**Communication**

**Circadian Rhythm of the Prokaryote *Synechococcus* sp. RF-1**

Tan-Chi Huang*, Jenn Tu, Te-Jin Chow, and Tsung-Hsien Chen  
*Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan 11529, Republic of China*

**ABSTRACT**

The prokaryotic *Synechococcus* sp. RF-1 exhibited a nitrogen fixation circadian rhythm with characteristics remarkably similar to the circadian rhythm of eukaryotes. The rhythm had a free-running period of about 24 hours when the length of the preentrained cycle did not differ too much from 24 hours, and it was insensitive to changes in temperature from 22°C to 33°C. Because the endogenous rhythm of nitrogen fixation was not affected by a phase-shift of its previous cycles, the circadian rhythm in *Synechococcus* sp. RF-1 was not considered to be controlled simply by a feedback mechanism.

Many biological properties of eukaryotes are known to fluctuate rhythmically, with either circadian (about 24 h) or noncircadian periods. The properties of the circadian rhythms of plants, animals, and eukaryotic microorganisms have been extensively studied and reviewed (1, 4, 7, 12, 14). However, since circadian rhythms had not been reported in prokaryotic organisms, it was proposed that the circadian rhythm is a unique property of eukaryotes (9). Grobbelaar et al. (5) in 1986 reported that a unicellular cyanobacterium *Synechococcus* sp. RF-1, a prokaryote, exhibits an endogenous N₂-fixing rhythm after being preconditioned to a diurnal light-dark regimen. Another aerobic N₂-fixing *Synechococcus (Synechococcus* sp. RF-1) (11) exhibits an endogenous rhythm similar to that of *Synechococcus* sp. RF-1. Recently, Sweeney and Borgese (15) reported that *Synechococcus* WH7803 possesses a circadian rhythm in cell division. In this report, data are presented to support the conclusion that the N₂-fixing rhythm of *Synechococcus* sp. RF-1 established in a light-dark regimen, is a circadian rhythm. Some physiological properties of the circadian rhythm of *Synechococcus* sp. RF-1 were also investigated.

**MATERIALS AND METHODS**

**Organism and Cultivation**

The axenic culture of *Synechococcus* sp. RF-1 described by Huang and Chow (8) was used in this study. The organism has been deposited at Pasteur Collection Center and designated PCC8801. Cultures were cultivated without aeration or shaking in Erlenmeyer flasks containing nitrate-free BG-11 medium (13) supplemented with 0.01 M EPPS buffer (pH 8.0). Unless stated otherwise, the culture was incubated at 28°C under about 35 μmol photon m⁻² s⁻¹ from white fluorescent tubes (Toshiba FL 20D/18, Taiwan Fluorescent Lamp Co.). They were grown in either an LL or an LD regimen depending on the requirements of the experiment. Cell concentration was estimated from measurements of optical density made with a Klett-Summerson photoelectric colorimeter fitted with a No. 42 blue filter. The total protein content in 10⁵ cells was 0.21 mg, determined by the Lowry method (10) after the cells were hydrolyzed with 1 N NaOH in a waterbath at 100°C for 10 min.

**Assay of Nitrogenase Activity**

Nitrogenase activity was assayed by the acetylene reduction method (3). Samples (1 mL) were removed from the cultures and placed into 14 mL test tubes. The test tubes were sealed with rubber stoppers and 1.4 mL commercial acetylene was added. A 0.5 mL gas sample was analyzed for its ethylene concentration by gas chromatography at the beginning of the incubation period and again 1 h later. During the incubation period the test tubes were kept under the same conditions of light and temperature as the parent cultures.

**RESULTS AND DISCUSSION**

Cultures of *Synechococcus* sp. RF-1 attained rhythmic N₂-fixing activity when they were grown under several LD regimens. The period length of the N₂-fixing cycle was entrained to 28, 24, 20, 16, or 12 h, respectively, when they were grown in 14:14, 12:12, 10:10, 8:8, or 6:6 LD cycles (Fig. 1). When the cultures which had been adapted to these different LD cycles were transferred to LL, the cultures which were entrained to 14:14, 12:12, or 10:10 LD all exhibited an endogenous nitrogen-fixing rhythm with the free-running period of about 24 h for at least four cycles (Fig. 1). Cultures which were entrained to LD cycles with a period considerably different from 24 h, such as 8:8 or 6:6 LD, exhibited an endogenous rhythm for one or two cycles only. It suggests that the endogenous clock of the N₂-fixing rhythm in *Synechococcus* sp. RF-1 has a period of 24 h. When the cells were entrained by cycles that differed considerably in length from 24 h, they

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1 Abbreviations: EPPS, N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid; LL, continuous light; LD, light-dark.
apparently found it difficult to adjust back from these extreme physiological conditions.

For an endogenous rhythm to qualify as a circadian rhythm, the most important requirement is that the rhythm must have a period of about 24 h in an unvarying environment, i.e., it must have a free running period of about 24 h. Like eukaryotes, the prokaryotic *Synechococcus* sp. RF-1 can adjust its N$_2$-fixation phase and generate a characteristic circadian rhythm when the length of the entrained LD cycle is not very different from 24 h.

When cultures of *Synechococcus* sp. RF-1, preadapted to 12:12 LD cycles at 28°C, were incubated in continuous light at temperatures ranging from 22 to 33°C, their rhythm remained in phase (Fig. 2) at the different temperatures, although the N$_2$-fixing activity at 33°C was about double that at 22°C (Q$_{10}$ about 2). These results indicate that the endogenous N-fixing rhythm of *Synechococcus* sp. RF-1 is insensitive to changes in temperature.

It is known that Ca$^{2+}$ is required for the N$_2$-fixing activity of *Synechococcus* sp. RF-1 (2). The N$_2$-fixing activity is suppressed in the presence of EGTA, and the activity can later be restored by the addition of additional Ca$^{2+}$ to the culture. As shown in Figure 3, when the N$_2$-fixation activity during the last dark period was delayed by 8 h, the first free running endogenous N$_2$-fixing peak exhibited under free running con-

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**Figure 1.** Rhythms of nitrogenase activity of *Synechococcus* sp. RF-1 in several different LD cycles followed by LL. The cultures were adapted to the LD conditions for one week before exposing them to LL.

**Figure 2.** Endogenous rhythm of nitrogenase activity of *Synechococcus* sp. RF-1 at several different temperatures. The cultures were all initially grown at 28°C under a 12:12 LD regimen for 1 week. They were then transferred to LL at 22, 26, 29, and 33°C, respectively. The N$_2$-fixing activity of these cultures incubated at various temperatures was assayed at 2 h interval for a period of 4 d.

**Figure 3.** Phase of the endogenous N$_2$-fixing rhythm was not affected by a shift of the last dark-entrained, nitrogen-fixing peak. *Synechococcus* sp. RF-1 adapted to 16:8 LD cycle was used in this experiment. EGTA (1 mM) was added to the culture at the onset of darkness, and 2 mM CaCl$_2$ was added 8 h later (indicated by arrow). (●), Control experiment without EGTA; (□), treated with EGTA and Ca$^{2+}$.
ditions was in phase with that of the control lacking EGTA. The results suggest that a simple feedback mechanism involving the products of the N₂-fixation process do not play an important role in regulating the timing of the endogenous nitrogen-fixing rhythm.

Thus, in addition to the 24 h free-running period, the N₂-fixing circadian rhythm of *Synechococcus* sp. RF-1 is similar to the circadian rhythms of eukaryotes in that its phases can be entrained by LD cycles of different lengths (see refs. 1, 7, and 14 for a review), and in that its endogenous rhythm is insensitive to changes in temperature (6). The remarkable similarity between the circadian rhythms of eukaryotes and the prokaryotic *Synechococcus* suggests that a better understanding of the control mechanism of the endogenous N₂-fixing circadian rhythm in *Synechococcus* will be highly beneficial for understanding the universal mechanism for circadian rhythm.

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**LITERATURE CITED**