Published in final edited form as:

Biotechnol Bioeng. 2012 September ; 109(9): 2190–2199. doi:10.1002/bit.24509.

Physiological Effects of Free Fatty Acid Production in Genetically Engineered *Synechococcus elongatus* PCC 7942

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Abstract

The direct conversion of carbon dioxide into biofuels by photosynthetic microorganisms is a promising alternative energy solution. In this study, a model cyanobacterium, *Synechococcus elongatus* PCC 7942, is engineered to produce free fatty acids (FFA), potential biodiesel precursors, via gene knockout of the FFA-recycling acyl-ACP synthetase and expression of a thioesterase for release of the FFA. Similar to previous efforts, the engineered strains produce and excrete FFA, but the yields are too low for large-scale production. While other efforts have applied additional metabolic engineering strategies in an attempt to boost FFA production, we focus on characterizing the engineered strains to identify the physiological effects that limit cell growth and FFA synthesis. The strains engineered for FFA-production show reduced photosynthetic yields, chlorophyll-*a* degradation, and changes in the cellular localization of the light-harvesting pigments, phycocyanin and allophycocyanin. Possible causes of these physiological effects are also identified. The addition of exogenous linolenic acid, a polyunsaturated FFA, to cultures of *S. elongatus* 7942 yielded a physiological response similar to that observed in the FFA-producing strains with only one notable difference. In addition, the lipid constituents of the cell and thylakoid membranes in the FFA-producing strains show changes in both the relative amounts of lipid components and the degree of saturation of the fatty acid side chains. These changes in lipid composition may affect membrane integrity and structure, the binding and diffusion of phycobilisomes, and the activity of membrane-bound enzymes including those involved in photosynthesis. Thus, the toxicity of unsaturated FFA and changes in membrane composition may be responsible for the physiological effects observed in FFA-producing *S. elongatus* 7942. These issues must be addressed to enable the high yields of FFA synthesis necessary for large-scale biofuel production.

Introduction

As the global demand for energy continues to rise and known oil reserves are depleted, the need for a sustainable energy source becomes increasingly urgent. Microalgal-based fuels are leading candidates for the replacement of petroleum fuels, yet many obstacles must be addressed before large-scale microalgal fuel production can be competitive with the petroleum industry. Initial efforts to develop microalgal-derived fuels focused primarily on eukaryotic algae which produce triacylglycerides (TAG), the precursors for biodiesel (Sheehan et al. 1998). Eukaryotic algal fuel production, however, is limited by natural TAG productivities, the cost of harvesting the algae and extracting the intracellular TAG, the provision of nutrients for large-scale production, and the biological and environmental

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Conflict of Interest

The authors have no conflict of interest to declare.

factors which lead to pond crash (Pienkos and Darzins 2009; Yang et al. 2011). Fuel production from engineered cyanobacteria has the potential to address many of these limitations.

Cyanobacteria offer the same advantages as eukaryotic algae, namely fast growth rates, reliance on a renewable energy source (sunlight), and consumption of CO₂ as the carbon source for fuel synthesis. Cyanobacteria also provide several advantages over eukaryotic algae, including facile genetic manipulation and the potential for fuel excretion. Through genetic engineering, fuel-producing pathways can be introduced to cyanobacteria; metabolic pathways can be engineered to improve fuel productivities; and other desirable traits can be added to tailor-design a biocatalyst for large-scale fuel production and help to prevent pond crash. With the ability to excrete potential fuel precursors such as free fatty acids (FFA) (Kaczmarzyk and Fulda 2010), cyanobacteria can enable a fuel production system whereby the fuel precursor is excreted extracellularly and recovered for further processing without the destruction of the biocatalyst. A fuel-excretion system such as this has the potential to simplify product recovery and allow for continuous fuel production in the stationary phase, alleviating the nutrient requirement for large-scale production. With these potential advantages over eukaryotic algae, there is increased interest in developing a cyanobacterial-based fuel production system.

Initial efforts to engineer cyanobacteria for fuel production have focused on several model species of cyanobacteria, as these species are the best characterized and the tools for their genetic manipulation are readily available. In 2009, Synthetic Genomics, Inc. filed a patent describing FFA production and excretion in three engineered cyanobacteria: *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942, and *Anabaena variabilis* ATCC 29413 (Roessler et al. 2009). The genetic engineering strategy included two main gene targets: knockout of the acyl-ACP synthetase and expression of an acyl-ACP thioesterase. Building upon this work, Roy Curtiss and colleagues improved FFA excretion by weakening the hydrophilic peptidoglycan layer in the cell membrane of *Synechocystis* 6803 (Liu et al. 2011b) and further enhanced FFA yields by expressing lipases to degrade membrane lipids and release FFA (Liu et al. 2011a). *Synechocystis* 6803 has also been engineered to produce other biofuels derived from the fatty acid biosynthesis pathway, including fatty alcohols and hydrocarbons (alkanes and alkenes) (Tan et al. 2011); however, the production of these biofuels is nearly 3 orders of magnitude lower than the amount of excreted FFA.

While many preliminary efforts have focused on *Synechocystis* 6803, other model cyanobacteria such as *S. elongatus* 7942 may be advantageous hosts for biofuel production. Unlike *Synechocystis* 6803, *S. elongatus* 7942 does not contain the pathway for polyhydroxybutyrate (PHB) synthesis (Miyake et al. 2000). As PHB synthesis competes with FFA synthesis for the available carbon flux, the PHB pathway is undesirable from a fuel-production perspective, making *S. elongatus* 7942 an attractive host candidate. In addition, *S. elongatus* 7942 has been successfully engineered for the production of short-chain alcohols, including ethanol and isobutanol (Atsumi et al. 2009; Deng and Coleman 1999). In this work, we investigate the potential of *S. elongatus* 7942 for FFA production and excretion. Our genetic engineering strategy is similar to previously reported efforts, but here, we focus on characterizing the physiological effects of engineering the cyanobacterium for FFA production. We also present a preliminary investigation of the underlying mechanisms causing the observed physiological effects. With the high production rates necessary for large-scale fuel production, negative physiological effects, such as those described in this work, may be prohibitive and must be addressed to realize the true potential for fuel production.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains used in this study are listed in Table 1, along with the plasmids constructed for genetic manipulation of *S. elongatus* 7942.

Genetic engineering of *S. elongatus* 7942 for FFA production

The plasmid pSE15 was constructed for gene knockout of Synpcc7942_0918 (Accession #: NC_007604). pSE15 is derived from pAM2991, generously provided by Susan Golden (Mackey et al. 2008). The 5' and 3' regions homologous to neutral integration site I (NSI) were sequentially removed from pAM2991 using the phosphorylated NSI primers listed in Table 1 and LongAmp Taq DNA polymerase. DNA fragments homologous to the regions upstream and downstream of Synpcc7942_0918 were amplified from *S. elongatus* 7942 genomic DNA using the primers in Table 1. These upstream and downstream DNA fragments were inserted into the modified pAM2991, replacing the previous 5' and 3' NSI regions. The resulting plasmid (pSE15) contains homologous regions for gene knockout of Synpcc7942_0918 and spectinomycin antibiotic resistance for selection. The plasmid pSE16 was constructed for simultaneous knockout of Synpcc7942_0918 and expression of a truncated thioesterase ('*tesA*') from *E. coli*. The truncated thioesterase was cloned from *E. coli* DH5 α genomic DNA using the primers listed in Table 3 and inserted into pSE15, downstream of the inducible *trc* promoter, to obtain pSE16. Plasmids pSE15 and pSE16 were transformed into *S. elongatus* 7942 as described in (Golden et al. 1987) to generate the FFA-producing strains SE01 and SE02. The transformation mixture was spread on BG-11/agar plates containing 1 mM of sodium thiocyanate and 40 μ g/mL of spectinomycin. Successful transformation and genome integration was confirmed via PCR amplification of Synpcc7942_0918 and '*tesA*'.

Cultivation of *S. elongatus* 7942 and engineered strains

S. elongatus 7942 was cultivated in BG-11 media at 30°C in a New Brunswick Scientific Innova 42R incubator shaker with photosynthetic light bank supplying illumination (60 μ mol photons m⁻² s⁻¹) from alternating cool white and plant fluorescent lights. From solid BG-11/agar media with 1 mM sodium thiosulfate, *S. elongatus* 7942 strains were inoculated into 4 mL of BG-11 with 40 μ g/mL of spectinomycin for the engineered strains and grown under conditions previously described with shaking at 150 rpm. After 5 to 7 days of cultivation, cultures were diluted 100x in 100 mL of BG-11 media (supplemented with spectinomycin for SE01 and SE02) in 500 mL baffled flasks and grown for 4 days to serve as inoculum. Inoculum cultures were added to 400 mL of BG-11 media in 1 L glass media bottles to an initial OD₇₃₀ of 0.15. The media bottles were sealed with 3-port Omnifit T series caps, including: a vent port containing a Whatman PolyVENT 4 filter to prevent contamination, a sampling port, and a port for bubbling filter-sterilized air supplemented with 1% CO₂. At an OD₇₃₀ of 0.6 – 0.8, IPTG was added to a final concentration of 1 mM. Samples were taken at 2 day intervals. For toxicity studies, emulsions of palmitic and stearic acid (10 mM) in DI water were sterilized by autoclaving and added to cultures of *S. elongatus* 7942 at an OD₇₃₀ > 1.0. The palmitic and stearic acid stock solutions were added at a ratio of 60:40 to final concentrations totaling 50, 100, and 300 μ M. Linolenic acid was also added to concentrations of 50, 100, and 300 μ M.

At the completion of each experiment, each culture was checked for contamination and genetic stability. To check for bacterial contamination, 100 μ L of each culture was spread on LB/agar plates, incubated at 30°C in the dark for up to 7 days, and checked daily for bacterial growth. To confirm stability of the genetic mutation, each culture was serially diluted and spread on BG-11/agar plates containing sodium thiocyanate. Single colonies

were selected from the plates after incubation at 30°C with illumination at 60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Approximately 50 single colonies were screened using PCR to confirm maintenance of the gene knockout (in SE01 and SE02) and the presence of *tesA* (SE02 only). Based on this PCR screening, the genetic modifications were confirmed to be stable in 88–100% of the viable colonies at completion of the experiments.

FFA and lipid analysis

For analysis of total FFA, 1 mL samples were centrifuged at $5,000 \times g$ for 5 min in 1.5 mL Eppendorf tubes. The supernatant was extracted and frozen at -20°C until analysis. Samples were diluted as necessary for analysis. Total FFA concentration was determined using the enzyme-based Free Fatty Acid Quantification Kit (Biovision). Fluorescence was measured using Corning black 96-well polypropylene assay plates and the Turner Biosystems Modulus Microplate Multimode Reader with green fluorescence module (Ex 525 nm; Em 580 – 640 nm). FFA concentrations were calculated using a standard curve for palmitic acid ranging from 0 to 0.02 nmol/ μL . To analyze the composition of excreted FFA, a 10 mL sample was extracted from the cultures at approximately 400 h. The sample was centrifuged at $4000 \times g$ for 10 min to remove the cell pellet, and FFA were extracted from the supernatant using the lipid extraction protocol detailed below. FFA extracts were derivatized into FAME's and analyzed using GC/MS at the Kansas Lipidomics Research Center.

Lipid samples were collected at 400 h and immediately frozen at -20°C until analysis. Frozen culture samples were thawed and 10 mL of culture was used for lipid extraction. Glass 50 mL Kimax culture tubes with PTFE-lined caps were used for lipid extraction. To each 10 mL sample, one volume of chloroform and one volume of methanol were added, followed by vortexing for 30 sec. One volume of chloroform and one volume of ultrapure water were added. After vortexing for 1 min, the culture tube was covered in foil and placed on a shaker at 300 rpm and room temperature for 30 min. The lower organic phase was removed using a Pasteur pipette and transferred to a new glass culture tube. An additional volume of chloroform was added to the aqueous phase to extract any residual lipid. Following vortexing, the lower organic phase was removed and combined with the first organic phase. The extracted lipid was washed with one volume of 1 M potassium chloride solution. The lower organic phase was transferred to a new tube and washed with one volume of ultrapure water. The organic phase containing the lipid extract was dried using a stream of nitrogen gas. Dried lipid extracts were sent to the Kansas Lipidomics Research Center for ESI-MS analysis of MGDG, DGDG, SQDG, and PG.

Photosynthetic yield and spectral measurements

Photosynthetic yield measurements were taken using a Waltz MINI-PAM Photosynthesis Yield Analyzer. For each measurement, triplicate readings were taken from different locations of the culture through the glass of the media bottle and averaged to obtain accurate values. Phycobiliprotein content in cyanobacteria can affect F_v/F_m measurements; however, because the phycobiliprotein content did not differ between the wild type and engineered strains (Figure 3), no correction was required for this data. Full absorbance spectra (300 – 800 nm) of diluted culture samples were measured using a Beckman Coulter DU-800 UV-Vis Spectrophotometer. The spectra were normalized with respect to OD₇₃₀ for comparison.

Hyperspectral fluorescence confocal imaging

Fluorescence hyperspectral imaging was carried out using a custom built imaging system designed and developed at Sandia National Laboratories (Sinclair et al. 2006). Six fluorescence images were collected for each strain (7942, SE01, and SE02) by exciting the cells with a solid state 488 nm laser (Sapphire 488–200, Coherent Incorporated). These images consisted of seven optical sections culminating into a spatial image cube with

dimensions $25 \times 25 \times 6 \mu\text{m}$. Each voxel within each image cube consisted of 512 emission wavelengths of native or endogenous fluorescence across a spectral range of 500–800 nm. The prism spectrometer incorporated in this imaging system allows for a spectral resolution of between 1–3 nm depending on the wavelength of emitted light. Images were collected using a 60x oil immersion lens (Nikon, 60x NA 1.4 plan apochromat) with an exposure time of 0.24 milliseconds to generate images that have diffraction limited spatial resolution (approx. 250 nm lateral and 600 nm axial).

Following the collection of the hyperspectral images, the images were preprocessed and analyzed using multivariate curve resolution (MCR) algorithms. Unwanted spectral and noise artifacts were removed from the spectral image data to improve the quality of the MCR results as described by (Jones et al. 2012). MCR was then used to extract the independently varying spectral components from the hyperspectral images. MCR provides a relative quantitative analysis of the image data without the need for spectroscopic standards and discovers the independently varying spectral species within an image with little or no *a priori* information (de Juan et al. 2008; Haaland et al. 2009; Vermaas et al. 2008). For this study, all of the images were combined together across the 3 strains to create a super image to be analyzed using MCR. This super image aids the MCR algorithm in extracting the independently varying spectral pure components by increasing the orthogonality of the spectral components contained within the image data set and furthermore provides a common set of spectral components across the different strains. The spectral components obtained from the *S. elongatus* 7942 images were compared to results obtained from imaging *Synechocystis* sp. PCC 6803 (Vermaas et al. 2008) to identify the fluorescence components. In addition, MCR creates a concentration image consisting of the relative intensities from each fluorescence component for each voxel within the hyperspectral image. These concentration images were later used to generate the corresponding colored RGB images used to illustrate the co-localization of the 3 main fluorescence components within the image (Figure 4).

Results

Metabolic engineering of *Synechococcus elongatus* PCC 7942 for FFA production

S. elongatus 7942 naturally produces fatty acids for synthesis of both cell and thylakoid membrane lipids (Figure 1), and any FFA released by membrane degradation are recycled for membrane synthesis via acyl-ACP synthetase (*aas*) (Kaczmarzyk and Fulda 2010). To allow for FFA accumulation, the acyl-ACP synthetase gene (*Synpcc7942_0918*) was targeted for gene knockout using pSE15 (see Materials and Methods), yielding the engineered strain SE01. While elimination of FFA metabolism via *aas* knockout will prevent product consumption, the FA biosynthesis pathway may be inhibited by accumulation of acyl-ACP, as demonstrated in *E. coli* (Jiang and Cronan 1994). Expression of a thioesterase, to cleave the FA moiety from the acyl-carrier-protein, was successful at alleviating feedback inhibition of the FA biosynthesis pathway in *E. coli*. Thus, a similar strategy was adopted to improve FFA production in *S. elongatus* 7942. A truncated thioesterase (*tesA*) was cloned from *E. coli* and simultaneously integrated into the genome of *S. elongatus* 7942 along with *aas* knockout using pSE16. The resulting engineered strain, SE02, expresses a thioesterase to reduce potential inhibition of FA biosynthesis and lacks the ability to metabolize FFA as a result of the *aas* knockout. The wild-type (7942) and two engineered strains (SE01 and SE02) were analyzed for FFA production and cell growth.

FFA production was assessed in each strain by two metrics: (1) determination of the chemical composition of excreted FFA (Table 2) and (2) measurement of the total excreted FFA on a per cell basis (Figure 2A). The first measurement provides insight into the

downstream fuel properties associated with using the FFA as feedstock for biodiesel production, while the second gives an estimate of the strain's capacity for FFA production.

Fuel properties are generally dictated by several parameters of the chemical composition, namely chain length and degree of saturation. The chain length of FFA produced by engineered *S. elongatus* 7942 is determined by the FA composition of the native cell and thylakoid membranes; so it is not surprising that the excreted FFA are mostly comprised of C16 and C18 hydrocarbon chains (Table 2). The degree of saturation will primarily depend on the mechanism by which the FA is released: acyl-ACP cleavage or membrane degradation. Cyanobacteria have been shown to contain only acyl-lipid desaturases (i.e. no acyl-ACP desaturases have been found) (Nishida and Murata 1996). Therefore, any FFA released from thioesterase cleavage of acyl-ACP will be saturated, and any unsaturated FFA must be derived from membrane degradation. In accordance with this theory, the thioesterase-expressing strain, SE02, has higher percentages of saturated FFA (16:0 and 18:0) and lower percentages of unsaturated FFA [16:1 and 18:1(Δ^9 cis)] compared to SE01. As the *S. elongatus* 7942 genome does not include any predicted thioesterases, all FFA excreted by SE01 are assumed to be from membrane degradation. In addition to changes in the degree of saturation, the FFA excreted by SE01 and SE02 include two FFA that are absent in the wild type (unknowns 1 and 2). The mass spectra of these two FFA correspond to 16:2 and 18:2 for unknowns 1 and 2, respectively. However, the peak retention times from GC analysis suggest that these FFA are not 16:2 and 18:2, for the peaks elute after their saturated counterparts instead of prior. While the exact structures of these FFA remain unresolved, they appear to be unique to the engineered strains.

The fuel-producing capability of a microalga is determined by the strain's productivity, which is defined by the organism's rate of fuel production and its growth rate. As expected, FFA excretion by wild-type *S. elongatus* 7942 is negligible, while elimination of FFA recycling allows for accumulation and excretion of FFA in SE01 (Figure 2A), primarily during the stationary phase (after 200 h). By removing feedback inhibition of FA biosynthesis through thioesterase expression in SE02, FFA production and excretion is further improved, particularly during the late exponential and early stationary phases (100 to 300 h). Under the testing conditions for FFA production, the wild-type culture grew with a specific growth rate of 0.014 h^{-1} in the exponential phase, reaching a final cell concentration over 1 g/L (Figure 2B). The specific growth rates of the engineered strains were similar to the wild-type, with values of 0.011 h^{-1} for SE01 and 0.012 h^{-1} for SE02. Cell growth in the late exponential phase varied significantly between the wild-type and engineered strains, however, resulting in 26% and 58% reductions in final cell concentration for SE01 and SE02, respectively. Some reduction in the growth rate is expected for the engineered strains, as the excreted FFA are extracted from the pathway for cell and thylakoid membrane synthesis (Figure 1). However, the amount of excreted FFA in SE01 and SE02 cultures accounts for only 18.5% and 5.9% of the loss in dry cell weight. This suggests that other factors contribute to the reduced cell concentrations of the FFA-producing strains. The reduction in final cell concentration will impact FFA productivity, particularly for large-scale biofuel applications. Thus, the underlying cause of impaired growth during FFA production warrants further investigation.

FFA production effects photosynthesis and photosynthetic pigments

Reduced cell growth can be caused by a myriad of factors. In *S. elongatus* 7942, photosynthesis is essential for cellular energy generation and subsequent cell growth, making photosynthetic damage a likely cause of reduced growth. The effect of FFA production on photosynthesis was analyzed through photosynthetic yield measurements (Campbell et al. 1998). The photosynthetic yield (F_v/F_m) of the wild-type (7942) decreased slowly over the course of the experiment from 0.45 to 0.3 (Figure 2C). The engineered

strains, on the other hand, show a dramatic drop in photosynthetic yield, with values approaching zero for both SE01 and SE02 at 500 h. A decrease in photosynthetic yield is indicative of cell stress and may signify damage to photosystem II.

Changes in photosynthetic pigments are often indicators of stress in cyanobacteria (Riethman et al. 1988) and may account for the observed decrease in photosynthetic yield. The amount of chlorophyll-*a* (Chl-*a*) and phycobiliproteins, the light harvesting pigments in cyanobacteria, remain relatively constant for the wild type, but SE01 and SE02 show a significant decrease in Chl-*a* during FFA synthesis, as illustrated by the absorption spectra (Figure 3). Over the course of FFA biosynthesis, the ratio of phycobiliprotein (625 nm) to Chl-*a* (680 nm) absorbance increases from 0.93 (± 0.0072) to 1.1 (± 0.021) for SE01 and from 0.91 (± 0.037) to 1.3 (± 0.072) for SE02, while the ratio does not exceed 1.0 for the wild type. These pigment changes are also reflected in the fluorescence spectra and likely contribute to the decrease in photosynthetic yield. Hyperspectral confocal fluorescence imaging was applied to determine localized changes in individual pigment fluorescence within the cell (Figure 4) (Sinclair et al. 2006). For the wild type, the fluorescence intensity of Chl-*a* is greater than the fluorescence of either phycobiliprotein, phycocyanin, or allophycocyanin, and the fluorescent pigments are predominantly located in the thylakoid membrane, which is structured in concentric layers adjacent to the inner cell membrane. This fluorescence signature is also observed for SE01 and SE02 during early time points in the synthesis reaction (data not shown). As FFA production increases over time, the fluorescence spectra and pigment localization changes for the engineered strains. At 350 h, SE01 shows decreased Chl-*a* and increased phycocyanin fluorescence. The increase in phycocyanin fluorescence is most likely due to the decrease in energy dissipation via photosynthesis (Figure 2C) because the phycobiliprotein content in SE01 does not differ significantly from 7942 (Figure 3). Moreover, the phycocyanin fluorescence is concentrated in aggregates along the thylakoid membrane (Figure 4B). SE02 images indicate further changes in pigment localization, with low levels of Chl-*a* and carotenoid and phycobiliproteins aggregating at the cell poles (Figure 4B). This suggests a degradation of thylakoid membrane structure or a reduced binding affinity between the phycobilisomes and the thylakoid membranes. Furthermore, the SE02 cell population shows a mixture of fluorescence signatures, with some cells resembling the wild type and other cells showing severe changes in pigment. This heterogeneity may represent differences in cell age or the amount of cellular FFA production. The absorbance and fluorescence data illustrate that significant changes in photosynthetic pigment concentration and localization accompany FFA production in the engineered strains, suggesting a radical change in cell physiology.

Underlying causes of the physiological effects induced by FFA production

Product toxicity is often a limiting factor in biofuel production, particularly for short-chain alcohols such as ethanol and butanol (Stephanopoulos 2007). In addition, FFA are known to have antimicrobial activity for Gram-negative and Gram-positive bacteria as well as several types of algae (Desbois and Smith 2010). To test for product toxicity, exogenous FFA were added to wild type cultures during the exponential growth phase. The excreted FFA profiles for SE01 and SE02 (Table 2) include mainly C16 and C18 fatty acids in both saturated and unsaturated forms. Therefore, palmitic (C16, saturated), stearic (C18, saturated), and linolenic (C18, unsaturated) acids were selected as representative FFA for this toxicity study. (Note: Linolenic acid (18:3) is not produced by the engineered strains but is readily available and often used in toxicity studies.) A mixture of palmitic and stearic acids was added to wild type cultures with final concentrations ranging from 50 to 300 μM . This concentration range is similar to the concentration of excreted FFA in SE02 cultures (approximately 200 μM). The mixture of saturated FFA had no detectable effect on cell growth, photosynthetic yield, or pigment concentration (data not shown). Using the same

concentration range, an unsaturated FFA, linolenic acid, was also added to exponentially growing cultures of the wild type. With all concentrations of linolenic acid tested, the wild type cultures showed reduced cell growth and photosynthetic yield along with decreased photosynthetic pigments. Data from a representative concentration of linolenic acid (100 μ M) are included in Figures 2B, 2C, and 3. These results suggest that unsaturated FFA released by SE01 and SE02 may have toxic effects on cell growth and photosynthesis.

Product toxicity may not be the only factor affecting the cellular physiology of the engineered strains. The genetic manipulation in SE01 and SE02 may inherently change the chemical composition of thylakoid and cell membranes. This is particularly relevant for SE02 which expresses the truncated *E. coli* thioesterase that may selectively release fatty acids of a specific chemical structure. The lipid profiles of the engineered strains were compared to the wild type to determine if changes in membrane composition accompany FFA production (Table 3). Overall, the engineered strains showed decreased amounts of digalactosyl diacylglycerol (DGDG) (7942 > SE01 > SE02) and sulfoquinovosyl diacylglycerol (SQDG) (7942 > SE01 > SE02) in the thylakoid membranes. For both thylakoid and cell membrane lipids, the amount of saturated fatty acids (SFA) increased in the engineered strains (7942 < SE01 < SE02), confirming that genetic manipulation of the fatty acid biosynthesis pathway has led to changes in lipid composition.

Discussion

The engineered strains constructed in this study demonstrate the potential for *S. elongatus* 7942 to be used for the production of biodiesel precursors such as FFA. The excreted FFA can be converted into biodiesel (i.e. fatty acid methyl esters) via acid-catalyzed transesterification (Fukuda et al. 2001), and the fuel properties of the resulting biodiesel are determined by the FFA composition. The chemical profile of FFA excreted by SE01 and SE02 indicate good fuel properties upon conversion to biodiesel. Predominantly comprised of C16 and C18 saturated and monounsaturated FFA, the resulting biodiesel should yield high cetane numbers and low iodine values, indicative of good ignition and combustion quality, lower deposit formation, and reduced lubricant degradation (Ramos et al. 2009). With low levels of polyunsaturated FFA, the excreted FFA should yield biodiesel that is relatively stable to oxidation, but this may also lead to cold-start problems due to high viscosity. Overall, the composition of FFA excreted by engineered *S. elongatus* 7942 appear to be promising for fuel production, yet the preliminary FFA production rates in SE01 and SE02 are too low to support large-scale production.

While FFA are successfully produced and excreted by the engineered strains of *S. elongatus* 7942, the overall cell health of these FFA-producing strains was shown to be compromised. A decrease in photosynthetic yield and Chl-*a* as well as a change in pigment localization was observed in the engineered strains. These detrimental effects on cell physiology must be analyzed and addressed before additional metabolic engineering strategies are applied to enhance FFA production. The preliminary investigation presented in this work suggests that the toxicity of unsaturated FFA may play a role in bringing about these physiological changes. Unsaturated FFA will readily oxidize into a variety of products known to be toxic (Ikawa 2004). As evidence of this toxicity, physiological changes, such as reduced cell growth, photosynthetic yield, and photosynthetic pigment, were observed when exogenous unsaturated FFA was added to wild type cultures. While these changes are reminiscent of those that accompany FFA production in the engineered strains, there is one notable difference: exogenous unsaturated FFA addition led to degradation of all photosynthetic pigments (Chl-*a*, phycocyanin, and allophycocyanin) while FFA production in the engineered strains resulted in selective degradation of only Chl-*a* pigment (Figure 3). This

indicates that the physiological changes cannot be solely attributed to the oxidation of unsaturated FFA.

Alteration of the chemical composition of fatty acids in the thylakoid and cell membranes may be another factor contributing to the physiological effects of FFA production. Lipid analysis confirms a change in the FA profile of membrane lipids in the engineered strains, most notably an increase in the degree of saturation. The increased saturation of membrane FA will affect membrane fluidity, leading to a more rigid membrane structure for both thylakoid and cell membranes (Nishida and Murata 1996). In turn, this may impact photosynthesis via several different mechanisms: (1) the change in membrane fluidity may affect the activity of membrane bound proteins, including those required for photosynthesis; (2) the change in chemical composition may disrupt the membrane structure itself, preventing formation of the thylakoid membranes; and (3) the increased saturation of FA may prevent the attachment of the light-harvesting phycobilisomes to the thylakoid membrane. The influence of fatty acid saturation on photosynthetic activity at low temperatures is well-documented (Gombos et al. 1991; Gombos et al. 1992; Sippola et al. 1998), illustrating that membrane composition impacts enzymatic activities in cyanobacteria. The reduced photosynthetic yields for FFA-producing *S. elongatus* 7942 may correspond to reduced activity of photosynthetic enzymes. Membrane structure may also be compromised, by either the increased degree of saturation of membrane lipids or changes in the ratio of the thylakoid membrane lipids: MGDG, DGDG, and SQDG. Supporting evidence for this mechanism includes the degradation of Chl-*a* and the change in pigment localization. The aggregation of phycobiliprotein at the cell poles may also indicate a reduced binding affinity between the phycobilisomes and the thylakoid membrane. Previous research has demonstrated that slight changes in membrane FA saturation can have a dramatic effect on phycobilisome attachment and diffusion across the membrane (Sarcina et al. 2001). Further exploration is necessary to determine which of these possible mechanisms contributes to the physiological effects observed in FFA-producing *S. elongatus* 7942.

Regardless of whether the observed physiological changes are caused by unsaturated FFA toxicity or changes in lipid composition, these changes must be addressed for cyanobacterial-derived FFA production to be realized at industrial scale. Metabolic engineering may be used to combat the detrimental physiological changes. For example, reactive oxygen species (ROS) are often responsible for the oxidation of unsaturated FFA and generation of toxic products (Bielski et al. 1983), and a metabolic engineering strategy of overexpressing ROS-degrading enzymes such as superoxide dismutase and catalase may repress the generation of toxic compounds. Curiously, changes in cell physiology were not reported for other FFA-producing strains of cyanobacteria, specifically engineered *Synechocystis* 6803. Both *S. elongatus* 7942 and *Synechocystis* 6803 excreted FFA at similar levels, 55.6 mg/L and 83.6 mg/L in comparably engineered strains (Liu et al. 2011b). However, the engineered *Synechocystis* 6803 strains were reported to only produce saturated FFA, indicating that membrane degradation provided a negligible contribution to the FFA pool. These reported variations may be attributed to differences in the host metabolism and if validated, may indicate *Synechocystis* 6803 or another cyanobacterium would be a more suitable host for FFA production compared to *S. elongatus* 7942. Lastly, the production of an alternative hydrocarbon product may alleviate the physiological changes associated with FFA production in *S. elongatus* 7942. Metabolic pathways have been identified for synthesis of a range of potential fuel hydrocarbons, including alkanes (Schirmer et al. 2010), fatty alcohols (Steen et al. 2010), and fatty acid ethyl esters (Kalscheuer et al. 2006). These pathways may be introduced to a host cyanobacterium and optimized for large-scale fuel production. Judicious selection of the target hydrocarbon and host cyanobacterium may be key for generating a production-ready strain, yet regardless of the target product or host strain, the product pathway will compete with essential metabolic

pathways for the available carbon flux. Therefore, an in-depth understanding of cyanobacterial metabolism and physiology is essential to engineer a fuel-producing cyanobacterium with minimal impact on cellular physiology.

Acknowledgments

This work was supported by the Harry S. Truman Fellowship in National Security Science and Engineering and the Laboratory Directed Research and Development program. Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy under Contract DE-ACO4-94AL85000. We are grateful to S. Golden (University of California, San Diego) for providing pAM2991, M. Raymer and O. Garcia (SNL) who assisted in the hyperspectral imaging and analysis, and M. Sinclair (SNL) for maintenance of the hyperspectral confocal fluorescence microscope. Instrument acquisition and method development at the Kansas Lipidomics Research Center was supported by NSF grants MCB 0455318 and DBI 0521587, K-INBRE (NIH Grant P20 RR16475 from the INBRE program of the National Center for Research Resources), and NSF EPSCoR grant EPS-0236913 with matching support from the State of Kansas through Kansas Technology Enterprise Corporation and Kansas State University.

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Figure 1.

Simplified schematic of the metabolic pathways in *S. elongatus* 7942. The grey, dashed X indicates gene knockout of the acyl-ACP synthetase in SE01 and SE02, and the grey, dashed arrow indicates heterologous expression of the truncated thioesterase in SE02.

Figure 2.

(A) FFA excreted on a dry cell weight basis; (B) cell growth profiles; (C) photosynthetic yield measurements. ● wild type (7942); □ SE01; ▲ SE02; × wild type (7942) with the addition of 100 μ M of linolenic acid at 100 h. Error bars represent the standard deviation of three biological replicates.

Figure 3. Normalized absorption spectra at 500 h for the wild type (7942) and engineered strains (SE01 and SE02) during FFA synthesis and for the wild type with addition of 100 μ M of linolenic acid (LA).

Figure 4.

Localization of photosynthetic pigments determined by hyperspectral confocal fluorescence imaging with samples collected at 350 h. Pure component spectra determined from MCR analysis (A). Relative concentration plots of allophycocyanin (APC), phycocyanin (PC), chlorophyll-a (Chl-a), and carotenoid. RGB images include the relative fluorescence intensities of APC (red), Chl-a (green), and PC (blue). The scale bar in each image is 5 μm .

Table 1

Strains, plasmids, and primers used in this study. Restriction enzyme sites in the primers are underlined.

Strain	Description	Reference
<i>Escherichia coli</i> DH5α	<i>E. coli</i> strain used for molecular cloning	New England Biolabs
<i>Synechococcus elongatus</i> PCC 7942	Wild type; a freshwater cyanobacterium	ATCC
SE01	<i>S. elongatus</i> 7942 with gene knockout of acyl-ACP synthetase (Synpcc7942_0918)	This study
SE02	<i>S. elongatus</i> 7942 with gene knockout of acyl-ACP synthetase (Synpcc7942_0918) and expression of a truncated thioesterase from <i>E. coli</i> ('tesA')	This study
Plasmids	Description	Reference
pAM2991	Plasmid constructed for <i>S. elongatus</i> 7942 genome integration at NSI and gene expression using the <i>trc</i> promoter	(Mackey et al. 2008)
pSE15	Plasmid derived from pAM2991 with NSI homologous regions replaced by upstream (961bp) and downstream (978bp) homologous regions of Synpcc7942_0918 for gene knockout of the acyl-ACP synthetase	This study
pSE16	Plasmid derived from pSE15 with a truncated thioesterase from <i>E. coli</i> ('tesA') expressed by the <i>trc</i> promoter	This study

Primers	Description
5'-P-CTGGAGATCTGACGAGCAGGGACTCGAAGCT-3'	Forward primer for removal of NSI 5' region from pAM2991
5'-P-GTCACTCGAGCGGCTGCCGGATATCTGCT-3'	Reverse primer for removal of NSI 5' region from pAM2991
5'-P-CTAGCTTAAGACTCACAGTCCAGTCCAGAAAAGCATCT-3'	Forward primer for removal of NSI 3' region from pAM2991
5'-P-GTCCACTAGTATCTTCTGCTCCAGAAAGCTCGAAA-3'	Reverse primer for removal of NSI 3' region from pAM2991
5'-GTACTTCTCGAGGCAGCTCCGTTGTGCGAGTGTGTCAGA-3'	Forward primer for cloning the region upstream of Synpcc7942_0918
5'-GAGTCGAGATCTGCTGTGGTCCCCGAGGTATAGATC-3'	Reverse primer for cloning the region upstream of Synpcc7942_0918
5'-GTCAGGACTAGTGAACCCCGCCGATTGAAGATGCCCT-3'	Forward primer for cloning the region downstream of Synpcc7942_0918
5'-GAGTTGCTTAAGAGACATCACTCAAGTCATCAGTCACAG-3'	Reverse primer for cloning the region downstream of Synpcc7942_0918
5'-GTGATGGAATTCGACGGGACACGTTATTGATTCTGG-3'	Forward primer for cloning 'tesA' from <i>E. coli</i> DH5α
5'-CGAGTCGGATCCTTATGAGTCATGATTACTAAAGGCTGC-3'	Reverse primer for cloning 'tesA' from <i>E. coli</i> DH5α

Table 2

Chemical composition of excreted FFA from GC/MS analysis.

	% of total peak area									
Strain	14:0	16:0	16:1	Unknown 1	18:0	18:1 ($\Delta 9$ cis)	18:1 ($\Delta 11$ cis)	Unknown 2	Total (%)	
7942	1.66 $\pm$ 0.93	42.8 $\pm$ 6.07	16.1 $\pm$ 4.46	ND	19.1 $\pm$ 6.60	6.78 $\pm$ 2.91	13.6 $\pm$ 6.86	ND	100	
SE01	0.82 $\pm$ 0.23	22.8 $\pm$ 7.38	19.8 $\pm$ 9.38	11.7 $\pm$ 3.59	11.4 $\pm$ 3.64	7.57 $\pm$ 1.25	17.9 $\pm$ 2.24	8.01 $\pm$ 2.72	100	
SE02	0.94 $\pm$ 0.11	26.3 $\pm$ 4.04	14.0 $\pm$ 4.02	11.2 $\pm$ 8.02	15.7 $\pm$ 6.27	5.99 $\pm$ 1.24	21.1 $\pm$ 2.62	4.79 $\pm$ 0.28	100	

Percentages are based on total peak area from GC analysis; MS spectra were used to confirm peak identities. Data are reported as averages of 3 biological replicates with standard deviation. While the amount of FFA excreted by the wild type is negligible due to recycling via acyl-ACP synthetase, FFA are detected from GC/MS analysis, most likely due to contaminating membrane components found in the supernatant.

Table 3

Lipid composition of thylakoid and cell membranes.

Strain	Thylakoid Membrane Lipids			Cell Membrane Lipid	Fatty Acid Side Chains of Membrane Lipids												MUFA	SFA	DUFA
	MGDG	DGDG	SQDG	PG	30:0	30:1	30:2	32:0	32:1	32:	34:	34:	34:	36:	36:2				
7942	62.6 ± 5.09	21.0 ± 2.43	2.53 ± 0.723	13.6 ± 2.08	0.20 ± 0.04	1.95 ± 0.40	0.42 ± 0.13	2.10 ± 0.79	35.3 ± 4.97	15. 6 ± 1.12	0.00 ± 0.00	14. 6 ± 1.06	18. 5 ± 2.41	0.70 ± 0.20	5.30 ± 1.64		52.5 ± 5.10	2.30 ± 0.79	39.9 ± 3.12
SE01	70.2 ± 9.02	17.5 ± 2.02	0.642 ± 0.216	9.86 ± 2.21	0.89 ± 0.61	4.43 ± 2.38	0.65 ± 0.50	4.92 ± 1.24	30.1 ± 6.41	15. 4 ± 4.13	0.08 ± 0.04	15. 9 ± 3.94	16. 3 ± 2.63	1.74 ± 0.72	4.78 ± 1.14		52.2 ± 7.92	5.90 ± 1.38	37.1 ± 5.05
SE02	72.8 ± 16.3	13.2 ± 3.00	0.649 ± 0.138	13.2 ± 3.28	1.44 ± 0.12	3.56 ± 3.21	0.39 ± 0.47	14.9 ± 6.79	30.9 ± 12.5	9.18 ± 5.60	0.67 ± 0.66	17. 8 ± 5.10	9.09 ± 2.00	3.74 ± 2.44	3.75 ± 1.89		56.0 ± 14.0	17.0 ± 6.82	22.4 ± 6.26

Values are listed as a percentage of the total signal for each sample. Data are reported as averages of 3 biological replicates with standard deviation.

Abbreviations: DGDG: digalactosyl diacylglycerol, DUFA: diunsaturated fatty acids, MGDG: monogalactosyl diacylglycerol, MUFA: monounsaturated fatty acids, PG: phosphatidylglycerol, SFA: saturated fatty acids, SQDG: sulfoquinovosyl diacylglycerol.