

# Precise Circadian Clocks in Prokaryotic Cyanobacteria

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## Abstract

**Prokaryotic cyanobacteria express robust circadian (daily) rhythms under the control of a timing mechanism that is independent of the cell division cycle. This biological clock orchestrates global regulation of gene expression and controls the timing of cell division. Proteins that may be involved in input pathways have been identified. Mutational screening has identified three clock genes that are organized as a gene cluster. The structure of cyanobacterial clock proteins, their phosphorylation, and regulation is described. A new model for the core clockwork in cyanobacteria proposes that rhythmic changes in the status of the chromosome underlie the rhythms of gene expression. Mixed-strain experiments demonstrate that this timekeeper confers adaptive value when different strains compete against each other.**

## Background: prokaryotes join the circadian club

Before 1985, it was believed that circadian programs were exclusively found among eukaryotes (Johnson *et al.*, 1996). The conclusion that only eukaryotes have circadian oscillators seemed reasonable, because it was assumed that an endogenous timekeeper with a period close to 24 h would not be useful to organisms that divide more rapidly than once every 24 h, as do many prokaryotes (Pittendrigh, 1993). However, two reports in 1986 on circadian rhythms in nitrogen fixing cyanobacteria overturned that confident assumption (Grobbelaar *et al.*, 1986; Mitsui *et al.*, 1986). Our laboratory and those of our collaborators—Drs. Takao Kondo, Susan Golden, and Masahiro Ishiura—extended the studies on circadian programming to cyanobacteria that were genetically malleable (Johnson *et al.*, 1996). Our first studies used a strain of *Synechococcus elongatus* PCC 7942 (previously named *Synechococcus* sp. strain PCC 7942) transformed with a luminescence reporter construct which is the fusion of the *psbAI* promoter with a bacterial luciferase cassette (*psbAIp::luxAB*; Kondo *et al.*, 1993). This strain of cyanobacteria is notable because of the ease in which exogenous DNA can be transformed and homologously recombined into the chromosome (Andersson *et al.*, 2000). Because the circadian clock turns this promoter on and off rhythmically, the reporter strain of *S. elongatus* glows rhythmically.

That choice of strain and promoter was due to the fact that Dr. Golden's laboratory had made this reporter strain for a completely different project. In retrospect, the choice

was a stroke of luck. Subsequent experiments using other strains/species of cyanobacteria have found rhythms (Aoki *et al.*, 1997), but the reporters in those strains are not bright. And even in *S. elongatus*, other promoters do not show such a robust rhythm of luminescence (see Class 3-5 rhythms in Figure 1—*psbAIp* is a strong Class 1 promoter; Liu *et al.*, 1995). The combination of the *psbAIp::luxAB* reporter and the *S. elongatus* strain remains one of the most robustly rhythmic combinations in cyanobacteria, even after ten years of intensive research. Dr. Kondo was able to exploit the bright and robust luminescence rhythm by designing clever apparatuses to monitor the rhythms. For liquid cultures, Dr. Kondo enlisted and modified the automated photomultiplier apparatus that was originally designed for endogenously bioluminescent algae (Kondo *et al.*, 1993). For monitoring colonies, Drs. Kondo and Ishiura discovered that the rhythms of single colonies could be tracked with a CCD camera (Kondo and Ishiura, 1994).

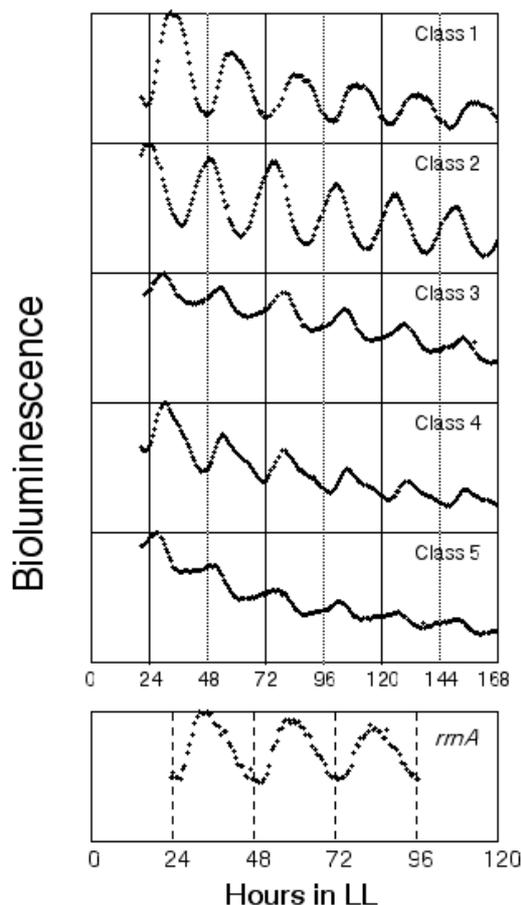


Figure 1. Global circadian regulation of promoter activities in *S. elongatus*. Upper five panels: representative traces of various classes of rhythmic waveforms resulting from the promoter trap experiment. Promoter activity is measured as bioluminescence. Lower panel: promoter activity of the rRNA gene, *rma*. Modified from Liu *et al.*, 1995.

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Dr. Kondo used those observations to design an innovative turntable/CCD camera apparatus for mutant screening of single colonies, enabling the simultaneous screening of up to 12,000 colonies in a single experiment! (Kondo *et al.*, 1994a).

The coincidence of good fortune, clever ideas, and hard work has transformed the *S. elongatus* system into one of the best characterized circadian clock systems, even though it is the newest comer to molecular clock analyses; only sixteen years ago, practically no one believed that prokaryotes were capable of circadian rhythmicity (Johnson *et al.*, 1996; Kippert, 1991). Therefore, the *S. elongatus* system has rapidly caught up with eukaryotic systems, and in some areas, it is leading the cutting edge—particularly in terms of structural aspects of clock proteins (Williams *et al.*, 2002; Mori *et al.*, 2002; Hayashi *et al.*, 2003), and of the rigorous assessment of adaptive significance (Ouyang *et al.*, 1998).

The issues of adaptive significance and occurrence of clocks in prokaryotes naturally lead to a consideration of evolutionary relationships. Later in this article, the genetics and function of the central clock gene cluster (*kaiABC*) will be discussed. These genes are widely distributed among cyanobacteria (Lorne *et al.*, 2000). There are putative homologs of KaiC in other bacteria, especially archaeobacteria, but no homologs to any of the Kai proteins have yet been discovered in eukaryotes (Johnson and Golden, 1999; DasSarma *et al.*, 2001; Dvornyk *et al.*, 2003). A genomic survey claimed that KaiC is a member of the bacterial RecA/DnaB family (Leipe *et al.*, 2000). RecA is an ATP-dependent DNA recombinase, and DnaB is the replication fork helicase in bacteria. The *kaiC* gene of *S. elongatus* appears to be an internally duplicated version of a RecA/DnaB-like gene; it has two parts that are very similar to each other (Leipe *et al.*, 2000; Iwasaki *et al.*, 1999). In Eubacteria (cyanobacteria *et al.*), *kaiC* has only been found in the “double-domain” version, whereas in Archaea, both single-domain and double-domain versions of *kaiC* have been found. These observations have stimulated hypotheses for the evolutionary origins of the *kai* genes (Leipe *et al.*, 2000; Dvornyk *et al.*, 2003).

## Outputs

A remarkable property of the circadian system in *S. elongatus* is that it globally regulates essentially all promoters in the genome, as shown by a sensitive and comprehensive “promoter trap” experiment (Liu *et al.*, 1995). A promoterless luciferase gene set was randomly inserted throughout the genome; whenever the luciferase gene set inserted into a genomic locus that was correctly positioned and oriented to a promoter, luciferase was expressed and the colonies glowed. The luminescent patterns of 3000 independent colonies were analyzed. Unexpectedly, all of the glowing colonies displayed circadian rhythms with the same period (Liu *et al.*, 1995). The pattern of the rhythmic expression differed among the promoters, both in terms of phasing and waveform, as shown in Figure 1. Figure 1 also shows that the promoter activity for a ribosomal RNA gene (*rrnA*) is likewise rhythmic. Apparently the cyanobacterial clock globally controls gene expression—in this case, the activity of promoters, and in a separate study

of *Synechococcus* RF-1, of protein abundances (Huang *et al.*, 1994). Moreover, when a heterologous *E. coli* promoter (*conIIP*) is tested in *S. elongatus*, it is not only transcribed, but it is rhythmically active (Katayama *et al.*, 1999). A model for this global control will be presented below.

As seen in Figure 1, there are several “classes” of promoter activity patterns. In particular, several of the classes are phased differently. Because most of the promoters are phased into a Class 1 phase, the other phases are especially interesting. Class 3 phasing has not been reproducible from experiment to experiment, but there are several promoters that reliably express in the Class 2 phase, i.e., in anti-phase to Class 1. This phasing means that expression will be largely in the night. One of the Class 2 genes that has been identified and characterized is *purF*, the rate-limiting enzyme of the purine biosynthetic pathway (Liu *et al.*, 1996). An interesting feature of the PurF enzyme is that its activity is inhibited by oxygen. Its nighttime expression might be adaptive to avoid the oxygen generated by photosynthesis in the daytime (Liu *et al.*, 1996). Another Class 2 promoter is that driving the expression of *opcA*, a gene that is involved in temporal control of pathways that generate reductants (Min and Golden, 2000).

There are other outputs besides gene expression of the cyanobacterial biological clock. In other cyanobacterial species there are rhythms of nitrogen fixation, amino acid uptake, and photosynthesis (Grobbelaar *et al.*, 1986; Mitsui *et al.*, 1986; Huang *et al.*, 1994; Chen *et al.*, 1991). *S. elongatus* is not capable of nitrogen fixation, but it also appears to have rhythms of photosynthetic capacity. There is a significant (but not very robust) rhythm of photosynthetic oxygen production that peaks in the subjective day (A. Dean and T. Mori, unpublished results). Probably related to the rhythm of photosynthesis is a rhythm of delayed chlorophyll fluorescence (Kondo *et al.*, 1994b).

Finally, another output of the circadian clock in cyanobacteria is the timing of cell division. As mentioned above, it had been assumed that a circadian oscillator would not be useful to organisms that divide more rapidly than once every 24 h (Pittendrigh, 1993). We now recognize that these assumptions were based on fallacious “eukaryote-centric thinking” (Mori and Johnson, 2000; Johnson and Kondo, 2001). Cyanobacteria express robust circadian rhythms of gene expression when dividing with doubling times of 6–12 h (Mori *et al.*, 1996; Kondo *et al.*, 1997). They also express excellent rhythms when they are not dividing at all (Mori and Johnson, 2001a). Therefore, the circadian clock is independent of the cell division cycle in cyanobacteria—unfettered by the status of cell division, the circadian clock “keeps on ticking”. However, the timing of cell division is not independent of the circadian clock; even when the cells are dividing rapidly, the circadian clock rhythmically slows the rate of cell division every night. Even in constant light, the rate of cell division is slowed in the early subjective night (Mori *et al.*, 1996). Apparently, the circadian clockwork is well buffered and stable against significant changes of the intracellular milieu. Consequently, the circadian clockwork in *S. elongatus* gates cell division and gene expression, but its timing circuit is independent of the cell division cycle.

## Inputs and Entrainment

Both light/dark and temperature cycles can entrain cyanobacterial clocks. In a study of *Synechococcus* RF-1, light/dark cycles appear to be a more potent zeitgeber than temperature cycles (Lin *et al.*, 1999). Which photopigments are involved in the entrainment by light/dark signals? A spectral study of the wavelengths of light that are effective phase-resetting cues would be useful. Light-induced phase shifting by the usual protocol (i.e., light pulses in a DD background) has been difficult to test in *S. elongatus* because the cells need to be in LL to allow robust circadian gene expression, and light pulses on an LL background have little phase-shifting efficacy. On the other hand, dark pulses on an LL background are very effective resetting agents of the *S. elongatus* clock (Kondo *et al.*, 1993). We therefore designed a protocol to determine which wavelengths of light would be effective in reversing a dark-pulse-induced phase shift. In other words, a dark pulse was compared with a blue, green, yellow, or red light pulse. Those wavelengths that have the same phase-shifting effect as a dark pulse are therefore those to which the clock is "blind." Wavelengths that provide input to the clock would be those that appear similar to the constant light control. In an action spectroscopy test of this protocol, we found that both blue and red light were effective input signals (peaks at 440 nm and 600-650 nm; Kondo and Johnson, unpublished results). Moreover, long exposures to the photosynthesis inhibitor, Diuron, can mimic the effect of dark pulses (Katayama *et al.*, 2003). Those observations suggest that photosynthetic electron transport could act as a clock entrainment input. However, the interpretation that photosynthesis can act as an input is premature, and better action spectrum data are required to answer this question.

A different strategy to identify input components used a transposon mutagenesis screen for disrupted phase resetting to a 5 h dark pulse (Katayama *et al.*, 2003; Schmitz *et al.*, 2000). This screen identified some period mutants, including two in the gene encoding an iron-sulfur protein named LdpA (Katayama *et al.*, 2003). One mutant identified in that screen showed a reduced phase-shifting response to the dark pulse and was identified as a phytochrome-like histidine protein kinase, called CikA (Schmitz *et al.*, 2000). CikA can autophosphorylate but does not bind bilin *in vivo*, as expected for a phytochrome (Mutsuda *et al.*, 2003). While CikA's phytochrome-like sequence implied its action as a photopigment, a surprising result is that CikA is required for response to phase-shifting temperature signals as well to light/dark signals (Mutsuda *et al.*, 2003). It is important to note that while *cikA* mutant strains do not phase shift in response to 5 h dark pulses (Schmitz *et al.*, 2000), it will entrain effectively to 12 h dark pulses. This result suggests that CikA does not mediate the only input pathway. Apparently, a variety of input signals can entrain the *S. elongatus* clock.

### Core mechanism: the *kaiABC* clock gene cluster

In work that was primarily performed in Dr. Kondo's lab in Japan, the turntable/CCD apparatus was used with the luciferase reporter strain of *S. elongatus* in EMS

mutagenesis screens to isolate more than 100 circadian mutants (Kondo *et al.*, 1994a). Some of these mutants were rescued by the introduction of libraries of wild-type *S. elongatus* DNA. Those rescue experiments pinpointed a cluster of three adjacent genes, named *kaiA*, *kaiB*, and *kaiC* (Ishiura *et al.*, 1998). The *kaiABC* cluster appears to be a clock-specific region of the chromosome in cyanobacteria because deletion of the entire cluster or of any one of the *kai* genes separately does not affect viability (in single-strain cultures), but causes arrhythmicity. Promoters were found in the upstream regions of both the *kaiA* and *kaiB* genes. The *kaiA* promoter (*kaiAp*) gives rise to a monocistronic *kaiA* mRNA, whereas the *kaiB* promoter (*kaiBCp*) produces a dicistronic *kaiBC* mRNA (Ishiura *et al.*, 1998).

As mentioned above, *kaiC* appears to be an internally duplicated version of a *recA/dnaB*-like gene; it has two parts that are very similar to each other (Iwasaki *et al.*, 1999). In each half of the *kaiC* gene, there is a Walker A or "P-loop" motif. This motif, [G or A]XXXXGK[T or S], is often a ATP/GTP nucleotide binding region. Indeed, KaiC binds ATP, and when the Walker A motifs are mutated, the nucleotide binding is eliminated and rhythmicity is severely disrupted or abolished (Nishiwaki *et al.*, 2000; Mori and Johnson, 2001b). Therefore, this motif is essential in both parts of the KaiC molecule for rhythmicity. As with eukaryotic clock proteins, interactions are important. KaiA, KaiB, and KaiC interact with each other (Iwasaki *et al.*, 1999; Xu *et al.*, 1999; Taniguchi *et al.*, 2001). KaiC also interacts with a histidine kinase, SasA, that is crucial for maintaining a high amplitude, robust rhythm of gene expression (Iwasaki *et al.*, 2000). These interactions appear to lead to the formation of complexes *in vivo* (Kageyama *et al.*, 2003). There is also evidence for the association of a population of KaiB and KaiC molecules with membranes (Kitayama *et al.*, 2003).

In some of the first structural work on clock proteins from any system, an NMR analysis indicated that the N-terminal domain of KaiA is a pseudo-receiver domain (Williams *et al.*, 2002). In work from our lab, electron microscopy coupled with analytical ultracentrifugation, chromatography, and native gel electrophoresis has demonstrated that purified KaiC will form ATP-dependent hexameric ring complexes *in vitro*, as shown in Figure 2 (Mori *et al.*, 2002; Hayashi *et al.*, 2003). These hexameric structures are similar to those found in DnaB and RecA and further supports the sequence analyses that placed KaiC in the RecA/DnaB superfamily (Leipe *et al.*, 2000). Moreover, these KaiC hexamers bind DNA (Mori *et al.*, 2002).

Some features of *kai* gene regulation are reminiscent of the regulation of eukaryotic clock genes. There are rhythms in the abundance of *kaiA* and *kaiBC* transcripts (Ishiura *et al.*, 1998) and the KaiB and KaiC proteins (Xu *et al.*, 2000; Iwasaki *et al.*, 2002). Continuous overexpression of KaiC repressed the *kaiBC* promoter, suggesting negative feedback of KaiC on its promoter in an analogous fashion to the case for eukaryotic clock proteins (Ishiura *et al.*, 1998). Pulsatile expression of the *kaiC* gene within physiological levels from an inducible promoter resets the phase of the rhythms (Ishiura *et al.*, 1998; Xu *et al.*, 2000). The observation that KaiC is rhythmically abundant and that pulses of KaiC expression elicit phase resetting led to the hypothesis that KaiC turnover might be an important determinant of clock period and phase (Xu *et al.*, 2000).

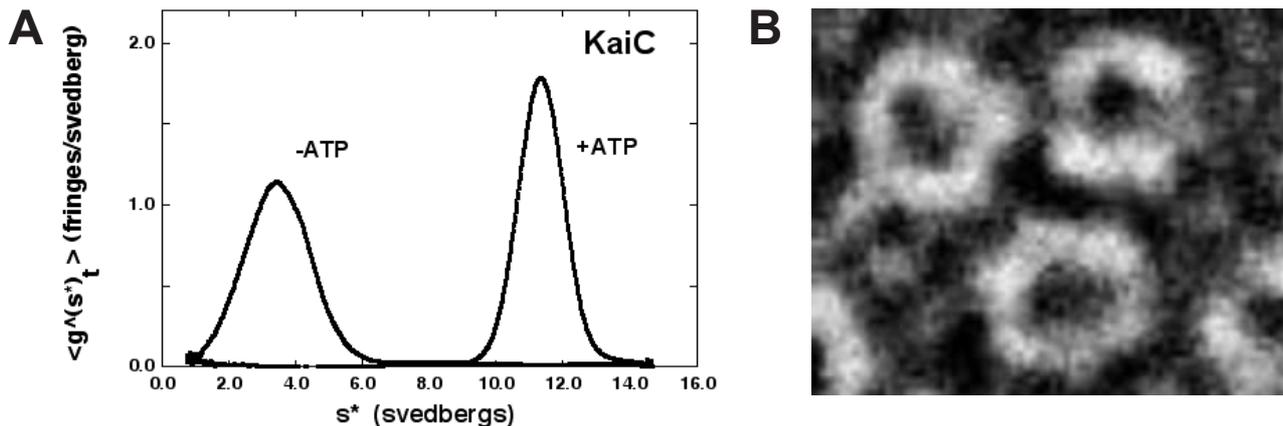


Figure 2. ATP-dependent protein complexes formed by the KaiC protein from *Synechococcus*. A. As determined by analytical ultracentrifugation, KaiC forms complexes in the presence of ATP that are hexamers (sedimentation coefficient of about 11.5 S). In the absence of ATP, KaiC is monomeric (sedimentation coefficient of about 3.6 S). B. Negatively stained hexameric ring complexes viewed by electron microscopy. Modified from Mori *et al.*, 2002.

This hypothesis was tested in a recent publication that found support for KaiC degradation rate as a partial determinant of period (Xu *et al.*, 2003).

KaiC exists in phosphorylated forms *in vivo* (Iwasaki *et al.*, 2002), suggesting another similarity to the posttranslational control of eukaryotic clock proteins. The ratio of phosphorylated to non-phosphorylated KaiC is correlated with the period at which the clock runs (Xu *et al.*, 2003). Unlike some eukaryotic systems, however, the extent of KaiC phosphorylation is not directly correlated with KaiC degradation (Xu *et al.*, 2003). Purified recombinant KaiC can autophosphorylate *in vitro* (Nishiwaki *et al.*, 2000; Iwasaki *et al.*, 2002; Williams *et al.*, 2002). This autophosphorylation is stimulated *in vitro* by KaiA (Iwasaki *et al.*, 2002; Williams *et al.*, 2002). On the other hand, KaiB antagonizes the effects of KaiA on KaiC autophosphorylation (Williams *et al.*, 2002; Kitayama *et al.*, 2003). KaiC will also *de*-phosphorylate *in vitro*, implying that KaiC will continuously turn over its phosphorylation status in the presence of ATP (Xu *et al.*, 2003). We found that dephosphorylation of KaiC *in vitro* is inhibited by KaiA, and this effect of KaiA is also antagonized by KaiB (Xu *et al.*, 2003). It is possible that the effects of KaiA and KaiB on KaiC dephosphorylation *in vitro* can account for the results of KaiA and KaiB on KaiC autophosphorylation (Xu *et al.*, 2003)—e.g., perhaps the apparent KaiA stimulation of KaiC autophosphorylation is due to an inhibition of dephosphorylation (Iwasaki *et al.*, 2002; Williams *et al.*, 2002; Kitayama *et al.*, 2003). However, for the time being, we will adopt the interpretation that KaiA both stimulates KaiC autophosphorylation and inhibits its dephosphorylation (Figure 3). The *in vitro* results are relevant to events happening within cells—*in vivo*, KaiA stabilizes KaiC in the phosphorylated form, and KaiB antagonizes the effect of KaiA (Iwasaki *et al.*, 2002; Xu *et al.*, 2003). Moreover, an extensive PCR mutagenesis of KaiA showed that the vast majority of KaiA mutations slowed the period of the clock (Nishimura *et al.*, 2002). The role of KaiA on stabilizing KaiC phosphorylation and the KaiA mutant analysis implies that phosphorylated KaiC is needed to speed the clock.

Not only is KaiC phosphorylated *in vivo*, but the proportion of phospho-KaiC to non-phospho-KaiC oscillates

over the circadian cycle (Iwasaki *et al.*, 2002). Our lab had previously reported that KaiC is rhythmically abundant (Xu *et al.*, 2000). Based on the work of Iwasaki and coworkers (Iwasaki *et al.*, 2002), we now realize that the multiple KaiC bands we observed in our previous time course study of wild-type cells (Xu *et al.*, 2000) are indicative of rhythms of KaiC phosphorylation in which P-KaiC predominates in the subjective night (upper bands). The abundance of nonP-KaiC is also rhythmic, although the nonP-KaiC band was obscured in our previous investigation by a cross-reacting antigen (Xu *et al.*, 2000). The rhythmic band that we identified as KaiC in our immunoblots is phospho-KaiC, and non-phospho-KaiC on our blots is a faster mobility band that co-migrates with a cross-reacting antigen (Xu *et al.*, 2000). Therefore, our results concur with those of Iwasaki and coworkers in demonstrating a circadian rhythm of the ratio of P-KaiC to nonP-KaiC *in vivo* (Iwasaki *et al.*, 2002; Xu *et al.*, 2003).

Figure 3 summarizes some of these data. The *kaiA*, *B*, and *C* genes are rhythmically transcribed. Probably the mRNAs are rhythmically translated, but KaiA is relatively stable so that its abundance is not rhythmic (Xu *et al.*, 2000; Kitayama *et al.*, 2002). The abundances of KaiB and KaiC are rhythmic (Xu *et al.*, 2000; Kitayama *et al.*, 2002). The Kai proteins interact and possibly form a complex *in vivo* (Kageyama *et al.*, 2003). KaiC autophosphorylates and forms a hexamer that may form the core of a complex (Mori *et al.*, 2002; Hayashi *et al.*, 2003). The ratio of phospho-KaiC to non-phospho-KaiC is regulated by KaiA, which stabilizes the phosphorylated form of KaiC, and by KaiB, which antagonizes the actions of KaiA (Iwasaki *et al.*, 2002; Williams *et al.*, 2002; Xu *et al.*, 2003). The extent of KaiC phosphorylation is correlated with clock speed *in vivo* (Figure 3; Xu *et al.*, 2003).

#### Core mechanism: a new model—the “oscilloid”

Taken together with analogous data from eukaryotic clocks, the results from studies up to the year 2000 had been used to support a model wherein there is negative feedback control of *kaiBC* expression by the KaiC protein acting upon *kaiBCp* to generate a circadian oscillation in which

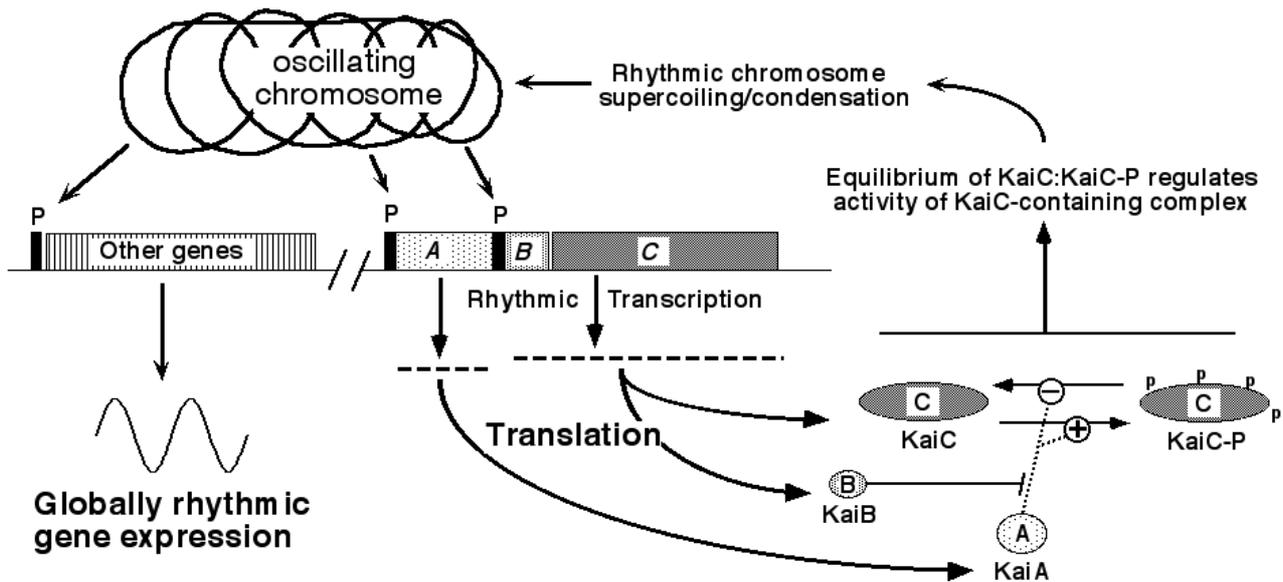


Figure 3. "Oscilloid" model for the circadian oscillator of *S. elongatus*. KaiA, KaiB, and KaiC are transcribed and translated from the *kaiABC* cluster using two promoters: *kaiAp* and *kaiBCp*. KaiA promotes the phosphorylation of KaiC and inhibits its dephosphorylation, while KaiB antagonizes the actions of KaiA. KaiC phosphorylation is coincident with the formation of a KaiC-containing complex that mediates rhythmic and global changes in the status of the chromosome. These changes in chromosomal status influence the transcriptional activity of all promoters in the chromosome so that there are global circadian changes in gene expression. "Other genes" includes heterologous genes (and heterologous promoters such as *trcp*). Modified from Xu *et al.*, 2003.

the stability of KaiC is influenced by its phosphorylation status and thereby modulates period length (Ishiura *et al.*, 1998; Nishiwaki *et al.* 2000). This model implies that the biochemistry of the clockwork in cyanobacteria is similar to that in eukaryotes. One of the predictions of that model is that the circadian feedback loop is dependent upon proper *kaiBCp* function. An ancillary expectation was that global gene expression might be mediated by changes in sigma subunits of RNA polymerase that would rhythmically turn on and off groups of genes. However, studies of sigma subunits in cyanobacteria have not yielded explanations for global regulation (Tsinoremas *et al.*, 1996; Nair *et al.*, 2002). Even more unexpectedly, we found that the *kaiBC* promoter is *not* specifically required for the circadian oscillator to operate in cyanobacteria—a non-specific heterologous promoter can drive *kaiBC* expression and obtain essentially wild-type circadian patterns (Xu *et al.*, 2003; Y. Nakahira and T. Kondo, submitted).

Three lines of evidence led us to propose a new model for the fundamental mechanism of the clockwork in *S. elongatus*: (1) global circadian regulation of promoters, even heterologous (e.g., *conI1p*) promoters; (2) when KaiB and KaiC expression is driven from a non-specific heterologous promoter (*trcp*) instead of the endogenous *kaiBC* promoter, rhythmicity persists; and (3) the key clock protein KaiC binds DNA and is related to bacterial recombinases and helicases, implying that KaiC might act directly on DNA (Mori and Johnson, 2001b). These observations emboldened us to consider that KaiC might mediate both its own negative feedback regulation (Ishiura *et al.*, 1998) and global regulation of the cyanobacterial genome (Liu *et al.*, 1995) by orchestrating oscillations in the condensation and/or supercoiling status of the entire cyanobacterial chromosome. The chromosome of most

bacteria is organized into a "nucleoid," which has a highly organized architecture based on condensation and coiling of DNA (Pettijohn, 1976; Trun and Marko, 1988). It is well known that changes in the local supercoiling status of DNA can affect the transcription rate of genes (Menzel and Gellert, 1987; Pruss and Drlica, 1989; Hsieh *et al.*, 1991).

We therefore proposed that a fundamental component of the cyanobacterial clockwork is that the condensation/supercoiling status of the chromosome rhythmically changes such that it becomes an oscillating nucleoid, or "oscilloid" (Figure 3; Mori and Johnson, 2001b). There is already precedence for daily rhythms of supercoiling status in the chloroplast chromosome of the eukaryotic alga *Chlamydomonas* (Salvador *et al.*, 1998). In cyanobacteria, we postulate that these condensation/supercoiling oscillations promote rhythmic modulation of the transcription rates of all genes, accounting for global regulation of gene expression. Therefore, gene-specific cis-elements that mediate rhythmic gene expression might be (at least partially) responsive to chromosomal status rather than exclusively to trans factors. In addition, heterologous promoters (or *trcp::kaiC* constructs) that are integrated into the chromosome are driven rhythmically since they are also subjected to the oscillating chromosomal status. A minor proportion of the genes oscillate in the opposite phase relationship (e.g., *purF*) to the majority of the genes, and this might be due to differential responses in different domains of the chromosome, or to the fact that different promoters react differently to negative vs. positive supercoiling (Drlica, 1992). Finally, we suggest that KaiC (or most likely, a KaiC-containing complex) is a key player in regulating these changes of chromosomal status, and that the phosphorylation status of KaiC is important in the

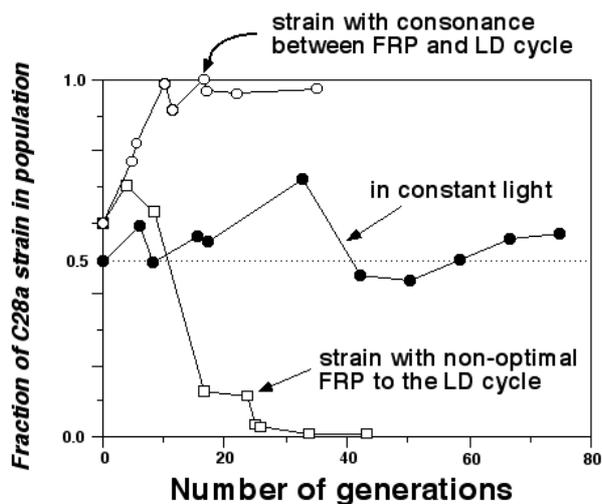


Figure 4. Competition of circadian strains in different light/dark cycles. Different strains of cyanobacteria were mixed and grown in competition under different light/dark cycles. The strain whose endogenous free-running period most closely matched that of the environmental light/dark cycle was able to out-compete strains with a non-optimal period. In constant light (non-selective conditions), all the strains were able to maintain their initial fraction in the population. FRP = free-running period, LD = light/dark cycle. These data show the competition between a 30-h mutant (C28a) and wild-type (FRP = 24 h). The upper trace is on a 30-h LD cycle, the middle trace is from constant light, and the lower trace is on a 24-h LD cycle. Ordinate, fraction of C28a mutant in the mixed population (modified from Ouyang *et al.* 1998).

regulation of this complex's activity (Figure 3; Xu *et al.*, 2003).

#### Does this clock enhance fitness?

The study of circadian rhythms has been dedicated to the proposition that temporal programs regulated by these clocks are adaptive. However, prior to 1998 there had been no direct, definitive tests for whether circadian clocks enhance fitness in any organism. We performed the first rigorous test of the adaptive significance of circadian programs by using competition experiments between different strains of the cyanobacterium *S. elongatus* (Ouyang *et al.*, 1998). For asexual microbes such as *S. elongatus*, differential growth of one strain under competition with other strains is a good measure of reproductive fitness. In pure culture, the strains grew at about the same rate in constant light and in light/dark cycles, so there did not appear to be a significant advantage or disadvantage to having different circadian periods when the strains were grown individually. The fitness test was to mix different strains together and to grow them in competition to determine if the composition of the population changes as a function of time. The cultures were diluted at intervals to allow growth to continue.

In experiments in which wild-type strains were competed with arrhythmic strains, the arrhythmic strains were rapidly defeated by wild-type strains in light/dark cycles, but under competition in constant light, the arrhythmic strains grew as well (and sometimes better than!) wild-type strains (Y. Ouyang and M. Woelfle, unpublished observations). These results indicate that having a

circadian clock improves fitness of cyanobacterial cells in a selective environment (i.e., a light/dark cycle), but not in a non-selective environment (i.e., constant light).

Different period mutants were used to answer the question, "does having a period that is similar to the period of the environmental cycle enhance fitness?" The circadian phenotypes of the strains used had free-running periods of about 22 h, 25 h (wild-type), and 30 h. When each of the strains was mixed with another strain and grown together in competition, a pattern emerged that depended upon the frequency of the light/dark cycle and the circadian period. When grown on a 22-h cycle (11 h light, 11 h dark), the 22 h-period mutant became the dominant cell type in the mixed cultures. On a 30-h cycle (15 h light, 15 h dark), the 30 h-period mutant ("C28a") could defeat either wild type or the 22 h-period mutant. On a "normal" 24 h cycle (12 h light, 12 h dark), the wild type strain could overgrow either mutant (Ouyang *et al.*, 1998). Figure 4 shows results from the competition between wild-type and the 30 h-period mutant C28a. Strains in which other clock genes were mutated showed the same effect (K. Phanvijhitsiri and M. Woelfle, unpublished data).

Clearly, the strain whose period most closely matched that of the light/dark cycle eliminated the competitor. Under a non-selective condition (in this case, constant light), each strain was able to maintain itself in the mixed cultures. Because the mutant strains could defeat the wild-type strain in LD cycles in which the periods are similar to their endogenous periods, the differential effects that were observed are likely to result from the differences in the circadian clock. A genetic test was also performed to demonstrate that the clock gene mutation was specifically responsible for the differential effects in the competition experiment (Ouyang *et al.*, 1998). This was the first rigorous demonstration in any organism of a fitness advantage conferred by a circadian system. What is the basis of this competitive advantage? The results of modeling studies favor the interpretation that the cyanobacteria rhythmically secrete an inhibitor (Gonze *et al.*, 2002). This interpretation has not yet been experimentally confirmed.

#### The significance of clock studies in cyanobacteria

Research on circadian programming in cyanobacteria has overturned at least two dogmas—(1) that prokaryotes cannot have circadian clocks, and (2) that organisms cannot express circadian rhythms when they divide more rapidly than once per 24 hours. These investigations have also provided the first rigorous evidence for a fitness advantage conferred by circadian programming, and for global orchestration of gene expression. The investigation of its central mechanism has progressed rapidly within a short time, but this clockwork remains mysterious. We proposed herein a new model to account for global gene regulation and for the role of the essential clock protein KaiC by envisioning an oscillating chromosome, the oscilloid. If this model is found to be true, it will introduce a new way in which circadian gene expression is regulated by DNA structure.

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## References

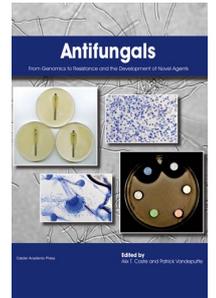
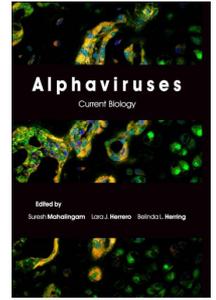
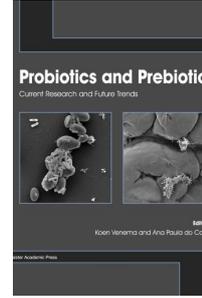
- Andersson, C.A., Tsinoremas N.F., Shelton, J., Lebedeva, N.V., Yarrow, J., Min, H., and Golden, S.S. (2000). Application of bioluminescence to the study of circadian rhythms in cyanobacteria. *Methods Enzymol.* 305, 527-542.
- Aoki, S., Kondo, T., Wada, H., and Ishiura, M. (1997). Circadian rhythm of the cyanobacterium *Synechocystis* sp. PCC 6803 in the dark. *J. Bacteriology* 179, 5751-5755.
- Chen, T.-H., Chen, T.-L., Huang, L.-M. and Huang, T.-C. (1991). Circadian rhythm in amino acid uptake by *Synechococcus* RF-1. *Plant Physiol.* 97, 55-59.
- DasSarma, S., Kennedy, S.P., Berquist, B., Ng, W.V., Baliga, N.S., Spudich, J.L., Krebs, M.P., Eisen, J.A., Johnson, C.H., and Hood, L. (2001). Genomic perspective on the photobiology of *Halobacterium* species NRC-1, a phototrophic, phototactic, and UV-tolerant haloarchaeon. *Photosynthesis Res.* 70, 3-17.
- Dvornyk, V., Vinogradova, O., and Nevo, E. (2002). Origin and evolution of circadian clock genes in prokaryotes. *Proc Natl Acad Sci USA* 100, 2495-2500.
- Drlica K. (1992). Control of bacterial DNA supercoiling. *Mol Microbiol* 6, 425-433.
- Gonze, D., Rousset, M.R., Goldbeter, A. (2002). A model for the enhancement of fitness in cyanobacteria based on resonance of a circadian oscillator with the external light-dark cycle. *J. Theor. Biol.* 214, 577-597.
- Grobbelaar, N., Huang, T.-C., Lin, H.Y., and Chow, T.J. (1986). Dinitrogen-fixing endogenous rhythm in *Synechococcus* RF-1. *FEMS Microbiol. Lett.* 37, 173-177.
- Hayashi, F., Suzuki, H., Iwase, R., Uzumaki, T., Miyake, A., Shen, J.-R., Imada, K., Furukawa, Y., Yonekura, K., Namba, K., and Ishiura, M. (2003). ATP-induced hexameric ring structure of the cyanobacterial circadian clock protein KaiC. *Genes to Cells* 8, 287-296.
- Hsieh, L.S., Rouviere-Yaniv, J., Drlica, K. (1991). Bacterial DNA supercoiling and [ATP]/[ADP] ratio: changes associated with salt shock. *J Bacteriol* 173, 3914-3917.
- Huang, T.-C., Chen, H.-M., Pen, S.-Y., and Chen, T.-H. (1994). Biological clock in the prokaryote *Synechococcus* RF-1. *Planta* 193, 131-136.
- Ishiura, M., Kutsuna, S., Aoki, S., Iwasaki, H., Andersson, C.R., Tanabe, A., Golden, S.S., Johnson C.H., Kondo, T. (1998). Expression of a gene cluster *kaiABC* as a circadian feedback process in cyanobacteria. *Science* 281, 1519-1523.
- Iwasaki, H., Taniguchi, Y., Kondo, T., Ishiura, M. (1999). Physical interactions among circadian clock proteins, KaiA, KaiB and KaiC, in Cyanobacteria. *EMBO J.* 18, 1137-45.
- Iwasaki, H., Williams, S.B., Kitayama, Y., Ishiura, M., Golden, S.S. and Kondo, T. (2000). A KaiC-interacting sensory histidine kinase, SasA, necessary to sustain robust circadian oscillation in cyanobacteria. *Cell* 101, 223-233.
- Iwasaki, H., Nishiwaki, T., Kitayama, Y., Nakajima, M., and Kondo, T. (2002). KaiA-stimulated KaiC phosphorylation in circadian timing loops in cyanobacteria. *Proc. Natl. Acad. Sci. USA* 99, 15788-15793.
- Johnson, C.H., Golden, S.S., Ishiura, M., Kondo, T. (1996). Circadian clocks in prokaryotes. *Mol. Microbiol.* 21, 5-11.
- Johnson, C.H. and Golden, S.S. (1999). Circadian programs in cyanobacteria: adaptiveness and mechanism. *Annu. Rev. Microbiol.* 53, 389-409.
- Johnson, C.H. and Kondo, T. (2001). Circadian rhythms in unicellular organisms. In: *Handbook of Behavioral Neurobiology*, Chapter 3. Plenum Press, p. 61-77.
- Kageyama, H., Kondo, T. and Iwasaki, H. (2003). Circadian formation of clock protein complexes by KaiA, KaiB, KaiC, and SasA in cyanobacteria. *J. Biol. Chem.* 278, 2388-2395.
- Katayama, M., Tsinoremas, N.F., Kondo, T., and Golden, S.S. (1999). *cpmA*, a gene involved in an output pathway of the cyanobacterial circadian system. *J. Bacteriol.*, 181, 3516-3524.
- Katayama, M., Kondo, T., Xiong, J. and Golden, S.S. (2003). IdpA encodes an iron-sulfur protein involved in light-dependent modulation of the circadian period in the cyanobacterium *Synechococcus elongatus* PCC 7942. *J. Bacteriol.* 185, 1415-22.
- Kippert, F. (1991). Essential clock proteins/circadian rhythms in prokaryotes—what is the evidence? *Bot. Acta* 104, 2-4.
- Kitayama, Y., Iwasaki, H., Nishiwaki, T. and Kondo, T. (2003). KaiB functions as an attenuator of KaiC phosphorylation in the cyanobacterial circadian clock system. *EMBO J.* 22, 1-8
- Kondo, T., Strayer, C.A., Kulkarni, R. D., Taylor, W., Ishiura, M., Golden, S. S. and Johnson, C. H. (1993). Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. *Proc. Natl. Acad. Sci. USA* 90, 5672-5676.
- Kondo, T. and Ishiura, M. (1994). Circadian rhythms of cyanobacteria: monitoring the biological clocks of individual colonies by bioluminescence. *J. Bacteriol.* 176, 1881-1885.
- Kondo, T., Tsinoremas, N. F., Golden, S. S., Johnson, C. H., Kutsuna, S. and Ishiura, M. (1994a). Circadian clock mutants of cyanobacteria. *Science* 266, 1233-1236.
- Kondo, T., Ishiura, M., Golden, S.S. and Johnson, C.H. (1994b). Circadian rhythms of cyanobacteria expressed from a luciferase reporter gene. In: *Evolution of Circadian Clock*. T. Hiroshige and K. Honma, eds. Hokkaido University Press, Sapporo. p. 59-73.
- Kondo, T., Mori, T., Lebedeva, N.V., Aoki, S., Ishiura, M., Golden, S.S. (1997). Circadian rhythms in rapidly dividing cyanobacteria. *Science* 275, 224-227.
- Leipe, D.D., Aravind, L., Grishin, N.V. and Koonin, E.V. (2000). The bacterial replicative helicase DnaB evolved from a RecA duplication. *Genome Res.* 10, 5-16.
- Lin, R.-F., Chou, H.-M., Huang, T.-C. (1999). Priority of light/dark entrainment over temperature in setting the

- circadian rhythms of the prokaryote *Synechococcus* RF-1. *Planta* 209, 202-206.
- Liu, Y., Tsinoremas, N.F., Johnson, C.H., Lebedeva, N.V., Golden, S.S., Ishiura, M. and Kondo, T. (1995). Circadian orchestration of gene expression in cyanobacteria. *Genes and Dev.* 9, 1469-1478.
- Liu, Y., Tsinoremas, N.F., Golden, S.S., Kondo, T. and Johnson, C.H. (1996). Circadian expression of genes involved in the purine biosynthetic pathway of the cyanobacterium *Synechococcus* sp. strain PCC 7942. *Mole. Microbiol.* 20, 1071-1081.
- Lorne, J., Scheffer, J., Lee, A., Painter, M. and Miao, V.P. (2000). Genes controlling circadian rhythm are widely distributed in cyanobacteria. *FEMS Microbiol Lett.* 189, 129-133.
- Menzel, R. and Gellert M. (1987). Modulation of transcription by DNA supercoiling: a deletion analysis of the *Escherichia coli* *gyrA* and *gurB* promoters. *Proc Natl Acad Sci USA* 84, 4185-4189.
- Min, H., Golden, S.S. (2000). A new circadian Class 2 gene, *opcA*, whose product is important for reductant production at night in *Synechococcus elongatus* PCC 7942. *J Bacteriol* 182, 6214-6221.
- Mitsui, A., Kumazawa, S., Takahashi, A., Ikemoto, H., and Arai, T. (1986). Strategy by which nitrogen-fixing unicellular cyanobacteria grow photoautotrophically. *Nature* 323, 720-722.
- Mori, T., Binder, B., Johnson, C.H. (1996). Circadian gating of cell division in cyanobacteria growing with average doubling times of less than 24 hours. *Proc Natl Acad Sci USA* 93, 10183-10188.
- Mori, T. and Johnson, C.H. (2000). Circadian control of cell division in unicellular organisms. In: *Progress in Cell Cycle Research*, Volume 4. L. Meijer, A. Jezequel, and B. Ducommun, eds. Kluwer Academic/Plenum Press, N.Y., p. 185-192.
- Mori, T. and Johnson, C.H. (2001a). Independence of circadian timing from cell division in cyanobacteria. *J. Bacteriol.* 183, 2439-2444.
- Mori, T. and Johnson, C.H. (2001b). Circadian programming in cyanobacteria. *Sem. Cell Develop. Biol.* 12, 271-278.
- Mori, T., Saveliev, S.V., Xu, Y., Stafford, W.F., Cox, M.M., Inman, R.B., and Johnson, C.H. (2002). Circadian clock protein KaiC forms ATP-dependent hexameric rings and binds DNA. *Proc. Natl. Acad. Sci. USA* 99, 17203-17208.
- Mutsuda, M., Michel, K.P., Zhang, X., Montgomery, B.L. and Golden, S.S. (2003). Biochemical properties of CikA, an unusual phytochrome-like histidine protein kinase that resets the circadian clock in *Synechococcus elongatus* PCC 7942. *J. Biol. Chem.* 278, 19102-19110.
- Nair, U., Ditty, J.L., Min, H., and Golden, S.S. (2002). Roles for sigma factors in global circadian regulation of the cyanobacterial genome. *J. Bacteriol.* 184, 3530-3538.
- Nishimura, H., Nakahira, Y., Imai, K., Tsuruhara, A., Kondo, H., Hayashi, H., Hirai, M., Saito, H. and Kondo, T. (2002). Mutations in KaiA, a clock protein, extend the period of circadian rhythm in the cyanobacterium *Synechococcus elongatus* PCC 7942. *Microbiol.* 148, 2903-2909.
- Nishiwaki, T., Iwasaki, H., Ishiura, M. and Kondo, T. (2000). Nucleotide binding and autophosphorylation of the clock protein KaiC as a circadian timing process of cyanobacteria. *Proc. Natl. Acad. Sci. USA* 97, 495-499.
- Ouyang, Y., Andersson, C.R., Kondo, T., Golden, S.S. and Johnson, C.H. (1998). Resonating circadian clocks enhance fitness in cyanobacteria. *Proc. Natl. Acad. Sci. USA* 95, 8660-8664.
- Pettijohn, D. (1976). Prokaryotic DNA in nucleoid structure. *CRC Critical Rev. Biochem.* 4, 175-202.
- Pittendrigh, C.S. (1993). Temporal organization: reflections of a Darwinian clock-watcher. *Annu. Rev. Physiol.* 55, 17-54.
- Pruss, G.J. and Drlica, K. (1989). DNA supercoiling and prokaryotic transcription. *Cell* 56, 521-523.
- Salvador, M.L., Klein, U. and Bogorad, L. (1998). Endogenous fluctuations of DNA topology in the chloroplast of *Chlamydomonas reinhardtii*. *Mole. Cell Biol.* 18, 7235-7242.
- Schmitz, O., Katayama, M., Williams, S.B., Kondo, T. and Golden, S.S. (2000). CikA, a bacteriophytochrome that resets the cyanobacterial circadian clock. *Science* 289, 765-768.
- Taniguchi, Y., Yamaguchi, A., Hijikata, A., Iwasaki, H., Kamagata, K., Ishiura, M., Go, M. and Kondo, T. (2001). Two KaiA-binding domains of cyanobacterial circadian clock protein KaiC. *FEBS Letters* 496, 86-90.
- Trun, N.J. and Marko, J.F. (1998). Architecture of a bacterial chromosome. *ASM News* 64, 276-283.
- Tsinoremas, N.F., Ishiura, M., Kondo, T., Andersson, C.R., Tanaka, K., Takahashi, H., Johnson, C.H. and Golden, S.S. (1996). A sigma factor that modifies the circadian expression of a subset of genes in cyanobacteria. *EMBO Journal* 15, 2488-2495.
- Williams, S.B., Vakonakis, I., Golden, S.S., and LiWang, A.C. (2002). Structure and function from the circadian clock protein KaiA of *Synechococcus elongatus*: a potential clock input mechanism. *Proc. Natl. Acad. Sci. USA* 99, 15357-15362.
- Xu, Y., Piston, D. and C.H. Johnson. (1999). A bioluminescence resonance energy transfer (BRET) system: Application to interacting circadian clock proteins. *Proc. Natl. Acad. Sci. USA* 96, 151-156.
- Xu, Y., Mori, T. and Johnson, C.H. (2000). Circadian clock-protein expression in cyanobacteria: rhythms and phase-setting. *EMBO J.* 19, 3349-57.
- Xu, Y., Mori, T. and Johnson, C.H. (2003). Cyanobacterial circadian clockwork: roles of KaiA, KaiB, and the *kaiBC* promoter in regulating KaiC. *EMBO J.* 22, 2117-2126.

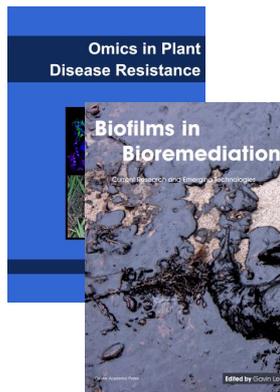
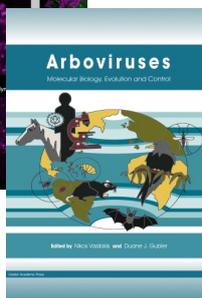
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