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Resetting the endogenous circadian N_2-fixing rhythm of the prokaryote *Synechococcus* RF-1.

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Abstract. *Synechococcus* RF-1 fixed N_2 rhythmically with the nitrogenase activity peak within the dark period when growing in a diurnal L/D regimen. When the light and dark phases were exchanged, the nitrogenase activity peak phase-shifted after one cycle of the new regimen to conform to the new (reverse phase) regimen. When a culture which was adapted to a 12h L/12h D regimen was exposed to the reverse phase regimen for two cycles, and then incubated in L/L, an endogenous N_2-fixing rhythm was obtained with the nitrogenase activity peaks coinciding with the dark periods of the reverse phase regimen. The results indicate that *Synechococcus* RF-1 can adapt to a phase resetting of the light-dark regimen under which it is growing with about the same efficiency as it can acquire an endogenous circadian rhythm when a L/L culture is exposed to a L/D regimen.

Key words: Circadian rhythm; Cyanobacteria; Nitrogen-fixation; Phase-resetting; Prokaryote; *Synechococcus.*

Introduction

Endogenous circadian rhythms have been found in many eukaryotic organisms, including plants, animals and eukaryotic micro-organisms. Because endogenous circadian rhythms had not been reported in prokaryotes, it was proposed that circadian rhythms are a unique property of eukaryotes (Kipper, 1988). Grobbelaar *et al.* (1986) reported that a prokaryotic unicellular cyanobacterium, *Synechococcus* RF-1, exhibits an endogenous N_2-fixing rhythm after being preconditioned to a diurnal light-dark regimen. A marine *Osillatoria* sp. (Stal and Krumbein, 1985) and *Synechococcus* Miami BG43511 (Leon *et al.*, 1986) exhibit an endogenous N_2-fixing rhythm similar to that of *Synechococcus* RF-1. Recently, Sweeney and Borgese (1989) reported that *Synechococcus* NH7803 possesses a circadian rhythm in cell division.

The characteristics of the endogenous circadian rhythms of eukaryotes have been well documented (Edmunds, 1988; Hillman, 1976; Sweeney, 1987). However, information about the circadian rhythms of the structurally and physiologically more primitive prokaryotes is rather limited. The characterization of the circadian rhythms of prokaryotes may provide fundamental knowledge which is essential to the understanding of the control mechanism of biological timekeeping. In this report, the adaptation of *Synechococcus* RF-1 to the resetting of the phases of a light-dark regimen was studied.

Materials and Methods

Organism and Culturing

The axenic culture of *Synechococcus* RF-1 described by Huang and Chow (1986) was used. It was cultured without shaking in 125 ml Erlenmeyer flasks containing 30 ml EPPS-buffered $BG\text{-}11_0$ medium (Stanier *et al.*, 1971). The cultures were kept at 28°C and were exposed to either a 12h light/12h dark regimen or to continuous white light from fluorescent tubes

(Toshiba FL 20D/18, Taiwan Fluorescent Lamp Co.) at about 35 μmol photons • m^{-2} • s^{-1}. Cell concentration was estimated according to the procedures described previously (Chou *et al.*, 1989).

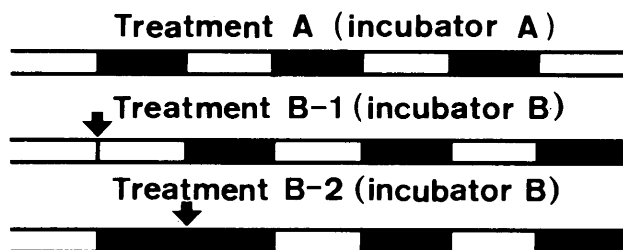
Nitrogenase Activity

Nitrogenase activity was assayed by the acetylene reduction method (Dilworth, 1966). Culture samples (0.5 ml) were sealed in 14 ml test tubes with rubber septums and 1.4 ml commercial acetylene was added per tube. Gas samples (0.2 ml) were analyzed for their ethylene content at the beginning of the incubation period and again one hour later by a Shimadzu GC-3BF gas chromatograph equipped with a flame ionization detector. The samples were incubated under the same conditions of light and temperature as the parent cultures.

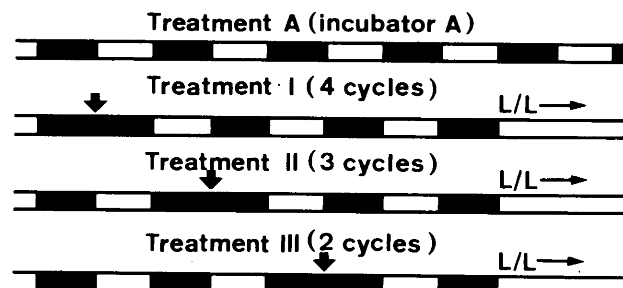
Resetting of the Light-Dark Phase

Two incubators (A and B) were used which had their 12h L/12h D cycles set so that when the lights of the one was on, the lights of the other was off.

Two experiments were carried out for examining the effects of the L/D phase-change on the rhythmic nitrogenase activity. For the first experiments, the parent culture was kept in incubator A for weeks and when the experiment was started, all the flasks were initially also kept in incubator A. One of the flasks was transferred to incubator B at the beginning (Treatment B-1) and another at the end (Treatment B-2) of one of the dark periods of incubator A. The light-dark periods of the cultures that were transferred were therefore exchanged by initially introducing either one double light period (Treatment B-1) or one double dark period (Treatment B-2) as shown in the diagram below:



In the second experiment, three flasks were transferred from incubator A to incubator B one by one at 24h intervals at the end of the dark periods of incubator A. The L/D periods of the cultures that were transferred were therefore exchanged as in the case of the B-2 treatment of the first experiment. After the transferred cultures were exposed to four, three, and two cycles (see diagram below) of the reversed L/D cycles respectively, they were exposed to continuous light and regularly monitored for their nitrogenase activity.



Results and Discussion

Synechococcus RF-1 fixes N_2 almost exclusively within the dark phase when growing in a diurnal 12h L/12h D regimen (Huang and Chow, 1986). In order to examine the effects of substituting light periods for dark periods and *vice versa*, the cells growing in an incubator set at 08h00-20h00 dark were transferred into another incubator set at 08h00-20h00 light at 08h00 and 20h00 respectively. As shown in Fig. 1, the first nitrogenase activity peak after the exchange of the L/D periods was delayed until the arrival of the second dark period of the new regimen. Initially the nitrogenase activity commenced about 2h before the beginning of the new dark period, but in all subsequent cases, the nitrogenase activity peaks fell wholly within the dark periods of the new regimen.

The results shown in Fig. 1 indicate that under L/D entraining conditions, *Synechococcus* RF-1 adapted its N_2-fixing rhythm to the new L/D regimen during the first cycle after the change. In order to see whether the re-adjusted cells have acquired a corresponding new endogenous N_2-fixing rhythm, they were transferred to continuous light after being exposed to four, three and two cycles of the new L/D regimen. As shown in Fig. 2, the cultures in all cases exhibited an endogenous circadian nitrogenase rhythm which corresponded to the latest L/D regimen to which they were exposed. However, after exposure to only two cycles (Fig. 2-III) of the new L/D regimen, the nitrogenase activity peaks were relatively broad and were not restricted to the times which corresponded to the dark periods of the

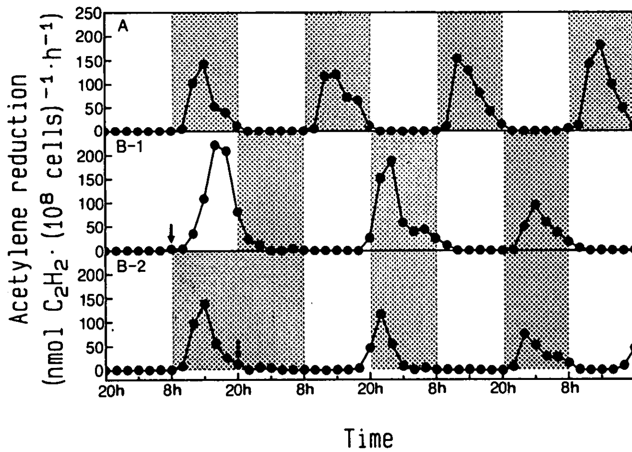


Fig. 1. Resetting the N_2-fixing rhythm of *Synechococcus* RF-1 which was adapted to a L/D regimen by exchanging the light and dark periods either at the beginning or end of one of the dark periods of the original regimen. Cultures adapted to a 12h L/12h D regimen (Treatment A; 08h00 -20h00 dark) was transferred to a reversed 12h L/12h D regimen either at the beginning of a dark period (Treatment B-1) or at the end of the same dark period (Treatment B-2) as indicated by arrows.

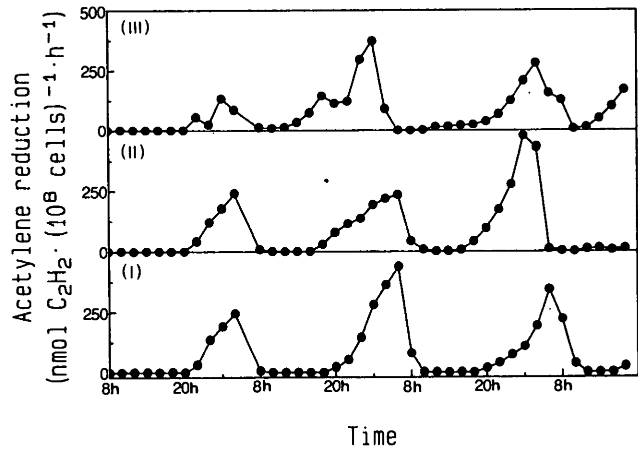


Fig. 2. Resetting the endogenous N_2-fixing rhythm of *Synechococcus* RF-1. Cultures adapted to a 12h L/12h D regimen (08h00-20h00 dark) were exposed to four (Treatment I), three (Treatment II) or two (Treatment III) reversed L/D cycles (08h00-20h00 light). The cultures were thereafter incubated in L/L and regularly assayed for nitrogenase activity.

latest L/D regimen. The cells established a well defined endogenous circadian N_2-fixing rhythm only after four cycles of the new regimen (Fig. 2-I).

Synechococcus RF-1 fixes N_2 continuously when growing under continuous light (Huang and Chow, 1986). It was shown in a previous report (Chou *et al.*, 1989), that when a culture that was growing in continuous light, is transferred to a diurnal 12h light /12h dark regimen, a periodic nitrogenase activity that coincides with the dark periods is induced in the culture within one L/D cycle. In order to examine how many L/D cycles are required to induce an endogenous N_2-fixing rhythm in a culture that had been adapted to growing in continuous light, such a culture was exposed to a L/D regimen for three, two and one cycle respectively before it was returned to continuous light. As shown in Fig. 3, one L/D cycle was sufficient to induce an endogenous circadian N_2-fixing rhythm. However, after such a short induction period, the nitrogenase activity was not completely restricted to the times that corresponded to the dark periods of the inductive regimen. After two L/D cycles, the nitrogenase activity fell almost exclusively within the periods that corresponded to the dark periods of the inductive regimen. A normal endogenous N_2-fixing rhythm was attained only after the culture received three cycles of the inductive regimen.

The results of Figs. 2 and 3 indicate that *Synechococcus* RF-1 can reset its endogenous N_2-fixing rhythm with an efficiency that is comparable to the efficiency with which a culture that was adapted to continuous light, can establish an endogenous N_2-fixing rhythm induced by diurnal light-dark entraining conditions.

The biological clock of eukaryotes has been known to have a number of common characteristics including the free-running rhythm, insensitiving to temperature, susceptibility to resetting of the phases by a change in the L/D regimen and a requirement for a signal from the environment to start the clock (Hall and Rosbash, 1987). From the results reported previously (Chou *et al.*, 1989; Huang *et al.*, 1990) and the results of this study, the endogenous N_2-fixing circadian rhythm of the prokaryotic *Synechococcus* RF-1 has the same characteristics as those mentioned above for the biological clock of eukaryotes. The remarkable

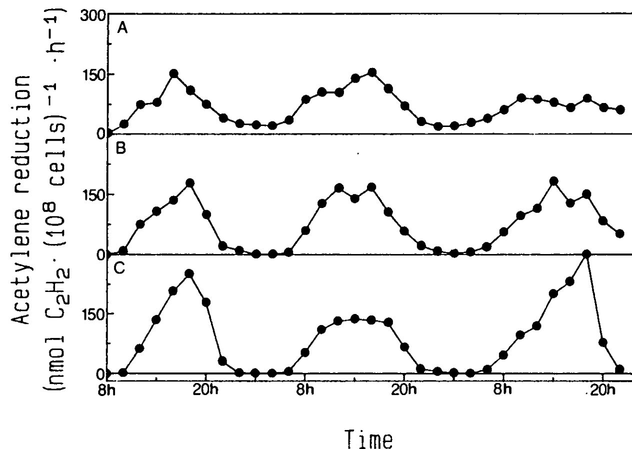


Fig. 3. The effect of the number of L/D induction cycles on the establishment of an endogenous N_2-fixing rhythm in *Synechococcus* RF-1. The culture which was adapted to growing in L/L was exposed to one (A), two (B) or three (C) 12h L/12h D cycles respectively whereafter they were transferred to L/L and their nitrogenase activity monitored regularly.

similarily between the circadian rhythm of a prokaryotic organism and the eukaryotes suggests that the time-keeping mechanism of the biological clocks of prokaryotes and eukaryotes are similar if not identical and that it does not involve organelles such as plastids and mitochondria which do not occur in prokaryotic cells.

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原核型聚球藻 RF-1 品系內生固氮週期韻律之再設定

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聚球藻 RF-1 品系培養於 12h/12h (8h00-20h00，暗)之光/暗週期下，其固氮活性在暗週期出現，如將光/暗週期互相對調(即 8h00-20h00，光)，則其固氮週期韻律發生位移，經一次新的光/暗週期後，即可與新的光/暗週期一致。當此藻經新的光/暗週期處理後移到連續照光，只要經過二次新的光/暗週期處理，即可建立新的內生固氮韻律。此報告亦提供資料證明再設定所需之時間與其建立週期韻律的時間大體相同。