Fitness Regulation by Timing Systems: Insight from Cyanobacteria and Purple Non-sulfur Bacteria

By

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To my parents

and

To my beloved husband and baby

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Chapter I

Introduction

Focus

Circadian clocks are endogenous timing mechanisms that allow organisms to anticipate daily changes in the environment, and they are found in a wide range of organisms including prokaryotic cyanobacteria. Cyanobacterial circadian clock is the simplest circadian machinery that has been discovered and comprehensively studied. To answer the question why circadian clocks are important, studies from Johnson lab conducted competition experiments between cyanobacterial strains with a functioning clock and strains with a disrupted clock or a clock with non-24-hour free running periods (Ouyang et al, 1998; Woelfle et al., 2004). When these strains were co-cultured under light-dark cycles, the one with a functioning clock rapidly out-competed the others, which clearly demonstrates the adaptive significance of circadian clocks. The underlying mechanism of the clock-mediated fitness enhancement, however, is still unclear. In the first part of this dissertation, experiments designed to uncover the potential mechanisms are described (**Chapter II**). Additionally, we unexpectedly found that the fitness of cyanobacteria was reduced at low temperatures when its circadian rhythms were enhanced by optimizing the codon of *kaiBC* genes, indicating that the “conditional” suppression of circadian clocks can be another way to enhance fitness (Xu et al., 2013).

Chapter III is an extension of Chapter II, where a novel hypothesis about the relationship between metabolism and circadian clocks is proposed and tested. I propose that the disruption of circadian clocks alters the metabolism under light-dark cycles, and that this altered metabolism contributes to the competition results. To test this hypothesis, the metabolic profiles of the wild-type cyanobacterium and the arrhythmic mutants were established and compared, and this hypothesis is supported by preliminary results (**Chapter III**).

Following the work done in cyanobacteria, **Chapter IV** focuses on answering another question: are there any other bacteria possessing a circadian clock? To address this question, a purple non-sulfur bacterium containing cyanobacterial circadian clock genes is investigated. This study revealed that this purple bacterium has a timing mechanism, and that this timing mechanism confers adaptive value under light-dark cycles.

Circadian rhythms

Circadian rhythms are found in a wide range of organisms from bacteria to mammals. They are usually defined by three criteria. First, they are endogenous, self-sustainable oscillations with the free running period (FRP) of around 24 hours, which means circadian rhythms persist even under constant conditions; second, they can be entrained by environmental cues; third, circadian rhythms are temperature-compensated within the physiological temperature ranges (Edmunds, 1983; Pittendrigh, 1981; Dunlap et al., 2004; Koukkari and Southern, 2006).

It was believed for a long time that prokaryotic organisms were incapable of generating circadian rhythms due to the short doubling time and lack of nuclear structures (Edmunds, 1983; Kippert, 1987). However, in 1986, endogenous rhythms of nitrogen fixation and photosynthesis were discovered in the cyanobacterial species *Synechococcus* RF-1 (Grobbelaar et al., 1986; Mitsui, 1986), which showed the existence of circadian rhythms in prokaryotes for the first time. Later on, benefiting from the available genetic tools, another cyanobacterial species, *Synechococcus elongatus* PCC 7942 (*S. elongatus*), was comprehensively studied as the model organism for circadian research (Golden, 1988; Golden et al., 1987; Kondo et al., 1993; Kondo et al., 1994). By introducing luminescence reporters to the genome, scientists monitored the gene expressions in *S. elongatus* and isolated several mutants that display altered circadian rhythms (Kondo et al., 1994), leading to the identification of core clock genes in *S. elongatus* (Ishiura, 1998). These pioneer studies established the foundation for circadian research in prokaryotic organisms, and they also opened the window for scientists to experimentally test the adaptive value of circadian clocks.

Cyanobacterial circadian clock

Circadian clocks are the endogenous timing mechanisms generating

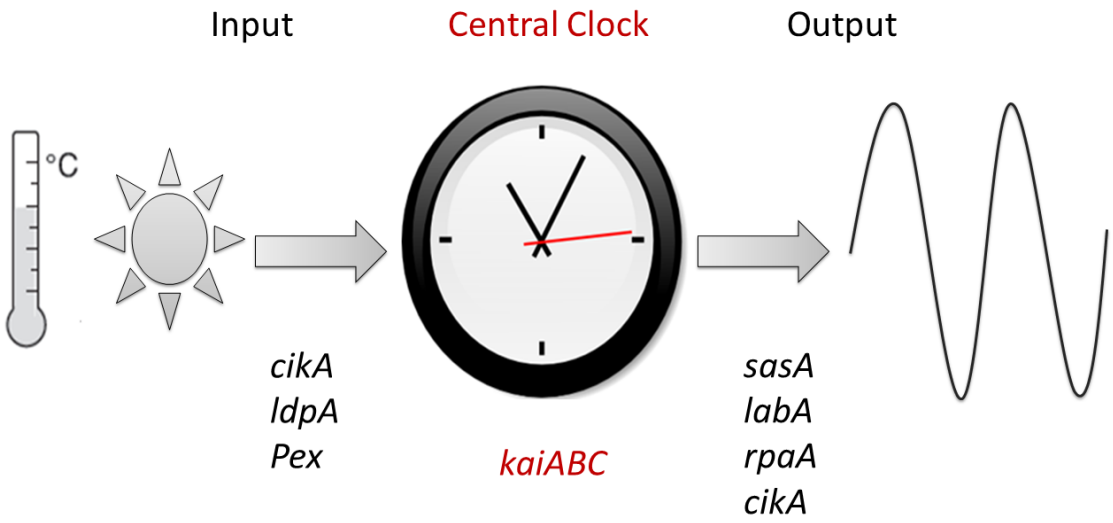


Figure 1.1 Simplified model for the *S. elongatus* PCC 7942 circadian clock. It includes the three conceptual designations for the circadian clock (the input pathway, the central clock, the output pathway), along with known *S. elongatus* genes involved in each pathway.

circadian rhythms. At the molecular level, a circadian system is composed of three basic elements: a central clock, an input pathway and an output pathway. The central clock is the core machinery connecting to the input and the output pathway. By receiving environmental signals from the input pathway, it can be entrained and then transmits the temporal information to the output pathway. The output pathway acts like a "hand" by which the downstream activities are regulated temporally (Ditty and Mackey, 2009).

In *S. elongatus*, the central clock is built up on the activities of three proteins, KaiA, KaiB and KaiC (Ishiura, 1998) (**Fig.1.1**). Deletions or mutations of any of these *kai* genes render the cells to be arrhythmic or alter their FRPs (Kondo et al., 1993; Ishiura, 1998). Among the Kai proteins, KaiC is the central

component with autokinase, autophosphatase and ATPase activities (Iwasaki et al., 2002; Kitayama et al., 2003; Nishiwaki, 2000; Xu et al., 2003; Terauchi et al., 2007). KaiA promotes the phosphorylation of KaiC, while KaiB antagonizes the action of KaiA (Johnson et al., 2008). For a long time the phosphorylation rhythms of KaiC are thought to be the basis of the circadian clock of *S. elongatus*, whereas some recent studies revealed that the ATPase activity of KaiC also plays an essential role in determining the timing for circadian clocks (Terauchi et al., 2007; Kitayama et al, 2013).

In the input pathway, three genes have been identified (**Fig.1.1**). LdpA senses the changes in the redox state of the cell as changes in light quantity (Ivleva et al., 2005), and Pex binds to the negative regulator sequence in the promoter of *kaiA* and is likely to repress the expression of *kaiA* (Arita et al., 2007). CikA is involved in the phase resetting in response to light or dark pulses (Schmitz et al., 2000). Recently, Gutu et al. (2013) reported that CikA dephosphorylates an output pathway protein, RpaA, indicating that *cikA* also plays a role in the output pathway. In the output pathway, *sasA*, *rpaA* and *labA* have been identified (**Fig.1.1**). SasA interacts with KaiC and then transfers its phosphoryl group to RpaA which regulates the global gene expressions (Smith and Williams, 2006; Takai et al., 2006; Markson et al., 2013). LabA is likely to function as a repressor of the activity of KaiC and RpaA (Taniguchi et al., 2007). In addition to these genes, it is likely that there are more genes participating in the circadian system. Identification of novel clock genes will help us understand the circadian mechanism and its evolutionary significance better.

The adaptive value of circadian clocks

It is believed that circadian clocks are evolved as an adaptation to the daily cycles of light and temperature driven by the rotation of the earth. To understand the adaptive value of circadian clocks, two questions have to be answered. First, what is the advantage of having a circadian clock? Second, is the circadian clock still adaptive under constant conditions or under non-24-hour cycles (Woelfle and Johnson, 2009)?

The most popular approach used by chronobiologists to address these questions is experimental manipulation of traits and/or ecology (Vaze and Sharma, 2013). By this approach, fitness is often measured by survival rates, longevity and growth rates. For instance, when the circadian system of chipmunks was disrupted by suprachiasmatic nucleus (SCN) lesion, their survival rates in the wild were significantly decreased, comparing to the chipmunks with intact circadian systems (DeCoursey et al., 2000). Studies of *Drosophila melanogaster* showed that the life span of the flies with altered circadian period was significantly reduced by up to 15% (Klarsfeld and Rouyer, 1998) and that the disruption of the circadian system also affected sperm production in males (Beaver et al., 2002). Besides manipulating circadian phenotypes, the adaptive value of circadian clocks was also confirmed by changing the light-dark (LD) cycles. Hillman (1960) reported that the growth rates of plants were reduced when they were grown under constant light (LL) conditions instead of under LD conditions. Moreover, when plants were reared under non-24-hour LD cycles,

their growth rates were also decreased, comparing to the growth rates under 24-hour LD cycles (Highkin and Hanson, 1954; Went, 1960).

In addition, many other approaches were employed to demonstrate the adaptive significance of circadian systems. For instance, some scientists study the correlation between trait and ecological/environmental variables (Vaze and Sharma, 2013), which mainly involves in investigations in constant environments, e.g., caves where it is constantly dark and arctic areas. In these environments, organisms that do not display circadian rhythms are found (Vaze and Sharma, 2013), indicating that circadian clocks may be not needed in these conditions. Moreover, by comparative analysis, researchers found that circadian clocks are not only present in numerous unrelated organisms, but also encoded by different genes (Bell-Pedersen et al., 2005; Vaze and Sharma, 2013), indicating that circadian clocks in different species are evolved convergently. Convergent evolution is a strong evidence of natural selection (Endler, 1986; Larson and Losos, 1996), thus supporting the idea that circadian clocks are adaptive.

Examples described here are only a small portion of the numerous studies targeted to illustrate the adaptive value of circadian clocks. However, among all of these studies, few of them have directly tested the relationship of the circadian clock and fitness. In evolutionary theory, “fitness” is defined as the capability that a genotype can be passed to the next generation, in other words, the ability of reproducing (Futuyma, 1998). Neither the surviving rates of the chipmunks nor the growth rates of plants can be considered as the measure of “fitness.” To

compare the fitness, multiple generations have to be tested and the productive ability has to be assessed, which is time-consuming and difficult to do in higher organisms. Fortunately, this problem was solved by studying the cyanobacterial circadian clock. Cyanobacteria reproduce asexually, and their doubling time is relatively shorter, thus allowing us to measure their reproduction in relatively short period. Since 1998, the Johnson group has conducted a series of competition experiments (Ouyang et al, 1998; Woelfle et al., 2004) to directly test the adaptive significance of circadian clocks in cyanobacteria. We will discuss them with more detail in the next section and **Chapter II**.

Testing the adaptive value of circadian clocks in cyanobacteria

The work done by Johnson's group has proved that *S. elongatus* can be used as a practical model system to test the adaptive value of circadian clocks (Ouyang et al, 1998; Woelfle et al., 2004). In 1998, Ouyang et al. addressed the adaptive significance of the circadian clock by competing the wild-type strain with period-altered mutants. When the period-altered mutants and the wild type were cultured separately under either LL conditions or LD cycles, no significant difference in their growth rates was observed. However, when the short-period mutant (C22a) was co-cultured with the wild type under LD 11:11, the wild type was defeated by C22a. Similarly, when the long-period mutant (C28a) and wild type were co-cultured under LD15:15, the wild type was out-competed by C28a. Moreover, when C22a and C28a were co-cultured, C22a won under LD11:11 and C28a won under LD15:15. In contrast, the fraction of each strain in the co-

cultures remained the same as the initial fraction when they were grown under a non-selective LL condition. This result indicated that the strain whose FRP was nearest to the environmental period was the most fit under selective conditions (co-culture and LD cycles) (Ouyang et al, 1998). In 2004, Woelfle et al. did another series of competition experiments between the clock-knockout strains and the wild type. This work showed that the wild-type strain out-competed the clock mutants when they were co-cultured under LD 12:12 cycles (selective condition), but not under LL conditions or in pure cultures, suggesting that the circadian clock confers adaptive value in cyanobacteria under cyclic conditions. Interestingly, when the clock mutant (CLAb) was co-cultured with the wild type under LL, the proportion of CLAb increased significantly from its starting proportion, suggesting that the clock might be a liability for cyanobacteria under constant conditions (Woelfle et al., 2004).

These two studies clearly demonstrated that the circadian clock confers an adaptive value in cyanobacteria when the period of the biological rhythms "resonate" with the period of environmental rhythms, nevertheless, the mechanism of the clock-mediated fitness enhancement remains elusive. Ouyang et al. proposed that the potential mechanisms might be either the competition for limiting resources, or some secreted inhibitors regulated by the circadian clock (Ouyang et al, 1998). Besides, a cell-to-cell communication model was also proposed by Woelfle et al. (Woelfle and Johnson, 2009). Although there are a number of hypotheses proposed, none of them has been tested rigorously. In **Chapter II**, I will report experiments that have been done to test these models

and their significance for future studies.

Circadian clocks and metabolism

In the last decade, many studies have been done to describe the relationship between circadian clocks and metabolism, mainly because of the increasing population with metabolic disorders. On one hand, circadian clocks regulate metabolism. For instance, in mice and humans, the blood pressure, body temperatures and many other metabolic events are regulated by circadian clocks. In plants, circadian clocks control the secretion of selected hormones involved in the defense against herbivores (Goodspeed et al., 2012). On the other hand, metabolism can also affect circadian clocks. For example, Stokkan et al. (2001) reported that circadian clocks in rat liver can be entrained by feeding at different times. Taken together, these studies suggest that the circadian clock and metabolism can form an autoregulatory feedback network.

For cyanobacteria, light plays an essential role in many of their physiological activities, including photosynthesis and the entrainment of circadian clocks, therefore we would expect the link between metabolism and circadian clocks to be particularly important in cyanobacteria. Unlike plants or many other organisms in which the light is sensed through photoreceptors (Liu, 2003; Millar, 2003), the cyanobacterial circadian clock appears to receive the entraining information directly from its metabolic processes (Rust et al., 2011; Ivleva et al., 2006; Kim et al., 2013; Pattanayak and Rust, 2014). In *S. elongatus*, the clock gene *cikA* is a key factor bridging the metabolic status and the circadian clock

(Ivleva et al., 2006). It senses the redox state of the plastoquinone pools which are the molecules involved in the electron transport chain in light reactions of photosynthesis, and through this process CikA is able to reset the phase of circadian rhythms by affecting the phosphorylation status of KaiC (Ivleva et al., 2006). In addition, the ATP/ADP ratio, which is one of fundamental factors determining the timing of the circadian clock, can also be altered by changing the light conditions, indicating the regulation of metabolism on the circadian clock (Rust et al., 2011). Kim et al. (2012) proposed that the redox state of quinone and the ATP/ADP ratio in the cell could work together to reset the phase of the clock, suggesting the importance of the coupling of circadian clocks and metabolism.

With these lines of evidence, it is clear that circadian clocks and metabolism are associated. However, several questions need to be answered. First, what is the global picture of metabolism under LD cycles and under LL conditions? Previous studies showed that gene expression of *S. elongatus* oscillate under both LD cycles and constant conditions (Liu et al., 1995; Ito et al., 2009). Will the level of metabolites also oscillate under these conditions? Given the fact that light plays a dominant role for photosynthesis, we would expect that metabolites show oscillations under LD cycles. But what about the metabolism under constant conditions? Second, will altering the clock phenotypes change the global picture of metabolism? For example, we would like to know if the metabolites of arrhythmic mutants oscillate the same way as the wild type under LD cycles. If not, do these different metabolic profiles contribute to the results of the competition experiments that we used to test the adaptive value of circadian

clocks? Some preliminary results addressing these questions will be described in **Chapter III**, which will provide direct evidence illustrating the connection between circadian clocks and metabolism.

KaiC in other prokaryotic organisms

As the circadian clock of *S. elongatus* is comprehensively investigated, another question arises: is this KaiABC-driven circadian clock a universal timing mechanism for prokaryotic organisms? To answer this question, we should first examine the prevalence of *kaiABC* genes in the eubacterial and archaeal domains. A bioinformatics study suggested that homologs of *kaiC* not only exist in almost all of the cyanobacteria, but also are present in archaea and many eubacteria (Dvornyk et al., 2003). In contrast, *kaiA* is only identified among cyanobacteria, whereas the distribution of *kaiB* is similar to *kaiC* (Dvornyk et al., 2003). A few studies have shown that some other cyanobacterial species possess *kai* clock genes that are homologous to those found in *S. elongatus*. For instance, *Synechocystis sp.* PCC 6803 is a freshwater cyanobacterium containing three *kaiC*, two *kaiB* and one *kaiA*, and 2-9% of the genes are regulated by its circadian clock (Kucho et al., 2005). In addition, circadian regulation by *kaiABC* genes is also reported in the filamentous cyanobacterium *Anabaena sp.* Strain PCC 7120, and this regulation happens in both vegetative cells and heterocysts (Kushige et al., 2013). On the other hand, not all of the cyanobacteria have all three *kai* genes. The marine cyanobacterium *Prochlorococcus*, for example, is thought to possesses an hour glass instead of a

circadian clock (Holtzendorff et al., 2008; Axmann et al., 2009). This might not be surprising because no *kaiA* is found in this cyanobacterium, and some features of the secondary structure of *proKaiC* may also prevent the formation of a self-sustainable timing mechanism in *Prochlorococcus* (Holtzendorff et al., 2008; Axmann et al., 2009; Axmann et al., 2014).

Although the study of circadian clocks has been intensively conducted in cyanobacteria, little is known about the timing mechanisms, if any, in other bacteria and archaea. Among the bacteria possessing *kaiC* homologs, the KaiC of some purple non-sulfur bacteria (PNSB) share a high similarity with one of the KaiC in *Synechocystis sp.* PCC 6803 (Dvornyk et al., 2003), suggesting the possibility that PNSB are able to generate circadian rhythms. Indeed, it was reported that one of the PNSB, *Rhodospirillum rubrum*, displayed 12-h rhythms in its uptake hydrogenase activity (Praag et al., 2000), and another PNSB, *Rhodobacter sphaeroides*, was also reported to show some oscillations in its luminescence reporter activity (Min et al., 2005). However, the authors in these two studies could not prove whether these oscillations were circadian or not. Furthermore, none of these two studies provided any evidence showing that these observed oscillations were under the control of *kaiC* or some other timing mechanisms. Therefore the function of *kaiC* and putative timing mechanisms are still a puzzle in these bacteria.

KaiC in the purple non-sulfur bacterium *Rhodopseudomonas palustris*

To understand the role of *kaiC* in other bacteria and to explore the

possible timing mechanisms driven by *kaiC*, the first step would be to find a suitable candidate for this study. This candidate should fulfill several criteria: first, it must have *kaiC* homologs in the genome; second, genetic tools must be available in order to construct loss-of-function mutants; third, it must perform some physiological activities that are easy to measure (or it can be transformed with reporters); fourth, it should be able to survive when isolated from a cyclic environment.

One candidate that satisfies all of these criteria is *Rhodopseudomonas palustris* (*R. palustris*). As a member of the PNSB group, *R. palustris* was recently considered as a model organism for bacterial communication study (Schaefer et al., 2008) as well as for bioenergy production (McKinlay et al., 2010). It is an anoxygenic photosynthetic bacterium that belongs to the alpha proteobacteria (Larimer et al., 2004). Although it performs photosynthesis, it has a much more complicated metabolism than cyanobacteria (**Fig. 1.2**). *R.palustris* adapts to different environments by switching among four different metabolic modes: photoautotrophic, photoheterotrophic, chemoheterotrophic and chemoautotrophic. In the absence of O_2, *R.palustris* can grow photoautotrophically by using CO_2 as the carbon source, or grow photoheterotrophically by using organic carbon sources. When it is exposed to O_2, it can rapidly shut down photosynthetic systems and switch its energy and carbon source to organic compounds (chemoheterotrophical), or to inorganic compounds and CO_2 (chemoautotrophical) (Larimer et al., 2004). Apparently the highly versatile metabolic system provides *R.palustris* a huge advantage that

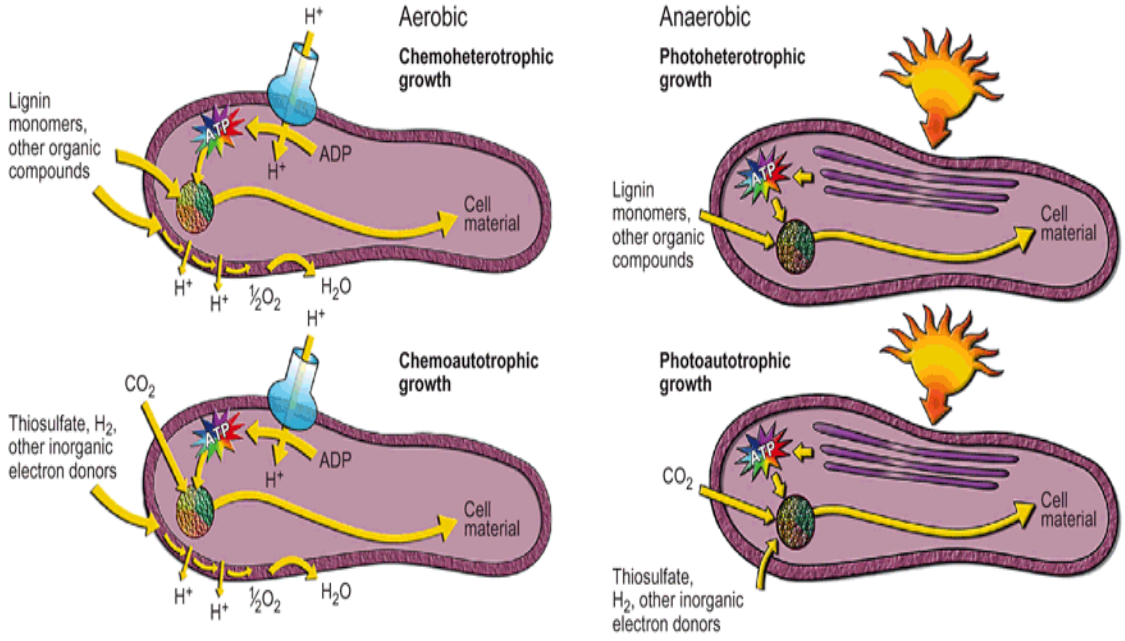


Figure 1.2 Overview of the physiology of *R. palustris* (Larimer et al., 2004). Schematic representations of the four types of metabolism that support its growth are shown. The multicolored circle in each cell represents the enzymatic reactions of central metabolism. Figure is from Larimer et al., 2004.

allows it to adapt to various conditions. Most importantly to a chronobiologist, *R. palustris* harbors *kaiB* and *kaiC* homologs (*kaiB*^{Rp} and *kaiC*^{Rp}, respectively). Studies in cyanobacteria show that the *kaiABC*-driven circadian clocks confers adaptive value under cyclic conditions (Ouyang et al., 1998; Woelfle et al., 2004), so it would be interesting to know how *kaiB*^{Rp} and *kaiC*^{Rp} function in *R. palustris*, and how their function contributes to its fitness. Moreover, *R. palustris* fixes nitrogen, which is easy to measure by well-established methods, and lots of genetic tools can be applied to it to perform DNA manipulation. The strain that I used in this study (Chapter IV) is *Rhodopseduomonas palustris* strain TIE-1, and it is recently isolated from a fresh water mat at Woods Hole, MA (Jiao et al.,

2005). In **chapter IV**, I will report the first study focusing on understanding the role of *kaiC* in *R. palustris*.

Nitrogen fixation

Nitrogen is an essential element for almost all of the organisms on the earth to build biomass and conduct biological activities. However, nitrogen gas in the air, the main source of nitrogen, is relatively inert. Organisms cannot utilize it until it is converted into ammonia, and the conversion from nitrogen to ammonia is known as nitrogen fixation (Wagner, 2011). Cyanobacteria and purple bacteria are among the major organisms that can perform nitrogen fixation. These small organisms express nitrogenases in their cells to catalyze the nitrogen fixation reaction, which initiates nitrogen cycles in the global ecosystem. And then the fixed nitrogen is available to plants and other organisms in the ecosystem as ammonium (Wagner, 2011).

For chronobiology researchers, nitrogen fixation is not only an essential biological activity, but was also the key that opened the door of circadian research in cyanobacteria. For a long time, scientists believed that prokaryotes are unlikely to have circadian clocks because of the short doubling time and lack of nuclear structures (Edmunds, 1983; Kippert, 1987). However, daily rhythms of nitrogen fixation was discovered when researchers studied the nitrogenase activities in cyanobacterium *Synechococcus* RF-1 (Grobbelaar et al., 1986), which soon brought attention from chronobiologists and led to the discovery of the first circadian clock in prokaryotic cyanobacteria.

Because of the important role that nitrogen fixation plays in agriculture and ecosystems, scientists have dedicated a lot of their effort to establish an accurate and convenient method to monitor nitrogen fixation activities. Previously, isotopes were mainly involved in the measurements of nitrogen fixation (Hardy et al., 1968). These measurements, however, are either relatively insensitive or limited by the short half-life of ^{13}N (~10min) (Hardy et al., 1968). After carefully studying the biochemical properties of nitrogenase, scientists found that nitrogenase is actually a versatile reductase (Hardy and Burns, 1968; Hardy and Knight, 1967), and that its activity can be measured by a parallel reaction catalyzed by nitrogenase (Dilworth, 1966; Hardy and Knight, 1967; Hardy et al., 1968). This parallel reaction is the well-known acetylene reduction assay.

The principle of measuring nitrogen fixation activity by the acetylene reduction assay is based on these two reactions catalyzed by nitrogenase:

$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$

C_2H_2 (acetylene) + $2H^+ + 2e^- \rightarrow C_2H_4$ (ethylene)

In the natural environment, bacteria express nitrogenase to fix nitrogen absorbed in the cells. Researchers found that acetylene can also be reduced to ethylene by nitrogenase when acetylene was incubated with the cell extracts of *Clostridium pasteurianum* (Dilworth, 1966), and that these two reactions are analogous, thus suggesting that the activity of nitrogenase can be represented by the reduction rates of acetylene (Dilworth, 1966; Hardy and Knight, 1967; Hardy

et al., 1968). In 1967, Hardy and Knight proposed that the amount of C_2H_2 and C_2H_4 could be quantified by hydrogen flame ionization after gas chromatography (Hardy and Knight, 1967; Hardy et al., 1968), which established the first sensitive assay for nitrogen fixation.

After so many years, the acetylene reduction assay has been tested by numerous studies and is widely applied to scientific research. When nitrogen fixation was tested in cyanobacterium *Synechococcus* RF-1 in 1986, acetylene was added to the headspace of the cell cultures and the reduction rates were quantified by gas-chromatography mass-spectrometry (GC-MS) with a flame ionization detector (Grobbelaar et al., 1986). Circadian rhythms of this cyanobacterium were discovered for the first time. In **Chapter IV**, the same protocol was used to test if the nitrogen fixation of *R. palustris* shows circadian rhythms or not.

As introduced in the first paragraph of this chapter, my thesis work has focused upon exploring the underlying mechanism of the clock-mediated fitness enhancement and investigating the occurrence of clocks or other putative timing mechanisms in prokaryotes. Currently, our knowledge of timing keeping mechanisms in prokaryotes is only limited in cyanobacteria, and little is known about the function of *kai* genes in other bacteria. By studying the function of *kaiC* in *R. palustris* and comparing cyanobacterial timing system with the timing system of purple bacteria, I hope my work can bring some insights and more attention to this field.

Chapter II *

An Evolutionary Fitness Enhancement Conferred by the Circadian System in Cyanobacteria

*This chapter is modified from Ma et al., 2013.

Abstract

Circadian clocks are found in a wide variety of organisms from cyanobacteria to mammals. Many believe that the circadian clock system evolved as an adaption to the daily cycles in light and temperature driven by the rotation of the earth. Studies on the cyanobacterium, *Synechococcus elongatus* PCC 7942, have confirmed that the circadian clock in resonance with environmental cycles confers an adaptive advantage to cyanobacterial strains with different clock properties when grown in competition under light-dark cycles. The results thus far suggest that in a cyclic environment, the cyanobacterial strains whose free running periods are closest to the environmental period are the most fit and the strains lacking a functional circadian clock are at a competitive disadvantage relative to strains with a functional clock. In contrast, the circadian system provides little or no advantage to cyanobacteria grown in competition in constant light. In addition, a recent study further confirmed the adaptive value of circadian clock in cyanobacteria under some-but not the all-envrionmental conditions by studying the non-optimal codon usage of clock genes.

To explain the potential mechanism of this clock-mediated enhancement in fitness in cyanobacteria, several models have been proposed; these include the limiting resource model, the diffusible inhibitor model and the cell-to-cell communication model. None of these models have been excluded by the currently available experimental data and the mechanistic basis of clock-mediated fitness enhancement remains elusive.

Introduction

Circadian clocks are endogenous timing mechanisms that function to regulate a variety of cellular, metabolic and behavioral activities over the course of the day-night cycle. Circadian systems allow organisms to anticipate daily changes in environmental signals such as light and temperature. Regulated by circadian clocks, organisms sustain roughly 24-hour rhythms even in the absence of environmental timing cues, and these clock-driven rhythms sustain stable free-running periods (FRPs) within the physiologically optimal temperature range (Johnson, 2004; Edmunds, 1983).

Circadian clocks have been found in a broad range of organisms from cyanobacteria to mammals. Given their ubiquity, circadian clocks are considered to be an evolutionary adaptation that enhances the fitness of organisms possessing them (Woelfle and Johnson, 2009). For instance, chipmunks with disrupted circadian clocks were more susceptible to predation in the wild than those with intact circadian systems. Ecological observations suggested that the nighttime restlessness of the arrhythmic chipmunks resulted in elevated detection

rates by predators (DeCoursey et al., 2000). Studies of *Drosophila melanogaster* showed that the life span of flies with altered circadian periods was significantly reduced by up to 15% (Klarsfeld and Rouyer, 1998), and that the disruption of the circadian clock also reduced sperm production in males (Beaver et al., 2002). Furthermore, *Arabidopsis* strains lacking a circadian clock showed lower viability, less carbon fixation and slower photosynthesis rates than wild-type strains (Dodd et al., 2005; Green et al., 2002). Moreover, *Arabidopsis* is more resistant to herbivory when plants were entrained in the same phase as the herbivores, indicating that the circadian system in *Arabidopsis* assists in defending against herbivory (Goodspeed et al., 2012).

Although these studies demonstrate that circadian regulation of cellular, metabolic and behavioral events is beneficial, few studies have rigorously tested the adaptive value of circadian clocks in terms of their contribution to fitness and adaptation. Fitness primarily describes reproductive success (Futuyma, 1998), whereas longevity, growth and development are secondary factors affecting the fitness of an organism. An adaptation is an acquired feature as a result of natural selection that enhances the fitness of an organism under certain selective pressures (Futuyma, 1998). An adaptation can only be presumed to be adaptive when it first emerges (Johnson, 2005). In the process of evolution, the adaptation may retain an “extrinsic” value only if the selective pressure remains. Alternatively, the adaptation may acquire an “intrinsic” value by becoming integrated with other processes. In this case, even if the original adaptation persists in the absence of the selective pressures, it is no longer considered to

be an adaptation (Futuyma, 1998). In order to fully test the adaptive value of circadian clocks, two questions must be addressed. Does the presence of a circadian clock (i) enhance the fitness and (ii) if so, is the adaptive value conferred by the circadian clock intrinsic or extrinsic (Woelfle and Johnson, 2009)? To date, most studies have only partially or indirectly addressed these questions. Furthermore, little if any research has addressed the potential mechanisms by which circadian clocks mediate fitness enhancement.

The cyanobacterium, *Synechococcus elongatus* PCC 7942 (*S. elongatus*) is an ideal model system to address these questions for several reasons (Woelfle and Johnson, 2009; Woelfle et al., 2004; Ouyang et al., 1998). First, the central clock mechanism is relatively simple; the core circadian clock in *S. elongatus* is composed of three proteins (KaiA, KaiB, and KaiC) that are encoded by the genes, *kaiA*, *kaiB* and *kaiC* (Ishiura et al., 1998) and a number of clock mutants have been generated (Kondo et al., 1994). Among these mutants are those with short and long periods as well as arrhythmic mutants. These mutant strains allow us to test the adaptive value of the cyanobacterial circadian clock by directly comparing them to the wild-type strain under different growth conditions. Second, *S. elongatus* reproduces asexually by binary fission and therefore growth rates are a direct measurement of fitness (Johnson, 2005). Third, the growth conditions of *S. elongatus* are relatively simple and therefore laboratory conditions can approximate the relevant features of natural conditions. Fourth, *S.elongatus* can grow in either constant light or in light/dark cycles, thus the extrinsic versus intrinsic adaptive value can be determined by artificially

introducing or removing selective pressures (Woelfle and Johnson, 2009). Finally, *S. elongatus* represents one of the most evolutionarily ancient organisms possessing a circadian system; therefore, elucidating the mechanisms of clock-mediated adaptation in this species could help us understand the selective pressures that may have led to the evolution of circadian clocks.

Although there are many advantages of *S. elongatus* for testing the adaptive value of circadian clocks, some limitations are unavoidable. On the one hand, cyanobacteria are the only prokaryotic organisms in which circadian clocks have been conclusively identified, so that whether circadian clocks are prevalent in bacterial and archaea domains is still a question. Therefore, even if its circadian clock confers an adaptive value for cyanobacteria, this is not sufficient to prove the adaptive significance of circadian clocks in other eukaryotic organisms. On the other hand, for organisms that propagate by sexual reproduction, the fitness enhancement by clocks is probably much more complicated, and many other physiological processes may be involved. Studying the clock-mediated fitness enhancement in *S. elongatus* may not be able to provide many insights to these organisms. Regardless of these limitations, *S. elongatus* is still one of the best model organisms that can be used for circadian research and for testing the adaptive significance of circadian clocks, as has already been proved in some pioneer studies (Ouyang et al., 1998; Woelfle et al., 2004).

In this chapter, the work that has been done to test the adaptive value of the circadian clock in *S. elongatus* will be described and the possible mechanisms that might explain how the cyanobacterial circadian system exerts its influence on overall fitness will be discussed.

Testing the Adaptive Value of the Circadian Clock in Cyanobacteria

The adaptive value of circadian clocks in cyanobacteria was tested by using growth in competition between the wild-type *S. elongatus* and several different clock mutant strains (Woelfle and Johnson, 2009; Woelfle et al., 2004; Ouyang et al., 1998). These clock mutants are due to point mutations in the *kaiA*, *kaiB* or *kaiC* genes respectively, resulting in altered clock properties such as arrhythmicity, or rhythmicity that exhibits FRPs that are longer or shorter than the wild-type value of ~24.5 hours (Kondo et al, 1994). In pure cultures, neither these mutant strains nor the wild-type strain have growth rates that are significantly different from each other in constant light (LL) or in light/dark (LD) cycles (Woelfle et al., 2004; Ouyang et al., 1998). This observation does not exclude the possibility that the circadian clock system enhances fitness in cyanobacteria; however the adaptive value may only be detectable under some selective circumstances such as competition. For this reason, competition experiments were designed to assess the reproductive fitness of the wild-type strain (WT) and the clock mutant strains under controlled environmental conditions (**Fig.2.1**) (Woelfle and Johnson, 2009; Woelfle et al., 2004; Ouyang et al., 1998). In these

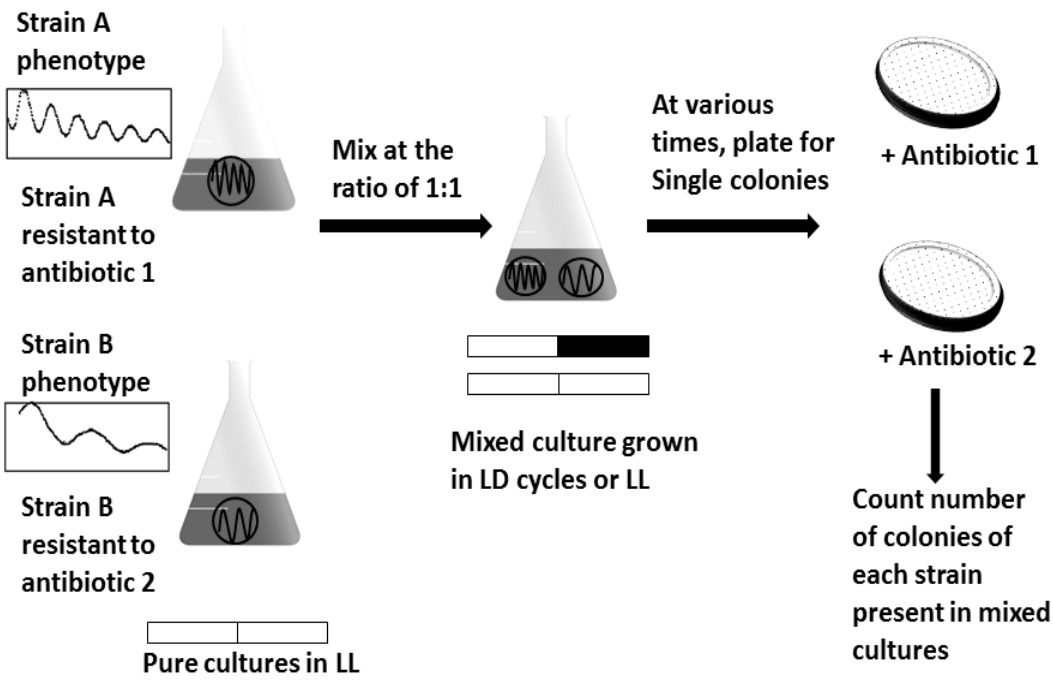


Figure 2.1 Competition experiment between *S.elongatus* strains with different clock phenotypes. The clock phenotypes of two strains, A and B, are shown as luminescence rhythms that report the promoter activity of *psbAI*. Strains A and B are resistant to different antibiotics such that their fractions in mixed cultures can be tracked by plating on selective media. Pure cultures of A and B were set up under LL, and when they reached log phase, equal numbers of A and B cells were mixed and cultured under different LD cycles or LL condition. Aliquots were taken from the mixed cultures every ~8 generations in LD and every ~16 generations in LL, and they were plated on selective media to count the number of colony-forming units (CFU) of each strain. Meanwhile, the mixed culture was diluted into fresh medium and grown for another ~8 generations (LD) and ~16 generations (LL). This process was repeated for 4 cycles to allow cells to grow for 40-50 generations. The fraction of each strain in the mixed culture was calculated by the number of colonies of each strain growing on selective media. Circadian phenotypes were confirmed by monitoring the luminescence rhythms of colonies of each strain at different sampling times. Figure modified from Woelfle and Johnson, 2009.

experiments, two cyanobacterial strains with different clock phenotypes were mixed and grown together in either constant light or in light/dark cycles and the composition of these mixed cultures was assayed over time as a test of reproductive fitness.

For example, to test whether the circadian clock enhances reproductive fitness in cyanobacteria, competition experiments were conducted between the WT strain with a FRP of approximately 24-25 hours and an arrhythmic mutant (CLAb) whose circadian clock was disrupted by a point mutation (G460E) in the *kaiC* gene (**Fig.2.2A**) (Woelfle et al., 2004; Ishiura et al., 1998; Kondo et al., 1994). In LD 12:12 (12 hours of light followed by 12 hours of darkness) cycles, the WT strain quickly (within 20 generations) became the predominant strain in mixed cultures (**Fig.2.2B**). As a control, the point mutation in CLAb was rescued by introducing a wild-type copy of the *kaiC* gene into the genome. When the rescued CLAb strain was grown in competition with the WT strain, the proportions of the WT and mutant strain remained approximately equal in the mixed cultures over many generations indicating that the reduction in fitness of CLAb was due to altered clock properties rather than some other difference in the genetic background of the two strains in competition (Woelfle et al., 2004).

This experiment confirmed that the circadian clock in cyanobacteria confers adaptive value in light/dark cycles, but it does not reveal whether this adaptive value is an intrinsic or extrinsic property of the clock. If the value is an intrinsic property of the clock, one would expect that the WT strain would also

defeat CLAb when grown in mixed cultures in constant conditions as well as when grown together in light/dark cycles. To address this question, the WT and arrhythmic strains were co-cultured and grown in constant light condition removing the presumed selective pressure of the day-night cycles. Surprisingly (at least, to some chronobiologists who favor the intrinsic adaptiveness of circadian timekeeping!), the CLAb strain was not only able to successfully maintain itself in mixed cultures with WT, but the proportion of CLAb significantly increased in these mixed population (p-value=0.01; **Fig.2.2B**). Additionally, competition experiments using the WT strain and a second *kaiC* mutant CLAc (T495A; which expresses a rapidly damped circadian oscillation (Ishiura et al., 1998; Kondo et al., 1994) yielded similar results. The WT strain once again became the predominant strain in mixed cultures in LD cycles, but when grown in constant light both strains maintained approximately equal proportions over many generations. Interestingly, the CLAc strain was able to remain as a small fraction of the mixed-culture even after 30 generations in light/dark cycles, while the fraction of the arhythmic CLAb mutant decreased rapidly within 20 generations (**Fig.2.2B**). Because CLAc is able to oscillate for one or two cycles in constant conditions (**Fig.2.2A**), the discrepancy in the competition kinetics may be due to the difference in their clock phenotypes, supporting the idea that even limited rhythmicity is of benefit to cyanobacteria in LD cycles. Based on these observations, it seems that the adaptive value of the circadian clock is of extrinsic value to *S. elongatus* cells rather than intrinsic, and additionally, the data in

constant light conditions implies that having a functional clock may not always be adaptive under non-selective conditions (e.g., constant light condition).

We wanted to determine if a circadian clock that is resonating with the environmental cycle confers higher fitness than a clock that is functional, but is entrained to the environmental cycle in a non-ideal phase relationship. The reproductive fitness for this scenario was tested by competition experiments between the WT strain and several mutants with altered FRPs (Ouyang et al., 1998). In one set of competition experiments, either a *kaiB* (B22a; R74W) or a *kaiC* mutant (C22a; A87V), both with a FRP of approximately 22 hours (Ishiura et al., 1998; Kondo et al., 1994), was grown in mixed cultures with the WT strain. In a second set of competition experiments, either a *kaiA* (A30a; R249H) or a different *kaiC* mutant (C28a; P236S), both with a FRP of 28-30 hours (Ishiura et al., 1998; Kondo et al., 1994), was grown in mixed culture with the WT strain (**Fig.2.3**). Neither of these mutants in pure cultures shows a significant difference in growth rate as compared to the WT strain either in constant light or in light/dark cycles (Woelfle et al., 2004; Ouyang et al., 1998). When the short period mutants, B22a or C22a, were grown in mixed cultures with the WT strain in LD 11:11 cycles (11 hours of light followed by 11 hours of darkness), the short period mutant (either B22a or C22a) out-competed the WT strain (**Fig.2.3B, top panel**). Similarly, both of the long period mutants, A30a and C28a were able to

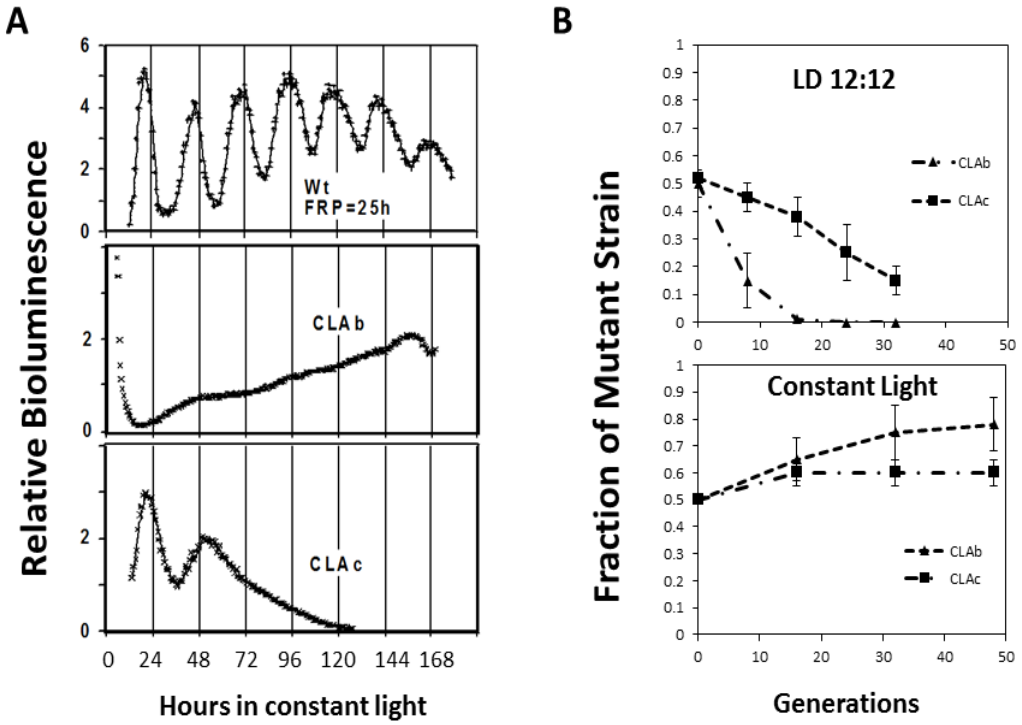


Figure 2.2 Competition of the WT strain with arhythmic strains (Woelfle et al., 2004). A, phenotypes of three strains used in the competition experiments. The WT strain (top) shows circadian rhythms with a ~25 h FRP. CLAb (middle), a clock-disrupted *kaiC* mutant, is arhythmic. Another *kaiC* mutant, CLAc (bottom), is also ultimately arhythmic but initially shows a rapidly damped oscillation. B, competitions between the WT strain and arhythmic mutants under LD 12:12 (upper) or LL conditions (lower). Data are plotted as the fraction of the mutant strain in mixed cultures (ordinate) versus the estimated number of generations (abscissa). Figure modified from Woelfle et al., 2004 and Woelfle and Johnson, 2009.

defeat the WT strain when these strains were co-cultured in LD 15:15 cycles (15 hours of light followed by 15 hours of darkness) (**Fig. 2.3B, bottom panel**). Conversely, the WT strain was the predominant strain in mixed cultures with short period mutants when grown in LD15:15 (**Fig.2.3B, bottom panel**) or when grown in mixed cultures with long period mutants in LD11:11 cycles (**Fig. 2.3B, upper panel**). Our analyses suggested that all of these mutants entrained to the LD cycles, but they entrained with different phase relationships relative to WT that were based on the difference between their FRP and the period of the LD cycle (Ouyang et al., 1998). It appears to be clear from these results that the cyanobacterial strain whose circadian clock was optimally entrained to the environmental cycle was more fit than the strains whose clock was entrained to the LD cycles in non-ideal phase relationships.

Furthermore, this fitness advantage is not dependent on which clock gene is mutated, i.e., *kaiA* vs. *kaiB* vs. *kaiC* (Woelfle et al., 2004). This result indicates that the difference in reproductive fitness is due to the clock phenotype itself, rather than to a mutation in a particular clock gene. One of the most persuasive features of these competition results is that mutants are able to out-compete WT when the period of the LD cycle dovetailed better with the mutants' FRP than with WT's FRP. When the period mutant were competed against the WT strain in constant light, the proportions of the WT and clock mutant in the mixed culture remained relatively constant, providing additional evidence that the adaptive value of the circadian clock is extrinsic rather than intrinsic. In many other cases

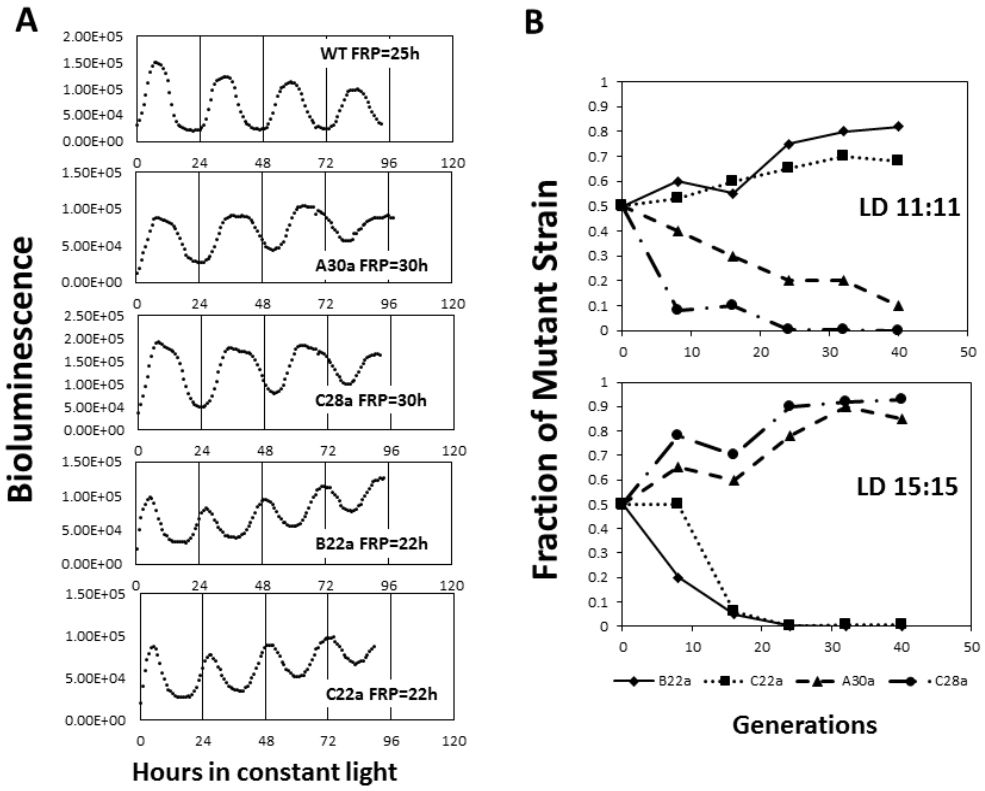


Figure 2.3 Competition of the WT strain with period-altered mutants under LD 11:11 and LD 15:15 cycles (Ouyang et al., 1998). A, circadian phenotypes of the WT strain and period-altered mutants used in these competition experiments. The short period mutants (FRP ~ 22 h) include the *kaiB* mutant B22a and the *kaiC* mutant C22a, and the long period mutants (FRP ~ 30 h) include the *kaiA* mutant A30a and the *kaiC* mutant C28a. All strains have a luciferase construct that reports the clock-regulated promoter activity of the *psbA1* gene by time-dependent luminescence intensity. B, competitions between the WT strain and the period-altered mutants under LD 11:11 cycles (upper) or LD 15:15 cycles (lower). Data are plotted as the fraction of the mutant strain in the mixed culture versus the estimated number of generations. Symbols for each strain are identified under the abscissa. Figure modified from Ouyang et al., 1998.

of tests of adaptive significance, mutant strains are uniformly out-competed by WT strains. But these studies of the cyanobacterial clock provide an example where mutants can out-compete the WT strain if their particular properties (e.g., FRP) resonant better than WT's with an imposed environmental condition (e.g., the period of the environmental light/dark cycle).

Taken together, the competition experiments between cyanobacteria with a normally functioning circadian clock and strains carrying mutations in clock genes have demonstrated (i) that the circadian clock enhances the reproductive fitness of cyanobacteria in cyclic environments but not in non-cyclic environments, and (ii) that this enhancement is the greatest when the period of the internal clock closely matches the period of the external cycle so that an ideal phase angle is achieved under entrainment (Woelfle et al., 2004; Ouyang et al., 1998).

Testing the adaptive value of cyanobacterial circadian clock in continuous cultures and on solid medium

The competition experiments have clearly demonstrated the adaptive value of circadian clocks in liquid batch cultures. Cyanobacteria, however, grow under many other conditions in its natural environments, e.g., the solid surfaces in soil, or in constantly mixed environments such as rivers. To test if circadian clocks enhance fitness under these conditions, competition experiments were performed on solid agar medium as well as in continuous cultures. As shown in

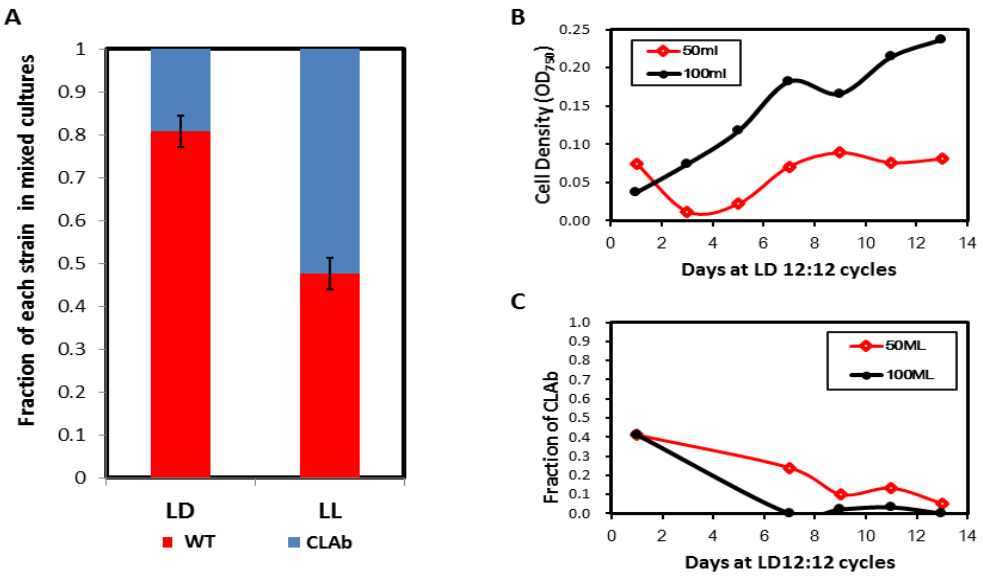


Figure 2.4 Competition experiments on solid agar medium and in continuous cultures. A, the competition experiment between the WT and ClAb on BG-11 agar plates. The WT cells was mixed with CLAb cells in the ratio of 1:1 and then plated on BG-11 agar plates. Cultures were incubated under LD 12:12 cycles or LL conditions. The fraction of each strain was quantified on 5th day. B, cell densities monitored in the continuous cultures. Red, 50ml cultures; Black, 100ml cultures. C, the fraction of CLAb in continuous co-cultures with the WT strain.

Fig.2.4A, after the WT was co-cultured with CLAb on agar medium for 5 days, the WT dominated the cultures under LD condition but not under LL condition, which is consistent with the results in batch cultures. Similarly, in continuous cultures where cell densities were relatively low and stable (**Fig. 2.4B**), CLAb was always out-competed by the WT under LD 12:12 cycles, regardless of the volume of the cultures (**Fig. 2.4C**). Altogether, these results suggest that the clock-mediated fitness enhancement can be extended to other growth conditions and that it is not limited to batch cultures.

Some cell physiological properties under the competition conditions

From the competition experiments we conclude that strains with a functioning clock out-compete strains without a functioning clock or with non-ideally entrained clocks in cyclic environments. Nevertheless, some details are missing. For instance, the decreasing proportion of the “loser” in mixed cultures could be due to much slower growth rates than the “winner,” or it can be the consequence of cell death. Understanding these details would help us uncover the mechanism of the competition. To address the question if the “loser” is still growing, a Yellow Fluorescence Protein (YFP) reporter (Chabot et al., 2007) was transformed into the WT such that two strains in mixed cultures can be distinguished by fluorescent microscopy and flow cytometry. The YFP strain shows yellow fluorescence upon excitation, while strains without YFP display red

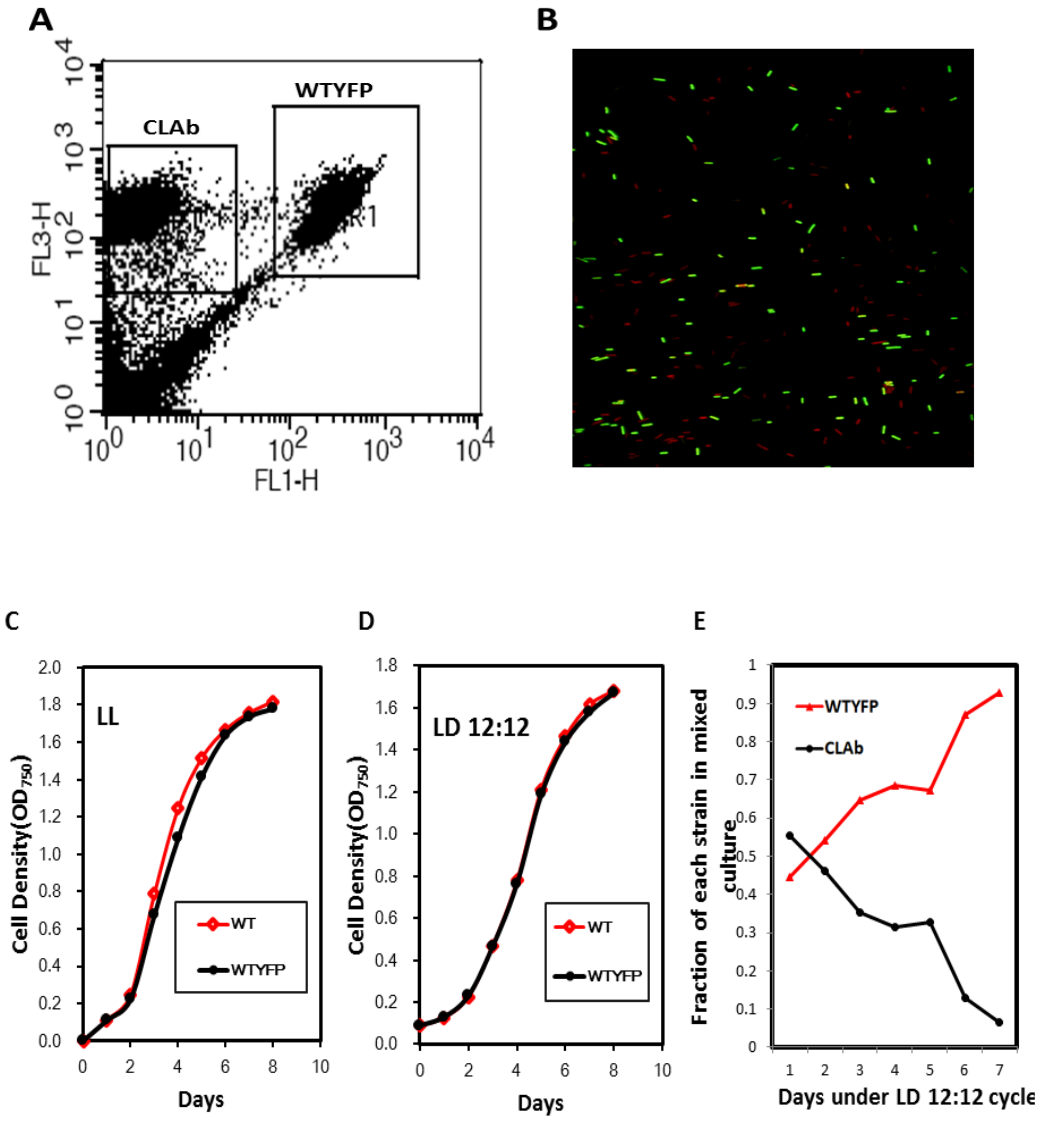


Figure 2.5 Competition experiments between the WTYFP strain and CLAb. A, flow cytometry plot used to quantify the ratio of WTYFP and CLAb in mixed cultures. The x-axis represents the fluorescence intensity around 650nm (red fluorescence), and the y-axis represents the fluorescence intensity around 540 nm (yellow fluorescence). Due to different fluorescence spectra, WTYFP and CLAb cells were located at different regions on the graph. By count the cell numbers through flow cytometer, the ratio of WTYFP and CLAb can be quantified in mixed cultures. B, image taken by a fluorescent microscope. WTFYP cells show yellow fluorescence due to the expression of *yfp*, and CLAb cells show red fluorescence due to chlorophyll. C, growth curves of the WT and WTYFP strains under LL conditions. D, growth curves of the WT and WTYFP strains under LD 12:12 cycles. E, fraction of the WTYFP (red) and CLAb (black) in co-cultures under LD 12:12 cycles quantified by flow cytometry.

fluorescence coming from the chlorophyll. These different fluorescence spectra can be separated by flow cytometry, which is used for cell counting, as shown in **Fig. 2.5A**. The cell morphology was measured and visualized by using fluorescent microscopy, as shown in **Fig. 2.5B**. No growth defect was observed in the YFP strain (**Fig. 2.5C&D**), and the WTYFP out-competed CLAb under LD 12:12 cycles (**Fig. 2.5E**), suggesting that the YFP reporter has no effect on the fitness of the WT. The WTYFP was then co-cultured with CLAb under LD 12:12 condition, and the growth rates of each strain in the mixed cultures were measured. As shown in **Fig. 2.6A**, CLAb kept growing in the first 5 days, but it grew significantly slower than the WT. The overall growth of the mixed cultures showed no significant difference from the pure cultures (**Fig. 2.6B**), suggesting that only CLAb was impaired by the competition.

Following the growth experiments, cell lengths of each strain and cell division rates were examined in pure and mixed cultures (**Fig. 2.6C&D**). Interestingly, in mixed cultures, the lengths of CLAb cells decreased during the process of culturing, and they are significantly shorter than cells in pure cultures after the 5th day. Consistently, cell division rates of CLAb were also reduced in mixed cultures, whereas in pure cultures both WTYFP and CLAb showed increasing division rates on the 3rd day (the exponential phase). Overall, the cell length and division rates of WTYFP were not influenced by co-culturing under LD 12:12 condition, whereas CLAb was weakened by co-culturing. Taken together, these results indicate that some of the physiological properties of CLAb

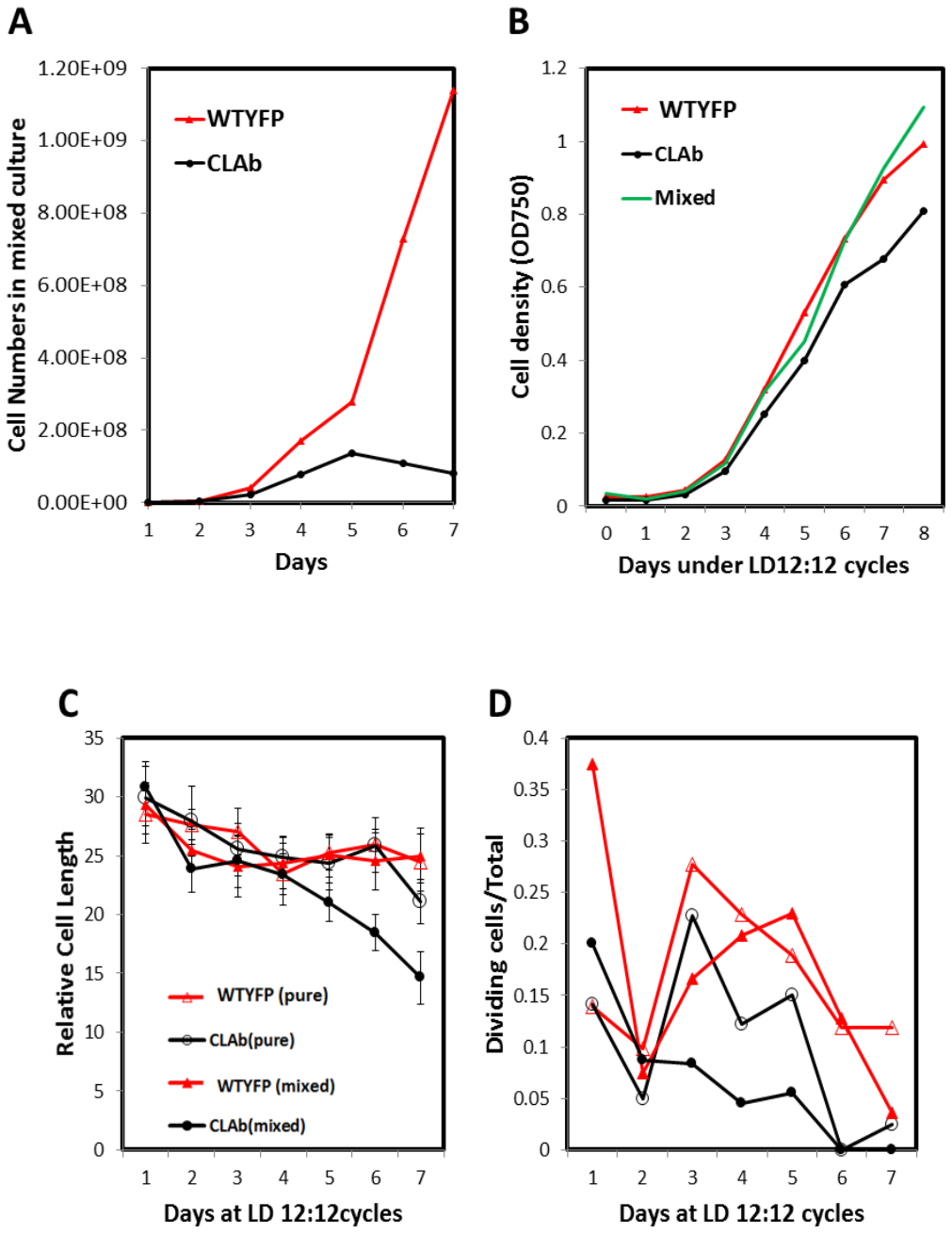


Figure 2.6 Dynamics of the competition experiments between WTYFP and CLAb. A, growth curves of WTYFP (red) and CLAb (black) in co-cultures under LD 12:12 cycles. B, growth curves of pure cultures and mixed-cultures under LD 12:12 cycles. Red, pure cultures of WTYFP; black, pure cultures of CLAb; green, co-cultures of WTYFP and CLAb. C, cell length of each strain in pure cultures or mixed-cultures. D, cell division events of each strain in pure cultures or mixed-cultures.

were adversely affected by co-culturing with the WT. However, no clear evidence showed that CLAb cells were undergoing cell death. Although with a significantly slower growth rate, these “losers” could still grow, indicating that the competition may be mediated by some growth inhibitors or limiting nutrients.

Another example: cyanobacterial circadian clock enhances fitness by non-optimal codon usage

The competition experiments have clearly demonstrated that the circadian clock enhances fitness of cyanobacteria in cyclic environments. Recently, another study confirmed the adaptive value of cyanobacterial circadian clock from a different perspective by studying the non-optimal codon usage of *kaiBC* genes (Xu et al., 2013). The circadian system regulates nearly all expression of the cyanobacterial genome (Liu et al, 1995; Ito et al., 2009), indicating the importance of *kai* genes in these organisms. A general observation from many organisms is that genes with high expression levels and functional importance are usually encoded by optimized codons (Ikemura, 1981) that have higher usage frequencies than other synonymous codons. Thus we might predict that *kai* genes should be encoded by optimized codons. An examination of the codon usage of *kaiBC* genes, however, revealed that the codon usage of *kaiBC* genes is not as translationally efficient as genes with high functional importance, e.g., ribosomal genes (Xu et al., 2013). To understand why *kaiBC* is not encoded by optimized codons, cyanobacterial strains expressing optimized codon *kaiBC* (OptkaiBC) were generated by Dr. Yao Xu in the Johnson lab. As predicted, the

expression levels of *kaiB* and *kaiC* genes are increased in OptkaiBC due to the optimization of codon usage. Interestingly, OptkaiBC showed no difference from the WT in its circadian rhythms at warm temperatures, while it displayed robust circadian rhythms at low temperatures where the wild-type strain tends to be arrhythmic or highly damped (Xu et al., 2013).

This result is quite surprising since we expected that better rhythms provide better fitness under rhythmic conditions, based on the conclusion of the previous competition experiments. From this line of reasoning, natural selection should prefer the OptkaiBC strain rather than the non-optimized codon strain. To address this question, I measured the growth rates of the WT, OptkaiBC and two arrhythmic strains (CLAb and CLAc) at different constant temperatures within the physiological range of temperatures (18 °C to 37°C) for this cyanobacterial species under LD 12:12 cycles. As shown in **Fig.2.7** and **Table2. 1**, at warm temperatures where both the WT and OptkaiBC show robust circadian rhythms, no significant difference was observed among their growth rates, including the two arrhythmic strains. However, as the temperature is reduced to 20 °C and 18 °C, OptkaiBC grew significantly slower than the other strains. Moreover, the two arrhythmic strains grew even slightly better than the WT (**Fig.2.7** and **Table 2.1**). From **Fig.2.8** we can see that the warm temperatures around 30 °C allow our cyanobacterial strains, both the WT and OptkaiBC, to grow at the fastest rates, while at cooler temperatures the growth rates were significantly reduced (**Fig. 2.8C**). Therefore we consider ~ 30 °C to be the optimal growth

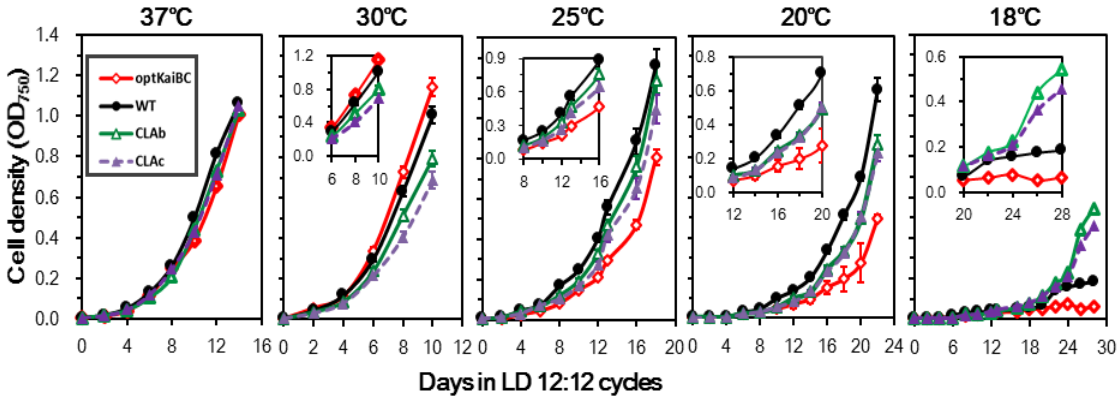


Figure 2.7 Growth curves of cyanobacterial strains at different temperatures. WT, optKaiBC, CLAb (arrhythmic) and CLAc (damped oscillation) strains were grown in LD 12:12 cycles at 37°C, 30°C, 25°C, 20°C, or 18°C with constant air bubbling and shaking. Cell densities were monitored by measuring OD750 every two days. Data are averages ± SEM from 2 to 6 independent experiments for each strain and condition. For a better comparison at 18°C, 20°C, and 25°C, the insets are a magnified portion for the specified times.

Strain	Doubling time (mean ± SEM; h) in LD at temperature							
	Time range at 37°C	Time range at 30°C	Time range at 25°C		Time range at 20°C		Time range at 18°C	
	0~14 days	0 ~ 12 days	0 ~ 20 days	8 ~ 16 days	0 ~ 24 days	10 ~ 20 days	0 ~ 28 days	20 ~ 28days
WT	60.00 ± 0.24	37.06 ± 1.00	57.31 ± 1.89	26.73 ± 5.32	63.84 ± 1.14	27.71 ± 1.48	131.32 ± 8.68	164.88 ± 9.15
optKaiBC	61.80 ± 1.08	36.41 ± 0.71	60.38 ± 1.23	38.24 ± 4.74*	79.04 ± 3.60**	96.76 ± 20.55**	226.32 ± 3.12	297.60 ± 3.63
CLAb	57.48 ± 0.12	38.22 ± 1.19	57.33 ± 1.23	27.40 ± 2.13	66.96 ± 1.59	39.68 ± 3.06**	90.96 ± 2.16	82.28 ± 2.12
CLAc	59.76 ± 2.64	39.20 ± 1.07	59.32 ± 2.03	33.70 ± 4.77	68.27 ± 2.32	43.80 ± 7.85*	98.64 ± 3.61	92.64 ± 4.30

Table 2.1 Doubling time of WT, optkaiBC, CLAb and CLAc strains at different temperatures under LD 12:12 cycles.

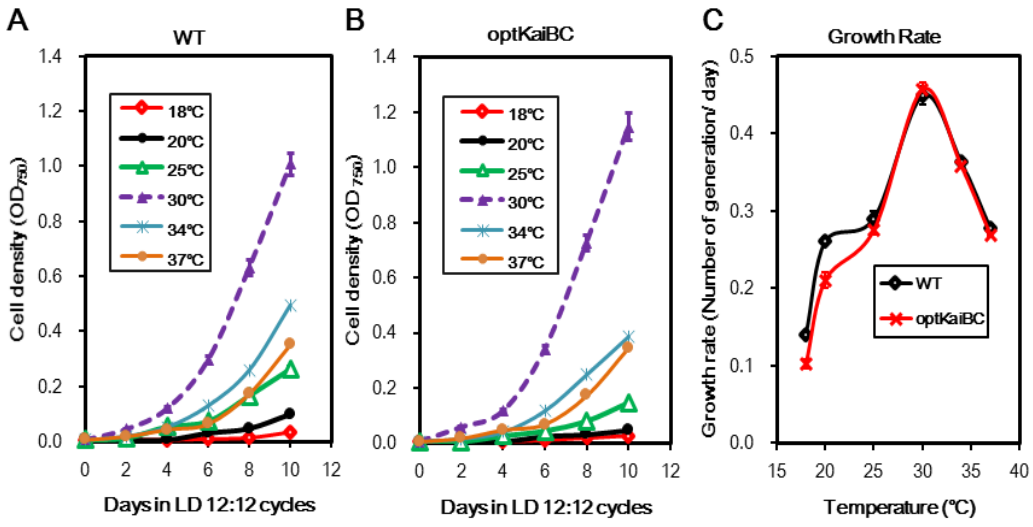


Figure 2.8 Growth of the WT and OptkaiBC strains at different temperatures under LD12:12 cycles. A, growth curves of WT at 37°C, 30°C, 25°C, 20°C, or 18°C . B, growth curves of OptkaiBC at 37°C, 30°C, 25°C, 20°C, or 18°C . C, growth rates of the WT (black) and OptkaiBC (red) strains was plotted as the function of temperatures.

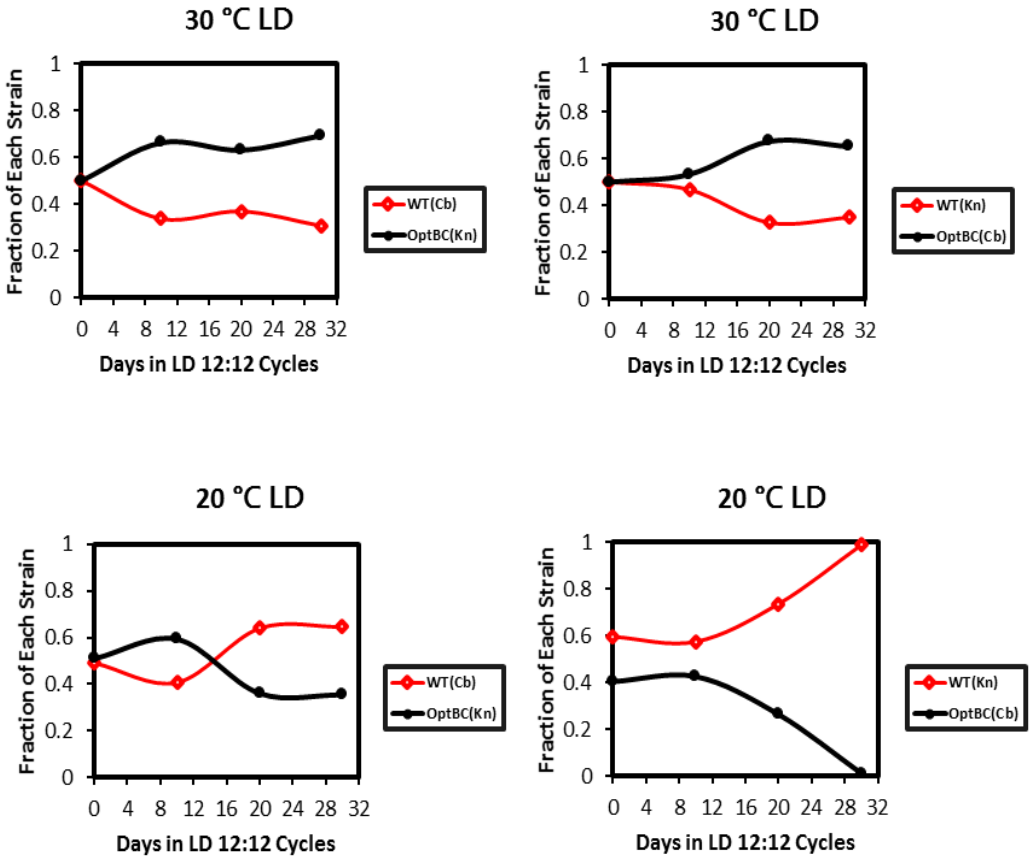


Figure 2.9 Competition experiments between the WT and OptkaiBC strains at 30 ºC or 20 ºC under LD 12:12 cycles. The WT strains either with Cb resistance (left) or Kn resistance (right) was competed against the OptkaiBC strains with the opposite resistance at 30 ºC (upper panels) or 20 ºC (lower panels).

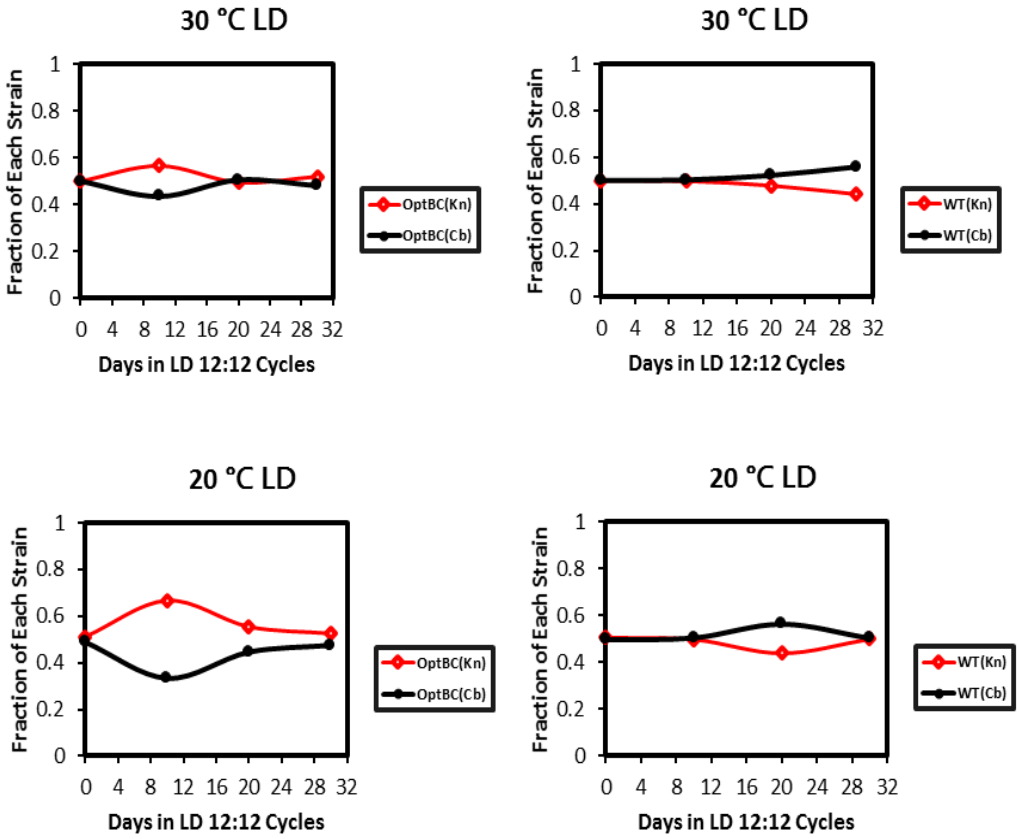


Figure 2.10 Same strains with different antibiotic resistances do not compete with each other.

temperatures for this particular species. These results indicate that a robust circadian rhythm at cool temperatures actually impacts the fitness, and that circadian rhythms provide an advantage only within a range of growth temperatures.

Although the OptkaiBC strain showed reduced growth rates in pure cultures at cool temperatures, perhaps the robust rhythms that it confers might be an advantage under competition conditions. To test this hypothesis, competition experiments between the WT and OptkaiBC strains were conducted at 30 ^{o}C and 20 ^{o}C under LD 12:12 cycles. As shown in **Fig.2.9**, the results were consistent with the observations of pure cultures (**Fig.2.7**): the OptkaiBC displayed a slight advantage over the WT at 30 ^{o}C (**Fig. 2.9 A&B**), while it was out-competed by the WT at 20 ^{o}C (**Fig. 2.9C&D**). To exclude the possibility that the competition results were compromised by the different antibiotic resistance genes incorporated in the genome of each strain, two sets of competition experiments, WT(Cb) vs. OptkaiBC(Kn) and WT(Kn) vs. OptkaiBC(Cb), were conducted, as shown in **Fig. 2.9**. No significant difference was observed between these two experiments. Furthermore, when strains with the same genotype but different antibiotic resistances {i.e., WT(Cb) vs. WT(Kn) and OptkaiBC (Kn) vs. OptkaiBC (Cb)} were competed against each other (**Fig. 2.10**), no competition was detected, thus indicating the competition resulted from the *kai* genotypes and not the antibiotic markers.

Results from this study suggest that having a robust circadian rhythm is not always an advantage. Under cold temperatures, cyanobacteria may face more challenges from the environment and simply surviving these environmental stresses might be a priority. Therefore, it is possible that running a robust timing mechanism is burdensome. By adapting the non-optimal codons to *kaiBC* genes, the circadian clock obtained the flexibility to guarantee the best fitness at different temperatures, suggesting another way whereby circadian clock enhances fitness, namely “conditionality” (Njus et al., 1977).

Potential Mechanisms of Clock-mediated Fitness Enhancement

While competition experiments have clearly demonstrated a clock-mediated fitness enhancement in cyanobacteria, the cellular mechanism remains unknown. Cyanobacterial strains with different clock properties all showed a similar growth rate when cultured alone; however, the reproductive fitness was negatively affected in mixed cultures in a way that is dependent on the light/dark cycles (Woelfle et al., 2004; Ouyang et al., 1998). To explain these observations, three models have been proposed: the “limiting resource model,” the “diffusible inhibitor model” and the “cell-to-cell communication model” (Woelfle and Johnson, 2009).

The Limiting Resource Model

The limiting resource model proposes that the circadian system enables individual cyanobacterial cells to maximally utilize some limiting environmental

resource by phasing their metabolism to the environmental cycle (Woelfle and Johnson, 2009). For instance, transcription of genes that encode components of the photosynthetic machinery in cyanobacteria is up-regulated during the day and down-regulated at night (Tomita et al., 2005), and this rhythmic gene expression may facilitate cyanobacterial cells to perform photosynthesis more efficiently and to consume less energy at night by limiting unnecessary transcription and translation. In contrast, cyanobacterial cells without a functioning circadian clock or with a non-optimally entrained clock may be less efficient metabolically, thereby having an inherent disadvantage when competing for a limited resource with cells that have a clock that is favorably entrained to the environmental cycle.

Our published results that the growth rates of the various pure cultures (WT and mutants) were experimentally indistinguishable led us to believe that the Limiting Resource Model was incorrect (Woelfle et al., 2004; Ouyang et al., 1998), but a more recent modeling paper from Hellweger has forced us to re-evaluate the experimental evidence for and against this model (Hellweger, 2010) (see below).

To show that the limiting resource model could be hypothetically true, Hellweger's mathematical modeling of the competition experiments attributed small differences in the growth rates between the cyanobacterial strains as a key determining factor in predicting the outcome of the competition (Hellweger, 2010). This model simulated the growth of the WT strain and period-altered

mutants in both pure and mixed cultures and was able to successfully reproduce the experimental outcomes (Hellweger, 2010). After examining parameters in the simulation, it was found that a circadian clock will have a higher amplitude when its FRP matches the period of the LD cycle, and this higher amplitude leads to greater expression of photosynthesis genes and ultimately to higher growth rates. The difference in predicted gene expression and growth rates was therefore suggested by this model to be the mechanism of the clock-mediated fitness enhancement. Although our experimental measurements of overall growth rates had detected no differences in pure cultures of the strains used in competition (Woelfle et al., 2004; Ouyang et al., 1998), Hellweger's model suggested that small differences may exist between the strains which are difficult to detect in the batch cultures used for the competition experiments; however, these hypothetically small differences in growth rates between the strains might be detectable when cyanobacteria are cultured using chemostats (Hellweger, 2010). Using a chemostat culturing method, cells can be grown in a physiological steady state where they grow at a constant rate, and all culture parameters remain constant (Harder and Kuenen, 1977). Previously, competition experiments between the WT strain and the long period mutant, C28a, conducted in chemostats produced results that are quite similar to the competition results using the batch culture method (Ouyang et al., 1998), but a re-evaluation of this approach was warranted based upon the modeling study of Hellweger (Hellweger, 2010).

Another way to reconcile the Limiting Resource Model with our experimental data is to consider the impact of transfers/dilutions in the competition assay. In our previous studies, we did not detect any significant differences in the growth rates between strains with different circadian phenotypes during exponential growth (Woelfle et al., 2004; Ouyang et al., 1998). We therefore considered the possibility that there could be small differences in the latency of these strains to start growing at the time of each transfer to new culture medium. If so, the initial growth rates might be different among the strains and these differences might accumulate over successive transfers. Therefore, to test the Limiting Resource Model experimentally, we focused on testing a hypothesis that small differences in initial growth rates may be cumulative over repeated dilutions of cell cultures and therefore are responsible for the result of the competition studies (Dr. Tetsuya Mori, personal communication). By analogy to a race, it is as if one runner gets started “off the blocks” from the starting line a little earlier than the other runners. If one strain has the innate ability to adapt to the new medium more quickly than the other such that it enters the exponential phase of growth in a shorter period of time after each transfer to new medium, small differences in this period of adaptation between strains with different circadian phenotypes could give rise to large differences in the composition of the mixed cultures after several generations (Woelfle and Johnson, 2009).

To test this hypothesis, we examined whether there were small differences in initial growth rates over several serial dilutions/transfers of pure cultures of the WT strain and of the arrhythmic mutant, CLAb. In previous

studies, the WT strain and clock mutants did not show any significant difference in their overall growth rates in pure cultures (Woelfle et al., 2004; Ouyang et al., 1998), however, the difference might have been too subtle to be observed with the sampling techniques that were used. If there were small differences in the ability of cells to adapt to new medium, we would expect that this difference could be enhanced by a series of dilutions to new medium over many generations. To experimentally test this version of the Limiting Resource Model, stationary phase cultures of the WT strain and CLAb cells were diluted one thousand fold every 8 days, and the growth in constant light or in a light/dark cycle of each of the two strains was monitored by measuring the optical density. As shown in **Fig.2.11**, during a series of four dilutions, no obvious differences were observed by eye in the growth curves of the WT and arrhythmic strains in pure cultures in either constant light or in LD 12:12 cycles. However, some small differences were detected when the initial growth rates were calculated (**Table 2.2**; the initial growth rates were calculated as the doubling time in the first 24 hours after dilution). In constant light, even though the initial doubling time of CLAb was significantly greater (i.e., slower) than that of the WT strain after the second dilution, no significant difference was observed after the third dilution, and after the fourth dilution, the direction of the difference was the opposite to that in the

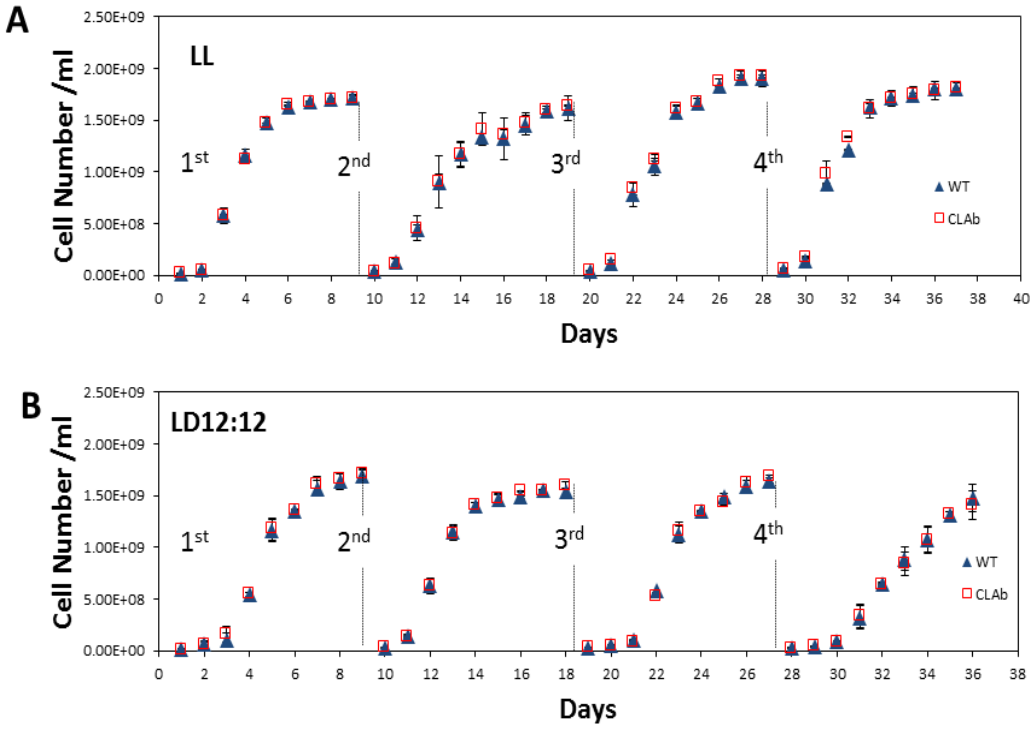


Figure 2.11 Growth curves of the WT strain and the clock mutant (CLAb) in pure cultures that were serially diluted four times. A, pure cultures of the WT strain (blue diamond) and CLAb (red squares) were set up under LL conditions. B, pure cultures of the WT strain and CLAb were set up under LD conditions. After the cells reached the stationary phase, they were diluted 1:1000 into fresh BG-11 medium. When the diluted cultures reached the stationary phase, they were diluted again. The cultures were serially diluted four times. Cell density was measured as OD_{750} value.

Initial doubling time (DT) in constant light conditions				
Dilutions	1st	2nd	3rd	4th
WT DT (h)	12.61 (1.24)*	13.89 (0.28)*	13.74 (2.07)*	19.05 (2.06)*
CLAb DT (h)	14.4 0(1.77)*	16.38 (0.25)*	13.90 (0.18)*	15.72 (0.12)*
p-value**	0.15	0.01***	0.88	0.02***
Initial doubling time (DT) in LD 12:12 cycles				
Dilutions	1st	2nd	3rd	4th
WT DT (h)	15.19 (1.12)*	9.35 (1.14)*	25.22 (4.95)*	27.45 (6.49)*
CLAb DT (h)	10.85 (1.58)*	10.54 (0.33)*	38.43 (1.86)*	33.06 (3.64)*
p-value**	0.01***	0.09	0.01***	0.18

* Numbers in ( ) represent standard deviation. n=4
** T-test, n=4
*** P<0.05, significantly different

Table 2.2 Initial doubling time of the WT and CLAb under LL conditions or LD 12:12 cycles.

second dilution. Therefore, the end result was that there is no difference in the initial growth rates between the WT and CLAb strains. In LD 12:12 cycles, the CLAb strain grew significantly faster than the WT strain in the lag phase after the first transfer to fresh medium, whereas in the subsequent dilutions, CLAb showed slower initial growth rates than the WT strain (and the difference was not significant after the fourth dilution).

Although these results indicate that the WT strain and the CLAb strain may have different abilities to adapt to new medium, the different initial growth rates in pure cultures were not amplified in a straightforward way by subsequent dilutions (**Fig.2.11**). Therefore, whether the outcome of these competition experiments is caused by small differences in the initial growth rates between strains remains unclear, and indicates that a better experimental design is needed to more rigorously test this model. Previous work utilizing chemostats to culture cyanbacterial cells in competition yielded results that were substantially the same as those obtained when competition is conducted in batch cultures (Ouyang et al., 1998). This culture method may allow us to more accurately determine whether there are differences between cyanobacterial strains in their ability to adapt to the introduction of new medium.

The Limiting Resource Model is supported by mathematical modeling that predicts there are differences in the physiological states between WT and clock mutants when grown in pure cultures versus mixed cultures. However, the clock mutants used in the competition experiments have not yet been found to differ

significantly from the WT strain in physiological properties that have been measured experimentally (Woelfle and Johnson, 2009). Further research is needed to address these questions.

The Diffusible Inhibitor Model

The Diffusible Inhibitor Model proposes that cyanobacterial cells rhythmically secrete a diffusible molecule that acts to inhibit the growth of other cells (i.e., cells of the same strain/species or cells of another strain/species) in the same environment (Woelfle and Johnson, 2009). If cyanobacteria are sensitive to their own inhibitory molecule, it would be advantageous for them to possess some mechanism to be insensitive to or to inactivate this inhibitor. For example, cyanobacterial cells could avoid inhibition by regulating both the timing of secretion of the inhibitor and their own sensitivity to the inhibitor. This model has two underlying assumptions: 1) the secretion of the inhibitor is light-dependent and is therefore limited to the light phase and the subjective day in constant conditions (clock and light dependent); 2) cyanobacterial cells are only sensitive to the inhibitor during the dark phase and in the subjective night (clock dependent) as depicted in **Fig. 2.12A** (Woelfle and Johnson, 2009). This hypothetical phenomenon could allow cyanobacterial cells that are entrained with the best phase angle to produce an inhibitor that retards the growth of competitors (inter- or intra-species competitors) without inhibiting themselves. The subjective day of the cyanobacterial strain whose clock is entrained to a cycling environment in the most appropriate phase angle will coincide with the

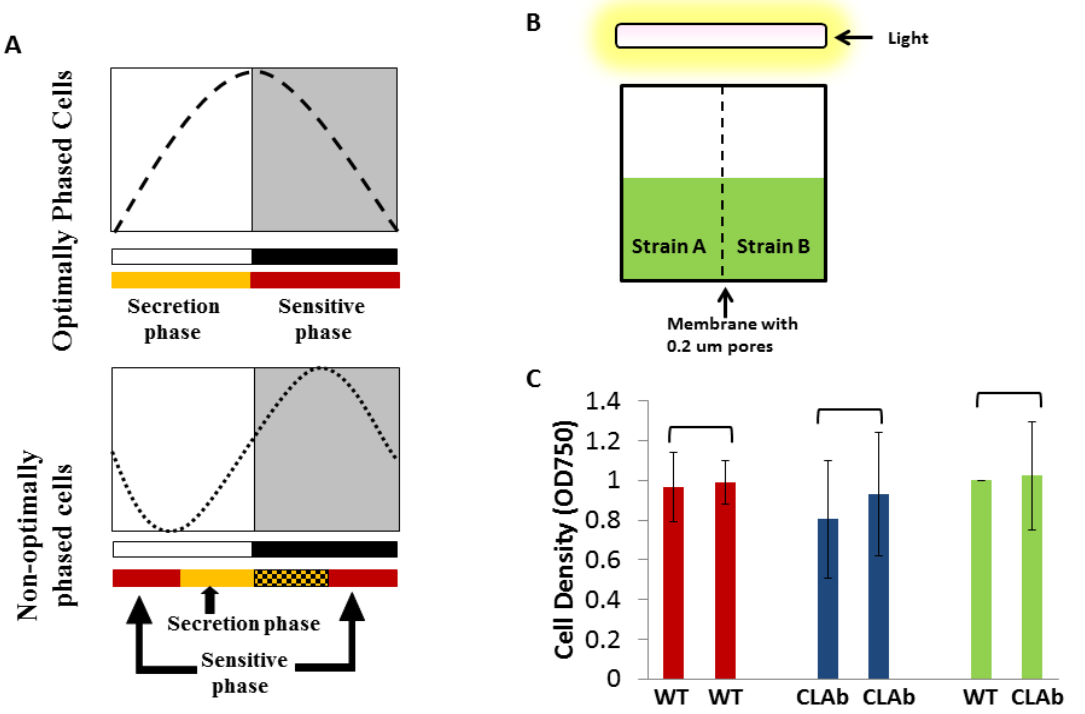


Figure 2.12 Test of the Diffusible Inhibitor Model. A, Depiction of the Diffusible Inhibitor Model. Entrained phases of two strains are modeled in terms of their entrained phase relationship to an LD 12:12 cycle. For the optimally-phased cells, the subjective day overlaps with the daytime (white box), and the subjective night phase overlaps with the nighttime (black box). Therefore, their secretion phase (yellow box) coincides with the daytime, and the sensitive phase (red box) coincides with the nighttime. For non-optimally-phased cells, their subjective day starts from middle of the daytime, and their subjective night starts from the middle of the night and ends in the middle of the daytime. Therefore, the secretion phase (yellow box) of the non-optimal-phased cells is only from the middle of the day to the end of the day, while the secretion-competent phase that overlaps with the dark phase (yellow-black box) does not result in secretion because the secretion is postulated to be light-dependent. The sensitive phase (red box) of the non-optimally-phased cells starts in the middle of the night and ends in the middle of the day. B, the semi-co-culture apparatus used to test the existence of a diffusible inhibitor. Two chambers (left and right) were separated by a membrane with 0.2 μm pores. Cells of different strains could be cultured separately in these two chambers, but their media passes freely through this membrane such that the putative inhibitor could diffuse to the other side. The cultures were illuminated by white fluorescent light from the top, and the light intensity was 50 $uE*m^{-2}*s^{-1}$. C, the WT strain and CLAb were semi-co-cultured in this apparatus under LD 12:12 cycles. Cell densities (OD_{750}) were measured on the fifth day. Bars which share the same color represent cultures in the same apparatus. Panel A modified from Woelfle and Johnson, 2009.

light phase,but strains that are entrained to a non-optimal phase relationship would have their subjective day in a different temporal regime (e.g., one hypothetical non-optimal phase relationship could be the subjective day starting in the middle of the light phase and ending in the middle of dark phase). In this scenario, the secretion of the inhibitor from these poorly entrained cells occurs during only part of the light phase and the secretion phase that overlaps with the dark phase does not result in active secretion (because the secretion is light-dependent, see underlying assumption # 1 above). However, the sensitive phase spans part of the subjective night starting in the middle of the night and ending during the middle of the day (**Fig.2.12A**) (Woelfle and Johnson, 2009).

Using this line of reasoning, in a mixed culture of an optimally entrained strain with a non-optimally entrained strain, one would expect the growth of the poorly entrained cells to be inhibited because their sensitive phase overlaps with the secretion phase of the optimally entrained strain. Therefore, the growth of the poorly entrained cells would be inhibited by the secretions from the optimally entrained cells (but not vice versa). Similarly, if an arrhythmic strain is grown in mixed culture with the WT cells in a LD 12:12 cycle, arrhythmic cells might be sensitive to the inhibitor all the time due to the lack of a functioning clock. There have been reports suggesting that cyanobacteria secrete secondary metabolites that are toxic to other species (Gleason and Paulson, 1984; Zaccaro et al., 2006) (and even to themselves). For example, a secreted secondary metabolite of the cyanobacterium *Scytonema hofmanni*, cyanobacterin, inhibits the growth of several other cyanobacterial species (Gleason and Paulson, 1984). A proteomic

study demonstrates that some proteins in *S. elongatus* are secreted into medium, but the function of these secreted proteins remains unknown (Koksharova et al., 2005). Therefore, it is possible that the competition between the WT and clock mutants could be mediated by secreted metabolites or proteins.

In contrast to Hellweger's mathematical modeling that best supports the Limiting Resource Model, an earlier modeling of the competition experiments provided support for the Diffusible Inhibitor Model (Roussel et al., 2000; Gonze et al., 2002). By postulating the existence of a diffusible inhibitor, Roussel et al. successfully reproduced all the experimental observations, whereas the theoretical model based on differences in resource exploitation did not lead to a satisfactory result (Roussel et al., 2000). Gonze et al. proposed and tested a more sophisticated model that supported the Diffusible Inhibitor Model and predicted that the outcome of the competition depends on the initial proportions of cells and on the FRPs of the different cyanobacterial strains (Gonze et al., 2002). However, this prediction was not supported by experimental testing–in contrast to the model predictions, competition experiments between the WT and CLAb strains found that CLAb was defeated by the WT strain even though the starting proportion of CLAb was as high as 90% of the whole culture (Woelfle et al., 2004).

As a different strategy to test the Diffusible Inhibitor Model experimentally, we designed a growth chamber in which two cyanobacterial strains are separated by a permeable membrane that allows the exchange of medium and

small molecules, but prevents the two strains from directly contacting each other (**Fig.2.12B**). Thus, if a diffusible inhibitor is secreted by one strain, it could pass through the membrane and affect the growth of the strain on the other side of the membrane. As discussed earlier, the WT strain rapidly becomes the predominant strain in mixed cultures with the arhythmic strain CLAb in LD 12:12 cycles (Woelfle et al., 2004); therefore, we conducted a series of experiments by using these two strains separated by a membrane. The pore size of the membrane was 0.2 μm that allows most molecules and small proteins to pass through but not the cyanobacterial cells themselves. If the Diffusible Inhibitor Model is correct, we would expect that the arhythmic strain would show a significantly slower growth rate; however, CLAb displayed the same growth rate as WT in LD 12:12 cycles (**Fig.2.12C**). One possible explanation for this result is that the putative diffusing molecule is too large to pass through the pores of the membrane; therefore, membranes with varying pore sizes could be tested (a pore size of 0.2 μm was used in the experiments whose results are depicted in **Fig. 2.12C**). Alternatively, the inhibitor could be a cell-surface molecule rather than a diffusible factor. Contact-dependent inhibition (CDI) has been reported in *E.coli* (Aoki et al., 2008; Aoki et al., 2005) and interestingly, a few potential homologs of genes involved in CDI in *E.coli* have been identified in *S. elongatus*. Knock-out mutants of these potential homologs could be constructed in order to address whether a contact-dependent inhibition mechanism is involved in the competitions between cyanobacterial strains.

The Cell-to-Cell Communication Model

The Cell-to-Cell Communication Model, which is a combination of the two hypotheses discussed above, postulates that circadian clocks regulate some pathways involved in cell to cell communication in cyanobacteria such that individual cells can cooperate as a group in order to adapt to the environment and/or best utilize a limiting resource (Woelfle and Johnson, 2009). This hypothesis postulates that when the circadian clock is disrupted or is not ideally entrained to the environment, cells do not effectively communicate due to the absence of proper circadian regulation. Under these conditions, cells with mutations in the circadian clock compete as individuals with WT cells that can act as a group, and thus are at a competitive disadvantage in utilizing a limited resource.

Quorum sensing is recognized as a mechanism by which many bacterial species communicate and cooperate (Ng and Bassler, 2009). Bacterial species that engage in quorum sensing secrete signaling molecules called autoinducers (AI), and the detection of the AI allows cells to switch between two distinct patterns of gene expression, depending upon cell density. When the cell density is high and AI reaches a threshold concentration, individual cells cooperate with others such that the entire population turns on a gene expression mode that triggers biological activity, for example the formation of a biofilm or virulence factor production (Ng and Bassler, 2009).

To date, there are only a few cyanobacterial species in which quorum-sensing has been reported (Sharif et al., 2008; Mooy et al., 2012). One of the AIs, N-octanoyl homoserine lactone (C8-AHL), was found in *Gloeothece* sp. PCC6909 cultures, and 43 genes were expressed differently in response to C8-AHL treatment. It was suggested that this quorum sensing may mediate the formation of a biofilm (Mooy et al., 2012). Another cyanobacterium, *Trichodesmium consortia*, was reported to respond to quorum-sensing molecules called acylated homoserine lactones (AHLs) that are produced by epibionts attached to its surface, and colonies of *Trichodesmium* that were treated by AHLs doubled their activity of alkaline phosphatases, which are enzymes used by epibionts in the acquisition of phosphate from dissolved-organic phosphorus molecules (Mooy et al., 2012). Homologs of *luxO* and *luxU*, genes that encode components of the quorum sensing pathway, are present in the cyanobacterial species *Synechocystis* sp. PCC 6803 (Sun et al., 2004). When quorum sensing genes from *Vibrio harveyi* (Ng and Bassler, 2009) were used to search the genome sequence of *S. elongatus*, potential homologs were identified; the homologs identified included the AI receptor *cqsS* (Ng et al., 2011), and *cqsA* (an enzyme involved in AI synthesis), as well as the signal transduction pathway component, *luxO* (Ng and Bassler, 2009) (**Table 2.3**). In addition, a homolog of *aphA*, the gene that encodes the transcription factor that is a master regulator of the quorum sensing pathway and is active at low cell density (LCD) in *V. harveyi* (Rutherford et al., 2011), is also present in the *S. elongatus* genome. Taken

QS gene in *V. harveyi* [26]	Function [26]	Potential Homologs in *S.elongatus*	E-value
LuxN	Receptor	periplasmic sensor hybrid histidine kinase	4e-22
LuxPQ	Receptor	N/A	N/A
CqsS	Receptor	periplasmic sensor hybrid histidine kinase	8e-22
LuxM	AI* synthesis	N/A	N/A
LuxS	AI* synthesis	N/A	N/A
CqsA	AI* synthesis	8-amino-7-oxononanoate synthase	6e-23
LuxO	components of the AI signaling pathway	nitrogen assimilation regulatory protein	2e-14
LuxU	components of the AI signaling pathway	N/A	N/A
LuxR	Transcriptional Regulator at HCD	N/A	N/A
AphA	Transcriptional Regulator at LCD	histone deacetylase/AcuC/AphA family protein-like	5e-67

* AI: autoinducer

Table 2.3 Homologs of quorum sensing genes of *V. harveyi* in *S. elongatus.*

together, the data from this bioinformatics approach suggest that *S. elongatus* may be capable of quorum sensing with clock-enhanced reproductive fitness mediated by this form of cell-to-cell communication. This possibility could be tested by knocking out these potential homologs.

Future Directions

Competition experiments have demonstrated the adaptive value of the circadian clock in cyanobacteria. It is clear that the circadian clock enhances the fitness of cyanobacterial cells in light/dark cycles, and that this enhancement in reproductive fitness is greatest when the circadian clock resonates with the environmental cycle. In contrast, the circadian clock in cyanobacteria provides little or no reproductive advantage under constant conditions, indicating that the adaptive value conferred by circadian clock is an extrinsic rather than intrinsic property (Woelfle et al., 2004; Ouyang et al., 1998).

To date, the underlying mechanism by which the circadian clock enhances reproductive fitness remains elusive. Although several models have been proposed and tested, each has some evidence that supports it and none can be excluded at this time. Each of the models discussed here will be examined further using a complementary approach. These include alternative culture methods aimed at accurately detecting small (but significant) differences in growth rates between wild-type and clock mutant strains. These small differences in growth rate could explain the competition outcomes. In addition, a genetic approach guided by bioinformatics and mathematical modeling could also be

used to identify genes involved in the pathway and define the mechanism of fitness enhancement.

Experimental Procedures

Bacterial strains and culture conditions

All cyanobacterial strains used in this study is listed in **Table 2.4** The cyanobacterial strains were grown in BG-11 medium (Bustos and Golden, 1991) at 30 °C, and illuminated by cool-white fluorescence bulbs (40-50 μE m^{-2} s^{-1}) with air bubbling. Spectinomycin (25μg/ml) and/or kanamycin (25μl/ml) were supplemented to the medium when necessary. For growth on solidified agar plates, BG-11 medium was supplemented with 1.5% agar and appropriate antibiotics.

Competition experiment

The clock phenotypes of cyanobacterial strains were determined by their luminescence rhythms that report the promoter activity of *psbAI*. Strains with different clock phenotypes and different antibiotic resistance, e.g., wild type vs. arhythmic, were selected for the competition experiments. Different antibiotics enable us to track their fractions in mixed cultures by plating on selective media.

Strain	Genotype	Reference
WT (AMC149)	Wild type S. elongatus with a luminescence reporter (*psbAIp::luxAB*).	(Kondo et al., 1993)
CLAb	Arhythmic mutant with a point mutation on *kaiC* of the WT.	(Kondo et al., 1994)
CLAc	Damped oscillation mutant with a point mutation on *kaiC* of the WT.	(Kondo et al., 1994)
C22a	Short period mutant with a point mutation on *kaiC* of the WT.	(Kondo et al., 1994)
C28a	Long period mutant with a point mutation on *kaiC* of the WT.	(Kondo et al., 1994)
A30a	Long period mutant with a point mutation on *kaiA* of the WT.	(Kondo et al., 1994)
B22a	Short period mutant with a point mutation on *kaiB* of the WT.	(Kondo et al., 1994)
WTYFP	WT transformed with a Yellow Fluorescence reporter (*ptrc::yfp*)	This study
CLAbYFP	CLAb transformed with a Yellow Fluorescence reporter (*ptrc::yfp*)	This study
OptkaiBC	Native kaiBC genes were replaced by Codon-optimized kaiBC genes.	(Xu et al., 2013)

Table 2.4 Cyanobacterial strains used in this chapter.

Pure cultures of two strains were set up under LL condition, and when they reached log phase (OD_{750} ~ 0.6), equal numbers of cells were mixed and cultured under different LD cycles or LL condition. Aliquots were taken from the mixed cultures every ~8 generations in LD and every ~16 generations in LL, and they were plated on selective media to count the number of colony-forming units (CFU) of each strain. Meanwhile, the mixed culture was diluted into fresh medium and grown for another ~8 generations (LD) and ~16 generations (LL). This process was repeated for 4 cycles to allow cells to grow for 40-50 generations. The fraction of each strain in the mixed culture was calculated by the number of colonies of each strain growing on selective media. Circadian phenotypes were confirmed by monitoring the luminescence rhythms of colonies of each strain at different sampling times. Similar procedure was applied in competition experiments on solid agar medium and in continuous cultures, and the continuous cultures were conducted in home-made apparatus with lamination and air bubbling.

Growth curves and calculation of doubling time

To generate growth curves, seed cultures of cyanobacterial strains were grown under LL conditions at 30°C until exponential phase before inoculation. To measure the initial growth rates of WT and CLAb, pure cultures of WT and CLAb strains were grown under either LD 12:12 cycles or LL conditions at 30°C with air bubbling. When cells reached stationary phase, cultures were diluted at the ratio of 1:1000 to fresh BG-11 medium, and this process was repeated for 4 times. To

compare the growth rates of WT, OptkaiBC, CLAb and CLAc strains at different temperatures, pure cultures of these strains were grown under LD 12:12 cycles at 37°C, 30°C, 25°C, 20°C and 18°C with air bubbling and shaking. Cell densities were monitored by measuring the optical density at 750nm (OD_{750}). OD_{750} was then plotted against time for a comparison of growth among strains. The growth constant *k* was generated by fitting exponential curves to the growth curves at log scale, and doubling time was calculated using this equation: doubling time (h) = (ln(2)/k) × 24. For initial growth rates calculation, the same equation was applied to the first 2-3 days of the data.

Membrane experiment

A semi-co-culture apparatus was used to test the existence of a diffusible inhibitor. Two chambers (left and right) were separated by a polyethersulfone membrane (Sterlitech, WA; Cat. # PES022005) with 0.2 μm pores. Cells of different strains can be cultured separately in these two chambers, but the medium passes freely through this membrane such that the putative inhibitor could diffuse to the other side. The cultures were illuminated by white fluorescent light from the top, and the light intensity was 50 $uE*m^{-2}*s^{-1}$. The WT strain and CLAb were semi-co-cultured in this apparatus under LD 12:12 cycles. Cell densities (OD_{750}) were measured on the fifth day.

Dynamics of the competition

A copy of the *yfp* gene (Chabot et al., 2007) driven by the *ptrc* promoter was incorporated into the Neutral Site II of the WT strain, generating an YFP reporter strain WTYFP. No effect was observed in the growth rates and FRP of the WTYFP, comparing to the wild-type strain, thus suggesting that WTYFP can be used to monitor the dynamic of the competition experiments. WTYFP was co-cultured with CLAb under LD12:12 cycles, and aliquots of cells was taken every two days. The growth of pure and mixed cultures was recorded by monitoring OD_{750}. Cell lengths and cell division rates were observed and quantified by using fluorescence microscopy. Fractions of each strain in mixed cultures were quantified by flow cytometry (**Fig. 2.5A**), and the OD_{750} value of the mixed cultures was converted to cell numbers by fitting to a standard curve. Cell numbers of each strain in mixed cultures were calculated by multiplying the fraction by the total cell numbers in the mixed cultures.

Bioinformatics analysis

Amino acid sequences of quorum sensing genes in *V. harveyi* were retrieved from the NCBI database (*http://www.ncbi.nlm.nih.gov/bioproject/?term=19857*). Homology search of these protein sequences was performed against the *S. elongatus* genome by using the BLASTP program (Altschul et al., 1997). The threshold of expected value is set up at 1e-10, and BLOSUM80 matrix and Low Complexity Filter were applied.

Chapter III

The global metabolic profiles of the wild-type cyanobacterium *Synechococcus elongatus* PCC 7942 and adaptive-fitness mutants

Abstract

Although numerous experiments have been done to test the potential mechanisms of the clock-mediated fitness enhancement, it is difficult to draw conclusions from these results. Recently, several studies have reported connections between metabolism and the circadian clock in *Synechococcus elongatus* PCC 7942. In addition, some of my preliminary results also revealed that the steady-state levels of metabolites in the clock mutant are different from the metabolite levels in WT. Therefore, in this study, I hypothesize that the clock-mediated fitness enhancement results from altered metabolism in the mutant strains. To test this hypothesis, I took advantage of the rapid development of metabolomics techniques and established the metabolic profiles for the WT strain and two clock mutant strains under light-dark cycles and in constant light. My results demonstrated that the global metabolic profiles were altered in the clock mutants, and that the metabolism of WT is dramatically different in light-dark cycles vs. constant light condition.

Introduction

Circadian clocks are endogenous timing mechanisms that regulate many physiological activities of organisms. The regulation of metabolic processes by

circadian clocks has been reported in a broad range of organisms ranging from bacteria to mammals (Bailey et al., 2014). In mice and humans, the blood pressure, body temperatures and many other metabolic events are regulated by circadian clocks. In plants, circadian clocks control the secretion of selected hormones involved in the defense against herbivores (Goodspeed et al., 2012). In the prokaryotic cyanobacteria, nitrogen fixation activities are separated from photosynthesis by circadian regulation to avoid the toxicity of oxygen to nitrogenase (Grobbelaar et al., 1986; Mitsui et al., 1986). On one hand, circadian clocks regulate metabolism; on the other hand, metabolism can also affect circadian clocks. This relationship implies that clocks and metabolism could form an autoregulatory feedback network. Recently, researchers proposed that the coupling of cyanobacterial circadian clocks and metabolism might be the mechanism through which circadian clocks receive entraining information from the environment (Rust et al., 2011; Kim et al., 2012). Kim et al. (2012) proposed that the redox state of quinone and the ATP/ADP ratio in the cell could work together to reset the phase of the clock, suggesting the importance of the coupling of circadian clocks and metabolism.

As one of the simplest circadian machineries, the cyanobacteria circadian clock confers adaptive significance in rhythmic environments, as indicated by a series of competition experiments in the cyanobacterium *Synechococcus elongatus* PCC 7942 (*S. elongatus*) (Ouyang, et al., 1998; Woelfle et al., 2004). We have been trying to understand for long time the mechanism by which the circadian clock enhances fitness under the competition conditions. Although

several models have been proposed and numerous experiments have been done to test these models, no definite answer is known at this time (Ma et al., 2013). However, because there is increasing evidence showing that the connection between circadian clocks and metabolism is important, I wish to test if metabolism can be altered by the disruption of the circadian clock, and whether the metabolic status under the competition conditions can help us to understand clock-conferred fitness. Answering these questions will not only help us to further understand the coupling of metabolism and circadian clocks, but may also provide clues to the underlying mechanism of the competition results. In particular, an experiment to study the metabolites in the cyanobacterial culture medium revealed differences between the wild type (WT) and the clock mutants, leading us to hypothesize that the metabolic profiles of clock mutants are altered, which could be the potential mechanism of the clock-mediated fitness enhancement. In this chapter, global metabolic profiles of the WT and two clock mutants, CLAb and CLAc, were examined by metabolomics techniques. From this study I demonstrated that metabolism varies in these three strains under light-dark (LD) conditions, while the regulation of metabolism by circadian clock is dominated by light conditions {LD vs. constant light (LL)}.

Results

The metabolic profiling is influenced by both clock phenotypes and light conditions

The clock phenotypes of three strains used in this study, WT, CLAb and

CLAc, are shown in **Fig. 3.1**. To establish the global metabolic profiles, cells of these three strains were collected every 6 hours under LD 12:12 cycles from Zeitgeber Time (ZT) 0 to ZT24. For the WT, cells under LL condition were also collected every 6 hours from Circadian Time (CT) 12 to CT36. After sample collection, cells were immediately frozen by liquid nitrogen and sent to Metabolon, Inc., which is a biotech company specialized in metabolomics, for metabolomics analysis. At Metabolon, samples were prepared by using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. Then intracellular metabolites were identified and quantified by liquid chromatography–mass spectrometry (LC-MS) and gas chromatography–mass spectrometry (GC-MS). Raw and normalized data was obtained from Metabolon after sample processing. I then analyzed these data by using R programming language (*http://www.R-project.org*). After removing metabolites with missing values during the 24-hour time course, 173 metabolites were included to build the metabolic profiles. These 173 metabolites are categorized into 6 super pathways and 37 sub pathways (**Fig. 3.2**).

To test if the metabolic profiles from the 4 groups (WTLD, CLAbLD, CLAcLD and WTLL) are statistically different from each other, the whole dataset was transformedg by natural logarithm and principal component analysis (PCA) was applied. PCA is a statistical procedure that reduces the dimensionality of the

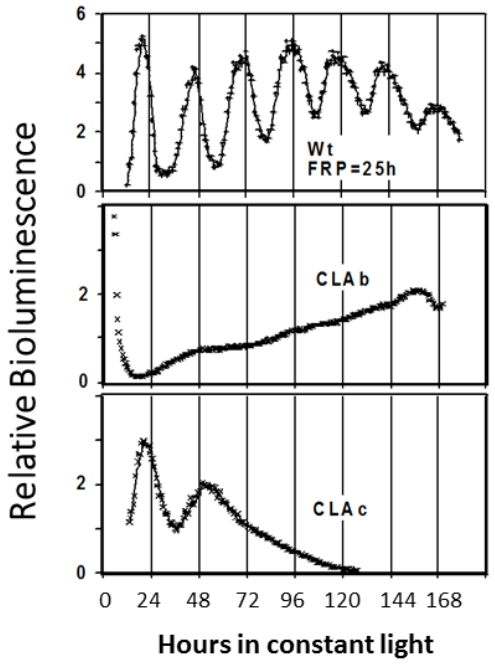


Figure 3.1 Clock phenotypes of three strains used in this study. Upper panel, WT whose FRP is about 25 hours; middle panel, the arrhythmic strain CLAb; bottom panel, CLAc with a damped rhythm.

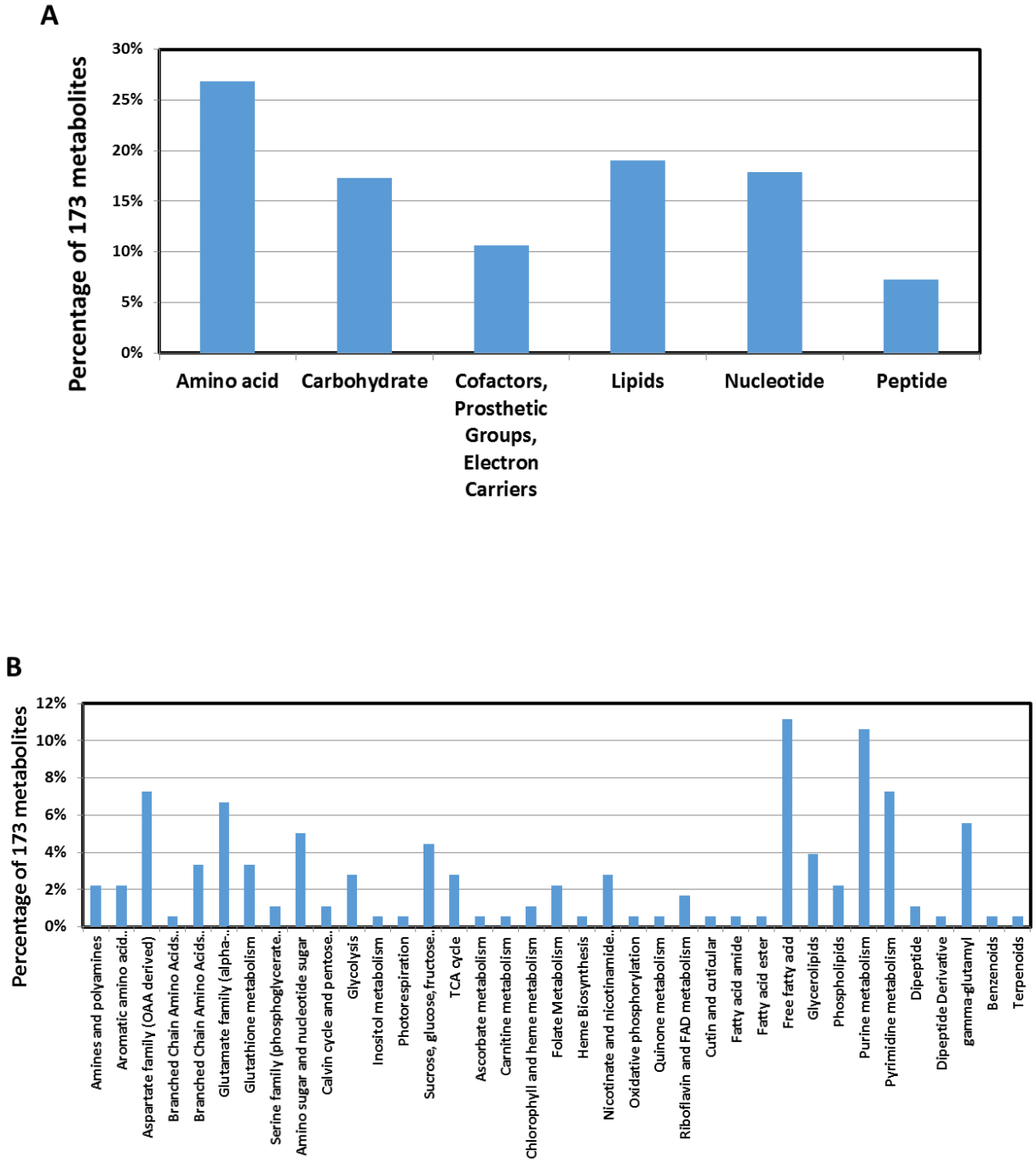


Figure 3.2 173 metabolites were included to establish the metabolic profiles. And they are categorized to 6 super pathways (A), and 37 sub pathways (B).

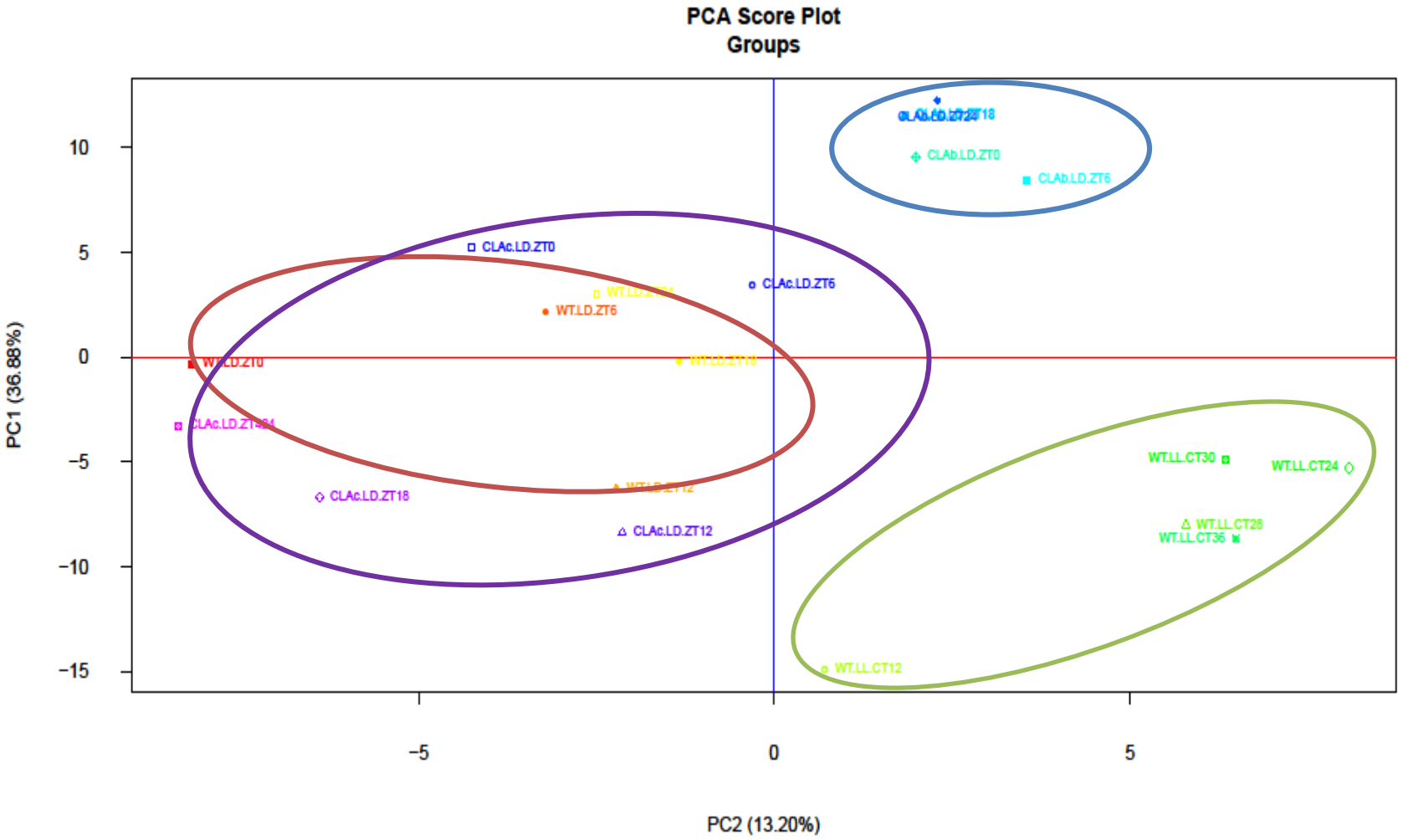

PCA Score Plot
Groups
PC1 (36.88%)
PC2 (13.20%)
10
5
0
−5
−10
−15
−5
0
5
CLAb.LD.ZT0
CLAb.LD.ZT6
CLAc.LD.ZT0
CLAc.LD.ZT6
WT.LD.ZT6
CLAc.LD.ZT18
CLAc.LD.ZT12
WT.LL.CT30
WT.LL.CT24
WT.LL.CT28
WT.LL.CT36
WT.LL.CT12

Figure 3.3 Principal component analysis (PCA) indicates that the metabolic profiling is associated with genotypes and light conditions. Green circle, WT samples under LL condition; orange circle, WT samples under LD condition; blue circle, CLAb samples under LD condition; purple circle, CLAc samples under LD condition.

data but the variations among samples can be preserved (Ringner, 2008). The first principal component (PC1) represents the direction that samples shows the largest variation of those metabolites levels. The second component (PC2) is uncorrelated with PC1, and it is the direction that samples show the second largest variation (Ringner, 2008). PCA enables the visualization of multi-dimensional data.

Our whole data set contains 40 samples, and each of these samples has 173 variables (173 metabolites) that define the property of this particular sample. By PCA, the property of one sample can be simply represented by two principal components with the largest variations among samples, and visualized by a two-dimensional plot, as shown in **Fig. 3.3**. For each strain and each condition, five data points are plotted to represent the five time points. As can be seen in **Fig. 3.3**, the clustering indicates that the metabolic profile is determined by both clock phenotypes and the light/dark conditions. For instance, samples from the WT under LD condition (orange circle) were clustered together, while samples from the WT under LL condition (green circle) were grouped together in another coordinate, suggesting that there are significant differences in their metabolic profiles when light conditions are different. When the samples were collected from different strains but under the same light condition, two situations were observed. The WT and CLAb displayed significant differences, as can be seen from **Fig. 3.3**. Interestingly, the five time points of CLAb (blue circle) did not show large variations among each other, while the five time points from other samples were spread out on the PCA plot, suggesting that the metabolic profile of CLAb

did not vary significantly at different time points. In contrast, samples from CLAc (purple circle) were not separated with the WT samples, but more variations were observed among the five time points of CLAc, indicating some small temporal differences between these two groups.

Global metabolic profiling was altered in CLAb and CLAc under LD 12:12 cycles

To visualize the metabolic profiles of these four groups, a heatmap was generated by hierarchical clustering, which shows the temporal patterns and the production levels of each metabolite in each group (**Fig. 3.4**). As can be seen in **Fig. 3.4**, the metabolic profiling was dramatically altered in the arrhythmic mutant CLAb. On one hand, production of most of the metabolites was down-regulated in CLAb (**Fig. 3.4**); on the other hand, it is apparent that some of the metabolites that display daily oscillation in the WT strain do not cycle in CLAb (**Fig. 3.4**).

I then quantified the number of cycling metabolites in these strains by using the modified cosiner method (Kucho et al., 2005; see methods). By fitting the detrended data to a 24-h cosine curve, an error factor (Ef) and the amplitude (Amp) was calculated to evaluate the fitting quality. For each metabolite, if its Ef is less than 0.2 and its Amp is greater than 1.2, it will be considered as a cycling metabolites (Kucho et al., 2005). As shown in **Fig. 3.4**, in WT, almost 40% of the metabolites were cycling in LD 12:12. In CLAb, however, only about 5% of the metabolites were oscillating under the same conditions. Besides the reduced numbers of cycling metabolites in CLAb, the peak time of these cycling

metabolites also differed from the WT (**Fig. 3.6 A&C**). In WT, about 70% of the cycling metabolites peaked around dusk (ZT12), whereas only 30% of the cycling metabolites peaked at this time in CLAb. In contrast, the majority of metabolites of CLAb peaked in the dark phase or near dawn, as shown in **Fig. 3.6A&C**. Interestingly, some of the cycling metabolites of CLAb are different from those in WT (**Table 3.1**). For instance, I did not observe glycerol oscillating in the WT, whereas it was cycling with the peak time at ZT22 in CLAb. In addition, although the majority of metabolites were down-regulated in CLAb, I detected a group of metabolites that were up-regulated and peaked in the dark phase (**Fig. 3.4** and **Table 3.2**). These up-regulated metabolites were highlighted in **Table 3.2**, and they belong to the free fatty acid pathway, indicating that the free fatty acid pathway was perturbed in CLAb.

In CLAc, which shows a damped rhythm under LL conditions and is also outcompeted by WT in LD but more slowly than is CLAb (Woelfle et al., 2004), the global metabolic profile was also affected. Unlike CLAb, I did not observe significant down-regulation of metabolite levels in CLAc (**Fig. 3.4**). However, some metabolites showed higher production levels during the dark phase in CLAc and were practically in antiphase to their pattern in WT, (i.e., at higher concentrations during the light phase in WT), as shown in the orange box in **Fig.**

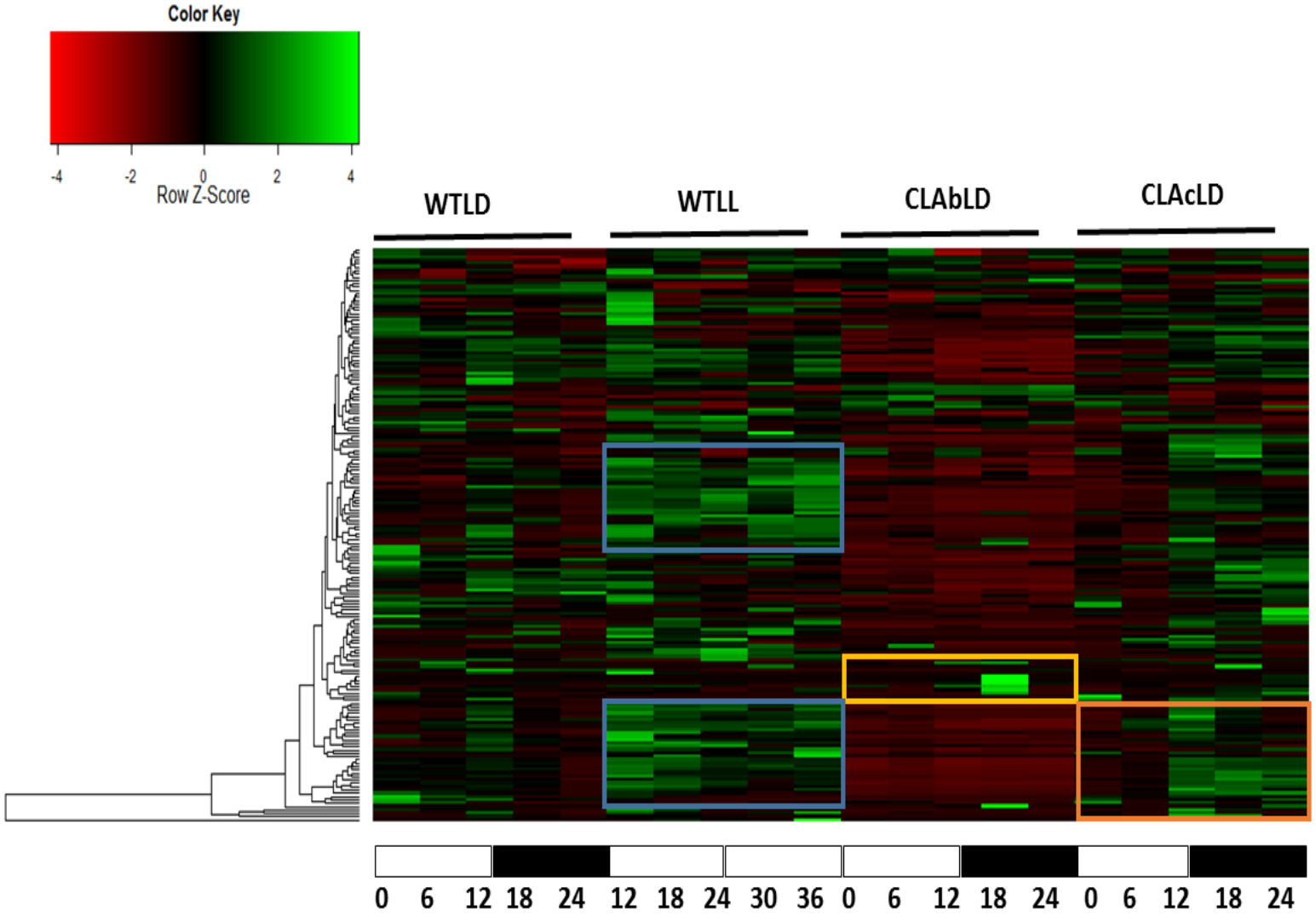

Color Key
-4
-2
0
2
4
Row Z-Score
WTLD
WTLL
CLAbLD
CLAcLD
0 6 12 18 24 12 18 24 30 36 0 6 12 18 24 0 6 12 18 24

Figure 3.4 Global metabolic profiling of 173 metabolites in WT and arrhythmic mutants strains under LD 12:12 conditions and LL condition (only for WT) for 24 hours. The white bars under the heatmap represent 12-hour light conditions, and the black bars represent 12-hour dark conditions. Metabolites are sorted by hierarchical clustering. The yellow and orange boxes highlight differences between the WT and CLAb (yellow) or CLAc (orange).

3.4. Furthermore, 15% of the metabolites displayed a 24-hour oscillation in CLAc, which is lower than the WT (40%), but is higher than CLAb (5%) (**Fig. 3.5**). Interestingly, almost all of the cycling metabolites of CLAc were also oscillating in the WT (**Table 3.1**), suggesting that the metabolic profile of CLAc preserved features of the WT profile. Even though the number of cycling metabolites was decreased in CLAc (**Fig. 3.5**), their peak time was not dramatically altered. As can be seen in **Fig. 3.6A &D**, the distribution of peak time in CLAc is quite similar to that of the WT, further confirming that CLAc is more similar to the WT than CLAb. These patterns are consistent with the observation that the "damped" strain CLAc is out-competed by WT more slowly than CLAb in the competition experiment (Woelfle et al., 2004). The probable explanation for that result is that the daily regulation of metabolism in CLAc is more "WT-like" than is that of CLAb, and therefore is able to compete more efficiently against WT than is the arrhythmic CLAb strain.

Taken together, the global profiles of these three strains, WT, CLAb and CLAc, revealed that their metabolic "signatures" are altered under LD 12:12 conditions when the clock phenotypes are changed, suggesting that the circadian clock plays a critical role for regulating metabolism of cyanobacteria over the daily cycle to enhance adaptation to the environment. It is likely that the altered metabolism may be a potential explanation for the results of the competition experiments (Woelfle et al., 2004).

Table 3.1 Metabolites that showed 24-hour rhythms in at least one group.

Metabolites	WTLD	WTLL	CLAbLD	CLAcLD
1,3-dihydroxyacetone	+	_	_	_
1-oleoylglycerophosphoglycerol*	+	_	_	_
1-palmitoylglycerol (1-monopalmitin)	_	_	_	+
1-palmitoylglycerophosphoglycerol*	+	_	_	_
2,3-dihydroxyisovalerate	+	_	+	_
2'-deoxyadenosine	_	_	_	+
2-palmitoylglycerophosphoglycerol*	+	_	_	_
2-phenoxyethanol	+	_	_	+
3-dehydrocarnitine*	_	_	+	_
3-hydroxymyristate	+	_	_	_
3-hydroxypalmitic acid methyl ester	+	_	_	_
3-methyl-2-oxovalerate	+	_	_	_
4-methyl-2-oxopentanoate	+	_	_	_
5-methylthioadenosine (MTA)	+	_	_	_
5-oxoproline	+	_	_	_
7-methylguanosine	+	_	_	+
8-hydroxyguanine	+	_	_	_
acisoga	+	+	_	+
adenosine	_	_	_	+
alanine	+	_	_	_
alpha-ketoglutarate	+	_	_	+
asparagine	+	_	_	_
benzoate	_	+	+	_
biopterin	_	_	_	+
citrulline	+	_	+	_
cytidine	+	_	_	+
cytidine 5'-monophosphate (5'-CMP)	_	_	_	+
dihydroxyacetone phosphate (DHAP)	+	_	_	_
erythronate*	+	_	_	+
flavin mononucleotide (FMN)	+	_	_	_
fructose	+	_	_	_
fumarate	_	+	_	_
gamma-glutamylalanine	+	_	_	_
gamma-glutamylglutamine	_	+	_	_
gamma-glutamylisoleucine*	+	_	_	+

gamma-glutamylmethionine	+	_	_	_
gamma-glutamylphenylalanine	_	+	_	_
gamma-glutamylthreonine*	+	_	_	_
gamma-glutamyltyrosine	_	+	_	_
glucose-6-phosphate (G6P)	_	_	+	_
glutamate	+	_	+	_
glutathione, oxidized (GSSG)	+	_	_	_
glycerol	_	_	+	_
glycolate (hydroxyacetate)	+	_	_	_
guanidine	+	_	_	_
guanine	+	+	_	+
guanosine	+	_	_	_
histidine	+	_	_	_
hypoxanthine	_	_	_	+
inosine	+	_	_	_
Isobar: UDP-acetylglucosamine, UDP-acetylgalactosamine	_	+	_	_
isoleucine	+	_	_	_
leucine	+	_	_	_
malate	+	_	_	_
maltose	+	_	_	+
maltotetraose	_	_	_	+
margarate (17:0)	_	_	_	+
methionine	+	_	+	_
methionine sulfoxide	_	_	_	+
myristate (14:0)	+	_	_	_
N2-acetyllysine	+	_	_	_
N6,N6-dimethyladenosine	+	_	+	_
N6-acetyllysine	+	+	+	+
N6-carbamoylthreonyladenosine	+	_	_	+
N-6-trimethyllysine	_	_	_	+
N-acetylaspartate (NAA)	+	_	_	+
N-acetylglucosamine	+	_	_	_
N-acetylglutamate	+	_	_	_
N-formylmethionine	+	_	_	_
nicotinamide	+	_	_	_
nicotinamide riboside*	+	_	_	_
norophthalmate*	+	_	_	_
ophthalmate	+	_	_	_
palmitate, methyl ester	+	_	_	_

palmitic amide	_	_	_	+
pentadecanoate (15:0)	+	_	_	_
phenylpyruvate	_	_	_	+
pheophorbide A	+	_	_	_
phosphate	+	_	_	_
proline	+	_	_	_
S-adenosylhomocysteine (SAH)	+	_	_	+
serine	+	_	_	+
succinate	+	_	_	_
symmetric dimethylarginine (SDMA)	+	_	+	_
threonate	+	_	_	+
threonine	+	_	_	_
thymidine 5'-monophosphate	+	_	_	_
uracil	+	_	_	_
uridine	+	+	_	_
valine	+	_	_	_
xanthine	_	+	_	_
xanthosine	+	+	_	_
Total cycling metabolites	**70**	**12**	**11**	**27**

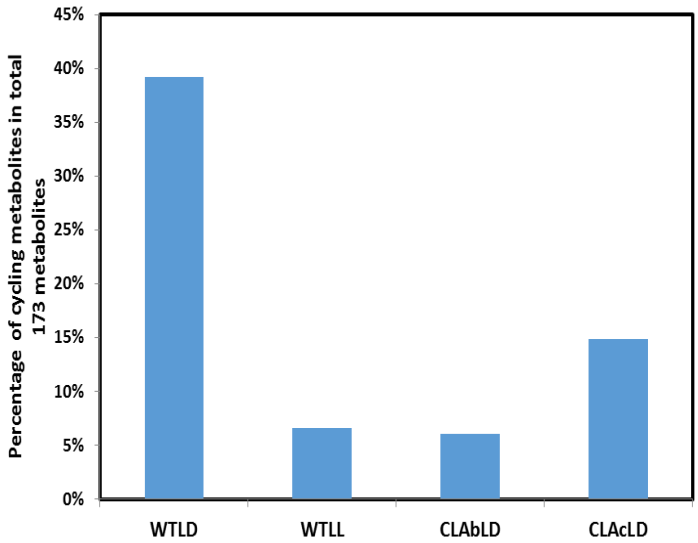


Figure 3.5 Percentage of cycling metabolites in total 173 metabolites detected. Criteria for distinguishing cycling vs. non-cycling metabolites are explained in the text and methods section.

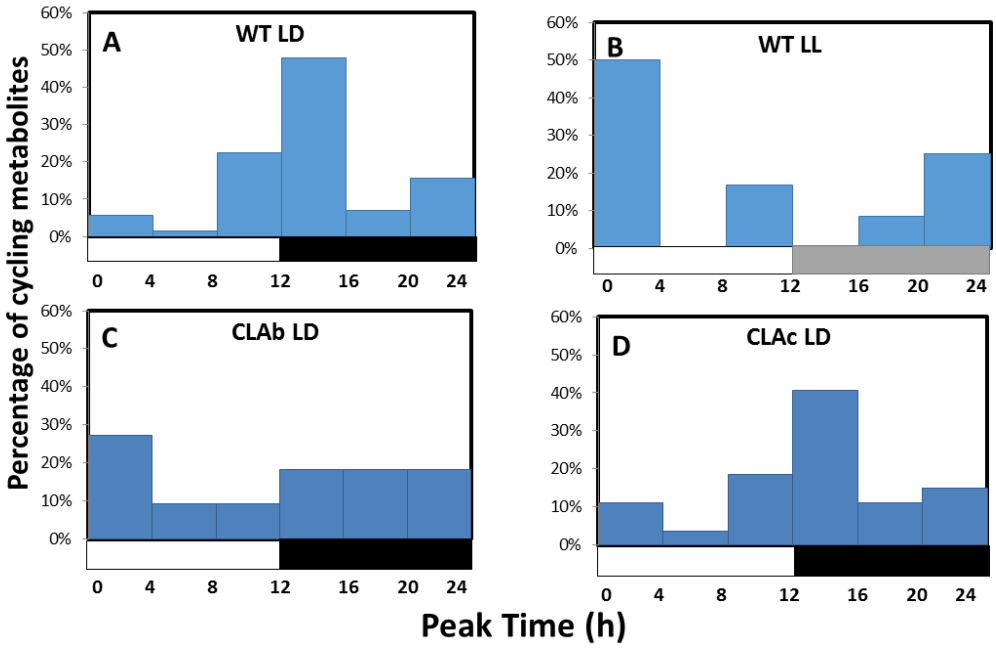


Figure 3.6 Peak time of cycling metabolites. A, WT under LD 12:12 cycles; B, WT under LL condition; C, CLAb under LD 12:12 cycles; D, CLAc under LD 12:12 cycles.

The metabolic profiles of WT are significantly different in LD vs. LL

For photosynthetic cyanobacteria, light plays an essential role in regulating metabolism through photosynthesis. My results from the clock mutants CLAb and CLAc suggest that circadian clock also regulates metabolic patterns beyond simple lights-on and light-off switches. Therefore I would like to know if circadian clock cycling is able to dominate the regulation of metabolism. If yes, I would expect that many metabolites that oscillate under LD cycles should also show circadian rhythms under LL conditions. In *S. elongatus*, the global gene expression continues oscillating under LL conditions (Liu et al., 1995; Ito et al., 2009). To investigate if the metabolism shows similar patterns as the gene expression under LD and LL conditions, I collected cells from the WT cultures every 6 hours in LL from CT12 to CT36. As shown in **Fig. 3.4**, some differences were observed when the metabolic profile of the WT under LL condition was compared with it under LD condition. For instance, some of the cycling metabolites under LD condition exhibited constant high levels under LL condition (blue boxes in **Fig. 3.4**). Moreover, the number of metabolites that cycle in LD was reduced to only 5% under LL conditions (**Fig. 3.5**), indicating that constant light over-rides the circadian control of gene expression as far as metabolite levels are concerned. In addition, under LL conditions, nearly 70% of the cycling metabolites reached the peak around CT0 or CT24, whereas under LD condition most of the cycling metabolites peaked around dusk (**Fig. 3.6 A&B**), once again demonstrating that environmental light conditions (i.e., cyclic vs. constant) mediate the production of cycling metabolites.

In summary, by comparing the metabolic profiles of the WT strain under LL conditions and LD conditions, I demonstrated that most of the metabolites were not oscillating when the cells were grown under LL conditions. This result is not totally unexpected. On one hand, photosynthesis is always activated when light is present, and the persistence of photosynthesis may not require simultaneous gene expression. On the other hand, the production of metabolites reflects enzyme activities, while microarray and promoter activity assays only detect gene expression. The metabolic profiles revealed in this study is more likely to predict the proteomics, therefore it is reasonable that the production of metabolites exhibits different daily patterns from the global gene expression.

In the competition experiments, the WT was only able to defeat CLAb and CLAc under LD conditions but not under LL conditions. Based on the metabolic profiles of the WT, it is likely that light also plays an important role on the metabolism of CLAb and CLAc under LL conditions. In this case, the defects of the circadian clocks in these two mutant strains might not be sufficient to cause any disadvantage to the growth of these two strains under LL conditions, which could be the possible reason that the competition was only observed under LD conditions. In particular, I hypothesize that the metabolic patterns of WT, CLAb, and CLAc are very similar in LL.

Metabolites of CLAb and CLAc that show significant differences from the WT are from a broad range of pathways

One of the aims of this study is to test the hypothesis that some changes in the metabolism of the clock mutants caused the competition results. My results have demonstrated that metabolism is altered in these clock mutants, providing preliminary evidence to support this hypothesis. By tracking the production levels and daily patterns of these metabolites, I expect to identify pathways that involve the majority of these altered metabolites. In this ideal situation, I would be able to target these pathways and eventually uncover the mechanism of the competition experiments. From this line of reasoning, I identified some metabolites that mainly contribute to the differences between the WT and CLAb or CLAc by examining their principal component scores. As presented in **Fig. 3.7** and **Fig. 3.8**, the PCA loading plots plot every metabolite according to their principal component scores, and metabolites with high scores were selected and listed in **Table 3.2** (CLAb) and **Table 3.3** (CLAc). For CLAb, 17 candidates from 5 super pathways and 9 sub-pathways were identified (**Fig. 3.9A** and **Table 3.2**), and for CLAc, 28 candidates were identified from 6 super pathways and 10 sub-pathways (**Fig. 3.9B** and **Table 3.3**).

As shown in **Fig. 3.9A**, nearly 30% of the candidates of CLAb are from the free fatty acid pathway. As I mentioned previously, these free fatty acids exhibited elevated concentrations in the dark phase in CLAb, whereas they were only produced in moderate levels and mostly in the light phase in WT (**Fig. 3.4**).

WT vs. CLAb under LD 12:12

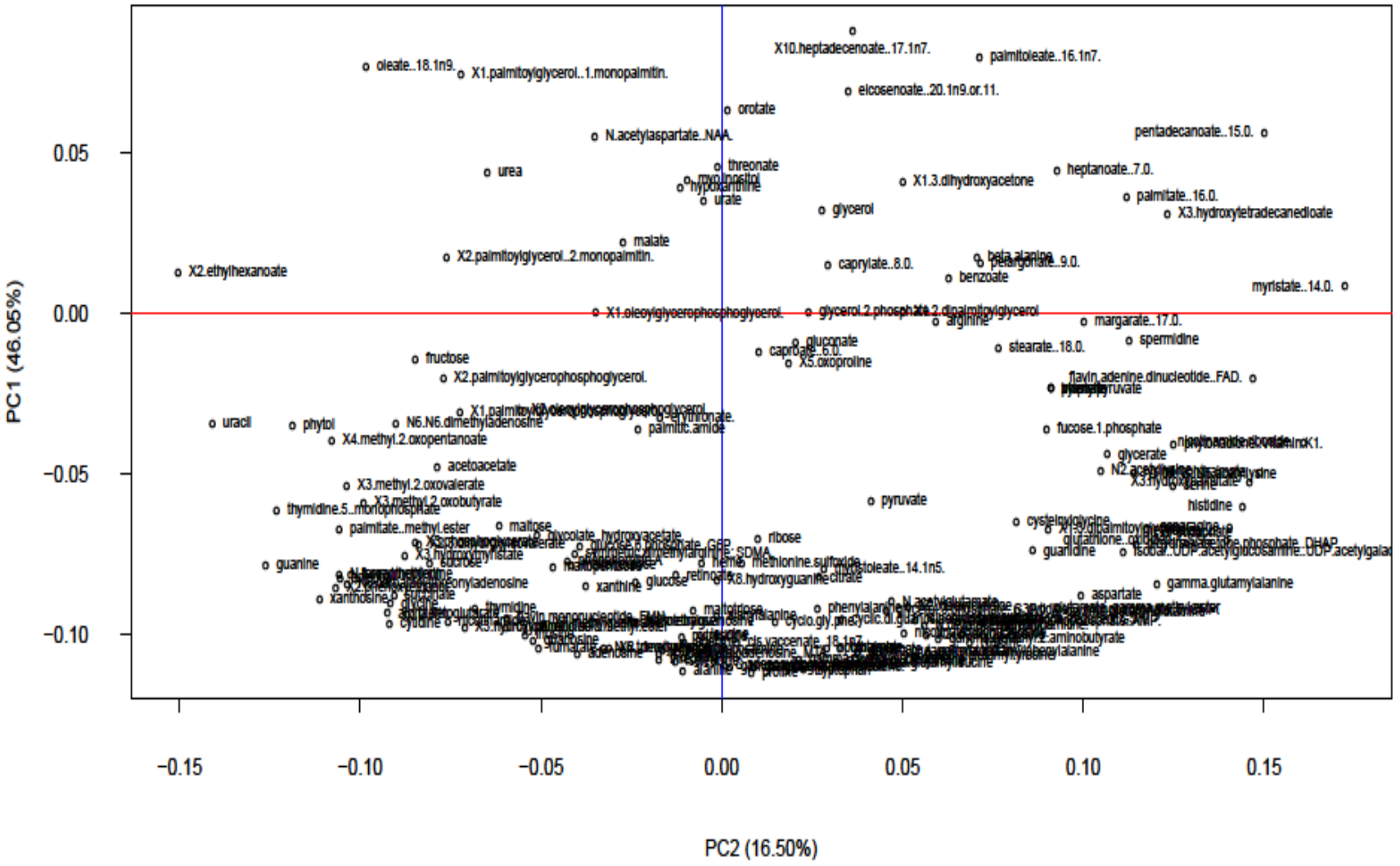


Figure 3.7 Loading plot of PCA based on the comparison between samples of WT and CLAb under LD 12:12 condition. Each dot represents a metabolite and the position of each metabolite is determined by the values of 5 time points of the metabolites.

WT vs. CLAc under LD 12:12

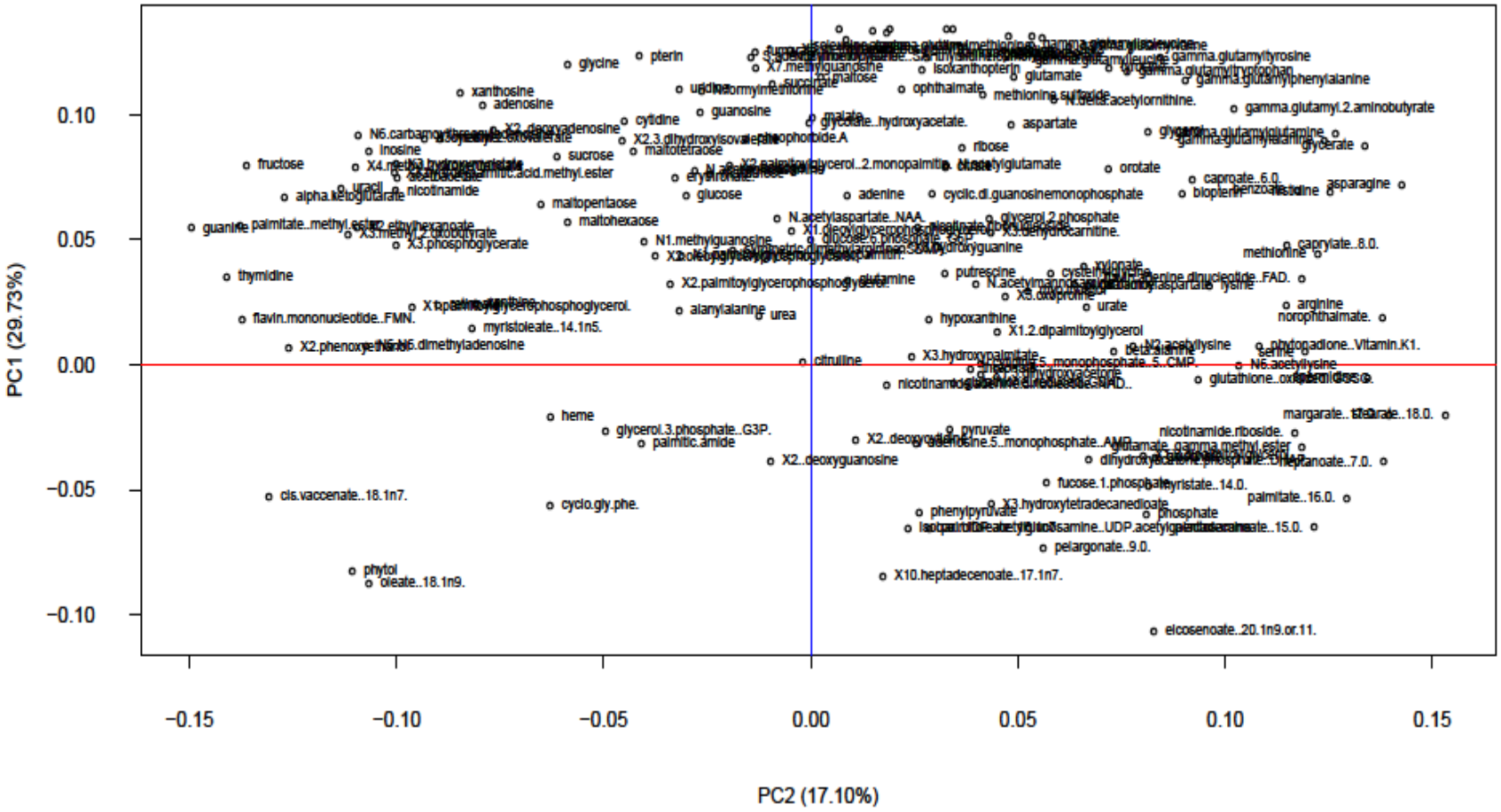


Figure 3.8 Loading plot of PCA based on the comparison between samples of WT and CLAc under LD 12:12 condition. Each dot represents a metabolite and the position of each metabolite is determined by the values of 5 time points of the metabolites.

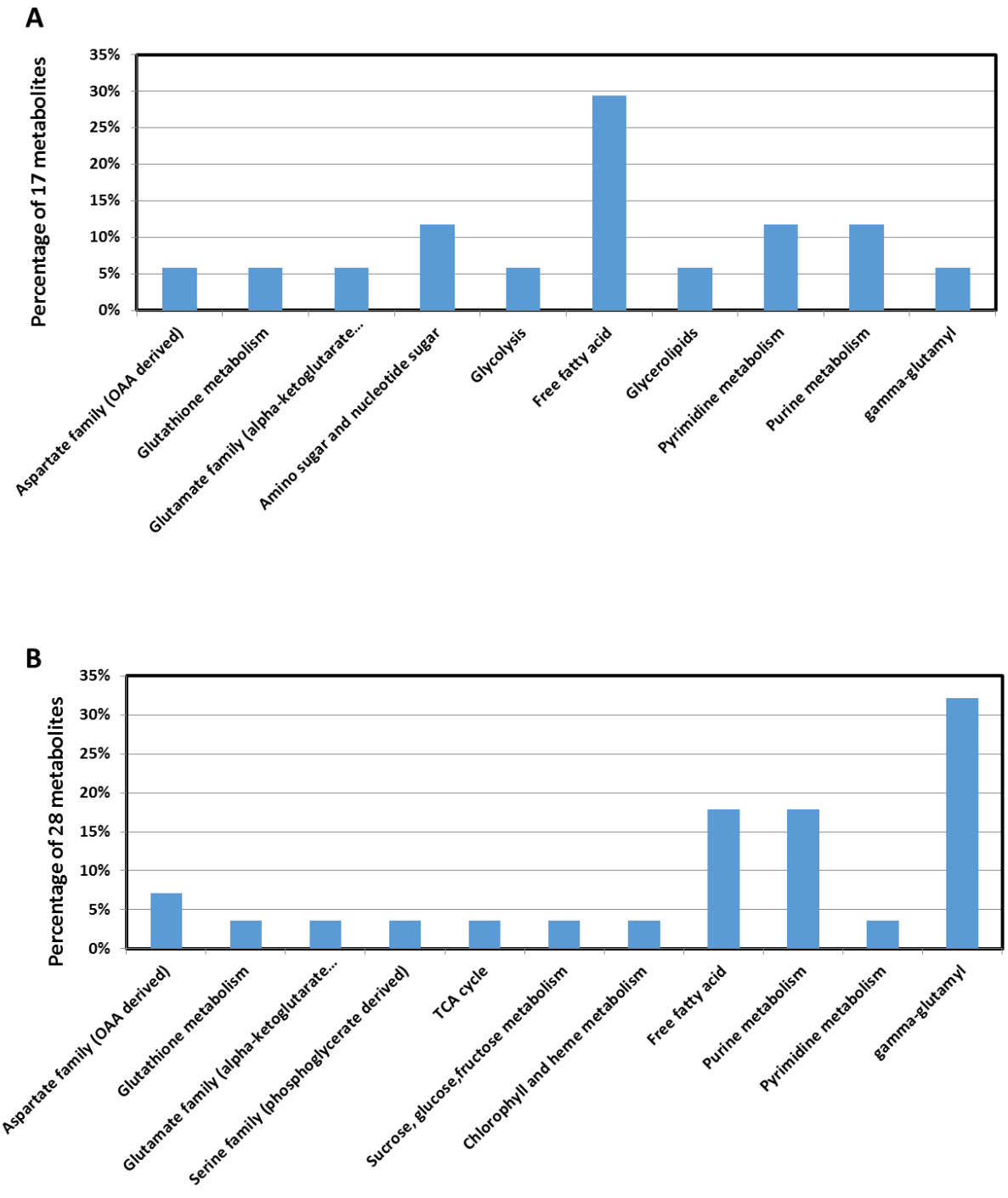


Figure 3.9 Metabolic candidates that showed significant difference from the WT. A, Percentage of metabolic candidates from CLAb. 17 metabolites were identified from 10 sub pathways. B, Percentage of metabolic candidates from CLAc. 28 metabolites were identified from 11 sub pathways.

Table 3.2. PCA loading plot revealed metabolites that show significant differences between samples from WT and CLAb under LD 12:12 condition.

Metabolites	Super Pathway	Sub Pathway
aspartate	Amino acid	Aspartate family (OAA derived)
glutathione, oxidized (GSSG)	Amino acid	Glutathione metabolism
histidine	Amino acid	Glutamate family (alpha-ketoglutarate derived)
fucose-1-phosphate	Carbohydrate	Amino sugar and nucleotide sugar
glycerate	Carbohydrate	Glycolysis
Isobar: UDP-acetylglucosamine, UDP-acetylgalactosamine	Carbohydrate	Amino sugar and nucleotide sugar
10-heptadecenoate (17:1n7)	Lipids	Free fatty acid
1-palmitoylglycerol (1-monopalmitin)	Lipids	Glycerolipids
eicosenoate (20:1n9 or 11)	Lipids	Free fatty acid
oleate (18:1n9)	Lipids	Free fatty acid
palmitoleate (16:1n7)	Lipids	Free fatty acid
pentadecanoate (15:0)	Lipids	Free fatty acid
cytidine	Nucleotide	Pyrimidine metabolism
guanine	Nucleotide	Purine metabolism
uracil	Nucleotide	Pyrimidine metabolism
xanthosine	Nucleotide	Purine metabolism
gamma-glutamylalanine	Peptide	gamma-glutamyl

Table 3.3 PCA loading plot revealed metabolites that show significant differences between samples from WT and CLAc under LD 12:12 condition.

Metabolites	Super Pathway	Sub Pathway
asparagine	Amino acid	Aspartate family (OAA derived)
aspartate	Amino acid	Aspartate family (OAA derived)
gamma-glutamyl-2-aminobutyrate	Amino acid	Glutathione metabolism
glutamate	Amino acid	Glutamate family (alpha-ketoglutarate derived)
glycine	Amino acid	Serine family (phosphoglycerate derived)
alpha-ketoglutarate	Carbohydrate	TCA cycle
fructose	Carbohydrate	Sucrose, glucose,fructose metabolism
phytol	Cofactors, Prosthetic Groups, Electron Carriers	Chlorophyll and heme metabolism
cis-vaccenate (18:1n7)	Lipids	Free fatty acid
heptanoate (7:0)	Lipids	Free fatty acid
margarate (17:0)	Lipids	Free fatty acid
palmitate (16:0)	Lipids	Free fatty acid
palmitate, methyl ester	Lipids	Free fatty acid
adenosine	Nucleotide	Purine metabolism
guanine	Nucleotide	Purine metabolism
inosine	Nucleotide	Purine metabolism
N6-carbamoylthreonyladenosine	Nucleotide	Purine metabolism
uracil	Nucleotide	Pyrimidine metabolism
xanthosine	Nucleotide	Purine metabolism
gamma-glutamylglutamine	Peptide	gamma-glutamyl
gamma-glutamylisoleucine*	Peptide	gamma-glutamyl
gamma-glutamylleucine	Peptide	gamma-glutamyl
gamma-glutamylmethionine	Peptide	gamma-glutamyl
gamma-glutamylphenylalanine	Peptide	gamma-glutamyl
gamma-glutamylthreonine*	Peptide	gamma-glutamyl
gamma-glutamyltryptophan	Peptide	gamma-glutamyl
gamma-glutamyltyrosine	Peptide	gamma-glutamyl
gamma-glutamylvaline	Peptide	gamma-glutamyl

In cyanobacteria, free fatty acids are mainly involved in lipid and membrane synthesis (Ruffing and Jones, 2012). Ruffing and coworkers reported that extra free fatty acids could damage the cell physiology in *S. elongatus* (Ruffing and Jones, 2012; Ruffing, 2013). For example, when *S. elongatus* was engineered to produce more free fatty acids, the permeability of cell membrane was increased, and photosynthesis was impaired (Ruffing and Jones, 2012). Furthermore, Ruffing also found that the level of reactive oxygen species (ROS) was significantly increased in strains that over-produced free fatty acids, indicating that these cells were under stress conditions (Ruffing, 2013). Although the production level of free fatty acids in CLAb may not be as high as that in the engineered strains, these over-produced molecules may still result in a stressful environment for CLAb cells.

Similarly to CLAb, some metabolites in the free fatty acid pathway were also over-expressed in CLAc (**Table 3.3** and **Fig. 3.9B**). In addition, 30% of the "aberrantly expressed" candidates of CLAc are gamma-glutamyl amino acids that belong to the peptide super pathway. Gamma-glutamyl amino acids are the products of amino acid gamma-glutamylation which is a process catalyzed by gamma-glutamyltranspeptidase (GGT). GGT is involved in glutathione (GSH) synthesis, and its function is conserved in a wide range of organisms from bacteria to mammals (Suzuki et al., 2007). GSH is an important antioxidant which prevents damages from ROS. Consistently, some metabolites involved in GSH metabolism were also among the candidates in both CLAb and CLAc (**Fig. 3.9**), indicating that GSH metabolism is affected by the disruption of the circadian

clock.

Besides the metabolites identified from the free fatty acid pathway and pathways involved in glutathione metabolism, candidates were also identified from many other pathways, but in relatively smaller degrees of difference (**Table 3.2**, **Table 3.3** and **Fig. 3.9**). It is possible that these pathways also contribute to the competition results, either directly or indirectly. Because the global metabolism is composed of numerous pathways and these pathways are all inter-connected, it is difficult to conclude whether or not specific pathways are critical for the competition at this moment. Experiments targeted to these pathways are in the planning stage.

Discussion

In this study, two questions are addressed. First, the two clock mutants, CLAb and CLAc that were out-competed by the WT under LD conditions, displayed different metabolic profiles from the WT, suggesting that the disruption of circadian clocks affected the metabolism, and that maladaptive metabolism of the clock mutants could be the potential mechanism of the competition. Second, the WT showed different metabolic profiles under LD vs. LL, indicating that constant light can over-ride the regulation of metabolism that is normally controlled by the circadian clock entrained to LD.

It has been more than a decade since scientists discovered that circadian clocks enhance the fitness of cyanobacteria in cyclic environments. The

underlying mechanism, however, remains elusive. Although several hypotheses have been proposed, none of them has been experimentally proven (Ma et al., 2013). Considering the fact that the cyanobacterial circadian clock regulates the transcription of almost all of the genes in the genome (Liu et al., 1995; Ito et al., 2009), we gradually realize that this competition phenomenon may be a global effect of the disruption of the circadian clock. In addition, an experiment designed to identify metabolites in the cyanobacterial culture medium discovered some differences between the WT and a clock mutant (data not shown), leading us to consider the global metabolic profiles of these strains.

As an ongoing project, results presented in this chapter open the window for us to reveal the mystery of the competition experiments. However, more work needs to done to fully address this question. For instance, in CLAb, the free fatty acid pathway displayed some abnormal production. Based on this observation, I hypothesize that the malfunction of the free fatty acid pathway in CLAb affects the metabolism of CLAb and reduces its fitness in under LD conditions. To test this hypothesis, first, I need to do a more specific experiment to confirm that this pathway is not functioning properly. An experiment targeted to specifically measure the metabolites in this pathway should be designed and conducted. Second, I would like to find a way to restore the normal metabolism back to the clock mutants, and then test if the competition effect can be eliminated or reversed. While the first step is relatively easy to conduct, the second step is challenging. Alternatively, I can test if the WT strain can display CLAb-like phenotypes when free fatty acid pathways are over-expressed.

In addition, studying period mutants could be another option. In the competition experiments, the WT out-competed not only the arrhythmic mutants, but also a long period mutant, (C28a, free running period ~ 30 hours) under LD 12:12 cycles. But when C28a was co-cultured with the WT under LD 15:15 cycles, it defeated the WT (Ouyang et al., 1998). Therefore, comparing to the arrhythmic mutants (CLAb and CLAc), it might be easier to manipulate the metabolism of WT vs. C28a by changing the light conditions between LD 12:12 (optimal for WT, non-optimal for C28a) and LD 15:15 (optimal for C28a, non-optimal for WT) to compare their metabolic profiles.

Methods

Sample collection

Samples were collected from the WT strain and two arrhythmic mutants CLAb (arrhythmic in LL from LL onset) and CLAc (damps in LL to arrhythmicity in 2-3 days). Cells were grown at LD 12:12 cycles for 4 days or until the OD_{750} value reached 0.5, then cultures were either kept in LD12:12 or released into LL conditions. 100ml cultures were collected at ZT0, ZT6, ZT12, ZT18 and ZT24 under LD 12:12 conditions or at CT12, CT18, CT24, CT30 and CT36 under LL conditions (only for WT). For each strain, samples from two parallel cultures were collected as duplicates. After sample collection, cells were immediately centrifuged at 4 ^{o}C in darkness for 15 mins at 4000 rpm, and cell pellets were immediately frozen by using liquid nitrogen and kept at -80 ^{o}C until the samples were processed.

Metabolomics measurement

Metabolites were measured in 40 samples including the WT strain, CLAb and CLAc by Metabolon, Inc. (Durham, NC, USA). To identify metabolites from samples, LC/MS and GC/MS were used by Metabolon. A total number of 182 metabolites were identified. Among these metabolites, 9 were excluded for further analysis due to missing values for more than 20% of the time points.

Statistical analysis and clustering

Raw and normalized LC/MS and GC/MS data was obtained from Metabolon. Statistical analysis and data visualization was performed by using R programming language with the packages “metabolomics” (De Livera and Bowne, 2013) and “gplots” (Warnes et al., 2014). ANOVA was applied to assess the statistical significance between duplicates or among samples from different genotypes and/or light conditions. To establish the metabolic profiling, PCA and hierarchical clustering were performed.

Identification of cycling metabolites

Cycling metabolites were identified by using the modified cosiner method (Kucho et al., 2005). In short, the normalized and natural log transformed LC/MS and GC/MS data was detrended linearly as described by Kucho et al.. Because I only have data in 24 hours, only metabolites cycling with a 24-hmy period were identified by fitting to the 24-hour cosine curve. Along with curve fitting, the peak time and amplitude were calculated as described (Kucho et al., 2005). I then

filtered the metabolites by two criteria: first, the cycling metabolites should have an error factor less than 0.2; second, the amplitude should be greater than 1.2.

Chapter IV

A New Kind of KaiC-based Biological Time Keeping Machanism in the Purple Bacterium *Rhodopseudomonas palustris* Strain TIE-1

Introduction

Circadian clocks are internal timing mechanisms that allow organisms to anticipate daily changes in the environment. In the cyanobacterium *Synechococcus elongatus* PCC 7942 (*S. elongatus*), the circadian clock is composed of three proteins, KaiA, KaiB and KaiC (Kondo et al., 1994), and it persists under constant conditions with a free-running period of about 24 hours. Although circadian clocks are ubiquitous among eukaryotes, in the domain of bacteria, cyanobacteria are the only phylum in which circadian clocks have been conclusively demonstrated (Kondo et al., 1993; Kondo et al., 1994; Liu et al., 1995; Johnson et al., 1996; Ito et al., 2009; Johnson et al., 2011; Kitayama et al., 2013). Homologs of *kaiB* and *kaiC* genes have been identified in many other bacteria and archaea (Dvornyk et al., 2003), thus suggesting that circadian clocks or similar timing mechanisms might exist in these microorganisms. In addition, studies in *S. elongatus* have clearly demonstrated that the circadian clock confers an adaptive value under light-dark (LD) cycles (Ouyang et al., 1998; Woelfle et al., 2004; Ma et al., 2013). One would expect that not only cyanobacteria, but other bacteria and archaea living under daily cycles, especially those performing photosynthesis, would also benefit from a timing mechanism to adapt to these daily changes. Therefore, it is reasonable to predict

that there should be other bacteria and/or archaea possessing a timing mechanism besides cyanobacteria.

Among non-cyanobacteria prokaryotes whose genome include *kaiB* and *kaiC* homologs, the purple non-sulfur bacterium *Rhodopseudomonas palustris* (*R. palustris*) attracted our attention due to its ability to perform photosynthesis. Unlike cyanobacteria, *R. palustris* is an anoxygenic phototrophic bacterium that belongs to alpha-proteobacteria, and it uses different electron donors during photosynthesis (Larimer et al., 2004; Jiao et al., 2005). Oxygen actually inhibits its growth and many other activities such as photosynthesis and nitrogen fixation (Larimer et al., 2004; Jiao et al., 2005). Despite this difference, *R. palustris* shares many similarities with cyanobacteria. First, it is as widely distributed as cyanobacteria, and in nature, it frequently shares the same locations with some cyanobacteria, but at a different depth of water or sand (Proctor, 1997; Larimer et al., 2004). A case where purple bacteria are located together with cyanobacteria is the photosynthetic mats near coastal areas, as can be seen in **Fig.4.1A**. Cyanobacteria reside in the top layer of the mat underneath a layer of sands to avoid high light intensities. Purple bacteria can be found under the cyanobacteria where concentrations of oxygen are relatively low (Stal, 1995). Second, *R. palustris* uses sunlight as the energy source, indicating that some of its physiological activities are regulated by light. Therefore, being able to anticipate light/dark changes could be adaptive.

Along with the presence of *kaiB* and *kaiC* homologs in *R. palustris* (*kaiB*^{Rp}

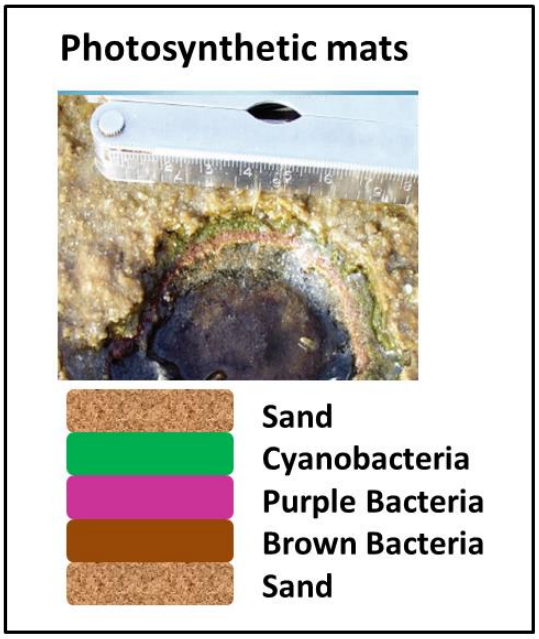
and *kaiC*^{Rp} thereafter) (Dvornyk et al., 2003; Larimer et al., 2004; Jiao et al., 2005), these lines of evidence suggest that *R. palustris* might have evolved a timing mechanism. In this study, I explored this possibility by measuring the nitrogen fixation activities of *R. palustris* under LD cycles and in constant light (LL) conditions. Furthermore, the function of *kaiC*^{Rp} was investigated in both *S. elongatus* and *R. palustris*. I report here that *R. palustris* possesses a timing mechanism regulated by *kaiC*^{Rp}, and that this timing mechanism might be an "hour glass" timer rather than a circadian clock. Consistent with the results in *S. elongatus*, this timing mechanism enhances the fitness of *R. palustris* under LD cycles but not LL conditions.

Results

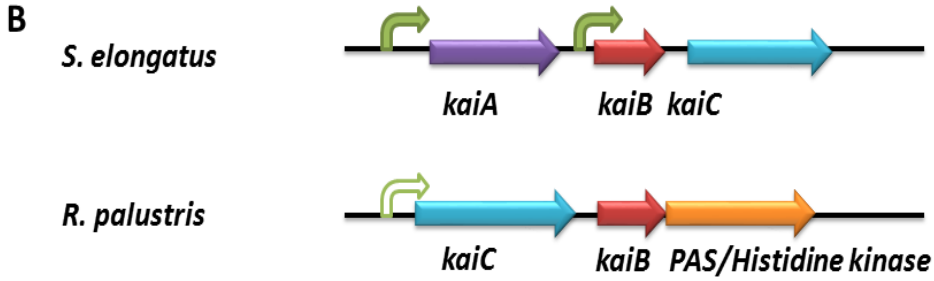
Homologs of *kaiB* and *kaiC* were identified in *R. palustris*

The three clock genes of *S. elongatus*, *kaiA*, *kaiB* and *kaiC*, (*kaiA*^{Se}, *kaiB*^{Se} and *kaiC*^{Se} thereafter) are essential for generating circadian rhythms in cyanobacteria (Kondo et al., 1994). In *R. palustris*, however, only *kaiB*^{Rp} and *kaiC*^{Rp} were identified, as depicted in **Fig.4.1B**. Instead of *kaiA*, a histindine kinase (*hk*^{Rp}) containing a PAS domain is closely located downstream of *kaiB*^{Rp} (**Fig. 4.1B**). It is not surprising that no *kaiA* exists in *R. palustris*; although *kaiC* and *kaiB* have been found across the bacteria and archaea domains, *kaiA* has only been identified in cyanobacteria (Dvornyk et al., 2003). The presence of *hk*^{Rp}

A


Photosynthetic mats
Sand
Cyanobacteria
Purple Bacteria
Brown Bacteria
Sand

B


S. elongatus
kaiA
kaiB
kaiC
R. palustris
kaiC
kaiB
PAS/Histidine kinase

C

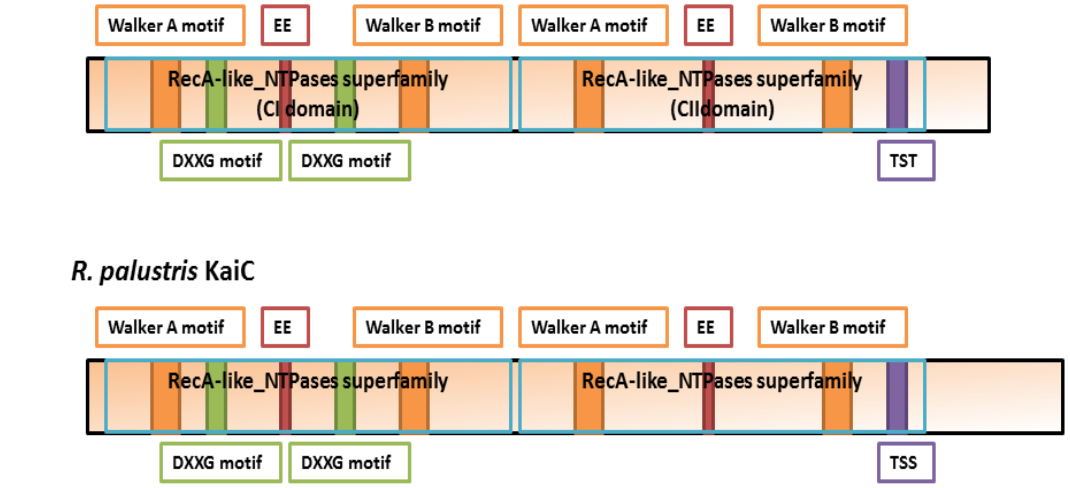
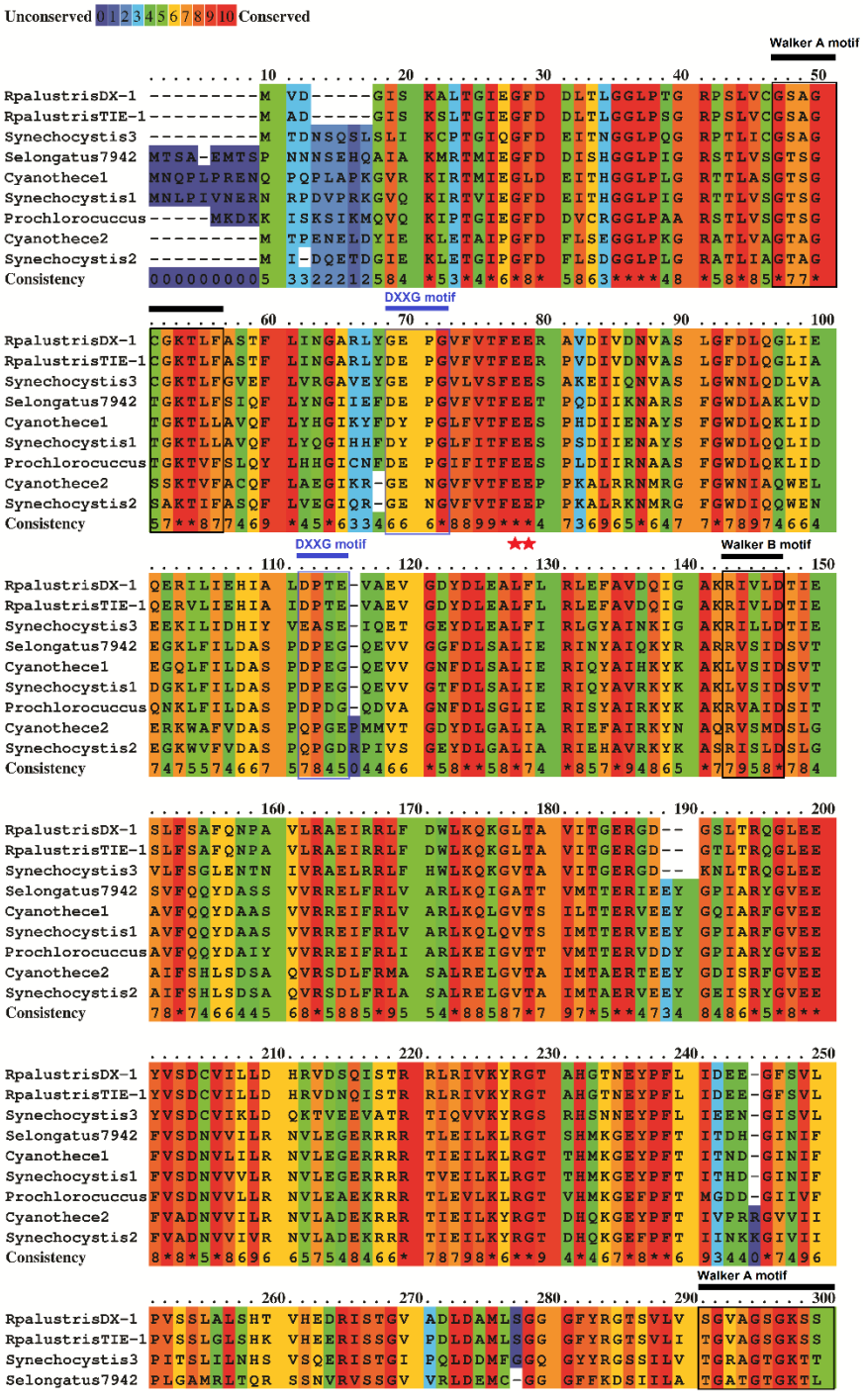

S. elongatus KaiC
Walker A motif
EE
Walker B motif
Walker A motif
EE
Walker B motif
RecA-like_NTPases superfamily (CI domain)
RecA-like_NTPases superfamily (CII domain)
DXXG motif
DXXG motif
TST
R. palustris KaiC
Walker A motif
EE
Walker B motif
Walker A motif
EE
Walker B motif
RecA-like_NTPases superfamily
RecA-like_NTPases superfamily
DXXG motif
DXXG motif
TSS

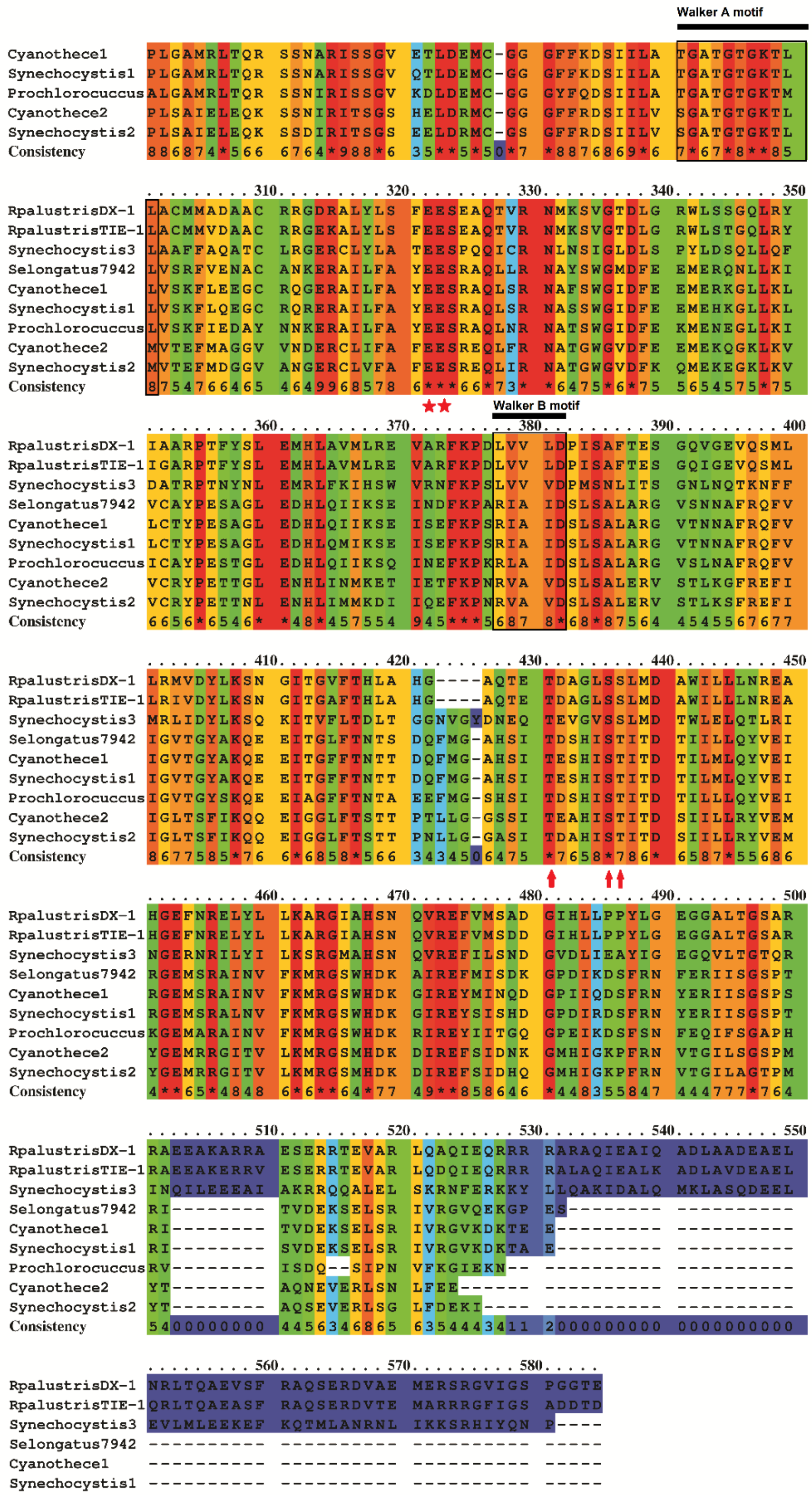
Figure 4.1 Homologs of *kaiC* and *kaiB* are identified in *R. palustris*. A, comparison of the *kaiABC* locus in *S. elongatus* with the *kaiBC* locus in *R. palustris*. In *S. elongatus*, *kaiB* and *kaiC* are co-transcribed by the same promoter. *KaiA* is located upstream of *kaiB* and is separately transcribed by another promoter (Ishiura et al., 1998). No *kaiA* was identified in *R. palustris*. Instead of *kaiA*, a histidine kinase containing PAS domain is located downstream of *kaiB*. Sequences for the primary sigma factor RpoD binding (rpoD promoter) were predicted to be the upstream of *kaiC* in *R. palustris*. B, comparison of predicted secondary structures of *S. elongatus* KaiC and *R. palustris* KaiC. Both of the KaiC proteins contain two RecA-like NTPase superfamily domains. In each of these domains, a Walker A and a Walker B motif is included as well as the EE residues that are the ATPase activity sites. In the first RecA-like NTPase superfamily domain (CI domain for *S. elongatus*), two DXXG motifs are present. Phosphorylation sites are located in the second RecA-like NTPase superfamily domain (CII for *S. elongatus*). In *S. elongatus*, these three phosphorylation sites are present as TST, while in *R. palustris* they are TSS. The C-terminus of *R. palustris* KaiC is about 50 amino acids longer than the *S. elongatus* KaiC.

suggests the possibility that *kaiC*^{Rp} functions through interactions with *kaiB*^{Rp} and *hk*^{Rp}. Promoter prediction suggested that a binding site of the primary sigma factor RpoD (Gruber and Gross, 2003) is present 17 bp upstream of *kaiC*^{Rp} (**Fig. 4.1B**), while no promoter was predicted upstream of *kaiB*^{Rp} or *hk*^{Rp}, indicating that these three genes might be coordinately transcribed.

The KaiC protein of *S. elongatus* (KaiC^{Se}) contains two RecA-like NTPase superfamily domains that are named the CI domain and the CII domain (Johnson et al, 2011). Each of these domains has an ATP/GTP-binding site known as the Walker motif, as well as catalytic carboxylates (EE) that are recognized to be the basis of autokinase and ATPase activity (Egli, et al., 2012). In addition, the CI domain possesses two DXXG motifs for GTP binding (Nishiwaki, et al., 2000). The sequence of the KaiC protein of *R. palustris* (KaiC^{Rp}) preserves all of the conserved domains of *S. elongatus* KaiC, as illustrated in **Fig.4.1C** and **Fig.4.2**. The C-terminus of KaiC^{Rp}, however, is about 50 amino acids longer than the KaiC^{Se}, and one of the phosphorylation sites of KaiC^{Se}, Thr432 (Xu et al., 2004), is present as a serine (**Fig. 4.1C** & **Fig.4.2**). Previous studies in cyanobacteria have shown that the C-terminal region of KaiC is essential for KaiA binding (Pattanayek et al., 2006; Vakonakis and LiWang, 2004). Given the fact that there is no *kaiA* identified in *R. palustris*, it is not surprising that the C-terminus is not conserved between KaiC^{Se} and KaiC^{Rp}.



Results colour-coded for amino acid conservation



Walker A motif
Walker B motif
Cyanothece1
Synechocystis1
Prochlorocuccus
Cyanothece2
Synechocystis2
RpalustrisDX-1
RpalustrisTIE-1
Synechocystis3
Selongatus7942
Consistency

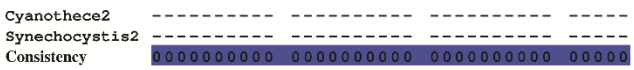


Figure 4.2 Alignment of KaiC proteins in some cyanobacteria species and two purple bacteria species. The Walker motifs are highlighted with black boxes. The DXXG motifs are highlighted with blue boxes. Red arrows indicate the phosphorylation sites, and the EE sites are labeled with red stars.

In addition to *S. elongatus*, *kaiABC* - driven timing mechanisms have also been described in other cyanobacteria (Kucho et al., 2005; Wiegard et al., 2013; Axmann et al., 2009; Holtzendorff et al., 2008; Gaudana et al., 2013; Cerveny et al., 2013). *Synechocystis sp.* Strain PCC 6803 (*Synechocystis*), for instance, has a circadian clock that regulates 2-9% of the genes in the genome under both cyclic and constant conditions (Kucho et al., 2005).Unlike *S. elongatus*, *Synechocystis* possesses three *kaiC*, two *kaiB* and one *kaiA* genes (Kucho et al., 2005; Wiegard et al., 2013). Interestingly, as shown in **Fig.4.2** and **Fig.4.3**, protein sequence alignment and phylogenetic analysis of KaiC showed that the KaiC (*Synechocystis1*) located together with KaiB and KaiA is grouped with KaiC of *S. elongatus*, and that the KaiC (*Synechocystis3*) located with KaiB alone is phylogenetically closer to $KaiC^{Rp}$ than any other cyanobacterial KaiC. Moreover, this KaiC (*Synechocystis3*) shares similar sequence and length with $KaiC^{Rp}$ in the C-terminus as well as the same phosphorylation sites (**Fig. 4.2**), indicating that this version of *kaiC* experienced horizontal gene transfer events and that it may contribute to the timing mechanism in a similar way to $KaiC^{Rp}$. In contrast, *Prochlorococcus*, a marine cyanobacterium with only *kaiB* and *kaiC*, and *Cyanothece sp.* ATCC 51142 with two *kaiC*, two *kaiB* and one *kaiA*, are clustered within the cyanobacteria group (**Fig.4.3**). The PAS domain histidine kinase is only identified in the *R. palustris* group (**Fig.4.3**), suggesting that hk^{Rp} may have a specific function in purple bacteria.

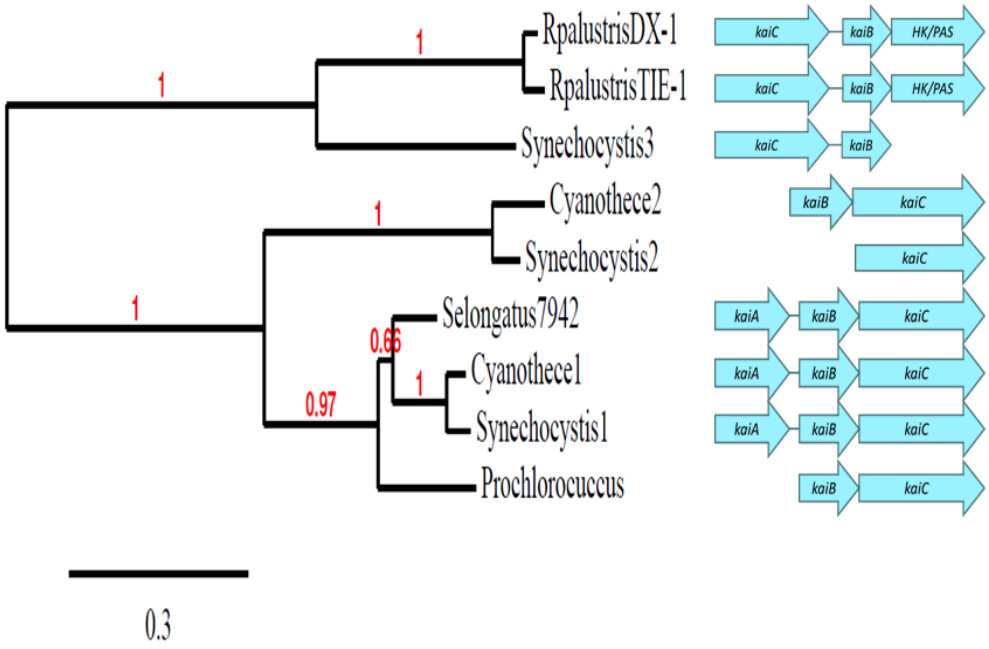


Figure 4.3 Phylogenetic tree of KaiC proteins in some cyanobacteria species and two purple bacteria species. Among these KaiC proteins, three copies are from *Synechocystis sp.* PCC 6803 (*Synechocystis* 1, 2 & 3), and two copies are from *Cyanothece sp.* ATCC 51142 (*Cyanothece* 1 & 2). The others have only one copy of *kaiC*. Along with *kaiC*, *kaiB*, *kaiA* or *hk* genes are illustrated in the blue arrows to compare the difference among these species. The *hk* genes are only identified in the purple bacteria, while *kaiA* are only present among cyanobacteria.

KaiC^{Rp} can influence the circadian clock of *S. elongatus*, but it is not sufficient to compensate the function of KaiC of *S. elongatus*

To investigate the function of KaiC^{Rp}, I first tested whether KaiC^{Rp} can influence the molecular clock of *S. elongatus* by expressing *kaiC^{Rp}* in the wild-type *S. elongatus* strain (AMC149) as well as the *kaiC*-deletion strain (DeltaC). AMC149 and DeltaC harbor luminescence reporters that allow clock-controlled promoter activities to be represented by luminescence levels (Kondo et al., 1993; Xu et al., 2000). AMC149 displays roughly 24-hour circadian rhythms under LL conditions, while DeltaC is arrhythmic due to the lack of *kaiC* (Kondo et al., 1993; Xu et al., 2000). When *kaiC^{Rp}* was expressed in AMC149 under the control of the *trc* promoter (Xu et al., 2003) by IPTG induction, the free running period of the luminescence rhythm was lengthened by about 1 hour, and the amplitude was reduced(**Fig. 4.4A** & **B**), suggesting some interference to the cyanobacterial circadian clock by KaiC^{Rp}. The over-expressing strain exhibited a slightly longer free running period even without IPTG induction, probably due to leaky expression from the *trc* promoter.

Moreover, an interesting phenomenon was observed when *kaiC^{Rp}* was overexpressed in the DeltaC strain. As can be seen in **Fig. 4.4C**, a transient peak was elicited in DeltaC by expressing *kaiC^{Rp}*, whereas DeltaC alone only showed declining luminescence over time. This peak was not observed in the absence of IPTG, even though a stronger reporter activity was detected (**Fig. 4.4C**), possibly due to leaky expression of *kaiC^{Rp}*.

As reported previously, overexpression of *kaiC*^{Se} in *S. elongatus* abolished the circadian rhythms of AMC149, and reestablished circadian rhythms in DeltaC at a critical concentration of IPTG (Xu et al., 2000). Overexpression of *kaiC*^{Rp}, however, only moderately influenced the circadian clock of *S. elongatus*, indicating that some differences between these two KaiC proteins are critical for functions in their corresponding species. Given the fact that no *kaiA* exists in *R. palustris*, the phenotypes observed here are reasonable. On one hand, KaiC^{Rp} is presumably not competent to interact with KaiA^{Se}; on the other hand, other factors in *R. palustris*, such as Hk^{Rp}, may be essential for KaiC^{Rp} function. Taken together, these results indicate that KaiC^{Rp} can partially interact with clock components (in AMC149) and thereby disrupt the normal system. Moreover, in DeltaC, KaiC^{Rp} can partially rescue the clock, but cannot fully restore clock function in *S. elongatus*.

Nitrogen fixation rhythms of *R. palustris* are regulated by *kaiC*^{Rp} under LD conditions

If KaiC^{Rp} drives a timing mechanism in *R. palustris*, we would expect that some physiological rhythms could be observed under either LD or LL conditions. The first circadian rhythm observed in cyanobacteria was that of nitrogen fixation (Grobbelaar et al., 1986). The methods to measure it, i.e., the acetylene reduction assay, are well-established and broadly applied (Hardy and Knight, 1967; Hardy et al., 1968). To test if there is a daily timing mechanism in *R. palustris*, nitrogen fixation activities of *R. palustris* were characterized under 12-

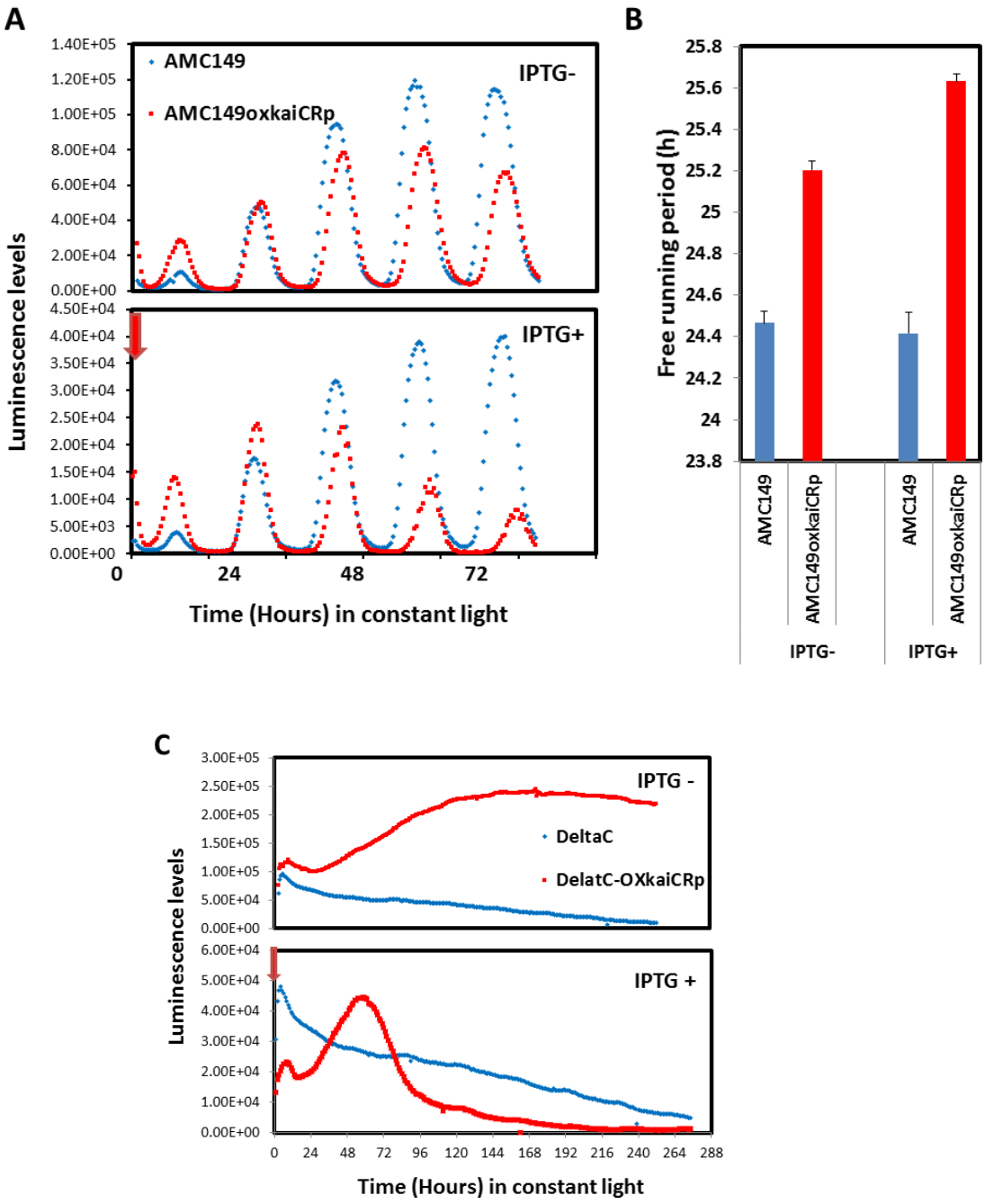


Figure 4.4 Luminescence rhythms of *S.elongatus* strains overexpressing of *kaiC*^{Rp}. A, *kaiC*^{Rp} was expressed in the wild type AMC149. Upper panel, no IPTG induction; lower panel, IPTG was applied to the cultures after entrainment. Comparing to the rhythms of AMC149 (blue dots), the luminescence rhythm was suppressed and the FRP was lengthened when *kaiC*^{Rp} was overexpressed (red dots). B, the quantification of FRPs in AMC149 (blue) and AMC149ox*kaiC*^{Rp} (red) strains with and without IPTG induction. C, *kaiC*^{Rp} was overexpressed in the arrhythmic strain DeltaC. Upper panel, no IPTG induction; lower panel, IPTG was applied to the cultures after entrainment. Red arrows indicate the time that IPTG was applied to the cultures.

hour-light-12-hour-dark cycles (LD 12:12). As shown in **Fig. 4.5A**, nitrogen fixation of the wild-type *R. palustris* (WT) exhibited an oscillating pattern under LD cycles at 30 °C. Each trace represents an individual culture. Nitrogen was mainly fixed during the day time, and this activity was shut down at night. At around Zeitgeber Time (ZT) 8, all of the individual wild-type cultures reached their maximal nitrogen fixation rates.

The observed oscillation could be one of the physiological activities controlled by a timing mechanism, whereas it is also possible that it is merely a response to light-dark signals. To distinguish between these two possibilities, a *kaiC*^{Rp}-deletion strain of *R. palustris* (RCKO) was generated. Surprisingly, the nitrogen fixation of RCKO showed similar fluctuations as the WT under LD condition except that the timing of the peaks varied dramatically among individual cultures (**Fig. 4.5B**). Quantitative RT-PCR further confirmed that no mRNA of *kaiC*^{Rp} was detected in the RCKO strain, and *kaiB*^{Rp} was transcribed normally as in WT (**Fig. 4.6A**). Furthermore, restoring a FLAG –tagged *kaiC*^{Rp} back to the RCKO genome rescued the wild-type phenotype (**Fig. 4.6B&C**). Taken together, these results indicate that deletion of *kaiC*^{Rp} resulted in a large increase in the variability of the nitrogen fixation peak of RCKO.

While *R. palustris* is usually cultured at 30 °C under laboratory conditions, it sometimes lives at lower temperatures in its natural environment (Jiao et al., 2005). To test if nitrogen fixation rhythms can be observed at lower temperatures, nitrogen fixation assays of the WT and RCKO strains were performed at 23°C

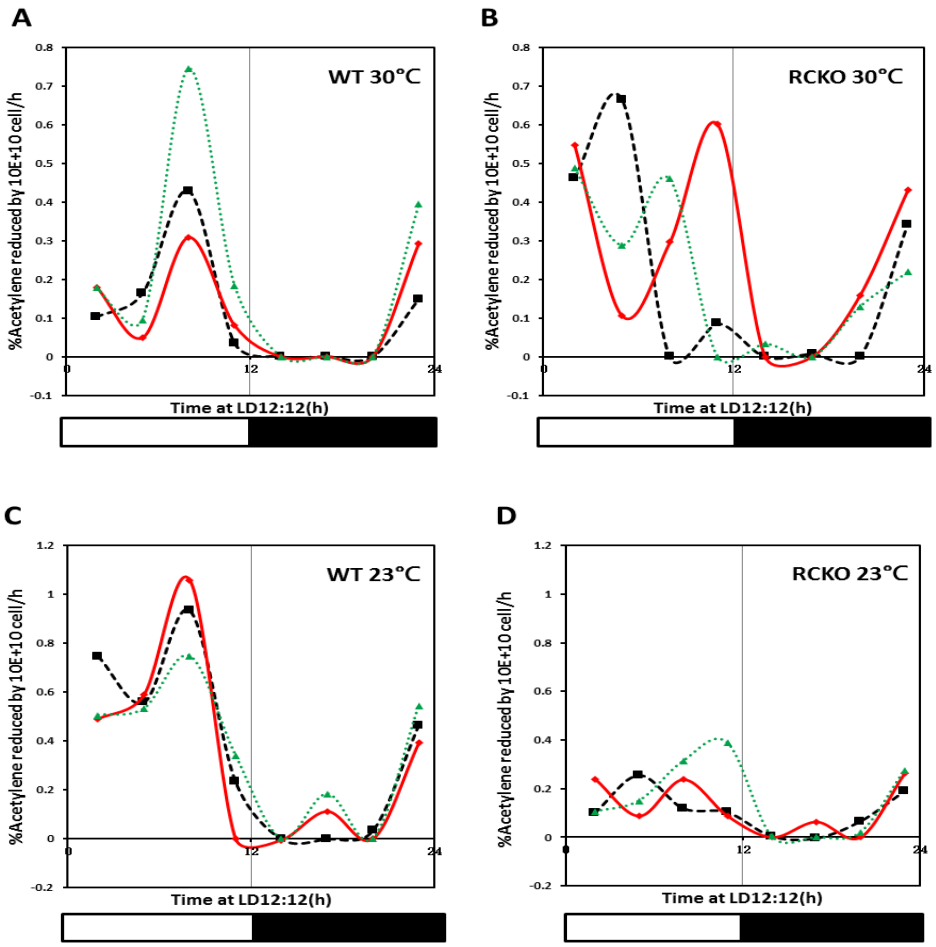


Figure 4.5 Nitrogen fixation rhythms of *R. palustris* under LD cycles. Samples were taken every 3 hours and incubated for 3 hours with acetylene under the same culture conditions. After 3 hours incubation, they were injected into GC to measure the acetylene reduction rates. A and C, nitrogen fixation rhythms of the wild-type *R. palustris* (WT) at 30 ^{o}C and 23 ^{o}C. Three traces represent three individual cultures. B and D, nitrogen fixation rhythms of the *kaiC*^{Rp}-deletion strain (RCKO) at 30 ^{o}C. The black and white bars underneath represent the light conditions.

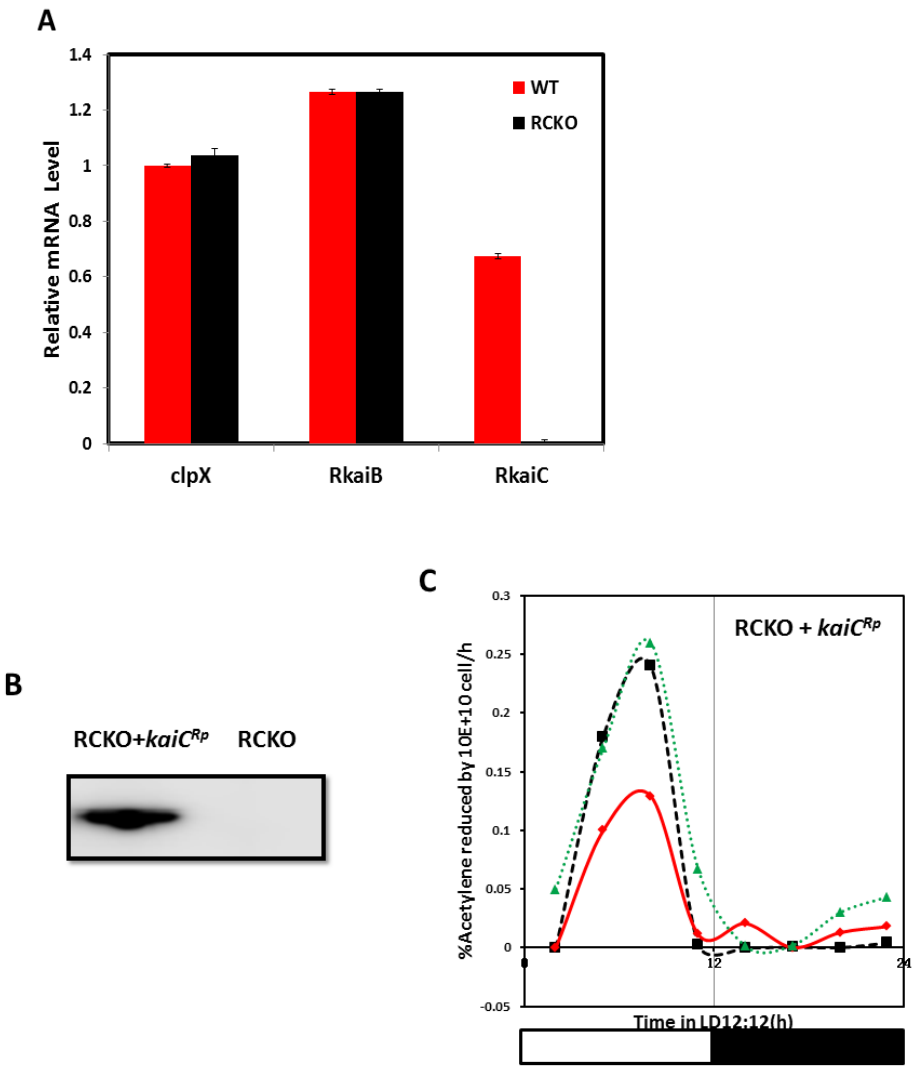


Figure 4.6 A, Quantitative PRC to confirm the deletion of *kaiC*^{Rp} and the expression of *kaiB*^{Rp}. The mRNA levels of *clpX* were included as the internal control. The mRNA levels of *kaiB*^{Rp} and *kaiC*^{Rp} were quantified in the WT (red) and RCKO (black) strains. B, western blot by anti-FLAG antibody to confirm the expression of KaiC^{Rp} in the rescue strain (RCKO+ *kaiC*^{Rp}). C, nitrogen fixation of the kaiCRp rescue strain under LD 12:12 cycles at 30 ^{o}C. Three traces represent three individual cultures.

under LD 12:12 conditions. Similar to the results obtained at 30°C, at 23°C nitrogen fixation rhythms of several WT cultures were in-phase with each other (**Fig. 4.5C**), whereas the individual RCKO cultures differed from each other(**Fig. 4.5D**). Furthermore, the total nitrogen fixation activities of the WT strain were two times higher than that of the RCKO strain at 23°C, suggesting some overall impact to the physiology of RCKO at low temperatures.

One of the fundamental properties of circadian clocks is that they can be entrained by environmental cues, whereby physiological processes adjust their periods and phases according to the external cycles (Dunlap et al., 2004). The stable phase angle observed in the WT strain suggests that WT is entrained to the environmental cycles, thus indicating that a timing mechanism exists in *R. palustris*, and that *kaiC*^{Rp} plays an important role in it.

Nitrogen fixation of *R. palustris* does not show clear rhythms under LL conditions

A timing mechanism could be a sophisticated circadian clock that persistently oscillates under constant conditions, or it could be an hour glass operating only under cyclic environments and which damps rapidly in constant conditions. As a continuation of the assays performed under LD cycles, the nitrogen fixation rates were measured from cells cultured under LL conditions to test if the rhythm persists without environmental signals. As shown in **Fig. 4.7A**, at 30°C, the apparent nitrogen fixation rhythm of the WT continued for two short-period cycles and then it damped out. The modified cosiner method (Kucho et al.,

2005) was applied to the data to statistically evaluate whether they conform to being an oscillation. Usually two criteria have to be satisfied for a rhythm: first, the error factor (Ef) of the curve fitting needs to be less than 0.2; second, the relative amplitude should be greater than 1.2. As can been seen in **Table 4.1**, the best fitting curve with the smallest Ef value (Kucho et al., 2005; see methods) of the WT data was the curve with a 19-hour cycle and the relative amplitude is 15.33. However, this Ef value is significantly greater than 0.2, suggesting that these data may not fit the criteria to qualify as a rhythm.

For the RCKO strain, possible oscillations with large variations were also observed at 30 °C. Similar to the WT, these data do not fulfill both of the two criteria, suggesting that no rhythm was detected in the RCKO strain at 30 °C. At 23°C, apparently no rhythm was detected in the RCKO strain (**Fig. 4.7B and Table 4.1**). In the WT strain, the nitrogen was fixed cyclically, but these data also failed the statistical test (**Fig. 4.7B and Table 4.1**).

Circadian rhythms usually exhibit periods that are nearly 24 hours in constant conditions. The nitrogen fixation rhythm of the WT strain, however, is not only significantly shorter than 24 hours, but also damped out after two cycles. These results indicate that the timing mechanism of *R. palustris* could be a highly damped circadian oscillator or possibly an hour glass that does not keep the rhythms running under constant conditions. However, it is also possible that the

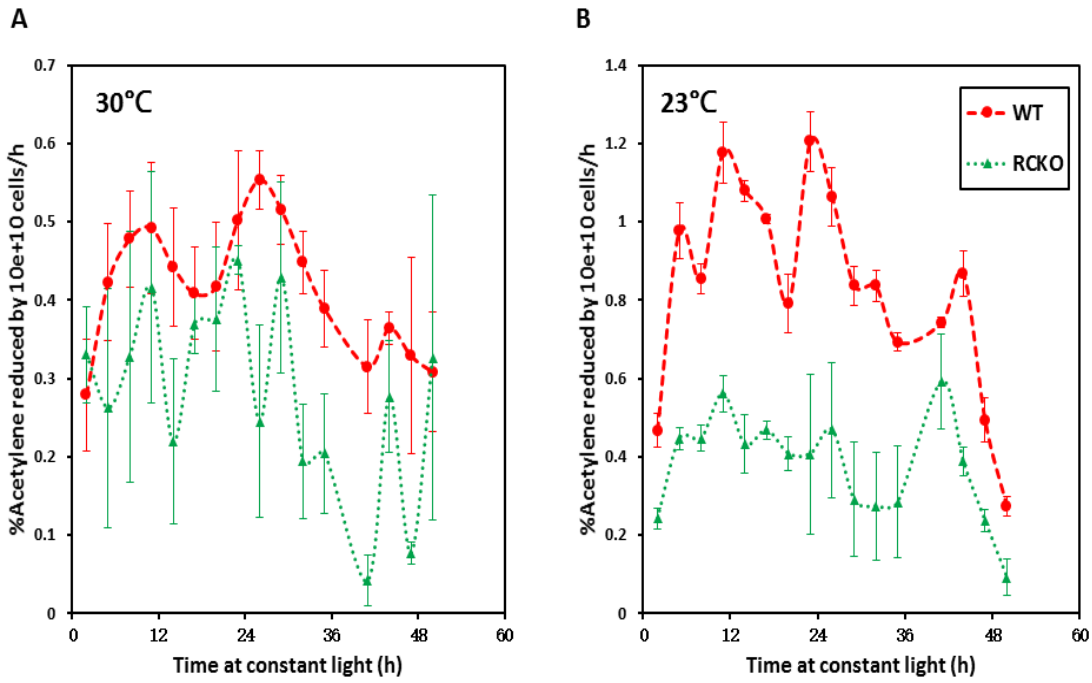


Figure 4.7 Nitrogen fixation activities of *R. palustris* under LL conditions. The WT strain and the RCKO strain were cultured under LD 12:12 cycles for 3 to 4 days, then they were released to LL condition. Under LL condition, samples were taken every 3 hours and incubated for 3 hours with acetylene under the same culture conditions before they were injected into GC to measure the acetylene reduction rates. A, nitrogen fixation activities of the WT (red) and RCKO (green) strains at 30 °C. B, nitrogen fixation activities of the WT (red) and RCKO (green) strains at 23 °C. Error bars were plotted as the standard deviation from three individual cultures (n=3).

Table 4.1 Statistical analysis of the nitrogen fixation data under LL conditions. Ef, error factor of the cosine curve fitting; Amp, relative amplitude; Peak, peak time calculated by curve fitting. The number after each category, e.g, Ef (24h), means the period of the cosine curve used for the fitting.

	Ef (24h)	**Ef (22h)**	**Ef (20h)**	**Ef (19h)**	**Ef (18h)**	**Ef (17h)**	**Ef (16h)**	**Ef (14h)**	**Ef (12h)**
WT(30 °C)	4.19	3.45	2.85	2.69	2.75	3.11	3.74	4.99	4.54
RCKO(30 °C)	0.04	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.03
WT(23 °C)	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.04
RCKO(23 °C)	0.13	0.12	0.11	0.11	0.10	0.09	0.09	0.08	0.07
	Amp (24h)	**Amp (22h)**	**Amp (20h)**	**Amp (19h)**	**Amp (18h)**	**Amp (17h)**	**Amp (16h)**	**Amp (14h)**	**Amp (12h)**
WT(30 °C)	16.00	15.84	15.91	15.33	14.08	12.29	10.37	8.36	9.19
RCKO(30 °C)	1.06	1.06	1.05	1.04	1.04	1.04	1.04	1.04	1.05
WT(23 °C)	1.06	1.06	1.07	1.06	1.06	1.06	1.05	1.05	1.04
RCKO(23 °C)	1.11	1.07	1.06	1.09	1.12	1.15	1.18	1.12	1.12
	Peak (24h)	**Peak (22h)**	**Peak (20h)**	**Peak (19h)**	**Peak (18h)**	**Peak (17h)**	**Peak (16h)**	**Peak (14h)**	**Peak (12h)**
WT(30 °C)	21.54	0.09	2.43	3.54	4.65	5.78	6.97	19.58	24.00
RCKO(30 °C)	13.43	14.49	15.38	15.72	15.98	16.17	16.33	6.91	6.00
WT(23 °C)	15.65	17.56	19.56	20.62	21.72	22.88	0.12	2.90	6.00
RCKO(23 °C)	14.78	14.69	9.07	7.91	7.32	7.00	6.82	6.51	6.00

damping rhythms are caused by some technical issues. The exponentially growing cells, for instance, may be entering the stationary phase where only a small amount of nitrogen is required, or the nitrogenase could be inhibited after fixing adequate nitrogen to the medium. Additionally, no clear patterns were observed in the RCKO strain, although some fluctuations were detected. These uncertainties leave this question unsolved.

The timing mechanism driven by KaiC^{Rp} enhances fitness of *R. palustris* under LD cycles.

Several studies in cyanobacteria have demonstrated that the *kaiC*-driven circadian clock enhances fitness under cyclic environments (Ouyang et al., 1998; Woelfle et al., 2004; Ma et al., 2013). To test whether this adaptive value of *kaiC* extends to *R. palustris*, growth rates of the WT and RCKO strains were measured under LD 12:12 cycles and LL conditions at 30°C and 23°C. As shown in **Fig. 4.8A&C**, at both temperatures, the RCKO strain grew in LL at about the same rate as did WT. However, a dramatic difference in their growth rates was observed when they were cultured under LD conditions (**Fig.4.8B&D**); the WT strain grew significantly faster than the RCKO strain. Furthermore, when a FLAG-tagged *kaiC^{Rp}* was restored into the RCKO genome, the rescued strain showed similar growth rates as the WT strain under both LL and LD conditions (**Fig. 4.8**), confirming that the reduced growth rate of RCKO is caused by the absence of *kaiC^{Rp}*. Taken together, these results suggest that a KaiC^{Rp}-dependent timing

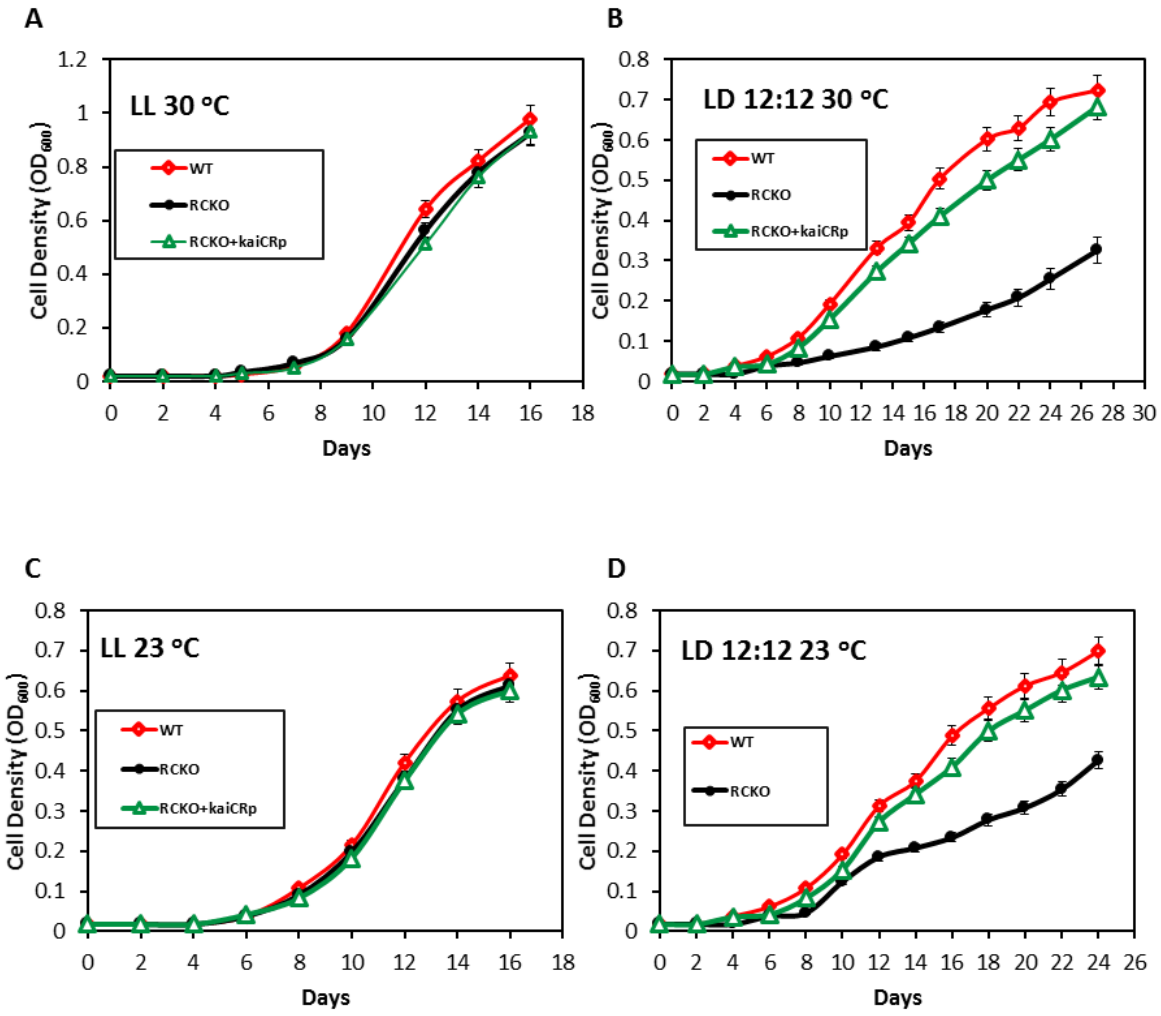


Figure 4.8 Growth curves of *R. palustris* under LL or LD 12:12 conditions. A, cell densities (OD_{600}) of the WT (red), RCKO (black) and the *kaiC*^{Rp} rescue strain (green) were measured every two days under LL condition at 30 °C. B, cell densities of the WT (red), RCKO (black) and the *kaiC*^{Rp} rescue strain (green) were measured every two days under LD 12:12 cycles at 30 °C. The OD_{600} value was plotted as the function of time (days).

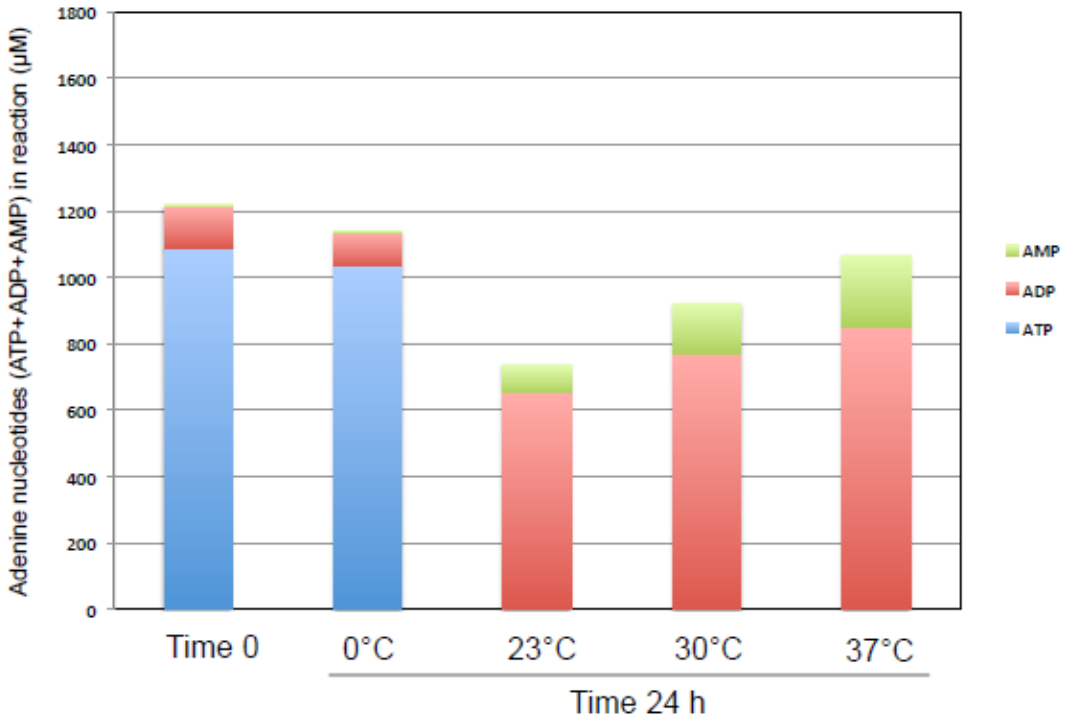


Figure 4.9 ATPase activity of KaiC^{Rp} *in vitro*. Purified KaiC^{Rp} was incubated with ATP at 0 ºC, 23 ºC, 30 ºC and 37 ºC for 24 hours. Then the amount of ATP/ADP/AMP was quantified by HPLC. Blue, ATP; red, ADP; green, AMP.

mechanism confers an adaptive value under cyclic conditions but not constant conditions.

KaiC^{Rp} displays ATPase activity *in vitro*

It is believed that the phosphorylation rhythm of KaiC along with the interactions of KaiA and KaiB is the basic mechanism of circadian clock in *S. elongatus* (Nakajima et al., 2005; Kageyama et al., 2006). Additionally, the ATPase acitivities of KaiC^{Se} was reported (Terauchi et al., 2007), and several studies suggested that three activities of KaiC^{Se} (ATPase, autokinase and autophosphatase) are coupled and that they are essential for generating circadian rhythms in *S. elongatus* (Kitayama et al, 2013).

The protein sequence alignment of KaiC suggested that the ATPase sites are highly conserved among different species (**Fig. 4.2**), indicating that the ATPase activity may also play an essential role for the function of KaiC in other bacteria. My results have demonstrated that KaiC^{Rp} drives a timing mechanism in *R. palustris*, but the biochemical basis of this timing mechanism remains elusive. Considering the importance of ATPase activity for KaiC function in *S. elongatus*, I hypothesize that KaiC^{Rp} has ATPase activity.

To test this hypothesis, *kaiC^{Rp}* was expressed in *E. coli* and the protein was purified. The purified KaiC^{Rp} was incubated with ATP at 0 ^{o}C, 23 ^{o}C, 30 ^{o}C and 37 ^{o}C for 24 hours, and then the reaction was terminated and measured by High-performance liquid chromatography (HPLC). As shown in **Fig. 4.9**, time 0

represents the initial composition of the reaction. After a 24-hour incubation at 0^{o}C, no ADP/AMP production was detected, while at 23 ^{o}C , 30 ^{o}C and 37 ^{o}C, all of the ATP was converted to ADP/AMP, suggesting that KaiC^{Rp} confers ATPase activities at these temperatures. Interestingly, compared to KaiC^{Se}, the ATPase activity of KaiC^{Rp} is at least 10 times higher (Terauchi et al., 2007). Taken together, these results indicate that KaiC^{Rp} is also an ATPase with a relatively high activity. Whether or not this ATPase activity plays a role in Kai^{Rp} function needs to be confirmed in future experiments

Discussion

Circadian clock vs. hour glass

A circadian clock is a sophisticated biochemical machinery whose oscillations persist even without external cues (Dunlap et al., 2004). An hour glass, on the contrary, is dependent on regular environmental stimulations to restart it every day, and it will not cycle without daily stimulation. Both a circadian clock and an hour glass need to be entrained by environmental signals to establish a stable phase angle (Rensing et al., 2001). In this study, the nitrogen fixation rhythms with a stable phase angle under LD12:12 cycles provide evidence for the presence of a timing mechanism in *R. palustris*, but it damped out under LL conditions after oscillating for two 18-hour cycles, indicating that this timing mechanism is more likely to be an hour glass that can trigger a second cycle or a highly damped oscillator rather than a persisting circadian clock. In *S. elongatus*, KaiA is essential for a functioning clock (Kondo et al., 1994), therefore

the lack of kaiA may prohibit *R. palustris* to form a self-sustained circadian clock.

Similar to *R. palustris*, the marine cyanobacterium *Prochlorococcus* only harbors *kaiB* and *kaiC* homologs, and possibly it possesses only an hour glass as the timing mechanism instead of a circadian clock (Holtzendoff et al., 2008; Axmann et al., 2009). *Prochlorococcus* cells are mainly found in the open ocean from the surface to depths of 100 to 200 meters, and they have not been found at high latitudes (Mullineaux and Stanewsky, 2009; Partensky et al., 1999). In contrast, *S. elongatus* inhabits fresh water where environmental changes such as shading due to vegetation or shifting sediments may be more dramatic and unpredictable than in the ocean areas (Mullineaux and Stanewsky, 2009). One can imagine that having a robust circadian clock will be beneficial when the environmental signals are not stable. In other words, a circadian clock will keep the physiological activities and gene expressions running normally when the environmental conditions are inconsistent. Therefore, for freshwater cyanobacterium *S. elongatus* an hour glass may be not sufficient. Although *R. palustris* is a freshwater bacterium, it is usually found at the bottom of ponds or under the cyanobacteria in mats to avoid stresses from oxygen and high light intensities(Proctor, 1997; Jiao et al., 2005). In these two conditions, environmental changes may be relatively consistant as compared with shallow areas, thus suggesting that an hour glass might be capable of maintaining normally phased daily activities of *R. palustris*.

Nevertheless, we still cannot exclude the possibility that a circadian clock

exists in *R. palustris*. First, the damping rhythms may be due to the culture conditions. For instance, I entrained these cells for at least 3 days and then monitored the nitrogen fixation activities for another 3 days under constant conditions. After being cultured for 6 days, cells may have grown to the stationary phase where the nitrogen fixation activity is not as robust as it in the exponential phase. To solve this problem and keep cells in the exponential stage, continuous cultures may be required for this experiment. Second, nitrogen fixation may not oscillate under constant conditions, but there may be other physiological activities or gene expressions that do oscillate persistently under constant conditions. To solve this problem, another assay needs to be developed. For example, microarrays could be applied to measure gene expressions in *R. palustris* under LD and LL conditions. Additionally, in *S. elongatus*, luminescence reporters are applied to track the circadian rhythms, allowing frequent sampling without interrupting the cell growth (Kondo et al., 1993). But neither this reporter nor other fluorescence reporters can be applied to *R. palustris* cultures because luminescence assays use luciferase, whose activity is dependent upon oxygen and no oxygen is present in our cultures. Recently, some luminescence reporters that do not require oxygen were published (Mukherjee et al., 2013). If we could apply these reporters to *R. palustris* cultures, we would be able to better test if this timing mechanism is a circadian clock or not.

Adaptive significance of timing mechanisms

In this study, we found that deletion of *kaiC*^{Rp} reduced the fitness of *R.*

palustris under LD cycles, suggesting that a *kaiC*-mediated timing mechanism confers adaptive value to the purple bacterium *R. palustris*. This discovery is consistent with the studies done in cyanobacteria (Ouyang et al., 1998; Woelfle et al., 2004), therefore indicating that the adaptive significance of timing mechanisms may be a universal property among different organisms. Interestingly, the timing mechanisms of *R. palustris* and *S. elongatus* are only adaptive under cyclic conditions. Under constant conditions, the wild type strains and the mutant strains grew equivalently in both *S. elongatus* and *R. palustris*, indicating that timing mechanisms are "extrinsic" adaptations rather than "intrinsic" adaptations in these two species (Woelfle and Johnson, 2009; Ma et al., 2013).

Diversity of KaiC

Bioinformatics studies showed that *kaiC* is widely distributed across the bacterial and archaeal domains (Dvornyk et al., 2003). Based on the sequence alignment (**Fig. 4.2**), most of the important domains are conserved among these KaiC, while some small differences are present (**Fig. 4.2**). I am curious how these small differences contribute to the discrepancies between their timing mechanisms. For instance, in my study, I found that the $KaiC^{Rp}$ has a longer C-terminus. In *S. elongatus*, the C-terminus of $KaiC^{Se}$ is critical for interacting with KaiA. Given the fact that there is no KaiA present in *R. palustris*, we would expect that this long tail of $KaiC^{Rp}$ may interact with other proteins or prevent $KaiC^{Rp}$ from interacting with other proteins. In addition, it is still a puzzle that some

organisms possess multiple *kaiC* genes. For example, the freshwater cyanobacterium *Synechocystis sp*. PCC 6803 has three copies of *kaiC* and one of its KaiC versions shares a similar long C-terminus structure with $KaiC^{Rp}$. For almost 20 years, comprehensive studies focused on the *S. elongatus* circadian system have been conducted, providing valuable knowledge about the *S. elongatus* KaiC (Johnson et al., 2011). However, little is known about KaiC in other bacteria and archaea. Along with some work done in other cyanobacteria (Kucho et al., 2005; Wiegard et al., 2013; Axmann et al., 2009; Holtzendorff et al., 2008; Gaudana et al., 2013; Cerveny et al., 2013), my study will help us to understand the diversity of KaiC as well as the diversity of microbial timing mechanisms, which will hopefully reveal the evolution of timing systems in bacteria and archaea.

Methods

Bacterial strains and culture conditions

All bacterial strains used in this study are listed in **Table 4.2**. *E.coli* was grown in Luria-Bertani (LB) broth at 37°C with shaking, and if necessary, gentamicin (50µg/ml) was supplemented. For photoheterotrophic growth, *R. palustris* strains were grown in Freshwater-Base (FW) medium (Jiao et al., 2005) supplied with 20mM sodium acetate, 50mM sodium bicarbonate, 20mM MOPS (PH7.2), 1mM potassium phosphate, 1mM sodium sulfate, multivitamin solution and trace elements solution. The medium was aliquoted into sealed serum bottles and the headspace was flushed with N_2 gas for 20 min before inoculating

cells. N_2 gas is used as the sole nitrogen source to ensure nitrogenase expression. The cultures were maintained at 30 °C (or 23 °C as specified) and illuminated by a cool-white fluorescent lamps (40-50 μE m^{-2} s^{-1}) with shaking at 120 rpm. For aerobic chemoheterotrophic growth, *R. palustris* strains were grown in YP medium containing 0.3% yeast extract and 0.3% peptone (Jiao et al., 2005) at 30 °C with shaking at 120 rpm, and if necessary, gentamicin (400μg/ml) was supplemented. The cyanobacterial strains were grown in BG-11 medium (Bustos and Golden, 1991) at 30 °C, and illuminated by cool-white fluorescence bulbs (40-50 μE m^{-2} s^{-1}) with air bubbling. The medium of AMC149 and DeltaC was supplemented with spectinomycin (25μg/ml), and the medium of the *kaiC*^{Rp} overexpressing strains was supplemented with spectinomycin (25μg/ml) and kanamycin (25μl/ml). For growth on solidified media, LB, FW or BG-11 medium was supplemented with 1.5% agar and appropriate antibiotics.

Construction of the *kaiC*^{Rp} deletion strain

All plasmids and primers used in strain constructions are listed in **Table 4.3** and **Table 4.4**. The *kaiC*^{Rp} deletion strain (RCKO) was constructed in *R. palustris* TIE-1 strain by overlap extension PCR and conjugation (Hirakawa et al., 2011, Bose and Newman, 2011). To delete the *kaiC*^{Rp} gene, the 1 kb upstream region and the 1 kb downstream region of the *kaiC*^{Rp} ORF were cloned from the genomic DNA of *R. palustris* TIE-1 strain and fused by overlap extension PCR. The 2-kb DNA fragment was ligated with the suicide vector pJQ-200KS (Quandt and Hynes, 1993). The resultant plasmid, pJQ-200KS-RCKO, was transformed

into *R. palustris* TIE-1 strain by conjugation with *E. coli* S17-1 (Simon et al., 1983; Hirakawa et al., 2011; Bose and Newman, 2011). The integration of the plasmid in either the upstream or the downstream regions of the *kaiC*^{Rp} locus was selected by gentamicin resistance and screened by PCR. Following the selection, the integrants were grown in non-selective YP medium for several generations and then plated on YP agar medium with 10% sucrose (Hirakawa et al., 2011) to induce double recombination. Among the survivors of the sucrose-YP medium, the double recombinants were selected by PCR screening. The deletion of the *kaiC*^{Rp} gene was confirmed by sequencing and Q-RT-PCR.

Construction of the FLAG-*kaiC*^{Rp} strain

To complement the *kaiC*^{Rp} gene deletion, a FLAG-tagged *kaiC*^{Rp} gene was restored to the genome of RCKO strain in the region surrounding the *glmUSX-recG* locus (Bose and Newman, 2011). The FLAG tag allows me to confirm the expression of KaiC^{Rp} in the rescued strain with an anti-FLAG antibody. To construct the insertion plasmid pJQ200KS-Insert, the 1 kb upstream and the 1 kb downstream regions of *glmUSX-recG* locus of *R. palustris* TIE-1 were cloned and fused by overlap extension PCR. A *NcoI* site was incorporated in the middle to allow the insertion of genes of interest (Bose and Newman, 2011). The resulting DNA fragment was ligated with pJQ200KS by *Sph*I and *Sma*I, as described by

Table 4.2 Bacterial strains used in this study.

Strain	Genotype and use	Reference
E.Coli S17-1	A conjugative donor strain [*thi pro hdsR hdsM+ recA*; chromosomal insertion of RP4-2 (Tc::Mu Km::Tn7)].	(Simon et al., 1983; Hirakawa et al., 2011)
R. palustris TIE-1	Served as the wild type strain of *R. palustris* TIE-1.	(Jiao et al., 2005)
RCKO	$kaiC^{Rp}$ knockout strain (1674bp deleted from $kaiC^{Rp}$ gene in *R. palustris* TIE-1)	This study
RCKO-FLAG$kaiC^{Rp}$	A FLAG-tagged $kaiC^{Rp}$ restored into the genome of RCKO.	This study
RCKO-HA$kaiC^{Rp}$	A HA-tagged $kaiC^{Rp}$ restored into the genome of RCKO.	This study
AMC149	Wild- type cyanobacterium *S. elongatus* with a luminescence reporter(*psbAIp::luxAB*).	(Kondo et al., 1993)
DeltaC	*S.elongatus* with an in-frame deletion in *kaiC gene* and a luminescence reporter(*psbAIp::luxAB*). Arhythmic strain.	(Xu et al., 2000)
AMC149ox$kaiC^{Rp}$	AMC149 overexpressing $kaiC^{Rp}$.	This study
DeltaCox$kaiC^{Rp}$	DeltaC overexpressing $kaiC^{Rp}$.	This study

Table 4.3 Plasmids used in this study.

Plasmid	Description	Reference
pJQ200KS	Mobilizable suicide vector; *sacB*, Gm^r	(Quandt and Hynes, 1993; Hirakawa et al., 2011)
pJQ200KS - RCKO	Overlap extension PCR was used to amplify homologous regions of 1-kb upstream and 1-kb downstream of $kaiC^{Rp}$ locus, then the 2-kb fragment was ligated with Pjq200ks by *BamHI* and *XbaI*. This plasmid was used to delete $kaiC^{Rp}$ gene in the wild-type *R. palustris.* Gm^r	This study
pJQ200KS -Insert	Overlap extention PCR was used to amplify the 2 kb region surrounding the *glmUSX-recG* locus of *R. palustris* TIE-1, a NcoI site was incorporated into the middle of this PCR fragment which was ligated to pJQ- 200KS by *Sph*I and *Sma*I, as described by Bose and Newman. This plasmid was used to restore tagged $kaiC^{Rp}$ gene into the RCKO strain. Gm^r	(Bose and Newman, 2011) This study
pJQ200KS – Insert-$PkaiC^{Rp}$	A 469-bp region upstream of $kaiC^{Rp}$ gene locus in *R. palustris* was amplified as the promoter region of $kaiC^{Rp}$ gene. This PCR fragment was ligated to the *NcoI* site of pJQ200KS – Insert. A *NdeI* and a *XbaI* sites were incorporated to the downstream of the promoter region. Gm^r	This study
pJQ200KS – Insert - $PkaiC^{Rp}$ - $FLAGkaiC^{Rp}$	$kaiC^{Rp}$ gene was amplified from the wild type *R. palustris*. A FLAG tag was incorporated into the N-terminus or the C-terminus. This PCR fragment was ligated to the downstream of promoter retion of $kaiC^{Rp}$ gene in pJQ200KS – Insert-$PkaiC^{Rp}$. This construct was used to restore FLAG-tagged $kaiC^{Rp}$ gene to the RCKO strain. Gm^r	This study
pJQ200KS – Insert - $PkaiC^{Rp}$ - $HAkaiC^{Rp}$	$kaiC^{Rp}$ gene was amplified from the wild type *R. palustris*. A HA tag was incorporated into the N-terminus or the C-terminus. This PCR fragment was ligated to the downstream of promoter retion of $kaiC^{Rp}$ gene in pJQ200KS – Insert-$PkaiC^{Rp}$. This construct was used to restore HA-tagged $kaiC^{Rp}$ gene to the RCKO strain. Gm^r	This study
Ptrc NSII	A vector containing homologous regions of the neutral site II of *S. elongatus* and an IPTG- inducible *Trc* promoter. Kan^r	(Xu et al., 2013)
Ptric NSII-$kaiC^{Rp}$	$kaiC^{Rp}$ gene was amplified from the wild-type *R. palustris* and ligated to Ptric NSII by *NdeI* under the control of IPTG- inducible *Trc* promoter. This plasmid was transformed into the cyanobacterial strains AMC149 and DeltaC to overexpress $kaiC^{Rp}$ gene in *S. elongatus*. Kan^r	This study

Table 4.4 Primers used in this study.

Primer	Sequence	Description/ Reference
CupF	ATAGGATCCACAGCTCGCCTCGCGGACCGGAA	1-kb upstream of *kaiC*^{Rp} gene
CupR56	TATGGCAATCGTGTGGATGGAGTCCGCCATCGCACCACAGG	1-kb upstream of *kaiC*^{Rp} gene
CdownF56	TCCATCCACACGATTGCCATAACGGACTAGGGGACGACGAT	1-kb downstream of *kaiC*^{Rp} gene
CdownR	AAATCTAGACGCGCTGCGACAGATCGACCAGG	1-kb downstream of *kaiC*^{Rp} gene
TIE-1upfor	GGCGCGCCGCATGCCACACCGGCAGGTTGTTGATGGCTG	(Bose and Newman, 2011)
TIE-1fusionrev(new)	CGGGTTAGTTACCACGCGTCATTACTAGTTCGCGACCATGGCTACCCGACCT TGTCCGGCGCCTTTTC	(Bose and Newman, 2011)
TIE-1dnrev	ACTAGTCCCGGGCGAGATCGATTTTCTGGTCGGCAC	(Bose and Newman, 2011)
TIE-1fusionfor(new)	GAAAAGGCGCCGGACAAGGTCGGGTAGCCATGGTCGCGAACTAGTAATGAC GCGTGGTAACTAACCCG	(Bose and Newman, 2011)
R10(PkaiF)	GCGGCTAGCCCATGGCGTCACGTCGCGCTTTGC	promoter region of *kaiC*^{Rp} gene
R30(PrkaicR2)	CTGACCATGGTCTAGACATATGCGCACCACAGGTCGTTTTCTGA	Promoter region of *kaiC*^{Rp} gene
RCup	ATGCAACATATGGCGGACGGCATATC	Forward primer of *kaiC*^{Rp} gene
RCdown	ATGCAATCTAGACTAGTCCGTGTCATCGGC	Reverse primer of *kaiC*^{Rp} gene
FlagCup	ATGCAACATATGGACTATAAGGACGACGACGACAAGGCGGACGGCATATC	Forward primer of *kaiC*^{Rp} gene
FlagCdown	ATGCAATCTAGACTACTTGTCGTCGTCGTCCTTATAGTCGTCCGTGTCATCG GC	Reverse primer of *kaiC*^{Rp} gene
HACup	ATGCAACATATGTATCCCTATGACGTGCCCGACTATGCGGCGGACGGCATAT C	Forward primer of *kaiC*^{Rp} gene
HACdown	ATGCAATCTAGACTACGCATAGTCGGGCACGTCATAGGGATAGTCCGTGTCA TCGGC	Reverse primer of *kaiC*^{Rp} gene
RCNdeF	ATACATATGagcATGGCGGACGGCATATC	Forward primer of *kaiC*^{Rp} gene
RCNdeR	ATACATATGagCTAGTCCGTGTCATCGGCCG	Reverse primer of *kaiC*^{Rp} gene

Bose and Newman (Quandt and Hynes, 1993; Bose and Newman, 2011). To include the native promoter region of the *kaiC*^{Rp} gene in the insertion plasmid, a 469-bp region upstream of the *kaiC*^{Rp} gene was cloned and ligated to the *NcoI* site of pJQ-200KS-Insert, resulting in pJQ200KS-Insert-PkaiC^{Rp}. A *NdeI-XbaI* site was incorporated downstream of the promoter region to allow the insertion of genes of interest. The *KaiC*^{Rp} gene was cloned from genomic DNA of the *R. palustris* TIE-1 strain, while a FLAG tag was fused to its N-terminus. This DNA fragment was ligated with the plasmid pJQ200KS-Insert-PkaiC^{Rp} by *NdeI* and *XbaI* where the FLAG- *kaiC*^{Rp} gene is under the control of the *kaiC*^{Rp} promoter. The resultant plasmid, pJQ200KS-Insert-PkaiC^{Rp}-FLAGkaiC^{Rp}, was then transformed into RCKO strain by conjugation with *E.coli* S17-1 (Simon et al., 1983; Hirakawa et al., 2011; Bose and Newman, 2011). The following selection procedures are similar to the construction of *kaiC*^{Rp} deletion strain. The integration of FLAG-kaiCRp was confirmed by sequencing and western blot.

Construction of *kaiC*^{Rp} overexpression strains in cyanobacterium *S. elongatus*

The *kaiC*^{Rp} gene was cloned from genomic DNA of *R. palustris* TIE-1 strain and ligated with the plasmid Ptrc-NSII by *NdeI* (Xu et al., 2013). The resulting plasmid, Ptrc-NSII-kaiC^{Rp}, was transformed into cyanobacterial strains including the wild-type AMC149 (Kondo et al., 1993) and an arhythmic strain DeltaC (Xu et al., 2000). By transformation, the *kaiC*^{Rp} gene was incorporated into the Neutral Site II of the genome of AMC149 and DeltaC under the control of

an IPTG-inducible promoter *trc* (Xu et al., 2013), resulting in two corresponding *kaiC*^{Rp} overexpression strains AMC149ox*kaiC*^{Rp} and DeltaCox*kaiC*^{Rp}. The *kaiC*^{Rp} overexpression strains inherit the *psbAIp::luxAB* luminescence reporter located on the Neutral Site I of AMC149 and DeltaC (Kondo et al., 1993; Xu et al., 2000).

Growth curve experiment

Batch liquid cultures of *R. palustris* strains including the wild-type *R. palustris* TIE-1, the *kaiC*^{Rp} knockout strain RCKO, and the *kaiC*^{Rp} complementation strain RCKO-FLAG*kaiC*^{Rp} were grown anaerobically in FW or YP medium. For growth curve experiments and doubling time calculations, cultures were grown at 30 °C or 23 °C with shaking under either LL conditions or LD 12:12 conditions. Seed cultures of these strains were grown anaerobically in FW medium under LL before inoculation. Growth was monitored by measuring the optical density (OD) at 600nm. OD_{600} was then plotted against time for a comparison of growth among strains. The growth constant *k* was generated by fitting exponential curves to the growth curves at log scale, and doubling time was calculated as this equation: doubling time (h) = (ln(2)/k) × 24.

Nitrogenase activity measurement

Nitrogenase activity of *R. palustris* was measured by acetylene reduction assay as described elsewhere (Hardy et al., 1973). Assays were carried out in sealed serum bottles containing the anaerobic cultures under LL or LD 12:12 conditions. 10% Acetylene (final concentration) was injected into the headspace

and incubated with cells under light (40-50 μE m^{-2} s^{-1}). For time-course experiments, samples were taken every 3 hours for 1 LD 12:12 cycle and/or 2 days (48 hours) in LL. 500μl gas in the headspace was analyzed by a gas chromatograph (Shimadzu GC-2010 Plus) fitted with a flame ionization detector and a Rt-Alumina BOND/MAPD PLOT column (Restech, PA). The temperatures of the injector, detector, and oven were 200°C, 200°C, and 130°C, respectively.

Phylogenetic analysis

The amino acid sequences of KaiC proteins of selected cyanobacterial species and purple non-sulfur bacterial species were retrieved from the NCBI GenBank. The phylogenetic tree for KaiC was constructed using Phylogeny.fr web service integrated MUSCLE alignment, Gblocks curation and PhyML method (Dereeper et al., 2010; Dereeper et al., 2008; Edgar, 2004; Castresana, 2000; Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006; Chevenet et al., 2006). The KaiC sequences were aligned using PRALINE multiple sequence alignment program with Homology-extended alignment strategy (Simossis et al., 2005; Heringa, 2000; Heringa, 2002; heringa, 1999; Simossis and Heringa, 2003; Simossis and Heringa, 2005).

Measuring luminescence rhythms in cyanobacterial strains

Cyanobacterial *kaiC*^{Rp} overexpressing strains, AMC149ox*kaiC*^{Rp} and DeltaCox*kaiC*^{Rp} , were cultured on BG-11 solid medium supplemented with appropriate antibiotics. Before measuring luminescence rhythms, toothpick

colonies of these strains were grown at 30 °C for two LD-cycles. After the cells were released to LL, the *trc* promoter inducer IPTG was added to the final concentrations of 0, 5, 10, 100, 250, 500 and 1000 μM to induce the expression of *kaiC*^{Rp}. Then the agar plates containing these strains were placed on a home-made luminescence monitoring machine to measure the luminescence rhythms for 5-7 days at 30 °C and constant light conditions (40-50 μE m^{-2} s^{-1}). The wild-type cyanobacterium AMC149 and the *kaiC*-deletion strain DeltaC were also included on the agar plates as controls.

Quantitative reverse-transcription PCR

RNA was isolated from 3-5 ml exponential growing cells with a NucleoSpin RNA II kit (Clontech). 300 ng of RNA was then used to synthesize cDNA by using the iScript cDNA synthesis kit (Bio-Rad). With 1ul of the synthesized cDNA as template, quantitative reverse-transcription PRC was conducted on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) by using iTaq SYBR green supermix (Bio-Rad). The program was running at 95 ^{o}C for 30s, followed by 40 cycles of 95 ^{o}C for 5s and 60 ^{o}C for 30s. A final melting curve was performed for each reaction to ensure that only a single peak was amplified. The primers were designed by using Primer3 web service (http://biotools.umassmed.edu/bioapps/primer3_www.cgi).

Immunoblot assay for FLAG-KaiC^{Rp}

The FLAG-*kaiC*^{Rp} strain was cultured under LD 12:12 cycles. When cell

density (OD_{600}) reached 0.4 in the exponential growth phase, 5 ml cells were collected every 3 hours for 1 LD 12:12 cycle and then the culture was released to LL where cell collection was continued for another 48 hours. After collection, cells were centrifuged at 4 ^{o}C, and cell pellets were immediately frozen by liquid nitrogen. Total protein was extracted from cell pellets resuspended in KaiC extraction buffer (Xu et al., 2000) by sonication, and the amount of total protein was quantified by the Lowry protein assay (Lowry et al., 1951). The extract was then separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10%) and transferred onto nitrocellulose membranes. The immunoblot was treated with FLAG antibody at 1:1000 dilutions and detected by using Pierce ECL Western Blotting Substrate (Thermo Scientific). The signal was captured by an Alpha Innotech gel image system (Alpha Innotech). Equal loadings were confirmed by Coomassie Brilliant Blue (CBB) staining in the gel, Ponceau Red staining on the membrane, and/or by the density of nonspecific bands on the immunoblots.

ATPase activity assay

The GST-tagged $KaiC^{Rp}$ protein was expressed and purified in DH5a *E.coli* cells following the protocol of expressing and purifying *S. elongatus* KaiC (Mori et al., 2007; Egli et al., 2012). GST fusion proteins were purified by affinity chromatography on glutathione-agarose resin (Pierce/Thermo Scientific) and cleaved from GST using human rhinovirus 3C protease. The proteins were further purified by ion-exchange chromatography on Q Sepharose with a gradient of NaCl. 1mM ATP was incubated with the purified protein (3.5uM) at 0 ^{o}C, 4 ^{o}C,

23 ^{o}C, 30 ^{o}C and 37 ^{o}C for about 24 hours. The hydrolysis of ATP by KaiCRp was quantified by using High-performance liquid chromatography (HPLC) as described (Sudo et al., 2000) by using a … HPLC column.

Statistical analysis

The modified cosiner method (Kucho et la., 2005) was used to determine if the data can be an oscillation/rhythm, or alternatively if they show non-cyclic variation. The nitrogen fixation data was first detrended linearly as described by Kucho et al.. And the detrended data was then fitted to a series of cosine curves with different periods ranging from 12 hours to 24 hours. Along with curve fitting, the peak time and amplitude were calculated as described (Kucho et al., 2005). An oscillation or rhythm was determined by two criteria: first, a true circadian rhythm should have an error factor (Ef) less than 0.2; second, the amplitude should be greater than 1.2.

Chapter V

Conclusions and Future directions

Conclusions

It was believed for a long time that prokaryotic organisms were not able to generate circadian rhythms due to the short doubling time and relatively simple cell structures (Edmunds, 1983; Kippert, 1987; Johnson et al., 1996). However, research from several groups have not only discovered that cyanobacteria (the most abundant prokaryotes on the earth) have circadian clocks, but also comprehensively demonstrated the molecular basis and the adaptive significance of cyanobacterial circadian clocks (Grobbelaar et al., 1986; Mitsui, 1986; Kondo et al., 1993; Kondo et al., 1994; Ishiura, 1998; Ouyang et al., 1998; Iwasaki et al., 2002; Woelfle et al., 2004; Johnson et al., 2008; Kitayama et al., 2013). Following these pioneer works, my dissertation mainly involves two studies: first, the potential mechanism by which the cyanobacteria circadian clock enhances fitness; second, the function of *kaiC*^{Rp} in the purple bacterium *Rhodopseudomonas palustris* (*R. palustris*) (to test if circadian clocks exist in another prokaryotic organism).

The mechanism by which the circadian clock system enhances fitness in cyanobacterium *Synechococcus elongatus* PCC 7942 (*S. elongatus*) cannot be explained by existing hypotheses

Circadian clocks are considered to be an adaptation to the daily cycles on the earth, and this theory has been rigorously tested in the cyanobacterium *S. elongatus* (Ouyang et al., 1998; Woelfle et al., 2004; **Chapter II**). Ouyang et al. (1998) reported that cyanobacterial strains whose free running periods (FRPs) were close to the environmental cycles were the most fit, as compared with strains whose FRPs were significantly longer or shorter than the environmental periods. Later, Woelfle et al. (2004) demonstrated the circadian clock-mediated fitness enhancement by competing the wild-type strain with arrhythmic mutants. In **Chapter II**, the clock-mediated fitness enhancement was tested in a variety of conditions, including on solid agar, in continuous cultures, and in a codon-optimized strain (OptkaiBC) (Xu et al., 2013). All of the results support the idea that cyanobacterial circadian clock confers adaptive significance under light-dark (LD) cycles within a physiological range of environmental conditions.

To explain the underlying mechanism of the clock-mediated fitness enhancement, several models have been proposed, including the “limiting resource model”, “diffusible inhibitor model” and “cell-cell communication model” (Woelfle and Johnson, 2009; Ma et al., 2013; **Chapter II**). Although numerous experiments have been done to test these models, none of them has been conclusively established. For example, when the WT strain and CLAb were

cultured and separated by a membrane which allows the diffusion of small molecules, no growth defect was observed in the CLAb culture under the competition conditions, as would have been predicted by the “diffusible inhibitor model.” In addition, no significant difference was observed in the initial growth rates between WT and CLAb, leaving the “limiting resource model” unconfirmed. Taken together, results in **Chapter II** suggested that the mechanism of the clock-mediated fitness enhancement cannot be explained by the existing hypotheses.

Metabolic profiles of the clock mutants are affected under LD cycles

In cyanobacteria, circadian clocks and metabolism form an autoregulatory feedback network. On one hand, circadian clocks regulate many metabolic processes; on the other hand, some metabolic processes may provide entraining information to the circadian clock (Rust et al., 2011; Kim et al., 2013). This relationship indicates that the coupling between circadian clock and metabolism may play an important role in cyanobacterial physiology. In **Chapter III,** I tested if the disruption of the circadian clock could alter metabolism in *S. elongatus*, and whether the metabolic status under the competition conditions can help us to understand the clock-conferred fitness.

As predicted, metabolic profiles of two clock mutants, CLAb and CLAc, displayed significant differences from the WT strain under LD cycles. Not only was the number of cycling metabolites largely reduced in these two mutants, but also abnormal production of metabolites were observed in some pathways, suggesting that the metabolic profiles were affected when the circadian clock is

disrupted. Especially in CLAb, some free fatty acids were over-expressed during the night, which might damage the cells and make this strain vulnerable. Although more work needs to be done to confirm that the altered metabolism contributes to the reduced fitness during the competition, these results definitely open a window for us to uncover the underlying mechanism of the clock-mediated fitness enhancement.

Metabolic profiles of the wild-type *S. elongatus* show dramatic difference in light-dark cycles vs. constant light (LL) condition.

For photosynthetic cyanobacteria, light is an essential factor that regulates their metabolism. Results in **Chapter III** demonstrated that ~40% of the metabolites in the WT strain were produced cyclically under LD cycles. Another question that I wanted to address was to test if constant light could over-ride the circadian regulation of the metabolism. To answer this question, the metabolic profiles of the WT strain were compared under different light conditions (LD vs. LL). Results in **Chapter III** showed that under LL conditions, the number of cycling metabolites was reduced, and that the global metabolic profiles was significantly different from that in LD cycles, indicating that the regulation by constant light exposure can over-ride the regulation by the circadian clock. Based on the results in the WT strain, it is possible that light also plays a dominant role in the regulation of metabolism in the arrhythmic mutants, which could be the reason that the WT strain only outcompetes CLAb or CLAc under LD cycles.

A new kind of timing mechanism driven by *kaiC^{Rp}* was discovered in the purple bacterium *R. palustris*

Although cyanobacteria are the only prokaryotic phylum in which circadian clocks have been conclusively demonstrated, one of the central clock genes, *kaiC*, has been identified in many other bacteria and archaea (Dvornyk et al., 2003). Little is known about the function of *kaiC* in these organisms. Considering the adaptive fitness conferred by the cyanobacterial circadian clock, it is reasonable to predict that other bacteria and/or archaea that inhabit cyclic environments, especially those performing photosynthesis, would also benefit from a daily timing mechanism. Therefore, in **Chapter IV**, I tested the hypothesis that the purple bacterium *R. palustris*, which possesses *kaiC* in its genome and also performs photosynthesis, has a timing mechanism driven by *kaiC^{Rp}*.

To test this hypothesis, a *kaiC^{Rp}* deletion strain (RCKO) was generated and nitrogen fixation activities were measured under LD cycles and in LL conditions. Under LD cycles, the nitrogen fixation activities of the wild-type strain (WT) were entrained to the environmental cycles and displayed a stable phase angle, while the RCKO strain did not exhibit a stable phase angle under LD cycles in its nitrogen fixation activity, indicating that a timing mechanism driven by *kaiC^{Rp}* might be present in *R. palustris*. However, in LL conditions, although some oscillations of nitrogen fixation activities were observed in both WT and RCKO, the rhythm was not confirmed to be a circadian rhythm by statistical

analysis, suggesting that this timekeeping mechanism is likely to be an “hour glass” timer or damped oscillator instead of a self-sustained circadian clock.

Moreover, consistent with the results in *S. elongatus*, data reported in **Chapter IV** confirmed that this *kaiC*^{Rp}-driven timing mechanism of *R. palustris* confers an adaptive value under LD cycles, indicating that the adaptive value of timing systems may be a universal feature.

Future Directions

Uncover the mechanism of the clock-mediated fitness enhancement by metabolic approaches

In **Chapter III**, I demonstrated that the metabolic profiles were affected when the circadian clock is disrupted, suggesting that circadian clocks regulate metabolism under LD conditions. While the altered metabolism may be the reason that these clock mutants were defeated by the WT strain during competition, more work needs to done to confirm this idea. First, targeted mass-spectrometry should be applied to confirm the identity of the metabolites that were produced abnormally. Second, once some pathways or specific metabolites are confirmed, they can be genetically manipulated in the WT strain to test if the competition could be affected. For example, some free fatty acids were over-expressed in CLAb. A competition experiment can be conducted between CLAb and an engineered WT strain that over-expresses the same free fatty acids. If the engineered “WT” strain no longer outcompetes CLAb, this result could indicate

that the over-production of free fatty acids could be involved in the mechanism of the competition experiment.

Besides focusing on these arrhythmic mutants (i.e., CLAb and CLAc), an alternative approach could use a long period mutant such that the metabolism can be manipulated by changing light conditions. Ouyang et al. (1998) reported that when the long period mutant C28a whose free running period is about 30 hours was co-cultured with the WT strain under LD 12:12 conditions, it was defeated by the WT strain. However, the WT strain was out-competed by C28a when the two strains were co-cultured under LD 15:15 conditions (Ouyang et al., 1998), indicating that both the WT and C28a have different metabolic profiles under different LD cycles. Therefore, if the metabolic profiles of C28a and WT can be characterized under LD 12:12 and LD 15:15 conditions, a clearer picture might be obtained to help us to understand how the circadian regulation of metabolism enhances the fitness of cyanobacteria.

The molecular basis of the timing system in *R. palustris*

The study in **Chapter IV** demonstrated that *R. palustris* has a timing mechanism driven by KaiC^{Rp}. The molecular basis of this timing system, however, remains elusive. In *S. elongatus*, both the phosphorylation status and the ATPase activity play essential roles in KaiC function (Kitayama et al., 2013). My preliminary result has shown that KaiC^{Rp} has ATPase activity, while the phosphorylation status of KaiC^{Rp} is unclear. Therefore, to further characterize this

new type of timing mechanism in *R. palustris*, the phosphorylation status of KaiC^{Rp} should be studied.

Besides *kaiC^{Rp}*, a homolog of *kaiB* is also present in *R. palustris*. Based on the essential role of *kaiB* in *S. elongatus*, I would predict that *kaiB^{Rp}* is also essential for this timing system. Because there is no *kaiA* present in *R. palustris*, the way that KaiB^{Rp} interacts with KaiC^{Rp} might be different from that in *S. elongatus*. *In vivo* and *in vitro* characterization of KaiB^{Rp} will be a critical step to fully understand the timing mechanism of *R. palustris*.

A functional timing system should be composed of three components: an input pathway, a pacemaker and an output pathway. In *S. elongatus*, each of these parts contains several genes and these genes work together to generate circadian rhythms (Ditty and Mackey, 2009). As the first step to study this new kind of timing mechanism in *R. palustris*, my work revealed only a small piece of information about the pacemaker. For future directions in the long term, more genes involved in this timing mechanism should be identified, which will hopefully help us to understand the evolution of microbial timing systems.

The diversity and evolution of microbial timing systems

After Dvornyk et al. (2003) reported that *kaiC* exists throughout eubacterial and archaeal species, many questions arose. What are the functions of *kaiC* in other bacteria? Is the *kaiC*-driven timing system ubiquitous among prokaryotic organisms? Why do some species have multiple copies of *kaiC*? My study in *R.*

palustris suggests that *kaiC*^{Rp} drives a new type of timing mechanism in *R. palustris*, but there are still numerous questions waiting to be answered.

Studies in the marine cyanobacterium *Prochlorococcus* indicates that this species may have only an “hour glass” timer, possibly due to the lack of *kaiA* (Holtzendorff et al., 2008; Axmann et al., 2009; Axmann et al., 2013). Together with the results in *R. palustris*, it is possible that *kaiA* is essential for a circadian clock but not for other kinds of timing systems. However, it is likely that the timing systems of *R. palustris* and *Prochlorococcus* are also quite different from each other. There is evidence showing that *Prochlorococcus* used to have a *kaiA* gene but lost it recently in the process of genome reduction (Axmann et al., 2009). In contrast, *R. palustris* possibly acquired *kaiB* and *kaiC* before the origin of *kaiA* in cyanobacteria (Mullineaux and Stanewsky, 2009).

Another interesting observation is that some organisms possess multiple copies of *kaiC*. In particular, the cyanobacterium *Synechocystis sp.* PCC 6803 (*Synechocystis*) possess three *kaiC* genes and one of them is phylogenetically closer to KaiC^{Rp} than any other cyanobacterial KaiC (**Chapter IV**). Kucho et al. (2005) reported that *Synechocystis* has a circadian clock that regulates 2-9% of the genes in its genome. However, little is known about the individual role that each KaiC plays in *Synechocystis*. Similar to *R. palustris*, *Synechocystis* is a metabolically versatile organism and they can both directly utilize some organic carbon sources from the environment (Anderson and McIntosh, 1991; Larimer et al., 2004). Therefore, it is possible that the KaiC^{Rp}-like KaiC in *Synechocystis*

functions under metabolic conditions that are different from *S. elongatus* while the KaiC^{Se}-like KaiC in *Synechocystis* functions similarly to KaiC in *S. elongatus*.

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