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CRISPR in Cyanobacteria: A System for Genomic Editing in *Synechocystis*

Abstract

CRISPR-system genomic editing is perhaps one of the most profound and far-reaching technological breakthroughs of the last decade; however, we have still yet to surpass the earliest stages of understanding this new technology. Through DNA manipulation achieved by the use of restriction digestion, Gibson Assembly, *E. coli* transformation, triparental conjugation and we established a foundation from which CRISPR experimentation can be achieved cheaply and easily in our laboratory in the Department of Microbiology & Molecular Genetics at Oklahoma State University. This foundation can be used as a template for further research and has been tested through the genetic knockout of the *cpcBACCD* operon (Figure 1) encoding the major light-harvesting antenna system of the cyanobacterial model organism *Synechocystis* sp. PCC6803.

Introduction

Precise genomic manipulation via CRISPR - **c**lustered **r**egularly-**i**nterspaced **s**hort **p**alindromic **r**epeats- systems is a revolutionary procedure with potential biotechnological applications spanning many species, including humans. This technology has the ability to efficiently and specifically edit the genes of the organisms that it has been tailored to. It allows for editing capabilities such as gene knockouts, which remove specific sequences of a gene, gene knock-ins, which insert sequences into the genome in place of or just adjacent to existing genes, and site-directed mutagenesis, which causes directed DNA alterations at the base pair precision level. However, the study and application of this technique is still in its infancy. CRISPR was first

hypothesized to act as an adaptive immunity system in prokaryotes 15 years ago (Mojica et al., 2005). It was not until 2013 that a team of researchers was able to successfully display the ability for a modified CRISPR system to accurately cleave target sequences in mouse and human cells (Cong et al., 2013). This system is not yet perfected for applications across all organisms and reasons for failures in application remain unresolved for many species. For the purposes of our lab, the CRISPR system needed to be able to be expressed in cyanobacteria, which are model organisms for studying photosynthesis in relation to fundamental and applied problems ranging from agricultural productivity and resilience, to creation of light-driven biorefineries; however, the utilization of a CRISPR system in cyanobacteria poses a problem when considering the generic DNA reagents needed for implementing the system. This is because the generic system utilizes a protein known as Cas9 (CRISPR-associated protein 9). The expression of this protein has been found to be toxic in cyanobacteria, resulting in cell death at elevated levels (Wendt et al., 2016). In order to circumvent this issue, another protein compatible with the CRISPR system was used, Cpf1 - CRISPR-associated endonuclease in *Prevotella* and *Francisella* 1 (Ungerer & Pakrasi, 2016). This enzyme is different from Cas9 in several ways, yet it still facilitates genomic editing without causing cell death in cyanobacteria. In this study, a plasmid encoding Cpf1 was used as the foundation for crafting a CRISPR system that could be utilized as a template for genetic manipulation of *Synechocystis* sp. PCC6803 (Ungerer & Pakrasi, 2016). In order to conform to the needs of our lab, the restriction sites used for the construction of the final CRISPR plasmid had to be modified, replacing the need for an expensive and difficult to attain restriction enzyme with a more economic choice. The goal was to create a final plasmid product that can be utilized by others through simply inserting so-called ‘guide RNA’ (gRNA) and repair template sequences specific to their experiments into it and then transforming or conjugating these genetic elements into *Synechocystis* to express them and thereby mediate the targeted genome editing reactions.

The system was tested through the knockout of the *cpcBACCD* operon, an experiment that has already been done through other methods (Lea-Smith et al., 2014) and now provides a

relatively easy screen to evaluate the success of the genome editing reactions. The elimination of the *cpcBACCD* operon was chosen because we sought a target gene that would produce outwardly visible changes to the appearance of colonies of transformed cells and thus would allow rapid scoring of whether the gene editing had functioned or failed. In other words, the visible phenotype recorded should directly report the occurrence of the correctly assembled CRISPR system within a population of candidate colonies that have been subjected to the CRISPR transformation procedure that we have worked out. After the re-construction of a plasmid that served as the starting material for my recombinant DNA project, the new plasmid was mobilized into a culture of the target cyanobacterium using bacterial conjugation techniques so that it was transferred into the cyanobacterial cell where we hoped that it would begin to replicate and begin the gene modification process that was programmed into the DNA sequence of the plasmid I constructed. These procedures and the ensuing results are described below. As a result of this work, the plasmid constructed can continue to be utilized as a recombinant vehicle for CRISPR editing.

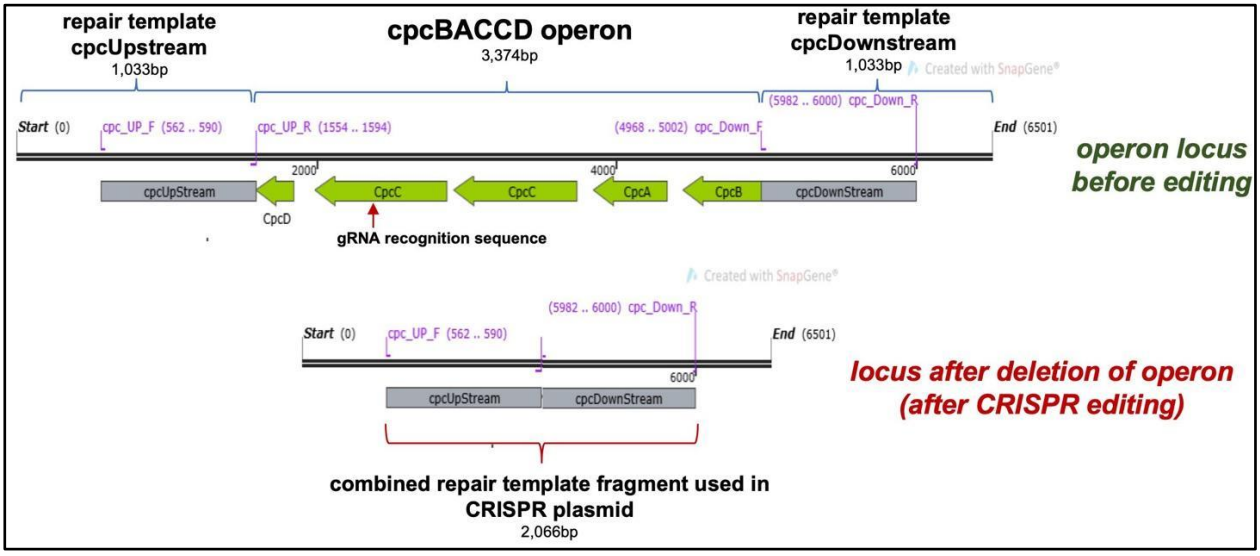

Figure 1. Map of the target cpcBACCD operon depicting the location of the cpc repair template upstream and downstream areas as well as the position of the gRNA recognition sequence. The purple text indicates the start of the repair template primers listed in Table 1. The green sequences are the target of this knockout experiment and are deleted if the repair template properly performs repair of the endonuclease cleavage of the Cpf1 enzyme guided by the gRNA to the recognition sequence location indicated with the red upward arrow and the combined repair template (bottom) is correctly utilized by the *Synechocystis* DNA repair enzymes to mediate the repair.

Methods

Plasmid Preparation

Cells of the *E. coli* strain DH5α contained the target plasmid, and were grown overnight at 37°C shaking at ~300 rpm in LB medium containing the appropriate antibiotic (either spectinomycin or kanamycin depending on the plasmid, see below). The cell suspension was transferred to a centrifuge tube and an Omega BIO-TEK plasmid kit was used to isolate the plasmid. First, the culture was centrifuged at a speed and time dependent on the culture volume at 4°C to form a pellet. The supernatant was discarded and Solution I, containing RNAse A, was added and used to resuspend the cells. Solution II was then added and gently mixed for 2 minutes to allow the cells to be lysed. Then Solution III was added to neutralize the reaction and the solution was centrifuged again. During the centrifugation, a Vac-Man Laboratory Vacuum Manifold was prepared by placing a Hi-Bind DNA column onto a suction spot and calibrated by allowing 1 mL of 3 M NaOH solution to flow through the column. Once the centrifugation was complete, the vacuum manifold was attached to a vacuum source and the solution was retrieved. Then, the supernatant was pipetted into the DNA column while the vacuum was running. Once all the supernatant had been filtered, HBC buffer diluted with 100% isopropanol was added and allowed to filter through. Next, DNA wash buffer was added two times and allowed to filter through. The column was then centrifuged again to ensure all traces of buffer were removed. The DNA column was then transferred to a sterile centrifuge tube. Warmed 10 mM Tris-HCl elution buffer was then added to the center of the DNA column matrix and allowed to sit for 1-3 minutes at room temperature. Afterwards, the centrifuge column was centrifuged to elute the plasmid from the DNA column. The DNA was then pipetted into a microcentrifuge tube and measured via Nanodrop for its concentration. The tube was then labelled and stored at -20°C.

DNA Precipitation

Frozen DNA was obtained from -20°C conditions and thawed. It was then spun in a desktop quick spin apparatus to reunite any stray droplets with the majority of the solution volume. First, 3M sodium acetate (1/10th the volume of DNA solution) was added, mixed and then 3 volumes of 100% ethanol. The sample was then mixed via gentle shaking and stored at -20°C for around 24 hours to allow the formation of a nucleic acid precipitate. Then the solution was centrifuged at 23,000 X g at 4°C for 30 minutes to collect the precipitate and then afterwards the supernatant was carefully pipetted out. Then 1 mL of 75% EtOH was carefully added to avoid disturbing the pellet and the sample was centrifuged under the same conditions again, only for 15 minutes. The EtOH was then discarded and the sample was run in a vacuum centrifuge with the cap open for 10 minutes to dry out any residual EtOH. Fifty microliters of elution buffer from the Omega BIO-TEK plasmid kit was then added and the sample was stored at -20°C.

Restriction Digest (RD)

To determine procedure specifics, such as ratios, buffers, and temperatures, NEBcloner was utilized (https://nebcloner.neb.com/#!/). The reaction was set up by adding 1 µg of target DNA, 1 unit of each enzyme, buffer, and purified water (nH_2O, $NanoH_2O$, purified using a Barnsted Inc multistage filtration unit) to a total volume of 20 µL. This sample was then mixed lightly and placed in a heated water bath according to the enzyme manufacturer's protocol. Once the reaction had run, the enzymes were heat inactivated, if possible, at high temperature.

Polymerase Chain Reaction (PCR)

The target DNA, forward and reverse primers, and Q5 High Fidelity 2X Master Mix (New England BioLabs), nH_2O were collected on ice. The PCR mix was then assembled by adding 20-30 µg of target plasmid DNA or 200-300 µg of chromosomal DNA, 1.25 µL of each 10 µM primer,

12.5 µL of Q5 polymerase, and water to a final volume of 25 µL in a 0.2 mL tube, with the water added first. The thermocycler was prepared prior to adding the tube by adjusting the programmed cycling according to the NEB protocol for the Q5 master mix. This protocol calls for the initial denaturation to take place at 98°C for 30 seconds. The PCR then takes place during 25-35 cycles of three temperatures, the first of which is 98°C and the last is 72°C. The middle (annealing) temperature of the cycle is determined based on primer sequences and concentration (http://tmcalculator.neb.com/#!/main). Once the program has been adjusted to the specifics of the PCR, the tube is placed in the thermocycler and the program is run to completion. Afterwards, the PCR solution was purified by adding 125 µL of Buffer PB (five times the volume of PCR solution) and mixing gently, resulting in one of two scenarios. If the liquid became yellow, the sample was added to a DNA binding column. If the liquid became orange or violet, 10 µL of 3M sodium acetate (~5.0pH) was added before transferring to the binding column. The column was then inserted into a centrifuge tube and centrifuged at 17,900 X g for 45 seconds. Then, 750 µL of Buffer PE were added to wash the sample and it was centrifuged at the same speed again for 45 seconds. The column was then transferred to a collection tube and the DNA was eluted by adding 50 µL of warm elution buffer and centrifuging for another 60 seconds. Afterwards, the liquid was transferred to a labelled tube and stored at -20°C.

Gibson Assembly (GA)

The linearized vector DNA, fragment DNA, and Gibson Assembly Master Mix (New England BioLabs), and nH_2O, were collected on ice. Two GA mixes were made: the target and a negative control. To assemble the target mix, fragment and vector DNA were added in an approximately 3:1 molar ratio, followed by 10 µL of GA master mix and nH_2O to a final volume of 20 µL. To assemble the control mix, no fragment DNA was added, the same amount of vector DNA and GA master mix was added, and nH_2O was added to bring the final volume to 20 µL. Both GA mixes were incubated in a thermocycler for 60 minutes and then stored at -20°C.

Gel Electrophoresis

The agarose gel was prepared by adding 0.3 g of agarose powder into 30 mL of 1 X TAE buffer and heating the solution in the microwave for 40-60 seconds until the powder was fully dissolved. It was then poured into a gel chamber fitted with a comb. Then it was allowed to solidify for at least 45 minutes. Once the gel was ready, the comb was removed and it was placed in an electrophoresis container filled with 1X TAE buffer. The samples and a molecular weight standard were prepared on a strip of Parafilm - each with a final volume of 6 µL. The samples were prepared with 1 µL of gel electrophoresis blue loading dye, a volume of plasmid DNA dependent on its concentration, and nH_2O to the final 6 µL volume. The standard was made with 1 µL of GeneRuler 1 kb Plus DNA Ladder, 1 µL of gel electrophoresis blue loading dye, and 4 µL of nH_2O. Afterwards the standard and samples were loaded into the wells of the gel. The lid was then placed on the container and hooked up to a voltmeter. The voltage was set at 25V for 5 minutes, then 75V for 30 minutes, then 25V for 5 minutes. While the electrophoresis was running, a dye was prepared to stain it by combining 30 mL of 1X TAE buffer and 9 µL of Gel Green Dye. The gel was placed in the staining solution and set on a shaker for 30 minutes. Afterwards, a picture of the gel was captured utilizing an E-Gel Imager and saved to the lab Dropbox cloud storage account.

Transformation of E. coli

Two strains of *E. coli* competent cells were used for transformation- DH5α for plasmid manipulation and HB101 for cells that would be involved in conjugation. To begin, two sets of cells were retrieved from a -80°C freezer and thawed on ice. One set was the GA target and the other was the negative control. Then 10-50 ng of target plasmid was added to the target while the GA negative control was added to a control tube containing competent cells. The thawed cells were then mixed with the added DNA via light flicking and allowed to sit on ice for 30 minutes. Afterwards, the samples were heat shocked at 42°C in a water bath for 30 seconds and

immediately moved back to ice for 5 more minutes. Nine hundred and fifty microliters of SOC outgrowth medium was added to each tube and then the contents were transferred to respective test tubes to incubate at 37°C on a shaker for 1 hour. After incubation, 200 µL of each sample was transferred and spread on to a respective LB plate with the appropriate antibiotic. The plates and test tubes were then incubated overnight at 37°C.

Conjugation of Synechocystis

Prior to the triparental conjugation, an HB101 *E. coli* strain containing CRISPR target plasmid (pCpf1_cpc_KO) along with the helper pRL623 plasmid and HB101 *E. coli* strain containing pRL443 plasmid were prepared using standard manufacturer transformation protocol (Promega, USA). After incubation overnight, 250 µL of culture was added to 10 mL of LB with appropriate antibiotic(s) and incubated for 2.5 hours at 37°C. Afterwards, the sample was gently washed three times with 10 mL of fresh LB medium containing no antibiotics. The supernatant was then discarded and the cells resuspended in 60 µL of LB medium. The sample containing the target CRISPR plasmid and the sample containing pRL443 were mixed and then allowed to sit at room temperature for 1-2 hours. While the mixed sample sat, the cyanobacterial cells (*Synechocystis* sp. PCC6803 nWT) were prepared by concentrating the cells via centrifugation at 6,000 X g for 7 minutes and then resuspended with 500 µL of DG BG-11. To estimate the amount of cyanobacterial cells to be used for conjugation, 100 µL of cell suspension was mixed with 900 µL of 100% methanol and mixed via vortex for 1 minute for the determination of the Chlorophyll (Chl) concentration. After centrifuging for 1 minute at 13,000 rpm, the Chlorophyll a concentration was measured using an extinction coefficient of 12.95 at 665 nm. 250 µL of cyanobacterial cell suspension (10 µg Chl total) was added to the *E. coli* mix and spread onto a nitrocellulose filter covering a DG BG-11+5% LB plate and stored under low light intensity for 3 hours. After 3 hours, the plate was kept under normal light conditions overnight- approximately 50 µmol (photons) m-2 s-1, provided by Cool White fluorescent lamps. The following day, the filters were transferred onto

DG BG-11 plates with no antibiotic and kept at normal light intensity for the day. On the third day, the filter was transferred onto a DG BG-11 plate containing 5 µg/mL gentamicin and 12.5 µg spectinomycin. Gentamicin was used to select for cyanobacterial cells over *E. coli*. On the sixth day, the filters were transferred to the DG BG-11 plates containing 5 µg/mL Gentamicin and 25 µg/mL spectinomycin and propagated until new colonies appeared. Once sufficient growth had occurred, cell samples were suspended in water at a concentration of 3.5 µg/mL and absorption spectra were recorded with a Shimadzu 2600 UV-VIS spectrophotometer. The spectra were then normalized from the readings at 665nm.

Results/Discussion

Replacement of AarI sites

The first step in manipulating the starting plasmid, pSL2680 (originally created by Ungerer & Pakrasi, 2016, obtained via AddGene) shown in Figure 2a, was to replace the AarI restriction enzymes sites with BsaI sites. The AarI enzymes proved difficult to acquire commercially and did not reliably produce enzymatic cuts and thus we chose to replace these two sites with alternative Type II enzyme recognition sequences for BsaI, as mentioned. To accomplish this, the plasmid DNA was isolated via plasmid preparation protocol. Fifteen microliters of plasmid (~1 µg) was then cut using SalI-HF and EcoRI-HF restriction enzymes in 1X CutSmart Buffer (New England BioLabs, USA). The resulting sample was then analyzed via gel electrophoresis in order to observe whether or not plasmid linearization via double digestion was successful. Figure 2b depicts the resulting gel. The smeared appearance of the uncut plasmid is due to its supercoiled state resulting in variable migration in response to the electrophoresis. The restriction digestion product migrated much more definitively than the uncut pSL2680, indicating that the DNA had linearized and a successful cut had taken place.

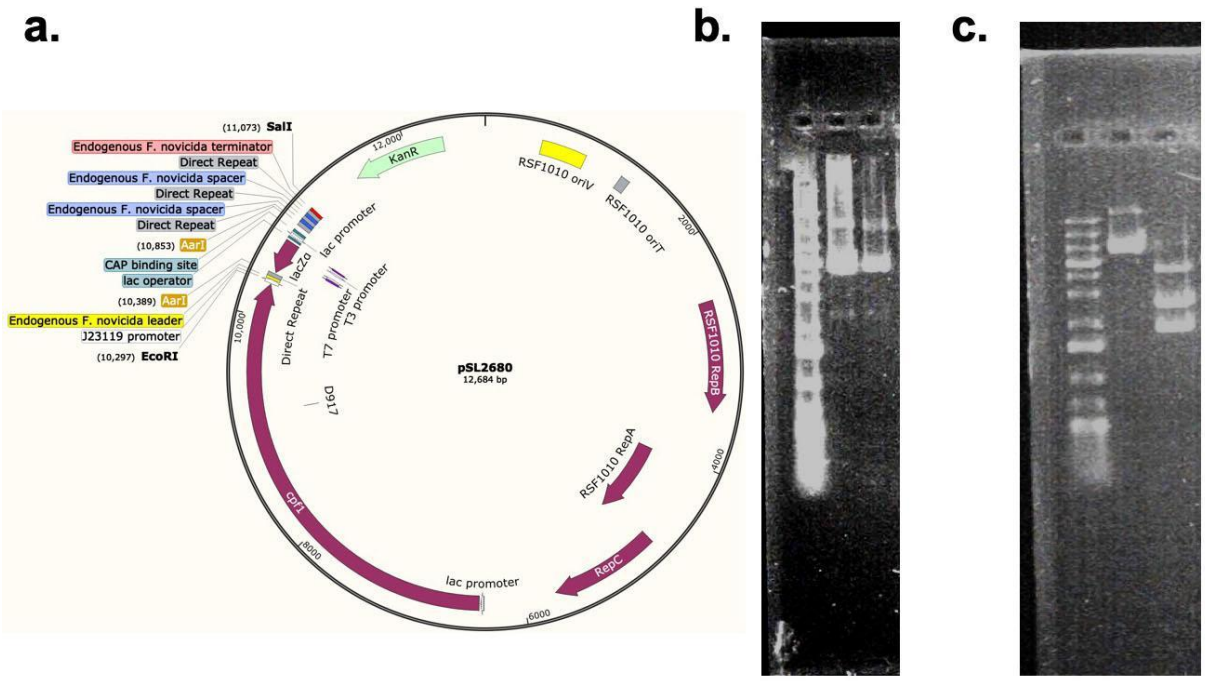

Figure 2. Initial work to introduce the BsaI sites into the pSL2680 plasmid: (a.) Plasmid map of the starter plasmid, pSL2680, depicting main CRISPR-related features and the AarI, EcoRI, and SalI restriction sites. The largest gene (cpf1) codes for the Cpf1 protein and the light green gene (KanR) is a kanamycin cassette. **(b.)** An image of the gel containing a DNA ladder in lane 1, uncut pSL2680 in lane 2, and the pSL2680 RD product in lane 3 using SalI-HF and EcoRI-HF restriction enzymes in 1X CutSmart Buffer (New England BioLabs, USA). Note that the loss of the ~800bp fragment did not appear to alter the mobility of the fragments, but subsequent cloning showed that at least a fraction of the plasmid was successfully cut since I could use the digestion product in GA **(c.)** A picture of the gel electrophoresis product following a vector containing a synthetic sequence for the guide RNA but with the alternative BsaI pUC57 RD. Lane 1 contains the DNA ladder, lane 2 contains the uncut pUC57, and lane 3 contains the pUC57 RD product.

pUC57 (GenScript) was obtained, a commercially synthesized plasmid containing a DNA region homologous to the region containing the AarI restriction enzyme sites in pSL2680, but with BsaI sites instead. It was isolated via plasmid preparation protocol and then digested using BspQI restriction enzymes in 1X NEBuffer 3.1 (New England BioLabs) using the manufacturer's protocol. The results of this restriction digestion are shown in Figure 2c. pUC57 has two BspQI restriction sites, therefore the appearance of three bands in the product row supports the conclusion that the cuts were successful. The sequence for the target fragment is 1,127 base pairs (bp), indicating that the middle band of the row containing cut pUC57 is the one that will be inserted into pSL2680.

To insert the region of interest containing the desired restriction sites into pSL2680, the obtained pieces of DNA were assembled via Gibson Assembly (GA) in one reaction tube. To have a control for false positive results, a negative control for the Gibson Assembly was used: a GA reaction containing only linearized pSL2680 and water, leaving out the target DNA piece obtained from pUC57. While the desired outcome for the assembly would be a new circular DNA containing pSL2680 backbone and insert sequence from pUC57, the negative control would only contain linear pSL2680 backbone and copies of non-digested pSL2680. The assembled product and negative control were then used to transform separate sets of DH5α *E. Coli* chemically competent cells and plated on separate LB agar plates with a 50 µL/mL kanamycin concentration. Six of the resulting colonies on the target plate were isolated and grown to be used for testing as sequencing samples. Plasmid preparations were conducted with each of the samples, and 500ng of each were sequenced with Cpf1_seq_F1 and Cpf1_seq_F2 primers (Table 1). The sequencing results indicated that the samples grown from colonies 2 and 5 contained the BsaI sites inserted into pSL2680. With the sites replaced, the plasmid was ready to receive the guide RNA (gRNA) and repair sequence needed to give the CRISPR system a target sequence to edit; however, this plasmid became superseded upon acquisition of an improved variant plasmid (next section). Nevertheless, having PCR-synthesized and introduced a new pair of BsaI restriction sites into pSL2680 and due to that region of the plasmid being similar to the new plasmid, called ***pCpf1-sp***, I was able to utilize the cloned DNA fragment with the pair of BsaI restriction sites in the improvement of the new vector.

Shift to pCpf1-sp

It is important to note that at the beginning of the project, I started work using plasmid pSL2680 with the first figures of the thesis describing the preparation of that plasmid. However, during the preparation of the new vector, the lab received an improved version of the original plasmid containing the *sacB* gene and allowing a counterselection procedure. Accordingly, after modifying pSL2680 to insert the gRNA and repair sequence, we inserted the modified sequence from pSL2680B into the new plasmid, which was a generous gift from Dr. Ju-Yuan Zhang in the laboratory of Professor Cheng-Cai Zhang, called pCpf1-sp, (Niu et al., 2018) of the Institute of Hydrobiology, Wuhan, China. The plasmid was essentially the same as pSL2680; however, it had a spectinomycin resistance cassette instead of a kanamycin resistance gene and contained a counter selectable marker gene, *sacB*. This *sacB* gene causes the toxicity to the target cyanobacterial host when exposed to sucrose, allowing for the selection of cells devoid of the plasmid once the intended genomic editing has taken place (Gay et al., 1983). This is achieved through the conversion of sucrose to levans, which inhibits growth or causes lysis from the presence of 5% sucrose in agar (Gay et al., 1985).

pCpf1-sp was accumulated and then digested using SpeI-HF and BamHI-HF restriction enzymes in 1X CutSmart buffer. The BsaI insert sequence was amplified from pSL2680B via polymerase chain reaction (PCR) utilizing the forward and reverse 'BsaI_Insertion' primers, along with the upstream and downstream sequences of the repair template with the forward and reverse 'cpc_UP' and 'cpc_DS' primers, respectively (Table 1). To verify specific PCR amplification of desired products, samples were processed on a 1% agarose gel via electrophoresis (Figure 3a). The upstream and downstream repair template amplicons were designed to be 1,033 bp long, and the bands on the gel clearly indicate specific amplification with the product size at around 1,000 bp. The BsaI-containing fragment from the previously engineered pSL2680 was expected to be 1,127 bp long. Overall, the results are indicative of successful amplification of all three target sequences.

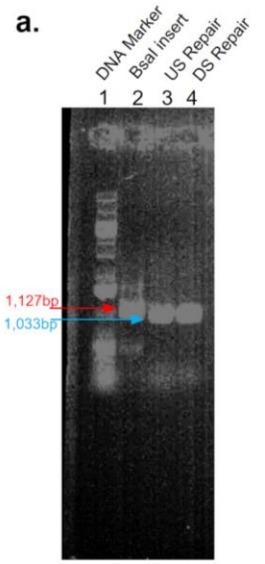

Figure 3a. A gel picture of PCR products. Row 1 contains DNA ladder, Lane 2 contains the BsaI insert amplification (1,127bp), lane 3 contains the upstream repair (USR) template amplification (1,033bp), and lane 4 contains the downstream repair (DSR) template amplification (1,033bp). These repair templates were Gibson assembled into the new plasmid in the following section. First, the BsaI insert was assembled into pCpf1-sp, and then the gRNA sequence was inserted.

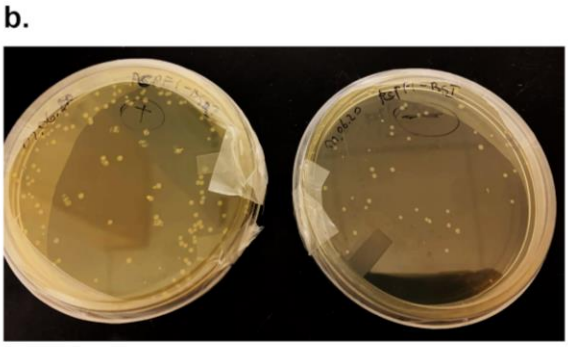

Figure 3b. Gibson assembly of the BsaI Insert into the pCpf1b-sp plasmid to produce pCpf1-BsaI. A picture of the target transformation plate (left) and the negative control (right). The presence of around double the amount of colonies on the target plate indicate that the GA and subsequent transformation were successful. Eight colonies were pick and screened by DNA sequencing to confirm.

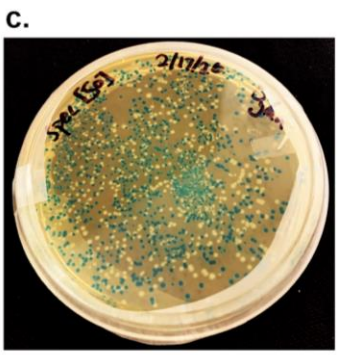

Figure 3c. Insertion of the gRNA into pCpf1-BsaI. Correct insertion could be scored by the blue-white selection on an X-Gal/IPTG plate. White colonies are assumed to contain the target sequence, while the blue pigment indicates the presence of the original *lacZ* gene.

The linearized pCpf1-sp and BsaI fragment from pSL2680 were then assembled via Gibson Assembly. The reaction was performed according to the manufacturer protocol with a molar ratio of insert to vector of 7 to 1. For the negative control, water was used instead of the insert DNA. The GA products were then used to transform two respective sets of DH5α *E. Coli* competent cells. The cells were then used to inoculate two separate sets of LB agar plates with a 50 µg/mL concentration of spectinomycin antibiotic. After incubation overnight, the plates revealed colonies as seen in Figure 3b. The growth on the negative control was representative of the relative amount of false-positive results. The presence of around double the number of colonies on the target plate compared to the negative control indicated a successful transformation. In order to fully ascertain if the assembly was accomplished, eight colonies were selected from the target plate. Plasmid preparations were conducted with each of the samples, and 500ng of each were sequenced with the forward and reverse 'BsaI_Insertion' primers (Table 1). Of the eight colonies, colony 6 yielded results signaling that the BsaI sites had been inserted into pCpf1-sp. This colony was picked and struck on fresh selective plates to obtain a pure isolate.

Insertion of sequences encoding the guide RNA (gRNA) via ligation

Plasmid DNA from colony 6 (pCpf1-BsaI) was isolated and then cut using BsaI restriction enzymes. The guide RNA sequence consisted of two primers. 10ng of each were combined to make the gRNA mix for the ligation reaction. The DNA backbone and gRNA insert were combined in a 1:15 molar ratio in 1X T4 ligase buffer with 2 units of T4 ligase and allowed to react for 2 hours at room temperature. 4 µL of this product was then spread onto an X-Gal/IPTG plate with a concentration of 50 µg/mL of spectinomycin antibiotic for blue-white selection and incubated overnight. The results are depicted in Figure 3c. The X-Gal/IPTG plate differentiates between colonies that contain the target sequence and the original plasmid via color of the colonies. Colonies that contain the *lacZ* gene that codes for ß-galactosidase (this gene is located between the BsaI sites and is the target to be replaced with gRNA), which hydrolyzes X-Gal and results in a blue pigment forming. Therefore, the white colonies can be assumed to contain the target sequence in place of the *lacZ* gene. The presence of white colonies indicates that the product ligation and transformation were successful, and that the plasmid has the gRNA sequence (Pcpf1-gRNA).

Insertion of repair template sequences into pCpf1-gRNA

The pCpf1-gRNA plasmid DNA was then accumulated via plasmid preparation and digested using the restriction enzyme BamHI in CutSmart buffer. The linearized plasmid was then mixed with the upstream and downstream repair template PCR products and combined via GA at a 2:2:1 inserts to vector molar ratio. A volume of water equal to the volume of the inserts was used for the negative control reaction. The GA products were then used to transform two respective sets of DH5α *E. Coli* competent cells. The cells were then used to inoculate two separate sets of LB agar plates with a 50 µg/mL concentration of spectinomycin. These plates were then incubated overnight. The target and negative control plates yielded very similar amounts of colonies, which did not support that the assembly and subsequent transformation

were successful; however, the sequencing results proved otherwise. Five colonies were selected from the target plate and their DNA was accumulated separately via plasmid preparation protocol. 500ng of each sample was then sequenced with the Cpf1_seq_F1 primer to detect the presence of the repair template (Table 1). The results showed that Colony 1 had indeed received the repair template. This Colony 1 DNA was then isolated via plasmid preparation to be used for conjugation. The final plasmid product was named pCpf1_cpc_KO and the map depicting the inserted gRNA and repair template, as well as the primer locations, can be seen in Figure 4.

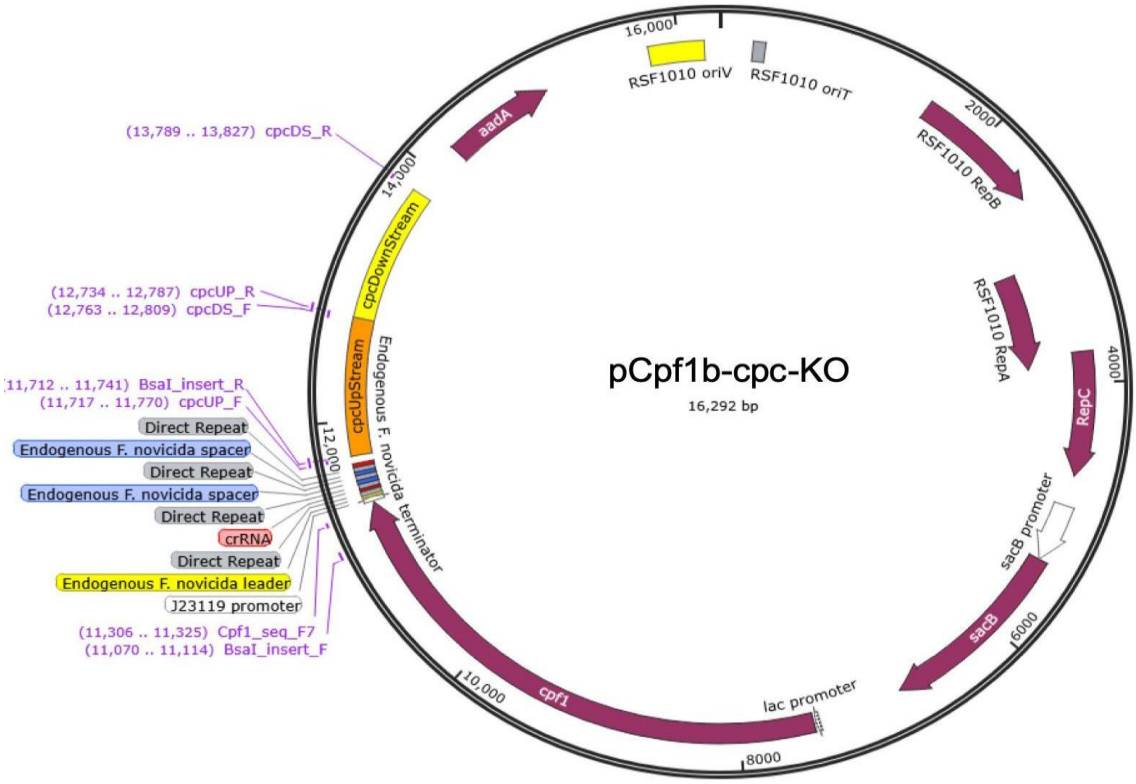

Figure 4. Plasmid map of the final plasmid product pCpf1b-cpc-KO. This plasmid has been manipulated to include the specific gRNA sequence as well as the fully constructed repair template to excise the cpc gene cluster encoding the highly blue pigmented light harvesting pigment proteins known as C-phycocyanin. See Fig. 3a for construction.

Conjugation and transformation of Synechocystis: Deletion of pigment-producing genes

The *Synechocystis* sp. PCC6803 cells were conjugated via the protocol described in Methods. The resulting colonies are depicted in Figure 5a. For comparison, a plate of wild type (WT) *Synechocystis* sp. PCC6803 cells is pictured to the right of the conjugation product plate. A

distinct difference can be observed. While the WT is a deep green color, the conjugation product is a much lighter, almost yellow color of green. This is exactly the phenotype expected as a result of successful CRISPR system assembly, indicating that the gene editing took place and the *cpcBACCD* operon had been knocked out. Though this visible change in appearance supported the conclusion that the experiment had functioned properly, absorbance spectra were still gathered in order to compound this assumption. The resulting spectra can be seen in Figure 6. The graph plots the absorbance spectra of both the WT and the knockout product, normalized from 665nm. The peak at ~620nm in the WT results from the pigment-protein complex phycocyanin (Glazer, 1989). This complex is encoded by the *cpcBACCD* operon, therefore the absence of this peak in the knockout product's spectra further shows that the operon was successfully removed.

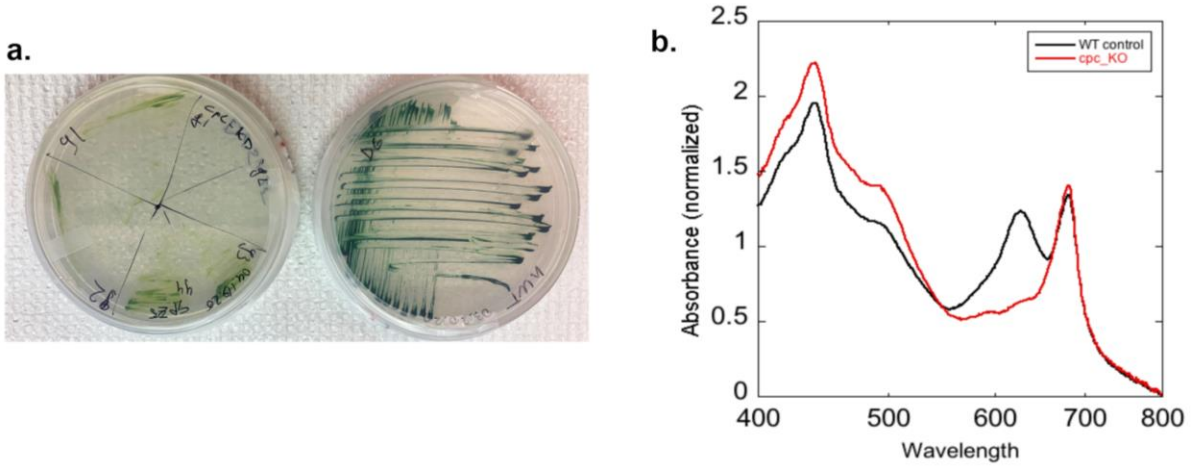

Figure 5. Phenotype of the edited *Synechocystis* cells: (a.) The conjugation products representing four transconjugant mutant cells (left) and WT control (right). Note the difference between the yellow green color of the **cpc** mutants versus the blue green color of the wild type, which is consistent with the loss of the blue C-phycocyanin light harvesting proteins encoded by the *cpcBACCD* operon. The yellow-green coloration of the conjugation product marks the absence of phycocyanin. **(b.) A graph depicting the absorption spectra of the knockout product and the wild type (WT) control.** The area of interest in ~620nm, where phycocyanin has maximal absorption. The absence of this peak in the cpc_KO supports the conclusion that the *cpcBACCD* was successfully removed. The data is normalized from 665nm.

To establish genetic evidence that this knockout had taken place, chromosomal DNA from the wild type species and the knockout target were isolated and then amplified via PCR. The primers

utilized were the forward and reverse primers of the upstream and downstream repair template sequences. The expected result of amplifying the unedited area of the operon is a 5,440 bp product, which is the sum of the repair template sequences (1,033 bp each) and the *cpcBACCD* operon (3,374 bp) as seen in Figure 1. The expected result of the knockout target amplification is a product containing only the repair template sequences, which is 2,066 bp total. Figure 6 depicts the resulting PCR products, which further indicate that the operon has been deleted.

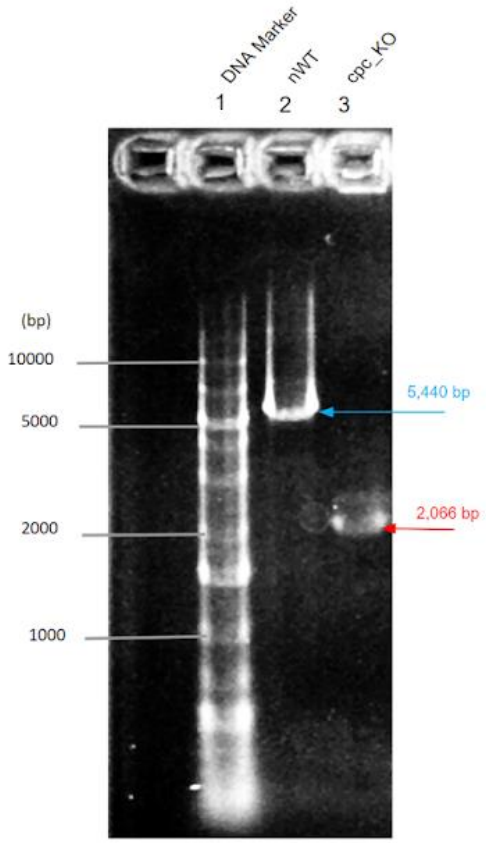

Figure 6. PCR products indicating genetic verification of successful knock out. Chromosomal DNA from the wild type species and the conjugated knockout species were amplified via PCR with the upstream and downstream cpc primers. The ~3,000 bp difference indicated that the *cpcBACCD* operon (3,374 bp) had been removed.

Conclusion

The purpose of this work was to create a CRISPR-Cpf1 system template plasmid that could be tailored for specific editing in *Synechocystis.* In order for this goal to be successful, the plasmid needed to be modified with convenient restriction enzyme sites and shown to be effective. To demonstrate its efficacy, the *cpcBACCD* operon was deleted from *Synechocystis* sp. PCC6803. The knockout was shown to have taken place visibly through expression of the olive-colored cell colonies phenotype, chemically through absence of phycocyanin absorption observed via analysis with a spectrophotometer, and genetically through PCR amplification of chromosomal DNA exhibiting a ~3,000 bp loss in the operon region. This template plasmid may now be confidently used to conduct further research involving genetic editing in bacteria.

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Table of Primers	
Name	Sequence
Cpf1_seq_F1	GGGCTATTTTGAGTTTAGTTTTGAT
Cpf1_seq_F2	GGGCAAAAAACTCAATTTGG
Cpf1(lacZ)_seq_F3	GGCGTAATCATGGTCATAGC
Bsa1_Insertion_Forward	GACAAAAAGTTTTTTGCTAAGCTAACTAGTGTCCTAAATACTATC
Bsa1_Insertion_ Reverse	GCAACGTTGTTGCCATTGCGGATCCCCCGGATTACAGATCCTCTAGAGTCGACG
gRNA_Forward	AGATCCCAAAGTTCTCTTGGTATTC
gRNA_Reverse	AGACGAATACCAAGAGAACTTTGGG
cpc_UP_Forward	ACTCTAGAGGATCTGTAATCCGGGGTAAAGTAGCCCAGAGTCGTTTTATTTCCC
cpc_UP_Reverse	GGAGATTAATTCAGCTATTTTGTTAATTACTATTTGAGCTGAGTGTAAAATACC
cpc_DS_Forward	TAACAAAATAGCTGAATTAATCTCCTACTTGACTTTATGAGTTGGGA
cpc_DS_Reverse	GCAACGTTGTTGCCATTGCGAGTAAACGCCTCAACCACG

Table 1. Lists primers and their respective sequences.

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