Title: Diel light:dark cycles significantly reduce FFA accumulation in FFA producing mutants of *Synechocystis* sp. PCC 6803 compared to continuous light

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Abstract: We examine the effect of diel light:dark cycles (12:12 LD) on the production of free fatty acids (FFAs) in *Synechocystis* sp. PCC 6803. Our results show that FFA titers in FFA producing strains of *Synechocystis* were significantly reduced in 12:12 LD compared to continuous light. On the transcriptional level, we saw no significant changes of the fatty acid biosynthesis pathway as a result of increased FFA production. In addition, we describe our efforts in increasing FFA production by introducing *tesA* from *E. coli*. Despite evidence of *tesA* mRNA transcript abundance, no measurable TesA enzymatic activity was observed in our *Synechocystis* mutants. Overall, our work demonstrates the importance of considering the effects of diel light:dark cycles in the metabolic engineering of cyanobacteria.

Key words: Cyanobacteria; *Synechocystis*; diel-cycles; light:dark cycles; Thioesterase I; free fatty acids; metabolic engineering

1. Introduction

Cyanobacterial technologies are promising platforms for efficient production of renewable chemicals from CO₂ and sunlight [1]. To date, a rapidly growing track record of industrially relevant molecules has been successfully produced in recombinant cyanobacteria (for review, see [2]). The current engineering approach is to design phototrophic bacteria in the same way as heterotrophic bacteria, such as *E. coli*, by transferring genetic components over and subsequently evaluating strains under laboratory simulated conditions. Yet, a growing body of knowledge into the physiology of cyanobacteria encourages a revised way of designing and evaluating them. It has been shown that many strains of cyanobacteria including *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) exhibit circadian rhythms [3-5], allowing for maximal productivity in daily sunlight fluctuations.

Sunlight is a daily “on-and-off” source of energy that governs the livelihood of cyanobacteria. Diurnal changes in transcription, primarily studied via microarrays, suggest that mRNA expression patterns change drastically throughout the day and are highly affected by light:dark cycles [6-9]. Not surprisingly, mRNA transcripts of genes involved in light harvesting, carbon fixation, nitrogen uptake, and photosynthesis peak during the day while the mRNA transcripts of genes involved in respiration, DNA replication, and cell wall biosynthesis peak during the night [8]. Consistent with microarray results, several studies have shown that glycolysis and pentose phosphate pathway related enzymes change dynamically throughout the diurnal LD cycle [10].

Several cyanobacterial cis-acting elements (i.e. promoter DNA elements) have been identified as light-responsive. Two examples are the *psa* and *psb* promoters, which are known to be induced under low-light conditions [11] and high-light conditions [12], respectively. Furthermore, the *lrfA* promoter is known to be dark-induced [13], and several sigma factors are redox-sensitive and attributed to wide-spread changes in transcription activation [14]. In *Synechococcus elongatus* sp. PCC 7942, there is evidence for global transcription activation/deactivation during circadian rhythm by altering chromosome topology via supercoiling [15]. Beyond the central dogma, metabolite changes are also

prevalent under diurnal cycles. For example, glycogen has been known to play a key role in energy storage: the glycogen stores are replete in the day-time and deplete in the night-time [16, 17].

The production of free fatty acids (FFA) in *Synechocystis* has been closely associated to acyl-ACP (Figure 1). Acyl-ACP is the product of the fatty acid biosynthesis pathway. As early as 2010, acyl-ACP synthetase (*aas*, EC 6.2.1.20, orf: slr1609) was first identified in *Synechocystis*, and its deletion lead to increased intracellular and extracellular FFA titers [18]. Another class of enzymes known as thioesterases cleaves the ACP moiety from acyl-ACP to yield FFAs. In 2011, the simultaneous introduction of thioesterases and deletion of endogenous genes (*aas* included) lead to increased FFA production in *Synechocystis* [19]. In 2013, another study in *Synechocystis* achieved increased FFA production by deleting *aas* and expressing *fatB* from *Arabidopsis thaliana* [20]. A common theme amongst all these studies is the characterization of FFAs production under continuous light conditions.

Given that cyanobacteria undergo dramatic changes in response to light availability, this study investigates the effect of light:dark diel cycles (12:12 LD) on the production of FFAs. In the first part of this work, we evaluated the production of FFAs in WT and an *aas* deletion mutant (Δaas strain) of *Synechocystis* in continuous light and in diel cycles (12:12 LD). The second part of this work focuses on our effort in increasing FFA production by optimizing the expression of a N-truncated thioesterase I (*'tesA^{CO}*) from *E. coli*. We evaluated all our *'tesA* expressing strains in both continuous light and in 12:12 LD. Although there were small increases in FFA titers as a result of expressing *'tesA* in our Δaas strain, we did not observe the large increase that we expected. Overall, this work serves to highlight the importance of evaluating FFA production in cyanobacteria under diel cycles and the need for careful evaluation of genetic modifications in *Synechocystis* in order to determine the contribution of each genetic change.

Figure 1. The fatty acid biosynthesis pathway in *Synechocystis*. Pathways in black represent common pathways in *E. coli* and cyanobacteria. Pathways in green represent exclusive routes in *Synechocystis*. Dashed lines indicate multiple steps leading to the desired metabolite.

2. Materials and Methods

2.1 Plasmids and strains

All strains and plasmids used in this study are listed in Table 1. Plasmids were constructed in *E. coli* 5 α (New England Biolabs). Gene sequences from *Synechocystis* were obtained from Cyanobase (<http://genome.kazusa.or.jp/cyanobase/Synechocystis>) [21]. The sequence for *tesA* was obtained from the sequenced genome of *E. coli* K12 MG1655 (<http://ecocyc.org/>). The segment encoding the *tesA* leader sequence was omitted (yielding '*tesA*') to allow localization of the protein in the cytoplasm [22]. The '*tesA*' sequence was subsequently codon optimized and chemically synthesized for expression in *Synechocystis* (Genscript, NJ, referred to as '*tesA*^{CO}' from here on, see Table S1 for sequence) in the plasmid pCO'TesA148. A *psbAII* promoter suite was previously developed in the lab (unpublished work). The *psbAII* promoter sequences used in this work can be obtained from NCBI as follows: *psbAII* promoter (KT162050), *psbAII* Δ Hex promoter (KT162051), and *psbAII* Δ Hex + HLR promoter (KT162053). All PCR primers used in this study are listed in Table S2. Plasmid p'TesA92 was constructed by inserting *psbAII* promoter (KT162050) and '*tesA*' between the PstI and Sall sites of the

94 plasmid pUC19m 1609 HR (from [23]). Plasmid pCO'TesA150 was constructed in a similar manner with
 95 the exception of 'tesA being replaced by 'tesA^{CO} (obtained from pCO'TesA148). Two variants of
 96 pCO'TesA150 were also subsequently constructed, this time with the *psbAII* promoter (KT162050)
 97 being replaced by *psbAII* ΔHex + HLR promoter (KT162053) and *psbAII* ΔHex promoter (KT162051) to
 98 yield plasmids pCO'TesA194 and pCO'TesA152 respectively. For plasmid pCO'TesA203, two sets of
 99 primers were designed to PCR the P_{psbAII complete Δhex}-'tesA^{CO} cassette from pCO'TesA152 and the pIGA4
 100 backbone from pIGA4maz-kan202 and combined via Gibson assembly to yield the final plasmid.

101 Transformation of these plasmids into *Synechocystis* and screening for desired mutants was
 102 performed as previously described [23]. Briefly, the strain WT NF1609 (from [23]) was transformed with
 103 plasmids p'TesA92, pCO'TesA150, pCO'TesA194, and pCO'TesA152 to yield strains AF1609, GG1.1,
 104 GG1.2, and GG1.3 respectively. Then GG1.3 was transformed with plasmid pIGA4maz-kan202 to yield
 105 strain GG1.3 NF0168. Finally, GG1.3 NF0168 was transformed with pCO'TesA203 to yield strain
 106 GG2.3. Completely segregated mutants were isolated as previously described [23]. In addition, the
 107 colony PCR products from the *Synechocystis* mutants were sequenced to verify that the desired DNA
 108 modifications were correct.

109 **Table 1:** Plasmids and strains used in this study

Strain	Genotype or plasmid	Source
<i>E. coli</i>		
DH5α	<i>E. coli</i> competent cells (WT <i>E. coli</i>).	New England Biolabs
Ec92	WT <i>E. coli</i> expressing 'TesA, P _{psbAII complete} (harbors plasmid p'TesA92).	This work
Plasmid		
p'TesA92	pUC19m, slr1609 homologous regions, 'tesA, P _{psbAII complete} .	This work
pCO'TesA148	pUC57-Kan, codon optimized 'tesA ('tesA ^{CO}).	Genscript
pCO'TesA150	pUC19m, slr1609 homologous regions, 'tesA ^{CO} , P _{psbAII complete} .	This work
pCO'TesA194	pUC19m, slr1609 homologous regions, 'tesA ^{CO} , P _{psbAII complete Δhex +HLR} .	This work
pCO'TesA152	pUC19m, slr1609 homologous regions, 'tesA ^{CO} , P _{psbAII complete Δhex} .	This work
pIGA4maz-kan202	pIGA4, mazF-kan.	This work
pCO'TesA203	pIGA4, 'tesA ^{CO} , P _{psbAII complete Δhex} .	This work
<i>Synechocystis</i>		

WT	WT <i>Synechocystis</i> sp. PCC 6803.	ATCC 27184
WT NF1609	WT with slr1609 replaced by <i>mazF/aphII</i> .	(Cheah <i>et al.</i> , 2012)
Δ Aas	WT with slr1609 deleted and <i>mazF/aphII</i> removed.	(Cheah <i>et al.</i> , 2012)
AF1609	WT with slr1609 replaced by ' <i>tesA</i> ', P_{psbAII} complete.	This work
GG1.1	WT with slr1609 replaced by ' <i>tesA</i> ^{CO} ', P_{psbAII} complete.	This work
GG1.2	WT with slr1609 replaced by ' <i>tesA</i> ^{CO} ', P_{psbAII} complete Δ hex +HLR.	This work
GG1.3	WT with slr1609 replaced by ' <i>tesA</i> ^{CO} ', P_{psbAII} complete Δ hex.	This work
GG1.3 NF0168	GG1.3 with slr0168 replaced by <i>mazF</i> -KanR cassette.	This work
GG2.3	GG1.3 NF0168 with slr0168 replaced by ' <i>tesA</i> ^{CO} ', P_{psbAII} complete Δ hex.	This work

2.2 Culturing conditions with bicarbonate feeding

Triplicate *Synechocystis* strains were inoculated to an OD_{730nm} of ~0.2 in 40 mL of BG-11 + 6xPhosphate (modified from BG-11 based on the known phosphate limitation [24]). All cultures were grown at 30 °C, 225 rpm in an INNOVA 44 Incubator Shaker (New Brunswick Scientific, Enfield, CT) equipped with 24 watt fluorescent grow lights with an average light intensity of 139.3 ± 5.7 μ mol photons/m²/s.

For continuous light studies, 800 μ L of 1 M sodium bicarbonate (NaHCO₃) was added to the cultures and the pH of the cultures was adjusted to 8.0 ± 0.2 with 6 N HCl. This step was repeated every 12 hrs for 6 days. Beginning day 2, 2 mL samples were taken daily for fatty acid extraction. To maintain the culture volume, equivalent volume of fresh media (BG-11 + 6xPhosphate) was replenished each time the cultures were sampled. For 12:12 LD cycle studies, the same procedure was repeated with one change: the lights in the incubator shakers were cycled on-and-off every 12 hrs.

2.3 Fatty acid extraction and derivatization

Aliquots of 2 mL cultures were stored at -80 °C prior to extraction. All samples were extracted via the Bligh and Dyer method [25] with the addition of a strong acid to acidify the aliquots [26]. Briefly, 200 μ L of acetic acid was added to each 2mL sample and vortexed. As an internal standard, 10 μ L of 4 g/L pentadecanoic acid (C15:0 FFA, Sigma) dissolved in hexane was added to each sample. Then 7.5 mL of chloroform-methanol (CHCl₃-CH₃OH, 1:2 [vol/vol]) was added to each sample and vortexed.

129 Subsequent addition of 2.5 mL of CHCl_3 causes the samples to partition into 2 layers. Then, 2.5 mL of
130 HPLC H_2O was added and vortexed again. The samples were left to phase separate overnight. The
131 following day, the lower layer was pipetted out into a new vial and evaporated to dryness under a
132 steady stream of nitrogen. Once the samples are dry, they were resuspended in 200 μL of hexane and
133 analyzed for FFAs on the GC.

134 Total fatty acid methyl esters (FAME) describe the sum of FFAs and the non-FFAs (i.e.
135 membrane phospholipids) in our samples. In our study, samples were derivatized with anhydrous
136 methanolic HCl [27] using the Supelco 3 N methanolic HCl kit (Sigma). Samples that were previously
137 dissolved in hexane were dried down under nitrogen again. Then 1 mL of 3 N methanolic HCl was
138 added to each sample and incubated at 80 °C in a sandbath for 2 hours. The reaction was halted by the
139 addition of 500 μL of HPLC H_2O . Subsequent addition of 500 μL of hexane and thorough vortexing
140 dissolved the FAME into the hexane layer. The hexane layer was extracted and analyzed for FAME on
141 the GC.

142 **2.4 GC-FID analysis**

143 All extracted samples were analyzed on a HP 5890 series II chromatograph equipped with an
144 FID at 250 °C, injector at 250 °C, and a Nukol capillary GC column (Sigma 25327) [28]. For FFA
145 analysis, 2.5 μL of each sample was injected using splitless injection with the following oven program:
146 0.5min at 90°C, 30°C/min ramp to 190 °C, and a final hold at 190 °C for 46 min. For FAME analysis, 2.0
147 μL of each sample was analyzed using splitless injection with the following oven program: 0.5 min at
148 90°C, 12 °C/ min ramp to 190 °C, and a final hold at 190 °C for 29.5 min. The standard curves for FFA
149 analysis were made with saturated & unsaturated fatty acid standard series (Nu-Chek Prep Inc,
150 Elysian, MN). For FAME analysis, standard curves were made with Supelco 37 FAME mix (Sigma).

151 **2.5 RNA extraction and qRT-PCR**

152 Cells were grown as in section 2.2. For cultures grown in continuous light, 15mL of
153 *Synechocystis* cells at an $\text{OD}_{730\text{nm}}$ of ~10 (approximately 6.8×10^{10} cells, assuming $\text{OD}_{730\text{nm}}$ of 1 equals
154 to 4×10^8 cells/mL; [19]) were harvested by centrifugation at 12, 000 xg at 4 °C. For cultures grown in

155 12:12 LD, 15mL of *Synechocystis* cells at an OD_{730nm} of ~5 (approximately 3.4×10^{10} cells) were
156 harvested. All samples were stored at -80°C prior to extraction. The RNA extraction procedure of Pinot
157 *et al.* 2009 was followed [29]. After RNA extraction, all samples were treated with DNase using the
158 TURBO DNA-free™ kit (Life technologies, Carlsbad, CA). To further improve the purity of RNA, all
159 samples were purified via phenol-chloroform extraction [30]. First strand cDNA was synthesized using
160 the GoScript™ Reverse Transcription System (Promega, Madison, WI).

161 For qRT-PCR, 5 ng of cDNA sample was used per reaction. *RnpB* was selected a reference
162 gene because it has been shown to be suitable in both continuous light and LD [31]. The primers for
163 each gene of interest (listed in Table S3) were ordered based on the best fit from Primer3
164 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Each reaction was prepared using
165 SsoAdvanced™ Universal SYBR® Green Supermix (Biorad, Hercules, CA), aliquot into 96 well plates,
166 and ran on a Biorad CFX Connect™ Real-Time PCR Detection System. No reverse transcriptase (-RT)
167 controls were ran for each gene of interest. All samples with reverse transcriptase were run in technical
168 quadruplicates. The data was analyzed by the relative quantification method. The Ct values of our
169 genes of interest were normalized to one reference gene (*rnpB*) and the fold changes were calculated
170 by $2^{-\Delta\Delta C_t}$.

171 2.6 TesA enzymatic activity assay

172 Our TesA enzymatic assay was adapted from Barnes *et al.* [32] and down-scaled to allow for
173 experiments in 96 well plates. Our crude protein isolation method was adapted from Wang *et al.* 2013
174 [33]. With cells grown in continuous light as in section 2.2, we harvested 10mL of day 3 *Synechocystis*
175 cells at an OD_{730nm} of ~10 (approximately 4.5×10^{10} cells) by centrifugation at 5,000 xg at 4 °C. As for
176 *E. coli* cells, 2 mL cultures of Ec92 and WT *E. coli* grown for 16 hrs were harvested in a similar manner.
177 All cell pellets were washed and resuspended in 1 mL 60 mM potassium phosphate (pH 7.4) and
178 treated with protease inhibitor cocktail (Sigma P8465). Following cell lysis via sonication (100 cycles of
179 3-s-on/3-s-off) on ice, the samples were centrifuged at 17,000 xg at 4 °C for 10 min and the
180 supernatant was harvested. Using a Bradford assay, the total protein concentrations of all samples

181 were determined and diluted to 1.0 ng/μL. Each reaction in a 96 well plate contains the following: 126.4
182 μL of 60mM potassium phosphate (pH 7.4), 5.6 μL of 0.4975 mM palmitoyl-CoA (Sigma P9716), 8.0 μL
183 of 2 mg/mL BSA, and 20 μL of 1 mM DTNB (Sigma D8130). The plate was incubated at 25 °C for 5 min
184 in the FLUOstar Omega Microplate reader (BMG Labtech, Cary, NC). Then with a multichannel pipet,
185 40 μL of crude extract was added to each reaction to make a total volume of 200 μL. The absorbance
186 at 412 nm was monitored for 30 min.

187 **2.7 Protein digestion for LC-MS/MS analysis**

188 One hundred milligrams of *Synechocystis* cell pellets were resuspended in 300 μL of lysis buffer
189 containing 50 mM triethylammonium bicarbonate (TEAB) and 8 M urea and homogenized by sonication
190 on ice (15 s pulse, three repeats, 60 s rest between pulse). Cell lysates were centrifuged at 4 °C for 300
191 s (15,000 rcf) and pelleted out cell debris. 20 μg of proteins in supernatant were reduced by
192 dithiothreitol and alkylated by iodoacetamide, according to the manufacturer's protocol (Pierce,
193 Rockford, IL, USA). Alkylated proteins were digested with 1 μg of trypsin (Promega) overnight at 37 °C.
194 The tryptic digests were cleaned up with C18 spin columns (Pierce, Rockford, IL, USA) prior to LC-
195 MS/MS analysis.

196 **2.8. LC-MS/MS analysis and identification of TesA**

197 One μg of tryptic digest was introduced to LC column (C18, 75 μm ID, 150 mm length, 120 Å,
198 Eksigent Technologies). NanoLC-Ultra-2D (Eksigent Technologies) was used to elute the peptides from
199 the LC column (2% ~ 80% acetonitrile gradient with 0.1% formic acid, flow rate: 300 nL/min). ESI-
200 tripleTOF 5600 (ABSciex) was used to analyze the peptides. LC-MS/MS raw data were converted into
201 mzML format and processed by PEAK 7.0 (BSI) against the TesA amino acid sequence and
202 *Synechocystis* database (<http://genome.kazusa.or.jp/cyanobase/Synechocystis>) [21].

203 **3. Results and Discussion**

204 **3.1 FFA titers are increased in Δ*aas* strain grown in continuous light conditions with**
205 **bicarbonate feeding**

206 In *Synechocystis*, acyl-ACPs are synthesized in two ways: (i) through the FA biosynthesis
207 pathway and (ii) through the recycling of FFAs released from membrane lipids (pathway in Fig 1).
208 Presently, little is known about genes/enzymes/pathways associated with membrane lipid recycling in
209 *Synechocystis*. Acyl-ACP synthetase (*aas*) is responsible for the recycling FFAs released from
210 membrane lipids back to Acyl-ACP; *aas* deletion mutants have been shown to secrete FFAs into the
211 media and cause an increase in FFA titer [18]. *LipA* (orf: sll1969) is a putative lipolytic enzyme that
212 purportedly releases fatty acids from diacylglycerols [34]. Acyl-ACP can also be converted to
213 heptadecane in *Synechocystis* [35], and Gao *et al.* demonstrated that *aas* expression effects both FFA
214 and heptadecane production [36].

215 Previously we constructed an *aas* deletion mutant in wild type (WT) *Synechocystis*, hereafter
216 Δaas [23]. In the present study, we characterized the total FFA titers (i.e. intracellular plus secreted) of
217 WT and Δaas strains across 6 days in continuous light conditions with bicarbonate feeding (Figure 2).
218 The Δaas mutant achieved a maximum total FFA productivity of ~19.5mg/L/day between days 2 and 3.
219 Beyond day 3, the productivity decreased (~4mg/L/day between days 3 and 4) before it reached a
220 plateau. Over the course of 6 days in continuous light, the WT strain achieved a maximum total FFA
221 titer of 22.9 ± 5.0 mg/L while the Δaas mutant achieved 44.3 ± 1.7 mg/L. Even though there were
222 significant increases in total FFA titers beyond day 3, FFA toxicity does not seem to heavily impact the
223 growth of the Δaas strain in continuous light cultivation (as seen by the OD_{730nm} in Figure 2). Consistent
224 with previous studies [18, 36], the most noticeable difference in FFA titers between the strains came
225 from increases in stearic acid (C18:0) with day 5 titers for Δaas strain being 6.0 ± 0.5 mg/L compared to
226 minimal amounts in WT (0.3 ± 0.3 mg/L, Figure 3). Other notable increases (of at least two fold) include
227 elaidic acid (C18:1n9t), linoleic acid (C18:2n6c), and γ -linolenic acid (C18:3n6) titers. Small differences
228 were observed in palmitic acid (C16:0) and palmitoleic acid (C16:1) titers.

Figure 2. Increased FFA titers by deletion of *aas* in continuous light conditions with bicarbonate feeding over 6 days. WT is shown in green and Δaas is shown in yellow. Standard deviation shown is across 3 biological replicates.

Figure 3. Detailed FFA profile titer showing significant changes in Δaas strain compared to WT when cultured in continuous light with bicarbonate feeding across 6 days. From day 3 onwards, significant increases were observed in stearic acid (C18:0), elaidic acid (C18:1n9t), linoleic acid (C18:2n6c), and γ -linolenic acid (C18:3n6). Standard deviation shown is across 3 biological replicates.

Next, we investigated several genes in the fatty acid biosynthesis pathway (*accC*, *fabF*, *fabG*, *fabI*, *fabZ*, *aas*, *plsX*, and *fabH*, see Figure 1) at the transcriptional level to elucidate potential pathway regulation as a result of *aas* deletion. In continuous light conditions, there were no significant changes of the transcripts in the pathway between WT and Δaas (Table S4). In addition, FFAs accounted for approximately 37% of the total FAME in the Δaas strain compared to 21% in the WT (Figure S1). It is

also worth noting that there was insignificant difference in the total FAME between the two strains (WT: 84.4 ± 4.3 mg/L, Δaas : 93.0 ± 7.2 mg/L, $p = 0.152$).

3.2 WT and Δaas FFA titers are similar when grown in 12:12 LD conditions with bicarbonate feeding.

Interestingly, total FFA titers (Figure 4) and species profiles (Figure 5) are significantly different from that seen in continuous light conditions (Figures 2 & 3). Between days 2 and 3, the Δaas strain attained a slightly higher FFA titer compared to WT. However, beyond day 3 there was little difference between the WT and Δaas strains. Over the course of 6 days in 12:12 LD, both WT and Δaas strains achieved similar titers of FFAs (Figure 4). A closer look at the distribution of FFA species between the strains showed little to no difference in FFA titers beyond Day 3 (Figure 5). This phenomenon was interesting as there was a significant cessation of FFA production in the Δaas strains under 12:12 LD (compared to continuous light). As far as we know, this phenomenon has yet to be described in literature. Hence we hypothesized that there may be differential transcriptional regulation in the fatty acid biosynthesis pathway in 12:12 LD vs continuous light which may lead to a decrease in FFA production.

We tested this hypothesis by comparing the fatty acid biosynthesis genes in WT vs Δaas strain during mid-day (day 2.75, Table S5A) and mid-night (day 3.25, Table S5B) via qRT-PCR. We found no significant differences in transcription of those genes. Surprisingly, we observed a significant increase ($p < 0.05$) in transcript abundance of *lipA* in both times and this result leads us to speculate that there may be changes occurring in the membrane lipid recycling pathway. *LipA* is a putative lipase gene in *Synechocystis* [37] and further investigation into its function may shed light onto its role in the cessation of FFA production. On the other hand, a comparison of fatty acid biosynthesis genes in mid-day vs. mid-night, in WT (Table S6A) and in the Δaas strain (Table S6B) showed a less surprising result: transcripts of fatty acid biosynthesis were more abundant in the light phase ($p < 0.05$) compared to dark phase. Several of our results (*fabG*, *fabF* and *acc*) agree with previously published literature (day at 10:00am versus dark at 0:00am in [8]).

Figure 4: Cessation of FFA production in the Δaas strain in 12:12 LD conditions with bicarbonate feeding. WT (green) and Δaas (yellow) cells were grown for six days. Grey regions represent periods of dark. Standard deviation shown is across 3 biological replicates.

Figure 5. Detailed FFA profile titers showing lack of changes in Δaas strain compared to WT in 12:12 LD conditions with bicarbonate feeding across 6 days. Standard deviation shown is across 3 biological replicates.

We compared the FFA content (as % of DCW) in our study to other published literature that reported FFA content in WT *Synechocystis*. In 2013, Hu *et al.* reported an intracellular FFA content of ~0.125% DCW and extracellular FFA content of ~0.2% DCW in WT *Synechocystis* [20]. Their total FFA content amounts to ~0.325% DCW. In 2011, Liu *et al.* reported FFA titers in a glucose tolerant (GT)

variant of *Synechocystis* in mass per cell (mg/cell) [19]. Using the reported FFA titers in their work and an OD_{730nm} to DCW correlation for WT *Synechocystis* in Wang *et al.* [38], we calculated a total FFA titer (intracellular plus secreted) of ~6.72 mg/L (at an OD₇₃₀ of 12.8) which amounts to approximately 0.231% DCW in GT *Synechocystis*. In 2010, Kaczmaryk *et al.* reported approximately 0.65 nmol/mL/OD₇₅₀ of total FFA titer (intracellular plus secreted) in WT *Synechocystis* [18]. Assuming an OD₇₅₀ of 1 is equivalent to 1.6×10^8 cells/mL [39], the estimated the total FFA titer of 0.417 mg/L (harvested at OD₇₅₀ of 2-3) which corresponds to ~0.153% DCW.

Assuming the same correlations as before, the total FFA content of WT *Synechocystis* in our continuous light study was $0.346 \pm 0.029\%$ DCW at day 6 (at an OD₇₃₀ of 24.2). Furthermore, we found that the total FFA content is highest at $1.32 \pm 0.58\%$ DCW at day 2 and decreases to $0.346 \pm 0.029\%$ DCW as the cultures approach day 6 (Figure 6A). Additionally, the day 3 total FAME content as % of DCW of our WT strain amounted to $3.72 \pm 0.14\%$, a value comparable to that in published literature (~4% [40]). Unlike WT, our Δaas strain maintained a FFA content close to 1% DCW in days 2 through 5 (Figure 6A). This value is different when compared to Δaas strains in other published literature (Hu *et al.* reported ~0.6% DCW [20], Kaczmaryk *et al.* was estimated to be 2% DCW [18]). The reason for these discrepancies between studies could be different growth conditions (we feed 20mM bicarbonate twice daily, Hu *et al.* uses gentle shaking in ambient CO₂ [20], Liu *et al.* uses bubbled 1% CO₂ [19], Kaczmaryk *et al.* uses aeration with sterile air [18]) or different variants of *Synechocystis* strains (we use ATCC 27184, Liu *et al.* uses GT *Synechocystis* [19]).

To get an insight on how the productivity difference occurred under different light conditions, we compared the FFA content in WT and Δaas strain in continuous light and 12:12 LD. In continuous light, the Δaas strain has a significantly higher FFA content ($p < 0.05$) compared to WT on days 2 through 5 (Figure 6A). However in 12:12 LD, the differences in FFA content between WT and Δaas were insignificant ($p > 0.05$) between days 2 through 5 (Figure 6B). The accumulation of FFAs could be attributed to enzymes such as lipases that can degrade phospholipids into FFAs. So far, two putative lipases have been identified in *Synechocystis* (*lipA*, slr1969 [34] and slr1994 [41]). In our study, we

311 found that *lipA* was significantly up-regulated in our Δaas strains during the dark phase (Table S5B).
312 Moreover, unlike the genes in fatty acid biosynthesis that were significantly down-regulated in the dark
313 phase, the mRNA transcripts of *lipA* remained unchanged in both WT and Δaas strains (Table S6).
314 Based on these observations, we hypothesize that the decrease in FFA content in Δaas during 12:12
315 LD could be due to differential regulation of enzymes such as lipases.

Figure 6: A) Comparison of FFA content (as % DCW) in WT to Δaas in continuous light across 6 days. B) Comparison of FFA content (as % DCW) in WT to Δaas in 12:12 LD across 6 days. B). Standard deviation shown is across 3 biological replicates. *Denotes significant difference ($p < 0.05$) compared to between the two strains.

3.3 Increasing transcript abundance of 'tesA^{CO} does not increase FFA production in continuous light and in 12:12 LD

Two genetic modifications are commonly made in *E. coli* to increase FFA production: (i) disabling FFA degradation (through β -oxidation) by deleting *fabD* [42], and (ii) expressing thioesterases that convert acyl-ACPs to FFAs [43-45]. Similar to approach (i), *aas* deletion has been applied to glucose tolerant (GT) *Synechocystis* [19] allowing the accumulation of FFAs (there is no known β -oxidation in *Synechocystis*). After multiple rounds of modifications, Liu *et al.* (2011) achieved the highest secreted FFA titer yet seen in cyanobacteria of 197mg/L. Unfortunately, genetic deletions and insertions in their work were coupled, thereby making it difficult to identify the individual contribution of each genetic modification. Yet, the dramatic increase in FFA titers seen after expression of thioesterases, especially 'tesA, was very promising. This led us to investigate the effect of varying the expression of codon optimized 'tesA (from here on, 'tesA^{CO}) on the production of FFAs in WT *Synechocystis*.

We used a *psbAII* suite of engineered promoters (unpublished results) to vary the expression strength of 'tesA^{CO} in our *Synechocystis* mutants. Since we knew the baseline production of our Δaas strain, we placed 'tesA^{CO} with the *psbAII* promoter suite in the loci of *aas* (orf: slr1609). We successfully developed three 'tesA^{CO} expressing strains in WT *Synechocystis*: GG1.1 (Δaas + 'tesA^{CO} under *psbAII* promoter), GG1.2 (Δaas + 'tesA^{CO} under *psbAII* Δhex +HLR promoter), and GG1.3 (Δaas + 'tesA^{CO} under *psbAII* Δhex promoter). A fourth strain was also made by adding a second copy of *psbAII* Δhex + 'tesA^{CO} into GG1.3, resulting in strain GG2.3 (Δaas , $\Delta 0168$ + 2x 'tesA^{CO} under *psbAII* Δhex promoter). 'tesA^{CO} mRNA transcript abundance was verified under continuous light. Our results confirmed that transcript abundance increases with increasing promoter strength and additional *tesA*^{CO} copies (Table 2).

Table 2: qRT-PCR of relative of 'tesA^{CO} transcript abundance in Δaas , GG1.1, GG1.2, GG1.3, and GG2.3 compared to GG1.1 (lowest promoter strength). *TesA*^{CO} transcript abundance increases with increasing promoter strength and additional *tesA*^{CO} copies. Standard deviation shown is across 3 biological replicates.

***tesA^{CO}* expression of various *Synechocystis* strains**

Strain	Description	Fold change relative to GG1.1
Δaas	Δaas	0.0 $\pm$ 0.0
GG1.1	Δaas <i>psbAII</i> ' <i>tesA^{CO}</i>	2.0 $\pm$ 2.3
GG1.2	Δaas <i>psbAII</i> Δhex +HLR ' <i>tesA^{CO}</i>	157.1 $\pm$ 35.0
GG1.3	Δaas <i>psbAII</i> Δhex ' <i>tesA^{CO}</i>	390.1 $\pm$ 131.5
GG2.3	Δaas $\Delta slr0168$ <i>psbAII</i> Δhex ' <i>tesA^{CO}</i> x2	667.0 $\pm$ 62.7

Unlike the pronounced difference in mRNA transcript abundance, there were either insignificant or small increases in FFA titers in '*tesA^{CO}* expressing strains under continuous light (Figure 7) or 12:12 LD (Figure 8) compared to the Δaas strain. Although there were marginal increases between in '*tesA^{CO}* expressing strains (GG1.2 and GG1.3) compared to the Δaas strain between days 2 and 3, we did not observe the large increase that we expected. The maximum titers (day 5) of '*tesA^{CO}* strains (48.9 ± 1.5 mg/L in GG1.3) were not greatly improved over that of the Δaas mutant (44.3 ± 1.7 mg/L, $p > 0.05$). Like the Δaas strain, all '*tesA^{CO}* mutants showed similar FFA productivity in continuous light (~ 17.6 mg/L/day in GG1.3 between days 2 and 3) and a significant cessation of FFA production in 12:12 LD (~ 6.0 mg/L/day in GG1.3 between days 2 and 3).

Figure 7: A) FFA titers in '*tesA*^{CO}' expressing strains of *Synechocystis* (GG1.1(black), GG1.2 (blue), GG1.3 (red), and GG2.3(purple)) and Δaas strain (yellow) compared to WT (green) when grown in

368 continuous light. B) OD_{730nm} of all *Synechocystis* strains monitored in continuous light light conditions
369 with bicarbonate feeding across 6 days. Standard deviation shown is across 3 biological replicates.

370

Figure 8: A) FFA production in all '*tesA*' expressing strains of *Synechocystis* (GG1.1(black), GG1.2 (blue), GG1.3 (red), and GG2.3(purple)) and Δaas strain in 12:12 LD. Grey regions represent periods of dark. B) OD_{730nm} of all *Synechocystis* strains monitored in 12:12 LD conditions with bicarbonate feeding across 6 days. Standard deviation shown is across 3 biological replicates.

3.4 Measurement of TesA enzyme activity and protein abundance.

To explain the lack of improvement in FFA titers of our '*tesA*^{CO}' strains, we measured their *in vitro* TesA enzyme activities (Table 3). As a control to show that the enzyme assay is working, we evaluated the TesA enzyme activities in an *E. coli* strain expressing '*tesA*' (*Ec92*, Table 1). The crude extracts of *Ec92* were obtained from overnight (16 h) cultivation. The TesA enzyme activity in *Ec92*, done in biological triplicates and technical duplicate, was $0.290 \pm 0.027 \times 10^{-3} A_{412}/\text{min}/\text{ng}$ crude protein. The enzyme activity of a *Synechocystis* strain expressing '*tesA*' (not codon optimized for *Synechocystis*) under the control of the *psbAII* promoter (Strain AF1609) was also evaluated. Table 3 summarizes our enzyme assay results. We found that the TesA enzyme activities of all *Synechocystis* strains were either insignificant or below the limit of detection.

Table 3: *In vitro* TesA crude enzyme assays of '*tesA*' expressing mutants of *Synechocystis*. TesA enzyme assays were conducted in biological triplicates with technical duplicates.

TesA activity of <i>Synechocystis</i> strains.			
Strain	TesA activity		
	($\times 10^{-3} A_{412}/\text{min}/\text{ng}$ crude protein)		
Δaas	0.0006	$\pm$	0.0003
AF1609	0.0000	$\pm$	0.0002
GG1.1	0.0004	$\pm$	0.0006
GG1.2	0.0007	$\pm$	0.0002
GG1.3	0.0008	$\pm$	0.0005
GG2.3	0.0006	$\pm$	0.0008

To investigate whether *tesA* was being actively translated into TesA proteins, we ran the soluble crude protein extracts of all '*tesA*' expressing strains of *E. coli* and *Synechocystis* on a SDS-PAGE stained with Coomassie Blue. We found no visible bands for TesA (~23kDa, data not shown). Further efforts to detect the presence of TesA proteins involved the use of LC-MS/MS. Crude protein extracts from day 3 of continuous light conditions with bicarbonate feeding were subjected to tandem MS

analysis. The N-terminal, C-terminal, and 6 internal peptides of TesA were identified in strains GG1.3 and GG2.1 (Table S7). This suggests that the entire 'tesA was expressed in protein level. Due to low signal strengths of TesA peptides, we were unable to quantify its relative abundance.

3.5 Importance of using industrial conditions to evaluate strains

The scale-up of photosynthetic organisms is dependent on light properties such as incident light source, wavelength, intensity, and light:dark cycles [46]. In scaling up, many biological systems often fail or have much worse performance when moving from lab to industrial scale [47]. Problems associated with scale-up may not be obvious on the bench scale and hence it is important to replicate industrial conditions inside the lab. While we acknowledge that our study does not adequately replicate industrial conditions, it does improve on continuous light conditions typically reported in literature.

On the lab scale, this contribution shows how diel cycles effect the production of FFAs in our cyanobacterial strains. Presently, we hypothesize that prominent changes in the cell's metabolism in 12:12 LD vs continuous light are likely the cause for the decrease in FFA production and will need to be studied in the future. Mathematical modeling approaches would be influential in understanding these metabolism changes. For *Synechocystis*, rapidly emerging approaches such as metabolic flux analysis [48] and flux balance analysis [49] are able to determine the steady-state flow of metabolites in the cell. This information can then be used to comprehend and describe the metabolism changes under diel cycles and guide further strain engineering efforts. Our lab's future research and collaborations will focus on better replicating industrial conditions in conjuncture with metabolic engineering and mathematical modeling.

4. Conclusions

We learn from this study that it is important to evaluate the production of desired molecules from photosynthetic microorganisms under light:dark cycles. Although we saw an increase in FFA titers in our FFA producing mutants under continuous light, its performance was starkly different in 12:12 LD. Transcriptionally, we found no changes in the fatty acid biosynthesis pathway as a result of aas

deletion. We postulate that changes beyond the fatty acid biosynthesis pathway (e.g. membrane lipid biosynthesis and membrane lipid recycling) are probable.

Based on previously published work [19], we initially set our sights on optimizing *tesA*^{CO} expression by varying promoter strengths and copy number. Despite the dramatic increases in *tesA*^{CO} mRNA abundance, our *Synechocystis* mutants did not have the desired enzyme activity. Our results lead us to conclude that *tesA* expression (in *Synechocystis*) does not likely contribute to increases in FFA production (as reported in previous work [19]). Instead, we hypothesize that the increase in FFA production is more likely due to strategic deletion of FFA associated genes and/or pathways. Since the *tesA* mRNA transcripts and TesA peptides were confirmed in our strains, future work should explore problems such as protein misfolding and solubility.

Overall our work is impactful in two ways: 1) to pave the way for future opportunities to understand the effects of circadian rhythm on the production of desired molecules and 2) to highlight the need to evaluate genetic modifications individually (particularly in *Synechocystis*) in order to determine the contribution of each genetic change.

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Appendix A. Supplementary material

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