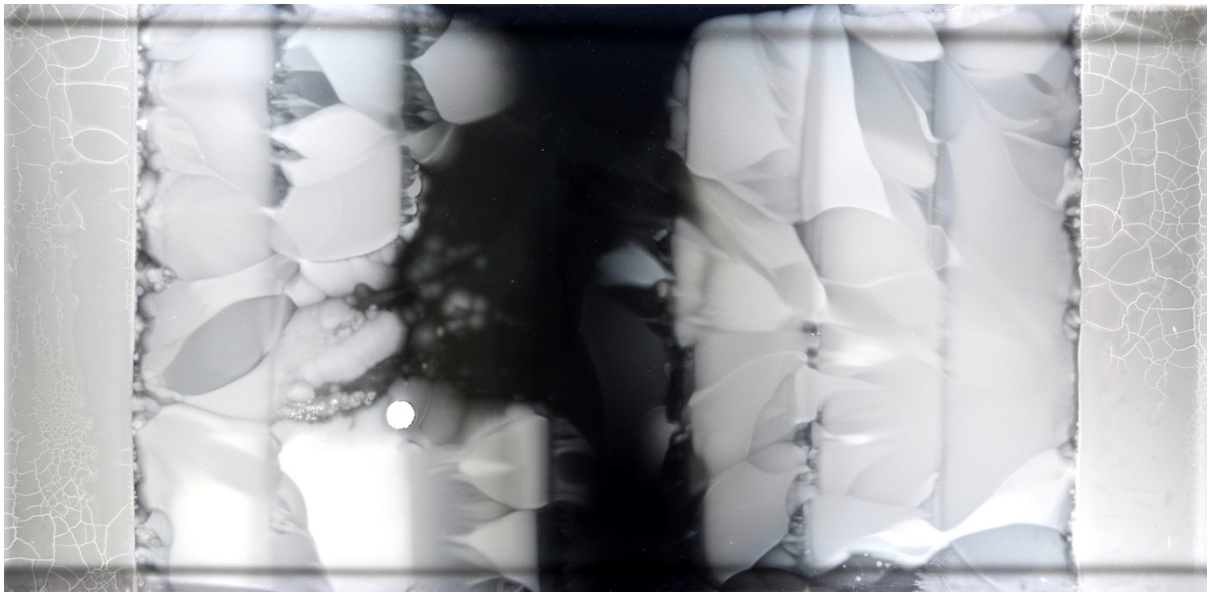
- ☐ *Ciprofloxacin interferes with DNA replication and Trimethoprim with the bacteria's metabolism.*
- ☐ *Ciprofloxacin inhibits membrane synthesis and Trimethoprim interferes with protein synthesis.*
- ☐ *Trimethoprim interferes with the cell wall synthesis, Ciprofloxacin by inhibiting DNA replication.*
- ☐ *Both interfere with the bacteria's metabolism.*

Let's have a look at the MEGA-Plate from above shortly after inoculation. Each of the sections is roughly 13 cm x 60 cm big. The bacteria will be seen as a white surface on the black agar.

Q5: What do you expect to happen within the next 240 hours?

- ☐ *E. coli will grow over the areas with no or low concentrations of antibiotic and stop there.*
- ☐ *E. coli will populate only the area without antibiotics.*
- ☐ *E.coli will populate a major part of the plate but unequally distribute within different sections.*
- ☐ *E. coli will start to grow over the whole MEGA-Plate and cover it equally.*

The following image shows the MEGA-plate after roughly ten days of incubation.



0 1 10 100 1000 100 10 1 0

Antibiotic Concentration

Q6: Take a close look at the picture and write down two things you notice about the plate. Remember the black part is agar which is still unpopulated and the white parts are bacteria.

First, we will check out different stages of growth in more detail. Watch the following video from the research group that invented the MEGA-Plate experiment and answer the following questions: **The Evolution of Bacteria an a Mega-Plate» Petri Dish**. https://www.youtube.com/watch?v=plVk4NVIUh8&t=2s

Q7: When do the bacteria start to mutate?

- ☐ *They always mutate.*
- ☐ *When there are no more nutrients.*
- ☐ *When they can no longer survive.*
- ☐ *When there is no more space.*

Q8: At some point several mutants which are resistant to the antibiotic to a certain extent, appear. How do these mutants interplay?

- ☐ *They compete with each other for nutrients.*
- ☐ *They compete with each other for space.*
- ☐ *They cooperate to get to the next boundary.*
- ☐ *They kill each other.*

Q9: What happens when a colony reaches the boundary to the next higher concentration of antibiotic?

- ☐ *Only a few bacteria can build a new colony because they acquired a mutation by chance.*
- ☐ *Only a few bacteria can build a new colony because they acquired a mutation on purpose.*
- ☐ *Only a few bacteria can build a new colony because of spatial shortage.*
- ☐ *Only a few bacteria can build a new colony because of nutrient shortage.*

As we look at the results of the experiment it is crucial to remember the following things:

1. All living organisms acquire mutations in their genome by a variety of mechanisms (UV, chemical mutagens, DNA replication errors etc.). In bacteria and archaea (as in all other organisms), this process is a major driver of evolution. The haploid state, the unicellularity, an error-prone DNA replication, the small size and very short generation times of these microorganisms lead to huge populations containing a considerable number of mutant cells on which selection can act. Under normal growth conditions, these mutants are often not discernable by phenotype and cells with detrimental mutations are not present in the population as the respective cells die and do not divide. If one applies changed growth conditions to such a population, however, some of the mutant cells might have an advantage and be able to do things that the other cells of the population are not - like for example deal with a certain amount of antibiotic.
2. Mutations never develop on purpose but by chance.
3. Organisms are constantly competing for resources like nutrients and space to live. Because they require the same resources members of the same species are often the biggest competition.

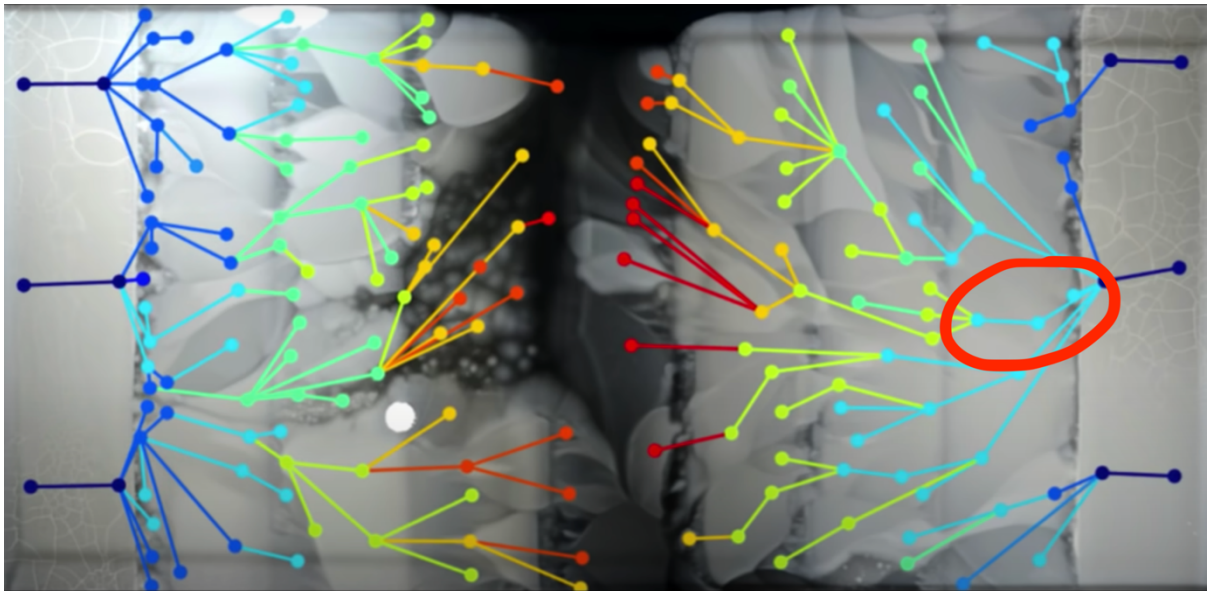
Looking at the final image of the MEGA-Plate again (see page 3), we can see that some bacteria proceeded to the middle of the plate where the concentration of the antibiotics is 1000 times higher than the wild-type strain of *E. coli* could barely tolerate.

Q10: What does this implicate for these bacteria?

- ☐ *These bacteria are genetically different from the wild-type E. coli that was inoculated at the start.*
- ☐ *These bacteria are have a better chance of survival in any of the regions of antibiotic concentrations than other colonies on the plate.*
- ☐ *These bacteria genetically differ from most other bacteria on the plate by just one crucial mutation.*
- ☐ *These bacteria are the only ones that developed resistances against the antibiotic.*

Developing Resistances

Let's have a look at the image from the end of the video depicting the bacterial lineages that can be followed on the plate.

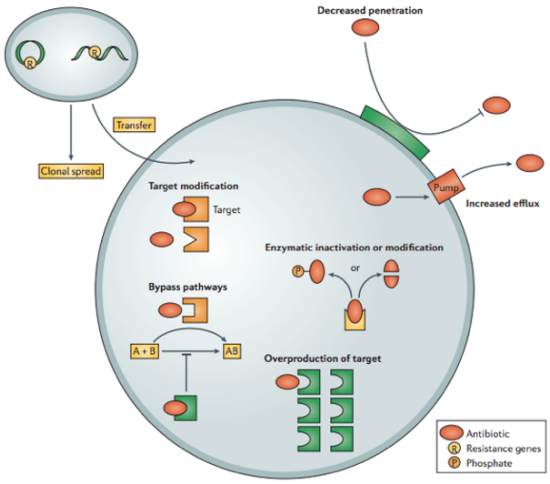


By sampling bacterial clones from the plate, researchers could track a path of accumulating mutations leading to antibiotic resistance by the colonies over the plate. They did so by taking bacteria from the plate and measuring the minimal inhibitory concentration (MIC) with Trimethoprim. They could then group the bacteria by their MICs (the different MICS are indicated by different colors in the figure), showing that the resistance increased within the lineages towards the center of the plate. They later also genotyped bacteria from the plate and found that bacteria accumulated mutations when following the lineages towards the center of the plate. This demonstrates that the resistant bacteria aquired serial mutations.

Q11: The MEGA-plate was designed in a way so that the concentration of the antibiotic increases from the sides, where the bacteria start, to the center. Why do you think it is important to have an increasing concentration of antibiotics? What would you expect to happen if there was no intermediate concentration (only no antibiotic and the highest concentration)?

As we focused on bacteria becoming resistant against a certain concentration of antibiotic, we would like to dig a little deeper into this observation. Remember, one of the antibiotics used in the experiment was called **Ciprofloxacin** the other one **Trimethoprim**.

Q12: How could the bacteria have developed a resistance against one of these drugs? Check out the graph on the right and the one on page 1 for some help.



In the actual experiment, researchers found frequent mutations in the gene *dnaQ*.

Q13: Google the function of this gene and explain why this mutation might be found so frequently.

The researchers also found frequent mutations in the following genes:

- *folA*, encodes dihydrofolate reductase and is the primary target of Trimethoprim. This is a classic case of target modification.

- stress response genes, e.g. *mar*, *sox,* these are known to be important in general antibiotic and toxin resistance.

Evolution at Warp Speed

The fact that bacteria have evolved a resistance against an antibiotic within 10 days indicates that evolution is taking place right in front of our eyes. In a next step, we will try to explain HOW bacteria evolved this resistance by using an Explanation Table.

We will look at four components of a complete explanation about evolution by "natural" selection and try to find evidence for them in this experiment. The four components are listed in the table below.

Q14: Write down a short description of each of these components ***not related*** *to our experiment yet.*

Q15: Now think about evidence from our experiment for the four components of evolution. Write down as many points as you can think of for each component. If you get stuck you may want to have a look at the following quick videos:

Bacterial Growth: https://www.youtube.com/watch?v=z43HNp3zVAA&t=1s

Penicillin Killing Bacteria: https://www.youtube.com/watch?v=l92fX0Yrnpc&t=1s

Condition	Description	Evidence
Variation		
Inheritance		
Differential survival and reproduction		
Adaptation		

Size Matters

Q16: The original MEGA-plate had a size of 120 cm x 60 cm. This large size serves two purposes. Before you continue, write down two ideas why the plate was designed that big?

The dimensions chosen by the researchers are one way of designing the experiment. What would you expect to change, if the dimensions were different?

Q17: What if the front of the plate would have been wider? So for example 120 cm x 80 cm instead of 120 cm x 60 cm.

- ☐ Due to the increased space, bacteria would have more spacial and nutritional supply and therefore compete less with each other, which leads to less selective pressure.
- ☐ The steps between regions of antibiotic concentrations would be bigger and therefore selection among adjacent colonies would increase.
- ☐ This aspect of dimension has no influence on the outcome of the experiment.
- ☐ The population size would be increased and therefore the supply with mutants is bigger.

Q18: What if the plate would have been longer? So for example 150 cm x 60 cm instead of 120 cm x 60 cm but with the same number of regions with different antibiotic concentrations.

- ☐ The population size would be increased and therefore the supply with mutants is bigger.
- ☐ Due to the increased space, bacteria would have more spacial and nutritional supply and therefore compete less with each other, which leads to less selective pressure.
- ☐ The steps between regions of antibiotic concentrations would be bigger and therefore selection among adjacent colonies would increase.
- ☐ This aspect of dimension has no influence on the outcome of the experiment.

We have covered many details about the experiment itself, the results and explanations thereof. If you are interested in the original publication by Baym et al., you can have a look at it here:

https://science.sciencemag.org/content/353/6304/1147

If you are interested in summary of this part, here we have Prof. Kishony himself explaining what happens during the experiment.

https://www.youtube.com/watch?v=Irnc6w_Gsas

As a final step, we would like to go back to one crucial point: the actual emergence of antibiotic resistances. To start, answer the following question:

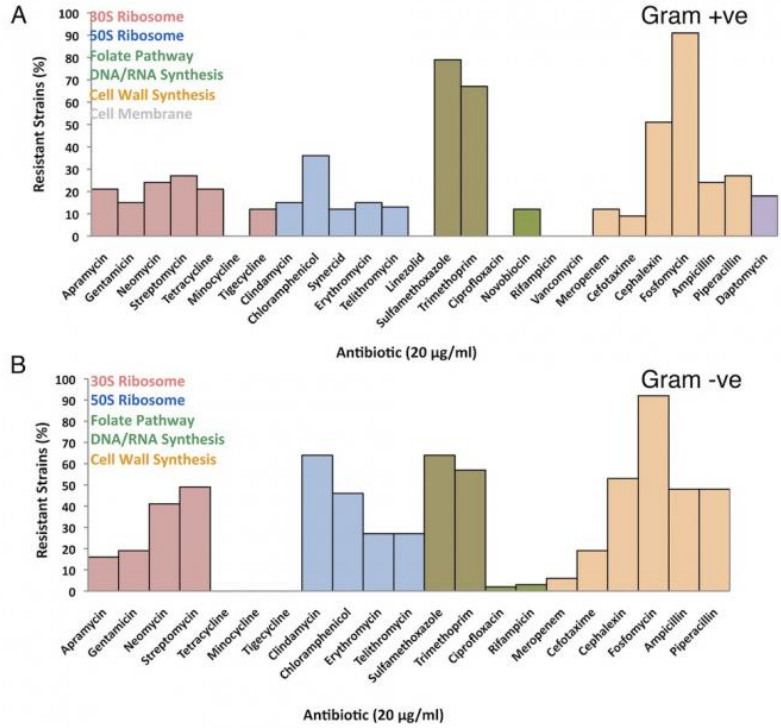
Q19: When do bacteria develop a resistance against a specific antibiotic? Tick all correct answers.

- ☐ When they are confronted with the corresponding antibiotic.
- ☐ When they compete against each other for resources.
- ☐ When there is any kind of selective pressure (e.g. other antibiotics).
- ☐ Whenever they can divide and grow.

Have a look at the following graph. Different strains of bacteria found in the Lechuguilla Cave in New Mexico, a location that had never been exposed to humans or modern antibiotics, were tested for resistance to a variety of antibiotics. The scientists collected over 500 unique bacterial strains from three locations in the cave. Of these, 93 culturable strains were randomly selected to be screened for resistance to modern-day antibiotics. Each strain of bacteria was tested by placing it on growth medium that contained a high concentration of an antibiotic. If the bacterial strain could reach a cell density of at least half that achieved by the same strain growing without antibiotic, it was considered resistant to the antibiotic.

The antibiotics are grouped by color to show the similar mode of action/target they employ to kill bacteria (as Prof. W..D. Hardt did on his slide on common molecular targets of antibiotics, in Prokaryotic cell biology II). Graph A shows the resistances in Gram-positive strains of bacteria, graph B the resistances in Gram-negative strains of bacteria.

Q20: What's striking about this experiment?



The rapid emergence of resistant bacteria worldwide threatens the efficacy of antibiotics. After being discovered by Sir Alexander Fleming in 1928, antibiotics were first prescribed in the 1940s and Penicillin was successful in controlling bacterial infections among soldiers in World War II.

However, shortly thereafter, penicillin resistance became a substantial clinical problem. Although new beta-lactam antibiotics like methicillin were discovered, it only took a few years before the first case of methicillin-resistant *Staphylococcus aureus* (MRSA) was identified in the UK in 1962. Unfortunately, resistance has eventually been seen to nearly all antibiotics that have been developed. As early as 1945, Sir Alexander Fleming raised the alarm regarding antibiotic overuse when he warned that the “public will demand [the drug and] … then will begin an era … of abuses.”

Overuse and misuse of antibiotics to treat infectious diseases as well as extensive use in agriculture contribute to the promotion of resistant bacteria. In the exercise by Prof. Hardt in the lecture “Fundamentals of Biology 2: cells” next week, you will discuss how antibiotic resistances emerge and how the antibiotic crises could be overcome.