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Discovery and characterization of *Synechocystis* sp. PCC 6803 light-entrained promoters in diurnal light:dark cycles

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ABSTRACT

Cyanobacteria are photosynthetic bacteria employed for production of valuable chemicals using sunlight and atmospheric carbon dioxide as substrates. Industrial production in outdoor facilities exposes cyanobacteria to daily light:dark (LD) cycles of sunlight availability. Strain engineers need genetic tools suited for diurnal LD cycles to maximize production in these conditions, but tools for engineering in diurnal LD cycles are extremely limited. Here, we discover native *Synechocystis* sp. PCC 6803 promoters which provide light-entrained expression in 12-hour light:12-hour dark (12:12) LD cycles. Promoters were characterized using bacterial luciferase bioluminescent promoter probes and RT-qPCR. P_{hliC}, P_{rbp1}, $P_{slr0006}$, and P_{sigA} provide light-entrained expression in 12:12 LD cycles when expressed from the *slr0168* chromosomal neutral site. None of the promoters provided free-running oscillations in continuous light (CL) following diurnal LD entrainment, but growth in CL resulted in constant mid-level bioluminescence. Transcripts from P_{rbp1}, $P_{slr0006}$, and P_{sigA} increased significantly following the onset of light, whereas transcripts from P_{hliC} exhibited no oscillations in 12:12 LD cycles. Furthermore, P_{hliC} bioluminescence induction at the onset of light increased at higher light intensities. Overall, our work provides additional genetic engineering tools for cyanobacterial strains for chemical production in diurnal LD cycles.

1. Introduction

Engineered microorganisms can be applied for the sustainable production of chemicals, fuels, and pharmaceuticals. Cyanobacteria are a promising chassis for development due to their innate ability to utilize solar energy and carbon dioxide as substrates for growth *via* photosynthesis. An assortment of chemicals have been produced in cyanobacteria (see Ref. 1 for review) with modest yield improvements from engineering advances [2–6], almost all of which have been conducted in continuous light conditions. However, industrial production of commodity chemicals will require outdoor cultivation where cyanobacteria cultures are exposed to natural 24-hour day/night, or diurnal, light:dark (LD) cycles. We recently demonstrated that free fatty acid productivity in *Synechocystis* sp. PCC 6803 (hereafter *S.* 6803) is significantly decreased in diurnal LD cycles as opposed to continuous light (CL) [7]. This observation is not entirely surprising given that the energy landscape of cyanobacteria changes drastically over the course of a 24-hour LD cycle [8].

Cyanobacteria synchronize global transcription and energy metabolism with diurnal LD cycles. During the day, light is harvested to produce energy cofactors (*e.g.*, ATP, NADPH) and storage molecules (*e.g.*, glycogen); during the night, these energy molecules are nearly depleted [8]. Transcripts within functional categories of translation, ATP synthase, photosynthesis, and CO_2 fixation are up-regulated during the day, while genes related to pentose phosphate pathway, respiration, and DNA replication are up-regulated at night [9]. Extensive work in *Synechococcus elongatus* PCC 7942 (hereafter *S.* 7942) has elucidated several mechanisms of circadian gene regulation. A post-transcriptional oscillator (PTO) comprised of KaiABC proteins mediates global transcriptional changes [10–13] and is re-set by cellular redox status [14,15] and ATP/ADP ratio [16]. Previous work has presented evidence for global transcriptional control *via* circadian-entrained changes in chromosome topology and thereby promoter accessibility [32]. However, mutations within transcriptional promoters can alter circadian phase, suggesting sequence specificity of *trans*-acting PTO transcription factors (TFs) and/or RNA polymerase sigma factors [17].

Abbreviations: 12-hour light:12-hour dark cycles of light, (12:12 LD cycles); *Synechocystis* sp. PCC 6803, (*S.* 6803); Promoter, (P); light:dark, (LD); continuous light, (CL)

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Less is known about *S.* 6803 regulation in daily LD cycles, but several studies describe genetic elements which contribute to daily LD cycle regulation. Deletion of *kaiABC* results in growth defects and differential gene regulation in LD cycles [18]. Of the nine putative sigma factors, many have differential regulation in response to light or dark conditions [19–22]. *S.* 6803 promoters are classified into three types based on conserved sigma factor recognition motifs; yet, transcription start points vary drastically even within type 1 promoters with conserved −10 and −35 hexamers [22]. Derivations of these conserved motifs have given rise to inducible non-native promoters for use in *S.* 6803 [23,24]. P*psbAII*, one of the most popular promoters in *S.* 6803, only functions as a light-inducible promoter from the native chromosomal locus and not from neutral site *slr0168* [25]. Engineering viable strains for chemical production in LD cycles requires additional modular genetic tools with diurnal temporal sensitivity.

Here, we evaluate nineteen native *S.* 6803 promoters using the bacterial luciferase reporter, LuxAB, in 12:12 LD cycles by monitoring bioluminescence. We identify P_{hliC}, P_{rbp1}, $P_{slr0006}$, and P_{sigA} as promoters which confer light-entrained expression in diurnal LD cycles from the *slr0168* chromosomal neutral site. Transcript increases were observed from P_{rbp1}, $P_{slr0006}$, and P_{sigA} at the onset of light in diurnal LD cycles. P_{hliC} is interesting due to the observations that mRNA abundance does not change despite a significant bioluminescence increase, and light-induced bioluminescence increased at higher light intensities. Overall, this work provides additional tools for temporal control of gene expression in diurnal LD cycles.

2. Materials and methods

2.1. Plasmids, strains and culture conditions

All strains and plasmids used in this study are listed in Table 1. All primers used in this study are listed in Table S1. Plasmids were constructed in *E. coli* DH5α (New England Biolabs). pAM1580 (Addgene, #40241) was modified by introducing a unique *Nde*I restriction site immediately upstream of *luxA* using the QuikChange Site Directed Mutagenesis Kit (Agilent, Santa Clara, CA) and flanking *slr0168* homologous regions to create pAZ1-Cam. Promoter regions were excised from *S.* 6803 genomic DNA and inserted into pAZ1 by cut-and-paste with *Nhe*I/*Nde*I restriction endonucleases. Chloramphenicol selection was ineffective as a selection marker in our strain of *Synechocystis* PCC 6803 [N-1] (American Type Culture Collection #27184); thus, all promoter:*luxAB* cassettes were subsequently inserted into pIGA4 [24] with InFusion Cloning (Clontech Labs., Mountain View, CA), resulting in the pAZ promoter probe suite (Fig. 1). Each of these plasmids were transformed into the *S.* 6803 *slr0168* neutral site *via* homologous recombination as previously described [24]. *S.* 6803 was cultured in 6× phosphate BG-11, pH adjusted with NaCl to 8.0, buffered with TES, and supplemented with 50 μg/mL kanamycin for selection.

Table 1
Strains and plasmids.

Name	Description	Source
DH5α	Chemically competent *E. coli*	New England Biolabs
S. 6803	Wild-type (WT) *Synechocystis* sp. PCC6803	ATCC 27184
S. 6803 #2–28	WT *Synechocystis* sp. PCC6803 with *slr0168* replaced with promoter:probe corresponding to pAZ2-28, respectively	This study
pAM1580	Promoterless *luxAB* cloning vector	Addgene
pAZ1	pAM1580 with native NdeI site removed and *Nde*I site introduced 5′ of *luxA*, Chloramphenicol resistance	This study
pIGA4	Cloning vector with *slr0168* homologous regions flanking kanamycin antibiotic resistance (Kan_R)	Albers et al. [24]
pAZ2	$P_{slr0749}$:*luxAB* flanked by *slr*0168 homologous regions and Kan_R, as with all pAZ plasmids to follow	This study
pAZ3	$P_{sll0947}$:*luxAB*	This study
pAZ6	$P_{sll1330}$:*luxAB*	This study
pAZ7	$P_{sll0543}$:*luxAB*	This study
pAZ8	$P_{slr1793}$:*luxAB*	This study
pAZ9	$P_{slr0623}$:*luxAB*	This study
pAZ11	$P_{slr1634}$:*luxAB*	This study
pAZ12	$P_{ssl1633}$:*luxAB*	This study
pAZ13	$P_{slr0006}$:*luxAB*	This study
pAZ14	$P_{sll0757}$:*luxAB*	This study
pAZ15	$P_{sll0517}$:*luxAB*	This study
pAZ16	$P_{sll1632}$:*luxAB*	This study
pAZ17	$P_{slr1347}$:*luxAB*	This study
pAZ20	$P_{sll1342}$:*luxAB*	This study
pAZ21	$P_{sll1732}$:*luxAB*	This study
pAZ22	$P_{slr0009}$:*luxAB*	This study
pAZ23	$P_{sll0807}$:*luxAB*	This study
pAZ26	$P_{slr0653}$:*luxAB*	This study
pAZ28	$P_{slr0756}$:*luxAB*	This study

2.2. Bioluminescence assays

Pre-cultures were inoculated from *S.* 6803 #2 through *S.* 6803 #28 freezer stocks into 20–50 mL BG-11 + Kan_{50} and grown for 2–4 days at 30 °C shaking at 225 rpm with 198 ± 36 μmole photons m^{-2} s^{-1} (μE) light delivered from GE Ecolux High Output 24 W T5 High Output fluorescent lights. Cultures were inoculated into 250 mL Erlenmeyer flasks at OD_{730} 0.2–0.5 and entrained to 12 hours light:12 hours dark (12:12) LD cycles or continuous light (CL) schemes for 2–3 days. The initial screen for functional promoters was conducted with biological duplicates; further characterization was conducted with biological triplicates. *In vivo* bioluminescence assays were performed as previously described [26]. Briefly, 150 μL of cell suspension was added to a black-sided flat- and clear-bottom 96-well plate (Corning #3631). Bioluminescence was recorded after > 2 min dark incubation on a FLOUstar Omega Microplate Reader at 520 nm for 10 s immediately following injection of 100 mM decanal in 50% (v/v) methanol:water. *In vitro* bioluminescence assays were performed as previously described [27]. Briefly, 500 μL of cell suspension was mixed with 400 μL cold lux buffer (50 mM sodium phosphate pH 7, 50 mM 2-mercaptoethanol, 2% bovine serum albumin) on ice and sonicated prior to addition of $FMNH_2$ to a final concentration of 5 mM. For both *in vitro* and *in vivo* measurements, average counts per second (cps) over 10 s were normalized to absorbance at 730 nm (Abs_{730}) for each technical triplicate; biological replicate averages were normalized to wild-type background, unless otherwise noted.

2.3. RNA extraction and RT-qPCR

Fifteen milliliters of cell culture were harvested after 7 days of growth in 12:12 L:D cycles, centrifuged at 10,000 *g* for 5 min, flash-frozen in liquid N_2, and stored at −80 °C for < 2 days before extraction. RNA was isolated using the PGTX method [28], treated with TURBO™ DNase kit (Invitrogen, Carlsbad, CA), and purified with the chloroform:phenol:isoamyl alcohol method [29]. cDNA was synthesized using the GoScript™ Reverse Transcription kit (Promega, Madison, WI) with random primers. Semi-quantitative real-time (q) PCR was performed with SsoAdvanced™ Universal SYBR® Green Supermix (Biorad, Hercules, CA) on a Biorad CFX Connect™ Real-Time PCR Detection System using *rnpB* as a reference gene [30]. Technical triplicates (3 ng of cDNA each) were run with *luxA* and *rnpB* primers. The relative quantification method was used to normalize Ct_{lux} to Ct_{rnpB} (ΔCt) and calculate fold change ($2^{-\Delta\Delta Ct}$) [31].