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Genetic Engineering of Cyanobacteria as Biodiesel Feedstock

Anne M. Ruffing, Christine A. Trahan, Howland D.T. Jones

Prepared by
Sandia National Laboratories
Albuquerque, New Mexico 87185 and Livermore, California 94550

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Anne M. Ruffing, Christine A. Trahan, Howland D.T. Jones
Bioenergy and Defense Technologies
Sandia National Laboratories
P.O. Box 5800
Albuquerque, New Mexico 87185-MS1413

Abstract

Algal biofuels are a renewable energy source with the potential to replace conventional petroleum-based fuels, while simultaneously reducing greenhouse gas emissions. The economic feasibility of commercial algal fuel production, however, is limited by low productivity of the natural algal strains. The project described in this SAND report addresses this low algal productivity by genetically engineering cyanobacteria (i.e. blue-green algae) to produce free fatty acids as fuel precursors. The engineered strains were characterized using Sandia's unique imaging capabilities along with cutting-edge RNA-seq technology. These tools are applied to identify additional genetic targets for improving fuel production in cyanobacteria. This proof-of-concept study demonstrates successful fuel production from engineered cyanobacteria, identifies potential limitations, and investigates several strategies to overcome these limitations. This project was funded from FY10-FY13 through the President Harry S. Truman Fellowship in National Security Science and Engineering, a program sponsored by the LDRD office at Sandia National Laboratories.

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Nomenclature

3-PGA	3-phosphoglycerate
aas	acyl-ACP synthetase
ACC	acetyl-CoA carboxylase
accA	ACC subunit, carboxyltransferase α subunit
accB	ACC subunit, biotin carboxyl carrier protein
accC	ACC subunit, biotin carboxylase subunit
accD	ACC subunit, carboxyltransferase β subunit
ACP	acyl-carrier-protein
APC	allophycocyanin
ASP	aquatic species program
C. reinhardtii CC-503	*Chlamydomonas reinhardtii* CC-503
cDNA	complementary DNA
Chl-*a*	chlorophyll-*a*
CO_2	carbon dioxide
CoA	coenzyme A
cpc	phycocyanin
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ESI-MS	electrospray ionization-mass spectrometry
F6P	fructose-6-phosphate
fat1	acyl-ACP thioesterase from *C. reinhardtii* CC-503
FFA	free fatty acid
F_m	maximum fluorescence
F_0	initial fluorescence
F_v	variable fluorescence
$\frac{F_v}{F_m}$	photosynthetic yield
GC/MS	gas chromatography/mass spectrometry
Glc	glucose
HCO_3	bicarbonate
IPTG	isopropyl-β-D-1-thiogalactopyranoside
MCR	multivariate curve resolution
OAA	oxaloacetate
OD	optical density
PBP	phycobiliprotein
PC	phycocyanin
Pcpc	phycocyanin promoter
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PLlacO1	LlacO1 promoter
PPP	pentose phosphate pathway
Prbc	RuBisCO promoter
psbAI	photosystem II reaction center D1 protein

Ptrc	*trc* promoter
PUFA	polyunsaturated fatty acid
PYR	pyruvate
qRT-PCR	quantitative reverse transcriptase PCR
rbc	RuBisCO
rbcL	RuBisCO large subunit
rbcS	RuBisCO small subunit
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	ribulose-1,5-bisphosphate
S. elongatus PCC7942	*Synechococcus elongatus* PCC7942
TAG	triacylglycerol
TCA	tricarboxylic acid
TE	thioesterase
‘*tesA*	truncated thioesterase from *E. coli*
UFA	unsaturated fatty acid

1. INTRODUCTION

This SAND report provides an overview of the research conducted under the Truman Fellowship project entitled 'Genetic Engineering of Cyanobacteria as Biodiesel Feedstock'. Detailed results and analyses can be found in publications that are currently available [1, 2] as well as manuscripts in preparation [3-5] (citation based on intended journal of publication, subject to change). The research described herein was conducted from January 25, 2010 through January 24, 2013.

1.1. Background and Motivation

With the limited availability of fossil fuels and increasing economic demand for oil, energy security is of the utmost importance for economic prosperity and national security. Among the alternative energy options under development, algal biofuels are prime candidates for the supplementation and replacement of conventional petroleum-based fuels. Algal-derived biodiesel is compatible with the existing infrastructure. Unlike corn ethanol, algal biofuels can be produced on marginal, non-arable land, reducing the impact on the agricultural industry [6]. The photosynthetic efficiency of microalgae often supersedes that of plant crops [7], allowing for higher rates of biomass production. And lastly, algae can convert carbon dioxide (CO_2) and sunlight directly into fuels or fuel precursors, simplifying the production process compared to cellulosic based biofuels (Figure 1.1) and offering the potential for greenhouse gas emission reduction due to CO_2 fixation.

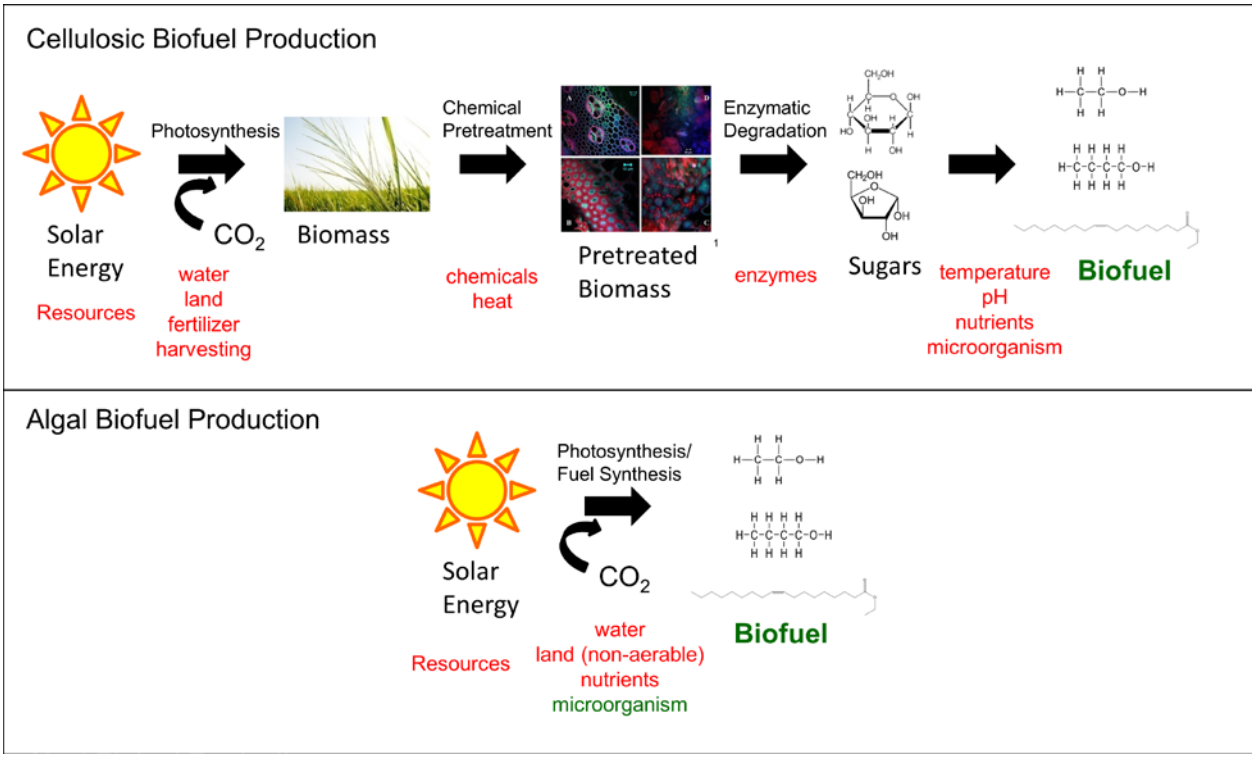

Figure 1.1. Schematic comparing cellulosic and algal biofuel production processes.

Despite the advantages of algal-based fuels, algal biofuel production is not economically competitive with conventional petroleum-based fuels. A major limiting factor in algal biofuel production is the rate of lipid (i.e. fuel-precursor) biosynthesis in microalgae. The natural ability of eukaryotic algae to accumulate large amounts of storage lipid makes them ideal candidates for biofuel production. However, the rate of lipid production is often insufficient for fuel production, and lipid accumulation is typically triggered by a stressor, such as nutrient limitation [8]. The stress-induced lipid accumulation mechanism requires a two-step production process for algal biofuels: a growth step and a stress/lipid accumulation step. Moreover, the lipid accumulates intracellularly, necessitating cell lysis during the extraction process. As a result, traditional algal biofuel production is a batch process, wherein additional biomass must be grown for each round of lipid production. This greatly increases the nutrient requirement for algal biofuels, particularly nitrogen and phosphorous resources which are also essential in the agricultural industry. Life cycle analyses of algal biofuel production often identify this nutrient requirement as a main factor limiting algal biofuel scale-up [9].

Cyanobacteria (formerly known as blue-green algae) are photosynthetic prokaryotic microorganisms, believed to be the progenitor of the chloroplast found in modern day algae and plants [10]. Like eukaryotic algae, cyanobacteria use energy from sunlight to fix CO_2 and form the molecular components required for cell growth, yet unlike eukaryotic algae, cyanobacteria are not known to accumulate significant amounts of storage lipid. In fact, this led the DOE's Aquatic Species Program (ASP) to deem cyanobacteria as 'not useful' for biofuel production [11].

While recombinant DNA technology was developed in the late 1970's and 1980's, the ASP did not consider targeted genetic manipulation until the end of the program in the mid-1990's [12]. With recombinant DNA technology, microalgae can be engineered to improve the production of fuel precursors. Unfortunately, genetic engineering is very challenging to apply to the lipid-accumulating eukaryotic algae due to complications with exogenous DNA transformation, the absence of homologous recombination in many algal species, and reduced gene expression due to RNA-mediated gene silencing or RNA interference (RNAi) [13]. On the other hand, cyanobacteria are very amenable to genetic engineering. Many species of cyanobacteria are naturally transformable, meaning they will naturally uptake exogenous DNA [14]. Like other prokaryotes, cyanobacteria use homologous recombination to integrate DNA into targeted regions of the genome, and their gene expression has not been shown to be regulated by complex mechanisms such as RNAi. The ability to easily engineer cyanobacteria offers many advantages, including the potential to optimize fuel production, to tailor the chemical composition of the fuel product, and to introduce other desirable production traits such as improved product tolerance, resistance to environmental stressors, and defense mechanisms against predators.

In addition to the benefits offered by genetic engineering, cyanobacteria provide a potential advantage for the overall production process: fuel precursor excretion. Cyanobacteria have been shown to excrete fuel precursors like free fatty acids (FFAs) outside the cell [15]. Fuel excretion will simplify the extraction process, and since the cell is not destroyed during fuel harvesting, a continuous production system is feasible. Moreover, this will substantially reduce the requirement for growth nutrients, particularly nitrogen and phosphorous. These genetic and process advantages make cyanobacteria attractive candidates for biofuel production.

1.2. Technical Approach and Objectives

The overall goal of this project is to investigate the feasibility of utilizing cyanobacteria for biodiesel production. For this proof-of-concept demonstration, a target biofuel and host strain must be selected.

1.2.1. Target Fuel

FFAs were selected as the target fuel precursor. FFAs can be easily converted into biodiesel using an acid-catalyzed transesterification [16], such as illustrated in Figure 1.2.

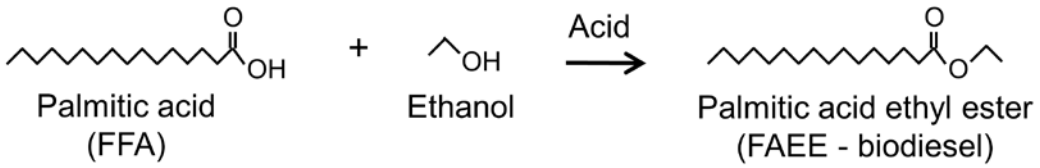

Figure 1.2. Example of acid-catalyzed transesterification of FFA into biodiesel.

Fatty acids are naturally produced by the cell for membrane biosynthesis, and therefore, the metabolic pathway for fatty acid biosynthesis is already present in the cyanobacterial host. Furthermore, FFA excretion has already been demonstrated in cyanobacteria which lack the gene for FFA recycling [15]. As described in the previous section, fuel precursor excretion may be preferable to intracellular product accumulation. These properties make FFAs an ideal target for cyanobacterial fuel production.

1.2.2. Host Selection

A model cyanobacterial strain, *Synechococcus elongatus* PCC7942, was selected as the host for FFA production. *S. elongatus* PCC7942 is naturally transformable, has tools developed for genetic engineering, and its genome sequence is available [17]. The growth rate of *S. elongatus* PCC7942 is conducive for fuel production, with a reported doubling time of 6 - 8 hours [18]. The natural metabolism of *S. elongatus* PCC7942 is more amenable for FFA biosynthesis compared to the other model species as it does not include the pathway for polyhydroxybutyrate biosynthesis, a potential carbon sink. With these desirable traits, *S. elongatus* PCC7942 was chosen as the initial host for engineering FFA production; however, other model strains were also investigated in this project.

1.2.3. *Project Objectives*

Five main objectives were addressed in this project:

1) To genetically engineer *S. elongatus* PCC7942 for biosynthesis and excretion of FFAs by targeting known rate-limiting steps in the cellular metabolism.
2) To characterize the physiology and fitness of the FFA-producing cyanobacterial strains.

3) To identify new targets for enhancing FFA productivity in the engineered cyanobacterial strains.
4) To investigate alternative cyanobacterial hosts for FFA production.
5) To determine the toxic effect of various biofuels on potential cyanobacterial hosts.

These objectives examine important aspects of cyanobacterial-based fuel production, and each objective will be discussed in detail in the following chapters of this SAND report.

1.3. Significance

This project provides a proof-of-concept demonstration for cyanobacterial-based, hydrocarbon fuel production and also probes several areas essential for strain development and fuel production, including host selection and product toxicity. Prior to the start of this project, only a few examples of cyanobacterial-based fuel production were reported [19-22], with most of these studies focusing on low energy density fuels like ethanol and butanol. Thus, this project helps to establish a foundation for high-energy density fuel production in cyanobacteria.

2. EXPERIMENTAL SETUP AND METHODS

2.1. Experimental Setup

The experimental setup illustrated in Figure 2.1 was used to test the engineered strains of *S. elongatus* PCC7942 for FFA production and excretion. *S. elongatus* PCC7942 is photoautotrophic, requiring both light and inorganic carbon (i.e. CO_2, HCO_3) for growth. A mixture of air and 1% CO_2 was humidified to prevent evaporation of the liquid culture, filter sterilized to prevent culture contamination, and bubbled through the liquid culture of BG-11 media. A filtered vent port allowed for pressure relief while also preventing contamination. A photosynthetic light bank, comprised of alternating cool white and plant fluorescent lights, provided illumination in the range of 60 – 70 µmol photons m^{-2} s^{-1}. The culture vessel was a 1 L glass media bottle with a 3-port cap, containing ports for ventilation, bubbling, and sampling. The culture was placed in an Innova 42R shaking incubator which provided mixing at 150 rpm and maintained the temperature at the growth optimum of 30°C. This setup was used for all FFA testing documented in this SAND report.

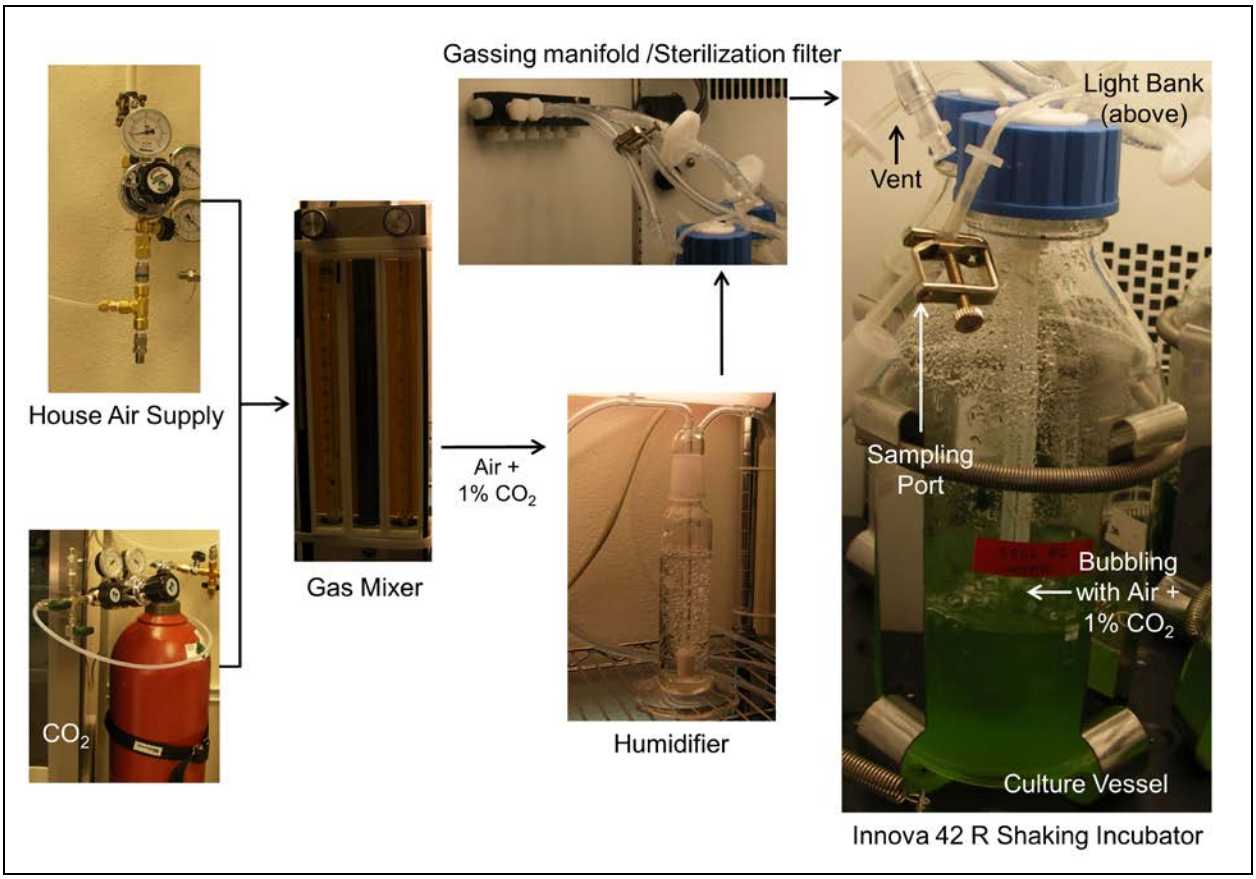

Figure 2.1. Experimental setup for cultivation and testing of *S. elongatus* PCC7942 strains engineered for FFA production.

2.2. Experimental Methods

All experimental methods and data analyses are described in [1-5]. The strains described in this report are listed in Table 2.1 for reference.

Table 2.1. Strains used and constructed in this project.

Strain Name	Description	Reference
Synechococcus elongatus PCC7942	Freshwater cyanobacterium, model organism	ATCC
Synechococcus sp. PCC7002	Marine cyanobacterium, model organism	ATCC
Synechocystis sp. PCC6803	Freshwater cyanobacterium, model organism	ATCC
SE01	*S. elongatus* PCC7942, Δ*aas*; gene knockout of acyl-ACP synthetase (*aas*)	[2]
SE02	*S. elongatus* PCC7942, Δ*aas*, Ptrc-‘*tesA*; expression of truncated thioesterase from *Escherichia coli* (‘*tesA*)	[2]
SE03	*S. elongatus* PCC7942, Δ*aas*, Ptrc-*fat1*; expression of acyl-ACP thioesterase from *Chlamydomonas reinhardtii* CC-503 (*fat1*)	[1]
SE04	*S. elongatus* PCC7942, Δ*aas*, Ptrc-*fat1*-*rbcLS*; overexpression of ribulouse-1,5-bisphosphate carboxylase/oxygenase (*rbcLS*)	[1]
SE05	*S. elongatus* PCC7942, Δ*aas*, Ptrc-*fat1*-*rbcLS*, PLlacO1-*accBCDA*; expression of chloroplast-associated acetyl-CoA carboxylase from *C. reinhardtii* CC-503 (*accBCDA*)	[1]
SE06	*S. elongatus* PCC7942, Δ*aas*, Ptrc-*fat1*-PpsbAI-*rbcLS*, insertion of *psbAI* promoter to drive *rbcLS* expression	[1]
SE07	*S. elongatus* PCC7942, Δ*aas*, Ptrc-*fat1*-PpsbAI-*rbcLS*, Prbc-*accBC*-Pcpc-*accDA*; insertion of *rbc* and *cpc* promoters to drive *accBC* and *accDA* expression	[1]
S01	*Synechococcus* sp. PCC7002, Δ*fadD*; gene knockout of acyl-CoA synthetase (Δ*fadD*)	[4]
S02	*Synechococcus* sp. PCC7002, Δ*fadD*, Ptrc-‘*tesA*; expression of truncated thioesterase from *Escherichia coli* (‘*tesA*)	[4]
S03	*Synechococcus* sp. PCC7002, Δ*fadD*, Ptrc-*fat1*; expression of acyl-ACP thioesterase from *C. reinhardtii* CC-503 (*fat1*)	[4]
S05	*Synechococcus* sp. PCC7002, Δ*fadD*, Ptrc-*tfat1*; expression of truncated acyl-ACP thioesterase from *C. reinhardtii* CC-503 (*tfat1*)	[4]
S06	*Synechococcus* sp. PCC7002, Δ*fadD*, Ptrc-‘*tesA*-*rbcLS*; expression of ribulouse-1,5-bisphosphate carboxylase/oxygenase from *S. elongatus* PCC7942	[4]

	(*rbcLS*)	
S07	*Synechococcus* sp. PCC7002, Δ*fadD*, Ptrc-‘*tesA*-PpsbAI-*rbcLS*; insertion of *psbAI* promoter from *S. elongatus* PCC7942 to drive *rbcLS* expression	[4]
S08	*Synechococcus* sp. PCC7002, Δ*fadD*, Ptrc-‘*tesA-rbcLS*, PLlacO1-*accBCDA*; expression of chloroplast-associated acetyl-CoA carboxylase from *C. reinhardtii* CC-503 (*accBCDA*)	[4]
S09	*Synechococcus* sp. PCC7002, Δ*fadD*, Ptrc-‘*tesA*-PpsbAI-*rbcLS*, Prbc-*accBC*-Pcpc-*accDA*; insertion of *rbc* and *cpc* promoters from *S. elongatus* PCC7942 to drive *accBC* and *accDA* expression	[4]
SE02a	SE02 with genome integration of the empty vector, pSA, at neutral integration site II (NSII)	[3]
SEB2632	SE02, PLlacO1-Synpcc7942_B2632; overexpression of Synpcc7942_B2632	[3]
SE1214	SE02, PLlacO1-Synpcc7942_1214; overexpression of Synpcc7942_1214	[3]
SE0437	SE02, PLlacO1-Synpcc7942_0437; overexpression of Synpcc7942_0437	[3]
SE1845	SE02, PLlacO1-Synpcc7942_1845; overexpression of Synpcc7942_1845	[3]
SE0900	SE02, PLlacO1-Synpcc7942_0900; overexpression of Synpcc7942_0900	[3]
SE1476	SE02, PLlacO1-Synpcc7942_1476; overexpression of Synpcc7942_1476	[3]
SE1655	SE02, PLlacO1-Synpcc7942_1655; overexpression of Synpcc7942_1655	[3]
SE0801	SE02, PLlacO1-Synpcc7942_0801; overexpression of Synpcc7942_0801	[3]
SE0122	SE02, PLlacO1-Synpcc7942_0122; overexpression of Synpcc7942_0122	[3]
SE1656	SE02, PLlacO1-Synpcc7942_1656; overexpression of Synpcc7942_1656	[3]
SE02Δ0444	SE02 with gene knockout of Synpcc7942_0444	[3]
SE02Δ2175	SE02 with gene knockout of Synpcc7942_2175	[3]
SE02Δ1224	SE02 with gene knockout of Synpcc7942_1224	[3]
SE02Δ1464	SE02 with gene knockout of Synpcc7942_1464	[3]
SE02Δ1607	SE02 with gene knockout of Synpcc7942_1607	[3]
7942a	*S. elongatus* PCC7942 with genome integration of the empty vector, pSA, at neutral site II (NSII)	[5]
7942_1214	*S. elongatus* PCC7942 with overexpression of Synpcc7942_1214	[5]
7942_1656	*S. elongatus* PCC7942 with overexpression of Synpcc7942_1656	[5]
7942_0437	*S. elongatus* PCC7942 with overexpression of	[5]

	Synpcc7942_0437	
7942_0801	*S. elongatus* PCC7942 with overexpression of Synpcc7942_0801	[5]
7002Δ*desB*	*Synechococcus* sp. PCC7002 with gene knockout of *desB* (SYNPCC7002_A0159)	[5]
7002Δ*desF*	*Synechococcus* sp. PCC7002 with gene knockout of *desF* (SYNPCC7002_A1989)	[5]
7002Δ*desE*	*Synechococcus* sp. PCC7002 with gene knockout of *desE* (SYNPCC7002_A2833)	[5]
7002ΔA0719	*Synechococcus* sp. PCC7002 with gene knockout of SYNPCC7002_A0719	[5]
7002ΔA1013	*Synechococcus* sp. PCC7002 with gene knockout of SYNPCC7002_A1013	[5]
7002ΔA0585	*Synechococcus* sp. PCC7002 with gene knockout of SYNPCC7002_A0585	[5]

3. ENGINEERING A MODEL CYANOBACTERIUM, *SYNECHOCOCCUS ELONGATUS* PCC7942, FOR FREE FATTY ACID (FFA) PRODUCTION

3.1. Introduction

Fatty acids, the major component of lipid molecules, are essential biomolecules within living cells. The fatty acid side chains of lipid molecules provide the characteristic hydrophobic nature of cell membranes and determine important properties of the membrane including viscosity and permeability. With such an essential and basic role in cellular structure and function, fatty acids and the metabolic pathways for their biosynthesis have been studied extensively [23, 24]. However, most of these studies have focused on plants and the model, Gram-negative bacterium *Escherichia coli* and have concentrated on the fundamental understand of fatty acid biosynthesis, rather than maximizing FFA production.

In this section, we report the successful engineering of the model cyanobacterium *Synechococcus elongatus* PCC7942 for FFA production. The metabolic pathways of *S. elongatus* PCC7942, shown in Figure 3.1, are engineered for FFA production by gene knockout of the FFA recycling enzyme, acyl-ACP synthetase (*aas*), and introduction of a thioesterase (TE) for release of the fatty acid from the acyl-carrier-protein (ACP). Additional improvement in FFA production is sought by targeting known rate-limiting steps of FFA biosynthesis, including carbon fixation via ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and the conversion of acetyl-CoA to malonyl-CoA, catalyzed by acetyl-CoA carboxylase (ACC). Lastly, we enhanced recombinant gene expression to further increase FFA production and excretion.

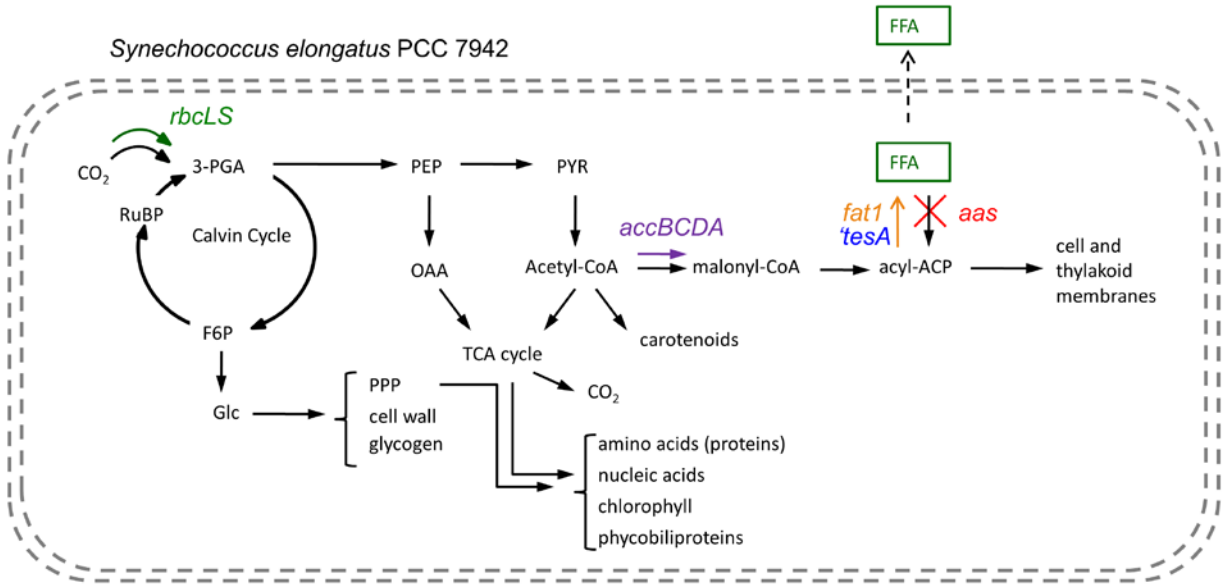

Figure 3.1. Simplified schematic of the metabolism of *S. elongatus* PCC7942 (in black) with genetic engineering targets highlighted in color. X indicates a gene knockout while the arrows indicate gene overexpression. Abbreviations are defined in the Nomenclature section.

3.2. Results and Discussion

3.2.1. Engineering S. elongatus PCC7942 for FFA Production

The first step to engineering FFA production in *S. elongatus* PCC7942 was to eliminate native mechanisms for FFA consumption and recycling. *S. elongatus* PCC7942 was previously shown to recycle FFAs through the acyl-ACP synthetase gene (*aas*) [15]. This enzyme is also highly homologous to acyl-CoA synthetase, which is responsible for FFA consumption through the β-oxidation pathway. Therefore, *aas* was deleted using gene knockout to yield the engineered strain SE01 (Table 3.1). Using the experimental setup described in Section 2, SE01 was shown to produce and excrete FFAs, primarily during the late stationary phase (after 200 hours, Figure 3.2). This accumulation of FFAs is likely due to membrane degradation, as *S. elongatus* PCC7942 does not contain a thioesterase.

Table 3.1. Strains of *S. elongatus* PCC7942 constructed for FFA production

Strain	Genetic Manipulation	Recombinant Operons
SE01	Gene knockout of acyl-ACP synthetase (Δ*aas*)	
SE02	Gene knockout of acyl-ACP synthetase (Δ*aas*); expression of truncated *E. coli* thioesterase (‘*tesA*)	Ptrc – ‘*tesA*
SE03	Gene knockout of acyl-ACP synthetase (Δ*aas*); expression of acyl-ACP thioesterase from *C. reinhardtii* (*fat1*)	Ptrc – *fat1*
SE04	Gene knockout of acyl-ACP synthetase (Δ*aas*); expression of acyl-ACP thioesterase from *C. reinhardtii* (*fat1*); overexpression of native ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcLS*)	Ptrc – *fat1* – *rbcLS*
SE05	Gene knockout of acyl-ACP synthetase (Δ*aas*) ; expression of acyl-ACP thioesterase from *C. reinhardtii* (*fat1*); overexpression of native ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcLS*); expression of chloroplast-associated acetyl-CoA carboxylase from *C. reinhardtii* (*accBCDA*)	Ptrc – *fat1* – *rbcLS* PLlacO1 – *accBCDA*
SE06	Gene knockout of acyl-ACP synthetase (Δ*aas*); expression of acyl-ACP thioesterase from *C. reinhardtii* (*fat1*); overexpression of native ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcLS*) from native *psbAI* promoter; expression of chloroplast-associated acetyl-CoA carboxylase from *C. reinhardtii* (*accBCDA*)	Ptrc – *fat1* – PpsbAI – *rbcLS*
SE07	Gene knockout of acyl-ACP synthetase (Δ*aas*) ; expression of acyl-ACP thioesterase from *C. reinhardtii* (*fat1*); overexpression of native ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcLS*) from native *psbAI* promoter; expression of chloroplast-associated acetyl-CoA carboxylase from *C. reinhardtii* from native *rbc* (*accBC*) and *cpc* (*accDA*) promoters	Ptrc – *fat1* – PpsbAI – *rbcLS* Prbc – *accBC* – Pcpc – *accDA*

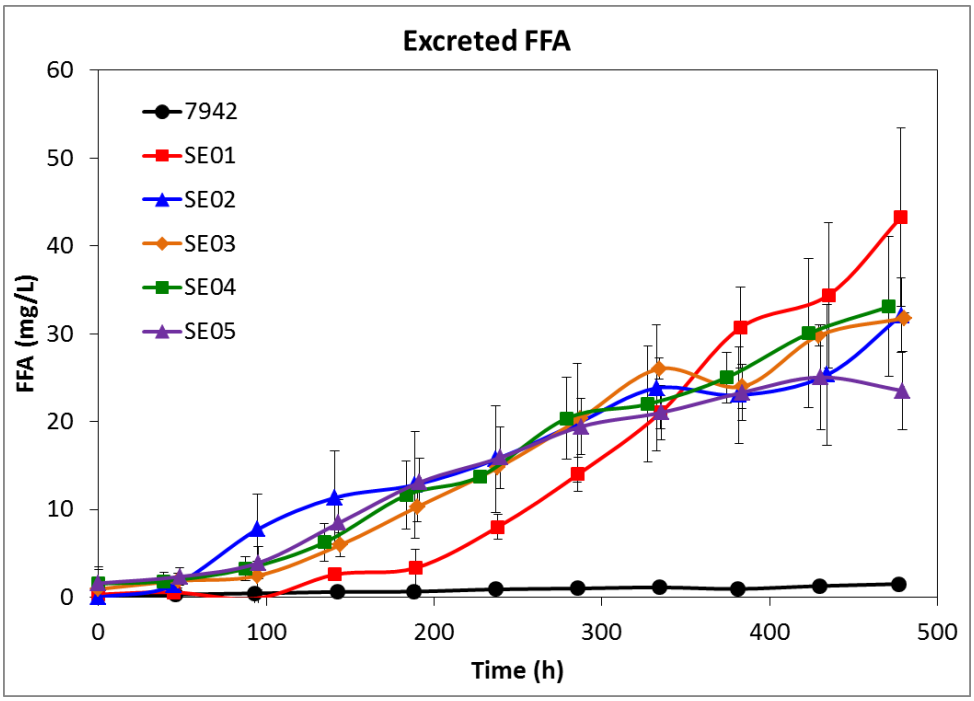

Figure 3.2. Excreted FFA concentration in cultures of wild type and engineered strains of *S. elongatus* PCC7942. Data are averages of three biological replicates and error bars indicate the standard deviation.

Organisms which contain membrane-bound organelles (i.e. eukaryotes) often contain thioesterases (TEs) to cleave fatty acids from the acyl-carrier-protein (ACP). In plants and eukaryotic algae, de novo fatty acid biosynthesis occurs in the chloroplast, a membrane-bound organelle derived from cyanobacteria [24]. Acyl-ACP TEs are therefore required to release the fatty acid for transport into the cytoplasm. In this study, TEs from two different sources were cloned and expressed along with knockout of *aas*. A periplasmic TE was discovered in *E. coli*, and with truncation, this TE was shown to be active in the cytoplasm as well [25]. This truncated *E. coli* TE (*'tesA*) was expressed in SE02 (Table 3.1). Similar to SE01, SE02 produced and excreted FFAs, but SE02 showed increased levels of excreted FFA during earlier time points (before 200 hours, Figure 3.2). Disappointingly, SE02 did not show an improvement in the total amount of excreted FFA at later time points. As discussed previously, eukaryotic algae contain acyl-ACP TEs to release bound fatty acids in the chloroplast; in fact, eukaryotic algae are shown to accumulate high levels of triacylglycerol (TAG), requiring an abundant supply of FFAs [8]. Based on this natural process, eukaryotic algae may possess very efficient acyl-ACP TEs. To test this hypothesis, the acyl-ACP TE from the green alga *Chlamydomonas reinhardtii* CC-503 (*fat1*) was cloned and expressed in *S. elongatus* PCC7942, producing strain SE03. SE03 produced and excreted FFAs, yet there was no detectable increase in the amount of excreted FFAs compared to the SE02 strain (Figure 3.2).

3.2.2. Targeting Rate-Limiting Steps in FFA Biosynthesis

FFA production and excretion was achieved in *S. elongatus* PCC7942 through gene knockout of *aas* and expression of a TE; however, the levels of excreted FFA were still too low to be used for

fuel applications. To boost FFA biosynthesis, we targeted known rate-limiting steps in FFA biosynthesis.

The fixation of CO_2 by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) has long been demarcated as a growth-limiting step in photosynthetic organisms due to the reversible nature of this enzyme and the enzyme's low affinity for CO_2 [26]. As such, RuBisCO improvement has been a long-standing goal in the agricultural industry, but attempts to improve the enzyme kinetics of RuBisCO have met with limited success [27]. In a recent effort to increase isobutryaldehyde production in cyanobacteria, the overexpression of RuBisCO subunits (*rbcLS*) in *S. elongatus* PCC7942 led to a 2-fold improvement isobutryaldehyde production [19]. Inspired by this success, we overexpressed the native RuBisCO subunits in the engineered strain SE04 (Table 3.1), along with TE expression and *aas* knockout. Unfortunately, RuBisCO overexpression in SE04 did not have a significant effect on FFA production and excretion (Figure 3.2), suggesting that another step within the FFA biosynthesis pathway is rate-limiting.

In *E. coli*, the first committed step of the fatty acid biosynthesis pathway, catalyzed by acetyl-CoA carboxylase (ACC), was found to be rate-limiting [28]. The fatty acid biosynthesis pathway interfaces with the primary metabolism at the acetyl-CoA node, and thus, ACC represents an important target for metabolic engineering because its activity will affect the carbon flux at the key metabolic node. Acetyl-CoA not only supplies carbon for fatty acid biosynthesis, it also feeds the tricarboxylic acid (TCA) cycle which plays a role in energy provision and synthesizes essential biomolecules for cell growth. The carbon flux distribution of acetyl-CoA must therefore be balanced for optimal FFA production without significantly compromising cell growth. In eukaryotic algae, there are 2 forms of ACC: a eukaryotic, cytosolic, and multi-domain ACC, and a prokaryotic, chloroplast-associated, multi-subunit ACC. In this work, we cloned the chloroplast-associated ACC, as this complex is responsible for *de novo* fatty acid biosynthesis and this form of the enzyme should be active within the cyanobacterial host (i.e. prokaryote). The model green alga *C. reinhardtii* CC-503 was used as the gene source, and the four ACC genes (*accBCDA*) were expressed in SE05 (Table 3.1). Once again, FFAs were produced and excreted by SE05, yet the excreted FFA concentration did not improve (Figure 3.2).

3.2.3. Improving Recombinant Gene Expression

Despite targeting the rate-limiting step in fatty acid biosynthesis, FFA production remains low in the engineered strains of *S. elongatus* PCC7942, indicating that another mechanism may be responsible for limiting these yields. While *S. elongatus* PCC7942 is a model organism, only a limited number of promoters have been used for recombinant protein expression in this strain. Furthermore, the recombinant operons in strains such as SE04 and SE05 rely on one promoter for the expression of multiple genes (Ptrc – *fat1* – *rbcLS*, PLlacO1 – *accBCDA*). To determine if recombinant gene expression may limit FFA production, quantitative reverse transcriptase PCR (qRT-PCR) was used to measure the relative transcript levels of the recombinant gene targets: *fat1, rbcL, rbcS, accB, accC, accD,* and *accA.*

Both promoters (Ptrc and PLlacO1) are IPTG inducible promoters constructed for expression in *E. coli*. To determine if these promoters are functionally inducible in *S. elongatus* PCC7942, transcript levels before (100 h) and after (288 h) IPTG addition were compared for each gene

(Figure 3.3A,B). Out of the 7 recombinant genes targeted for inducible expression, only *fat1* had significant gene expression after induction (Figure 3.3A). This suggests that the expression of downstream genes in the recombinant operon (*rbcL* and *rbcS*) is not subject to regulation by the *trc* promoter. In addition, these results indicate that the *LlacO1* promoter does not yield inducible gene expression in *S. elongatus* PCC7942 (Figure 3.3B).

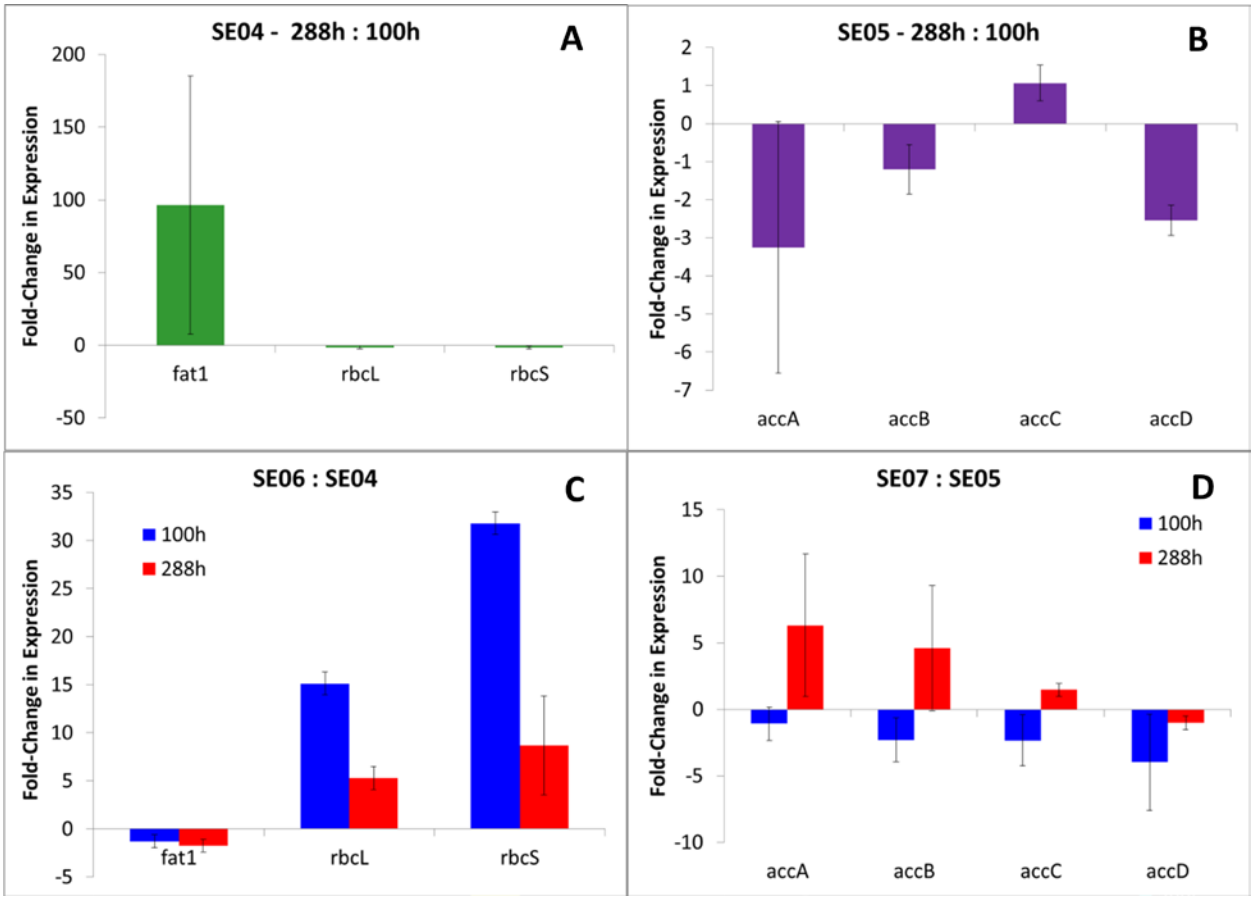

Figure 3.3. A comparison of gene expression levels (A,B): before (100 h) and after (288 h) IPTG induction in SE04 and SE05 and (C,D): in strains expressing native promoters (SE06, SE07) vs inducible promoters (SE04, SE05). Data are averages of three biological replicates and error bars indicate the standard deviation.

To improve recombinant gene expression of *rbcLS* and *accBCDA*, native promoters were cloned from *S. elongatus* PCC7942 and integrated into the recombinant operons. In the SE06 strain (Table 3.1), the promoter for *psbAI*, a protein component of photosystem II, was inserted upstream of *rbcLS* to enhance gene expression, and in SE07 (Table 3.1), the promoters for RuBisCO (*rbc*) and the phycocyanin operon (*cpc*) were integrated upstream of *accBC* and *accDA*, respectively. Expression of the RuBisCO subunits *rbcL* and *rbcS* increased with addition of the *psbAI* promoter in SE06 (Figure 3.3C), yet expression of the ACC genes *accB, accC, accD,* and *accA* did not improve significantly with addition of the *rbc* and *cpc* promoters (SE07), relative to the LlacO1 promoter (SE05) (Figure 3.3D). These results indicate that *rbcLS* expression was limited in the Ptrc-*fat1*-*rbcLS* operon and that *accBCDA* expression in the PLlacO1-*accBCDA* operon is adequate but not regulated by IPTG induction. Despite the

improvement in gene expression in the SE06 and SE07 strains, excreted FFA levels did not show improvement.

3.3. Conclusions

The work described in this section of the SAND report constitutes a proof-of-concept demonstration of FFA production and excretion in *S. elongatus* PCC7942. Several strategies for improving FFA production were also investigated, including the overexpression of predicted rate-limiting steps (*rbcLS* and *accBCDA*) and the improvement of recombinant gene expression through the use of native, constitutive promoters (PpsbAI, Prbc, and Pcpc). Both strategies failed to increase the FFA yield from the engineered strains of *S. elongatus* PCC7942, suggesting that other factors must limit FFA production. In the next section, the FFA-producing strains are characterized in an attempt to identify these other limiting factors.

4. CHARACTERIZATION OF FFA-PRODUCING *SYNECHOCOCCUS ELONGATUS* PCC7942

4.1. Introduction

In the previous section, *S. elongatus* PCC7942 was successfully engineered for FFA production; however, FFA levels failed to improve with additional metabolic engineering efforts. To identify other potential limiting factors, the FFA-producing strains of *S. elongatus* PCC7942 were analyzed to assess physiological changes, including changes in cell growth, stress, membrane integrity, photosynthetic yield, and photosynthetic pigments. In addition to uncovering the factors limiting FFA production, this characterization also provides valuable information for the implementation of large-scale fuel production, as these physiological properties will also affect overall biomass yield and strain robustness. Biomass yield will directly impact the productivity of FFA production, while strain robustness will dictate the frequency of culture collapse or 'pond crash'. While optimizing FFA production is the primary objective for fuel production, we must do so without significantly compromising cell health and fitness. Strain characterization is therefore an essential phase in strain development. This section details the characterization of the FFA-producing strains constructed in Section 3 of this SAND report: SE01, SE02, SE03, SE04, SE05, SE06, and SE07, along with the wild type, *S. elongatus* PCC7942.

4.2. Results and Discussion

4.2.1. FFA Production Effects Cellular Health: Growth, Stress, and Membrane Permeability

The overall FFA productivity of a culture is determined by the rate of FFA biosynthesis and the growth rate of the cell. FFA production and excretion inherently impacts cell growth because the FFAs are siphoned directly from the pathway for cell and photosynthetic (i.e. thylakoid) membrane biosynthesis (Figure 3.1). Hence, some decrease in cell growth is expected with FFA production, yet with the low levels of excreted FFA observed for the engineered strains (< 50 mg/L), the decrease in cell growth should not be very significant. The measured cell concentrations of the FFA-producing strains are severely decreased compared to the wild type, with the final cell concentrations reduced by as much as 80% in some strains (Figure 4.1). Clearly, the 880 mg/L reduction in cell concentration for SE05 cannot be accounted for by the 20 mg/L of excreted FFAs. The reduction in cell concentration appears to correlate with the degree of genetic manipulation, as SE05 and SE07, strains expressing the most potential rate-limiting steps in FFA biosynthesis, have the greatest reduction in cell concentration. This implies that the rate of FFA production, particularly during the exponential growth phase, has a detrimental effect on cell growth. Alternatively, this may suggest that the metabolic burden of recombinant protein production negatively effects cell growth, for SE05 and SE07 also have the most recombinant genes targeted for expression (Table 3.1). The growth inhibition concomitant with FFA production is potentially a showstopper for cyanobacterial-based fuel production. To investigate what may be causing the reduced cell concentration in the FFA-producing strains, cellular stress and membrane permeability were assessed.

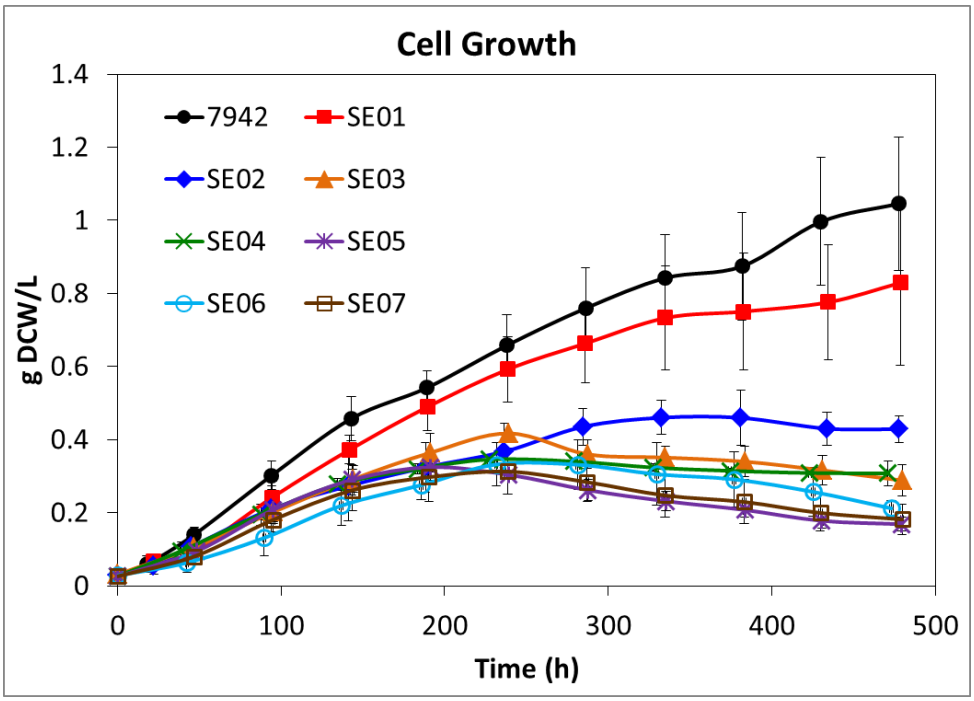

Figure 4.1. Cell concentration, grams of dry cell weight (DCW) per liter, in cultures of wild type and engineered strains of *S. elongatus* PCC7942. Data are averages of three biological replicates and error bars indicate the standard deviation.

The generation of reactive oxygen species (ROS) is often an indicator of cell stress. Elevated ROS levels have been shown to correlate with a variety of stress conditions including nutrient limitation, high light stress, heat stress, and even programmed cell death [29]. ROS levels were measured in the FFA-producing strains SE01 and SE02 along with the wild type for comparison (Figure 4.2A). ROS levels were elevated in the SE02 strain, with approximately 35-55% of the cell population staining positive for ROS after induction. Less than 10% of the SE01 population tested positive for ROS despite the high level of FFA accumulation at later time points (Figure 3.2). While ROS levels do not appear to correlate to the amount of excreted FFA, there is good correlation between the ROS and cell concentration data (Figure 4.1). From this, we conclude that oxidative stress, indicated by ROS, contributes to the reduced cell concentrations in FFA-producing strains; however, it cannot be determined whether this stress is resulting from FFA production or recombinant protein production.

FFA excretion may be directly responsible for the observed decrease in cell growth and elevated oxidative stress. While FFA transport across cell membranes has been documented in several organisms [28, 30], the mechanism(s) for this transport remains unknown. There has been extensive kinetic analyses of FFA membrane transport, but scientists have drawn conflicting conclusions in these studies, some advocating passive diffusion as the sole mechanism and others claiming active transport plays a role [31]. Regardless of the possible contribution from active transporters, FFAs will likely integrate into the cell and photosynthetic membranes due to their hydrophobic side chains. In turn, this will affect the activity of membrane-bound proteins, like those responsible for photosynthesis, as well as the structural integrity of the cell membrane. A membrane-impermeable nucleic acid stain, SYTOX Green, was used to interrogate the cell membrane permeability of the wild type (7942) and FFA-producing strains SE01 and SE02

(Figure 4.2B). Membrane permeability increased in both SE01 and SE02 populations. Interestingly, membrane permeability and ROS levels were well correlated for SE01, but for SE02, the percentage of membrane permeable cells is much lower than the percentage of ROS positive cells. This indicates that increased membrane permeability did not cause the increase in cellular stress. There is some correlation between membrane permeability and excreted FFA concentration, yet membrane permeability does not exclusively regulate FFA excretion, as excreted FFA concentrations for SE01 are higher than for SE02 at 480 h (Figure 3.2) but the percentage of membrane permeable cells is lower for SE01 at this time point (Figure 4.2B).

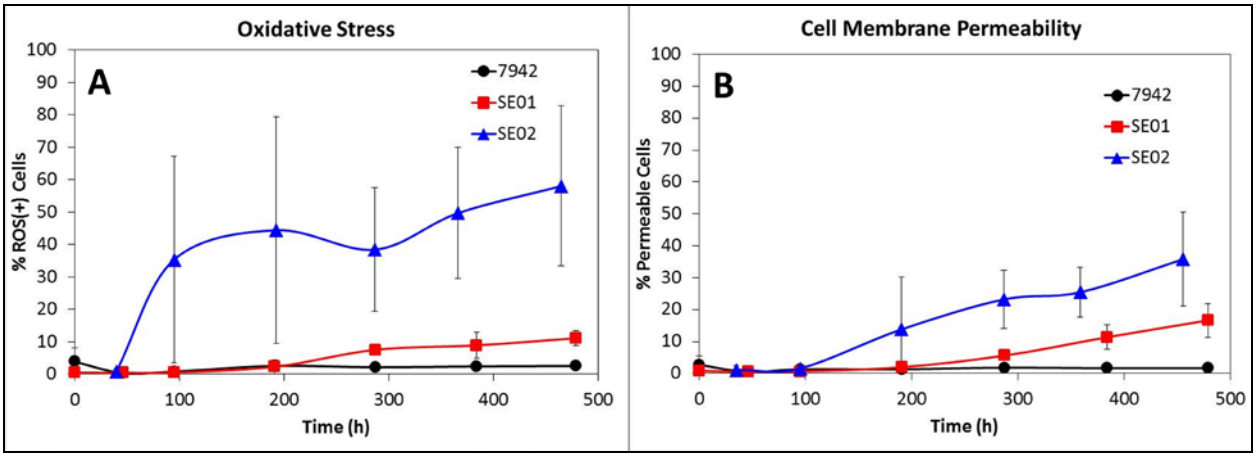

Figure 4.2. Oxidative stress (A) and membrane permeability (B) measurements of cell populations for the FFA-producing SE01 and SE02 strains along with the wild type, 7942. Data are averages of three biological replicates and error bars indicate the standard deviation.

The strain characterization presented in this section reveals some general trends between data sets: (1) cell growth and cell stress are negatively correlated and (2) excreted FFA concentration and cell membrane permeability are positively correlated. However, the analysis is complicated by the fact that these physiological measurements do not relate to the quantitative level of excreted FFA. The highest concentration of excreted FFA (~ 45 mg/L) is measured for SE01 at approximately 480 h, but SE02 shows a greater reduction in cell growth as well as higher levels of stress and membrane permeability. This suggests that the detrimental physiological effects are either specific to fatty acid release during the exponential growth phase or due to recombinant protein production.

4.2.2. Photosynthetic Effects of FFA Production in S. elongatus PCC7942

For cyanobacterial-based fuel production, photosynthesis determines the rates of energy production and carbon fixation, thereby controlling the growth rate and rate of FFA biosynthesis. Interruption or damage to the photosynthetic process must be closely monitored and eliminated for optimal fuel production. To assess the effects of FFA production on photosynthesis, we measured photosynthetic yields and analyzed changes in photosynthetic pigments.

Photosynthetic yield indicates the efficiency of electron transfer within photosystem II and is calculated from a fluorescence measurement using the following equation:

$$\frac{F_v}{F_m} = \frac{F_m - F_0}{F_m} \quad (2)$$

where F_v is the variable fluorescence, F_m is the maximum fluorescence from a saturating pulse of light, and F_0 is the initial fluorescence under actinic light. All of the FFA-producing strains of S. *elongatus* PCC7942 had a significant drop off in photosynthetic yield after induction at 100 h (Figure 4.3A). During later time points (300 – 500 h), photosynthesis was severely compromised, with photosynthetic yields near zero for many of the engineered strains.

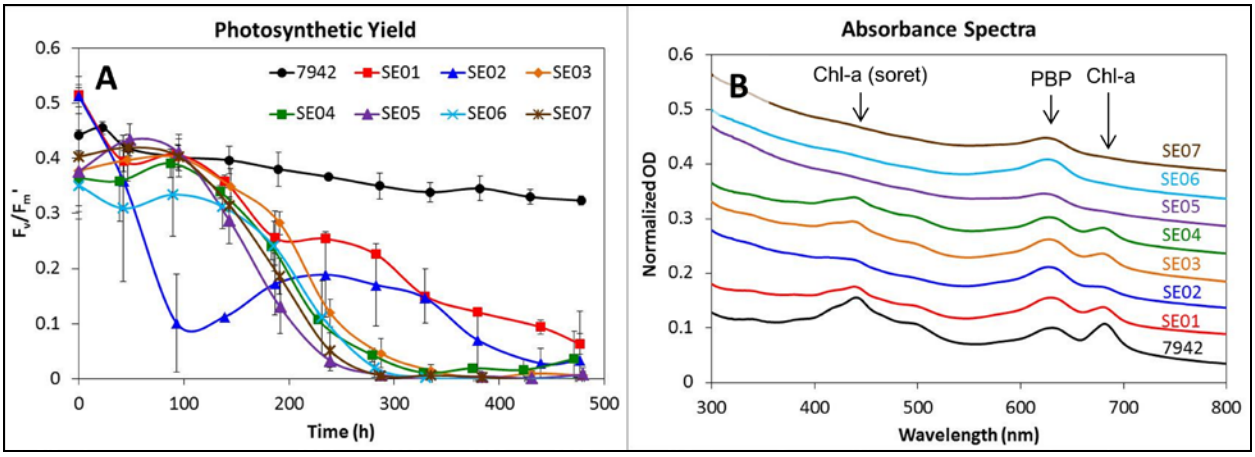

Figure 4.3. Photosynthetic yield (A) and photosynthetic pigment absorption (B) for the FFA-producing strains and wild type, 7942. The photosynthetic yield data are averages of three biological replicates and error bars indicate the standard deviation.

The photosynthetic pigments in *S. elongatus* PCC7942, chlorophyll-a (Chl-a) and the light-harvesting phycobiliproteins (PBP), were measured from their characteristic absorption peaks (Figure 4.3B). Interestingly, the FFA-producing strains showed reduced Chl-a levels but had little variation in the amount of PBP. To gain further insight into this change in photosynthetic pigments, we utilized Sandia's capability of hyperspectral confocal fluorescence microscopy and multivariate curve resolution (MCR) analysis to obtain information on the subcellular location of the photosynthetic pigments. The hyperspectral imaging confirmed the reduced levels of Chl-a in the FFA-producing SE02 strain compared to the wild type (Figure 4.4). The phycobiliproteins, phycocyanin (PC) and allophycocyanin (APC), appeared to be aggregated at the cell poles (Figure 4.4), suggesting that either the phycobilisomes could not attach to the thylakoid membrane or the structure of the thylakoid membrane was compromised.

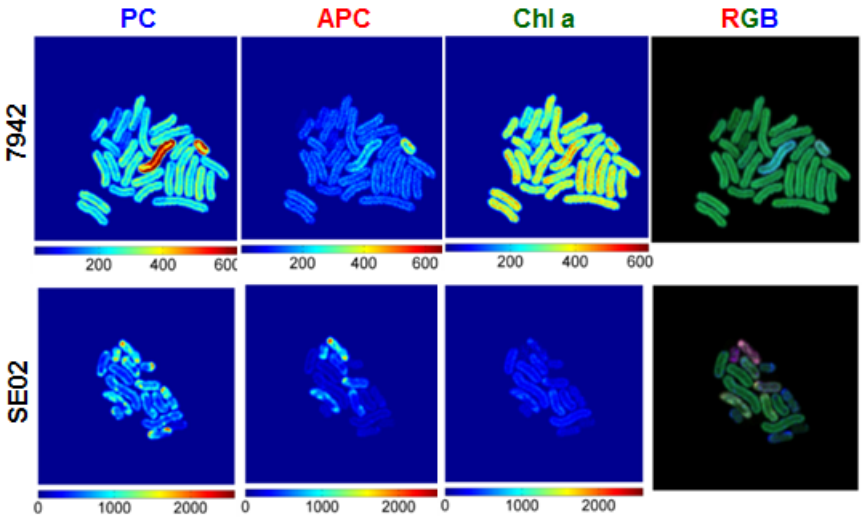

Figure 4.4. Concentration plots of the photosynthetic pigments, phycocyanin (PC), allophycocyanin (APC) and chlorophyll-a (Chl a), in wild type *S. elongatus* PCC7942 and the FFA-producing strain SE02. The images are constructed with data obtained from hyperspectral confocal fluorescence microscopy and analyzed using MCR analysis.

The changes in photosynthetic yield and pigments in the FFA-producing strains of *S. elongatus* PCC7942 suggest that photosynthesis is severely impaired. This will have cascading effects on cellular energy production, carbon fixation, cell growth, and overall cell health. The underlying mechanisms leading to these physiological effects must be identified and addressed before improvement in FFA production can be realized.

4.2.3. Exploring the Underlying Mechanisms of FFA-Induced Effects

Several mechanisms may contribute to the observed physiological effects of FFA production in *S. elongatus* PCC7942. In this study, we investigated two of these mechanisms: (1) altered membrane composition and (2) FFA toxicity.

In the engineered strains of *S. elongatus* PCC7942, the native metabolism has been modified to extract fatty acids from the pathway for membrane biosynthesis (Figure 3.1). The thioesterases ('TesA and Fat1) which cleave the fatty acid from the acyl-carrier-protein (ACP) may selectively act on specific chemical forms of fatty acid, thereby changing the chemical composition of fatty acids that are supplied for cell and thylakoid membrane biosynthesis. The hyperspectral imaging data (Figure 4.4) provides some indirect evidence to support this mechanism. The aggregation of phycobiliproteins at the cell poles may indicate that the phycobilisomes cannot attach to the thylakoid membranes, possibly due to changes in the chemical composition. To further explore this potential mechanism, the chemical composition of membrane lipids was analyzed using electrospray ionization mass spectrometry (ESI-MS). Compared to the wild type, the FFA-producing strain, SE02, had higher levels of saturated and monounsaturated fatty acid moieties in the thylakoid membrane lipids and lower levels of polyunsaturated fatty acid chains (Figure 4.5). This change in the degree of membrane saturation will result in increased membrane viscosity, which may influence the attachment of phycobilisomes and the activity of membrane-bound proteins such as those involved in photosynthesis.

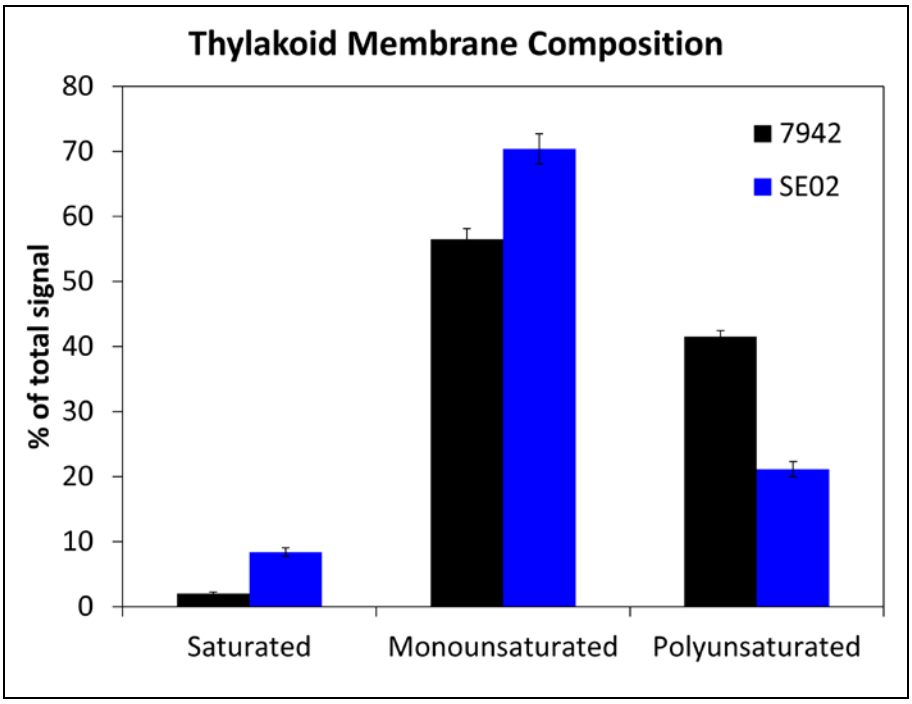

Figure 4.5. Degree of saturation of fatty acids in thylakoid membrane lipids for wild type, 7942, and FFA-producing SE02. Data are averages of three biological replicates and error bars indicate the standard deviation.

In addition to altering membrane composition, the FFAs themselves may be toxic to the host cell. The antimicrobial activity of FFAs is well-documented in the literature, with cytotoxic effects observed for organisms including microalgae, Gram-positive and Gram-negative bacteria, fungi, viruses, and even multi-cellular organisms [32, 33]. The potential toxicity of FFAs was tested by adding exogenous FFAs to cultures of the wild type, *S. elongatus* PCC7942. Addition of saturated fatty acids, palmitic (C16:0) and stearic (C18:0) acids, had no observable effect on cell growth, photosynthetic yield, or photosynthetic pigments (Figure 4.6A). Addition of the unsaturated linolenic acid (C18:3), however, led to reduced cell growth and photosynthetic yield as well as loss of both PBP and Chl-a pigments (Figure 4.6B,C). While this toxic effect of unsaturated fatty acids may contribute to the physiological changes observed in the FFA-producing strains, the physiological response of exogenous linolenic acid addition differs from that of the FFA production. In FFA-producing strains, the Chl-a pigment is selectively degraded (Figure 4.3B), while exogenous linolenic acid addition results in the degradation of all photosynthetic pigments (Figure 4.6C).

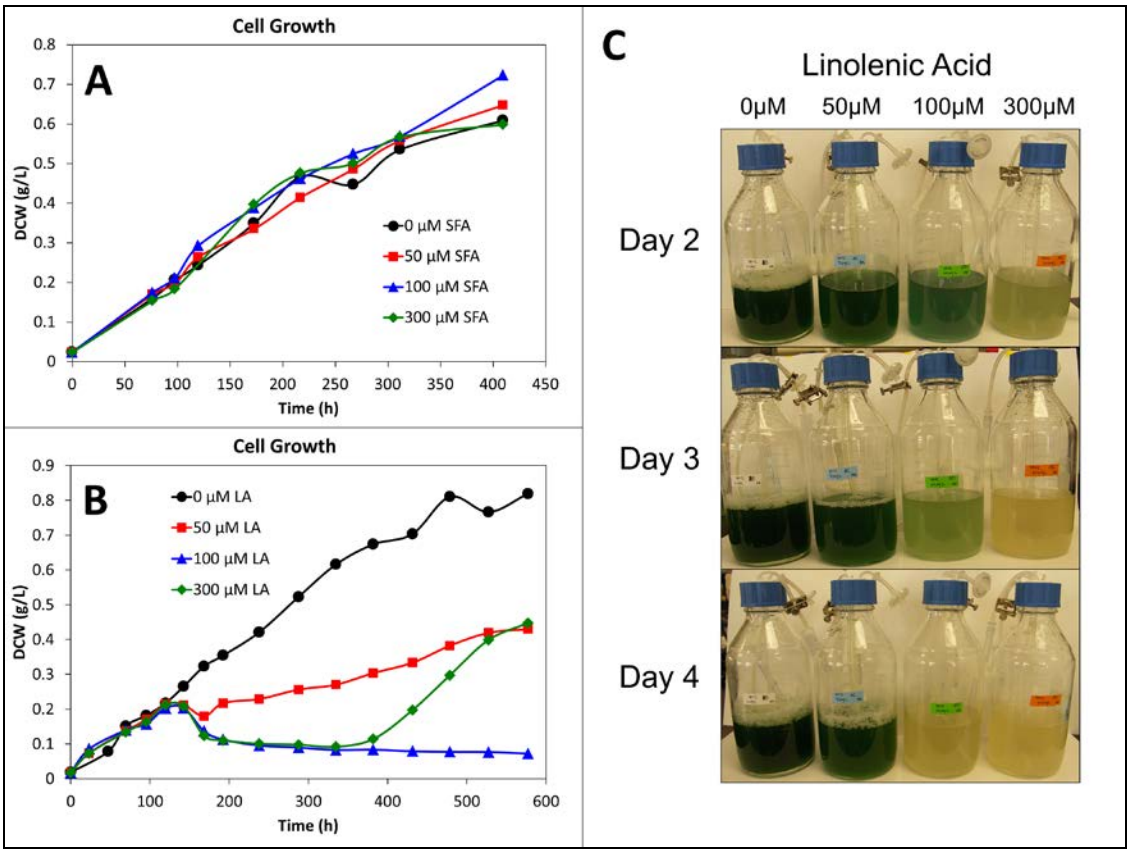

Figure 4.6. Cell growth of S. elongatus PCC7942 with exogenous addition of saturated (palmitic and stearic) (A) and unsaturated (linolenic) (B) fatty acids. The loss of photosynthetic pigments following linolenic acid addition is visually observed (C).

From this preliminary investigation, it is possible that both mechanisms, altered membrane composition and FFA toxicity, contribute to the physiological effects of FFA production in *S. elongatus* PCC7942.

4.3. Conclusions

Numerous physiological changes accompany the production of FFAs in *S. elongatus* PCC7942. This section of the SAND report details these changes, including reduced cell concentration, elevated levels of ROS, increased cell membrane permeability, decreased photosynthetic yield, Chl-a degradation, and aggregation of phycobiliproteins at the cell poles. Two potential mechanisms may cause these physiological changes: altered membrane composition and FFA toxicity. Preliminary evidence supporting these mechanisms includes an increased degree of saturation for membrane fatty acids in a FFA-producing strain and reduced cell growth and pigment degradation with addition of exogenous unsaturated FFA. Metabolic engineering strategies to address these mechanisms must be developed to enable high levels of FFA production in *S. elongatus* PCC7942.

5. SEQ-ING GENETIC TARGETS FOR IMPROVING FFA PRODUCTION IN *SYNECHOCOCCUS ELONGATUS* PCC7942

5.1. Introduction

RNA-seq is a powerful method for measuring transcript levels across the entire genome of an organism. In this technique, RNA is purified from a culture, converted into cDNA, sheared to generate fragments, ligated to adaptors, and sequenced using high-throughput DNA sequencing [34]. RNA-seq is made possible by the much-improved capacity and reduced cost of next-generation sequencing technologies. RNA-seq technology is touted as being more accurate for transcriptomics than its predecessor, microarrays. In this project, we applied RNA-seq technology to investigate the transcriptional response to FFA production in *S. elongatus* PCC7942. The goal was not only to determine the genetic changes associated with FFA production, but to also identify genes as potential targets for improving FFA production. By discovering the natural host response to FFA production, we could potentially boost this response using metabolic engineering to enhance cell recovery and improve survival. Additionally, RNA-seq may uncover FFA exporters, and by overexpressing these genes, we can promote FFA secretion and minimize the detrimental effects of intracellular FFA accumulation. While this RNA-seq effort is largely exploratory, it will undoubtedly provide a better understanding of the cellular impact of FFA production.

5.2. Results and Discussion

5.2.1. RNA-seq Analysis of FFA Production in S. elongatus PCC7942

To investigate the transcriptional response to FFA production in *S. elongatus* PCC7942, the RNA-seq experiment was designed to analyze multiple strains across several time points. Three strains were analyzed: the wild type (7942), SE01, and SE02. SE01 only produced FFAs during the stationary growth phase, while SE02 produced FFAs following thioesterase induction at 100 h (Figure 5.1). Samples were collected during two time points: following induction (100 h) and during FFA production (240 h), and three biological replicates were included for statistical analyses. From these samples, five conditions of high v. low FFA were compared for differential gene expression:

A: SE02, 100 h (high) v. SE01, 100 h (low)
B: SE02, 100 h (high) v. 7942, 100 h (low)
C: SE01, 240 h (high) v. 7942, 240 h (low)
D: SE02, 240 h (high) v. 7942, 240 h (low)
E: SE01, 240 h (high) v. SE01, 100 h (low)

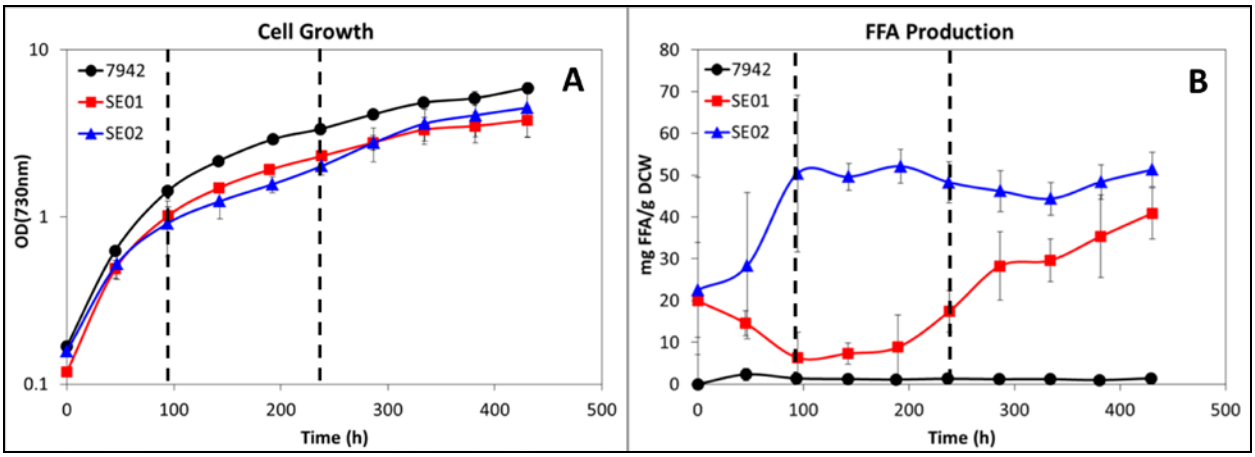

Figure 5.1. Cell concentration (A) and excreted FFA (B) for the wild type (7942) and FFA-producing strains (SE01 and SE02) used for RNA-seq analysis. Sampling times are indicated by the dashed lines. Data are averages of three biological replicates and error bars indicate the standard deviation.

Combining the results from these 5 comparisons, 150 genes were found to be significantly up-regulated and 204 genes were down-regulated under high FFA conditions. Only genes with a fold change greater than 2 and a p-value less than 0.05 were considered to be differentially expressed. A majority of the differentially expressed genes are designated as hypothetical proteins (Figure 5.2). As expected, stress response genes are significantly up-regulated during high FFA production. Cell wall biosynthesis genes are also up-regulated, presumably due to the reduced supply of fatty acid precursors. Interestingly, genes associated with nitrogen limitation were up-regulated despite the adequate supply of nitrate in the media. In agreement with this perceived nitrogen limitation, genes involved in chlorophyll metabolism were down-regulated. Chlorophyll degradation is a common response in nitrogen limitation due to the nitrogen content in the chlorophyll molecule. This also agrees with previous physiological measurements that show a selective degradation of Chl-a (Figure 4.3B). Carbon metabolism genes and regulatory proteins are also significantly down-regulated under high FFA concentrations (Figure 5.2). These transcriptional changes confirm the previous biochemical measurements, indicating elevated stress levels and pigment degradation. Yet, can this transcriptome analysis be used to identify genes which can help overcome these negative physiological effects of FFA production?

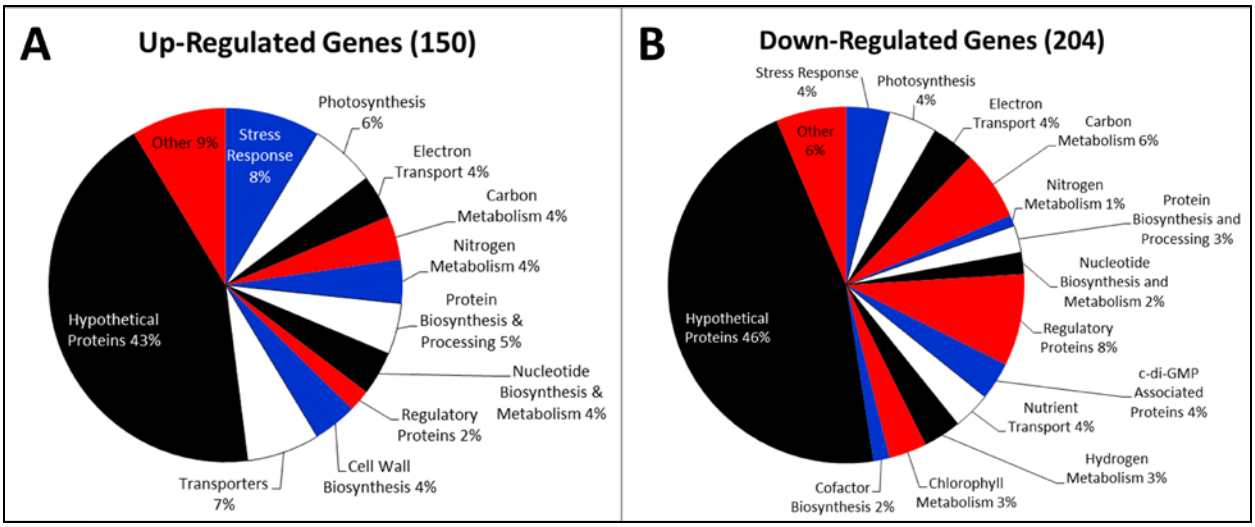

Figure 5.2. Differentially expressed genes (fold change > 2, p-value < 0.05) during high FFA concentrations. Each gene was manually categorized corresponding to its metabolic function.

5.2.2. Identification of Genes Influencing FFA Production and Cell Physiology

Out of the 354 genes found to be differentially expressed under high FFA conditions, 17 genes were targeted for genetic manipulation (Table 5.1). A control strain, SE02a, was also constructed for analysis of the overexpression and knockout mutants. SE02a is similar to SE02 but includes integration of an empty vector into the neutral integration site II (NSII). This integration site was also used for construction of the overexpression mutants, and the selective marker (kanamycin resistance) is the same marker used to select the knockout mutants. Therefore, SE02a served as the control for both the overexpression and knockout mutants.

A total of 9 hypothetical proteins were differentially expressed in 4 out of the 5 comparisons of high vs. low FFA (Table 5.1); the 3 hypothetical proteins showing increased expression were targeted for gene knockout while the 6 hypothetical proteins with reduced expression were cloned for overexpression. If these hypothetical proteins play a role in either FFA production or cellular physiology, the mutants should show changes in FFA production, cell growth, and/or photosynthetic yield. For the hypothetical protein mutants, 7 out of the 9 were successfully constructed; gene knockout of Synpcc7942_1561 and Synpcc7942_1023 failed despite repeated attempts at transformation. Synpcc7942_1561 and Synpcc7942_1023 may therefore be essential for cell growth in *S. elongatus* PCC7942. To test the mutants for changes in growth, photosynthetic yield, and FFA production, two transformed colonies were screened for each mutant. As shown in Figure 5.3A,B, the overexpression of 3 hypothetical proteins (S1655#2 and #5, S0122#4, and S0900#2) led to improved photosynthetic yields and cell growth after induction (165 and 239 h). However, the extracellular FFA concentration for these mutants was not significantly different from the control (SE02a) (Figure 5.4A). As expected, the overexpression of some hypothetical proteins led to reduced FFA concentrations (SB2632#1 and #2, S0122#3, S1476#1, and S0900#1); however, the reduced FFA values do not fall outside the standard deviation due to the large variation among biological replicates. While the hypothetical proteins Synpcc7942_1655, Synpcc7942_0122, and Synpcc7942_0900 may not effect FFA

concentration, these proteins are important for maintaining cell health under the stress of FFA production.

Table 5.1. List of genes targeted for genetic manipulation (gene overexpression or gene knockout) along with the fold-change in gene expression for each comparison of high v. low FFA concentration. Data are averages of three biological replicates; p-values are less than 0.05 (not shown).

Locus	Product	Fold-change						Overexpress or Knockout
		A	B	C	D	E	Average	
Hypothetical Proteins								
0444	hypothetical protein	3.19	3.83		2.01	4.05	3.27	Knockout
1561	hypothetical protein	2.43	3.69	2.14	2.40		2.67	Knockout
1023	hypothetical protein	2.02	2.39	2.11	2.06		2.15	Knockout
1476	hypothetical protein		-8.08	-5.41	-4.87	-2.38	-5.18	Overexpress
1655	hypothetical protein	-3.07	-4.03	-2.60		-2.22	-2.98	Overexpress
0900	hypothetical protein	-2.74	-4.03	-2.03	-2.90		-2.92	Overexpress
B2632	hypothetical protein	-2.66	-3.50	-2.26	-2.30		-2.68	Overexpress
0122	hypothetical protein	-2.06	-3.38	-2.03	-2.65		-2.53	Overexpress
1845	hypothetical protein		-2.34	-2.14	-2.64	-2.01	-2.28	Overexpress
ROS Degrading Proteins								
1214	glutathione peroxidase	2.04	3.22				2.63	Overexpress
0437	glutathione peroxidase		2.83		2.25		2.54	Overexpress
0801	superoxide dismutase		2.70	2.42			2.56	Overexpress
1656	catalase/peroxidase		-2.38				-2.38	Overexpress
Potential FFA Exporters								
2175	transport system substrate-binding		2.23	3.76			2.99	Knockout
1224	ABC-transporter membrane fusion	2.23	3.26				2.74	Knockout
1464	porin				2.28		2.28	Knockout
1607	porin/major outer membrane protein			2.16			2.16	Knockout

Reactive oxygen species (ROS) may play an important role in the mechanism of FFA toxicity. Unsaturated fatty acids (UFAs) are known to react with ROS to form toxic degradation products such as hydroperoxides, which may explain the increased toxic effect observed with UFAs [35]. Moreover, ROS are often generated under stress conditions and have been reported to serve as signaling molecules to trigger cellular stress responses [29]. ROS may therefore play a beneficial role in the cellular response to FFA-induced stress. To determine if ROS contributes to either FFA toxicity or a FFA-induced stress response, four major ROS-degrading enzymes were targeted for overexpression in SE02 (Table 5.1). Overexpression of these ROS-degrading proteins should reduce the intracellular levels of ROS which were found to accumulate in SE02 (Figure 4.2). For each ROS-degrading protein mutant, two transformants were screened for changes in cell growth, photosynthetic yield, and FFA production. Three strains showed improved photosynthetic yield measurements at 165 h: S1214#1, S0801#1, and S1656#1, but

these strains did not have any significant change in cell growth (Figure 5.3C,D). Some strains had reduced levels of FFA production (S1214#2, S0437#1, and S1656#2), but again, the decreased level of FFA is not outside the range of the standard deviation. There appears to be some detrimental effect of ROS on photosynthesis, as the overexpression of ROS-degrading proteins improved photosynthetic yield, but the minimal effect on cell growth and FFA production indicates that ROS does not play a major role in FFA toxicity.

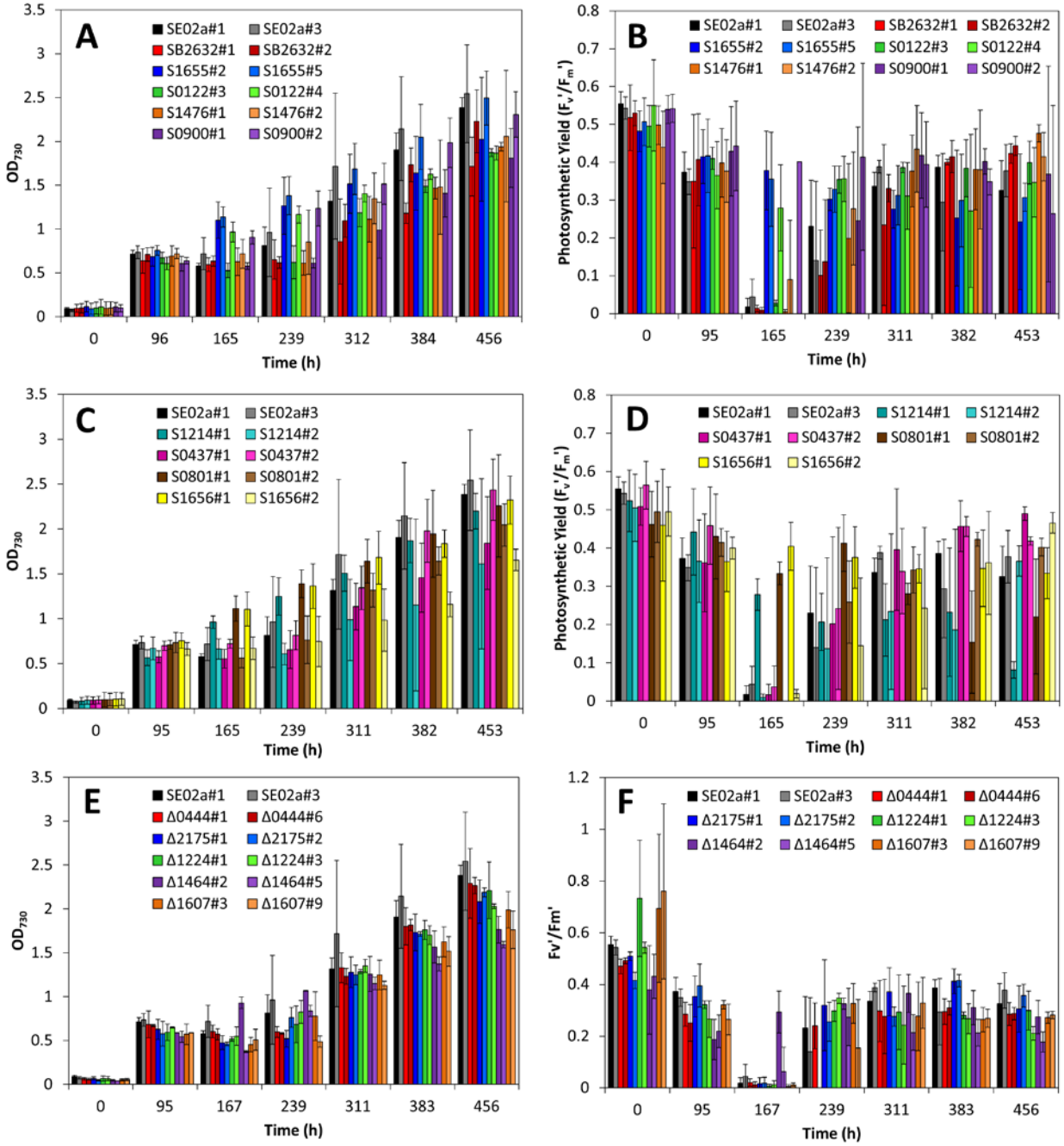

Figure 5.3. Cell growth (A, C, E), and photosynthetic yield (B, D, F) for overexpressed hypothetical protein mutants (A, B), overexpressed ROS-degrading enzyme mutants (C, D), and knockout mutants of a hypothetical protein and potential FFA exporters (E, F).

Data are averages of three biological replicates and error bars indicate the standard deviation.

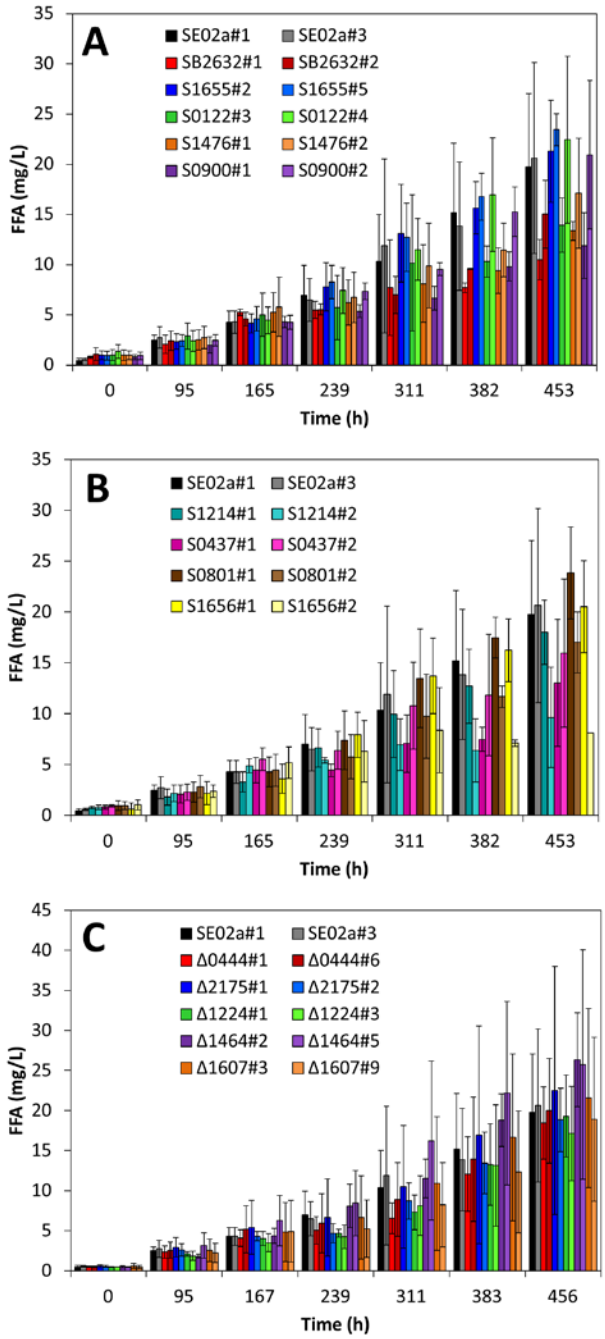

Figure 5.4. Excreted FFA concentration for overexpressed hypothetical protein mutants (A), overexpressed ROS-degrading enzyme mutants (B), and knockout mutants of a hypothetical protein and potential FFA exporters (C). Data are averages of three biological replicates and error bars indicate the standard deviation.

The identification of FFA exporters was a main objective of this experiment. The RNA-seq analysis revealed four transport proteins that were up-regulated during FFA production (Table 5.1), and these four genes were targeted for knockout to determine their effect on extracellular

FFA accumulation. Analysis of these transport protein mutants suggests that porins are important for cell growth during FFA production and FFA excretion. Gene knockout of porins Synpcc7942_1464 and Synpcc7942_1607 led to reduced cell concentration during late time points (383 h and 456 h) (Figure 5.3E). The reduced cell concentration may be due to the build-up of toxic FFAs inside the cell with gene knockout of the porin protein. Unexpectedly, knockout of porin Synpcc7942_1464 also led to improved photosynthetic yield following induction (167 h, Figure 5.3F) and a corresponding improvement in cell concentration at this time point. The concentration of extracellular FFA did not change significantly for any of the transport protein mutants (Figure 5.4C); however, there was a slight increase in extracellular FFA for the SE02Δ1464 mutants. This contradicts the hypothesis that the porin protein allows for enhanced FFA excretion. It is possible that the Synpcc7942_1464 porin allows the FFAs to re-enter the cell after they are excreted. With this proposed function, gene knockout of Synpcc7942_1464 would prevent FFA uptake, leading to higher extracellular FFA concentrations and possibly improved photosynthetic yield. While this study failed to identify an active FFA exporter, the results suggest that porin proteins may play an important role in regulating FFA transport across the cell membrane.

With the overexpression and knockout mutants, there was significant variability among transformants of the same gene target. The transformants were confirmed to be genetically similar using PCR amplification of the target mutation and are therefore expected to produce similar experimental results. The diversity of transformant responses suggests that gene expression and perhaps genetic manipulation within *S. elongatus* PCC7942 are not stable. This instability complicates the analysis of these mutants, and consequently, the results presented in this report should be viewed as preliminary.

5.3. Conclusions

RNA-seq technology was applied to determine the transcriptional response of *S. elongatus* PCC7942 to FFA production. Analysis of the data reveals a cell-wide response to FFA production which includes up-regulation of stress response genes and down-regulation of carbon metabolism, regulatory genes, and chlorophyll metabolism. Specific genes were targeted for either gene knockout or gene overexpression to generate 15 mutant strains. These mutants include hypothetical proteins, ROS-degrading proteins, and transport proteins that were differentially expressed under high FFA conditions. Characterization of these mutant strains identified genes affecting cell growth: hypothetical proteins (Synpcc7942_1655, Synpcc7942_0122, and Synpcc7942_0900) and porin proteins (Synpcc7942_1464 and Synpcc7942_1607); photosynthetic yield: hypothetical proteins (Synpcc7942_1655, Synpcc7942_0122, and Synpcc7942_0900), ROS-degrading enzymes (Synpcc7942_1214, Synpcc7942_0801, and Synpcc7942_1656), and a porin protein (Synpcc7942_1464); and FFA production: hypothetical proteins (Synpcc7942_B2632, Synpcc7942_0122, Synpcc7942_1476, and Synpcc7942_0900), ROS-degrading proteins (Synpcc7942_1214, Synpcc7942_0437, and Synpcc7942_1656), and a porin protein (Synpcc7942_1464). Unexpectedly, the level of FFA production did not change significantly for any of the 15 mutant strains, suggesting that these target genes do not play an essential role in FFA production or excretion. The most significant changes were observed in the photosynthetic yield, and the genes affecting photosynthetic yield may be important targets for addressing the negative physiological effects of FFA production.

Additional research is needed to determine if these gene targets can, in fact, eliminate the physiological effects of FFA production in *S. elongatus* PCC7942.

6. ENGINEERING FFA PRODUCTION IN ANOTHER MODEL STRAIN: *SYNECHOCOCCUS* SP. PCC7002

6.1. Introduction

The engineering of *Synechococcus elongatus* PCC7942, described in Sections 3 and 4 of this SAND report, resulted in low levels of FFA production and excretion, and despite repeated attempts at improving FFA yields, the physiological effects associated with FFA production prevented any additional improvement. While FFAs have been reported to be cytotoxic for many microorganisms [32], toxic effects were not reported in other microbial hosts such as *E. coli* and *Synechocystis* sp. PCC6803 [30, 36]. Therefore, in this section, another model cyanobacterium, *Synechococcus* sp. PCC7002, is engineered for FFA production to determine if the physiological effects of FFA production are specific to the host strain.

Synechococcus sp. PCC7002 has many advantageous traits for large-scale biofuel production. Isolated from a marine environment, *Synechococcus* sp. PCC7002 has moderate salt tolerance, growing in salt concentrations up to 3 times higher than seawater [37]. Salt tolerance is beneficial for biofuel production in open ponds, which may be subject to higher salt concentrations due to evaporation, and also for the use of brackish and oceanic water sources to reduce the requirement for freshwater [38]. Algal fuel production systems are also subject to high light conditions, which can cause photoinhibition in many algal strains [39]. *Synechococcus* sp. PCC7002 has been shown to have high light tolerance, growing at light intensities higher than the peak intensities of natural sunlight [40]. Lastly, *Synechococcus* sp. PCC7002 can grow at elevated temperatures; in fact, its temperature optimum is 38°C [41]. This high temperature tolerance makes *Synechococcus* sp. PCC7002 ideal for algal production systems in the desert southwest region of the United States and in enclosed photobioreactor systems, where water temperatures can reach as high as 45°C [42], a lethal temperature for many algal strains. With the salt, light, and temperature tolerances of *Synechococcus* 7002, engineering this organism for efficient FFA production may yield a desirable strain for industrial application.

6.2. Results and Discussion

6.2.1. Temperature Influences FFA Production and Cell Physiology in Synechococcus sp. PCC7002

For preliminary analysis of FFA production in *Synechococcus* sp. PCC7002, two engineered strains were constructed: S01 with gene knockout of acyl-CoA synthetase (*fadD*) and S02 with *fadD* gene knockout and expression of the *E. coli* thioesterase, *'tesA*. The wild type and engineered strains were analyzed for FFA production at two temperatures: 38°C, the reported optimal growth temperature for *Synechococcus* sp. PCC7002, and 30°C, the optimal growth temperature of *S. elongatus* PCC7942 and a more realistic temperature for large-scale, outdoor fuel production. Unexpectedly, cell growth and final cell concentration were greater at 30°C for the wild type, rather than the reported optimum of 38°C (Figure 6.1A). On the other hand, FFA production for S02 was slightly higher at 38°C compared to 30°C, but this difference is not very

significant and is most likely due to the kinetic advantage associated with the higher temperature (Figure 6.1B). Interestingly, the negative physiological effects of FFA production appeared to be mitigated at the lower growth temperature (30°C). Both the photosynthetic yield and the photosynthetic pigment concentrations in the FFA-producing S02 strain were similar to the wild type at 30°C (Figure 6.1C,D). At 38°C, the photosynthetic yield decreased throughout the course of the experiment, even for the wild type (7002). This reduction in photosynthetic yield is even more pronounced for the FFA-producing S02 strain (Figure 6.1C). The higher growth temperature also led to decreased levels of photosynthetic pigments, as illustrated by the absorption spectra (Figure 6.1D). These results suggest that temperature regulation can be used to mitigate the detrimental effects of FFA production with *Synechococcus* sp. PCC7002 as host.

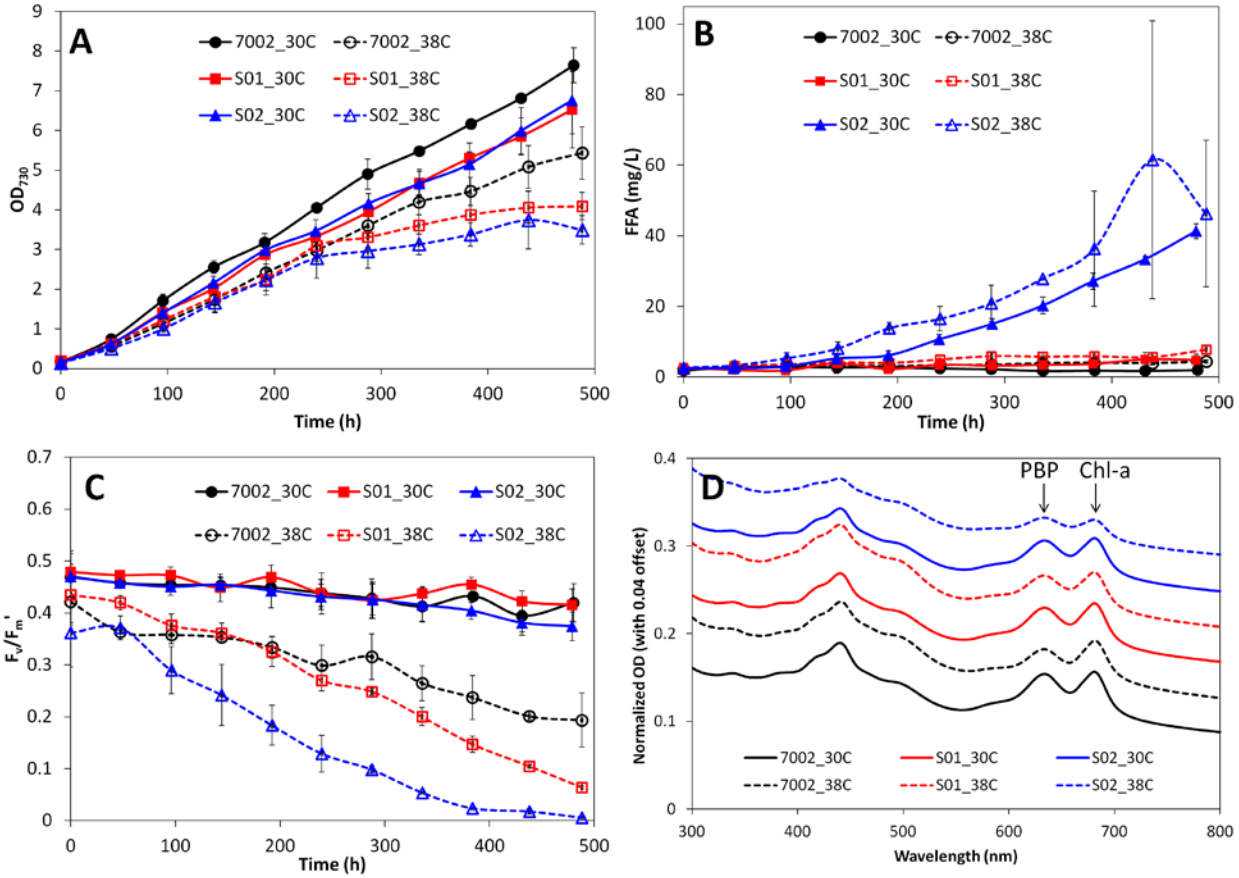

Figure 6.1. Temperature effects on cell growth (A), extracellular FFA concentration (B), photosynthetic yield (C), and absorbance spectra (D) for the wild type (7002) and FFA-producing strains (S01 and S02) of *Synechococcus* sp. PCC7002. Data are averages of three biological replicates and error bars indicate the standard deviation.

Previous studies have investigated the transcriptional changes within *Synechococcus* sp. PCC7002 under varying growth temperature conditions [43-45]. Desaturases were found to be an important factor in temperature tolerance for *Synechococcus* sp. PCC7002, for changing the degree of membrane saturation alters its viscosity, allowing membranes to remain fluid at lower temperatures. This change in membrane saturation may also help to confer the temperature-induced FFA tolerance of *Synechococcus* sp. PCC7002 at 30°C. To test this hypothesis, the ω3-

acyl-lipid desaturase, *desB*, was targeted for gene knockout in the FFA-producing S02 strain. In *Synechococcus* sp. PCC7002, *desB* is not expressed at the temperature optimum of 38°C but is highly expressed at lower growth temperatures [43]. The FFA-producing *desB* mutant (S02Δ*desB*) was analyzed for FFA production at 30°C, but surprisingly, there were no significant changes in FFA production, cell growth, or photosynthetic yield for S02Δ*desB* compared to S02 (data not shown). While this does not conclusively prove that membrane saturation is not responsible for the FFA tolerance of *Synechococcus* sp. PCC7002 at 30°C, it does suggest that another mechanism may be responsible for the temperature-associated FFA tolerance.

6.2.2. Engineering Synechococcus sp. PCC7002 for Enhanced FFA Production

The reduced physiological effects of FFA production at 30°C allows for additional genetic engineering to further improve FFA production in *Synechococcus* sp. PCC7002. Two proposed rate-limiting steps were targeted: thioesterase cleavage of the FFA and CO_2 fixation by RuBisCO.

We have previously shown that expression of the acyl-ACP thioesterase from *C. reinhardtii* CC-503, *fat1*, improved FFA production in *S. elongatus* PCC7942 on a dry cell weight basis [1]. To determine if *fat1* expression will improve FFA yields in *Synechococcus* sp. PCC7002, *fat1* was expressed in the S03 strain. While cell growth and photosynthetic yield for S03 were similar to the '*tesA*-expressing S02 strain, the extracellular FFA concentration for S03 was more than 4-fold lower than S02 (Figure 6.2A). In fact, FFA production in S03 was not significantly greater than that of S01 with no thioesterase expression, suggesting that Fat1 may not be active in *Synechococcus* sp. PCC7002. One possible explanation for the lack of Fat1 activity in S03 is that the chloroplast-targeting signal was not removed from *fat1*. As a nuclear-encoded gene, *fat1* includes a chloroplast-targeting signal peptide to enable selective transport of Fat1 protein across the chloroplast membrane; during the transport process, the chloroplast-targeting peptide signal is cleaved to yield a functional enzyme. Therefore, Fat1 activity in S03 may be reduced due to either steric hindrance from the chloroplast-targeting signal or export of Fat1 outside the cell. To determine the effect of the chloroplast-targeting signal, the predicted signal sequence was removed to construct a truncated *fat1* (*tfat1*), which was expressed in strain S05. S05 showed a significant improvement in extracellular FFA accumulation compared to S03, with nearly a 2.5-fold increase (Figure 6.2A). Unexpectedly, S05 also had reduced cell growth and photosynthetic yield compared to S03 and S02. It is unclear why these physiological effects are manifested in S05, as the amount of FFA produced by S05 is still lower than that produced by S02. Despite the improved activity of tFat1 in S05, the *E. coli* thioesterase 'TesA yielded the highest level of extracellular FFA and was used for additional genetic engineering efforts.

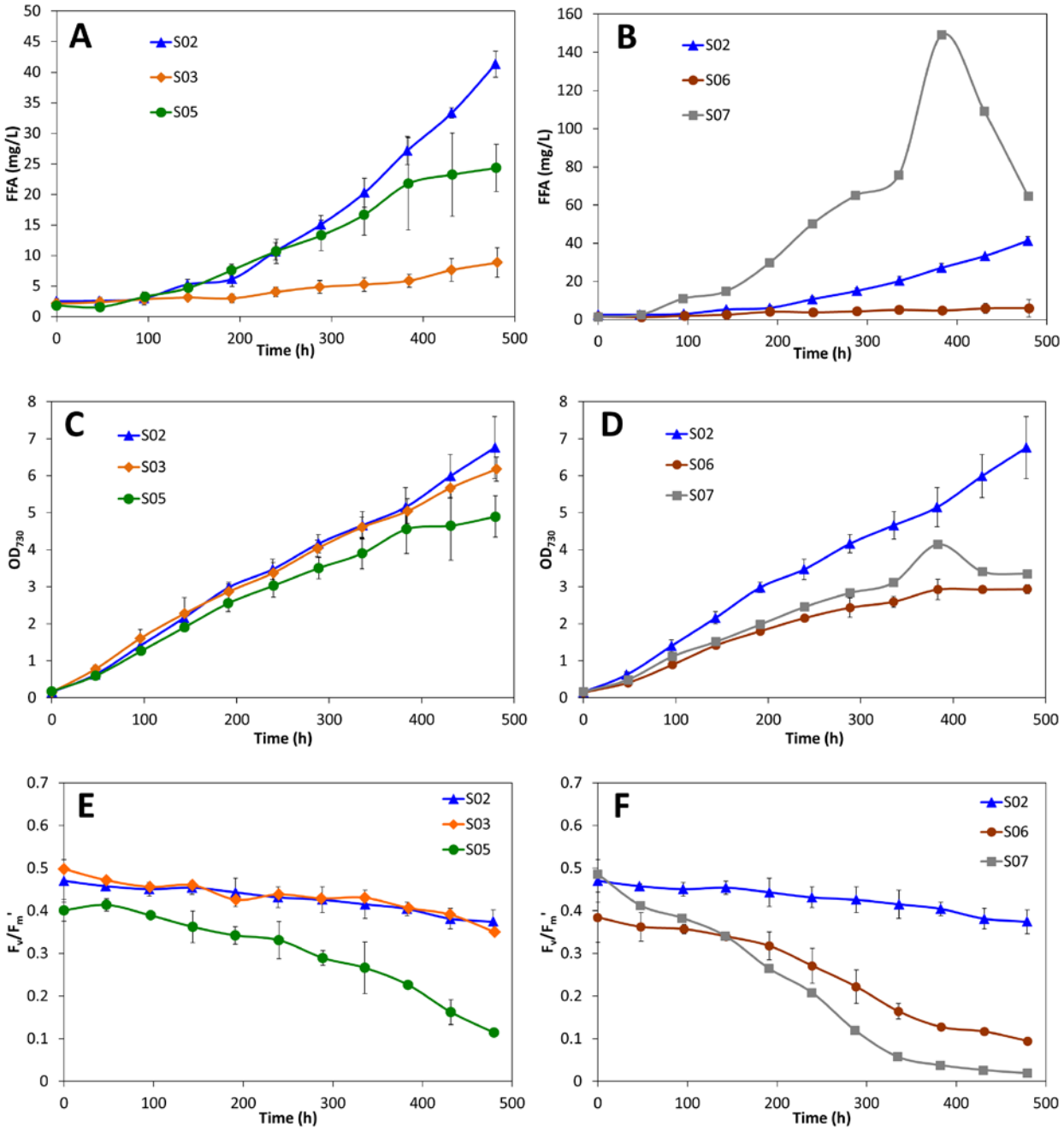

Figure 6.2. Extracellular FFA concentration (A,B), cell growth (C, D), and photosynthetic yield (E, F) measurements comparing the effects of thioesterase expression (A, C, E) and RuBisCO expression (B, D, F). All cultures were grown at 30°C. Data are averages of two or three biological replicates and error bars indicate the standard deviation for S02, S03, S05, and S06 data. For S07, only one biological replicate is included in the data.

The fixation of CO_2 by RuBisCO determines the total flux of carbon into the cell and is therefore an important factor affecting the rate of FFA production. To improve the carbon flux for cell growth and FFA production, RuBisCO was targeted for overexpression. The large and small RuBisCO subunits from *S. elongatus* PCC7942 (*rbcLS*) were cloned and expressed along with *'tesA* and *fadD* gene knockout in S06. Surprisingly, S06 showed reduced cell growth,

photosynthetic yield, and FFA production compared to S02. It is unclear why S06 suffered from these negative physiological effects without any apparent improvement in FFA production. We have previously shown that expression of *rbcLS* from the recombinant operon Ptrc-*fat1*-*rbcLS* is low and not actively controlled by the *trc* promoter in *S. elongatus* PCC7942. Therefore, it seems to reason that *rbcLS* expression from the recombinant operon Ptrc-'*tesA*-*rbcLS* may also be poorly expressed in S06. To improve *rbcLS* expression, the *psbAI* promoter from *S. elongatus* PCC7942 was cloned and inserted upstream of *rbcLS* to construct the recombinant operon Ptrc-*'tesA*-PpsbAI-*rbcLS*. This improved operon was transformed into *Synechococcus* sp. PCC7002 to create strain S07. With *rbcLS* expression from PpsbAI, FFA production was greatly improved in S07, yielding 3.6-fold more extracellular FFAs compared to S02 (Figure 6.2B). The rate of FFA production for S07 was also improved compared to S02 (0.27 mg FFA L^{-1} h^{-1} vs. 0.12 mg FFA L^{-1} h^{-1}). Furthermore, S07 produced FFAs at such a high concentration that the FFAs precipitated from the media and floated to the top, forming a white layer. Along with this increase in FFA production, S07 also showed increased physiological effects with reduced cell growth and photosynthetic yield (Figure 6.2D,F). Even at the lower growth temperature of 30°C, the negative physiological effects of FFA production were observed for the *Synechococcus* sp. PCC7002 host.

6.3. Conclusions

This section of the SAND report describes the engineering and characterization of another model cyanobacterial species, *Synechococcus* sp. PCC7002, as host for FFA production. The negative physiological effects of FFA production observed in *S. elongatus* PCC7942 were abated in *Synechococcus* sp. PCC7002 by using a growth temperature of 30°C. While the physiological effects were temperature dependent in *Synechococcus* sp. PCC7002, membrane desaturation via *desB* is not responsible for the improved FFA tolerance. The green algal acyl-ACP thioesterase, Fat1, had reduced activity in *Synechococcus* sp. PCC7002 compared to *S. elongatus* PCC7942, and despite the improved activity with removal of the chloroplast-targeting signal from Fat1, FFA production was highest with the *E. coli* thioesterase 'TesA. The overexpression of *S. elongatus* PCC7942 RuBisCO subunits from the *psbAI* promoter had the greatest impact on FFA yield in *Synechococcus* sp. PCC7002, with extracellular FFA concentrations reaching as high as 150 mg/L. In fact, the concentration of FFA may be even higher, as FFA measurement was complicated by FFA precipitation. These results demonstrate that *Synechococcus* sp. PCC7002 is a superior host for FFA production compared to *S. elongatus* PCC7942.

7. EXPLORING BIOFUEL TOXICITY IN THREE MODEL CYANOBACTERIAL SPECIES

7.1. Introduction

Biofuel toxicity is a major obstacle in achieving economical, large-scale biofuel production. The limitations imposed by biofuel toxicity is evident throughout this SAND report, most notably in Section 4, which describes the physiological effects associated with FFA production in *S. elongatus* PCC7942. While the physiological effects were reduced in the alternative host, *Synechococcus* sp. PCC7002 (Section 6), the detrimental effects on cell growth and photosynthesis are still likely to limit FFA production. Therefore, in this section, we explore the mechanisms of biofuel toxicity in three model cyanobacterial species: *S. elongatus* PCC7942, *Synechocystis* sp. PCC6803, and *Synechococcus* sp. PCC7002. These three species are the most common host candidates for cyanobacterial biofuel production due to the availability of tools and protocols for the genetic manipulation of these organisms, the knowledge of metabolic pathways and processes in these microalgae, and the availability of their genome sequences. A wide range of potential biofuel products are investigated in this study, including short and long-chain alcohols (ethanol, isobutanol, and hexadecanol), saturated and unsaturated FFAs (palmitic, stearic, and linolenic acids), and long chain alkanes and alkenes (pentadecane and heptadecene). The objectives of this investigation are (1) to identify which cyanobacterial strain has the highest tolerance for each potential fuel product, (2) to identify which biofuel is the best target product (i.e. has low toxicity), and (3) to determine the underlying mechanisms responsible for biofuel tolerance.

7.2. Results and Discussion

7.2.1. Screening 3 Model Cyanobacterial Species for Biofuel Toxicity

The 3 model cyanobacterial species were screened for biofuel toxicity by adding the potential biofuel products exogenously to the media and measuring the effect on cell growth. The results of this screening are shown in Figure 7.1. In general, the short-chain alcohols, ethanol and isobutanol, were found to inhibit cell growth for all 3 cyanobacterial species (Figure 7.1A,B). This is not surprising, as the toxicity of ethanol and isobutanol is well documented [46]. The polyunsaturated fatty acid (PUFA), linolenic acid, was also found to inhibit cyanobacterial growth (Figure 7.1D), yet the saturated fatty acids, palmitic acid and stearic acid, had no significant effect on cell growth (Figure 7.1E,F). Again, these results agree with previous reports of the antimicrobial activity of fatty acids and the greater toxicity of unsaturated fatty acids (UFAs) as compared to saturated fatty acids [32]. Minimal cytotoxic effects were observed for the long-chain alcohol: 1-hexadecanol, the alkane: pentadecane, and the alkene: heptadecene. These results suggest that long-chain alcohols or alkanes/alkenes are good metabolic targets for biofuel production, at least from a toxicity perspective.

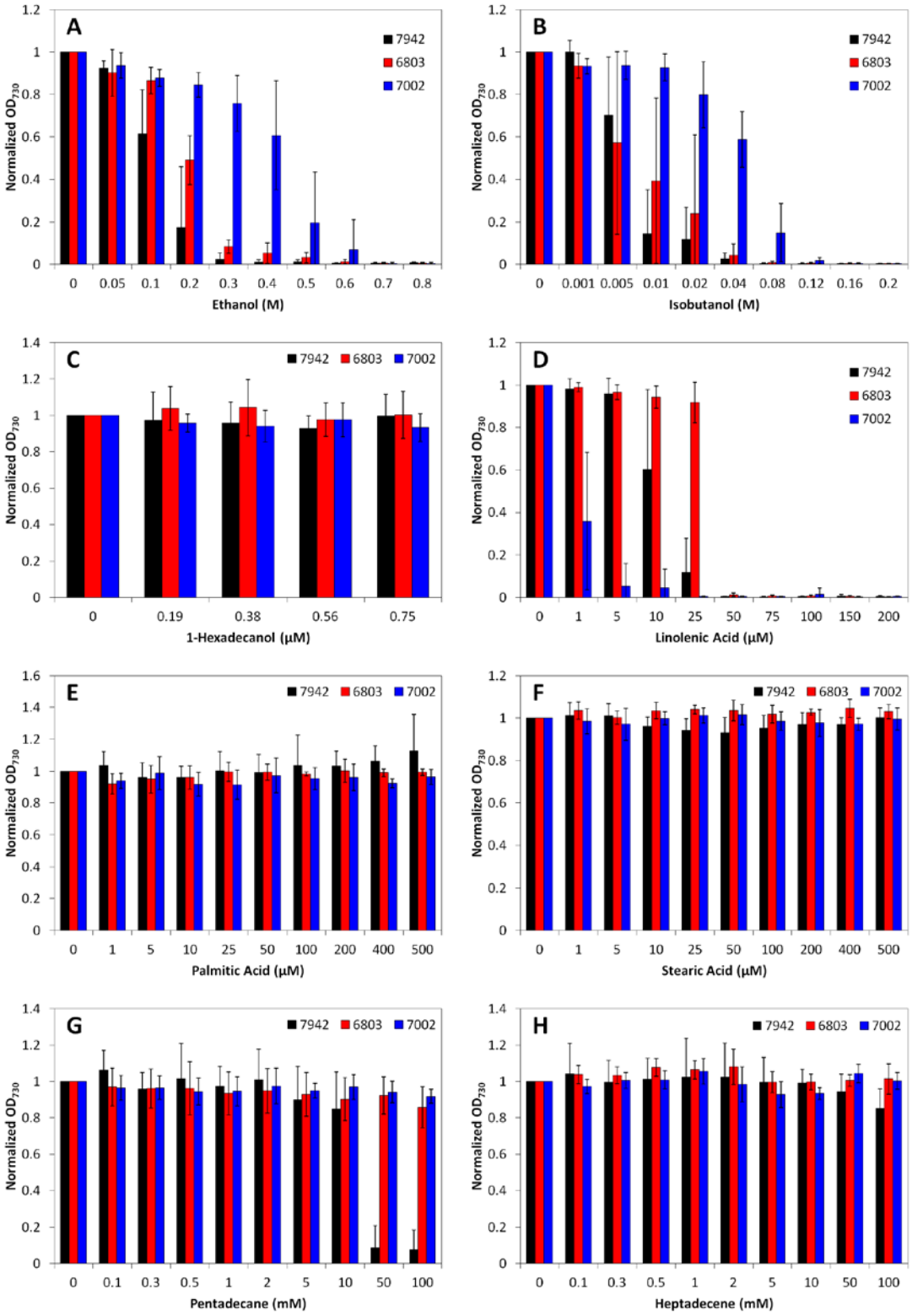

Figure 7.1. Growth inhibition of 3 model cyanobacterial species with biofuel addition: Ethanol (A), Isobutanol (B), 1-Hexadecanol (C), Linolenic acid (D), Palmitic acid (E), Stearic acid (F), Pentadecane (G), and Heptadecene (H). Data are averages of three biological replicates and error bars indicate the standard deviation.

The 3 model cyanobacterial species demonstrated varying responses of biofuel tolerance. *Synechococcus* sp. PCC7002 showed the highest tolerance of short-chain alcohols, while *S. elongatus* PCC7942 had the lowest. This trend did not hold true for UFA toxicity. *Synechococcus* sp. PCC7002 was the most susceptible to linolenic acid toxicity, while *Synechococystis* sp. PCC6803 was the most resistant. The growth of *S. elongatus* PCC7942 was inhibited at high concentrations of pentadecane (Figure 7.1G). Not only was this toxic effect not observed for the other 2 cyanobacterial species, but the concentration at which the toxicity was observed is well above the predicted solubility of pentadecane in water (0.013 μM). The distinct response of each cyanobacterial species to these potential biofuels suggests that there may be multiple mechanisms by which the biofuels exert their toxic effect, and correspondingly, multiple mechanisms of biofuel tolerance.

7.2.2. Comparative Genomics: Searching for Biofuel Tolerance

In an effort to identify mechanisms of biofuel tolerance in the model cyanobacterial species, comparative genomics tools were applied to analyze their genomic sequences. Specifically, genes associated with four known mechanisms of biofuel tolerance were investigated: 1) efflux proteins, which pump the biofuel out of the cell, 2) desaturases, which play a role in membrane fluidity and temperature tolerance, 3) ROS-degrading proteins, which eliminate damaging ROS that may be generated under stress, and 4) general stress response proteins (Table 7.1).

Overall, the number of efflux protein genes present in the genome seems to correlate with the tolerance to short-chain alcohols (*Synechococcus* sp. PCC7002 > *Synechocystis* sp. PCC6803 > *S. elongatus* PCC7942). This may explain the high ethanol and isobutanol tolerance of *Synechococcus* sp. PCC7002; the increased number of efflux pumps enhances the organism's capability to expel toxic alcohols from the cell. *Synechococcus* sp. PCC7002 also has the highest number of desaturase genes present in its genome. By changing the membrane fluidity with these desaturases, *Synechococcus* sp. PCC7002 may be able to lower the cell membrane's permeability to the exogenous biofuels, thereby improving its tolerance. Both efflux pumps and desaturases may play a role in the short-chain alcohol tolerance of *Synechococcus* sp. PCC7002.

Cyanobacterial tolerance to the PUFA, linolenic acid, also differed between the 3 model species (*Synechocystis* sp. PCC6803 > *S. elongatus* PCC7942 > *Synechococcus* sp. PCC7002). One proposed mechanism of UFA toxicity is the degradation of UFAs into toxic products via reaction with ROS [35]. The expression of ROS-degrading proteins may therefore reduce UFA degradation and minimize the associated toxic effects. Both *Synechocystis* sp. PCC6803 and *S. elongatus* PCC7942 have an additional gene coding for a ROS-degrading protein, which may confer higher UFA tolerance compared to *Synechococcus* sp. PCC7002 (Table 7.1).

Table 7.1. Comparative genomics of potential mechanisms for biofuel tolerance in 3 model cyanobacterial species: *Synechococcus elongatus* PCC7942 (locus prefix: Synpcc7942_), *Synechocystis* sp. PCC6803 (locus prefix: SYNGTS_), and *Synechococcus* sp. PCC7002 (locus prefix: SYNPCC7002_). Grey highlighted genes indicate low homology with the target gene (efflux proteins, desaturases, ROS-degrading proteins, and stress response proteins).

7942		6803		7002	
Locus	**Description**	**Locus**	**Description**	**Locus**	**Description**
Efflux Proteins					
1869	cation efflux system protein	1991	cation or drug efflux system protein	A0587	cation efflux system protein CzcA
1938	multidrug-efflux transporter	1831	hypothetical protein	A0589	arsenite efflux pump ACR3
2032	multidrug-efflux transporter quinolene resistance protein NorA	1260	quinolene resistance protein NorA	A0087	major facilitator transporter
2369	hydrophobe/amphiphile efflux-1 HAE1, AcrB, TtgB, MexF BLAST hit	1494	cation or drug efflux system protein, AcrB, TtgB, MexF BLAST hit	A1013	hydrophobe/amphiphile efflux-1 (HAE1) family protein, AcrB, TtgB, MexF BLAST hit
2368	secretion protein HlyD	2464	hypothetical protein	A1574	RND family efflux transporter MFP subunit
1989	cation diffusion facilitator family transporter	1569	hypothetical protein	A2463	cation efflux system protein
0553	secretion protein HlyD	0814	hypothetical protein	A2552	RND family efflux transporter MFP subunit
1761	hypothetical protein	1696	hypothetical protein	A0585	Outer membrane efflux protein
1870	secretion protein HlyD	1992	hypothetical protein	A0591	RND family efflux transporter MFP subunit
0985	hypothetical protein	2022	hypothetical protein	A0719	multidrug efflux transporter
1699	MATE efflux family protein	2483	Probable multidrug resistance protein norM		
0792	multidrug efflux MFS transporter				
		2125	cation or drug efflux system protein		
		2737	cation or drug efflux system protein		
		3105	cation or drug efflux system protein		
				A1483	RND family efflux transporter MFP subunit
				A1723	HlyD family secretion protein, homology to SYNPCC7002_A1483
Desaturases					
2561	delta-9 acyl-phospholipid desaturase	2538	acyl-CoA desaturase, desC	A2198	delta-9 acyl-lipid desaturase, desC

1713	hydrocarbon oxygenase MocD	1594	fatty acid desaturase, homology to sll1441, desA	A2756	homology to SYNPCC70025_A0159, desA
1713	hydrocarbon oxygenase MocD	1727	delta 15 desaturase, homology to slr1350, desB	A0159	omega-3 acyl-lipid desaturase, homology to SYNPCC7002_A2756, desB
		1931	delta-6 desaturase, desD		
				A1989	syn-2, delta 9 acyl-lipid fatty acid desaturase, desF
				A2833	fatty acid desaturase, desE
ROS-Degrading Proteins					
0801	superoxide dismutase	1451	superoxide dismutase	A0242	Mn-superoxide dismutase
1214	glutathione peroxidase	1769	glutathione peroxidase	A0117	glutathione peroxidase
1656	catalase/peroxidase HPI	1399	catalase HPI	A2422	catalase/peroxidase HPI
1937	peptide methionine sulfoxide reductase	0046	methionine sulfoxide reductase A (protects against oxidative stress)	A0215	methionine sulfoxide reductase A (protects against oxidative stress)
2190	methionine sulfoxide reductase B	0218	methionine sulfoxide reductase B (protects against oxidative stress)	A0672	methionine-R-sulfoxide reductase
		1305	glutathione peroxidase	A0970	glutathione peroxidase
0437	glutathione peroxidase				
B2620	putative catalase				
		0239	methionine sulfoxide reductase A (protects against oxidative stress)		
Stress Response Proteins					
1923	RNA polymerase sigma factor RpoE	0897	RNA polymerase sigma factor RpoE	A1970	RNA polymerase sigma factor RpoE
2072	heat shock protein GrpE	2328	heat shock protein GrpE	A0695	heat shock protein
0559	heat shock protein 33	1398	heat shock protein 33	A1123	Hsp33-like chaperonin, active under oxidative stress
1813	heat shock protein 90	3115	heat shock protein 90	A1902	heat shock protein 90
2580	molecular chaperone DnaK, heat shock protein 70	0548	molecular chaperone DnaK, heat shock protein 70	A2160	molecular chaperone DnaK, heat shock protein 70
2468	molecular chaperone DnaK, heat shock protein 70	2096	molecular chaperone DnaK, heat shock protein 70	A2418	molecular chaperone DnaK, heat shock protein 70
2073	molecular chaperone DnaK, heat shock protein 70	2327	molecular chaperone DnaK, heat shock protein 70	A0694	molecular chaperone DnaK, heat shock protein 70
2074	molecular chaperone DnaJ, heat shock protein 40	2897	molecular chaperone DnaJ, heat shock protein 40	A0693	chaperone protein DnaJ, heat shock protein 40
2401	heat shock protein Hsp20	0420	chaperone	A0654	small heat shock protein

7.2.3. Identifying Genes Responsible for Biofuel Tolerance

To determine which genes are responsible for biofuel tolerance in the model cyanobacterial species, genes identified through the comparative genomics analysis were targeted for either gene overexpression or gene knockout.

The comparative genomics analysis identified efflux pumps and desaturases as likely candidates for the short-chain alcohol tolerance of *Synechococcus* sp. PCC7002. Three efflux pumps were targeted for gene knockout in *Synechococcus* sp. PCC7002: SYNPCC7002_A1013, SYNPCC7002_A0585, and SYNPCC7002_A0719. The efflux pump protein A1013 is homolgous to the AcrB efflux pump, which is responsible for solvent tolerance in *E. coli* [47]. The other two efflux pump proteins A0585 and A0719 are unique to *Synechococcus* sp. PCC7002 (Table 7.1) and may be responsible for the enhanced alcohol tolerance of this strain. Three desaturases were also targeted for gene knockout in *Synechococcus* sp. PCC7002: SYNPCC7002_A0519 (*desB*), SYNPCC7002_A1989 (*desF*), and SYNPCC7002_A2833 (*desE*). DesB was shown to be important for temperature tolerance in *Synechococcus* sp. PCC7002 [43], and DesE and DesF are not found in the other 2 model cyanobacterial species. The mutant strains were tested for growth inhibition with increasing ethanol concentration (Figure 7.2A,B). The efflux pump protein mutants did not show any significant changes in growth inhibition compared to the wild type. This is consistent with literature reports which demonstrate that efflux pumps do not confer alcohol tolerance in *E. coli* [47, 48]. The desaturase mutants, 7002Δ*desE* and 7002Δ*desF*, also do not deviate from the growth inhibition observed for the wild type; however, the *desB* mutant shows increased growth inhibition at 0.5 M ethanol (Figure 7.2B). This suggests that the degree of membrane saturation may play a role in the enhanced alcohol tolerance of *Synechococcus* sp. PCC7002.

While the mechanism of UFA toxicity has yet to be determined, many have proposed that UFA degradation via reaction with ROS generates compounds that are toxic to the cell [35]. To investigate this possible mechanism, four ROS-degrading proteins were overexpressed in *S. elongatus* PCC7942: Synpcc7942_0801 (*sodB*), Synpcc7942_1214, Synpcc7942_0437, and Synpcc7942_1656. These mutant strains were tested for UFA-induced growth inhibition along with the wild type (Figure 7.2C). While there appears to be some improvement in UFA tolerance with overexpression of the ROS-degrading proteins, the variability between biological replicates is so large that this improvement does not fall outside the standard deviation. Therefore, ROS may play some role in UFA toxicity, but the influence of ROS is not likely to be the main cause of UFA toxicity.

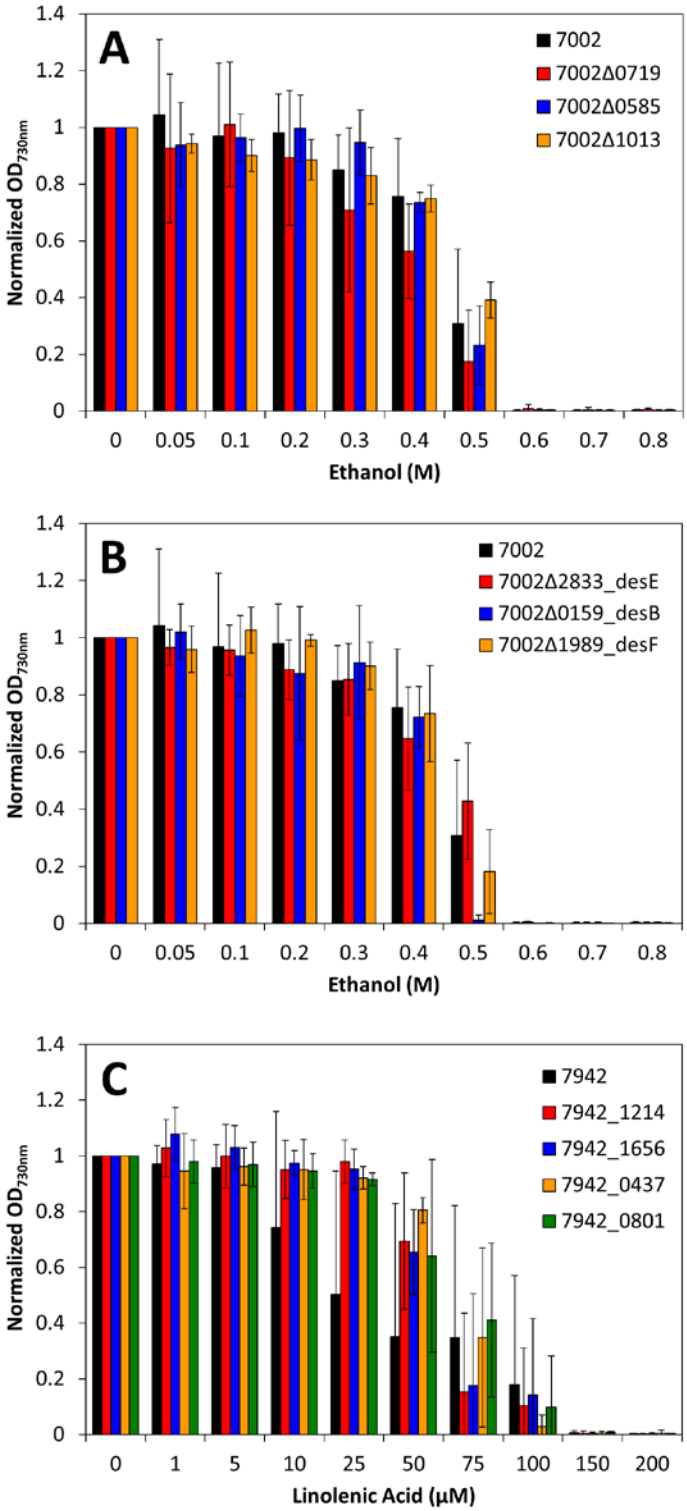

Figure 7.2. Growth inhibition of efflux pump (A), desaturase (B), and ROS-degrading protein (C) mutants with addition of ethanol (A, B) and linolenic acid (C). Data are averages of three biological replicates and error bars indicate the standard deviation.

7.3. Conclusions

Biofuel toxicity is a major obstacle in the commercialization of cyanobacterial fuel production. In this section of the SAND report, we explored the toxicity of various biofuel products and the tolerance of 3 model cyanobacterial strains. Low toxicity was observed for the long-chain alcohol: 1-hexadecanol, saturated long-chain fatty acids: palmitic and stearic acid, the long-chain alkane: pentadecane, and the long-chain alkene: heptadecene. From a toxicity perspective, these metabolic targets are optimal for biofuel production. On the other hand, the short-chain alcohols, ethanol and isobutanol, and long-chain unsaturated fatty acid, linolenic acid, resulted in significant growth inhibition, making these targets less than ideal. Host selection is also an important consideration for reducing the negative effects of biofuel toxicity. *Synechococcus* sp. PCC7002 showed the highest tolerance to short-chain alcohols, while *Synechocystis* sp. PCC6803 had the highest tolerance to linolenic acid. Three potential mechanisms of biofuel tolerance were investigated by constructing targeted mutants: efflux pumps, the degree of membrane saturation, and ROS-degradation. Of the genes targeted in this study, only the desaturase, DesB, was found to influence biofuel tolerance. While the degree of membrane saturation may improve strain tolerance to exogenous biofuel addition, it is not likely to improve tolerance of the biofuel-producing microbe, as microbial-based biofuel production occurs intracellularly. New mechanisms must be identified to address intracellular biofuel toxicity.

8. CONCLUSIONS AND FUTURE WORK

8.1. Significant Contributions

This project explored the genetic engineering of cyanobacteria for the production of a biodiesel precursor, FFAs. The results of this work provide valuable insight into cyanobacterial-based biofuel production and include several significant contributions to this emerging field:

1) Cyanobacterial FFA production results in detrimental physiological effects, including reduced cell growth, decreased photosynthetic yield, and changes in photosynthetic pigment concentration and subcellular location. Other reports of FFA production in *E. coli* and cyanobacteria did not report these physiological effects [22, 28, 30, 36, 49], but the observation of these effects in both strains of FFA-producing cyanobacteria investigated in this study (*S. elongatus* PCC7942 and *Synechococcus* sp. PCC7002) suggests that the physiological effects are conserved rather than host-specific. As a conserved microbial response to FFA production, these physiological effects represent a major obstacle to large-scale FFA production. The reduced cell growth and impaired photosynthetic process will limit productivities and compromise strain robustness.

2) Using RNA-seq and targeted genetic engineering, we identified several candidate genes which may improve cell physiology during FFA production. These genes include hypothetical proteins (Synpcc7942_1655, Synpcc7942_0122, and Synpcc7942_0900), ROS-degrading enzymes (Synpcc7942_1214, Synpcc7942_0801, and Synpcc7942_1656), and a porin protein (Synpcc7942_1464). Furthermore, these preliminary results suggest that porin proteins may play an important role in FFA transport across the cell membrane.

3) Temperature was determined to play an important role in cyanobacterial FFA tolerance. In *Synechococcus* sp. PCC7002, the physiological effects of FFA production were abated by lowering the growth temperature from 38°C to 30°C. The lower temperature may reduce FFA solubility, leading to lower FFA levels in solution. Alternatively, the change in cell membrane saturation at the lower temperature may also play a role. The underlying mechanism for the enhanced FFA tolerance remains to be determined, but once discovered, this offers another possible solution for overcoming the physiological effects of FFA production.

4) *Synechococcus* sp. PCC7002 was determined to be an advantageous host for cyanobacterial FFA production. In addition to the reduced physiological effects of FFA production at 30°C, *Synechococcus* sp. PCC7002 demonstrated an enhanced capacity for FFA synthesis and excretion. Engineered strain S07 produced high levels of soluble, excreted FFAs (~ 150 mg/L) and yielded significant quantities of precipitated FFA, as captured in Figure 8.1. This illustrates the potential process design advantages associated with cyanobacterial fuel excretion, as the precipitated product is physically separated from the culture and can be removed without destroying the biomass. While these results are promising, it should be noted that this high level of FFA production was not stable. After repeated rounds of culturing, FFA production in S07 was significantly reduced,

suggesting that the recombinant genes for high FFA production were mutated due to the selective pressure against FFA production. With high FFA production in S07, the negative physiological effects of FFA production were evident by the reduction in cell growth and photosynthetic yield. Once again, these effects must be addressed to achieve strain stability with high FFA production.

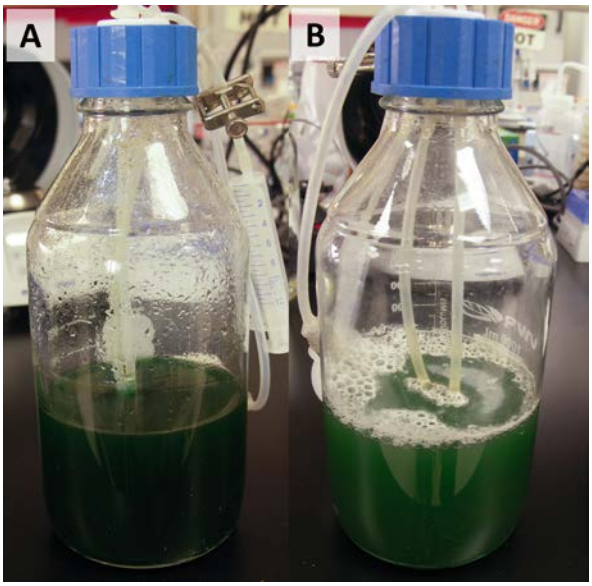

Figure 8.1. Wild type *Synechococcus* sp. PCC7002 (A) and FFA-producing S07 (B) cultures at 500 h. The white layer at the top of the S07 culture is precipitated FFA.

5) Through biofuel toxicity screening, we identified potential biofuel targets with low toxicity as well as host cyanobacterial strains with enhanced biofuel tolerance. Low toxicity biofuel targets include saturated long-chain FFAs, long-chain fatty alcohols, and long-chain alkanes and alkenes. *Synechococcus* sp. PCC7002 showed improved tolerance of short-chain alcohols, while *Synechocystis* sp. PCC6803 had increased tolerance of PUFAs. This fundamental knowledge of cyanobacterial biofuel tolerance may inform future efforts of strain development.

8.2. Future Work

While research efforts, such as the work presented in this report, have made significant contributions to the advancement of cyanobacterial based biofuels, large-scale production of cyanobacterial fuels remains to be demonstrated. For strain development, additional research is necessary regarding the fundamental understanding of cyanobacterial metabolism and genetics, the optimal metabolic product for fuel production, cyanobacterial strain stability, strain robustness, and potential contamination or predator issues. While these areas are too broad to address completely, we will discuss a few of these issues in greater detail along with some proposed future work.

FFAs were targeted as a fuel precursor in this project to demonstrate the potential for hydrocarbon-based fuel production in cyanobacteria. For large-scale fuel production, however, drop-in fuel targets are more desirable than precursors such as FFAs. The metabolic pathway for

alkane production was recently reported in cyanobacteria [50], offering a route for converting FFAs into a drop-in fuel replacement. However, preliminary efforts aimed at engineering alkane production in cyanobacteria have resulted in low yields [49]. We proposed that alkane production may be improved by transferring the alkane production pathway into a high FFA producing strain, such as the S07 strain constructed in this project. Moreover, the two steps of the alkane synthesis pathway must be optimized. These enzymes may be improved by identifying homologous genes in other organisms with improved activities or by using random mutagenesis and screening. The alkane-producing cyanobacterial strains must also be characterized to assess any physiological effects of alkane production. If successful, the development of an efficient alkane-producing cyanobacterium will bring cyanobacterial fuel production one step closer to commercialization.

While the genetic engineering of cyanobacteria is relatively straightforward, particularly compared to the genetic manipulation of eukaryotic algae, the time and effort required for traditional genetic engineering applications currently limits cyanobacterial strain development. High-throughput metabolic engineering methods, such as multiplex automated genome engineering (MAGE) and global transcriptional machinery engineering (gTME) [51, 52], have recently been successful at targeting multiple genes and pathways in *E. coli*. We proposed that the application of genome engineering techniques to cyanobacterial strain development may rapidly accelerate cyanobacterial-based biofuel production.

Cyanobacterial fuel excretion is advantageous for large-scale production as this simplifies the fuel extraction process and the cellular biocatalyst is not destroyed in the fuel harvesting process. Because the cell is not destroyed, a continuous production process is feasible, reducing the amount of nutrients (N & P) required for cell growth and decreasing the time delay associated with growing a new batch of biomass. Lowering the nutrient requirement is particularly beneficial for industrial-scale fuel production, for life cycle analyses of the current batch processes for algal fuel production have predicted that nutrient resource limitations will render the process unsustainable at large scales [9]. While fuel excretion is favorable in this respect, it also exacerbates another impediment in the fuel production process: contamination. The fuel or fuel precursor that is produced and excreted by the cyanobacterial host is a potential carbon source for contaminants. Even under sterile conditions in the laboratory, several contaminants were detected in the FFA-producing cultures of *S. elongatus* PCC7942 (Figure 8.2). The pink/orange colored contaminant did not appear to effect FFA production, but it did produce an unpleasant odor. The white contaminant consumed the FFAs produced by the cyanobacterial culture and was identified as a *Streptomyces* species. These common environmental contaminants present a significant challenge in cyanobacterial fuel production. A potential solution to address contamination is to engineer the cyanobacterial host to survive under adverse environmental conditions. These conditions may include high salt or high pH. High salt conditions are likely for large-scale fuel production. Marine water sources are desirable for fuel production due to the limited availability of freshwater sources, and since evaporation rates are high for open pond systems, the salt concentration within the pond is likely to increase. While this presents challenges for microalgal growth, it will also help to prevent contaminant growth. High pH is another option for controlling contamination. This strategy has been successfully used to grow the cyanobacterium, *Spirulina*, in open ponds for nutritional products. Genetic targets have been identified for salt tolerance which may be used to transfer this trait to the fuel-

producing microalgal host [53], and additional studies on alkaline resistance may reveal the mechanism of high pH tolerance in *Spirulina*. Alternatively, microalgal strains with natural high salt or high pH tolerance may be used as the host. These adverse environmental conditions should help to minimize contaminant growth in cyanobacterial fuel production.

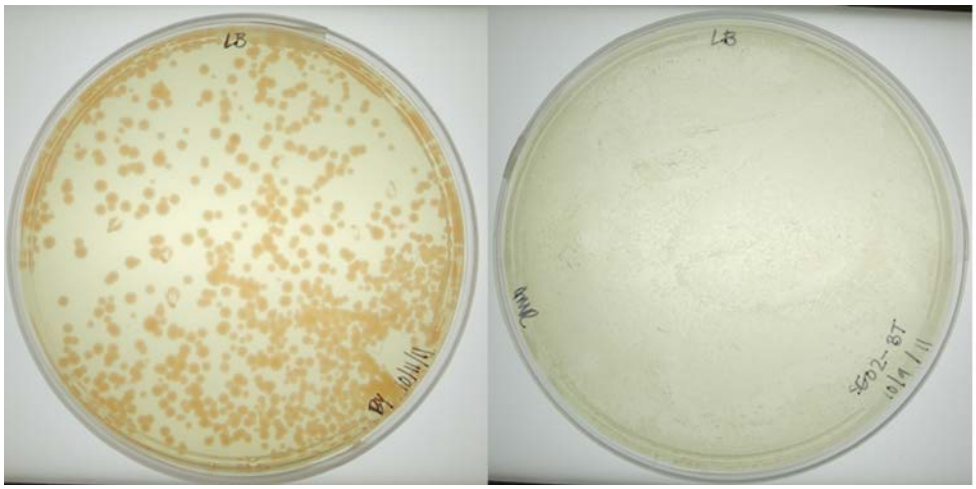

Figure 8.2. Contaminants isolated from FFA-producing cultures of *S. elongatus* PCC7942.

A number of obstacles clearly remain to be addressed to enable profitable, large-scale cyanobacterial fuel production. Fortunately, many of these obstacles have viable solutions, but a continued investment in research and development for cyanobacterial biofuels is necessary to ensure successful commercialization of this technology.

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