Biohacking: Resistant *Escherichia coli* Transformation through CRISPR Gene Editing

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Introduction

- Josiah Zayner is a global leader in the Biohacker movement and the CEO of the Open Discovery Institute (ODIN).
- The ODIN DIY CRISPR Genome Engineering Kit created streptomycin antibiotic resistant *E. coli* cells by mutation of the ribosomal subunit protein rpsL.

- Bio-Rad is a global leader in developing innovative products for life science research and education.
- Bio-Rad educational kits are used in college biology lab courses
- The Out of the Blue CRISPR kit created *E. coli* that have mutations in the *lacZ* gene, displayed through color

Objectives

I. Successfully perform a CRISPR gene editing experiment and simultaneously learn how the CRISPR-cas9 system operates.
II. Compare and evaluate the ODIN DIY CRISPR Genome Engineering Kit to the Bio-Rad Out of the Blue CRISPR Kit.
III. Discover to what extent CRISPR gene editing can be applied in the future of therapeutic medicine.

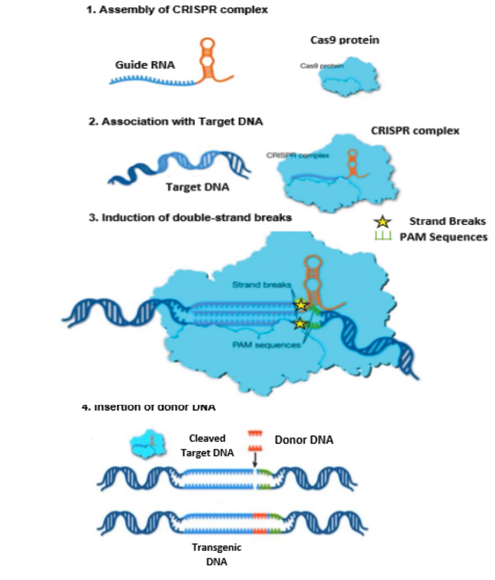
CRISPR and Cas9

- Clustered regularly interspaced palindromic repeats (CRISPR) were first described as part of the genome of *E. coli* bacteria.
- Cas9 is a restriction enzyme (cutting enzyme) that can cut specific sequences in DNA.

What is the CRIPSR-Cas9 System?

- The CRISPR-Cas9 System is a programmable gene editing tool that utilizes guide RNA (gRNA) and a cas9 protein to form a CRISPR-complex. (see Figure 1.1)
- Next, the Cas9 enzyme uses a guide RNA (gRNA) to find a specific site in DNA (see Figure 1.2) and cleaves both strands of DNA (see Figure 1.3)
- A designed template DNA is used to introduce a specific mutation (insertion, deletion, or alteration) where Cas9 cleaved the DNA. In this representation, it is the insertion of a Donor DNA. (see Figure 1.4)

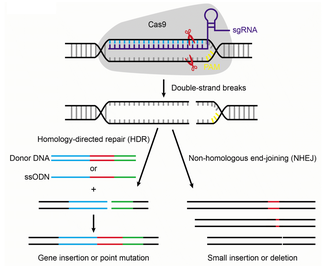
Figure 1. CRISPR-cas9 system



DNA Repair

- DNA repair can happen by two mechanisms: homology directed repair (HDR) and non-homologous end joining (NHEJ) displayed in Figure 2.
- HDR- specific proteins patch a double stranded DNA using a donor template DNA
- NHEJ- specific proteins connects the ends of a double-strand DNA break. This process may randomly insert or delete bases that can disrupt gene function

Figure 2. HDR and NHEJ DNA repair mechanisms



Methods

ODIN DIY CRISPR Genome Engineering Kit

- The modification of the DNA base adenine (a) to cytosine (c) enables the bacteria to code for Threonine amino acids instead of Lysine amino acids at position 43 in the protein makes the bacteria streptomycin-resistant.
- A specific gRNA was used to program Cas9 to cut the *rpsL* gene in *E. coli* (see Figure 3)
- A specific DNA template was used to introduce a single letter change in the *rpsL* gene (see Figure 4)

Figure 3. Guide RNA directing Cas9 to cut the *rpsL* gene in *E. coli*

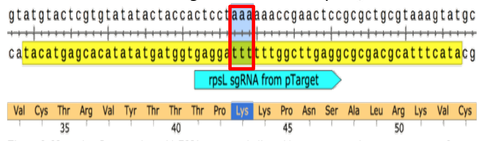
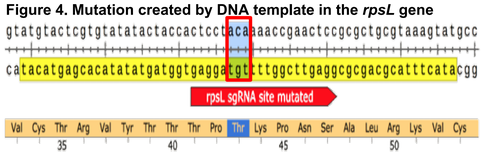


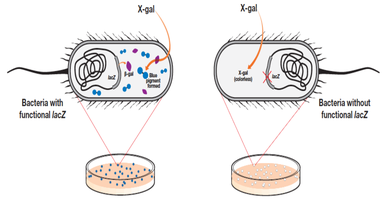
Figure 4. Mutation created by DNA template in the *rpsL* gene



Bio-Rad Out of the Blue CRISPR Kit

- This experiment uses CRISPR-Cas9 to cut the *E. coli* chromosome DNA within the lacZ gene. The lacZ gene codes for the enzyme β-galactosidase (β-gal) that catalyzes the hydrolysis of lactose into its sugar monomers, glucose and galactose.
- A blue-white screening was used to determine which bacteria were expressing β-gal. β-gal hydrolyzes X-gal, a colorless pigment, to yield an insoluble blue pigment. If resulting bacterial colonies were edited successfully, they showed blue pigmentation (see Figure 5)

Figure 5. blue-white screening for functional lacZ gene



Results

ODIN DIY CRISPR Genome Engineering Kit

- The initial attempt during the fall 19 semester was relatively unsuccessful (see Table 1). An example of a successful experiment is shown in Figure 6.
- During the initial attempt, several ways to improve the protocol were discovered.
  - Mixing the bacteria and DNA more to increase interactions
  - Incubating bacteria longer
  - Full, 1/2, 1/10, and 1/100 strength streptomycin
- ODIN provided a free kit to try again during the spring 2020 semester. The results improved in this second attempt by implementation of the protocol improvements (see Figure 7)
- *E. coli* bacteria are white or cream in color. At each strength interval, both cream colored and orange colored bacteria grew on the streptomycin media. The orange colored bacteria are likely contaminant resistant bacteria that may have been introduced during the manufacturing process of the ODIN kits. This suggests the kits are not made in as sterile of an environment as the Bio-Rad kits.

Figure 6. Fall 2019 Experiment

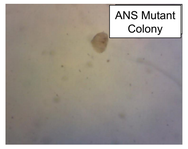
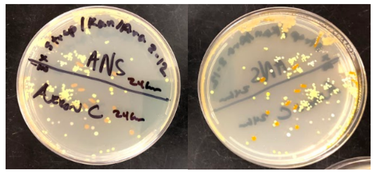


Table 1. Fall 2019 Results

# of successful experiments	# of unsuccessful experiments
2	7
Success Rate:	22%

Figure 7. ODIN CRISPR plates at ½ strength streptomycin



Bio-Rad Out of the Blue CRISPR kit

- Bio-Rad is an educational kit, available for purchase only by educators and authorized subscribers
- A harmless edit of the *lacZ* gene is made . This results in the cell becoming less-viable, instead of creating a "superbug"
- The Bio-Rad Out of the Blue kit is unique in having controls and figures that clearly indicate how bacteria is affected by each variable
- The results are more visual from the use of a color test which provides a simple, visual way to indicate if insertion and editing were a success
- The expected transformations (see Figure 8) are show below next to the experimental transformations made through CRISPR by the Bio-Rad Kit (see Figure 9)
- Plate D Shows white and blue colonies, displaying examples of the two existing types of DNA repair (see Figure 2 in bottom left)

Figure 8. Expected Transformations on plates A – D

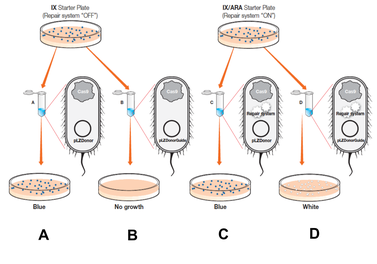


Figure 9. Experimental transformations on plates A – D

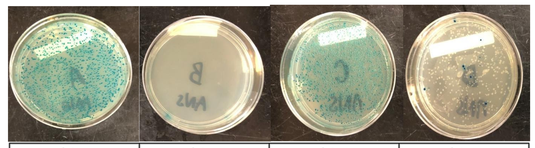


Plate A	Plate B	Plate C	Plate D
No gRNA No DNA Repair System	Has gRNA No DNA Repair System	No gRNA Has DNA Repair System	Has gRNA Has DNA Repair System
Conclusions: Control plate where no repair or CRISPR editing is happening bacteria are blue like the starter bacteria	Conclusions: Control plate where gRNA with Cas9 cuts DNA but DNA Repair System is not present so broken DNA causes bacteria to die	Conclusions: Control plate without gRNA showing that Cas9 and the DNA Repair system is not toxic to the bacteria.	Conclusions: gRNA with Cas9 cuts DNA and DNA Repair System fixes *lacZ* gene using template with stop codon making mutant white colonies

Ingredients in all plates:
1. Kanamycin – Antibiotic that forces the bacteria to hold on to the DNA Repair System plasmid
2. IPTG – Induces expression of β-galactosidase enzyme
3. X-gal – Substrate similar to lactose for β-galactosidase enzyme that becomes a blue pigment product when the enzyme reacts with it
4. Spectinomycin – Antibiotic that forces the bacteria to hold on to the pLZDonor or pLZDonorGuide plasmid DNA

Conclusions

- The ODIN kit is sold as a biohacker kit and gives off a shock-value notion that the editing is fairly simple and that anyone could do it. The editing in this kit is of the rpsL gene to make bacteria stronger by antibiotic-resistance. The protocol was quite faulty and therefore required provision. With ODIN, it was more difficult to determine if the gene editing was a success. The results suggest that the ODIN kit was contaminated with an antibiotic-resistant bacteria.
- Bio-Rad kit is sold as an educational tool. The alteration of the lacZ gene makes the bacteria weaker and less-viable. While there is less sensational promotion with Bio-Rad, the kit proved to be more functional and the gene editing could be identified by color change. The Bio-Rad kit made it possible to see different types of CRISPR editing - blue colonies on plate D.
- The truth is that while kits may be advertised as DIY anywhere, it is not likely an average person without lab equipment would have success at home.
- The kits are supplying non-pathogenic bacteria and streptomycin at a strength that if they grew would be no match for a prescribed antibiotic for bacterial infection.

The New Frontier

- Cystic Fibrosis (CF) is a disease that causes the build up of viscous mucus in the lungs, pancreas, and small intestine.. Today, there are approximately 70,000 individuals worldwide with CF, but many go untreated and do not survive past their 20s.
- CF is caused by a deletion of the transmembrane conductance regulator (CFTR). This deletion results in a loss of the amino acid phenylalanine in the protein at the 508th position (see Figure 10).
- Ideally, CRISPR technology could be used to create a cut in a cell's DNA near the CFTR mutation and through homologous recombination, mediated by CRISPR, a new healthy version of the gene would take the place of the mutation.
- Although cas9 is effective in making precise cuts in the DNA where programmed, CRISPR utilizes the cells own repair system in modification which can potentially make errors. For this reason, CRISPR is not used in treating human disease today.
- In this research, two different genes were modified with CRISPR. CRISPR has considerable potential in therapeutic gene editing because of its unspecialized ability to modify, insert, or delete any genes of any organism at any point throughout its development.

Figure 10. Absence of CFTR by phenylalanine deletion