ORIGINAL ARTICLE

Investigating the Roles for Essential Genes in the Regulation of the Circadian Clock in *Synechococcus elongatus* Using CRISPR Interference

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Abstract Circadian rhythms are found widely throughout nature where cyanobacteria are the simplest organisms, in which the molecular details of the clock have been elucidated. Circadian rhythmicity in cyanobacteria is carried out via the KaiA, KaiB, and KaiC core oscillator proteins that keep ~24 h time. A series of input and output proteins—CikA, SasA, and RpaA—regulate the clock by sensing environmental changes and timing rhythmic activities, including global rhythms of gene expression. Our previous work identified a novel set of KaiC-interacting proteins, some of which are encoded by genes that are essential for viability. To understand the relationship of these essential genes to the clock, we applied CRISPR interference (CRISPRi) which utilizes a deactivated Cas9 protein and single-guide RNA (sgRNA) to reduce the expression of target genes but not fully abolish their expression to allow for survival. Eight candidate genes were targeted, and strains were analyzed by quantitative real-time PCR (qRT-PCR) for reduction of gene expression, and rhythms of gene expression were monitored to analyze circadian phenotypes. Strains with reduced expression of SynPCC7942_0001, *dnaN*, which encodes for the β-clamp of the replicative DNA polymerase, or SynPCC7942_1081, which likely encodes for a KtrA homolog involved in K^+ transport, displayed longer circadian rhythms of gene expression than the wild type. As neither of these proteins have been previously implicated in the circadian clock, these data suggest that diverse cellular processes, DNA replication and K^+ transport, can influence the circadian clock and represent new avenues to understand clock function.

Keywords circadian clock, cyanobacteria, CRISPRi, KaiABC, dnaN, ktrA

Circadian rhythms, regulated by a 24-h biological clock, allow organisms to anticipate predictable environmental changes allowing for the coordination of temporal programs of cellular physiology and facilitate adaptation to daily environmental changes in diverse organisms (Bell-Pedersen et al., 2005). Cyanobacteria are the simplest organisms known to possess a robust and rigorously tested circadian

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clock, where *Synechococcus elongatus* PCC 7942 has emerged as the premier model system to elucidate the molecular details of the cyanobacterial circadian clock. The cyanobacterial circadian clock is governed by 3 core oscillator proteins: KaiA, KaiB, and KaiC. KaiC is a hexameric protein consisting of CI and CII domains and functions as an autokinase, autophosphatase, and ATPase (Nishiwaki et al., 2004; Xu et al., 2004). The CII domain of KaiC contains A-loops extensions that bind to KaiA during the day. The binding of KaiA to the A-loops at dawn promotes KaiC autophosphorylation (Chang et al., 2012; Kim et al., 2008) on serine and threonine amino acids located in the CII domain in an ordered manner where threonine is phosphorylated first, followed by serine (Nishiwaki et al., 2007, 2004; Rust et al., 2007). Once in the fully phosphorylated state, a conformational change exposes the KaiB binding site, allowing KaiB to bind to the CI domain of KaiC and KaiA, sequestering KaiA away from the A-loops, and promoting KaiC's autophosphatase activity (Chang et al., 2012; Kim et al., 2008). This cycle of KaiC phosphorylation and dephosphorylation occurs over a ~24-h period and functions as the basic timekeeping mechanism in cyanobacteria. Deletion or overexpression of any of the *kai* genes abolishes the circadian clock (Ishiura et al., 1998).

The core oscillator receives input from the environment, through input proteins, and transmits temporal information, through output proteins, to clock controlled activities including timing of cell division (Dong et al., 2010; Mori et al., 1996), compaction of the chromosome (Smith and Williams, 2006; Woelfle et al., 2007), natural transformation (Taton et al., 2020), and regulation of global patterns of gene expression (Ishiura et al., 1998; Kondo et al., 1994; Liu et al., 1995; Nakahira et al., 2004). CikA is a protein that plays key roles in both circadian input and output (Gutu and O'Shea, 2013; Schmitz et al., 2000). CikA monitors cellular redox through the binding of quinones in the membrane that allows the oscillator to synchronize with the environment, specifically signaling the night-time state (Ivleva et al., 2006; Kim et al., 2012). SasA, a histidine kinase, and RpaA, the cognate response regulator for SasA, are part of a 2-component regulatory system that transmit signals from the oscillator to clock-controlled outputs. SasA interacts with phosphorylated KaiC through its KaiB-like sensory domain (Iwasaki et al., 2000), which allows for SasA to autophosphorylate itself and transfer the phosphate group to RpaA (Takai et al., 2006). Phosphorylated RpaA, RpaA-P, is a DNA-binding transcription factor and is also known as the master regulator of rhythmic gene expression (Markson et al., 2013). Without RpaA, there would be no transmission of time from the KaiABC proteins to the clock outputs. CikA functions as a phosphatase that removes the phosphate group from RpaA, inactivating RpaA and driving rhythms of RpaA phosphorylation. SasA and CikA activity drive circadian oscillation in RpaA phosphorylation (Gutu and O'Shea, 2013).

In addition to changes in protein levels that occur over the course of the day (Kitayama et al., 2008), the clock undergoes an elegant orchestration in its subcellular localization patterns where KaiA and KaiC are found diffuse throughout the cell during the day and localized to a single pole of the cell at night, in a circadian fashion (Cohen et al., 2014). In order to determine the mechanism of KaiC localization, immunoprecipitation followed by mass-spectrometry was performed to identify proteins that interact specifically with KaiC in either a localized or delocalized state. Mutant variants of a Yellow Fluorescent Protein (YFP)-KaiC fusion that displayed constitutive polar localization, KaiC-AA, mutation of the phosphorylated serine and threonine residues to alanine, or constitutive cytoplasmic localization, KaiC-AE, mutation of the phosphorylated serine and threonine residues to alanine and glutamic acid, were used. Twenty-nine proteins were identified to interact with diffuse KaiC or localized KaiC, but not both (McKnight et al., 2023). 15 of these proteins mapped to genes that were identified as essential (Rubin et al., 2015), meaning that they are crucial for cell viability and replacement with an antibiotic-resistant cassette, was unsuccessful. Another two genes, SynPCC7942_0001 and SynPCC7942_1081, hereafter referred to as *dnaN* and *ktrA*, were identified as non-essential and beneficial, meaning that a growth defect was observed when mutated in standard laboratory conditions (Rubin et al., 2015); however, complete deletion via replacement with an antibiotic-resistant cassette was not achieved, suggesting that these genes are indeed essential for viability. Here, we target 8 of these genes for additional characterization using CRISPR interference (CRISPRi) to determine how reduced expression of these genes effects the circadian clock.

CRISPR encodes Cas9, an RNA-guided nuclease, and single-guide RNA (sgRNA), consisting of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). Cas9 is then guided to the target genes by the single guide where the targeted DNA is cleaved (Ran et al., 2013). CRISPRi was repurposed in 2013 for genome regulation rather than genome editing, by inactivating the catalytic function of Cas9 and using it for RNA-guided transcription regulation without genetically altering the targeted sequence (Qi et al., 2013). Unlike Cas9 used in CRISPR-Cas9, the deactivated Cas9, dCas9, protein does not possess nuclease activity. CRISPRi continues to form a partial fusion of crRNA and tracrRNA, known as the sgRNA, to mimic the natural duplex making up the dCas9-sgRNA

complex. This complex binds to the desired sequence, such as the promoter, which blocks transcription initiation, or within the 5′ untranslated region (UTR) or coding sequence, which blocks transcription elongation, resulting in the suppression of transcription. CRISPRi has been used to reduce the expression of essential and non-essential genes in various organisms, including cyanobacteria (Gordon et al., 2016; Huang et al., 2016; Knoot et al., 2020).

Here we report that strains with decreased expression of two essential genes, *dnaN*, which encode for the β-clamp of the replicative DNA polymerase, and *ktrA*, a hypothetical protein suggested to be part of the potassium transport system, display long circadian rhythms of gene expression compared to the wild type (WT). Disruption to DNA replication or potassium transport has not been previously implicated in clock function; thus, our results highlight how these previously unknown cellular processes may be connected to the circadian clock.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

S. elongatus PCC 7942 was obtained from the Golden lab at the University of California, San Diego [UCSD] UCSD (Golden and Sherman, 1984) and grown in BG-11 media (17.6 mM $NaNO_3$, 0.23 mM K_2HPO_4, 0.3 mM $MgSO_4 \bullet 7H_2O$, 0.24 mM $CaCl_2 \bullet 2H_2O$, 0.031 mM citric acid$\bullet H_2O$, 0.021 ferric ammonium citrate, 0.0027 mM $Na_2EDTA \bullet 2H_2O$, 0.19 mM Na_2CO_3) supplemented with appropriate antibiotics, at 30 °C under 50-300 μE of light for 3-5 days (Clerico et al., 2007). Strains were constructed by expressing genes in one of three *S. elongatus* neutral sites (NSs): NS1, NS2, or NS3.

CRISPRi Plasmid Construction

Plasmids expressing dCas9 or sgRNAΦ, a plasmid expressing the sgRNA handle that binds to dCas9 without a target sequence, were obtained from the Hu lab at the National Tsing Hua University [NTHU] (Huang et al., 2016). dCas9 is expressed from NS1, under the P_{smtA} promoter. dCas9 expression can be induced with zinc chloride ($ZnCl_2$) from the P_{smtA} promoter (Huang et al., 2016). sgRNAΦ is expressed from NS2 under the P_{J23119} constitutive promoter (Huang et al., 2016).

Primers to amplify the psgRNAΦ plasmid were designed to add a 20-bp target sequence, which targets either the 5′ UTR or within the open reading frame (ORF) of the genes of interest and are listed in Supplementary Table S1. For most genes, multiple target sequences were tested within the 5′UTR, ORF, or both. Q5 DNA polymerase (NEB Biolabs) was used for PCR amplification, followed by Dpn1 digest to remove the psgRNAΦ template lacking the target sequence. Linear PCR fragments were then assembled into circular plasmids using a GeneArt Seamless Cloning and Assembly Kit (Thermo Fisher Scientific) and propagated in *Escherichia coli* XL1 Blue cells. Plasmids were verified by Sanger sequencing. Plasmids generated are described in Supplementary Table S2.

Circadian Bioluminescence Monitoring

Bioluminescence was monitored using a P_{kaiB}-luciferase fusion reporter inserted into NS3 (AMC2158) (Cohen et al., 2018) under constant light and temperature (30 °C). Cultures were entrained to 2-3 cycles of 12 hours of light and 12 hours of darkness at 30 °C prior to synchronizing the population as previously described (Mackey et al., 2007). In the cases where $ZnCl_2$ was added, a final concentration of 8-μM $ZnCl_2$ was added after entrainment. Bioluminescence was monitored every 2 h from a TECAN Spark bioluminescence plate reader for 5-7 days. Data were plotted in Excel and analyzed for rhythmicity using a MFourFit algorithm in BioDare2 (https://biodare2.ed.ac.uk) (Zielinski et al., 2014).

RNA Extraction

Twenty-four hours prior to extraction, $ZnCl_2$ was added to a final concentration of 8 μM. Ten milliliters of culture at optical density (OD_{750}) 0.2-0.4 was collected on ice and centrifuged at −10 °C at 10,000 × g for 10 minutes. Pellets were re-suspended with 1 mL of TriZol reagent (Invitrogen), and cells were lysed by 10 rounds of vortexing for 30 seconds and incubating on ice for 30 seconds. RNA was isolated using a Direct-zol RNA Miniprep Kit (Zymo Research) according to manufacturer's instructions. DNase treatment was performed by adding 5 μL of DNase I (Thermo Fisher Scientific) along with 75 μL of DNA Digestion Buffer to the spin column and letting it sit at room temperature for 15 min. RNA concentration is determined via nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific), and the quality of the RNA was determined by observing the 23S and 16S rRNA bands on 1.2% agarose gel.

Quantitative Real-Time PCR Analysis

cDNAs were synthesized from 1 μg of total RNAs with Superscript IV Reverse Transcription Kit (Ambion) following the manufacturer's protocol.

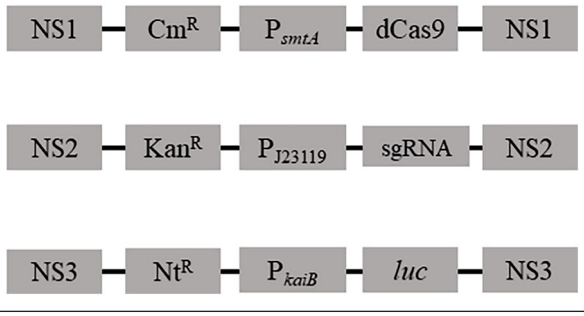

Figure 1. CRISPR interference schematic. dCas9 is expressed from NS1 under the $ZnCl_2$ inducible promoter P_{smtA} and is selected for with the chloramphenicol (Cm) antibiotic-resistant cassette. The single-guide RNAs are expressed from NS2 under the constitutive promoter, P_{J23119}, and selected for with kanamycin (Km) antibiotic resistance. The firefly luciferase reporter, P_{kaiB}-*luc*, used to monitor circadian rhythms of gene expression is expressed from NS3 with the nourseothricin (Nt) antibiotic-resistant cassette.

Primers were designed using Primer3 (Koressaar et al., 2018; Koressaar and Remm, 2007; Untergasser et al., 2012) and listed in Supplementary Table S3. Quantitative real-time PCR (qRT-PCR) was carried out using Maxima SYBR Green qPCR Master Mix (2X) (Molecular Biology) and Eppendorf Realplex System (Eppendorf) following the manufacturers' protocol. *rpoA* Gene was used as the endogenous control, and genomic DNA contamination was assessed by running a no-RT control PCR with total RNA. The threshold cycle (Ct) values were obtained, and the relative gene expression to the WT strain was calculated by the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using the transcript level of *rpoA* as the endogenous control (Kim et al., 2020). Statistical analysis was determined via Student *t* test.

RESULTS

Construction of CRISPRi Strains and Evaluation by qRT-PCR

In order to establish CRISPRi, we expressed dCas9 from NS1 under the P_{smtA}-inducible promoter and the sgRNA from NS2 under a constitutive promoter. In addition, a luciferase reporter was expressed from NS3, which allowed us to monitor rhythms of gene expression (Figure 1). CRISPRi can be used to block either transcription initiation or transcription elongation, depending on where the sgRNA is designed to bind. In order to prevent possible off-target effects, primers were designed to block transcription elongation. sgRNA plasmids containing 20 bp of the target sequence homologous to where dCas9 was targeted, either within the 5′ UTR or within the ORF of the genes of interest, were generated (Table 1). Of the 17 essential proteins identified as associating with KaiC, we identified 8 candidate genes to test for roles in the circadian clock using CRISPRi. Notably, we decided to not pursue ribosomal genes, as ribosomal proteins are typically highly abundant. Strains were generated to target both these regions to maximize the chance of obtaining at least one strain with sufficient reduction in gene expression to be able to observe a phenotype, as differences in gene expression depending on where the sgRNA was targeted have been observed (Huang et al., 2016). Two of the genes of interest were located within operons. For *ktrA*, the second gene of a 2-gene operon, and *frr*, the second gene of a 4-gene operon, several targets within the ORF were selected so not to disrupt expression of the upstream genes; although it is likely that gene expression was reduced for the genes present downstream of *frr*.

To ensure that reduced expression of the target gene was observed in CRISPRi strains, qRT-PCR was performed. Relative expression of the target genes in the CRISPRi strains compared to the WT strain expressing dCas9 (CRISPRi WT) was calculated using *rpoA* as the endogenous control. We observed that the expression of target genes was significantly reduced in all CRISPRi strains under inducing conditions, although the magnitude of reduction varies depending on the sgRNA used (Figure 2).

Reduced Expression of *dnaN* or *ktrA* Results in Altered Circadian Phenotypes

We were able to successfully generate and propagate all CRISPRi strains, with the notable exception of 0001-5′UTR10, 1743-5′UTR3, 1743-5′UTR1, 2378-5′UTR22, which grow poorly and are difficult to propagate even without inducing dCas9 with $ZnCl_2$. As noted previously, there is leaky expression of dCas9 under non-inducing conditions, and reduced gene expression was observed for candidate genes tested previously (Huang et al., 2016). This suggests that even mildly reduced expression of target genes in these strains' effects viability. All strains were monitored for changes in circadian rhythms of gene expression using a luciferase reporter. Conditions where dCas9 expression was induced by the addition of $ZnCl_2$ or non-induced resulting in low, leaky expression from the *smtA* promoter were both tested. We observed that the addition of 8-μM $ZnCl_2$, required to induce dCas9 expression, did not affect circadian rhythms of gene expression (Figure 3). Of the 18 strains tested, 15 showed no phenotypic changes under non-induced conditions (Supplementary Table S4). Strains that targeted either *dnaN* or *ktrA* resulted

Table 1. CRISPRi strain constructs.

SynPCC7942	Gene	Strain Name	Protein Roles
0001	*dnaN*	0001ORF943	DNA polymerase III, beta subunit
		0001ORF749	
		0001-5′UTR10	
1743	*ndhH*	1743-5′UTR3	NADH dehydrogenase (ubiquinone)
		1743-5′UTR1	
		1743-5′UTR2	
1552	*ilvC*	1552-5′UTR1	Ketol-acid reductoisomerase
		1552-5′UTR15	
1379	*accC*	1379-5UTR1	Acetyl-CoA carboxylase, biotin carboxylase subunit
		1379-5′UTR39	
1081	*ktrA*	1081ORF2029	Hypothetical protein, homologous to KtrA protein responsible in the potassium uptake system
		1081ORF1408	
0507	*frr*	0507ORF1104	Ribosome recycling factor
		0507ORF922	
1501	*serA*	1501ORF11	D-3-phosphoglycerate dehydrogenase
		1501ORF747	
2378	*ftsZ*	2378-5′UTR22	Bacterial tubulin homolog involved in cell division
		2378-5′UTR53	

Abbreviations: sgRNA = single-guide RNA; UTR = 5′ untranslated region; ORF = open reading frame; CRISPRi = CRISPR interference. sgRNA targets were constructed to bind to target sequences either within the UTR or the ORF of the gene of interest. The first column represents the SynPCC7942 gene number. The second column represents the gene name for the genes of interest, if available. The third column represents the CRISPRi strains constructed and can be broken down as such: the first 4 numbers are the SynPCC7942 gene number followed by the location to where the sgRNA guides the dCas9. The sgRNA targets were identified based on their position relative to the start codon of the gene of interest if the sgRNA targets the ORF, or to the transcription start site if the sgRNA targets within the 5′ UTR. The fourth column provides a description of the functionality of the proteins associated with the genes of interest.

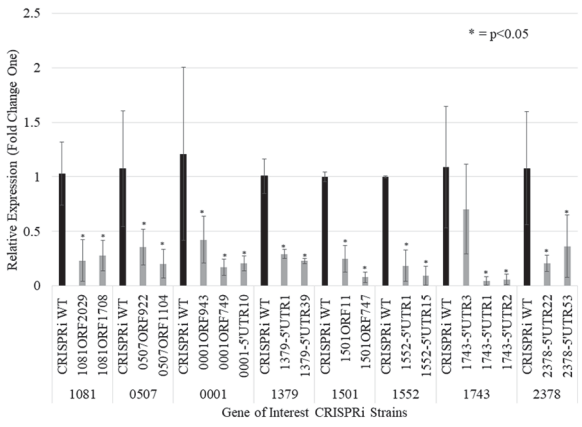

Figure 2. Confirmation of reduced gene expression by CRISPRi using quantitative real-time PCR. For each gene targeted, noted on the bottom of the x-axis, triplicate quantitative real-time PCR experiments were performed. Expression of each gene in strains expressing CRISPRi components, dCas9 and sgRNAs, (gray) is plotted relative to CRISPRi WT (AMC2158 + dCas9) (black) under conditions where dCas9 is induced with $ZnCl_2$. Expression of each gene in the CRISPRi WT strain was normalized to 1, and expression in strains expressing sgRNAs was normalized to the corresponding CRISPRi WT strain. Results represent confirmation of reduced gene expression from CRISPRi strains. Standard deviations are marked by error bars, significance calculated by one-sample Student's *t* test, asterisk (*) indicated *p* values less than 0.05. Abbreviations: CRISPRi = CRISPR interference; PCR = polymerase chain reaction; sgRNA = single-guide RNA; WT = wild type.

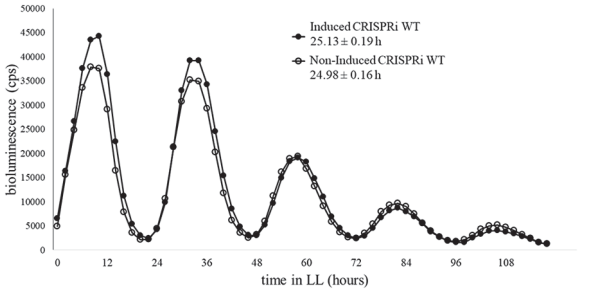

Figure 3. Induction of dCas9 with zinc chloride ($ZnCl_2$) does not affect the circadian clock. Circadian rhythms of gene expression are monitored from a P_{kaiB}-luciferase reporter either without dCas9 induction (open circle) or with dCas9 induction (closed circle) in the CRISPRi WT strain (AMC2158 + dCas9). Bioluminescence in counts per second (cps) is on the y-axis and time in constant light (LL) is on the x-axis. Period analysis shows that under induced conditions, CRISPRi WT has a period of 25.13 ± 0.19 h compared to non-induced conditions with a period of 24.98 ± 0.16 h, demonstrating that the addition of $ZnCl_2$ does not affect the circadian period. Abbreviations: CRISPRi = CRISPR interference; WT = wild type.

in long-period rhythms of gene expression compared to the WT even without the induction of dCas9 (Figures 4 and 5). In order to determine if induction of dCas9 could lead to enhanced circadian phenotypes, dCas9 was induced with $ZnCl_2$. Indeed, more predominant phenotypic changes were observed when

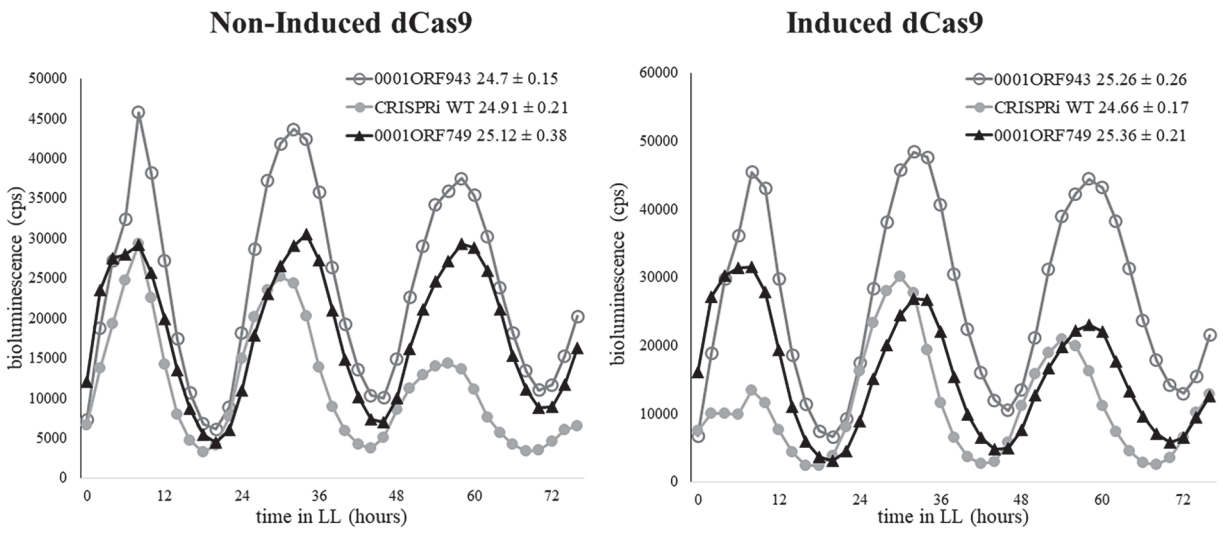

Figure 4. Targeting *dnaN* with CRISPRi results in long circadian rhythms of gene expression. Circadian rhythms of gene expression were monitored from a luciferase reporter either without dCas9 induction (non-induced dCas9) or with dCas9 induction (induced dCas9). CRISPRi strains (0001ORF943 & 0001ORF749) under non-induced conditions display a period of 24.7 ± 0.15 h (open, dark gray circle) and 25.12 ± 0.38 h (black, triangle) compared to the CRISPRi WT 24.91 ± 0.21 h (closed, light gray circle). CRISPRi strains (0001ORF943 & 0001ORF749) under induced conditions have a period of 25.26 ± 0.26 h (open, dark gray circle) and 25.36 ± 0.21 h (black triangle) compared to the CRISPRi WT 24.66 ± 0.17 h (closed, light gray circle). Bioluminescence in counts per second (cps) is on the y-axis and time in constant light (LL) is on the x-axis. Abbreviations: CRISPRi = CRISPR interference; WT = wild type.

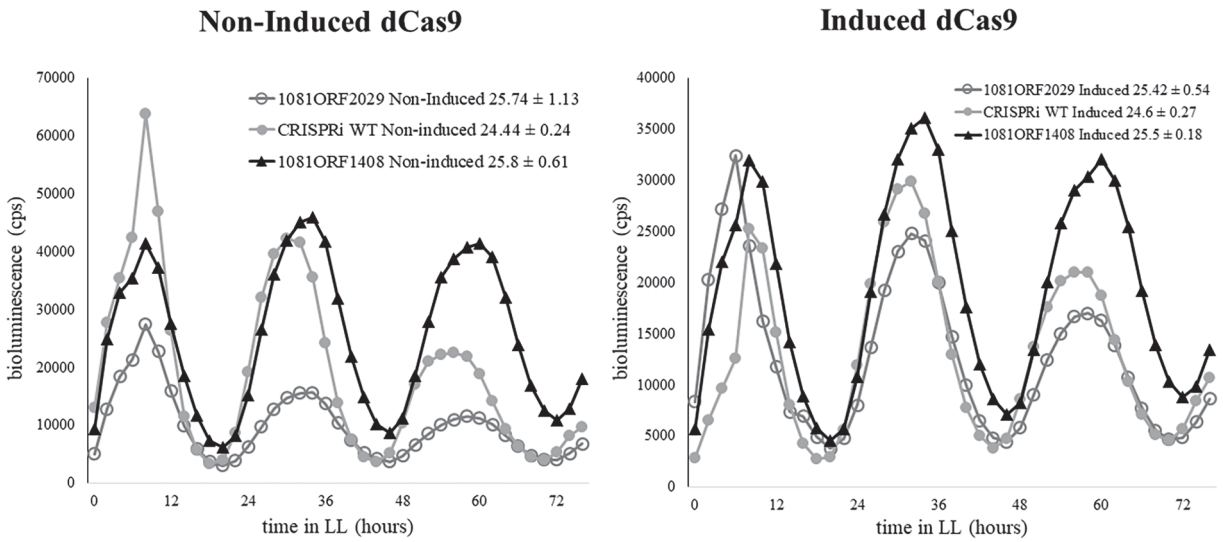

Figure 5. Circadian analysis of CRISPRi strains targeting *ktrA*. Circadian rhythms of gene expression monitored from a luciferase reporter either without dCas9 induction (non-induced dCas9) or with dCas9 induction (induced dCas9). CRISPRi strains (1081ORF2029 & 1081ORF1408) under non-induced conditions have a period of 25.74 ± 1.13 h (open, dark gray circle) and 25.8 ± 0.61 h (black triangle) compared to the CRISPRi WT 24.44 ± 0.24 h (closed, light gray circle). CRISPRi strains (1081ORF2029 & 1081ORF1408) under induced conditions have a period of 25.42 ± 0.54 h (open, dark gray circle) and 25.5 ± 0.18 h (black triangle) compared to the CRISPRi WT 24.6 ± 0.27 h (closed, light gray circle). Bioluminescence in counts per second (cps) is on the y-axis and time in constant light (LL) is on the x-axis. Abbreviations: CRISPRi = CRISPR interference; WT = wild type.

dnaN was targeted (Figure 4). These data demonstrate that two of the candidate genes displayed phenotypic changes when targeted by the CRISPRi system, suggesting that reduced gene expression of these targets led to changes in the circadian clock.

Three CRISPRi strains were designed to target *dnaN*: 0001-5′UTR10, which targets within the 5′ UTR, and 0001ORF749 and 0001ORF943, both of which target within the ORF. As mentioned earlier, the strain 0001-5′UTR10 grew poorly and was difficult to

Table 2. SynPCC7942_1081 likely encodes a KtrA homolog.

Protein	% Identity to SynPCC7942_1081
E. coli TrkA	17.56
PCC7002_TrkA	29.57
PCC6803_KtrA	45.61
PCC6803_KtrB	24.42
PCC6803_KtrE	14.47

Amino acid sequence of SynPCC7942_1081 was compared to TrkA from *E. coli* and *Synechococcus sp.* PCC 7002 along with KtrABE from Synechocystis PCC 6803. SynPCC7942_1081 has the highest percentage identity to the KtrA protein from Synechocystis PCC 6803 at 45.61%.

propagate long enough to gather sufficient data. This suggests that targeting the 5′UTR has a greater effect on gene expression than targets within the ORF, a trend we noticed for several genes. Period analysis shows that when *dnaN* is targeted, a longer circadian period is observed for the two CRISPRi strains that targeted the ORF. Without dCas9 induction, we see that the strain 0001ORF749 has a circadian period of 25.12 ± 0.38 h and strain 0001ORF943 has a period of 24.7 ± 0.15 h as compared to CRISPRi WT (AMC2158 expressing dCas9) which has a period of 24.91 ± 0.21 h (Figure 4). Upon dCas9 induction, strain 0001ORF749 has a circadian period of 25.36 ± 0.21 h, and strain 0001ORF943 has a period of 25.26 ± 0.26 h compared to CRISPRi WT which has a period of 24.66 ± 0.17 h (Figure 4).

Since SynPCC7942_1080 and SynPCC7942_1081, which encodes for *ktrA*, are part of an operon and share the 5′ UTR, we designed the CRISPRi strains targeting the ORF of *ktrA* so not to disrupt the expression of SynPCC7942_1080. Two CRISPRi strains targeting *ktrA* were tested, 1081ORF2029 and 1081ORF1408. Without dCas9 induction, we see period changes in both strains; strain 1081ORF2029 has a period of 25.74 ± 1.13 h, and strain 1081ORF1408 has a period of 25.8 ± 0.61 h as compared to CRISPRi WT which has a period of 24.44 ± 0.24 h (Figure 5). Upon dCas9 induction, strain 1081ORF2029 has a period of 25.42 ± 0.54 h, and strain 1081ORF1408 has a period of 25.5 ± 0.18 h compared to CRISPRi WT which has a period of 24.6 ± 0.27 h (Figure 5). These data demonstrate that targeting *ktrA* results in changes to the circadian clock, where long-period rhythms of gene expression were observed.

SynPCC7942_1081 Likely Encodes for a *ktrA* Homolog

SynPCC7942_1081, hereafter referred to as *ktrA*, is listed as a hypothetical protein; however, genome annotation websites list peripheral membrane proteins, TrkA and KtrA, involved in K^+ transport as possible homologs. TrkA is essential for the activity of the Trk potassium uptake system and has been described as mediating constitutive, low-affinity, high-rate K^+ transport energized by the proton-motive force (Rhoads and Epstein, 1977). Ktr is a sodium-dependent potassium transport system crucial in playing a role in the response to hyperosmotic stress (Matsuda and Uozumi, 2006). Through protein alignments, we observed that KtrA/SynPCC7942_1081 had 17.56% identity to TrkA from *E. coli* and 29.57% identity to TrkA from *Synechococcus sp.* PCC 7002, a marine *Synechococcus* species, whereas *S. elongatus* PCC 7942 is a freshwater cyanobacterium (Table 2). We then aligned KtrA/SynPCC7942_1081 to the Ktr proteins KtrA, KtrB, and KtrE from *Synechocystis sp*. PCC 6803. We observed that KtrA/SynPCC7942_1081 has a 45.61% amino acid identity to the KtrA protein from *Synechocystis sp.* PCC 6803 (Table 2). This suggests that KtrA/SynPCC7942_1081 of *S. elongatus* most likely encodes for a KtrA homolog that functions within the Ktr potassium transport system rather than the Trk potassium transport system. KtrA, a peripheral membrane-associated protein, is proposed to regulate the potassium transport activity of KtrB, the membrane transporter, through conformational changes (Zulkifli et al., 2010).

DISCUSSION

The utilization of CRISPRi has allowed us to investigate the roles for 8 essential genes in regulating the circadian clock in *S. elongatus*. Prior to CRISPRi, ways to reduce the expression of essential genes often resulted in inconsistent reduction in gene expression. Here, we targeted 8 essential candidate genes, which had been identified as co-purifying with KaiC, and determined their effects on the cyanobacterial circadian clock. We were able to confirm through qRT-PCR that reduced gene expression was observed for all CRISPRi strains generated. We found that reduced expression of 2 genes, *dnaN* and *ktrA*, resulted in longer circadian period phenotypes. For strains in which phenotypic changes were not observed, it is possible that either the genes targeted do not affect the circadian clock or that gene expression needs to be reduced even further for a phenotype to be observed. It is possible that targeting different regions within these genes could potentially lead to enhanced reduction of gene expression, allowing for the observation of additional circadian phenotypes.

We found that CRISPRi strains targeting *dnaN* resulted in long-period circadian rhythms of gene expression, under the condition where dCas9 was either induced or non-induced. SynPCC7942_0001, *dnaN*, encodes for the β subunit, which makes up

the β processivity clamp, of the replicative DNA polymerase III and is required for DNA replication (McHenry, 1985). Interestingly, it has been shown that the circadian clock schedules rhythmic assembly of the replisome, where high-level initiation of DNA replication is observed at dawn, in order to minimize incomplete replication forks at night (Liao and Rust, 2021). Since we observed changes to the circadian clock when the expression of *dnaN* is reduced, it suggests that DNA replication may also regulate the circadian clock. While there are many possible explanations for why changes to the circadian clock are observed when expression of *dnaN* is reduced, it is possible that KaiC, which is localized to the cell pole at night, sequesters the beta clamp at night as a mechanism to prevent DNA replication from occurring.

Through protein alignment, we were able to provide evidence that SynPCC7942_1081, which was annotated as a hypothetical protein, likely encodes for a KtrA homolog, which is part of the Ktr potassium transport system observed in other cyanobacteria like *Synechocystis* sp. KtrA is a membrane-associated protein that associates with the membrane-bound K^+ transporter KtrB. Interestingly, KtrA was identified as interacting with KaiC in a localized state, suggesting KtrA could be part of the night-time clock complex that forms a focus at or near the poles of cells (McKnight et al., 2023). The cyanobacterial circadian clock synchronizes with the environment indirectly by monitoring cellular redox. CikA and KaiA bind to oxidized quinones at night (Ivleva et al., 2006), signaling the onset of darkness (Ivleva et al., 2006; Kim et al., 2012; Wood et al., 2010). However, KaiC binds to adenosine triphosphate (ATP) and can sense the changes in the ratio of ATP to adenosine diphosphate (ADP), which gradually declines throughout the night and determines the length of night-time period (Lin et al., 2014; Rust et al., 2011). As KtrA binds to Nicotinamide adenine dinucleotide [NAD^+/NADH] (Zulkifli et al., 2010), it is possible KtrA association with the core oscillator represents another mechanism by which redox signals are conveyed to the clock.

In summary, we have successfully applied CRISPRi as a tool to study the roles for essential genes in the circadian clock in *S. elongatus*. We generated several sgRNAs targeting 8 essential genes, previously shown to co-purify with KaiC, and were able to demonstrate that reduced expression of 2 genes resulted in circadian phenotypes. It is possible that the observed phenotypes arise from disruption of essential processes rather than the essential gene directly regulating the circadian clock. While the molecular mechanisms by which these factors regulate clock function are not investigated here, our work sheds light on previously unknown cellular factors that influence the clock and paves the way for investigating the roles for other essential genes in circadian clock function.

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CONFLICT OF INTEREST STATEMENT

The authors have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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NOTE

Supplementary material is available for this article online.

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