METABOLIC AND PHYSIOLOGICAL ENGINEERING OF PHOTOSYNTHETIC MICROORGANISMS FOR THE SYNTHESIS OF BIOENERGY FEEDSTOCKS: DEVELOPMENT, CHARACTERIZATION, AND OPTIMIZATION

by

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ABSTRACT

Biological processes are the reason for Earth’s hydrocarbon reservoirs and its oxygenated atmosphere. Life would be different today if not for the advent of photosynthetic carbon reduction by cyanobacteria 3 billion years ago. Oxygenic photoautotrophy uses carbon dioxide (CO₂) from the air, the radiant energy of sunlight, and reductive potential abstracted from water to derive structural carbon. The simplicity of this concept is rivaled by the complexity of its constituent mechanisms, but this has not inhibited the vast diversity of plantlife throughout the ages. Photosynthetic organisms thrive in most photic environments in unicellular and multicellular forms.

It is evolutionarily remarkable that anthropologic efforts now seek to understand these life forms for their biosynthetic, photophysiological, and adaptive properties. Of particular interest is the possibility to direct the metabolism of CO₂-reducing organisms into bioproducts of practical significance in modern human lifestyle. One perception is that combustible molecules could be synthesized by these organisms, recovered for conversion to a fuel source, and once utilized as such, the products (CO₂ and water) could be reconverted into the same reduced carbon compounds by the same organisms. Taking the model of carbon neutrality to its fruition is as sensitive, multifaceted, and profoundly complex an endeavor as bringing the concept of photosynthesis into reality. The characterization of photosynthetic microorganisms (PSMs) has been widely pursued for over 50 years, and the study of photosynthesis even longer. Still requiring much clarity, research has begun to manipulate photosynthetic metabolism for desired effects. This thesis defines the physiologies of two distinct classes of PSMs, green algae and cyanobacteria, under conditions to assess the strains’ capabilities and adaptations toward bioenergy-related productivities.

The green alga *Chlamydomonas reinhardtii* was genetically engineered to eliminate one of its major biomolecular constituents, polyglucan carbohydrates, such as starch and amylose. The purpose was to determine the possibility of reallocating fixed carbon into another basic component, fatty acid-containing lipids such as triacylglycerol (TAG). The results were mildly favorable, but partitioning was drastically altered when the ability to properly synthesize starch was reintroduced by complementation. Effects of nitrogen deprivation, a known starch- and
lipid-accumulation trigger, were assessed, but significantly, complemented mutants accumulated greater amounts of both starch and storage lipid during nutrient replete cultivation than wildtype or starchless strains during nitrogen stress. This hyperaccumulation phenotype is promising for the possibility of tuning photosynthetic metabolism to the synthesis of specific molecules.

The cyanobacterium Synechococcus sp. PCC 7002 was likewise modified for the interruption of glucose activation to higher glucans and was also engineered for the secretion of fatty acids. Carboxylated hydrocarbons of medium chain length such as lauric acid (C12) are drop-in fuel precursors that require minimal processing to derive the combustible product. When conferred with a C12-secreting capability, this organism dedicated 10% of its fatty acid portfolio to lauric acid, most of which was released from the cell into the culture medium without further persuasion. Though eliminating higher carbohydrates did not change the amount of C12 generated, a small increase in total fatty acyl lipids was observed. Aside from a severe decrease in reducing carbohydrate content, the most dramatic effects of removing this important pathway occurred in photosynthesis and during nitrogen deprivation. Rearrangements were observed in electron transport from photosystem II and through the plastoquinone pool, and the photoprotective abilities of this organism are illustrated by wildtype levels of O₂ being generated by the inhibited strain despite a lower growth rate. When nitrogen starved, a buildup of metabolic precursors resulted in organic acids being secreted into the culture medium, which are also valuable biocommodities. Synechococcus sp. PCC 7002 is a robust platform for metabolic engineering and physiological investigation, and it may be emerging as a feedstock organism for targeted bioproducts.

The task of re-engineering photosynthetic metabolism can be likened to domesticating an agricultural plant. We can begin the process, but its outcome will be dictated by the ancient biology on which it is based. The results of this work can be progressively adjusted in the pursuit of renewable and sustainable energy sources, an endeavor that appears to be a viable possibility.

To those that photosynthesize, we salute you.
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ACKNOWLEDGMENTS

Randor Radakovits
Jonathan Meuser
Robert Jinkerson
Sarah D’Adamo
Lee Elliott
Patrick Eduafo
Devin Karns
Fiona Davies
Dongxu (Tom) Li
Travis Saari
Huiya Gu
Lee Stanish
Ahriel Godoy
Erin Stephens
Tarryn Miller
Christopher Gallahar
Stephanie Ives
Tom Giordano
Matthew Scholz
Brian Vogler
Dylan Franks
Matthew Posewitz
Maria Ghirardi
Lieve Laurens
Narayana Murthy
G. Charles Dismukes
David Vinyard
Casey McAlpin
Ed Dempsey
Roy Curtiss III
Matthew Melnicki
Eric Hill
Leo Kucek
Sergey Liubinetsky
Bree Reed
Alexander Beliaev
Allan Konopka
Kurt Weiss
David Kennedy
Heather Brewer
Young-Mo Kim
Donald A. Bryant
Gaozhong Shen
Shuyi Zhang
Anthony Dean
Junko Munakata-Marr
Ronald Cohen
John Spear
Barabara & William Demming-Adams
Laura Beer
Stephanie Carr

This work would not have been completed without the faith and constant positivity of my family and friends. Thank you for your enduring support.
For family
CHAPTER 1
INTRODUCTION

1.1 Photosynthesis

Photobiology is the process by which an organism transforms radiant energy in sunlight into the physicochemical carbon (C) bonds that are used in the structure and function of the organism. Carbon bonds exist in most biological elements in just as many conformations and are found most often singly or doubly bonded (C-C, C=C), sharing one or two valence pairs, respectively, leaving three or two free electrons often shared with the other macronutrients oxygen (O), hydrogen (H), nitrogen (N), phosphorus (P) via phosphate, or sulfur (S) atoms. Micronutrients and osmolytes such as iron (Fe), manganese (Mn), magnesium (Mg), calcium (Ca), potassium (K), sodium (Na), zinc (Zn), copper (Cu), and molybdenum (Mo) – necessary cofactors, electrolytes, and electron exchangers in metabolism but at lesser concentrations – are complexed with biological elements and are influential in signal transduction for intercellular trafficking of the small carbon-based molecules (metabolites) that compose the cell [DellaPenna 1999, Berdanier and Zempleni 2008]. Organisms capable of photobiology, known as phototrophs, have developed different methods for translating light energy into the electrochemical gradient that runs the cell’s energy currency apparatus, ATP synthase [Pfennig 1977, Müller et al. 1992, Ort et al. 1996]. Once the gradient has been established, the cycle used by green plants to fix CO$_2$ into biological reduced carbon is called the Calvin-Benson-Bassham (CBB) cycle [Bassham et al. 1949]. Phototrophic carbon assimilation that abstracts electrons from water with oxygen as a byproduct is stoichiometrically represented as CO$_2$ and water being transformed into glucose, a reduced six-carbon compound (hexose carbohydrate) with O$_2$ as the byproduct, such that

$$6 \text{CO}_2 + 6 \text{H}_2\text{O} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6 \text{O}_2.$$ 

Beginning with light gathering (harvesting), a gradient is formed by a photoenergy-facilitated electron transport chain (ETC) within a glyco- and phospholipid membrane, in which electron carriers facilitate the movement of a photon-excited electron down its electrochemical gradient while the energy released is used to translocate protons from one side of the membrane to the other [Müller et al. 1992]. In oxygenic green plants, this takes place in the chloroplast
across the thylakoid membrane [Ort et al. 1996]. The ETC concentrates protons in the lumenal compartment, and ATP synthase uses their movement back into the stroma to phosphorylate ADP to ATP (adenosine triphosphate). The hydrolysis of ATP back to ADP is the primary exergonic reaction that supports cellular function [Atkinson 1971, Bunik 2011]. Additional reducing equivalents are generated during this and other metabolic processes, including NADPH via the oxidation of ferredoxin-NADP⁺ reductase at the terminus of photosynthetic electron transport (PET) [San Pietro and Lang 1956, Shin et al. 1963, Kresge et al. 2005], NADH from glycolysis and the tricarboxylic acid cycle (TCA) [Krebs and Lowenstein 1960, Romano and Conway 1996, Bar-Even et al. 2012], and FADH₂ from fatty acid oxidation and the TCA [Garland et al. 1967].

Oxygenic photosynthetic electron transport requires water (H₂O), the initial electron donor [Bassham et al. 1949, Spikes et al. 1954]. It is oxidized when the appropriate potential is reached within the responsible complex, which then facilitates the photoactivation of abstracted electrons for passage between carriers [Cheniae 1970]. PET involves proteins and complexes arranged in a “Z-scheme” of reductive potential, starting with the water-splitting complex and photosystem II (PSII) [Arnold and Azzi 1968]. A manganese-containing protein structure separates water molecules into O₂, protons, and electrons that are passed singly to the P680 pair of chlorophyll a in the PSII reaction center [Cheniae and Martin 1969, Grundmeier and Dau 2012]. Excited by a single photon at 680nm, P680* transfers the activated electron to pheophytin, through a plastoquinone proton-pumping cycle, and to plastocyanin which enters the electron into photosystem I (PSI) containing a P700 reaction center [Rochaix 2011]. The electron is excited by another photon and accepted by ferredoxin, a mobile iron-sulfur protein capable of providing reductant for cyclic electron transport [Tagawa et al. 1963, Cleland and Bendall 1992, Hertle et al. 2013], hydrogen production (anoxic) [Benemann et al. 1973, Melis and Happe 2001], and the formation of NADPH by ferredoxin-NADP⁺ reductase, which is the typical terminus of PET under conditions favorable for oxygenic photosynthesis.

The purpose of PET is to establish an energetic environment optimal for the intensive reactions of carbon fixation via the CBB cycle. Three stages of CO₂ assimilation can be defined: 1) fixation of one CO₂ to the 5-carbon substrate ribulose-1,5-bisphosphate (RuBP) and hydroxylation to two molecules of 3-carbon 3-phosphoglycerate (PGA), 2) reduction of PGA by NADPH and phosphorylation by ATP to glyceraldehyde-3-phosphate (GAP), and 3) a) release of
some GAP to central metabolism, which can be polymerized to a hexose carbohydrate such as glucose or sucrose for immediate or stored energy, while b) the remaining GAP is used to regenerate the 5-carbon substrate for further CO₂ fixation. Stoichiometrically,

\[3 \text{ CO}_2 + 3 \text{ RuBP} \rightarrow 6 \text{ PGA} + 6 \text{ ATP} + 6 \text{ NADPH} \rightarrow \\
1 \text{ GAP (catabolic)} + 5 \text{ GAP (regenerative)}.\]

The enzyme Rubisco, or Ribulose-1,5-bisphosphate carboxylase/oxygenase, is activated by ATP and binds CO₂ to RuBP via a carbamoylated lysine residue [Weissbach et al. 1956, Portis and Parry 2007]. Due to the similarity of CO₂ and O₂, Rubisco also performs an auxiliary reaction leading to one molecule of PGA, and one molecule of two-carbon 2-phosphoglycolate which must be recycled through the anabolic (respiratory) glycolate pathway [Bowes et al. 1971]. Photorespiration, reductant requirement, and the slow reaction time of Rubisco (3 per second), make the CBB cycle a demanding process for the cell. From light harvesting to fixed carbon, maximal observed energy conversion efficiencies in oxygenic photosynthesizers reach only 6% in microorganisms and around 2% in crop plants during the day [Blankenship et al. 2011].

Pigments, often chlorophylls, are the light-harvesting molecules that translate photons into excited electrons and toward a concentrated location, the reaction centers of photosystems [Van Amerongen et al. 2000]. They exist throughout photosynthetic membranes such as those of the chloroplast and thylakoids and contain, as do the membranes, hydrocarbon components that in pigments are unsaturated to facilitate resonance. Light harvesting complexes (LHCs) are migratable collections of pigments that can associate with photosystems and are found in myriad conformations depending on the type of organism, light and nutrient availability, growth stage, energy requirements, and many other factors [Armond et al. 1977]. Major pigments in oxygenic photosynthesizers include chlorophylls (acylated porphyrins), carotenoids (polyene tetraterpenoids), and phycobilins (water soluble tetrapyrrole chromophores), their biosynthesis regulated closely with the type and amount of incident light [Horton et al. 1996, Tomitani et al. 1999]. When incident light exceeds the reducing capacity of PET, excited pigments accumulate without electron acceptors. As O₂ readily accepts electrons to become toxic superoxide, a multifaceted system of photoprotection is invoked to dissipate excess electrons as heat or transfer them to innocuous compounds [Demmig-Adams 1990, Demmig-Adams and Adams 1992]. When light levels are low, membranes become enriched with LHCs [Larsson et al. 1987].
Interestingly however, repressing LHCs by mutation in the green alga *Chlamydomonas reinhardtii* improves light penetration and increases oxygen production and density of cultures [Polle et al. 2003, Melis 2009]. Much research exists and is still needed to model these processes, and photosynthetic microorganisms (PSMs) such as green algae, among others, have been studied to great effect for this purpose.

The manner in which photosynthesis occurs in unicellular green algae, which are nucleated organellar eukaryotes as are differentiated plants, is highly conserved between the two classes, affording researchers a simple and fast-growing organism that can be enquired to understand the greater natural world [Wodniok et al. 2011]. This is also true for much of cellular metabolism, compartmentalization, and genetics [De Smet and Beeckman 2011]. Widely studied eukaryotic algae include *C. reinhardtii*, *Chlorella variabilis*, *Nannochloropsis* spp., and *Phaeodactylum tricornutum*, as well as representatives from the genera Tetraselmis, Scenedesmus, and Botryococcus [Elliott et al. 2010]. In turn, two different classes of microorganisms are used to understand sub-cellular componentry of eukaryotic organisms [Rochaix et al. 1998]. Mitochondria, membranous respiratory organelles that are central to ATP formation, are thought to be descendent from a symbiotic heterotrophic (organic carbon-respiring) bacterium similar to *Escherichia coli*, the study of which provides much of our current understanding of heterotrophic metabolism [Orth et al. 2011]. Chloroplasts, the photosynthetic organelle of higher plants, are believed to be the result of a similar endosymbiotic event with a cyanobacterium [McFadden 2014]. Extensively characterized cyanobacteria include the Synechocystis and Synechococcus genera, and representatives from Prochlorococcus, Anabaena, Oscillatoria, Nostoc, and Arthrospira are also widely studied [Hess 2011].

Phototrophic and heterotrophic metabolisms share many of the same biosynthetic and anabolic pathways. Glycolysis and gluconeogenesis, the tricarboxylic acid cycle (TCA), fatty acid synthesis and oxidation, and the formation of amino acids, isoprenoids, and pentose phosphates are conserved across trophic and complexity levels. Central metabolites, several of which being anapleurotic to allow their integration into different compounds, are allocated into these pathways for the synthesis of other metabolites and structures. These include PGA, GAP and its isomer dihydroxyacetone phosphate (DHAP), pyruvate and its precursor phosphoenolpyruvate (PEP), activated acetate (acetyl-CoA), malonyl-CoA, phosphorylated fructose and glucose, and nucleotide (adenosine or uracil) diphosphate-activated glucose (nDP-
Gluc), as well as TCA intermediates oxaloacetate (OA) and alpha-ketoglutarate (aKG). The integration of these metabolites in photosynthetic metabolism is discussed and illustrated in the following chapters. The products of these pathways are carbon-dense and good sources of organic energy. The potential to harness their energy is the topic of this dissertation.

An introduction to thesis projects appears below, and an extensive introduction to photosynthetic microorganisms in the literature is provided in Chapter 2. The background chapter contains four journal articles that illustrate the fundamental pathways of central metabolism and examine how the capture, storage, and release of basic biomolecules are being investigated in photosynthetic organisms concerning their value in anthropologic occupations. Primary research done for this dissertation is then presented in two parts, Chapter 3 regarding algae and Chapters 4–6 regarding cyanobacteria.

1.2 Technical Introduction

The research described in this thesis aimed to evaluate the acclimations of two particular PSMs to modifications for biofuel-related physiologies and biomolecules. Both the green alga *Chlamydomonas reinhardtii* and the planktonic cyanobacterium *Synechococcus* sp. PCC 7002 are model laboratory strains amenable to genetic manipulation [Williams and Szalay 1983, Kindle 1990, Buleon *et al.* 1997, Siaut *et al.* 2011, Merchant *et al.* 2007, Ludwig and Bryant 2011]. Deleterious alterations were performed to interrogate the effects of disrupted carbohydrate synthesis on lipid metabolism, and heterologous metabolic elements were introduced to determine the feasibility and physiology of generating non-native products.

Presented in Chapter 3 is a study of lipid and carbohydrate partitioning in *C. reinhardtii* and associated genetic mutants. Four metabolic variants and one wildtype strain were cultivated to investigate the content of lipid and reducing carbohydrates resulting from genetic modifications in which enzymes of starch synthesis were modulated. Strains with impaired starch synthesis are the starchless mutants, *sta6* lacking the glucose-activating ADP-glucose pyrophosphorylase (AGPase) enzyme and *sta7* lacking the debranching isoamylase enzyme [Mouille *et al.* 1996]. Two strains of *sta7* with isoamylase reinstated include the complemented strains c5 and c19. The five strains exhibited different metabolic partitioning behavior, which is discussed with reference to conditions, specifically nitrogen deprivation, that expose these phenotypes. In green algae, nitrogen deprivation is a trigger for accumulation and storage of
reduced carbon, particularly starch (a higher carbohydrate) and the neutral lipid triacylglycerol (TAG) [Libessart et al. 1995, Alonso et al. 2000]. It was hypothesized that during nitrogen stress the starchless mutants could redirect carbon flux from the disrupted starch pathway into that of TAG, a biodiesel feedstock [Knothe et al. 2005]. Acclimations are discussed with reference to culture density, photosynthetic performance, starch and lipid content, and utilization of a heterotrophic carbon source. Epifluorescence microscopy was also used to illustrate morphological changes.

Similar studies were performed using the cyanobacterium *Synechococcus* sp. PCC 7002 and metabolic mutants thereof. The objectives of this work were to engineer and quantify the secretion of non-endogenous fatty acids from the cyanobacterium and understand fixed carbon partitioning in variants lacking AGPase. In this organism, AGPase is encoded by the *glgC* gene and activates glucose for polymerization to glycogen, which is the primary reduced carbon store formed during stress [Sakamoto and Bryant 1997]. A similar cyanobacterium *Synechocystis* sp. PCC 6803 has been engineered [Liu et al. 2011] based on findings that *Escherichia coli* conferred with a eukaryotic thioesterase released fatty acids into the culture medium [Voelker and Davies 1994]. In this dissertation, Chapter 4 introduces techniques for genetic engineering of *Synechococcus* sp. PCC 7002, Chapter 5 chronicles the behavior of genetic variants over a range of growth conditions, and Chapter 6 distills the observed physiologies of parent and modified strains.
CHAPTER 2
BACKGROUND

2.1 Microalgae as a feedstock for the production of biofuels: Microalgal biochemistry, analytical tools, and targeted bioprospecting


2.1.1 Authors’ contributions

L.G. Elliott compiled this manuscript and wrote the sections detailing oleaginous algae, select algal bioproducts, FACS, bioprospecting, and the DoE ASP, and provided microscopy and data figures. V.H. Work wrote the section on algal lipid biochemistry. P. Eduafo wrote the section on algal lipid classes. R. Radakovits and R.E. Jinkerson provided writings on algal bioproducts and genomes and contributed to schematic artwork. A. Darzins and M.C. Posewitz oversaw, edited, and funded the preparation.

2.1.2 Abstract

There are currently intensive research efforts aimed at increasing the accumulation of lipids, alcohols, hydrocarbons, H₂, polysaccharides, and other energy storage compounds in algae for use as renewable biofuels. Photosynthetic microorganisms (PSMs), more commonly known as cyanobacteria and algae, have emerged as promising platforms for the production of renewable biofuels. Compared to terrestrial crops, PSMs are typically more efficient solar collectors, use less or no land, can be converted into liquid fuels including JP-8 and other military grade fuels, are arguably more economically viable than lignocellulosic biomass, and offer other secondary uses that fossil fuels do not provide. Additionally, PSMs have evolved unique metabolic capabilities that allow the production of a greater diversity of biofuels. Their rapid generation times and extensive natural diversity, along with established genetic techniques,
make water-oxidizing PSMs particularly well suited for the physiological and genetic manipulations required to optimize the conversion of sunlight, water, and CO₂ into forms of chemical energy that can be used as transportation fuels. Recently, many improvements have been realized, including increased lipid and carbohydrate production, improved H₂ yields, and the diversion of central metabolic intermediates into fungible biofuels. The application of genetic engineering to enhance biofuels production in PSMs is advancing rapidly with the development of more sophisticated algal genetic manipulation techniques. It is likely that many of these advances can be extended into industrially relevant organisms in order to improve algae and cyanobacteria as a feedstock for the production of biohydrogen, starch-derived alcohols, diesel fuel surrogates, and/or alkanes.

2.1.3 Introduction

As the use of petroleum-based fuels has grown, so have concerns over the environmental effects of greenhouse gas (GHG) emissions, increased worldwide demand for energy, and international energy security. To ameliorate these concerns, there has been a recent surge in renewable energy research and technology development. One promising renewable energy scenario of considerable interest involves employing microalgae, which are an extraordinarily diverse group of photosynthetic microorganisms, as biological solar cells to capture and convert the photon energy of sunlight into an array of chemicals that can be readily harvested, transported, stored, and seamlessly integrated into the current fuel distribution infrastructure. This chapter is intended to (a) discuss the various microalgal chemical intermediates, especially lipids, which can be converted into biofuels, (b) present the underlying biochemistry behind the synthesis of these intermediates, and (c) highlight the analytical tools and targeted bioprospecting efforts used for the development of these organisms into a feedstock for the production of biofuels.

The recent surge of interest in developing microalgae for biotechnology follows many years of successful exploitation of a small number of well-studied microalgae as sources of bioactive compounds, nutraceuticals, human and animal food supplements, vitamins, polyunsaturated fatty acids, polysaccharides, proteins, and fluorescent pigments among others [León et al. 2007, Spolaore et al. 2006]. Demonstrated to be more efficient than most terrestrial plants at oxygenic photosynthesis, in which photon energy is harvested to generate a chemical
energy potential, microalgae fix inorganic carbon into renewable, energy-rich hydrocarbon stores [Hu et al. 2008, Beer et al. 2009]. As such, microalgae are capable of producing a variety of chemical intermediates, many of which can be readily converted into biofuels: lipids, hydrocarbons, polyglucans, alcohols, terpenes, terpenoids, and H2.

2.1.4 Microalgal Carbohydrates and Hydrocarbons

Microalgae predominantly produce two bioenergy carriers that are applicable for biofuel production: carbohydrates and hydrocarbons. Carbohydrates have long been used for the production of renewable biofuels through their conversion to alcohols and hydrocarbons, such as terpenes, terpenoids, and alkanes that can be used to produce diesel and gasoline fuel surrogates.

2.1.4.1 Starch Synthesis

Microalgae store glucans in a variety of distinct polyglucan macromolecules. The most common glucan found in nature, D-glucose, is polymerized in algae to serve as a transient energy store (e.g. glycogen and starch) or in structural polymers such as cellulose [Robyt 1998]. Chlorophyta (green algae), Dinophyta (dinoflagellates), Glaucophyta, and Rhodophyta (red algae) store glucans in α-1,4 and α-1,6 glycosidic linkages [Ball and Deschamps 2009], but Phaeophyceae (brown algae) and Bacillariophyceae (diatoms) store glucans in β-1,3 and β-1,6 linkages [Hirokawa et al. 2008]. Starch synthesis in Chlorophyta is the most well characterized algal polyglucan biosynthetic pathway due to the extensive study of the green alga *Chlamydomonas reinhardtii* [Ball and Deschamps 2009, Ball and Morel 2003]. In green algae, glucans are stored in linear α-1,4 and branched α-1,6 glycosidic linkages that make up insoluble starch granules. These semi-crystalline granules are composed of two fractions: amylopectin (70-90%) and amylose (10-30%). Amylopectin is semi-crystalline and moderately branched (5% α-1,6 linkages), whereas amylose is a linear amorphous polyglucan with very few α-1,6 branches and is synthesized by granule-bound starch synthase I (GBSSI) [van de Wal et al. 1998]. Semi-crystalline amylopectin is a more complex molecule that consists of amorphous branching regions and crystalline regions of linear glucans arranged into double helices. Amylopectin synthesis requires more enzymatic processing than amylose and is thought to follow the glucan trimming model proposed by Ball and coworkers [Ball et al. 1996, Mouille et al. 1996].
Starch synthesis (Figure 2.1) begins when fructose 6-phosphate from photosynthetic carbon fixation is converted to glucose 6-phosphate by a phosphoglucone isomerase (PGI1). Next, glucose 6-phosphate is converted to glucose 1-phosphate by a plastidial phosphoglucomutase (PGM). ADP-glucose pyrophosphorylase (AGPase) then catalyzes a major rate-controlling step of polysaccharide synthesis in plants: the reaction of glucose 1-phosphate with ATP, resulting in ADP-glucose and pyrophosphate. AGPase is a heterotetramer comprised of large regulatory and small catalytic subunits. It is allosterically activated by 3-phosphoglyceric acid (3-PGA), linking starch synthesis to photosynthesis [Stark et al. 1992]. This enzyme is inhibited by orthophosphate, preventing the storage of glucans during times of cellular energy debt.

The elongation of starch molecules proceeds when starch synthases (SS) transfer the glucose from the glycosyl nucleotide ADP-glucose to the non-reducing end of an α-1,4 linked glucan, synthesizing a crystalline layer of the growing starch granule [Ball 1998]. When enough α-1,4 linked glucans are assimilated, a branching enzyme (BE) hydrolyzes an α-1,4 linkage of a nearby donor glucan chain which is transferred to the acceptor chain via a new branching α-1,6 glycosidic bond. These newly formed amylopectin branches are disordered and loosely packed. A proofreading step is needed to form more optimally packed starch structures. Isoforms of the debranching enzymes (DBE) pullulanase and isoamylase hydrolyze tightly and loosely packed α-1,6 linkages respectively, resulting in a tightly packed amorphous region of α-1,6 linkages that are ready to be elongated by starch synthases [Mouille et al. 1996, Dauvillée et al. 2001]. This process is repeated, creating alternating layers of crystalline and amorphous regions, forming the semi-crystalline starch granule.

2.1.4.2 Starch Degradation

Starch catabolism in algae is largely unknown [Ball and Deschamps 2009], but putative degradation mechanisms have been inferred from known pathways in other plants such as Arabidopsis thaliana [Smith et al. 2005]. Hydrolytic and phosphorolytic starch degradation mechanisms are thought to exist in green algae. Hydrolytic starch degradation proceeds when the semi-crystalline glucans undergo enzymatic hydrolysis at the insoluble starch granule surface. In Arabidopsis, this is catalyzed by the chloroplast-targeted α-amylases (αAMY) [Smith et al.]

Figure 2.1. Overview of the metabolites and pathways involved in microalgal starch biosynthesis and degradation (black). Enzymes shown in red. During amylopectin synthesis glucans are added to the non-reducing end of the growing polysaccharide by $\alpha$-1,4 glycosidic linkages. Once elongated, a branching enzyme targets the ends of the growing polysaccharides by forming new $\alpha$-1,6 glycosidic linkages. Some of these branches are trimmed preferentially, and this process is repeated until a starch granule is formed. Hydrolytic and phosphorolytic [Starch-(P)$_n$] degradation pathways are shown. $\alpha$AMY, $\alpha$-amylase; AGPase, ADP-glucosepyrophosphorylase; $\beta$AMY, $\beta$-amylases; BE, branching enzymes; DBE, debranching enzymes; DEP, disproportionating enzyme (1 and 2) $\alpha$-1,4glucanotransferase; Glc, glucose; GWD, glucan-water dikinases; ISA, isoamylases; MEX1, maltose transporter; MOS, malto-oligosaccharides; PGM, plastidial phosphoglucomutase; PGI1, phosphoglucose isomerase; P, phosphate; Pi, inorganic phosphate; PPI, pyrophosphate; PWD, phosphoglucan-water dikinases; SS, starch synthases.
Starch can also be degraded in Arabidopsis via phosphorolytic mechanisms even with all three α-amylases are knocked out [Yu et al. 2005]. Phosphorolytic degradation is initiated by glucan-water dikinases (GWD) which catalyze the transfer of β-phosphate in ATP to the C-6 position of a glucan in a crystalline amylopectin double helix [Ritte et al. 2006]. Glucans can also be phosphorylated by the phosphoglucon-water dikinases (PWD) at the C-3 position. These phosphorylated glucans are thought to disrupt the crystalline structure of the amylopectin helices to allow access to glucan-metabolizing enzymes. Several approaches of attenuating starch degradation have been proposed to increase algal starch levels [Radakovits et al. 2010]. Knocking out key enzymes, such as GWD or PWD, may limit algal starch degradation and lead to a starch accumulation phenotype.

2.1.4.3 Terpenes and Terpenoids

Terpenes and terpenoids are a large class of compounds synthesized by algae that could be used for biofuel production. Terpenes are hydrocarbons, while terpenoids, also known as isoprenoids, are similar to terpenes except they contain functional groups. Two isoprene building blocks are used for terpene and terpenoid synthesis: isopentylidiphosphate (IPP) and dimethylallyl diphosphate (DMAPP). They are produced via the mevalonate or the non-mevalonate pathways from acetyl-CoA and pyruvate precursors, respectively. These activated isoprenes are then used to build terpene and terpenoids of various sizes and configurations. Terpenes follow the general molecular formula of \((C_5H_8)_n\), where \(n\) is the number of linked isoprenes [Fortman et al. 2008, Lee et al. 2008].

2.1.4.4 Alcohols

Traditionally, ethanol biofuels have been produced from the fermentation of food starches and sugars. Algal starches also have been shown to be a suitable feedstock for ethanol production via yeast fermentation [Nguyen et al. 2009]. Many algae have native fermentative metabolic pathways that produce ethanol and other alcohols; however, directly coupling ethanol production to photosynthetic carbon fixation would be preferred. This has been demonstrated in cyanobacteria where the introduction of pyruvate decarboxylase and alcohol dehydrogenase from Zymomonas mobilis has created a pathway for ethanol biosynthesis [Deng and Coleman 1999, Dexter and Fu 2009]. Other pathways for the direct, non-fermentative production of higher
alcohols has been demonstrated in \textit{E. coli} and could be employed in algae. These approaches utilize native amino acid biosynthetic pathways to produce 1-propanol, 1-butanol, 2-methyl-1-butanol, isobutanol, and phenylethanol [Atsumi \textit{et al.} 2008, Connor and Liao 2009].

2.1.4.5 \textbf{Botryococcus Hydrocarbons}

\textit{Botryococcus braunii} is an oleaginous microalga that has been well studied and produces a variety of hydrocarbons including alkanes, fatty acids, terpenes, and terpenoids. Several strains of \textit{B. braunii} are known and are classified by the type of hydrocarbons they produce. For example, the Race A strain produces dienes and trienes from C23 to C33; the Race B strain produces very long chain triterpenoid hydrocarbons, C30–C37 botryococcenes, and C31–C34 methylated squalenes; and the Race L strain produces lycopadiene [Metzger and Largeau 2005]. Race A has been observed to accumulate a hydrocarbon content of up to 61\% of their dry weight, while Race B and L have been noted to accumulate hydrocarbons up to 40\% and 8\% dry weight, respectively [Metzger and Largeau 2005].

2.1.5 \textbf{Microalgal Hydrogen Production}

Many eukaryotic microalgae and cyanobacteria have the ability produce \( \text{H}_2 \) either from dark, fermentative pathways coupled to saccharide catabolism or in the light from the electron transport chain at the level of ferredoxin [Hankamer \textit{et al.} 2007, Posewitz \textit{et al.} 2009, Hemschemeier \textit{et al.} 2008]. It is now clear that many microalgae can withstand extended periods of anoxia and that hydrogenase activity is an integral component of this metabolism [Grossman \textit{et al.} 2007]. This anaerobic metabolism supports the secretion of alcohols and organic acids, as well as \( \text{H}_2 \) production during anaerobiosis [Hankamer \textit{et al.} 2007, Dubini \textit{et al.} 2009]. Currently, the most significant hurdles hindering \( \text{H}_2 \)-production yields include (a) the sensitivity of hydrogenases to \( \text{O}_2 \), which prevents sustained \( \text{H}_2 \)-photoproduction coupled to the photosynthetic electron transport chain [Ghirardi \textit{et al.} 1997], (b) the preferential use of competing metabolic pathways during aerobic metabolism, especially \( \text{CO}_2 \) fixation [Cinco \textit{et al.} 1993], and (c) the Thauer limit, which restricts dark, fermentative \( \text{H}_2 \) production to 4 moles of \( \text{H}_2 \) per mole of glucose [Thauer \textit{et al.} 1977]. To date, the use of sulfur deprivation remains the most effective means to sustain \( \text{H}_2 \) photoproduction [Melis \textit{et al.} 2000]. This physiological stress attenuates photosynthesis to levels below respiration, allowing the establishment of anaerobiosis in sealed
cultures and residual photosynthetic activity to provide reductant to the hydrogenase enzyme. Genetic techniques have been applied with the aim of increasing H$_2$-photoproduction activity by decreasing light harvesting antennae size, inhibiting state transitions, manipulating competing pathways, and engineering the hydrogenase [Hankamer et al. 2007, Posewitz et al. 2009, Hemschemeier et al. 2008]. Physiological, genetic, and biochemical approaches have rapidly advanced our understanding of H$_2$ metabolism and enzyme maturation in green microalgae, and several strategies are emerging to further advance our ability to optimize H$_2$ production in these eukaryotic organisms.

2.1.6 Oleaginous Microalgae

It is well known that microalgae are capable of producing various storage products that are of potential commercial value, and certain oleaginous species have been documented to produce high levels of non-polar triacylglycerols (TAGs) [Hu et al. 2008]. As a result, many research efforts have focused on utilizing these organisms as a biofuel feedstock and developing methods to readily harvest lipids [Spolaore et al. 2006, Griffiths and Harrison 2009, Chisti 2008, Ben-Amotz et al. 2009]. It is also well documented that some species of microalgae, particularly under environmental stress, can accumulate up to 50 percent of their dry weight as neutral storage lipids. The level of TAG accumulation achievable appears to be dependent on both environmental conditions and the metabolism of individual species [Huesemann and Benemann 2009]. Notably, Chlamydomonas has been shown to accumulate TAGs in the laboratory during nutrient deprivation and under high light [Riekhof and Benning 2009].

Algal lipids can be harvested by various techniques and readily converted into diverse biofuels [Amin 2009]. Many well-studied microalgae have been shown to produce high levels of lipids and theoretical calculations of maximum algal oil production from these organisms offer strong support that in best case scenarios they are capable of outcompeting traditional oilseed crops in terms of oil productivity [Harwood and Guschina 2009]. However, as not all species are oleaginous, lipid productivity is a critical factor to consider for species selection, and maximizing lipid productivity is essential to the development of an economically viable algal biofuels industry [Griffiths and Harrison 2009]. Consequently, many current research efforts are directed towards genetically engineering well-characterized microalgae to optimize TAG production, which requires a significant investment of time and resources. Alternatively, some of
the hurdles encountered in genetic optimization procedures can be bypassed, or at least lowered, by taking advantage of natural selection to identify native strains of microalgae that already possess more efficient metabolic traits desirable for commercial feedstock development.

2.1.6.1 **Consideration of Carbon Source in Microalgal Lipid Accumulation**

As evidenced by their broad distribution, microalgae have adapted biochemical mechanisms to withstand extended periods of unfavorable conditions such as nutrient deprivation, high light conditions, and drought. To a large extent, these adaptations manifest into a diverse composition of cellular lipids which are critical for the ability to survive a broad range of dynamic environmental conditions [Hu *et al.* 2008, Harwood and Guschina 2009]. As the environment becomes less favorable, many microalgae begin to accumulate lipids as a consequence of a limited supply of key nutrients. This likely occurs by two possible mechanisms, the first of which would entail de novo lipid synthesis by which extracellular carbon is actively fixed and stored in the form of lipids. The second mechanism would involve a regulatory pathway that when activated causes the termination of de novo carbon fixation and initiation of intracellular reorganization of previously fixed carbon. Here, carbon would be actively scavenged from various non-essential cellular components for biochemical conversion into energy-dense storage products such as TAGs. It is conceivable that a combination of the two proposed pathways occurs, although from the feedstock perspective, productivity would be greater if a constitutive lipid trigger could be found.

2.1.6.2 **Algal Lipid Biochemistry**

To narrow the range of biomolecules classified as lipids, this term will be used to reference fatty acid-containing or otherwise highly non-polar molecules that are associated with energy storage, cell membrane structure, and signaling. There have been relatively few recent investigations of microalgal lipid biochemistry. However, sequence homology comparisons of genes involved in lipid metabolism in Arabidopsis and putative genes in Chlamydomonas indicate that lipid synthesis is likely very similar but may be less complex in microalgae [Riekhof and Benning 2009, Hu *et al.* 2008, Stern and Harris 2009]. Algal lipid biochemistry may eventually be found to deviate from the pathways detailed below, but these models give informed insight into the basic biochemistry involved.
2.1.6.2.1 **Fatty Acids**

The fundamental building-block of many lipids is the fatty acid, which is a carboxylic acid connected to a hydrocarbon with a methyl group at its terminus. The carboxyl carbon is in position C1 and subsequent carbons are defined as alpha (α), beta (β), and gamma (γ) carbons, respectively. Fatty acid chain length ranges from six carbons (hexanoic acid) to very-long-chain fatty acids (VLCFAs) containing over 22 carbons. Carboxylic acids containing five or fewer carbons are generally water-soluble and not classified as lipids. Carbon-carbon double bonds can be introduced within the hydrocarbon chain to confer structural diversity. Unsaturation is commonly found at positions toward the methyl terminus, or the omega (ω) carbon. Generally, sites of unsaturation are not seen before the γ carbon (C4), and multiple double bonds are usually found separated either by one or two single bonds. The majority of naturally derived unsaturated fatty acids are found in the cis conformation. However, some organisms produce fatty acids with trans unsaturation. This is most often found in conjugated fatty acids: those with two or more double bonds. Conjugated fatty acids with trans unsaturation are found in ruminants, some flower petals and seeds, some vegetables, and the red alga Bossiella orbigniana [Willet and Mazaffarian 2008, Cahoon et al. 1999, Burgess et al. 1991]. Also, partial hydrogenation, an industrial method of removing double bonds from unsaturated fatty acids, often results in byproducts with trans double bonds (trans fats).

2.1.6.2.2 **Biosynthesis**

Fatty acids (FAs) are a fundamental component of cellular structure and operation. The synthesis of algal lipids containing fatty acyl chains occurs via fatty acid synthase (FAS). In plants and photosynthetic algae, FA synthesis occurs predominately within the chloroplast and is catalyzed by a set of discrete enzymes collectively known as FAS II [Riekhof and Benning 2009]. Many other eukaryotic organisms synthesize FAs in the cytosol [Smith 1994], utilizing the multi-subunit enzyme complex FAS I. The discrete plastidial enzymes in plant and algal FA synthesis carry out analogous processes to their subunit counterparts in FAS I, but do not occur as single enzyme subunits.

Acetyl-CoA Carboxylase (ACCase). The committed and highly regulated initial step in fatty acid synthesis is the formation of malonyl-CoA (3-C) via carboxylation of acetyl-CoA (2-C) by ACCase. This irreversible reaction takes place in the chloroplast and involves an ATP-
dependent carboxylation of a biotin carrier followed by transcarboxylation of acetyl-CoA to form malonyl-CoA, which is the primary source of carbon in fatty acid synthesis. Six distinct enzymatic reactions follow, resulting in the formation of a fatty acid [Sul and Smith 2008].

Malonyl-CoA/ACP Transacylase (MAT). Malonyl-CoA is loaded onto an acyl carrier protein (ACP), resulting in the loss of coenzyme A (CoA-SH) and the formation of a thioester bond between malonate and the ACP, forming malonyl-ACP [Baud and Lepiniec 2009].

β-ketoacyl-ACP Synthase (KAS). To begin fatty acid synthesis, KAS III is required to condense malonyl-ACP with acetyl-CoA, forming acetoacetyl-ACP (a ketoacid) and releasing one CO₂. Acetoacetyl-ACP then undergoes a reduction-dehydration-reduction sequence (see below) to generate butyryl-ACP (4:0), which is then elongated. After the first condensation, malonyl-CoA is used as the sole carbon source. This requires a different type of β-ketoacyl-ACP synthase, KAS I, which adds two carbons from malonyl-CoA to the elongating fatty acyl chain and releases the third as CO₂. The new acyl-ACP is recycled back to KAS I for condensation with malonyl-CoA and reduction-dehydration-reduction. This forms an acyl-ACP elongated by two carbons, and the cycle continues to 16:0. Plastidial fatty acid synthesis typically terminates at palmitic acid (16:0) [Schmid and Ohlrogge 2008]. Once the fatty acid has reached 16 carbons, a thioesterase cleaves the bond between the fatty acyl chain and its carrier protein. Fatty acyl-ACP thioesterase (FAT) catalyzes this hydrolysis, releasing water, ACP-SH, and the fatty acid [Voelker et al. 1992, Voelker and Davies 1994].

First, β-ketoacyl-ACP reductase (KAR) reduces the ketoacid to a hydroxyacid. This reaction requires one NADPH molecule, which is oxidized to NADP+. In the first round of reactions, this forms β-hydroxybutyryl-ACP from acetoacetyl-ACP. Second, β-hydroxyacyl-ACP dehydratase (HD) releases one water molecule as it removes the hydroxyl group and introduces an α-β double bond to form a trans-2-Enoyl-ACP. This forms crotonyl-ACP in the first round. Third, Enoyl-ACP reductase (ENR) reduces the trans-2-Enoyl-ACP molecule’s double bond to form a saturated acyl-ACP. One proton is used, and the reductant is again NADPH, which is oxidized to NADP+. Crotonyl-ACP is reduced to butyryl-ACP (4:0) after the first cycle of reactions [Heath et al. 2001].
2.1.6.2.3 **Plastidial Modification**

Acyl chains both shorter and longer than 16 carbons are necessary components of many lipids. To form FAs shorter than 16 carbons, specialized thioesterases truncate fatty acid synthesis at a chain length specific to that thioesterase [Schmid and Ohlrogge 2008, Voelker *et al.* 1992, Voelker and Davies 1994]. Plants and algae contain a wide range of FA chain lengths, and 18-carbon chains are made within the chloroplast. Further elongation and modification occurs within the endoplasmic reticulum (ER) and export of fatty acids from the chloroplast is done predominantly as FFAs [Durrett *et al.* 2008].

The longest FA chain synthesized within the chloroplast contains 18 carbons; its elongation is catalyzed by a third type of β-ketoacyl-ACP synthase, KAS II [Schmid and Ohlrogge 2008]. In this case, the thioesterase responsible for cleaving ACP from palmitate is regulated, allowing KAS II to condense malonyl-CoA with palmitoyl-ACP to form stearoyl-ACP (18:0) [Wilson and Burton 2002]. Both 16- and 18-carbon chains are highly prevalent in membrane and storage lipids [Thompson 1996]. Unsaturation can be introduced in the plastid both by soluble and integral membrane desaturases, or in the ER by integral membrane desaturases [Riekhof and Benning 2009, Schmid and Ohlrogge 2008]. They are named for the location of the double bond they form. A $\Delta^9$-desaturase introduces a double bond at C9; whereas an $\omega$-3 desaturase forms a double bond at the third carbon from the methyl end. Plastidial desaturation of stearoyl-ACP is performed by a soluble stearoyl-ACP $\Delta^9$-desaturase, forming oleoyl-ACP (18:1). It has been found that a significant portion of the thioesterases present within the chloroplast are specific for 18:1-ACP, indicating that a large amount of 18:1 FAs are transported outside the plastid for modification. Similar fractions of 16:0 and 18:0 are also exported [Schmid and Ohlrogge 2008].

2.1.6.2.4 **TAG Synthesis**

A significant portion of lipids contain fatty acids esterified to a specialized head group. Because the hydrocarbon chains are relatively simple and not particularly differentiable, a head group allows the cell to diversify lipid function while using common building blocks to construct the basic form of the lipid. Head groups can serve to direct the lipid to specific cellular locations and engage in cellular communication and signal transduction [Carruthers and Melchoir 1986]. In the synthesis of storage lipids, glycerol is the most important backbone for acyl chain
attachment. Each carbon is hydroxylated, allowing derivatization at three locations. If glycerol is phosphorylated, the phosphoryl group can either be replaced by an acyl group or it can be bound by another functional group for further derivatization. TAGs contain three acyl groups linked by ester bonds to a glycerol backbone. Glycerol-3-phosphate (G3P), a product of glycolysis, is a primary substrate to begin constructing complex lipids.

The right-hand side of Figure 2.2 shows one common fate of activated acyl groups and glycerol-3-phosphate in an algal cell. During periods when the algal cell requires supplemental energy and can’t photosynthesize, (e.g. dark conditions), this pathway would be inhibited, and lipids within the TAG droplet shown at the bottom right would be metabolized as a source of energy through β-oxidation (see below).

Acyl-CoA synthetase. In order for a FFA to be available for attachment to a head group, it must first be activated. Once exported into the cytosol, FFAs headed to the ER for modification are derivatized by coenzyme A (CoA) via a thioester bond. Acyl-CoA synthetase catalyzes this two-step process, which involves an adenylate intermediate. The FFA displaces diphosphate from ATP, forming pyrophosphate (PPI) and an acyl-AMP (acyl adenylate) intermediate. The immediate and highly exergonic hydrolysis of PPI pulls this reaction toward the products. Once the acyl adenylate has been formed, CoA-SH displaces AMP, forming an acyl-CoA. This reaction requires a net of two ATPs, since ATP is taken to AMP [Schneider et al. 2005].

Fatty acid elongase complex (FAE). ER-mediated elongation is facilitated by FAE, which recognizes acyl-CoAs and condenses them with malonyl-CoA. The FAE complex carries out functions similar to KAS, KAR, HD, and ENR, adding two carbons sequentially to the carboxyl end of the acyl chain [Baud and Lepiniec 2009].

Glycerol-3-phosphate acyltransferase (GPAT) links an activated acyl group (acyl-CoA) to G3P, forming lysophosphatidic acid (LPA). This monoacyl phospholipid is itself a signaling molecule and a precursor to membrane lipids, other signaling molecules, and nonpolar storage lipids [Radakovits et al. 2010, Moolenaar 1995, Ryu 2004].

Lysophosphatidic acid acyltransferase (LPAAT) is specific for LPA and adds another activated acyl group to form phosphatidic acid (PA), which is an intermediate for either polar or neutral lipids. As a polar lipid precursor, PA can be derivatized by one of several head groups including choline, inositol, ethanolamine, or serine, to become a glycerophospholipid. For
neutral lipids, a third acyl group can replace PA’s phosphate group to form a TAG [Radakovits et al. 2010].

Phosphatidic acid phosphatase (PAP) is the first step in forming a TAG from PA. This enzyme hydrolyzes the phosphoryl group from PA, forming diacylglycerol (DAG) [Nakamura and Ohta 2010].

Diacylglycerol acyltransferase (DAGAT) catalyzes the addition of the third acyl group to DAG, maximizing glycerol’s capacity to carry acyl chains. This yields a highly nonpolar TAG, an efficient storage molecule that forms protein-coated lipid droplets (oleosomes) within the cytosol [Radakovits et al. 2010].

2.1.6.2.5 **Fatty Acid Oxidation**

Although the mobilization of TAGs is not yet well understood, it is believed that the interaction of lipases with the hydrophilic surface of lipid droplets exposes TAGs to lipase, an esterase that cleaves the acyl chain from its glycerol backbone. The products are FFAs that can be derivatized and shuttled into the mitochondrion for oxidation and energy generation [Huang 1992]. Fatty acids are very energy dense. When completely oxidized under standard conditions, long chain (12C-20C) saturated FAs amount to an energy release of around 37 kJ/g (about 9790 kJ/mol) [Turkish and Sturley 2006]. On a per weight basis, this is twice the energy content of carbohydrate or protein. The oxidation of one saturated palmitate (16:0) molecule requires 2 ATP for activation, and subsequently yields 8 acetyl-CoA, 7 FADH$_2$, and 7 NADH. Through the TCA cycle, 8 acetyl-CoAs yield 80 ATP. Each FADH$_2$ generates 1.5 ATP through the electron transport chain and oxidative phosphorylation, resulting in 10.5 ATP. Similarly, each NADH results in the formation of 2.5 ATP, producing 17.5 ATP. Overall, oxidation of palmitate yields 108 ATP, with a net of 106 ATP. However, there is not a perfect conversion of this energy into usable cellular energy. Under standard conditions, the energy stored in 106 ATP only amounts to 3233 kJ/mol, but complete oxidation of palmitate releases almost 10,000 kJ/mol of energy, amounting to 33% conversion efficiency. However, under the conditions at which the cell operates, conversion efficiencies can be higher. Even with less-than-perfect conversion, the oxidation of fatty acids is a huge contributor to cellular energy.
Figure 2.2. Overview of the metabolites and pathways involved in microalgal lipid biosynthesis (black). Enzymes shown in red. Free fatty acid synthesis occurs in the chloroplast, while TAG assembly may occur at the ER. ACCase, acetyl-CoA carboxylase; ACP, acylcarrier protein; ACSase, Acyl-CoA synthetase; CoA, coenzyme A; DAGAT, diacylglycerol acyltransferase; ENR, enoyl-ACP reductase; FAE, fatty acid elongase; FAT, fatty acyl-ACP thioesterase; GPAT, glycerol-3-phosphate acyltransferase; HD, 3-hydroxyacyl-ACP dehydratase; KAR, 3-ketoacyl-ACP reductase; KAS, 3-ketoacyl-ACP synthase; LPAAT, lyso-phosphatidic acid acyltransferase; MAT, malonyl-CoA:ACP transacylase; PAP, Phosphatidic acid Phosphatase; PDH, pyruvate dehydrogenase complex; TAG, triacylglycerols.

2.1.6.2.6 Localization

FA oxidation generally occurs in the mitochondria, though it can also happen in the peroxisomes via an oxidase instead of a dehydrogenase [Winkler et al. 1988]. However, there is no mechanism that can directly transport acyl-CoAs across the mitochondrial membranes; thus, a shuttling system regulated by malonyl-CoA is required to allow acyl-CoAs into the mitochondria for oxidation. Carnitine, a small carrier molecule, replaces CoA and facilitates transport of the
acyl chain into the mitochondrial matrix. Once inside, the carnitine carrier is replaced by CoA and the acyl-CoA goes into β-oxidation [Bourdin et al. 2007].

Carnitine acyltransferase I (CAT I), located within the outer mitochondrial membrane, facilitates the conversion of acyl-CoA to acyl-carnitine. CoA-SH is released back into the cytosol. Carnitine, CoA-SH, acyl-CoA, and acyl-carnitine move through the outer mitochondrial membrane without the aid of a transport protein. Thus, exchange of CoA with carnitine can occur either on the cytosolic or intermembrane side of the mitochondrion.

Carnitine-acylcarnitine translocase (CACT) passively facilitates the movement of acyl-carnitine and carnitine across the inner mitochondrial membrane. Generally, when the cell is undergoing oxidation and the generation of ATP, acyl-carnitine moves into the matrix and after delivering its acyl group, carnitine moves back into the cytosol.

Carnitine acyltransferase II (CAT II), located on the matrix side of the inner mitochondrial membrane, removes carnitine from the FA and re-derivatizes it with CoA from the mitochondrial CoA pool. The acyl-CoA is now localized and activated.

2.1.6.2.7 β-Oxidation

The term β-oxidation derives from the process of sequentially removing two-carbon units from the carboxyl end of the acyl chain. This results in a new FA minus 2C, where the β-carbon has become the carboxyl carbon. It is essentially the reverse of FA synthesis: an oxidation-hydration-oxidation scheme followed by thiolysis to yield acetyl-CoA and an acyl-CoA minus 2C.

Acyl-CoA dehydrogenase (ADH) forms a double bond between the α and β carbons via reduction of FAD to FADH₂. The product is a trans-Δ²-Enoyl-CoA.

Enoyl-CoA hydratase (ECH) adds water across the double bond, hydroxylating the β carbon and forming a methylene group at the α carbon. This forms β-hydroxyacyl-CoA.

β-hydroxyacyl-CoA dehydrogenase (HADH) converts the β carbon’s hydroxyl group to a keto group, reducing NAD⁺ to NADH and releasing a proton to form β-ketoacyl-CoA. This molecule has two carbonyl groups separated by one methylene group, an intrinsically unstable condition that readily leads to thiolysis.

Thiolase (Acyl-CoA acetyltransferase) facilitates the nucleophilic attack of a free CoA-SH to the β carbon. This cleaves the α-β bond and releases C1, the α carbon, and the attached
CoA as acetyl-CoA. A new CoA-SH attaches to the shortened acyl chain, forming acyl-CoA minus two carbons. The new acyl-CoA returns to ADH and the cycle continues until the entire chain is converted to acetyl-CoA. In order to completely oxidize odd-chain or unsaturated fatty acids, other enzymes are required.

2.1.6.2.8 Unsaturated Fatty Acid Oxidation

\( \Delta^3,\Delta^2 \)-Enoyl-CoA isomerase (ECI). In the instance of a \( \Delta^9 \)-monounsaturated FA, three cycles of \( \beta \)-oxidation will occur before the double bond interferes. After three acetyl-CoAs have been released, the cis double bond comes into the \( \beta-\gamma \) position, inhibiting ECH. ECI is responsible for repositioning the \( \beta-\gamma \) double bond to form an \( \alpha-\beta \) double bond. In other words, ECI shifts \( \Delta^3 \) unsaturation to the \( \Delta^2 \) position. This provides ECH with a trans double bond in the correct position for \( \beta \)-oxidation to continue.

2,4-dienoyl-CoA reductase (DECR). Oxidation of polyunsaturated fatty acids requires ECI and a second enzyme, DECR. For example, after three turns of \( \beta \)-oxidation with a \( \Delta^9,\Delta^{12} \) diunsaturated FA, ECI shifts the \( \Delta^9 \) double bond to allow for ECH, HADH, and thiolase to release one more acetyl-CoA. However, the \( \Delta^{12} \) double bond has now moved into a position between C4 and C5 (\( \Delta^4 \)). ADH is able to oxidize this molecule to form a trans-\( \Delta^2 \)-Enoyl-CoA with cis-\( \Delta^4 \) unsaturation. In order for \( \beta \)-oxidation to continue, the \( \Delta^4 \) double bond must be reduced by DECR, resulting in a \( \Delta^3 \) double bond. This is isomerized by ECI and the newly formed trans-\( \Delta^2 \)-Enoyl-CoA reenters \( \beta \)-oxidation [Nelson and Cox 2005, Chensworth et al. 1998].

2.1.6.2.9 Odd-Chain Fatty Acid Oxidation

Although the majority of FAs contain an even number of carbons, some organisms synthesize odd-chain fatty acids. Breakdown of these FAs occurs in the same manner as even-chain FAs, except at the very end. The products at the end of odd-chain FA oxidation are acetyl-CoA and the three-carbon activated product propionyl-CoA. Unlike acetyl-CoA, propionyl-CoA cannot go through the TCA, and must be converted to succinyl-CoA, a four-carbon activated TCA intermediate.

Propionyl-CoA carboxylase (PCCase) requires bicarbonate (\( \text{HCO}_3^- \)) and one ATP to facilitate the binding of a single carboxyl group to the \( \alpha \) carbon of propionyl-CoA, forming
methylmalonyl-CoA. This reaction is facilitated by a biotin cofactor in a reaction similar to that of ACCase.

Methylmalonyl-CoA epimerase (MCE) rearranges methylmalonyl-CoA to its levorotary optical configuration, effectively forming a 3-carbon chain with a carboxyl group at its terminus and the middle carbon linked to carbonyl-CoA.

Methylmalonyl-CoA mutase (MCM) uses a vitamin B12 cofactor to move the carbonyl-CoA group and bind it to the terminal carbon [Nelson and Cox 2005, Chensworth et al. 1998]. This forms succinyl-CoA, which is a TCA intermediate.

2.1.6.3 Algal Lipid Classification

The lipid composition of microalgae is not as well-studied as that of higher plants. However, there is substantial information about algal lipid structure, biochemistry, and metabolism. Microalgae contain a range of glycolipids and phospholipids, the major lipids of higher plants, including significant quantities of the glycosylglycerides monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and large amounts of the phosphoglycerides phosphatidylcholine [Drapcho et al. 2008], phosphatidylethanolamine (PE), and phosphatidylglycerol (PG). These comprise the main lipids found in the membranes of thylakoids, which dominate the intracellular membrane fraction in algae [Harwood 1998]. Microalgae have MGDG as their primary lipid, with smaller amounts of DGDG and two anionic lipids, SQDG and PG. MGDG is often present at fractions ranging from 40-55%, DGDG at 15-35%, SQDG at 10-20% and PG at 10-20% of the total lipid content [Harwood 1998]. The relative abundance of these lipids is highly dynamic and dependent on culturing conditions, but C16 and C18 fatty acids usually dominate. Algal lipids are often highly enriched in polyunsaturated fatty acids (PUFAs), especially in glycosylglycerides. Unusual lipid classes are present in some species of algae. For example, red algae contain sulfolipids such as phosphatidylsulfocholine and chlorosulfolipids, while some marine algae have the arsenic-containing lipids arsenoribosylphosphatidylglycerol. Most green alga have the betaine lipids diacylgyceryltrimethylhomoserine (DGTS) and diacylglycerol hydroxymethyltrimethyl-β-alanine (DGTA) [Harwood 1998].
Betaine lipids belong to a class of complex polar lipids called glycerolipids. They can be found in lower plants (bryophytes), fungi, photosynthetic bacteria, and many algae [Christie 2010]. Three primary betaine lipids are known: diacylglyceryltrimethylhomoserine (DGTS), diacylglyceryl hydroxymethyltrimethyl-β-alanine (DGTA), and diacylglyceryl carboxyhydroxymethylcholine (DGCC). DGTS and DGTA are the most common naturally-occurring betaine lipids and are widely distributed among bryophytes and algae. Labeling experiments with algae suggest that betaine lipids may play a metabolic role in the transfer of fatty acids between the cytoplasm and the chloroplast and that they may be involved in fatty acid biosynthesis by first accepting the fatty acids formed de novo, before they are processed and redistributed to other cellular lipids [Christie 2010]. DGTS is a primary membrane lipid of some green algae (C. reinhardtii, Volvox cateri and Dunaliella) and algae that contain chlorophyll $a$ and $c$ [Sato 1992]. Most algae with betaine lipids do not contain phosphatidylcholine; thus, it has been suggested that DGTS is a substitute for phosphatidylcholine. In green algae, the concentrations of DGTS and PC seem to be reciprocal, suggesting an apparent substitution of DGTS for PC as a membrane component. Interestingly, it has been found that C. reinhardtii accumulates high levels of DGTS and no PC [Giroud et al. 1988]. In contrast, organisms such as Ulva petusa and Chaetomorpha spirilis have very low levels of PC and high amounts of DGTS [Sato 1992]. It can be established from this inverse relationship that DGTS has progressively been replaced by PC during the evolution of green plants. DGTA is primarily found in the brown algae Fucales and Dictyotales [Sato 1992]. DGTS is usually absent in algae that contain DGTA, and only Crytomonas and Ochromonas are known to produce both of these betaine lipids [Eichenberger et al. 1993]. It has been demonstrated in Ochromonas daninca and Cryptomonas sp. that DGTA can be uniquely synthesized from DGTS; therefore, species of algae that contain DGTA may contain low levels of DGTS as a precursor to DGTA [Sato 1992].

2.1.6.4 Lipid Chemistry Affects Fuel Properties

One difference between higher plants and algae is that many algae are capable of producing long-chain polyunsaturated fatty acids (PUFAs), which are suggested to be beneficial for human health and nutrition, but can have an adverse impact on lipid-based biofuel performance. FA chemistry is important to consider for biofuel applications because chemical structure determines important biodiesel properties such as cold flow, oxidative stability, and
cetane number. Increasing the PUFA content of the feedstock organism would improve fuel performance at the lower temperatures encountered in colder climates as a higher degree of unsaturation decreases the cloud point; however, this would also decrease the oxidative stability of the fuel, as unsaturated double bonds are prone to oxidation [Hu et al. 2008, Drapcho et al. 2008]. With these effects in mind, the FA composition of the chosen feedstock organism(s) must be analytically determined, and will need to be tailored to achieve a reliable feedstock that can be readily incorporated into the current transportation fuel infrastructure.

Fatty acid synthesis can be manipulated by genetic modification; alternatively, variations in culture conditions have been shown to greatly affect microalgal FA composition, making it possible to direct FA synthesis towards more commercially valuable chemical structures within a controlled environment. Evidence also suggests that due to natural adaptations to particular environments, some algal species are better suited for producing commercially valuable FAs. For example, a Parietochloris incisa strain isolated from an alpine environment was found to contain the highest levels of long chain PUFAs than any other plant source described previously [Bigogno et al. 2002], alluding to the potential benefits of bioprospecting efforts directed towards discovering new, unique species of microalgae.

2.1.6.5 Qualitative Lipid Analysis of Microalgae

Upon microscopic analysis, some microalgae visibly produce oil bodies or vesicles that contain lipid in the form of TAG (Figure 2.3A). The extent of lipid accumulation can usually be assessed qualitatively in situ by using fluorescence microscopy and staining with neutral, lipophilic fluorophores such as Bodipy 493/503 (Molecular Probes, Invitrogen) (Figure 2.3B) or Nile Red. Notably, it is typical for cells to remain viable after exposure to Bodipy dye, a trait that can potentially be leveraged for high-throughput screening techniques to rapidly identify, and then culture, cells that produce neutral lipids.

Unfortunately, some microalgae are recalcitrant to staining. This is likely due to the chemistry and composition of the cell wall and extracellular polysaccharide (EPS) matrices. Depending on the chemical makeup of these structures, dye uptake can be completely or partially occluded. However, protocols using heat shock, freeze fracturing, and/or treatment with chemicals like DMSO, methanol, ethanol, acetone, isopropanol, and glutaraldehyde have been shown to overcome this recalcitrance [Chen et al. 2009], but this is often at the cost of
solubilizing chlorophyll. For example, DMSO treatment can enhance dye uptake but can make a structural analysis of the cell impossible as the solvent visibly disrupts lipid bodies and chloroplasts in some algae (Figure 2.4). Once solubilized, the chlorophyll phase-separates directly into the lipid bodies, which can be verified by fluorescence microscopy. Here, phase-separation is visualized as overlapping chlorophyll and Bodipy fluorescence signals, appearing as yellow/orange to orange/red droplets.

Figure 2.3. Image of a diatom species that visibly contains large oil bodies (arrow) (A). Fluorescence image of another diatom species where lipids were stained with Bodipy 493/503 (B). Chlorophyll is red and Bodipy is green. Scale bars are 10 µM.

Figure 2.4. Comparison of Bodipy stained cells without and with the fixative DMSO. Fixative clearly disrupts intracellular structure and solubilizes chlorophyll. Chlorophyll is red and Bodipy is green, and overlapping signals are yellow/orange to orange/red. Scale bars are 10 µM.
Electroporation can also be used to enhance dye uptake where the field strength of the electric pulse will determine the degree of dye penetration in recalcitrant cells. This method can preserve the fine structure of the cell and maintain viability. Unfortunately though, this technique is not well adapted for high-throughput analysis as the electroporation conditions require optimization for different microalgal strains and growth conditions.

An alternative to electroporation is high-pressure freezing-freeze substitution (HPF-FS), which fixes the cells but preserves the fine structure. Here, the biological sample is rapidly frozen at rates that approach $-20,000^\circ C/sec$, wherein a fixative replaces the cellular water, preventing damaging ice crystals from forming and resulting in nearly perfect preservation of the cellular architecture. Frozen samples could be stained and imaged using electron microscopy. This has been used to elucidate a semi-crystalline secondary structure of TAG lipid bodies in Schizochytrium sp. and Thraustochytrium sp. that was not found in Neochloris oleoabundans and other oleaginous species [Ashford et al. 2000].

2.1.6.6 **Semi-Quantitative Lipid Analysis of Microalgae and FACS**

Semi-quantitative lipid analysis can be performed using fluorophores such as Bodipy when coupled to flow cytometry. Using this technique, relative changes in fluorescence between populations of cells can be easily determined in addition to differences in light scattering properties. This is very useful for detecting both large and small changes within and between populations of cells. However, if cells in the population are recalcitrant to staining, this technique becomes much more challenging as fixation or electroporation must be carried out prior to analysis. Not only are these both time-consuming endeavors, but treatment with permeabilizing agents will also make the gathered data incomparable to analyses where such techniques were not employed. The true value of this type of analysis is realized when coupled to flow cytometry using a fluorescent activated cell sorter (FACS). This instrument facilitates the physical collection of individual cells or populations of cells into test tubes or the wells of microtiter plates. If for example, a desired group of traits, like intensity of Bodipy fluorescence on particles that also contain chlorophyll, are distinguishable by fluorescence, side scatter (a measure of intracellular complexity), and/or forward scatter (a measure of particle size), they can be used as gating criterion to physically sort cells with those combined traits [Mattanovich and Borth 2006,

### 2.1.6 Fully Quantitative Lipid Analysis of Microalgae

Qualitative information regarding lipid accumulation is useful for a rapid assessment of lipid productivity. However, a more detailed quantitative analysis is required to accurately assess the feedstock potential of the organisms. Quantitative determination of lipid composition can be revealed by fatty acid fingerprinting methods using gas chromatography [Dexter and Fu 2009] coupled to mass spectrometry (MS). Analysis by GC requires that the analyte be volatile, which is achieved most often by acid-catalyzed trans-esterification with methanol into the corresponding fatty acid methyl esters (FAMEs). Traditional analysis techniques involve lengthy extractions and long run times that result in a very low sample throughput. Fortunately, analysis can be accelerated significantly with the use of recently developed sample preparation and analysis techniques. For instance, methods have been developed that greatly decrease sample preparation time by performing *in situ* methylation or direct thermal desorption. Furthermore, an even greater sample throughput can be achieved if these methods are coupled to shortened GC run times, which is accomplished by manipulating the column chemistry, column length, carrier gas, and temperature ramp [Laurens 2008]. Despite the development of these sophisticated analytical methods, differences in TAG accumulation level and TAG chemistry remain largely uncharacterized for most microalgae.

### 2.1.7 Molecular Tools for Algal Feedstock Engineering

The development of tools for genetic engineering of algae has been steadily progressing for more than twenty years and many methods for transformation have been developed and tested in a wide range of algal model systems. These developments are likely to accelerate due to recent efforts to sequence algal genomes. Expressed sequence tag (EST) databases have been created; also, nuclear, mitochondrial, and chloroplast genomes from several microalgae have been sequenced and many more are reportedly being sequenced. While earlier efforts were focused mainly on genetic manipulation of the green alga *C. reinhardtii*, tools have also been developed for other algae that may be of greater interest for industrial applications. Transgene over-expression is becoming a routine procedure in many common algal model systems and
several genetic selection markers have been proven to work well in algae. Some of the biggest challenges in algal genomics concern the knockout of endogenous genes, but recent progress with homologous recombination and RNA silencing may provide a way forward.

2.1.7.1 Methods for Transformation and Expression

To date, more than 30 different strains from several different phyla of microalgae have been successfully transformed. These include green algae (Chlorophyta) such as *C. reinhardtii*, *Chlorella ellipsoidea*, *Chlorella saccharophila*, *Chlorella vulgaris*, *V. carteri*, *Chlorella sorokiana*, *Chlorella kessleri*, *Ulva lactuca* and *Dunaliella viridis* [Shimogawara et al. 1998, Jarvis and Brown 1991, Maruyama et al. 1994, Dawson et al. 1997, Schiedlmeyer et al. 1994, El-Sheekh 1999, Huang et al. 1996, Sun et al. 2006]. Heterokontophyte algae that have been transformed include *Nannochloropsis oculata*, diatoms such as *Thalassiosira pseudonana* and *Navicula saprophila*, and brown algae (Phaeophyta) such as *Laminaria japonica* and *Undaria pinnatifida* [Chen et al. 2008, Poulsen et al. 2006, Dunahay et al. 1995, Qin et al. 1999, Qin et al. 2003]. Red algae, (Rhodophyta) including *Cyanidoschyzon merolae*, *Porphyra yezoensis*, *Kappaphycus alvarezi*, *Gracilaria changii*, and *Porphyridium* sp. have also been transformed [Minoda et al. 2004, Cheney et al. 2001, Kurtzman and Cheney 1991, Gan et al. 2003, Lapidot et al. 2002]. Dinoflagellates that have been transformed include *Amphidinium* sp. and *Symbiodinium microadriaticum* [Michael and Miller 1998]. The only euglenoid that has been transformed to date is *Euglena gracilis* [Doetsch et al. 2001].

Several methods for introducing transgenes into algae exist, including agitation in the presence of glass beads or silicon carbide whiskers [Michael and Miller 1998], electroporation [Shimogawara et al. 1998, Maruyama et al. 1994, Chen et al. 2008], biolistic microparticle bombardment [Jarvis and Brown 1991, El-Sheekh 1999, Dunahay et al. 1995], and *Agrobacterium tumefaciens* mediated gene transfer [Cheney et al. 2001]. The efficiency of these transformation methods vary between different algal species, and the method of transformation must be carefully tested and optimized for each species of algae. In many cases, transformation has resulted in stable expression of transgenes, either from the nuclear or the chloroplast genome, but in some cases only transient expression has been achieved. Methods developed primarily in *C. reinhardtii* [Eichler-Stahlberg et al. 2009] demonstrate that the stability of expression can be
improved through proper codon usage, the use of strong endogenous promoters, and inclusion of species specific 5’, 3’ and intronic sequences.

Many different genetic markers have been used to facilitate efficient isolation of genetic transformants. These markers comprise antibiotic resistance genes and fluorescent/biochemical markers. Many algae are resistant to a wide range of antibiotics and the choice of antibiotic will vary between different species of algae. Antibiotic resistance genes that have been used for algal transformant selection include bleomycin [Lumbreras et al. 1998], spectinomycin and streptomycin [Doetsch et al. 2001], paromomycin [Sizova et al. 2001], G418 [Eichler-Stahlberg et al. 2009] and hygromycin [Zhao et al. 2009]. Fluorescent and biochemical markers have also been used to confirm genetic transformation in algae. These markers include luciferase [Jarvis and Brown 1991], β-glucuronidase [El-Sheekh 1999, Cheney et al. 2001], β-galactosidase [Qin et al. 2003, Gan et al. 2003], and green fluorescent protein (GFP) [Cheney et al. 2001].

Homologous recombination events are rare in algae; thus, transformation generally results in a random integration of transgenes into the nuclear genome. While this may be suitable for transgene expression or for random mutagenesis screens, it makes it difficult to delete specific target genes. Homologous recombination has been reported in the nuclear genomes of C. reinhardtii and C. merolae at a very low efficiency, and further optimization is needed to improve successful homologous recombination [Minoda et al. 2004, Zhao et al. 2009]. RNA silencing is another option for gene inactivation that has been studied in algae as RNA silencing has been shown to be a feasible strategy in both C. reinhardtii and P. tricornutum. Recent improvements in gene knockdown strategies include the development of high throughput artificial micro-RNA (amiRNA) techniques in C. reinhardtii that are reportedly more target-specific and stable than traditional RNAi approaches [Zhao et al. 2009].

Transgene expression and protein localization in the chloroplast are needed for the proper function of many genes involved in metabolic pathways. In C. reinhardtii, it is possible to achieve transformation of the chloroplast through homologous recombination [Marin-Navarro et al. 2007]. Chloroplast transformation has not been demonstrated in diatoms. However, plastid targeting sequences in diatoms have been characterized, which allow for translocation of proteins into the chloroplast [Gruber et al. 2007].
2.1.7.2 Microalgal Genomes

Algal genetic modifications are greatly facilitated by the availability of microalgal genome sequences, and the recent developments in rapid large-scale sequencing technology represent a revolution in microalgal research. Some of the past and current microalgal genome sequencing projects include *C. reinhardtii* [Shrager et al. 2003], *Ostreococcus tauri* [Derelle et al. 2006], *Fragilariopsis cylindrus, Pseudo-nitzschia, Thalassiosira rotula, Botryococcus braunii, C. vulgaris, D. salina, Micromonas pusilla, Galdieria sulphuraria, Porphyra purpurea, V. carteri, and Aureococcus annophageferrens* [Liolios et al. 2008]. In addition, there are several completed and ongoing efforts to sequence plastid and mitochondrial genomes, as well as dynamic transcriptome analysis from many different microalgae [Shrager et al. 2003].

2.1.8 Bioenergy-Focused Bioprospecting for Microalgal Diversity

At least 40,000 algal species have been described to date with some conservative and more generous estimates of actual diversity ranging from several hundred thousand to millions of extant microalgae and macroalgae species combined [Norton et al. 1996, Mann and Droop 1996]. Only a fraction of this enormous biological and genetic diversity has been examined to date, as it traditionally requires a significant investment of time and resources to perform conventional taxonomic and biological analyses. However, in conjunction with classical techniques and a recent surge in interest, the advent of molecular tools such as rDNA gene sequencing will help to rapidly expand the list of known and described species.

Microalgae thrive in a broad range of diverse natural habitats with many species being predominantly aquatic. With such an abundant diversity of species, it is feasible that there are undiscovered microalgae capable of producing and accumulating useful co-products. As such, metabolic diversity, especially with respect to differences in TAG metabolism, is likely to be enormous, and the need to assess this unknown potential has rapidly grown with the increasing interest of using these organisms for biofuels. This idea was extensively explored during the 1980’s by the U.S. Department of Energy (DOE) during the Aquatic Species Program (ASP) directed from the Solar Energy Research Institute (SERI), later established as the National Renewable Energy Laboratory (NREL). Initial efforts were geared towards isolating strains from diverse environments throughout the U.S. Subsequent screening and characterization of these isolates revealed several strains of high interest for biofuels production. The program was
eventually canceled as a result of low petroleum prices and federal budget cuts. Fortunately, information was gathered regarding all aspects of microalgal biology, process development, and the feasibility of using algae as a biofuel feedstock, which is presented in the ASP Close-Out report discussed below [Sheehan et al. 1998]. Most notably, this report provides compelling evidence that large-scale microalgal bioprospecting efforts targeting a broad spectrum of environments can uncover commercially valuable oleaginous species.

2.1.8.1 DOE’s Former Aquatic Species Program: Establishment of a Microalgal Culture Collection for Biofuels Applications

From 1979 to 1996 the US Department of Energy (DOE) supported a relatively small algal biofuel research effort project known as the Aquatic Species Program (ASP). The major focus of this DOE program was the development of technologies for producing biodiesel from oil accumulating microalgae grown in large, open raceway ponds using the CO$_2$ derived from coal-fired power plants. The activities of the ASP included the isolation and characterization of microalgal strains, the study of the physiology and biochemistry of lipid accumulation, genetic engineering of microalgae for enhanced lipid production, and the demonstration of open raceway pond systems for the cultivation of microalgal biomass. Despite the significant advancements made in the biological and process engineering areas, the program was terminated in 1996. In 1998, a close-out report was prepared detailing the research accomplishments of ASP. One of the most important accomplishments was the establishment of microalgal culture collection which represented a large genetic resource with the potential for oil production [Sheehan et al. 1998].

At the beginning of the ASP, the information available in the general field of microalgal biology was rather limited. In addition, researchers exploring the feasibility of developing microalgal-derived biofuels did not have the benefit of accessing existing microalgal culture collections from which to identify microalgal strains that could serve as candidates for a commercial oil production process. Therefore, a major undertaking by ASP researchers from 1983-1987 was to build and characterize a large microalgal strain collection. Over the four-year period, microalgal strains were collected primarily from inland, shallow saline aquatic environments in the West, Northwest and Southeastern regions of the continental U.S. and from Hawaii. The rationale for collecting microalgal strains from these diverse locations was that these habitats, largely characterized by predominantly high solar irradiation and temperatures,
would harbor strains that could naturally withstand substantial variations in temperature and salinity throughout the year. It was, therefore, hypothesized that these strains would be better able to withstand the fluctuating conditions that would be experienced at a commercial cultivation facility. The major goals of the strain collection work was to 1) isolate microalgal biodiversity from a variety of US aquatic environments; 2) characterize the isolated strains for their ability to grow rapidly under high light, high temperature, and saline conditions; 3) analyze the strains for lipid components; and 4) investigate the effects of various stress conditions on lipid composition.

The strain collection effort resulted in the identification of approximately 3,000 microalgal strains that were isolated from within the continental US (Alabama, Arizona, California, Colorado, Mississippi, Nevada, New Mexico, Florida, Nebraska, Utah, and Washington) and Hawaii. Since most of the strains in the culture collection had not been characterized with respect to lipid content, a rapid screening method based on the lipophilic fluorescent dye Nile Red was developed [Cooksey et al. 1987]. In the presence of a neutral lipid environment (i.e., lipid droplets), Nile Red fluoresces yellow. Subsequent method development studies were able to demonstrate that an increase in Nile Red fluorescence (575nm) was correlated with an increase in total lipid content after nutrient deprivation. The Nile Red assay was then coupled with a screen for rapid growth of microalgal strains under high conductivity, high temperature (30°C), and high light conditions. Of the ten fastest growing strains, six were identified as cyanobacterial species belonging to the genus Oscillatoria and Synechococcus. The fastest growing eukaryotic algae identified belonged to the genus Chlorococcum (CHLOC4; 3.47 doublings/day), Amphora (AMPHO46; 2.84 doublings/day), Nannochloris (NANNO13; 2.78 doublings/day) and Chlorella (CHLOR23; 2.66 doublings/day) [Sheehan et al. 1998]. However, none of these fast-growing strains made the list of the ten highest Nile Red fluorescing strains. All ten of the strains identified as having the highest Nile Red fluorescence were diatoms comprising the genus Nitzchia, Amphora (AMPHOR45 and 27), and Fragilaria. The best microalgal strains that combined both high Nile Red fluorescence and rapid growth were Chaetoceros muelleri (CHAET9), Navicula saprophila (NAVIC2), and Nitzchia pusilla (NITZS12). Despite the identification of these superior strains through the ASP strain screening procedure, many of the 3000 strains in the collection were never characterized [Sheehan et al. 1998].
Between the years of 1983-1987 SERI published three Culture Collection Catalogs (Solar Energy Research Institute, SERI/SP-231-2486; SERI/SP-232-2863; SERI/SP-232-3079). The 1984-1985, 1985-1986, and 1986-1987 catalogs contained 11, 10, and 29 microalgal strains, respectively [Sheehan et al. 1998]. The 1987 catalog contained a total of 50 strains comprising a broad range of different microalgal classes including Chlorophyceae (26%), Bacillariophyceae (60%), Chrysophyceae (8%), and Eustigmatophyceae (6%) (121). By the end of the ASP in 1996, approximately 37 of the 50 strains in the 1987 strain collection catalog were still viable. These strains, combined with an additional 260 strains that were not extensively characterized, brought the collection to approximately 300 strains [Sheehan et al. 1998] and were transferred to the Marine Bioproducts Engineering Center (MarBEC) headquartered at the University of Hawaii. MarBEC was established as a National Science Foundation (NSF) Engineering Research Center in 1998 through a partnership between the University of Hawaii at Monoa and the University of California at Berkeley. Since NSF funding for MarBEC ran out in 2004, the current status of the SERI culture collection is largely unknown. However, in the final report (http://mybiofuels.org/Pubs/MarBEC_Final_2004.pdf) a summary of the MarBEC Culture Collections stated that former NREL Marine Biofuels Collection is comprised of 183 microalgal strains. Based on this information, it is likely that only a fraction of the original 300 microalgal strains transferred to the University of Hawaii are viable today. The Executive Summary of the ASP close-out report emphasized the desire that future researchers should be able to make use of the culture collection; however, no additional publically available information regarding the current status of this largely untapped source of microalgal genetic diversity can be found.

2.1.8.2 Establishment of a Bioenergy-Focused Microalgal Strain Collection with FACS

The ASP strain screening effort was successful in that promising microalgae were identified, but was incredibly labor-intensive and time-consuming as it utilized traditional isolation and screening techniques. Approaches utilizing state-of-the-art technology to rapidly screen large communities of microalgae collected directly from the environment for TAG productivity and chemistry are greatly needed. One such approach employs FACS to rapidly screen large heterogeneous populations of cells for oleaginous microalgae and then subsequently sort those cells for cultivation. This technique utilizes the physical properties of various isomers of chlorophyll, which make algal cells highly autofluorescent at characteristic light wavelengths.
Consequently, it is possible to identify and sort algae by coupling flow cytometry with a cell-sorting. Furthermore, by staining samples with fluorescent neutral lipid probes like Bodipy or Nile Red, it is possible to identify microalgal cells that accumulate TAGs. Together, these traits can be leveraged for bioprospecting using FACS to isolate oleaginous algae from large heterogeneous populations of microorganisms.

Recently, a new bioprospecting effort funded by the Colorado Center for Biorefining and Biofuels (C2B2) was undertaken using high-throughput FACS to rapidly re-establish a new bioenergy-focused microalgal strain collection at the National Renewable Energy Laboratory (NREL). Forty-five water samples were collected from a broad range of environments throughout the Southwestern states of Colorado, New Mexico, Arizona, and Utah. Differences in water chemistry were compared in terms of pH and conductivity, which is related to the total ions in solution. Conductivity measurements ranged from freshwater to brine, and pH ranged from near neutral to slightly basic.

Stained water samples were directly analyzed on a BD FACSAria to identify populations with both positive chlorophyll and Bodipy 493/503 fluorescence (Figure 2.5). These populations were gated and single cells were sorted. The sort protocol was devised to isolate a representative number of the naturally-occurring microalgae from a particular water sample. This process resulted in the isolation of 245 robust, unialgal cultures followed by the successful cryopreservation of over 96% of the collection with methanol or dimethyl sulfoxide (DMSO) as a cryoprotectant. However, most species isolated using FACS were fresh water green algae despite the observation that the dominant species present in many of the water samples were diatoms. This selection bias likely occurred because diatoms, possessing a silica cell wall, are more fragile and may be less likely to survive the sorting process as there are considerable forces generated within the sample stream required to sort particles in a high throughput liquid platform. It is also possible that suboptimal cultivation conditions were responsible. Furthermore, dye recalcitrance, as discussed above, may have caused some oleaginous algal species to be overlooked during the sorting process.

A great deal of valuable information has been collected throughout this project resulting in the successful establishment of a bioenergy focused microalgal strain collection, which can be used to refine future high-throughput bioprospecting efforts. It can be concluded that FACS is
very useful for analyses of algal populations and for subsequent physical separation of robust cells from such populations.

Figure 2.5. FACS process of algal isolation from a natural water sample depicting data gathering and analysis, gating, and growth after sorting, left to right respectively. Relative chlorophyll and Bodipy fluorescence of particles detected in stained water sample. Scatter plot of all events, where gate P1, P2, and P3 defines the sort criterion of particles with both positive chlorophyll and Bodipy fluorescence. Gated cells are sorted into a 96 well microtiter plate and growth is later characterized to identify unique isolates.

2.1.9 Conclusion

The development of microalgae into a feedstock for the production of biofuels is a promising renewable energy scenario that many believe capable of providing a substantial fraction of our transportation energy needs. However, viable commercial development of this technology will ultimately require the use of algal strains that have much higher and consistent production of chemical intermediates than any species described to date. Many efforts are currently focused on genetically optimizing metabolic pathways to increase lipid productivities in well-studied organisms, which are laborious and time consuming tasks. However, the difficulty could be lessened by taking advantage of natural selection and identifying native strains of microalgae that already possess the metabolic traits necessary for commercial feedstock development.

2.1.10 Acknowledgements

The authors acknowledge support from the Air Force Office of Scientific Research grant FA9550-05-1-0365 and the Office of Biological and Environmental Research, GTL program,
2.2 Improving photosynthesis and metabolic networks for the competitive production of phototroph-derived biofuels

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2.2.1 Authors’ contributions

V.H. Work compiled this manuscript, wrote the sections on photosynthetic efficiency, and designed the schematic artwork. S. D’Adamo wrote the section on hydrogen, R. Radakovits wrote the section on lipids, and R.E. Jinkerson wrote the section on carbohydrates. M.C. Posewitz oversaw, edited, and funded the preparation.

2.2.2 Abstract

To improve bioenergy production from photosynthetic microorganisms it is necessary to optimize an extensive network of highly integrated biological processes. Systematic advances in pathway engineering and culture modification have resulted in strains with increased yields of biohydrogen, lipids, and carbohydrates, three bioenergy foci. However, additional improvements in photosynthetic efficiency are necessary to establish a viable system for biofuel production. Advances in optimizing light capture, energy transfer, and carbon fixation are essential, as the efficiencies of these processes are the principal determinants of productivity. However, owing to their regulatory, catalytic, and structural complexities, manipulating these pathways poses considerable challenges. This review covers novel developments in the optimization of photosynthesis, carbon fixation, and metabolic pathways for the synthesis of targeted bioenergy carriers.

2.2.3 Introduction

Interest in the use of photosynthetic microorganisms (PSMs) as feedstocks for bioenergy applications has been emphasized and demonstrated repeatedly, especially within the past fifteen
years [Elliott et al. 2011, Scott et al. 2010, Wijffels and Barbosa 2010, Radakovits et al. 2010, Kruse et al. 2005, Hu et al. 2008, Dismukes et al. 2008]. Their manifold attributes include high photoautotrophic productivity, diversity of environments conducive to growth, production of simple biomolecules easily converted to fungible fuels, an extensive suite of techniques for genetic manipulation, and potential for non-cropland growth. However, an industrially competitive system has yet to be established. An aggressive approach is needed to develop strains suited for the production of desired fuel surrogate molecules, optimize the accumulation of these molecules, and establish systems for large-scale cultivation and extraction. The efficiency with which light energy is converted to chemical energy is a fundamental obstacle precluding biological systems from offsetting any significant fraction of fossil fuels consumption, and improvements in light energy capture and conversion are essential to the development of economically viable bioenergy platforms. Lipids, carbohydrates, and hydrogen, three focal derivatives from PSMs, have great potential as bioenergy feedstocks, as nominal reconfiguration would be required for their integration into modern energy infrastructure. Significant laboratory advances have been made in directing metabolism into these feedstock molecules and must now be coupled with improved photon conversion efficiencies.

2.2.4 Photosynthetic efficiency

Photosynthesis is the fundamental system required for the production of all potential bioenergy surrogates from PSMs. However, it is a relatively low-efficiency process in terms of energy conversion when compared to the downstream synthesis of targeted products. More than 90% of the photon energy delivered to a given photosynthetic footprint can be dissipated as heat or fluorescence, and current estimates for realistic photosynthetic conversion efficiency fall around 6% of total incident light energy [Ort et al. 2011, Blankenship et al. 2011]. Maximization of photosynthetic potential is one of the most important and complex challenges in current efforts to employ primary productivity in bioenergy applications (Figure 2.6).

2.2.4.1 Antenna complexes

One well studied strategy involves truncation of the photosystem antenna complex to minimize excess photon capture and enhance light penetration (and thus availability) into PSM cultures. It has been shown that truncated light-harvesting antenna complex (tlh) mutants of
Chlamydomonas reinhardtii exhibit higher cell densities and increased O\(_2\) evolution maxima [Melis 2009, Polle et al. 2003]. Similar effects have been demonstrated in the same alga through RNA interference of the light-harvesting antenna complexes (LHC) [Mussgnug et al. 2007] or by heterologous expression of a constitutive variant of NAB1, an LHC translation repressor [Beckmann et al. 2009].

2.2.4.2 Carbon concentrating mechanisms

Widely proposed as a method to improve crop yield is the implementation of C4-type carbon concentrating mechanisms (CCM) into C3 plants [Murchie et al. 2009]. Though true C4 metabolism relies on differentiated cell types, a C4-like system has been observed in the diatom Thalassiosira weissflogii, but is not yet well understood (for a comprehensive review of algal CCM, see [Reinfelder 2011]). Simpler CCMs have been detected in many microalgae, primarily relying on carbonic anhydrase (CA), which catalyzes the reversible conversion of CO\(_2\) to bicarbonate (HCO\(_3^-\)) [Yamano et al. 2010, Spalding 2008]. Bicarbonate requires specific transporters to cross membranes, and thus the compartmentalization of CA influences its function in carbon concentration. External CA can dehydrate HCO\(_3^-\) to provide CO\(_2\) for diffusion through the cell membrane. Cytoplasmic CA can produce HCO\(_3^-\) either for transport into the chloroplast or for direct incorporation into C4 metabolites in C4-like CCM. Chloroplastic CA can produce CO\(_2\) in the vicinity of RuBisCO from actively transported HCO\(_3^-\). Due to CA’s reversible concentration-dependent functioning and CO\(_2\)’s pH-dependent speciation, manipulating this system is highly complex. Fundamentally, however, carefully designed introduction of high-activity versions of CAs, as well as a chloroplastic HCO\(_3^-\) transporter could greatly augment CO\(_2\) concentration around RuBisCO, thus improving its carboxylation to oxygenation ratio. This directly enhances the efficiency of energy transfer from photosynthesis to carbon fixation. Improvements to RuBisCO’s specificity and turnover rate would further promote this effect. Though efforts to engineer a more efficient RuBisCO have been pursued for over twenty years, only moderate advances have been made [Whitney et al. 2011, Parikh et al. 2006, Greene et al. 2007].

2.2.4.3 Calvin cycle optimization

Downstream of RuBisCO, optimizing the number of Calvin cycle enzymes present in the
chloroplast has been suggested as a method to maximize productivity. Metabolic modeling by Zhu et al. using an ‘evolutionary algorithm’ indicated that more effective enzymatic resource partitioning has the potential to greatly improve photosynthetic efficiency [Zhu et al. 2007]. One of the principal enzymes predicted by the model to have this effect is sedoheptulose-1,7-bisphosphatase (SBPase). Found to exist predominantly at low levels, SBPase is a key enzyme in ribulose-1,5-bisphosphate (RuBP) regeneration. Several studies have confirmed that SBPase overexpression increases Calvin cycle intermediates, leading to higher carbon fixation and dry weights. Enhanced photosynthetic capacity was also observed [Miyagawa et al. 2001, Lefebvre et al. 2005, Tamoi et al. 2006].

2.2.4.4 Photoprotection and the water–water cycle

Photoprotection is an immensely complex system that facilitates safe dissipation of excess photon energy through light avoidance, nonphotochemical quenching, and scavenging of reactive oxygen species (ROS), among other reactions and regulations. One of the key components of photoprotection is the xanthophyll cycle, or violaxanthin–antheraxanthin–zeaxanthin (VAZ) cycle. Upon interaction with overexcited triplet chlorophyll (Chl*), violaxanthin is de-epoxidized to zeaxanthin, releasing the energy as heat and thus diminishing the potential for Chl* to reduce O₂ to ROS. Though this pathway competes with photosynthesis for light energy, its careful manipulation would optimize energy flow through the photosystems, and could be especially useful when coupled with truncated antenna size [Murchie et al. 2009, Murchie and Niyogi 2011]. At PSI, as part of the water–water cycle, overaccumulation of photosynthetic electrons drives the Mehler reaction, reducing O₂ to superoxide (O₂•−). Superoxide can then be detoxified to water through superoxide dismutase and ascorbate peroxidase. An organism engineered to perform these reactions at optimal levels may exhibit less oxidative stress and improved fitness [Waring et al. 2010, Asada 2000].

2.2.4.5 Light quality, quantity, and capture

The type and intensity of incident light is also a major determinant of photosynthetic efficiency. The discovery of a novel chlorophyll f has prompted investigation into engineering chlorophyll to extend the spectrum of light that PSMs are able to capture [Chen et al. 2010, Chen and Blankenship 2011]. Establishment of co-cultures capable of capturing a broader range of light
has also been suggested as a method to maximize the utilization of solar energy [Scott et al. 2010]. Fine-tuning these systems could improve cell density and whole-culture photosynthetic functioning. Similar results could be achieved in bioreactors that facilitate tight control of light availability, especially with mixing [Wijffels and Barbosa 2010]. Additionally, in a patented method designed to reduce the detrimental effects of UV exposure and provide more useful wavelengths to the photosynthetic apparatus, Gressel et al. report a technique to express a fluorescence protein in algae or cyanobacteria that absorbs UV and emits photosynthetically active radiation (PAR) [Gressel et al. 2010]. This could be particularly effective when used in tandem with optimized native photoprotective mechanisms. Interestingly, a study by Forjan et al. demonstrates improved productivity in *Nannochloropsis gaditana* upon exposure to low-level UV-A irradiation [Forján et al. 2011]. As compared to control cells illuminated with 140 µE/m²s PAR, cultures exposed to 140 µE/m²s PAR supplemented with 6 µE/m²s UV-A exhibited increases in growth rate, nitrate uptake, quantum yield of O₂ per chlorophyll, dry weight, and carotenoid content, notably beta-carotene and zeaxanthin (2-fold). Chlorophyll content was slightly reduced in the UV-exposed cultures, suggesting an effect similar to LHC attenuation. It is possible that this study supports the plausibility of optimizing the xanthophyll cycle for improved algal productivity.

### 2.2.5 Lipids

Triacylglycerol (TAG), an attractive biofuel feedstock, is a major storage lipid produced by microalgae. Accordingly, much effort has gone into investigating lipid biosynthetic pathways in algae and several attempts have been made to increase their lipid productivity. Several studies investigated the possibility of forcing more photosynthate into lipid production at the expense of starch by genetically interrupting starch biosynthesis in *C. reinhardtii*. Four out of five of these studies demonstrated that, relative to wildtype, the starchless mutants exhibited increased levels of both total lipids (2-fold to 5-fold) and TAG (2-fold to 8-fold) on a per cell basis [Wang et al. 2009, Work et al. 2010, Li et al. 2010a, James et al. 2011]. The amount of lipid was also shown to increase on a dry weight and a culture volume basis. Various cell lines and controls were used, and one study reported that no connection could be found between the cellular starch and lipid content on a per cell basis but an increased amount of lipid was produced on a dry weight basis [Siaut et al. 2011]. Mirroring previous work done in higher plants, other efforts aim to increase
Figure 2.6. Generic chloroplast of a green alga showing placement of fuel-relevant primary metabolites and their integration into bioenergy production. Also depicted are the major components of photosynthesis and carbon fixation, including elements with the potential to be engineered for optimization of these pathways, as described in the text (specifically BT, CA, FP, HYD, LHC, RuBisCO, SBPase, VAZ, water–water cycle). APX: ascorbate peroxidase, BT: bicarbonate transporter, CA: carbonic anhydrase, Cyt b6f: cytochrome b6f, FDX: ferredoxin, FFA: free fatty acids, FNR: ferredoxin-NADP+ reductase, FP: fluorescent protein, G3P: glyceraldehyde 3-phosphate, HCO₃⁻: bicarbonate, HYD: hydrogenase, LHC: light-harvesting complex, PAR: photosynthetically active radiation, PC: plastocyanin, PS: photosystem, PQ pool: plastoquinone pool, SBPase: sedoheptulose-1,7-bisphosphatase, SOD: superoxide dismutase, SST: soluble sugar transporter, TAG: triacylglycerol, UV: ultraviolet light, VAZ: xanthophyll cycle.
the lipid content of algal cells through overexpression of key enzymes in TAG biosynthesis, including diacylglycerol acyltransferase (DGAT), glycerol-3-phosphate dehydrogenase (G3PDH), and acetyl-CoA carboxylase (ACCase).

Algal TAG production tends to increase dramatically on a cellular basis during nitrogen limitation, leading to speculation on the existence of a nitrogen-controlled genetic ‘lipid accumulation trigger.’ Ideally, this could be over-expressed for increased lipid production during normal growth [Jinkerson et al. 2011]. Attempts to find this elusive trigger have been made through comparative transcriptome analysis between normal and nitrogen-limited C. reinhardtii, Phaeodactylum tricornutum, and Thalassiosira pseudonana [Miller et al. 2010, Maheswari et al. 2005]. Identification of genes that are upregulated upon nitrogen depletion resulted in the elucidation of a putative transcription factor that acts as a lipid accumulation trigger in C. reinhardtii (accession number XP_001692719). Overexpression of the gene resulted in an approximately 50% increase in lipid production in some cases [Yohn et al. 2011].

In terms of TAG droplet architecture, little is known regarding the possible function of scaffolding proteins found in lipid droplets and what effect, if any, they may have on TAG accumulation. Lipid body proteomics was used successfully to identify a ‘major lipid droplet protein’ (MLDP) in C. reinhardtii, and RNAi was used to knock down its expression. Enlarged lipid droplets were observed, but there was no change in total cellular TAG content [Moellering and Benning 2010].

The lipid biosynthesis pathways in algae have been thought to be similar to higher plants. However, recent findings indicate that in addition to the TAG assembly pathways in the endoplasmic reticulum that exist in higher plants, yeast, and mammals, C. reinhardtii also has the ability to assemble TAG in the chloroplast [Fan et al. 2011]. The precise pathway involved in this novel TAG synthesis pathway is currently unknown. Cyanobacteria have been genetically engineered to produce TAG through the overexpression of a bacterial diacylglycerol acyltransferase, a phosphatidate phosphatase, and an acetyl-CoA carboxylase [Roberts et al. 2010]. Easily harvestable filamentous TAG-producing cyanobacteria could prove to be an excellent system for biodiesel production. It remains to be seen whether the TAG accumulation rates in engineered cyanobacteria can compete with those of microalgae.

While the majority of the work on algal lipid biosynthesis pathways has been done with C. reinhardtii, some progress has been made using the diatom P. tricornutum. In addition to
engineering microalgae for increased lipid production, it is also desirable to improve the quality of algal lipids for use as biodiesel surrogates. The carbon chain length of fatty acids affects the cold flow properties of biodiesel, with shorter chain lengths being preferred. Two medium-chain acyl-ACP thioesterases from *Umbellularia californica* and *Cinnamomum camphora* have been overexpressed in *P. tricornutum* resulting in production of lauric acid and increased production of myristic acid [Radakovits et al. 2011]. Interestingly, the overexpression of these thioesterases also resulted in an increased production of total fatty acids on a per cell basis. This was, however, offset by a reduced growth rate. Recently, a novel native thioesterase was cloned from *P. tricornutum* and overexpression resulted in increased total fatty acid production on a per cell basis [Gong et al. 2011], but there was no report on the effect on total culture productivity.

Several species of Nannochloropsis are emerging as models for algal lipid production. Many strains are exemplary biomass and lipid producers, and successful genetic manipulation of Nannochloropsis genomes has been demonstrated. Bailey *et al.* report a possible deletion of the alternative oxidase gene (AOX) through homologous recombination [Bailey *et al.* 2011, Kilian *et al.* 2011]. This deletion resulted in an approximate 10% increase in total fatty acid production, presumably due to decreased lipid oxidation. Currently, strong efforts are underway to sequence several Nannochloropsis genomes, as this alga is predicted to have great potential in the bioenergy sector.

### 2.2.6 Hydrogen

A wide range of fermentative and photosynthetic organisms have been proposed as potential feedstocks in the production of hydrogen (H\textsubscript{2}) for bioenergy applications. Biological H\textsubscript{2} production falls into three major categories: biophotolysis, photofermentation, and dark fermentation. Biophotolysis of water, carried out by green microalgae and cyanobacteria, is the most desirable route, as it couples photosynthesis to H\textsubscript{2} evolution, requiring only water and sunlight [Oh *et al.* 2011]. In green algae, biophotolysis occurs directly, relying on both photosystems (PSII and PSI) and the H\textsubscript{2}-producing hydrogenases. Several cyanobacteria utilize an indirect pathway wherein storage carbohydrates generated through photosynthesis are used fermentatively to produce H\textsubscript{2}. Efforts to optimize biophotolysis focus primarily on the modification of three main components: the light-harvesting antenna complexes, the electron transport system, and the hydrogenases [Oh *et al.* 2011]. Hydrogen yield has been shown to
respond positively to truncation of antenna complexes, reduction of chlorophyll content [Kosourova et al. 2011], suppression of the LHC regulatory gene tla1 [Mitra and Melis 2010], and overexpression of NAB1 [Beckman et al. 2009] (for comprehensive reviews, see [Srirangan et al. 2011, Brentner et al. 2000]). Further, the *C. reinhardtii* mutant stm6 shows impaired cyclic electron transport around PSI. This results in increased electron flow to the hydrogenase enzymes [Kruse et al. 2005]. A D1 protein mutant exhibits higher carbohydrate storage capacity and a sustained contribution to H₂ production through PSII [Torzillo et al. 2009, Scoma et al. 2011]. Further elucidation of alternative anaerobic pathways [Hemschemeier and Happe 2011], auxiliary electron transport [Peltier et al. 2010], and other distinct anaerobic H₂ metabolisms [Meuser et al. 2009] will aid the development of novel methods to optimize H₂ production in these systems.

The extreme O₂ sensitivity of most hydrogenases remains one of the greatest challenges facing the establishment of an industrial biophotolysis process. To address this problem, several studies have focused either on controlling O₂ levels [Melis et al. 2000, Surzycki et al. 2007, Wang et al. 2011] or on protein engineering to create enzymes with greater O₂ tolerance. The [FeFe]-hydrogenases present in green algae and some bacteria are highly sensitive to O₂ and irreversibly inhibited by it. However, several membrane-bound [NiFe]-hydrogenases have been found to be more O₂ tolerant and are presently under investigation [Weyman et al. 2011, Goris et al. 2011, Liebgott et al. 2011, Wu et al. 2011]. A two-stage process was developed to allow temporal separation between oxygenic photosynthesis and photobiological H₂ production. In the first stage, cells are grown phototrophically in nutrient-replete medium. In the second stage, the cells are deprived of sulfur, which inhibits the synthesis of sulfur-containing polypeptides including some proteins of the PSII reaction center (e.g. D1). This results in reduced O₂ production, and the low levels of O₂ are quickly consumed by respiration, establishing anaerobiosis [Melis et al. 2000]. To date, designs for H₂ photobioreactors are primarily based on this two-stage process, and several additional approaches have been proposed to further stimulate H₂ photoproduction in these sulfur-deprived cultures [Antal et al. 2011].

Research into indirect biophotolysis by cyanobacteria is currently focused on identification of new H₂-producing strains, optimization of culturing conditions, and engineering of specific strains to enhance H₂ production [McKinlay and Harwood 2011, Quintana et al. 2011]. Modification techniques are similar to those described for direct biophotolysis, such as
redirecting electron flow to the hydrogenases, developing O₂-tolerant hydrogenases, and eliminating auxiliary pathways that consume reducing equivalents. Mutants of *Synechococcus* sp. PCC 7002 deficient in lactate dehydrogenase were shown to exhibit a five-fold increase in total H₂ production as compared to the wildtype [McNeely *et al.* 2010].

### 2.2.7 Carbohydrates

Carbohydrates are a primary store of photosynthate in many species of algae and serve structural (e.g. cellulose) and transient energy storage roles (e.g. starch, glycogen, or chrysolaminarin). Carbohydrates such as cornstarch have many industrial applications and have long been used as feedstock for biofuels through conversion to alcohols [Santelia and Zeeman 2011]. The predominant energy storage polysaccharide in Chlorophyta (green algae), Dinophyta (dinoflagellates), Glaucohyta, and Rhodophyta (red algae) is starch, while Phaeophyceae (brown algae) and Bacillariophyceae (diatoms) store glucans in laminaran and chrysolaminarin, respectively. Algal polysaccharides can be hydrolyzed and then either fermented to ethanol by yeast or used as a heterotrophic carbon source for the production of a variety of biofuels by most industrially relevant microorganisms [Nguyen *et al.* 2009, Harun *et al.* 2010].

Increasing carbohydrate production in algae would be advantageous for biofuel production, and several strategies have been proposed to do so [Radakovits *et al.* 2010]. These include overexpression of key enzymes in starch biosynthesis (e.g. ADP-glucose pyrophosphorylase or isoamylase), knockout of starch degrading enzymes (e.g. glucan-water dikinases and amylases), and secretion of soluble carbohydrates. Recently, complementation of the native isoamylase in mutants of *C. reinhardtii* defective in this enzyme has resulted in ‘starch excess’ strains capable of producing starch on the order of 3-fold to 4-fold over wildtype, but at the cost of cell division and protein synthesis [Work *et al.* 2010]. Interestingly, photosynthetic O₂ evolution in the starch excess strains is greater than the wildtype during nitrogen deprivation. Furthermore, the starch excess strains produce starch at the same level in nutrient replete medium as the wildtype does under nitrogen deprivation. This is advantageous from a bioenergy standpoint to the potential to forgo nitrogen deprivation while maintaining biomass and starch yields. This would improve cultivation time, as nitrogen-deprived wildtype cultures grow significantly more slowly than nutrient-replete cultures of the starch excess strains, and nutrient deprivation has been shown in many studies to attenuate photosynthesis. Further investigation is
required to determine the role of isoamylase in starch production and how the apparent overexpression of this gene alters starch biosynthesis.

Apart from polysaccharides, soluble carbohydrates are also produced by PSMs. One strategy to facilitate their use in biofuel applications is to engineer a transport system to allow for carbohydrate secretion. This could eliminate the need to harvest the cells, improve product recovery, and possibly relieve product feedback inhibition. Such an approach has been pursued in Synechococcus cyanobacteria, in which a glucose- and fructose-facilitated diffusion transporter was heterologously expressed along with an invertase to convert sucrose to glucose and fructose [Niederholtmeyer et al. 2011]. Hexose sugars were secreted and yields were improved by overexpressing genes responsible for sucrose biosynthesis (e.g. UDP-glucose phosphorylase). This photosynthetic production and secretion of sugars was enough to support E. coli growth without any additional carbon source. Such a model could likely be adapted for an algal platform.

2.2.8 Perspectives

In algal biofuels research, major advances are being made in pathway engineering for desired products. And while it is a focal point, the task of optimizing the photosynthetic efficiency of PSMs has not been as aggressively pursued. This could be due in part to the fact that conversion efficiencies in algae are already higher than land plants, or it may be stymied by the complexity of manipulating the photosynthetic apparatus. However, even a fraction of a percent increase in efficiency would have a significant effect on productivity, and would be highly relevant on a commercial scale as it would decrease the footprint required for biofuels production. The algal biofuels community has begun to narrow its focus on high-productivity strains with improved metabolic flux toward specific molecules tailored for fuels use; and exciting new possibilities in the use of PSMs in bioenergy will emerge as efforts to optimize photosynthesis and carbon fixation begin to mature.

2.2.9 Acknowledgements

This work was supported by the Air Force Office of Scientific Research (FA9550-11-1-0211), the U.S. Department of Energy (EERE funding under DOE Award # DE-EE0003372), and the Genomic Science Program (GSP) Office of Biological and Environmental Research
(BER) originating from the GSP Biofuels Scientific Focus Area (BSFA) of the Pacific Northwest National Laboratory. R.E. Jinkerson was supported by a Graduate Research Fellowship from the National Science Foundation.

2.3 Microbial hydrocarbons: back to the future

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The defining challenge of energy research in the 21st century is the development and deployment of technologies for large-scale reconfiguration of global energy infrastructure. Modern society is built upon a concentrated yet finite reservoir of diverse hydrocarbons formed through the photosynthetic transformation of several hundred million years of solar energy. In human history, the fossil energy era will be short lived and never repeated. Although the timing of peak oil is extensively debated, it is an eventuality. It is, therefore, imperative that projections for both when it will occur and the degree to which supply will fall short of demand be taken into serious consideration, especially in the sectors of energy technology development, political and economic decision making, and societal energy usage. The requirement for renewable energy systems is no longer a point for discussion, and swift advances on many fronts are vital to counteract current and impending crises in both energy and the environment. Biology will again be tapped to provide energy-dense molecules, but we can no longer avariciously reap the benefits of historic biological processes. Instead, we must investigate these systems to develop our own sustainable platforms for desired products.

Presently, one of our greatest assets is a scientific understanding of the diversity of biological hydrocarbons synthesized with exquisite compositional and stereochemical control. The two most prevalent biosynthetic pathways for long-chain hydrocarbons are acetyl-CoA condensation to fatty acyls and isopentenyl pyrophosphate (IPP) condensation to produce higher isoprenoids. The latter is responsible for over 40,000 different isoprene-derived compounds, many of which are appealing candidates for fuels or fuel additives due to their desirable cetane
and cold-flow properties [Fortman et al. 2008]. The low- to zero-oxygen content of isoprenoids results in energy densities similar to the alkanes in current diesel fuels and a diversity of ring structures affords lower cloud points [Joyce and Stewart 2011, Jovanovic et al. 2010]. Additionally, it has been found that slight modifications to enzymes involved in the final steps of higher isoprenoid synthesis can result in subtle product variants with distinct thermochemical and thermophysical properties [Keeling et al. 2008]. For most of these molecules, direct precursors occur natively in photosynthetic microorganisms (PSMs), and engineering the full biosynthetic pathway in PSMs would require only one or two additional genes. Although studies regarding the use of starch, cellulose, fatty acids and triacylglycerols for conversion to fuels are widespread, active investigation into the seemingly boundless potential of isoprenoids for renewable energy has only just begun.

Two distinct pathways are known to lead to IPP and dimethylallyl pyrophosphate (DMAPP) – the isomeric activated building blocks of higher isoprenoids. The mevalonate (MVA) pathway, found in eukaryotes and some bacteria [Lombard and Moreira 2011], begins with acetyl-CoA and goes through hydroxymethylglutaryl-CoA and several mevalonic acid intermediates to produce IPP, which can be isomerized to DMAPP. The more recently characterized plastidial non-MVA pathway is observed in plants, bacteria (including cyanobacteria) and some protozoa [Hunter 2007]. Also called the methyerythritol phosphate (MEP) or deoxyxylulose phosphate (DOXP) pathway, this takes glyceraldehyde-3-phosphate and pyruvate through several cytidine diphosphate-activated sugar alcohol phosphate intermediates to generate IPP and DMAPP. Studies have reported that cyanobacteria are also able to complete this pathway independent of pyruvate and DOXP by incorporating pentose phosphate sugars, but the exact entry points and constituent enzymes of this alternative shunt are not yet clear [Ershov et al. 2002, Poliquin et al. 2004].

At present, several isoprenoids are of particular interest as promising biofuel feedstocks. Isoprene itself, also called terpene, is a volatile five-carbon compound not directly suitable for fuel use, but amenable to polymerization for higher-order fuel molecules, sealants, rubbers and adhesives [Whited et al. 2010]. The terminal step in isoprene synthesis is catalyzed by isoprene synthase, which has recently been engineered into cyanobacteria enabling isoprene to be captured in the headspace of bioreactors [Bentley and Melis 2011]. Beta-caryophyllene, a major component in the resin of the ‘diesel tree’ (Copaifera langsdorffii), is a highly sought-after
sesquiterpene (C15) ideal for use in diesel fuel. Heterologous expression of only one gene, β-caryophyllene synthase, is required for its synthesis in the cyanobacterium Synechococcus sp. PCC 6803 [Reinsvold et al. 2010]. Limonene, a terpenoid commonly used in natural flavoring, also exhibits desirable fuel properties [Tracy et al. 2009]. Its synthesis requires a one-step cyclization via limonene synthase of geranyl pyrophosphate, which is formed through the condensation of IPP and DMAPP. Bisabolane is a fully reduced monocyclic branched terpinane suitable for transportation-grade diesel. Recently, production of its precursor, bisabolene, was engineered into Escherichia coli, with the highest yield being observed in strains transformed with the Abies grandis (a fir) bisabolene synthase [Peralta-Yahya et al. 2011]. Relative to commercial bisabolane, a comparatively pure fraction of bisabolane was generated through chemical hydrogenation of bisabolene extracted from the transgenic bacteria. This study was based upon the synthesis of bisabolene through the MVA pathway, the use of heterotrophic carbon and a lethal extraction technique; however, it could conceivably be engineered into an MEP-based photoautotrophic system since farnesyl pyrophosphate, the sole substrate for bisabolene synthase, occurs natively in most PSMs. A technique for microbial formation of isoprenyl alkanoates has also been developed, which involves the condensation of higher prenols with fatty acids [Lee et al. 2010]. Other isoprenoids, such as myrcene, ocimene, pinene, cymene and terpinene, and several prenols and prenylated organic acids, also exhibit desirable fuel properties, but their synthesis in phototrophic microorganisms has yet to be studied in detail.

A significant amount of research has gone into determining the steps in both the MVA and MEP pathways with the highest impact on IPP/DMAPP production [Anthony et al. 2009, Pitera et al. 2007]. Of particular interest are the rate-limiting enzymes DOXP synthase (MEP pathway) and MVA kinase (MVA pathway), but investigations are ongoing into almost every enzyme involved [Keasling 2010]. Further downstream, overexpression of farnesyl pyrophosphate synthase is commonly used to improve yields of higher-order isoprenoids. Conversely, knockout or knockdown of nonessential pathways that compete for isoprenoid precursors may also be necessary. Interrupting carotenoid synthesis has been suggested in this context, as a large amount of isoprene flux goes into pigments. However, these phenotypes could prove severe unless light levels are strictly controlled. Thus, determining the roles of lesser isoprenoids will greatly inform the possibilities of redirecting isoprene flux into desirable compounds. Additionally, confounding effects have been encountered upon increasing the
amount of intermediates for either the MVA or MEP pathways. High levels of hydroxymethylglutaryl-CoA were found to inhibit fatty acid biosynthesis and decreased isoprenoid titers are observed during overexpression of MEP pathway enzymes downstream of DOXP synthase [Keasling 2010, Martin et al. 2003, Kizer et al. 2008]. Pursuant to these results, it is clear that much research is still required in order to optimize the expression levels of these genes, as this task is an essential first step toward the development of efficient, high-output phototrophic strains.

An important consideration in engineering photoautotrophs for large-scale isoprenoid production is the efficient linkage of photosynthetic carbon fixation to the desired pathway. Starting materials for both the MVA and MEP pathways can be derived from the Calvin–Benson cycle, forgoing the need for sugar catabolism required in a heterotrophic system. The potential for pentose phosphates to be incorporated into the MEP pathway is also an appealing mechanism for this reason, and further study of this process will provide insight into effective linking of photosynthesis with isoprenoid production. In particular, a cyanobacterial platform for terpenoid fuel production is appealing, as these PSMs are highly amenable to genetic engineering through homologous recombination and utilize the MEP and MEP-based pentose phosphate pathways for isoprenoid biosynthesis. Although the MEP pathway may be particularly sensitive to feedback inhibition, it has been shown that careful control of enzyme levels can result in efficient flux through the pathway [Ajikumar et al. 2010].

There is great interest and almost unbounded possibility in this budding field and, as scientists turn to biology in new contexts for hydrocarbons with biotechnological applications, significant advances toward scaled production of phototrophic renewable fuels are on the horizon.

2.4 Biocommodities from photosynthetic microorganisms

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2.4.1 Authors’ contributions

V.H. Work and F.K. Bentley wrote the section on cyanobacteria, and V.H. Work designed and constructed the schematic artwork. M.J. Scholz wrote the section on algal cell walls, S. D'Adamo on photosynthetic antennae and dark metabolism, H. Gu on starch and lipid accumulation, B.W. Vogler and L.F. Stanish on some of eukaryotic algae and new model systems, D.T. Franks on RNA interference, and R.E. Jinkerson on starch and new model systems. M.C. Posewitz oversaw, edited, and funded the preparation.

2.4.2 Abstract

Photosynthetic microorganisms are able to produce a diverse array of renewable biochemical commodities. Although promising platforms for the accumulation of targeted products, these organisms must be optimized in solar energy conversion, carbon capture and utilization, and the partitioning of metabolic flux to the requisite biosynthetic pathways. Metabolic engineering efforts are systematically addressing these obstacles and demonstrate the potential to develop consolidated bioprocessing organisms that are able to efficiently transform the energy in sunlight directly to refined chemicals of economic value. Particularly intriguing are mechanisms to synthesize and secrete bioproducts of interest from cyanobacteria, thereby eliminating the need to dewater and process cellular biomass. Significant advances in more classical approaches to triacylglycerol and carbohydrate accumulation in algae have also recently been realized. Importantly, genetic tools and sequenced genomes are emerging for some of the most biotechnologically relevant strains.

2.4.3 Introduction

It is estimated that 1 million years’ worth of net photosynthate stored as fossil fuels is combusted every year [Falkowski 2012]. Our ability to readily extract and utilize these ancient energy reserves has enabled a unique but unsustainable period in the Earth’s history—a time in which abundant energy carriers are used to provide relatively inexpensive electricity, heating, and transportation fuels, as well as sustain current agricultural practices. This in turn has facilitated a rapid expansion in the human population, which is projected to rise from 7 to 8 billion within the next 25 years [Bloom 2011]. Energy and food are inextricably linked to a society’s well-being, and the 21st century will likely see dramatic changes in how these products
are generated and distributed, as a growing human population encounters diminishing fossil energy reserves, climate change, and terrestrial ecosystem degradation. A key component in this transformation will be leveraging photosynthesis in novel ways to satisfy aspects of the world’s energy and nutritional demands [Blankenship et al. 2011, Dismukes et al. 2008, Hu et al. 2008].

Photosynthetic microorganisms (PSMs), which include algae and cyanobacteria, have the potential to fill an important role in this transformation, and PSMs are currently being intensively investigated for the efficient production of several targeted bioenergy carriers (Figure 2.7). These include, but are not limited to, triacylglycerols (TAGs), carbohydrates (e.g., starch, glycogen, and sucrose), H$_2$, free fatty acids (FFAs), omega-3-fatty acids and other nutraceuticals, vitamins, amino acids and proteins, terpenoids, alcohols, alkanes, aldehydes, and organic acids. PSMs are capable of exemplary photosynthetic yields and can thrive in a diverse range of ecosystems, including saltwater [Blankenship et al. 2011, Dismukes et al. 2008, Merchant et al. 2012, Radakovits et al. 2010, Work et al. 2012]. As potable water is a highly prized and limited resource, the development of marine systems for bioenergy and food production will likely prove critical in satisfying anthropologic demands. Contemporary agriculture required centuries of evolution and breeding, whereas targeted biomass improvements in PSMs is a relatively new concept that is in the transformative stages of development. Fortunately, rapid advances in genome sequencing, molecular biology, and analytical chemistry are allowing scientists to dissect and improve PSM metabolic networks to more efficiently synthesize a diversity of targeted products.

Although the conversion of sunlight and CO$_2$ into basic precursors suitable for nutritional, biofuel and other specialty products is an inherent property of PSMs, improvements in product yields and processing technologies are necessary to attain environmental and economic sustainability. Currently, PSM productivities are limited by well-recognized shortcomings that include (a) inefficiencies in coupling light capture to photosynthetic processes, (b) the absorption of excess excitation energy relative to the amount used in photochemical processes, (c) insufficient CO$_2$ availability, trafficking, and reduction, and (d) limited metabolic flux directed to biocommodity pathways [Blankenship et al. 2011, Hu et al. 2008, Merchant et al. 2012, Radakovits et al. 2010, Mitra and Melis 2008]. Additionally, harvesting PSMs from media where they typically represent approximately 1% of the total mass remains a significant technoeconomic challenge, as does the deconstruction and separation of PSM biomass into
purified or enriched products of value.

Genetic engineering and strain evolution will likely be used to overcome factors currently limiting the large-scale deployment of PSMs. Ultimately, a self-contained bioprocessing platform in a single organism may emerge—a “photons to fuels” PSM that is capable of highly efficient light utilization, CO₂ assimilation, and metabolic partitioning into biomolecules that are readily harvested and transformed into commodity products. This would uncouple the current need for a secondary organism (e.g., yeast or bacterium) to convert photosynthate (e.g., cellulose) to biofuels, thereby significantly simplifying costly integration steps. The deployment of these organisms should be designated to footprints that do not compete with agriculture or require substantial freshwater or fertilizer inputs. Recent years have seen both rapid growth in PSM research and significant advances in improving PSM productivities.

2.4.4 Cyanobacteria

As the ancient organisms responsible for oxygenating Earth’s atmosphere, cyanobacteria are efficient autotrophs, spending little fixed carbon on aerobic respiration. Ancestral to photosynthetic endosymbionts, they share many similarities with chloroplasts of green algae and higher plants, differing biosynthetically at certain points in the tricarboxylic acid (TCA) cycle and acyl trafficking [Kaczmarzyk and Fulda 2010, Sato and Wada 2010, Jansson 2012, Zhang and Bryant 2011, Steinhauser et al. 2012]. Instead of starch, cyanobacteria typically store glycogen [Yoo et al. 2002, Nakamura et al. 2005, Yoo et al. 2007]; and instead of triacylglycerol, accumulation of the lipid-like polymer polyhydroxybutyrate (PHB), derived from acetyl-CoA, is often seen under stress conditions [Bhati et al. 2010].

Bioenergy from cyanobacteria is a topic that is gaining popularity in research communities focused on photosynthesis, metabolic biology, and growth systems engineering [Machado and Atsumi 2012, Rosgaard et al. 2012, Tan et al. 2012, Daroch et al. 2013, Liu et al. 2011, Melnicki et al. 2013, Melnicki et al. 2012, Deng and Coleman 1999]. Novel cyanobacterial cultivation systems are being developed both for strain characterization and as scaling models [Brennan and Owende 2010, Singh et al. 2011, Wiley et al. 2011, Chen et al. 2011, Carvalho et al. 2006]. Ideally, targeted bioenergy carriers will be nontoxic, synthesized at high levels with minimal auxiliary energy expenditure, and exported from the cell in a harvestable and directly utilizable form.
Figure 2.7. Primary metabolic networks from photosynthesis to targeted biocommodities. 3-Phosphoglycerate (3-PG) is produced by the CO$_2$-fixing enzyme ribulose-1,5-bisphosphate carboxylase oxygenase and is subsequently allocated to the indicated pathways for the biosynthesis of proteins, lipids, or carbohydrates. Shown are some of the products targeted by metabolic engineering approaches to improve biofuel yields, including organic acids and alcohols, terpenoids, acyl-based lipids, and carbohydrates. Boxes indicate oxygenated energy carriers, reduced straight-chain hydrocarbons requiring a committed step at the production of malonyl-CoA, or energy dense branched hydrocarbons from the 2-C-methylerythritol 4-phosphate (MEP) pathway. Additional abbreviations include α-ketoglutarate (αKG), 1-deoxyxylulose 5-phosphate (DOX5P), dihydroxyacetone phosphate (DHAP), dimethylallyl pyrophosphate (DMAPP), farnesyl pyrophosphate (FPP), fructose 6-phosphate (F6P), geranyl pyrophosphate (GPP), geranylderivanyl pyrophosphate (GGPP), glyceraldehyde phosphate (GAP), isopentenyl pyrophosphate (IPP), phosphoenolpyruvate (PEP), oxaloacetate (OA), and uridine or adenosine diphosphate-glucose (nDP-gluc).
The cell thus serves as a biofuel production catalyst instead of a biomass-accumulating feedstock that requires harvesting and deconstruction. In systems biology, advances in steady-state culturing have provided reproducible sampling of distinct and often transient growth stages. Controlled parameters ranging from light and nutrient availability to salinity, temperature, and pH can be used to find maximal productivity, suspend cells at a particular doubling rate, or determine the limits of cell viability under stress, among many other applications [Melnicki et al. 2013, Melnicki et al. 2012, Vu et al. 2012].

Importantly, several types of planktonic cyanobacteria capable of rapid cell division have emerged as useful platforms for genetic engineering and physiological investigation, including *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7002, and *Synechococcus elongatus* PCC 7942 [Jansson 2012, Wang et al. 2012, Lindblad et al. 2012, Xu et al. 2011, Kuhlemeier et al. 1983]. Metabolically, these three model organisms are encouragingly simple to manipulate, as they are naturally transformable via homologous recombination and readily incorporate and express exogenous DNA. Transgenes can be targeted to any part of the genome and are commonly introduced as part of a cassette that affords isolation of mutants through antibiotic selection. Recent studies have introduced pathways for the heterologous synthesis of hydrocarbons, terpenoids, and alcohols in cyanobacteria, while others have identified perturbations resulting in overproduction or secretion of soluble sugars, FFAs, terpenoids, and organic acids. Such advances are pertinent to renewable energy, as these organisms can be grown in fresh or salt water with supplemental CO$_2$ as the sole carbon source.

### 2.4.4.1 Free Fatty Acid Secretion

Acyl-based hydrocarbons, a major constituent of diesel fuel, are essential cellular membrane components. The rate-limiting step in fatty acyl biosynthesis is acetyl-CoA carboxylation to malonyl-CoA by acetyl-CoA carboxylase (ACCase), which has been targeted for overexpression to improve the yields of fatty acid-derived lipids [Dunahay et al. 1996]. While the chloroplasts of eukaryotic algae are responsible for fatty acid synthesis (FAS) and subsequent FFA export to the cytoplasm for trafficking and modification, cyanobacteria do not typically contain thioesterases (TEs) that generate FFAs. Instead, the majority of fatty acyl chains are directly transferred from the acyl carrier protein (ACP) to the membrane lipid backbone. It was found that adding heterologous TEs to a bacterial host such as *E. coli* or
cyanobacteria catalyzes the cleavage of acyl hydrocarbons from ACP, resulting in the release of FFAs that then readily move across the cell membrane into the culture medium by unknown mechanisms [Liu et al. 2011, Voelker and Davies 1994, Lu et al. 2008, Liu et al. 2010, Ruffing and Jones 2012]. This approach typically requires deletion of the acyl-ACP synthetase, which is able to covalently link FFAs to ACP for metabolic reincorporation. Improved yields of secreted FFA from cyanobacteria were observed by introducing multiple thioesterases and modulating membrane integrity [Liu et al. 2011].

2.4.4.2 Secretion of Carbohydrates and Central Metabolites

Sugars and organic acids can be converted to alcohols for use in fuels and are both diverse and abundant metabolites. The secretion of pyruvate and α-ketoglutarate during nitrogen deprivation was observed from a glycogen-deficient mutant of *Synechocystis* 6803 (ADP-glucose pyrophosphorylase knockout) [Carrieri et al. 2012, Gründel et al. 2012]. As this strain is incapable of polymerizing glucose into glycogen, and since glycogen is preferentially synthesized under stress conditions, this “energy spilling” phenotype is attributed to relieving a buildup of central metabolic intermediates in this mutant. Sucrose, glucose, fructose, glucosylglycerate, and lactate export from transgenic cyanobacteria has also been achieved [Niederholtmeyer et al. 2010, Ducat et al. 2012, Xu et al. 2013, Hickman et al. 2013]. For these secretion-based systems, further investigation is necessary to better understand the movement of molecules across PSM membranes and the potential reuptake and utilization of secreted products.

2.4.4.3 Synthesis of Terpenoids in Cyanobacteria

Terpenoids represent the largest and most chemically diverse family of naturally occurring organic compounds. More than 40,000 individual terpenoids have been identified [Keeling and Bohlmann 2012], the majority of which are plant secondary metabolites with important roles in defence against herbivory, pollinator attraction, and seed dispersion [Gershenzon and Dudareva 2007]. All terpenoids are derived from the monomeric five-carbon isopentenyl pyrophosphate (IPP) building block and its isomer dimethylallyl pyrophosphate (DMAPP) and classified based on the number of five-carbon units they comprise: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30),

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tetraterpenes (C40), and polyterpenes (greater than C40). This diverse suite of bioactive compounds represents a valuable resource for bioprospectors. Plant terpenoids are found in products ranging from perfumes to pesticides to medicines, and the energy-rich hydrocarbons are ideally suited for development as biofuels to blend with current fuel supplies or as feedstock for the synthetic chemistry industry [Peralta-Yahya et al. 2011, Bohlmann and Keeling 2008].

The harvesting and purification of terpenoids from plant material is labor-intensive and costly with relatively small yields. Chemical synthesis of terpenoids at industrial scale is also difficult due to the chemical complexity of the products, and this method is reliant on diminishing petrochemical resources. Photosynthetic microorganisms offer a more suitable platform for the renewable biosynthesis of terpenoids when grown in open ponds or enclosed bioreactors, and the ability to simultaneously harvest solar energy and store it in the form of an energy-rich hydrocarbon via photosynthesis is economically significant [Dismukes et al. 2008, Angermayr et al. 2009, Melis 2009, Melis 2012]. However, because cyanobacteria and microalgae were not subjected to the same evolutionary pressures as plants to synthesize a diverse array of secondary metabolites for the purposes of defence and reproduction, they are not endowed with many of the terpene synthase (TPS) enzymes that plants utilize for terpenoid biosynthesis. Therefore, much of the recent pioneering work on the engineering of photosynthetic microorganisms for terpenoid production has focused on the cloning and introduction of plant TPS genes into cyanobacterial systems.

The first demonstration of the heterologous expression of a plant TPS in a photosynthetic microbe was described in *Synechocystis* sp. PCC 6803 upon transformation with the *Pueraria montana* isoprene synthase gene [Lindberg et al. 2010]. The volatile hemiterpene product, isoprene (C5H8), was synthesized at a rate of 4 mg isoprene L⁻¹ h⁻¹ [Bentley and Melis 2012]. Similar advances saw *Synechocystis* successfully transformed with the β-caryophyllene synthase from *Artemisia annua* [Reinsvold et al. 2011] and the β-phellandrene synthase from *Lavandular angustifolia* [Bentley et al. 2013], permitting accumulation of the sesquiterpene β-caryophyllene and the monoterpene β-phellandrene. All three TPSs were cloned into the *Synechocystis* genome at the *psbA2* locus via double homologous recombination, with expression driven by the native *psbA2* promoter to promote high expression in a light-dependent manner [Lindberg et al. 2010]. The *psbA2* locus is well established as a suitable “neutral” site for transgene integration in cyanobacteria because the deletion of *psbA2* is compensated by a strong upregulation of the
homologous *psbA3* gene [Mohamed *et al.* 1993]. Optimization of the TPS transgene for codon usage in *Synechocystis* increased isoprene synthase protein expression 10-fold [Lindberg *et al.* 2010], highlighting the importance of incorporating codon usage information in the design of heterologous gene expression. Importantly, all cyanobacterial strains reached homoplasmy for the introduced TPS transgene, a scenario where all genomic DNA copies contain the transgene, and a requirement for any robust industrial strain.

Natural product separation from biomass is an important economic factor in a microbial production system, as it can alleviate the need for costly dewatering and purification processes and also reduce product toxicity, which is important for maintaining optimal growth rates for maximum yields. Isoprene (C5) and β-phellandrene (C10) were both found to separate from cyanobacterial biomass. Isoprene as a small, hydrophobic molecule diffuses through cellular membranes and accumulates as a volatile product in the headspace of an enclosed bioreactor [Bentley and Melis 2012], while β-phellandrene, with a higher boiling point, accumulates as a nonmiscible product floating on the culture surface [Bentley *et al.* 2013]. It remains to be seen, however, if C15 or larger terpenoids are capable of moving across biological membranes, or if the engineering of heterologous transporters is required.

The purity of a targeted terpenoid in a microbial production system will be dependent on the promiscuity of the heterologously expressed TPS. Mechanistically, TPSs catalyze the formation of highly reactive carbocation intermediates from a prenyl diphosphate substrate [Chen *et al.* 2011], and subsequent rearrangements of the carbon backbone result in a large array of cyclic and acyclic products. A single TPS often yields multiple products from a single substrate [Bohlmann *et al.* 1999, Dmissie *et al.* 2011, Schilmiller *et al.* 2009], as was observed upon expression of the β-phellandrene synthase in *Synechocystis*, where small amounts of β-myrcene and limonene were detected in addition to β-phellandrene as the major product [Bentley *et al.* 2013]. It appears inevitable that small amounts of minor products, with similar chemical structures, will form in such production systems, but purity is likely to be superior to plant or petrochemically derived terpenoids.

The greatest challenge remains to improve the titer of targeted products by increasing the flux of photosynthetically assimilated carbon toward the terpenoid biosynthetic pathway. The methylerythritol phosphate (MEP) pathway utilizes glyceraldehyde-3-phosphate and pyruvate for the synthesis of the terpenoid precursors (IPP and DMAPP) in bacteria, cyanobacteria, several

2.4.4 Future Research

Although it is energetically unfavorable for cells to relinquish fixed carbon, secretion of diesel precursors is an appealing scenario, eliminating the requirement for cell harvesting, extraction, and waste processing. To realize this goal, improvements in the preferential synthesis of select molecules are needed, as is a mechanistic understanding of secretion. Metabolomics, along with protein and transcript profiling, are being used with increasing efficacy to assess cellular physiology during generation of heterologous compounds and identify strategies for more efficient production and growth [Tan et al. 2012, Vu et al. 2012, Hamilton and Reed 2012, Hu et al. 2013]. After introducing the desired transgene(s), optimization can include native gene overexpression, diverting carbon flux from alternative carbon sinks (e.g., glycogen), protein engineering for more enzymatically efficient isoforms, and relieving product inhibition, potentially by modulating allosteric regulatory domains or by cellular product removal (e.g., secretion). Substantial increases in yield will also likely require the complex task of engineering photosynthetic processes [Work et al. 2012, Rosgaard et al. 2012]. Some advances have been made in photon capture and carbon concentration [Price et al. 2008, Price et al. 2013], and successful demonstrations of improved carbon fixation have resulted from overexpression of key Calvin cycle enzymes [Ducat and Silver 2012, Maurino and Weber 2013, Zarzycki et al. 2013].

In sum, several cyanobacteria are already prodigious producers of economically tractable molecules such as glycogen and PHBs and are exceptional photosynthetic platforms for the production and secretion of high-value metabolites from engineered strains. The synthesis and secretion of sugars, FFA, terpenoids, α-ketoacids, and organic acids have all been recently reported, and this approach is highly attractive as dewatering and biomass separation steps are eliminated, and product feedback inhibition is reduced. Further research challenges include improving metabolic flux to targeted pathways, deciphering and enhancing secretion mechanisms, and developing cost-effective strategies to expediently remove product from the
culture medium prior to assimilation by contaminating organisms.

2.4.5 Eukaryotic Algae

In contrast to cyanobacteria, eukaryotic algae can accumulate high levels of intracellular starch or chrysolaminarin, as well as TAGs, particularly when stressed for nutrients such as nitrogen. Significant research efforts are therefore focused on studying the metabolic rearrangements induced by nutrient stress, as well as the biosynthetic routes to TAGs, storage carbohydrates, and H₂. Hundreds of distinct algal strains from existing and newly established [Elliott et al. 2012] culture collections have been evaluated for their biofuel or nutraceutical production potential. Contemporary algal-biofuels research is divided among approaches that either (a) characterize and manipulate biofuel-relevant processes in established model laboratory organisms, which typically have low productivities outdoors [Merchant et al. 2012, Lu et al. 2011, Mussgnug et al. 2007, Radakovits et al. 2011, Rasala and Mayfield 2011, Rupprecht 2009, Wang et al. 2009, Work et al. 2010], or (b) study industrially promising organisms that have high productivities, but are not as fully characterized and often do not have established genomes or robust genetic manipulation techniques [Dismukes et al. 2008, Hu et al. 2008, Anandarajah et al. 2012, Chisti 2007, Hu and Gao 2003, Van Wagenen et al. 2012, Wawrik and Harriman 2010]. Importantly, the barriers to genome sequencing, annotation, and assembly have diminished in recent years and many new algal genomes are now emerging, several with biofuels or biocommodity production being the principal driver for investigation.

Genetic manipulations that alter algal metabolism or introduce transgenes can be used to increase production of a desired molecule. Although successful homologous recombination in eukaryotic algae is reported for only a select few species [Kilian et al. 2011, Minoda et al. 2004], several organisms can be successfully transformed using exogenous DNA that is ectopically incorporated into the genome, enabling the transgenic expression of proteins [Radakovits et al. 2010]. Random integration of marker genes can disrupt endogenous genes for forward genetic screens.

Gene silencing by RNA interference (RNAi) has also emerged as a powerful genetic tool to attenuate targeted transcript levels and is particularly well developed in C. reinhardtii [Casas-Mollano et al. 2008, Cerutti and Ibrahim 2011, Cerutti et al. 2011, De Riso et al. 2009, Huang et al. 2011, Molnar et al. 2009, Molnar et al. 2007, Rohr et al. 2004]. RNAi in algae typically relies
on either microRNA (miRNA) or small interfering RNA (siRNA) molecules to generate small single-stranded RNAs that are complimentary to portions of the targeted transcript sequence [Cerutti and Ibrahim 2011, Cerutti et al. 2011]. miRNAs usually arise from endogenous, noncoding single-stranded RNA transcripts with imperfect complementarity that fold into defined stem-loop structures, whereas siRNAs originate from long regions of double-stranded RNA that display near-perfect complementarity and can be attained from a variety of diverse sources [Cerutti and Ibrahim 2011, Cerutti et al. 2011]. These double-stranded RNA molecules are recognized by the Dicer enzyme, which binds and cleaves the substrates into small RNAs (~20–30 nt in length). The RNA “passenger strand” is then removed and the single remaining “guide strand” coordinated by the protein Argonaute enables the formation of an effector known as the RNA-induced silencing complex (RISC) [Cerutti and Ibrahim 2011, Cerutti et al. 2011]. RISC uses the small single-stranded RNA guide fragment for the recognition and binding of complementary mRNA, which is degraded.

The existence of RNAi pathways has been experimentally confirmed in C. reinhardtii and P. tricornutum [Casas-Mollano et al. 2008, Cerutti and Ibrahim 2011, Cerutti et al. 2011, De Riso et al. 2009, Huang et al. 2011, Molnar et al. 2009, Molnar et al. 2007, Rohr et al. 2004], and homologs to the key components of RNAi machinery have been identified from genome and transcriptome sequences in Volvox carteri, Dunaliella salina, Porphyra yezoensis, Ectocarpus siliculosus, Nannochloropsis oceanica, Vaucheria frigida, and Euglena gracilis. Gene silencing through the introduction of exogenously synthesized dsRNAs has long been used as a transient method for probing gene function. The above studies also describe techniques to express inverted repeat (IR) transgenes that code for dsRNA molecules or artificial miRNAs (amiRNA).

2.4.5.1 Intracellular Triacylglycerol and Starch Accumulation

In the model green alga C. reinhardtii, notable advances have enabled starch hyperaccumulation [Work et al. 2010, Spalding 2013], reduction of light-harvesting antennae for more efficient light harvesting [Polle et al. 2003, Kirst et al. 2012a, Kirst et al. 2012b, Mitra et al. 2012], and increased TAG accumulation in starchless mutants [Wang et al. 2009, Work et al. 2010, Li et al. 2010a, Li et al. 2010b, Siaut et al. 2011]. In the model diatom Phaeodactylum tricornutum, medium chain fatty acid synthesis and incorporation into TAG has been accomplished [Radakovits et al. 2011]. Medium chain fatty acid derivatives (C10–C14) have
lower melting points relative to the C16 and C18 fatty acids that typically dominate algal biomass and are therefore better suited for biofuel applications.

Starch serves as the primary substrate of human caloric intake, and green algal starch is similar to the starch of higher plants [Ball and Morell 2003]. Starch is readily hydrolyzed to glucose, which can be fermented into a variety of fuel and nutritional products by secondary organisms. Differential expression of the isoamylase gene caused three- to five-fold starch overaccumulation in C. reinhardtii in nutrient replete media as compared to the wild type [Work et al. 2010]. Interestingly, starch was synthesized at the expense of cell division and protein synthesis, providing a case study for pulling metabolic intermediates into alternative pathways, rather than pushing metabolism via genetic disruption. The starch overaccumulating strains were generated by complementing the sta7 mutant (isoamylase 1 disruption) with the wild-type isoamylase 1 gene [Posewitz et al. 2005, Posewitz et al. 2004]. Complementation restored the starchless phenotype, but isoamylase gene expression was likely altered outside of its native context with respect to the wild type, as DNA transformed into C. reinhardtii is randomly integrated at other chromosomal loci. To generate algal strains that produce more lipids, the sta7 mutant as well as the sta6 (ADP-glucose pyrophosphorylase disrupted) starchless mutant have been investigated. These strains direct metabolism away from carbohydrate storage into lipid biosynthesis, resulting in increased TAG production during nitrogen limitation [Wang et al. 2009, Work et al. 2010, Li et al. 2010b].

2.4.5.2 Photosynthetic Antennae

Several studies in C. reinhardtii have reported reductions in the size of photosystem antennae upon diminishing or eliminating the accumulation of key light-harvesting-complex (LHC) proteins, regulatory proteins influencing the assembly of the photosynthetic apparatus or pigment biosynthesis enzymes [Beckman et al. 2009, Kosourov et al. 2011, Mitra and Melis 2010]. These efforts are focused on improving photosynthetic efficiencies and have yielded laboratory improvements in photosynthetic O2 evolution, biomass accumulation, and/or H2 production yields. Specifically, a mutant lacking chlorophyll b showed a reduced PSII antenna size, no PSII antenna size heterogeneity, and chlorophyll a substitution in LHC proteins [Polle et al. 2000]. While the quantum yield, variable to maximal chlorophyll fluorescence yield ratios (Fv=Fmax), and the light-saturated rate of photosynthesis (Pmax) are all lower in cells grown in
mixotrophic tris-acetate-phosphate medium, the chlorophyll b-less mutant exhibits a 2.5-fold higher Pmax in medium containing bicarbonate as the sole carbon source.

Using RNAi technology, the expression of 20 genes encoding LHCI, LHCII, CP26, and CP29 homologs were simultaneously and strongly downregulated in the stm3LR3 mutant, resulting in an altered thylakoid ultrastructure [Mussgnug et al. 2007]. Reduced chlorophyll content and antennae size, an increase in photosynthetic quantum yields, and a decrease in photoinhibition were all observed in this mutant. Photosynthetic pigment biosynthesis was not adversely affected, and importantly the remaining core LHC complexes afforded an increase in cell growth rates at high light (1000 mmol photons/m²/s) [Mussgnug et al. 2007].

In C. reinhardtii, the TLA1 gene is proposed to influence chloroplast development and affects chlorophyll biosynthesis rates and antenna size. Attenuation of TLA1 mRNA transcripts, and corresponding protein accumulation, results in less total chlorophyll in both photosystems and lower levels of LHC proteins [Polle et al. 2003]. With respect to the wild type, O₂-evolution rates in the tla1 mutant required higher light intensities for saturation, indicating improved photosynthetic performance at high light [Polle et al. 2003], and displayed a sixfold increase in H₂ photoproduction at medium-light intensities (350 µE/m²/s) during a 250-h experiment [Kosourov et al. 2011].

The C. reinhardtii NAB1 protein influences the size and composition of the LHCII antenna complex by binding and sequestering mRNA encoding components of this complex [Mussgnug et al. 2005]. A recombinant NAB1 gene containing mutations in codons for two cysteines that regulate activity in the wildtype protein was expressed behind the strong PSAD promoter. Expression of this permanently active NAB1 variant led to a 10–17% reduction in LHC antenna size, improved light transmission into the culture, and higher light conversion efficiencies [Beckmann et al. 2009].

2.4.5.3 Dark Metabolism

*Chlamydomonas reinhardtii* is the model algal system to study eukaryotic phototroph fermentative metabolism. Most PSMs encode a suite of fermentative pathways that are readily activated at night during a diel cycle when cellular respiration can deplete O₂ levels. In the case of *C. reinhardtii*, formate, acetate, ethanol, and H₂ are major products from starch catabolism [Meuser et al. 2012, Mus et al. 2007]. *Chlamydomonas reinhardtii* mutants deficient in
fermentative enzymes readily redirect metabolic flux, and in the case of a pyruvate formate lyase/ alcohol dehydrogenase double mutant, this resulted in a complete rerouting of fermentation to produce primarily glycerol and lactate, which are not observed to be secreted from the wild type [Catalanotti et al. 2012, Magneschi et al. 2012].

Although still in the nascent stages of development, genetic tools available in *C. reinhardtii* have been effectively used to alter algal metabolic networks and photosynthetic properties and in some cases improve target product yields. These efforts demonstrate promising advances in reprogramming algal cells for biotechnological purposes.

### 2.4.6 New Model Systems

While model organisms such as *C. reinhardtii* and *P. tricornutum* have been instrumental for studying aspects of photosynthesis, nutrient acquisition, and algal metabolism, these organisms have yet to emerge in successful large-scale outdoor cultivation platforms. Researchers are now focusing on algal species with the demonstrated ability to grow outdoors at meaningful scales, where they are currently used in nutraceutical and aquaculture industries.

Among these are highly oleaginous marine species of Nannochloropsis. Recently, Nannochloropsis genomes have been published for *N. gaditana* [Radakovits et al. 2012] and *N. oceanica* [Pan et al. 2011, Vieler et al. 2012], with several others in progress [Jinkerson et al. 2013] and transformation methods in development [Kilian et al. 2011, Radakovits et al. 2012, Cha et al. 2011]. Nannochloropsis species are particularly attractive as new model systems due to their exemplary lipid production characteristics [Boussiba et al. 1987, Converti et al. 2009, Simionato et al. 2011]. In outdoor culture, Nannochloropsis is projected to produce more than 90 kg of oil per hectare per day [Rodolfi et al. 2009]. Although lipid production for biofuels has largely driven the use of Nannochloropsis species as model organisms, additional biocommodity prospects exist, including the production of eicosapentaenoic acid and a number of valuable pigments [Lubián et al. 2000].

*Nannochloropsis gaditana* is a small (2–4 mm), nonmotile, marine eustigmatophyte related to diatoms and brown algae [Radakovits et al. 2012, Andersen et al. 1998, Rocha et al. 2003]. It is spherical in shape with a rugged cell wall and large lipid droplets. The lipid profile of Nannochloropsis species is rich in C14, C16, and C18, with variable amounts of C20 [Radakovits et al. 2012, Vieler et al. 2012, Hodgson et al. 1991]. With nuclear transformation
becoming a routine process in Nannochloropsis, it is expected that rapid progress in investigating and manipulating lipid metabolic pathways, cell wall biosynthesis, and light-harvesting complexes will ensue and that the addition of exogenous elements for the production of other marketable chemicals will be undertaken. Homologous recombination has been reported in one strain of Nannochloropsis (W2JB3) [Kilian et al. 2011]. In N. gaditana, a number of genetic ontology (GO) terms associated with RNAi were identified [Radakovits et al. 2012, Jinkerson et al. 2013], indicating that Nannochloropsis may also be amenable to manipulation by RNAi techniques.

To effectively enable industrially promising organisms, such as Nannochloropsis, to become useful “chassis” for further strain improvements, molecular tool kits must be expanded. Many of these newly emerging organisms require extensive time for phototrophic growth on agar plates, thereby delaying the subsequent selection and study of genetic transformants. Enabling heterotrophy is a potential mechanism to accelerate research in these cases. Zaslavskai et al. demonstrated a successful trophic conversion of P. tricornutum by introducing a human glucose transporter (Glut1) into the nuclear genome [Zaslavskai et al. 2001]. Similar approaches have been applied to C. reinhardtii and Volvox carteri by expressing the hexose=H1 symporter (HUPI) gene from Chlorella [Hallmann and Sumper 1996, Doebbe et al. 2007] and in cyanobacteria by expressing the glucose transporter gene (glcP) from Synechocystis PCC 6803 [Zhang et al. 1998]. Although heterotrophy is frequently established by simply expressing an appropriate transporter, other enzymes, for example α-ketoglutarate dehydrogenase in the tricarboxylic acid cycle (TCA cycle) or isocitrate lyase in glyoxylate cycle, may have to be introduced in some cases [Smith et al. 1967, Mendzhul et al. 2000, Neilsonm et al. 1972, Pearce et al. 1969, Van Dien et al. 2003].

2.4.7 Algal Cell Walls

In contrast to cyanobacterial approaches that are focused on metabolite secretion, the majority of algal biofuels research is centered on harvesting cells then deconstructing and segregating algal biomass to attain the end product, typically starch or TAG. Understanding and manipulating algal cell walls is likely to improve both cell harvesting and processing methodologies. To be economically viable, high oil species must effectively resist predation and endure the environmental changes inherent to outdoor growth facilities, as well as be amenable
to efficient separation and extraction. To a great extent, these traits are embedded in the structure of the cell wall. Biofuel production processes will benefit from a deeper understanding of cell wall biosynthesis, composition, architecture, dynamism, and vulnerabilities. This will allow molecular biologists to genetically engineer more compliant strains, cue harvesters to intelligent flocculant options, guide chemists’ choices of enzymes and chemicals for biomass extraction, and provide engineers with a physical basis for modeling the bulk flow properties of algal slurries.

Even at the ultrastructural level, eukaryotic algae present a diversity of cell wall types, including some algae, such as species of Dunaliella, that do not have a cell wall [Ben-Amotz and Avron 1973]. In *C. reinhardtii*, the cell wall comprises five distinct layers of hydroxyproline-rich glycoproteins [Adair *et al.* 1987]. Yamada and Sakaguchi [Yamada and Sakaguchi 1982] found three distinct wall architectures among the thin-section electron micrographs of 12 surveyed species of Chlorella that contained either two layers—with or without a trilaminar outer sheath—or one microfibrillar layer. The chrysophycean algae, Pleurochrysis scherfellii, exhibits a wall of cellullosic plates embedded in a gelatinous, pectic matrix [Brown *et al.* 1970]. A thin retaining wall that is studded with tubules and freeze-fractures into two distinct layers surrounds colonies of *Botryococcus braunii* (Race B) cells, which are themselves each surrounded by a bipartite, polysaccharide wall [Weiss *et al.* 2012].

As is the case with higher plants, algal walls assemble a panoply of molecules. Typically, polysaccharides, proteins, and glycoproteins are believed to form the bulk of the wall, but few mass balances of algal cell wall composition have been published [Loos and Meindl 1982]. Other classes of molecules found in the wall of some algae include algaenans [Allard *et al.* 1998, Gelin *et al.* 1997, Kodner *et al.* 2009, Simpson *et al.* 2003, Blokker *et al.* 1998], phlorotannins [Koivikko *et al.* 2005], and lignin [Martone *et al.* 2009].

Cell wall polysaccharides including cellulose [Delmer 1987], chitin or chitosan-like molecules [Kapaun and Reisser 1995], hemicelluloses [Domozych *et al.* 1980], pectins [Domozych *et al.* 2007], fucans [Berteau and Mulloy 2003], alginates [Michel *et al.* 2010], ulvans [Lahaye and Robic 2007], carrageans [Michel *et al.* 2003], and lichenins [Ford and Percival 1965] have all been described in various algae. Some of these have industrial applications: fucans are proposed anticoagulants [Dore *et al.* 2012], alginates are used by the pharmaceutical and cosmetic industries [Tonnesen and Karlsen 2002], and cellulose can be
converted to biofuel. Chemical modifications of the polysaccharides, such as acetylation [Selig et al. 2009, Wu and Zivanovic 2008] and sulfonation [Berteau and Mulloy 2003, Michel et al. 2003], help determine their properties and how they are cross-linked into larger networks. A large number of enzymes have been identified that attack both the primary linkages and cross-links of these networks [Bauer et al. 2006]. Ionic chelators, such as EDTA and EGTA, may also disrupt ionic linkages among pectins, which are important determinants of cell wall rigidity and susceptibility to enzymatic attack in higher plants and likely some algae as well [Wehr et al. 2004].

Identification of polysaccharides often begins with determination of their monomeric constituents, and an abundance of literature addresses these compositions in several species. Notably, Takeda catalogued monomeric sugars to classify 40 species of Chlorella and nine species of Scenedesmus [Takeda 1991, Takeda 1996]. Species of Chlorella were grouped into cells with “rigid” walls (resistant to 2N TFA) of either glucosamine or galactose and mannose, and wall “matrix” material (hydrolyzed in 2N TFA) that contained a broad suite of pentose and hexose sugars. Scenedesmus species exhibited rigid walls of glucose, galactose, and mannose (but no glucosamine) and wall matrices of mannose, galactose, and glucose.

Although surveys of amino acid profiles of algal walls are available [Punnett and Derrenbacker 1966, Burczyk et al. 1999], characterization of the corresponding proteome is still in its infancy. Structural glycoproteins are common scaffolding material in the extracellular matrices of plants and animals, and plants often express hydroxyproline-rich glycoproteins (HRGPs), including the covalent and insoluble network of extensins and the GPI-anchored, soluble arabinogalactan proteins (AGPs) [Kieliszewski et al. 2010]. Unsurprisingly, this is true of algae as well. The cell wall of C. reinhardtii, for example, comprises self-assembling, extensin-like HRGPs [Adair et al. 1987], and HRGPs are also found in other green algae [Domozych et al. 1980, Baylson et al. 2001]. The susceptibility of the Chlorella vulgaris cell wall to trypsin digestion suggests one way in which understanding the structural role of algal wall proteins might lead to improved biomass extraction methods [Gerken et al. 2012].

Many algal genomes have now been published, and, in consort with proteomics efforts, that data is being mined for insight into cell wall metabolism. Wang et al. [Wang et al. 2004] isolated 81 protein orthologs from the cell wall fraction of Haematococcus pluvialis, including proteins involved in cell wall construction, modification, and hydrolysis and others involved in
signaling, transport, motility, and heat shock. Putative cell wall proteins in the brown alga Ectocarpus siliculosus have also been identified [Michel et al. 2010].

Algaenans represent an intriguing class of molecules present in the cell walls of some algae [Allard et al. 1998, Gelin et al. 1997, Kodner et al. 2009, Simpson et al. 2003, Blokker et al. 1998]. These long-chain and typically saturated hydrocarbons are highly resistant to hydrolysis and solvation, resembling the exine material of pollen grains and the cutin and cutan of higher plants [Atkinson et al. 1972, Jeffree 2007]. Resolving whether these compounds are truly distinct from one another will require greater understanding of how each is biosynthesized. Some of the more recalcitrant algal cell walls, such as those found in species of Chlorella, Scenedesmus, and Nannochloropsis, contain algaenans, and it is tempting to hypothesize that their presence makes these cell walls difficult to breach. What little is known of algaenans largely comes from the organic geochemistry literature, where they have been studied as a potential source of diagenic material. Allard et al. concluded that the algaenans of *Chlorella emersonii*, *Tetraedron minimum*, and *Scenedesmus communis* were at least in part monomeric, extremely long-chain (up to C120) carboxylic acids and alcohols [Allard et al. 2002]. On the other hand, Blokker et al. determined that polymeric, long-chain, α-hydroxy fatty acids form the structural basis of algaenan in some green algae and are found ester-bound to the cell walls of several species [Blokker et al. 1998]. In *Nannochloropsis salina*, Gelin et al. also determined an algaenan polymeric structure, but comprising mid-chain and terminal ether linked, unbranched n-alkanes and n-alkenes with dominant lengths of C28–C34 [Gelin et al. 1997]. While such structural disparities may imply distinct modes of algaenan biosynthesis, structural determinations to date have used severe acid hydrolysis steps at elevated temperature to strip away material attached to the more refractory algaenan, and these steps can produce artifacts [Koivikko et al. 2005]. A “softer” approach to isolating algaenans, such as enzymatic removal of contaminating carbohydrates and proteins, might produce a more relevant material for characterization.

Other valuable molecules associated with the cell wall are the carotenoids. Although carotenoids are typically found in the plastid where they mediate light and heat transfer, a few studies have found them tightly associated with cell walls. It is not clear whether the carotenoids are integral to the wall or if free carotenoids are simply adventitiously binding to shed or otherwise disrupted walls. Canthaxanthin, astaxanthin, lutein, and other pigments have been
described in preparations of the cell walls of *Scenedesmus obliquus* [Burczyk *et al.* 1999], and pigment bodies are strongly associated with the shed walls of a *Nannochloropsis* sp. [Rodolfi *et al.* 2003]. Some algae divide by eleutheroschisis, wherein the parental cell wall is shed into the media as the daughter cells emerge. Owing to their resilient nature, these often pigmented, shed walls persist and accumulate in the culture where they likely affect light transmittance.

Algae with by robust cell walls that buffer the inner cell from external threat possess an inherent advantage, yet these are barriers not only against predators but also to biofuel processors who must contend with refractory biomass when trying to extract commodities of interest from the intracellular compartment. An expanded understanding of algal cell wall monomeric composition, polymeric architecture, and dynamic compositional changes are necessary to inform research regarding manipulation of cell walls into more compliant harvesting and deconstruction phenotypes, which will doubtlessly yield more cost-effective approaches to algal biofuel production.

### 2.4.8 Conclusions

Continued expansion of the human population combined with ambitions for higher living standards must be reconciled with climate change, ecosystem degradation, and the finite reserves of fossil fuels. Photosynthesis provides the caloric, nutritional, and oxygenic requirements for human metabolism and established the fossil energy reserves that are now deeply integrated into our society. A transition to more sustainable means of providing the energy resources required for current and future anthropologic lifestyle is necessarily imminent and requires new photosynthetic platforms. Compared to traditional agricultural crops, relatively little time and effort have been devoted to PSM strain improvements for societal gain. The last decade has seen explosive interest in gaining a better understanding of PSM physiology, metabolism, and photosynthesis, and dramatic advances have been made in manipulating these organisms. Several PSMs are already highly productive, genetically tractable, and likely more straightforward to manipulate than higher plants. Considerable opportunities remain to improve photosynthetic efficiencies and carbon utilization, as well as modulating product titers to enable economic feasibility. Given the rapid rate of progress in these areas at present, meaningful advances using these metabolically versatile organisms are likely to emerge in the coming years.
2.4.9 Acknowledgments

The authors gratefully acknowledge financial support from the U.S. Department of Energy, Office of Science, Basic Energy Sciences (grant DE-FG02-12ER16339), and the Air Force Office of Scientific Research (grant FA9550-11-1-0211).
CHAPTER 3
INCREASED LIPID ACCUMULATION IN THE CHLAMYDOMONAS REINHARDTII STA7-10 STARCHLESS ISOAMYLASE MUTANT AND INCREASED CARBOHYDRATE SYNTHESIS IN COMPLEMENTED STRAINS

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3.2 Authors’ contributions

V.H. Work constructed the c5 complemented strain, performed a majority of the replicate cultivations and sample preparation, and collected data for cell density, chlorophyll content, and lipid content. R. Radakovits performed starch assays and assisted with lipid quantification. R.E. Jinkerson compiled data graphs and tables, performed the iodine starch assay, and assisted with lipid quantification. J.E. Meuser performed photosynthetic O₂ evolution experiments. L.G. Elliott provided microscopy images. D.J. Vinyard performed PAM fluorometry. L.M.L. Laurens performed select confirmatory lipid quantification and U.M.N. Murthy (not listed) is gratefully acknowledged here for contributing acetate quantification. G.C. Dismukes and M.C. Posewitz oversaw, edited, and funded the preparation.

3.3 Abstract

The accumulation of bioenergy carriers was assessed in two starchless mutants of Chlamydomonas reinhardtii (the sta6 [ADP-glucose pyrophosphorylase] and sta7-10 [isoamylase] mutants), a control strain (CC124), and two complemented strains of the sta7-10 mutant. The results indicate that the genetic blockage of starch synthesis in the sta6 and sta7-10 mutants increases the accumulation of lipids on a cellular basis during nitrogen deprivation relative to that in the CC124 control as determined by conversion to fatty acid methyl esters.
However, this increased level of lipid accumulation is energetically insufficient to completely offset the loss of cellular starch that is synthesized by CC124 during nitrogen deprivation. We therefore investigated acetate utilization and O$_2$ evolution to obtain further insights into the physiological adjustments utilized by the two starchless mutants in the absence of starch synthesis. The results demonstrate that both starchless mutants metabolize less acetate and have more severely attenuated levels of photosynthetic O$_2$ evolution than CC124, indicating that a decrease in overall anabolic processes is a significant physiological response in the starchless mutants during nitrogen deprivation. Interestingly, two independent sta7-10:STA7 complemented strains exhibited significantly greater quantities of cellular starch and lipid than CC124 during acclimation to nitrogen deprivation. Moreover, the complemented strains synthesized significant quantities of starch even when cultured in nutrient-replete medium.

3.4 Introduction

Microalgae are able to efficiently convert sunlight, water, and CO$_2$ into a variety of products suitable for renewable energy applications, including H$_2$, carbohydrates, and lipids [Dismukes et al. 2008, Ghirardi et al. 2007, Hankamer et al. 2007, Hu et al. 2008, Posewitz et al. 2009, Radakovits et al. 2010, Schenk et al. 2008]. The unicellular green alga *Chlamydomonas reinhardtii* has emerged as a model organism for studying algal physiology, photosynthesis, metabolism, nutrient stress, and the synthesis of bioenergy carriers [Ghirardi et al. 2007, Grossman et al. 2007, Hemschemeier et al. 2009, Kruse et al. 2005, Merchant et al. 2007]. During acclimation to nitrogen deprivation, *C. reinhardtii* cells accumulate significant quantities of starch and form lipid bodies [Ball and Deschamps 2009, Chochois et al. 2009, Dauvillee et al. 2001, Li et al. 2010a, Libessart et al. 1995, Martin and Goodenough 1975, Mouille et al. 1996, Sager and Granick 1954, Wang et al. 2009, Zabawinski et al. 2001]. Despite the significance of these products in algal physiology and in biofuels applications, the metabolic, enzymatic, and regulatory mechanisms controlling the partitioning of metabolites into these distinct carbon stores in algae are poorly understood. Several *C. reinhardtii* starch mutants with various phenotypic changes in starch content and structure have been isolated [Ball 1998, Ball 2002, Ball and Deschamps 2009]. Two of these, the sta6 and sta7 mutants, contain single-gene disruptions that result in “starchless” phenotypes with severely attenuated levels of starch granule accumulation [Ball 1998, Ball and Deschamps 2009, Mouille et al. 1996, Posewitz et al. 2005,
The disrupted loci in the two isolated starchless mutants are distinct and each mutant has a unique phenotype [Dauvillee et al. 2001, Posewitz et al. 2004]. In the \textit{sta6} mutant, the small, catalytic subunit of ADP-glucose pyrophosphorylase (AGPase-SS) is disrupted [Ball 1998, Ball and Deschamps 2009, Zabawinski et al. 2001], and this mutant accumulates less than 1\% of the starch observed in wild-type (WT) cells under conditions of nitrogen deprivation. The \textit{sta7} mutant contains a disrupted isoamylase gene [Dauvillee et al. 2001, Dauvillee et al. 1999, Dauvillee et al. 2000, Posewitz et al. 2005, Posewitz et al. 2004] and also has severely attenuated levels of starch, but it accumulates a soluble glycogen-like product [Ball and Deschamps 2009, Dauvillee et al. 1999]. In this study, we conducted an examination of the unique physiological acclimations that are utilized by these mutants to adapt to the loss of starch synthesis. As the genetic lesions in these two mutants are distinct and block starch synthesis via two very different mechanisms, we investigated the physiological consequences of starch inhibition in both of these mutants from a holistic bioenergy perspective, which included photosynthetic parameters and the overall yields of lipids and carbohydrates, the two primary bioenergy carriers in \textit{C. reinhardtii}. Specifically, we examined whether the inability to synthesize starch would result in the accumulation of additional lipid, alter cellular growth or cell size, affect acetate utilization, and/or influence photosynthetic O$_2$ evolution. Our data indicate that both the \textit{sta6} (BAFJ5) and \textit{sta7} (\textit{sta7-10}) mutants accumulate more lipid than the CC124 control during nitrogen deprivation. However, the additional lipid does not completely offset the loss of starch synthesis from a complete energetic perspective. Increased lipid accumulation during nitrogen stress has also been reported for a variety of starch mutants in recent papers [Li et al. 2010a, Li et al. 2010b, Wang et al. 2009]. A significant feature in both of the starchless mutants studied here is that O$_2$ evolution and acetate utilization are diminished during nitrogen stress, which is undesirable from an overall bioenergy perspective. Remarkably, complementation of \textit{sta7-10} with genomic DNA encoding the wildtype isoamylase gene resulted in cells that were larger than those of the \textit{sta6}, \textit{sta7-10}, and CC124 strains, exhibited the highest total lipid levels during nitrogen deprivation, and over-accumulated starch even in nutrient-replete medium.
3.5 Materials and Methods

This section describes experimental protocols for this study.

3.5.1 Strains and culturing conditions

CC124 was obtained from the Chlamydomonas Center, the sta6 mutant (BAFJ5) was kindly provided by Steven Ball [Zabawinski et al. 2001], and the sta7 (sta7-10) mutant in the CC425 background was isolated as described previously [Posewitz et al. 2004]. The sta7-10 complemented strains were obtained after transformation of the sta7-10 mutant with a construct carrying the WT STA7 gene (BamHI/KpnI fragment), which was cloned along with a Ble’ zeocin resistance cassette [Lumbreras et al. 1998] into pUC19. The sta7-10[c19] clone was isolated previously [Posewitz et al. 2004], and the sta7-10[c5] clone was isolated as a part of this study from a separate transformation.

Cultures were grown to late log phase in nitrogen-replete Tris-acetate-phosphate (TAP) liquid medium and resuspended at 2.0 x 10^6 to 2.5 x 10^6 cells/ml in parallel in nitrogen-replete TAP medium or nitrogen-depleted TAP (TAP–N) medium, in which NH₄Cl was omitted [Gorman and Levine 1965, Harris 2009, Harris 1989]. Cells were grown under 50 µmol photons/m²s (50 µE/m²s) photosynthetically active radiation (PAR) constant illumination on an orbital shaker. Samples for analysis were taken immediately after resuspension (0 h) and at the indicated times. Cell counts and cell sizes were assessed using a Z2 Coulter Counter cell and particle counter (Beckman-Coulter, Brea, CA). Cells were assumed to be spherical for diameter calculations, and background and cellular debris were excluded in all cellular count, volume, and diameter assessments. Coulter cell counts were verified for representative samples using microscopy.

3.5.2 Chlorophyll measurements

Chlorophyll was determined using ethanol extraction. One milliliter of culture was centrifuged at 6,000 x g for 5 min at room temperature (RT), the supernatant was saved for acetate quantification (see below), and the cell pellets resuspended in 95% ethanol and vortexed to extract pigments. Cellular debris was pelleted by centrifugation (14,000 x g) for 3 min, and absorption was read at 665 nm and 649 nm using a Jenway 6505 UV-visible spectrophotometer (Barloworld Scientific Ltd., Essex, United Kingdom). Calculations of total chlorophyll (µg/ml)
were performed as described previously [Harris 1989].

3.5.3 **Microscopy**

The effects of nitrogen deprivation on nonpolar lipid accumulation were visually assayed using laser scanning confocal microscopy. After 96 h in TAP–N medium, all strains were stained with the nonpolar lipid fluorophore Bodipy 493/503 [Gocze and Freeman 1994] (Molecular Probes, Invitrogen Corporation). To prepare the cells for imaging, 3 ml of each culture was centrifuged at 4,000 x g at RT for 5 min. The supernatant was removed, and 100 µl of the supernatant was used to resuspend the cell pellet. The concentrated cells were stained with 10 µg/ml Bodipy 493/503 for 5 min. To immobilize cells, 1% low-melting-temperature (LMT) agarose was heated to 65°C for use as mounting medium, and 5 µl of stained cell suspension was rapidly mixed with 5 µl of molten 1% LMT agarose. Five microliters of this mixture was immediately transferred to a coverslip, which was then inverted on a microscope slide and allowed to solidify. Coverslips were sealed with a clear epoxy (nail polish) to prevent evaporation of the mounting medium during the imaging process.

Images were acquired using a Nikon Eclipse E800 microscope equipped with a Nikon D-Eclipse C1 laser scanning confocal imaging system using a Melles Griot Kyma 488 series 85-BCD-010 solid-state laser for fluorescence excitation and light transmission as well as a SPOT RT KE color mosaic charge-coupled device (CCD) camera for bright-field imaging. The laser output power was 10 mW, with an emission wavelength of 488 ± 0.5 nM. Laser emission was controlled at 25% of maximum with an ND4 optical filter. Chlorophyll autofluorescence was detected using a 685/70 band-pass optical filter, and Bodipy 493/503 fluorescence was detected using a 515/30 band-pass optical filter. The small-pinhole confocal configuration was used to filter out-of-focus fluorescence emission light.

3.5.4 **Starch assays**

Cellular glucose levels contained in starch were determined using amyloglucosidase digestion and the Sigma glucose (HK) assay kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions. Cells were concentrated by centrifugation of 10 ml of culture at 3,600 x g for 10 min. The supernatant was discarded, and cells were frozen at –80°C. Samples were then resuspended in 100 mM sodium acetate (pH 4.5), autoclaved to solubilize starch, and
then digested with amyloglucosidase overnight at 60°C to liberate glucose. To visually assess starch content in colonies on agar plates, iodine vapor staining was performed by placing solid I2 pellets on the surfaces of agar plates to initiate sublimation.

3.5.5 FAME quantification

Glycerolipids were converted to fatty acids for GC-FID analysis as described previously [Pendergrass and Jensen 1997, Pendergrass 1998]. Lipids were extracted and derivatized from liquid culture at the indicated times. Briefly, 1.0 ml methanol saturated with NaOH was added to 0.5 ml culture and heated in tightly sealed vials at 100°C for 2 h, resulting in cell lysis and lipid saponification. Acid-catalyzed methylation was accomplished by adding 2 ml 1:1.2 6 N HCl/MeOH and incubating at 80°C for 2 h, followed by overnight incubation at 60°C. Fatty acid methyl esters (FAMEs) were extracted into 1 ml 1:1 hexane-methyl tertiary butyl ether (MTBE) via gentle inversion. Extracts were washed with distilled water and analyzed directly by gas chromatography-flame ionization detection (GC-FID) using an Agilent 7890A gas chromatograph with a DB5-ms column (Agilent Technologies, Santa Clara, CA). Tridecanoic acid (13:0 fatty acid) was also spiked into representative samples, and recovery of this internal standard converted to FAME was above 90%. Although the majority of FAME analyses for algae involve the use of dried biomass, the sta6 and sta7-10 cells after nitrogen deprivation were extremely fragile and difficult to centrifuge into cellular pellets due to their low density, resulting in significant sample losses. Therefore, we modified existing protocols [O’Fallon et al. 2007, Pendergrass and Jensen 1997, Pendergrass 1998] for lipid extraction and FAME analysis from wet biomass directly from culture, which also significantly improved sample throughput. FAME recovery from representative samples from nutrient-replete cultures, which are easily harvested by centrifugation, was analyzed using the protocol described here and compared to that with a more conventional lipid extraction and derivatization technique consisting of lyophilizing cells pellets, solubilizing the lipids in chloroform-methanol (2:1, vol/vol), and subsequently transesterifying the lipids with HCl-methanol (5%, wt/vol) for 1 h at 80°C in the presence of a tridecanoic acid (C13) methyl ester internal standard [25]. The resulting FAMEs were extracted with hexane at room temperature for 1 h and analyzed by GC-FID (method adapted from that of Lepage and Roy [Lepage and Roy 1986] and optimized with regard to reaction conditions and extraction time and temperature). Although the wet biomass extraction and derivatization
protocol resulted in diminished FAME recovery (by approximately 15 to 30% variation when expressed on a volume basis), the difference in FAME recovery was consistent for all strains used and was deemed to provide an acceptable proxy for relative changes in lipid accumulation.

3.5.6 Acetate utilization

Acetate remaining in culture media was quantified using high-pressure liquid chromatography (HPLC) as described previously [Datar et al. 2007]. Both nitrogen-replete and nitrogen-depleted TAP contained 17.5 mM acetate prior to culturing (0 h). One milliliter of medium supernatant was filtered through a 0.45-μm nylon membrane prior to HPLC analysis and injected into an Agilent 1200 HPLC (Agilent Technologies, Santa Clara, CA) equipped with an Aminex HPX-87H (Bio-Rad, Hercules, CA) column (45°C), using a 0.6-ml/min flow rate and 4 mM H₂SO₄ as an isocratic mobile phase. UV-visible and refractive index detectors were used to detect and quantify acetate levels by comparison with standards.

3.5.7 Photosynthetic oxygen evolution rates

Oxidogenic photosynthesis was assessed by measuring in vivo O₂ production using a custom-built Clark-type apparatus equipped with YSI5331 platinum electrodes (YSI Incorporated, Yellow Springs, OH). Cells were grown to late log phase under nitrogen-replete conditions, whereupon cultures were resuspended at approximately 1 x 10⁷ cells/ml in TAP–N medium. Cell suspensions (0.8 ml) were taken immediately after removal from the orbital shaker and added to a temperature-controlled, water-jacketed glass reaction cell (Allen Scientific Glass, Boulder, CO) based on an earlier design by Gilson Inc. Probe polarization (0.6 V) and digital signal amplification were accomplished using a custom-built digital picoammeter circuit and acquired using a digital data acquisition card (National Instruments, Austin, TX) and custom software [Karns 2009]. Calibration of the electrode signal was done at the initiation of each experiment using 50 mM air-saturated TAP medium and argon-purged (General Air, Denver, CO) reference buffer. Oxygen photoproduction of the stirred cell suspensions was measured during a 3-min illumination with approximately 80 μE/m²s from an incandescent Fiber-Lite high-intensity illuminator (Dolan-Jenner Industries, Lawrence, MA). Oxygen photoproduction rates were calculated from the change in O₂ concentration over the 3-min illumination phase. Normalizations were done based on the amount of chlorophyll (μg) or the cell density of each
sample.

3.5.8 **Chlorophyll fluorescence measurements**

The intensity of chlorophyll a fluorescence emission was measured during excitation at 618 ± 10 nm with 695-nm (high-pass) and 780-nm (low-pass) cutoff filters using the Photon Systems Instrument (PSI) FluorCam 800MF (Crno, Czech Republic). The light intensity at the sample surface (500 µE/m²/s) was sufficient to saturate the variable yield of fluorescence emission, Fv, as evidenced by the lack of change in maximum fluorescence signal upon further increasing actinic light intensity. Fv fluorescence arises from photosystem II (PSII) and reaches a maximum yield (Fm = Fv + Fo) when PSII primary charge separation occurs and closes the reaction center by preventing further photochemical quenching [Shinkarev 2004]. Fo reports on the nonvariable yield of chlorophyll a emission. The CC124, sta6, and sta7-10 strains (five replicates each) were plated on TAP and TAP–N agar and dark adapted initially for 15 min before any measurements and subsequently for a 1-min dark time following the initial flash sequence to reestablish a dark-adapted state (open reaction centers). Fo and Fm were measured directly from each colony and are reported here as Fv/Fm, where Fv = Fm – Fo.

3.6 **Results**

This section describes the results of this study.

3.6.1 **Distinct growth rates and cell sizes in the starchless mutants and complemented strains**

To examine the unique physiological acclimation mechanisms used in the absence of starch synthesis, we precultured the control strain (CC124), the starchless mutants (sta6 and sta7-10), and two sta7-10:STA7 complemented strains (denoted sta7-10[c5] and sta7-10[c19]) to late log phase in TAP medium. Cultures were then centrifuged and resuspended in either TAP or TAP–N medium at standardized cell numbers (~ 2.0 x 10^6 to 2.5 x 10^6 cells/ml). CC124 was used as a control since the parental strains of the sta6 and sta7-10 mutants (330 and CC425, respectively) are auxotrophic for arginine, which could be used as a cellular nitrogen/carbon source. It should be noted that CC124 has an intact cell wall and that the mutants and complements lack a cell wall. The growth curves and cell diameter data in Figure 3.1 and Table
3.1, respectively, show several distinguishing features. In nitrogen-replete medium, distinct final cell densities are achieved, with \( \text{sta6} > \text{CC124} > \text{sta7-10} > \text{sta7-10[c5]} > \text{sta7-10[c19]} \).

![Figure 3.1](image)

Figure 3.1. Cell counts for CC124, the \( \text{sta6} \) and \( \text{sta7-10} \) mutants, and the \( \text{sta7-10:STA7} \) complemented \( \text{sta7-10[c5]} \) and \( \text{sta7-10[c19]} \) strains. Growth curves from a representative experiment are shown and were constructed from cell counts at resuspension (0 h) until 96 h, as indicated, in TAP (A) or nitrogen-depleted TAP–N (B) medium. Each data point represents three replicates, except for \( \text{sta7-10[c5]} \), which represents two biological replicates. Error bars indicate standard deviations.

Although the highest cell concentrations are attained in the \( \text{sta6} \) mutant, this strain has the smallest average cell diameter (5.6 \( \mu \)m). However, it appears that this phenotype may be restricted to cells cultured in TAP medium, as \( \text{sta6} \) cells cultured in HS medium do not exhibit smaller diameters [Ursula Goodenough, personal communication]. In contrast, the \( \text{sta7-10[c5]} \) (6.2 \( \mu \)m) and \( \text{sta7-10[c19]} \) (6.8 \( \mu \)m) complemented strains have the largest average cell diameters but achieve the lowest cell densities. Intermediate cell diameters and final cell counts are observed for CC124 (5.9 \( \mu \)m) and \( \text{sta7-10} \) (6.0 \( \mu \)m). The average cell diameters for each strain remained relatively stable for the entire 96-h culturing period in nitrogen-replete medium, with the exception of the \( \text{sta7-10[c5]} \) and \( \text{sta7-10[c19]} \) strains, which enter stationary phase prior to the other strains and become noticeably larger at the end of the 96-h culturing period (Table 3.1).

Additionally, the results in TAP medium indicate that decreased rates of cell division correspond well with increased average cell diameters. In nitrogen-depleted medium, an increase
in cell numbers was observed only for CC124 (~1.7-fold increase), and none of the cell wall-less strains \((sta6, sta7-10, sta7-10[c5], \text{and} sta7-10[c19])\) showed any significant change in cell number. This indicates an arrest of cell division in the cell wall-less strains in nitrogen-depleted medium during the assay (Figure 3.1B), which is consistent with a previous study [Li et al. 2010b]. In contrast to the case for culturing in nutrient-replete medium, the average cell diameters increased for each strain during nitrogen stress. After 96 h of culturing in TAP–N, the largest cell diameters were observed for the \(sta7-10[c5] (7.5 \mu m)\) and \(sta7-10[c19] (7.5 \mu m)\) strains, followed by the CC124 (7.2 \mu m), \(sta7-10 (6.5 \mu m)\), and \(sta6 (6.0 \mu m)\) strains. Each strain exhibits an increase in average cell diameter during acclimation to nitrogen deprivation (Table 3.1), which is suggestive of an increase in cellular carbon product accumulation. It should be noted that all cultures contained a distribution of cell sizes for both culturing conditions, the majority of which were within 1.0 \mu m of the average cell diameter (Table 3.1). Additionally, cell diameters are calculated based on the assumption that the displaced fluid volume in the Coulter Counter corresponds to a sphere, which tends to underestimate the size of \(C. \text{reinhardtii}\) cells observed using transmission microscopy, as \(C. \text{reinhardtii}\) cells are not perfectly spherical.

Table 3.1. Average cell sizes for the CC124, \(sta6, sta7-10, sta7-10[c5], \text{and} sta7-10[c19]\) strains cultured in TAP (N+) or TAP–N (N–) medium.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Strain</th>
<th>0 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cell diam ((\mu m))(^a)</td>
<td>Total cellular vol ((\mu l/m) culture)</td>
</tr>
<tr>
<td>N+</td>
<td>CC124</td>
<td>5.9 ± 1</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>sta6</td>
<td>5.6 ± 0.7</td>
<td>0.15 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>sta7-10</td>
<td>6 ± 0.9</td>
<td>0.18 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>sta7-10 [c5]</td>
<td>6.2 ± 0.9</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>sta7-10 [c19]</td>
<td>6.8 ± 1.1</td>
<td>0.27 ± 0.11</td>
</tr>
<tr>
<td>N–</td>
<td>CC124</td>
<td>5.2 ± 0.7</td>
<td>0.21 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>sta6</td>
<td>4.9 ± 0.7</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>sta7-10</td>
<td>5.6 ± 0.9</td>
<td>0.15 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>sta7-10 [c5]</td>
<td>6.3 ± 0.9</td>
<td>0.18 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>sta7-10 [c19]</td>
<td>6.4 ± 1.1</td>
<td>0.16 ± 0.08</td>
</tr>
</tbody>
</table>

\(^a\) Cell diameter data are reported as the mean value ± standard deviation of the normal distribution of cell diameters, which is representative of the distribution of cell sizes in each culture. Cell diameter data represent a minimum of at least four independent biological replicates, in which the standard deviation of replicate average diameters is less than 0.7 \mu m.

\(^b\) The number of cells was assessed with a Z2 Coulter Counter cell and particle counter at resuspension (0 h) and after 96 h in TAP and TAP–N media.
3.6.2 Excess starch accumulation in the sta7-10 complemented strains

To assess whether the observed differences in growth rates and cell sizes could be correlated with the accumulation of starch and lipid, we first measured the levels of starch-derived glucose after treatment with amyloglucosidase (Figure 3.2).

![Figure 3.2. Starch analyses.](image)

In TAP medium, CC124 cells contained 7.8 ± 1.0 μg starch/10^6 cells, measured as glucose equivalents, whereas after 4 days in nitrogen-depleted medium, an approximately 6-fold cellular increase to 41.9 ± 13.0 μg starch/10^6 cells was observed, values that are consistent with those recently recorded by Chochois et al. [Chochois et al. 2009]. As reported previously [Dauvillee et al. 2001, Mouille et al. 1996, Posewitz et al. 2005, Posewitz et al. 2004], both sta6 and sta7-10 mutant cells contained severely attenuated levels of starch and essentially no starch-derived glucose was detected in the sta6 mutant, while sta7-10 cells contained 1.7 ± 0.2 μg starch/10^6 cells in TAP medium and 4.0 ± 0.3 μg starch/10^6 cells after 4 days in TAP–N medium. Interestingly, after 96 h in nutrient-replete medium, the sta7-10[c5] and sta7-10[c19] complemented strains had 51.6 ± 2.1 μg starch/10^6 cells and 55.1 ± 6.3 μg starch/10^6 cells, respectively, cellular levels that are near those observed in CC124 only after transfer to nitrogen-depleted medium. In fact, as shown in Figure 3.2B, the highest yields of starch on a culture
volume basis were attained in nitrogen-replete sta7-10[c5] and sta7-10[c19] cultures, with yields exceeding 400 mg/liter after 4 days in cultures inoculated at 2.5 x 10^6 cells/ml. These data indicate that complementation of the sta7-10 mutant causes significantly altered starch accumulation and that despite transformation with a genomic copy of the STA7 gene containing native 5’ and 3’ untranslated regions (UTRs) and promoters, enzyme activity is occurring outside the native context, resulting in modulated starch accumulation.

### 3.6.3 Increased cellular lipid levels in sta6, sta7-10, and complemented strains

The other major carbon resource observed during nitrogen stress in *C. reinhardtii* is the formation of lipid bodies [Chochois et al. 2009, Li et al. 2010a, Mouille et al. 1996, Wang et al. 2009, Zabawinski et al. 2001]. Since the blockage of starch synthesis in the starchless mutants creates the potential for diverting metabolic precursors into lipid biosynthetic pathways, we investigated whether lipids were differentially accumulated in the starchless mutants by quantifying lipid-derived fatty acid methyl esters (FAMEs) using GC-FID. Lipids were extracted, derivatized, and quantified from liquid cultures of the CC124, sta6, sta7-10, sta7-10[c5], and sta7-10[c19] strains at the indicated times of acclimation to nitrogen deprivation (Figure 3.3).

![Figure 3.3](image)

Figure 3.3. GC-FID quantification of fatty acid methyl esters derived from CC124, sta6, sta7-10, sta7-10[c5], and sta7-10[c19] lipids at the indicated times of culturing in nitrogen-depleted TAP–N medium. Values are representative of triplicate biological samples. Lipid was quantified per million cells (A) or by ml of culture (B). Error bars indicate standard deviations.
The results indicate that the greatest levels of lipid amenable to conversion into FAMEs using the described transesterification protocol are observed in the sta7-10[c19], sta7-10[c5], and sta7-10 strains, followed by the sta6 and CC124 strains. The starchless mutants contained approximately 2- to 4-fold more lipids per cell than CC124, indicating that additional lipid accumulated in these strains relative to CC124. This is consistent with recent reports regarding increased lipid synthesis in the sta6 mutant relative to control strains [Li et al. 2010a, Li et al. 2010b, Wang et al. 2009]. However, on a bioenergetic basis, it should be noted that CC124 cell numbers continued to increase in nitrogen-depleted medium. Overall, the increased lipid content in the starchless mutant cultures does not completely offset the loss of starch (140 µg starch/ml of culture at 96 h in TAP–N CC124 cultures) from an energetic standpoint, despite the increased energy density of lipids relative to starch. It should also be noted that our cultures were inoculated at low cell densities and that by standardizing to cell counts, the smaller sta6 cells are slightly underrepresented from an initial biomass perspective in these experiments.

The major fatty acids observed in all strains cultured in either TAP or TAP–N medium were 16:0, 16:1, 18:0, 18:1, 18:2, and 18:3, which is consistent with previously reported results [Li et al. 2010a, Moellering and Benning 2010, Wang et al. 2009]. A representative GC-FID chromatogram with a C13:0 internal standard is shown in Figure 3.4. We observed only minor differences in the fatty acid profiles among the different strains under the culturing conditions used (Table 3.2).

Table 3.2. Fatty acid composition during early (22 h) and late (96 h) nitrogen deprivation

<table>
<thead>
<tr>
<th>Strain</th>
<th>h¹</th>
<th>Total lipid (µg)²</th>
<th>Fatty acid (% of total lipids)³</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C16:0</td>
<td>C16:1</td>
</tr>
<tr>
<td>CC124</td>
<td>22</td>
<td>1.7 ± 1</td>
<td>37.2 ± 0.9</td>
<td>3.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.6 ± 0.1</td>
<td>44.1 ± 1.7</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>sta6</td>
<td>22</td>
<td>0.6 ± 0.1</td>
<td>33.2 ± 0.5</td>
<td>6.4 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.3 ± 0</td>
<td>31.7 ± 1.2</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>sta7-10</td>
<td>22</td>
<td>1.1 ± 0.3</td>
<td>32.3 ± 0.6</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.4 ± 0.1</td>
<td>37.0 ± 0.7</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>sta7-10[c5]</td>
<td>22</td>
<td>1.5 ± 0.2</td>
<td>40.1 ± 0.7</td>
<td>5.2 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.3 ± 0.0</td>
<td>35.5 ± 0.7</td>
<td>5.6 ± 0.0</td>
</tr>
<tr>
<td>sta7-10[c19]</td>
<td>22</td>
<td>0.5 ± 0.1</td>
<td>37.6 ± 0.5</td>
<td>6.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.3 ± 0.0</td>
<td>31.5 ± 1.1</td>
<td>7.3 ± 0.1</td>
</tr>
</tbody>
</table>

¹ Hours of culture in nitrogen-depleted medium. ²Total lipid-derived FAME per ml culture.
³Values are averages of triplicate measurements, with standard deviations, obtained using GC-FID for quantification. ⁴C18:1, C18:2, and C18:3 together.

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3.6.4 Lipid body formation during nitrogen deprivation visualized by fluorescence imaging

To investigate lipid droplet formation after acclimation to nitrogen deprivation, all strains were visually assayed for nonpolar lipid accumulation using laser scanning confocal microscopy after incubation with the nonpolar lipid fluorophore Bodipy 493/503. As shown in Figure 3.5, nonpolar lipid body formation, depicted by green Bodipy 493/503 fluorescence, increases in nitrogen-stressed cells relative to cells in nutrient-replete medium, which is consistent with previous reports on the induction of lipid droplet formation in *C. reinhardti* as a consequence of nitrogen limitation [Li et al. 2010a, Moeller and Benning 2010, Wang et al. 2009]. Although the greatest density of lipid droplet formation appears in *sta6* cells, substantial accumulation of nonpolar lipid bodies is observed in all strains (Figure 3.5).

3.6.5 Attenuation of photosynthetic oxygen evolution and acetate uptake in the starchless mutants during nitrogen deprivation

To further probe the underlying mechanisms of differential carbohydrate and lipid accumulation during nitrogen deprivation and to determine whether these parameters are correlated to lipid and starch synthesis, we assessed the utilization of acetate and quantified the
From a holistic bioenergy perspective, it is critical that high levels of photosynthetic activity are maintained during illumination and that culturing conditions are optimized for water oxidation and CO$_2$ reduction. Metabolic pathway engineering strategies must focus on improving the overall yields of all algal bioenergy carriers, and efforts that optimize the accumulation of a single bioenergy feedstock should not sacrifice overall fitness and energy carrier yields.

The underlying hypothesis of this study was that by genetically blocking the synthesis of starch, the dominant carbon storage product in nutrient-deprived $C. reinhardtii$ cells, major compensatory mechanisms would result, providing new insights into the acclimation mechanisms used in starchless mutants relative to control strains during nitrogen deprivation. These insights could then be leveraged to further engineer this alga for improved bioenergy phenotypes using rational or random approaches. Moreover, because $sta6$ and $sta7^{-10}$ represent the most severe carbohydrate mutations but block starch synthesis at different levels, it was anticipated that unique acclimation strategies would be employed.

Figure 3.5. Laser scanning confocal fluorescence microscopy images merged with transmitted light images. CC124, sta6, sta7-10, sta7-10[c5], and sta7-10[c5] cells are shown. The two leftmost columns show cells stained with the nonpolar lipid fluorophore Bodipy 493/503. Nonpolar lipid bodies are visualized by green Bodipy fluorescence after 96 h (left, TAP; right, TAP–N). The two rightmost columns show differential interference contrast images after 96 h (left, TAP; right, TAP–N). Oil bodies are visible inside the cells as blue-green-tinted vesicles. Chlorophyll autofluorescence is red. All scale bars represent 10 µm.
levels of photosynthetic O₂ evolution of each strain during acclimation to nitrogen deprivation (Figure 3.6). Interestingly, significant quantities of acetate remained in the medium for all strains after 96 h of nitrogen deprivation when cultures were inoculated at 2.0 x 10⁶ to 2.5 x 10⁶ cells/ml (Figure 3.6A). More complete acetate utilization would be anticipated if cultures were inoculated at higher cell densities. Under nutrient-replete conditions, acetate is completely consumed within 48 h (data not shown) when cells are inoculated at 2.0 x 10⁶ to 2.5 x 10⁶ cells/ml, and acetate is presumably used to synthesize proteins, membrane lipids, and nucleic acids or to support respiration. These results indicate an overall attenuation of anabolic processes in all strains during nitrogen deprivation. However, in accordance with the starch and lipid accumulation data, acetate utilization was the greatest in the sta7-10[c5] and sta7-10[c19] strains, followed by the CC124, sta7-10, and sta6 strains. The increased use of acetate in the complemented sta7-10[c5] and sta7-10[c19] strains relative to the other strains at the cell concentrations used indicates that anabolic processes in the sta7-10[c5] and sta7-10[c19] strains are less severely affected during nitrogen stress.

Photosynthetic O₂ evolution was also monitored, and all strains exhibited attenuated levels of O₂ evolution (50 to 70% reduction [data not shown]) after 24 h of nitrogen deprivation (Figure 3.6B) relative to those during nutrient-replete culturing. This is consistent with previous observations that demonstrated attenuated levels of O₂ evolution in C. reinhardtii and other algae as a consequence of a variety of nutrient (N, P, and S) stresses [Herzig and Falkowski 1989, Martin and Goodenough 1975, Melis et al. 2000, Wykoff et al. 1998]. As shown in Figure 3.6B, O₂ evolution in the sta6 and sta7-10 strains was more severely attenuated than in the sta7-10[c5], sta7-10[c19], and CC124 strains during the first 24 h of nitrogen deprivation.

These O₂ evolution data, in combination with the acetate utilization results, indicate diminished anabolic activity in the starchless mutants relative to CC124, activity that is reestablished and possibly even augmented in the complemented strains. Although sta6 cultures showed the lowest levels of acetate utilization and photosynthetic activity, it should be noted again that because the average diameter of these cells is less than those of the other strains and because we standardized cultures according to cell number, less cellular volume and chlorophyll were initially present in the sta6 cultures. However, even when the acetate utilization and photosynthetic activity data are adjusted to the same starting chlorophyll, O₂ evolution and acetate uptake are still attenuated in the sta6 strain relative to the CC124, sta7-10[c5], and sta7-
As shown in Figure 3.7, chlorophyll levels in the *sta6* and *sta7-10* strains decrease faster than in the CC124, *sta7-10[c5]*, and *sta7-10[c19]* strains during culturing in TAP–N medium. The accelerated loss of chlorophyll in the starchless mutants is consistent with the more severely attenuated O\(_2\) evolution activities observed in these strains. Under nutrient-replete conditions, similar amounts of chlorophyll are attained in all strains.

**Figure 3.6.** (A) Concentrations of acetate remaining in CC124, the *sta6* and *sta7-10* mutants, and the *sta7-10:STA7* complemented *sta7-10[c5]* and *sta7-10[c19]* strains in TAP–N medium at the indicated culturing times. Cultures were inoculated at approximately 2.0 x 10\(^6\) to 2.5 x 10\(^6\) cells/ml. The initial acetate concentration in TAP and TAP–N media was 17.5 mM. Values are representative of triplicate biological samples. (B) Oxygen evolution from 0.8-ml aliquots of the indicated strains after 24 h in nitrogen-depleted TAP–N medium. Oxygen evolution is shown on the basis of cell number and chlorophyll (Chl). Cultures were inoculated at approximately 1.0 x 10\(^7\) cells/ml to produce O\(_2\) at levels sufficient for detection. Error bars indicate standard deviations.

### 3.6.6 Chlorophyll fluorescence is able to distinguish the starchless mutants from CC124

It is clear that photosynthetic and anabolic processes are significantly modulated in the starchless mutants under stress conditions. Nevertheless, the blockage of starch synthesis and the ability to channel metabolic precursors into other biosynthetic pathways, including lipid synthesis, will make these mutants very useful platforms for further metabolic engineering. The ability to assess photosynthetic performance in a high-throughput fashion should allow the screening of secondary mutant populations in the starchless backgrounds with the aim of...
isolating strains that have water oxidation capacity restored as the result of increased CO₂ fixation.

Figure 3.7. Chlorophyll content per ml of culture in a representative experiment at the specified time points for the CC124, sta6, sta7-10, sta7-10[c5], and sta7-10[c19] strains in nitrogen-replete (TAP) (A) or nitrogen-depleted (TAP–N) (B) medium. Values are representative of triplicate biological samples. Cells were precultured to late log phase and then resuspended at 2.0 x 10⁶ to 2.5 x 10⁶ cells/ml in either TAP or TAP–N medium. Error bars indicate standard deviations.

Of particular interest is generating strains that divert starch precursors to the production and secretion of targeted hydrocarbons [Atsumi et al. 2009, Radakovits et al. 2010]. To ascertain whether the starchless phenotype could be discriminated from that of wild-type cells using chlorophyll fluorescence techniques, we probed the variable fluorescence ratio Fv/Fm [Kolber et al. 1998] in the sta6, sta7-10, and CC124 strains. Measurements were recorded periodically after plating cells using the PSI FluorCam imager, which is amenable to high-sample-throughput applications. As shown for a representative experiment in Figure 3.8, when strains are plated on TAP–N agar, Fv/Fm ratios are consistently higher for the starchless mutants (sta7-10 slightly higher than sta6), whereas Fv/Fm ratios are similar on nutrient-replete TAP plates (data not shown). This increase indicates differential acclimation of the photosynthetic apparatus in the starchless mutants relative to the wild type after several days of nitrogen stress.
Figure 3.8. (A) Variable chlorophyll \( \alpha \) fluorescence (Fv/Fm) of the CC124, \( \text{sta}6 \), and \( \text{sta}7-10 \) strains plated on TAP–N agar (five replicate colonies for each strain) as measured by the PSI FluorCam imager indicated. (B) Nonvariable chlorophyll \( \alpha \) emission (Fo) of the CC124, \( \text{sta}6 \), and \( \text{sta}7-10 \) strains plated on TAP–N agar (five replicate colonies for each strain) as measured by the PSI FluorCam imager. Error bars indicate standard deviations.

3.7 Discussion

Significant remodeling of metabolic processes occurs both in the starchless mutants and, surprisingly, in the \( \text{sta}7-10:ST A7 \) complemented strains. From a holistic bioenergy perspective, it is critical that high levels of photosynthetic activity are maintained during illumination and that culturing conditions are optimized for water oxidation and CO\(_2\) reduction. Metabolic pathway engineering strategies must focus on improving the overall yields of all algal bioenergy carriers, and efforts that optimize the accumulation of a single bioenergy feedstock should not sacrifice overall fitness and energy carrier yields.

The underlying hypothesis of this study was that by genetically blocking the synthesis of starch, the dominant carbon storage product in nutrient-deprived \( \text{C. reinhardtii} \) cells, major compensatory mechanisms would result, providing new insights into the acclimation mechanisms used in starchless mutants relative to control strains during nitrogen deprivation. These insights could then be leveraged to further engineer this alga for improved bioenergy phenotypes using rational or random approaches. Moreover, because \( \text{sta}6 \) and \( \text{sta}7-10 \) represent the most severe carbohydrate mutations but block starch synthesis at different levels, it was anticipated that unique acclimation mechanisms that could be uniquely leveraged in bioenergy applications would emerge in these strains.

To investigate the acclimation mechanisms used in the starchless mutants, we focused
primarily on two potential metabolic outcomes: (i) the partitioning of carbon precursors normally used for starch synthesis to lipid biosynthetic pathways and (ii) the attenuation of cellular anabolic processes (e.g., photosynthesis and carbon storage product synthesis/accumulation). A third possible outcome, the secretion of soluble sugars, was briefly assessed, but under our experimental conditions we were unable to detect evidence for the secretion of significant quantities of soluble sugars (data not shown). However, both of the first two potential acclimation mechanisms were observed during nitrogen deprivation, when the greatest levels of starch and nonpolar lipid accumulation occur. First, increased lipid accumulation on a cellular basis was seen in both the sta6 and sta7-10 mutants relative to CC124 during nitrogen deprivation. Lipid-derived FAMEs under our experimental conditions were approximately 2- and 4-fold greater in the sta6 and sta7-10 mutants, respectively, than in CC124 on a per-cell basis. This increase was more modest on a culture volume basis (~ 1.4- to 1.8-fold, respectively), as CC124 cells continued to undergo limited cell division during the first 24 h of acclimation to nitrogen stress. It is currently unclear why CC124 cell numbers continued to increase relative to those of the cell wall-less strains used in this study, but is likely a consequence of CC124 having a fully assembled cell wall and not due to an aspect of carbohydrate metabolism, as the sta7-10[c5] and sta7-10[c19] complemented strains did not continue to divide in nitrogen-deprived medium. Attempts to cross sta7-10 into a cell wall background have been unsuccessful to date in our hands.

The second significant acclimation mechanism observed during nitrogen stress in the starchless mutants was a decrease in overall anabolic processes, reflected by decreased levels of O2 evolution activity and acetate utilization. Additional research is required to understand the regulatory mechanisms controlling these processes, which must be reversed to fully realize the potential of shifting metabolic flux to alternative pathways in the absence of starch synthesis.

Fv/Fm parameters are higher for the starchless mutants than for CC124 after several days on TAP–N agar plates. In liquid media during nitrogen stress, chlorophyll is degraded faster in the starchless mutants. The decrease in Fv/Fm under nitrogen-deplete conditions in CC124 during the first several days after plating (Figure 3.8A) is primarily a consequence of an increase in Fo, the baseline-level fluorescence not involving PSII charge separation (Figure 3.8B): Fv/Fm reaches its minimum on day 7, when Fo is at its maximum. Fo first increases, presumably due to the dissociation of PSII antenna complexes from the reaction center, then subsequently decreases
as the cells lose pigmentation under nitrogen-depleted conditions. This effect is most pronounced in CC124, while the sta6 and sta7-10 mutants show a more gradual decline in Fo. These results indicate that the starchless mutants have distinct fluorescent signatures that will be useful in future studies both examining perturbations in the photosynthetic electron transport chain that occur as a consequence of the blockage in starch synthesis and identifying mutants in the starchless backgrounds that have improved photosynthetic properties.

During the preparation of this paper, studies by Wang et al. and Li et al. characterized the effects of nitrogen depletion on the accumulation of nonpolar lipid bodies in the sta6 mutant [Wang et al. 2009, Li et al. 2010a, Li et al. 2010a]. Our results regarding the sta6 mutant are consistent with the results reported in those studies and further extend the characterization of lipid overaccumulation to the sta7-10 mutant, which under our culturing conditions accumulates even more cellular lipid than the sta6 mutant. Increased levels of lipid accumulation have also been observed in a starchless mutant of the alga Chlorella pyrenoidosa [Ramazanov and Ramazanov 2006]. The study by Li et al. used light intensities 4-fold higher than those used here during acclimation to nitrogen deprivation and reports a 10-fold increase in triacylglycerol (TAG) content relative to that in the control strain, CC1690 [Li et al. 2010a], as a function of dry weight, which is a consequence of both increased TAG synthesis and loss of carbohydrate mass. The study by Wang et al. reported an approximately 2-fold increase in lipid bodies relative to their control (a potential revertant of 330 that is no longer an arginine auxotroph [Wang et al. 2009]), which is more reflective of the increase of overall lipid in the sta6 mutant that we observed.

Complementation of sta7-10 resulted in an unanticipated phenotype displaying significant differences in cell morphology, starch synthesis, and lipid accumulation relative to those for CC124 and the sta7-10 mutant. The sta7-10[c5] and sta7-10[c19] strains accumulate levels of cellular starch in nutrient-replete medium that are similar to those achieved in nitrogen-deprived CC124 cells. The mechanistic reasons for this phenotype are outside the scope of the present study but will be pursued in future research. It is possible that the isoamylase enzyme, which is typically part of a larger protein complex [Dauvillee et al. 2001], is no longer regulated in the proper context or that enzyme levels have been perturbed, resulting in increased starch synthesis. The complemented strains also had the greatest quantities of lipid on a cellular basis, which is likely the result of larger cells with more volume for accumulation and/or additional
quantities of membrane that can be converted into FAMEs. The increased accumulation of both starch and lipid in the complemented strains is consistent with increased acetate utilization and oxygenic photosynthesis in these strains during nitrogen deprivation. It should be noted that although sta7-10:STA7 complemented strains were analyzed for H₂ production in a previous study [Posewitz et al. 2004], starch levels were assessed only visually using a qualitative iodine assay to determine whether starch accumulation was restored relative to the that in sta7-10 mutant. Starch levels in the complemented strains were not quantified as a part of the previous study, and the starch-excess phenotype was not readily observed until incorporation of the culturing and analytical methods used in this study.

The increases in starch content in the complemented strains during nutrient-replete culturing correlate with decreased growth rates but larger cell sizes. This phenotype is of particular interest as it represents a mechanism to increase the synthesis of a primary bioenergy carrier (starch) at the expense of other cellular constituents used for cell division (proteins and nucleic acids) without transferring cells to a nutrient-depleted medium.

It is important to reinforce that our cultures were standardized at relatively dilute cell density (2.5 x 10⁶ cells/ml). As a consequence of the complete arrest of cell division in the cell wall-less strains cultured in nitrogen-deprived medium, the total amount of bioenergy carriers is smaller than those reported in other studies on a culture volume basis, but those levels could be achieved if we resuspended our cultures at higher cell numbers.

Our initial experiments have been conducted in continuous light using acetate as a heterotrophic boost. Although biased toward the accumulation of bioenergy carriers, these conditions provide insights into the capacity of these strains to synthesize starch and lipids and will serve as a useful reference point for further dissecting the metabolic pathways used for carbon product synthesis and examining metabolic adjustments as a consequence of genetic manipulation. Under the current growth conditions, significant quantities of acetate (320 µg/ml in CC124 [Figure 3.6A]) are metabolized, which could account for a substantial quantity of the products observed during nitrogen deprivation.

In summary, our results indicate that from a bioenergy perspective (i) nitrogen deprivation is advantageous as it represents an effective means to organize cellular metabolites into the two principal biofuels feedstocks, starch and TAG, but a significant drawback of nitrogen deprivation is that it results in attenuated rates of photosynthesis and acetate uptake, as
overall anabolic processes are diminished; (ii) the disruption of starch synthesis is an effective means to overaccumulate lipid in the sta6 and sta7-10 starchless mutants during nitrogen deprivation, but this is tempered by decreases in overall anabolic processes (primarily starch accumulation) in comparison to the other strains; and (iii) the isoamylase complemented strains accumulate significantly higher levels of lipids and starch on a cellular basis during nitrogen deprivation, and starch overaccumulation can be achieved in the sta7-10[c5] and sta7-10[c19] strains in nutrient-replete medium while these cells are actively dividing, albeit at a lower rate than the other strains in nitrogen-replete medium.

The single-gene alterations reported here result in dramatic carbon product accumulation phenotypes. The metabolic engineering of algae for improved biofuel productivity is just beginning, and it is noteworthy that the relatively simple genetic manipulations reported here resulted in metabolic alterations that significantly affected the accumulation of starch or lipid, the two most relevant algal bioenergy carriers. As engineering approaches in C. reinhardtii and other algae become more refined, additional advances are likely.

3.8 Acknowledgments

We thank Steven Ball for generously providing his sta6 mutant previously. We acknowledge support from the Air Force Office of Scientific Research (grant FA9550-05-1-0365) (M.C.P.) and the U.S. Department of Energy BES and BER programs.
CHAPTER 4
MOLECULAR TOOLS FOR METABOLIC ENGINEERING OF THE CYANOBACTERIUM
SYNECHOCOCCUS SP. PCC 7002

4.1 Cassettes for chromosomal integration by homologous recombination

Generating novel cyanobacterial strains has been demonstrated by homologous recombination of naturally transformable Synechocystis and Synechococcus species, among others [Grigorieva and Shestakov 1982, Williams and Szalay 1983, Chauvat et al. 1983, Frigaard et al. 2004, Xu et al. 2011, Liu et al. 2011]. Facilitated by genome sequences of these fast-growing planktonic photoautotrophs, the ability to genetically modify these model organisms opens many avenues for investigation of photosynthetic metabolism.

The plasmids described are derived from the pAQ1Ex expression vector designed by Xu et al. (cited above) for homologous recombination (HR) at an uncompromising locus in the genome of Synechococcus sp PCC 7002 (Synechococcus 7002 hereafter). The pAQ1Ex plasmid integrates into the organism’s smallest endogenous extra-chromosomal plasmid, pAQ1, and confers high-level expression of a target gene (YFP, Figure 4.1) driven by the cpcBA promoter from Synechococcus sp. PCC 6803 that is highly active and involved in the promotion of several genes (Zhou et al. 1992, Swanson et al. 1992). The restriction sites at either end of each element in this plasmid make swapping genetic material very simple. The basic methods are described in Section 4.1.1.

Two new vectors for expression by HR at separate chromosomal loci were constructed using pAQ1Ex as a template: one to interrupt an endogenous gene, and the other to integrate into a noncoding, neutral region of the genome. The former is targeted to knock out the native fadD gene, a putative acyl-acyl carrier protein (ACP) synthetase, and has been used to confer secretion of lauric acid by simultaneous introduction of the heterologous fatB1 thioesterase specific to 12-carbon fatty acids (Chapters 5, 6, and 8). The latter, termed NSI for neutral site integration, recombines between two hypothetical proteins and itself does not have a metabolic effect (Section 4.3 and Chapter 8). Since their construction, these vectors have been used successfully to express several different genes and promoters in Synechococcus 7002.
Figure 4.1. The pAQ1Ex vector facilitating HR of *Synechococcus* 7002 via the pAQ1 plasmid. The lefthand elements between brown flanking regions are integrated into the genome. The righthand Ori/pMB1 (purple) and AmpR (green) elements assist with cloning through *E. coli* and are lost during HR and spectinomycin selection by SpecR/aadA (green). The vector contains the high-level PpcpBA promoter (orange) that drives a YFP reporter (red).

4.1.1 Materials and Methods

The pAQ1Ex plasmid was kindly provided by D.A. Bryant (Figure 4.1) [Xu *et al.* 2011], and related primer sequences can be found in Appendix A. To replace elements of pAQ1Ex with other genetic elements, primers were designed containing the appropriate restriction sites: one at the beginning of the forward primer, and one at the end of the reverse primer. The desired element was amplified by PCR using the Phusion high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA) and amplicons were purified using a PCR reaction cleanup kit (Qiagen, Germantown, MD). The amplicons and pAQ1Ex plasmid were independently digested with the corresponding restriction enzymes and purified either by gel extraction or enzymatic reaction cleanup kit (Qiagen). The isolated vector was recircularized with the new insert by ligation with T4 DNA Ligase (Fermentas, Pittsburgh, PA). The following vectors were rebuilt to target loci in
the main chromosome of *Synechococcus* 7002, specifically 1) across a gene targeted for knockout, and 2) at a neutral site between two genes.

4.1.2 **Vector for interruption of the putative acyl-ACP synthetase *fadD***

The *Synechococcus* 7002 gene A0675, or *fadD*, is annotated as a long-chain acyl-CoA ligase. It shares 56% amino acid identity with the *Synechocystis* 6803 gene *slr1609*, an acyl-ACP synthetase (Aas) that has been shown to facilitate metabolic integration of fatty acids from culture medium [Kaczmarzyk and Fulda 2010]. Figure 4.2 shows the pLAS plasmid modified from pAQ1Ex to contain 750-bp regions flanking A0675 to interrupt this gene while simultaneously expressing a transgenic thioesterase (*fatB1*) from *Umbellularia californica* specific to lauroyl-acyl carrier protein (ACP) that liberates 12-carbon (C12) fatty acids (FA) that then move across the cell membrane into the culture medium [Liu *et al.* 2011], as described in Chapters 5 and 6. Knocking out *fadD* was found to be essential for C12 synthesis and secretion. The Uc *fatB1* gene was kindly provided by R. Curtiss III. The integrating cassette, *cpcBA-fatB1-aadA*, is termed the lauric acid secretion (LAS) module.

4.1.3 **Neutral site integration (NSI) and other constructs**

Several new expression vectors were made as described above, and primers that were used to construct them are listed in Appendix A. The plasmid for neutral site integration (NSI) contains flanking regions that homologously recombine with the ends of two genes, A0935 and A0936, both of indeterminate function, such that the transformed cassette seats between them and does not disrupt any known coding sequence. This vector has been used to introduce transgenic terpene synthase genes into *Synechococcus* 7002 (Section 4.3) and to overexpress an endogenous malonyl-CoA:ACP transacylase (Chapter 8).

Other elements that have been inserted into the described expression vectors include an alkane biosynthesis operon (red, gene site), alternate antibiotic resistance genes (green, marker site), and a suite of different promoters (orange, promoter site) described in the following section.
Figure 4.2. Modified expression vector for knockout of *fadD* (A0675) with simultaneous expression of a desired gene driven by the *cpc* promoter (PcpcBA, orange), in this case the *fatB1* thioesterase from *U. californica* (UcfatB1, red).

4.2 **pAQ1Ex promoter library**

This study was the senior undergraduate thesis project of Erin R. Stephens at the Colorado School of Mines during 2011-2012, assisted by Victoria H. Work who provided materials and procedures. Plasmid reconstruction, transformations, and data collection and reduction were done by E. R. Stephens who also compiled this report. The results are reproduced with permission from Erin R. Stephens. The aim was to develop and characterize a collection of expression vectors for *Synechococcus* 7002 containing promoters of different types.

4.2.1 **Promoter Selection**

Promoters of *Synechococcus* 7002 genes were chosen by NCBI Basic Local Alignment Search Tool cross-references to *Synechocystis* 6803 genes reported in a project compiled by Bryant *et al.* [Bryant 2012]. The Kyoto Encyclopedia of Genes and Genomes was used for sequence data [Kanehisa 2012]. The promoters were selected from various regions of
metabolism to provide a range of promotion strengths and inducibility. The promoters are listed in Table 4.1, along with their predicted strength and role in cyanobacterial metabolism.

The promoters were identified using the prediction programs PPP [van Hijum and Zomer 2012] and Softberry BPROM [Softberry, Inc. 2012] as well as known prokaryotic promoter features: the Pribnow Box (TATA) at -10 bp from the respective gene’s start codon, the Shine-Dalgarno sequence (AGGAGG) at -35 bp, and the optional UP element [Estrem et al. 1998]. It was assumed that promoters would be relatively short sequences (<1000 bp) and occur nearby/upstream of the respective gene.

The promoters (eepF, narB, and tstR) were each reported to be inducible by nutrient stress: eepF by phosphate starvation [Ray et al. 1991], narB by nitrogen source alteration, and tstR by sulfur starvation [Laudenbach et al. 1991]. The initial experiments were designed to measure the promoter strength in regular A+ media. Future experiments will alter the levels and types of nutrients available to the cyanobacteria containing these promoter constructs to determine the effects on gene expression.

4.2 Cloning

Once the sequences for the selected promoters had been determined, primers were designed to contain an EcoRI restriction site on the 5’ end of the forward primer and NcoI restriction site on the 3’ end of the reverse primer. The promoters were amplified from wildtype *Synechococcus* 7002 using Phusion high fidelity DNA polymerase (New England Biolabs, Ipswich MA) and validated by gel electrophoresis to compare amplicon size to ensure the proper sequence had been amplified (Figure 4.3).

Figure 4.3. Agarose gel of amplified promoter DNA. Left to right: ladder, cruP, eepF, hemH ldhA, mutT, narB, prdE, rbcX, rvtR, tstR1, tstR2.
Table 4.1. Promoters identified from the *Synechococcus* 7002 genome. Several of the promoters were chosen based on reports of inducibility and others by their roles in the natural metabolism of cyanobacteria.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Gene</th>
<th>Metabolic role</th>
<th>Strength</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>cruP</td>
<td>Lycopene cyclase</td>
<td>Carotenoid biosynthesis, catalyzes the double cyclization reaction of lycopene to β-carotene</td>
<td>Medium</td>
<td>281</td>
</tr>
<tr>
<td>eepF</td>
<td>Endonuclease/Exonuclease/Phosphatase family protein</td>
<td>DNA replication and repair, suggested to be inducible by phosphate stress</td>
<td>Inducible</td>
<td>993</td>
</tr>
<tr>
<td>hemH</td>
<td>Ferrochelatase</td>
<td>Protoheme formation, dependent on porphoryn and chlorophyll metabolism</td>
<td>Low</td>
<td>277</td>
</tr>
<tr>
<td>ldhA</td>
<td>Fermentive Lactate Dehydrogenase</td>
<td>TCA cycle, converts pyruvate to lactate, consumes NADH</td>
<td>Medium</td>
<td>244</td>
</tr>
<tr>
<td>mutT</td>
<td>Mutator protein, 7,8-dihydro-8-oxoguanine triphosphatase</td>
<td>DNA proofreading, should show a peak in transcription each cell cycle</td>
<td>Low</td>
<td>189</td>
</tr>
<tr>
<td>narB</td>
<td>Nitrate reductase</td>
<td>Nitrate metabolism, expression is dependent on nitrate concentrations in the cell</td>
<td>Inducible</td>
<td>273</td>
</tr>
<tr>
<td>prdE</td>
<td>Pyruvate dehydrogenase</td>
<td>Catalyzes the decarboxylation of pyruvate to acetyl CoA, central metabolism</td>
<td>Medium</td>
<td>344</td>
</tr>
<tr>
<td>rbcX</td>
<td>Rubisco chaperone</td>
<td>Large operon of Rubisco chaperones, used in photosynthesis and carbon fixation</td>
<td>High</td>
<td>300</td>
</tr>
<tr>
<td>rvtR</td>
<td>Reverse transcriptase</td>
<td>RNA-dependent DNA polymerase, transcribes DNA from an RNA template</td>
<td>High</td>
<td>447</td>
</tr>
<tr>
<td>tstR</td>
<td>Thiosulfate sulfurtransferase</td>
<td>Sulfur metabolism (used in the detoxification of cyanide molecules, dependent on sulfur levels)</td>
<td>Inducible</td>
<td>1013</td>
</tr>
</tbody>
</table>

The PCR products were purified using a gel purification kit (Qiagen, Germantown, MD). The isolated promoter DNA was digested using EcoRI and NcoI restriction enzymes and purified using a reaction cleanup kit from Qiagen. Alongside, the pAQ1EX-Pcpc-YFP plasmid was likewise digested, separated by agarose gel electrophoresis, and the larger band purified.

The purified, restriction-digested promoter DNA and the restriction-digested promoterless pAQ1Ex-YFP backbone DNA were ligated using T4 DNA ligase (Fermentas). The ligated promoter-backbone reactions were used to transform *E. coli* competent cells by electroporation and chemical transformation. The transformed bacteria were plated on solid LB medium containing 50 µg/mL each of ampicillin and spectinomycin and screened for antibiotic resistance. The cultures showing resistance were picked and grown overnight in 5 mL liquid LB media, whereupon a Plasmid Miniprep Kit from Qiagen was used to isolate the plasmids. Verification of promoter incorporation into the plasmids was done using restriction digests, PCR, and sequencing. Once the promoters were confirmed to have been successfully cloned into the
pAQ1Ex-YFP plasmid, the constructs were purified using the Plasmid Maxi Prep Kit (Omega Bio-Tek, Norcross, GA) from 200-mL LB cultures of transformed *E. coli* to obtain significant amounts of plasmid DNA.

4.2.3 Genetic transformation

Wildtype *Synechococcus 7002* was transformed with each of the newly constructed pAQ1Ex-[promoter]-YFP expression vectors, which also contained the spectinomycin resistance gene *aadA*. The transformants were cultured with 2-3 µg of respective plasmid DNA at 37°C and 1% CO₂ for six hours and then plated on 50 µg/mL spectinomycin A+ plates. Colonies were transferred to fresh selective plates and the selected strains were cultivated with antibiotic pressure throughout the course of experimentation.

4.2.4 Results

Of the ten promoters initially chosen for cloning, seven were successfully cloned ahead of the YFP gene on the pAQ1 directed construct: eepF, hemH, ldhA, narB, prdE, rbcX, and rvtR. The seven successful constructs were also successfully transformed into the pAQ1 plasmid of wildtype *Synechococcus 7002*.

YFP fluorescence of the cells was verified using a light microscope. The transformed cultures were grown in A+ media to mid-log phase for microscopy. Measurements were taken on a Nikon Eclipse 80i, using a Nikon Intense Light C-HGFI as a light source with a GFP filter cube. The transformed cells were observed to fluoresce, examples of which are shown in Figure 4.4.

![Figure 4.4. YFP fluorescence (40X magnification) of transformants containing the promoter-YFP-SpecpAQ1-directed constructs. A) rbcX, B) hemH, C) ldhA.](image)
The qualitative measurements of YFP expression by microscopy was followed with quantitative measurement of expression using a Nanolog Horiba Jobin Yvon Fluorimeter in vivo. Liquid cultures of the transformants were standardized to OD = 1. Fluorescence in the YFP range was measured using an excitation wavelength of 510 nm (Figures 4.5 and 4.6). The emission and excitation wavelengths of YFP are narrowly grouped, so emission was measured from 520 nm to 550 nm.

Promoter strength reported by fluorometry (Figure 4.5) shows that rbcX promoter produced the highest levels of YFP expression. Although ldhA and prdE are central to metabolism, the promoters resulted in relatively low expressions of YFP, which was corroborated by slower growth in antibiotic supplemented media. The gene driven by rvtR has two annotations and, based on its relatively high levels of promotion, more likely encodes the DNA polymerase than the reverse transcriptase. The inducible promoters, eepF and narB, show low levels of promotion, indicating a high potential for optimizable, induced expression. Figure 4.6 shows YFP fluorescence under the control of selected promoters relative to the cpcBA promoter, a high-level phycocyanin promoter from Synechocystis sp. PCC 6803, which expresses the gene over an order of magnitude more than the native promoters.

Figure 4.5. YFP fluorescence by promoter in Synechococcus 7002 pAQ1Ex transformants. Experimental values were corrected by subtracting background values of wildtype and blank media curves.
4.2.5 Section Conclusions

The development of a promoter library for the optimization of gene expression in *Synechococcus* 7002 was successful. Promoters of different strengths were characterized using YFP expression as a reporter. New genes and target loci can be cloned into plasmids with a desired promoter for further genetic engineering and characterization of cyanobacterial physiology, photosynthetic metabolism, and the pursuit of renewable energy. The range of promoter strengths and sensitivities affords the capability of fine-tuning enzyme levels and modulating expression by physiology. These are powerful multifaceted tools that could be used to elicit a desired reaction from the organism under conditions that are not naturally conducive to it. For example, stress responses in PSMs often cause preferential synthesis of certain valuable molecules such as lipids and polyglucans, but it may not be economical to continuously stress a feedstock culture. With an inducible promoter, a nutrient trigger can be introduced that causes similar rearrangements without the global effects of nutrient deprivation.
4.3 Manipulation of carbon partitioning in limonene- and bisabolene-producing strains of *Synechococcus* sp. PCC 7002

In a recent publication by Davies *et al.*, the NSI cassette described in Section 4.1.3 was used as a transformation vector to introduce heterologous terpene synthase genes into *Synechococcus* sp. PCC 7002 [Davies *et al.* 2014]. The study describes synthesis of terpenes by *Synechococcus* 7002 conferred via the neutral-locus chromosomal integration module. Exerpts are reproduced with permission from Fiona K. Davies.

4.3.1 Authors and Affiliations

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2Civil and Environmental Engineering Division, Colorado School of Mines, Golden, CO, USA.
3Systems Biology Group, Pacific Northwest National Laboratory, Richland, WA, USA. 

4.3.2 Authors’ Contributions

F.K. Bentley transformed the TPS strains, performed batch cultivations and biochemical assays, designed the schematic artwork, and compiled the manuscript. V.H. Work designed and constructed the vector for neutral chromosomal integration and facilitated the procurement of the bisabolene synthase. A.S. Beliaev and M.C. Posewitz oversaw, edited, and funded the preparation.

4.3.3 Abstract

Limonene (C₁₀H₁₆) and α-bisabolene (C₁₅H₂₄) plant terpenoids are hydrocarbon precursors to a vast range of industrially-relevant chemicals. High-titer microbial synthesis of limonene and α-bisabolene feedstock would pave the way for advances in *in vivo* engineering of tailor-made hydrocarbons, and production at commercial scale. We have engineered the fast-growing marine cyanobacterium *Synechococcus* sp. PCC 7002 to produce yields of 4 mg/L limonene and 0.6 mg/L α-bisabolene through heterologous expression of *Mentha spicata* L-limonene synthase and *Abies grandis* (E)-α-bisabolene synthase genes, respectively. Titers were significantly higher when a 5% dodecane overlay was applied during culturing, suggesting that dodecane may trap large quantities of volatile limonene and α-bisabolene that would otherwise be lost to evaporation, and/or dodecane continuously milked the products from the cell,
alleviating negative feedback inhibition, to promote higher rates of synthesis. We describe attempts to rewire cyanobacterial carbon-sink partitioning to increase metabolic flux though the terpenoid pathway for higher yields of limonene and α-bisabolene. The simultaneous blocking of glycogen and protein biosynthesis (through inactivation of the glgC ADP-glucose pyrophosphorylase and deprivation of a nitrogen supply) resulted in the secretion of a suite of organic acid overflow metabolites, which were apparently not directed toward terpenoid metabolism, but provide a suitable platform for downstream terpenoid pathway engineering efforts.

4.4 Outlook

The techniques and materials described in this chapter have been used to construct many novel strains of the cyanobacterium Synechococcus sp. PCC 7002. These variants are studied to understand their growth and molecular phenotypes, supplementing a greater knowledge of the physiological behaviors, metabolic functions, and acclimation mechanisms employed by the cyanobacterium when modified and cultivated for bioproducts. The following chapters characterize growth and molecular profiles of engineered strains with reference to the secretion of lauric acid and organic acids.
CHAPTER 5
PHYSIOLOGICAL SURVEYS OF SYNECHOCOCUS SP. PCC 7002

5.1 Batch cultivation

Growing photosynthetic microorganisms is accomplished in myriad ways. If a quantity of live cells is placed in finite growth medium and cultivated in a viable manner until (or before) the medium is depleted of necessary nutrients, this is referred to as a batch culture and often follows an S-like growth-curve of which Figure 5.1 is an example. This technique was employed to characterize the behavior of strains of the cyanobacterium Synechococcus sp. PCC 7002 under select conditions and to establish a standard procedure for repeatable physiology. Cultivation of Synechococcus 7002 strains was done at 34-38 °C in rotated aseptic flasks under constant illumination in an atmosphere of 1% CO₂ in incubation chambers. A reference for the strains is provided in Table 5.1. Different medium types, inoculum densities, and light schemes are discussed. Similar studies have been undertaken previously [Liu et al. 2011, Ruffing and Jones 2012, Ruffing 2014] based on seminal work by Voelker and Davies [Voelker and Davies 1992, Voelker and Davies 1994].


<table>
<thead>
<tr>
<th>Genotype</th>
<th>Description</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔfadD::Pcpc-YFP-aadA</td>
<td>wildtype Synechococcus sp. PCC 7002</td>
<td>wt, wt 1, wt 2, 7002</td>
</tr>
<tr>
<td>ΔfadD::Pcpc-fatB1-aadA a</td>
<td>YFP-expressing Aas ko</td>
<td>ΔfadD</td>
</tr>
<tr>
<td>ΔglgC::aphII</td>
<td>C12:0-secreting Aas ko</td>
<td>las1, SA01</td>
</tr>
<tr>
<td>ΔglgC::aphII, ΔfadD::Pcpc-fatB1-aadA</td>
<td>RC-deficient AGPase ko</td>
<td>ΔglgC</td>
</tr>
<tr>
<td></td>
<td>RC-deficient, C12:0-secreting AGPase ko</td>
<td>gls3, SA13</td>
</tr>
</tbody>
</table>

a LAS module (lauric acid secretion)
Figure 5.1. Growth phases of a wildtype culture of *Synechococcus* 7002 that has not been previously conditioned to liquid medium. Lag: ~0-30 h, log or exponential: ~30-90 h, late log: ~90-110, and entry into stationary: ~120+ h. Conditions of 38 °C, 250 μE/m²s PAR, and 1% CO₂ were used.

Free fatty acids secreted by modified strains of *Synechococcus* 7002 during nutrient replete or nitrogen deprived cultivation are shown in Figure 5.2. Phenotypic evidence for a secreted detergent-forming product is shown in Figure 5.3, in which a layer of bubbles appears atop aqueous cultures of the modified strains, but not wildtype. The control mutant (ΔfadD) secretes a small amount of free fatty acids, which translates to fewer bubbles than those expressing the C12 thioesterase (las1 and las3). No FFA secretion was observed during nitrogen deprivation.

Figure 5.2. Free fatty acids (FFA) in culture medium during nutrient replete (A+) and nitrogen deprived (-N) cultivation (BioVision FFA kit). Figure provided by Travis Saari.
5.1.2 Effects of inoculum density and nitrogen deprivation on lauric acid-secreting, carbohydrate-deficient, and doubly modified Synechococcus sp. PCC 7002

This section describes batch cultures of wildtype (wt), carbohydrate-deficient (∆glgC), and lauric acid-secreting (LAS-modified) mutants of the two backgrounds (lasI and gls3, respectively) (Table 5.1). The section is divided by the two sets of conditions that were employed for cultivation. Materials and methods are described in Chapter 6, Section 6.5. Reducing carbohydrates (RC) are reported as glucose equivalents, and 12- to 18-carbon fatty acyl chains (FA) are reported as fatty acid methyl esters (FAME). In certain cases, organic acids (OA, Figure 6.7) and proteins (Figure 5.23) were quantified. This was done directly by HPLC or BCA assay kit (Pierce Biotechnology, Rockford IL), respectively.

5.1.2.1 High temperature, high light (HT/HL): 36 °C, 250 µE/m²s, 1% CO₂

The cultivations, sampling, assays, analysis, and compilation presented in this section were performed by Victoria H. Work. A Percival I-30BLL (Percival Scientific, Perry IA) controllable incubation chamber fit with 24-Watt 3500K and 6500K T5 fluorescent lights and an orbital shaker was used for these experiments. Cultures were conditioned for two generations and experimental replicates were grown at 36 °C in 150-mL flasks under constant 250 µE/m²s illumination in an atmosphere containing 1% CO₂. The next section (Section 5.1.2.2) describes similar experiments under lower light and temperature. The conditions in the present section are abbreviated HT/HL and those in the next section LT/LL.
The figures below represent liquid batch cultures sampled every 24 h for 72 h. The dry mass of a cyanobacterial culture and its chlorophyll \( a \) content are used as indicators of cell growth and viability. Both were measured for three treatments: nutrient replete and low inoculum density (Figure 5.4), nutrient replete and high inoculum density (Figure 5.5), and nitrogen deplete with a high-density inoculum (Figure 5.6).

Figure 5.4. Content of chlorophyll \( a \) (A) and dry cell weight (B) in batch cultures of wt, \( las1 \), \( \Delta glgC \), and \( gls3 \) over 72 h starting at low inoculum density.

Figure 5.5. Content of chlorophyll \( a \) (A) and dry cell weight (B) in batch cultures of wt, \( las1 \), \( \Delta glgC \), and \( gls3 \) over 72 h starting at high inoculum density.

Figure 5.6. Content of chlorophyll \( a \) (A) and dry cell weight (B) in batch cultures of wt, \( las1 \), \( \Delta glgC \), and \( gls3 \) over 72 h in nitrogen deplete medium.
Conclusions from the previous section are reinforced here, though the present set of treatments was only run for 72 h and the chlorophyll \( a \) content plateau for these cultures is not known. Under nitrogen deplete conditions, no increase in chlorophyll \( a \) was observed in any strain over the time course (Figure 5.6A). No dry mass was gained in cultures of the AGPase-disrupted background over 72 h but the wildtype background showed a 1.5-fold increase in dry cell weight (Figure 5.6B), possibly from the accumulation of reducing carbohydrates (RC).

The content of RC in cultures for two of the above treatments appear in Figure 5.7. These values are normalized by chlorophyll \( a \) (Figure 5.8) and by dry cell weight (Figure 5.9) to offer further insight. The lefthand graphs represent the high-density nutrient replete treatment and the righthand graphs illustrate the nitrogen deplete set. With abundant nutrients, the amount of RC increases with growth until a sharp increase between 48 h and 72 h. This could suggest entry into nutrient-limited stationary phase resulting in carbohydrate storage. Accumulation of RC in wildtype during nitrogen deprivation on a dry weight basis is more accurately depicted in Figure 5.15, in which RC is accumulated to 33% of dry cell weight and the AGPase-disrupted control achieves only 6%. Also shown in the next section, organic acids secreted from the carbohydrate-deficient background during nitrogen deprivation make up for a significant portion of dry cell weight lost by the AGPase disruption but a detriment to the proportion of RC in relation to dry cell weight remains (Figure 5.15).

For the above high-density and nitrogen-deplete treatments, an examination of the FA content measured as fatty acid methyl esters (FAME) appears in Figures 5.10, 5.11, and 5.12. It is possible that metabolic reallocation is observable, as the carbohydrate-deficient background exhibits increased FA content on the basis of chlorophyll \( a \) content and dry cell weight, particularly in the nitrogen deplete group. Remarkably, even during nutrient replete cultivation, the AGPase-disrupted background exhibited higher FA content than the wildtype background on all accounts at 72 h. Lauric acid generated by LAS-modified strains during high-density cultivation is shown in Figure 5.17A.

5.1.2.2 **Low temperature, low light (LT/LL): 34 °C, 160 µE/m²s, 1% CO₂**

The cultivations, sampling, assays, analysis, and compilation in this section were performed by Victoria H. Work, assisted by Fiona K. Davies in RC quantification and by Sarah D’Adamo for OA quantification. An ATR Multitron (ATR Inc., Laurel MD) incubation chamber
Figure 5.7. Reducing carbohydrates (RC) in batch cultures of wt, las1, ΔglgC, and gls3 over 72 h. A: high inoculum density, nutrient replete. B: nitrogen deplete.

Figure 5.8. RC normalized to chlorophyll \( a \) in batch cultures of wt, las1, ΔglgC, and gls3 over 72 h. A: high inoculum density, nutrient replete. B: nitrogen deplete.

Figure 5.9. RC normalized to dry cell weight (dcw) in batch cultures of wt, las1, ΔglgC, and gls3 over 72 h. A: high inoculum density, nutrient replete. B: nitrogen deplete.
Figure 5.10. Fatty acids (FA) reported as FAME in batch cultures of wt, las1, ΔglgC, and gls3 over 72 h. A: high inoculum density, nutrient replete. B: nitrogen deplete.

Figure 5.11. FA normalized to chlorophyll a in batch cultures of wt, las1, ΔglgC, and gls3 over 72 h. A: high inoculum density, nutrient replete. B: nitrogen deplete.

Figure 5.12. FA normalized to dry cell weight (dcw) in batch cultures of wt, las1, ΔglgC, and gls3 over 72 h. A: high inoculum density, nutrient replete. B: nitrogen deplete.
fit with 15-Watt 4200K T8 fluorescent lights and an orbital shaker was used for batch
cultivation. The cultures were grown in 250-mL flasks under constant illumination from the
fluorescent lights at 160 µE/m²s, a temperature of 34 °C, and an atmosphere containing 1% CO₂.
The conditions in this section are abbreviated LT/LL. This section contains supplementary
material to Chapter 6.

As shown in Figures 5.13 and 5.14, growth profiles are severely affected by nitrogen
starvation during which both the wildtype and carbohydrate-deficient backgrounds maintain
inoculum levels of chlorophyll $a$ and dry cell weight. In nutrient replete medium the LAS-
modified single mutant contains less chlorophyll $a$ at 48 h and an impairment to dry cell weight
of the AGPase-disrupted control is evident after 72 h.

Figure 5.13. Chlorophyll $a$ content over 72 h in batch cultures of wt, $las1$, $ΔglgC$, and $gls3$ under
LT/LL conditions in nutrient replete (A) or nitrogen deplete (B) medium.

Figure 5.14. Dry cell weight over 72 h of wt, $las1$, $ΔglgC$, and $gls3$ batch cultures under LT/LL
conditions in nutrient replete (A) or nitrogen deplete (B) medium.
Reducing carbohydrates (RC) and organic acids (OA) in cultures of the four strains during nitrogen deprivation are shown in Figure 5.15. The top panel is RC content normalized to average dry cell weight and the bottom shows raw RC content. On a culture volume basis, RC content was severely limited in strains of the AGPase-disrupted background which accumulated 10-fold less RC than wildtype backgrounds over the first 48 h. A rapid increase in RC in the wildtype background was observed over the first 24 h of nitrogen deprivation, which maintained over the next 24 h and appears to diminish by 72 h. The carbohydrate-deficient strains secreted organic acids, which improved the dry cell weight percent of carbohydrate but did not fully make up for RC loss. The changes at 72 h could be the strains utilizing energy stores or passing away.

![Graph A](image1)

**Figure 5.15.** Profiles of reducing carbohydrates (RC) and secreted organic acids (OA) for wt, las1, ΔglgC, and gls3 batch cultures over 72 h in nitrogen deplete medium. A: RC normalized to dry cell weight (dcw). B: RC content by culture volume.
Quantification of FAs was done at the 48-h time point and can be found along with the chain length profile in Chapter 6, Figures 6.4 and 6.5 and Table 6.2. The ratio of total FA to chlorophyll \( a \) is remarkably similar between all strains at this time point (Figure 5.16). A comparison of the lauric acid present in cultures grown under the HT/HL and LT/LL conditions described above appears in Figure 5.17. The amount of secreted lauric acid is not available for the first set (HT/HL), and it is evident that the second set (LT/LL) conferred a minor advantage to overall C12 content.

Figure 5.16. Ratio of FA (represented by FAME) to chlorophyll \( a \) in cultures of wt, las1, \( \Delta \) glgC, and gls3 at 48 h of nutrient replete high-density batch cultivation.

Figure 5.17. Transesterifiable lauric acyl (C12) content of cultures from two separate experiments. A: HT/HL, high-density nutrient replete batch cultures. B: LT/LL, low density nutrient replete. s: secreted
5.2 Steady-state cultivation

This section contains supplementary material for Chapter 6, which also contains further details of the growth system. The characterizations presented in this section were facilitated by a photobioreactor (PBR) cultivation system [Melnicki et al. 2013] operated by Eric A. Hill of the Systems Biology group at the Pacific Northwest National Laboratory. The PBR can be operated as a turbidostat that holds the culture at a set optical density or as a chemostat that holds the culture at a set dilution rate. Aside from Figure 5.25, all results represent turbidostatic operation for the investigation of physiology when the culture’s maximum stable doubling rate was achieved at a set light intensity. The range of illumination values and their designations is provided in Table 5.2. The PBR was run as a chemostat at the lowest non-fatal nitrogen concentration (determined previously by E.A. Hill) for which Figure 5.25 illustrates the reducing carbohydrate content. FAME quantitation was assisted by Karl Weiss.

Physiologies of wildtype and the las1 and Δg/gC mutants of Synechococcus 7002 were investigated once cultures had reached a steady growth rate at each light intensity. A previous wildtype experiment (wt 1) is plotted with the present study to show reproducibility of the method. The biomass profiles in Section 5.2.2 describe wt 2, which is the wildtype strain discussed in Chapters 4-6.

Table 5.2. Reference for light intensity designations. All units are µE/m²s.

<table>
<thead>
<tr>
<th>630 nm</th>
<th>680 nm</th>
<th>Total, linear</th>
<th>Total, spherical</th>
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</thead>
<tbody>
<tr>
<td>5</td>
<td>5</td>
<td>10</td>
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<td>462</td>
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<td>610.5</td>
</tr>
<tr>
<td>170</td>
<td>60</td>
<td>230</td>
<td>759</td>
</tr>
</tbody>
</table>
5.2.1 Doubling rate and $O_2$ evolution

The conversion to doubling time (h) is $\ln(2)/$dilution rate. The fastest doubling times observed over the 30 °C light course were 3.5 h for wt, 3.8 h for $las1$, and 4.6 h for $\Delta glgC$ (Figure 5.18). Improvements were achieved under select conditions, and maximum sustainable doubling times observed at 36 °C were 1.9 h for wt (Eric Hill, personal communication), 2.2 h for $las1$, and 2.1 h for $\Delta glgC$. The lauric acid-secreting strain generated more $O_2$ despite lower doubling rates and, curiously, $O_2$ generated by the carbohydrate-deficient strain continued to increase even after growth rate had saturated (Figure 5.19).

![Graph showing dilution rate vs. incident light]

Figure 5.18. Dilution rate (growth rate proxy) of turbidostatic wt, $las1$, and $\Delta glgC$ cultures over the PBR light course at 30 °C and two 230 µE/m²s values at 36 °C. Error bars are vanishingly small. Figure provided by EAH.

5.2.2 Biomass profiles

Parameters for growth and productivity were measured over the 30 °C light course including dry cell weight and the content of reducing carbohydrates (RC), proteins, and fatty acids (FAs). It appears that dry cell weight is dynamic (Figure 5.20) and that protein level is the most constant parameter maintained by the turbidostat (Figure 5.23). The increase in dry cell weight with increasing light intensity possibly reflects RC accumulation in the wildtype background (Figure 5.23) that could be a protective or stress-type response. Biomass profiles are
shown in Figure 5.23 followed by a side-by-side comparison of mass balance between the strains at 462 µE/m²·s (Figure 5.24).

Figure 5.19. Dissolved O₂ generated by turbidostatic wt, las1, and ΔglgC cultures over the PBR light course at 30 ºC. Error bars are vanishingly small. Figure provided by EAH.

Figure 5.20. Dry cell weight over the PBR light course for wt, las1, and ΔglgC cultures.
Cultures are maintained at a dilute concentration in the PBR for complete light perfusion and only trace amounts of FA were detected for which estimated concentrations at the 396 µE/m²s light level are shown in Figure 5.21. Whole-culture (total, T) and cell-free culture medium (filtrate, F) samples were derivatized and FAME detected by GC-MS. Only 16- and 12-carbon acyls were observable and it appears that las1 experiences a detriment to C16 that C12 does not make up for. When normalized to doubling rate, both las1 and ΔglgC show compromised FAME yield relative to wt on a unit time basis due to lower growth rates but similar levels of FAME on a per cell basis, ΔglgC containing slightly more than wt (Figure 5.22) which supports observations from batch experiments.

![Figure 5.21](image1)

Figure 5.21. Estimated quantity of FA as reported by FAME at 396 µE/m²s (60 µE/m²s 630 nm and 680 nm each). T: total cell culture, F: cell-free culture medium (filtrate).

![Figure 5.22](image2)

Figure 5.22. Estimated FAME quantity in untreated wt, las1, and ΔglgC PBR cultures at 396 µE/m²’s normalized with doubling rate. Primary y-axis: FAME divided by h/doubling, secondary y-axis: total FAME divided by doublings/h.
Figure 5.23. Reducing carbohydrates (RC), protein, and dry cell weight (DCW) for PBR cultures of wt, lasI, and ΔglgC. The protein value for lasI at 165 µE/m²/s is an estimate and dry cell weight for lasI and ΔglgC at 759 µE/m²/s are not available.
Cultures of \textit{las1} and \textit{ΔglgC} were grown chemostatically in nitrogen-limited medium containing 0.9 mM NH$_4$Cl using a protocol established previously by E.A. Hill and M.R. Melnicki. This technique provides only enough nitrogen (N) to prevent fatal starvation, suspending cellular productivity at this level. During N limitation the wildtype background (\textit{las1}) accumulates about 10-fold more RC than the AGPase-disrupted background (Figure 5.25), corroborating observations from batch cultivation.

![Graph: Analyte (mg/L)](image1)

**Figure 5.24.** Dry cell weight (DCW) and partitioning of reducing carbohydrates (RC) and protein in PBR cultures of wt, \textit{las1}, and \textit{ΔglgC} at the 496 $\mu$E/m$^2$s light point (70 $\mu$E/m$^2$s 630 nm and 680 nm each).

![Graph: RC (mg/L glucose eq)](image2)

**Figure 5.25.** Reducing carbohydrates (RC) present in PBR cultures during nitrogen limited chemostatic cultivation of \textit{las1} and \textit{ΔglgC}. No information is available for wt.
5.2.3 Temperature and pH optima

The PBR facilitated the determination of optimal temperature and pH for the two Synechococcus 7002 mutants las1 and ΔglgC. Two representations of the growth profiles during cultivation at different temperatures are shown, Figure 5.26 by change in dilution rate and Figure 5.27 by doubling time. The same investigation was done at different pH points, for which Figure 5.28 gives the change in dilution rate compared to starting rate. For both strains, 36 °C and pH 8 were found to be conditions optimal for growth rate.

![Graph showing change in dilution rate as % of 30°C growth rate for las1 and ΔglgC strains of Synechococcus sp. PCC 7002 at different temperatures.](image)

Figure 5.26. Turbidostatic growth rates represented by change in dilution rate of las1 and ΔglgC strains of Synechococcus sp. PCC 7002 at different temperatures. Error bars are vanishingly small. Figure provided by EAH.

5.3 Chapter conclusions

The characterizations presented in this chapter illustrate that the described genetic variants of the cyanobacterium Synechococcus sp. PCC 7002 are amenable to cultivation and their altered metabolic activity can be quantified. In batch culture, bioproducts were observed to be secreted from engineered cells; and a turbidostatic PBR, a powerful tool for investigating empirical physiology, was used to understand the dynamic range of metabolic adjustments to lauric acid secretion and carbohydrate deficiency in this PSM.

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Figure 5.27. Turbidostatic growth rates represented by doubling time of las1 and ΔglgC strains of *Synechococcus* sp. PCC 7002 at different temperatures. Error bars are vanishingly small. Figure provided by EAH.

Figure 5.28. Change in turbidostatic growth rates (by dilution rate) of las1 and ΔglgC strains of *Synechococcus* 7002 over a pH range. Error bars are vanishingly small. Figure provided by EAH.
6.1 Authors and Affiliations

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6.2 Authors’ contributions

The LAS module was constructed by V. H. Work, who also transformed and developed the LAS strains, designed batch experiments, performed batch cultivations and a majority of the biochemical assays, and compiled this manuscript. M. R. Melnicki performed PAM fluorometry measurements and contributed to PBR research design. F. K. Davies identified and helped quantify organic acids, and assisted with quantitation of reducing carbohydrates. E. A. Hill designed, built, and operated the LED-photobioreactor to generate \( \text{O}_2 \) and dilution rate measurements, and assisted with dry weights. L. A. Kucek contributed to PAM fluorometry data collection. S. Zhang (acknowledged) transformed the \( \Delta glgC \) mutant used in this study. A. S. Beliaev and M. C. Posewitz were responsible for the project’s conception and provided laboratory resources. All authors revised the manuscript for intellectual content.

6.3 Abstract

The cyanobacterium \textit{Synechococcus} sp. PCC 7002 was genetically engineered to secrete biofuel compatible medium-chain fatty acids during photoautotrophic growth. By introducing a lauroyl-acyl carrier protein (C12:0-ACP) thioesterase to interrupt an endogenous putative acyl-ACP synthetase, secretion of 3-4 mg/L•d transesterifiable C12 was achieved in CO\textsubscript{2} supplemented batch cultures. Grown at steady-state over a range of light intensities in an LED photobioreactor operated as a turbidostat, the secreting mutant exhibited a modest reduction in growth rate and increased \( \text{O}_2 \) evolution relative to wildtype. Inhibition of i) glycogen synthesis by deletion of ADP-glucose pyrophosphorylase (AGPase, encoded by \textit{glgC}) and ii) protein synthesis by nitrogen deprivation were investigated as strategies for metabolite redistribution to
fatty acid synthesis. When the modification was introduced into a carbohydrate-deficient (glgC- or AGPase-disrupted) background, the resulting strain secreted comparable amounts of C12 during nutrient replete growth and no C12 was generated in either background when nitrogen starved. The AGPase-disrupted strains accumulated reducing carbohydrates to 7-13% of wildtype levels during nitrogen deprivation and secreted several types of organic acids consistent with an energy spilling phenotype. At steady-state, the ΔglgC modification caused growth rate saturation at a lower light intensity than the wildtype background, but surprisingly, O2 evolution was not compromised on a cellular basis and continued to increase with irradiance. Photophysiological properties of the ΔglgC mutant suggest energy dissipation from photosystem II and redox effects to the plastoquinone pool. Sustained oxygenic electron flux may imply a capturable movement of energy.

6.4 Introduction


In photosynthetic eukaryotes, after fatty acid (FA) chains have elongated to the appropriate length, thioesterase enzymes hydrolyze FAs from acyl carrier protein (ACP) to facilitate derivatization with a target head group and the free fatty acids (FFA) move out of the
chloroplast into the cytoplasm where they are activated by CoA for modification [Radakovits et al. 2010, Elliott et al. 2012, Li et al. 2013]. However, most bacteria including cyanobacteria bypass the FFA intermediate when assembling newly synthesized FA into metabolically integrated lipids [Sato and Wada 2010, Jansson 2012]. When certain bacteria are conferred with non-native thioesterases, most of the hydrolyzed FFA is found either in the culture medium or associated with the outside of the cell [Voelker and Davies 1994, Liu et al. 2011, Zhang et al. 2011, Ruffing and Jones 2012, Ruffing 2014]. Though the mechanism of secretion is not understood, it is known that extracellular FFA can move back across the membrane and be reincorporated into metabolism by an acyl-acyl carrier protein synthetase (Aas) [Kaczmarzyk and Fulda 2010]. If this enzyme is disrupted, FFAs remain in the medium unaffected and under certain conditions can separate to the top of aqueous cultures. The present study describes physiologies of the cyanobacterium Synechococcus sp. PCC 7002 engineered with modifications to carbon distribution, specifically medium-chain (C12:0) fatty acid secretion from both wildtype and carbohydrate-deficient genetic backgrounds.

6.5 Materials and Methods

This section describes experimental protocols for this study.

6.5.1 Genetic engineering of Synechococcus sp. PCC 7002

Transformation and vector design protocols were adapted from methods and materials described previously [Frigaard et al. 2004, Xu et al. 2011]. The ΔglgC mutant [Guerra et al. 2013] and pAQ1Ex plasmid were kindly provided from the laboratory of Donald A. Bryant. In Synechococcus sp. PCC 7002 (Synechococcus sp. 7002 hereafter), the glgC gene (A0095) encodes ADP-glucose pyrophosphorylase (AGPase), which activates glucose for polymerization. The gene fadD (A0675) encodes a putative acyl-ACP synthetase (Aas) with homology to the Synechocystis sp. PCC 6803 gene slr1609 [Kaczmarzyk and Fulda 2010, Gao et al. 2012]. The thioesterase that hydrolyzes 12-carbon chains from acyl carrier protein (ACP) during fatty acid (FA) synthesis is encoded by fatB1 from Umbellularia californica codon optimized for Synechocystis sp. 6803 [Liu et al. 2011] and was generously provided from the laboratory of Roy Curtiss III. The pAQ1Ex expression vector was modified to target flanking regions of the fadD gene for knockin of a cassette containing the cpcBA promoter from Synechocystis sp. 6803, the
fatB1 thioesterase, and a resistance gene for spectinomycin selection. The strain SA01 contains this lauric acid secretion (LAS) module in a wildtype background, and the strain SA13 contains the module in a carbohydrate-deficient background (Table 6.1). Homoplastic integration was achieved in transformants by successive cultivation on plates with increasing antibiotic concentrations and was confirmed by PCR for allele segregation (not shown) and long-term stable phenotype without antibiotic pressure.

Table 6.1. *Synechococcus* sp. 7002 strains used in this study. Antibiotic markers confer resistance to spectinomycin (*aadA*) or kanamycin (*aphII*). The *cpcBA* promoter drives *fatB1* expression. RC: reducing carbohydrate, glucose equivalent.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>wildtype <em>Synechococcus</em> sp. 7002</td>
<td></td>
</tr>
<tr>
<td>SA01</td>
<td>Secretes lauric acid (C12:0 FA)</td>
<td>∆fadD::Pcpc-fatB1-aadA a</td>
</tr>
<tr>
<td>∆glgC</td>
<td>AGPase disrupted (RC-deficient)</td>
<td>∆glgC::aphII</td>
</tr>
<tr>
<td>SA13</td>
<td>RC-deficient, secretes C12:0 FA</td>
<td>∆glgC::aphII, ∆fadD::Pcpc-fatB1-aadA</td>
</tr>
</tbody>
</table>

a LAS module

6.5.2 Batch cultivation

The saltwater medium A+ used in batch experiments contains, per L, 18 g NaCl, 5 g MgSO₄•7H₂O, 1 g NaNO₃, 0.6 g KCl, 0.05 g KH₂PO₄, 0.03 Na₂-EDTA, 0.27 g CaCl₂, 1 g Trizma base (Tris), 1 mL/L of a 3.89 g/L FeCl₃•6H₂O stock in 0.1 N HCl, and 1 mL/L of a P1 metals micronutrient solution. The P1 stock solution contains, per liter, 34.26 g H₃BO₃, 4.32 g MnCl₂•4H₂O, 0.315 g ZnCl, 0.03 g MoO₃ (85%), 12.15 mg CoCl₂•6H₂O, and 3 mg CuSO₄•5H₂O. For A+ medium without nitrogen (-N), NaNO₃ was replaced by an equimolar amount of NaCl. The sole carbon source was CO₂/HCO₃⁻ by atmospheric CO₂ supplementation.

Cells were grown on a rotary shaker under constant illumination in an atmosphere of 1% CO₂ in 250-mL Erlenmeyer flasks with soft caps to facilitate gas exchange (VWR, Radnor PA). Cultures were standardized to 2.5 mg/L chlorophyll a at the beginning of each experiment and grown at 34°C and 160 µmol photons/m²/s (µE/m²/s) photosynthetically active radiation (PAR). Precultures were similarly normalized and grown to mid-log phase (15-25 µg/mL chlorophyll a), whereupon cells were concentrated by centrifugation and resuspended in fresh medium for experimental replicates. Samples were taken every 24 h over a time course.
6.5.3 **Turbidostat cultivation**

Steady-state physiology was assayed in an LED photobioreactor (PBR) that maintains constant optical density (turbidostasis) over a range of 630- and 680-nm light intensities, as described previously [Melnicki et al. 2013]. Cells were cultivated in A+ medium containing 0.9 g/L NH₄Cl as the nitrogen source, and Tris was omitted as pH was maintained independently. Doubling times by ln(2)/dilution rate, O₂ evolution by percent air saturation, photophysiology by PAM fluorometry, and biochemical composition were measured in wildtype (wt), SA01, and ΔglgC strains at light intensities of 5/5 (33), 10/10 (66), 15/15 (99), 20/20 (132), 25/25 (165), 40/40 (264), 60/60 (396), 70/70 (462), 125/60 (610), and 170/60 (759) as incident 630-nm/680-nm each and (in parentheses) total spherical µE/m²/s. A 2π incident sensor was used to measure individual wavelengths, and total spherical illumination was reported by a 4π sensor. Absorbance scans of total cell culture were measured over a 350-nm to 900-nm spectral range using a Shimadzu BioSpec 1601 spectrophotometer (Shimadzu, Kyoto, Japan).

6.5.4 **Biochemical analyses**

Batch flask cultures were grown in quadruplicate and normalized by chlorophyll a content. Chlorophyll a was measured by absolute methanol extraction of a 1-mL cell pellet from centrifugation of liquid culture at 10,000 x g for 3 min. Cell-free supernatant was removed, filtered, and stored frozen for secreted metabolite analyses. Following thorough resuspension of the cell pellet in methanol, the alcohol soluble fraction was cleared of debris by centrifugation and its absorbance read at 663nm using a Beckman DU 800 spectrophotometer (Beckman Coulter, Brea CA). Absorbance values were divided by 78.74 to give mg/mL chlorophyll [Meeks and Castenholz 1971, Porra et al. 1989].

Dry cell weight of batch cultures was measured by concentrating cells from 2 mL liquid culture by centrifugation, removing the medium, and resuspending the pellet in 1 mL of 1 g/L Tris wash buffer. Cells were repelleted by centrifugation and the wash solution removed. Another 1 mL of the wash buffer was added to the cell pellet, which was resuspended and the mixture dried at 80°C overnight in a pre-weighed aluminum dish. The weight of 1 mL 1 g/L Tris likewise treated was subtracted from dry cell mass. Dry weights of PBR cultures are represented as ash-free weight from 400 mL steady-state culture concentrated by centrifugation, resuspended
in distilled water, dried at 105°C overnight in a pre-weighed dish, and burned at 550°C for 4 h. Ash-free weight was calculated as mass lost between drying and burning.

Reducing carbohydrates (RC) were measured as glucose equivalents by an anthrone-sulfuric acid assay described previously [Meuser et al. 2012]. Briefly, a 100-µL sample of liquid culture was mixed with 900 µL of 2 g/L anthrone in 29:71 % v/v distilled water:concentrated H₂SO₄, and the colorimetric hydrolysis reaction was developed in a boiling water bath for 12 min. Absorbance was read at 625 nm and correlated to a glucose standard curve run alongside the samples.

Organic acids (OA) were quantified via HPLC (Surveyor Plus, Thermo Scientific, Waltham MA) by 25-µL injection of 0.45-µm filtrate from culture supernatant onto a 150 x 7.8 mm fermentation monitoring column (BioRad, Hercules CA) at 0.5 mL/min 8 mM H₂SO₄ eluent, 45°C column operating temperature, and 50°C refractive index (RI) detector operating temperature, in parallel with a photodiode array detector (PAD) for absorbance at 210 nm. A standardized mix of acetate, pyruvate, succinate, α-ketoglutarate, and α-ketoisocaproate was used for quantification, and all samples were held at 10°C in a thermostated sample tray before injection.

Fatty acyl hydrocarbon content was measured as transesterifiable fatty acid methyl esters (FAMEs) representing carbon chain lengths of C8-C24 by lytic saponification and acid-catalyzed methylation modified for liquid culture and quantitation from methods described previously [Radakovits et al. 2011]. First, 0.5 mL of liquid culture was hydrolyzed and lipids saponified at 100 ºC for 2 h in 1 mL 95:5 % v/v absolute methanol:0.8 g/L KOH (in H₂O). Then 1.5 mL 94.2:5.8 % v/v methanol:12N HCl was added for acid-catalyzed methylation at 80 ºC for 5 h. FAMEs were extracted into 1 mL n-hexane and 1 µL of the extract was analyzed using an Agilent 7890A gas chromatograph and DB5ms column with flame ionization detection (Agilent Technologies, Santa Clara CA). A flow rate of 1.15 mL/min of H₂ carrier gas was used to separate FAMEs by carbon chain length at 20 ºC/min to 230 ºC for a 1-min hold, then 20 ºC/min to 310 ºC for a 5-min hold. A standard mix of FAMEs was used for quantification and retention time correlation (37-component FAME mix, Supelco, Bellefonte PA). The GC-FID peak that elutes just before C16:1 is unknown and labeled “unk”. Lauric acid in the text designates transesterifiable 12-carbon acyl chains, possibly also in the form of laurate or C12-derivatized lipids.
6.5.5 Pulse amplitude modulation (PAM) fluorometry

Variable chlorophyll fluorescence was measured using pulse amplitude modulated (PAM) fluorometry in a DUAL-PAM-100 system (Walz GmbH, Effeltrich, Germany) with a photodiode detector and RG665 filter [Schreiber 1986]. Red measuring light (620 nm) at the lowest power was pulsed at 1000 Hz during the dark and at 10,000 Hz during 635 nm actinic illumination at 98 µE/m²s. From PBR cultures, 3 mL cells were immediately transferred to a cuvette and fluorescence induction was measured with a programmed script consisting of 15 s darkness, 30 s actinic illumination, application of a saturating pulse at 2000 µE/m²s for 200 ms, 5 s of only far-red light (730 nm), another 15 s of actinic light, and 30 s of darkness. Variable fluorescence observed during the O-J-I-P-S induction provided the basis to compare changes in the electron transport processes downstream of PSII.

The effective quantum yield of PSII (YII’ ) was measured by transient fluorescence changes after induction with a 200 ms saturating pulse at 2000 µE/m²s, 5 s far-red light (730 nm), another 15 s actinic light, and 30 s darkness. The maximum theoretical quantum yield of PSII (Fv/Fm) was calculated from maximal fluorescence (Fm-true) recorded with 15 µM DCMU during actinic illumination, and minimal fluorescence (Fo) after 10 min incubation with only far-red (730 nm) light [Campbell et al. 1998]. The estimated redox status of the plastoquinone (PQ) pool was determined by the rise from “I” to “P” level during the O-J-I-P-S induction, normalized to the total variable fluorescence observed over this period, and subtracted from 1 [Chylla and Whitmarsh 1989]. Relative changes in electron transport downstream of the PQ pool were measured by P>>S quenching as the drop from “P” to “S” states relative to the variable fluorescence observed during the polyphasic induction [Serrano et al. 1981]. The relative rate of cyclic electron flow was obtained from the slope of the post-illumination fluorescence rise in the dark (RFp) [Deng et al. 2003], and the relative dark rate of PQ oxidation was obtained from the declining slope of post-illumination fluorescence, calculated from between 10 and 20 s after the level had peaked [Ryu et al. 2003]. Dark-adapted measurements were taken after cells were held in the dark for 20 min and then acclimatized in actinic light for 90 s before induction.

6.6 Results

This section describes the results of this study.
6.6.1. **Nutrient availability affects chlorophyll a content and dry cell weight to a greater extent than genetic modifications**

When cultures of wt, SA01, \(\Delta glgC\), and SA13 were grown in the presence or absence of nitrate in the medium, all exhibited similar growth characteristics. During nitrogen deprivation, chlorophyll \(a\) was not accumulated (Figure 6.2), and dry cell weight remained near inoculum levels (Figure 6.3). During nutrient replete cultivation, SA01 exhibited a ~15% decrease in chlorophyll \(a\) content and dry cell weights reached 3.1 ± 0.3 g/L (wt), 2.6 ± 0.5 g/L (SA01), 2.6 ± 0.5 g/L (\(\Delta glgC\)), and 2.4 ± 0.5 g/L (SA13) after 48 h. Over 48 h of nutrient replete growth, the strains accumulated 30-37 mg/L chlorophyll \(a\) and 1.9-2.5 g/L dry mass. Additionally, layer of surfactant bubbles was apparent on the surface of cultures secreting lauric acid (Figure 6.1).

![Figure 6.1](image)

**Figure 6.1.** A layer of bubbles is visible atop the culture medium of lauric acid-secreting strains of *Synechococcus* sp. PCC 7002 (SA01 far left, SA13 second from right).

![Figure 6.2](image)

**Figure 6.2.** Chlorophyll \(a\) content in (A) nutrient replete and (B) nitrogen deprived cultures over 48 h of growth in 1% CO\(_2\) at 34ºC under 160 \(\mu\)E/m\(^2\)/s PAR.
6.6.2 Fatty acid profiles are altered by C12 secretion and AGPase disruption

Secretion conferred by the LAS modification of transesterifiable lauric acid (C12:0, C12 hereafter) into batch culture media is demonstrated in Figure 6.4. Lauric acid was neither generated during nitrogen starvation nor when fatB1 was expressed at a neutral locus without concurrent deletion of the putative Aas fadD (not shown). The highest C12 content in batch cultures was observed after 48 h in nutrient replete medium at which point SA01 and SA13 had accumulated, respectively, 9.1 ± 0.4 mg/L and 8.7 ± 0.6 mg/L C12 (Figure 6.5) corresponding to 10.3 ± 0.4 % and 8.4 ± 0.6 % of total FAME (Table 6.2), of which 6.5 ± 0.6 mg/L (SA01) and 5.9 ± 0.2 mg/L (SA13) were secreted into the culture medium.

After 48 h of nutrient replete cultivation, native FA content as measured by FAME was slightly elevated on a culture volume basis in the AGPase-disrupted background and negatively affected by the LAS mutation with C12 appearing to make up for the difference. Normalized by average dry cell weight, FAME content is elevated in all three mutants: both ΔglgC and the double mutant SA13 exceed wt and SA01, and some C12 is generated by SA01 in addition to wildtype levels of native FAs. The latter trend is less obvious in SA13, in which LAS appears to offset native FA content. Despite these differences, the strains show a remarkably similar ratio of 2.5 FA per chlorophyll at this condition.

Profiles of FAME in the four strains after 48 h nutrient replete growth are presented in Table 6.2. Aside from the unknown, all GC-FID peaks are consistent with previous reports of Synechococcus sp. 7002 FAs (ie. 16:0, 16:1, 18:0, 18:1, 18:2) [Kenyon 1972, Sakamoto et al.
1997]. Though it occurs at a position to suggest further unsaturated C16, previous reports indicate that the desaturase that forms C16:2 is absent from *Synechococcus* spp. [Sakamoto *et al.* 1997, Sarcina *et al.* 2001]. The LAS modification appears to result in diminished contents of C16:1, C18:1, and the unknown species. The fraction of C18:2 is decreased in SA01 but occurs at wildtype levels in SA13. Possibly related to this neutralizing phenomenon, ΔglgC contains two-fold more C18:0 than wt and comparable C18:1 and C18:2 levels. In the glgC-disrupted background, the LAS modification confers a lower fraction of C12 relative to total FAME, but the percent secreted was consistent between SA01 and SA13 at 71-72%.

Figure 6.4. Demonstration of C12 fatty acid secretion conferred by the lauric acid secretion (LAS) module. GC-FID chromatograms of FAME extraction/derivatization from representative (A) wt cell culture, (B) SA01 cell culture, (C) wt cell-free supernatant, (D) SA01 cell-free supernatant. RT: retention time (polarity separation), pA: picoamps (detection level).

Figure 6.5. Fatty acyl content after methyl esterification to FAMEs of 48-h nutrient replete batch cultures of wildtype (wt) or engineered *Synechococcus* sp. 7002 grown in 1% CO₂ at 34°C under 160 µE/m²/s PAR, represented by volume (left) and as a function of average dry cell weight (right). Grey: native fatty acyl hydrocarbons, white: non-native C12 fatty acids.
Table 6.2. Percent of total FAME by chain length in 48-h nutrient replete cultures, corresponding to Figure 6.5. The two values for C12:0 represent total and secreted FAs and are not additive.

<table>
<thead>
<tr>
<th>Strain</th>
<th>12:0</th>
<th>16:0</th>
<th>16:1</th>
<th>unk</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>nd</td>
<td>48.5 ± 0.7</td>
<td>10.7 ± 0.3</td>
<td>8.7 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>12.2 ± 0.3</td>
<td>17.2 ± 0.5</td>
</tr>
<tr>
<td>SA01</td>
<td>10.3 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.6 ± 0.9</td>
<td>6.9 ± 0.3</td>
<td>5.3 ± 0.1</td>
<td>2.9 ± 0.4</td>
<td>10.6 ± 0.2</td>
<td>15.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>7.4 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔglgC</td>
<td>nd</td>
<td>49.2 ± 0.8</td>
<td>9.4 ± 0.6</td>
<td>7.9 ± 0.4</td>
<td>3.4 ± 0.6</td>
<td>12.0 ± 0.6</td>
<td>17.0 ± 1.0</td>
</tr>
<tr>
<td>SA13</td>
<td>8.4 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.6 ± 0.4</td>
<td>7.6 ± 0.7</td>
<td>4.6 ± 0.1</td>
<td>3.0 ± 1.0</td>
<td>10.9 ± 0.4</td>
<td>17.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>6.0 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> cell culture; <sup>b</sup> cell-free supernatant of respective cell culture; nd: none detected

6.6.3 **Absence of AGPase causes carbohydrate deficiency and organic acid secretion during nitrogen starvation**

Chlorophyll a content did not change from initial levels (2.5 mg/L) over 48 h of nitrogen deprivation (–N) during batch cultivation (Figure 6.2B). Dry cell weights began at 0.63 ± 0.3 g/L wt, 0.75 ± 0.3 g/L SA01, 0.75 ± 0.3 g/L ΔglgC, and 0.67 ± 0.4 g/L SA13, and differences over the time course did not exceed the range of error (Figure 6.3B). By glucose equivalents, ΔglgC accumulated 7% of wt reducing carbohydrate (RC) levels on a culture volume basis after 24 h of nitrogen deprivation and 11% after 48 h, while SA13 reached 6% after 24 h and 13% after 48 h (Figure 6.6). Organic acids (OA) were secreted into the culture medium of nitrogen starved AGPase-disrupted strains, and including these improves the yields such that the RC-OA total relative to wt RC levels during –N reached 31% after 24 h and 39% after 48 h in ΔglgC and 28-38% in SA13 (Figure 6.6). Organic acids quantified from ΔglgC and SA13 culture supernatants after 48 h –N are shown in Figure 6.7. Neither wt nor SA01 secreted detectable amounts of OA over the -N time course, and OA were not detected in nutrient replete growth media. Acetate was the most abundant OA secreted from the AGPase-disrupted background, followed by succinate and α-ketoisocaproate. Lesser concentrations of pyruvate and α-ketoglutarate were also observed, and levels of secreted metabolites do not appear to be affected by the LAS mutation.

After 24 h and 48 h of nitrogen deprivation, respectively, the content of reducing carbohydrates (RC) relative to dry cell weight reached 31-29% in wt and 32-33% in SA01. The AGPase-disrupted backgrounds accumulated substantially less RC: 4-5% of dry cell weight in ΔglgC and 4-7% in SA13. In the carbohydrate-deficient background, organic acids were secreted...
at the same fraction of dry cell weight, to a total of 14-15% after 24 h and 12-13% after 48 h. Including these, ΔglgC accumulated both RC and OA under –N conditions to 19% of dry cell weight after 24 h and 18% after 48 h, and SA13 reached 18-20%.

Figure 6.6. RC content and secreted OA by volume in wt, SA01, ΔglgC, and SA13 cultures during nitrogen deprivation shown at the beginning of the time course (0 h) and after 24 and 48 h. Nitrogen deplete cultures were standardized to 2.5 mg/L chlorophyll a and grown with 1% CO₂ at 34ºC under 160 µE/m²s illumination.

6.6.4 Steady-state cultivation reveals similarities in O₂ evolution despite growth defects from the genetic modifications

Maximum growth rates calculated from dilution rates were 3.5 h/doubling (wt), 3.8 h/dbl (SA01), and 4.6 h/dbl (ΔglgC). Despite displaying lower bulk O₂ evolution and growth rates, on a per-doubling basis O₂ evolution from ΔglgC does not differ from wt (Figure 6.8B, C) and in fact exceeds wt levels at 396 µE/m²s and above. The SA01 mutant exhibits consistently elevated O₂ evolution, both on per-volume and per-doubling bases, but attains lower growth rates than wt. With increasing irradiance, the wildtype background accumulated RC up to 51% (wt, 610 µE/m²s) and 43% (SA01, 610 µE/m²s) of dry cell weight whereas ΔglgC attained a maximum of only 9.7% (264 µE/m²s) (Figure 6.8D-F). Interestingly, ΔglgC maintained dry cell weights between 78-101% of wt over the light course, and SA01 varied between 86-104% of wt.
Due to low cell density, fatty acid content in PBR cultures was below quantifiable detection (< 1 mg/L), but in a tested culture of SA01 at 396 µE/m²s, C16:0 and C12:0 peaks were present at a ratio of 3:1 (not shown). All detected C12 was also observed in SA01 cell-free filtrate and no C12 was produced by wt or \( \Delta glgC \) at this light point. Using peak area as a proxy, \( \Delta glgC \) contained ~90% of wt C16 by volume and SA01 only ~50%. Addition of C12 levels brought SA01 to ~80% of wt. Normalized by doubling rate, this translates to a ~10% increase in \( \Delta glgC \) and an ~8% decrease in SA01 of total observable FAME relative to wt (see Figure 5.22).

![Figure 6.7](image)

Figure 6.7. (A) Pigmentation differences between wildtype and carbohydrate-deficient backgrounds during nitrogen deprivation. (B-F) Organic acids secreted from \( \Delta glgC \) and SA13 strains after 48 h of nitrogen deprivation.

6.6.5 AGPase disruption causes photophysiological rearrangements

Changes in photophysiology from low to high light intensities between wt, SA01, and \( \Delta glgC \) strains at steady state are depicted in Figure 6.9. With increasing irradiance, \( \Delta glgC \) exhibits a more reduced PQ pool and a higher PSII quantum yield than the wildtype background. The P-to-S rate of electron transport downstream of PQ is also adversely affected in \( \Delta glgC \), as less quenching occurs with higher light. Compared to the wildtype background, more rapid rates
of dark PQ oxidation were observed in dark-adapted ΔglgC and a greater fraction of non-transmitted 680-nm light by ΔglgC cultures was observed whereas 630-nm absorbance by cell culture was unaffected between backgrounds.

Figure 6.8. Growth rates, \(O_2\) evolution, dry weights, and RC in steady-state PBR cultures over increasing light intensities. Left panel: (A) bulk \(O_2\) evolution, (B) doubling rate, and (C) \(O_2\) produced on the basis of doubling rate (normalized). Right panel: dry cell weight and glucose equivalents over the light course in (D) wt, (E) SA01, and (F) ΔglgC cultures. The glucose value for 33 \(\mu\)E/m²s and biomass values for 759 \(\mu\)E/m²s were not available.
Figure 6.9. Photophysiology by pulse amplitude modulation (PAM) fluorometry from steady-state PBR cultures of wt, SA01, and ΔglgC over a range of light intensities. (A) PSII quantum yield and (B) relative PQ redox status (more positive is more reduced). Relative (C) electron transport rates after PQ and (D) dark PQ oxidation rates in dark-adapted cultures. Non-transmitted fractions of (E) 630-nm and (F) 680-nm light by whole cell culture.

6.7 Discussion

Saturated fatty acids, particularly C6 to C14 FFAs, are valuable biodiesel precursors that can be synthesized phototrophically and secreted by genetically engineered cyanobacteria [Liu et al. 2011, Ruffing and Jones 2012, Ruffing 2014]. The results are consistent with the recent report
of 3-4 mg/L•d FFA secreted during early growth stages by Synechococcus sp. 7002 cultures engineered with a bacterial thioesterase [Ruffing 2014]. Rates of 10-12 mg/L•d are described by Liu et al in analogous studies using Synechocystis sp. 6803, and all three works investigate further modifications such as overexpression of Rubisco or acetyl-CoA carboxylase, deletion of competing metabolites, and weakening of the outer membrane and achieve long-term yields up to 131 mg/L in Synechococcus sp. 7002, 49.3 mg/L in Synechococcus elongatus 7942, and 197 mg/L in Synechocystis sp. 6803. The present study aimed first to quantify during rotated batch cultivation the lauric FA that is synthesized and secreted from wildtype or carbohydrate-deficient backgrounds of Synechococcus sp. 7002 when a heterologous thioesterase for lauric acid (C12 FFA) is expressed by knockin to disrupt FA recycling, second to determine effects on the fatty acid profile due to these modifications, and third to assess the physiologies of metabolic mutants at turbidostatic steady-state.

It was speculated that a strain unable to synthesize the ADP-glucose precursor for glycogen may favor FA synthesis, and a genetic modification for lauric fatty acid secretion (LAS) was conferred to wildtype Synechococcus sp. 7002 and the corresponding ΔglgC (ΔAGPase) carbohydrate-deficient mutant. This resulted in 10% of total cellular FAs represented as C12:0 in the wildtype background and 8% in the glgC-disrupted background, both serving to neutralize a volumetric detriment to native FA content seeming to result from the LAS modification. About 70% of the esterifiable C12 FAs were secreted into the culture medium. A small increase in total FAME content by volume and by dry cell weight was evident in cultures of the carbohydrate-deficient background, suggesting natural redirection of metabolism to FA synthesis. The LAS modification alone appears to increase FAME on a dry cell weight basis but this was less apparent in the AGPase-disrupted background and it is still unclear whether de novo synthesis is conferred. The observed FAME contents compare well to previous evaluations of the Synechococcus sp. 7002 FA profile [Sakamoto et al. 1997, Sakamoto and Bryant 2002], with C16:0 comprising the majority, moderate levels of C18:1 and C18:2, slightly less C16:1, very little 18:0, and C18:3 absent possibly due to a higher culturing temperature. In batch cultivation, doubling rates are restricted and shading effects may alter FA synthesis for photosynthetic membrane components. A more thorough understanding of the associations between FA synthesis and growth rate, light intensity, nutrient availability, and temperature in this cyanobacterium is being developed to further optimize lipid production.
The lower chlorophyll $a$ content in the single LAS mutant relative to the other strains could be a mild bleaching response causing deconstruction of photosynthetic components to provide nutrients to other metabolic pathways, which occurs to a greater degree during nitrogen deprivation. Nitrogen enhancement might correct this, but the chlorophyll $a$ deficiency could be due to detergent toxicity and membrane damage, non-stress resource allocation, and/or a lower doubling rate. In turn, the carbohydrate-deficient LAS mutant exhibits wildtype levels of chlorophyll $a$ but generates fewer FFAs. Molecular trafficking in the carbohydrate-deficient strains is discussed below with reference to nitrogen deprivation, and the observation of pigment preservation by this background may also assist chlorophyll $a$ protection during nutrient replete growth.

Since reducing carbohydrates are accumulated in the wildtype background during nitrogen deprivation (-N), it was considered that C12 secretion from the carbohydrate-deficient background may increase under these conditions. However, C12 was not detectable in -N batch cultures with the LAS modification. This may be due to cessation of protein synthesis or, since a phycocyanin-related promoter is responsible for fatB1 expression, the gene may be downregulated in times of nitrogen stress, as phycobiliproteins can be metabolized as an intracellular nutrient source [Sauer et al. 1999, Richaud et al. 2001]. Conversely, a non-bleaching phenotype was observed in ΔglgC batch cultures when nitrogen deprived, and based on spectral analysis, higher absorbances in the 580-650nm phycobilin range suggest these proteins are not deconstructed for nutrients in the AGPase-disrupted background as they are in wildtype [Davies et al. 2014]. This behavior has also been described in carbohydrate-deficient mutants of *Synechocystis* sp. 6803 and *Synechococcus elongatus* 7942 [Carrieri et al. 2012, Gründel et al. 2012, Hickman et al. 2013]. Similarly, while reducing carbohydrates in the wildtype background exceed ΔglgC levels over the steady-state light course, significantly more scattered or absorbed 680nm light by ΔglgC cultures suggests a molecule or process more prevalent and sustained in this background. Among other potential storage products and photoprotective elements that may be related to this phenotype, phycocyanobilin is a phycobiliprotein that absorbs at 680nm and is involved in free radical scavenging [Ge et al. 2013].

During nitrogen deprivation, central metabolites pyruvate (C3) and α-ketoglutarate (C5) have been reported in culture supernatants of AGPase-disrupted *Synechocystis* sp. 6803 mutants...
carrieri et al. 2012, gründel et al. 2012; and α-ketoglutarate, succinate (c4), and fumarate (c4) were secreted by the analogous mutants of synechococcus elongatus pcc 7942 under the same conditions [hickman et al. 2013]. these central metabolites are closely related to the tricarboxylic acid cycle (tca) and provide insight into metabolic redistribution caused by nitrogen deprivation compounded with carbohydrate deficiency. in many cyanobacteria including synechococcus sp. 7002, conversion of α-ketoglutarate to succinate occurs in two steps through a succinic semialdehyde intermediate, which is central to the interconversion between pyruvate, oxaloacetate, and the amino acids alanine, glutamate, and aspartate [zhang and bryant 2011, steinhauser et al. 2012]. pyruvate is a ubiquitous and versatile central metabolite, and in synechococcus elongatus 7942, α-ketoglutarate has been demonstrated as an effector of the nitrogen regulator ntcA [vázquez-bermúdez et al. 2001, tanigawa et al. 2002]. lower tolerance to osmotic stress in agpase-disrupted cyanobacteria has also been shown [suzuki et al. 2010, guerra et al. 2013].

in this study, the glgC-disrupted strains of synechococcus sp. 7002 secreted pyruvate, α-ketoglutarate, and succinate, as well as acetate (c2) and α-ketoisocaproate (C6H9O3), a biosynthetic precursor of the amino acid leucine. several of these are gluconeogenic metabolites. possibly from protein oxidation, α-ketoisocaproate can be converted via enzymes similar to those of the tca and β-oxidation to anapleurotic acetyl-CoA and acetoacetate, which along with pyruvate and acetate are direct biosynthetic precursors of FAs, isoprenoids, and higher alcohols, as well as reduced storage polymers such as poly-3-hydroxybutyrate (PHB) and polyhydroxyalkanoate (PHA). though biosynthetic enzymes for the latter two have not been identified in synechococcus sp. 7002, synthesis of a similar product other than glycogen could be accumulated in the carbohydrate-deficient background to offset the compromised levels of reducing carbohydrates [Mcneely et al. 2010, xu et al. 2013]. Many additional processes or products in the glgC-disrupted background could be acting to manage excess energy by storing it in reduced carbon, cycling it by futile carbon oxidation [Badger et al. 2000], scattering or absorbing it with pigments or other molecules [Zhu et al. 2010], employing redox coenzyme acceptors, secreting small metabolites, and/or neutralizing reactive oxygen species [Nomura et al. 2006]. Advanced cultivation and photophysiological techniques will make it possible to characterize the empirical behaviors of these novel strains.
Static growth in an LED-photobioreactor of *Synechococcus* sp. 7002 wildtype (wt), C12-secreting (SA01), and carbohydrate-deficient (ΔglgC) strains facilitated maximum photoautotrophic doubling times of 3.5 h (wt), 3.8 h (SA01), and 4.6 h (ΔglgC), which are close to the most rapid rates observed for this organism [Ludwig and Bryant 2012]. The wildtype background accumulated reducing carbohydrates with increasing irradiance, and both growth rate and O₂ production began to plateau around 396 µE/m²s. The ΔglgC mutant reached its maximum growth rate at a lower light intensity than the wildtype background and incurred deficiencies in dry cell weight and reducing carbohydrates. However, O₂ production by ΔglgC on a cellular level was unchanged relative to wt. In fact, O₂ evolution by ΔglgC continued to rise after growth rate saturated. With increasing irradiance, photophysiology revealed the ΔglgC mutant to exhibit higher PSII quantum yields and a more reduced PQ pool with lower rates of downstream electron transport than the wildtype background. The ΔglgC modification may affect the availability of electron acceptors for photosynthetic reductant, causing a compound reducing effect on the PQ pool from electrons unable to move out of the photosynthetic electron transport chain and those entering PQ. The severity of these redox alterations appears to activate mechanisms to protect PSII, and dissipation of excess electrons by ΔglgC is suggested in an elevated PSII quantum yield, a lower fraction of transmitted 680-nm light, and higher rates of dark PQ oxidation (mechanism unknown). The first two observations may be indicative of more per-cell PSII complexes in the ΔglgC mutant relative to wildtype. Additionally, in *Synechococcus* sp. 6803 the activity of succinate dehydrogenase has been demonstrated as a contributor to respiration through the PQ pool [Vermaas 2001], which may also occur in *Synechococcus* sp. 7002. As the PQ pool in the AGPase-disrupted mutant is overreduced with lower electron transfer rates, these could be related to accumulating succinate during nutrient stress. As mentioned above, many additional processes or products in the glgC-disrupted background could be acting to manage excess energy. Organic acids in steady-state cultures were not quantifiable due to low detection levels but might be expected to secrete from ΔglgC, possibly assisting to relieve reductant, when exposed to irradiances at which the wildtype background accumulates more carbohydrates. Comparatively moderate changes to photosynthetic electron transport are brought about by the LAS modification, in which PSII quantum yield and post-PQ electron transport rates appear to be similar but less dynamic than wt. At the highest light intensity tested, changes to ΔglgC O₂ production and wt PSII quantum yield,
PQ redox status, and post-PQ electron transport suggest physiological shifts for further investigation.

Based on biomolecular and photosynthetic physiologies of three mutants engineered for biofuel production, *Synechococcus* sp. 7002 presents an amenable and robust platform for research in phototrophic metabolism. Secretion of heterologous lauric acid was achieved in both wildtype and carbohydrate-deficient backgrounds, and adjustable metabolic partitioning was observed by disrupting AGPase. Understanding these mechanisms will greatly inform the design of next generation bioenergy feedstock strains. The results are encouraging for studies of increased FA synthesis in this organism, and efforts could seek to reallocate fixed carbon, particularly the central metabolites orphaned by AGPase disruption, into desired pathways.

6.8 Acknowledgments

The authors thank Shuyi Zhang and Dr. Donald A. Bryant for providing genetic materials and the ΔglgC mutant, and Dr. Roy Curtiss III for providing the *fatB1* gene. We gratefully acknowledge the work of Gaozhong Shen to provide technical advice, Dr. Sarah D’Adamo for assistance with HPLC analysis, Dr. Robert Jinkerson for optimizing the anthrone protocol, and Dongxu (Tom) Li and Dr. Jinkerson for contributions to FAME methodology. This research was supported by the Genomic Science Program (GSP), Office of Biological and Environmental Research (OBER), U.S. Department of Energy, and is a contribution of the PNNL Biofuels Scientific Focus Areas.
CHAPTER 7
CONCLUSIONS

Photosynthetic microorganisms are amenable to research-scale metabolic engineering by molecular genetics and mass cultivation for bulk production of desired compounds. Almost all of the chemical energy needed to sustain modern life is derived from biological components. Particularly, coal and hydrocarbon resources (fossil energy) are the remnants of ancient phototrophs that reduced CO$_2$ from the air into their physical structures, which then decayed and transformed over time. This thesis tested the feasibility of using photosynthetic microorganisms (PSMs) during normal growth, specifically green algae and cyanobacteria, to generate fixed carbon moieties directly integratable into current transportation fuel processing infrastructure. The results were favorable for endeavors to enhance the productivity of fatty acid (FA) metabolism in these organisms, which is the primary pathway for the straight-chain hydrocarbons of diesel fuel. Triacylglycerol (TAG) content and storage was modulated in the green alga *Chlamydomonas reinhardtii*, and secretion of lauric acid, a 12-carbon FA (C12), from the cyanobacterium *Synechococcus* sp. PCC 7002 was achieved. The goal of this research was to examine the physiological effects of synthesizing, storing, and exporting these and other biofuel precursors such as carbohydrates, organic acids, and isoprenoids on CO$_2$-assimilating organisms.

The biological membranes of organisms belonging to the domains Bacteria and Eukarya are derived from the successive condensation of two-carbon acetyl-CoA units in fatty acid synthesis (FAS), and in organellar PSMs this occurs in the chloroplast [Rawsthorne 2002]. Also the site of photosynthetic electron transport, carbon fixation, and carbohydrate synthesis, chloroplasts are the photosynthetic compartment of eukaryotic PSMs and are believed to be derived from cyanobacterial endosymbionts. During FAS, the hydrocarbon chain, or acyl group, is bound to acyl carrier protein (ACP) until it reaches a target size, usually 16 carbons (C16), whereupon it is released from ACP and moves via unknown mechanisms out of the chloroplast and into the cytoplasm where it is further modified for metabolic integration.

In *C. reinhardtii*, it was discovered that two different genetic alterations producing a starchless (*sta*) phenotype resulted in greater partitioning to lipid bodies, particularly during nitrogen stress. This nutrient signal causes cessation of cell division and accumulation of reduced
energy storage molecules, which normally include starch and TAG that are stored in the chloroplast [Ball et al. 1990, Libessart et al. 1995, Goodson et al. 2011]. When the ability to synthesize starch was disrupted, by deletion of either ADP-glucose pyrophosphorylase (AGPase) as in the sta6 strain or the debranching isoamylase enzyme as in sta7, photosynthetic capacity was diminished and more severe growth effects were observed during nutrient stress, but the amount of TAG per cell and per unit volume was increased. These organisms are useful in determining the consequences of attempted metabolic redistribution to products of interest, but they would not be viable feedstocks for several reasons. First, their outermost membranes are fragile and would not withstand the harsher conditions of mass cultivation. Second, they do not accumulate large amounts of TAG unless nutrient stressed, which produces the unfavorable effects of decreased growth rates and photosynthetic activity. Third, C. reinhardtii grows slowly under photoautotrophic conditions. Finally, the reduced carbon accumulated by strains in this study was partially derived from added acetate, which would not be economical for scaled production and would introduce the potential for contamination by heterotrophs. Though these strains of C. reinhardtii would not provide the necessary resources for mass production of biofuel precursors, they are valuable research tools, and more promising yields may be achievable by modulating rate-limiting enzyme levels, optimizing culture conditions, or epigenetic control over the carbon storage response.

When the isoamylase gene was reintroduced into sta7, the resulting strains exhibited extreme phenotypes including significantly elevated O2 evolution, heterotrophic carbon utilization, and accumulation of both starch and TAG above all other strains tested, even when compared to nitrogen deplete conditions. These strains are of particular utility in defining metabolic flux adaptations, and unlike for sta6 and sta7, systems should be investigated for their large-scale cultivation and extraction. The hyperaccumulation of desirable biomolecules in these complemented strains during normal growth is a significant asset, which is compounded with the observation that cellular fitness and photosynthesis are not severely compromised. Though C. reinhardtii has been classically used as a research model, constructing scaled apparati based on the physiology of its mutants would be a worthwhile endeavor. The extensive collection of C. reinhardtii mutants could be used to great effect in testing the feasibility of such systems for PSMs even outside of the species.
Though intimately related, the physiologies of eukaryotic algae and cyanobacteria, photosynthetic prokaryotes, exhibit myriad differences. The energetic requirements, modes of growth, and constituent molecules are variable and diverse, providing an expansive world of possibility. The extent of photobiological plasticity is likely an endless pursuit, for the diversity of PSMs alone reaches far beyond our current definitions and is further multiplied by higher plants. Nonetheless, the basic processes of photosynthesis, carbon fixation, and central metabolism are highly conserved and well characterized, facilitating investigation of the wide-ranging metabolic behaviors of phototrophs.

One particular cyanobacterial isolate, *Synechococcus* sp. PCC 7002, was investigated for its metabolic and physiological preferences during acclimation to biophysical perturbations by genetic and energetic modifications. Particularly, the aim was to introduce the organism to factors of significance in biofuel production. Metabolically, these include alterations to basic biomolecular pathways toward glucan carbohydrates and fatty acids. Physiologically, conditions of low to high light, nutrient limitation, and different growth stages were also investigated. This PSM proved highly amenable to these manipulations, adapting with facility to the challenges presented. Previously described accounts of the photosynthetic and photoprotective capabilities of *Synechococcus* sp. 7002 are corroborated, and evidence is provided for similar robustness in its catabolic metabolism. The organism was found to be amenable to secreting medium-chain fatty acids, managing nutrient stress, and remaining viable at high light intensities.

When an enzyme of fatty acid activation, the putative acyl-ACP synthetase *fadD*, was deleted from the *Synechococcus* 7002 genome, the organism was able to retain its fitness, possibly through increased use of similar enzymes. It was discovered that this gene product is important in FA activation, as concurrently introducing a heterologous enzyme capable of hydrolyzing 12-carbon (lauric) fatty acid chains from ACP resulted in the release of FAs into the culture medium. Though FAs have been shown to be toxic to certain cyanobacteria [Ruffing and Jones 2012], *Synechococcus* 7002 was not significantly adversely affected, showing only a slight decrease in maximal growth rate as compared to the wildtype strain. The modification caused 10% of fatty acids to be represented in the engineered chain length (C12), the majority of which were found outside of the cells. This result is exciting in its possibility for sustainable generation of renewable bioproducts, as costly harvesting and lytic procedures could be avoided. Secretion of high levels of a hydrophobic compound is also a desirable characteristic, as mass cultures
could conceivably produce superaqueous layers of the desired product that could be skimmed off the surface. The possibilities simply in adjusting this organism’s lipid productivity are manifold, and many other products have also been identified and studied for their tractability as bioenergy feedstocks.

Carbohydrate partitioning in *Synechococcus* 7002 was found to be a significant contributor to the organism’s overall metabolism, and interrupting the glucose activation enzyme AGPase resulted in many divergent and revealing physiologies, particularly in photosynthetic electron transport. A significant decrease in reducing carbohydrates was observed overall, which further affected the organism’s ability to accumulate its usual storage molecule, glycogen, during nitrogen stress. This necessitated an altered response to nutrient deprivation, specifically a conservation of phycobilin protein stores and secretion of organic acids. The latter observation is likely due to several factors from both the catabolic and anabolic sides of this acclimation. Intermediates of glycogen synthesis cannot be fully converted and could be evident in the buildup of otherwise transient TCA components, as this cycle is used to generate reducing equivalents for catabolic reactions. The secretion of acetate and α-ketoisocaproyate are intriguing, as they are not directly integratable into carbohydrate stores but are instead more closely related to proteins and lipids. Acetate can be derived from pyruvate or the oxidation of fatty acids, and α-ketoisocaproyate is an oxidation product of the amino acid leucine. It is conceivable that the cyanobacterium naturally begins depleting its protein stores during nutrient stress for allocation into other molecules. These results are noteworthy for both biocommodity applications and investigations of the organism’s preferred rearrangements during a stringent modification such as the elimination of a major cellular constituent.

Photophysiological effects of AGPase disruption were observed at almost every node of photosynthesis investigated in this study and exemplify the adaptable nature of *Synechococcus* 7002 even, or maybe especially, in its photosynthetic functioning. Electron transport is affected by this modification at both photosystems and the redox-sensitive plastoquinone (PQ) pool and suggests a general abatement in electron flux, requiring energy dissipation. Locations of excess energy release are indicated at PSII with a higher quantum yield and at PQ with higher rates of dark oxidation. Also possibly associated with PSII is a smaller transmitted fraction of 680-nm light by AGPase-disrupted cultures. Additionally, a buildup of unused electrons is apparent in an overreduced PQ pool and an inability to properly move electrons out of this pool. This could be
thought of similarly to the accumulation of metabolic intermediates in this organism, suggesting blockages that prevent otherwise transient moieties from moving uninhibited through their respective pathways. The AGPase mutant indeed doubles more slowly and reaches a lower maximal growth rate than the wildtype but curiously maintains similar rates of $O_2$ evolution. The consequences of this perturbation are of great value in determining this organism’s tendencies in the highly complex and multifaceted matter of managing excess photic energy.

The *Synechococcus* 7002 cyanobacterium is versatile and robust, exhibiting tolerance to many different conditions [Ludwig and Bryant 2012]. It provides a promising template for further modifications and could be of great utility in the design of bulk cultivation platforms. It is viable under a wide range of salinities, which make it an attractive candidate for studies in scaled oceanic systems. It is capable of utilizing high light intensities to enhance growth and is efficient in dissipating unneeded photic energy, both of which are also desirable characteristics in outdoor growth settings, along with retained viability under a wide range of temperatures. These traits and others have positioned *Synechococcus* 7002 as a valuable research tool and potentially as a scalable bioenergy feedstock organism.

In small climate-controlled batch quantities, PSMs genetically engineered for metabolic carbon redistribution yielded promising results for fatty acid based straight-chain hydrocarbon products triacylglycerol (TAG) and lauric acid (C12) as well as more oxidized fermentable compounds such as polyglucan carbohydrates and organic acids. Ideally, biodiesel precursors would be synthesized by large consolidated quantities of the feedstock organism using atmospheric or scrubbed $CO_2$, wastewater or seawater, and ambient light. Scaled systems require high titres from robust organisms that research efforts have only just begun to develop for preliminary testing. With the availability of genome sequences and improved techniques for strain construction and screening, the near future is likely to see a great expansion of the current portfolio of PSMs specialized for renewable and sustainable bioenergy concepts.

It is well advised that PSMs altered in metabolic partitioning be further investigated and engineered for the potential to direct energy and carbon flux toward desired pathways. Though this task is only beginning in PSM-based biofuel applications, this premise is already illustrated in the agricultural domestication of higher plants. With an ever-improving suite of bioinformatic and molecular tools, along with a wide variety of cultivable organisms, this endeavor will likely find great success in many different facets of anthropologic and environmental sustainment.
8.1 Metabolomics

These studies were performed using the following strains of the cyanobacterium *Synechococcus* sp. PCC 7002. The wildtype is designated as wt. The AGPase-deficient mutant is referred to either as ΔglgC or simply glgC. The lauric acid secreting (LAS-modified) strain is referred to as las1, which contains the “C12” cassette positioned to interrupt the native *fadD* gene, a putative acyl-ACP synthetase. The cassette confers expression of the *fatB1* gene from *U. californica* driven by the *cpcBA* promoter. The strain containing the C12 cassette in a ΔglgC background is referred to as gls3.

Metabolites were isolated and detected as described previously [Huang *et al.* 2014, Bennette *et al.* 2011, Brauer *et al.* 2006], and data was generated in collaboration with Dr. Young-Mo Kim at the Pacific Northwest National Laboratory. Only the compounds exhibiting differences between strains or conditions are shown. Results are from turbidostatic, chemostatic, or batch cultivation and are compared either between strains in the same condition or between conditions in the same strain. Values are not quantified but are relative, based on raw abundance. They are normalized empirically by turbidostat or chemostat cultivation, which was performed in the LED-PBR described in Chapters 5 and 6. No wildtype information is available for PBR measurements. For batch cultures, metabolites are normalized to chlorophyll *a* content.

Differences in percent abundance between las1 and glgC metabolites under different PBR conditions are shown in Table 8.1. Aspartate, glutamate, pyroglutamate, serine, and valine are all elevated in glgC as compared to las1 (a wildtype background) during normal growth, and glucosyl moieties are decreased. Lactate is also slightly increased in glgC. During nitrogen deprivation, glutamate exhibits lower abundance and aspartate increases in glgC relative to las1. The relative increase in glgC glucosylglycerol (or decrease in las1) could not be explained, but it may not be synthesized at high levels by the wildtype background under this condition.

The notable changes appear to occur in TCA-associated amino acids, which is in agreement with results from Chapter 6 describing the activity of the TCA in glgC during nitrogen deprivation. This cycle seems to be heavily relied upon in this background to assist with
a diminished capacity for glucose polymerization. These results give insight into TCA regulation and use during AGPase disruption and should be further investigated to determine specific products generated and more detailed flux characteristics.

When compared across conditions within the same strain, many trends become apparent, the extent and interpretation of which are beyond the scope of these preliminary observations. Results are shown in Tables 8.2 (las1) and 8.3 (ΔglgC). The metabolites that seem to be significantly affected by alteration of conditions in the las1 mutant include lauric acid, lactate, gluconate, benzoic acid (amino acid-related), adenine (nucleic acid), proline, and the TCA intermediates α-ketoglutarate, fumarate, and succinate. Adenine, proline, lactate, laurate (source unknown), gluconate, α-ketoglutarate, and succinate also appear to be altered by changing conditions in the ΔglgC mutant, in addition to gulose (monosaccharide) and four other amino acids: methionine, phenylalanine, pyroglutamate, and tyrosine. The modulation of a greater number of amino acids in glgC further suggests this background’s increased reliance on the TCA and possibly protein synthesis in the absence of polymerizable glucose.

Comparisons of metabolite abundance between the four strains under several conditions in batch culture are shown in Figure 8.1. The TCA intermediates succinate, α-ketoglutarate, isocitrate, fumarate, and malate, as well as the TCA precursor pyruvate, are elevated in all strains during nitrogen and sulfur stress, suggesting a buildup of these compounds possibly due to slowed metabolic rate and less protein synthesis. Increases in metabolites at late (70-h) time points during nutrient replete cultivation with ammonium (NH$_4^+$) as the nitrogen source are likely suggestive of nutrient deprivation, as these cultures attained higher density sooner than those with nitrate (NO$_3^-$), possibly depleting NH$_4^+$ more quickly than NO$_3^-$.

Interestingly, two anapleurotic molecules phosphoenolpyruvate (PEP) and dihydroxyacetone phosphate (DHAP) are accumulated during sulfur stress, PEP at similar levels between all strains but slightly elevated in the C12-secreting mutants las1 and gls3, and DHAP at lesser concentrations in the secreting strains. PEP is a precursor of pyruvate derived from 3-phosphoglycerate (3PG), and DHAP, an isomer of glyceraldehyde-3-phosphate (GAP), is a precursor to fructose- and glucose-6-phosphates. Both PEP and DHAP are closely associated with the carbon-fixing CBB cycle. This result is one of very few indications observed during this study of the metabolic effects of lauric acid synthesis in Synechococcus 7002. The amount of lauric acid produced by these strains under sulfur deprivation was not determined but may be
diminished due to conservation of metabolic activity. Nonetheless, the effects of the genotype may indicate global characteristics, in which partitioning to pyruvate and acetyl-CoA (to fatty acid synthesis) may be enhanced through PEP and concurrently decreased toward polyglucan carbohydrate precursors, indicated by decreased DHAP. Enzymes of the CBB cycle could be overexpressed to enhance this phenotype and determine the extent of these metabolites’ contributions to the biomolecular profiles of the strains.

Table 8.1. Differences in metabolite abundance between cultures of las1 and ΔglgC at selected light and nutrient conditions in the LED-PBR described in Chapters 5 and 6. Conditions 15/15, 60/60, and 170/60 are turbidostatic light points indicating 630-nm/680-nm μE/m²’s irradiances. The LL and –N conditions designate chemostatic low light and nitrogen limitation, respectively. Those marked with an asterisk indicate that strains were adapted to high light and high O₂ prior to measurement. A threshold of ≥ 3% difference was used.

<table>
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<th>*60/60</th>
<th>*170/60</th>
<th>*LL</th>
<th>*-N</th>
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</tbody>
</table>

Positive values (red) indicate a larger amount in las1
Negative values (green) indicate a larger amount in ΔglgC
* indicates strains adapted to high light and high O₂

8.2 Overexpression of malonyl-CoA:ACP transacylase fabD

The A2559 gene in the Synechococcus 7002 genome (Kyoto Encyclopedia of Genes and Genomes) was chosen for overexpression by insertion into a neutral chromosomal locus to investigate its effects on fatty acid secretion. This is annotated as fabD, a malonyl-CoA:ACP transacylase (MCAT). Provided this is correct, the gene is involved in loading malonyl-CoA onto ACP for fatty acid synthesis by polymerization with further malonyl-CoA. Therefore, overexpression was hypothesized to affect the synthesis and/or secretion of lauric fatty acids in the ΔfadD::C12 strains. The las1 and gls3 mutants were transformed with an NSI cassette containing the native fabD gene inserted between the cpcBA promoter and a gentamycin (Gm) resistance gene. Homoplasmic integration has not been confirmed by PCR, but after several
Table 8.2. Differences in percent metabolite abundance between conditions in the lasI strain. For a description of conditions, see Table 8.1. A threshold of ≤ 3% was used, but non-zero values were left in for reference.

<table>
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<tr>
<th>Metabolite</th>
<th>Condition &gt;</th>
<th>Compared to 15/15</th>
<th>Compared to 60/60</th>
<th>Chemostats</th>
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<td>15 to *N</td>
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<td>-1</td>
</tr>
<tr>
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<td>-1</td>
<td>-1</td>
</tr>
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<td>L(-) lactic acid</td>
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<td>-1</td>
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<td>succinic acid</td>
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<td>-1</td>
</tr>
</tbody>
</table>

Positive values (blue): greater amount of metabolite in the first condition listed

Negative values (yellow): greater amount of metabolite in the second condition

* strain treated with high light & O₂
Table 8.3. Differences in percent metabolite abundance between conditions in the ΔglgC strain. For a description of conditions, see Table 8.1. A threshold of \( \leq 3\% \) was used, but non-zero values were left in for reference. Color explanations in Table 8.2.

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</tbody>
</table>
Figure 8.1 Metabolites detected in equal sample volumes of wildtype and engineered *Synechococcus* 7002 batch cultures after 50 and 70 h of growth in nutrient replete (NO+ [nitrate] or NH+ [ammonium]), nitrogen-deplete (-N), or sulfur-deplete (-S) A+ medium. Cultures were standardized to equal starting chlorophyll *a* and y-axes represent raw abundance normalized to chlorophyll *a* content of the sample. Cells were grown in Erlenmeyer flasks on a rotary shaker under constant 200 µmol/m² s PAR at 35°C in 1% CO2. Values are not available for glgC after 70 h in NH+. wt (wildtype), las1 (C12 secretion), glgC (ΔglgC), gls3 (ΔglgC and C12 secretion).
generations without antibiotic, strains are still able to grow with 800 µg/mL Gm, suggesting the genotype is not lost.

The results were surprising, in that both synthesis and secretion of lauric acid were diminished in strains containing the overexpression module (Figure 8.2). The rate of fatty acid synthesis may have been enhanced by this modification, also resulting in a higher rate of fatty acid recycling, which is decreased but not completely abolished by the absence of fadD. The MCAT enzyme may also be capable of a fatty acid activating function, but an assessment of its flexibility in catalyzing reactions with longer-chain acyls would be necessary to determine this possibility.

The wildtype and ΔglgC strains were also transformed with this cassette, and a curious but subtle phenotype arose. The AGPase-interrupted backgrounds (ΔglgC and gls3) seemed to develop a deeper green coloration on plates than the wildtype backgrounds (wt and las1), suggesting this possible upregulation in fatty acid synthesis confers an advantage when glycogen and higher glucan carbohydrates are rendered unavailable (Figure 8.3).

Though lauric acid secretion was not increased but was in fact decreased by MCAT overexpression, these results are valuable in understanding this organism’s lipid trafficking behavior. Further investigation should seek to identify other fatty acid recycling enzymes and to assess the fitness of the different backgrounds conferred with increased malonyl-CoA:ACP loading. Metabolite flux and partitioning may also be affected, which would be highly useful both in identifying pathways and enzymes for improved lipid productivity and in further discovery of how *Synechococcus* 7002 adapts to metabolic alterations in its basic biomolecular portfolio.

8.3 Transformations for expression of an alkane operon, β-caryophyllene synthase, and the bacterial thioesterase ‘tesA

The neutral-site integration (NSI) cassette (Chapter 4) was used to introduce other genes into wildtype *Synechococcus* 7002: 1) an operon for the biosynthesis of alkanes from *Synechococcus elongatus* PCC 7942, 2) a synthase for the sesquiterpene β-caryophyllene from *Artemisia annua*, and 3) the *E. coli* thioesterase ‘tesA. The anticipated molecular products were largely unachieved, one explanation for which is genetic adaptation to cease a toxic effect, possibly exacerbated by the high promoter strength of *cpcBA*. Transformants did not appear over
Figure 8.2. Fatty acid methyl esters (FAME) of lauric acid (C12) secreted into the culture medium of engineered Synechococcus 7002 strains grown in batch culture at 34°C under continuous 160 µE/m²/s PAR with 1% CO₂. The las1 (L) mutant contains the LAS module (ΔfadD::C12) and gls3 (G) contains ΔglgC and LAS modifications. M designates overexpression of fabD by the cpcBA promoter in a gentamycin resistant NSI cassette. Grey bars are C12 content in supernatants (triplicate), and red dots are C12 content in one representative FAME extraction of whole culture (cells and medium).

Figure 8.3. Cultures of Synechococcus 7002 wildtype and mutants plated on solid A+ medium. Two plates are shown per strain: nonselective A+ (lefthand) and A+ with 800 µg/mL gentamycin (righthand). The four transformants (righthand) contain the fabD-overexpression cassette, whereas the single culture (lefthand) does not. A: wildtype, B: ΔglgC, C: las1, D: gls3.
several attempts to introduce the alkane operon. Only one initial assay yielded a positive result for transgenic β-caryophyllene synthesis (Figure 8.4), which was not able to be repeated. Supernatants of strains transformed with ‘tesA contained only trace amounts of C16, and C12 secretion from strains also containing LAS was not affected. A weaker promoter may give more favorable results. Sequence information for the alkane operon and β-caryophyllene synthase can be found in Appendix A.

Figure 8.4. GC-FID chromatograms showing apparent accumulation of β-caryophyllene in a representative chloroform/methanol total lipid extraction [Bligh and Dyer 1959] from a 15-mL cell pellet of *Synechococcus* 7002 transformed at a neutral site (NSI) with *cpcBA* driving expression of β-caryophyllene synthase.
8.4 Airlift cultivation system

Construction and testing was done for a bubble-column cultivation system in which PSMs were grown in long glass tubes with air slowly sparged from the bottom. The rising bubbles provide constant mixing and aeration of the culture, possibly affording better access to CO₂ or other gaseous nutrients than in batch culture.

Plans for a custom tube holder are shown in Figure 8.5 and the constructed version with starter cultures is depicted in Figure 8.6. The design was intended to provide maximum exposure to a side-mounted light source while securing the growth columns for connection to an air pump. The configuration and SL-3500-W-J cool white LED light source (Photon Systems Instruments, Brno, Czech Republic) were placed inside an incubation chamber where conditions were set to 35 °C, 170 µE/m²s irradiance, and 1% CO₂. An Aquatop MA-600 aquarium pump (Aquatop, Brea, CA) was used to circulate air, which was purified through a 0.2-µm in-line filter before entering the narrow interior glass tubing into the bottom of the columns.

Regulation of bubble rate and culture pH were found to be important criteria, with a high bubbling rate causing rapid evaporation and acidification of the cultures. A surprisingly small amount of air (1 bubble per second) was needed to maintain visibly viable (unprecipitating) cultures, and a modification to A+ medium was tested for longterm pH maintenance. During preparation of A+, the stock solution of Tris buffer was not corrected (normally to pH 8.2) before it was added, which resulted in a starting A+ medium pH of 9. In regular A+, the pH began at 8.2 and dropped to ~6.5 over 4 days, but in the modified A+ medium, pH decreased only from 9 to 7.9 over 4 d without an apparent effect on cellular viability. Curiously, airlift cultures took on a reddish pigmentation, which could be due to the type of light with which they were grown. Testing with other light sources is necessary to determine the extent of this phenotype, but it appears to be a significant metabolic alteration. A comprehensive assessment of growth rates and other productivities has not yet been undertaken, but it is conceivable that conditions could be more finely and favorably controlled in this type of system than can be achieved with rotary agitation of flask cultures.
Figure 8.5. Technical drawing of a hybridization tube holder for algal cultivation by airlift. Drawings and subsequent construction (Figure 8.6) were done by Ed Dempsey.

Figure 8.6. Glass tubes prepared for airlift cultivation in the custom plexiglass holder shown in Figure 6.21. Each contains a 10-mL glass pipet fitted through a foam stopper. These will be connected to an air pump and placed in front of a light source in a climate-controlled incubator.
8.5 Photos and Artwork

The following material (Figures 8.7 through 8.13) was designed and captured by Victoria H. Work during graduate studies at the Colorado School of Mines (Golden, CO) and the Pacific Northwest National Laboratory (Richland, WA) between 2009-2014.

Figure 8.7. The task of engineering photosynthesis into non-photosynthetic organisms.

Figure 8.8. Late-log stage nutrient replete cultures of *Synechococcus* sp. PCC 7002 wildtype (wt) and associated genetic variants (Table 5.1). A layer of bubbles is visible atop culture medium of the lauric acid-secreting strains.
Figure 8.9. Nitrogen-limited batch cultures of wildtype (wt) *Synechococcus* sp. PCC 7002 and associated genetic variants. The *tes4* and *las1-tesA* cultures contain the bacterial thioesterase ‘tesA’ (Section 8.3). See Table 5.1 for other strain descriptions.

![Image of cultures](image)

Figure 8.10. Schematic of the *Synechococcus* 7002 Δ*glgC* mutant genotype and phenotype under nutrient replete and nitrogen deprived conditions. ADP-Gluc: ADP-glucose (activated), AGPase: ADP-glucose pyrophosphorylase (*glgC*), Gluc-1-P: glucose-1-phosphate, TCA: tricarboxylic acid cycle.

**Organic Acids**

- Acetate
- Succinate
- Ketoisocaproate
- Pyruvate
- Alpha-ketoglutarate

**Atmosphere of glycogen storage**

Secretion of organic acids:

- Gluc-1-P → Intermediates of TCA & protein synth
Figure 8.11. Schematic of the *Synechococcus* 7002 las1 (∆fadD::C12) mutant genotype and phenotype under nutrient replete and nitrogen deprived conditions. ACP: acyl carrier protein, C12: lauric acid, C16: palmitic acyls joined with a lipid head group, hTE: heterologous thioesterase (*fatB1*), PDH: pyruvate dehydrogenase.

Figure 8.12. Schematic of the *Synechococcus* 7002 gls3 (∆gglC, ∆fadD::C12) mutant genotype and phenotype under nutrient replete and nitrogen deprived conditions. Accase: acetyl-CoA carboxylase, C12: lauric acid, C16: palmitic acyls joined with a lipid head group, hTE: heterologous thioesterase (*fatB1*), 3PG: 3-phosphoglycerate (from carbon fixation).
Figure 8.13. L: Schematic of *Synechococcus* 7002 and metabolic elements during nutrient replete and nitrogen-deprived cultivation. R: Schematic of photosynthetic metabolism.
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APPENDIX A
DNA SEQUENCES

Primers for modified pAQ1Ex plasmids

Neutral site (NSI) for homologous recombination (HR) between A0935 and A0936
Upstream flank (NsiI/EcoRI)
F: 5’ agttcacATGCATAaatcataattctcgagtcagaatttacaacag 3’
R: 5’ cgtatagGAATTCTtaactcttttaagtaattcagacaaattcatcatgcatctctct 3’
Downstream flank (SalI/SphI)
F: 5’ gcatacGTGACctatttatactctttctccagagtagcaggacattt 3’
R: 5’ agttcacGCATGCagaggtgagtagtccagactaactaaacgaagtt 3’

Integration site to interrupt the Synechococcus 7002 A0675 fadD gene, a putative acyl-ACP synthetase, by HR
Upstream flank (NsiI/EcoRI)
F: 5’ agttcacATGCATggctaggttcgtaatctttgggggta 3’
R: 5’ cgtatagGAATTGccgaatctgctacagtctctttt 3’
Downstream flank (SalI/SphI)
F: 5’ gcatacGTGACgacccgagagggtagcgcggctgctt 3’
R: 5’ agttcacGCATGCgtgctcgttttttcaacctttg 3’

Primers for integration of metabolic genes into pAQ1Ex-based vectors
(NcoI/BamHI)

Uc fatB1: C12 (lauroyl)-ACP thioesterase from Umbellularia californica, codon optimized for Synechocystis sp. PCC 6803
F: 5’ aatctagCCATGGCCctaccacctcttttagctttttctgc 3’
R: 5’ aagtcatGGATCCttacacgchgtgcggggc 3’
BCS: β-caryophyllene synthase from *Artemisia annua*, codon optimized for *Synechococcus* 7002
F: 5’ aggtctcCCATGGGCagctaaaag 3’
R: 5’ gggtctcGGATCCttaatgggaatgc 3’

*AgBIS*: bisabolene synthase from *Abies grandis* codon optimized for *Synechococcus* 7002
F: 5’ aatctagCCATGGccgtgtgcagctgaagtaaag 3’
R: 5’ aagtcatGGATCCttataacggcaacggtcgtgagacactctttg 3’

Malonyl-CoA:ACP transacylase (S-malonyltransferase, *fabD*, A2559), endogenous to *Synechococcus* 7002
F (NcoI): 5’ aatctagCCATGGGCGgtaaatcggtgtctttcgggccaa 3’
F (NdeI gives His tag in pAQ1Ex): 5’ tggagggCATATGggtaaatgctgggttctttcgggccaa 3’
R (BamHI): 5’ aagtcatGGATCCctagccataactggtgaggtatgacactttg 3’

**Other primers and genes**

*glgC*: ADP-glucose pyrophosphorylase of *Synechococcus* 7002
F: 5’ tggttctacgtctgtcgtgctgctgagtaa 3’
R: 5’ tagctcgtcgtcgtcgtgctgctgagtaa 3’

*aadA*: Spectinomycin resistance, from pAQ1Ex (BamHI/Sall)
F: 5’ aagtcatGGATCCggtctgtaacaagcc 3’
R: 5’ tagactaGTCGACctagctcgagctgacttttagcttgcagttgccttgcag 3’

*aphII*: Kanamycin resistance from pJ201 (DNA 2.0 cloning vector) with restriction sites for insertion into pAQ1Ex (BamHI/Sall)
F: 5’ aagtcatGGATCCatgagccatattcagctcagctgaccgcc 3’
R: 5’ agtttcaGTCGACTtagaaaaactcatcagctcagctcagctcagctc 3’
Gene sequence of the *Synechococcus elongatus* PCC 7942 two-gene alkane operon, codon optimized for *Synechococcus* sp. PCC 7002 and containing NcoI/BamHI restriction sites for pAQ1Ex integration. The sequence contains two genes: 1594 is a putative acyl-ACP reductase, and 1593 is a putative alkanal decarbonylase/alkanal decarboxylative monooxygenase [Schirmer et al. 2010].

CCATGGG

Promoter library

Information for genes associated with the promoters described in Chapter 4 appears below and was provided by Erin R. Stephens.

Promoter Name: cruP
Gene Name: cruP
Description: Lycopene cyclase
NCBI-GI: 170076678
NCBI-GenelID: 6056726
Gene Location in Genome: 46832…48373

Promoter Name: eepF
Gene Name: <none>
Description: Endonuclease/exonuclease/phosphatase family protein
NCBI-GI: 170076537
NCBI-GenelID: 6054884
Gene Location in Genome: pAQ7 68503…73563
Promoter Name: hemH
Gene Name: hemH
Description: Ferrochelatase
NCBI-GI: 170079183
NCBI-GeneID: 6056609
Gene Location in Genome: 2709800…2710960

Promoter Name: ldhA
Gene Name: ldhA
Description: Fermentative lactate dehydrogenase
NCBI-GI: 170076634
NCBI-GeneID: 6054991
Gene Location in Genome: pAQ7: 184913…185905

Promoter Name: mutT
Gene Name: mutT
Description: Mutator mutt protein
NCBI-GI: 170076695
NCBI-GeneID: 6057585
Gene Location in Genome: 65831…66229

Promoter Name: narB
Gene Name: narB
Description: Nitrate reductase
NCBI-GI: 170077929
NCBI-GeneID: 6056923
Gene Location in Genome: 1368906…1371134

Promoter Name: prdE
Gene Name: <none>
Description: Pyruvate dehydrogenase E1 component subunit alpha
NCBI-GI: 170076981
NCBI-GeneID: 6056987
Gene Location in Genome: 372151…373182

Promoter Name: rbcX
Gene Name: rbcX
Description: RbcX protein, rubisco chaperone
NCBI-GI: 170078403
NCBI-GeneID: 6055340
Gene Location in Genome: complement (1882276…1882680)
Promoter Name: rvtR
Gene Name: <none>
Description: Reverse transcriptase (RNA-dependent DNA polymerase)
NCBI-GI: 170079539
NCBI-GeneID: 6057956
Gene Location in Genome: pAQ5: 19438…20457

Promoter Name: tstR
Gene Name: <none>
Description: Thiosulfate sulfurtransferase
NCBI-GI: 170077529
NCBI-GeneID: 6056558
Gene Location in Genome: 940327…941604
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Biofuels (Future Science)

Environmental Progress and Sustainable Energy (American Institute of Chemical Engineers)
Beliaev, Alex S.

Victoria Work <work@mymail.mines.edu>

Mar 7

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Victoria Work
Colorado School of Mines
work@mines.edu

Beliaev, Alexander S <Alex.Beliaev@pnnl.gov>

Mar 11

Yes to the Biofuels review. I also don’t have any problems with data generated by you while working at PNNL that directly relates to the Synechococcus 7902 mutant strains.

However, I will need more information on any other material from posters and presentations that you want to include in your thesis before providing you with a decision. There can be certain sensitivities when it comes to unpublished data that were generated by other researchers and are not directly related to your project.

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To: Beliaev, Alexander S
Subject: Permission to include coauthored material in my thesis

Victoria Work <work@mymail.mines.edu>

May 5

Hello Dr. Beliaev,

With regard to your request for more information, I plan to include the turbidostat growth, O2, PAM, and biochemical assays in my thesis. These correspond to the material presented in this manuscript:


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Thank you very much.

Beliaev, Alexander S

May 6

7:15 PM (17 hours ago)

yes

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Beliaev, Alexander S

May 6

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Sarah D’Adamo <sdadamo@mines.edu>

Mar 7

Dear Victoria,

Of course yes. Neither need to ask for.

Sincerely,

Sarah

On: Sarah D’Adamo (PostDoc researcher)

Colorado School of Mines

Chemistry Dept.

Golden, CO - USA

Darzins, Al

Al darzins@st.com uk@mc.ac.com

to me

Dear Victoria,

Yes, you have my permission. Good luck with the thesis.

Best regards,

Al

Sent from my iPhone

On Mar 7, 2014, at 5:03 PM, Victoria Work <vwork@mysmall.mines.edu> wrote:

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vwork@mimnes.edu
Davies, Fiona K.

Victoria Work <work@mymail.mines.edu>

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Fiona Davies <f Davies@mymail.mines.edu>

Mar 9

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Dismukes, G. Charles

Charles Dismukes <cdismukes@rcrutgers.edu>

Mar 7

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All the best,

Charles Dismukes

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work@mymail.mines.edu
Eduafo, Patrick

Patrick Eduafo <peduafo@mymail.mines.edu>

You have my permission.

Best of luck

Patrick

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Victoria Work
Colorado School of Mines
vwork@mines.edu

Elliott, Lee G.

Lee Elliott <lee.elliott@gmail.com>

Hi Victoria,

Yes, you absolutely have my permission to include all of this material in your thesis. Good luck on the defense!

Lee

From: Victoria Work <mailto:vwork@mymail.mines.edu>
Sent: Friday, March 7, 2014 12:38 PM

Subject: Re: Permission to include coauthored material in my thesis

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Victoria Work
Colorado School of Mines
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223
Franks, Dylan T.

Franks, Dylan <dyfranks@castlemail.columbia.edu>
Mar 7

That's an affirmative! YES!

-----

On Fri, Mar 7, 2014 at 2:13 PM, Victoria Work <vwork@mymail.mines.edu> wrote:

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Victoria Work
Colorado School of Mines
vwork@mines.edu

-----

Gu, Huiya

Victoria Work <vwork@mymail.mines.edu> Mar 7

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Co-authored posters, presentations, and/or other preparations (e.g. progress reports).

Thank you and please let me know if you have any questions.

-----

Huiya Gu <hgu@mymail.mines.edu> Mar 7

To: me

Yes!
Hill, Eric A.

Victoria Work <vwork@mymail.mines.edu>  Mar 12

to eric.Hill :

Hello Mr. Hill,

I am writing to obtain permission from you to include in my doctoral thesis material from presentations and preparations of work we have done together. If you agree please reply to this email; a yes is sufficient.

The material, including figures and text, is from the following:


Data logsheets, experimental write-ups, and/or progress reports.

Thank you and please let me know if you have any questions.

Victoria Work
Colorado School of Mines
vwork@mines.edu

Hill, Eric A <Eric.Hill@pmml.gov>  Mar 12

to Hilda, Alexander, Me :

Hilda

Does this get entered into ERICA?

Victoria

I don’t know what permissions I am able to grant, so I’ll defer to Alex.

Eric Allen Hill, CCET, MS
Scientist II
Biological Sciences Division/Microbiology Group
Pacific Northwest National Laboratory

Jinkerson, Robert E.

Robert Jinkerson <robert.jinkerson@gmail.com>  Mar 7

to me :

Dear Victoria,

You have my permission to include any co-authored material into your thesis.

-Robert Jinkerson

On Fri, Mar 7, 2014 at 9:23 AM, Victoria Work <vwork@mymail.mines.edu> wrote:

Hello Dr. Jinkerson,

I am writing to obtain permission from you to include in my doctoral thesis material from publications that we coauthored together. If you agree please reply to this email; a yes is sufficient.

The material, including figures and text, is from the following publications:


Including presented materials:


Co-authored posters, presentations, and/or other preparations (eg. progress reports).

Thank you and please let me know if you have any questions.

Victoria Work
Colorado School of Mines
vwork@mines.edu
Konopka, Allan

Victoria Work <work@myemail.mines.edu>

to Allan.konopka @

Mar 7

Hi Dr. Konopka,

I am writing to obtain permission from you to include in my doctoral thesis material from publications that we coauthored together. If you agree please reply to this email; a yes is sufficient.

The material, including figures and text, is from the following publications:


And presented material:

Co-authored posters, presentations, and other preparations (eg, progress reports)

Thank you and please let me know if you have any questions.

Victoria Work
Colorado School of Mines
work @ mines.edu

Konopka, Allan E <Allan.Konopka@ornl.gov>

Mar 9

Hello Victoria,

Yes, it will be fine to use these materials.

Smooth sailing in finishing your dissertation.

Best regards,

Allan Konopka

Kucek, Leo A.

Victoria Work <work@myemail.mines.edu>

Mar 11

Hi Leo,

I am writing to obtain permission from you to include in my doctoral thesis material from presentations and preparations of work we have done together. If you agree please reply to this email; a yes is sufficient.

The material, including figures and text, is from the following:


Related data, graphs, experimental write-ups, manuscripts, and/or progress reports.

Thank you and please let me know if you have any questions.

Victoria Work
Colorado School of Mines
work @ mines.edu

Leo Kucek

Mar 11

Yes, sounds great. Good luck with the thesis.

Leo

Leo A. Kucek

MS/PhD Student
Cornell University
Biological & Environmental Engineering
Apparatus Lab
511 Newman Hall
Ithaca, NY 14853
(607) 255-0552 (home)
leakue@cornell.edu
engineeringbox.eeornel.edu
(607) 255-0401 (home)
Laurens, Lieve M.

Laurens, Lieve <lieve.laurens@nrel.gov>

To me:

Yes, no problem. Good luck!

Lieve

From: Victoria Work <vwork@mines.edu>
Date: Friday, March 7, 2014 12:50 PM
To: Laurens Laurens <lieve.laurens@nrel.gov>
Subject: Permission to include coauthored material in my thesis

Hello Dr. Laurens,

I am writing to obtain permission from you to include in my doctoral thesis material from publications that we coauthored together. If you agree please reply to this email; a yes is sufficient.

The material, including figures and text, is from the following publications:


Thank you and please let me know if you have any questions.

Victoria Work
Colorado School of Mines
vwork@mines.edu

Melnicki, Matthew R.

Victoria Work <vwork@mines.edu>

To: matthew.melnicki @mines.edu

Hello Mr. Melnicki,

I am writing to obtain permission from you to include in my doctoral thesis material from presentations and preparations of work we have done together. If you agree please reply to this email; a yes is sufficient.

The material, including figures and text, is from the following:


Data logs, experimental write-ups, and/or progress reports.

Thank you and please let me know if you have any questions.

Victoria Work
Colorado School of Mines
vwork@mines.edu

Matt Melnicki

To: me

I, matt melnicki, hereby consent to your using this material in your dissertation.

Thank you for the request, and I wish you the best!

Matt.

227
Meuser, Jonathan E.

Victoria Work <vwork@mines.edu>
Mar 7

Hello Dr. Meuser,

I am writing to obtain permission from you to include in my doctoral thesis material from publications that we coauthored together. If you agree please reply to this email; a yes is sufficient.

The material, including figures and text, is from the following publications:

Work, Redaković, Jinkerson, Meuser, Elliott, Vinyard, Laurens, Dimovski, and Posewitz, 2010. Increased lipid accumulation in the Chlamydomonas reinhardtii star-10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains. Eukaryotic Cell 9:1251-1261

And presented material:


Co-authored posters, presentations, and other preparations (e.g., progress reports).

Thank you and please let me know if you have any questions.

Victoria Work
Colorado School of Mines
vwork@mines.edu

Jonathan Meuser <jonathan.meuser@gmail.com>
Mar 7

Yes

Jonathan E. Meuser, Ph.D.
e-mail: jonathanmeuser@gmail.com
phone: (eight five eight) seven nine four - two nine four eight
mobile phone: (eight five eight) nine five six - nine one four six

Posewitz, Matthew C.

Victoria Work <vwork@mines.edu>
Mar 18

Dr. Posewitz,

I am writing to obtain permission from you to include in my doctoral thesis material from publications that we coauthored together. If you agree please reply to this email; a yes is sufficient.

The material, including figures and text, is from the following publications:


Including presented material:


Co-authored posters, presentations, and/or other preparations (e.g., progress reports, abstracts, proposals, etc.).

Thank you and please let me know if you have any questions.

Matthew Posewitz <mposewitz@mines.edu>
Mar 18

Yes
Radakovits, Randor

Hello Dr. Radakovits,

I am writing to obtain permission from you to include in my doctoral thesis material from publications that we coauthored together. If you agree please reply to this email; a yes is sufficient.

The material, including figures and text, is from the following publications:


And references may be made to:


Thank you and please let me know if you have any questions.

Victoria Work
Colorado School of Mines
work@mines.edu

Randor Radakovits <randor.radakovits@gmail.com> to me Feb 29

Yes, that would be fine.
Congratulations regarding the thesis! When will you defend?

Randor.

Saari, Travis

Hello Mr. Saari,

I am writing to obtain permission from you to include in my doctoral thesis material from presentations and preparations of work we have done together. If you agree please reply to this email; a yes is sufficient.

The material, including figures and text, is from the following:


Data logs, experimental write-ups, and/or progress reports.

Thank you and please let me know if you have any questions.

Victoria Work
Colorado School of Mines
work@mines.edu

Travis Saari to me 9:05 PM (2 hours ago)

Yes
Scholz, Matthew J.

Victoria Work <vwork@mymail.mines.edu>

Mar 7   Mar 7

Hello Dr. Scholz,

I am writing to obtain permission from you to include in my doctoral thesis material from publications that we coauthored together. If you agree please reply to this email; a yes is sufficient.

The material, including figures and text, is from the following publications:


Thank you and please let me know if you have any questions.

Matt Scholz <matscholz@gmail.com>

Mar 5

Work if Permission granted.

Stanish, Lee F.

Victoria Work <vwork@mymail.mines.edu>

Mar 7   Mar 7

Hello Dr. Stanish,

I am writing to obtain permission from you to include in my doctoral thesis material from publications that we coauthored together. If you agree please reply to this email; a yes is sufficient.

The material, including figures and text, is from the following publications:


Thank you and please let me know if you have any questions.

Lee Stanish <lstanish@gmail.com>

Mar 7   Mar 7

Hey there Victoria,
Of course!
Good luck on your thesis, let me know when you present! If it's public then I'd like to try and attend.
Best,
-Lee
Stephens, Erin R.

Victoria Work <vwork@mymail.mines.edu>

to vwork@mines.edu

11:30 PM (10 hours ago)

Hello Ms. Stephens,

I am writing to obtain permission from you to include in my doctoral thesis material from presentations and preprints of work we have done together. If you agree please reply to this email; a yes is sufficient.

The Material, including figures and text, is from the following:


Data logs, experimental write-ups, and/or progress reports.

Thank you, and please let me know if you have any questions.

Victoria Work
Colorado School of Mines
vwork@mines.edu

---

Erin Stephens

to me

7:20 AM (8 hours ago)

Hello Ms. Work,

I gladly give you permission to include work from my Undergraduate Research Project in your thesis, providing for proper citation. I’ve attached three Word documents with the basic summary of what I did, although it is possible that you already have these via other emails.

Best of luck on your defense!

Erin Stephens

---

Vinyard, David J.

Vinyard, David <david.vinyard@yale.edu>

to me

March 7, 2014 3:14 PM

Dear Victoria,

Yes, you have my permission. Congrats on finishing up and all the best.

David

---

From: Victoria Work <vwork@mymail.mines.edu>
Sent: Friday, March 07, 2014 3:14 PM
To: Vinyard, David
Subject: Re: Permission to include coauthored material in my thesis

Hello Dr. Vinyard,

I am writing to obtain permission from you to include in my doctoral thesis material from publications that we coauthored together. If you agree please reply to this email; a yes is sufficient.

The Material, including figures and text, is from the following publications:


Thank you, and please let me know if you have any questions.

Victoria Work
Colorado School of Mines
vwork@mines.edu
On Mar 7, 2014 1:10 PM, "Victoria Work" <vwork@mymail.mines.edu> wrote:

Hello Mr. Vogler,

I am writing to obtain permission from you to include in my doctoral thesis material from publications that we coauthored together. If you agree please reply to this email: a yes is sufficient.

The material, including figures and text, is from the following publications:


And presented material:

Co-authored posters, presentations, and/or other preparations (eg. progress reports).

Thank you and please let me know if you have any questions.

Victoria Work
Colorado School of Mines
vwork@mines.edu