DISSERTATION

METABOLIC ENGINEERING OF THE CYANOBACTERIUM *SYNECHOCYSTIS SP*. PCC 6803 FOR THE PRODUCTION OF ASTAXANTHIN

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ABSTRACT

METABOLIC ENGINEERING OF THE CYANOBACTERIUM *SYNECHOCYSTIS SP.* PCC 6803 FOR THE PRODUCTION OF ASTAXANTHIN

Synechocystis sp. PCC 6803 is a photosynthetic eubacterium capable of using light energy to generate biomass from atmospheric CO_2 and is considered to be the model organism of photosynthetic microbes. Much of the knowledge accumulation related to this organism has centered on the cellular photosynthetic process because this organism has many similarities to the chloroplasts of higher order plants. *Synechocystis* also shows great promise as a microbial cell factory, as scientific studies describing metabolite production from this organism continue to accumulate in the literature. While these studies highlight the considerable amount of gains made in regards to production in *Synechocystis*, they also shed light on the considerable amount of gaps in knowledge regarding many aspects of this organism. As the field of metabolic engineering continues to grow within *Synechocystis*, researchers must continue to develop production pathways that leverage comprehensive engineering strategies that help in shedding light on critical engineering hurdles. This information is critical for the successful development of photosynthetic microbes as cellular production platforms capable of generating titers similar to those seen in other cellular systems utilized to generate economically viable metabolites for humankind.

In this work, we utilized several metabolic engineering strategies to manipulate the carotenoid biosynthesis pathway in *Synechocystis* for the production of the non-native carotenoids, astaxanthin as well as canthaxanthin. A *Synechocystis* mutant was engineered with

an insertion of a β-carotene di-ketolase gene *crtW148* from *Nostoc punctiforme*, insertion of an additional copy of the endogenous β-carotene hydroxylase gene *crtR* from *Synechocystis*, and an open reading frame disruption of the endogenous β-carotene mono-ketolase gene *crtO*. These manipulations generated a mutant capable of an increase in the overall carotenoid content by 178 ± 10% % of that seen in wild type cells as well as astaxanthin titers that reached production rates of 1.11 ± 0.07 mg/l/day and canthaxanthin titers reaching 1.49 ± 0.05 mg/l/day. To add upon this work, we leveraged several promoters, the $P_{SCA6\text{-}2}$ promoter as well as the P_{sigA} promoter to control the expression of the *crtW148* gene within several constructs. These promoters were generated in a research study we performed that leveraged rational design strategies to develop a suite of promoters capable of driving gene expression as various strengths within *Synechocystis*. This study generated a library of 10 promoter-constructs capable of a dynamic range of expression strength, exhibiting a 78 fold change between the lowest expressing promoter, $P_{sca8\text{-}2}$ and the highest expressing promoter, $P_{sca3\text{-}2}$ when tested within *Synechocystis*. Use of the $P_{SCA6\text{-}2}$ promoter within the carotenoid pathway engineering experiment increased carotenoid production of target carotenoids by 150% to 197% over production seen from the same constructs run by the promoter P_{sigA}.

In addition to engineering of the carotenoid biosynthesis pathway, we also tested the impacts of diel cycle light conditions on carotenoid production and accumulation. When exposed to 12 hour light/dark conditions, the mutant *crtR::cruB::ΔcrtO-*$P_{SCA6\text{-}2}$*::crtW* generates carotenoids at rates of 43 ± 14.8 % of that of the same culture grown in constant light conditions. We hypothesized that this lag was caused by the endogenous cellular control of the carotenoid pathway initiated by the metabolic burden placed on the cell. We also hypothesize that this

metabolic burden was caused by the engineered constitutive expression of the astaxanthin producing genes during dark conditions.

To address potential concerns of constitutive expression of pathway genes during stress conditions like the dark conditions highlighted in the astaxanthin work, our lab constructed a chemically inducible construct for use in *Synechocystis* that is based on the *tac* repressor. Upon chemical induction with IPTG, this same mutant strain was capable of exhibiting an average 24X increase in GFP expression over that of the repressed state. In addition to this work, we studied several light induced promoters to better understand their ability to control gene expression during various light conditions in neutral locations within the *Synechocystis* genome. We identified that the P_{psbAII} promoter functions very differently in light and dark conditions when it is moved from its native location within the genome. As many researchers utilize this promoter to control gene expression, this information may be critical to fully understanding gene expression of pathways leveraging this promoter construct. Three additional promoter constructs, the $P_{psbAIII}$. P_{groEL2}, *and* P_{sigD} promoters were also tested for differential expression in light and dark conditions within the neutral region *slr0168*. Additionally, nucleotide mutations were made to regions within the P_{psbAII} promoter, to better understand this promoter's sensitivity to varying light intensities.

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CHAPTER 1

Introduction

The use of photoautotrophic microbes as cell factories

Microbial production of valuable byproducts has been a staple of man's ingenuity for centuries. While fermenting grain with yeast to brew beer has been traced back to the ancient Egyptians, only in the last century has science been able to leverage this skill to further develop the library of microbes capable of generating valuable products for mankind. In particular, the utilization of photoautotrophic microbes, organisms capable of converting the electromagnetic energy found in sunlight into the chemical energy that is then used to fix CO_2 into biomass and other byproducts, has only begun to be refined over the last 30+ years. Of particular interest are photoautotrophic bacteria, or cyanobacteria. These single celled eubacterial microbes have many attributes that make them valuable platforms for product production. Many of these organisms exhibit extremely rapid doubling times, a tractable genetic structure, and are capable of generating a wide array of products that include fatty acids, short and long chain alcohols, alkanes, hydrogen, lactic acid, sucrose, as well as pigment molecules like carotenoids, figure 1 (for a review, see Gronenberg, et. Al. (2013) and Berla, et. Al. (2013)).

While many attributes of cyanobacteria mirror those found in heterotrophic systems like yeast and *Escherichia coli (E. coli)*, photosynthetic microbes exhibit unique cellular conditions and challenges that can substantially change the strategies pursued in the development of these organisms into viable production platforms. As demonstrated in figure 2, essentially two growth conditions occur in photosynthetic cells exposed to natural circadian light conditions. Gene

expression is drastically altered as a consequence of changing light conditions, with anywhere between 30%-65% of the transcriptome changing expression profiles as a result (Kopf et al., 2014; Lenz and Sogaard-Andersen, 2011). During light conditions, autotrophic growth is driven by oxygenic photosynthesis, while excess generated energy is stored as the carbohydrate glycogen (Ball and Morell, 2003). Also, light conditions generate more favorable redox potential and cyclic electron flow conditions for heterologous product production.

During dark conditions, stored glycogen is utilized as a carbohydrate source to drive expression and function of essential oxygen sensitive, nitrogen utilization and general maintenance genes (Lenz and Sogaard-Andersen, 2011). When the ability of glycogen storage and/or utilization is removed from photosynthetic cells via pathway knockout mutants, cell viability plummets after prolonged dark periods compared to wild type cells in the same conditions (Scanlan et al., 1995). In addition, stress recovery is impacted when overall glycogen stores are decreased from what is normally found in cells (Gründel et al., 2012). Glycogen defective mutants challenged with salt or oxidative stress during light conditions exhibit a decrease in overall growth rate, while wild-type cells are largely unaffected (Suzuki et al., 2010). Salt and oxidative stresses activate stress signal transduction pathways that up-regulate specific stress genes which requires excess ATP. This excess ATP is generated via glycogen catabolism which makes glycogen stores essential for peak fitness of photosynthetic cells. While many research facilities provide 24-hour light exposure to cyanobacterial cultures grown for experimental research, a detailed understanding of the diurnal nature of these microbes must be taken into account in order to design better production systems capable of harnessing sunlight as the main energy input, a critical component involved in any economical large scale production system.

The cyanobacterium *Synechocystis sp.* PCC 6803 (hereafter referred to as *Synechocystis*) has become a model organism for many areas of cyanobacterial research. *Synechocystis* is a photosynthetic gram-negative eubacterium that shows an extremely high homology to the chloroplasts of higher order plants (Abdallah et al., 2000). *Synechocystis* has been extensively studied within the scientific community and was the first photosynthetic microbe to have a fully sequenced and annotated genome (Kaneko et al., 1996). A considerable amount of knowledge began to accumulate around the study of this organism, which lead to the development of critical research tools and techniques. The development DNA manipulation techniques based on single stranded DNA uptake and homologous recombination allowed researchers to incorporate exogenous DNA into the genome and better understand gene regulation within this organism. Optimal culturing techniques and best fit strategies for cellular growth and DNA transformation have been streamlined which has been critical for the development of this organism as a "model organism" within photosynthetic microbes (Barten and Lill, 1995; Williams, 1988a). The highly annotated and sequenced genome of *Synechocystis* also allows for identification of target sequences, increasing the ability for genetic engineering strategies to be incorporated into the system (Kaneko et al., 1996). While the basic research tools related to *Synechocystis* continues to accumulate in the scientific literature, new areas of research have also demonstrated that *Synechocystis* can be utilized as a "microbial cell factory" capable of partitioning a significant amount carbon flux towards the generation of products not associated with cell viability and maintenance. Theoretical studies have argued that *Synechocystis* metabolism is capable of directing over 50% of the carbon flux towards the synthesis of carbon containing products while still maintaining cell viability (Angermayr et al., 2014). Additionally, simple manipulations performed by Zhou and colleagues (2014b) were capable of driving the production of two

functional proteins within *Synechocystis* to concentrations that totaled over 15% of the total soluble protein found in the cell.

While support for the potential of *Synechocystis* as a production platform is accumulating, a large majority of the studies leveraging *Synechocystis* have performed minimal manipulations to the genomes in question. Over the last several decades, research in *Synechocystis* has accumulated concerning metabolites that can be utilized as fuel replacements, commonly known as biofuels (Lindberg et al., 2010; Liu et al., 2012; Machado and Atsumi, 2012; Zhu et al., 2013). A considerable amount of research interest has focused on genetic manipulation for the production of ethanol, as research began as early as the late 1990's on ethanol production in other species of cyanobacteria. Researchers Dexter and colleagues (2009) engineered a strain of *Synechocystis* capable of generating ethanol at 5.2 mmol/OD_{730}/liter/day. Their work was based on similar research in which two heterologous genes from an ethanol producing *Zymomonas* were inserted into a *Synechococcus* species. Dexter and colleagues inserted these genes into the genomic region of *Synechocystis* and controlled their expression with the P_{psbAII} promoter. While several new variables were introduced into the work that were not incorporated into the original work, this *Synechocystis* mutant generated ethanol at an order of magnitude lower than the *Synechococcus* mutant, and was hypothesized to be caused by cellular redirection of carbon flux away from the inserted pathway and not by ethanol toxicity. While this work demonstrates that ethanol production from *Synechocystis* is attainable, it also highlights the considerable amount of additional knowledge required for efficient engineering within this species. Since this work was published, a considerable amount of research has accumulated in the scientific literature that is focused on understanding the impacts of ethanol exposure and production within *Synechocystis*. In an effort to better understand the cellular response to exposure to ethanol, multiple labs have

performed transcriptomic studies as well as metabolic modeling studies on *Synechocystis* reprogrammed to generate ethanol (Dienst et al., 2014; Fu, 2009; Wang et al., 2012). This work is critical to building upon the current knowledge set so that future engineers have a better understanding of the metabolic network at play in strains capable of ethanol production.

While ethanol is an enticing biofuel target because of its use as an oxygenate in gasoline fuel, biofuels that mimic the long chain carbons found in diesel fuels is difficult. Production of fatty acids, carboxylic acids with long chain hydrocarbon tails, is a natural target because of its universal presence across all domains of life. Liu and colleagues (2011) were capable of generating a strain of *Synechocystis* that produced high levels of fatty acids via manipulation of multiple genes run by a nickel induced promoter, P_{nrsB} as well as the P_{psbAII} promoter. Cheah and colleagues (2015) build upon the work performed by Liu and placed a heterologous thioesterase gene under the transcriptional control of several engineered promoters, in an effort to drive expression and therefore, fatty acid accumulation to higher levels. While transcription rates of the thioesterase gene were increased considerably, enzyme accumulation was unchanged from wt levels.

While these discussed research projects are only several examples of engineering *Synechocystis* for metabolite production, they highlight the potential of metabolite production within *Synechocystis* while also highlighting the need for comprehensive engineering strategies to fully realize the potential of utilizing these microbes as metabolite production platforms.

Carotenoid Production within *Synechocystis*

While pathway engineering for the goal of producing metabolites like ethylene, isoprene, acetone, lactic acid, alcohols, sugars, fatty acids, biofuels, and other valuable molecules has

occurred in *Synechocystis*, few studies have looked to increase the production of carotenoids within this organism. The family of carotenoid molecules comprises a diverse group of over 600 structurally different 40 carbon chain length molecules that contain multiple conjugated double bonds. The pathway originates from the terpenoid biosynthesis pathway, a pathway that is ubiquitous amongst all life on earth. The conversion of geranyl di-phosphate to phytoene via phytoene synthase is considered to be the first committed step in the development of downstream carotenoids (Dibari et al., 2012). The carotenoids play diverse roles in protecting photosynthetic organisms from deleterious effects caused by cellular metabolism. Carotenoids act in quenching reactive photo-oxidative elements that include triplet chlorophyll reactive species, reactive singlet oxygen species (ROS) and lipid peroxides, all of which are generated during stress conditions and all are capable of reducing the overall fitness of the organism (Auldrige et al., 2006; Lu and Li, 2008). Carotenoids also are active as photosynthetic antennae molecules capable of harvesting light photons and transferring this energy to chlorophyll molecules as well as quenching excess excitation energy during high light conditions. This group of molecules also plays an integral role in the structural rigidity of cyanobacterial cell outer membranes, impacting adhesion properties, protection against pathogens and high light conditions, overall membrane rigidity, and aiding in the control of intracellular pH. *Synechocystis* harbors a functioning carotenoid biosynthesis pathway and generates a small suite of carotenoids (myxoxanthophyll, β-carotene, zeaxanthin, and echinenone) in observable amounts, figure 3.

One specific carotenoid of note that is not generated naturally in *Synechocystis* is the diketolated and dihydroxylated derivative of β-carotene, astaxanthin. Over the last several decades, astaxanthin has increased in commercial value, with considerable amounts of the molecule being utilized as a colorant in the nutraceuticals, cosmetics, and aquaculture industries.

In addition to these benefits, the molecule has also been investigated for potential anti-inflammatory, anticancer, as well as anti-diabetic effects within humans because of the molecules ability to act as one of the most capable free radical quenchers found within cellular systems (Higuera-Ciapara et al., 2006; Huang et al., 2002; Palozza and Krinsky, 1992). Market values of the molecule fluctuate anywhere from $2000 to $7000 per kg, with an overall market size of $250M per year and growing to an estimated $1.5B by 2020, which has generated considerable interest in production of this molecule from natural as well as synthetic sources (Lorenz and Cysewski, 2000; Nguyen, 2013) Synthetic production of the molecule dominates the market currently, with the most common mechanism for production being the modifications of naturally derived precursor molecules, canthaxanthin, zeaxanthin, and lutein (Huang et al., 2002). While synthetic production strategies are well developed, non-natural stereoisomers of the molecule are generated and therefore considered to be less pure than natural sources of the molecule (Higuera-Ciapara et al., 2006).

Natural production of astaxanthin mainly occurs from two distinct organisms, the eukaryotic alga, *Haematococcus pluvialis* and the eukaryotic fungus, *Xanthophyllomyces dendrorhous.* The heterotrophic red yeast, *Xanthophyllomyces dendrorhous* is fungal species that has gained considerable interest in astaxanthin production over the last several decades. This organism naturally generates low rates of astaxanthin (below 0.1%) and as of late, has been the focus of a considerable amount of research (Johnson and Schroeder, 1996). Strategies like chemical mutagenesis and pathway engineering have generated hyper producing strains capable of substantial increases in production of astaxanthin. Gassel and colleagues (2014) were able to leverage strains identified via chemical mutagenesis to further engineer these strains with genes that increased the expression of four identified enzymes considered to be limiting steps within

the pathway. Three endogenous genes from the committed carotenoid biosynthesis pathway, geranylgeranyl diphosphate synthase, phytoene synthase, as well as an astaxanthin synthase, were upregulated along with a coenzyme-A reductase gene, also from *Xanthophyllomyces.* Combinations of these manipulations were capable of increasing overall astaxanthin production by close to an order of magnitude within the mutants (Gassel et al., 2014). While astaxanthin increases within this organism have shed light on effective strategies to increase overall production, heterotrophic organisms like *Xanthophyllomyces* utilize external energy sources generated from other organisms, which adds additional inputs that can impact the overall economics of large scale production.

Another microbe which exhibits endogenous production of astaxanthin is the photosynthetic alga *Haematococcus pluvialis*. Current commercial production systems leverage *Haematococcus'* cellular response to stress conditions like high salt exposure, high light exposure, increased growth temperatures as well as nutrient deprivation to induce cells produce anywhere between 1-3% astaxanthin (Guedes et al., 2011; Olaizola, 2000). Production employs a batch growth system, which allows the freshwater cells to reach high cell densities in natural light conditions prior to introduction of stress conditions, which causes them to encyst and generate higher concentrations of astaxanthin. Once cells encyst, cells must be aggregated via centrifugation, dried, and cracked to gain access to the astaxanthin. While *Haematococcus pluvialis* employs energy derived from the capture of light to drive reactions that generate astaxanthin within the cells, intrinsically slow growth rates as well as low cell yield have been a major barrier to efficient production within this cell system. Productivities in industrial systems typically reach 2.2 mg/l, while production in bench scale research has been reported to be as high as 11 mg/l/day (Aflalo et al., 2007; Olaizola, 2000). The presented gains were made by

modulating the amount of astaxanthin generated by cells via exposure to the various stress conditions known to impact astaxanthin production as well as changes to the overall culture densities found in the various types of growth systems used to grow the cells. Very few studies have leveraged genetic engineering techniques to manipulate astaxanthin titers in *Haematococcus*, mostly because of the lack of genetic tools capable of delivering deliberate manipulations to the genome of the cell as well as a lack of genetic stability found in long term studies of transgenic *Haematococcus* cells. In 2015, Kathiresan and colleagues (2015) were capable of increasing native production of astaxanthin by 2-3 fold via agrobacterium-mediated insertion of a second copy of the β-carotene ketolase gene, BKT. While this research demonstrates the nascent capability of genetic manipulation in *Haematococcus,* considerably more information is needed to fully understand the reasons behind the recalcitrance of genetic engineering within this organism.

While no previously identified research has investigated astaxanthin production in *Synechocystis*, relatively few research investigations have studied *Synechocystis* to better understand this organism's ability to produce economically viable titers of carotenoids. Specific changes in the environmental conditions like changes in nutrient composition of the media as well as varying the exposure to various light conditions have been able to modulate the production of native carotenoids in *Synechocystis*. Steiger and colleagues (1999) observed an increase in the de-novo synthesis of carotenoids when cells were shifted from low light conditions to high intensity illumination conditions. While the carotenoid biosynthesis pathway was upregulated, an overall decrease in the carotenoid titer was observed, which was due to the rapid breakdown of existing carotenoid concentrations at high light intensities. Paliwal and colleagues (2015) were able to increase β-carotene concentrations by simply exposing cells to

BG-11 media containing a 1M concentration of sodium chloride. Additionally, this same research highlighted how overall carotenoid concentrations changed dependent upon specific media type.

While research has demonstrated that overall carotenoids titers can be modulated via environmental changes, manipulation of native pathways via metabolic engineering strategies has also shown promise. In the hopes of driving carbon flux towards zeaxanthin and β-carotene, several research studies have knocked out genes that drive flux towards competing products with varying levels of success. As a strategy to assess impacts to myxoxanthophyll production, Abdelwahab (2015) was able to knockout a probable glucosyl transferase within *Synechocystis*, which severely impacting the fitness of the culture, but also increased myxoxanthophyll precursor concentrations as well as myxoxanthophyll itself. Lagarde and colleagues (2000) were capable of knocking out the endogenous β-carotene ketolase gene *crtO*, thus knocking out the production of echinenone. While this knockout mutant did increase the overall titer of the carotenoid myxoxanthophyll, the mutant also accumulated less overall carotenoids when compared to the wt culture. In addition to this mutation, this same research group duplicated several additional endogenous genes within the carotenoid pathway, phytoene synthase (*crtB*), phytoene desaturase (*crtP*), and β-carotene hydroxylase (*crtR*). While *crtP* has been considered to be the rate limiting step in the downstream pathway leading to carotenoid production, the researchers saw no increase in myxoxanthophyll and zeaxanthin production when over-expressing this enzyme, but were able to increase myxoxanthophyll and zeaxanthin production by 50% when over-expressing the *crtB* gene. This same group was able to increase zeaxanthin from a wild-type content of 25% of overall carotenoid fraction to over 50% of overall carotenoid content by overexpressing *crtR*. While this research demonstrates changes are capable of being

made to overall carotenoid titers within *Synechocystis*, it also provides clues to understanding how metabolic engineering strategies can be leveraged to further impact carotenoid production within *Synechocystis*.

While targeted manipulation of specific genes within the carotenoid biosynthesis pathway have begun to show increases in the overall titers capable of being generated in *Synechocystis*, endogenous regulation of the carotenoid pathway is critical to understand if further engineering approaches are to build upon recent successes. Endogenous regulation of the carotenoid pathway allows organisms to manage the production of these multifunctional metabolites for when conditions are essential for their production. Alternatively, when stress conditions like nutrient starvation, low CO_2 concentrations, extreme light conditions, high salt media concentrations are absent from culture conditions, carotenoid metabolites are non-essential and thus, down-regulated within the cell (Cazzonelli and Pogson, 2010; Demmig-Adams and Adams, 1996).

Identifying, understanding, and modulating the critical rate-limiting enzymes within the methylerythritol 4-phosphate (MEP) pathway that generate the critical precursor molecules isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) have shown to have substantial impacts on the overall production of carotenoids. Several enzymes have been identified as being rate limiting within this pathway (Cordoba et al., 2009). The first committed enzyme within this pathway, 1-deoxy-D-xylulose-5-phosphate synthase (DXS), has been shown to play a major role in regulation of the MEP pathway, while the last committed step in this pathway, IPP:DMAPP isomerase (IDI), has also been shown to have substantial pathway regulatory control within eukaryotic systems. (Chang et al., 2013; Harker and Bramley, 1999; Kuzuyama et al., 2000). Ajikumar and colleagues (2010) were capable of balancing the expression of the enzymes *dxs, and idi* that alleviated pinch-points within the pathway and thus

increased the overall titers of IPP and DMAPP, which increased downstream terpenoid production by over 5 fold within the study.

Further downstream regulation of the carotenoid biosynthesis pathway has also been identified as control points for production of the colored carotenoids. Specifically, the enzyme phytoene synthase (PYS) that generates phytoene, the colorless precursor to the carotenoids, and acts as the first committed step in the carotenoid biosynthesis pathway has been identified as controlling carotenoid production within multiple species(Bramley, 2002; Fraser and Bramley, 2004). Additionally, the enzyme that converts phytoene into lycopene, phytoene desaturase (PDS), has been separately identified as critical rate-limiting steps within this pathway. Chamovitz and colleagues (1993) as well as Misawa and colleagues (1993) were able to generate PDS mutants in the cyanobacterium *Synechococcus* as well as in tobacco that identified phytoene desaturase as a clear rate limiting step in the production of xanthopylls. These studies demonstrate that endogenous regulation of upstream pathways which contribute precursors to the carotenoid biosynthesis pathway are critically important to understand when targeting increases in overall carotenoid production. They also bring to light the need for comprehensive strategies to realize the full capability of utilizing these microbes as production platforms. It is critical to understand the endogenous cellular control that occurs in these organisms as well as the multiple strategies that can be utilized to manipulate pathways as well as gene expression. Understanding the impacts of these strategies on the overall cellular fitness is also a critical component in the design of further iterations on this pathway.

Metabolic Engineering Strategies within Microbial Species

Generating multiple pathway manipulations capable of producing valuable molecules in microbial platforms is a complex undertaking. Multiple strategies must be pursued to achieve titers of commercial significance. While genome scale modeling of metabolic networks in *Synechocystis* and other cyanobacteria has helped genetic engineers better understand the complexities and impacts of multiple gene manipulations, *in vivo* management of entire pathways to push product titers towards theoretical levels have only recently begun to surface in the research literature (Fu, 2009; Pei et al., 2014; Wang et al., 2013). The development of carbon sinks that act to pull carbon through specific pathways has succeeded in various degrees within cyanobacterial species. The development of a carbon sink may increase overall productivity by starving essential pathways of carbon, thus stimulating cells to increase primary metabolism in order to manage the carbon shortfall via metabolic feedback regulation. Oliver and colleagues (2013) were capable of increasing overall carbon yield within *Synechococcus* by inserting genes capable of generating 2,3-butanediol. This sink pathway partitioned pyruvate away from participating in cellular growth and in doing so, increased carbon yield by 1.8X within the cell. While carbon sinks may have a beneficial impact on overall production, studies that focus directly on understanding how to manage and mitigate bottlenecks upstream of the final target molecule has shown promise to increase overall titers within *Synechocystis* as well as other species (Angermayr et al., 2015; Atsumi et al., 2009). In an effort to increase the overall titers of the terpenoid precursor molecules, dimethyalyl diphosphate (DMAPP) to isopentyl diphosphate (IPP), Gao and colleagues (2016) introduced heterologous genes to specific steps of the methylerythritol phosphate pathway within Synechococcus, which substantially increased the overall IPP and DMAPP, which translated into higher titers of the target metabolite, isoprene.

The management of pathways generating the main precursors of secondary metabolism products like pyruvate and acetic acid have been instrumental in further increasing metabolite titers. Angermeyr and colleagues (2014) were able to demonstrate higher lactic acid titers within *Synechocystis* by inserting a second copy of a pyruvate kinase gene capable of increasing the pools of pyruvate. The management of carbon flux through branch points within metabolic networks has also been a useful tool to increase overall product titers in *Synechocystis*. Specifically, utilizing rational design engineering to push carbon flux down determinate network paths via enzyme knockouts has proven to be a valuable tool. Zhou and colleagues (2012) were capable of synthesizing appreciable titers of acetone in *Synechocystis* by inserting a synthetic acetone synthesis pathway as well as knocking out the endogenous acetate synthesis pathway. Titers were only observed when both modifications occurred, simple insertion of the synthetic pathway itself was incapable of generating titers of acetone. When redirecting flux towards a specific metabolite, full gene knockouts are viable strategy for decreasing flux down non-essential pathways. This strategy is not functional when enzymes and metabolites are essential for cellular viability. Flux can be driven away from these pathways by manipulating the expression strength of specific genes within the essential pathway via the use of specialized promoters. While a multitude of engineering strategies can be leveraged within cellular systems, understanding the impacts these strategies have on cellular fitness and viability are as critical as understanding how they impact metabolite production.

Metabolic Burden within Microbial Species

While advances in understanding the manipulation of multiple pathways components within *Synechocystis* is continuing to accumulate in the literature, critical importance must be

taken in understanding how these complex manipulations impact overall cellular fitness. Over the last several decades, advances have been made in understanding the metabolic burden caused by pathway engineering to produce high titers of valuable products in heterotrophs. Metabolic burden is most often described as the amount of additional cellular resources withdrawn from central metabolism that is required to manage and maintain exogenous DNA. Heterologous gene expression can cause physiological changes within cells that often mimic responses that occur during extreme temperatures, amino acid depletion, and resource starvation. While identified as occurring in cyanobacterial species harboring engineered pathways, few studies have looked to lessen metabolic burden via further genetic engineering (Akiyama et al., 2011; Fu, 2009; Wijffels et al., 2013). In contrast, metabolic burden has been intensively studied and mitigated in heterotrophic production systems (Mattanovich et al., 2004; Rozkov et al., 2004). Tools capable of controlling gene expression via promoter engineering, codon optimization, and heterologous gene insertion have shown to be vital components to managing the adverse conditions generated during heterologous gene expression (Ajikumar et al., 2008; Santos and Stephanopoulos, 2008a). Specifically, understanding how gene expression can be modulated via promoter control tools is critical in managing carbon flow through pathways. Research in heterotrophic microbes has shown that mutagenesis of strong constitutive promoter constructs to produce a wide range of varying strength promoters has proven to be a useful strategy to reduce overall cellular stress which increases population growth and product production (Mattanovich et al., 2004). Additionally, the development of promoters capable of chemical induction at specific cell densities has been used successfully to activate gene expression during growth conditions best suited for decreased burden and increased overall titers in heterotrophic systems (Fass et al., 1991). Delayed induction of targeted gene expression allows cells to produce and accumulate

cell products essential to the fitness of the cell prior to the redirection of carbon flux towards the production of target molecules (Ramirez and Bentley, 1995).

Understanding of Native Promoters found Within Microbial Species

While ongoing research continues to increase the body of knowledge concerning metabolic burden in heterotrophs, only recently have research labs begun to better understand the toolkits capable of controlling gene expression within *Synechocystis*. The cyanobacterial promoter is composed of a cis-acting element made up of nucleotides capable of recruiting RNA polymerase and other trans-acting elements that play a role in initiating gene transcription. Cyanobacterial promoter elements have evolved over billions of years to exhibit a wide range of transcription initiation rates as well as being controlled by a wide range of activation conditions. Understanding how specific promoter elements function and the conditions at which they initiate transcription are critical to efficient pathway engineering of increased titer of products. Various native promoters within *Synechocystis* have been utilized to drive gene expression within experimental studies. While use of native promoters has met some success in controlling gene expression, clear elucidation of the full functionality of the native promoters is usually outside the scope of research. While many of the native promoters leveraged in pathway engineering studies within *Synechocystis* are assumed to be driving expression in a constitutive manner, few promoters are scrutinized for full functionality across a multitude of cellular conditions. This strategy can prove to be problematic, especially within a cyanobacterial system capable of varied gene expression during various growth and stress conditions. Studies have used promoters that drive native production of the large subunit of the carbon concentrating enzyme Rubisco, the ribonuclease p subunit B gene, the plastocyanin gene, the ATP synthase subunit A gene,

phycocyanin β-subunit gene, amongst others to control gene expression within manipulated systems. One native promoter in particular, the P_{psbAII} promoter, has been utilized in multiple research studies to drive gene expression within *Synechocystis* (Bentley et al., 2014; Englund et al., 2015; Formighieri and Melis, 2014; Lagarde et al., 2000). The P_{psbAII} promoter region responsible for generating the D1 protein in the photosystem II complex (Mulo et al., 1998; Sicora et al., 2006). This promoter region is highly active during high-light conditions while being down regulated during low light conditions. Therefore it exhibits quite a dynamic range of expression during various light conditions (El Bissati and Kirilovsky, 2001; Eriksson et al., 2000). While 24 hour light growth was utilized in a vast majority of studies, few if any studies take note of the dynamic transcription activity observed within P_{psbAII} platform. This dynamic may impact the overall productivity of constructs leveraging this promoter.

Inducible promoters are another type of native promoter leveraged in metabolic engineering studies within *Synechocystis*. Inducible promoters remain inactive until specific chemical or environmental conditions initiate transcription. A majority of the inducible systems identified in *Synechocystis* function to regulate transition metal abundance within the cell. Promoters that respond to changes in the metals cadmium, cobalt, copper, iron, nickel, and zinc have all been identified and utilized within engineered expression systems in *Synechocystis* (for a full review, see Berla et. al. (2013)). Regulation of these systems is critical for cell viability, and therefore expression is tightly regulated by the cell. These types of promoters can be quite beneficial to the metabolic engineer, but only when a comprehensive understanding of the promoter activity is understood across a wide range of cellular and environmental conditions. It is critical to understand that in addition to transcriptional initiation, gene expression and product production can be impacted by post-transcriptional control as well as post-translational control,

which adds multiple layers of control to a gene system. These systems of control have evolved alongside the evolution of the species themselves, and thus the multiple layers of gene control can be difficult to understand and conceptualize, when designing a promoter system capable of predictable control. In an effort to focus on promoter systems that are theoretically decoupled from the endogenous control mechanisms within *Synechocystis*, some metabolic engineers have begun to look to heterologous promoter systems from heterotrophic microbes like *E. coli*. The scientific community has studied, annotated, and leveraged promoter systems within *E. coli* for decades, which provides engineers with a wealth of understanding of these systems. While the use of heterologous promoters can alleviate the potential endogenous control systems found within *Synechocystis*, a further structural understanding of the components that interact with promoter regions is just as critical to the functionality of promoters systems moved between the cell types. While both *E. coli* and *Synechocystis* are eubacterial in nature, several key structural differences in the eubacterial RNA polymerase are important to highlight. While *E. coli* RNA polymerase contains the α_2, β, and β' core enzymes, the β' core enzyme in *Synechocystis* is split into two parts, β' and γ, making the RNA polymerase structurally distinct between the two cell types (Bergsland and Haselkorn, 1991; Imamura et al., 2003b; Schneider and Hasekorn, 1988). This additional enzyme subunit physically elongates the holoenzyme, and therefore changes the binding affinity dynamics within the organism relative to the spacing of the cis-regions found on the DNA that recruit components of RNA polymerase (Imamura et al., 2003b; Yoshimura et al., 2002). Changes in the spacing of these elements also may play a role in the stability of melted DNA and therefore the retention of sigma factors within these regions, which can have a significant impact on promoter driven-gene expression control (Koo et al., 2009). Recently, engineers have begun to utilize several heterologous promoter elements within *Synechocystis*

with varying degrees of success. While the promoters for bacteriophage lambda as well as the *E. coli* tetracycline operon have been tested in *Synechocystis*, promoter strength was considerably lower when compared to expression control within the native *E. coli* system. The P_{tac} suite of promoters, originally designed and utilized in *E. coli*, is the most commonly utilized heterologous promoter suite in *Synechocystis*. Although the suite does show functionality in *Synechocystis*, expression trends within the suite is considerably different from what is seen in *E. coli* cell lines. This only further highlights the need for metabolic engineers to gain a better understanding of the promoter elements to be utilized in specific systems as well as how those elements interact with the native mechanisms driving gene expression within cells.

Genetic Engineering of Promoters within *Synechocystis*

As metabolic engineers begin to develop the foundation of promoter engineering within *Synechocystis*, it is critical to understand the two major approaches used to develop engineered promoter constructs within microbial cell lines. Two of the major approaches that have been leveraged to design promoter elements are random mutagenesis and rational design. Random mutagenesis of promoter elements exposes promoters to treatments capable of adding, deleting, or substituting nucleotides within the promoter sequence in a random manner and thus impacting recruitment of trans-acting elements or modulating transcription initiation rates. While in both *Synechocystis* as well as in other organisms, random mutagenesis has been leveraged to further understand the specific control elements present within promoters of interest. Extensive work within heterotrophic systems has taken this technique one step further and has leveraged random mutagenesis to develop a "library" of promoters that show a range of transcriptional initiation strength which can be utilized to fine tune gene expression within a target pathway. Alper and

colleagues (2005) were one of the first groups to use random mutagenesis to develop, test and demonstrate the applicability of a suite of promoters that were capable of driving metabolite production. This initial work was performed in *Saccharomyces* and leveraged error prone PCR to generate a suite of promoters that drove GFP expression. The various strength promoters were selected by the amount of fluorescence generated by the mutated constructs. Examples of promoter library development via random mutagenesis in cyanobacteria can only be found within closely related species. Markley and colleagues (2014) generated a suite of promoters via error prone PCR techniques that were capable of exhibiting a 48-fold dynamic range of transcription strength within *Synechococcus*. While the researchers were able to demonstrate functionality using a modified GFP reporter construct, the generated promoter suite was not leveraged to run pathways capable of targeted metabolite production.

While random mutations to the promoter region have generated suites of promoters capable of "tuning" gene expression in various species, the first genetic engineers to understand the value of engineering promoters utilized a basic understanding of the functionality of various parts of a promoter region. They then used this information to manipulate or combine various regions to build promoters capable of modulating gene expression in a deliberate manner. The use of rational design strategies that leverage science knowledge to make deliberate nucleotide manipulations that have a high probability of delivering predictive expression results have been used successfully for decades within the scientific community. Early research in *E. coli* performed by Deboer and colleagues (1983) initiated the concepts of rational promoter design when they leveraged knowledge of the P_{lacUV5} and the P_{trp} promoter constructs. They used that knowledge to develop the hybrid P_{tac} promoter, a promoter capable of several states of activity, which is dependent on the presence or absence of specific chemical inducers in the cell system.

These researchers were able to build a hybrid promoter that contained the consensus -10 nucleotide region, the pribnow box region, from the P_{trp} promoter as well as a consensus -35 nucleotide region from the P_{lacUV5} promoter,. This hybrid promoter increased overall transcription rates by 3x over that of the native P_{trp} promoter and 11x over that of the P_{lacUV5} promoter. Brosius and colleagues (1985b) built upon this research to better understand the impacts of changing nucleotide spacing within the -10 and -35 cis-acting regions within the P_{tac} promoter construct. They were capable of generating a small library of promoters, which was subsequently named the P_{tac} suite of promoters. This suite exhibited various transcriptional initiation strengths within *E. coli*. The P_{tac} suite of promoters have been utilized to drive engineered gene expression within multiple species for over 30 years.

Understanding rational design strategies for promoter library development within *Synechocystis* has begun in earnest within the last several years. Several research groups have focused on how changes in nucleotides within the core components of specific promoter regions impact transcription initiation and overall product production. Huang and Lindblad (2013) focused on how nucleotide substitutions within the region that separates the transcription initiation site and the -10 pribnow box region of the P_{TetR} repressible promoter from *E. coli* would impact fluorescence of a GFP reporter system. While initial substitutions of nucleotides within this region increased overall fluorescence, additional changes to the sequences produced very similar expression to the initial substitution. In addition, no clear correlations related to the type of nucleotides present and the corresponding amount of fluorescence were able to be made. Similar to this work, Qi and colleagues (2013) were able to insert additional single nucleotides into the spacer region that separates the -10 and -35 region of the promoters that run the *Synechocystis* genes *psbAII*, *petE,* and *psbD*. While successful at nucleotide insertion within

these regions, little to no change in promoter strength was observed from that of the native promoters generated. Zhou and colleagues (2014b) leveraged rational design strategies that leveraged multiple transcription factor binding sites (TFBS) to develop a promoter system capable of high rates of transcriptional initiation within *Synechocystis*. While this group was able to develop a promoter that drove production of a targeted functional protein to titers of over 15% of total protein found in the cell, the group did not pursue the development a suite of promoters based upon the newly constructed promoter.

While research has been slow to demonstrate how rational design changes can positively impact overall constitutive promoter control in *Synechocystis*, rational design has been successfully leveraged to manipulate promoters utilized in chemical induction systems, like the P_{trc} promoter system. Promoter research from Uppsala University has focused on understanding the variations of promoter and their ability to impact gene expression in *Synechocystis*. The Lindblad group (2010b) has focused on understanding how the P_{trc} promoter system can control gene expression system while utilizing the lacI repression system. Their first area of research focused on understanding how the introduction of operator regions into the P_{trc} promoter impacted repression and induction. The operator regions are cis-acting nucleotide sequences found within the P_{trc} promoter that are capable of binding trans-acting repressor proteins, which has the effect of decreasing the binding efficiency of the RNA polymerase and thus decreasing transcription rates. Efficient repression within the LacI repressor model is dependent on the formation of a LacI protein tetramer binding onto the operator regions within the promoter (Kramer et al. 1987). This efficiency is also considered to be dependent on periodic spacing distances between the two operator regions, while Muller and colleagues (1996) demonstrated that periodic spacing of 11.5 nucleotides starting with a 70.5 base pair separation distance exhibit

the highest levels of repression. Huang and colleagues (2010b) introduced successive operator regions into the P_{trc} system within *Synechocystis*. Initial work spaced the centers of the two operator regions 88 base pairs from one another, and while near complete repression resulted from their construct, very little induction, 7.5% of the promoter's activity without the repressor, was obtained. The group continued their research and focused on making deliberate changes in the nucleotide spacing between cis-acting operator regions, in an effort to further "fine tune" inducibility. While their work was able to demonstrate that deliberate changes in the spacer length between operator regions led to a predictable trend in repression and induction strength within the constructed promoter suite, it did not lead to the development of a promoter construct capable of efficient repression as well as clear and strong induction.

Scope of the Dissertation

While the production of commercially valuable molecules within heterotrophic cell systems is well established, the use of cyanobacteria and more specifically, *Synechocystis sp.* PCC 6803 presents unique benefits as well as unique challenges. This organism exhibits a considerable amount of benefits over its heterotrophic relatives. It's capable of utilizing sunlight as an energy driver, utilizing carbon dioxide as a carbon source, and also exhibits rapid doubling times. Research into the utilization of this organism to generate commercially valuable metabolites like carotenoids is still in its infancy. Carotenoid molecules have many benefits to mankind; they are capable of replacing petrochemical pigments and dyes with natural alternatives, they play vital structural roles within cells of many aquatic organisms, and have been shown to have considerable protective properties in regards to human health. While tools capable of driving heterologous and endogenous gene expression in cell lines like *E. coli* and

Saccharomyces are well established and understood, this is not the case in *Synechocystis*. Microbes leveraging photosynthesis as a main energy source exhibit unique cellular conditions and cell control mechanisms that have evolved to tightly control gene expression during appropriate stimuli, which can be problematic to the metabolic engineer.

This work highlights development of a suite of constitutively expressed promoter constructs based on the P_{tac} suite of promoters that show a wide range of varied gene expression control within *Synechocystis*. Our work leveraged rational design strategies to modulate the nucleotide gap between the -10 and -35 binding regions within this promoter suite. In addition, changes were made to the $P_{SCA6\text{-}2}$ promoter that included cis-acting control elements that generated a chemically inducible promoter construct. This construct demonstrates tight repression capabilities as well as inducibility when the chemical inducer IPTG is introduced.

This work also discusses the P_{psbAII} promoter and the insights obtained when utilizing this promoter within two locations in the genome of *Synechocystis*. The P_{psbAII} promoter is a widely utilized promoter within genetic engineering in *Synechocystis* and is considered to be a promoter that is regulated by the absence or presence as well as by the intensity of light received by the cell. While many other researchers utilize the promoter in its native location our research highlights that when the core promoter is moved to a neutral location on the *Synechocystis* genome, light inducibility is lost. Additionally, this research highlights critical changes to cis-acting regions on the P_{psbAII} core promoter that changes the overall initiation strength of the promoter itself.

Lastly, this dissertation describes the genetic engineering that was performed to produce the non-native carotenoid astaxanthin within *Synechocystis*. This work describes the manipulation of several endogenous genes as well as the insertion of two heterologous genes

within the carotenoid biosynthesis pathway and how combinations of these manipulations impacted native and non-native carotenoid metabolite production. Promoters from the previously described research were leveraged to further increase production of canthaxanthin as well as astaxanthin within various mutants. In addition to the manipulations described here, the wt and an astaxanthin producing mutant were exposed to diel light cycle conditions for the duration of the experiment. Impacts of overall carotenoid production, rates of production as well as cellular biomass accumulation were measured and analyzed for cultures exposed to 24 hour light conditions as well as cultures exposed to periodic 12 hour light/12 hour dark conditions.

Product	Organism	Overall Production	Authors
Fatty Acids	Synechocystis	197 mg/l	Liu, Curiss
Manoyl Oxide	Synechocystis	0.45 mg/gDCW	Englund, Lindberg
Isobutyraldehyde	Synechcococcus	450 mg/l	Atsumi, Liao
Isoprene	Synechocystis	50 µg/gDCW	Lindberg, Melis
Zeaxanthin	Synechocystis	2.49 µg/ml	Lagarde, Vermass
Ethanol	Synechococcus	1710 µM	Deng, Coleman
Acetone	Synechocystis	36 mg/l	Zyou, Ma
Fatty Alcohols	Synechocystis	120 µg/l	Tan, Lu
Squalene	Synechocystis	0.67 mg/OD_{730}/l	Englund, Lindberg
β-phellandrene	Synechocystis	3.2 mg/gDCW	Formighieri, Melis

Figure 1. Selected examples of metabolite engineering in photosynthetic microbes

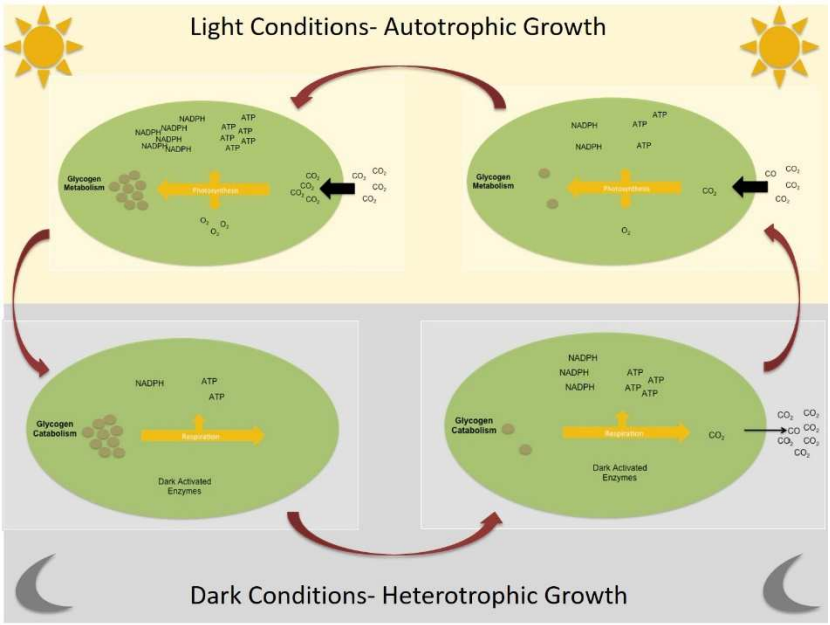

Figure 2. The diurnal nature of photosynthetic single-celled microbial cells.

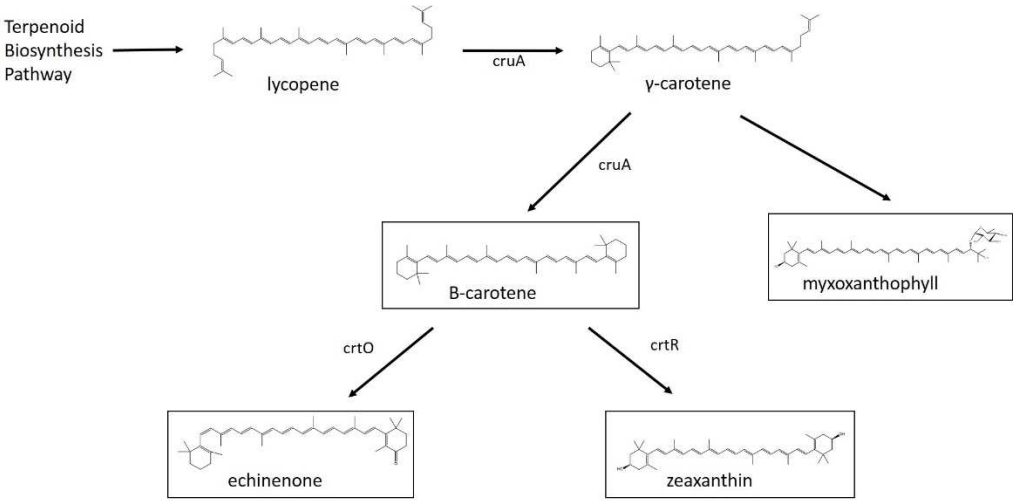

Figure 3. A simplified representation of the carotenoid biosynthesis pathway within *Synechocystis sp.* PCC 6803. Boxed metabolites represent carotenoids that accumulate in measurable amounts within *Synechocystis*.

CHAPTER 2

Engineering of Genetic Control Tools in *Synechocystis sp.* PCC 6803 using Rational Design Techniques[1]

Chapter 2 Brief Introduction:

Cyanobacteria show promise as photosynthetic microbial factories capable of harnessing sunlight andCO2to produce valuable end products, but few genetic control tools have been characterized and utilized in these organisms. To develop a suite of control elements capable of gene control at a variety of expression strengths, a library of 10 promoter-constructs were developed and built via rational design techniques by adding individual nucleotides in a step-wise manner within the −10 and −35 cis-acting regions of the tac promoter. This suite produced a dynamic range of expression strength, exhibiting a78 fold change between the lowest expressing promoter, Psca8-and the highest expressing promoter, $P_{sca3\text{-}2}$ when tested within *Synechocystis sp.* PCC 6803. Additionally, this study details the construction of a chemically inducible construct for use in *Synechocystis* that is based on the tac repressor system most commonly used in Escherichia coli. This research demonstrates the construction of a highly expressed inducible promoter that is also capable of high levels of gene repression. Upon chemical induction with IPTG, this same mutant strain was capable of exhibiting an average 24X increase in GFP expression over that of the repressed state.

[1] Albers, S. C., Gallegos, V. A., Peebles, C. A., 2015. Engineering of genetic control tools in *Synechocystis sp.* PCC 6803 using rational design techniques. J Biotechnol. 216, 36-46.

CHAPTER 3

Evaluating light-induced promoters for the control of heterologous gene expression in *Synechocystis sp.* PCC 6803[2]

Chapter 3 Brief Introduction:

Cyanobacteria are enticing microbial factories, but little is understood how their gene control elements respond to the periodic availability to light. This research tested the capability of the P_{psbAII} *promoter* to control gene expression during light/dark conditions when moved to a neutral location within the *Synechocystis sp. PCC 6803* genome. When the *eYFP* reporter gene was run by the P_{psbAII} promoter in the promoter's native genomic location, mutants exposed to 12 hour light conditions experienced a 15.8X increase in transcript abundance over that observed from the same construct exposed to 12 hour dark conditions. When this same construct was moved to the hypothetical coding region *slr0168* in the genome, transcripts generated during 12 hour light conditions accumulated to 1.67X of the levels of transcripts generated by the same construct during 12 hour dark conditions. Three additional promoter constructs, the $P_{psbAIII}$, P_{groEL2}, *and* P_{sigD} promoters were also tested for differential expression in light and dark conditions within the neutral region *slr0168*. While little activity was observed from P_{groEL2} *and* P_{sigD}, the $P_{psbAIII}$ promoter construct accumulated 5.79X more transcripts when compared to transcript abundance during dark conditions, which highlights the potential of this promoter to control gene expression during diel-cycle light conditions. Additionally, nucleotide mutations

[2] Submitted to the academic journal: Biotechnology Progress

were made to regions within the P_{psbAII} promoter. Mutations to the cis-acting hexo-nucleotide region increased expression 3.71 X over that of the native promoter, while the addition of the "*HLR*" nucleotide region to the $P_{psbAII::\Delta Hex}$ promoter construct increased expression 2.76 X over that of the native promoter.

CHAPTER 4

Carotenoid Pathway Engineering and Diel Light Cycle Impacts on the Production of Astaxanthin within the Cyanobacterium *Synechocystis sp.* PCC 6803[3]

Chapter 4 Brief Introduction:

The cyanobacterium *Synechocystis, sp.* PCC 6803 is considered to be a model organism for the production of many value added molecules in photosynthetic microbes. While many studies have leveraged *Synechocystis* to produce a multitude of valuable metabolites, little combinatorial engineering work has been performed on the endogenous carotenoid biosynthesis pathway. We leveraged metabolic engineering techniques to develop strains of *Synechocystis* sp. PCC 6803 capable of the production of two valuable carotenoids not naturally found in *Synechocystis*, astaxanthin and canthaxanthin. A *Synechocystis* mutant was engineered with an insertion of a β-carotene di-ketolase gene *crtW148* from *Nostoc punctiforme*, insertion of an additional copy of the endogenous β-carotene hydroxylase gene *crtR* from *Synechocystis*, and an open reading frame disruption of the endogenous β-carotene mono-ketolase gene *crtO*. These manipulations generated a mutant capable of an increase in the overall carotenoid content by 178 ± 10% % of that seen in wild type cells as well as astaxanthin titers that reached production rates of 1.11 ± 0.07 mg/l/day and canthaxanthin titers reaching 1.49 ± 0.05 mg/l/day. Additionally, this work also highlights the ability to increase production of target carotenoids by varying promoter strength driving the expression of *crtW148*. Use of the $P_{SCA6\text{-}2}$ promoter increased

[3] To be submitted to: Journal of Metabolic Engineering

carotenoid production of target carotenoids by 150% to 197% over production seen from the same constructs run by the P_{sigA} promoter. In addition to engineering of the pathways, we also tested the impacts of diel cycle light conditions on carotenoid production and accumulation. When exposed to 12 hour light/dark conditions, the mutant *crtR::cruB::ΔcrtO-*$P_{SCA6\text{-}2}$*::crtW generates carotenoids at rates of* 43 ± 14.8 *% of that of the* same culture grown in constant light conditions.

CONCLUSION

The utilization of *Synechocystis sp.* PCC 6803 as a metabolite production platform is continuing to progress. *Synechocystis* is an enticing microbial cell factory because the organism's is capability to: 1) utilize light as an energy source, 2) capture CO_2 from the atmosphere to use as the building blocks for growth and metabolite production, and 3) grow at some of the highest rates found within photosynthetic organisms, amongst other attributes. While many researchers have developed *Synechocystis* strains capable of generating valuable metabolites, this dissertation describes the first instance of *Synechocystis* producing the commercially valuable carotenoid metabolite, astaxanthin. One aspect that limits the success of metabolic engineering is the need for well characterized control elements capable of driving gene expression within target cell lines. This dissertation describes the development of constitutive, chemically inducible, as well as environmental inducible promoters and leverages a subset of these tools to make a number of manipulations in the carotenoid biosynthesis pathway in *Synechocystis* for the production of astaxanthin.

Chapter 2 demonstrates the use of rational design techniques to engineer a suite of promoters based on the *E. coli* derived P_{tac} promoter capable of an 87X dynamic range of transcription initiation within *Synechocystis*. The promoter suite was developed by modulating of the number of nucleotides present in between the cis acting -35 and -10 binding regions within the target promoter. Additionally, this work demonstrates the refinement of the LacI repression system for the $P_{sca6\text{-}2}$ promoter and validates the ability of LacI to repress expression within the cell line while also allowing for promoter induction with the chemical inducer, IPTG. The development of these promoters substantially increases the number of characterized promoters

for *Synechocystis,* and adds a functional inducible system able to manage the impacts of metabolic burden through the modulation of gene expression and timing. While many promoter constructs engineered for use in heterotrophic microbes exhibit poor functionality in *Synechocystis,* the development of the presented promoter suite further validates the use and functionality of rational design techniques when engineering control elements for *Synechocystis*. Further validation of the full capability of the promoter suite within a wide range of environmental conditions will only add to the functionality of this promoter suite. Changes in light concentrations, nutrient availability, co-factor availability, as well as conditions that increase cellular stress may impact how these promoters function within the cell. To do this, a more dynamic and real-time reporter system must be utilized in conjunction with this promoter suite. While the currently utilized fluorescent reporter molecule eYFP is well positioned within the suite of fluorescent molecules within *Synechocystis* in terms of excitation and emission spectra, subtle changes in expression strength are difficult to identify. Thus, other reporter systems like the luciferase system may be more appropriate for this organism. This will deliver critical information to metabolic engineers looking to fully leverage promoter libraries to drive distinct and controllable metabolite production within *Synechocystis*.

While the inducible promoter construct is capable of controlling gene expression upon the addition of the molecule IPTG, addition of chemical inducers can complicate commercial production. Efficient access to the inducer molecule within large systems and the additional cost of the molecule itself can be problematic in industrial scale systems. To further understand the functionality of an often used putative environmentally induced promoter construct, chapter 3 investigated the functionality of the endogenous P_{psbAII} promoter driving expression of the modified green fluorescent protein reporter gene, *eYFP,* in two distinct locations with the

Synechocystis genome. While this promoter is often used in engineered systems in *Synechocystis*, little work has been performed to understand the full functionality of this promoter within a wide range of cellular conditions and genomic locations. This work highlights changes in the overall activity of the core P_{psbAII} promoter during diel light cycle conditions when moved from its native location within the genomic DNA of *Synechocystis*. When *eYFP* gene replaces the *psbAII* gene and is run by the P_{psbAII} promoter, transcription initiation is severely repressed when exposed to dark conditions and shows a ~15X increase in overall eYFP transcript abundance when cultures were returned to light conditions. Interestingly, when the P_{psbAII}*::eYFP* construct is moved from the native location to a hypothetical coding region within the genome, only a 1.5X change in *eYFP* transcript abundance is seen when shifting from dark to light conditions. These results clearly highlight that light activated induction and dark induced repression of the P_{psbAII} promoter is controlled by more than just the core cis-acting elements on the promoter itself and that the location of the promoter-gene construct impacts transcription.

Chapter 3 also highlights the effect of several mutations to putative cis-acting regions within the P_{psbAII} promoter that are demonstrated to increase overall promoter strength, while also decreasing the light induced sensitivity of the P_{psbAII} promoter. In addition to the work performed on the P_{psbAII} promoter region, the $P_{psbAIII}$ promoter was tested for diel cycle sensitive transcription initiation when utilized in a neutral location on the genome. This promoter demonstrated functionality in generating mature eYFP proteins and also exhibited a 6X increase in the transcript abundance when mutants were moved from dark to light conditions. Future work could utilize the data presented in chapter 3 to generate a suite of promoters capable of light inducibility for the control of gene expression in *Synechocystis*. In addition, this promoter

suite is well positioned to further add understanding to the management of metabolic burden caused when expressing genes and pathways during diel cycle light conditions. While more work needs to be performed to fully understand the P_{psbAII} promoter and its light inducibility in a multitude of genomic and plasmid based locations within *Synechocystis*, this work demonstrates the potential pitfalls of utilizing poorly understood control elements for targeted metabolic engineering.

Chapter 4 highlights the use of several metabolic engineering strategies to manipulate the endogenous carotenoid biosynthesis pathway for the production of astaxanthin, a valuable commercial metabolite not naturally produced within *Synechocystis*. Insertions of *crtW148*, a heterologous gene from the organism Nostoc, generated a fully ketolated derivative of β-carotene, canthaxanthin, but was unable to utilize the endogenous pool of zeaxanthin to generate astaxanthin. Only when the cellular pool of zeaxanthin was increased via insertion of a second copy of the endogenous gene *crtR* did astaxanthin production occur within *Synechocystis*. In an attempt to further increase the pool of β-carotene, a vital precursor molecule of the carotenoid pathway, the endogenous b-carotene ketolase *crtO* was knocked out. This theoretically decreases carbon flux used to generate echinenone, a carotenoid in direct competition with zeaxanthin for β-carotene pools. An additional strategy was pursued to increase β-carotene titer that included the introduction of a second heterologous gene *cruB*, a γ-carotene cyclase from the green sulfur bacteria *Chlorobium phaeobacteroides*. While both the *crtO* knockout mutant as well as the cruB insertion mutant did statistically increase the overall available pools of β-carotene, increases in titers of canthaxanthin and astaxanthin were not observed. This data suggests that β-carotene pools are tightly regulated by the cell and thus direct manipulation of these pools has little overall impact. In addition to the discussed manipulations, two promoters developed in the

research from chapter two, P_{sigA} and $P_{SCA6\text{-}2}$, were used to drive the expression of *crtW148* within multiple constructs. Use of the more active $P_{SCA6\text{-}2}$ promoter increased carotenoid production of target carotenoids by 150% to 197% over production seen in from constructs harboring the P_{sigA} promoter construct. This clearly correlated to the differences seen between these same two promoters from the work performed in chapter two, in which the $P_{SCA6\text{-}2}$ promoter increased eYFP production by 180% over that seen by the same construct under the control of the P_{sigA} promoter. While the research presented here demonstrates the capability to generate astaxanthin within *Synechocystis*, expanded use of the $P_{SCA6\text{-}2}$ promoter to drive *crtR* expression as well as other pathway genes would be warranted. Additionally, further understanding and utilization of the various β-carotene hydroxylases as well as other β-carotene diketolases present in the scientific literature may be capable of further driving carbon flux towards astaxanthin to reach commercially viable titers within *Synechocystis*.

In summary, *Synechocystis sp.* PCC 6803 is a theoretically viable cellular platform for the production of metabolites valuable to mankind. While considered a "model organism" of the photosynthetic microbe research world, very little work has been performed to optimize the cellular machinery capable of deliberate control within the organism. The work presented here highlights potential pitfalls of the endogenous P_{psbAII} promoter, a well utilized promoter in which expression characteristics have only been evaluated within strict laboratory based conditions within the research community. In an effort to build out the promoter library available to metabolic engineers working in *Synechocystis*, we built a library of promoters capable of varied expression strength and also optimized an inducible construct system capable of controlling expression via chemical cues. This work culminated in demonstrating the functionality of *Synechocystis* as a production platform by leveraging several metabolic engineering strategies to

generate astaxanthin within *Synechocystis*. We used endogenous gene duplication, pathway gene knockouts, heterologous gene insertion, as well as varying transcription initiation strength via modulation of two constructed promoters to develop a highly engineered carotenoid biosynthesis pathway in *Synechocystis*.

While the insights revealed and tools generating during this research will help push the field of metabolic engineering in *Synechocystis* further, additional work must be performed to fully develop *Synechocystis* into a capable industrial microbial production platform. Considerable amounts of work must be performed to better understand the dynamic nature of gene expression within this organism during a wide range of environmental conditions. At the genome level, circadian rhythms oscillations continue to occur, regardless of light exposure, and thus have considerable impacts on gene expression. Understanding these oscillation impacts will be critical if metabolic engineers hope to design rational and predictable systems within single celled photosynthetic microbes. While the simplicity of cyanobacteria like *Synechocystis* is of benefit to the metabolic engineer in a multitude of ways, it can also hinder. Generating high titers of specific metabolites that naturally play a structural role within the components of the organism will be increasingly difficult unless mechanisms that allow for metabolite delivery out of the cell are identified and included into any strategy moving forward. While eukaryotic systems contain storage organelles capable of sequestering an overabundance of naturally derived metabolites, prokaryotic cells run the risk of damaging critical cell systems as excessive amounts of products are added and disrupt the natural composition of these systems.

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