Winter 12-15-2016

Genetic Circuits for Transcriptional Regulation in Synechocystis sp. PCC 6803

Cheryl M. Immethun
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

School of Engineering & Applied Science

Department of Energy, Environmental and Chemical Engineering

Dissertation Examination Committee:
Tae Seok Moon, Chair
Gautam Dantas
Marcus Foston
Elizabeth Haswell
Himadri Pakrasi
Fuzhong Zhang

Genetic Circuits for Transcriptional Regulation in *Synechocystis* sp. PCC 6803

by
Cheryl M. Immethun

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

December 2016
St. Louis, Missouri

Table of Contents

List of Figures

Chapter 2

Chapter 3

Chapter 4

List of Tables

Chapter 2

Chapter 3

Chapter 4

Acknowledgements

First, I need to thank Professor Tae Seok Moon for giving me the opportunity to be his first student. It has been quite a journey. I also need to thank Professor Moon and all of my committee members, Professors Gautam Dantas, Marcus Foston, Elizabeth Haswell, Himadri Pakrasi, and Fuzhong Zhang, for being willing to take on someone with no lab experience, and who learns and moves slower than 30 years ago. The attributes I had to offer were just a perpetual desire to learn and to share that with other students. I am grateful for your time and patience.

I also need to thank so many mentors who are not on my committee. Professor Richard Axelbaum was my first research rotation advisor. His confidence in me was an energy source throughout my first year. Professor Yinjie Tang was also a research rotation advisor. He continues to be an on-going resource, providing guidance and support that I know I can always rely on. Dr. Arul Varman introduced me to synthetic biology and cyanobacteria, and provided me with the groundwork from which I built this dissertation. His patience in my ignorance provided me a starting place. Dr. Bert Berla always had time to talk about my results and their meaning. Dr. Deng Liu helped me develop research questions that are meaningful to the cyanobacteria community. I appreciate all of your contributions to my continuing growth as a scientist.

I cannot overlook the contributions of my peers. Dr. Alireza Faghaninia patiently helped me fill in the holes in my background that enabled me to succeed in our first year classes. My fellow lab members, Drew DeLorenzo, Ray Henson, Allie Hoynes-O'Connor, Charlie Johnson, Soo Ji Kim, Ryan Lee, Kenneth Ng, and Tatenda Shopera, are always a source of support, solidarity and critical analysis of my research. Dr. Siqin He, Dr. He Jing, Begum Karakocak,

and Agnes Terenyi knew how to pick me up, dust me off and put me on my way to the next challenge. You have been my ballast.

I also need to acknowledge the students and iGEM teams, Blake Actkinson, David Ayeke, Charlotte Bourg, Drew DeLorenzo, Drew Ells, Caroline Focht, Zach Glick, Harley Greene, Dinesh Gupta, Ben Huang, Jeffrey Lee, Kevin Lingard, Charlie Johnson, Soo Ji Kim, Kenneth Ng, Arshag Mooradian, Gillian Myers, Mike Toomey and Ben Waldron-Feinstein, I mentored while at WashU. You embody the reason I pursued this degree at this point in my life. Working with you, talking with you, and being challenged by you is my source of energy. I learned as much from you as you did from me. I appreciate the energy, enthusiasm and curiosity you shared with me.

Last, but not least, I have to thank my family. Thank you for believing in me when I didn't, listening to me when I was in despair and reminding me of what's important. Thank you for understanding my non-traditional pursuit, the call that I could contribute more. Your support has grown by more than the years and the miles that have passed. I am fortunate to have your love and support.

As you can tell by reading this acknowledgement, I am truly blessed. So many people are responsible for me reaching this point. This milestone is not mine, but all of ours. Thank you!

This work was supported by a NSF Graduate Research Fellowship, DGE-1143954 and the NSF NITROGEN program, grant MCB-1331194.

Cheryl Immethun

Washington University in St. Louis

December 2016

Dedicated to my husband, Greg

ABSTRACT OF THE DISSERTATION

Genetic Circuits for Transcriptional Regulation in *Synechocystis* sp. PCC 6803

by

Cheryl M. Immethun

Doctor of Philosophy in Energy, Environmental and Chemical Engineering

Washington University in St. Louis, 2016

Professor Tae Seok Moon, Chair

Microbial biosynthesis has produced a variety of complex compounds using processes that are more environmentally-friendly than many conventional methods. The most common hosts are heterotrophs, which require the addition of an organic carbon source; while cyanobacteria possess many traits that make them a more sustainable biotechnology platform. As phototrophs, cyanobacteria can employ sunlight and carbon dioxide to create many value-added compounds. A wealth of tools has been developed to engineer the commonly used heterotrophs for higher yields and titers; yet, few synthetic biology tools have been designed for cyanobacteria. Furthermore, many of the tools created for heterotrophs do not function as designed in the photosynthetic organisms. We developed a multi-input and several single-input transcriptional regulators for the model cyanobacterium *Synechocystis* sp. PCC 6803 to address this problem. These circuits were designed to respond to industrially-relevant signals, including oxygen, light and the cells' nitrogen status, in addition to an inexpensive sugar. The two-input AND logic gate we built adds more sophisticated heterologous gene expression to the cyanobacterium's synthetic biology toolbox. The addition of these regulators provides engineers more options when looking for a part that meets the needs of the situation. This was

demonstrated by our use of the oxygen-responsive promoter to express, in a heterologous host, genes from a cluster that encodes nitrogenase. This new device can be used to probe the regulation of nitrogen fixation in a photosynthetic cell. Our development of genetic circuits for transcriptional regulation in *Synechocystis* sp. PCC 6803 improves the viability of this photosynthetic host in biotechnology.

Chapter 1: Introduction

1.1 Background

The production of value-added compounds through microbial biosynthesis offers a sustainable alternative to biomass extraction and chemical synthesis (Immethun et al., 2013). The extraction of natural compounds from biomass is energy intensive and can depend on large quantities of environmentally-unfriendly solvents (Sasidharan et al., 2011; Wang and Weller, 2006). Variation in the composition and concentration of the product can be introduced by the native host, often plants, depending on environmental and climatic conditions (Smirnoff, 1995; Violle et al., 2007). Furthermore, yields from biomass extraction are often low (Chemat et al., 2012; Rates, 2001) and maintaining populations of rare plants to harvest is not maintainable (Manohar, 2012). Chemical synthesis struggles with producing complex compounds, especially when they include multiple centers of chirality (Ajikumar et al., 2010; Engels et al., 2008). Microbes have been used to produce highly-functionalized and specific compounds as a sustainable alternative to these more conventional methods (Cheong et al., 2016; Hong and Nielsen, 2012).

While heterotrophs are commonly used for compound synthesis, key characteristics of phototrophic cyanobacteria make these organisms a promising biotechnology platform. Cyanobacteria can convert carbon dioxide into compounds of interest using energy harvested from sunlight, while the common heterotrophic hosts rely on the addition of organic carbon sources. Aquatic phototrophs, including cyanobacteria, are distinguished from terrestrial plants by their higher solar energy to biomass conversion efficiency (Dismukes et al., 2008). Cyanobacteria have also adapted to a broad range of environmental factors, including imbalances of light, nutrients, metal ions, salts, and temperature (Tandeau de Marsac and Houmard, 1993), which can be used to reduce the risk of contamination (Ducat et al., 2011) and improves their tolerance to fluctuations in production conditions. Many strains of cyanobacteria can be genetically modified by integration into the chromosome or the introduction of plasmids (Berla

et al., 2013), and thus have been used to produce a number of value-added compounds (Angermayr et al., 2015; Oliver and Atsumi, 2014). Despite these aforementioned advantages, product yields and titers are often lower for cyanobacterial hosts than heterotrophic hosts (Chen and Nielsen, 2013; Markley et al., 2014). Synthetic regulation of gene expression can be used to boost production (Chubukov et al., 2016; Lo et al., 2013), but most of the tools have been designed for common heterotrophs, including *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (Immethun et al., 2013; Peralta-Yahya et al., 2012). In addition, many of these genetic devices do not function as designed in cyanobacteria (Camsund et al., 2014).

1.2 Current Status of the Field

To achieve their potential as biotechnology platforms, synthetic biology tools that provide precise, specific and complex regulation need to be designed for cyanobacteria. Regulating transcription can reduce the metabolic burden of production by limiting expression to a defined set of optimal conditions (Bradley et al., 2016). Therefore, I have focused on transcriptional regulation and the tools that are needed to improve cyanobacteria as production hosts. Recently, the clustered regularly interspaced short palindromic repeats interference (CRISPRi) system from *Streptococcus pyogenes* has been used to precisely control transcription of native genes in both *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 (Gordon et al., 2016; Yao et al., 2016). CRISPRi is a powerful tool for modifying a host's metabolism, but is not currently useful for heterologous gene expression in cyanobacteria.

Both of these groups expressed the catalytically-inactive dCas9 using an aTc-inducible promoter that had been engineered in their strain of cyanobacteria (Huang and Lindblad, 2013; Zess et al., 2016). Inducible promoters can be used to sense and respond to only the relevant conditions, providing stringent control of heterologous gene expression. A number of

endogenous metal-inducible promoters have been used to control gene expression in cyanobacteria (Berla et al., 2013). While these transcriptional regulators often produce a large dynamic range of expression, they can have problems with specificity, responding to more than one metal ion. The native light-inducible promoters for *psbA2* (Lindberg et al., 2010) and *cpcG2* (Miyake et al., 2014) have also been used for heterologous gene expression in *Synechocystis* sp. PCC 6830, and the promoter for nitrite reductase has been employed in multiple strains (Desplancq et al., 2005; Omata et al., 1999; Qi et al., 2005). Induction of these promoters required very high intensity light, specifically colored light, or changing the nitrogen source in the media, respectively. None of these options for transcriptional regulation provide specific control of heterologous gene expression in industrially-relevant conditions.

Promoters that respond to chemical signals have recently been engineered for *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002. Anhydrotetracycline (aTc)-inducible promoters use the repressor protein TetR to control transcription. For both of the promoters recently engineered (Huang and Lindblad, 2013; Zess et al., 2016), TetR binds to two operator sites, one directly upstream of the -35 region and one between the -35 and -10 regions, which prevent RNA polymerase from binding to the promoter sequence in the absence of aTc. The addition of aTc causes a conformational change in TetR. This change in conformation prevents the protein regulator from binding to the DNA, allowing transcription (Ramos et al., 2005). Huang and Lindblad started with a TetR-repressible promoter available in the Registry of Standard Biological Parts, BBa_R0040 (http://parts.igem.org/Part:BBa_R0040). They changed the -10 region to *Synechocystis* sp. PCC 6803's consensus sequence and systematically changed the second and third bases immediately downstream of the -10 region. Their best performing promoter produced a 239-fold change between the uninduced and fully induced states, using 10

μg/mL aTc, in light-activated heterotrophically grown cultures (LAHG). Zess et al. used a truncated version of the promoter for *Synechocystis* sp. PCC 6803's phycocyanin beta subunit, CpcB, as the core of their aTc-inducible promoter (Markley et al., 2014), replacing the sequence on either side of the -35 region with the sequence for the Tet operator. Their construct was fully induced with ten times less aTc than the Huang and Lindblad promoter, possibly the result of using a weaker constitutive promoter to express TetR. Their construct produced a 32-fold change between the uninduced and fully induced states. aTc is degraded by light, which is also clearly shown in Huang and Lindblad's work, limiting the uses for this promoter in cyanobacteria to primarily a laboratory tool.

Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoters from *E. coli* have been used with some success in cyanobacteria, including *Synechococcus elongatus* PCC 7942 (Geerts et al., 1995; Niederholtmeyer et al., 2010; Nozzi and Atsumi, 2015) and *Synechocystis* sp. PCC 6803 (Guerrero et al., 2012). Repression of all of these promoters depends on LacI binding to the promoter sequence in the absence of the inducer, IPTG. IPTG causes a conformational change of the repressor molecule, inhibiting its ability to bind to the DNA (Taraban et al., 2008), thus allowing transcription. Many variations of the IPTG-inducible promoter have been created for *E. coli* by varying the number and location of the LacI operator sites. Of the variations tested in cyanobacteria, some are not repressible and some are not inducible (Camsund et al., 2014; Huang et al., 2010). To overcome these problems, IPTG-inducible promoters have been developed for *Synechococcus* sp. PCC 7002 (Markley et al., 2014) and *Synechocystis* sp. PCC 6803 (Albers et al., 2015). Markley et al. truncated the promoter for *Synechocystis* sp. PCC 6803's phycocyanin beta subunit, CpcB, to use as the core of their construct. They added IPTG-inducibility by introducing two different LacI operator sites, Oid and O1, one 42 bases upstream

of the -35 region and the other directly downstream of the -10 region. Their final construct achieved a 48-fold dynamic range, after also changing the promoter for the repressor protein, making a single amino acid substitution in LacI and mutating the core promoter sequence. Albers et al. started with a common IPTG-inducible *Ptac*. They first varied the number of nucleotides between the -35 and -10 regions from 16 to 18, motivated by the structural differences between *E. coli*'s and cyanobacteria's RNA polymerase. The construct that used 18 nucleotides between the two regions produced the highest level of expression. They next placed the O1 LacI operator site between the -10 region and the transcription start site and the Oid LacI operator site 72 bases upstream, which introduced responsiveness to IPTG with a dynamic range of 78-fold. All of the engineered regulators respond to a single input, enabling basic gene expression. The ability to employ more sophisticated synthetic regulation in cyanobacteria depends on developing additional parts, including those that respond to industrially-relevant conditions, from which complex devices can be built.

1.3 Contributions to the Field

My work concentrated on the development of single and multi-input transcriptional regulators for the model cyanobacterium, *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). Most of this work was designed to support the NSF NITROGEN project. The project focuses understanding the principles of nitrogen-fixation in a photosynthetic cell by transferring the nitrogen-fixing enzyme nitrogenase to the non-diazotrophic *Synechocystis*. The sensors for oxygen, nitrogen, and light, as well as the two-input AND logic gate, were developed with the NITROGEN project in mind. Replacing a native regulatory region in the gene cluster for nitrogenase with synthetic regulation, to begin to unravel complex control mechanisms, was also in support of this grant. While working on the two-input AND gate, it became clear that the

choice of available parts was insufficient for creating more than a few complex circuits with robust performance, especially for phototrophic conditions. This led to characterizing *E. coli*'s *PBAD* and building a family of arabinose-responsive promoters in *Synechocystis*.

While many of the regulators were developed for a specific project, they have multiple applications. The oxygen, nitrogen and light sensors are all well-suited for biological hydrogen formation, a potential source of clean energy (Hallenbeck et al., 2012). The light sensors can reduce the competition for cellular resources between the cells' metabolism and heterologous pathway expression by not allowing that expression to occur in the light, when many cyanobacteria carry out the majority of their metabolic processes (Beck et al., 2012). The arabinose sensors are tightly off when not induced, a sought after property of recombinant protein expression systems that limits toxicity to the host (Rosano and Ceccarelli, 2014; Saida et al., 2006). The status of nitrogen assimilation, as detected by the nitrogen sensors, could be an important signal for the production of any number of proteins. Native systems often control gene expression in response to more than one signal (Mitrophanov and Groisman, 2008); the two-input AND logic gate could provide synthetic systems with similar control. My work, enabled by all of the many contributors (see the Acknowledgements), provides a number of new tools for regulating transcription in *Synechocystis*, helping it achieve its potential as a biotechnology platform.

1.4 Structure of the Dissertation

Chapter two covers the development of oxygen-responsive genetic circuits in *Synechocystis*. This includes a single-input inducible promoter and its use to build a two-input AND logic gate. The effect of genetic context on the performance of the circuit was also explored. Chapter two was published in *Biotechnology and Bioengineering* 2016; **113**: 433–442.

Chapter three focuses on the building of physical, chemical and metabolic state sensors to control transcription in *Synechocystis.* This suite of sensors includes light sensors, a family of arabinose sensors and a library of nitrate sensors. Chapter three has been submitted to *Biotechnology and Bioengineering*.

Chapter four covers the expression of a synthetically-regulated *nif* cluster in the non-diazotrophic *Synechocystis* sp. PCC 6803. The single-input oxygen-responsive promoter developed in chapter two replaced what is believed to be a native regulatory region in *Cyanothece* sp. ATCC 51142's *nif* cluster. The work in this chapter is not published.

Chapter five discusses the contributions of the work to the field and suggested future work. This includes suggestions for the completion of the work in chapter four as well as recommendations for reducing *Synechocystis*' intracellular oxygen, to provide an environment more suitable for nitrogenase. I also suggest testing protocol changes for a nitrogen and oxygen-responsive two-input AND logic gate.

1.5 References

Ajikumar PK, Xiao WH, Tyo KEJ, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G. 2010. Isoprenoid pathway optimization for taxol precursor overproduction in Escherichia coli. Science 330.

Albers SC, Gallegos VA, Peebles CA. 2015. Engineering of genetic control tools in Synechocystis sp. PCC 6803 using rational design techniques. J Biotechnol 216:36-46.

Angermayr SA, Gorchs Rovira A, Hellingwerf KJ. 2015. Metabolic engineering of cyanobacteria for the synthesis of commodity products. Trends in Biotechnology 33(6):352-361.

Beck C, Knoop H, Axmann IM, Steuer R. 2012. The diversity of cyanobacterial metabolism: genome analysis of multiple phototrophic microorganisms. BMC Genomics 13(1):1-17.

Berla BM, Saha R, Immethun CM, Maranas CD, Moon TS, Pakrasi HB. 2013. Synthetic biology of cyanobacteria: unique challenges and opportunities. Front Microbiol 4:246.

Bradley RW, Buck M, Wang B. 2016. Tools and Principles for Microbial Gene Circuit Engineering. Journal of Molecular Biology 428(5, Part B):862-888.

Camsund D, Heidorn T, Lindblad P. 2014. Design and analysis of LacI-repressed promoters and DNA-looping in a cyanobacterium. J Biol Eng 8(1):4.

Chemat F, Vian MA, Cravotto G. 2012. Green Extraction of Natural Products: Concept and Principles. International Journal of Molecular Sciences 13(7):8615-8627.

Chen Y, Nielsen J. 2013. Advances in metabolic pathway and strain engineering paving the way for sustainable production of chemical building blocks. Current Opinion in Biotechnology 24(6):965-972.

Cheong S, Clomburg JM, Gonzalez R. 2016. Energy- and carbon-efficient synthesis of functionalized small molecules in bacteria using non-decarboxylative Claisen condensation reactions. Nat Biotechnol 34(5):556-61.

Chubukov V, Mukhopadhyay A, Petzold CJ, Keasling JD, Martín HG. 2016. Synthetic and systems biology for microbial production of commodity chemicals. Npj Systems Biology And Applications 2:16009.

Desplancq D, Bernard C, Sibler AP, Kieffer B, Miguet L, Potier N, Van Dorsselaer A, Weiss E. 2005. Combining inducible protein overexpression with NMR-grade triple isotope labeling in the cyanobacterium Anabaena sp. PCC 7120. Biotechniques 39(3):405-11.

Dismukes GC, Carrieri D, Bennette N, Ananyev GM, Posewitz MC. 2008. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Curr Opin Biotechnol 19(3):235-40.

Ducat DC, Way JC, Silver PA. 2011. Engineering cyanobacteria to generate high-value products. Trends Biotechnol 29(2):95-103.

Engels B, Dahm P, Jennewein S. 2008. Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metab Eng 10(3-4):201-6.

Geerts D, Bovy A, de Vrieze G, Borrias M, Weisbeek P. 1995. Inducible expression of heterologous genes targeted to a chromosomal platform in the cyanobacterium Synechococcus sp. PCC 7942. Microbiology 141 (Pt 4):831-41.

Gordon GC, Korosh TC, Cameron JC, Markley AL, Begemann MB, Pfleger BF. 2016. CRISPR interference as a titratable, trans-acting regulatory tool for metabolic engineering in the cyanobacterium Synechococcus sp. strain PCC 7002. Metabolic Engineering 38:170-179.

Guerrero F, Carbonell V, Cossu M, Correddu D, Jones PR. 2012. Ethylene synthesis and regulated expression of recombinant protein in Synechocystis sp. PCC 6803. PLoS One 7(11):e50470.

Hallenbeck PC, Abo-Hashesh M, Ghosh D. 2012. Strategies for improving biological hydrogen production. Bioresource Technology 110:1-9.

Hong KK, Nielsen J. 2012. Metabolic engineering of Saccharomyces cerevisiae: a key cell factory platform for future biorefineries. Cell Mol Life Sci 69(16):2671-90.

Huang HH, Camsund D, Lindblad P, Heidorn T. 2010. Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. Nucleic Acids Res 38(8):2577-93.

Huang HH, Lindblad P. 2013. Wide-dynamic-range promoters engineered for cyanobacteria. J Biol Eng 7(1):10.

Immethun CM, Hoynes-O'Connor AG, Balassy A, MOON TS. 2013. Microbial production of isoprenoids enabled by synthetic biology. Frontiers in Microbiology 4.

Lindberg P, Park S, Melis A. 2010. Engineering a platform for photosynthetic isoprene production in cyanobacteria, using Synechocystis as the model organism. Metab Eng 12.

Lo T-M, Teo WS, Ling H, Chen B, Kang A, Chang MW. 2013. Microbial engineering strategies to improve cell viability for biochemical production. Biotechnology Advances 31(6):903-914.

Manohar PR. 2012. Sustainable harvesting of medicinal plants: Some thoughts in search for solutions. Ancient Science of Life 32(1):1-2.

Markley AL, Begemann MB, Clarke RE, Gordon GC, Pfleger BF. 2014. Synthetic Biology Toolbox for Controlling Gene Expression in the Cyanobacterium Synechococcus sp. strain PCC 7002. ACS Synth Biol.

Mitrophanov AY, Groisman EA. 2008. Signal integration in bacterial two-component regulatory systems. Genes Dev 22(19):2601-11.

Miyake K, Abe K, Ferri S, Nakajima M, Nakamura M, Yoshida W, Kojima K, Ikebukuro K, Sode K. 2014. A green-light inducible lytic system for cyanobacterial cells. Biotechnology for Biofuels 7(1):1-8.

Niederholtmeyer H, Wolfstädter BT, Savage DF, Silver PA, Way JC. 2010. Engineering cyanobacteria to synthesize and export hydrophilic products. Appl Environ Microbiol 76.

Nozzi NE, Atsumi S. 2015. Genome Engineering of the 2,3-Butanediol Biosynthetic Pathway for Tight Regulation in Cyanobacteria. ACS Synth Biol 4(11):1197-204.

Oliver JWK, Atsumi S. 2014. Metabolic design for cyanobacterial chemical synthesis. Photosynthesis Research 120(3):249-261.

Omata T, Price GD, Badger MR, Okamura M, Gohta S, Ogawa T. 1999. Identification of an ATP-binding cassette transporter involved in bicarbonate uptake in the cyanobacterium Synechococcus sp. strain PCC 7942. Proc Natl Acad Sci U S A 96(23):13571-6.

Peralta-Yahya PP, Zhang F, Del Cardayre SB, Keasling JD. 2012. Microbial engineering for the production of advanced biofuels. Nature 488(7411):320-328.

Qi Q, Hao M, Ng WO, Slater SC, Baszis SR, Weiss JD, Valentin HE. 2005. Application of the Synechococcus nirA promoter to establish an inducible expression system for engineering the Synechocystis tocopherol pathway. Appl Environ Microbiol 71(10):5678-84.

Ramos JL, Martínez-Bueno M, Molina-Henares AJ, Terán W, Watanabe K, Zhang X, Gallegos MT, Brennan R, Tobes R. 2005. The TetR Family of Transcriptional Repressors. Microbiology and Molecular Biology Reviews 69(2):326-356.

Rates SMK. 2001. Plants as source of drugs. Toxicon 39(5):603-613.

Rosano GL, Ceccarelli EA. 2014. Recombinant protein expression in Escherichia coli: advances and challenges. Frontiers in Microbiology 5:172.

Saida F, Uzan M, Odaert B, Bontems F. 2006. Expression of highly toxic genes in E. coli: special strategies and genetic tools. Curr Protein Pept Sci 7(1):47-56.

Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga Latha L. 2011. Extraction, Isolation and Characterization of Bioactive Compounds from Plants' Extracts. African Journal of Traditional, Complementary, and Alternative Medicines 8(1):1-10.

Smirnoff N. 1995. Metabolic flexibility in relation to the environment. Environment and plant metabolism: flexibility and acclimation. Oxford: Bios Scientific publishers:1-13.

Tandeau de Marsac N, Houmard J. 1993. Adaptation of cyanobacteria to environmental stimuli: new steps towards molecular mechanisms. FEMS Microbiology Letters 104(1):119-189.

Taraban M, Zhan H, Whitten AE, Langley DB, Matthews KS, Swint-Kruse L, Trewhella J. 2008. Ligand-Induced Conformational Changes and Conformational Dynamics in the Solution Structure of the Lactose Repressor Protein. Journal of molecular biology 376(2):466-481.

Violle C, Navas M-L, Vile D, Kazakou E, Fortunel C, Hummel I, Garnier E. 2007. Let the concept of trait be functional! Oikos 116(5):882-892.

Wang L, Weller CL. 2006. Recent advances in extraction of nutraceuticals from plants. Trends in Food Science & Technology 17(6):300-312.

Yao L, Cengic I, Anfelt J, Hudson EP. 2016. Multiple Gene Repression in Cyanobacteria Using CRISPRi. ACS Synth Biol 5(3):207-12.

Zess EK, Begemann MB, Pfleger BF. 2016. Construction of new synthetic biology tools for the control of gene expression in the cyanobacterium Synechococcus sp. strain PCC 7002. Biotechnology and Bioengineering 113(2):424-432.

Chapter 2: Oxygen-Responsive Genetic Circuits Constructed in *Synechocystis* sp. PCC 6803

This chapter was published in *Biotechnology and Bioengineering* 2016;**113**: 433–442.

Abstract

As photoautotrophic prokaryotes, cyanobacteria are promising platforms for producing value-added bioproducts. However, few regulatory genetic parts and devices (e.g., inducible promoters and regulatory circuits) have been developed for these potential hosts. Furthermore, the devices that have been created respond only to a single input. To address these issues, we developed an inducible genetic circuit that generates heterologous proteins in response to oxygen, an environmental signal. To test its performance and utility in *Synechocystis* sp. PCC 6803, a model cyanobacterial strain, we connected this circuit to either heterologous *nifHDK* genes, which encode oxygen-sensitive nitrogenase's structural proteins, or a fluorescent protein gene. The circuit was transcriptionally activated to generate *nifHDK* transcripts or fluorescent output only in low oxygen conditions. We expanded the oxygen-responsive circuit into a more complex circuit by building a two-input AND gate, which allows *Synechocystis* to specifically control expression of the fluorescent reporter in response to two signals, low oxygen and high anhydrotetracycline. To our knowledge, the AND gate is the first complex logic circuit built in a cyanobacterial strain. This work expands the synthetic biology tools available for complex gene expression in cyanobacteria, increasing their potential as biotechnology platforms.

2.1 Introduction

As interest in biochemical production grows, cyanobacteria have emerged as promising candidates for the sustainable synthesis of fuels and commodity chemicals. Unlike organisms commonly used in industrial biotechnology, cyanobacteria perform oxygenic photosynthesis, harnessing sunlight and carbon dioxide to power their cellular metabolism. While eukaryotes, such as plants and algae, have traditionally been utilized for their photosynthetic capacity, prokaryotic cyanobacteria possess a number of key advantages, including a higher solar energy-to-biomass conversion efficiency and ease of genetic manipulation (Berla et al., 2013; Ducat et al., 2011; Gimpel et al., 2013). Cyanobacteria, including the model cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter called *Synechocystis*), have been engineered to produce valuable products, including fatty acids, alcohols, hydrocarbons, and biodegradable plastic monomers (Gronenberg et al., 2013; Oliver and Atsumi, 2014; Varman et al., 2013b).

Despite cyanobacteria's potential in biotechnology, it is less well explored than commonly-used heterotrophic organisms such as *Saccharomyces cerevisiae* and *Escherichia coli*. Engineering a microbial host for chemical production involves fine-tuning the natural or heterologous pathway through the use of genetic tools, enabled by knowledge of the host's genome, metabolic pathways, and regulatory networks. Scalable and stringent control of gene expression is crucial to ensure compatibility with the host's metabolism, as imbalances can lead to suboptimal production. Chemical synthesis through heterologous pathways in cyanobacteria has indeed resulted in titers below what has been achieved in hosts such as *E. coli* (Oliver and Atsumi, 2014). While comprehensive genomic information is available for many cyanobacterial strains, including *Synechocystis*, the tools for precise control of heterologous genes in these organisms are still limited (Camsund et al., 2014; Heidorn et al., 2011b; Markley et al., 2014).

In contrast to the wealth of options available for engineering *E. coli*, few promoters, degradation tags, and fluorescent reporters function as required in cyanobacteria (Huang et al., 2010; Markley et al., 2014). Promoters that control transcription in response to specific inputs can provide defined, tunable gene expression. The *lac* family of inducible promoters from *E. coli* is widely used in cyanobacterial applications; however, their performance is suboptimal (Camsund et al., 2014; Guerrero et al., 2012; Oliver et al., 2013; Varman et al., 2013a). The structural sub-units of RNA polymerase differ between *E. coli* and cyanobacteria (Imashimizu et al., 2011; Schyns et al., 1998), which may cause many *E. coli* promoters to function poorly in cyanobacteria (Camsund et al., 2014; Heidorn et al., 2011b). The inducible pTet (Huang and Lindblad, 2013) promoter has been engineered for use in *Synechocystis* sp. PCC 6803, as well as the inducible pTrc (Markley et al., 2014) for use in *Synechococcus* sp. PCC 7002; however, more inducible promoters need to be developed, including ones that respond to environmental signals.

Living systems employ sense-and-respond cycles in order to adapt to their fluctuating environment. For example, *Cyanothece* sp. ATCC 51142's nitrogenase, which permits nitrogen fixation in the photosynthetic host, is oxygen sensitive (Welsh et al., 2008). *Cyanothece* sp. ATCC 51142 (hereafter called *Cyanothece*) regulates transcription of nitrogenase genes with its circadian rhythm to protect the enzyme from oxygen generated during photosynthesis (i.e., transcriptional activation of nitrogenase genes only at night when intracellular oxygen levels are low) (Stockel et al., 2008). Additionally, *Cyanothece* activates transcription of nitrogenase genes only in nitrogen-fixing conditions (i.e., in the absence of fixed nitrogen) (Bandyopadhyay et al., 2010). Since this *Cyanothece* strain is difficult to genetically manipulate (Aryal et al., 2013), understanding the sophisticated transcriptional regulation in the native host is a challenging task. By expressing *Cyanothece*'s nitrogenase in the closely-related, non-diazotrophic *Synechocystis*

strain, we could determine the cellular requirements for nitrogen fixation. Yet, sophisticated regulation is often missing when genes are expressed in heterologous hosts. Synthetic, multi-input control of gene expression (e.g., in response to oxygen and fixed nitrogen) could allow engineered cells to mimic native sensing-responding behaviors.

In this work, we developed an oxygen-responsive genetic circuit in *Synechocystis* by introducing *E. coli*'s fumarate and nitrate reduction (FNR) system (Kang et al., 2005) into the photosynthetic host. Using a fluorescent protein gene (*fbfp*) or the genes encoding the structural proteins of *Cyanothece*'s nitrogenase (*nifHDK*), we demonstrate that the oxygen sensor is activated only in low oxygen. Additionally, by integrating the oxygen sensor into a two-input AND logic gate, we show the implementation of a complex, synthetic genetic circuit in a cyanobacterial strain for the first time. This programmed genetic circuit couples heterologous gene expression to multiple inputs, providing specific control. Furthermore, we explore the effect of genetic context on the circuit's performance. This work expands the available tools for engineering *Synechocystis*, facilitating more sophisticated cyanobacterial biotechnology.

2.2 Materials and Methods

2.2.1 Strains, Plasmids, and Growth Conditions

All plasmids were constructed in *E. coli* DH10B which was grown in LB (Miller, AMRESCO) at 37°C and 250 rpm in 14 mL BD Falcon™ round-bottom tubes. Kanamycin (kan) (20 μg/mL) and spectinomycin (spec) (100 μg/mL) were added as appropriate. Plasmids were constructed using the Golden Gate assembly method (Engler et al., 2008) or blunt-end ligation-based cloning, and were sequence-verified at the Protein and Nucleic Acid Chemistry Laboratory, Washington University in St. Louis, School of Medicine, or at GENEWIZ, Inc. The plasmids are summarized in Table 2.1, and the gene sequences are listed in Table 2.2. Enzymes were

purchased from New England BioLabs, Inc. We obtained *fnr* (Gene ID: 945908; http://www.ncbi.nlm.nih.gov/gene) from the genomic DNA of *E. coli* MG1655 and obtained *nifHDK* (Gene IDs: 6167590, 6167591, and 6167592, respectively; http://www.ncbi.nlm.nih.gov/gene) from the genomic DNA of *Cyanothece* sp. ATCC 51142.

Table 2.1 Plasmids used in this work.

Name	Parts	Type
pCI053	f1/pBR322 ori; kan^R; *Bba J23100-tetR*; *pTet-fbfp f37t*	Integrative (*psbA1*)
pCI061	f1/pBR322 ori; kan^R; *Bba J23104-fbfp f37t*	Integrative (*psbA1*)
pCI063	f1/pBR322 ori; kan^R; FNR-*activated promoter-sicA**; *Bba J23104-fnr*; *pTet-invF* (original RBS); *Bba J23100-tetR*	Integrative (*psbA1*)
pCI064	f1/pBR322 ori; kan^R; *Bba J23100-fbfp f37t*	Integrative (*psbA1*)
pCI065	f1/pBR322 ori; kan^R; *psicA-fbfp f37t*	Integrative (*psbA1*)
pCI066	RSF1010 replicon; $spec^R$; *psicA-fbfp f37t*	Replicative
pCI067	RSF1010 replicon; $spec^R$; FNR-*activated promoter-sicA**; *Bba J23104-fnr*; *pTet-invF* (original RBS); *Bba J23100-tetR*	Replicative
pCI068	f1/pBR322 ori; kan^R; FNR-*activated promoter-nifHDK*; *Bba J23104-fnr*	Integrative (*psbA1*)
pCI069	RSF1010 replicon; $spec^R$; FNR-*activated promoter-sicA**; *Bba J23104-fnr*; *pTet-invF* (weakened RBS); *Bba J23100-tetR*	Replicative
pCI072	f1/pBR322 ori; $spec^R$; FNR-*activated promoter-sicA**; *Bba J23104-fnr*; *pTet-invF* (original RBS); *Bba J23100-tetR*	Integrative (NSC1)
pCI076	M13/pBR322 ori; $spec^R$; *psicA-fbfp f37t*	Integrative (NSP1)
pCI077	f1/pBR322 ori; kan^R; *Bba J23100-eyfp*	Integrative (*psbA1*)
pCI078	f1/pBR322 ori; kan^R; *Bba J23104-eyfp*	Integrative (*psbA1*)
pCI079	RSF1010 replicon; $spec^R$; FNR-*activated promoter-sicA**; *Bba J23104-fnr*; *pTet-invF* (weakened RBS); *Bba J23100-tetR*; *psicA-fbfp f37t*	Replicative
pCI080	M13/pBR322 ori; $spec^R$; FNR-*activated promoter-sicA**; *Bba J23104-fnr*; *pTet-invF* (weakened RBS); *Bba J23100-tetR*	Integrative (NSP1)
pCI081	M13/pBR322 ori; $spec^R$; FNR-*activated promoter-sicA**; *Bba J23104-fnr*; *pTet-invF* (weakened RBS); *Bba J23100-tetR*; *psicA-fbfp f37t*	Integrative (NSP1)
pCI083	RSF1010 replicon; kan^R; FNR-*activated promoter-sicA**; *Bba J23104-fnr*; *pTet-invF* (weakened RBS); *Bba J23100-tetR*	Replicative
pCI084	M13/pBR322 ori; kan^R; FNR-*activated promoter-sicA**; *Bba J23104-fnr*; *pTet-invF* (weakened RBS); *Bba J23100-tetR*	Integrative (NSP1)

pCI088	f1/pBR322 ori; kan^R; *pTrc10-fbfp f37t*	Integrative (*psbA1*)
pKN003	f1/pBR322 ori; kan^R; FNR-*activated promoter-fbfp f37t*; *Bba J23104-fnr*	Integrative (*psbA1*)
pKN004	f1/pBR322 ori; kan^R; FNR-*activated promoter-fbfp f37t*; *pTet-fnr*; *Bba J23100-tetR*	Integrative (*psbA1*)

Table 2.2 List of genetic parts used in this work.

Part name	Type and source	DNA sequence
fnr	Gene *E. coli* MG1655	atgatcccggaaaagcgaattatacggcgcattcagtctggcggttgtgctatccattgccaggattgcagcatcagccag ctttgcatcccgttcacactcaacgaacatgagcttgatcagcttgataatatcattgagcggaagaagcctattcagaaagg ccagacgctgtttaaggctggtgatgaacttaaatcgctttatgccatccgctccggtacgattaaaagttataccatcactga gcaaggcgacgagcaaatcactggtttccatttagcaggcgacctggtgggatttgacgccatcggcagcggccatcacc cgagcttcgcgcaggcgctggaaacctcgatggtatgtgaaatcccgttcgaaacgctggacgatttgtccggtaaaatgc cgaatctgcgtcagcagatgatgcgtctgatgagcggtgaaatcaaaggcgatcaggacatgatcctgctgttgtcgaaga aaaatgccgaggaacgtctggctgcattcatctacaacctgtcccgtcgttttgcccaacgcggcttctcccctcgtgaattc cgcctgacgatgactcgtggcgatatcggtaactatctgggcctgacggtagaaaccatcagccgtctgctgggtcgcttc cagaaaagcggcatgctggcagtcaaaggtaaatacatcaccatcgaaaataacgatgcgctggcccagcttgctggtca tacgcgtaacgttgcctga	
	5’ UTR	cttctctgctgttaaggtttgcttagacttacttgctccctaaaaagatgttaaaattgacaaatatcaattacggcttgagcaga cct
FNR-*activated promoter*	Promoter (Grainger et al., 2007)	ggaattcgcggccgcttctagagtttgatttacatcaattacggctagctcagtcctaggtattatgctagctactagaga
fbfp f37t	Gene (Mukherjee et al., 2012)	atgagaggatcgcatcaccatcaccatcacggatccatgatcaacgcaaaactcctgcaactgatggtcgaacattccaac gatggcatcgttgtcgccgagcaggaaggcaatgagagcatccttatctacgtcaacccggccactgagcgcctgaccg gctactgcgccgacgatattctctatcaggacgcacgttttcttcagggcgaggatcacgaccagccgggcatcgcaatta tccgcgaggcgatccgcgaaggccgcccctgctgccaggtgctgcgcaactaccgcaaagacggcagcctgttctgga acgagttgtccatcacaccggtgcacaacgaggcggaccagctgacctactacatcggcatccagcgcgatgtcacagc gcaagtattcgccgaggaaagggttcgcgagctggaggctgaagtggcggaactgcgccggcagcagggccaggcc aagcactga	
	5’ UTR	atgagaattcacagaattcattaaagaggagaaattaact
eyfp	Gene (Landry et al., 2013)	atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccac aagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggc aagctgcccgtgccctggcccaccctcgtgaccaccttcggctacggcctgcaatgcttcgcccgctaccccgaccacat gaagctgcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggca actacaagacccgcgccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttca aggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgtctatatcatggccgacaagc agaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccgaccactacc agcagaacaccccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagctaccagtccgccctgagca aagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacg agctgtacaagtaa	
	5’ UTR	tcacactcaagcggataacaatttcacacatactagagaaagaggagaaatactag
pTet	Promoter (Moon et al., 2012)	ttttcagcaggacgcactgacctccctatcagtgatagagattgacatccctatcagtgatagagatactgagcacatct

tetR	Gene (Moon et al., 2012)	atgtccagattagataaaagtaaagtgattaacagcgcattagagctgcttaatgaggtcggaatcgaaggtttaacaacccgtaaactcgcccagaagctaggtgtagagcagcctacattgtattggcatgtaaaaaataagcgggctttgctcgacgccttagccattgagatgttagataggcaccatactcacttttgcccttagaaggggaaagctggcaagatttttacgtaataacgctaaaagttttagatgtgctttactaagtcatcgcgatggagcaaaagtacatttaggtacacggcctacagaaaaacagtatgaaactctcgaaaatcaattagccttttatgccaacaaggtttttcactagagaatgcattatatgcactcagcgctgtggggcattttacttaggttgcgtattggaagatcaagagcatcaagtcgctaaagaagaaagggaaacacctactactgatagtatgccgccattattacgacaagctatcgaattatttgatcaccaaggtgcagagccagccttcttattcggccttgaattgatcatatgcggattagaaaaacaacttaaatgtgaaagtgggtcctaa
	5’ UTR	tcacacaggaaaggcctcg
nifH	Gene (Bandyopadhyay et al., 2011)	atgggacgcaactcccaaggtttcctaaccacaatacgaatcatcgttacgtcagcaacggatcaaccttccaactttatacactcactgtctaacaagcgagaatcaactatgcgtcagattgcattttacggaaaaggcggtatcggtaagtctaccacttctcagaataccattgctgcgttagctgaaaccaaccgcatcatgattgttggttgtgaccctaaagctgattctacccgcttaatgcttcacaccaaagcacaaaccaccattctgcacttagcagcagaacggggaaccgttgaagacatcgaactcgaagaagtattactcgaaggataccaaggagtcaagtgtgttgagtccggtggtcctgagcctggagttggatgtgcgggtcgtggtattatcaccgccattaacttcttagaagaagaaggtgcttacgaagacctagacttcgtatcctacgacgtattaggagacgttgtatgtggtggtttcgctatgcctatccgtgaaggaaaagcacaagaaatctacatcgtaacctccggggaaatgatggcgatgtacgctgcaaacaacattgctcgtggtattttaaaatacgctcacactggtggtgttcgtttaggtggtttaatttgtaacagccgtaacgttaactgtgaagctgagttaatcgaagaattagctcgtcgtctcggaacccaaatgattcacttcgtaccccgttctaagcaggtacaagaagctgaattacgtcgtatgactgttatcgaatattctcctgatcaccctcaggctcaggaataccgtgagttatctcgcaaaatcgagaataacaccaacctcgttattcctactcctatcaccatggaagaactcgaagaactcttagttgacttcggtattctcggtggtgaagacgagtatgagaaagctcttcaagctgataaagctgctaccaaagcttag
	5’ UTR	ctagctcggaagacatctactgacgtagcgttcgagggttcta
nifD	Gene (Bandyopadhyay et al., 2011)	atggcaacagttgaagacaataagaagctcattgccgacgttctgtcgacttatcccgaaaaagctgctaaaaaacgcgcaaaacacttaggtgtttacgaagaaggcgaagctgattgtggcgtaaaatccaataaacaatccttacctggggttatgaccgctcgtggttgtgcctatgcaggatcaaaaggggttgtttggggtccgattaaggatatggttcacatctcccacggtcctgtggggttgtggttactactcctggtctggtcgtcgtaactactacatcggaaccacaggggttgactccttcggtaccatgcagtttacctccgatttccaagaacgggacatcgtttttggtggagacaaaaaactcgctaaaatcattgatgaaatcgaagaattattcccctaaacggtggtgtttctgttcagtcagaatgtcccgttggtttaattggagatgacatcgaatccgttgctcgtaccaagagcaaagaaaccggcaaatccgttgttcctgttcgttgtgaagggttccgtggggtttctcagtccttaggacaccacatcgctaacgacatgattcgtgactgggttttccccactgcggataaagaaaacgcagaaaaaggatttgaaggcactccttatgacgtagccatcattggtgactacaacatcggtggagatgcttggtctagccgtatcttactcgaagaaatcggtcttcgtgtggtagcacaatggtctggtgatggtaccttaaccgagatgaaggcaacccccaacgttaagttgaacctcatccactgttatcgttccatgaactacatcagtcgtcacatggaagaaaaatatggtattccctggttagaatacaacttcttcggtcctagcaagattgctgcttctttaagagaaatcgcttcacgcttcgatgaaaaaatccaagccaaagctgaagaagtcatcgagaagtacagaaagcaatctgaagagatcattgctaagtaccgtcctcgtttagaaggcaaaactgtaatgatgatggtgggtggtttacgtcccccgtcacgttgttcctgctttcaaagacttaggcatggaaatcatcggtaccggatacgaattcgctcacggtgacgactacaaacggaccactggctacgttgaagatgctaccctcatctatgatgacgtaactggttatgagtttgaagagttcgttaaagaacttaagcctgacttagtagctgccggtattaaagagaagtatgtcttccaaaagatggctcttcccttccgtcaaatgcactcttgggattactccggaccttaccacggttatgatggcttcgctatcttcgcccgtgacatggacttagccctcaatagtcccacctggggattaattggcacccccttggaataaataa
	5’ UTR	actgaatctcgcttagtaggattgaggatcattctaaggcaaaagtgaataggaaatagtgacaaactatcaactatcaacttttaaactccttttgccttcccctctttcccttttctctaatttttcttctacaccaactttgagaggaacacc

nifK	Gene (Bandyopadhyay et al., 2011)	atggctcaaaacgtaaacaatattaaggatcacgttgatctattccatcagcctgaataccaagaactgtttgagaacaagaa acagtttgaaggtatgcccactgctgagaaagttcaagaagtagctgaatggaccaaaagctgggaatatcgggaaaaga actttgctcgtgaagcgttaaccgttaaccctgctaaagcttgtcaacctttaggtgcattattagctgctattggctttgaagg aaccttacccttcgttcacggttctcaaggttgtgtggcttacttccgtacccacttaacccgtcacttcaaagagcctgtaagt gctgtttcttcttccatgactgaaaacgctgcggttttcggtggattaaagaacatggttgatggtctacaaaactcctatgctc tttacaagcccaaaatgatcgctgtctgtacaacttgtatggctgaggttattggggatgacttaggtgctttcttaggaaatgc tcgtcaagacggcgttattcctgacgatttacctgttcctttcgctcatacacctagttttgtgggttctcacatcactggttatga cagcatgatgaaatcgattctttctaccttaactgaaggtaagaagaaagaaaccaccaacggtaaaatcaacttcatcgct ggatttgaaacctacatcggtaacgtccgtgcgataaaaaatatcatctctgcgtttgatttagaaggtactcttttaagcgaca ccgaaatgtacttagattctcccaacttaggggaattcaaaatgtaccatgaaggaacttccttagaagatgcagctgattcta tcaacgctgaagcgaccgttacccttcaaacctacaccactcctaaaactcgtgaatacatcgagaagaagtggggacaa aaaacctacacctatcgtccttggggtgttaaaggtactgatgagttcttaatgggcttatctgagttaactggcaaaccgatt cctaaagagtttgaaattgctcgtggacgtgctgttgacgcaatgaccgatactcaagcttgggtacatggtaagcgtgctg cagtttacggtgatcctgatctcgtaatgggcttattacagttcatgttagaaatgggtatcgagcctgttcacgtcttggttaac aacagcaccaaggaatttgaagaagaagctaaagctctcttagctgctagtccctacggacaacaagctaccgtttggggt ggtaaagacttatggcatatgcgctctttactcttcactgagcctgttgacttcttagttggtaattcctacgctaagtatttacag cgtgataccaagactcctttaatccgtattggatatcctatctttgatcgtcaccacttacaccgttactctaccatcggttatga aggggctatcaacctactcaactggatcgccaatggactaatggatgaattagatcgcaagacagatactccttctgtaacg gatatttcttttgacttagttcgttaa
	5’ UTR	gagggaggatgatcatggggaaccagggaagtcagaagtcagaagtcagaagtcagaagtattaaagtcataatattctc tacctttcttttcgacttactctctccaaccttccccctctccctctcctgtccaagtttgtttgtctgagatatccatcctatactgg agttaccaccaatggctcaaaacgtaaacaatattaag
*sicA**	Gene (Moon et al., 2012)	atggattatcaaaataatgtcagcgaagaacgtgttgcggaaatgatttgggatgccgttagtgaaggcgccacgctaaaa gacgttcatgggatccctcaagatatgatggacggtttatatgctcatgcttatgagttttataaccagggacgactggatgaa gctgagacgttctttcgttacttatgcatttatgatttttacaatcccgattacaccatgggactggcggcagtatgccaactga aaaaacaatttcagaaagcatgtgaccttatgcagtagcgtttacgttacttaaaaatgattatcgccccgttttttttaccggg cagtgtcaattattaatgcgtaaggcagcaaaagccagacagtgttttgaacttgtcaatgaacgtactgaagatgagtctct gcgggcaaaagcgttggtctatctggaggcgctaaaaacggcggagacagagcagcacagtgaacaagaaaaggaat aa
	5’ UTR	ctaggagctcggtacctcacacaggaaaggcctcg
invF	Gene (Moon et al., 2012)	atgctaaatacgcaggaagtacttaaagaaggagagaagcggaaaatccgcagcccggaagcatggtttatacagacgt gttccgcgcaaaagctgcatatgtcattttctgaaagccgacacaatgaaaattgcctgattcaggaaggcgcgctgctttttt gcgagcaggccgttgtcgcaccagtatcaggagacctggtttttcgaccgttaaaaattgaagtactcagcaaattactggc atttatcgatggcgcaggattagtggacacgacatatgctgaatccgataaatgggttttgctgagtcctgagtttcgcgctat ttggcaagatcgtaaacgctgcgagtactggtttttgcagcaaattattacgccttctccggccttcaataaggtactggcgct gttacgaaaaagcgagagttactggttggttggctatttactcgctcagtcaaccagcggcaacacgatgagaatgctggg agaagactatggcgtttcttatacccatttttcgtcgtttgtgcagcagagcgttgggcggaaaagcgaagagtgaattacga aactggcgtatggcgcaatcgctgctgaatagtgtagaaggccacgagaacatcacccaattagccgttaatcatggttact catcgccttcacatttttctagtgagatcaaagagctgatcggcgtttcgccgcggaaattatcaaatattattcaattggcaga caaatga
	original 5’ UTR (Moon et al., 2012)	atctcgagctcatcccgtcagcgttcctatggatggtacaact
	100 TIR 5’ UTR (Espah Borujeni et al., 2014; Salis et al., 2009)	atctcgagccggagtacacgaagtctcactccaacttcacat

	250 TIR 5' UTR (Espah Borujeni et al., 2014; Salis et al., 2009)	atctcgagcatcactcgcattttagttttatacgacaaactcta
	350 TIR 5' UTR (Espah Borujeni et al., 2014; Salis et al., 2009)	atctcgagctaatatagaacatcactacagtaccatca
	425 TIR 5' UTR (Espah Borujeni et al., 2014; Salis et al., 2009)	atctcgagccaaggtcaagcgcaagatagacaacaagaacgttc
psicA	Promoter (Moon et al., 2012)	ccacaagaaacgaggtacggcattgagccgcgtaaggcagtagcgatgtattcattgggcgttttttgaatgttcactaacc accgtcggggtttaataactgcatcagataaacgcagtcgttaagttctacaaagtcggtgacagataacaggagtaagta
pTrc10	Promoter (Huang et al., 2010)	ttgacaattaatcatccggctcgtataatgtgtggaattgtgagcggataacaatttcacaca

A glucose-tolerant strain of *Synechocystis*, a derivative of the Kazusa strain obtained from Dr. Himadri Pakrasi, was naturally transformed by plasmids (Zang et al., 2007), plated onto solid BG-11 medium (http://www-cyanosite.bio.purdue.edu/media/table/BG11.html) supplemented with kanamycin (10 μg/mL) (Taton et al., 2014) and spectinomycin (50 μg/mL) (Pitt et al., 2010) as appropriate, and grown at 30°C under continuous white light (50 μmol of photons $m^{-2}s^{-1}$). Single colonies were picked onto new BG-11 plates that had a higher concentration of antibiotic, kanamycin (20 μg/mL) and spectinomycin (100 μg/mL) as appropriate, and also grown at 30°C under continuous white light (50 μmol of photons $m^{-2}s^{-1}$). Successful plasmid transformation, and segregation as appropriate, was confirmed by colony PCR, as outlined in the Supplemental Material. Oligonucleotides for colony PCR were purchased from Integrated DNA Technologies and are summarized in Table 2.3. The *Synechocystis* strains engineered in this work are summarized in Table 2.4. *Synechocystis* liquid cultures (25 – 50 mL, dependent on the particular test being run) were grown in BG11 media in

250 mL baffled flasks at 30°C and 160 rpm, under continuous white light (50 μmol of photons $m^{-2}s^{-1}$) in ambient air.

Table 2.3 Oligonucleotides used to assess *Synechocystis* transformation and segregation.

Name	Sequence (5' to 3')	Strains Checked
6803_psbu_for 6803_pppsbd_rev	gaggttagcaccgtggttaactc caccactgctccgattaaatgg	All using the integrative site (NS1)
6803_kanpsbd_for 6803_kan_rev	aaaaagccgtttctgtaatgaagg ggcctgttgaacaagtctgg	CI067, CI082, CI090, CI091, CI130, CI131
6803_FNR_for 6803_psbuFNR_rev	catctacaacctgtcccgtcg ggacaggttgtagatgaatgc	CI061, CI086, CI107, CI114, CI115, CI132
6803_pp_for 6803_ppFNR_rev	ggtgtgatggacaactcgttc gcatcaccatcaccatcac	CI061, CI107, CI132
6803_tet_for 6803_psbutet_rev	ccagaagctaggtgtagagcagc gctccatcgcgatgacttag	CI086, CI114, CI115
6803_pppsbd_for 6803_pp_rev	ggttgacgtagataaggatgctctc aactcctgcaactgatggtcg	CI090
nif_sap2_seq_f nif_sap1_seq_r	ggtgttcctctcaaagttgg gctacgttgaagatgctaccc	CI107
NSC1US_for NSC1DS_rev	cttaacgtccaccccaatcc cgtcaagttggagttccgaattg	CI115
PCC5.2_seq_f PCC5.2_seq_r	ctctgataccgtatccactgaatc gatttgtcagggtctaggtgtc	All using the integrative site (NSP1)
spec_seq_for2 invf_seq_rev	gttacaaccaattaaccaattctg gtttggtgtggggtttagc	All using a replicative plasmid

Table 2.4 *Synechocystis* strains used in this work.

Name	Plasmids	sp. PCC 6803 Location
sp. PCC 6803	glucose tolerant wild-type	
CI061	pKN004	*psbA1*
CI067	pCI053	*psbA1*
CI082	pCI061	*psbA1*
CI086	pCI063	*psbA1*
CI090	pCI065	*psbA1*
CI091	pCI064	*psbA1*
CI092	pCI067 (AND gate inputs) pCI065 (AND gate output)	replicative *psbA1*

CI094	pCI063 (AND gate inputs) pCI066 (AND gate output)	*psbA1* replicative
CI106	pCI069 (AND gate inputs) pCI065 (AND gate output)	replicative *psbA1*
CI107	pCI068	*psbA1*
CI113	pCI076	NSP1
CI114	pCI063 (AND gate inputs) pCI076 (AND gate output)	*psbA1* NSP1
CI115	pCI072 (AND gate inputs) pCI065 (AND gate output)	NSC1 *psbA1*
CI124	pCI079 (AND gate inputs and output)	replicative
CI125	pCI081 (AND gate inputs and output)	NSP1
CI126	pCI080 (AND gate inputs) pCI065 (AND gate output)	NSP1 *psbA1*
CI127	pCI084 (AND gate inputs) pCI066 (AND gate output)	NSP1 replicative
CI128	pCI083 (AND gate inputs) pCI076 (AND gate output)	replicative NSP1
CI130	pCI077	*psbA1*
CI131	pCI078	*psbA1*
CI132	pKN003	*psbA1*
CI138	pCI088	*psbA1*

2.2.2 Oxygen Sensor and AND Gate Testing Conditions

Synechocystis liquid cultures were diluted to an optical density of 0.4 at 730 nm in a 25 – 50 mL culture and grown in 250 mL baffled flasks at 30°C and 160 rpm, under continuous white light (50 μmol of photons $m^{-2}s^{-1}$) in ambient air. After 24 hours, 2 mL per tube was aliquoted into 14 mL BD Falcon™ round-bottom tubes with 5 mM D-Glucose (Sigma-Aldrich Co. LLC). aTc (anhydrotetracycline from Sigma-Aldrich Co. LLC) dissolved in absolute ethanol was added as appropriate, with a final concentration range of 10 – 10^4 ng/mL. The equivalent volume of absolute ethanol was added to the 0 aTc (control) cultures. For dark conditions, the tubes were wrapped in foil. For aerobic conditions, a 17 gauge, 2 inch hypodermic needle was inserted into each culture tube's lid. For anaerobic conditions, the culture tubes were opened in a vinyl anaerobic chamber (Coy Laboratory Products). Five minutes after the oxygen concentration

reached 0 ppm, the culture tubes were sealed. Wild type cultures were grown in each combination of testing conditions (light/dark, aerobic/anaerobic, 0-10^4 ng/mL aTc) to correlate with the recombinant cultures. All tubes were grown for 72 hours at 30°C and 250 rpm, under continuous white light (50 μmol of photons $m^{-2}s^{-1}$).

2.2.3 Fluorescence Measurements

200 μL of each culture, as well as 200 μL of the media (in triplicate), was loaded into black 96-well plates (flat bottom, chimney well, μclear (Greiner Bio-One)). Measurements were taken using a Tecan Infinite 200 Pro plate reader (absorbance at 730 nm, gain set to 100). For FbFP, excitation was at 450 nm and emission at 495 nm, and for EYFP, excitation was at 485 nm and emission at 528 nm. Normalization was done as follows. The average fluorescence of the media was subtracted from the fluorescence of all cultures (recombinant (RC) and wild type (WT)). The media-adjusted fluorescence for each culture was divided by its associated absorbance ($(F-F_{media})/A$). This calculation for the wild type cultures ($(F-F_{media})/A$) was averaged and subtracted from the adjusted fluorescence divided by absorbance for each well of recombinant culture, in the same combination of testing conditions. This resulted in the normalized fluorescence $[((F-F_{media})/A)_{RC} - ((F-F_{media})/A)_{average\ WT}]_{set\ of\ testing\ conditions}$. The average normalized fluorescence and standard deviation (based on the entire population) were calculated for each combination of testing conditions. The recombinant culture fluorescence, $((F-F_{media})/A)_{RC}$, which was within one standard deviation of the average wild type fluorescence, $((F-F_{media})/A)_{average\ WT}$, in the equivalent set of testing conditions was considered to be background fluorescence and indicated by an asterisk (*) on the graph.

2.2.4 RT-qPCR

Cultures were grown following the testing conditions described in the Oxygen Sensor and AND Gate Testing Conditions section. RNA for each 2 mL biological replicate in its set of testing conditions was isolated following the protocol for TRIzol® Reagent (Life Technologies™), with modifications listed in the Supplemental Material. After the RNA pellet was dried and re-suspended in DEPC-treated water, the concentration and purity (A_{260}/A_{280}) were measured with a Nanodrop 2000 UV-Vis Spectrophotometer. The RNA was immediately stored at -80°C.

DNA was removed within four days of freezing the extracted RNA, using the DNA-free™ Kit (Life Technologies™) according to the manufacturer's instructions. To check for the complete removal of DNA, the DNase-treated RNA was used as the PCR template, as outlined in the Supplemental Material, and oligonucleotides used for RT-qPCR are listed in Table 2.5. The DNase treatment protocol was repeated for any RNA samples that produced a band after PCR and gel visualization. The DNase-treated RNA was stored at -80°C.

Table 2.5 Oligonucleotides used for RT-qPCR. The "expected value of amplicon" is a measure of the specificity of the amplicon. The likelihood of a match, other than the amplicon being tested, decreases as the "expected value of amplicon" approaches zero.

Name	Gene checked	Amplicon size (bp)	Expected Value of Amplicon (Altschul et al., 1997)	Sequence (5' to 3')
F37T_F F37T_R	*fbfp f37t* (Mukherjee et al., 2012)	147	no significant similarity found	caaaactcctgcaactgatg cgtcctgatagagaatatcg
nifh2_F nifh2_R	*nifH* (Bandyopadhyay et al., 2011)	129	no significant similarity found	ttagctcgtcgtctcggaacc cacggtattcctgagcctgagg
nifd2_F nifd2_R	*nifD* (Bandyopadhyay et al., 2011)	188	no significant similarity found	gttcctgttcgttgtgaagggttcc ctagaccaagcatctccaccgatg
nifk2_F nifK2_R	*nifK* (Bandyopadhyay et al., 2011)	144	no significant similarity found	ctcgtggacgtgctgttgacg ccaagacgtgaacaggctcgatacc

rrn16S2_F rrn16S2_R	*rrn16Sa* (Pinto et al., 2012)	189	6E-104	gcaggattccagggatgtcaagc tactaggcgtggcttgtatcgacc
rnpB2_F rnpB2_R	*rnpB* (Pinto et al., 2012)	160	1E-86	gcgtgaggacagtgccacag agtacctctcgatactgctggtgc
petB2_F petB2_R	*petB* (Pinto et al., 2012)	181	3E-99	accctgacctgcttcctgatc ggagaatcatcatcagcaccatcatgc

Within four days of storing the DNase-treated RNA, up to 3µg of it was converted to a cDNA library, for each biological replicate in its set of testing conditions, using the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies) with random primers (300 ng) in a 20µL reaction according to the manufacturer's instructions. The thermal cycler's settings were 25°C for 5 minutes, 42°C for 15 minutes, and 95°C for 5 minutes. The cDNA concentration and purity (A_{260}/A_{280}) were measured with a Nanodrop 2000 UV-Vis Spectrophotometer. The cDNA library concentrations ranged from 1700 – 4000 ng/µL. Since the copy number of the gene of interest was less than 50 (Griese et al., 2011), the concentration of cDNA needed for RT-qPCR was initially estimated by varying the cDNA concentration in 25 µL PCR reactions, as outlined in the Supplemental Material. The cDNA was stored at -20°C.

RT-qPCR was performed using Power SYBR® Green PCR Master Mix (Life Technologies^{TM}) according to the manufacturer's instructions, as outlined in the Supplemental Material. To find ΔC_q, the quantification cycle (C_q) for each gene of interest was subtracted from the geometric mean of the quantification cycles of the reference genes *petB*, *rnpB*, and *rrn16S* (Pinto et al., 2012) (sequence accession number NC_020286) for each replicate in its set of testing conditions. ΔC_q for each gene of interest was then standardized following a series of sequential corrections, which included log transformation, mean centering, and autoscaling, to account for biological variability across all replicates (Bustin et al., 2009; Willems et al., 2008).

Biological and technical replicates were averaged for each gene of interest in each set of testing conditions, and the standard deviation (based on the entire population) was calculated.

2.3 Results and Discussion

2.3.1 Development of an oxygen-responsive transcription system in *Synechocystis*

The genetic devices previously employed in cyanobacteria were designed to respond to chemicals (Camsund et al., 2014; Huang and Lindblad, 2013; Markley et al., 2014). An inducible promoter that responds to oxygen, an environmental signal, can be used to express oxygen-sensitive heterologous proteins without wasting cellular resources. The facultative anaerobe *E. coli* uses its fumarate and nitrate reduction (FNR) system to transition between aerobic and anaerobic conditions (Kang et al., 2005). The dimerized FNR protein acts as a global regulator in the absence of oxygen, turning on expression of the genes needed for anaerobic growth, as well as repressing genes used for aerobic growth (Crack et al., 2012; Grainger et al., 2007). An FNR-activated promoter could therefore be used to control expression of oxygen-sensitive enzymes in *Synechocystis*. Since *Synechocystis* does not have a FNR protein homolog, *fnr* from *E. coli* MG1655 (Gene ID: 945908; http://www.ncbi.nlm.nih.gov/gene) was chosen to produce the transcription factor. The FNR protein and FNR-activated promoter (Table 2.2) are the core components of our gene regulation system, activating transcription in the absence of oxygen.

To test the oxygen-responsive system, a reporter was needed that could perform in both aerobic and anaerobic conditions, as well as be detected amidst *Synechocystis*' fluorescent background. Fluorescent proteins, including Cerulean, GFPmut3B, and EYFP, have all performed adequately in *Synechocystis* despite the organism's background fluorescence (Huang et al., 2010). Yet, all GFP variants, including these three, require molecular oxygen for the

synthesis of their chromophore (Yang et al., 1996), making them unsuitable for testing oxygen-responsive transcription. Flavin mononucleotide binding fluorescent proteins (FbFP), developed from bacterial photoreceptors, fluoresce similarly in both the absence and presence of oxygen (Drepper et al., 2007). In addition, FbFP's excitation spectrum (maximum at 450 nm) and emission spectrum (maximum at 495 nm) do not significantly overlap with the fluorescence spectra for *Synechocystis*' pigments, chlorophyll a, and phycocyanin (French et al., 1956). We tested the engineered, oxygen-independent FbFP F37T (Mukherjee et al., 2012) (hereafter called FbFP) as the reporter for our transcription system (Materials and Methods).

FbFP was expressed from the constitutive promoters Bba J23100 (http://parts.igem.org/Part:BBa_J23100) or Bba J23104 (http://parts.igem.org/Part:BBa_J23104), with the parts integrated into the neutral site *psbA1* (Gene ID: 14618584; http://www.ncbi.nlm.nih.gov/gene) in *Synechocystis*' chromosome by double homologous recombination (Golden et al., 1987) (Figure 2.1a). This neutral site, called NS1 in this work (Table 2.4), has been demonstrated to be silent in most conditions (Mohamed et al., 1993). However, it has been shown that *psbA1* is up-regulated in oxygen depleted conditions (Summerfield et al., 2011). To avoid potential interference from the native regulation, the gene and its promoter were removed during recombination, eliminating response to the transcriptional regulator ChlR (Aoki et al., 2012). When constitutively transcribed from Bba J23100 or Bba J23104 in the same aerobic conditions in *Synechocystis*, FbFP performed comparably to EYFP (Figure 2.1b). Expression of *fbfp* from Bba J23104 was higher than from Bba J23100, while expression of *eyfp* was similar for both promoters. Each of the reporter genes has its unique 5' UTR (Table 2.2), which can lead to different expression trends (Espah Borujeni et al., 2014; Lou et al., 2012). Applying the same conditions, FbFP was further used to characterize the aTc-

inducible promoter pTet (Figure 2.1c and 2.1d). The solubility limit of aTc in absolute ethanol restrained the aTc concentration range to a maximum of 10^4 ng/mL.

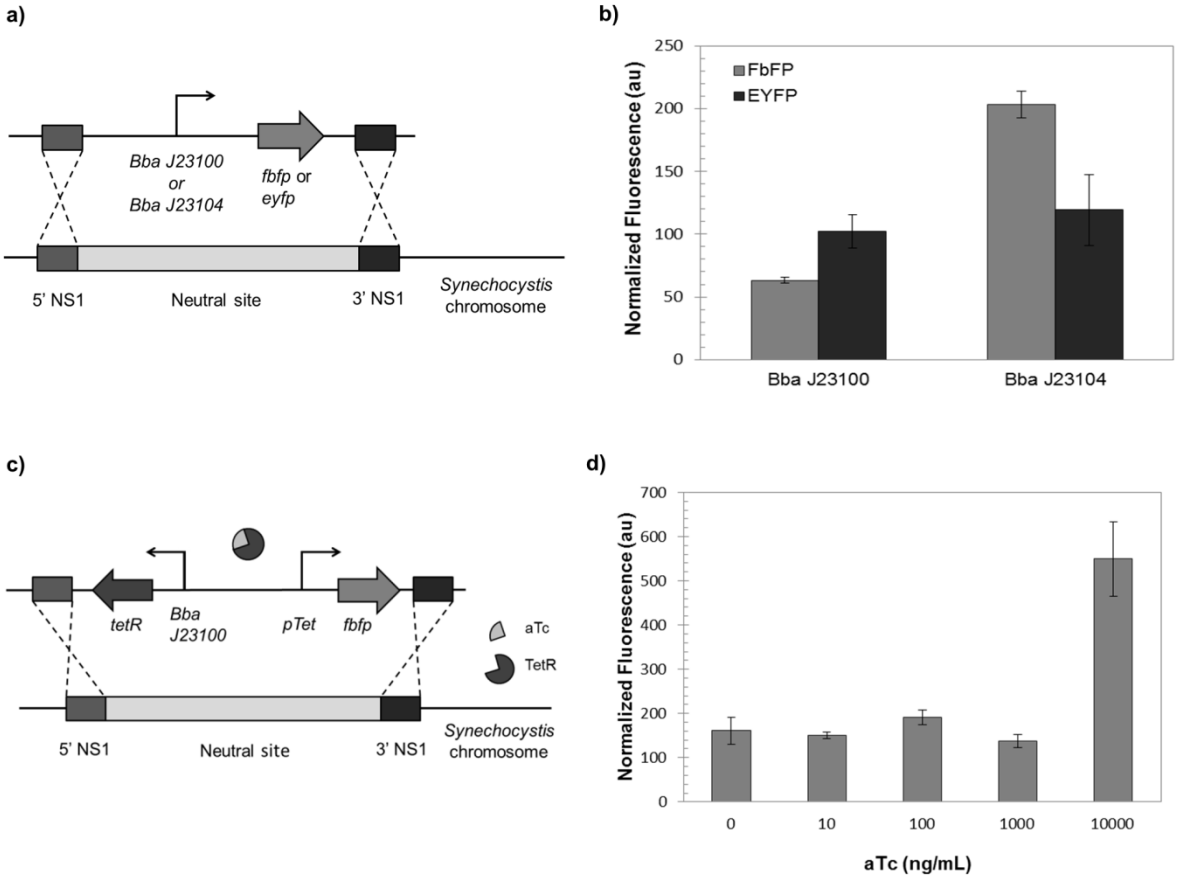


Figure 2.1 Promoter and reporter characterization in *Synechocystis*. a) Schematic of constitutive promoters controlling a flavin binding fluorescent protein (FbFP) (excitation at 450 nm and emission at 495 nm) or enhanced yellow fluorescent protein (EYFP) (excitation at 485 nm and emission at 528 nm). The genes and promoters were integrated into a neutral site in *Synechocystis*' chromosome (*psbA1*, called NS1 in this work) by double homologous recombination. b) Normalized fluorescence, $((F\text{-}F_{media})/A)_{RC}$ - $((F\text{-}F_{media})/A)_{average\ WT}$, of FbFP and EYFP expressed from Bba J23100 or Bba J23104 (Materials and Methods). RC are recombinant cultures and WT are wild type cultures. All cultures were grown heterotrophically in the dark. The data reported are the average of three biological replicates. Error bars indicate one standard deviation. c) Schematic of anhydrotetracycline (aTc)-inducible pTet promoter expressing *fbfp* integrated into NS1 in *Synechocystis*' chromosome by double homologous recombination. d) Normalized fluorescence, $[((F\text{-}F_{media})/A)_{RC}$ - $((F\text{-}F_{media})/A)_{average\ WT}]_{aTc\ concentration}$, of FbFP expressed from pTet (Materials and Methods). All cultures were grown

heterotrophically in the dark. The data reported are the average of three biological replicates. Error bars indicate one standard deviation.

Table 2.6 Flanking regions used for double homologous recombination.

Name	DNA sequence	Location
5' NS1	cggaacaggaccaagccttgatgcgaggatcaaaaacatcctccacaatgcccgctcgaatgggtcgatggagg gtcagaccatggccggtggcggatctttcccggtcaggacagaccactgtgacttcatggcctgcggcgactagg gtgttggccaaggtacgtacccccttgggaaaaaatgccgtcatcgttactgacaaggagattgaggaccggggatg gggtcataggacaccaaagaatcgagattgtgcctgccagccttaacgatatcaggctatgtcccgcttaaactctga ttcttaccaggtaactcctgggctccatggggccacaaccaggcagtattttgttcctttggccaatggggcgatcgg ggaaaaatggcttgatctggcatttacgagaaaaatttttatttttaatgatttatttttcctattaaaatctttttttaccttt ggaaaccaactgcaatctgagaaaccatcc	chromosome *psbA1*
3' NS1	tgtgccattgccataactgctttcggttagacttcgtttcatttggttaatcaagggcactctcgcaatggggtgccttta tggtccaaggttaaagttaagccagtaaagtttaagtctatttctagggtgaaatgtaatgaatcaatttagggactggg gtttttccactgattggtggcaaggcaaaaaaggggaatattgggtacttgggcaaacaattctctccgttggatttgta ctgttaccggtttatactcctactaatttacagttactagctgaacaaaatcagtggattgcctgggctggtactctgttttt tggcatgattgcggcagttttgctgattgggggggtctgcatttaggggaaaatctgactcccttgccccatcctaaa aaggatagtcaaa	chromosome *psbA1*
5' NSC1	gcaatgcccactcctccacaggcggtgagggtaaagctcagcagtaaagttaccaggactacagcagtggatagg gacttttcaacatgggagaaagggaagaactgggcaagaaggcaaaattacctttccttacccattaaacctccaat ggttgaccagaaactagaggtagaatgattcccgccagaaaaagaagtttaacaatttgccatgggcactgttctga agcggggaggaaggctatggcctcctcaaccaatttatttcctgcctaacccaggggacgttgttgaggctataagtt gaggctataaatttaacttattaaaaggttcgacaaatttgagatagttttgtggcaaagatactgcttaggaaccaaat attgcataaacttagagatatagtttttctaaaaaaatagtcttatttctatctattgaatcggggcaatttaaactcagaat agattagttgttcccagctgaaaccatcgtgtgctttttccagaggcgtttttggcaatttttcctctggtaaatttcaccga ctttggggcaatgctcataatcaccatagagtgaaatccatgaacaagtttgaatcaagacaatcgg	chromosome intergenic region
3' NSC1	ttcccacgcttgctctttttcctgggttgtggtgtcggcttactactgtcggtggtttgggtcaatgttgctcgccatagtc ctccgctagaatcctccccagtcaaggtctcgcctcccttccaggtcgactagtcacaacaatttaaaaatcagaaaa attgtcccattgatcaacttacaggggccattgagcaaaatccggggtcaccatctagtccccaaaaagctggcga tcgccaaataatagtaaaacttatcattcaaatttaaaattacttagcagatccaggggacaactgcaaaattggtcg gatttacatatagactttagcttatagatttcaagacataggcattcaaacctgcatagacaagagtctatacagagcga agccaatggggttcattgcccctggaaagatcaagcaaactgccgaagattcagggccaagctttactaccccaatc cccataaatttcaaccaaggagacaatttacattatggatttttgtccaatttcttgacggacttcgtgggacaattgca gtccccaaccctagcctttctgattgggggatggttattgccg	chromosome intergenic region
5' NSP1	atataatcccgttaacaggctaaacccatgcagggcatagataaacccaaaggggaaggggacatcatgccccg tatctcaccactcacctaagagtcccgataccagttaaggatgaagtgcagggcattattgaccgctacagggaaga cttactggcaggaagacagctccaagatgttcccagctcctacgaggtcaaaacggcgatcgccattctgacggag gcactcagccttaaagccaatgccggtggagccatcaaagcaaaaatcagagaagccctagctatcttggaaagg aactgaaatgagaaaactaaaacatcgccgccgccgctatcccgaaattgagtttgagattgtccaggcgttagagc aatttgggcccctgtccatccccgagatatttcaccacttgaggggtcgtcatcctccccgggccgtttttgattctctttt atcggctggaggaagtcgggggaatccataaagaaaagctgcaaataactctatccgcaccaccaccaccaccac acacacctatttctcaaaatgacagggaagggcaattgctggggaactgatagaaatctctgat	endogenous plasmid pCC5.2
3' NSP1	gacaggagtttagccatgggattagtcagagtccctagccattgctgatgcgagattatttaaactttgccaagggata ttgaccagacgggccgtgctaccaagccgttgctgtcgcaagtgtcgcttaacccgcttctgggctatccaatggtta ttaatcttgtaaacgcttatcctgccctgctcactggcaatgtagaaccgcttatggactctaagccagttcttccagttt ggactctccaaactcagagggtacttagtccctgcaccgtgcaagtatccgtcaatcaccgtcggttgaggctccata cctacattgcccctatcaagccagttttagcacgctgggaagcacgataatctactgcgactaccctagggtactccc gtagaaaagcgttatgaagtatgaccggcaaaaaaccacattgaccctatcagagcaaagctagcacggtaggga acgcgataatctactgcaacacctataggtctgttgctgacacgcacgatgacgtatgacccttttagcacggtaggg agcgtgataatcttctgcaacacctatatagtattgttgcgatcgcgagcgatggcgt	endogenous plasmid pCC5.2

The first version of the oxygen sensor developed in *Synechocystis* consists of the constitutively transcribed *fnr* (under control of the Bba J23104 promoter; Figure 2.1b) and the oxygen-independent *fbfp* reporter (under control of an FNR-activated promoter), integrated into the chromosome (neutral site NS1) by double homologous recombination (Figure 2.2a). To test in four different environments, cultures were grown in either aerobic or anaerobic conditions, and either in the light (oxygenic photosynthesis) or in the dark (Materials and Methods). The highest normalized fluorescence occurred in dark, anaerobic cultures, and the lowest normalized fluorescence occurred in light, aerobic cultures (Figure 2.2b). This result indicates that *fbfp*'s promoter was activated in the absence of oxygen. To confirm that the fluorescent response was due to transcriptional activation in the absence of oxygen, RT-qPCR (following the MIQE guidelines (Bustin et al., 2009)) was used. Figures 2.2c and 2.2d show the mRNA concentration of *fbfp* and *Cyanothece*'s *nifHDK*, respectively, relative to three reference genes in each of the four culture conditions (Materials and Methods). *nifHDK* encode dinitrogenase reductase (*nifH*) and dinitrogenase (*nifDK*), which are nitrogenase's oxygen-sensitive structural proteins (Rubio and Ludden, 2005). The highest relative mRNA concentration occurred in dark, anaerobic cultures for all of the RT-qPCR results, while all other oxygen conditions had very low transcript levels. These results together demonstrate a relative correlation between testing conditions and sensor output, indicating that the oxygen sensor activates transcription only in low oxygen conditions. Furthermore, the maximum expression level from the oxygen sensor (pO_2) is four times greater than that of pTrc10 (Huang et al., 2010) (Figure 2.3), which is commonly used as a strong synthetic promoter in *Synechocystis.*

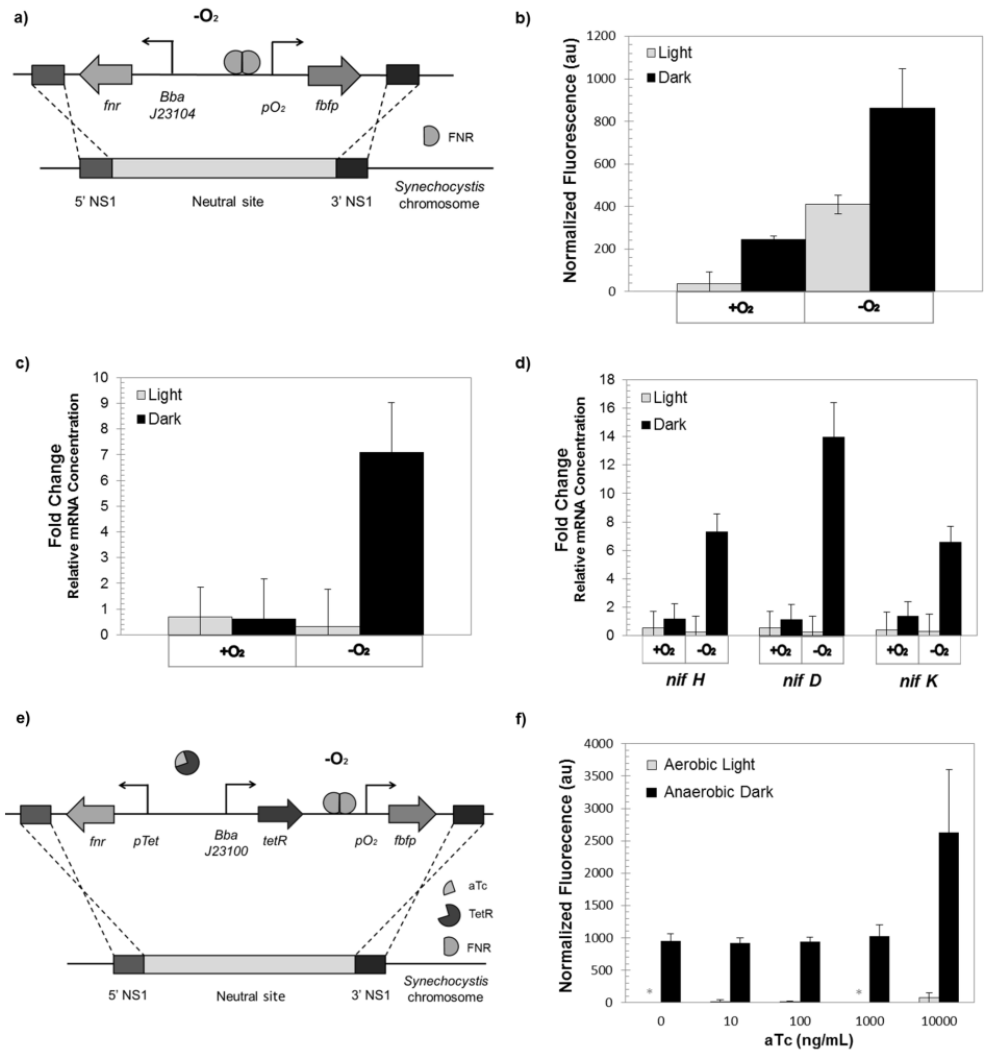


Figure 2.2 Oxygen-responsive transcription system built in *Synechocystis*. a) Schematic of *E. coli*'s fumarate and nitrate reduction (FNR) system expressing *fbfp* in oxygen depleted conditions, integrated into neutral site NS1 in *Synechocystis*' chromosome by double homologous recombination. b) Normalized fluorescence, $[((F\text{-}F_{media})/A)_{RC} - ((F\text{-}F_{media})/A)_{average\ WT}]_{culture\ condition}$, of FbFP expressed from the FNR system, in different culture conditions (Materials and Methods). Aerobic cultures are represented by $+O_2$ and anaerobic cultures by $-O_2$. Cultures grown in the light perform oxygenic photosynthesis, while cultures grown in the dark do not generate oxygen. The data reported are the average of a minimum of three biological replicates performed on different days. Error bars indicate one standard deviation. The dissolved oxygen level (%) of the aerobic light and anaerobic dark cultures is shown in Figure 2.4. The normalized fluorescence for the phototrophic cultures grown at two different light intensities is shown in Figure 2.4. c) Relative mRNA concentration (*fbfp* relative to *petB, rrn16S*, and *rnpB*) in different culture conditions (Materials and Methods). The geometric mean of quantification cycles (C_q) of the reference genes was subtracted from the C_q of each gene of interest for each replicate in its set of testing conditions, followed by a series of sequential

corrections, which included log transformation, mean centering, and autoscaling, to account for biological variability across all replicates (Bustin et al., 2009; Willems et al., 2008). Two biological and two technical replicates were averaged for each gene of interest in each set of testing conditions, and the standard deviation (based on the entire population) was calculated. d) Relative mRNA concentration (*nifHDK*, replacing *fbfp* in Figure 2.2a, relative to *petB*, *rrn16S*, and *rnpB*) in different culture conditions (Materials and Methods). The data reported are the average of two biological replicates with two technical replicates each (four replicates total). Error bars indicate one standard deviation. e) Schematic of aTc-inducible control of the FNR regulatory protein, with the FNR system expressing *fbfp* in oxygen depleted conditions. The genes and promoters were integrated into NS1 in *Synechocystis*' chromosome by double homologous recombination. f) Normalized fluorescence, $[((F\text{-}F_{media})/A)_{RC} - ((F\text{-}F_{media})/A)_{average\ WT}]_{oxygen\ condition\ and\ aTc\ concentration}$, of *Synechocystis* expressing *fbfp* from the FNR system, with aTc-inducible control of the FNR regulatory protein, in anaerobic dark and aerobic light conditions. The data reported are the average of three biological replicates performed on different days. Error bars indicate one standard deviation. Asterisks (*) indicate that the recombinant culture fluorescence is within one standard deviation of the average wild type fluorescence (Materials and Methods).

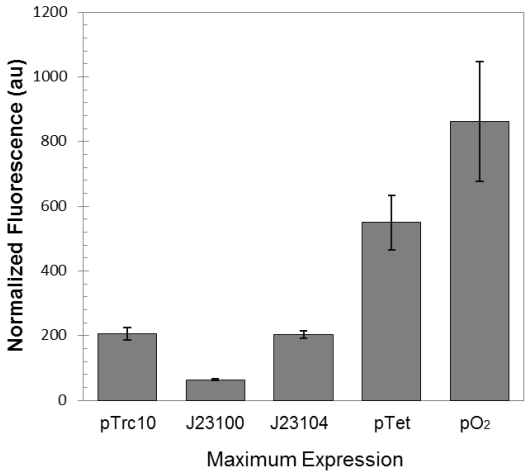


Figure 2.3 Comparison of diverse promoters expressing *fbfp.* All of the promoters and reporter were integrated into NS1 in *Synechocystis*' chromosome. All cultures were grown heterotrophically in the dark. pTrc10, commonly used as a strong constitutive promoter (without inducer) in *Synechocystis.* pTet, induced with 10^4 ng/mL aTc. pO_2, the first version of the oxygen sensor with *fnr* under control of the Bba J23104 promoter (Figures 2.2a). The data reported are the average of a minimum of three biological replicates. Error bars indicate one standard deviation (Materials and Methods).

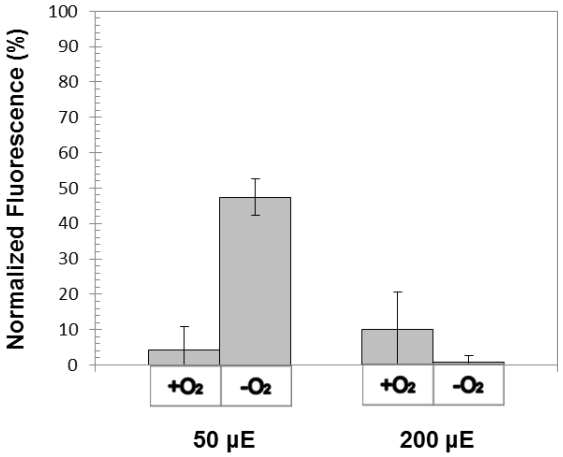


Figure 2.4 Normalized fluorescence (%) of the phototrophic oxygen sensor cultures, containing the constitutively transcribed *fnr* (under control of the Bba J23104 promoter) and the oxygen-independent *fbfp* reporter (under control of an FNR-activated promoter), grown at different light intensities (Supplemental Materials and Methods). To obtain the normalized fluorescence (%), the normalized fluorescence of each phototrophic oxygen sensor culture was divided by the average normalized fluorescence of the corresponding anaerobic dark cultures, and then multiplied by 100. Aerobic cultures are represented by $+O_2$ and anaerobic cultures by $-O_2$. The data reported are the average of three biological replicates. Error bars indicate one standard deviation.

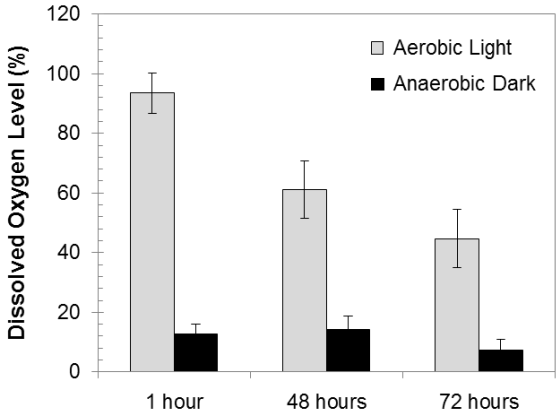


Figure 2.5 Dissolved oxygen levels (%) of the oxygen sensor cultures, containing the constitutively transcribed *fnr* (under control of the Bba J23104 promoter) and the oxygen-independent *fbfp* reporter (under control of an FNR-activated promoter) (Supplemental Materials and Methods). The dissolved oxygen level (%) was calculated by dividing the measured value for each oxygen sensor culture by the measured value of saturated room temperature water, and then by multiplying by 100. From Figure 2.2c and this figure, the threshold dissolved oxygen level (%) required for the oxygen sensor activation was estimated to be ~20% or lower (a conservative estimate). The data reported are the average of three biological replicates. Error bars indicate one standard deviation.

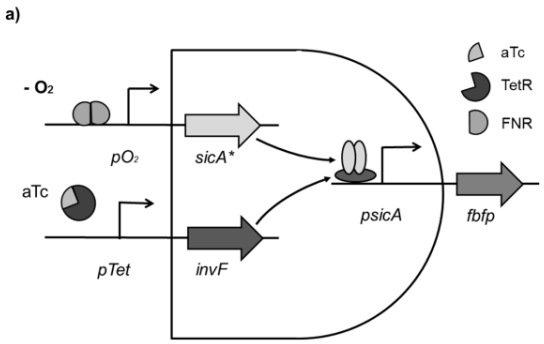
2.3.2 Expanding the oxygen sensor's dynamic range

To improve the dynamic range of the oxygen sensor, we changed the promoter for the transcription factor FNR to pTet (Moon et al., 2012) (Figure 2.2e). As compared to Bba J23014, the normalized fluorescence of FbFP was approximately 2.5 times greater when pTet was induced at 10^4 ng/mL aTc (Figures 2.1b and 2.1d). We tested this new version of the oxygen sensor in anaerobic dark and aerobic light conditions, with 0 to 10^4 ng/mL aTc. The maximum normalized fluorescence for the second oxygen sensor (Figure 2.2f) was three times greater than that of the original version (with FNR constitutively expressed, Figure 2.2b) in the anaerobic dark condition, while maintaining very low normalized fluorescence in the aerobic light condition. Replacing Bba J23104 with pTet for the expression of *fnr* therefore improved the oxygen sensor's dynamic range, making the sensor applicable to a broader array of oxygen-responsive transcription applications.

2.3.3 Creation of a two-input AND gate

The ability to regulate transcription with specificity in response to multiple signals can be achieved through multi-input logic gates. Using endogenous genetic circuits, bacteria function robustly in response to environmental fluctuations, while efficiently allocating cellular resources to the specific transcription of diverse genes. Although inducible transcriptional control has been previously demonstrated in cyanobacteria (Camsund et al., 2014; Huang and Lindblad, 2013; Markley et al., 2014), combining more than one inducible input into a transcriptional circuit has not been shown. We used the oxygen-responsive FNR-activated promoter and the aTc-inducible pTet promoter to build, for the first time, a two-input AND gate in *Synechocystis*, which is based on the *Salmonella* Pathogenicity Island 1 type III secretion system (Moon et al., 2012) (Figure

2.6a). In oxygen depleted conditions, *sicA** is transcribed, while aTc induces the transcription of *invF*. The chaperone SicA* and the transcription factor InvF form a transcription-activating complex, turning fluorescence on only in the absence of oxygen ($-O_2$) and the presence of 10^4 ng/mL aTc, representing the ON (1 1) state. Expression of *fbfp* should be in the OFF state in the absence of aTc, regardless of oxygen level (0 0) or (1 0), as well as in the presence of both oxygen and 10^4 ng/mL aTc (0 1) (Figure 2.6b). The cultures were grown for three days heterotrophically in the dark, following the protocols used for the oxygen sensor (Materials and Methods), so possible degradation of aTc by light (Huang and Lindblad, 2013) did not affect the circuit's performance.



b)

- O₂	aTc	FbFP
0	0	0
1	0	0
0	1	0
1	1	1

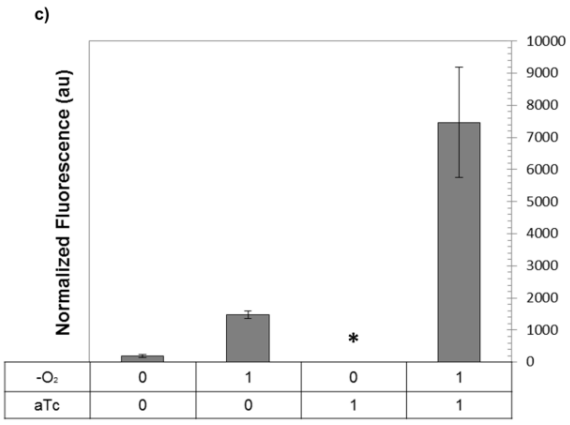


Figure 2.6 Two-input AND gate built in *Synechocystis*. a) Schematic of a two-input AND gate. The gene for the chaperone, *sicA**, and for the transcription factor, *invF*, are from the *Salmonella* Pathogenicity Island 1 type III secretion system (Moon et al., 2012). FNR is produced

constitutively from Bba J23104, for the FNR system expressing *sicA** in oxygen depleted conditions. TetR is produced constitutively from Bba J23100, for the aTc-inducible transcription of *invF*. The anaerobic dark culture condition is represented by $-O_2$. aTc denotes 10^4 ng/mL anhydrotetracycline. b) AND gate truth table. For $-O_2$, 0 represents the aerobic culture condition, and 1 is the anaerobic culture condition. For aTc, 0 indicates the solvent, absolute ethanol, without aTc, and 1 is 10^4 ng/mL aTc. c) Normalized fluorescence, $[((F\text{-}F_{media})/A)_{RC} - ((F\text{-}F_{media})/A)_{average\ WT}]_{oxygen\ condition\ and\ aTc\ concentration}$, of *Synechocystis* expressing *fbfp* from the two input AND gate (Materials and Methods). The inputs, *pO₂-sicA** and *pTet-invF* (with weakened RBS for *invF* (229 TIR); see Figure 2.7), are located together in the replicative plasmid. The output, *psicA-fbfp*, is located in the chromosome, NS1. All cultures were grown heterotrophically in the dark. The data reported are the average of three biological replicates. Error bars indicate one standard deviation. The asterisk (*) indicates that the recombinant culture fluorescence is within one standard deviation of the average wild type fluorescence (Materials and Methods).

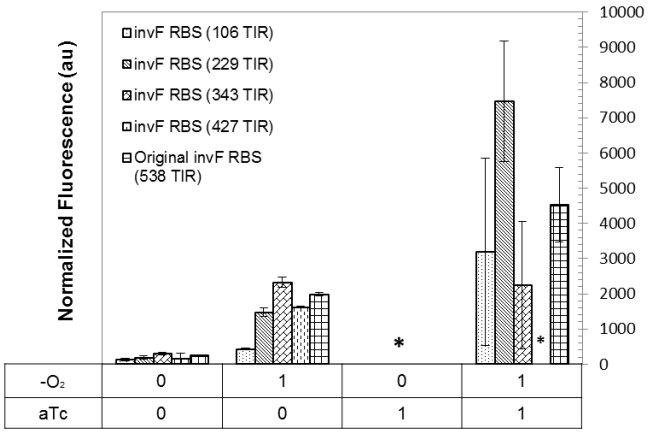


Figure 2.7 Normalized fluorescence of *Synechocystis* expressing *fbfp* from the two-input AND gate (Materials and Methods), using different *invF* ribosome binding sites (RBS). The inputs, *pO₂-sicA** and *pTet-invF*, are located together in the replicative plasmid. The output, *psicA-fbfp*, is located in the chromosome, NS1. For $-O_2$, 0 represents the aerobic culture condition, and 1 is the anaerobic culture condition. For aTc, 0 signifies the addition of only the solvent, absolute ethanol, and 1 is 10^4 ng/mL aTc. All cultures were grown heterotrophically in the dark. The data reported are the average of three biological replicates. Error bars indicate one standard deviation. Asterisks (*) indicate that the recombinant culture fluorescence is within one standard deviation of the average wild type fluorescence (Materials and Methods). Among the RBS variants tested, the RBS with a translation initiation rate (TIR, au) of 229 (Espah Borujeni et al., 2014; Salis et al., 2009) decreased the leaky expression in the absence of the inducer aTc (1 0), when compared to that of the original RBS ($P < 0.02$ for two sample, unequal variances, two-tail t-test), while maintaining high expression at the ON state (1 1). This RBS was used for all subsequent AND gate constructs with their inputs located in the replicative plasmid or the endogenous plasmid.

The oxygen-responsive *sicA** and the aTc-inducible *invF* (i.e., the inputs of the AND gate) were introduced together into a broad host-range replicative plasmid containing the

RSF1010 replicon (Landry et al., 2013). The SicA*/InvF complex-responsive *fbfp* was integrated into the NS1 neutral site in the chromosome by double homologous recombination. In the absence of oxygen and the presence of 10^4 ng/mL aTc, which is the ON state (1 1), the normalized fluorescence of FbFP (Materials and Methods) was the strongest (Figure 2.6c). The normalized fluorescence in aerobic conditions, without or with aTc, (0 0) or (0 1), was less than 3% of the normalized fluorescence of the ON state (1 1), which indicates that very little *sicA** was transcribed (from pO_2) in the presence of oxygen. The normalized fluorescence of the anaerobic condition without aTc (1 0) was 20% of the normalized fluorescence of the ON state (1 1), suggesting leaky expression of *invF* from the pTet promoter. As shown in Figure 2.1d, pTet was also leaky when directly controlling *fbfp*. Note that this working AND gate, with a 5-fold difference between (1 1) and (1 0), was built by optimizing the ribosome binding site (RBS) strength for *invF* (Table 2.2), which was designed to be approximately 20%, 40%, 60% and 80% of the original RBS strength (Espah Borujeni et al., 2014; Salis et al., 2009). For the circuit that used *invF*'s original RBS, the normalized fluorescence in the anaerobic condition without aTc (1 0) was 44% of the normalized fluorescence of the ON state (1 1) (Figure 2.7). All subsequent constructs with their inputs located in the replicative plasmid or the endogenous plasmid used this improved version with the weakened RBS for *invF*.

2.3.4 Dependence of two-input AND gate performance on genetic context

Genetic context can affect circuit performance through copy number (Kittleson et al., 2011), as well as through possible interactions with the surrounding DNA/RNA (Lou et al., 2012) or metabolites (Arkin, 2008). In *Synechocystis*, heterologous DNA can be expressed in the chromosome (Berla et al., 2013) or an endogenous plasmid (Berla and Pakrasi, 2012), as well as in a broad host-range replicative plasmid (Heidorn et al., 2011b) (Table 2.7). Copy number

varies among these different options. *Synechocystis* is a polyploid with up to 50 copies of its chromosome per cell, depending on its growth phase (Griese et al., 2011). Up to this point, all genetic parts used in this work were integrated into *Synechocystis*' chromosome, replacing *psbA1* and its promoter (Varman et al., 2013a). Other neutral sites in the chromosome have been used for heterologous expression, including *slr0168* encoding a hypothetical protein (Angermayr et al., 2012; Kunert et al., 2000; Viola et al., 2014); *slr2031* encoding a phosphatase expressed in instances of nitrogen or sulfate starvation (Ejima et al., 2012; Yu et al., 2013); and several other regions (Wang et al., 2013), including NSC1 (personal communication with Dr. Himadri Pakrasi, Table 2.7) identified from a transcription start site map (Mitschke et al., 2011), *psbA2*, and *sqs* (Englund et al., 2015). Only one replicon, RSF1010, has been shown to function in *Synechocystis*. The copy number for replicative plasmids containing the broad host-range replicon RSF1010 has been reported to be one to three per chromosome (thus up to 150 copies per cell) (Heidorn et al., 2011b). Copy number could be increased further if the genetic part is integrated into one of *Synechocystis*' endogenous plasmids. Depending on growth phase and nutrition condition, the copy number for the endogenous plasmid pCC5.2 can vary from one to more than seven per chromosome (thus up to ~350 copies per cell) (Berla and Pakrasi, 2012). Therefore, there are multiple options for expressing the two-input AND gate with different copy numbers and other possible contextual effects.

Table 2.7. Options for expressing heterologous genes in *Synechocystis*.

Location of expression	Copy number per cell	Example
Neutral site in the chromosome	Up to 50; Growth phase dependent[a]	*slr 2031*[b], *psbA1*[b], *slr0168*[c], intergenic regions[d,e], *psbA2*[f], *sqs*[f]
Replicative plasmid	Up to 150[g]	RSF1010 replicon[g]
Neutral site in an endogenous plasmid	Up to 350; Growth phase & nutrition condition dependent[h]	*myo_rs18470*[d]

a. (Griese et al., 2011). b. (Yu et al., 2013). c. (Kunert et al., 2000). d. Personal communication with Dr. Himadri Pakrasi. e. (Wang et al., 2013). f. (Englund et al., 2015). g. (Heidorn et al., 2011b). h. (Berla and Pakrasi, 2012).

The AND gate's inputs, both the oxygen-responsive *sicA** and the aTc-inducible *invF*, were introduced together into the chromosome (neutral site NS1 or NSC1), into a replicative plasmid with the RSF1010 replicon, or into the neutral site NSP1 in pCC5.2 (Personal communication with Dr. Himadri Pakrasi, Table 2.6). Similarly, the AND gate's output, the SicA*/InvF complex-responsive *fbfp*, was introduced separately into the chromosome (NS1), into a replicative plasmid with the RSF1010 replicon, or into the NSP1 in pCC5.2. In all, nine different combinations were constructed and tested in four conditions: aerobic and no aTc (0 0), anaerobic and no aTc (1 0), aerobic and 10^4 ng/mL aTc (0 1), and anaerobic and 10^4 ng/mL aTc (1 1) (Figure 2.8). The cultures were grown for three days heterotrophically in the dark, following the protocols used for the oxygen sensor (Materials and Methods).

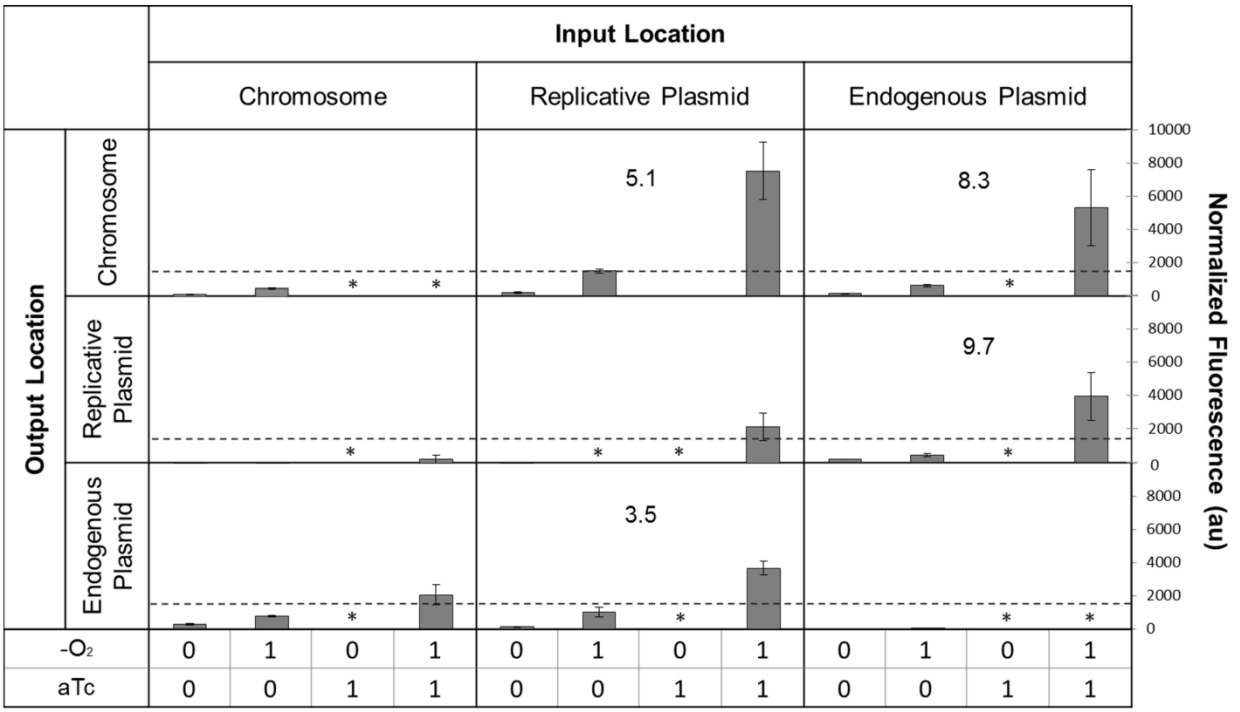


Figure 2.8 Effect of genetic context on circuit performance. Normalized fluorescence, $[((F-F_{media})/A)_{RC} - ((F-F_{media})/A)_{average\ WT}]_{oxygen\ condition\ and\ aTc\ concentration}$, of *Synechocystis* expressing *fbfp* from the two-input AND gate is shown in different genetic contexts (Materials and Methods). The inputs, *pO₂-sicA** and *pTet-invF*, are located together in the chromosome (neutral site NS1 or NSC1), replicative plasmid, or endogenous plasmid PCC5.2 (neutral site NSP1). The output, *psicA-fbfp*, is located in the chromosome (NS1), replicative plasmid, or endogenous plasmid PCC5.2 (NSP1). For $-O_2$, 0 represents the aerobic culture condition, and 1 is the anaerobic culture condition. For aTc, 0 indicates the solvent, absolute ethanol, without aTc, and 1 is 10^4 ng/mL aTc. All cultures were grown heterotrophically in the dark. The data reported are the average of three biological replicates. Error bars indicate one standard deviation. Asterisks (*) indicate that the recombinant culture fluorescence is within one standard deviation of the average wild type fluorescence (Materials and Methods). The dashed line indicates the leakiest OFF level. This line defines the minimum ON level required for a working AND gate and is drawn as a guide to the eye. The fold change between the leakiest OFF state and the ON state is listed for each of the working circuits.

The performance of two-input AND gates depended on the location of the genetic circuit's parts. The threshold level for the ON state (1 1) of a functional circuit (the dashed line in Figure 2.8) was defined by the leakiest OFF state, which occurred when the inputs were located in the replicative plasmid and the output was in the chromosome (NS1). None of the

constructs functioned as an AND gate when the inputs were integrated into the chromosome, regardless of the output location, or when the inputs and output were located together. The construct with the inputs in the replicative plasmid and the output in the chromosome (NS1) produced a 5-fold change between the ON state (1 1) and the leakiest OFF state (1 0) (Figures 2.6 and 2.8). When the inputs were integrated into NSP1 of pCC5.2, two of the three combinations also functioned as designed. With the output in the chromosome, the fold change between the ON state (1 1) and the (1 0) condition, the leakiest of the OFF states, improved to ~8. With the output in the replicative plasmid, the fold change improved further to ~10. These results led us to speculate that the inputs required a higher copy number than the output. The exception occurred when the inputs were in the replicative plasmid and the output was in the endogenous plasmid; however, this case produced only a 3.5-fold change. As discussed earlier, the copy number of the endogenous plasmid has been shown to vary from one to more than seven, depending on the culture conditions or growth phase (Berla and Pakrasi, 2012). This variation could explain the exception, together with other complex genetic context effects. The AND gate performance's dependence on part location demonstrates the importance of genetic context in synthetic circuit construction, including how genetic context complicates precise and tunable control of gene expression.

2.4 Conclusions

We developed an oxygen-responsive regulation system that transcriptionally controlled the oxygen-sensitive structural proteins from *Cyanothece*'s nitrogenase in *Synechocystis*. The system transcribed *nifHDK* only in the absence of oxygen, mimicking their regulation in *Cyanothece*. When fused to a fluorescent reporter, the oxygen sensor fluoresced in the anaerobic light and aerobic dark conditions, both less than 50% of the anaerobic dark ON state, while the

associated relative mRNA concentrations were less than 10% of the ON state (Figures 2.2b and 2.2c). Differences in mRNA and protein stability, as well as changes in translational modifiers in the varied conditions, such as regulatory proteins and small regulatory RNAs, could result in variations between mRNA and protein abundance (Maier et al., 2009; Mitschke et al., 2011).

We employed the inducible pO_2 and pTet to control expression of heterologous regulators (SicA* and InvF), forming the first two-input AND gate built in *Synechocystis*. As the number of inducible promoters that function in cyanobacteria grows, genetic circuits will not need to rely on parts that perform sub-optimally in cyanobacteria, such as the leaky pTet promoter. Robust and predictable engineering of genetic circuits is complicated by genetic context. Part location of the AND gate affected the expression of the fluorescent reporter and the leakiness of pTet, as well as the functionality of the circuit. Improved understanding of complex gene regulation, supported by our bottom-up approach, and development of new genetic parts and devices will further increase cyanobacteria's utility as biotechnology platforms.

2.5 Acknowledgements

The authors thank Dr. Himadri Pakrasi for his comments on the manuscript and providing *Synechocystis* sp. PCC 6803, the *eyfp* gene, the replicative plasmid, the integrative plasmids for NSC1 and NSP1, and the use of the Clarke electrode; Dr. Charles Schroeder for the *fbfp f37t* gene; Dr. Yinjie Tang for the integrative plasmid for *psbA1* and for the use of the anaerobic chamber; and Dr. Gautam Dantas for the use of the real-time thermal cycler. We also thank Dr. Arul Varman and Dr. Bert Berla for their advice. This work was supported by National Science Foundation grant MCB-1331194 and Gates Foundation grant OPP1087549. CMI is the recipient of an NSF Graduate Research Fellowship, DGE-1143954.

2.6 Supplemental Material

2.6.1 Materials and Methods

Colony PCR

Colony PCR was used to verify successful plasmid transformation, and segregation as appropriate, in *Synechocystis*. The reaction components included a culture grown from a single colony on solid media as the template, 500 nM forward and reverse primers, and 1.5 µL GoTaq® DNA Polymerase (Promega Corporation) in a 50 µL reaction according to the manufacturer's instructions. The thermal cycler settings were 94°C for 10 minutes, 35 cycles of (94°C for 30 seconds, annealing temperature determined by New England BioLabs Tm Calculator (http://tmcalculator.neb.com/#!/) for 30 seconds, and 72°C for 1 minute/kb), followed by 72°C for 10 minutes. Oligonucleotides for colony PCR were purchased from Integrated DNA Technologies and are summarized in Table 2.3. The primers 6803_psbu_for/6803_pppsbd_rev (for NS1) and NSC1US_for/NSC1DS_rev (for NSC1) bind to the chromosome, and PCC5.2_seq_f/PCC5.2_seq_r (for NSP1) bind to the endogenous plasmid PCC5.2, all outside the flanking regions used for double homologous recombination (Table 2.6). Two amplicons are possible when using these primers, one from the inserted DNA and one from the wild type chromosome or endogenous plasmid. There was only one band during gel visualization, corresponding to the length of the inserted DNA when segregation was complete. The other primers listed in Table 2.3 bind to the heterologous DNA, thereby increasing confidence that the parts were inserted as designed.

RNA extraction

RNA for each 2 mL biological replicate in its set of testing conditions was isolated according to the protocol for TRIzol® Reagent (Life Technologies^{TM}), with the following modifications.

After re-suspending the pellet of cells harvested by centrifugation in 1 mL TRIzol, the samples were heated to 95°C for 5 minutes and then cooled in ice for 5 minutes (Pinto et al., 2009). After vigorously shaking the samples upon addition of chloroform, the samples were left at room temperature for 10-15 minutes (Pinto et al., 2009). After adding isopropanol and letting the samples sit for 10 minutes at room temperature, the tubes were centrifuged at 12,000 g and 4°C for 30 minutes. The ethanol used for the RNA wash was ice cold.

PCR of DNase-treated RNA

To check for the complete removal of DNA, the DNase-treated RNA was used as the PCR template, along with the primers for RT-qPCR and 0.5 μL GoTaq® DNA Polymerase (Promega Corporation) in a 50 μL reaction according to the manufacturer's instructions. The thermal cycler settings were 95°C for 2 minutes, 40 cycles of (95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 12 seconds), followed by 72°C for 5 minutes. The primer concentration had been previously determined by running PCR following the above protocol using 50-100 ng of gDNA or water as the templates. The primer concentration that produced only the correct band using gDNA, but no band using water, was selected. The RT-qPCR primers had also been verified to be specific to the gene of interest by PCR (following the above protocol), using 50-100 ng gDNA from the recombinant culture and from the wild type culture, followed by gel visualization. The oligonucleotides were purchased from Integrated DNA Technologies and are summarized in Table 2.5.

PCR of cDNA

The concentration of cDNA needed for RT-qPCR was initially estimated by varying the cDNA concentration in 25 μL PCR reactions. Other reaction components included RT-qPCR primers (100 – 250 nM, as determined for the verification of the DNase treatment) and 0.25 μL GoTaq®

DNA Polymerase (Promega Corporation), following the manufacturer's instructions. The thermal cycler settings were 95°C for 2 minutes, 40 cycles of (95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 12 seconds), followed by 72°C for 5 minutes. The gel visualization also verified the correct amplicon size.

RT-qPCR

RT-qPCR was performed using Power SYBR® Green PCR Master Mix (Life Technologies™) according to the manufacturer's instructions. The reaction components included 25 μL Power SYBR® Green PCR Master Mix, RT-qPCR primers (100 – 250 nM, as determined for the verification of the DNase treatment), and 0.5 or 1 μL of the cDNA library (for each biological replicate in its set of testing conditions) in a 50 μL reaction. The volume of the cDNA library was varied between technical replicates to look for inhibition. There were two biological replicates and two technical replicates for each set of testing conditions. The reaction conditions for the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) were 95°C for 10 minutes and 40 cycles of (95°C for 15 seconds and 60°C for 1 minute). After all of the cycles were complete, a melting curve was run, from 65-95°C, incrementing 0.5°C every 5 seconds. Fluorescence measurements were taken by the thermal cycler after each of the 40 cycles and every 5 seconds during the melting curve run. Melting curve analysis was used to verify the accuracy and specificity of the PCR product. No template controls (four replicates for each set of primers) were run to verify the absence of unintended PCR products (primer dimers) (Bustin et al., 2009). The CFX96 Touch™ Real-Time PCR Detection System software (Bio-Rad Laboratories, Inc.) used the baseline subtracted curve fit method to set the baseline and the single threshold method to determine the quantification cycle, C_q.

Measurement of dissolved oxygen levels

Oxygen sensor cultures, with the constitutively transcribed *fnr* (under control of the Bba J23104 promoter) and the oxygen-independent *fbfp* reporter (under control of an FNR-activated promoter), were grown following the testing conditions described in the Oxygen Sensor and AND Gate Testing Conditions (Materials and Methods), in triplicate for each time point. The dissolved oxygen for each culture was measured with a Clarke electrode at 1 hour, 48 hours, and 72 hours after induction. The membrane of the Clarke electrode was tested (for the verification) by measuring the dissolved oxygen of room temperature water that had been shaken for two minutes (saturation), followed by the addition of sodium hydrosulfite to remove the dissolved oxygen. Each liquid culture of the oxygen sensor strain was loaded into the electrode and the dissolved oxygen was recorded. When measuring the dissolved oxygen of the cultures that had been grown in the dark, the electrode was kept in the dark. The electrode was washed a minimum of six times after the addition of sodium hydrosulfite and a minimum of three times between each culture. The dissolved oxygen level (%) was calculated by dividing the measured value for each oxygen sensor culture by the measured value of saturated room temperature water, and then by multiplying by 100.

Testing the oxygen sensor at different light intensities

Oxygen sensor cultures, with the constitutively transcribed *fnr* (under control of the Bba J23104 promoter) and the oxygen-independent *fbfp* reporter (under control of an FNR-activated promoter), were grown, in triplicate, following the testing conditions described in the Oxygen Sensor and AND Gate Testing Conditions (Materials and Methods), except for the light intensity. All cultures were grown under continuous white light, at either 50 μmol of photons $m^{-2}s^{-1}$ (μE) or 200 μE. Normalized fluorescence values were calculated: $[((F\text{-}F_{media})/A)_{RC}$ - ((F-

$F_{media})/A)_{average\ WT}]_{set\ of\ testing\ conditions}$. This normalized fluorescence value for the phototrophic cultures was then divided by that of the corresponding anaerobic dark cultures (and then multiplied by 100) to obtain the normalized fluorescence (%), as shown in Figure 2.4.

2.7 References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25(17):3389-402.

Angermayr SA, Paszota M, Hellingwerf KJ. 2012. Engineering a cyanobacterial cell factory for production of lactic acid. Appl Environ Microbiol 78(19):7098-106.

Aoki R, Takeda T, Omata T, Ihara K, Fujita Y. 2012. MarR-type transcriptional regulator ChlR activates expression of tetrapyrrole biosynthesis genes in response to low-oxygen conditions in cyanobacteria. J Biol Chem 287(16):13500-7.

Arkin A. 2008. Setting the standard in synthetic biology. Nat Biotechnol 26(7):771-4.

Aryal UK, Callister SJ, Mishra S, Zhang X, Shutthanandan JI, Angel TE, Shukla AK, Monroe ME, Moore RJ, Koppenaal DW and others. 2013. Proteome analyses of strains ATCC 51142 and PCC 7822 of the diazotrophic cyanobacterium Cyanothece sp. under culture conditions resulting in enhanced H(2) production. Appl Environ Microbiol 79(4):1070-7.

Bandyopadhyay A, Elvitigala T, Welsh E, Stockel J, Liberton M, Min H, Sherman LA, Pakrasi HB. 2011. Novel metabolic attributes of the genus cyanothece, comprising a group of unicellular nitrogen-fixing Cyanothece. MBio 2(5).

Bandyopadhyay A, Stockel J, Min H, Sherman LA, Pakrasi HB. 2010. High rates of photobiological H2 production by a cyanobacterium under aerobic conditions. Nat Commun 1:139.

Berla BM, Pakrasi HB. 2012. Upregulation of plasmid genes during stationary phase in Synechocystis sp. strain PCC 6803, a cyanobacterium. Appl Environ Microbiol 78(15):5448-51.

Berla BM, Saha R, Immethun CM, Maranas CD, Moon TS, Pakrasi HB. 2013. Synthetic biology of cyanobacteria: unique challenges and opportunities. Front Microbiol 4:246.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL and others. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55(4):611-22.

Camsund D, Heidorn T, Lindblad P. 2014. Design and analysis of LacI-repressed promoters and DNA-looping in a cyanobacterium. J Biol Eng 8(1):4.

Crack JC, Green J, Thomson AJ, Le Brun NE. 2012. Iron-sulfur cluster sensor-regulators. Curr Opin Chem Biol 16(1-2):35-44.

Drepper T, Eggert T, Circolone F, Heck A, Krauss U, Guterl JK, Wendorff M, Losi A, Gartner W, Jaeger KE. 2007. Reporter proteins for in vivo fluorescence without oxygen. Nat Biotechnol 25(4):443-5.

Ducat DC, Way JC, Silver PA. 2011. Engineering cyanobacteria to generate high-value products. Trends Biotechnol 29(2):95-103.

Ejima K, Kawaharada T, Inoue S, Kojima K, Nishiyama Y. 2012. A change in the sensitivity of elongation factor G to oxidation protects photosystem II from photoinhibition in Synechocystis sp. PCC 6803. FEBS Lett 586(6):778-83.

Engler C, Kandzia R, Marillonnet S. 2008. A one pot, one step, precision cloning method with high throughput capability. PLoS One 3(11):e3647.

Englund E, Andersen-Ranberg J, Miao R, Hamberger B, Lindberg P. 2015. Metabolic Engineering of Synechocystis sp. PCC 6803 for Production of the Plant Diterpenoid Manoyl Oxide. ACS Synth Biol.

Espah Borujeni A, Channarasappa AS, Salis HM. 2014. Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites. Nucleic Acids Res 42(4):2646-59.

French CS, Smith JHC, Virgin HI, Airth RL. 1956. Fluorescence-Spectrum Curves of Chlorophylls, Pheophytins, Phycoerythrins, Phycocyanins and Hypericin. Plant Physiology 31(5):369-374.

Gimpel JA, Specht EA, Georgianna DR, Mayfield SP. 2013. Advances in microalgae engineering and synthetic biology applications for biofuel production. Current Opinion in Chemical Biology 17(3):489-495.

Golden SS, Brusslan J, Haselkorn R. 1987. Genetic engineering of the cyanobacterial chromosome. Methods Enzymol 153:215-31.

Grainger DC, Aiba H, Hurd D, Browning DF, Busby SJ. 2007. Transcription factor distribution in Escherichia coli: studies with FNR protein. Nucleic Acids Res 35(1):269-78.

Griese M, Lange C, Soppa J. 2011. Ploidy in cyanobacteria. FEMS Microbiology Letters 323(2):124-131.

Gronenberg LS, Marcheschi RJ, Liao JC. 2013. Next generation biofuel engineering in prokaryotes. Curr Opin Chem Biol 17(3):462-71.

Guerrero F, Carbonell V, Cossu M, Correddu D, Jones PR. 2012. Ethylene synthesis and regulated expression of recombinant protein in Synechocystis sp. PCC 6803. PLoS One 7(11):e50470.

Heidorn T, Camsund D, Huang HH, Lindberg P, Oliveira P, Stensjo K, Lindblad P. 2011. Synthetic biology in cyanobacteria engineering and analyzing novel functions. Methods Enzymol 497:539-79.

Huang HH, Camsund D, Lindblad P, Heidorn T. 2010. Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. Nucleic Acids Res 38(8):2577-93.

Huang HH, Lindblad P. 2013. Wide-dynamic-range promoters engineered for cyanobacteria. J Biol Eng 7(1):10.

Imashimizu M, Tanaka K, Shimamoto N. 2011. Comparative Study of Cyanobacterial and E. coli RNA Polymerases: Misincorporation, Abortive Transcription, and Dependence on Divalent Cations. Genet Res Int 2011:572689.

Kang Y, Weber KD, Qiu Y, Kiley PJ, Blattner FR. 2005. Genome-wide expression analysis indicates that FNR of Escherichia coli K-12 regulates a large number of genes of unknown function. J Bacteriol 187(3):1135-60.

Kittleson JT, Cheung S, Anderson JC. 2011. Rapid optimization of gene dosage in E. coli using DIAL strains. J Biol Eng 5:10.

Kunert A, Hagemann M, Erdmann N. 2000. Construction of promoter probe vectors for Synechocystis sp. PCC 6803 using the light-emitting reporter systems Gfp and LuxAB. J Microbiol Methods 41(3):185-94.

Landry BP, Stockel J, Pakrasi HB. 2013. Use of Degradation Tags To Control Protein Levels in the Cyanobacterium Synechocystis sp. Strain PCC 6803. Appl Environ Microbiol 79(8):2833-5.

Lou C, Stanton B, Chen Y-J, Munsky B, Voigt CA. 2012. Ribozyme-based insulator parts buffer synthetic circuits from genetic context. Nat Biotech 30(11):1137-1142.

Maier T, Guell M, Serrano L. 2009. Correlation of mRNA and protein in complex biological samples. FEBS Lett 583(24):3966-73.

Markley AL, Begemann MB, Clarke RE, Gordon GC, Pfleger BF. 2014. Synthetic Biology Toolbox for Controlling Gene Expression in the Cyanobacterium Synechococcus sp. strain PCC 7002. ACS Synth Biol.

Mitschke J, Georg J, Scholz I, Sharma CM, Dienst D, Bantscheff J, Voss B, Steglich C, Wilde A, Vogel J and others. 2011. An experimentally anchored map of transcriptional start sites in the model cyanobacterium Synechocystis sp. PCC6803. Proc Natl Acad Sci U S A 108(5):2124-9.

Mohamed A, Eriksson J, Osiewacz HD, Jansson C. 1993. Differential expression of the psbA genes in the cyanobacterium Synechocystis 6803. Mol Gen Genet 238(1-2):161-8.

Moon TS, Lou C, Tamsir A, Stanton BC, Voigt CA. 2012. Genetic programs constructed from layered logic gates in single cells. Nature 491(7423):249-53.

Mukherjee A, Weyant KB, Walker J, Schroeder CM. 2012. Directed evolution of bright mutants of an oxygen-independent flavin-binding fluorescent protein from Pseudomonas putida. J Biol Eng 6(1):20.

Oliver JW, Machado IM, Yoneda H, Atsumi S. 2013. Cyanobacterial conversion of carbon dioxide to 2,3-butanediol. Proc Natl Acad Sci U S A 110(4):1249-54.

Oliver JWK, Atsumi S. 2014. Metabolic design for cyanobacterial chemical synthesis. Photosynthesis Research 120(3):249-261.

Pinto F, Pacheco CC, Ferreira D, Moradas-Ferreira P, Tamagnini P. 2012. Selection of suitable reference genes for RT-qPCR analyses in cyanobacteria. PLoS One 7(4):e34983.

Pinto F, Thapper A, Sontheim W, Lindblad P. 2009. Analysis of current and alternative phenol based RNA extraction methodologies for cyanobacteria. BMC Molecular Biology 10(1):1-8.

Pitt FD, Mazard S, Humphreys L, Scanlan DJ. 2010. Functional Characterization of Synechocystis sp. Strain PCC 6803 pst1 and pst2 Gene Clusters Reveals a Novel Strategy for Phosphate Uptake in a Freshwater Cyanobacterium. Journal of Bacteriology 192(13):3512-3523.

Rubio LM, Ludden PW. 2005. Maturation of nitrogenase: a biochemical puzzle. J Bacteriol 187(2):405-14.

Salis HM, Mirsky EA, Voigt CA. 2009. Automated design of synthetic ribosome binding sites to control protein expression. Nat Biotechnol 27(10):946-50.

Schyns G, Jia L, Coursin T, Tandeau de Marsac N, Houmard J. 1998. Promoter recognition by a cyanobacterial RNA polymerase: in vitro studies with the Calothrix sp. PCC 7601 transcriptional factors RcaA and RcaD. Plant Mol Biol 36(5):649-59.

Stockel J, Welsh EA, Liberton M, Kunnvakkam R, Aurora R, Pakrasi HB. 2008. Global transcriptomic analysis of Cyanothece 51142 reveals robust diurnal oscillation of central metabolic processes. Proc Natl Acad Sci U S A 105(16):6156-61.

Summerfield TC, Nagarajan S, Sherman LA. 2011. Gene expression under low-oxygen conditions in the cyanobacterium Synechocystis sp. PCC 6803 demonstrates Hik31-dependent and -independent responses. Microbiology 157(Pt 2):301-12.

Taton A, Unglaub F, Wright NE, Zeng WY, Paz-Yepes J, Brahamsha B, Palenik B, Peterson TC, Haerizadeh F, Golden SS and others. 2014. Broad-host-range vector system for synthetic biology and biotechnology in cyanobacteria. Nucleic Acids Res 42(17):e136.

Varman AM, Xiao Y, Pakrasi HB, Tang YJ. 2013a. Metabolic engineering of Synechocystis sp. strain PCC 6803 for isobutanol production. Appl Environ Microbiol 79(3):908-14.

Varman AM, Yu Y, You L, Tang YJ. 2013b. Photoautotrophic production of D-lactic acid in an engineered cyanobacterium. Microb Cell Fact 12:117.

Viola S, Ruhle T, Leister D. 2014. A single vector-based strategy for marker-less gene replacement in Synechocystis sp. PCC 6803. Microb Cell Fact 13:4.

Wang B, Pugh S, Nielsen DR, Zhang W, Meldrum DR. 2013. Engineering cyanobacteria for photosynthetic production of 3-hydroxybutyrate directly from CO2. Metabolic Engineering 16:68-77.

Welsh EA, Liberton M, Stockel J, Loh T, Elvitigala T, Wang C, Wollam A, Fulton RS, Clifton SW, Jacobs JM and others. 2008. The genome of Cyanothece 51142, a unicellular diazotrophic cyanobacterium important in the marine nitrogen cycle. Proc Natl Acad Sci U S A 105(39):15094-9.

Willems E, Leyns L, Vandesompele J. 2008. Standardization of real-time PCR gene expression data from independent biological replicates. Anal Biochem 379(1):127-9.

Yang F, Moss LG, Phillips GN, Jr. 1996. The molecular structure of green fluorescent protein. Nat Biotechnol 14(10):1246-51.

Yu Y, You L, Liu D, Hollinshead W, Tang YJ, Zhang F. 2013. Development of Synechocystis sp. PCC 6803 as a Phototrophic Cell Factory. Marine Drugs 11(8):2894-2916.

Zang X, Liu B, Liu S, Arunakumara KK, Zhang X. 2007. Optimum conditions for transformation of Synechocystis sp. PCC 6803. J Microbiol 45(3):241-5.

Chapter 3: Physical, Chemical, and Metabolic State Sensors Expand the Synthetic Biology Toolbox for *Synechocystis* sp. PCC 6803

This chapter was has been submitted to *Biotechnology and Bioengineering*

Abstract

Many under-developed organisms possess important traits that can boost the effectiveness and sustainability of microbial biotechnology. Photoautotrophic cyanobacteria can utilize the energy captured from light to fix carbon dioxide for their metabolic needs while living in environments not suited for growing crops. Many value-added compounds have been produced by cyanobacteria in the laboratory; yet, the products' titers and yields are often not industrially relevant and lag behind what have been accomplished in heterotrophic microbes. Genetic tools for biological process control are needed to take advantage of cyanobacteria's beneficial qualities, as tool development also lags behind what has been created in common heterotrophic hosts. To address this problem, we developed a suite of sensors that regulate transcription in the model cyanobacterium *Synechocystis* sp. PCC 6803 in response to metabolically relevant signals, including light and the cell's nitrogen status, and a family of sensors that respond to the inexpensive chemical, L-Arabinose. Expanding the synthetic biology toolbox with more complex and precise control of gene expression for this cyanobacterium improves our ability to utilize this important under-developed organism in biotechnology.

3.1 Introduction

Many value-added compounds can be created through microbial biotechnology, including plastics, pharmaceuticals, nutraceuticals, beauty products, fuels, and commodity chemicals (Immethun et al., 2013; Lopes, 2015). However, producing these compounds causes competition for finite cellular resources. Strict control of transcription can reduce the metabolic burden and improve process stability (Weisse et al., 2015). Genetic sensors and circuits have provided stringent pathway control through gene regulation (Bradley et al., 2016; O'Connor and Adams, 2010), improving product yields and titers (Chubukov et al., 2016; Du et al., 2011; Lo et al., 2013). As biological processes transition from lab scale to industrial scale production, biological sensors are needed to link control of transcription to specific and relevant signals (Hoynes-O'Connor and Moon, 2015).

Most genetic control systems have been created for heterotrophs, primarily *Escherichia coli* and *Saccharomyces cerevisiae* (Immethun et al., 2013; Peralta-Yahya et al., 2012). Phototrophs harness light for their energy needs, making them a more sustainable biotechnology platform than heterotrophs, which must be supplied with a source of reduced carbon (Berla et al., 2013; Ducat et al., 2011). Cyanobacteria are fast-growing phototrophs (Yokoo et al., 2015) that can live in conditions that do not support the growth of crops; thus, they do not compete for arable land (Hollinshead et al., 2014; Pade and Hagemann, 2015). The model cyanobacterium, *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) has been used to produce many compounds of interest (Angermayr et al., 2015; Oliver and Atsumi, 2014) due to the availability of comprehensive genomic information and the ease of genetic manipulation (Ducat et al., 2011). Yet, the titers achieved in *Synechocystis*, and other cyanobacteria, are lower than those achieved in the model heterotrophs (Chen and Nielsen, 2013; Markley et al., 2014).

The availability of genetic control systems, which can boost production through precise regulation of heterologous gene expression, is limited in cyanobacteria (Berla et al., 2013; Huang et al., 2010; Ramey et al., 2015; Zess et al., 2016). Inducible promoters provide a defined transcriptional response upon sensing specific inputs. For example, metal-inducible promoters have been used in cyanobacteria (Berla et al., 2013; Huang and Lindblad, 2013). However, these regulators often suffer from cross talk, responding to multiple ions; furthermore, controlling the concentration of metal ions can be difficult due to their ubiquitous nature. Exogenous inducible promoters have recently been engineered for *Synechocystis*. The TetR-regulated P_{tet} (Huang and Lindblad, 2013) and the LacI-regulated P_{tac} (Albers et al., 2015) respond to chemical inducers, and the FNR-regulated P_{O_2} responds to oxygen (Immethun et al., 2016). While the number of synthetic regulators has grown recently, there are still few that can be used reliably in *Synechocystis*.

To expand the synthetic biology toolbox for *Synechocystis*, we developed sensors that regulate transcription in response to different classes of inputs, including physical, chemical and metabolic state signals. In *E. coli*, a chimeric light sensor has been shown to turn on in the dark, but not the light (Levskaya et al., 2005). We adapted this chimeric system for use in *Synechocystis*, showing that it responds to light, an important physical signal for cyanobacteria. P_{BAD} expresses the genes responsible for arabinose catabolism in *E. coli* (Schleif, 2010). Since *Synechocystis*' growth was not enhanced by L-Arabinose (hereafter arabinose), we utilized this well-studied *E. coli* promoter to create a family of transcriptional regulators that are controlled by the inexpensive chemical. Regulators that sense and respond to a cell's metabolic state can control the synthesis of a desired product to the optimal level without imposing severe burden. We employed native promoters from *Synechocystis* that respond to a 2-oxoglutarate-activated

transcription factor NtcA or the NtcA-activated transcription factor NrrA to create a library of nitrogen status regulators. These new sensors increase the number and type of devices that can be used to reliably engineer *Synechocystis*, expanding the number of potential applications for this promising biotechnology platform.

3.2 Materials and Methods

3.2.1 Strains, Plasmids, and Growth Conditions

All plasmids were constructed in *E. coli* DH10B. *E. coli* strains were grown in LB (Miller, AMRESCO) at 37°C and 250 rpm in 14 mL BD Falcon™ round-bottom tubes. Kanamycin (kan) (20 μg/mL), chloramphenicol (cm) (34 μg/mL) and spectinomycin (spec) (100 μg/mL) were added as appropriate. Plasmids were constructed using blunt-end ligation-based cloning or the Golden Gate assembly method (Engler et al., 2008). The plasmids were sequence-verified at the Washington University in St. Louis, School of Medicine's Protein and Nucleic Acid Chemistry Laboratory. The plasmids are summarized in Table 3.1. The gene sequences are listed in Table 3.2. Enzymes were purchased from New England BioLabs, Inc.

Table 3.1 Plasmids used in this work.

Name	Parts	Type
pCI094	RSF1010 replicon; spec^R; *P$_{gapI}$-fbfp f37t*	Replicative
pCI095	RSF1010 replicon; spec^R; *P$_{slr1852}$-fbfp f37t*	Replicative
pCI116	RSF1010 replicon; spec^R; *P$_{nrrA}$-fbfp f37t*	Replicative
pCI117	RSF1010 replicon; spec^R; *P$_{ntcA}$-fbfp f37t*	Replicative
pCI118	RSF1010 replicon; spec^R; *P$_{sigE}$-fbfp f37t*	Replicative
pCI119	RSF1010 replicon; spec^R; *P$_{glnN}$-fbfp f37t*	Replicative
pCI120	RSF1010 replicon; spec^R; *P$_{icd}$-fbfp f37t*	Replicative
pCI125	M13/pBR322 ori; kan^R; *P$_{gapI}$-nrrA 51142*	Integrative (NSP1)
pCI126	M13/pBR322 ori; kan^R; *P$_{slr1852}$-nrrA 51142*	Integrative (NSP1)
pCI128	RSF1010 replicon; spec^R; *P$_{BAD}$-eyfp; Pc-araC*	Replicative

pCI129	RSF1010 replicon; spec^R; *PBAD-eyfp O2-; Pc-araC*	Replicative
pCI130	RSF1010 replicon; spec^R; *PBAD-eyfp O1-; Pc-araC*	Replicative
pCI131	RSF1010 replicon; spec^R; *PBAD-eyfp I1-; Pc-araC*	Replicative
pCI132	RSF1010 replicon; spec^R; *PBAD-eyfp I2-; Pc-araC*	Replicative
pCI133	RSF1010 replicon; spec^R; *PBAD-eyfp CAP-; Pc-araC*	Replicative
pCI136	RSF1010 replicon; spec^R; *PBAD-eyfp O1- +22 1; Pc-araC*	Replicative
pCI143	RSF1010 replicon; spec^R; *PBAD-eyfp O1- +22 2; Pc-araC*	Replicative
pCI145	f1/pBR322 ori; kan^R; *Bba J23104-ompR*	Integrative (NS1)
pCI146	M13/pBR322 ori; kan^R; *Bba J23104-ompR*	Integrative (NSP1)
pCI148	RSF1010 replicon; cm^R; *PTet-cph8; PompC-eyfp*	Replicative

Table 3.2 Genetic parts used in this work.

Part name	Type and source	DNA sequence
ompR	Gene *E. coli* MG1655 Gene ID: 947913[a]	atgcaagagaactacaagattctggtggtcgatgacgacatgcgcctgcgtgcgctgctggaacgttatctcaccgaacaa ggcttccaggttcgaagcgtcgctaatgcagaacagatggatcgcctgctgactcgtgaatctttccatcttatggtactgga tttaatgttacctggtgaagatggcttgtcgatttgccgacgtcttcgtagtcagagcaacccgatgccgatcattatggtgac ggcgaaaggggaagaagtggaccgtatcgtaggcctggagattggcgctgacgactacattccaaaaccgtttaacccg cgtgaactgctggcccgtatccgtgcggtgctgcgtcgtcaggcgaacgaactgccaggcgcaccgtcacaggaagag gcggtaattgctttcggtaagttcaaacttaacctcggtacgcgcgaaatgttccgcgaagacgagccgatgccgctcacc agcggtgagtttgcggtactgaaggcactggtcagccatccgcgtgagccgctctcccgcgataagctgatgaaccttgc ccgtggtcgtgaatattccgcaatggaacgctccatcgacgtgcagatttcgcgtctgcgccgcatggtggaagaagatcc agcgcatccgcgttacattcagaccgtctggggtctgggctacgtctttgtaccggacggctctaaagcatga
	5’ UTR	cttctctgctgttaaggtttgcttagatacaaattgttgcgaacctttgggagtacaaaca

cph8	Gene (Levskaya et al., 2005)	atggccaccaccgtacaactcagcgaccaatccctccgtcagctagaaaccctcgccatccacaccgcccacctgattca gccccacggtttagtggtggtcctgcaggaaccagacctcaccatcagccaaattagcgccaactgcaccggcattttag ggcgatcgccagaggatttgttgggcagaaccctaggggaagtgtttgatagctttcagattgatcccatccagagtcgcct aacggccggacaaatcagcagcctcaaccccagtaaactttgggcgcgggtcatgggggacgactttgtcatttttgacg gggtttttcatcgcaacagtgacggtttattggtatgtgaactcgagccagcctacacttccgataatctgcccttcctcggttt ttatcacatggccaacgctgccctgaatcggttgcgccaacaagctaatctacgggatttctacgatgttattgtcgaagaag tccgccgtatgactggctttgaccgggtgatgctataccgctttgatgaaaataaccacggtgatgtcattgccgaagataaa cgggatgatatggaaccctatttgggcctgcactatcccgaatcggatattccccaacccgcccgtcggctatttatccaca accccattcgagtaattcccgatgtttatggtgtggcggtgcccctgaccccagcggttaaccccagcaccaaccgagcg gtggatttaacagaatccattctgcgcagtgcgtaccattgccacttgacctatctgaaaaatatgggggtaggagcgtcttt aaccatttccctaattaaggacggccatctctgggggctcattgcctgccaccatcaaacccccaaagtaattccctttgaac tgcgtaaagcctgcgaatttttggtcgggtggtgtttagcaacatttccgcccaggaagatacggaaaccttcgattaccgg gtgcagctggcggagcatgaagcggttttattggacaaaatgaccacggcggcggattttgtcgaaggattaactaatcat cccgatcgcctgttgggattaacgggctcccaggggcggccatttgctttggggaaaaattgattttagtaggggaaacc ccggacgagaaagcagtgcaatatttactgcaatggttggagaatcgggaagtgcaagacgttttcttcacctcttccctctc acaaatttatcctgatgcagtgaattttaaatccgtggccagtggcttattggccattcccattgcccgtcacaacttttgctct ggtttcgccctgaagtgttgcaaacggttaattggggcggtgacccaaatcatgcttacgaagctacccaggaagacggta aaatcgagctccatccccgccaatcctttgacctctggaaagaaattgtccgactccaatctttgccctggcaatcggtgga aatccaaagtgccctggccctgaaaaaggcgatcgtcaacctcattttgcgccaggcagaagaattgcatatggcggctg gtgttaagcaactggcggatgaccgcacgctgctgatggcgggggtaagtcacgacttgcgcacgccgctgacgcgtat tcgcctggcgactgagatgatgagcgagcaggatggctatctggcagaatcgatcaataaagatatcgaagagtgcaac gccatcattgagcagtttatcgactacctgcgcaccgggcaggagatgccgatggaaatggcggatcttaatgcagtactc ggtgaggtgattgctgccgaaagtggctatgagcgggaaattgaaaccgcgctttaccccggcagcattgaagtgaaaat gcacccgctgtcgatcaaacgcgcggtggcgaatatggtggtcaacgccgcccgttatggcaatggctgggtcaaagtc agcagcggaacggagccgaatcgcgcctggttccaggtggaagatgacggtccgggaattgcgccggaacaacgtaa gcacctgttccagccgtttgtccgcggcgacagtgcgcgcaccattagcggcacgggattagggctggcaattgtgcagc gtatcgtggataaccataacgggatgctggagcttggcaccagcgagcggggcgggctttccattcgcgcctggctgcc agtgccggtaacgcgggcgcagggcatgacaaaagaagggtaa
	5’ UTR	atgagcacatcagcaggacgcactgaccgaattcattaaagaggagaaaggtaccc
P_{Tet}	Promoter (Moon et al., 2012)	tccctatcagtgatagagattgacatccctatcagtgatagagatactgagcacat
P_{ompC}	Promoter *E. coli* MG1655 GenBank: CP014225.1: 510030 to 510137[b]	tcccttgcatttacattttgaaacatctatagcgataaatgaaacatcttaaaagttttagtatcatattcgtgttggattattctgca tttttggggagaatggact
P_{BAD}, including P_C	Promoter (Moon et al., 2012)	aagaaaccaattgtccatattgcatcagacattgccgtcactgcgtcttttactggctcttctcgctaaccaaaccggtaaccc cgcttattaaaagcattctgtaacaaagcgggaccaaagccatgacaaaaacgcgtaacaaaagtgtctataatcacggca gaaaagtccacattgattatttgcacggcgtcacactttgctatgccatagcatttttatccataagattagcggatcctacctg acgctttttatcgcaactctctactgtttctccatac
P_{BAD} *O1-*	Promoter	aagaaaccaattgtccatattgcatcagacattgccgtcactgcgtcttttactggctcttctcgctaaccaaaccggtaaccc cgcttattaaaagcattctgtaacaaagcgggaccaaagccatgacaaaaacgcgtaacaaaagtgtctataatcatatttg cacggcgtcacactttgctatgccatagcatttttatccataagattagcggatcctacctgacgctttttatcgcaactctctac tgtttctccatac
P_{BAD} *O1-+22 1*	Promoter	aagaaaccaattgtccatattgcatcagacattgccgtcactgcgtcttttactggctcttctcgctaaccaaaccggtaaccc cgcttattaaaagcattctgtaacaaagcgggaccaaagccatgacaaaaacgcgtaacaaaagtgtctataatcaaatttc ataaagattgctttggtatttgcacggcgtcacactttgctatgccatagcatttttatccataagattagcggatcctacctgac gctttttatcgcaactctctactgtttctccatac

PBAD O1-+22 2	Promoter	aagaaaccaattgtccatattgcatcagacattgccgtcactgcgtcttttactggctcttctcgctaaccaaaccggtaacccc cgcttattaaaagcattctgtaacaaagcgggaccaaagccatgacaaaaacgcgtaacaaaagtgtctataatcagtaatc atagctgactttatttttatttgcacggcgtcacactttgctatgccatagcatttttatccataagattagcggatcctacctgacg ctttttatcgcaactctctactgtttctccatac
PBAD O2-	Promoter	aagaaattgcatcagacattgccgtcactgcgtcttttactggctcttctcgctaaccaaaccggtaaccccgcttattaaaag cattctgtaacaaagcgggaccaaagccatgacaaaaacgcgtaacaaaagtgtctataatcacggcagaaaagtccaca ttgattatttgcacggcgtcacactttgctatgccatagcatttttatccataagattagcggatcctacctgacgctttttatcgc aactctctactgtttctccatac
PBAD CRP-	Promoter	aagaaaccaattgtccatattgcatcagacattgccgtcactgcgtcttttactggctcttctcgctaaccaaaccggtaaccc cgcttattaaaagcattctgtaacaaagcgggaccaaagccatgacaaaaacgcgtaacaaaagtgtctataatcacggca gaaaagtccacattgattatttgcacggcgtcacactatagcatttttatccataagattagcggatcctacctgacgcttttat cgcaactctctactgtttctccatac
PBAD I1-	Promoter	aagaaaccaattgtccatattgcatcagacattgccgtcactgcgtcttttactggctcttctcgctaaccaaaccggtaaccc cgcttattaaaagcattctgtaacaaagcgggaccaaagccatgacaaaaacgcgtaacaaaagtgtctataatcacggca gaaaagtccacattgattatttgcacggcgtcacactttgctatgccatacataagattagcggatcctacctgacgcttttat cgcaactctctactgtttctccatac
PBAD I2-	Promoter	aagaaaccaattgtccatattgcatcagacattgccgtcactgcgtcttttactggctcttctcgctaaccaaaccggtaaccc cgcttattaaaagcattctgtaacaaagcgggaccaaagccatgacaaaaacgcgtaacaaaagtgtctataatcacggca gaaaagtccacattgattatttgcacggcgtcacactttgctatgccatagcatttttatccataagattagcctgacgcttttat cgcaactctctactgtttctccatac
araC	Gene (Moon et al., 2012)	atggctgaagcgcaaaatgatcccctgctgccgggatactcgtttaatgcccatctggtggcgggtttaacgccgattgag gccaacggttatctcgatttttttatcgaccgaccgctgggaatgaaaggttatattctcaatctcaccattcgcggtcagggg gtggtgaaaaatcagggacgagaatttgtttgccgaccgggtgatattttgctgttcccgccaggagagattcatcactacg gtcgtcatccggaggctcgcgaatggtatcaccagtgggtttactttcgtccgcgcgcctactggcatgaatggcttaactg gccgtcaatatttgccaatacggggttctttcgcccggatgaagcgcaccagccgcatttcagcgacctgtttgggcaaatc attaacgccgggcaaggggaagggcgctattcggagctgctggcgataaatctgcttgagcaattgttactgcggcgcat ggaagcgattaacgagtcgctccatccaccgatggataatcgggtacgcgaggcttgtcagtacatcagcgatcacctgg cagacagcaattttgatatcgccagcgtcgcacagcatgtttgcttgtcgccgtcgcgtctgtcacatcttttccgccagcagt tagggattagcgtcttaagctggcgcgaggaccaacgtatcagccaggcgaagctgcttttgagcaccacccggatgcct atcgccaccgtcggtcgcaatgttggttttgacgatcaactctatttctcgcgggtatttaaaaaatgcaccggggccagccc gagcgagttccgtgccggttgtgaagaaaaagtgaatgatgtagccgtcaagttgtcataa
	5’ UTR	gcgtttttgtcatggctttggtcccgctttgttacagaatgcttttaataagcggggttaccggtttggttagcgagaagagcca gtaaaagacgcagtgacggcaatgtctgatgcaatatggacaattggtttcttctctgaatggcgggagtatgaaaagt
eyfp	Gene (Landry et al., 2013)	atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccac aagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggc aagctgcccgtgccctggcccaccctcgtgaccaccttcggctacggcctgcaatgcttcgcccgctaccccgaccacat gaagctgcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggca actacaagacccgcgccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttca aggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgtctatatcatggccgacaagc agaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccgaccactacc agcagaacaccccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagctaccagtccgccctgagca aagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacg agctgtacaaggctgcgaattga
	5’ UTR for *PompC* construct	tgccgactttcacacatactagagaaagaggagaaatactag
	5’ UTR for *PBAD* constructs	cagcggataaagtagcaaagagaaggaggttagga

P_{sigE}	Promoter *Synechocystis* sp. PCC 6803 GenBank: CP012832.1: 1302116 to 1302203[b]	attaaggaaagctgacaaaaatggcgatcaaatcaagtttgtatcacgaattacactgccgtgaaaatttaacgatattttggacaga
P_{glnN}	Promoter *Synechocystis* sp. PCC 6803 GenBank: CP012832.1: 2126505 to 2126631[b]	ttcatattgtgatccaacgatgtcgacccatttacgcttccccttgggaggcaaagaaatgggtcgttgtttttgtatctatattgtctattttaaaaaatcatcttgcgtatgattgggggggtttg
P_{ntcA}	Promoter *Synechocystis* sp. PCC 6803 GenBank: CP012832.1: 1589135 to 1589324[b]	ggaatttatcagtgtgcctatcagaaatattcagggcgagtacatggtggtgcgaccctcggcgatcgttgccattcgggtggaaccaattttctttggcagtgtggagcgcatgtaatccgttgtgtataatcttgatacagaatggggttttgcacttcccttgatgccccccattaccgtgaacgtgc
P_{nrrA}	Promoter *Synechocystis* sp. PCC 6803 GenBank: CP012832.1: 3292160 to 3292307[b]	ggaaatggtacgaacaattaacattatggcgagcaaattctttatatttttttgaagatttgccccccccatgcaggtaactgttgttacaaagccttgacattgactttgttagattaacagggaactttcagtcaggggtctagatcg
P_{icd}	Promoter *Synechocystis* sp. PCC 6803 GenBank: CP012832.1: 282885 to 283036[b]	caagcatgaagataaccaaatagaagaacctagggtctgttatagaaattctccttactagtggagtgattttcagttccgcaatgggataaaaatttcgtaacagccaatgcaatcagagcctccagaaaggattatgatctgctccgtgc

$P_{slr1852}$	Promoter *Synechocystis* sp. PCC 6803 GenBank: CP012832.1: 1190756 to 1191122[b]	tcttccctagcttaggcttagtacgtttacaaatatttacatggttaaattttggcgatcgccaatggacagtctagggacaatta aaatgtcaagaattattgactaattgtcaaggaatgatagctgcaaaacgtcgaaatgggtcgagctggatttcctcagtaac atttcgttacagtggcaatttagtcgggagattatgctatgctttgcaccaaattagttctctttgaatttttctttcggagtgcgga gtattctggttctgaacaggtgaatttttttcaagcccgtcacccgtcggaactgacccccgctttgcttaaattaaactgattac ttttagttatcttggcaaatcatct
P_{gapI}	Promoter *Synechocystis* sp. PCC 6803 GenBank: CP012832.1: 1146992 to 1147289[b]	aatttagcagtttacagaggcgatttatcggcgggtaagactatacagtatcgggaaaaaattaagaacggtcaaagaatct ggacatatcacaacccacaatctagtattcaaaatccttctgcctggccttatttggtcgtatttacccattgtgcccaaatccg accattgttgccaattattccccaggtaaccacggcgatcgccaaggaaagatttaagtatttttttcccattctccctaatcctg cggccaaggagctgggttaacgttagggcaagtcggatgtcctggt
fbfp f37t	Gene (Mukherjee et al., 2012) 5’ UTR	atgagaggatcgcatcaccatcaccatcacggatccatgatcaacgcaaaactcctgcaactgatggtcgaacattccaac gatggcatcgttgtcgccgagcaggaaggcaatgagagcatccttatctacgtcaacccggccactgagcgcctgaccg gctactgcgccgacgatattctctatcaggacgcacgttttcttcagggcgaggatcacgaccagccgggcatcgcaatta tccgcgaggcgatccgcgaaggccgcccctgctgccaggtgctgcgcaactaccgcaaagacggcagcctgttctgga acgagttgtccatcacaccggtgcacaacgaggcggaccagctgacctactacatcggcatccagcgcgatgtcacagc gcaagtattcgccgaggaaagggttcgcgagctggaggctgaagtggcggaactgcgccggcagcagggccaggcc aagcactga atgagaattcacagaattcattaaagaggagaaattaact
nrrA	Gene *Cyanothece* sp. ATCC 51142 Gene ID: cce_1808[c] 5’ UTR	gtgagtctcatttataccgctattgttgagggaaatcctcatctacgctcattattaggttggcatctgcaacaggcaggatac acagcgcaacaatgtgcgaatctgcaacaagctcgtactgtctttacccgtcgccaacccaccttagtgatccttgacgctg atctatctgacggagatcctctagaattgtgccggtggcttcaccaacaacggcaatccatgattctcattttatcagcccgta acacagaaaaggatattgtcagaggattaaatgcaggggctgacgattatttgactaaacccttcggaatgcaagaatttat ggccagggttgaagccctcatcaggcgggtgcgtgttgcttctgctcctttgttccttgattatggggacttaaaaattgatttg gtacaacgccgagttcagtttcaagggcattttgtggatttaacccctcaagaatttagcttattgtatgtcctagctcaagcag aaggaaaccccctcagtcgttcagaattgctgcaaagagcttggccagaggcgatcgataatcctcgtaccattgacaccc atgttttgtccttacggaagaaaattgaaaccgatccccgacaaccgagtttaattcaaacagttcggaatgtaggttatcgct tcaactcagaagttatccaagagtcctccgcttcccccaccacttcatctcaaactcaatcagcaaaaatggcagccgttga acccccttaaatttaataagttatcgtcttga acgctaaggactgtcaaactagaaggacgaaaactaag

[a] http://www.ncbi.nlm.nih.gov/gene

[b] https://www.ncbi.nlm.nih.gov/nuccore

[c] http://genome.microbedb.jp/cyanobase/CCE/genes

Plasmids were naturally transformed into a glucose-tolerant strain of *Synechocystis* sp. PCC 6803 following the previously defined protocol (Immethun et al., 2016). The flanking regions used for integration into neutral sites in *Synechocystis* genome and endogenous plasmid are listed in Table 3.3. Colony PCR, as described previously, was used to confirm successful plasmid transformation, and segregation as appropriate (Immethun et al., 2016). Oligonucleotides for colony PCR, summarized in Table 3.4, were purchased from Integrated DNA Technologies. Engineered *Synechocystis* strains for this work are summarized in Table 3.5. Seed cultures of the *Synechocystis* strains were grown in 50 mL of BG11 media (http://www-cyanosite.bio.purdue.edu/media/table/BG11.html) in 250 mL baffled flasks at 30°C and 160 rpm, under continuous white light (50 μmol of photons $m^{-2}s^{-1}$(μE)) in ambient air.

Table 3.3 Flanking regions used for double homologous recombination.

Name	DNA sequence	Location
5' NS1	cggaacaggaccaagccttgatgcgaggatcaaaaacatcctccacaatgcccgctcgaatgggtcgatggagg gtcagaccatggccggtggcggatctttcccggtcaggacagaccactgtgacttcatggcctgcggcgactagg gtgttggccaaggtacgtacccccttgggaaaaaatgccgtcatcgttactgacaaggagattgaggaccggggatg gggtcataggacaccaaagaatcgagattgtgcctgccagccttaacgatatcaggctatgtcccgcttaaactctga ttcttaccaggtaactcctgggctccatggggccacaaccaggcagtattttgttcctttggccaatggggcgatcgg ggaaaaatggcttgatctggcatttacgagaaaaatttttatttttaatgatttatttttcctattaaaatctttttttaccttt ggaaaccaactgcaatctgagaaaccatcc	chromosome *psbA1*
3' NS1	tgtgccattgccataactgctttcggttagacttcgtttcatttggttaatcaagggcactctcgcaatggggtgccttta tggtccaaggttaaagttaagccagtaaagtttaagtctatttctagggtgaaatgtaatgaatcaatttagggactggg gtttttccactgattggtggcaaggcaaaaaaggggaatattgggtacttgggcaaacaattctctccgttggatttgta ctgttaccggtttatactcctactaatttacagttactagctgaacaaaatcagtggattgcctgggctggtactctgttttt tggcatgattgcggcagttttgctgattgggggggtctgcatttaggggaaaatctgactcccttgccccatcctaaa aaggatagtcaaa	chromosome *psbA1*
5' NSP1	atataatcccgttaacaggctaaacccatgcagggcatagataaacccaaaggggaaggggacatcatgccccg tatctcaccactcacctaagagtcccgataccagttaaggatgaagtgcagggcattattgaccgctacagggaaga cttactggcaggaagacagctccaagatgttcccagctcctacgaggtcaaaacggcgatcgccattctgacggag gcactcagccttaaagccaatgccggtggagccatcaaagcaaaaatcagagaagccctagctatcttggaaagg aactgaaatgagaaaactaaaacatcgccgccgccgctatcccgaaattgagtttgagattgtccaggcgttagagc aatttgggcccctgtccatccccgagatatttcaccacttgaggggtcgtcatcctccccgggccgtttttgattctctttt atcggctggaggaagtcgggggaatccataaagaaaagctgcaaataactctatccgcaccaccaccaccaccac acacacctatttctcaaaatgacagggaagggcaattgctggggaactgatagaaatctctgat	endogenous plasmid pCC5.2

3’ NSP1	gacaggagtttagccatgggattagtcagagtccctagccattgctgatgcgagattatttaaactttgccaagggata ttgaccagacgggccgtgctaccaagccgttgctgtcgcaagtgtcgcttaacccgcttctgggctatccaatggtta ttaatcttgtaaacgcttatcctgccctgctcactggcaatgtagaaccgcttatggactctaagccagttcttccagttt ggactctccaaactcagagggtacttagtccctgcaccgtgcaagtatccgtcaatcaccgtcggttgaggctccata cctacattgcccctatcaagccagttttagcacgctgggaagcacgataatctactgcgactaccctagggtactccc gtagaaaagcgttatgaagtatgaccggcaaaaaaccacattgaccctatcagagcaaagctagcacggtaggga acgcgataatctactgcaacacctataggtctgttgctgacacgcacgatgacgtatgacccttttagcacggtaggg agcgtgataatcttctgcaacacctatatagtattgttgcgatcgcgagcgatggcgt	endogenous plasmid pCC5.2

Table 3.4 Oligonucleotides used to assess *Synechocystis* transformation and segregation.

Name	Sequence (5’ to 3’)	Strains Checked
6803_psbu_for 6803_pppsbd_rev	gaggttagcaccgtggttaactc caccactgctccgattaaatgg	CI242, CI276
6803_kanpsbd_for 6803_kan_rev	aaaaagccgtttctgtaatgaagg ggcctgttgaacaagtctgg	CI242, CI276
PCC5.2_seq_f PCC5.2_seq_r	ctctgataccgtatccactgaatc gatttgtcagggtctaggtgtc	All using the integrative site NSP1
PCC5.2_seq_f 6803_kan_rev	ctctgataccgtatccactgaatc ggcctgttgaacaagtctgg	CI225, CI226, CI227, CI228, CI243, CI277
pompcNseq_F pompcNseq_R	cgagggtttctagctgacggagg gaaacgcctggtatctttatagtcctg	CI275, CI276, CI277
pBADseq_F pBADseq_R	gacgaccgtagtgatgaatctctcc acttcagggtcagcttgccgtag	CI231, CI232, CI233, CI234, CI237, CI265, CI274
ptetseq_for pgap1seq_rev	gcaaatcaatatcactgtgtggcttc gcagcgaatagaccagctatcc	CI164, CI165, CI225, CI226, CI227, CI228
fbfpseq_F fbfpseq_R	gtaaccagcaaatcaatatcactgtgtg ttgacgtagataaggatgctctcattg	CI203, CI204, CI205, CI206, CI207

Table 3.5 *Synechocystis* strains used in this work.

Name	Plasmids	sp. PCC 6803 Location
sp. PCC 6803	glucose tolerant wild-type	
CI164	pCI094	replicative
CI165	pCI095	replicative
CI203	pCI119	replicative
CI204	pCI120	replicative
CI205	pCI116	replicative
CI206	pCI117	replicative
CI207	pCI118	replicative
CI225	pCI095 pCI126	replicative NSP1
CI226	pCI095 pCI125	replicative NSP1

CI227	pCI094 pCI126	replicative NSP1
CI228	pCI094 pCI125	replicative NSP1
CI231	pCI129	replicative
CI232	pCI130	replicative
CI233	pCI132	replicative
CI234	pCI133	replicative
CI237	pCI131	replicative
CI242	pCI145	NS1
CI243	pCI146	NSP1
CI265	pCI143	replicative
CI274	pCI128	replicative
CI275	pCI148	replicative
CI276	pCI145 pCI148	NS1 replicative
CI277	pCI146 pCI148	NSP1 replicative

3.2.2 Testing Conditions

Seed cultures of *Synechocystis* were diluted to an optical density of 0.2 at 730 nm in a 50 mL culture, 24 hours before starting each test. They were grown in 250 mL baffled flasks at 30°C and 160 rpm, under continuous white light (50 μE) in ambient air. All three sensors were tested in clear 48-well cell culture plates (Greiner Bio-One), 600 μL per well. 600 μL of sterile water was added to all empty wells in the plate to reduce evaporation. The water was refilled daily. All plates were grown for 72 hours at 30°C and 285 rpm, under continuous white light (50 μE), except as noted for the light sensors. Testing conditions unique to each sensor are listed in the Supplemental Material.

3.2.3 Fluorescence Measurements

200 μL of each culture and 200 μL of the media (in triplicate), was loaded into black 96-well plates (flat bottom, chimney well, μclear (Greiner Bio-One)). Measurements were taken using a Tecan Infinite 200 Pro plate reader (absorbance at 730 nm, gain set to 100). For EYFP,

excitation was at 485 nm and emission at 528 nm, and for FbFP, excitation was at 450 nm and emission at 495 nm. Measurements were normalized as described previously (Immethun et al., 2016). Curves were fit using a modified Hill equation, while minimizing the root mean squared error (RMSE). The equations can be found in the Supplemental Material.

3.3 Results and Discussion

3.3.1 Physical sensors

Physical signals can link relevant conditions to the synthesis of a desired product. When utilizing photosynthetic organisms in biotechnology, light is an important physical signal. *Synechocystis* carries out the majority of its metabolic processes in the light (Saha et al., 2016); therefore, limitations of cellular resources (Cardinale and Arkin, 2012) could be especially acute if the heterologous pathway is expressed during the day. A dark-inducible sensor could also be beneficial for expressing enzymes inactivated by photosynthetic oxygen, including nitrogenase (Stockel et al., 2008) and hydrogenase (Melis et al., 2000).

The light (dark-inducible) sensors utilized the chimeric transmembrane Cph8, consisting of the histidine kinase domain from *E. coli*'s EnvZ (abbreviated as EnvZ*) linked to the photoreceptor from *Synechocystis*' Cph1 (abbreviated as Cph1*) (Levskaya et al., 2005; Olson et al., 2014). In the dark, as detected by Cph1*, EnvZ* auto-phosphorylates, subsequently phosphorylating *E. coli's* OmpR. The phosphorylated OmpR (p-OmpR) activates transcription from P_{ompC} (Figure 3.1a). In this work, *cph8* and *ompR* were expressed constitutively, from *pLtetO-1* (Moon et al., 2012) and from *Bba J23104* (http://parts.igem.org/Part:BBa_J23104), respectively. *Bba J23104-ompR* was integrated into a neutral site in *Synechocystis*' chromosome, called NS1 (Table 3.3), replacing *psbA1* and its promoter (Gene ID: 14618584; http://www.ncbi.nlm.nih.gov/gene) (Varman et al., 2013a), or a neutral site in the endogenous

plasmid pCC5.2, called NSP1 (Table 3.3) (Ng et al., 2015). *cph8* and *eyfp* were expressed in a broad host-range replicative plasmid containing the RSF1010 replicon (hereafter called the replicative plasmid) (Heidorn et al., 2011a). *PompC* controlled expression of *eyfp*, enhanced yellow fluorescent protein gene, when activated by a p-OmpR (Figure 3.1a).

When the cultures were grown in 50 μE white light, the normalized fluorescence was within one standard deviation of the average wild type fluorescence (Figure 3.1b). The strain with *Bba J23104-ompR* integrated into NSP1 also did not produce a fluorescent response above the wild type background when tested in 20 μE white light, while the expression from the strain with *Bba J23104-ompR* integrated into NS1 was leaky. Possible interactions with nearby DNA or RNA sequences (Lou et al., 2012) or other context dependencies (Arkin, 2008) could be responsible for the difference in behavior between the two strains when tested in 20 μE white light. In contrast, the cultures grown in the dark produced a significant fluorescent signal, regardless of the location of the constitutively-expressed transcription factor OmpR.

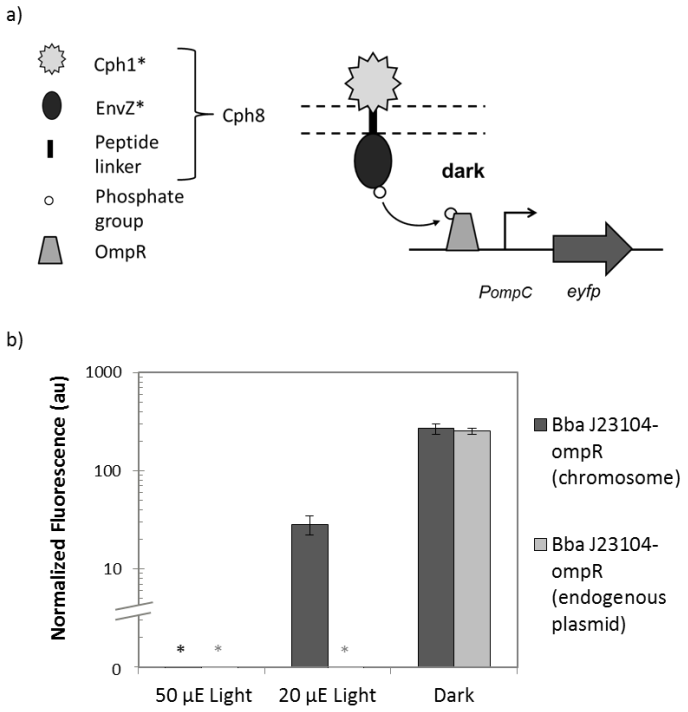


Figure 3.1 *E. coli*'s P_{ompC} responds to light in *Synechocystis* sp. PCC 6803. (**a**) Schematic of the gene for enhanced yellow fluorescent protein (*eyfp*) expressed from the chimeric light sensor (Levskaya et al., 2005). The histidine kinase domain of *E. coli*'s EnvZ (EnvZ*) is autophosphorylated in the absence of light, as detected by the photoreceptor of *Synechocystis*' Cph1 (Cph1*). EnvZ* phosphorylates OmpR, which then activates transcription from *E. coli*'s P_{ompC} promoter. (**b**) Normalized fluorescence $\left(\frac{(F_{RC}-F_{media})}{A_{RC}} - \left(\frac{(F_{WT}-F_{media})}{A_{WT}}\right)_{average}\right)_{light\ intensity}$ of *eyfp* controlled by P_{ompC} in a replicative plasmid containing the RSF1010 replicon in *Synechocystis*. The gene encoding the transcription factor OmpR was expressed constitutively from *Bba J23104*, and was integrated by double homologous recombination into a neutral site either in the chromosome (*psbA1*, called NS1 in this work) or the endogenous plasmid pCC5.2 (called NSP1 in this work). F is fluorescence (excitation at 485 nm and emission at 528 nm). A is absorbance (730 nm). RC is the recombinant culture. WT is wild type *Synechocystis*. All strains were grown in BG-11 media supplemented with 5 mM D-Glucose. The data reported are the average of at least three biological replicates measured three days after induction (Materials and Methods). Error bars indicate one standard deviation. The asterisk (*) indicates that the recombinant culture fluorescence is within one standard deviation of the average wild type fluorescence. Growth data is shown in Figure 3.2.

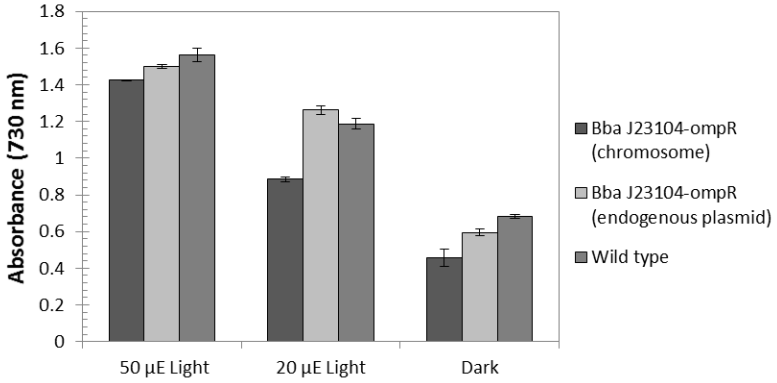


Figure 3.2 Photomixotrophic and heterotrophic growth of the *Synechocystis* strains with the light sensors. Absorbance was measured at 730 nm. All cultures were grown in BG-11 media supplemented with 5 mM D-Glucose. The data reported are the average of at least three biological replicates measured three days after induction (Materials and Methods). Error bars indicate one standard deviation.

Since expression occurred only in the dark for the strain with *Bba J23104-ompR* integrated into NSP1, this strain is well suited for regulating proteins that perform best in the night. Furthermore, this strain did not require special conditions for induction, as compared to previously reported light sensors. For example, the native light-inducible promoter for *psbA2* has been used for a synthetic system that responds to high-intensity light. A change from 10 to

500 μE induced expression of *Pueraria montana*'s isoprene synthase in *Synechocystis* (Lindberg et al., 2010). The native light-inducible promoter for *cpcG2* has been used to produce T4 bacteriophage-derived lysis genes when cultures were grown in a combination of red and green light (Miyake et al., 2014). Both of these alternate systems tested in *Synechocystis* require the additional expense of a colorized light system or the extra energy to produce high-intensity light.

3.3.2 Chemical sensors

Since *Synechocystis*' growth was not affected by arabinose (Figure 3.3), this sugar was used as a chemical signal for a family of sensors based on the P_{BAD} promoter from *E. coli* (Schleif, 2010). The transcription factor AraC and the cAMP-cAMP receptor protein complex (cAMP-CRP) control transcription from P_{BAD}, as well as transcription of *araC* from the *Pc* promoter (Figure 3.4a). The specific mechanism of P_{BAD} and *Pc* regulation by AraC and cAMP-CRP in *E. coli* has been thoroughly studied (Dunn et al., 1984; Hahn and Schleif, 1983; Schleif, 2010). The different repression and activation states of P_{BAD} in *E. coli* can be found in Figures 3.5-3.7. To check if expression from P_{BAD} would be affected by catabolite repression in *Synechocystis*, the cells containing P_{BAD}-*eyfp* in the replicative plasmid were grown phototrophically in 0, 5 and 56 mM D-Glucose, while varying the concentration of arabinose in the media (Figure 3.4b). No glucose catabolite repression was evident. For all cultures, the normalized fluorescence was within one standard deviation of the average wild type fluorescence at low arabinose concentrations, while a significant and similar fluorescent signal was produced at arabinose concentrations at or above 5 mM. *Synechocystis*' adenylyl cyclase, Cya1, the enzyme that converts ATP to cAMP, is upregulated in blue light (Terauchi and Ohmori, 2004) and inhibited by bicarbonate (Masuda and Ono, 2004), but no work has indicated that Cya1 is also inhibited by glucose.

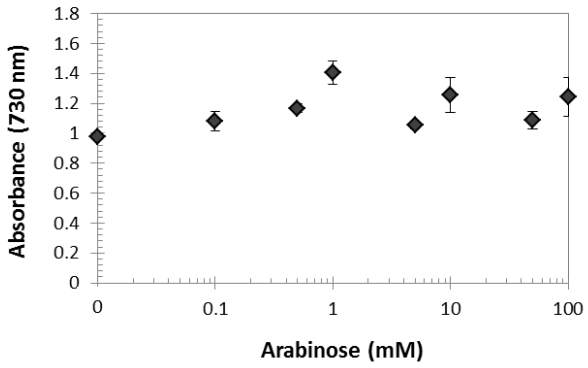


Figure 3.3 Arabinose did not significantly affect *Synechocystis*' phototrophic growth. Absorbance was measured at 730 nm. The cultures with arabinose sensors were grown photoautotrophically. This graph is from culture expressing the original *PBAD-eyfp*, and is representative for all of the variations of the arabinose sensor and wild type *Synechocystis*. The data reported are the average of three biological replicates measured three days after induction (Materials and Methods). Error bars indicate one standard deviation.

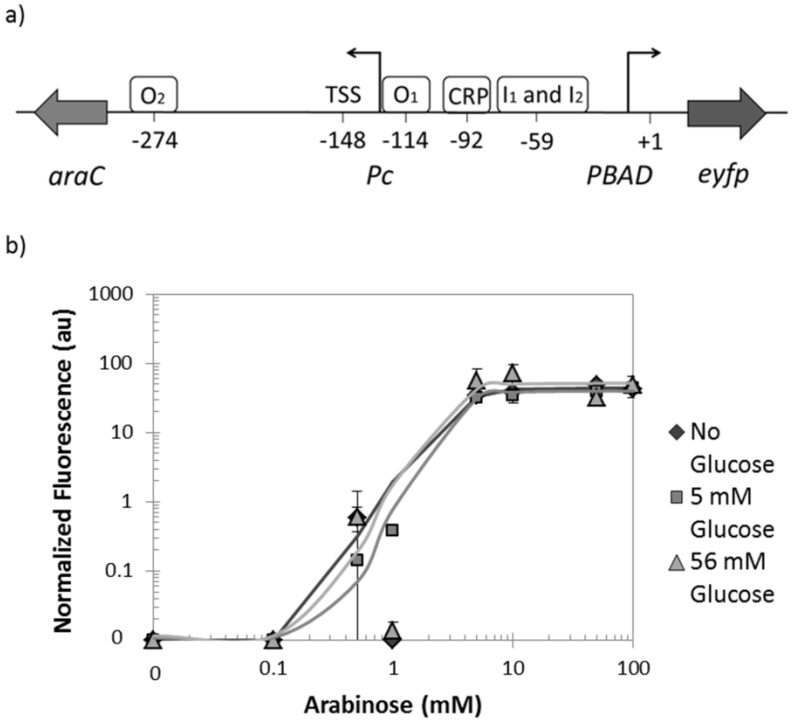


Figure 3.4 *E. coli*'s *PBAD* responds to L-Arabinose in *Synechocystis* independent of the media's glucose concentration. (**a**) Schematic of the gene for enhanced yellow fluorescent protein (*eyfp*) expressed by *E coli*'s *PBAD* promoter. The gene for the transcriptional regulator, *araC*, is controlled by its native promoter *Pc*. O_1, O_2, I_1 and I_2 are AraC binding sites. CRP is a cAMP-cAMP receptor protein complex (cAMP-CRP) binding site. cAMP-CRP activates transcription from both *Pc* and *PBAD* in *E. coli* (Schleif, 2010). (**b**) Normalized fluorescence ($\frac{(F_{RC}-F_{media})}{A_{RC}}$ –

$\left(\frac{(F_{WT}-F_{media})}{A_{WT}}\right)_{average}$)$_{[L-Arabinose\ and\ D-Glucose]}$ of *eyfp* expressed from *PBAD* in the replicative plasmid containing the RSF1010 replicon in *Synechocystis*. F is fluorescence (excitation at 485 nm and emission at 528 nm). A is absorbance (730 nm). RC is the recombinant culture. WT is wild type *Synechocystis*. All strains were grown phototrophically at the indicated glucose concentrations. The data reported are the average of three biological replicates measured three days after induction (Materials and Methods). Error bars indicate one standard deviation. The curve was fit using the modified Hill equation while minimizing the root mean squared error (RMSE) (Materials and Methods).

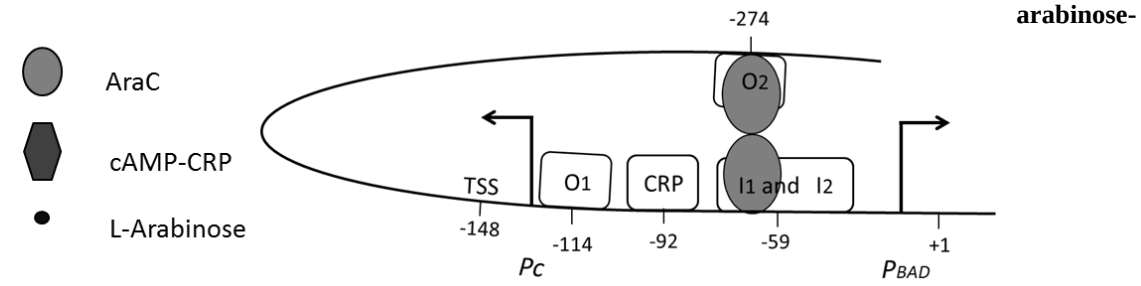


Figure 3.5 When AraC binds to O_2 and I_1 operator sites in the absence of arabinose, DNA looping prevents transcription of *araC* from *Pc* and *eyfp* from *PBAD* (Lobell and Schleif, 1990).

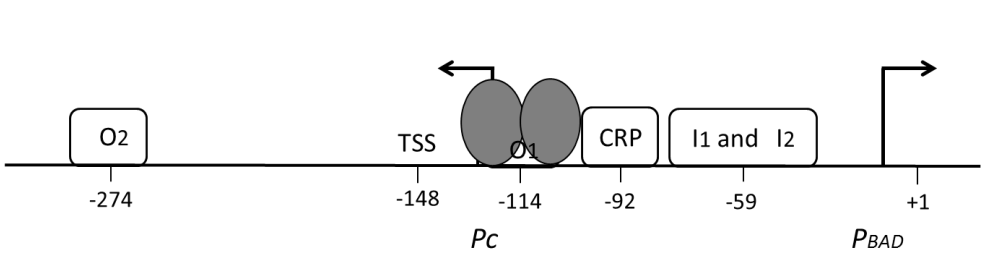


Figure 3.6 When AraC binds to the two half sites of O_1 in the absence of arabinose, steric hindrance prevents the transcription of *araC*. Transcription of *eyfp* from *PBAD* does not occur without AraC (Casadaban, 1976).

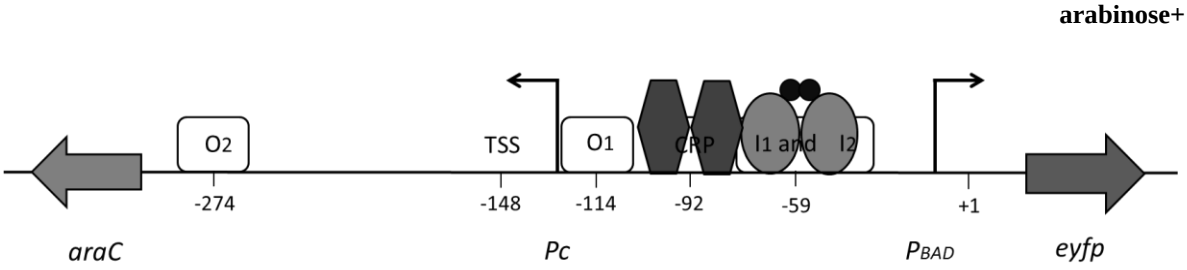


Figure 3.7 When an arabinose-AraC complex binds to I_1 and I_2 and a dimer of cAMP-CRP binds to the CRP site, *eyfp* is transcribed from *PBAD* (Ogden et al., 1980).

Individual operator sites were altered to create a family of promoters with different responses to arabinose (Figure 3.8a). Removing 22 nucleotides from the O_1 operator site created the *O1-* promoter, which in *Synechocystis* required higher concentrations of arabinose for activation than the original P_{BAD} (Figure 3.8b). When the nucleotides from the O_1 site were replaced with 22 random nucleotides, the strain that used *O1- +22 1* (Table 3.2) did not generate fluorescence above one standard deviation of the average wild type fluorescence at any arabinose concentration in either *Synechocystis* or *E. coli* (data not shown). However, the *Synechocystis* strain that used the *O1- +22 2* promoter (Table 3.2) responded with a maximum fluorescent output twice as high as that of the original P_{BAD} in *Synechocystis* (Figure 3.8b). When 13 nucleotides were removed from the O_2 operator site to create the *O2-* promoter, the corresponding *Synechocystis* strain produced higher fluorescence at a lower concentration of arabinose than the culture that contained the original P_{BAD} promoter, and achieved maximum expression at greater than 1 mM arabinose. Altering these operator sites, O_2 and O_1, produced arabinose-responsiveness in both *Synechocystis* and *E. coli* (Figure 3.8b and Figure 3.9).

The response to arabinose was different between the two organisms when the CRP site was altered by removing ten nucleotides, to create the *CRP-* promoter (Figure 3.8b and Figure 3.9). The *E. coli* strain with the *CRP-* promoter only achieved 40% of the maximum expression of the *E. coli* strain with the original promoter, as seen previously (Miyada et al., 1984). In contrast, the *Synechocystis* strain with the *CRP-* promoter achieved the maximum expression of the *Synechocystis* strain with the original promoter, but did not start to respond to arabinose until the concentration was 50 mM. In *E. coli*, cAMP-CRP is believed to help open the loop that represses transcription (Lobell and Schleif, 1990) and help RNA polymerase bind to and/or open

the DNA (Schleif, 2010), while the effects of *Synechocystis*' SYCRP1 (Yoshimura et al., 2002) on the *E. coli* CRP site could be different from those of *E. coli*'s CRP.

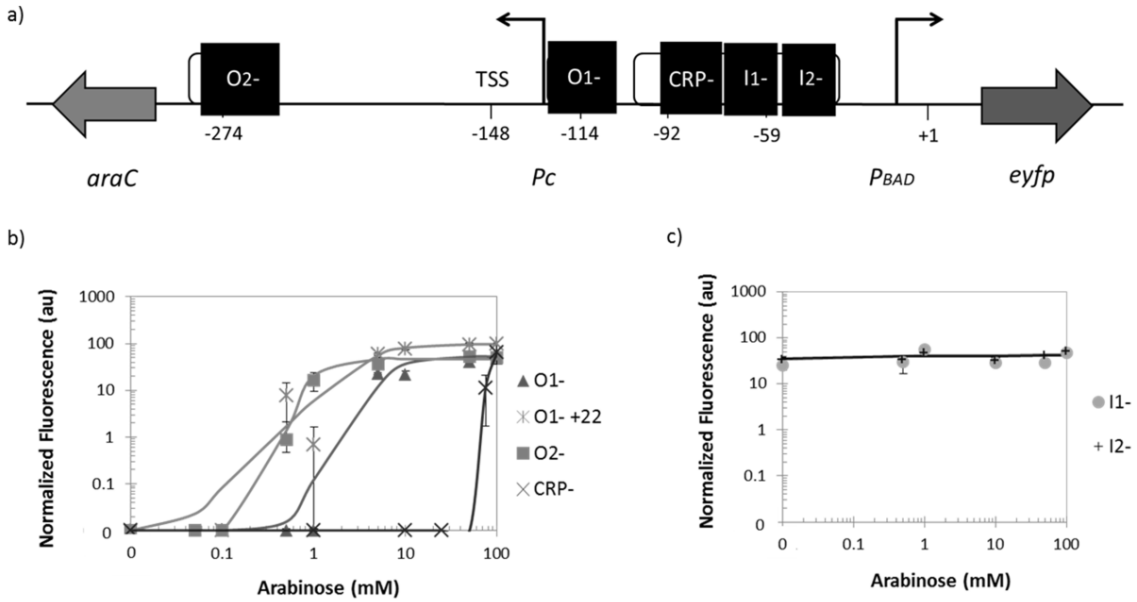


Figure 3.8 Engineering the AraC and cAMP-CRP binding sites varies *PBAD*'s response to L-Arabinose in *Synechocystis*. (**a**) Schematic of changes made to the regulatory region of the arabinose sensor. The black boxes indicate the position of the nucleotides removed in the associated operator sites. The 22 nucleotides removed to create the *O1-* promoter were replaced with 22 random nucleotides for the *O1- +22* promoter (Table 3.2). (**b**) Normalized fluorescence $\left(\frac{(F_{RC}-F_{media})}{A_{RC}} - \left(\frac{(F_{WT}-F_{media})}{A_{WT}}\right)_{average}\right)_{[L-arabinose]}$ of *eyfp* controlled by the engineered variations of the arabinose sensor (*O1-*, *O1- +22*, *O2-*, and *CRP-*) in the replicative plasmid containing the RSF1010 replicon in *Synechocystis*. F is fluorescence (excitation at 485 nm and emission at 528 nm). A is absorbance (730 nm). RC is the recombinant culture. WT is wild type *Synechocystis*. All strains were grown photoautotrophically. The data reported are the average of three biological replicates measured three days after induction (Materials and Methods). Error bars indicate one standard deviation. The curves were fit using the modified Hill equation while minimizing the RMSE (Materials and Methods). (**c**) Normalized fluorescence of *eyfp* controlled by the engineered variations of the arabinose sensor (*I1-* and *I2-*) in the replicative plasmid containing the RSF1010 replicon in *Synechocystis*. All strains were grown photoautotrophically. The data reported are the average of three biological replicates measured three days after induction (Materials and Methods). Error bars indicate one standard deviation. The curves were fit using the modified Hill equation while minimizing the RMSE (Materials and Methods).

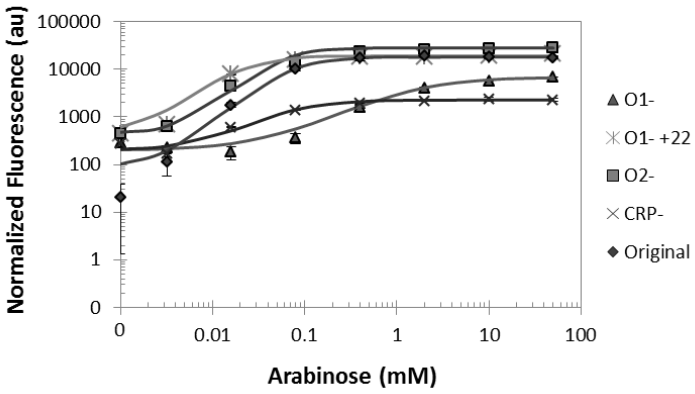


Figure 3.9. Normalized fluorescence $\frac{F_{RC}}{A_{RC}} - \left(\frac{F_{WT}}{A_{WT}}\right)_{average}$ of *eyfp* controlled by the engineered variations of the arabinose sensor (*O1-*, *O1- +22*, *O2-*, and *CRP-*) and the original sensor (*PBAD*) in *E. coli* DH10B, using the same plasmids as was tested in *Synechocystis* (Figures 3.4b and 3.8b). F is fluorescence (excitation at 485 nm and emission at 528 nm). A is absorbance (600 nm). RC is the recombinant culture. WT is wild type *E. coli* DH10B. The data reported are the average of three replicates (Materials and Methods). Error bars indicate one standard deviation. The curves were fit using the modified Hill equation while minimizing the RMSE (Materials and Methods).

When either the I_1 or I_2 operator site was removed from the original *PBAD* promoter, the response to arabinose was eliminated in *Synechocystis*, resulting in *PBAD* variants with constitutive high expression (Figure 3.4c). In *E. coli*, removing the I_1 operator site resulted in constitutive low expression, compared to expression from the original promoter. Similar low expression resulted when the I_2 operator site was removed in *E. coli*, while the resultant promoter was somewhat responsive to arabinose (Figure 3.10). The differences in expression of the two promoters in the two different organisms could be due to many factors including, for example, the holoenzyme of *Synechocystis*' RNA polymerase is longer than the enzyme found in other eubacteria, including *E. coli* (Imamura and Asayama, 2009). Full transcriptional activation from *pBAD* is due to cAMP-CRP and both the distal and proximal AraC all interacting together with the C-terminal domain of RNA polymerase's alpha subunit, resulting in the DNA being bent

approximately 90°. The extreme bending allows each of the three transcription factors to be in contact with the polymerase (Saviola et al., 1998). *Synechocystis*' longer polymerase could change this bending, altering the expression from *PBAD* in *Synechocystis*. Detailed molecular-level investigation would elucidate the mechanism in the future.

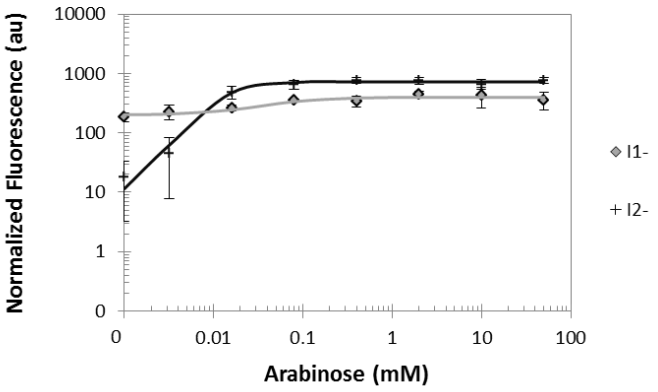


Figure 3.10 Normalized fluorescence $\frac{F_{RC}}{A_{RC}} - \left(\frac{F_{WT}}{A_{WT}}\right)_{average}$ of *eyfp* controlled by the engineered variations of the arabinose sensor (*I1*- and *I2*-) in *E. coli* DH10B, using the same plasmids as was tested in *Synechocystis* (Figure 3.8c). F is fluorescence (excitation at 485 nm and emission at 528 nm). A is absorbance (600 nm). RC is the recombinant culture. WT is wild type *E. coli* DH10B. The data reported are the average of three replicates (Materials and Methods). Error bars indicate one standard deviation. The curves were fit using the modified Hill equation while minimizing the RMSE (Materials and Methods).

Five of the seven promoters in the *PBAD* family are tightly off when not induced in *Synechocystis* (Figures 3.4b and 3.8b). Non-leaky expression can be beneficial when expressing genes whose product is toxic to the cells. For instance, when highly toxic proteins are produced in *E. coli* using *PBAD*, glucose is added to media to cause catabolite-repression and thus low basal expression (Rosano and Ceccarelli, 2014; Saida et al., 2006). The addition of glucose to the media is not necessary for low basal expression in *Synechocystis* (Figure 3.4b), allowing its photoautotrophic growth to be exploited. It has been shown that DNA looping effectively

represses transcription in *Synechocystis* (Camsund et al., 2014), which may be why our arabinose sensors have a tight off state in the cyanobacterium.

3.3.3 Metabolic state sensors

Controlling expression of the desired product in response to the host's metabolic state has been shown to increase process stability, reduce toxic byproducts, and improve product yield and titer (Farmer and Liao, 2000; Zhang et al., 2012). Since nitrogen is a required nutrient for cell growth, a library of regulators that respond to the culture's nitrogen status could be used in a number of applications. Cyanobacterial hydrogen formation, a potential fuel source, increases during nitrogen deprivation when the hydrogenase and nitrate reductase do not compete for electrons (Burrows et al., 2008; Gutekunst et al., 2014; Gutthann et al., 2007). Since biological nitrogen fixation by nitrogenase is regulated by the cell's nitrogen status (Halbleib and Ludden, 2000), expression of a heterologous nitrogenase in a non-diazotrophic host may require synthetic regulation which responds to the host's nitrogen status (Hoynes-O'Connor and Moon, 2015)

NtcA-activated sensors

Microbes use a build-up of 2-oxoglutarate to signal low nitrogen availability (Huergo and Dixon, 2015). 2-Oxoglutarate produced from the tricarboxylic acid (TCA) cycle is the carbon skeleton for nitrogen assimilation reactions, connecting the carbon and nitrogen metabolisms. In cyanobacteria, the binding of 2-oxoglutarate to the global regulator NtcA favors interaction with the co-activator PipX (Espinosa et al., 2006; Huergo and Dixon, 2015). This 2-oxoglutarate activated NtcA/PipX complex promotes the transcription (Figure 3.11a) of several genes associated with nitrogen assimilation (Mueller et al., 2016) and regulators of sugar catabolism (Osanai et al., 2006).

We tested the expression of *fbfp*, the flavin-binding fluorescent protein F37T (Mukherjee et al., 2012), from NtcA-activated promoters (Table 3.2) in the replicative plasmid by varying the initial concentration of nitrate in the media (hereafter nitrate will be used when referring to the initial concentration of nitrate in the media) of photoautotrophic cultures (Material and Methods) (Figure 3.11b). The NtcA-activated regulators included the promoters for the RNA polymerase group-2 sigma factor *sigE* (Muro-Pastor et al., 2001a; Su et al., 2005), isocitrate dehydrogenase (*icd*), which is responsible for converting isocitrate to 2-oxoglutarate in the TCA cycle (Muro-Pastor et al., 1996; Su et al., 2005), the nitrogen-starvation specific glutamine synthetase (*glnN*) (Reyes et al., 1997), the nitrogen response regulator *nrrA* (Azuma et al., 2011; Liu and Yang, 2014), and the nitrogen-responsive (0.8 kb) *ntcA* transcript (Alfonso et al., 2001). There are two transcripts for *ntcA* (0.8 kb and 1.2 kb), each with different 5' ends (Alfonso et al., 2001). The number of shorter transcripts has been shown to increase during nitrogen deprivation while the number of longer transcripts remained constant. When tested (Figure 3.11b), the promoter for the shorter *ntcA* transcript produced constitutively high fluorescence, implying that many factors, including sequence context, may affect its regulation in a complex manner (Alfonso et al., 2001).

The fluorescent response for the rest of the NtcA-activated promoters tested in this work was highest for low nitrate concentrations, while the lowest fluorescent response occurred when the nitrate concentrations were at or above what is normally found in BG11 media, which is 17.6 mM. These four inducible promoters responded to nitrate with a variety of dynamic ranges (Figure 3.11b). The strain containing P_{sigE} produced the strongest fluorescence, and the strain with P_{nrrA} produced the lowest maximum fluorescence. The strain using P_{glnN} also produced a strong maximum fluorescent response, but the dynamic range of expression was not as large as that of the strain using P_{sigE}. The transition from high to low expression occurred at similar

nitrate concentrations (Figure 3.11b). Bleaching, as denoted by the yellowish color of the cultures (Ogawa and Sonoike, 2016), was evident at the nitrate concentrations that generated the maximum fluorescence (Figure 3.12). During nitrogen stress, non-diazotrophic cyanobacteria first deplete the nitrogen stored in cyanophycin granules and then degrade their nitrogen-rich light-harvesting antenna called phycobilisomes, causing the color change (Allen and Smith, 1969; Grossman et al., 1993).

2-Oxoglutarate connects carbon and nitrogen metabolisms (Huergo and Dixon, 2015). We were therefore interested in the effect of glucose on the nitrate-responsiveness of P_{sigE}, the best NtcA-activated promoter tested in this work. For both the photoautotrophic and photomixotrophic cultures expressing P_{sigE}-*fbfp*, the fluorescent response was highest for low nitrate concentrations and lowest when the nitrate concentrations were at or above what is normally found in BG11 media, and the dynamic ranges were similar (Figure 3.11c). Bleaching was evident in the P_{sigE}-*fbfp* photomixotrophic cultures at higher concentrations of nitrate, up to 7 mM nitrate, versus only up to 5 mM nitrate for the photoautotrophic cultures (Figures 3.12 and 3.13). The nitrate sensors' maximal fluorescence occurred at the nitrate concentrations associated with bleaching, a phenotypic indicator of nitrogen stress. The addition of glucose to the media did not change the dynamic range, but it did increase the nitrate concentration threshold below which high fluorescence and bleaching occurred. 2-Oxoglutarate did not increase in *Synechocystis* when glucose was added to phototrophic cultures (Yoshikawa et al., 2013), implying that the change in the responsiveness to nitrate could be the result of the available nitrogen being consumed more for biomass during photomixotrophic growth (Figure 3.14), not a build-up of 2-oxoglutarate due to glucose catabolism.

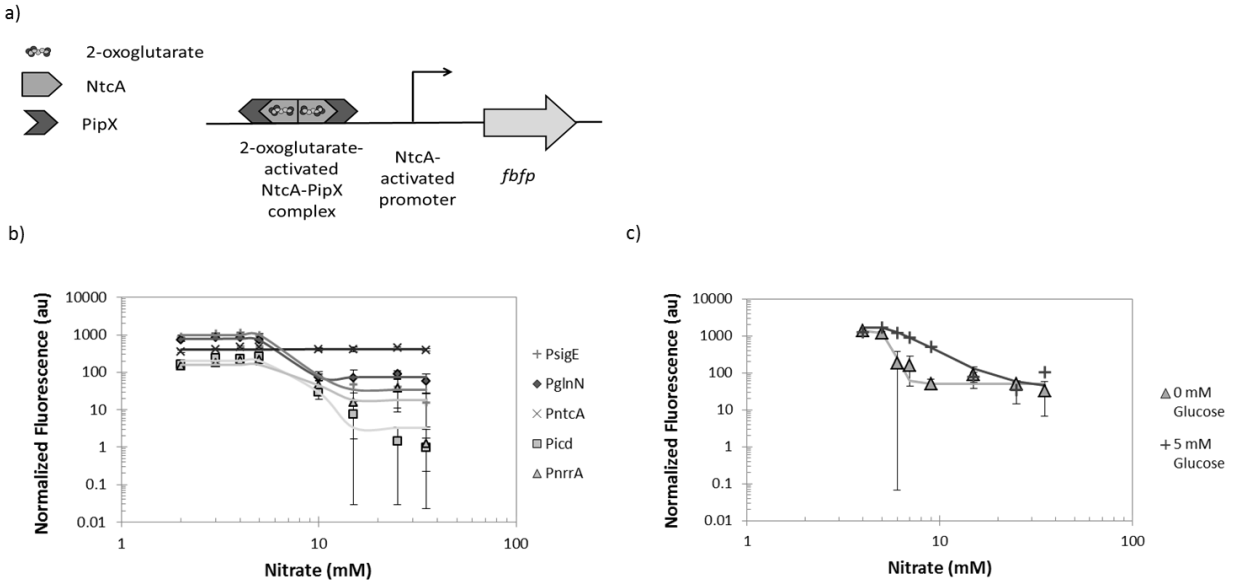


Figure 3.11 NtcA-activated promoters respond to nitrogen deprivation. **(a**) Schematic of the gene for the flavin-binding fluorescent protein (*fbfp*) expressed from promoters that have been shown to be activated by the global regulator NtcA during nitrogen deprivation (Muro-Pastor et al., 2001b; Su et al., 2005). When 2-oxoglutarate binds to NtcA, interaction with the co-activator PipX is favored, promoting transcription (Espinosa et al., 2006; Forcada-Nadal et al., 2014). (**b**) Normalized fluorescence $(\frac{(F_{RC}-F_{media})}{A_{RC}} - \left(\frac{(F_{WT}-F_{media})}{A_{WT}}\right)_{average})_{[nitrate]}$ of *fbfp* controlled by NtcA-responsive promoters in the replicative plasmid containing the RSF1010 replicon in *Synechocystis*. F is fluorescence (excitation at 450 nm and emission at 495 nm). A is absorbance (730 nm). RC is the recombinant culture. WT is wild type *Synechocystis*. All strains were grown photoautotrophically. The data reported are the average of three biological replicates measured three days after induction (Materials and Methods). Error bars indicate one standard deviation. The curves were fit using the modified Hill equation while minimizing the RMSE (Materials and Methods). (**c**) Normalized fluorescence $(\frac{(F_{RC}-F_{media})}{A_{RC}} - \left(\frac{(F_{WT}-F_{media})}{A_{WT}}\right)_{average})_{[glucose\ \&\ nitrate]}$ of *PsigE-fbfp* expressed in the replicative plasmid in *Synechocystis*. All strains were grown phototrophically. The data reported are the average of three biological replicates measured three days after induction (Materials and Methods). Error bars indicate one standard deviation. The curves were fit using the modified Hill equation while minimizing the RMSE (Materials and Methods).

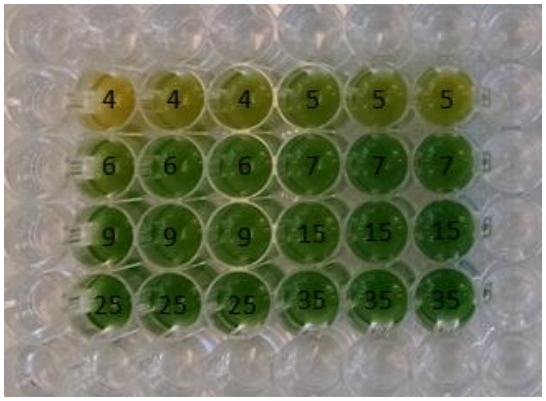


Figure 3.12 Bleaching was observed in the photoautotrophic cultures when the initial nitrate concentration of *Synechocystis'* media was 5 mM or less. The photograph is representative of all photoautotrophic cultures, recombinant or wild type *Synechocystis*, three days after induction (Materials and Methods). The strain pictured is *Synechocystis* expressing P_{sigE}-*fbfp*. The media's initial nitrate concentration is listed for each well, in mM.

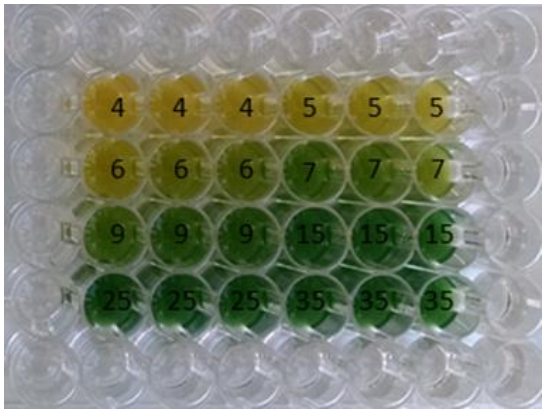


Figure 3.13 Bleaching was observed in the photomixotrophic cultures (supplemented with 5mM D-Glucose) when the initial nitrate concentration of *Synechocystis'* media was 7 mM or less. The photograph is representative of all photomixotrophic cultures, recombinant or wild type *Synechocystis*, three days after induction (Materials and Methods). The culture pictured is *Synechocystis* expressing P_{sigE}-*fbfp*. The media's initial nitrate concentration is listed for each well, in mM.

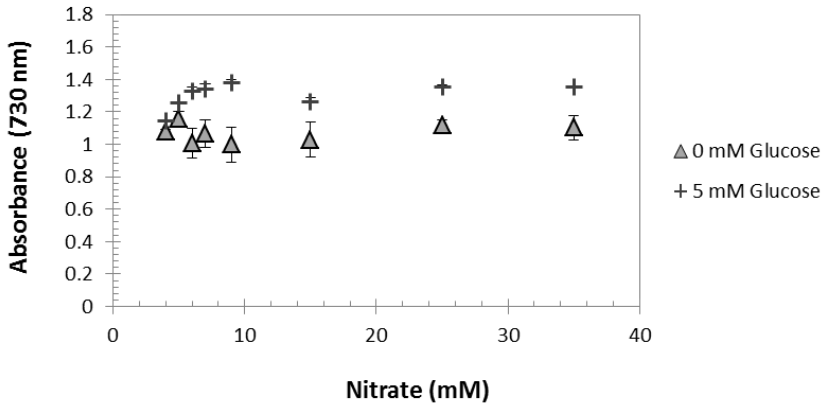


Figure 3.14 *Synechocystis*' photoautotrophic and photomixotrophic growth at different initial nitrate concentrations. Absorbance was measured at 730 nm. This graph is for the culture expressing P_{sigE}-*fbfp*, and is representative for all of the variations of the nitrate sensor and wild type *Synechocystis*. The data reported are the average of three biological replicates measured three days after induction (Materials and Methods). Error bars indicate one standard deviation.

NrrA-activated sensors

NrrA, whose expression is activated by NtcA in *Synechocystis*, regulates expression of genes involved in glycolysis, glycogen degradation, and arginine synthesis (Liu and Yang, 2014). Arginine is used for the synthesis of the nitrogen storage granules called cyanophycin (Allen, 1984). We tested the expression of *fbfp* from the NrrA-activated promoters for glyceraldehyde-3-P dehydrogenase (*gapI*) and the *icfG* operon (slr1852-slr1861) (Liu and Yang, 2014) at varying nitrate concentrations, using the replicative plasmid. The promoter for *gapI* produced the strongest fluorescence at low nitrate concentrations, while the fluorescent response for nitrate concentrations greater than or equal to 8 mM was less than the average wild type background fluorescence (Figure 3.15a). In contrast, the promoter for *slr1852* did not respond to nitrogen deprivation, instead producing constitutively high fluorescence.

Transcriptional regulators often regulate their own expression (Wall et al., 2004). The global regulator NtcA has been shown to be positively autoregulated in *Synechocystis* (Alfonso et al., 2001), as well as other species of cyanobacteria (Luque et al., 1994; Ramasubramanian et al.,

1996). The nitrogen response regulator NrrA is positively autoregulated in four *Cyanothece* species (ATCC 51142, PCC 8801, PCC 7822, and PCC 7424), but not in *Synechocystis* sp. PCC 6803 (Liu and Yang, 2014). Genes for enzymes with the same function, such as glycogen phosphorylase, phosphofructokinase and argininosuccinate synthetase, are regulated by NrrA in both *Synechocystis* and *Cyanothece* sp. ATCC 51142. In addition, *Cyanothece* sp. ATCC 51142's *nrrA* is 77% identical to *Synechocystis*' *nrrA*, with the C terminus 100% identical, which includes the DNA-binding domain of NrrA (Liu and Yang, 2014).

We therefore introduced *Cyanothece* sp. ATCC 51142's *nrrA*, expressed either from the promoter for *gapI* or *slr1852*, into the NSP neutral site to produce different response curves from the NrrA-activated promoters (Figure 3.15b). When $P_{slr1852}$-*fbfp* was used, the addition of *nrrA* did not change its response curve significantly due to its high constitutive expression state (Figures 3.15a and 3.15b). In contrast, the strain expressing both *fbfp* and *nrrA* from P_{gapI} (P_{gapI}-*fbfp*/P_{gapI}-*nrrA*) produced a reduced maximum fluorescence with a lower nitrate threshold (~5 mM), compared to the ~7 mM threshold for the strain without *nrrA* autoregulation (P_{gapI}-*fbfp*). When the strain with P_{gapI}-*fbfp*/$P_{slr1852}$-*nrrA* was used, the response to nitrate became more gradual than the strain without *nrrA* overexpression. These results show that *Cyanothece* sp. ATCC 51142's NrrA, in addition to *Synechocystis*' native NrrA, can change the response curves of NrrA-activated promoters, building a more diverse set of nitrogen sensors.

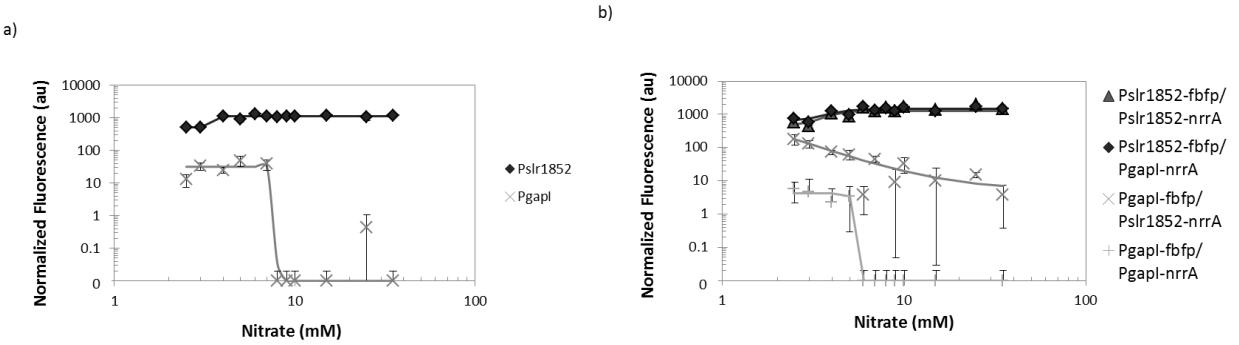


Figure 3.15. Promoters, which have been shown to be activated by the nitrogen-responsive regulator NrrA during nitrogen deprivation (Azuma et al., 2011; Liu and Yang, 2014), controlled expression of *fbfp* with and without the heterologous expression of *nrrA*. (**a**) Normalized fluorescence $\left(\frac{(F_{RC}-F_{media})}{A_{RC}} - \left(\frac{(F_{WT}-F_{media})}{A_{WT}}\right)_{average}\right)_{[nitrate]}$ of *fbfp* controlled by NrrA-responsive promoters in the replicative plasmid containing the RSF1010 replicon in *Synechocystis*. F is fluorescence (excitation at 450 nm and emission at 495 nm). A is absorbance (730 nm). RC is the recombinant culture. WT is wild type *Synechocystis*. All strains were grown photoautotrophically. The data reported are the average of three biological replicates measured three days after induction (Materials and Methods). Error bars indicate one standard deviation. The curves were fit using the modified Hill equation while minimizing the RMSE (Materials and Methods). (**b**) Heterologous expression of *nrrA* alters the response of the NrrA sensors to nitrogen deprivation. Normalized fluorescence was measured for devices using NrrA-responsive promoters (either *Pslr1852* or *PgapI*) to express *fbfp* in the replicative plasmid containing the RSF1010 replicon, with either *Pslr1852* or *PgapI* controlling *Cyanothece* sp. ATCC 51142's *nrrA*. (*Pslr1852* or *PgapI*)-*nrrA* was integrated into NSP1 in the endogenous plasmid pCC5.2 (Ng et al., 2015). All strains were grown photoautotrophically. The data reported are the average of three biological replicates measured three days after induction (Materials and Methods). Error bars indicate one standard deviation. The curves were fit using the modified Hill equation while minimizing the RMSE (Materials and Methods).

3.4 Conclusions

While the number of synthetic biology tools available for building circuits in cyanobacteria is growing (Nozzi and Atsumi, 2015; Zess et al., 2016), engineering complex gene expression is still limited by a shortage of well-characterized regulators that sense and respond to specific inputs. We developed a number of sensors for three separate classes of signals: physical, chemical and metabolic state. The chimeric light sensor and a library of nitrogen status sensors could be used to control cellular processes that require dark conditions and/or low available

nitrogen, including hydrogen production and nitrogen fixation. Our arabinose sensors were tightly off in the absence of arabinose, making them well-suited for the production of proteins that are toxic to the host. This suite of new sensors expand the tools available for transcriptional regulation in response to diverse signals in *Synechocystis*, enabling the control of complicated cellular pathways and thus improving the viability of cyanobacteria as a biotechnology platform.

3.5 **Acknowledgements**

The authors thank Dr. Himadri Pakrasi for providing *Synechocystis* sp. PCC 6803, the *eyfp* gene, the replicative plasmid, and the integrative plasmid for NSP1; Dr. Charles Schroeder for the *fbfp f37t* gene; and Dr. Yinjie Tang for the integrative plasmid for *psbA1*. We also thank Dr. Deng Liu and Dr. Bert Berla for their advice, and Ben Waldron-Feinstein for building and testing an earlier version of the arabinose sensor. This work was supported by National Science Foundation grant MCB-1331194. CMI is the recipient of an NSF Graduate Research Fellowship, DGE-1143954.

Since this work is not yet published, I would like to also acknowledge the authors who made significant contributions to this chapter, Caroline M. Focht, Drew M. DeLorenzo, Dinesh Gupta, Charles B. Johnson and Tae Seok Moon.

3.6 **Supplemental Material**

3.6.1 Materials and Methods

Light Sensor Testing Conditions

D-Glucose (Sigma–Aldrich Co. LLC) was added to each culture in the 48 well plates, for a final concentration of 5 mM. The cultures were grown in either 20 μE or 50 μE continuous white light, or in the dark. For dark conditions, the plate was wrapped in foil.

Arabinose Sensor Testing Conditions

L-Arabinose (Sigma–Aldrich Co. LLC) was added as appropriate to the cultures in the 48 well plates, with a final concentration range of 0.1-100 mM. D-Glucose (Sigma–Aldrich Co. LLC) was also added as appropriate for a final concentration of 5 or 56 mM for Figure 3.4b.

Nitrate Sensor Testing Conditions

Before aliquoting the cultures into the 48 well plates, each culture was washed twice with BG11o. Starting from the recipe for BG11 media, we did not add the sodium nitrate, and replaced the ferric ammonium citrate with 5.6 mg/L ferric citrate (Sigma–Aldrich Co. LLC) and the cobalt (II) nitrate hexahydrate from the Trace metal mix A5 with 40.4 mg/L cobalt (II) chloride (Sigma–Aldrich Co. LLC) to create BG11o. After re-suspending each pellet in 50 mL BG11o and adding antibiotic as appropriate, 600 μL per well was aliquoted into the clear 48-well cell cultures plates. Sodium nitrate (Sigma–Aldrich Co. LLC) was added as appropriate, with a final concentration range of 2-35 mM. D-Glucose (Sigma–Aldrich Co. LLC) was also added as appropriate for a final concentration of 5 mM for Figure 3.11c and Figure 3.13.

3.6.2 Equations

For the arabinose sensors, the Hill equation is modified as follows:

$$P_{BAD} = \frac{(F_{max} - F_{min}) * [Ara]^n}{[Ara]^n + K^n} + F_{min} \quad (3.1)$$

P is the calculated promoter activity, F_{max} is the maximum promoter activity, F_{min} is the basal promoter activity, [Ara] is the concentration of L-Arabinose, n is the Hill coefficient, and K is inducer concentration for half-maximal promoter activity.

For the nitrate sensors, the Hill equation is modified as follows:

$$P_{NO_3} = \frac{(F_{max} - F_{min}) * K^n}{[NO_3^-]^n + K^n} + F_{min} \quad (3.2)$$

where $[NO_3^-]$ is the concentration of nitrate.

The equation used for the root mean squared error (RMSE) is

(3.3) $$\sqrt[2]{\frac{\sum_{i=1}^{n}(P-F)^2}{(n-2)}}$$

P is the calculated promoter activity, F is the experimentally measured promoter activity, and n is the number of inducer concentrations tested. The Hill equation parameters are summarized in Table 3.6.

Table 3.6 Hill equation parameters in this study. Data fitting was done to provide a guide to the eye, rather than to obtain biologically meaningful parameters.

Figure	Strain	Fmax	Fmin	n	K	RMSE
3.4	No Glucose	44	0	2.6	3.2	3.0
	5 mM Glucose	40	0	2.9	3.4	2.2
	56 mM Glucose	51	0	2.7	3.3	13.5
3.8	O1-	53	0	3.0	7.5	8.9
	O1- +22	97	0	1.9	4.2	3.8
	O2-	47	0	4.7	1.0	5.5
	CRP-	64	0	30.4	78.9	0.7
	I1-	55	34	0.1	200	13.5
	I2-	49	35	0.1	200	5.8
3.9	O1-	6848	200	1.0	1.4	191.1
	O1- +22	19067	454	1.6	0.02	1003.8
	O2-	28103	450	1.8	0.05	3068.3
	CRP-	2264	201	1.3	0.06	121.2
	Original	18045	87	1.7	0.07	100.2
3.10	I1-	398	201	1.3	0.04	34.9
	I2-	723	5	1.9	0.01	43.5
3.11	P_{sigE}	984	34	19.9	8.6	90.7
	P_{glnN}	784	74	58.3	5.2	45.7
	P_{ntcA}	405	415	48.3	6.8	61.3
	P_{nrrA}	158	18	90.7	9.8	33.7
	P_{icd}	202	3	23.5	9.2	44.4
	P_{sigE} 5 mM Glucose	2905	41	3.3	5.4	39.9
3.15	$P_{slr1852}$	1117	511	64.3	3.2	98.5
	P_{gapI}	31	0	150.7	7.6	9.0
	$P_{slr1852}$-fbfp / $P_{slr1852}$-nrrA	1267	482	9.0	3.8	476.4
	$P_{slr1852}$-fbfp / P_{gapI}-nrrA	1482	606	5.9	3.9	222.0
	P_{gapI}-fbfp / $P_{slr1852}$-nrrA	2210	6	1.9	0.7	8.4
	P_{gapI}-fbfp / P_{gapI}-nrrA	4.2	0	157.7	5.0	0.8

3.7 References

Albers SC, Gallegos VA, Peebles CA. 2015. Engineering of genetic control tools in Synechocystis sp. PCC 6803 using rational design techniques. J Biotechnol 216:36-46.

Alfonso M, Perewoska I, Kirilovsky D. 2001. Redox control of ntcA gene expression in Synechocystis sp. PCC 6803. Nitrogen availability and electron transport regulate the levels of the NtcA protein. Plant Physiol 125(2):969-81.

Allen MM. 1984. Cyanobacterial cell inclusions. Annu Rev Microbiol 38:1-25.

Allen MM, Smith AJ. 1969. Nitrogen chlorosis in blue-green algae. Arch Mikrobiol 69(2):114-20.

Angermayr SA, Gorchs Rovira A, Hellingwerf KJ. 2015. Metabolic engineering of cyanobacteria for the synthesis of commodity products. Trends in Biotechnology 33(6):352-361.

Arkin A. 2008. Setting the standard in synthetic biology. Nat Biotechnol 26(7):771-4.

Azuma M, Osanai T, Hirai MY, Tanaka K. 2011. A response regulator Rre37 and an RNA polymerase sigma factor SigE represent two parallel pathways to activate sugar catabolism in a cyanobacterium Synechocystis sp. PCC 6803. Plant Cell Physiol 52(2):404-12.

Berla BM, Saha R, Immethun CM, Maranas CD, Moon TS, Pakrasi HB. 2013. Synthetic biology of cyanobacteria: unique challenges and opportunities. Front Microbiol 4:246.

Bradley RW, Buck M, Wang B. 2016. Tools and Principles for Microbial Gene Circuit Engineering. Journal of Molecular Biology 428(5, Part B):862-888.

Burrows EH, Chaplen FWR, Ely RL. 2008. Optimization of media nutrient composition for increased photofermentative hydrogen production by Synechocystis sp. PCC 6803. International Journal of Hydrogen Energy 33(21):6092-6099.

Camsund D, Heidorn T, Lindblad P. 2014. Design and analysis of LacI-repressed promoters and DNA-looping in a cyanobacterium. J Biol Eng 8(1):4.

Cardinale S, Arkin AP. 2012. Contextualizing context for synthetic biology – identifying causes of failure of synthetic biological systems. Biotechnology Journal 7(7):856-866.

Casadaban MJ. 1976. Regulation of the regulatory gene for the arabinose pathway, araC. J Mol Biol 104(3):557-66.

Chen Y, Nielsen J. 2013. Advances in metabolic pathway and strain engineering paving the way for sustainable production of chemical building blocks. Current Opinion in Biotechnology 24(6):965-972.

Chubukov V, Mukhopadhyay A, Petzold CJ, Keasling JD, Martín HG. 2016. Synthetic and systems biology for microbial production of commodity chemicals. Npj Systems Biology And Applications 2:16009.

Du J, Shao Z, Zhao H. 2011. Engineering microbial factories for synthesis of value-added products. J Ind Microbiol Biotechnol 38(8):873-90.

Ducat DC, Way JC, Silver PA. 2011. Engineering cyanobacteria to generate high-value products. Trends Biotechnol 29(2):95-103.

Dunn TM, Hahn S, Ogden S, Schleif RF. 1984. An operator at -280 base pairs that is required for repression of araBAD operon promoter: addition of DNA helical turns between the operator and promoter cyclically hinders repression. Proc Natl Acad Sci U S A 81(16):5017-20.

Engler C, Kandzia R, Marillonnet S. 2008. A one pot, one step, precision cloning method with high throughput capability. PLoS One 3(11):e3647.

Espinosa J, Forchhammer K, Burillo S, Contreras A. 2006. Interaction network in cyanobacterial nitrogen regulation: PipX, a protein that interacts in a 2-oxoglutarate dependent manner with PII and NtcA. Mol Microbiol 61(2):457-69.

Farmer WR, Liao JC. 2000. Improving lycopene production in Escherichia coli by engineering metabolic control. Nat Biotech 18(5):533-537.

Forcada-Nadal A, Forchhammer K, Rubio V. 2014. SPR analysis of promoter binding of Synechocystis PCC6803 transcription factors NtcA and CRP suggests cross-talk and sheds light on regulation by effector molecules. FEBS Letters 588(14):2270-2276.

Grossman AR, Schaefer MR, Chiang GG, Collier JL. 1993. The phycobilisome, a light-harvesting complex responsive to environmental conditions. Microbiol Rev 57(3):725-49.

Gutekunst K, Chen X, Schreiber K, Kaspar U, Makam S, Appel J. 2014. The bidirectional NiFe-hydrogenase in Synechocystis sp. PCC 6803 is reduced by flavodoxin and ferredoxin and is essential under mixotrophic, nitrate-limiting conditions. J Biol Chem 289(4):1930-7.

Gutthann F, Egert M, Marques A, Appel J. 2007. Inhibition of respiration and nitrate assimilation enhances photohydrogen evolution under low oxygen concentrations in Synechocystis sp. PCC 6803. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1767(2):161-169.

Hahn S, Schleif R. 1983. In vivo regulation of the Escherichia coli araC promoter. J Bacteriol 155(2):593-600.

Halbleib CM, Ludden PW. 2000. Regulation of biological nitrogen fixation. J Nutr 130(5):1081-4.

Heidorn T, Camsund D, Huang H-H, Lindberg P, Oliveira P, Stensjo K, Lindblad P. 2011. Synthetic Biology in Cyanobacteria: Engineering and Analyzing Novel Functions. San Diego, CA; Waltham, MA; London: Elsevier, Inc. 678 p.

Hollinshead WD, Varman AM, You L, Hembree Z, Tang YJJ. 2014. Boosting D-lactate production in engineered cyanobacteria using sterilized anaerobic digestion effluents. Bioresource Technology 169:462-467.

Hoynes-O'Connor A, Moon TS. 2015. Programmable genetic circuits for pathway engineering. Current Opinion in Biotechnology 36:115-121.

Huang HH, Camsund D, Lindblad P, Heidorn T. 2010. Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. Nucleic Acids Res 38(8):2577-93.

Huang HH, Lindblad P. 2013. Wide-dynamic-range promoters engineered for cyanobacteria. J Biol Eng 7(1):10.

Huergo LF, Dixon R. 2015. The Emergence of 2-Oxoglutarate as a Master Regulator Metabolite. Microbiol Mol Biol Rev 79(4):419-35.

Imamura S, Asayama M. 2009. Sigma factors for cyanobacterial transcription. Gene Regul Syst Bio 3:65-87.

Immethun CM, Hoynes-O'Connor AG, Balassy A, MOON TS. 2013. Microbial production of isoprenoids enabled by synthetic biology. Frontiers in Microbiology 4.

Immethun CM, Ng KM, DeLorenzo DM, Waldron-Feinstein B, Lee YC, Moon TS. 2016. Oxygen-responsive genetic circuits constructed in Synechocystis sp. PCC 6803. Biotechnol Bioeng 113(2):433-42.

Landry BP, Stockel J, Pakrasi HB. 2013. Use of Degradation Tags To Control Protein Levels in the Cyanobacterium Synechocystis sp. Strain PCC 6803. Appl Environ Microbiol 79(8):2833-5.

Levskaya A, Chevalier AA, Tabor JJ, Simpson ZB, Lavery LA, Levy M, Davidson EA, Scouras A, Ellington AD, Marcotte EM and others. 2005. Synthetic biology: Engineering Escherichia coli to see light. Nature 438(7067):441-442.

Lindberg P, Park S, Melis A. 2010. Engineering a platform for photosynthetic isoprene production in cyanobacteria, using Synechocystis as the model organism. Metab Eng 12.

Liu D, Yang C. 2014. The nitrogen-regulated response regulator NrrA controls cyanophycin synthesis and glycogen catabolism in the cyanobacterium Synechocystis sp. PCC 6803. J Biol Chem 289(4):2055-71.

Lo T-M, Teo WS, Ling H, Chen B, Kang A, Chang MW. 2013. Microbial engineering strategies to improve cell viability for biochemical production. Biotechnology Advances 31(6):903-914.

Lobell RB, Schleif RF. 1990. DNA looping and unlooping by AraC protein. Science 250(4980):528-32.

Lopes MSG. 2015. Engineering biological systems toward a sustainable bioeconomy. Journal of Industrial Microbiology & Biotechnology 42(6):813-838.

Lou C, Stanton B, Chen Y-J, Munsky B, Voigt CA. 2012. Ribozyme-based insulator parts buffer synthetic circuits from genetic context. Nat Biotech 30(11):1137-1142.

Luque I, Flores E, Herrero A. 1994. Molecular mechanism for the operation of nitrogen control in cyanobacteria. The EMBO Journal 13(12):2862-2869.

Markley AL, Begemann MB, Clarke RE, Gordon GC, Pfleger BF. 2014. Synthetic Biology Toolbox for Controlling Gene Expression in the Cyanobacterium Synechococcus sp. strain PCC 7002. ACS Synth Biol.

Masuda S, Ono TA. 2004. Biochemical characterization of the major adenylyl cyclase, Cya1, in the cyanobacterium Synechocystis sp. PCC 6803. FEBS Lett 577(1-2):255-8.

Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M. 2000. Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga Chlamydomonas reinhardtii. Plant Physiol 122(1):127-36.

Miyada CG, Stoltzfus L, Wilcox G. 1984. Regulation of the araC gene of Escherichia coli: catabolite repression, autoregulation, and effect on araBAD expression. Proceedings of the National Academy of Sciences of the United States of America 81(13):4120-4124.

Miyake K, Abe K, Ferri S, Nakajima M, Nakamura M, Yoshida W, Kojima K, Ikebukuro K, Sode K. 2014. A green-light inducible lytic system for cyanobacterial cells. Biotechnology for Biofuels 7(1):1-8.

Moon TS, Lou C, Tamsir A, Stanton BC, Voigt CA. 2012. Genetic programs constructed from layered logic gates in single cells. Nature 491(7423):249-53.

Mueller TJ, Welsh EA, Pakrasi HB, Maranas CD. 2016. Identifying Regulatory Changes to Facilitate Nitrogen Fixation in the Nondiazotroph Synechocystis sp. PCC 6803. ACS Synthetic Biology 5(3):250-258.

Mukherjee A, Weyant KB, Walker J, Schroeder CM. 2012. Directed evolution of bright mutants of an oxygen-independent flavin-binding fluorescent protein from Pseudomonas putida. J Biol Eng 6(1):20.

Muro-Pastor AM, Herrero A, Flores E. 2001a. Nitrogen-regulated group 2 sigma factor from Synechocystis sp. strain PCC 6803 involved in survival under nitrogen stress. J Bacteriol 183(3):1090-5.

Muro-Pastor MI, Reyes JC, Florencio FJ. 1996. The NADP+-isocitrate dehydrogenase gene (icd) is nitrogen regulated in cyanobacteria. J Bacteriol 178(14):4070-6.

Muro-Pastor MI, Reyes JC, Florencio FJ. 2001b. Cyanobacteria perceive nitrogen status by sensing intracellular 2-oxoglutarate levels. J Biol Chem 276(41):38320-8.

Ng AH, Berla BM, Pakrasi HB. 2015. Fine-Tuning of Photoautotrophic Protein Production by Combining Promoters and Neutral Sites in the Cyanobacterium Synechocystis sp. Strain PCC 6803. Appl Environ Microbiol 81(19):6857-63.

Nozzi NE, Atsumi S. 2015. Genome Engineering of the 2,3-Butanediol Biosynthetic Pathway for Tight Regulation in Cyanobacteria. ACS Synth Biol 4(11):1197-204.

O'Connor CM, Adams JU. 2010. Essentials of Cell Biology. Cambridge, MA: NPG Education.

Ogawa T, Sonoike K. 2016. Effects of Bleaching by Nitrogen Deficiency on the Quantum Yield of Photosystem II in Synechocystis sp. PCC 6803 Revealed by Chl Fluorescence Measurements. Plant and Cell Physiology 57(3):558-567.

Ogden S, Haggerty D, Stoner CM, Kolodrubetz D, Schleif R. 1980. The Escherichia coli L-arabinose operon: binding sites of the regulatory proteins and a mechanism of positive and negative regulation. Proc Natl Acad Sci U S A 77(6):3346-50.

Oliver JWK, Atsumi S. 2014. Metabolic design for cyanobacterial chemical synthesis. Photosynthesis Research 120(3):249-261.

Olson EJ, Hartsough LA, Landry BP, Shroff R, Tabor JJ. 2014. Characterizing bacterial gene circuit dynamics with optically programmed gene expression signals. Nat Methods 11(4):449-55.

Osanai T, Imamura S, Asayama M, Shirai M, Suzuki I, Murata N, Tanaka K. 2006. Nitrogen induction of sugar catabolic gene expression in Synechocystis sp. PCC 6803. DNA Res 13(5):185-95.

Pade N, Hagemann M. 2015. Salt Acclimation of Cyanobacteria and Their Application in Biotechnology. Life 5(1):25-49.

Peralta-Yahya PP, Zhang F, Del Cardayre SB, Keasling JD. 2012. Microbial engineering for the production of advanced biofuels. Nature 488(7411):320-328.

Ramasubramanian TS, Wei TF, Oldham AK, Golden JW. 1996. Transcription of the Anabaena sp. strain PCC 7120 ntcA gene: multiple transcripts and NtcA binding. J Bacteriol 178(3):922-6.

Ramey CJ, Barón-Sola Á, Aucoin HR, Boyle NR. 2015. Genome Engineering in Cyanobacteria: Where We Are and Where We Need To Go. ACS Synthetic Biology 4(11):1186-1196.

Reyes JC, Muro-Pastor MI, Florencio FJ. 1997. Transcription of glutamine synthetase genes (glnA and glnN) from the cyanobacterium Synechocystis sp. strain PCC 6803 is differently regulated in response to nitrogen availability. J Bacteriol 179(8):2678-89.

Rosano GL, Ceccarelli EA. 2014. Recombinant protein expression in Escherichia coli: advances and challenges. Frontiers in Microbiology 5:172.

Saha R, Liu D, Hoynes-O'Connor A, Liberton M, Yu J, Bhattacharyya-Pakrasi M, Balassy A, Zhang F, Moon TS, Maranas CD and others. 2016. Diurnal Regulation of Cellular Processes in the Cyanobacterium Synechocystis sp. Strain PCC 6803: Insights from Transcriptomic, Fluxomic, and Physiological Analyses. MBio 7(3).

Saida F, Uzan M, Odaert B, Bontems F. 2006. Expression of highly toxic genes in E. coli: special strategies and genetic tools. Curr Protein Pept Sci 7(1):47-56.

Saviola B, Seabold RR, Schleif RF. 1998. DNA bending by AraC: a negative mutant. J Bacteriol 180(16):4227-32.

Schleif R. 2010. AraC protein, regulation of the l-arabinose operon in Escherichia coli, and the light switch mechanism of AraC action. FEMS Microbiol Rev 34(5):779-96.

Stockel J, Welsh EA, Liberton M, Kunnvakkam R, Aurora R, Pakrasi HB. 2008. Global transcriptomic analysis of Cyanothece 51142 reveals robust diurnal oscillation of central metabolic processes. Proc Natl Acad Sci U S A 105.

Su Z, Olman V, Mao F, Xu Y. 2005. Comparative genomics analysis of NtcA regulons in cyanobacteria: regulation of nitrogen assimilation and its coupling to photosynthesis. Nucleic Acids Research 33(16):5156-5171.

Terauchi K, Ohmori M. 2004. Blue light stimulates cyanobacterial motility via a cAMP signal transduction system. Mol Microbiol 52(1):303-9.

Varman AM, Xiao Y, Pakrasi HB, Tang YJ. 2013. Metabolic engineering of Synechocystis sp. strain PCC 6803 for isobutanol production. Appl Environ Microbiol 79(3):908-14.

Wall ME, Hlavacek WS, Savageau MA. 2004. Design of gene circuits: lessons from bacteria. Nat Rev Genet 5(1):34-42.

Weisse AY, Oyarzun DA, Danos V, Swain PS. 2015. Mechanistic links between cellular trade-offs, gene expression, and growth. Proc Natl Acad Sci U S A 112(9):E1038-47.

Yokoo R, Hood RD, Savage DF. 2015. Live-cell imaging of cyanobacteria. Photosynthesis Research 126(1):33-46.

Yoshikawa K, Hirasawa T, Ogawa K, Hidaka Y, Nakajima T, Furusawa C, Shimizu H. 2013. Integrated transcriptomic and metabolomic analysis of the central metabolism of Synechocystis sp. PCC 6803 under different trophic conditions. Biotechnology Journal 8(5):571-580.

Yoshimura H, Yanagisawa S, Kanehisa M, Ohmori M. 2002. Screening for the target gene of cyanobacterial cAMP receptor protein SYCRP1. Mol Microbiol 43(4):843-53.

Zess EK, Begemann MB, Pfleger BF. 2016. Construction of new synthetic biology tools for the control of gene expression in the cyanobacterium Synechococcus sp. strain PCC 7002. Biotechnology and Bioengineering 113(2):424-432.

Zhang F, Carothers JM, Keasling JD. 2012. Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. Nat Biotechnol 30(4):354-9.

Chapter 4: Synthetic Regulation of *Cyanothece* sp. ATCC 51142's Nitrogenase in the Non-Diazotrophic *Synechocystis* sp. PCC 6803

This chapter covers worked started but not finished, and thus unpublished.

4.1 Introduction

All living organisms require nitrogen for growth. Many organisms, including plants, depend on forms of fixed nitrogen, such as ammonium and nitrate. Without ample sources of fixed nitrogen, plant growth is limited, reducing crop yields (Charpentier and Oldroyd, 2010). Synthetic fertilizers can provide fixed nitrogen to crops. In fact, nearly 50% of the world's current population is fed by crops nourished by synthetic nitrogen fertilizers (Erisman et al., 2008). As the world's population continues to climb, robust crop yields will be needed to ensure an adequate food supply.

While synthetic fertilizers have largely enabled the Green Revolution, their synthesis and use are a source of their own problems. The Haber-Bosch process generates ammonia by combining nitrogen from the air and hydrogen from natural gas at high temperature and pressure. Five percent of the natural gas produced and two percent of the energy produced annually in the world are consumed by the Haber Bosch process (Ritter, 2008). When applied, ammonium fertilizer is converted to nitrate by soil microbes (Bernhard, 2012). While plants can utilize nitrate, the negatively charged ion does not adsorb onto soil particles, allowing it to be easily washed into the water supply. Heavy rains following a drought raised the nitrate level above safe drinking water limits in Des Moines, Iowa for 74 days in 2013 (Van Metre et al., 2016). Nitrate accumulation in waterways also contributes to hypoxic marine dead zones. The benefits of applying synthetic fertilizer to crops come with significant economic and environmental costs.

Some prokaryotes fix atmospheric nitrogen to produce ammonia with the enzyme nitrogenase (Mauseth, 2014). Nitrogen is fixed when and where the organism needs it, without the run off problems associated with synthetic fertilizers. Prokaryotic nitrogen fixation is widely distributed across the archaeal and bacterial domains. Despite its requirement for large amounts

of ATP and sensitivity to oxygen, nitrogenase is still the sole means of biological nitrogen fixation (Dos Santos et al., 2012; Latysheva et al., 2012). Diazotrophic (nitrogen-fixing) bacteria protect the enzyme from oxygen either spatially or temporally, even among single-celled photosynthetic organisms like cyanobacteria. Efforts to engineer nitrogen fixation in plants focus on some of the same strategies used by diazotrophic bacteria.

To help determine the minimum requirements for nitrogen fixation in a photosynthetic cell, nitrogenase from the unicellular, diazotrophic *Cyanothece* sp. ATCC 51142 has been expressed in the unicellular, non-diazotrophic *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) (Deng Liu, et al., in preparation). *Cyanothece* sp. ATCC 51142 (hereafter *Cyanothece*) has strong nitrogenase activity in both anaerobic and aerobic conditions (Bandyopadhyay et al., 2011). Minimal activity was achieved when expressing the full *nif* cluster in *Synechocystis* in anaerobic conditions (Deng Liu, et al., in preparation). This activity improved when the cluster was reduced by eleven genes, leaving the contiguous *nifT* to *hesB* (Sequence ID: CP000806.1: 556790 to 576716), in the synthetic replicative plasmid pSL2397.

Deng Liu et al. succeeded in attaining nitrogenase activity in the non-diazotrophic host, yet little is known about the transcriptional regulation of the cluster in *Cyanothece* or *Synechocystis*. Previous work in *Cyanothece* looked at changes in the transcription of the *nif* genes in light and dark cycles of varying lengths and periods (Cerveny et al., 2013; Colon-Lopez et al., 1997; Elvitigala et al., 2009; Gaudana et al., 2013; Stockel et al., 2008; Toepel et al., 2008; Toepel et al., 2009) and during mixotrophic growth (Krishnakumar et al., 2015). The relative *nif* protein concentrations in response to nitrate have also been studied (Aryal et al., 2013). It is unknown which genes in the cluster have their own promoter. There is a 958 base pair sequence between the divergent *cysE2* and *nifB* that is expected to include a promoter for each gene on

opposite strands. To help elucidate the transcriptional regulation of the *nif* cluster in *Synechocystis*, we replaced the sequence between *cysE2* and *nifB* with the oxygen-responsive P_{O2} (Immethun et al., 2016). Replacing this native sequence with the divergent P_{O2}s did not significantly alter nitrogenase activity, thus providing a tool to explore the cluster's transcriptional regulation in *Synechocystis*. In previous work, P_{O2} only allowed transcription in the absence of oxygen (Immethun et al., 2016). Therefore, any *nif* genes transcribed in aerobically-grown cultures should have their own promoter. This new synthetic biology tool can be used to investigate the regulation of the *nif* cluster in a non-native host.

4.2 Materials and Methods

4.2.1 Strains and Plasmids

The integrative plasmid that replaced the DNA sequence between *nifB* and *cysE2* of pSL2397 were constructed in *E. coli* DH10B, which was grown in LB (Miller, AMRESCO) at 37°C and 250 rpm in 14 mL BD Falcon™ round-bottom tubes. Spectinomycin (spec) (100 μg/mL) was added as appropriate. Plasmids were constructed using the Golden Gate assembly method (Engler et al., 2008) and were sequence-verified at the Protein and Nucleic Acid Chemistry Laboratory, Washington University in St. Louis, School of Medicine. The plasmid is listed in Table 4.1, and the gene sequences are listed in Table 4.2. Enzymes were purchased from New England BioLabs, Inc.

Table 4.1 Plasmid used in this work.

Name	Parts	Type
pCI092	f1/pBR322 ori; $spec^R$; P_{O2} (one on each strand), *Bba J23104-fnr*	Integrative (*pSL2397*)

Table 4.2 List of genetic parts used in this work.

Part name	Type and source	DNA sequence
fnr	Gene (Immethun et al., 2016)	atgatcccggaaaagcgaattatacggcgcattcagtctggcggttgtgctatccattgccaggattgcagc atcagccagctttgcatcccgttcacactcaacgaacatgagcttgatcagcttgataatatcattgagcgga agaagcctattcagaaaggccagacgctgtttaaggctggtgatgaacttaaatcgctttatgccatccgctc cggtacgattaaaagttataccatcactgagcaaggcgacgagcaaatcactggtttccatttagcaggcga cctggtgggatttgacgccatcggcagcggccatcacccgagcttcgcgcaggcgctggaaacctcgat ggtatgtgaaatcccgttcgaaacgctggacgatttgtccggtaaaatgccgaatctgcgtcagcagatgat gcgtctgatgagcggtgaaatcaaaggcgatcaggacatgatcctgctgttgtcgaagaaaaatgccgag gaacgtctggctgcattcatctacaacctgtcccgtcgttttgcccaacgcggcttctcccctcgtgaattccg cctgacgatgactcgtggcgatatcggtaactatctgggcctgacggtagaaaccatcagccgtctgctgg gtcgcttccagaaaagcggcatgctggcagtcaaaggtaaatacatcaccatcgaaaataacgatgcgctg gcccagcttgctggtcatacgcgtaacgttgcctga	
	5’ UTR	cttctctgctgttaaggtttgcttagacttacttgctccctaaaaagatgttaaaattgacaaatatcaattacgg cttgagcagacct
PO2	Promoter (Immethun et al., 2016)	ggaattcgcggccgcttctagagtttgatttacatcaattacggctagctcagtcctaggtattatgctagctac tagaga

A glucose-tolerant strain of *Synechocystis* was transformed previously with the pSL2397 plasmid, which includes *Cynaothece* sp. ATCC 51142’s *nif* cluster, *nifT* to *hesB* (Deng Liu et al., in preparation). This strain, T2397, was naturally transformed as described previously (Immethun et al., 2016) by the integrative plasmids. The flanking regions used to transform into pSL2397 in *Synechocystis* are listed in Table 4.3. Colony PCR, as described previously, was used to confirm successful segregation (Immethun et al., 2016). Oligonucleotides for colony PCR, summarized in Table 4.4, were purchased from Integrated DNA Technologies. Engineered *Synechocystis* strains for this work are summarized in Table 4.5

Table 4.3 Flanking regions used for double homologous recombination.

Name	DNA sequence	Location
5’ NSnif	catgttctatttctacccacaaggagccatcttcaggacaccagcagggaatggttaattcttggagacgttggtaaacac tccaacgatcgcaacgagaaactttctcaacttgactgattagagcatcagaatggtaaggttgcatcataattgtaaagt aaaggtcaaggtagtttttgtatcttttgttccaattcttcaattcggtctagtaaactccgaatgacttggccctcaacatcg ggtaatttgccgtgttctaaaggacaaccccgaccactacgggagataatacgaccaggaacgcctactaccgtgcaa tcatggggaacatcgcttaaaacgattgatccggctccaatacggacatgattgccaatttcgatgttgcctaatacttttg ccccggctcccacaatgactgaattgcctagagtgggatggcgtttaccactttctttacccgtccccctagggtgacat tttggtaaatgaggcaataatcaccaataatcgctgtttccccaatgaccacccccataccatgatcgataaacacccctt ggccaagagtcgcccctggatgaatttcgattcccgtaagaaaacgggcaatatgagagagaaaacggggaaaaaa aggcaaattgcgatgccataattcgtgggaaagacgatggatcgccaaagcgtgtaaacctggatagcaacacacca cttctaaccaattacgggctgcgggatctctttcaaaaatgatttgaaaatccttccgcaaaaagcttaataggttaggtcg agaagtgcgcggttcagaaatagagctttctgacggacttccacgacgctctctgacagggagtgatccaaacattaaa ttccgtgcttgcgcatagacttagttgttatgcttatacc	*pSL2397*
3’ NSnif	caggctttagctcaccctctaacttacggaattcaatgttacaatctacaggtctcattaacactgacgcatctacggctaat ccaacaactgcgccgggaacctcatttattcccaaagcaaaaggagattgtgcttgtccctcaagtggggatagtacac aaaccatagatgcaaagattcaagaaagaattgccaaacacccttgttacagtgaagatgcccatcatcactatgcacg gatgcacgtcgccgtcgcccctgcttgtaacattcaatgcaactattgtaaccgcaaatacgactgtgccaacgaaagtc ggccaggagtggttagtgaagttttaaccccagaagaagcagcccataaagcattagtaattggtggaaaaattcccca aatgactgtattaggtattgccggtcctggtgatcctttagcgaaccccaaacaaacctttcgcacctttgaattagtagca gataaagcccccgatattaaactctgcttatccagtaacggattaatgttaccggaatacgttgatcgcattaaagaactca acatcgatcatgtcaccctcaccattaatatgatcgaccccgaaattggggaaaaaatttatccttgggttcggtataatcg gaaacgatataaaggcatagaaggggttaagatacttcatgagaaacaaatggagtcccttgatgccctcaaagaagc ggatatcctctgcaaagtgaactccgtcatgattccaggaatcaacgacgaacacttagccgaagttaatgaagtcattc gttctaaaggtgcatttctccacaatattatgcccttgatttctgcccctgaacacggtactcatttcggtttgaccggacaa agaggccccacccctaaagagctaaaagctttacaggataagtgttctggcaacatgaaaatgatgcgtcactgccgtc aatgtcgcgctgatgctgttgggctactc	*pSL2397*

.

Table 4.4 Oligonucleotides used to assess *Synechocystis* transformation and segregation.

Name	Sequence (5’ to 3’)
2397reg_F	agcttaataggttaggtcgagaagtgc
2397reg_R	ctttcttgaatctttgcatctatggtttgtgtactatc
2397regmid_F	catgctggcagtcaaaggtaaatacatcac
2397regmid_R	gtgatgaaccttctgccagatc

Table 4.5 *Synechocystis* strains used in this work.

Name	Plasmids
T2397	pSL2397
CI182	pCI092 integrated into pSL2397

4.2.2 Nitrogenase Activity

Growth Conditions

Seed cultures of the *Synechocystis* strains were grown in 50 mL of BG11 media (http://www-cyanosite.bio.purdue.edu/media/table/BG11.html), with kanamycin (kan) (40 μg/mL) or spectinomycin (spec) (100 μg/mL) as appropriate, in 250 mL baffled flasks at 30°C and 160 rpm, under continuous white light (50 μmol of photons $m^{-2}s^{-1}$(μE)) in ambient air until they reach an OD_{730} of 6. The cultures were washed two times with BG11o (Chapter 3 Materials and Methods). After the final wash, each pellet was resuspended in 500 mL BG11o in one liter roux bottles. Antibiotic was added as appropriate. The cultures were bubbled with ambient air, 30°C, cycling 50 μE white light for 12 hours and dark for 12 hours. After the cultures yellowed, which indicates nitrogen stress (Allen and Smith, 1969; Grossman et al., 1993), 20 mL of BG11 was added to each culture. When the cultures yellowed a second time, 25 mL were moved into 125 mL testing bottles three – four hours before the beginning of the light cycle. For the anaerobic cultures, DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) (Sigma–Aldrich Co. LLC) was added, for a final concentration of 20 μM. The anaerobic cultures were sealed and flushed with argon for 15 minutes. The aerobic and anaerobic cultures were placed on a shaker, back into cycling light conditions.

Acetylene Reduction Assay

At the beginning of the dark period, approximately 15 hours after the cultures were transferred into testing bottles, the cultures' 100 mL headspace were injected with 5ml pure acetylene. Gas samples were drawn from the headspace at zero and three hours after acetylene injection and the ethylene production measured as described previously (Bandyopadhyay et al., 2010).

Total chlorophyll *a* (ch) was extracted with methanol. 1 mL of the culture used for the acetylene reduction assay was centrifuged at 10,000 g for 10 minutes. The pellet was resuspended vigorously in 1 mL methanol (Sigma–Aldrich Co. LLC) and centrifuged at 16,000g for 3 minutes. 500 µL of the supernatant was added to 500 µL methanol and quantified spectrophotometrically as described previously (Bandyopadhyay et al., 2010).

(4.1) $((16.29 * Abs_{665\,nm} - 8.54 * Abs_{652}) * 2)$ µg/mL

Nitrogenase activity (µmol/mg ch/ hour) was calculated as follows:

(4.2)

$$\frac{\left(Area_{Ethylene_{3\ hours}} - Area_{Ethylene_{0\ hours}}\right) * 100\ mL\ Argon}{\left(4225.5\ \frac{Area_{Ethylene}}{\left(\frac{Volume_{Ethylene}}{Volume_{\ Argon}}\right)}\right)^{a} * 24.86 \frac{mL}{\mu mol} Ethylene\ at\ 30°C * chlorophyll\ a\left(\frac{mg}{mL}\right) * 25\ mL\ culture * 3\ hours}$$

[a] Determined by a standard curve

4.3 Results and Discussion

Regulation of *Cyanothece* sp. ATCC 51142's nitrogenase has been well studied, but the location of the transcriptional regulators and their response to environmental signals has yet to be clarified. This strain of *Cyanothece* has proven to be recalcitrant to genetic analysis, making in-depth study problematic (Aryal et al., 2013). Furthermore, the differences in transcriptional regulation between the native organism and a heterologous host are just beginning to be probed. It is logical to expect that the region between the divergent *cysE2* and *nifB* contains two promoters, one on each strand. Therefore, replacing this region with synthetic regulation could be a step towards clarifying the native system and how it is regulated in a heterologous host.

Synechocystis had previously been transformed by *Cyanothece*'s *nif* cluster on a replicative plasmid (Deng Liu et al., in preparation). We introduced, between the divergent *cysE2* and *nifB* by double homologous recombination, two oxygen-responsive P_{O2} promoters on opposite strands, separated by the gene for their transcription factor (FNR) controlled by the constitutive *Bba J23104* (http://parts.igem.org/Part:BBa_J23104) (Figure 4.1a) and a different gene for antibiotic resistance. Nitrogenase activity was similar in both strains of *Synechocystis* expressing the 24 gene *nif* cluster, with *Cyanothece*'s native sequence and with the P_{O2} promoters (Figure 4.1b). This was true whether the cultures were tested in aerobic or anaerobic conditions. Nitrogenase is irreversibly inactivated by oxygen (Dixon and Kahn, 2004), so the lack of activity in aerobic conditions was expected and serves as a negative control.

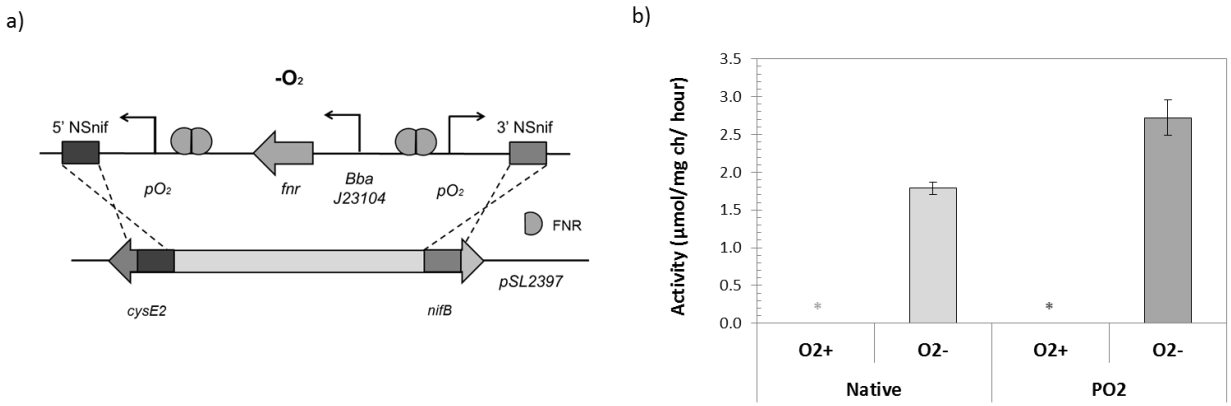


Figure 4.1 Synthetic regulation does not significantly alter nitrogenase activity of *Cyanothece* sp. ATCC 51142's 24 gene *nif* cluster expressed in *Synechocystis* sp. PCC 6803. (**a**) Schematic of the oxygen-responsive P_{O2} replacing the sequence between the divergent *nifB* and *cysE2* of *Cynanothece* sp. ATCC 51142's 24 gene *nif* cluster in the synthetic plasmid pSL2397 (Materials and Methods). P_{O2} was built from *E. coli*'s fumarate and nitrate reduction (FNR) system (Immethun et al., 2016). (**b**) *Synechocystis'* nitrogenase activity of the 24 gene *nif* cluster using native regulation and using the oxygen-responsive P_{O2} was determined by the acetylene reduction assay (Materials and Methods). The data reported are the average of two replicates measured on the same day. Error bars indicate one standard deviation. The asterisk (*) indicates that there was no activity when the cultures were tested in aerobic conditions.

Replacing the sequence between the divergent *cysE2* and *nifB* with two P_{O2} promoters did not significantly alter nitrogenase activity in *Synechocystis*. We have therefore introduced a new synthetic biology tool that can be used to probe the *nif* cluster's regulation in *Synechocystis*. It has been shown that transcription from P_{O2} only occurs in low oxygen in *Synechocystis* (Immethun et al., 2016). Thus, any genes transcribed in aerobic conditions should have their own oxygen-insensitive promoter, or be transcribed polycistronically, with a gene(s) preceding it that has its own oxygen-insensitive promoter. Deng Liu et al., will show in the up-coming publication which genes of the native *nif* cluster are transcribed in aerobic cultures of *Synechocystis*, providing further data on the effect of the oxygen-sensitive promoters on the transcription of the genes in the cluster.

4.4 Conclusions

Increasing what we know about the transcriptional regulation of the *nif* cluster, in both *Cyanothece* and *Synechocystis*, could help us tune the cluster's expression to optimal conditions in non-native hosts. Nitrogenase activity in *Synechocystis* was maintained when the sequence between the divergent *cysE2* and *nifB* was replaced with two oxygen-responsive promoters, supplying a new synthetic biology tool that can be used to probe transcriptional control of the cluster. This new tool can help identify promoters within the cluster that control transcription in *Synechocystis*. By replacing the oxygen-responsive promoters with nitrate-responsive promoters, we could also investigate the transcriptional regulation in various stages of nitrogen deprivation. This work strengthens our ability to unravel the principles of nitrogen fixation in a photosynthetic host. While it was not finished before my defense, the groundwork has been laid for its completion. My suggestions for experiments with this new tool are outlined in the future directions section of chapter 5.

4.5 Acknowledgements

The authors thank Dr. Himadri Pakrasi for providing *Synechocystis* sp. PCC 6803 strain T2397 and Dr. Deng Liu for patiently teaching me the protocol, running the acetylene reduction assays, and discussing the scientific relevance of this work. This work was supported by National Science Foundation grant MCB-1331194. Cheryl Immethun is the recipient of an NSF Graduate Research Fellowship, DGE-1143954.

4.6 References

Allen MM, Smith AJ. 1969. Nitrogen chlorosis in blue-green algae. Arch Mikrobiol 69(2):114-20.

Aryal UK, Callister SJ, Mishra S, Zhang X, Shutthanandan JI, Angel TE, Shukla AK, Monroe ME, Moore RJ, Koppenaal DW and others. 2013. Proteome analyses of strains ATCC 51142 and PCC 7822 of the diazotrophic cyanobacterium Cyanothece sp. under culture conditions resulting in enhanced H(2) production. Appl Environ Microbiol 79(4):1070-7.

Bandyopadhyay A, Elvitigala T, Welsh E, Stockel J, Liberton M, Min H, Sherman LA, Pakrasi HB. 2011. Novel metabolic attributes of the genus cyanothece, comprising a group of unicellular nitrogen-fixing Cyanothece. MBio 2(5).

Bandyopadhyay A, Stockel J, Min H, Sherman LA, Pakrasi HB. 2010. High rates of photobiological H2 production by a cyanobacterium under aerobic conditions. Nat Commun 1:139.

Bernhard A. 2012. The Nitrogen Cycle: Processes, Players, and Human Impact. Nature Education Knowledge 3(10).

Cerveny J, Sinetova MA, Valledor L, Sherman LA, Nedbal L. 2013. Ultradian metabolic rhythm in the diazotrophic cyanobacterium Cyanothece sp. ATCC 51142. Proc Natl Acad Sci U S A 110(32):13210-5.

Charpentier M, Oldroyd G. 2010. How close are we to nitrogen-fixing cereals? Curr Opin Plant Biol 13(5):556-64.

Colon-Lopez MS, Sherman DM, Sherman LA. 1997. Transcriptional and translational regulation of nitrogenase in light-dark- and continuous-light-grown cultures of the unicellular cyanobacterium Cyanothece sp. strain ATCC 51142. J Bacteriol 179(13):4319-27.

Dixon R, Kahn D. 2004. Genetic regulation of biological nitrogen fixation. Nat Rev Micro 2(8):621-631.

Dos Santos PC, Fang Z, Mason SW, Setubal JC, Dixon R. 2012. Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. BMC Genomics 13:162.

Elvitigala T, Stockel J, Ghosh BK, Pakrasi HB. 2009. Effect of continuous light on diurnal rhythms in Cyanothece sp. ATCC 51142. BMC Genomics 10.

Engler C, Kandzia R, Marillonnet S. 2008. A one pot, one step, precision cloning method with high throughput capability. PLoS One 3(11):e3647.

Erisman JW, Sutton MA, Galloway J, Klimont Z, Winiwarter W. 2008. How a century of ammonia synthesis changed the world. Nature Geosci 1(10):636-639.

Gaudana S, Krishnakumar S, Alagesan S, Digmurti M, Viswanathan G, Chetty M, Wangikar P. 2013. Rhythmic and sustained oscillations in metabolism and gene expression of Cyanothece sp. ATCC 51142 under constant light. Frontiers in Microbiology 4(374).

Grossman AR, Schaefer MR, Chiang GG, Collier JL. 1993. The phycobilisome, a light-harvesting complex responsive to environmental conditions. Microbiol Rev 57(3):725-49.

Immethun CM, Ng KM, DeLorenzo DM, Waldron-Feinstein B, Lee YC, Moon TS. 2016. Oxygen-responsive genetic circuits constructed in Synechocystis sp. PCC 6803. Biotechnol Bioeng 113(2):433-42.

Krishnakumar S, Gaudana SB, Digmurti MG, Viswanathan GA, Chetty M, Wangikar PP. 2015. Influence of mixotrophic growth on rhythmic oscillations in expression of metabolic pathways in diazotrophic cyanobacterium Cyanothece sp. ATCC 51142. Bioresour Technol 188:145-52.

Latysheva N, Junker VL, Palmer WJ, Codd GA, Barker D. 2012. The evolution of nitrogen fixation in cyanobacteria. Bioinformatics 28(5):603-6.

Mauseth JD. 2014. Botany: An Introduction to Plant Biology. Burlington, Massachusetts: Jones & Bartlett Learning LLC. 696 p.

Ritter SK. 2008. The Haber-Bosch Reaction: An Early Chemical Impact On Sustainability. Chemical & Engineering News: American Chemical Society.

Stockel J, Welsh EA, Liberton M, Kunnvakkam R, Aurora R, Pakrasi HB. 2008. Global transcriptomic analysis of Cyanothece 51142 reveals robust diurnal oscillation of central metabolic processes. Proc Natl Acad Sci U S A 105.

Toepel J, Welsh E, Summerfield TC, Pakrasi HB, Sherman LA. 2008. Differential transcriptional analysis of the cyanobacterium Cyanothece sp. strain ATCC 51142 during light–dark and continuous-light growth. J Bacteriol 190.

Toepel JR, McDermott JE, Summerfield TC, Sherman LA. 2009. TRANSCRIPTIONAL ANALYSIS OF THE UNICELLULAR, DIAZOTROPHIC CYANOBACTERIUM CYANOTHECE SP. ATCC 51142 GROWN UNDER SHORT DAY/NIGHT CYCLES(1). J Phycol 45(3):610-20.

Van Metre PC, Frey JW, Musgrove M, Nakagaki N, Qi S, Mahler BJ, Wieczorek ME, Button DT. 2016. High Nitrate Concentrations in Some Midwest United States Streams in 2013 after the 2012 Drought. Journal of Environmental Quality 45:1696-1704.

Chapter 5: Conclusions and Future Directions

5.1 Conclusions

Utilizing cyanobacteria's photoautotrophy to produce compounds of interest at significant titers requires the development of new tools, including ones that can regulate expression in response to specific signals. I developed transcriptional regulators for the model cyanobacterium *Synechocystis* sp. PCC 6803, each responding to a different type or combination of signals. The promoter based on *E. coli*'s fumarate and nitrate reduction system could be used for processes sensitive to oxygen, including hydrogen formation, nitrogen fixation and the production of biofuels through fermentative pathways. Light is a fundamental requirement for cyanobacteria and their use as sustainable hosts. The chimeric light sensors could be used to establish separation between processes that should only occur either in the light or in the dark. Arabinose is not tied to *Synechocystis*' growth, leaving it available as an inexpensive chemical signal. The family of arabinose sensors is tightly off when uninduced, an important characteristic when producing compounds toxic to the host. The library of nitrate sensors responds to the intracellular nitrogen status, which is vital to protein production. All of these inducible promoters respond to a single signal, while gene expression in a native host often responds to multiple signals. The two-input AND gate now provides a source of complex control of transcription in heterologous hosts. These additional choices of transcriptional regulators can help engineers find one that better suits the needs of their situation. Our work expressing *Cyanothece* sp. ATCC 51142's *nif* cluster in *Synechocystis* demonstrates how the right tool can facilitate larger goals.

5.2 Future Directions

5.2.1 Current limitations of biotechnology

Microbes have been employed for the manufacture of food products and other goods for centuries. The uses of microbial biotechnology have grown as we learn more about the inner workings of the production hosts. Yet, our incomplete understanding of biology often limits the choices of platform organisms to either natural producers or commonly engineered microbes, instead of organisms that possess unique and beneficial traits. The shortage of biological knowledge also contributes to long design cycles, which are frequently relegated to trial and error instead of forward engineering (Kwok, 2010). Huge advancements have been made in the sequencing and annotation of microbial genomes; although, annotation is still incomplete, especially for less commonly employed organisms. Even less is known about the genomics; including gene regulation and interactions. In addition, enzyme activity and substrate affinity data may not be available for pathways of interest (Chubukov et al., 2016). Applications of microbial bioprocessing are promising, but still limited by current biological information.

Process yields, titers, and productivity of microbial bioprocessing can reduce profitability. An inadequate supply of the appropriate cofactors and substrates can contribute to low production of the compound of interest (Oliver and Atsumi, 2014). Product creation competes for available cellular resources, including replication machinery, RNA polymerases and ribosomes, as well as substrates and co-factors (Weisse et al., 2015). Normal cellular processes can suffer as a result, limiting the growth and health of the organisms. Toxicity of the final product or process intermediates can impair the fitness of the host, also adversely affecting production (Oliver and Atsumi, 2014). Organisms evolved to maximize their own growth, not make products which do not contribute to this objective. Strategies that allow a host to maintain normal cellular functions while maximizing production are needed.

Manufacturing requires robust processes. Microbes need to be able to tolerate the fluctuations that can occur in large vats, as the organisms might not always experience ideal conditions (Chubukov et al., 2016). The stress of non-ideal conditions or even the stress of production should not result in mutations, which can eliminate or alter the process. Fluctuations can also occur within cultures due to uneven distribution of cellular resources (Xiao et al., 2016). Furthermore, other bacteria, fungi and phages can reduce productivity or even crash a culture (Ducat et al., 2011). Process and strain development could help reduce the sources of variation and maximize the culture's ability to withstand deviations.

Scaling up a successful process from laboratory scale to production scale often introduces new problems. In addition to the differences in nutrient mixing, the availability of oxygen, or light for phototrophic hosts, can be reduced during production conditions (Chubukov et al., 2016). These fluctuations can shift the organism's metabolic state, and thus the available substrates, cofactors and energy, which subsequently alters production. Bioprocessing has also not been commercialized as long as industrial chemical processing. The development tools, such as process modeling and statistical-based design of experiments, are not as well established (Neubauer et al., 2013). Therefore scaling up production can take a long time, five to ten years, and cost more than traditional chemical processes. Specific attention to these obstacles, including the investment of time and money, could help bioprocessing become more industrially relevant.

5.2.2 Promises of biotechnology

Bioprocessing can be a sustainable alternative to traditional chemical processing. The feedstocks for the microbial hosts are commonly derived from renewable sources (Erickson et al., 2012). Waste products from other processes can even be consumed in some bioprocesses, while the

waste products of microbial cultures are inherently biodegradable (Cheong et al., 2016). Microbial biosynthesis often needs fewer steps to create the product than chemical synthesis and uses less energy from external sources (Erickson et al., 2012). It is also well suited for the creation of complex compounds without producing a racemic mixture (Hong and Nielsen, 2012). Enzyme engineering can even be used to create new molecules with unique properties. Furthermore, biotechnology is not usually geographically constrained, which can reduce transportation costs. Bioprocessing can provide both environmental and economic benefits.

As the molecular biology revolution progresses, many of the limitations related to our incomplete understanding of biology will diminish. This can enable forward engineering instead of trial and error. Synthetic biology can assist the building of production hosts from parts that best fit the needs to the process, regardless of the parts' origin, magnifying the realm of what's is possible beyond what nature has created. Problems with the application of engineering principles to manufacturing processes are not unique to biotechnology. When wave soldering was introduced in the manufacturing of printed circuit boards, engineers at AT&T Technologies in Lee's Summit, Missouri struggled to apply engineering principles for process control due to a lack of knowledge of how the process variables interacted. It was commonly stated that wave soldering was art or even black magic. As the knowledge base grew, over a number of years, the ability to control the process, and thus yields and productivity, improved. The growth of the biology knowledge base will facilitate the application of engineering principles to biotechnology. There are already a number of commercially viable bioprocesses. Dupont manufactures a variety of products, from cosmetics to antifreeze to carpets, from microbially produced 1, 3 propanediol. NatureWorks makes food grade plastics from polylactic acid produced by microbes. The number of success stories will continue to grow along with the expansion of the knowledge base.

5.2.3 Promises and limitations of cyanobacteria

Cyanobacteria offer several advantages over commonly used heterotrophic hosts. As photoautotrophs, they utilize sunlight to power their metabolism, which includes fixing carbon from carbon dioxide. Engineering cyanobacteria to directly consume flue gas from power plants would provide the organisms with a significant feedstock source while concurrently reducing the emission of the greenhouse gas. Through their billions of years of evolution, cyanobacteria have adapted to a large range of conditions. Many species are tolerant to a range of salt (Joset et al., 1996), metal (Los et al., 2008), and nitrogen (Klotz et al., 2016) concentrations, as well as alkaline pH (Summerfield and Sherman, 2008). The organisms could therefore withstand fluctuations that can occur in industrial settings. Furthermore, the tolerance could be exploited to create conditions suitable for the production host, but not sources of contamination (Ducat et al., 2011).

The adaptability of cyanobacteria can also be a limitation. Many species are naturally competent. While this is very useful in the lab, horizontal gene transfer could be a problem in the production setting (Ruffing, 2011). Cyanobacteria have adapted to a number of growth conditions. Many species employ photoautotrophic and photomixotrophic growth, while some species are also capable of heterotrophic or even anaerobic growth (Stal and Moezelaar, 1997). Switching between these metabolisms in production conditions could diminish productivity. Cyanobacterial metabolisms are also complicated by diurnal and/or circadian cycling of gene expression (Saha et al., 2016). Also, as discussed in the introduction, tool development for cyanobacteria has lagged behind heterotrophic hosts (Berla et al., 2013). These limitations need to be addressed to improve the relevance of cyanobacterial biotechnology, also providing a number of opportunities.

5.2.4 The NITROGEN project

Cyanothece sp. ATCC 51142's nitrogenase has been shown to have strong activity in both aerobic and anaerobic conditions, as discussed in Chapter 4. Despite significant interest in this organism's ability to fix nitrogen and produce hydrogen, 12 of the 35 genes in the cluster are not annotated and the function of others are not clearly defined (Bandyopadhyay et al., 2011). Furthermore, the lack of genetic tractability of this species limits the study of the cluster's regulation in the native host (Aryal et al., 2013). Studying the principles of nitrogen fixation in a photosynthetic cell with this enzyme would be improved with the addition of annotation for all of the cluster's genes and clarification of the cluster's regulation. Expressing the cluster in a heterologous host, aided by synthetic biology, can help elucidate this cluster's missing biological information.

Mimicking the natural intracellular environment in the heterologous host could improve nitrogenase's activity and help clarify the cluster's regulation. For instance, it has been shown that the activity of *Paenibacillus* sp. WLY78's nitrogenase expressed in *E. coli* 78-7 increased significantly when the genes for *Paenibacillus*' electron transport were also expressed in *E. coli* (Li et al., 2016). *Cyanothece* sp. ATCC 51142's nitrogenase is irreversibly inactivated by oxygen (Toepel et al., 2008). The native host boosts respiration by upregulating its glycogen catabolism to create a microoxic environment, as well as increase ATP (Colon-Lopez et al., 1997; Schneegurt et al., 1994; Stockel et al., 2008). A heterologous host, which is *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) in the current studies, does not create a microoxic environment or boost ATP production for nitrogenase activity naturally. The addition of synthetic regulation in response to light, to express the genes only in the dark, or in response to oxygen could help protect the enzyme from irreversible inactivation. Work included in Chapter

3 indicated that *Cyanothece* sp. ATCC 51142's *nrrA* was recognized by a *Synechocystis* NrrA-activated promoter. NrrA has been shown to activate genes responsible for glycogen catabolism in *Synechocystis*. Therefore, *nrrA* over-expression could provide a way to upregulate *Synechocystis* glycogen catabolism, mimicking the approach the native host uses to reduce intracellular oxygen and increase ATP. Synthetic regulation, as discussed in Chapter 4, could also provide methods to investigate how the cluster is regulated by its native sequence in the new host.

In free-living, not symbiotic, native hosts, nitrogenase is regulated by the availability of fixed-nitrogen (Dixon and Kahn, 2004). We saw nitrogenase activity in *Synechocystis*, from *Cyanothece*'s nitrogenase, increase with increasing nitrogen stress (unpublished data). This was true for both the *Synechocystis* culture expressing *Cyanothece*'s native sequence and when the P_{O2} promoters were introduced, Chapter 4. This indicates that the sequence responsible for regulating the cluster in response to nitrogen is not between *cysE2* and *nifB* and is recognized by *Synechocystis*. We could therefore probe regulation of *Cyanothece*'s native *nif* cluster sequence in *Synechocystis* by using one of the metabolic state sensors (Chapter 3) to express *cysE2* and *nifB* (Chapter 4). Expressing nitrogenase in *Synechocystis*, aided by the new synthetic biology tools developed in this work can help unravel the principles if nitrogen fixation in a photosynthetic cell.

5.2.5 Nitrogen-fixing chloroplasts

Transferring *Cyanothece*'s *nif* cluster to the non-diazotrophic *Synechocystis*, investigating the cluster's regulation and optimizing the enzyme's activity can help determine the principles for creating nitrogen-fixing chloroplasts. Chloroplasts have been successfully engineered to express heterologous genes for herbicide, insect, pathogen, drought and salt resistance, as well as for

amino acid synthesis (Daniell et al., 2002). Furthermore, gene flow to other plants and toxicity to non-targeted insects is minimized when foreign genes are expressed in chloroplasts (Daniell, 2002), making them strong candidates for expressing the *nif* cluster. The development of nitrogen-fixing chloroplasts could reduce the use of synthetic fertilizers, and the energy required to produce and transport it. In countries whose food production is limited by inadequate infrastructure, reducing the infrastructure required for the production, distribution and application of fertilizers could have a significant impact (Godfray et al., 2010). In addition to the economic impact, nitrogen-fixing chloroplasts would also have an environmental impact. In free-living hosts, nitrogenase is only active when the organism requires more fixed nitrogen. This would limit excess nitrogen that could end up in the soil, where it can run off into waterways after being converted to nitrate. Nitrogen-fixing chloroplasts could contribute to the next green revolution.

5.2.6 Possibilities for the advancement of cyanobacterial biotechnology

Genetic stability is required for hosts in biotechnology. While cyanobacteria's adaptability can yield robust cultures, an altered genotype can reduce production. This result has been seen in my unpublished work as well as the work of other scientists working with cyanobacteria, especially when the cultures experience stress from production conditions, toxic products or over-expression (Jones, 2014). For machines used in traditional manufacturing, the limits of the equipment need to be understood and operated within. Running a piece of machinery near its limits results in processes that are harder to control and broken equipment. This is also true for hosts in biotechnology. Understanding the specific mechanisms which cause cellular stress will lead to process design and control that minimizes the mutations. The *nif* cluster was stably

maintained on a plasmid for months in bubbling cultures of *Synechocystis* (Chapter 4), indicating that even large gene clusters can be stable in the right conditions.

Deletion of gene(s) responsible for homologous recombination, after integration of the genes of interest into a production host, could promote genetic stability. The deletion of *E. coli*'s *recA*, which is associated with DNA repair and maintenance, increased genetic stability ten-fold, leading to a 60% improvement in lycopene yield (Tyo et al., 2009). A homolog was identified in *Synechocystis* sp. PCC 6803, but its deletion only produced viable cultures in low light conditions (Minda et al., 2005). Homologs of other *E. coli* genes associated with DNA repair and mutations have been identified in cyanobacteria (Jones, 2014); although it is not clear whether the mechanisms are the similar to those in *E. coli*. This provides numerous opportunities for studying DNA repair and maintenance mechanisms in cyanobacteria and how they can be utilized to improve genetic stability.

Even with the explosion of biological information now available as a result of high-throughput techniques, there is still much to be learned, especially for less-commonly studied organisms like cyanobacteria. A clearer understanding of the gene annotation, regulation and interactions will allow the forward engineering of production processes in cyanobacteria. The number of well-studied cyanobacterium is also relatively low. Therefore, potential hosts with desirable traits might have not been characterized yet. For instance, a fast-growing cyanobacterium was just recently discovered in the Pakrasi lab that may become an excellent production host (Yu et al., 2015). Furthermore, the number of tools for heterologous gene expression is still limited in cyanobacteria. Each of these subjects offers opportunities for significant advancement.

To date most products from biotechnology are made by monocultures (Sabra et al., 2010), yet microbial co-cultures have demonstrated improved product titers (Cui et al., 2011; Qu et al., 2012; Xu and Tschirner, 2011). In microbial consortia, species can interact, sharing substrates, growth factors and metabolites, often with more than one species capable of supplying a particular component (Escalante et al., 2015). The division of labor can reduce metabolic burden, improving genetic stability and product titers (Weisse et al., 2015; Wu et al., 2016). Members of consortia can remove toxic byproducts from the culture, also improving genetic stability and health of the cultures (Escalante et al., 2015). Cyanobacteria are found in natural systems with other species (Crits-Christoph et al., 2016; Wong et al., 2015) and have recently been used in an engineered system (Fistarol et al., 2016). Microbial consortia that include cyanobacteria could provide a robust and efficient method of production in biotechnology.

Commercial biotechnology, especially cyanobacterial biotechnology is still a new field. As we search for sustainable alternatives to conventional processes and products, microbial biosynthesis provides specific and efficient choices that are environmentally balanced. While there currently are a number of limitations, they are also opportunities for development. This is a function of the field's youth. With the dedication of research and development to biotechnology, the discipline's contributions will continue to grow and expand to new product lines, providing both economic and environmental benefits.

5.3 References

Aryal UK, Callister SJ, Mishra S, Zhang X, Shutthanandan JI, Angel TE, Shukla AK, Monroe ME, Moore RJ, Koppenaal DW and others. 2013. Proteome analyses of strains ATCC 51142 and PCC 7822 of the diazotrophic cyanobacterium Cyanothece sp. under culture conditions resulting in enhanced H(2) production. Appl Environ Microbiol 79(4):1070-7.

Bandyopadhyay A, Elvitigala T, Welsh E, Stockel J, Liberton M, Min H, Sherman LA, Pakrasi HB. 2011. Novel metabolic attributes of the genus Cyanothece, comprising a group of unicellular nitrogen-fixing Cyanothece.

Berla BM, Saha R, Immethun CM, Maranas CD, Moon TS, Pakrasi HB. 2013. Synthetic biology of cyanobacteria: unique challenges and opportunities. Front Microbiol 4:246.

Cheong S, Clomburg JM, Gonzalez R. 2016. Energy- and carbon-efficient synthesis of functionalized small molecules in bacteria using non-decarboxylative Claisen condensation reactions. Nat Biotechnol 34(5):556-61.

Chubukov V, Mukhopadhyay A, Petzold CJ, Keasling JD, Martín HG. 2016. Synthetic and systems biology for microbial production of commodity chemicals. Npj Systems Biology And Applications 2:16009.

Colon-Lopez MS, Sherman DM, Sherman LA. 1997. Transcriptional and translational regulation of nitrogenase in light-dark- and continuous-light-grown cultures of the unicellular cyanobacterium Cyanothece sp. strain ATCC 51142. J Bacteriol 179(13):4319-27.

Crits-Christoph A, Gelsinger DR, Ma B, Wierzchos J, Ravel J, Davila A, Casero MC, DiRuggiero J. 2016. Functional interactions of archaea, bacteria and viruses in a hypersaline endolithic community. Environ Microbiol 18(6):2064-77.

Cui F, Li Y, Wan C. 2011. Lactic acid production from corn stover using mixed cultures of Lactobacillus rhamnosus and Lactobacillus brevis. Bioresour Technol 102(2):1831-6.

Daniell H. 2002. Molecular strategies for gene containment in transgenic crops. Nat Biotechnol 20(6):581-6.

Daniell H, Khan MS, Allison L. 2002. Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. Trends Plant Sci 7(2):84-91.

Dixon R, Kahn D. 2004. Genetic regulation of biological nitrogen fixation. Nat Rev Micro 2(8):621-631.

Ducat DC, Way JC, Silver PA. 2011. Engineering cyanobacteria to generate high-value products. Trends Biotechnol 29(2):95-103.

Erickson B, Nelson JE, Winters P. 2012. Perspective on opportunities in industrial biotechnology in renewable chemicals. Biotechnology Journal 7(2):176-185.

Escalante AE, Rebolleda-Gomez M, Benitez M, Travisano M. 2015. Ecological perspectives on synthetic biology: insights from microbial population biology. Front Microbiol 6:143.

Fistarol GO, Rosato M, Thompson FL, do Valle Rde A, Garcia-BlairsyReina G, Salomon PS. 2016. Use of a marine microbial community as inoculum for biomethane production. Environ Technol 37(3):360-8.

Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas SM, Toulmin C. 2010. Food Security: The Challenge of Feeding 9 Billion People. Science 327(5967):812-818.

Hong KK, Nielsen J. 2012. Metabolic engineering of Saccharomyces cerevisiae: a key cell factory platform for future biorefineries. Cell Mol Life Sci 69(16):2671-90.

Jones PR. 2014. Genetic Instability in Cyanobacteria – An Elephant in the Room? Frontiers in Bioengineering and Biotechnology 2:12.

Joset F, Jeanjean R, Hagemann M. 1996. Dynamics of the response of cyanobacteria to salt stress: Deciphering the molecular events. Physiologia Plantarum 96(4):738-744.

Klotz A, Georg J, Bucinska L, Watanabe S, Reimann V, Januszewski W, Sobotka R, Jendrossek D, Hess WR, Forchhammer K. 2016. Awakening of a Dormant Cyanobacterium from Nitrogen Chlorosis Reveals a Genetically Determined Program. Curr Biol.

Kwok R. 2010. Five hard truths for synthetic biology. Nature 463(7279):288-90.

Li XX, Liu Q, Liu XM, Shi HW, Chen SF. 2016. Using synthetic biology to increase nitrogenase activity. Microb Cell Fact 15:43.

Los DA, Suzuki I, Zinchenko VV, Murata N. 2008. Stress Responses in Synechocystis: Regulated Genes and Regulatory Systems. In: Herrero A, Flores E, editors. The Cyanobacteria: Molecular Biology, Genomics, and Evolution. Norfolk, UK: Caister Academic Press. p 117-158.

Minda R, Ramchandani J, Joshi VP, Bhattacharjee SK. 2005. A homozygous recA mutant of Synechocystis PCC6803: construction strategy and characteristics eliciting a novel RecA independent UVC resistance in dark. Mol Genet Genomics 274(6):616-24.

Neubauer P, Cruz N, Glauche F, Junne S, Knepper A, Raven M. 2013. Consistent development of bioprocesses from microliter cultures to the industrial scale. Engineering in Life Sciences 13(3):224-238.

Oliver JWK, Atsumi S. 2014. Metabolic design for cyanobacterial chemical synthesis. Photosynthesis Research 120(3):249-261.

Qu Y, Feng Y, Wang X, Logan BE. 2012. Use of a coculture to enable current production by geobacter sulfurreducens. Appl Environ Microbiol 78(9):3484-7.

Ruffing AM. 2011. Engineered cyanobacteria: teaching an old bug new tricks. Bioeng Bugs 2(3):136-49.

Sabra W, Dietz D, Tjahjasari D, Zeng A-P. 2010. Biosystems analysis and engineering of microbial consortia for industrial biotechnology. Engineering in Life Sciences 10(5):407-421.

Saha R, Liu D, Hoynes-O'Connor A, Liberton M, Yu J, Bhattacharyya-Pakrasi M, Balassy A, Zhang F, Moon TS, Maranas CD and others. 2016. Diurnal Regulation of Cellular Processes in the Cyanobacterium Synechocystis sp. Strain PCC 6803: Insights from Transcriptomic, Fluxomic, and Physiological Analyses. MBio 7(3).

Schneegurt MA, Sherman DM, Nayar S, Sherman LA. 1994. Oscillating behavior of carbohydrate granule formation and dinitrogen fixation in the cyanobacterium Cyanothece sp. strain ATCC 51142. J Bacteriol 176(6):1586-97.

Stal LJ, Moezelaar R. 1997. Fermentation in cyanobacteria1. FEMS Microbiology Reviews 21(2):179-211.

Stockel J, Welsh EA, Liberton M, Kunnvakkam R, Aurora R, Pakrasi HB. 2008. Global transcriptomic analysis of Cyanothece 51142 reveals robust diurnal oscillation of central metabolic processes. Proc Natl Acad Sci U S A 105.

Summerfield TC, Sherman LA. 2008. Global transcriptional response of the alkali-tolerant cyanobacterium Synechocystis sp. strain PCC 6803 to a pH 10 environment. Appl Environ Microbiol 74(17):5276-84.

Toepel J, Welsh E, Summerfield TC, Pakrasi HB, Sherman LA. 2008. Differential transcriptional analysis of the cyanobacterium Cyanothece sp. strain ATCC 51142 during light–dark and continuous-light growth. J Bacteriol 190.

Tyo KE, Ajikumar PK, Stephanopoulos G. 2009. Stabilized gene duplication enables long-term selection-free heterologous pathway expression. Nat Biotechnol 27(8):760-5.

Weisse AY, Oyarzun DA, Danos V, Swain PS. 2015. Mechanistic links between cellular trade-offs, gene expression, and growth. Proc Natl Acad Sci U S A 112(9):E1038-47.

Wong HL, Smith DL, Visscher PT, Burns BP. 2015. Niche differentiation of bacterial communities at a millimeter scale in Shark Bay microbial mats. Sci Rep 5:15607.

Wu G, Yan Q, Jones JA, Tang YJ, Fong SS, Koffas MA. 2016. Metabolic Burden: Cornerstones in Synthetic Biology and Metabolic Engineering Applications. Trends Biotechnol 34(8):652-64.

Xiao Y, Bowen CH, Liu D, Zhang F. 2016. Exploiting nongenetic cell-to-cell variation for enhanced biosynthesis. Nat Chem Biol 12(5):339-44.

Xu L, Tschirner U. 2011. Improved ethanol production from various carbohydrates through anaerobic thermophilic co-culture. Bioresour Technol 102(21):10065-71.

Yu J, Liberton M, Cliften PF, Head RD, Jacobs JM, Smith RD, Koppenaal DW, Brand JJ, Pakrasi HB. 2015. Synechococcus elongatus UTEX 2973, a fast growing cyanobacterial chassis for biosynthesis using light and CO(2). Sci Rep 5:8132.

Cheryl M. Immethun

Washington University in St. Louis * Energy, Environmental & Chemical Engineering
One Brookings Drive * 3044 Brauer Hall * St. Louis, MO 63130
(816) 550-1030 * cheryl.immethun@wustl.edu

EDUCATION

Doctor of Philosophy in Energy, Environmental & Chemical Engineering October 2016
Washington University in St. Louis

Dissertation title: "Genetic Circuits for Transcriptional Regulation in *Synechocystis* sp. PCC 6803"
Committee: Gautam Dantas, Marcus Foston, Elizabeth Haswell, Himadri Pakrasi, Fuzhong Zhang, Tae Seok Moon (Chair)
GPA: 3.83

Bachelor of Science in Environmental Science, Minor in Chemistry May 2011
University of Missouri-Kansas City

Summa Cum Laude, GPA: 4.00

Bachelor of Science in Mechanical Engineering May 1985
University of Missouri-Columbia

Magna Cum Laude, GPA: 3.84
Engineer in Training (EIT) – Passed the Fundamentals of Engineering Exam

RESEARCH EXPERIENCE

Graduate Research Assistant July 2012 – October 2016
Department of Energy, Environmental & Chemical Engineering (EECE), Washington University in St. Louis

- Design, build and optimize the performance of synthetic transcriptional regulators in cyanobacteria. This includes synthetic regulation of a nitrogen fixation cluster in the non-diazotrophic *Synechocystis* sp. PCC 6803.
- As the first graduate student in the Moon Research Group, I set up the lab. This included purchasing the equipment and supplies, as well as developing the safety protocols and lab procedures.

Graduate Rotation Student Spring 2012
EECE, Washington University in St. Louis

- Tested and analyzed the ability of different fungi to decompose municipal solid waste under varied environmental conditions. To help elucidate the important interactions between the conditions in a manageable number of tests, I utilized a Fractional Factorial Design of Experiment (DOE) for five factors, two levels per factor (2_V^{5-1}).
- Optimized an isobutanol collection system for a genetically engineered strain of *Synechocystis* sp. PCC 6803.

RESEARCH EXPERIENCE (Continued)

Graduate Rotation Student Fall 2011
EECE, Washington University in St. Louis

- Studied the scale-up of sequestering carbon dioxide from flue gas using *Chlorella* sp. in 100 liter photobioreactors.

People, Prosperity and the Planet Student Design Competition for Sustainability 2011
University of Missouri – Kansas City (UMKC)

- Tested desiccants for their ability to absorb atmospheric moisture.
- Designed, built and tested a solar incubation and condensation system, to retrieve water from the desiccant.
- The system was built from low-cost parts (sheet metal, motorcycle radiator, etc.).

PUBLICATIONS

CM Immethun, CM Focht, DM DeLorenzo, D Gupta, CB Johnson, TS Moon. Physical, Chemical and Metabolic State Transcriptional Regulators Expand the Synthetic Biology Toolbox for *Synechocystis* sp. PCC 6803, (submitted).

N Wan, DM DeLorenzo, L He, L You, **CM Immethun**, G Wang, EEK Baidoo, W Hollinshead, JD Keasling, TS Moon, YJ Tang. Cyanobacterial Carbon Metabolism: Fluxome Plasticity and Oxygen Dependence, *Biotechnol Bioeng.* (under review).

CM Immethun, WR Henson, X Wang, DR Nielsen, TS Moon. in *Biotechnology for Biofuel Production and Optimization* Ch. 1. Engineering Central Metabolism for Production of Higher Alcohol-based Biofuels, 1 - 34 (Elsevier, 2016).

CM Immethun, KM Ng, DM DeLorenzo, YC Lee, B Waldron-Feinstein, TS Moon. Oxygen-Responsive Genetic Circuits Constructed in *Synechocystis* sp. PCC 6803, *Biotechnol Bioeng.* **113**:2 (2016).

BM Berla, R Saha, **CM Immethun**, CD Maranas, TS Moon, HB Pakrasi. Synthetic Biology of Cyanobacteria: Unique Challenges and Opportunities**,** *Front. Microbio*. **4**:246 (2013).

CM Immethun, AG Hoynes-O'Connor, A Balassy and TS Moon. Microbial Production of Isoprenoids Enabled by Synthetic Biology, *Front. Microbio*. **4**:75 (2013).

GRANTS

People, Prosperity and the Planet Student Design Competition for Sustainability 2010
United States Environmental Protection Agency
"Safe Drinking Water from Atmospheric Moisture using Desiccants and Solar Radiation"

- Wrote the grant awarded to the UMKC team for $10,000.

CONFERENCE PROCEEDINGS

CM Immethun, KM Ng, DM DeLorenzo, YC Lee, B Waldron-Feinstein, TS Moon. Oxygen-Responsive Genetic Circuits Constructed in *Synechocystis* sp. PCC 6803, *Poster Presentation, 12th Workshop on Cyanobacteria*, Tempe, AZ, USA, May 19-22, 2016.

CM Immethun, KM Ng, DM DeLorenzo, YC Lee, B Waldron-Feinstein, TS Moon. Oxygen-Responsive Genetic Circuits Constructed in *Synechocystis* sp. PCC 6803, *Poster Presentation, Monsanto Fellows Symposium*, St. Louis, MO, USA, April 13-14, 2016.

CM Immethun, KM Ng, DM DeLorenzo, YC Lee, B Waldron-Feinstein, TS Moon. Oxygen-Responsive Genetic Circuits Constructed in *Synechocystis* sp. PCC 6803, *Oral Presentation, AIChE Annual Meeting*, Salt Lake City, NV, USA, November 8-13, 2015.

CM Immethun, KM Ng, YC Lee, B Waldron-Feinstein, TS Moon. Engineering cyanobacteria as sustainable biotechnology platforms through synthetic biology tools, *Oral Presentation, IBE Annual Conference*, St. Louis, MO, USA, March 5-7, 2015.

CM Immethun, KM Ng, YC Lee, B Waldron-Feinstein, TS Moon. Engineering cyanobacteria as sustainable biotechnology platforms through synthetic biology tools, *Oral Presentation, AIChE Annual Meeting*, Atlanta, GA, USA, November 16-21, 2014.

CM Immethun, KM Ng, YC Lee, TS Moon. Engineering *Synechocystis* sp. PCC 6803 as a sustainable biotechnology platform through synthetic biology tools, *Poster Presentation, SIMB Annual Meeting*, St. Louis, MO, USA, July 20-24, 2014.

CM Immethun, KM Ng, YC Lee, TS Moon. Engineering *Synechocystis* sp. PCC 6803 as a sustainable biotechnology platform through synthetic biology tools, *Poster Presentation, Synberc Spring Symposium*, Berkeley, CA, USA, March 24-26, 2014.

ACADEMIC AWARDS

National Science Foundation Graduate Research Fellowship	2013 - 2016
Registration Scholarship for the 12th Workshop on Cyanobacteria	2016
EECE Graduate Student Teaching Award	2014
Curators Scholar	1981-1985
Engineering Club of Kansas City Scholarship	1984
American Business Women's Association Scholarship	1984
University of Missouri College of Engineering Scholarship	1983
3M Scholarship	1983

TEACHING & ADVISING

Teaching Assistant, Chemical Process Dynamics and Control Fall 2013
EECE, Washington University in St. Louis

- The course covered the mathematical analysis of processes' dynamic behavior and different control strategies.
- Led the recitation section, including preparation of the material that supplemented class lectures, for 45 senior level undergraduates.
- Held bi-weekly office hours, assisted in the creation of course assessments, and graded homework and exams.
- Received the EECE Graduate Student Teaching Award, based on student evaluations.

Teaching Assistant, Environmental Nanochemistry Spring 2013
EECE, Washington University in St. Louis

- The course covered the nanochemistry of various environmental interfaces, focusing on colloid, nanoparticle, and surface reactions.
- Held weekly office hours for 20 graduate students, and graded homework and exams.

Teaching Assistant, Introduction to Energy, Environmental & Chemical Engineering
EECE, Washington University in St. Louis Fall 2012

- The course provided an overview of chemical engineering, including material balances, fluid flow, mass transfer, heat transfer, and reaction engineering.
- Held weekly office hours for 70 freshman level undergraduates, and graded homework and exams.

Advisor, International Genetically Engineered Machine (iGEM) Competition 2014- Present
Washington University in St. Louis and Pennsylvania State University

- Recruited, trained, and mentored the 2015 and 2016 teams. These teams are comprised of undergraduates conducting research in three different labs at two different universities.
- Mentored a pair of undergraduates that worked in the Moon Research Group on the 2014 team.

Graduate Mentor, First-Year Rotation Students 2013 - 2015
EECE and Division of Biology and Biomedical Sciences, Washington University in St. Louis

- Developed independent research projects for five first-year graduate students.
- Provided the training the students needed to complete, analyze and present their project.
- Four of the students produced work that is included in my publications.

Graduate Mentor 2013 - 2016
EECE, Washington University in St. Louis

- Developed independent research projects for six undergraduate students.
- Provided the ongoing training the students needed to complete, analyze and present their project.
- Three of the students produced work that is included in my publications.

TEACHING & ADVISING (Continued)

Fellowship Workshop Mentor Fall 2013 - 2015

School of Engineering, Washington University in St. Louis

- Guided first and second-year graduate students as they created their essays for the National Science Foundation's Graduate Research Fellowship.
- One of the students I mentored received the fellowship and three students earned an honorable mention.

Teaching Assistant Orientation Discussion Leader August 2015

The Teaching Center, Washington University in St. Louis

- Planned and led a discussion section for engineering graduate students during their teaching assistant orientation.

PROFESSIONAL DEVELOPMENT

WU-CIRTL Associate 2016

The Center for the Integration of Research, Teaching, and Learning, and The Teaching Center - Washington University in St. Louis

- Completed six STEM pedagogy workshops, three TA training workshops, and two teaching and professional development workshops.

INSTITUTIONAL SERVICE

Graduate Council 2013 - 2014

The Graduate School, Washington University in St. Louis

- Served on the council that acts as the legislative branch for the Graduate School.

Faculty Search Committee 2014

EECE, Washington University in St. Louis

- Served on the student committee that interviewed faculty candidates.

TEACHING & ADVISING – COMMUNITY INVOLVEMENT

Judge for Giant Jamboree October 2016

International Genetically Engineered Machine (iGEM) Competition

- Evaluate the poster and oral presentations of collegiate, high school, and community lab teams from across the world at the Giant Jamboree.
- Provide teams constructive feedback on the science, communication, creativity, and impact of the projects, to maximize the learning potential of the competition.

Teacher Workshop Instructor June 2016

BioBuilder® Educational Foundation

- Led a three day workshop for educators that investigated the field of synthetic biology and how cutting-edge science can be incorporated into their classrooms.

TEACHING & ADVISING – COMMUNITY INVOLVEMENT (Continued)

Kits Coordinator January 2014 – June 2016
Young Scientist Program, Washington University in St. Louis

- Developed kits covering basic scientific concepts for middle and high school teachers to use in their classrooms, and managed the checkout system.

Verbal & Written Communication Tutor Summer 2013 - 2015
Young Scientist Program, Washington University in St. Louis

- Led high school students participating in the summer research program, Summer Focus, in weekly discussions about their research and their experience in the program.
- Developed and taught a workshop to prepare the Summer Focus students for the English section of the ACT.

Teacher Workshop Instructor June 2014
Moon Research Group, Washington University in St. Louis

- Co-developed the curriculum that introduced high school teachers to synthetic biology and the use of genetic circuits.
- Co-taught the two day workshop.
- Co-developed a genetic circuits kit that area teachers can check out for use in their classrooms.

Teacher-Leader Development Day Presenter 2013 - 2014
Teach For America

- Shared resources that are available from Washington University in St. Louis programs with area Teach for America science teachers.

Volunteer for Women in Science Day 2011 - 2012
Association for Women in Science, St. Louis Chapter

- Assisted with the "Getting Ahead" College Prep Panel.

Troop Leader, Canoe and Kayak Instructor, Older Girl Coordinator, and Day Camp Program Director 1997 - 2011
Girl Scouts of NE Kansas and NW Missouri

- Created programming to help girls develop the confidence and integrity to make positive societal contributions.
- Mentored girls, first grade – high school, in small and large groups.

Confirmation Catechist 1992 - 2011
St. Margaret of Scotland Catholic Church

- Created programming that encouraged high school students to examine their Catholic faith.
- Led discussion groups of twelve students, as well as classes and all day workshops for 50 – 70 high school students and their adult mentors.

INDUSTRY EXPERIENCE

Design Engineer 1997 - 2002
Digby Willard Engineering, Inc.

- New product development and machine design - from design to product/equipment delivery. Projects included a folding bleacher system and an automated bag-sealing system.

Chapter Chairman 1995 - 1996
Society of Manufacturing Engineers, Kansas City Chapter 1989 - 2003

- Other positions held included Professional Development Chairman, Technical Programs Committee, Engineers' Week Chairman, Advertising Chairman, Membership Recruitment Committee, Membership Retention Chairman, Audit Committee, Nominating Committee, Suppliers Night Committee, and Honor Awards Chairman.

Project Engineer 1985 - 1987
AT&T Technologies

- Special projects for the plant's various product lines, including piece part and fixture design through implementation. I also led teams that planned and implemented major operational changes, including starting the plant's first Just-in-Time (JIT) line.

Summer Engineer 1984
Texas Instruments

- Material testing and documentation for the HARM missile.

Student Engineer 1983 - 1984
University of Missouri - Columbia Research Reactor

- Designed fixtures for the research scientists.

PROFESSIONAL AND COMMUNITY SERVICE AWARDS

Outstanding Service Award 2010
Girl Scouts of NE Kansas and NW Missouri

International Award of Merit 2002
Society of Manufacturing Engineers

- For contributions to the international society's professional activities and growth.

President's Award 1990, 1993 & 1994
Society of Manufacturing Engineers

- For outstanding service to the Kansas City Chapter.