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BACTERIAL GENETICS LAB: BASIC SKILLS

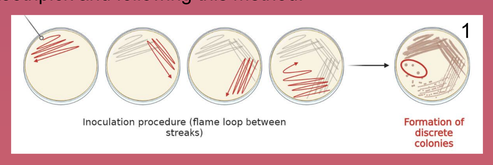
In order to be able to carry out different experiments in our lab, we had to acquire some basics skills:

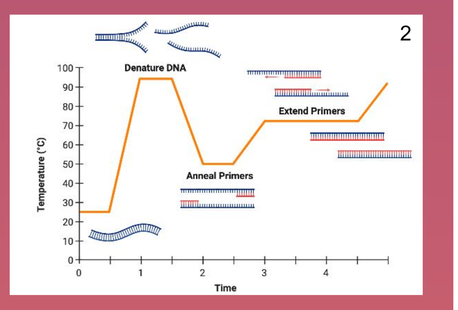
- **How to mix LB broth powder, sometimes agarose and water in the right proportions (20g/L each) to make media for the bacterial cultures.**

- **How to pour LB agar plates with and without antibiotics, including pouring gradient antibiotic plates that let us see at which antibiotic concentration the colonies stop growing.**
  For that, we have to pour the warm and still liquid LB agar (with a certain concentration of a specific antibiotic if necessary) in the empty plate with a 10mL pipette and a pipette gun.
  For the gradient plates, we first pour the liquid LB antibiotic agar on a tilted plate and when it solidifies, then we pour the plain LB agar and finally we let the antibiotic diffuse vertically inside the LB agar in other to get a large concentration of antibiotic in one side of the plate and almost none on the other.

- **How to inoculate culture tubes**
  We pick colonies from an LB agar plate or a liquid culture with a sterile toothpick and put them in a tube with liquid LB mix, then let them grow overnight at 37 degrees.

- **How to streak LB agar plates**
  In order to obtain well-isolated colonies, we pick bacteria cells from a culture tube or another LB agar plate and streak them on new plates using a toothpick and following this method:





- **How to get DNA copies of a specific locus using polymerase chain reaction (Colony PCR with a Taq polymerase)**
  PCR consists of a series of cooling and heating processes that allow the DNA to melt (when heated) and the polymerase enzyme to replicate said DNA (when cooled down). This way, the chosen sequence of DNA can be quickly and exponentially amplified. For that to happen, we have to put a small sample of the target DNA, reverse and forward primers, the Taq polymerase and some other components into a tube that will go to the thermocycler, where it will experience drastic temperature changes.

- **How to visualize the difference in length of different fragments of DNA using the gel electrophoresis technique**
  This technique is used to separate DNA fragments according to their size. An electric current is applied to an agarose gel that has wells in which we can load the samples of DNA. These samples are negatively charged, so they are pulled towards the positive electrode and they move more or less depending on their mass. That way, we can compare longer samples of DNA (that have more mass and therefore wove less) with shorter ones. We can also compare them to a DNA ladder, a standardized mix of DNA fragments of known sizes.

TRANSDUCING A GENE DELETION VIA P1 BACTERIOPHAGE:

Transducing an antibiotic resistance gene via P1 bacteriophage consists of moving an antibiotic resistance gene that has replaced (deleted) a gene of interest from one strain of bacteria to another using a viral vector; in this case, the **bacteriophage P1**. This virus infects **Escherichia coli** cells by injecting its own DNA into the cell, who will be replicating the viral genome along with the bacterial genome as the cell divides - a process known as "**lysogeny**". However, there is another process called **"lysis"** in which one the cell's replication machine is hijacked and creates many copies of the virus genome. The amount of copies will eventually overflow the interior of the cell and make it explode and release the newly created bacteriophages into the environment.

To **transduce the antibiotic resistance gene**, we will take advantage of this process. The phage's DNA will be replicated and packed into the protein shell of the capsule, but, because this is an imperfect process, some bacterial genes been chopped into same size fragments of the phage's DNA, will be packed instead of the phage's DNA. Then, when these capsules go to infect another cell, they will inject the **bacterial genome**. If the fragment contains the **Kanamycin resistance gene**, we will be able to select the population of recipient cells who integrated that gene using an antibiotic selection step. To stop the phage's activity and avoid the collapse of the culture, as the P1 phage needs Calcium ions for infection, we will remove the available Calcium ions via chelation with Sodium Citrate.

In our project, our donor strains were KEIO, **JW2662 luxS** (ΔluxS) and **JW2703 mutS** (ΔmutS), and our recipient strains were **+lexA ind-** and **MG1655 ΔtolC, ΔfliC, prmCherry:attp21** (ΔtolC). The goal was to create four new strains that had the Kanamycin resistance gene of our donor strains.

STRAINS		Donors	
		ΔluxS	ΔmutS
Recipient	+LexA ind-	+lexA ind-, ΔluxS:KanR	+lexA ind-, ΔmutS:KanR
	ΔtolC	ΔtolC, ΔluxS:KanR	ΔtolC, ΔmutS:KanR

First of all, we prepared the phage lysate of the donor strains by collapsing the cultures with the P1 phage. We transferred the donor overnight cultures into Eppendorf tubes and centrifuged them. Finally, we collected the supernatant in fresh tubes and we stored them at 4 degrees, those were our phage lysates.

To transduce the antibiotic resistance marker, we made a media with 5M $MgSO_4$ solution, 5M $CaCl_2$ solution and LB. We centrifuged our overnight culture of our recipient strains and we re-suspended them in the new media. We added the phage lysate of the donor's strains and we incubated the tubes for 1 hour at 37 degrees. Then we centrifuged and resuspended the tubes again, but in a 1M Na-citrate and LB solution, in order to remove the unbound phage particles. We incubated our tubes for 60 minutes at 37 degrees to allow the expression of the antibiotic resistance gene. Eventually, we resuspended the cell pellets in 100mM Na-citrate media and we spread the re-suspended pellets onto **Kanamycin plates**. We let them grow in an incubator all night at 37 degrees. The next day we re-streaked them onto a Kanamycin plate with a chelator to make sure there's no phage contamination in our cultures.

In order to ensure that the new strains had the mutations in different genes, we ran a **PCR** and a **gel electrophoresis** of the region of the LuxS gene. Looking at the results we could see that the ones whose donor strain was KEIO ΔluxS were longer than the other two strains whose donor was KEIO ΔmutS. That's because the Kanamycin resistance gene is longer than the luxS gene.

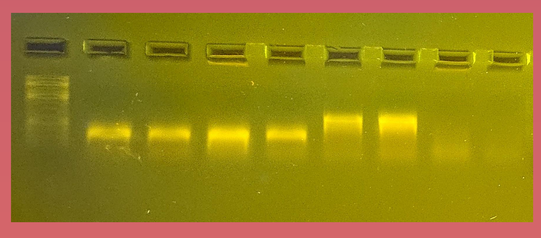
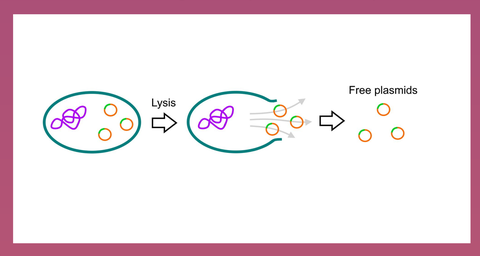


Figure 1: Results of the gel electrophoresis on the samples amplified via PCR with primers that bind to the luxS genetic region. In the first well we can see the ladder. In the second and third, and fourth and fifth, we found the samples whose donor was the KEIO ΔmutS. The next two are one of the strains whose donor was KEIO ΔluxS, and the last two holes, we can't see any result because the PCR didn't work well.

TRANSFORMATION AND FLUORESCENT BACTERIAS:

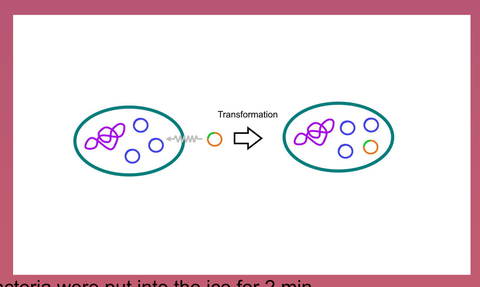
1. Isolation of plasmid DNA and gel electrophoresis
Isolation of plasmid DNA is a process when we break bacterial membranes and get free plasmids in supernatant.

1.1 Isolation of plasmid DNA
At first we centrifuged bacterial culture and discarded supernatant. Next thing was using a plasmid resuspension buffer to mix bacterial cells. Then we added 200 µl of plasmid lysis and 100 µl plasmid neutralization buffers to crack the bacterial envelopes. After that lysate has been centrifuged for 5 min. Supernatant contained **free plasmids**.



1.2 Gel electrophoresis
For **making gel** we diluted agarose powder into TAE Buffer by 100 in order to get 1% the weight. Then we boiled the solution, mix it and place into a gel box. When the gel was ready we put there result of our PCR and switched on the electrophoresis-machine to 100 V and one hour.

2. Transformation
Transformation is a process of absorbing a plasmid from an external environment by bacteria and into the bacterial envelope.
We made two types of transformation - chemical and electrical. The beginning was preparing competent cells into 100 mM solution of $CaCl2$ and into $H2O$ as such.



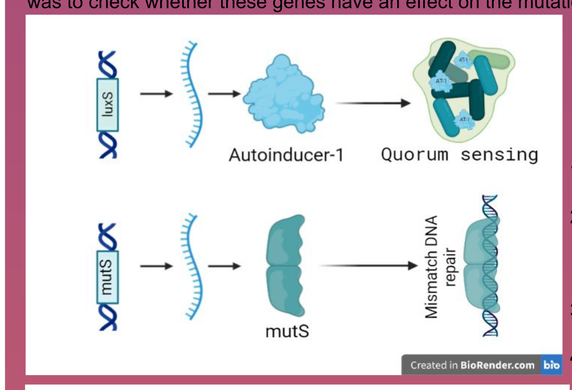
2.1 Chemical transformation
First of all we add 50 ng of plasmid DNA to tubes with bacterias and heat shocked the cells by placing them into a heating block for 42°C and 30 sec. After that, bacteria were put into the ice for 2 min.

2.2 Electrical transformation
We add 50 ng of plasmid DNA to bacterial cells and created an electrical-shock by putting them to electroporator (P1-1800V)

3. Fluorescent bacteria
Fluorescence has been added to transformed bacterias. Then we mixed a solution with them and 500 µl of LB. After that we could draw with fluorescent bacterias. These are our paintings.

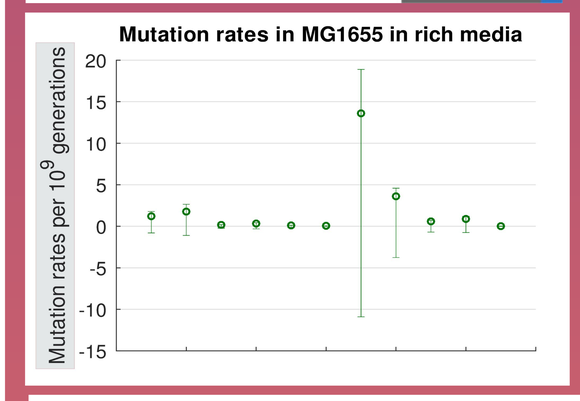
FLUCTUATION TEST:

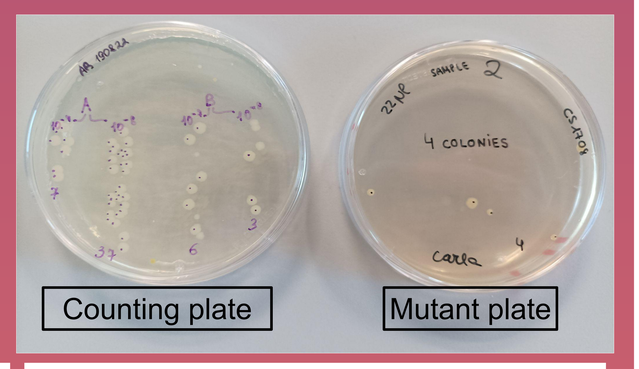
The genes which we used in transduction are responsible for quorum sensing and a DNA repair system. Our **main goal** of research was to check whether these genes have an effect on the mutation rate. A fluctuation test allows you to measure the mutation rate.

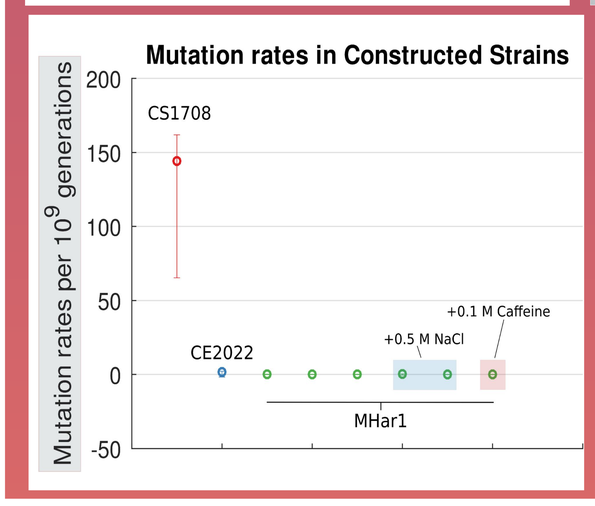


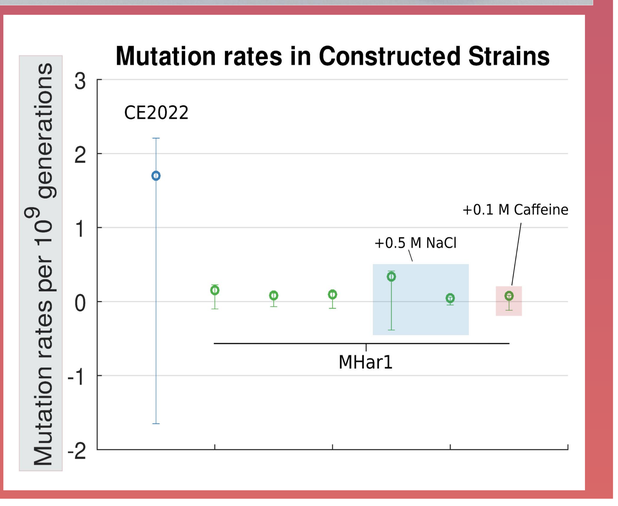
Our **hypothesis** was that the mutation rate would be higher in +lexA ind-, ΔmutS:KanR and ΔtolC, ΔmutS:KanR because of deletion of DNA repair protein. In +lexA ind-, ΔluxS:KanR and ΔtolC, ΔluxS:KanR it would be lower.

Fluctuation Method to examine rate of Rifampicin mutants:
1. Plate the 100 µl of undiluted culture to the plate with antibiotic medium and incubate overnight at 37 degrees.
2. Make a serial dilution of the culture by adding 10 µl of it one by one into 8 wells filled with 90 µl of LB. Then collect 10 µl of culture from wells 7 and 8 (they are diluted by factors of 10^{-7} and 10^{-8}) and make a drop on a plate without an antibiotic.
3. Count the colonies on the both plates and calculate the number of CFUs and mutants per 1 ml, respectively.
4. Use the tool FALCOR[3] to calculate the mutation rate using the MSS maximum likelihood method.









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