Analysing the growth of antibiotic-resistant CRISPR-transformed *Escherichia coli* under different concentrations of streptomycin.

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Abstract

We set out to explore if *Escherichia coli* transformed with CRISPR/Cas9 to become resistant to the antibiotic streptomycin will present different growth levels(total number of colonies) to different concentrations of streptomycin in their agar plates. We plated the transformed and untransformed E.coli in separate Petri-dishes containing 0, 25, and 50 ug/ml of streptomycin. Each of these treatments was repeated 3 times(for the sake of statistical significance) for a total of 18 plates. We observed a total of 4 colonies of transformed E.coli growing in the plates containing 50 ug/ml of streptomycin. We also observed bacterial lawns in all of our 18 plates. The lawn growth in all of our plates is due to a contaminating bacteria unidentified to date. We hypothesize that this bacteria is not affected by streptomycin, and the fact that this bacteria grew lawns in all of our plates rendered our experiment to be skewed by an unaccounted factor. We therefore cannot conclude if E.coli transformed with CRISPR/Cas9 to become resistant to streptomycin grows at the same rate regardless of the antibiotic's concentration, since the E.coli growth may have been limited by the growth of the unknown bacteria due to less available space and nutrients.

Introduction

One of the most important tools in biological research is genome editing for site-specific chromosome changes. While traditional approaches normally deal with one genomic region at a time, developing microbial cell factories frequently necessitates the modification of numerous

genomic targets (Feng et al., 2018). Genetic engineering is the process of changing an organism's DNA to change a characteristic for a specific purpose. CRISPR is a gene-editing technology that is a simple technique for locating a specific piece of DNA within a cell. After that, altering that piece of DNA is usually the next step in CRISPR gene editing (Feng et al., 2018).

To begin with, pathogenic bacteria such as Escherichia coli O157:H7 and Listeria monocytogenes can cause microbial outbreaks when raw fish is consumed(Ozer & Demirci,2006). E. coli is a possible pollutant indicator, it comes in a variety of forms. E. coli is most often acquired by fish when they eat feces-contaminated food (Hicks et al. 2008). E. coli was also found in the intestines of farm-raised tilapia and rainbow trout, according to Hicks et al. The source of the E. coli was not the fish; rather, it was their food, which had been contaminated by pigeon droppings (Hicks et al. 2008). As a result, raw fish must be treated to inactivate pathogenic bacteria(Ozer & Demirci,2006).

What we are trying to accomplish is to demonstrate the power of the CRISPR Cas9 system by modifying the genomic DNA of an E. coli strain to allow it to grow and survive in conditions it would not normally be able to survive in. To survive, bacteria and other organisms must produce proteins(Jiang et al. 2015). Cas9 and all other proteins are made by the ribosome, a nucleic acid and protein complex in the cell. Streptomycin binds to the ribosome and inhibits it from producing proteins, stopping the bacteria from replicating and reproducing (Yifan et al. 2015). This experiment creates a mutation in the ribosomal subunit protein rpsL that prevents streptomycin from binding to the bacteria, allowing them to grow on streptomycin media. It converts the Lysine amino acid at position 43 (K43) to Threonine by changing a single DNA base (Jiang et al. 2015).

We wanted to see if antibiotic-resistant bacteria modified with CRISPR showed varying levels of resistance to different antibiotic doses. We predicted that as the antibiotic doses increased, only transformed bacterial colonies would be able to grow, but there would be no statistical difference in the number of colonies among the transformed bacteria and the different concentration levels of streptomycin. The genes that code for streptomycin resistance can have a big impact on the distribution of streptomycin MICs (minimum inhibitory concentrations) in E. coli. The strA-strB genes are likely involved in providing high-level streptomycin resistance, but the aadA gene cassettes appear to have the opposite effect (Sunde & Norström, 2005). The inclusion of aadA gene cassettes in integrons causes low-level streptomycin resistance, which makes it difficult to identify E. coli as susceptible or resistant to streptomycin (Sunde & Norström, 2005).

Method

We used the CRISPR Cas 9 Bacterial Genomic Editing Kit in this experiment and followed the protocols listed in the accompanying guide. An overview of the experiment is briefly outlined below but a detailed procedure on how to make the agar plates, make competent bacterial cells for transformation, perform DNA transformation and the general CRISPR experiment follow the protocol provided in the kit guide (The ODIN, 2020). Any changes differing from the experimental protocol listed in the guide were noted below.

Preparation

Prepare the experimental agar plates and grow the initial bacterial culture by streaking the bacteria onto the plate. Leave this plate for 12-18 hours to allow the bacteria to grow to sufficient amounts. As we prepared our experimental plates, we labeled each dish according to its treatment group. Treatment groups differed in terms of streptomycin concentration and/or

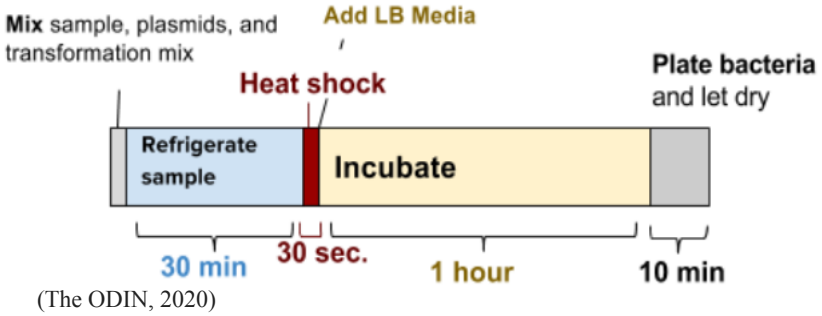
E.coli type (i.e., transformed vs. untransformed). Each group was separated into three plates, each of which served as a single trial, allowing for multiple treatments to be tested. We had a total of six treatment groups:

UT - 0 ug/mL, UT - 25ug/mL, UT - 50ug/mL

T - 0 ug/mL, T - 25ug/mL, T - 50ug/mL | T - 0 ug/mL, T - 25ug/mL, T - 50ug/mL

Then we started growing our E.coli colonies that had not been transformed (UT). As a result, we prepared the necessary CRISPR components. To create competent bacterial cells for transformation, we'll combine bacteria, plasmids, and transformation mix together in a bacterial transformation mix. After that, the sample solution will be refrigerated for about 30 minutes before being heat-shocked for about 30 seconds. After that, the bacterial solution will be plated and left to air dry for 10 minutes before being incubated. For growth, we will give a 24-48 hour incubation time.

Using an inoculation loop, we transferred part of our E.coli to microcentrifuge tubes, then mixed it with the CRISPR components, water, and powdered agar. For the bacteria that would be the untransformed groups, we simply mixed our E.coli with water and powdered agar. These bacterial mixtures were allowed to settle for 24 hours. After waiting for this incubation period, we plated our bacterial mixes to their corresponding plates, and allowed them to grow for 48 hours. At the end of this final incubation period we observed their growth to draw our results and conclusions.



(The ODIN, 2020)

Result

In the first treatment, where we transformed the E.coli bacteria with Cas9 protein, gRNA and template DNA, the bacteria showed growth in the form of white rounded dots on the plates under different concentrations of streptomycin, with 0 micrograms per millilitre, 25 micrograms per millilitre and 50 micrograms per millilitre, respectively. In the second treatment, which represents the negative control, the untransformed E.coli bacteria had growth under different concentrations of streptomycin with 0 micrograms per millilitre, 25 micrograms per millilitre, and 50 micrograms per millilitre, respectively.In both transformed and untransformed treatments, the repeated groups under concentrations of streptomycin of 0 micrograms per millilitre, 25 micrograms per millilitre, and 50 micrograms per millilitre all had bacterial growth(lawns). A total of 4 transformed E.coli colonies were identified amidst the bacterial lawns on plates T1,T2 and T3 of the 50 ug/ml of streptomycin. No transformed colonies were observed in the lawn of plates T1,T2 or T3 of the 25 ug/ml of streptomycin. This result conflicts with the hypothesis in this paper, which predicts that only the transformed E.coli bacteria would be able to grow under the various concentrations of streptomycin.

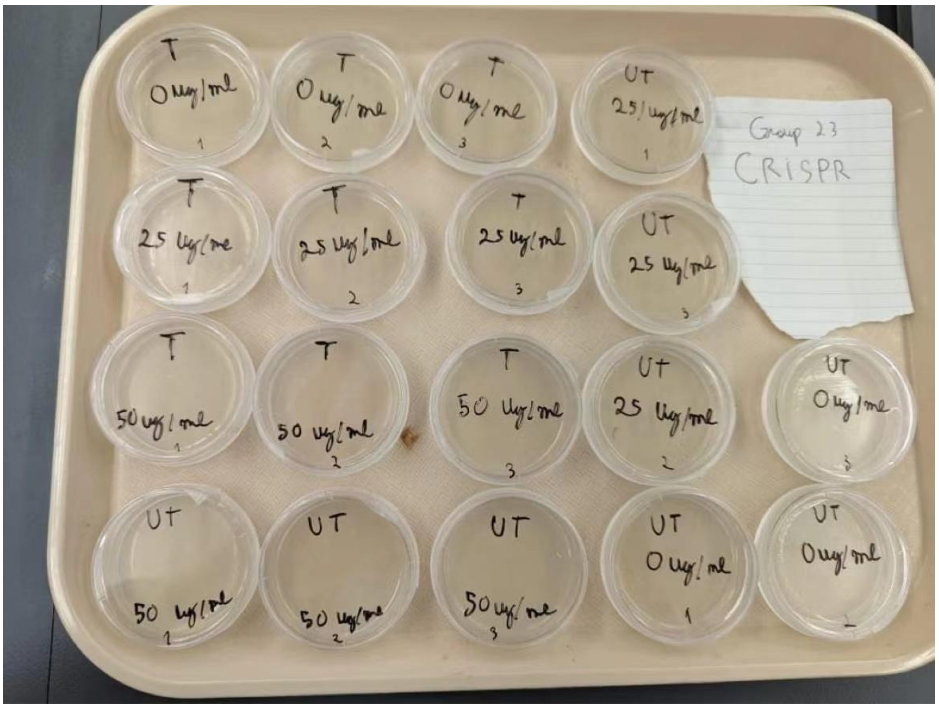


Figure 1. All plates with transformed and untransformed E.coli bacteria under concentration streptomycin, with 0 micrograms per millilitre, 25 micrograms per millilitre and 50 micrograms per millilitre. Picture before incubation.

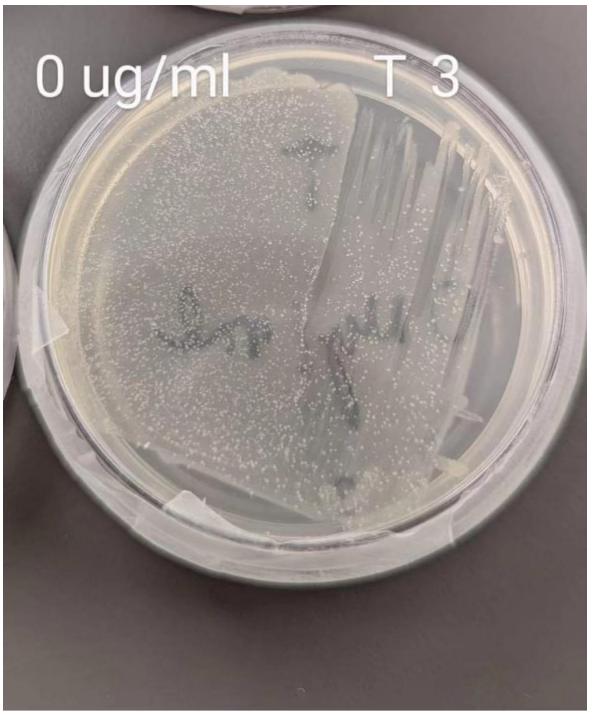


Figure 2. E.coli bacteria growth, this photo indicates the growth of E.coli under 0 micrograms per millilitre concentrations of streptomycin (on plate number 3). The incubation period of 48h. Incubation temperature of 30°C.

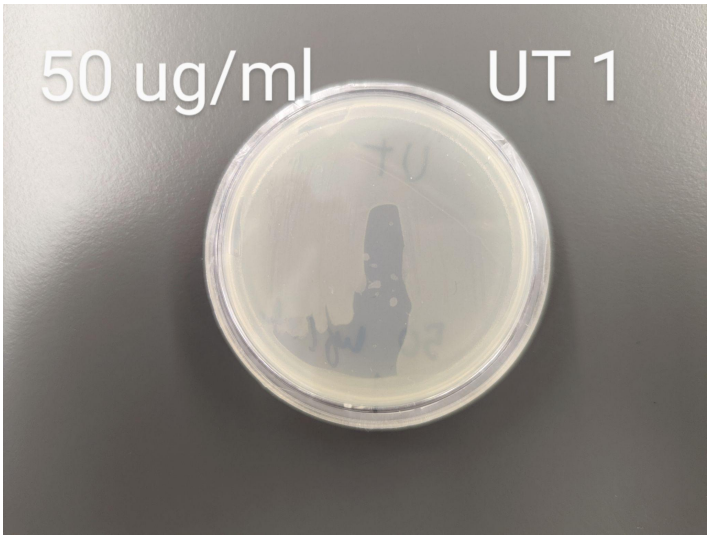


Figure 3. Bacteria lawn on plate number 1 of the untransformed E.coli treated with streptomycin at a concentration of 50 micrograms per milliliter. Incubation period of 48h. Incubation temperature of 30°C**.** The bacterial lawn is not composed of E.coli.

Discussion

Our objectives with this experiment were two-fold. The first part consisted of successfully transforming our E.coli with CRISPR/Cas 9. This in and of itself doesn't tell us anything about our bacteria, but it allowed for a fun exercise in genetic manipulation. The second part of the experiment consisted in observing if the transformed bacteria would present different growth levels (number of total colonies) based on different concentrations of the antibiotic streptomycin. Our rationale is that making a genetic change that causes streptomycin to be unable to bind to the E.coli's 16s ribosomal RNA component (Montandon et al., 1986) would mean that the concentration of the antibiotic should not be a factor in the total number of colonies that we observe, given that the streptomycin would not be able to bind to the E.coli at all. While we were successful in transforming our E.coli and we did observe growth of our transformed bacteria in the streptomycin filled plates, we failed to demonstrate the correlation(or lack thereof) of the number of colonies of E.coli and antibiotic concentration in our plates. We therefore cannot reject or support our null hypothesis. This is mostly due to the fact that we also had growth of an unknown bacteria that prevented any sort of qualitative analysis to be conducted because it formed a lawn in all of our plates and made colony counting extremely challenging. It also may have limited the growth of our transformed bacteria in the plates that contained antibiotics.

We investigated if the lawns present in all of our plates were actual bacterial growth or if they could be explained by some other cause. Because our methods consisted of mixing our transformed and untransformed bacteria with a small quantity of LB broth (short for Luria Broth, Lysogeny Broth), or Luria Bertani Broth dissolved in water and then pouring them on our plates, we hypothesized that our apparent lawns could simply be a different agar layer on top of our previously prepared agar dishes. To test this, we prepared an “empty” solution containing only water and the LB broth, with no bacteria, to be plated in a new dish containing the antibiotic-mix agar. After an incubation period of 48h we observed colonies growing in our ‘’blank’’ plate, confirming that we had contaminating bacteria. This bacteria appears to be unaffected by streptomycin as it was able to grow normally regardless of the quantity of the antibiotic. Interestingly enough, this bacterium seemed to be outcompeted by E. coli in the plates that contained no antibiotics. When observing our plates under a microscope, we found that the lawns in the antibiotic-free plates were composed almost exclusively of E. coli, while the lawns in the streptomycin-containing plates were composed entirely of the unknown bacteria. This could be due to E. coli being generally more capable at reproducing than our mysterious bacteria (Gangwe Nana et al.,2018), or it could simply be that the initial E.coli colony used for plating as larger than the initial population of the unknown bacteria. Further analysis on this issue is required for conclusions to be drawn. We would firstly need to identify our contaminating bacteria; we would then recommend that the experiment be replicated with different concentrations of the initial bacterial colonies. The growth of our unknown bacteria also leads to the hypothesis that our transformed bacteria were unable to grow due to the resources and space in the dish being consumed by the contaminating bacteria (Contois, 1959). We also recommend this to be studied further so that we can better understand the competitive relationship between these two bacteria.

Because our unknown bacteria may have affected the growth of our E.coli in all of our plates, we cannot confidently conduct a qualitative analysis of our colonies because we clearly have an extraneous factor that we did not account for in our experiment. We therefore cannot derive any meaningful results about CRISPR transformed E.coli growth under different antibiotic concentrations.

Overall, we had relative success in transforming our E. coli using CRISPR/Cas9, having observed a total of 4 E. coli colonies growing in our antibiotic-filled plates. We recommend that, in the event that this experiment is replicated, the water used should be boiled beforehand and stored in a sterilized container to minimize the chances of contamination. We also recommend that a larger range of antibiotic concentrations be used, to further demonstrate if there is any association between the antibiotic concentration and the number of transformed colonies that are able to grow. We recognize that this may have been a limitation in our study, given that our "antibiotic concentration spectrum" was not very ample and should we replicate the experiment, we would employ a larger number of different concentrations, focusing in particular in larger increases(10x to 40x the base amount we used) to provide a more comprehensive range that allows for more reliable results to be derived.

Conclusion

We set out to investigate if CRISPR-modified antibiotic-resistant bacteria would present various levels of resistance to different antibiotic concentrations. We hypothesized that untransformed E.coli would be unable to grow in the antibiotic containing plates, but we expected that the transformed E.coli would be equally able to grow regardless of the antibiotic concentration. Our results however, cannot reject or support our hypothesis presented in this

research, due to the fact that a contaminating bacteria grew a lawn in all of our plates, and prevented any sort of meaningful quantitative analysis to be conducted.

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