

THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF CHEMICAL ENGINEERING

DEVELOPMENT OF THE MARINE CYANOBACTERIUM *SYNECHOCOCCUS* SP. PCC
7002 AS A SCALABLE LOW-COST PHOTOAUTOTROPHIC PROTEIN PRODUCTION
PLATFORM

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ABSTRACT

This work describes the development of a novel protein expression platform which allows for low-capital, high-level expression of functional proteins in the marine cyanobacteria *Synechococcus* sp. PCC 7002. While significant research has focused on high-level expression of genes in cyanobacteria, the platform cannot effectively be scaled due to the need to maintain antibiotic selection pressure to prevent loss of transformed genes. In order to improve this expression platform, this work employs the CRISPR/Cas9 genome editing system to selectively cause double stranded breaks (DSBs) in untransformed copies of a native high copy number expression plasmid, thereby providing selection pressure against the native plasmid to facilitate its elimination. Cloning genes of interest and the CRISPR system into separate endogenous plasmids provides for a platform approach to generate genetically stable, high level expression strains of cyanobacteria. Once the native plasmid is eliminated, these strains can be used for large-scale protein production without the presence of antibiotic selection pressure. This platform can in turn be implemented as a value-added approach to production of biomass for conversion to hydrocarbon biofuels; as well as facilitate low-capital production of high-value proteins. This work details the development of a CRISPR/Cas9 complex capable of providing selection pressure against the native plasmid, as well as progress towards integrating this complex into a cyanobacterial cloning vector.

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Chapter 1

Background Information:

1.1 Protein expression in cyanobacteria

The recent push to develop biofuels and cost-effective biological protein production platforms has resulted in extensive characterization of cyanobacteria. Perhaps best known as the culprit of toxic aquatic blue-green algal blooms, cyanobacteria are a highly adaptable class of photosynthetic bacteria. Research on this broad class of organisms has revealed that they produce a wide range of biologically active compounds with antiviral, antibacterial, antifungal and anticancer properties (Abed, Dobretsov, & Sudesh, 2009).

In addition to the potential for a wide range of new pharmaceutical and commodity compounds, cyanobacteria present an alternative opportunity for high-level protein expression in a photoautotrophic, CO₂-fixing organism. Many existing protein production platforms including *E. coli* require comparatively high-value sugar feed substrates, limiting their practicality to high-value products. In addition, the utilization of sugars as a feed stock makes heterotrophic bioreactors inherently more permissive to contamination, and therefore require high-capital cost sterile operating bioreactors or pre-sterilized disposable alternatives. As a photoautotrophic bacteria, the growth substrate requirements of cyanobacteria are comparatively minimal, and therefore competitive growth is minimized. Nitrogen and phosphorus supplementation could ultimately be supplied by recycled wastewater effluent, making only trace nutrients necessary to facilitate large-scale production. Development of a marine strain of cyanobacteria also presents

an opportunity to eliminate need for high volumes of fresh water for protein production. This expression platform could be grown in coastal regions or even off-shore with nutrient-supplemented ocean water as their primary substrate.

One feature of cyanobacteria that makes it particularly interesting to study is its ability to acquire new DNA from its surroundings, a trait referred to as “natural competency”. As an evolutionary asset, the ability to acquire new genes from other organisms provides the opportunity to gain a selective advantage over surrounding bacteria. From a research perspective, this provides us the opportunity to insert and express genes from other organisms in an easy-to-grow platform. Moreover, the cyanobacterium *Synechocystis* sp. PCC 6803 was the first completely sequenced photosynthetic organism, and it continues to be a valuable and well-characterized model organism. The strain used in this study, *Synechococcus* sp. strain PCC 7002, is also naturally highly competent for homologous recombination, and its genomic DNA and plasmids have been completely sequenced.

Several expression plasmids have been developed to take advantage of this natural competency and allow simple genetic transformation into *Syn.* sp. 7002 using basic cloning techniques. This strain contains six native endogenous plasmids, denoted pAQ1, pAQ3, pAQ4, pAQ5, pAQ6, and pAQ7 (in order of ascending size). While exact copy number of each plasmid varies, it has been reported that pAQ6 and pAQ7 have the same copy number as the chromosome, while the net copy numbers are as follows: [copy number per cell in exponential phase--chromosome : pAQ1 : pAQ3 : pAQ4 : pAQ5 : pAQ6 : pAQ7 = 6 : ~50 : 27 : 15 : 10 : 6 : 6] (Xu et al., 2011). As a result, research has focused on insertion of genes into pAQ1 and pAQ3 in hopes of achieving high-level expression associated with the elevated copy number of these plasmids.

Presently, the major drawback to this expression platform is the inability to cost-effectively scale up production of a protein once the organism is transformed. While highly competent to transformation via homologous recombination, only a fraction of the native plasmids are transformed with a gene of interest, resulting in transformed strains retaining copies of both transformed and untransformed native plasmid. Theoretically, optimal protein production could be achieved by "curing" a strain of the native plasmid, therefore maximizing the copy number of the expressed gene of interest. Moreover, the natural competency of *Syn. sp.* means that strains containing both transformed and untransformed copies of the plasmid can readily revert to the native plasmid, losing the cloned gene of interest. At present, the only way to ensure the gene of interest is maintained is to grow the strain under antibiotic selection pressure and thus far researchers have not been successful in curing strains of the native plasmid aside from pAQ4 (Bryant, unpublished result). However, Algenol recently filed a patent involving a method of curing strains of *Syn. sp. PCC 7002* of both pAQ1 and pAQ3 of their native plasmids using antibiotic concentrations in excess of 3000 ng/ μ L (US Patent 20140272949 A1). It is interesting that such a patent would be awarded based on a technique that is somewhat obvious to those familiar with plasmid selection, but it none-the-less reflects the difficulty of achieving this goal of native plasmid removal. While this technique successfully eliminates the native plasmid to generate a stable transformed strain, the process is highly involved and any new gene-containing strain must be slowly acclimatized to increasing concentrations of antibiotic.

In order to address these concerns, this work seeks to create an economically feasible platform approach to cloning new genes into cyanobacteria then selectively eliminating the native plasmid. The platform will allow for the creation of genetically stable transformed strains which can then be utilized for high-level protein expression without the use of antibiotics, while

allowing freedom to operate without infringing on the aforementioned patent. Specifically, the CRISPR/Cas9 endonuclease system will be introduced to the organism to selectively cause a double stranded break (DSB) in the native pAQ1 plasmid. Because the native plasmid will be subject to frequent DSBs, copies of the transformed plasmid carrying our gene of interest will be favorably retained over the native plasmid. Within several generations, this selection pressure may be sufficient to completely eliminate the native plasmid thereby producing a genetically stable protein expression platform suited for large-scale production.

An additional characteristic of this approach is that the use of pAQ3 as a vector to introduce the CRISPR/Cas9 system will create a system in which the plasmid is unstable to its native pAQ3 counterpart. As a result, after elimination of the native pAQ1 plasmid, a relaxation of the selection pressure provided for the pAQ3-CRISPR/Cas9 construct will result in its associated elimination. One can envision that eventually a transformation strain of *Synechococcus* could be developed carrying a reporter such as pAQ1-YFP in which the subsequent transformation would replace the YFP so that a CRISPR/Cas9 could be developed to target YFP and allow for visual confirmation of the complete replacement of genes for expression in the pAQ1 plasmid.

1.2: Understanding the CRISPR/Cas9 genome editing system

CRISPR/Cas9 genome editing has quickly become regarded as one of the most promising new tools for genetic engineering for applications ranging from developing genetic knock-outs to potential applications for repairing single nucleotide polymorphisms (SNPs) responsible for crippling diseases in the human genome. In nature this system is used as a form of adaptive

immunity against foreign DNA including phages in bacteria. The mechanism of CRISPR, which stands for Clustered Regularly-Interspaced Short Palindromic Repeats, while outside the scope of this work, can be generally understood through the application of three primary components: Cas9 endonuclease, crRNA (or CRISPR RNA), and tracrRNA.

For the purpose of this work, the CRISPR/Cas9 system described is that which was originally adapted from *Streptococcus pyogenes*, and contains separate crRNA and tracrRNA sequences. An alternative version of this complex utilize a chimeric crRNA:tracrRNA protein in which these two components are fused and therefore need only associate with the target DNA and the Cas9 protein complex.

A simplified figure depicting these major components of the system and their mechanism of cutting DNA is included in Figure 1.

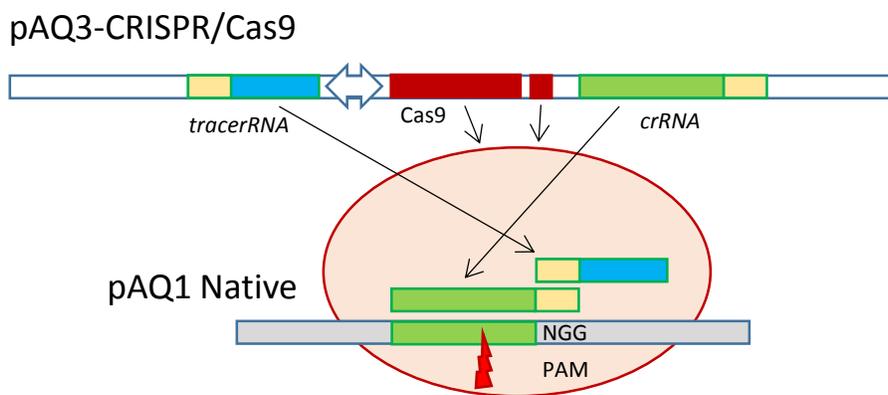


Figure 1: Overview of the CRISPR/Cas9 System. The crRNA::tracrRNA duplex recruits the Cas9 enzyme complex to facilitate the double stranded DNA break at the target sequence

Cas9 or Cas9 endonuclease is a protein which contains two nuclease domains, and is primarily responsible for cutting DNA. This protein complex is depicted in red in Figure 1, and

will not cut DNA unless it associates with the other two components. tracrRNA is a transcribed RNA product which associates with the crRNA and recruits Cas9 endonuclease, and is depicted here in blue. In simplified terms, tracrRNA acts as a "bridge" between the crRNA which binds to the target DNA and the Cas9 protein complex. Finally, the crRNA (depicted in green) is the actual piece of the system responsible for specific binding to target DNA and ultimately cutting that DNA with Cas9. The crRNA consists of a short sequence specific to foreign DNA; for the purposes of utilizing CRISPR/Cas9 for genome editing this is the sequence in which target DNA is inserted. Once transcribed, the crRNA associates with target DNA with a matching sequence based on highly specific DNA/RNA homology. In addition, the target DNA must be flanked by a 3 nucleotide PAM (or protospacer-adjacent motif, in this case NGG where N is any nucleotide followed by two guanines) for the system to cut. Once the crRNA is bound to the target DNA, tracrRNA binds to it and recruits Cas9. This "scaffold" then directs Cas9 to cut slightly upstream of the PAM sequence.

For the native CRISPR/Cas9 system of *Streptococcus pyogenes*, there are additional enzymes following CAS9 that are involved in processing multiple tandem target RNAs which is not needed in this application. Should future applications seek to include additional targets for CRISPR/Cas9, these could be incorporated without re-cloning the entirety of the Cas9 complex. An additional note is that the system utilized here is driven by the native *S. pyogenes* promoters. While this work relies on the assumption that the native promoters will be expressed in cyanobacteria, a separate project is underway utilizing the chimeric crRNA:tracrRNA CRISPR/Cas9 system with endogenous cyanobacterial promoters.

Chapter 2

Materials and Methods:

2.1 Bacterial Strains:

All work concerning “cyanobacteria” was conducted using *Synechococcus* sp. strain PCC 7002 unless otherwise noted. Plasmid proliferation of all plasmids was performed in TOP10 *E. coli*.

2.2 Plasmids and Expression Systems:

All plasmid maps are included in Appendix A: Plasmid Maps; FASTA DNA sequences are included in Appendix B: Sequences.

To optimize protein expression, the two highest copy number plasmids present in *Syn. sp* 7002 were selected for transformation. These native plasmids are pAQ1 and pAQ3. Expression systems, defined as plasmids designed for the insertion of genes into the native plasmids of *Syn. sp* 7002, were provided by Dr. Donald Bryant, Professor of Biochemistry and Molecular Biology at the Pennsylvania State University. These expression systems include pAQ1-Ex and pAQ3-Ex, which are comprised of a multiple cloning site (MCS) and antibiotic resistance genes, flanked on either side by regions homologous to their respective native plasmids to allow cloning of cyanobacteria via homologous recombination.

The level of protein expression is critically dependent upon the strength of the promoter; in this case the promoter utilized is the strong constitutive promoter of the *c*-phycoyanin CPC operon. Phycoyanin is involved in light- harvesting and represents one of the most highly

accumulated proteins in cyanobacteria. Interestingly this promoter (P_{pcp}) has been independently reported for the application of high-level expression in both *Synechococcus* and *Synechocystis* which is discussed in detail in [Appendix C](#). This work has been with the version of this promoter developed by Dr. Donald Bryant.

For cloning of the CRISPR/Cas9 endonuclease system, the plasmids pCAS9 and pCRISPR were supplied by Dr. Edward Dudley, Associate Professor of Food Science at the Pennsylvania State University. pCAS9 and pCRISPR were gifts from Luciano Marraffini (AddGene plasmids #42875 and #42876 respectively).

2.3 Primers and Oligonucleotides.

All primers and oligonucleotides were custom designed for cloning; sequences are compiled in [Appendix B: Sequences](#).

2.4 Cloning Methods:

2.4.1: Cloning methods for manipulation of homologous recombination transformation vectors

Cloning the aforementioned genes of interest into pAQ1-Ex was performed as previously described (Xu et al., 2011). An abbreviated method is outlined below:

- 1) Genes of interest are isolated from DNA via PCR. Cloning primers are designed with restriction overhangs including BamHI and either NdeI or NcoI. NcoI retains a HIS tag for protein purification while NdeI removes it as per design of the MCS.

- a) If the gene contains restriction sites matching these enzymes, primers can be designed using a compatible restriction endonuclease site (e.g. BglII instead of BamHI)
 - b) When designing primers for restriction-based cloning, an appropriate number of additional basepairs beyond the enzyme restriction sequence must be incorporated. Refer to [New England Biolab \(NEB\) for cutting efficiency](#) close to the end of a DNA fragment.
- 2) Both the PCR product and purified pAQ1-Ex plasmid are double digested to generate compatible nucleotide overhangs.
- a) Enzyme/buffer compatibility must be considered when double or sequential digesting. For SacII/SacII, an adjustment solution was prepared to allow sequential digestion in NEBuffer 2.1 and NEBuffer 3.1 by adjusting buffer salt concentrations. Recipe for this solution is included in Appendix D: Media and Reagent Recipes.
- 3) Digested products are ligated together generating pAQ1-Ex plus a gene of interest.
- 4) Ligation product is used to transform Top10 *E. coli* and transformants are selected for using either ampicillin or spectinomycin antibiotics based on the resistances included on the plasmid.
- a) Spectinomycin resistance is included for ultimate selection pressure in cyanobacteria; the appropriate gene is expressed in *E. coli* and can be used to avoid formation of satellite colonies typical of beta-lactamases.
 - b) While endogenous to *Synechocystis*, the CPC promoter used for high-level expression in cyanobacteria is also expressed in *E. coli*. As a result, proteins should be expressed in transformed *E. coli*.

- c) pAQ1-Ex and pAQ3-Ex both have versions which contain YFP in the MCS, which is expressed in *E. coli* by the CPC promoter. Clones containing plasmid which did not ligate with the new desired protein can be screened by visualization of fluorescence.
- d) For the following experiments, YFP fluorescence was visualized using a Clare Chemical Research DarkReader Transilluminator (Model DR-88X).
- 5) After confirmation of correct gene insertion, the purified plasmid is linearized via restriction digest and used to transform cyanobacteria.

2.4.2: Cloning methods for manipulation of CRISPR/Cas9 and insertion into pAQ3-Ex

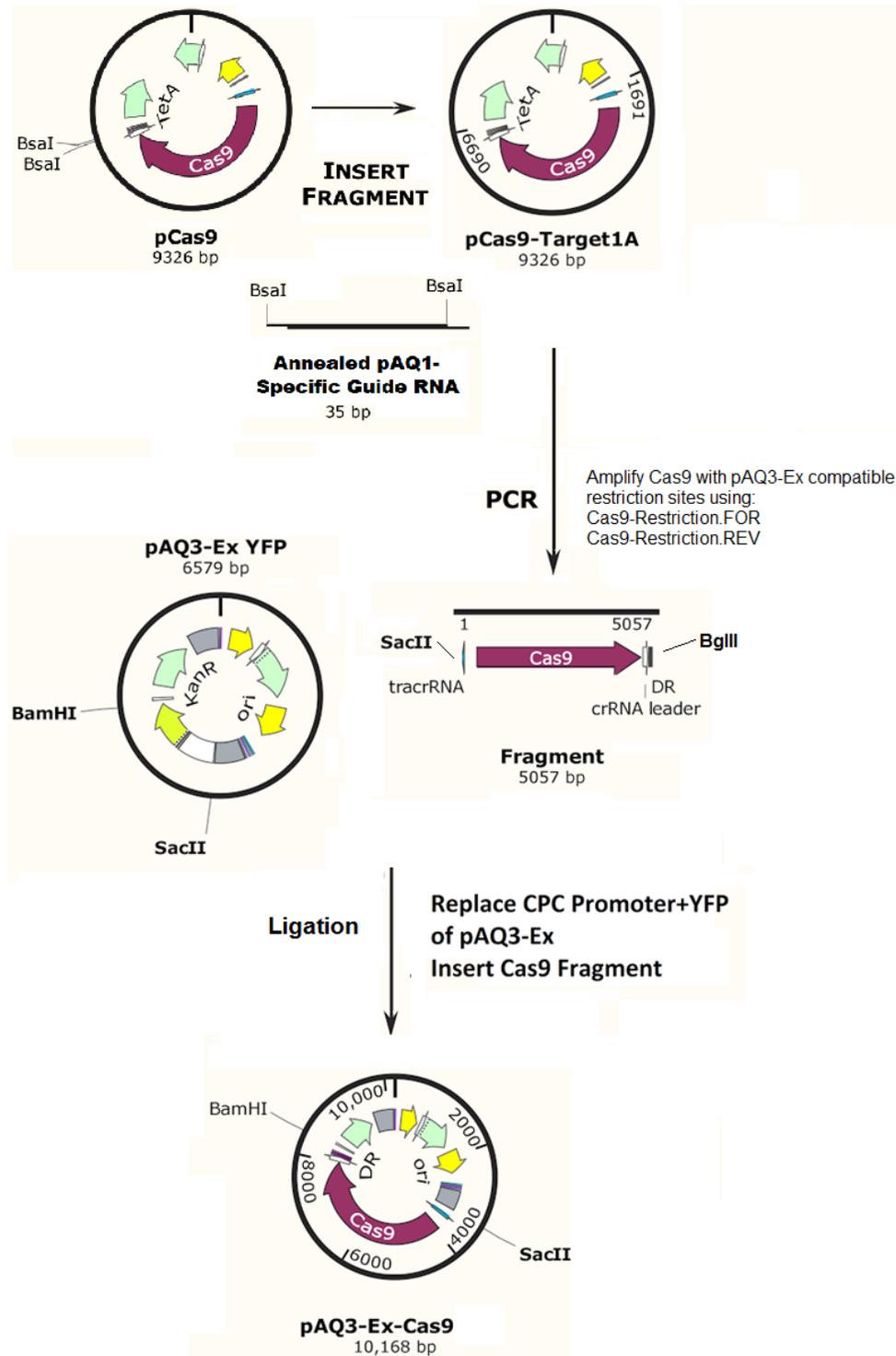


Figure 2: Overview of the cloning process used to generate pAQ3-Ex Cas9 from pCAS9 and pAQ3-Ex YFP plasmids

Figure 2 details the abbreviated cloning process of creating pAQ3-Ex Cas9 starting with the CRISPR construct from *S. pyogenes*. The pCAS9 plasmid contains genes for the CRISPR/Cas9 endonuclease genome editing system where the approach is to rely on the native promoters from *Streptococcus pyogenes* to drive the components of the CRISPR/Cas9 contained on the pCAS9 plasmid. CRISPR uses a highly specific crRNA scaffold that is unique to the DNA of its target, in this case the native pAQ1 plasmid. Once assembled, the pAQ3-Ex Cas9 plasmid can be used to transform cyanobacteria by homologous recombination where the CRISPR/Cas9 and kanamycin antibiotic resistance genes will be inserted into the native pAQ3 plasmid.

The first step in utilizing the CRISPR/Cas9 system for genome editing is adding a target DNA sequence to the crRNA (refer to Figure 1 for an overview of the CRISPR/Cas9 complex). For this application, the desired target sequence must be specific to the native pAQ1 plasmid but not the transformed plasmid. As depicted in Figure 3, there is a roughly 1 kb region in the native pAQ1 plasmid which is between the flanks used for homologous recombination with pAQ1-Ex (which are shown in green). This 1 kb region is therefore eliminated when homologous recombination occurs, and makes an ideal region to target with Cas9 to cause DSBs in only the native plasmid.

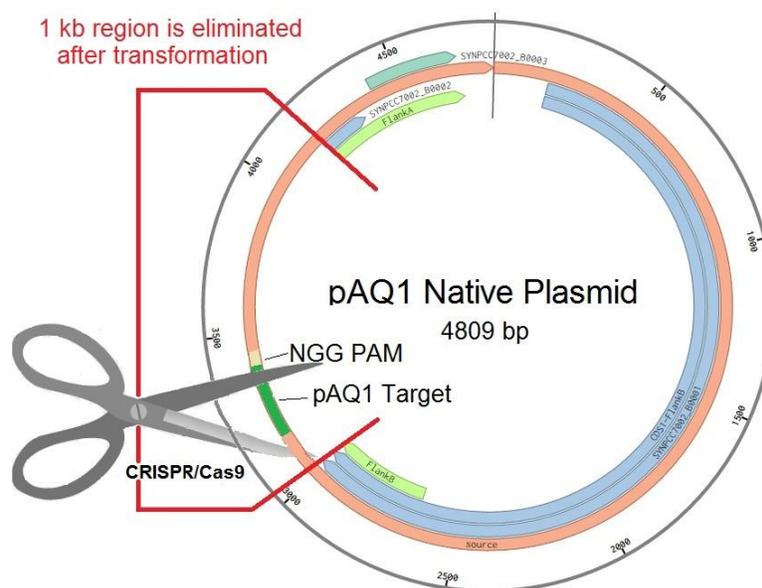


Figure 3: The target sequence selected for CRISPR/Cas9 is contained in a region that is eliminated from the transformed plasmid

The oligonucleotides containing the sequence selected for this construct is included in [Appendix B](#) as gRNA_pAQ1_Top and gRNA_pAQ1_Bot. This sequence was selected based on the presence of an NGG PAM sequence which is critical for successful targeting by the CRISPR/Cas9 complex, and this particular target is placed in the only predicted gene in the region. In the case that this gene offers a selective advantage to the cell, causing a DSB in this region should remove that advantage by causing a frame-shift mutation and interfering with translation of the gene. A permissive BLAST was performed against the *Synechococcus* sp. PCC 7002 genome in order to ensure there are no off-target cutsites. In addition to the target sequence, these oligonucleotides contain BsaI restriction site overhangs. Cloning this target into pCAS9 was performed as described in the Marraffini pCRISPR protocol available on AddGene (Wenyan Jiang, David Bikard, David Cox, Feng Zhang, 2013). The procedure is summarized in brief:

- 1) The oligonucleotides gRNA_pAQ1_Top and gRNA_pAQ1_Bot are annealed by heating to 95 °C and slowly cooling to room temperature.
- 2) pCAS9 is digested with BsaI which cuts twice leaving restriction overhangs matching the annealed oligonucleotides.
- 3) Digested plasmid and annealed oligonucleotides are ligated together.
- 4) The ligated plasmid product is used to transform *E. coli* via electroporation .
- 5) Transformants are screened via colony PCR to confirm successful insertion of the target sequence using the internal primers pCAS9_gFWD and pAQ1-Target.FOR (sequences included in [Appendix B](#)).

2.5 *Synechococcus* sp. 7002 transformation procedures

All cyanobacterial transformation procedures were carried out as described by Xu et. al. (Xu et al., 2011) with the addition of co-transforming cyanobacteria with the pAQ1-Ex and pAQ3-Ex expression platforms. It was determined that aeration of liquid cultures could be performed using air without supplemented CO₂ without significantly delaying transformation. Primary transformation was performed using several potential genes of interest including yellow fluorescence protein (YFP), TcPLA (*Theobromo cacao* phtocyanin-like arabinogalactan protein [PLA]), and OsPLA (*Oryza sativa* PLA) (Legenski, 2016). Subsequent transformation of these pAQ1-transformed strains will be carried out using the pAQ3-Ex Cas9 plasmid. This transformation is performed as described previously, however liquid culture is carried out with spectinomycin antibiotic to maintain selection pressure on the transformed pAQ1 plasmid. After

transformation with pAQ3-Ex Cas9 the product is plated on A+ media containing both spectinomycin and kanamycin to select for colonies which are co-transformed.

To verify insertion of genes into the pAQ1 and pAQ3 plasmids, flanking primers were designed for each transformation plasmid. These primers are included in [Appendix B](#), and should amplify the regions of overlap between the native flanks and the newly inserted genes of interest. An additional primer pair was included to screen for presence of the native plasmid.

Chapter 3

Results and Discussion

3.1 Cyanobacteria culture maintenance

Significant effort was expended obtaining sufficiently optimizing growth of *Synechococcus* to carry out transformation procedures. The peak reported doubling times for this organism vary; the highest reported doubling time was under conditions of 38 °C, 1% V/V CO₂ supplemented air, irradiation of 250 μmol photons m⁻²s⁻¹ giving a doubling time of 2.6 hours (Ludwig & Bryant, 2012). Other sources indicate maximum growth at 30 °C with a peak doubling time of 3.5 hours with 275 μmol photons m⁻²s⁻¹ (Bernstein et al., 2014).

3.1.1 Solid culture maintenance

For this study, initial culture conditions were carried out in a 30 °C incubator supplemented with 1% V/V CO₂ and irradiated under 1-4X 65W incandescent light bulbs. Under these growth conditions strains subcultured on plates of solid A+ media containing antibiotics showed minimal growth. As observed in Figure 4, the phenotype under these growth conditions was moderate growth at high subculture density with low to no separation of colonies. Depicted growth is after nearly two weeks.

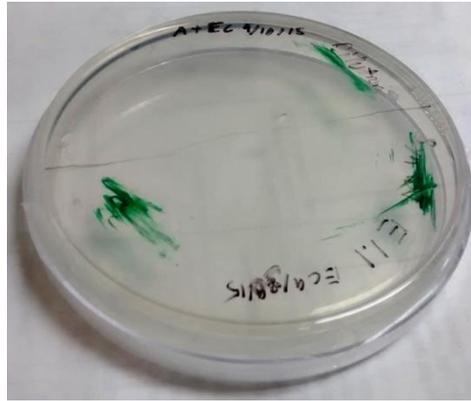


Figure 4: Poor colony growth and separation due to sub-optimal conditions

Alternative growth conditions of room temperature ($\sim 25\text{ }^{\circ}\text{C}$) and irradiance provided by T9 fluorescent bulbs suspended 0.5 m above the cultures in a reflective Mylar-lined chamber ($\sim 110\text{ }\mu\text{mol photons m}^{-2}\text{s}^{-1}$) were examined in the Pilot Plant "Chlamy hut". Under these conditions, significantly improved growth and isolation of single colonies was achieved. Figure 5 indicates growth after one week under these conditions.

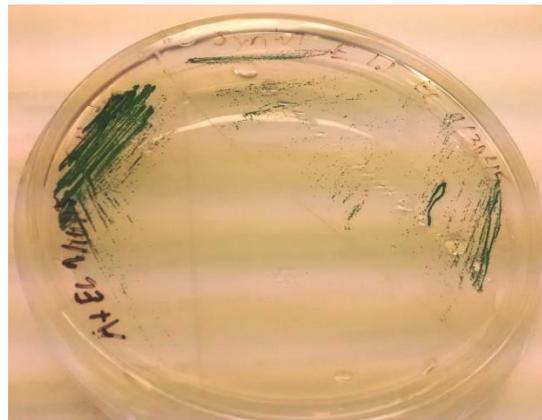


Figure 5: Improved colony separation under increased irradiance, room temperature and no CO₂ supplementation

In addition to light conditions, it was concluded that degradation of growth medium plays a critical role in cyanobacterial growth rate. The recipe for A+ growth medium is included in

[Appendix D: Media and Reagent Recipes](#). It was concluded that degradation of vitamin B12 is primarily responsible for decreased growth rate between growth experiments. Because vitamin B12 is photo- and heat-sensitive, degradation both during storage and moreover during growth quickly makes B12 a limiting nutrient for long-term growth experiments and culture maintenance. This concern is further elaborated upon in [Chapter 4: Future Work](#).

While poor growth is observed for solid cultures under reduced light conditions, this reduced metabolic rate is ideal for moderate-term culture storage. To optimize growth rate cultures were first grown to maturity under high irradiance and increased temperature for 4-7 days, then for long-term culture maintenance they were placed under ambient room light and temperature (~22 °C) for maintenance.

3.1.2 Liquid culture maintenance

Liquid culture of cyanobacteria significantly improves growth rate and is the ultimate intended growth mode for large-scale culture and protein expression. Liquid cultures are fairly robust in terms of growth conditions: liquid cultures have been grown with and without shaking, at temperatures ranging from 20-37 °C, with and without air supplementation, with and without CO₂ supplementation. For the purposes of this work, conditions which maximized growth rate will be described.

Liquid cultures for transformation and preparation for cryostocks were grown up in a high-light, 37 °C temperature-mediated water bath with 1-2% CO₂-supplemented sterile air bubbled into the cultures. Gas flow-rate was adjusted to achieve ~0.5-1 bubbles per second,

which achieves sufficient culture mixing to keep the culture suspended. Under these conditions, OD 3 can be achieved in roughly three days from single colony inoculation.

3.1.3 Cryostock preservation

Cryostocks of wild type and all transformed strains were prepared and maintained in at -80 °C. The procedure was adapted from a generalized cyanobacteria cryopreservation procedure (Brand, 2012).

3.2 Observable variable obsolescence of transformed strains

The inspiration for using the CRISPR/Cas9 system to provide selection pressure against the native plasmid was the result of an observation of general maintenance of transformed strains. While crude, Figure 6 lead to the original observation that the same strain of cyanobacteria, transformed with the same gene and maintained on the same plate clearly “age” differently; the only difference between the three strains depicted is the time at which the culture was transformed. The darkest two colonies were transformed in January 2015; the intermediate two colonies were transformed in June 2015 and the lightest four colonies were transformed in December 2015. The difference in cultures only became apparent after over a month of culture maintenance when they began to photo-bleach due to stress. Based on the observed phenotype, it was concluded that transformations must be slowly altering the transformed plasmids despite maintenance on antibiotic selection pressure. The most recently transformed colonies likely contain more copies of the transformed strain; while it confers antibiotic resistance, over-production of the gene of interest (squid ring-tooth protein or SRT in this case) induces

significant metabolic stress. While it is not clear the mechanism of action, these cultures suggest some form of genetic "shuffling" is occurring over time which allows the transformed strain to optimize its growth under the new metabolic stress. Based on the high degree of natural competence observed in *Syn. sp.* 7002, a logical interpretation of this observation is that the highly expressed gene of interest is being homologously recombined with the native plasmid, losing expression and in turn reducing stress on the bacteria.

This phenotype represents the inherent genetic instability of allowing copies of both the native and transformed plasmids to remain in culture. To develop a consistent, marketable protein expression platform this instability must be remedied by elimination of the native plasmid.



Figure 6: Variation in transformations maintained under identical conditions with the exception of culture age. Colonies from three transformations with the same gene inserted in pAQ1 are easily distinguished by color variations.

3.3 Cloning genes into pAQ1-Ex

pAQ1-Ex as-supplied contained the gene encoding yellow fluorescence protein (YFP), which was used as the basis for initial proof-of-concept transformations and as a positive control when cloning the PLA genes due to the ease of screening colonies based on UV fluorescence. Cyanobacteria was subsequently transformed with pAQ1-Ex containing the TcPLA and OsPLA genes.

When cloning pAQ1-Ex YFP and pAQ3-Ex YFP into Top10 *E. coli* for plasmid proliferation, Figure 7 indicates that the YFP fluorescent marker is still expressed, despite using a high level expression cyanobacteria-specific promoter (see [Appendix C: Understanding the CPC promoter used for over expression](#)). This observation offers a convenient means of screening for religated or undigested DNA backbone when incorporating new genes into the multiple cloning site (MCS) of either expression platform.

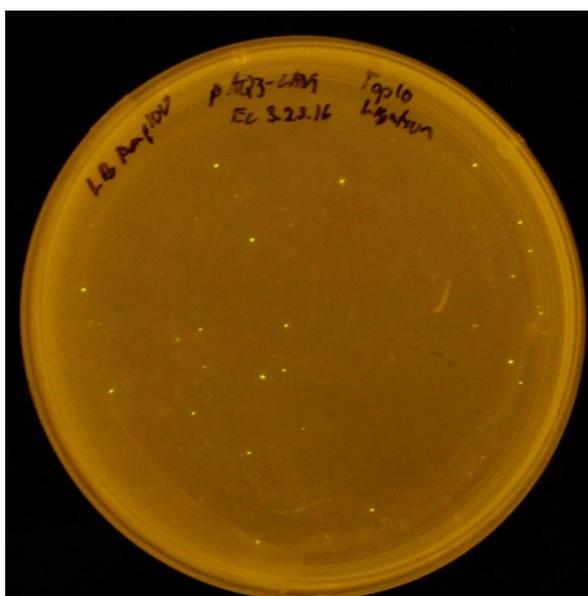


Figure 7: YFP expressed by the cyanobacterial CPC promoter is expressed in *E. coli*, vastly simplifying screening negative religated backbone transformation products

3.4 Cloning pAQ3-Ex Cas9

3.4.1 Inserting the native pAQ1-specific crRNA target into pCAS9

As previously described, the CRISPR/Cas9 system requires a 30-basepair crRNA target sequence which is unique to the native pAQ1 plasmid and is flanked by a PAM. This crRNA sequence forms a highly specific RNA/DNA complimentary scaffold which directs the cutting of Cas9. The sequence selected is contained in the ~1100 bp region between the homologous flanks of the native plasmid which is lost when the native sequence is “overwritten” by homologous recombination. The oligonucleotides containing this 30-bp target and dual BSAI restriction enzyme overhangs are included in [Appendix B: Sequences](#). A permissive BLAST of this sequence against the *Synechococcus* sp. PCC 7002 genome was performed in order to ensure there were no off-target cut sites with significant homology.

Successful insertion of the pAQ1-specific target into pCAS9 was confirmed via colony PCR using the primer pair pCAS9-gFWD and pAQ1-Target.REV, the sequences of which are included in [Appendix B: Sequences](#). pAQ1-Target.REV binds inside the pAQ1 target sequence, thus, successful PCR should produce a 225 bp band. The results of this PCR are shown in Figure 8; 18 total transformed colonies were screened, 15 of which are positive.

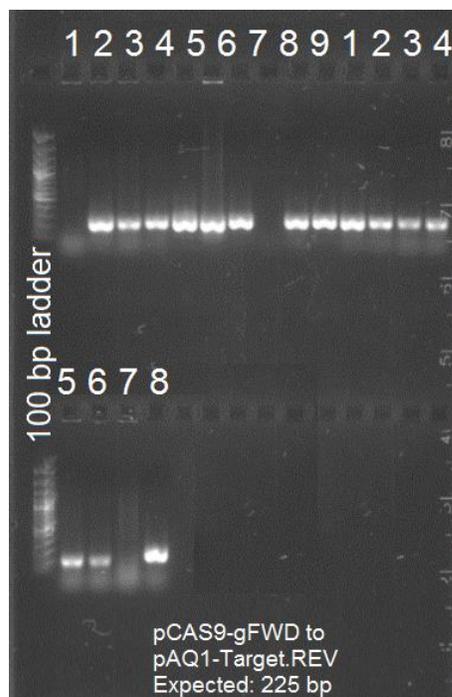


Figure 8: Colony PCR of 18 *E. coli* strains transformed with pCas9 after digestion and ligation. Ligation was performed at ratio of 1:8 vector:insert; of 18 colonies screened 15 are positive

3.4.2 Amplification, isolation and digestion of genes encoding CRISPR/Cas9

Initially the cloning procedure for producing pAQ3-Cas9 was designed utilizing Gibson assembly, the primers for which are included in [Appendix B: Sequences](#) as Cas9-GB.FOR and Cas9-GB.REV. While both PCR of the linear Cas9 fragment with appropriate overhangs homologous to pAQ3-Ex and digestion of pAQ3-Ex with BamHI and SacII were successful, the final pAQ3-Cas9 assembly product was not transformed. The procedure was repeated twice; once in which both products were gel extracted and once in which both were column purified. Transformation was attempted with the assembly product using both chemically competent Top 10 *E. coli* cells as well as electroporation using both homemade and purchased competent Top 10 cells. Despite these efforts, no transformed strain was ever produced via this method. Most

notably, not even background undigested pAQ3-Ex YFP or religated backbone colonies were produced. The root cause of these difficulties was never determined, however due to the high cost of Gibson assembly reagents and a relative lack of experience with Gibson troubleshooting the procedure was abandoned. It is worth noting that the following procedures utilize the same reagents and strains as the failed Gibson assembly cloning method. As an alternative, a more traditional restriction-based cloning procedure was developed.

For restriction-based cloning of pAQ3-Cas9, purified pCAS9 plasmid containing the pAQ1 crRNA target was used as template to amplify Cas9 and add SacII and BglII restriction sites for cloning into pAQ3-Ex. SacII digests between the forward pAQ3 native flank and the CPC promoter; therefore both YFP and the CPC promoter are cleaved out by this digestion process. Three independent *S. pyogenes* promoters drive transcription of the components of Cas9; thus this construct relies on the assumption that these promoters will remain functional in *Syn. sp.* PCC 7002. Because BamHI has a cut site inside Cas9, a complimentary restriction enzyme BglII was used for secondary digestion of the Cas9 linear fragment to allow for insertion into pAQ3-Ex MCS.

The primers used for amplification of this linear fragment (from now on referred to as "Cas9-Restriction") are Cas9-Restriction.FOR and Cas9-Restriction.REV and are included in [Appendix B: Sequences](#). For the first attempt at cloning, this PCR product was directly column-purified with an Omega Biotech Cycle Pure kit. However, due to non-specific binding and amplification of numerous extraneous bands by the cloning primers, this product was later re-purified via gel extraction using a Qiagen Gel Extraction Kit.

Gel electrophoresis of the initial PCR product and digested pAQ3-Ex YFP is depicted in Figure 9. Based on this gel, it is evident that while the fragment most likely amplified correctly,

there is nonspecific primer binding leading to small fragments which will compete with the Cas9-Restriction PCR product for ligation into pAQ3-Ex unless removed by gel extraction.

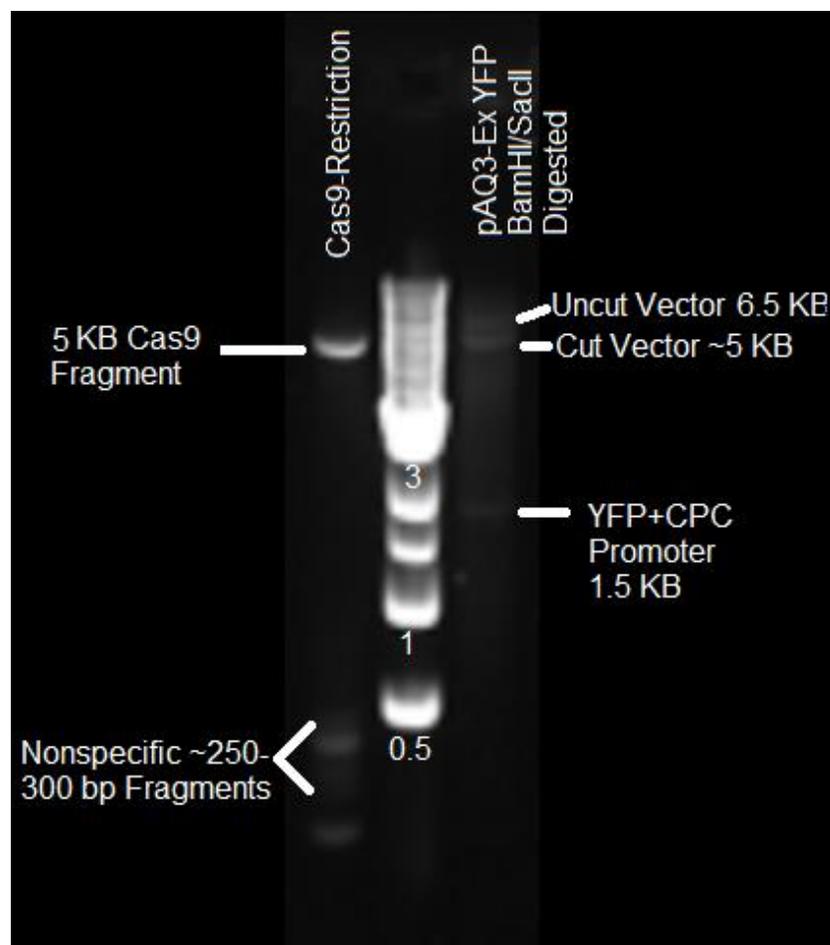


Figure 9: Cas9-Restriction PCR product and the digested pAQ3-Ex plasmid. The top band in the Cas9-Restriction PCR product lane represents the correctly amplified 5 kb linear fragment, while the <500 bp bands indicate products of non-specific cloning primer binding

While a small degree of nonspecific binding was evident, the PCR product was column purified under the assumption that the smaller products would be washed out preferentially to the correct 5 kb product, and that the large product would comprise the majority of DNA in the final ligation. In addition, several experiments using identical cloning procedures with the exception of gel extraction versus column purification indicated that the process of gel extraction interferes with downstream ligations when using otherwise functional DNA. The mechanism of action of

this observed interference was not investigated, however it was concluded that the "sticky ends" produced by digestion may be degraded during gel electrophoresis, or that residual gel red which is used for staining may interfere with downstream processing. If the former hypothesis is true, gel extraction of products which will later be restriction digested may prove less problematic for downstream applications. As an alternative approach, gel extracted DNA can be used as the template for a second PCR reaction using the same primers which would eliminate any non-specific products that result from binding elsewhere on the plasmid. However, for a product of this size (~5 kb) accumulation of errors due to sequential PCR may lead to an unacceptably high number of mutations.

Both the vector (pAQ3-Ex YFP) and insert (Cas9-Restriction) were double digested and re-column purified prior to ligation. The vector was double digested with BamHI and SacII in CutSmart Buffer, while the insert was sequentially digested with SacII in NEBuffer 2.1, adjusted to NEBuffer 3.1 (see [Appendix D: Media and Reagent Recipes](#)) and digested with BglIII. The vector and insert were ligated in a 1:1.5 vector:insert ratio using 20 fmol of vector in order to achieve optimal DNA concentration based on NEB's T4 DNA ligation procedure, and because the vector and insert sizes are both ~5 kb.

3.4.3 Screening for successfully transformed pAQ3-Cas9 clones and troubleshooting incorrect cloning products

Transformation was performed by electroporation with 2 μ L of the final ligation product. A single transformation yielded 35 total colonies, 22 of which were visually screened out based on expression of YFP leaving 13 possible transformants. Colony PCR was carried out using three sets of primers: pCAS9-gFWD to pAQ3-MCS.REV, pAQ3-Flank.FOR to Cas9.REV, and

pCAS9-gFWD to pAQ1-Target.REV. Each primer set should yield a positive band for successfully cloned pAQ3-Cas9: the first two sets are flanking primers and the last set is the same set used to confirm successful insertion of the pAQ1 targeting sequence in pCAS9. The gels containing the results of these PCR reactions are included in Figure 10.

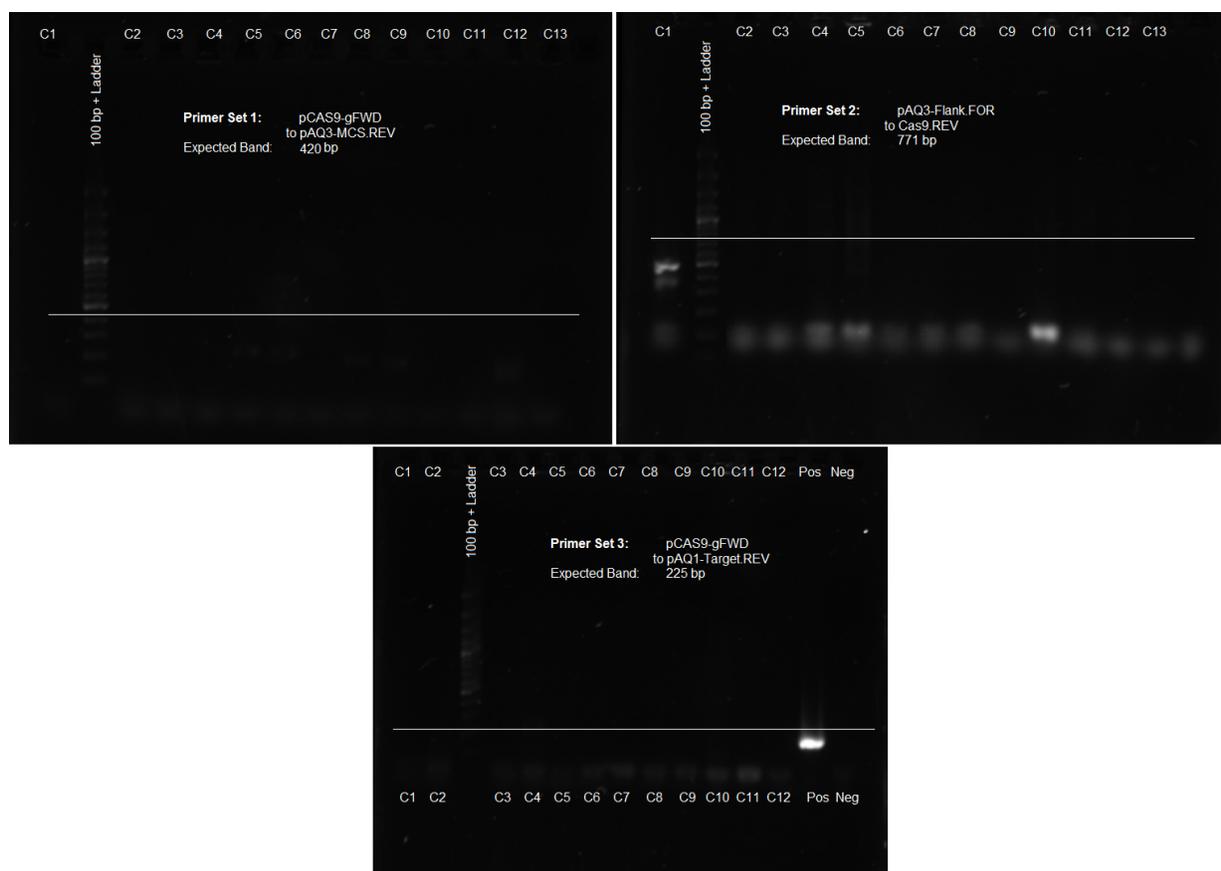


Figure 10: Colony PCR results on 13 pAQ3-Cas9 candidates using three sets of primers. Colonies 1, 4, 5, 6, 9, and 12 indicate partial positives

The faint bands for colonies 1, 4, 5, 6, 9, and 12 indicated partial positives, and pure plasmid was prepared from each of these colonies for further screening. Note the positive control included here is pCAS9-gFWD to pAQ1-Target.REV with previously confirmed pCAS9 with paQ1 target pure plasmid as template.

Next, pure plasmid was run on a gel to determine total plasmid size. pAQ3-Ex is 6.5 kb; pAQ3-Ex without YFP and the CPC promoter is 5 kb, and pAQ3-Cas9 is 10 kb. To further elucidate plasmid identity, each plasmid was digested with BsaXI restriction enzyme. pAQ3-Ex without Cas9 should be cut twice yielding two bands of 2.2 kb and 4.4 kb, while pAQ3-Cas9 should be cut four times with bands of 2.2 kb, 2.3 kb, and 3.5 kb. Both results are depicted in Figure 11.

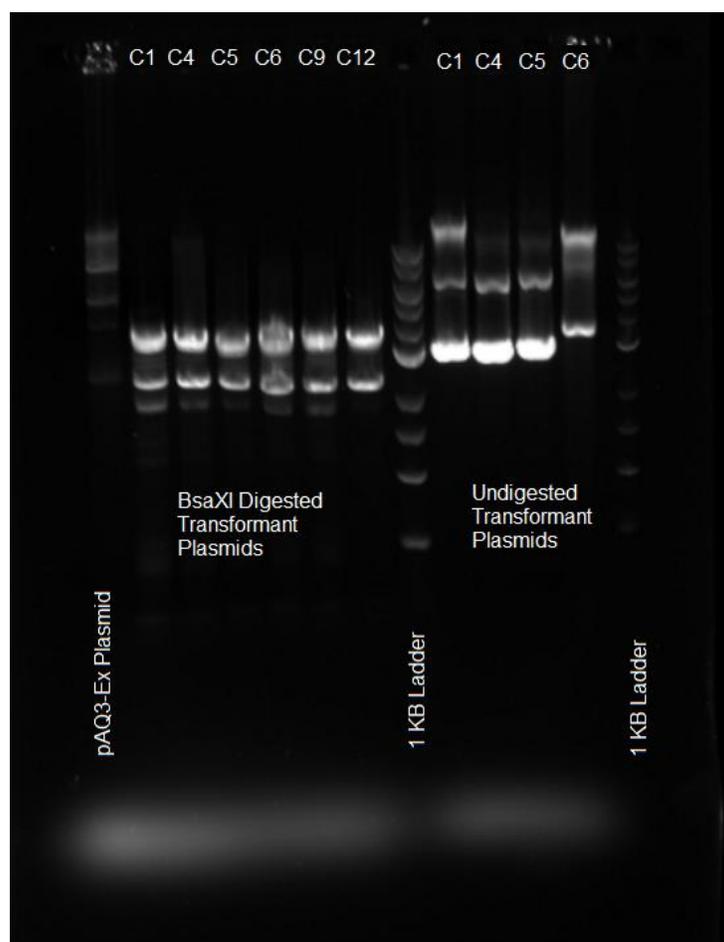


Figure 11: Digested and undigested transformant plasmids. Undigested plasmids contain 3 bands: circular nicked (largest), linearized (true size), and super-coiled (smallest). Both results indicate Cas9 is not present in the transformed plasmids

While the resolution of bands in this gel are fairly complex, both cut and uncut results indicate that Cas9 is not present in the transformed plasmids. Actual plasmid size is roughly 6-7 kb, 3 kb short of the expected total plasmid size.

An additional 30 colonies were screened to determine whether any of the transformation products may include the correct pAQ3-Cas9 construct. All 30 colonies are negative for containing both Cas9 and the pAQ1 target sequence based on colony PCR.

To elucidate what product was actually cloned so as to determine an alternative strategy, colony PCR was carried out on 5 clones using the MCS flanking primers pAQ3-Flank.FOR to pAQ3-MCS.REV, the results of which are included in Figure 12.

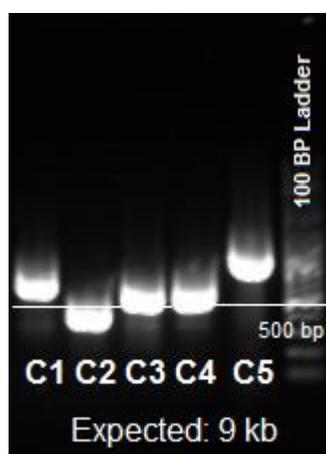


Figure 12: Colony PCR of five potential pAQ3-Cas9 clones with pAQ1-Flank.FOR and pAQ3-MCS.REV. The small 250-700 bp inserts in the MCS indicate small nonspecific PCR products from the amplification of Cas9-Restriction preferentially ligated into pAQ3-Ex

The result indicates at least 3 different 300-700 bp nonspecific PCR products from the original Cas9-Restriction PCR amplification have been cloned into pAQ3-Ex rather than the complete 5 kb Cas9 fragment. These small products were generated using the same Cas9-Restriction primers as the desired product, and therefore also include the restriction sites required to clone into pAQ3-Ex. It was concluded that the relative probability of both ends of a 300-700

bp band annealing and recircularizing the plasmid versus a vastly larger 5 kb band resulted in a near complete "washout" of viable plasmid with small nonspecific inserts.

3.4.4 Revised cloning procedure for obtaining pAQ3-Cas9

Based on the previous results, the necessity of isolating only the correctly amplified Cas9 fragment prior to ligation into pAQ3-Ex is evident. As a result, the following amendments to the previously described cloning procedure were implemented:

1. Cas9-Restriction PCR product is gel extracted using a Qiagen Gel Extraction kit prior to sequential digestion, taking care to pipette slowly to avoid damage to the purified insert.
2. pAQ3-Ex YFP backbone is alkaline phosphatase-treated during double digestion.

By eliminating small nonspecific PCR products and preventing the YFP backbone from religating by removing the DNA's 5' phosphate groups, all undesired ligation products should be eliminated. In the original transformation this step was omitted due to the simplicity of screening for religated pAQ3-Ex YFP. However the relative probability of incorporating the smaller 1.5 kb CPC promoter and YFP gene is much higher as opposed to the 9 kb Cas9 fragment into the cut vector. Inhibiting religation of this undesired small fragment should improve odds of successfully incorporating Cas9.

Chapter 4

Future Work

4.1 Ongoing Work

The cloning procedure as described in Chapter 3.4.4 is currently underway.

Economic analysis of protein production in cyanobacteria versus the traditional *E. coli* expression platform will be performed once the native pAQ1 is eliminated to determine whether cyanobacteria truly is a more cost-effective production platform. The key information needed for this analysis is the pAQ1 protein expression levels once the native plasmid is eliminated. Once expression is quantified, the economic viability of the platform will be evaluated.

As previously discussed, observed phenotypes between similarly transformed strains of cyanobacteria indicate there may be a difference in copy number of the transformed plasmid. To quantify levels of protein expression and plasmid copy number, quantitative PCR (qPCR) is currently being investigated. This technique uses a fluorescence-based detection system to determine how many copies of a gene are present in a PCR sample. By comparing copies of transformed plasmid to a control of genomic DNA (for which there is only one copy per cell) the final copy number of transformed plasmids can be quantified.

In addition to qPCR for quantifying copy number, colony PCR will be utilized to determine if and when a strain of cyanobacteria has been completely cured of the native plasmid. Upon complete elimination of the native plasmid, colony PCR using primers specific to the same region targeted by the CRISPR/Cas9 endonuclease system will be negative indicating the original target native plasmid DNA has been eliminated from the cell line. This cell line will

therefore be incapable of reverting to the native plasmid via homologous recombination, and can thus be used for protein expression without antibiotic selection pressure.

4.2 Incorporation of a tag allowing low-cost protein purification

To meet the ultimate goal of developing a cost-effective, low-capital protein production system, a comparably cost-effective protein purification will also be investigated. Many common protein purification techniques utilize expensive resins, including the resin used for the existing histidine-tag included in the pAQ1-Ex expression system. In this case, purification of the protein requires lysis of the cell material followed by affinity chromatography through a nickel/cobalt resin.

To decrease processing and capital costs of protein purification, the existing histidine tag present on the pAQ1-Ex expression platform could be replaced with an self-cleaving elastin-like polypeptide (ELP)/intein protein tag. A recently developed self-cleaving intein tag technology, depicted in Figure 13, utilizes pH and salt-concentration shifts to cause selective precipitation of proteins (Wu, Mee, Califano, Banki, & Wood, 2006). Acidification of the product cyanobacteria cell solution could allow the high-value PLA protein to be precipitated out of solution, eliminating the capital costs associated with traditional column and affinity based chromatography. This approach is particularly viable for proteins such as embryogenic PLA which do not require extremely high purity for application. Similarly there are plans to implement this system for expression and purification of a topical anti-HIV protein, which can safely be applied without achieving purities required of injection or ingestion.

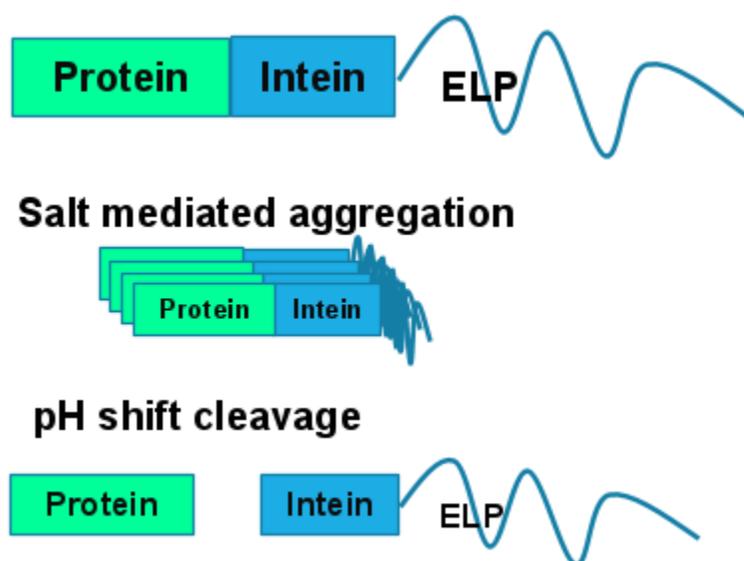


Figure 13: Overview of ELP-Intein based protein purification. The ELP-intein protein tag is incorporated on the desired protein product. Purification is carried out by precipitating the ELP and therefore intein tag by adding salt, cleaving the tag by adjusting pH, and finally re-precipitating the ELP tag to isolate pure protein product.

4.3 Addressing vitamin B12 requirements:

At present, there are only two unstable, costly components to this expression platform: antibiotics and vitamin B12. While this work aims to eliminate dependence of this system on antibiotics by eliminating the requirement for selection pressure on the transformed pAQ1 plasmid, vitamin B12 poses an independent challenge. Vitamin B12 is both temperature- and light sensitive, and therefore is a critical concern when attempting to achieve low-capital, low-cost protein expression with this photosynthetic system.

While outside the scope of this work, developing a B12-protrophic strain of cyanobacteria would be a significant stride towards making a vastly improved, low-capital production system. As an alternative, co-culture of cyanobacteria with a B12 synthesizing organism could be considered. By selecting a chemoautotrophic or heterotrophic B12-

synthesizing organism, growth of this secondary organism could be closely controlled to produce the low levels of B12 required for cyanobacterial photoautotrophy by manipulating the feedstock for that organism independently of cyanobacteria culture medium requirements.

As an alternative technology, there is ongoing work to generate a non-vitamin B12-auxotrophic strain of cyanobacteria by replacing the genes for which vitamin B12 is an essential cofactor (Bryant, unpublished manuscript). The platform developed by this work could be implemented in such a strain, and would provide an additional capital and operating cost reduction for by eliminating the need to maintain a feed of fresh vitamin B12, especially for long-term UV exposure associated with photoautotrophic growth.

Appendix A

Plasmid Maps

Sequences can be found in [Benchling](#), an open-source molecular biology tool.

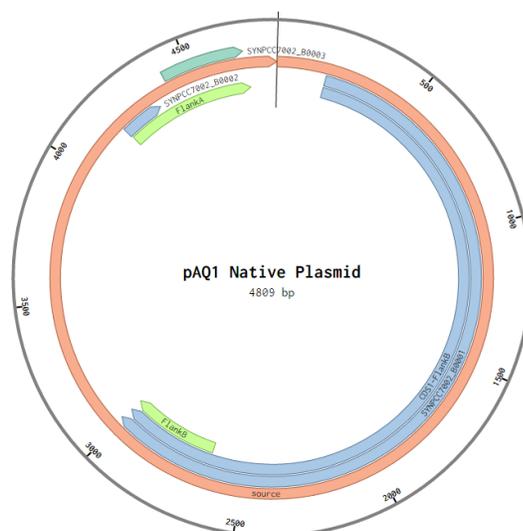


Figure 14: *Syn. sp.* PCC 7002 pAQ1 Native Plasmid

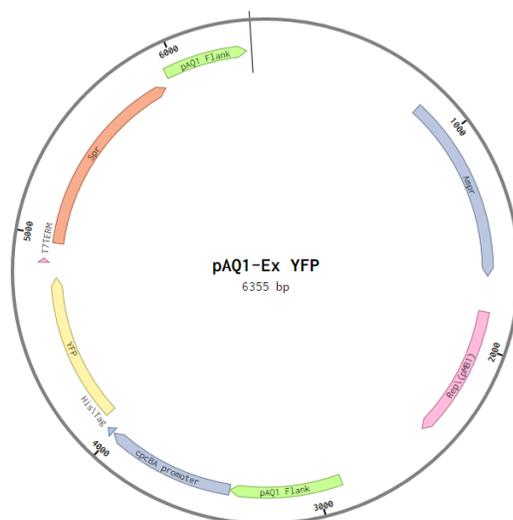


Figure 15: pAQ1-Ex YFP Transformation Plasmid

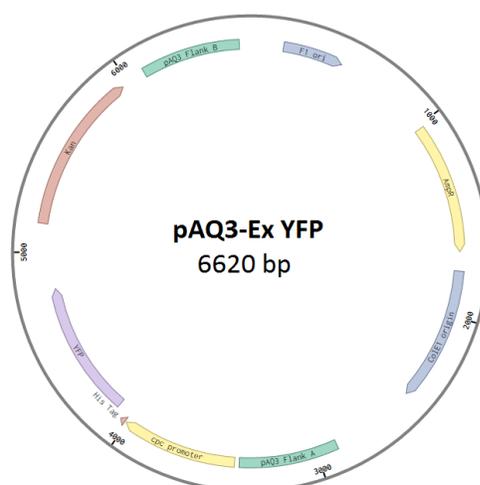


Figure 16: pAQ3-Ex YFP Transformation Plasmid

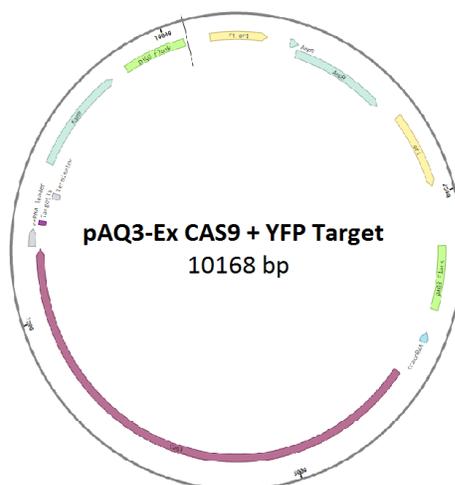


Figure 17: pAQ3-Ex Cas9 Plasmid. Co-transforming *Syn. sp.* PCC 7002 with this plasmid will selectively cause DSBs in the native pAQ1 plasmid.

Appendix B

Sequences

Table 1: Primer and Oligonucleotide Sequences

Primer Name	Sequence (FASTA)
pCAS9 gFWD	agctaggaggtgactgaagt
pCAS9 gREV	CTCAACAAGTCTCAGTGTGC
pAQ1 Target REV	tgaggcttatgatctggcac
pAQ3 Flank FWD	CTGCCTACCCTCATCGAAATC
CPC Promoter REV	CAGATTGTCCTGGATTGGGAAG
pAQ3 Flank REV	ggttacggtctttgtcgcata
CAS9 REV	CGACGCTATTTGTGCCGATA
pAQ3 MCS REV	gcttccttcgggctttgtt
Cas-GB.FOR	acgaggaattgcaggcagcagccgCGGCATCCTGTGGAGCTTAGTAGGT
Cas-GB.REV	cttcgggctttgtagcagccggtaccGGTATTAACCCTCTTTCTCAAGTTATCATCGG
gRNA_pAQ1_Top	aaacgtgccagatcataagcctcactgagggtag
gRNA_pAQ1_Bot	aaaactcaccctcagtgaggcttatgatctggcac
Cas9-Restrict.FOR	taatccgCGGCATCCTGTGGAGCTTAGTAGGT
Cas9-Restrict.REV	attaagatctATCCGACTGCTGGTATTAACCC
Cy1	GCGACCGGGCGAAATATGACCA
Cy2	gggattgccaaggagagctgc
Cy3	gtgagcgcttcattgccctgt
Cy4	CCTCCTTCGCCTTGACGATCATG
Cy5	TGGGGTAGGGAAGACTGTTGCC

Cy6	GAAGAAGATCGCTTGGCCTCGC
Cy7	GGTTTTGCCGTCCCTGTTTGCT

Appendix C

Understanding the CPC Promoter used for Over-Expression

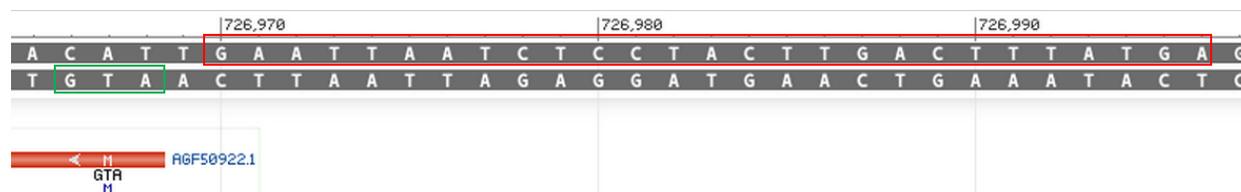
The *Synechocystis* phycocyanin operon promoter (Pcpc) has been shown to achieve high level expression in cyanobacteria. This was described in detail by the laboratory of Don Bryant (REF) and more recently published as the discovery of a “super strong” promoter which is nearly identical as described below. Utilizing genomic sequences and the sequences of the provided PRC primers, Dr. Curtis had examined these promoter sequences in detail. A summary of that analysis is provided here due to its relevance in achieving the goal of this thesis – high level protein expression. It is noteworthy that the group in China indicated that they were trying to patent the promoter, while the Pcpc promoter developed by the Bryant lab is in the Public Domain.

In both cases, the promoter was from Syn PCC 6803. Ironically, the rationale for the Bryant lab was to achieve stability by utilizing a heterologous promoter, while the China group chose to then utilize this promoter homologously in the same cyanobacterial strain.

DON BRYANTS cpcBA promoter:

REVERSE Primer (near the ATG since the cpcB operon is on the – strand)

cpcBproR CGTCGACCATGGAATTAATCTCCTACTTGACTTTATG



Note there will be a change of T → G adjacent to the start codon when this is digested
This is the same length and therefore corresponds to Genome base # 726969

FORWARD Primer (near the ATG since the cpcB operon is on the – strand)

cpcBproF AGGAATTCGTTATAAAATAAACTTAACAAATCTAT



There will be an extra AA tacked onto the beginning of the promoter from the restriction overhang.

SUMMARY: Bryant vector is from sequenced genome base pair number 727,562 to 726,969 (negative strand) with the base preceding the ATG changed from T → G and an extra AA at the start of the promoter.

Functionally this is $(727562 - 726968 = 594)$ bases preceding the ATG start of the cpcB gene.

Reverse compliment of the negative strand ... starting at the amplification of forward primer.

BRYANT PRIMER: **AGGAA**TTCGTTATAAAAATAAACTTAACAAATCTAT

CHINA PRIMER: CTGACTCTCGAG**ACCTGTAGAGAAGAGTCCCTGAA**

CATAAAGTCAAGTAGGAGATTAATTCCA**IGGTCGACG** (reverse compliment) **XX**
removed

CHINA cpc560 promoter:

FORWARD Primer Pcp560F(XhoI)

CTGACTCTCGAGACCTGTAGAGAAGAGTCCCTGAA

There will be an extra GAGTC tacked onto the beginning of the promoter from the restriction overhang

I am not sure what the reverse primer was for amplification of this promoter ... but assuming it captured similar to BRYANT (this should be checked) ... from 727,528 to 726,969 (negative strand)

Functionally this is $(727,528 - 726,968 = 560)$ base pairs preceding the promoter.

Appendix D

Media and Reagent Recipes

A+ Medium for growth of *Synechococcus* 7002

<u>Powder or stock solution</u>	<u>1 Liter</u>
NaCl (300 mM final conc.)	18g
KCl (8 mM final conc.)	0.6g
NaNO ₃ (11.8 mM final conc.)	1.0g
MgSO ₄ x 7 H ₂ O (20.3 mM final conc.)	5.0g
KH ₂ PO ₄ (50 g/L)	1 mL
CaCl ₂ (37 g/L)	7.2 mL
Na ₄ -EDTA (tetra Na salt) (3 g/L)	10 mL
FeCl ₃ x 6 H ₂ O (3.89 g/L liter 0.1 N HCl)	1 mL
Tris (100 g/L, pH 8.0)	10 mL
P1 Metals (1000x)	1 mL
<u>Only for Agar plates: Na₂S₂O₃</u>	2 g
<u>Only for glycerol-containing A+:</u>	80% glycerol
for 11 mM final conc.:	1 mL
for 27.5 mM final conc.:	2.5 mL
ddH ₂ O	to 1 L
Autoclave and cool down to room temperature, then add	
Vitamin B12 (4 mg/L)	1 mL
[final conc. in medium: 4 µg/L!!!]	
For agar plates: Bacto Agar	14 g/L

Wait to cool down to 50-55 °C, and then add Vitamin B12 stock solution and appropriate antibiotic(s).

Suggested final concentration* of antibiotic (s) for A+ plates:

Kanamycin	100 µg/mL
Streptomycin/spectinomycin	50 µg/mL
Erythromycin	20 µg/mL
Chloramphenicol	10 µg/mL

Gentamycin

20 µg/mL

*Transformed strains have been observed to grow at normal growth rate in up to 200 µg/mL Spectinomycin. Increasing antibiotic concentration is likely to aid in maintaining the transformed plasmid.

P1 Metals (to make 1 liter 1000x stock solution)

H ₃ BO ₃ (554 mM)	34.26 g
MnCl ₂ x 4 H ₂ O (21.8 mM)	4.32 g
ZnCl ₂ (2.31 mM)	0.315 g
MoO ₃ (85%)	0.03 g
CuSO ₄ x 5 H ₂ O (12.0 µM)	0.003g
CoCl ₂ x 6 H ₂ O (51.1 µM)	0.01215 g

ddH₂O to 1 L

KH₂PO₄ stock

KH₂PO₄ → 0.367 M 50 g/L

CaCl₂ stock

CaCl₂ → 0.333 M 37 g/L

Na-EDTA stock

Na₄-EDTA → 7.89 mM 3 g/L

FeCl₃ stock

FeCl₃ x 6 H₂O → 14.4 mM 3.89 g/L

Dissolve FeCl₃ in 0.1 N HCl

Tris/HCl stock

Tris → 0.826 M 100 g/L

Adjust pH to 8.0 with conc. HCl

NEBuffer 2.1-NEBuffer 3.1 Adjustment Solution

Table 2: NEBuffer 2.1 vs. NEBuffer 3.1 Composition

	NEBuffer 2.1	NEBuffer 3.1
NaCl (mM)	50	100
Tris-HCl (mM)	10	50
MgCl ₂ (mM)	10	10
BSA (ug/mL)	100	100
pH (25C)	7.9	7.9

Final adjustment solution:

Add 5% V/V adjustment solution to final concentration NEBuffer 2.1 to achieve suitable NEBuffer 3.1 salt concentrations.

Sterile H ₂ O	1 mL
NaCL	0.03214 g
Tris-HCL	0.0694 g

UV sterilize or autoclave the resulting solution (UV sterilization prevents concentration by evaporation) prior to use.

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