Circadian Rhythm of the Cyanobacterium *Synechocystis* sp. Strain PCC 6803 in the Dark

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The cyanobacterium *Synechocystis* sp. strain PCC 6803 exhibited circadian rhythms in complete darkness. To monitor a circadian rhythm of the *Synechocystis* cells in darkness, we introduced a *P* _dnaK1::luxAB* gene fusion (S. Aoki, T. Kondo, and M. Ishiura, J. Bacteriol. 177:5606–5611, 1995), which was composed of a promoter region of the *Synechocystis dnaK1* gene and a promoterless bacterial luciferase *luxAB* gene set, as a reporter into the chromosome of a dark-adapted *Synechocystis* strain. The resulting *dnaK1*-reporting strain showed bioluminescence rhythms with a period of 25 h (on agar medium supplemented with 5 mM glucose) for at least 7 days in darkness. The rhythms were reset by 12-h-light–12-h-dark cycles, and the period of the rhythms was temperature compensated for between 24 and 31°C. These results indicate that light is not necessary for the oscillation of the circadian clock in *Synechocystis*.

Circadian rhythms are biological oscillations with a period of about 1 day and are found ubiquitously in organisms from cyanobacteria to humans. Since circadian rhythms in diverse organisms persist under constant conditions (i.e., constant light or darkness at a constant temperature), an endogenous mechanism called “circadian clock” that generates the rhythms is postulated.

Light resets the phases of circadian rhythms in all organisms examined so far. In green plants, a phase-resetting light signal or signals are supposed to be mediated by phytochrome (8, 23) or a blue-light receptor (21). Light is also required for sustaining circadian rhythms in plants and algae (7, 10, 11, 15, 20, 22, 24), because circadian rhythms are dampened in complete darkness (DD), even in the presence of exogenously added energy sources. In *Lemna gibba* (12), *Samanea saman* (23), and *Albizia julibrissin* (20), daily exposure to brief red-light pulses sustains the circadian rhythms in darkness. In wheat, a red-light pulse prevents the damping of the circadian rhythms of *CAB-1* gene expression in DD, and far red reverses the red-light effect (17). In tobacco, overexpression of a rice phytochrome makes the circadian rhythms of *CAB* gene expression persistent in DD (10). Therefore, a light signal mediated by phytochrome has an important role in sustaining the expression of the circadian rhythms in these plants. However, whether such a light signal is necessary (i) for the oscillation of the circadian clock itself, (ii) for the expression of a specific gene (for example, the *CAB* gene family), or (iii) for the ability to utilize exogenously added energy sources which are necessary for sustaining the expression of circadian rhythms remains to be resolved.

Previously, by using a bacterial luciferase *luxAB* gene set as a reporter, we demonstrated that a circadian clock controls the expression of the *dnaK1* gene, which encodes a heat shock protein, DnaK, in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (2). The *dnaK1*-reporting strain CFC2 cultured under continuous illumination (LL) exhibited circadian rhythms of bioluminescence, which reflect the rhythmic activation of the *dnaK1* promoter (2). However, the bioluminescence declined completely within 1 or 2 days when the cells were maintained in DD. In cyanobacteria, it remains to be determined whether a light signal is necessary for the oscillation of the circadian clock.

We took advantage of the fact that *Synechocystis* can grow heterotrophically on glucose in darkness when the cells are exposed to a daily, brief light pulse (light-activated heterotrophic growth [LAHG] [1]). The *Synechocystis* cells grown under LAHG conditions (dark-adapted cells) can continue to grow for 6 to 8 days even in DD (1). We constructed a dark-adapted *dnaK1*-reporting strain and found that this strain showed a persistent circadian rhythm of bioluminescence even in DD, indicating that the oscillation of the clock does not need any light signal in *Synechocystis*.

**MATERIALS AND METHODS**

*Bacterial strains, media, and cultures.* Wild-type cells of *Synechocystis* sp. strain PCC 6803 were maintained in BG-11 liquid medium (19) or on BG-11 agar that contained 1 mM sodium thiosulfate, 10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES [pH 8.2]), and 1.5% (wt/vol) Bacto-Agar (Difco Laboratories, Detroit, Mich.) under LL (intensity of about 35 μmol m~2~ s~−1~ from white fluorescent lamps), as described previously (2). A bioluminescent *dnaK1*-reporting strain of *Synechocystis* CFC2 (2), was maintained in BG-11 medium (or agar) supplemented with 40 μg of spectinomycin sulfate per ml. Dark-adapted cells of *Synechocystis* were maintained under LAHG conditions, in which cells were grown in the presence of 5 mM glucose in darkness except for a 15-min light pulse of 40 μmol m~−2~ s~−1~ every 24 h (1). (Although Anderson et al. [1] used daily 5-min light pulses, we used 15-min light pulses for technical convenience.) A *dnaK1*-reporting strain of the dark-adapted *Synechocystis* constructed in this study, CFC4, was maintained under the LAHG conditions in the presence of 40 μg of spectinomycin sulfate per ml. Unless otherwise stated, cells were cultured at 27°C.

*Construction of a dnaK1-reporting strain of dark-adapted *Synechocystis*.* We first adapted wild-type cells of *Synechocystis* and CFC2 cells gradually to LAHG conditions as follows (24a). Cells were inoculated into 100 ml of BG-11 liquid medium containing 5 mM glucose and cultured with vigorous shaking (rotation speed of about 180 rpm) under constant illumination (LL) of 35 μmol m~−2~ s~−1~ on the first day. The light period was then decreased stepwise by 2 h a day. On the 13th day, the cells were transferred to and then maintained under LAHG conditions and used as dark-adapted cells. The dark-adapted cells grew continuously under LAHG conditions, both in liquid medium and on agar medium.
Next, we transformed the dark-adapted *Synechocystis* cells with a *dnaK1* reporter plasmid pCF5 to introduce a *P* _dnaK1::luxAB* gene fusion into a specific targeting site of the genome (2), as described previously (25) with some modifications. pCF5 carries a *P* _dnaK1::luxAB* gene fusion, namely, the promoter region of the *Synechocystis dnaK1* gene (*P* _dnaK1_) (15) connected to the coding region of the *Vibrio harveyi* luciferase gene set (*luxAB*) (4), and the Ω fragment (18) that contains the spectinomycin-streptomycin resistance gene (2). The dark-adapted strain, CFC4. The cells were cultured under LAHG conditions and most cells lost viability rapidly in DD.

We examined further whether light-dark cycles entrain the bioluminescence rhythms from these two cultures to three 12-h-light–12-h-dark (LD) cycles (light intensity of about 1.2 μmol m$^{-2}$ s$^{-1}$ from white fluorescent lamps) that were 180° out of phase and then maintained them in DD to monitor their bioluminescence (Fig. 2).

We found that the bioluminescence from these two cultures oscillated with opposite phases which depended on the timing of the preceding LD cycles, although the level of bioluminescence declined. The peak of bioluminescence occurred about 18.7 h after the onset of DD.

Entrainment of the bioluminescence rhythms to daily light-dark cycles and the phase shift of the rhythms by illumination. We examined further whether light-dark cycles entrain the bioluminescence rhythms of CFC4 cells. We exposed two cultures to three 12-h-light–12-h-dark (LD) cycles (light intensity of about 1.2 μmol m$^{-2}$ s$^{-1}$ from white fluorescent lamps) that were 180° out of phase and then maintained them in DD to monitor their bioluminescence (Fig. 2).

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Temperature compensation of the bioluminescence rhythms.

We examined the effects of temperature on the period length of the bioluminescence rhythms. CFC4 cells were cultured under LAHG conditions for 3 days, and then the cells were maintained in DD at three different temperatures (24, 27, and 31°C) to have their bioluminescence (photon counts per second) monitored. Bioluminescence rhythms observed at the three different temperatures are shown. Other conditions were the same as those described in the legend to Fig. 1.

We found that the period lengths of the rhythms were 25.7, 25.0, and 27.2 h at 24, 27, and 31°C, respectively. The Q10 values (temperature coefficients) for 24 to 27°C and for 27 and 31°C were calculated separately to be 1.1 and 0.8, respectively. The phases of the rhythms observed at 24 or 31°C significantly differed from those observed at 27°C. The rhythms were unstable at temperatures higher than 31°C or lower than 24°C. These data indicate that the period length of the rhythms was compensated against changes in ambient temperature.

The data described above indicate that the bioluminescence rhythms of CFC4 cells maintained in DD satisfied the three criteria for circadian rhythms, as did those of CFC2 cells cultured under LL (2), namely, persistence under constant conditions (Fig. 1), entrainment to daily LD cycles (Fig. 2), and temperature compensation of the period (Fig. 4). Thus, we can conclude that the bioluminescence rhythms of CFC4 cells maintained in DD are circadian rhythms. Furthermore, the magnitude and direction of the phase shifts induced by an illumination depended on the time of day at which the light illumination was administered (Fig. 3). This observation represents the basic character of light responsiveness of circadian rhythms.

We demonstrated here that no light signal was necessary for the oscillation of the circadian clock or for the expression of bioluminescence rhythms in the dark-adapted Synechocystis strain CFC4. Strain CFC2 could not grow heterotrophically on glucose and therefore lost viability in DD (Fig. 1).

In contrast to cyanobacteria, several higher plants require a light signal which is supposed to be mediated by a phytochrome to sustain the expression of circadian rhythms (10, 12, 17, 20, 23). It is uncertain whether such a light signal is necessary to sustain the oscillation of the circadian clock itself in these plants, because downstream events which are used for monitoring the clock in these plants may depend on light. In the case of the CAB gene reporter, we cannot monitor the expression of the CAB gene in DD because a light signal is required for its expression (10, 17). Thus, it is possible that no light signal is required for the oscillation of the clock in DD because the expression of the dnaK1 gene occurs in DD.

There are several differences between the bioluminescence rhythms of the CFC2 strain cultured under LL (2) and the CFC4 strain maintained in DD. First, the free-running period of CFC4 cells maintained in DD (25 h) is longer than that of

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**Figures:**
- FIG. 2. Entrainment of the bioluminescence rhythms of CFC4 cells to LD cycles. To synchronize the circadian clock, two CFC4 cultures were subjected to LD cycles (light intensity was about 1.2 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) from white fluorescent lamps) which were 180° out of phase, and then the cells were maintained in DD for monitoring of bioluminescence (photon counts per second). The last 12-h light periods in the LD cycles (open bars) and the DD (filled bars) are shown on the upper abscissa for the open circles and lower abscissa for the solid circles. The lower portions of the vertical axes are omitted for precise comparison of the phases of the two rhythms. Other conditions were the same as described in the legend to Fig. 1.
- FIG. 3. Phase response induced by light. A 3-h illumination (1.2 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) was given to CFC4 cells at various times during the first day after the last LAHG pulse. Other conditions were the same as those described in the legend to Fig. 1. The magnitude of phase shifts was calculated as the difference between the times when the fourth peak occurred in control (without illumination) and experimental cultures and is shown as a function of the time of onset of illumination after the last LAHG pulse. Positive and negative values in the ordinate indicate phase advances and delays, respectively. Data points are means for four replicate experiments, and the bars on each point indicate the standard deviation. We did not draw lines between the data points because it is not clear whether the Synechocystis PRC is continuous.
- FIG. 4. Temperature compensation during the period of the bioluminescence rhythms. After culture of CFC4 cells under LAHG conditions for 3 days, cells were maintained in DD at three different temperatures (24, 27, and 31°C) to have their bioluminescence (photon counts per second) monitored. Bioluminescence rhythms observed at the three different temperatures are shown. Other conditions were the same as described in the legend to Fig. 1.
CF2 cells cultured under LL (22.6 ± 0.1 h; n = 4 [2a]) by about 2.4 h at 27°C. The free-running period of circadian rhythms in many organisms observed in DD is often different from that observed under LL (3). An extreme example of this phenomenon is seen in Arabidopsis seedlings, in which the period in DD is greater than 30 h, while the period under LL is about 25 h (15). Mutations of Arabidopsis seedlings that activate phototransduction pathways shorten the period length of circadian rhythms in CAY2 gene expression, suggesting that activation of phototransduction pathways is supposed to affect the period of circadian rhythms (15).

Second, the rhythms of strain CFC4 maintained in DD persisted stably for a week, whereas those of strain CF2 cultured under LL persisted only for 5 days with significant dampening (the peak/trough ratios of the bioluminescence rhythms observed in strain CF2 were about 3 and 1.3 on the first day and the fifth day of LL, respectively [2]). At present, we do not know why the rhythms of strain CF2 cultured under LL were dampened more rapidly. In the unicellular cyanobacterium Synechococcus sp. strain PCC 7942, the bioluminescence rhythms persist very stably for more than 2 weeks under LL (14).

Third, the average level of bioluminescence from the CFC4 strain maintained in DD (Fig. 1) was about 10 times higher than that from strain CF2 cultured under LL (2). This increase in bioluminescence observed in DD was mainly due to the increase in a nonoscillating component of bioluminescence. Previously, we demonstrated by Northern blotting analyses that the level of luxAB mRNA in CF2 cells immediately after a dark-to-light transition was significantly higher than that in the cells at later times under LL (2). This suggests that the level of luxAB mRNA may be down-regulated by light.

Furthermore, the bioluminescence reaction catalyzed by bacterial luciferase is also affected by changes in the levels of its substrates, n-decanal, reduced flavin mononucleotide (FMNH2), and O2. The levels of the latter two substrates may be different, because the metabolic states of cells should be different between CF2 cells cultured under LL and CFC4 cells maintained in DD.

We have demonstrated that single light pulses induce a significant phase shift in the bioluminescence rhythm of strain CFC4 (Fig. 3). We entrained the Synechocystis circadian clock by LAHG light pulses to obtain a PRC (Fig. 3). Thus, it is clear that the strains CFC2 and CFC4 (Fig. 3). We entrained the cyanobacterium Synechocystis sp. strain PCC 6803: a blue-light-resetting process. J. Bacteriol. 172:2761–2767.


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