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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 *Escherichia 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.

1. Introduction

Cyanobacterial technologies are promising platforms for efficient production of renewable chemicals from CO_2 and sunlight [1]. To date, a rapidly growing track record of industrially relevant molecules has been successfully produced in recombinant cyanobacteria (for review, see Ref. [2]). The current engineering approach is to design phototrophic bacteria in the same way as heterotrophic bacteria, such as *Escherichia 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 *lrtA* promoter is known to be dark-induced [13], and several sigma factors are redox-sensitive and attributed to widespread 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 (Fig. 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

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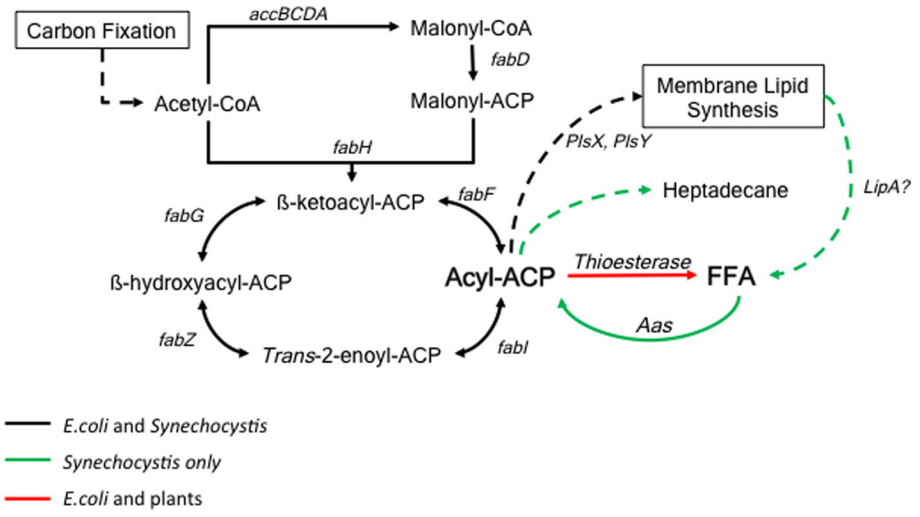

Fig. 1. The fatty acid biosynthesis pathway in *Synechocystis*. Pathways in black represent common pathways in *E. coli* and cyanobacteria. Pathways in green represent exclusive routes in *Synechocystis*. Dashed lines indicate multiple steps leading to the desired metabolite.

[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 among 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 (Δ*aas* 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 Δ*aas* 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.

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* 5α (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* ΔHex promoter (KT162051), and *psbAII* ΔHex + 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 SalI sites of the plasmid pUC19m 1609 HR (from [23]). Plasmid pCO'TesA150 was constructed in a similar manner with the exception of '*tesA* being replaced by '*tesA*^{CO} (obtained from pCO'TesA148). Two variants of pCO'TesA150 were also subsequently constructed, this time with the *psbAII* promoter (KT162050) being replaced by *psbAII* ΔHex + HLR promoter (KT162053) and *psbAII* ΔHex promoter (KT162051) to yield plasmids pCO'TesA194 and pCO'TesA152 respectively. For plasmid

Table 1
Plasmids and strains used in this study.

Strain	Genotype or plasmid	Source
E. coli		
DH5α	*E. coli* competent cells (WT *E. coli*).	New England Biolabs
Ec92	WT *E. coli* 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}, $P_{psbAII\ complete\ \Delta hex\ +HLR}$.	This work
pCO'TesA152	pUC19m, slr1609 homologous regions, '*tesA*^{CO}, $P_{psbAII\ complete\ \Delta hex}$.	This work
pIGA4maz-kan202	pIGA4, mazF-kan.	This work
pCO'TesA203	pIGA4, '*tesA*^{CO}, $P_{psbAII\ complete\ \Delta hex}$.	This work
Synechocystis		
WT	WT *Synechocystis* sp. PCC 6803.	ATCC 27184
WT NF1609	WT with slr1609 replaced by *mazF/aphII*.	(Cheah et al., 2012)
ΔAas	WT with slr1609 deleted and *mazF/aphII* removed.	(Cheah et al., 2012)
AF1609	WT with slr1609 replaced by '*tesA*, $P_{psbAII\ complete}$.	This work
GG1.1	WT with slr1609 replaced by '*tesA*^{CO}, $P_{psbAII\ complete}$.	This work
GG1.2	WT with slr1609 replaced by '*tesA*^{CO}, $P_{psbAII\ complete\ \Delta hex\ +HLR}$.	This work
GG1.3	WT with slr1609 replaced by '*tesA*^{CO}, $P_{psbAII\ complete\ \Delta hex}$.	This work
GG1.3 NF0168	GG1.3 with slr0168 replaced by mazF-KanR cassette.	This work
GG2.3	GG1.3 NF0168 with slr0168 replaced by '*tesA*^{CO}, $P_{psbAII\ complete\ \Delta hex}$.	This work