JOURNAL OF UNDERGRADUATE RESEARCH

Utilizing CRISPR/Cas9 Gene Editing Techniques for Knockout of Genes in *Escherichia coli* Bacteria

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Abstract

This research explored the potential of CRISPR/Cas9 to knockout genes in *Escherichia coli* that encode for virulence factors such as efflux pumps and biofilms. Specifically, this study focused on the ability of CRISPR/Cas9 plasmid BPK764 to express CRISPR/Cas9 proteins in *E. coli* and subsequently edit red fluorescent protein (*RFP*) plasmids and endogenous *E. coli* biofilm gene *LuxS*. This study required the design of single guide RNA (sgRNA) oligos which target genes of interest. These were then ligated into the plasmid via linearization of BPK764, gel extraction, and annealing. These plasmids were grown on Luria-Bertani (LB) agar and sequenced to confirm insertion. The edited plasmids were then electroporated into T7 Express *E. coli* cells and grown on LB plates to select colonies. These colonies were picked, induced, and grown on LB plates with selective antibiotics to determine if the Cas9 was able to edit the target gene. The experimental plates had editing efficiencies of 98%, 88.6%, and 100%. The control plates all had editing efficiencies of 0.0%. Sequencing data from *LuxS* suggested successful knockout, however further testing is necessary. The data collected from this study suggests that CRISPR/Cas9 plasmids, specifically BPK764, can be utilized to knockout genes in *E. coli*. Further research is required to determine if BPK764 can be used to completely knockout endogenous *E. coli* genes.

Keywords: CRISPR/Cas9, Antibiotic Resistance, Bacterial Gene Editing

Background

Antibiotic resistance is becoming an increasingly prevalent problem as more antibiotics are becoming obsolete. Antibiotic resistance occurs when antibiotics are overused and incorrectly prescribed, which allows the bacteria that they target to develop and mount defenses (Frieri et al., 2017). Through natural selection and evolution, strains that are resistant to antibiotics can become dominant and spread their genes to other strains through horizontal gene transfer (Frieri et al., 2017). This can result in bacterial strains that are resistant to a majority of current antibiotics, such as Methicillin-Resistant *Staphylococcus aureus* and multidrug-resistant *Mycobacterium tuberculosis*. In the case of *Escherichia coli*¸ a gram-negative human pathogen, there are multiple virulence factors utilized to resist antibiotics, including biofilms and efflux pumps (Alshammari et al., 2023; Swick et al., 2011). Biofilms are a community of bacteria enclosed in a matrix made of extracellular polymeric substances (Alshammari et al., 2023). They are highly resistant to antibiotic attacks as they do not allow antibiotics to reach the bacterial cells inside of the matrix (Alshammari et al., 2023). Efflux pumps, located in the membrane of the bacteria, contribute to antibiotic resistance by exporting antibiotics that have successfully entered the bacterial cell (Su et al., 2006). Biofilms and efflux pumps are two of the main methods used by *E. coli* strains to confer antibiotic resistance.

The genes that encode for virulence factors in *E. coli* can be impacted and disrupted by CRISPR/Cas9 gene editing techniques. CRISPR, which stands for Clustered Regularly Interspaced Short Palindromic Repeats, is a highly effective method for editing genomes (Vigliotti & Martinez, 2018). CRISPR technology can be used to create a double-stranded break, known as cis-cleavage, in the genomic DNA of *E. coli*. This occurs through the Cas9 protein binding to a protospacer adjacent motif (PAM) sequence located on the 5' end of the antisense strand next to the gene of interest (Redman et al., 2016). Cas9 binds to the DNA using a single guide RNA (sgRNA) to locate the target sequence and then creates a double-stranded break. The break in the genome can then be repaired by introducing donor DNA to fill the gaps in a process known as homology-directed repair (HDR), or it can be repaired by allowing the endogenous DNA repair systems to fix the gap, known as non-homologous end joining (NHEJ) (Redman et al., 2016).

One study researched the use of CRISPR/Cas9 to knockout biofilm forming genes in *E. coli*, as biofilms are one of the main causes of catheter-associated nosocomial infections (Alshammari et al., 2023). Another study demonstrated success in reducing efflux pump activity using CRISPR interference (CRISPRi), but not using CRISPR/Cas9 (Wan et al., 2022). The ability to use CRISPR/Cas9 to effectively knockout genes in *E. coli* can be visualized by targeting the red-fluorescent protein (*RFP*) gene, as its deactivation will result in a loss of fluorescence in previously fluorescent bacterial colonies (Pieczynski et al., 2019). Plasmids that use CRISPR/Cas9 to knockout genes in *E. coli* are still being identified and could be used to reinstate the bacterium's susceptibility to antibiotics, if effective.

Aims and Hypotheses

The first aim of this research study was to transform engineered *RFP* BPK764 into *E. coli* and visualize decreased *RFP* expression as compared to a control. The second aim was to transform engineered *LuxS* BPK764 into *E. coli* and determine its ability to achieve *LuxS* knockout through sequencing. These aims are visualized in Figure 1.

The first hypothesis of this research study was if the edited *RFP*-targeting BPK764 plasmid is induced and expressed in *E. coli* containing the *RFP* gene, then there will be a lower percentage of red fluorescent colonies, as compared to a control. The second hypothesis was if the edited *LuxS*-targeting BPK764 plasmid is induced and expressed in *E. coli*, there will be knockout of the endogenous biofilm gene *LuxS*, as confirmed by sequencing.

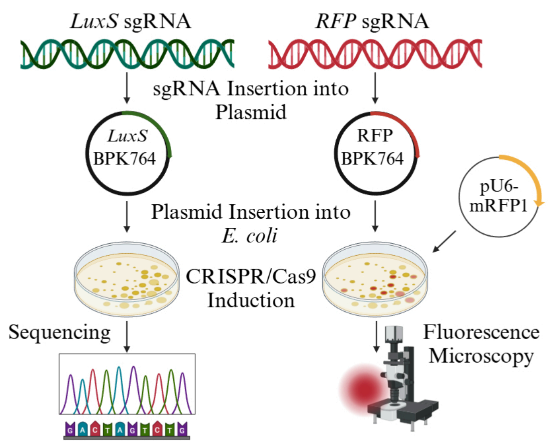

Figure 1. Overall project workflow with various aims showing differences between the *RFP* and *LuxS* sgRNA procedures. Created with Biorender.com (*BioRender*, n.d.).

Method

Plasmids and Oligos

The authors ordered plasmids BPK764 (from Keith Joung; Addgene #65767) and pU6-mRFP1 (from David Liu, Addgene #132777) from ADDGENE. Oligos such as sgRNAs, for insertion into the plasmid, and primers were ordered from IDT. Primers were designed in SnapGene and NCBI Primer BLAST. *E. coli* NEB10β and T7 Express cells were used for analysis.

Creating Electrocompetent *E. coli* T7 Express Cells

A 50 µL frozen stock of *E. coli* T7 Express cells was added to 40 mL of room temperature Luria-Bertani (LB) broth. The broth was incubated overnight in a shaker at 200 rpm and 37ºC. The next day, 2 mL of overnight culture was added to new LB broth and grown to an optical density (OD600) around 0.6. Once grown to the correct OD600, the culture was centrifuged for 5 minutes at 3500 × g at 4ºC. The following procedure was carried out on ice. The supernatant was discarded, and the pellet resuspended in 20 mL of cold 10% glycerol. The cells were then centrifuged for 5 minutes at 3500 × g at 4ºC and the supernatant was discarded. They were then resuspended in 10 mL of cold 10% glycerol and the centrifuge step was repeated. The supernatant was discarded, and the cells were resuspended in 5 mL of cold 10% glycerol. The centrifuge step was repeated and the supernatant discarded. Finally, the cells were resuspended in 1 mL of cold 10% glycerol and aliquoted into tubes of 50 µL each for immediate use or storage at 4ºC.

Making LB Agar Plates for *E. coli* Growth and Experimentation

Chloramphenicol 25 mg/mL stock was made by diluting 0.5 g of chloramphenicol in 20 mL of 100% ethanol. LB agar plates were made by adding 25 g/L LB powder and 15 g/L Difco agar to 1 liter of deionized water. This was done twice for two separate bottles of media. The media was autoclaved for sterilization and allowed to cool on a shaker. After cooling, chloramphenicol (25 µg/mL) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (500 nM) were added to one bottle. Chloramphenicol (25 µg/mL) and ampicillin (50 µg/mL) were added to the second bottle. Media was poured into petri dishes and allowed to set before being stored at 4ºC. Some plates were poured containing only chloramphenicol.

Cloning the sgRNA Sequences into the BPK764 Plasmid

The BPK764 plasmid was first digested with BsaI-HF restriction enzyme. It was then incubated at 37ºC for 20 minutes and 15 µL of gel loading dye was added before incubating again at 80ºC for 5 minutes. Then, the product was run on a 1% agarose gel for 30 minutes at 140 volts. A blue light box was used to remove the larger DNA fragment (~8000 base pairs) from the agarose gel. This fragment was removed to ensure that the sequence removed by BsaI-HF was completely separated from the rest of the plasmid. This allowed for the insertion of the sgRNA that targets *RFP* and *LuxS*. After removal, the large plasmid gel fragments were processed using a Monarch gel extraction kit. The product was split into two tubes and ligated with the *RFP* and *LuxS* sgRNAs, with one sgRNA in each tube to create two separate plasmids. These were incubated at room temperature for 10 minutes, then inactivated at 65ºC for 10 minutes. They were then transformed into *E. coli* NEB10β cells via heat-shock. The products were added to the cells and sat on ice for 30 minutes, dipped into a 42ºC water bath for 30 seconds, then sat on ice for another 10 minutes. After this, the cells recovered in 1 mL of warm Super Optimal Broth with Catabolite repression (SOC) media for 1 hour in a shaker at 200 rpm. Then, they were plated on LB agar plates containing only chloramphenicol (25 µg/mL). These plates were incubated overnight at 37ºC.

Nanopore Sequencing of the Edited BPK764 Plasmid

The next day, colonies were picked from the plates and prepared for Nanopore sequencing using the Nanopore sequencing protocol, as previously reported (Oxford Nanopore Technologies, 2025). This was performed to determine if the target sgRNAs for *RFP* and *LuxS* were successfully ligated into the BPK764 plasmid. This resulted in two different edited BPK764 plasmids: one with the *RFP* sgRNA (*RFP* BPK764) and one with the *LuxS* sgRNA (*LuxS* BPK764).

Electroporation of *E. coli* T7 Express with BPK764 Plasmid and pU6-mRFP1 Plasmid

After the ligation of the target sgRNAs was confirmed, the colonies from the previously mentioned plates were grown in LB media with selective antibiotics at 37ºC overnight and miniprepped using the Monarch plasmid miniprep kit. The *RFP* BPK764 plasmid was then added to the electrocompetent *E. coli* T7 Express cells along with the plasmid pU6-mRFP1, which inserted the *RFP* gene into the *E. coli*. Each plasmid was added at a concentration of 100 ng. The cells were electroporated at 2500 volts, then 1 mL of warm SOC media was immediately added. They recovered for 1 hour in the shaker at 37ºC before being plated on the chloramphenicol and ampicillin plates. Additionally, the non-targeting experiment was performed, in which unedited BPK764 plasmid and pU6-mRFP1 plasmid were electroporated into T7 Express cells. Furthermore, the *LuxS* BPK764 plasmid was electroporated into T7 Express cells, without being co-transformed with pU6-mRFP1, then plated on LB plates containing chloramphenicol. This is because the *LuxS* gene is already present in the *E. coli* genome. This resulted in three separate plates of cells: one containing *RFP* BPK764 and pU6-mRFP1, one containing unedited BPK764 and pU6-mRFP1, and one containing *LuxS* BPK764.

Inducing CRISPR/Cas9 and Counting *RFP* Colonies

The plate containing *RFP* BPK764 and pU6-mRFP1 was used to make the experimental, or "targeting", plates. One colony was picked from the plate and added to 1 mL of warm LB broth. Then, 20 µL was transferred to another 1 mL of LB broth for one dilution. Next, 20 µL of the first dilution was transferred to another 1 mL of LB broth for the second dilution. From each dilution, 50 µL was plated on an LB plate with chloramphenicol and IPTG to induce CRISPR/Cas9 expression. This was repeated two more times with a total of three separate colonies. Then, this procedure was repeated for the control, or "non-targeting", plate containing unedited BPK764 and pU6-mRFP1 and the plate containing the *LuxS* BPK764 plasmid. These plates were incubated overnight at 37ºC.

The next day, the plates containing *RFP* BPK764 and those containing unedited BPK764 were visualized using a UVP GelStudio Plus gel viewer. They were analyzed under green light to view the red fluorescent colonies. The number of red colonies was counted along with the total number of colonies on each plate with a reasonable amount of bacterial growth. For one non-targeting plate, a small section of the plate was counted to determine the percentage of red colonies since there were too many colonies to count on the entire plate.

Analysis of T7 Express Cells Electroporated with the *LuxS* BPK764 Plasmid

Colonies were picked from the plate containing the *LuxS* BPK764 plasmid and polymerase chain reaction (PCR) was performed using KOD DNA polymerase. The colonies were added to water and then denatured at 90ºC for 5 minutes. After PCR, the products were processed using the Monarch PCR Cleanup Kit and sent for Sanger sequencing to determine if editing occurred.

Results

Nanopore sequencing of the BPK764 plasmid after the sgRNA was inserted showed successful editing. Three targeting *RFP* plates and three non-targeting plates were imaged. As shown in Figure 2, the *RFP* BPK764 plates consisted of T7 Express *E. coli* on LB agar containing chloramphenicol and IPTG.

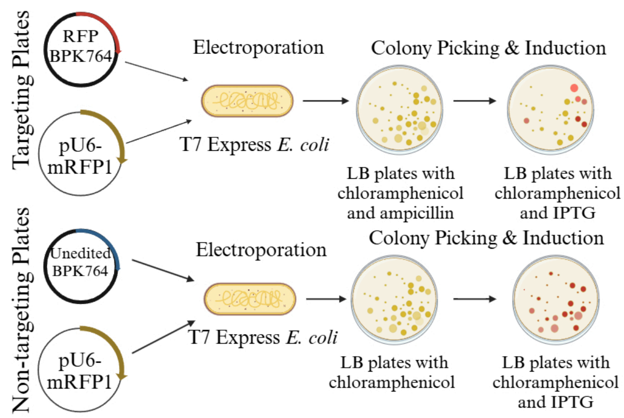

Figure 2. Map of protocol for creation of targeting and non-targeting plates showing expected difference in number of red colonies in the final LB plates. Created with Biorender.com (*BioRender*, n.d.).

The bacteria on the targeting plates contained both an edited BPK764 plasmid and pU6-mRFP1. The maps of these plasmids are shown in Figures 3 and 4. The non-targeting plates were

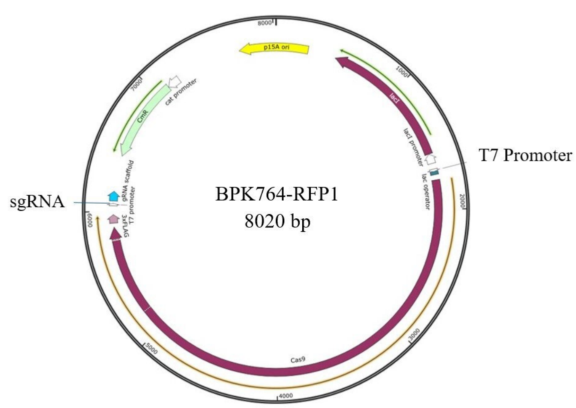

Figure 3. Plasmid map of BPK764 showing the location of sgRNA insertion for *RFP* and *LuxS* sgRNA. Plasmid is a gift from Keith Joung; Addgene plasmid # 65767 (Kleinstiver et al., 2015).

the same, however the BPK764 plasmid contained no sgRNA and therefore did not target any genes, as shown in Figure 2.

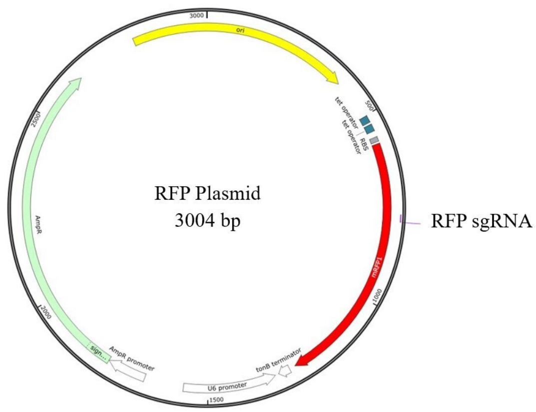

Figure 4. Plasmid map of *RFP* plasmid pU6-mRFP1 used for insertion of RFP gene into *E. coli*. Plasmid is a gift from David Liu; Addgene plasmid #13277 (Anzalone et al., 2019).

The first targeting plate had 1 red fluorescent colony out of a total of 51 colonies, which is approximately 2% red, or a 98% editing efficiency. The second and third experimental plates showed editing efficiencies of 88.6% and 100%, respectively. The mean percentage from the targeting plates was 4.5% red colonies with a standard deviation of $\pm$ 6.1%. All the colonies on all three control plates were 100% red fluorescent colonies, as shown in Figure 5. The mean percentage of these plates was 100% red colonies with a standard deviation of 0.0%. This data is visualized in Figure 5 and shown in Table 1.

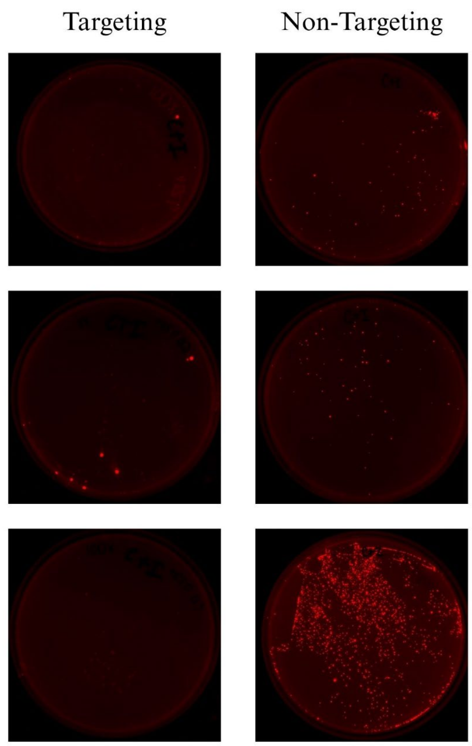

Figure 5. Fluorescence images of the *RFP* targeting and non-targeting plates. Note that for the third non-targeting plate, a small section was counted as there were too many colonies to count on the entire plate. Made on Canva.com (*Canva,* n.d.)

Table 1. Colony Data from Targeting and Non-Targeting Plates

Plate	Colonies		% Red	% Editing Efficiency
	Red	Total		
Targeting Plate 1	1	51	1.96	98.04
Targeting Plate 2	8	70	11.43	88.57
Targeting Plate 3	0	44	0	100
Non-Targeting Plate 1	95	95	100	0
Non-Targeting Plate 2	73	73	100	0
Non-Targeting Plate 3	138	138	100	0

At 4 degrees of freedom, an unpaired t-test resulted in a p-value of less than 0.0001 at α = 0.05, seen in Figure 6.

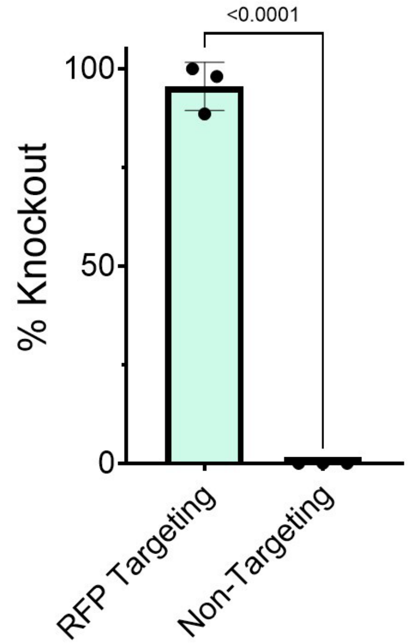

Figure 6. Percent knockout for the *RFP* targeting and non-targeting plates with triplicate data points, standard deviation error bars, and unpaired t-test p-value result.

Sequencing was performed on the edited *LuxS* strains to determine if editing was achieved. The sequencing results showed elevated levels of background noise in the expected editing location, as shown in Figure 7, however more testing is needed to confirm editing. The presence of the highlighted “N” sequence and high “peaks”, or background noise, in Figure 7 in the targeting bacteria means that the sequencing machine could not determine which base pairs, if any, were present, suggesting successful knockout of the sequence. If editing had not occurred, the “N” sequence and background noise would not likely be present and it would look similar to the wild-type data. Confirming this data could involve running an agarose gel on the *LuxS* BPK764 PCR product and gel extracting prior to sending for sequencing again. Once obtained, a minimum inhibitory concentration assay and biofilm staining could be performed on the edited and non-edited bacteria. The edited bacteria should have a higher susceptibility to antibiotics and less biofilm activity. Unofficially, it was observed that the wild-type bacteria had more adhesion to the agar plates in comparison to the targeting bacteria while removing colonies for analysis. This could also suggest that the knockout of biofilm gene *LuxS* was successful and reduced biofilm adhesion activity in the targeting bacteria, but a formal biofilm staining assay is necessary. In conjunction with the sequencing result, these tests could confirm that the *LuxS* gene was successfully knocked out.

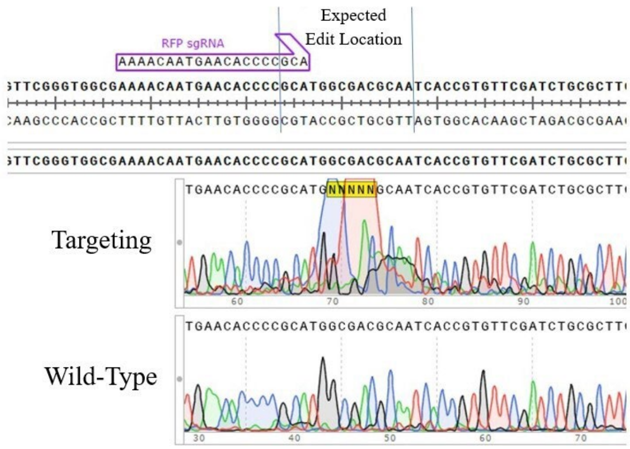

Figure 7. Sequencing data from the *LuxS* gene, showing that colonies from the targeting plate have significantly greater background noise than the wild-type gene.
result.

Discussion

The results of the unpaired t-test performed on this data suggest that the difference between the means of the targeting and non-targeting plates is highly statistically significant. This suggests that the editing that occurred on the targeting plates was not due to chance. The colonies on the non-targeting plates were all red, suggesting 100% *RFP* expression. This is because the BPK764 plasmid did not contain any sgRNA, so the Cas9 did not cut the *RFP* gene, which was inserted by pU6-mRFP1. In the targeting plates, the proportion of red colonies was significantly lower. This is because the BPK764 plasmid contained sgRNA that targeted the *RFP* gene. These results show that the BPK764 plasmid can express Cas9 post-induction in *E. coli* and cut inserted genes. Furthermore, the *LuxS* sequencing data from the colonies where Cas9 was induced had significant background noise compared to the unedited gene sequence. While this is not conclusive data, this does warrant further research into the ability of BPK764 to edit endogenous genes in *E. coli*, as the background noise suggests some editing may have occurred. This data supported the first hypothesis of this study as there were fewer red fluorescent colonies in bacteria with edited BPK764 plasmid. The second hypothesis was partially supported, but more analysis is necessary to confirm if the BPK764 plasmid actually knocked out *LuxS*.

CRISPR/Cas9 gene editing *in vivo* is still being widely researched. This research tentatively aligns with previous literature findings which showed successful editing of *LuxS* and *FimH* genes (Alshammari et al., 2023). *E. coli* editing was previously achieved using two different plasmids (Alshammari et al., 2023). If editing can be performed using only one plasmid, such as the BPK764 plasmid, this would create a much simpler and streamlined editing process. Creating simpler gene editing protocols would make it easier for CRISPR/Cas9 to be delivered into eukaryotic systems. Delivering CRISPR/Cas9 into eukaryotic systems would allow for gene editing of virulence factors in pathogenic bacteria that are actively infecting a host. This could be an incredible alternative to antibiotics, as long as there are no off-target effects to the host. One limitation of this study is that the bacteria were grown on a plate and not in a live host, so there is uncertainty about this process in a living system. Furthermore, the fluorescent expression of *RFP* in bacteria, while extremely efficient, can occasionally vary. While variability did not seem to be present in this study, due to the results from the non-targeting control plates, it is possible that some of the decrease in *RFP* expression in the experimental bacteria could be due to variability in *RFP* expression instead of CRISPR/Cas9. This could be validated through bulk Next-Generation Sequencing on both the treated and untreated bacteria to quantify *RFP* expression. Time constraints limited the ability to do this, therefore future testing would involve confirming both the *RFP* expression results and the *LuxS* sequencing results with further sequencing, biofilm assays, and minimum inhibitory concentration assays. Overall, this experiment showed that a single plasmid has the potential to edit genes in live bacteria using CRISPR/Cas9.

Acknowledgements

I would like to thank the members of the Jain Research Lab for their assistance and support. Specifically, I would like to express my gratitude for the instrumental guidance provided by Noah Rakestraw and for the help of Dr. Piyush K. Jain. Funding was provided by the UF University Scholars Program and NIH-NIGMS MIRA R35GM147788 via Piyush K. Jain.

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