Rhythmic oscillations in KaiC1 phosphorylation and ATP/ADP ratio in nitrogen-fixing cyanobacterium *Cyanothece* sp. ATCC 51142

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(Received 3 October 2015; accepted 29 October 2015)

Cyanobacterial circadian clock composed of the Kai oscillator has been unraveled in the model strain *Synechococcus elongatus* PCC 7942. Recent studies with nitrogen-fixing *Cyanothece* sp. ATCC 51142 show rhythmic oscillations in the cellular program even in continuous light albeit with a cycle time of ~11 h. In the present study, we investigate correlation between cellular rhythms, KaiC1 phosphorylation cycle, ATP/ADP ratio, and the redox state of plastoquinone pool in *Cyanothece*. KaiC1 phosphorylation cycle of *Cyanothece* was similar to that of *Synechococcus* under diurnal cycles. However, under continuous light, the cycle time was shorter (11 h), in agreement with physiological and gene expression studies. Interestingly, the ATP/ADP ratio also oscillates with an 11 h period, peaking concomitantly with the respiratory burst. We propose a mathematical model with C/N ratio as a probable signal regulating the clock in continuous light and emphasize the existence of a single timing mechanism regardless of the cycle time.

**Keywords:** ultradian rhythm; systems biology; post-translational oscillator; circadian clock; mathematical model

1. Introduction

Cyanobacteria are the simplest organisms displaying circadian rhythm (Grobbelaar et al. 1986). Cyanobacterial circadian clock has been widely studied in *Synechococcus elongatus* PCC 7942 (henceforth *Synechococcus*), a non-nitrogen-fixing unicellular cyanobacterium (Kondo et al. 1993; Dong & Golden 2008). In this cyanobacterium, the KaiC post-translational oscillator (PTO) forms the central pacemaker of the clock along with KaiA and KaiB proteins (Iwasaki et al. 2002; Xu et al. 2003). The Kai proteins are present in most cyanobacterial strains (except a few like *Gloeobacter violaceus* PCC 7421), with many strains having multiple homologs of *kaiB* and *kaiC* genes (Lorne et al. 2000). UCYN-A, a cyanobacterium with a reduced genome, lacks *kaiA* and *kaiB* but has *kaiC*, perhaps emphasizing the critical role of KaiC in clock functioning (Zehr et al. 2008; Axmann et al. 2014). However, the significance of KaiA and KaiB in sustaining the PTO is marked by the absence of robust oscillations in *Prochlorococcus sp.* which lacks *kaiA* gene (Axmann et al. 2009).

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Redox state of plastoquinone (PQ) pool and ATP/ADP ratio are reportedly the two major input signals which reset the circadian clock in *Synechococcus* (Ivleva et al. 2006; Kim et al. 2012). PQ pool undergoes rapid oxidation at the onset of dark. Oxidized quinone binds to KaiA and potentially inhibits it from stimulating kinase activity of KaiC, activating KaiC dephosphorylation (Wood et al. 2010). In addition, KaiC phosphorylation is directly influenced by the ATP/ADP ratio, since ADP acts as a competitive inhibitor of autokinase activity of KaiC (Rust et al. 2011). Further, *in vitro* experiments under different ATP and ADP concentrations have shown phase shifts and departure from 24-h cycle time for the KaiC phosphorylation/dephosphorylation cycle (Goda et al. 2014). The cellular ATP/ADP ratio reflects the metabolic status of the cell. Non-photosynthetic micro-organisms like *Escherichia coli* and *Bacillus* have a higher (>0.8) adenylate energy charge during exponential growth (Chapman et al. 1971) (Table 1). In cyanobacteria, the cellular ATP levels vary with growth conditions like light intensity, medium pH, and carbon dioxide concentration (Kallas & Castenholz 1982; Dietz & Heber 1984). In addition, the ATP/ADP ratio is reportedly lower in nitrogen-fixing cells as compared to vegetative cells in cyanobacteria (Upchurch & Mortenson 1980; Privalle & Burris 1983).

*Cyanothece* sp. ATCC 51142 (henceforth *Cyanothece*) is a unicellular diazotrophic cyanobacterium, which temporally separates the occurrence of oxygen-evolving photosynthesis and oxygen-sensitive nitrogen fixation (Reddy et al. 1993; Schneegurt et al. 1994). Circadian rhythm in transcription and metabolism has been widely studied in *Cyanothece* (Stockel et al. 2008; Krishnakumar et al. 2013). *Cyanothece* has two homologs for KaiC-KaiC1 and KaiC2, which are 82 and 56% identical, respectively, to KaiC of *Synechococcus*. While kaiAB1C1 in *Cyanothece* lie in tandem, kaiB2C2 cluster lacks an upstream kaiA gene (Dvornyk et al. 2003). In addition, *Cyanothece* KaiC2 lacks DXXG motif and aligns proline against the crucial glutamine115 of KaiC of *Synechococcus* (Axmann et al. 2014), suggesting a non-functional or sub-functional role of KaiC2 in *Cyanothece*.

*Cyanothece* have a cycle time of 24 h under diurnal cycles, which reduces to ~11 h under constant illumination with alternating photosynthetic and respiratory cycles (Cerveny et al. 2013; Gaudana et al. 2013). Sustained ultradian rhythms have been observed in global gene expression and metabolism under a growth condition of high external irradiance and high turbulence to simulate flashing light effect (Krishnakumar

| Organism                  | ATP/(ATP + ADP)          | References                                   |
|---------------------------|--------------------------|-----------------------------------------------|
| *E. coli*                 | >0.8                     | (Tran & Unden 1998)                          |
| *K. pneumonia*            | 0.45                     | (Upchurch & Mortenson 1980)                  |
| *Synechococcus* Y-7c-s   | 0.6 (6klux)–0.8 (18–20klux) | (Kallas & Castenholz 1982)                  |
| *S. elongatus* sp. 7942   | 0.4 (night)–0.9 (day)     | (Rust et al. 2011)                           |
| *Anabaena* sp. strain 7120| 0.4 (heterocysts), 0.78 (filament) | (Privalle & Burris 1983)                  |
| *Gloeothoece* sp. ATCC 27152| 0.25 (D6-D12)–0.7 (L0-D6) | (Gallon et al. 1988)                        |
| Spinach leaves            | 0.6–0.74                 | (Dietz & Heber 1984)                         |
| *Cyanothece* sp. ATCC 51142| 0.3 (day)–0.6 (night); 0.7 (nitrate sufficient); 0.65 (day)–0.85 (night) (mixotrophic) | Present study |
et al. 2015). Another group however reported that the ultradian rhythm under continuous light shifts to circadian rhythm under reduced light availability suggesting that the ultradian rhythm results from a mechanism independent of the core clock (Cerveny et al. 2013). Experimental knowledge about the KaiC PTO and the role of input signals in *Cyanothece* remains elusive, understanding of which will help determine if the timing mechanisms are indeed independent. In the present study, we unravel the circadian and ultradian clock in *Cyanothece* by investigating the KaiC1 phosphorylation–dephosphorylation cycle under diurnal and continuous light condition, and study its correlation with the cellular rhythms, ATP/ADP ratio, and redox state of PQ pool. Further we propose a mathematical model to simulate the underlying clock mechanism, which explains the variation in cycle time and complements published microarray results.

2. Materials and methods

2.1. Growth conditions

Axenic cultures of *Cyanothece* sp. ATCC 51142 were grown and maintained in ASP2 medium as previously reported (Reddy et al. 1993; Gaudana et al. 2013). For mixotrophic cultivation of *Cyanothece*, ASP2 medium (without nitrate) was supplemented with 30 mM glycerol (Alagesan et al. 2013). Axenic cultures of *S. elongatus* sp. PCC 7942 were cultivated in BG-11 medium in Erlenmeyer flasks under conditions similar to that used for *Cyanothece*. To measure the intracellular ATP and ADP concentrations from flask experiments, *Cyanothece* and *Synechococcus* cultures were grown under alternating 12-h light/dark condition at 30 °C and 125 rpm, and samples were collected at 6-h intervals after three diurnal cycles. All samples were directly quenched in two volumes of 60% methanol with 0.85% ammonium bicarbonate (−40 °C) (Sellick et al. 2011), centrifuged and stored in −80 °C till analysis.

*Cyanothece* culture was grown as reported previously in 2L Biostat B plus bioreactor (Sartorius, Goettingen, Germany) (Gaudana et al. 2013), with a surface illumination of 180 μmol m−2 s−1. The cells were washed with nitrate-free ASP2 medium and inoculated into 1.8L ASP2 medium (without nitrate) such that the initial O.D730nm was 0.3. The culture in the photobioreactor was allowed to grow under diurnal cycles for 96 h followed by continuous light (Supplementary Figure S1). Nine samples were collected during the last diurnal cycle: 1 h before and after switch on/off of light, and two samples each within the light and dark phase. Thirteen samples were collected in three cycles of ultradian oscillations in continuous light (Gaudana et al. 2013). Samples taken for ATP/(ADP + ATP) and PQ estimation were directly quenched in two volumes of 60% methanol with 0.85% ammonium bicarbonate (−40 °C) (Sellick et al. 2011), centrifuged and stored in −80 °C till analysis.

2.2. ATP–ADP estimation

Sample volume such that the product of the volume and O.D730 = 10 was taken for extraction and estimation of ATP and ADP levels in the cells. The culture was centrifuged and 300 μl of 0.8 M perchloric acid was added to the pellet. The suspension was sonicated for 60 s with two 20 s pulses. The lysate was centrifuged and the supernatant was neutralized with 5 M KOH on ice for 30 min (Napolitano & Shain 2005; Faijes et al. 2007). The neutralized extract was used for estimation using EnzyLight ADP/ATP ratio assay kit (BioAssay Systems, Hayward, CA).
2.3. **PQ extraction and redox state estimation**

Sample volume such that the product of the volume and O.D$_{730} = 10$ was drawn and centrifuged at 12,000 g for 10 min. Ice-cold methanol (500 μl) (containing 1 mg/ml of pyrogallol to prevent changes in PQ redox state) was added to the pellet. Following resuspension of the pellet, 500 μl of heptane was added, and vortexed. The samples were given a short spin to separate out the two phases. The upper heptane fraction containing PQ was transferred to a fresh tube. The process was repeated twice, and the heptane fractions were pooled, and dried in a vacuum concentrator (Lominski & Rienits 1981). The dried extract was dissolved in absolute ethanol, and the absorbance was measured at 255 and 290 nm. The amount of oxidized and reduced PQ was estimated using the molar extinction coefficients $\varepsilon_{255} = 17.94 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\varepsilon_{290} = 3.39 \text{ mM}^{-1} \text{ cm}^{-1}$, respectively (Kruk & Karpinski 2006).

2.4. **Protein extraction**

Culture volume equivalent to O.D$_{730} = 15$ was taken for protein extraction. Total protein from the cells was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. Following acetone precipitation and washing to remove salts, protein pellet was air dried and resuspended in resuspension buffer containing 8 M urea and 2 M thiourea (Reddy et al. 2013). The protein concentration was estimated using Bradford reagent (Biorad, Hercules, CA).

2.5. **Gel electrophoresis and western blotting**

Protein sample (3 μg) was mixed with 5X loading buffer and heated at 95 °C for 5 min. The sample was resolved on 9% SDS-PAGE gels (acylamide: bisacrylamide ratio of 37.5:1). Following activation of PVDF membrane with methanol, protein bands were transferred to membrane using wet-transfer at 100 V for 80 min.

For generating antibodies against KaiC1, a peptide from KaiC1 sequence (QPLPRENQPQPQLAPK-C) was selected and synthesized at USV limited (Mumbai, India). The peptide was conjugated with a carrier protein and polyclonal antibodies were raised against KaiC1 peptide at Abexome Biosciences Private Limited (Bangalore, India). Anti-NifH antibodies were received as a kind gift from Prof Louis A. Sherman (Colon-Lopez et al. 1997). For probing the protein bands with anti-KaiC1 and anti-NifH antibody, blocking was carried out at room temperature in 5% skimmed milk in TBST buffer. The membrane was incubated overnight at 4 °C in primary antibody (anti-KaiC1 diluted 1:2000 or anti-NifH to 1:5000) diluted in blocking buffer. After washing, the blots were incubated with secondary antibody (anti-rabbit goat IgG-HRP conjugated diluted 1:5000) for 1 h at RT. Membrane was developed using TMB-enhanced one component HRP membrane solution (Sigma-Aldrich, St. Louis, MO). For probing KaiC1 bands with anti-phosphoserine antibody (Enzo Life Sciences, Farmingdale, NY), 1% bovine serum albumin in TBST was used as the blocking buffer and the secondary antibody (anti-rabbit goat IgG-HRP conjugated) was diluted in TBST. The protein bands in the gel were confirmed using in-gel digestion followed by LC–MS/MS (Supplementary data).

2.6. **Mathematical model formulation**

A mathematical model for regulation KaiC1 phosphorylation was developed based on a published model for Neurospora (Cheng et al. 2009). The model is based on the
presumption that the C/N ratio feedback regulates the phosphorylation/dephosphorylation of KaiC1 protein, in turn causing a switch between day/night metabolism by transcription regulation (Figure 5). In the model, unphosphorylated and phosphorylated KaiC1 are assumed to activate the transcription of dawn peaking and dusk peaking genes (Krishnakumar et al. 2015), respectively, which is characterized by Hill’s equation. The dawn and dusk peaking genes are involved in day and night metabolism, respectively, resulting in imbalance in C/N (ratio of cellular carbon to nitrogen content) ratio which in turn is assumed to feedback regulate the KaiC1 phosphorylation cycle. Therefore, the model assumes that though the PTO is critical to global transcription regulation, the shift is indeed driven by C/N imbalance in order to maintain cellular C/N homeostasis. A simplified differential equation model incorporating these regulations was formulated as follows:

$$\frac{dA}{dt} = k_1 \frac{C^n}{K^n_1 + C^n} - \beta_A * A$$

(1)

$$\frac{dB}{dt} = k_2 \frac{C_p^n}{K^n_p + C_p^n} - \beta_B * B$$

(2)

$$\frac{dCN}{dt} = k_3 * A - k_4 * B$$

(3)

$$\frac{dC}{dt} = k_5 \frac{C_p}{K_3 + C_p} - k_6 * \frac{CN}{K_4 + C}$$

(4)

where $A$, $B$ – transcript abundance of dawn and dusk peaking genes, respectively; $CN$ – cellular C/N ratio; $C$, $C_p$ – concentration of unphosphorylated and phosphorylated form of KaiC1 protein, respectively; $\beta_x$ – the degradation constants for mRNA and $K_x$ – the Michaelis–Menten constants. The total KaiC1 ($=C + C_p = 3.4 \mu M$) concentration was assumed to be constant, production, and degradation terms were ignored. The values for the parameters were obtained from experimental data and from published models (Table 2) (Cheng et al. 2009; Rust et al. 2011). The model was solved using ODE45 solver in Matlab R2009b.

The model output was used to simulate the cellular ATP level using the following relation:

$$\text{ATP level} = \left( \frac{3A}{C^n} - 0.3 \frac{CN}{C^n} \right) + \left( \frac{6B}{C_p} - 1.6 \frac{CN}{C_p^n} \right)$$

3. Results and discussion

Nitrogenase gene, *nifH*, shows robust oscillations in transcript and protein abundance under diurnal cycles and continuous light (Colon-Lopez et al. 1997; Gaudana et al. 2013). Hence, in the present study NifH protein was used as a positive control in immunoblot analysis for optimizing the sampling time and sample processing steps.
Under diurnal cycles, NifH protein was detected at the end of light phase and during the dark phase (Figure 1), validating the experimental methodology followed. Under continuous illumination, ultradian rhythm in NifH levels correlates with the reduced cycle time observed in physiological and gene expression studies. Sequence analysis of KaiC homologs suggests a non-functional or sub-functional role of KaiC2 clock protein in *Cyanothece*. Further, the transcript abundance of *kaiC1* in microarray data was more than 10-fold higher than that of *kaiC2* (Krishnakumar et al. 2015). Therefore, the present study focused on deciphering the phosphorylation pattern of KaiC1 homolog only. Nevertheless, a similar analysis for KaiC2 under diverse growth conditions should be interesting to unravel the possible need for two KaiC homologs in *Cyanothece*.

### 3.1. Rhythms in KaiC1 phosphorylation

Under diurnal cycles, three distinct protein bands were observed at the end of dark phase (71 and 95 h) with anti-KaiC1 antibody; lower bands corresponding to hypophosphorylated KaiC1, while the top band corresponding to hyper-phosphorylated form (Figure 1). The lower bands became fainter during the day signifying phosphorylation, while the appearance of lower bands in the dark period suggested dephosphorylation at night. This is also signified using densitometric analysis (Figure 1). Using a general anti-phosphoserine (anti-pS) antibody, the transient dephosphorylation stage of KaiC1 could be accurately captured. The distinct two bands at D4.3 corresponded to the doubly phosphorylated and singly phosphorylated (phosphoserine) KaiC1. The identity of the bands was re-affirmed by in-gel digestion followed by LC/MS–MS analysis (Supplementary data). Five protein bands (three bands corresponding to KaiC1, and the bands above and below as negative controls) on the SDS–PAGE gel were analyzed.

These results show that phosphorylation–dephosphorylation cycle in *Cyanothece* is broadly similar to that in *Synechococcus*. However, while in *Synechococcus* KaiC is completely dephosphorylated at night; in *Cyanothece* a significant portion of KaiC1 remained phosphorylated at D11 (Figure 1). We speculate that higher KaiA–KaiC affinity leads to enhanced KaiC1 phosphorylation in *Cyanothece*. The KaiA protein in *Cyanothece* is only 45% identical to KaiA in *Synechococcus* (Supplementary data), and lacks two C–terminal residues similar to the truncated-KaiA mutant of *Synechococcus* which shows enhanced KaiC phosphorylation due to stronger KaiA–KaiC affinity (Chen et al. 2009; Rust et al. 2011).

### Table 2. Values for the parameters used in the C/N model to describe the rhythms in *Cyanothece* sp. ATCC 51142 (Cheng et al. 2009; Rust et al. 2011).

| Parameter | Value |
|-----------|-------|
| $k_1$     | 10 μM h $^{-1}$ |
| $k_2$     | 10 μM h $^{-1}$ |
| $k_3$     | 2.7 h $^{-1}$ |
| $k_4$     | 2.7 h $^{-1}$ |
| $k_5$     | 9 μM h $^{-1}$ |
| $k_6$     | 0.7 h $^{-1}$ |
| $K_1$     | 5 μM |
| $K_2$     | 5 μM |
| $K_3$     | 0.5 μM |
| $K_4$     | 0.5 μM |
| $\beta_x$ | 0.17 h $^{-1}$ |
| $n$       | 4     |
et al. 2009; Dong et al. 2010; Nakajima et al. 2010; Hosokawa et al. 2013). However, this speculation is based solely on sequence analysis and would be interesting if verified experimentally.
The three distinct bands observed under diurnal cycles with anti-KaiC1 antibody were not observed in continuous light and KaiC1 majorly existed in the doubly phosphorylated form (Supplementary Figure S2). This is possibly due to the shorter respiratory phase (~4 h), which is insufficient for complete dephosphorylation of KaiC1. However, with anti-pS antibody, distinct two bands with darker lower band (singly phosphorylated serine KaiC1) were observed in the middle of the respiratory phase (similar to D4.3 in diurnal cycles) in the two ultradian cycles, implying ultradian rhythms in KaiC1 PTO (Figure 1).

These results suggest KaiC1 phosphorylation follows circadian rhythm under diurnal cycles and ultradian rhythm under constant illumination with phosphorylation and dephosphorylation occurring during the photosynthetic and respiratory phases, respectively. The observed ultradian rhythms of the PTO motivated us to explore the rhythms in input signals to the clock, and study their variation under continuous light. Further, in vitro experiments with recombinant KaiC1 and KaiC2 would be helpful in re-affirming the clock rhythm and determining the role of KaiC2 in Cyanothece.

3.2. ATP/ADP ratio in nitrogen-fixing Cyanothece

During the day, ATP required for carbon fixation by the Calvin cycle is supplemented by photosynthetic light reaction. Nitrogen-fixation is an energy-intensive process, which is possibly supplemented by the respiratory breakdown of glycogen reserves at night. A previous study showed that exit gas profile complements precisely the gene expression and biochemical estimates, and adequately represents the real time metabolic status of the cells (Krishnakumar et al. 2013). Hence, we used the changes in the exit gas oxygen concentration as a measure of the light reaction during the day and respiration at night to estimate the cellular ATP levels in Cyanothece during light and dark condition, respectively (Figure 2). While during the day, light reaction seems to provide a continuous supply of ATP for the Calvin cycle, the respiratory burst at the onset of dark perhaps results in a rapid spike in the cellular ATP levels. Therefore, though the total ATP produced during the day and night is approximately same, higher cellular ATP level is expected at the onset of dark phase due to the respiratory burst.

On experimental analysis, robust rhythm in cellular ATP/ADP ratio was observed both under diurnal cycles and continuous light. As predicted, higher ATP/ADP ratio was noted during the respiratory burst (Figure 3), possibly due to the breakdown of glycogen reserves. The dip in ATP levels at D4.3 can be attributed to the maximal nitrogenase activity occurring 4–6 h into the dark period (Schneegurt et al. 1994). During the day, ATP/ADP levels declined perhaps due to enhanced carbon fixation. Under continuous light, similar profile for the ATP/ADP ratio was observed during the respiratory phase, however, with a reduced cycle time. Though the observed values for ATP/ADP ratio were lower than that reported for Synechococcus, the values were similar to that observed in other nitrogen-fixing cyanobacteria like Gloeothecae (Gallon et al. 1988). Since the ATP profile complemented our predictions based on the exit gas, the cellular ATP levels in Cyanothece indeed reflects the metabolic status of the cell.

3.3. ATP/ADP ratio under different growth conditions

The correlation between ATP/ADP ratio and metabolic rhythm in Cyanothece was further explored under different growth conditions (Figure 4). In nitrate-sufficient growth, low-amplitude, and arrhythmic profile for ATP /ADP ratio were observed, which agrees
with the lower intracellular glycogen levels and loss of rhythms in gene expression previously observed in this condition (Gaudana et al. 2013). Further, the higher ATP/ADP ratio values in nitrate-replete cultures compared to nitrogen-fixing condition complemented that reported for vegetative cells (~78%) and heterocysts (~40%) in Anabaena sp. (Privalle & Burris 1983) (Table 1). However, in mixotrophic nitrogen-fixing condition, the enhanced ATP levels at night emphasized the role of nitrogen

Figure 2. Predicted cellular ATP levels in Cyanothece 51142.
Notes: Exit gas oxygen concentration (Supplementary data) was used to estimate the amount of ATP produced during the day by photosynthetic light reaction and at night by the respiratory breakdown of glucose. The calculations give the total ATP produced (area under the curve) during the day and night phase.

\[
\text{DAY:}
\]

ATP produced by photosynthetic light reaction during the day

\[12\text{H}_2\text{O} + 12\text{NADP} + 18\text{ADP} \rightarrow 6\text{O}_2 + 12\text{NADPH} + 18\text{ATP}\]

Therefore, ATP produced during the day = Area under curve = 0.68 mmoles/L

\[
\text{NIGHT:}
\]

ATP produced by respiratory breakdown of glucose residues at night

\[6\text{O}_2 + \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 6\text{CO}_2 + 6\text{H}_2\text{O} + 36\text{ATP}\]

Therefore, ATP produced during the night = Area under curve = 0.6 mmoles/L
fixation in regulating the rhythms in *Cyanothece* (Figure 4). The ATP ratio values were higher in mixotrophic condition as the enzymes involved in ATP regeneration are more active in the presence of a constant carbon substrate (i.e. glycerol) as opposed to in photoautotrophic condition.

In similar experiments for *Synechococcus*, the cellular ATP levels increased during the later part of the dark period, possibly due to breakdown of carbon reserves following catabolic processes (Figure 4) and remained high till the mid of light phase, attributed to the photosynthetic light reactions. Previous studies have reported a drop in ATP/ADP ratio values during a 5-h dark pulse in continuous light in *Synechococcus* following entrainment in one light–dark cycle (Rust et al. 2011). The spike in ATP levels towards the end of dark phase observed in our study may have been missed in the previous studies, which monitor the levels for a shorter duration (5 h). Further the overall lower ATP levels in *Synechococcus* observed in our study may be attributed to the difference in light/dark adaptation, growth conditions, and extraction protocol.

### 3.4. Redox state of PQ pool

Under diurnal cycles the PQ pool was reduced during the day (Figure 3). PQ pool was oxidized at the onset of dark, eventually becoming reduced in the late dark phase, similar to that observed in *Synechococcus* (Kim et al. 2012). Since PQ in the thylakoid membrane is shared during photosynthesis and respiration, it undergoes reduction both
during the day and night (Rabouille et al. 2013). Under continuous illumination, PQ pool was oxidized during the respiratory burst followed by reduction in the late respiratory phase (Figure 3). However, in the photosynthetic phase, it was majorly oxidized. Though redox state of PQ pool is usually measured by fluorescence-based method, in the present study, the oxidized and reduced forms were extracted from the cells and measured spectrophotometrically. Despite limitations due to instability of PQ pool during spectrophotometric analysis, consistent rhythms in the redox state were observed under diurnal cycles and continuous light, which was verified statistically. The similar redox changes in *Cyanothecae* and *Synechococcus* suggests the role of PQ pool in regulating the clock timing in *Cyanothecae*.

The ultradian rhythms in redox state of PQ pool and ATP/ADP ratio under continuous light indeed reflect the metabolic rhythms of the cell. Further, the loss of rhythm in ATP/ADP levels in nitrate-supplemented *Cyanothecae* implies the significance of nitrogen fixation in regulating the clock. It has also been noted previously that diazotrophic cyanobacteria shift between day and night metabolisms, where the glycogen (Krishnakumar et al. 2013) and C/N ratio (GroBkopf & LaRoche 2012) (Supplementary Figure S3) increase in the light phase and decline during the dark phase. A recent report on *Synechococcus* suggested a possible feedback regulation of the circadian clock by

Figure 4. (Colour online) Cellular ATP/ADP profiles in *Cyanothecae* sp. ATCC 51142 and *Synechococcus elongatus* sp. 7942.
Notes: Cultures were grown in 250 ml Erlenmeyer flasks under diurnal cycles at 30 °C. Samples were collected at 6-h intervals. The top panel gives the illumination conditions (white-day, black-night). Black-nitrate-replete condition (*Cyanothecae*); Blue–mixotrophic diazotrophic growth (*Cyanothecae*); Red–for *Synechococcus*.
glycogen/carbon reserves (Pattanayak Gopal et al. 2014). Since Cyanothece oscillates between day and night metabolism in order to carry out the two mutually incompatible processes of carbon and nitrogen fixation, respectively, we speculate that the internal C/N ratio (ratio of cellular carbon to nitrogen content) acts as a feedback signal to regulate the clock timing.

3.5. Mathematical model for regulation of rhythms in Cyanothece

We hypothesize that the cellular C/N imbalance generated by alternating day/night metabolism acts as a feedback signal for the clock PTO in order to achieve C/N homeostasis, in turn locking the system into sustained oscillations. Under diurnal cycles, the external light regime plays a role in resetting the clock; however, in continuous light the C/N ratio possibly acts as the major input signal for the clock regulation. Hence, a mathematical model for the regulation of KaiC1 phosphorylation cycle by the C/N ratio under continuous light was developed (Figure 5). The model makes the following assumptions, (1) unphosphorylated KaiC1 activates dawn peaking genes (involved in day metabolism) leading to enhanced carbon accumulation and increase in C/N ratio, (2) the C/N ratio beyond the cellular cut-off limit stimulates phosphorylation of KaiC1 during the photosynthetic phase, (3) phosphorylated KaiC1 in turn activates the dusk peaking genes (involved in night metabolism), and (4) the decrease in C/N ratio due to respiration and nitrogen fixation during night metabolism promotes KaiC1 dephosphorylation during the respiratory phase.

Due to the lower fold change in transcript levels of kaiC1 as compared to nif genes under diurnal cycles and continuous light (Figure 5) (Stockel et al. 2008), the total KaiC1 concentration in the model was assumed to be constant. In order to simplify the model, reactions for phosphorylation of individual residues, and the transcription of clock and other metabolic genes were not included. Due to limitation of available experimental estimates, parameters for the model (Table 2) were taken from preliminary experiments and existing reports (Cheng et al. 2009; Rust et al. 2011). Nonlinear functions for activation of transcription and phosphorylation/ dephosphorylation cycle, along with the negative feedback loop ensure sustained limit cycle oscillations (Supplementary Figure S3) with cycle time of ~11 h in agreement with published microarray data (Krishnakumar et al. 2015). Further, the ATP profile obtained using the model output adequately matches the experimentally observed ATP profile in continuous light (Figure 5).

The model highlights the existence of a single timing mechanism, which explains the probable dependence of cycle time on light availability and the shift from ultradian rhythm to circadian rhythm in dense cultures. Enhanced photosynthesis in dilute cultures due to higher light availability results in rapid increase in C/N ratio leading to shorter cycle time, as compared to dense cultures, where reduced light penetration leads to a gradual increase in C/N ratio. This explanation complements the reported dissolved O2 and CO2 profiles, which show a flatter, prolonged photosynthetic phase with a brief respiratory period in dense cultures following circadian rhythm, while higher amplitude shorter duration photosynthetic phases are observed in ultradian cycles (Cerveny et al. 2013). Hence, the model represents the probable architecture of the clock mechanism in Cyanothece under continuous light. Knowledge about the actual signaling molecule detecting the C/N imbalance will help in further expanding the model for detailed understanding of the timing mechanism.
4. Conclusion

This study provides new insights into the underlying clock mechanism in nitrogen-fixing unicellular cyanobacteria. Despite experimental limitations of in vivo and in vitro study of the PTO in *Cyanothece*, the circadian and ultradian rhythm in KaiC1 phosphorylation under diurnal cycles and continuous light, respectively, suggests the existence of a single timing mechanism in *Cyanothece*. Further, the rhythms in PQ redox state and ATP/ADP ratio strongly suggest the reciprocal interaction between the clock and metabolic status of the cell which we describe using a mathematical model. The proposed model precisely complements the published microarray data, and explains the observed shift from ultradian to circadian period in dense cultures.

**Supplemental material**

Supplemental material for this article can be accessed at [http://dx.doi.org/10.1080/09291016.2015.1116737](http://dx.doi.org/10.1080/09291016.2015.1116737).
Acknowledgements
This work was partially supported by grant from Department of Biotechnology, Ministry of Science and Technology, Government of India (grant number BT/EB/PAN IIT/2012) awarded to P.P.W. Authors acknowledge Prof Louis A. Sherman, Purdue University for providing anti-NifH antibody.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
This work was supported by the Department of Biotechnology, Ministry of Science and Technology, Government of India [grant number BT/Indo-Aus/04/04/2009].

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