REVIEW
ARTICLEThe circadian clock in the prokaryote
Synechococcus RF-1Tan-Chi Huang¹ and Nathanaël Grobbelaar²

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Circadian rhythms in prokaryotes

The term 'circadian rhythm' is used to denote an endogenous oscillation in a behavioural or biochemical phenomenon which (1) persists for at least several days under uniform environmental conditions, (2) has a periodicity of about 24 h (*circa* = approximately; *diem* = day) and (3) is unique amongst rhythms in living organisms in that it has a period of oscillation that shows a high degree of temperature compensation, so that it changes but little at different constant ambient temperatures (Wilkins, 1992). The term 'diurnal rhythm' is reserved for conditions in which there is a direct input of external periodic signals. Consequently 'diurnal rhythms' do not manifest themselves under uniform environmental conditions. Many biological parameters are coupled to the circadian clock mechanism and therefore exhibit daily rhythmic fluctuations (Edmunds, 1988). Some parameters which have been intensively studied include conidiation in *Neurospora* (Feldman, 1988; Dunlap, 1993), bioluminescence in *Gonyaulax* (Johnson & Hastings, 1986), eclosion activity in *Drosophila* (Hall, 1990; Young *et al.*, 1989), leaf movement (Satter *et al.*, 1988) and photosynthesis (Hennessey & Field, 1991; Wilkins, 1992) in plants, and body temperature, hormone and enzyme levels and sensitivity to drugs in man (Moore-Ede *et al.*, 1982; Arend *et al.*, 1989). Because of the unsuccessful attempts to observe circadian rhythms in *Escherichia coli*, halobacteria or cyanobacteria, it was believed that circadian rhythms do not occur in prokaryotes (Kippert, 1988). The nonprokaryote dogma was overturned in 1986 by a report that the prokaryotic *Synechococcus* RF-1 displays endogenous rhythms in nitrogenase activity (Grobbelaar *et al.*, 1986). Since then, circadian rhythms of cell division (Sweeney & Borgese, 1989), nitrogenase activity (Huang *et al.*, 1990), amino acid uptake (Chen *et al.*, 1991), *nif* gene expression (Huang & Chow, 1990) and *psbA* gene expression (Kondo *et al.*, 1993) in the prokaryotic *Synechococcus* have been reported. The prokaryote is structurally and functionally simpler than the eukaryote

and therefore it is potentially a useful model system for studying the control mechanism of circadian rhythms. Although the chemical or physical nature of the oscillator in prokaryotes, as well as in eukaryotes, is still unknown, the physiological properties of circadian rhythms have been well characterized. In future the prokaryotic system should play an important role in elucidating fundamental questions regarding circadian rhythms. Thus far, most of the studies on prokaryote circadian rhythms have been carried out on *Synechococcus* RF-1. As a consequence, this review deals mainly with studies involving this particular prokaryote. A summary of the characteristics of *Synechococcus* RF-1 should therefore provide a useful background to the remainder of this review.

Characteristics of *Synechococcus* RF-1

Synechococcus RF-1 (pcc 8801) is an aerobic nitrogen-fixing unicellular cyanobacterium isolated from a rice field in Taiwan (Huang & Chow, 1986). It is a sheathless organism with an average size of $3.8 \times 3.0 \mu\text{m}$ (Huang & Chow, 1988b). Structurally, it contains many uncommon polyglucan granules with a size varying from 0.1 to 0.4 μm which are found only in the sheathless nitrogen-fixing unicellular cyanobacteria (Chou *et al.*, 1994). *Synechococcus* RF-1 is a strict photoautotroph having a generation time of about 3 d when cultivated in sodium-nitrate-free BG-11 medium (Stanier *et al.*, 1971) at 28 °C under 35 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of light from white fluorescent lamps (Huang & Chow, 1986). The physiological temperature range for *Synechococcus* RF-1 is 20 °C–42 °C when growing in BG-11, or 20 °C–37 °C in the sodium-nitrate-free BG-11. The cells can survive in a 'suspended state' for more than 2 weeks in constant darkness (D/D) (Huang & Pen, 1994). *Synechococcus* RF-1 has peroxidase activity (Huang & Chow, 1986), but catalase and superoxide dismutase activity could not be detected (T.-C. Huang, unpublished data). Two forms of intracellular α -amylase, one EGTA sensitive and the other EGTA insensitive, exist in the

crude cell extract (Chou *et al.*, 1994). Enzymes related to ammonia assimilation, such as glutamine synthetase (GS), ferredoxin-dependent glutamate synthase (Fd-GOGAT), glutamate dehydrogenase (GDH) and alanine dehydrogenase (ADH), have been detected in the crude extracts with very high specific activity (H.-F. Yuan, personal communication). A novel class II restriction endonuclease (*Ssp*RF1) has been isolated from *Synechococcus* RF-1 (Li & Tu, 1991). The enzyme recognizes the palindrome 5'-TT/CGAA-3' and generates 5'-protruding CG-dinucleotides. Low-salt conditions enhance the activity of *Ssp*RF1 whilst the methylation of its inner adenine residue inhibits its activity.

Synechococcus RF-1 fixes nitrogen continuously under constant light (L/L). It fixes nitrogen mainly during the dark periods when the L/L culture is transferred to diurnal light/dark (L/D) cycles (Huang & Chow, 1986). Calcium ions are required in the medium for nitrogenase activity *in vivo* (Chen *et al.*, 1988). Since O₂ will form during photosynthesis and nitrogenase is O₂-labile, the relationship between photosynthesis and nitrogenase activity in the aerobic nitrogen-fixing unicellular cyanobacteria has attracted much attention (Gallon, 1992). In *Synechococcus* RF-1, increasing the ambient CO₂ concentration will increase the rate of CO₂ assimilation but significantly decrease nitrogenase activity, presumably due to competition for the energy and reducing power required for both reactions (Grobbelaar *et al.*, 1992). The nitrogenase activity of a L/L culture is drastically reduced by CO₂ concentrations higher than 1% – a phenomenon which is uncommon among the cyanobacteria. 3-(3,4-Dichlorophenol)-1,1-dimethylurea (DCMU), which inhibits photosynthetic CO₂ assimilation, temporarily stimulates the nitrogenase activity of both L/L and L/D cultures in the light (Grobbelaar *et al.*, 1992).

Circadian nitrogenase activity and leucine-uptake rhythms in *Synechococcus* RF-1

The oscillator in *Synechococcus* RF-1 controls the circadian rhythms of its nitrogenase activity (Huang *et al.*, 1990), leucine uptake (Chen *et al.*, 1991), photosynthesis (Y.-J. Shieh, personal communication), ATP level (T.-H. Chen, personal communication) and protein synthesis activity (Huang *et al.*, 1994). Among the overt rhythms, the properties of the circadian nitrogenase activity and leucine-uptake rhythms have been well characterized. A *Synechococcus* RF-1 culture fixes nitrogen and takes up leucine arrhythmically when adapted to continuous illumination under aerobic conditions. When the arrhythmic culture is entrained by a suitable diurnal L/D or temperature regimen, the culture simultaneously exhibits a circadian nitrogenase activity and leucine-uptake rhythm, but the leucine-uptake peaks are about 12 h out-of-phase compared to the nitrogenase activity peaks (Huang *et al.*, 1994). In addition to leucine, the uptake rate of L-valine, L-isoleucine, L-proline, L-phenylalanine, L-tyrosine, L-methionine and L-tryptophan may also be controlled by the oscillator (Chen *et al.*, 1991).

The circadian rhythms of *Synechococcus* RF-1 can be reset by changing the diurnal L/D phases (Huang *et al.*, 1991) or by exposing the culture to a pulse of low temperature (Chen *et al.*, 1991; Grobbelaar & Huang, 1992). The free-running periods of the nitrogenase activity and leucine-uptake rhythms were found to be temperature compensated when the temperature was changed within the physiological range (Huang *et al.*, 1990; Chen *et al.*, 1991).

There is a close association between the nitrogenase activity and the dark respiration rate in *Synechococcus* RF-1. The dark respiration rate increased considerably a few hours before the increase in the nitrogenase activity after the establishment of the circadian rhythm (Grobbelaar *et al.*, 1991). However, there does not appear to be a fixed relationship between the height of the nitrogenase activity peak and the increase in the respiration rate. When the nitrogenase is repressed by nitrate, an increase in the dark respiration rate corresponding to that observed for the nitrate-free treatment could not be detected (Chen *et al.*, 1989; Grobbelaar *et al.*, 1991). These results indicate that the increase in the dark respiration rate is not directly controlled by the clock because nitrate does not prevent the establishment of the clock-controlled leucine-uptake rhythm (Chen *et al.*, 1991). Instead, increased respiration is a reaction coupled to nitrogen fixation. Since nitrogenase is O₂-labile, the increase in the respiration rate has been suggested to be essential for the protection of the nitrogenase activity in *Synechococcus* RF-1 (Grobbelaar *et al.*, 1987).

The presence of calcium ions in the medium is essential for nitrogenase activity (Chen *et al.*, 1988) and for the increase of the dark respiration rate (Chen *et al.*, 1989). The nitrogenase activity is inhibited within 10 min after the calcium ions in the medium are chelated by EGTA. This inhibition can be completely abolished by a replenishment of the calcium ions immediately or several hours after EGTA application (Chen *et al.*, 1988). By inhibiting the nitrogenase activity with EGTA, and then reversing the inhibition by adding a calcium ion supplement, one nitrogenase activity peak can be selectively delayed for several hours, without affecting the position in time of the subsequent nitrogenase activity peaks (Huang *et al.*, 1990). The results indicate that the circadian nitrogenase activity rhythm in *Synechococcus* RF-1 is different from an ordinary feed-back reaction.

The *in vivo* requirement for calcium ions is interesting because calcium ions are not required for nitrogenase activity *in vitro*. When O₂ in the gas phase above the culture is below 1% (v/v), the inhibition of the nitrogenase activity by EGTA is reduced to less than 20% of the control value (Chen *et al.*, 1988). The results indicate that calcium ions are essential for nitrogenase activity only when the culture is growing aerobically. It has therefore been suggested that calcium ions are required to protect the nitrogenase in the cell against O₂ (Chen *et al.*, 1988). Further studies on the effects of calcium ions on the *nif* gene expression indicate that the role of extracellular calcium ions is not at the transcription level (Huang & Chow, 1990).

Induction of circadian rhythms in *Synechococcus* RF-1

The circadian nitrogenase activity rhythm in *Synechococcus* RF-1 can be induced by a diurnal L/D regimen varying from 20 h L/4 h D to 6 h L/18 h D (Chou *et al.*, 1989). The establishment of a circadian rhythm is a gradual process which is influenced by the intensity of the induction treatment and by the organism's physiological condition. The rhythm in *Synechococcus* RF-1 can be induced by one cycle of a L/D treatment, such as exposing a L/L culture to 8 h darkness. However, for maximal induction more than one consecutive L/D cycle is required. Total darkness is not required for the induction of a circadian rhythm by means of L/D treatments. A circadian rhythm can be induced in *Synechococcus* RF-1 by lowering the intensity of the light source, e.g. three consecutive cycles of a 12 h 3000 lx/12 h 1000 lx regimen will be effective (Chen *et al.*, 1993). During the L/D entraining process, a brief (30 min) interruption of the dark or light period did not have a significant effect on the phase of nitrogenase activity (T.-C. Huang, unpublished data). The circadian rhythm of nitrogen fixation can also be induced by exposing a culture which had been preadapted to continuous red light (680 nm; 10 nm half-band width) to a single 12 h dark period (Chen *et al.*, 1993). In plants, phytochrome is involved in regulating some circadian rhythms (Lumsden, 1991). For example, the circadian expression of the wheat *cab-1* gene, which encodes the major light-harvesting chlorophyll-binding protein of chloroplasts, is affected if the plant is exposed to far-red light for 30 min during the dark period (Nagy *et al.*, 1988). In *Synechococcus* RF-1, however, the rhythmicity was not affected by a 30 min pulse of 730 nm far-red light applied at various intervals during the dark treatment (Chen *et al.*, 1993).

The overt rhythms in *Synechococcus* RF-1 can also be induced by a small temperature change within the physiological range. For instance, the exposure of a 28 °C culture to either a 16 h 28 °C/8 h 35 °C, or a 16 h 28 °C/8 h 20 °C regimen will be quite effective in inducing a nitrogenase circadian rhythm (Huang *et al.*, 1994). The rhythm can be induced in continuous light by periodically lowering the oxygen concentration of the culture by either bubbling nitrogen through it, or by treating the culture with 100 µM DCMU (Grobbelaar *et al.*, 1987; Huang & Chow, 1990). A 4 h–8 h diurnal low temperature (0 °C–15 °C) treatment on several consecutive days in continuous light also induces the rhythm in a 28 °C culture (Grobbelaar & Huang, 1992).

The cell's physiological condition during the induction period is also important for the successful establishment of a circadian rhythm. It has been suggested that rhythmic nitrogen fixation and photosynthesis in the prokaryotic *Synechococcus* BG43511 are linked to different phases of the cell division cycle under synchronized conditions (Mitsui *et al.*, 1986). *Synechococcus* RF-1 can be maintained in a 'suspended state' for more than 2 weeks while it is kept in continuous darkness at 28 °C. It was also found that the circadian nitrogenase activity rhythm in *Synechococcus* RF-

1 could be induced by a raised or lowered temperature regimen within the physiological range while the cells were in the suspended state (Huang & Pen, 1994). Therefore, cell division and/or cell growth are not essential for the successful induction of the circadian nitrogenase activity rhythm in *Synechococcus* RF-1.

The nitrogen-fixing patterns of *Synechococcus* RF-1 cultures that were exposed to L/D cycles greater or less than 24 h have also been studied (Chou *et al.*, 1989). When a *Synechococcus* RF-1 culture was exposed to 6 h/6 h, 8 h/8 h, 10 h/10 h, 14 h/14 h, 16 h/16 h or 18 h/18 h L/D cycles, the nitrogenase activity changed rhythmically with a period length corresponding to that of the L/D cycle employed. When the culture was exposed to 10 h/10 h, 8 h/8 h or 6 h/6 h L/D cycles, the nitrogenase activity commenced after the onset of darkness and continued into the subsequent light period. When the culture was exposed to 14 h/14 h, 16 h/16 h or 18 h/18 h L/D cycles, the nitrogenase activity commenced about 13 h after the onset of illumination, that is about 1 h, 3 h or 5 h, respectively, before the onset of darkness (Chou *et al.*, 1989). The results indicate that in addition to darkness, the accumulation of photosynthetic products probably has a strong influence on the initiation of nitrogenase activity, especially when the culture is exposed to a L/D regimen with a very long light phase (Chou *et al.*, 1989). When the cultures which had been exposed to L/D cycles other than 24 h were transferred to L/L, a culture which was exposed to either a 10 h/10 h or 14 h/14 h L/D regimen exhibited a circadian nitrogenase activity rhythm with a free-running period of about 24 h (Huang *et al.*, 1990). However, the nitrogenase activity of a culture which was exposed to a L/D cycle that differed considerably in length from 24 h, such as a 6 h/6 h, 8 h/8 h, 16 h/16 h or 18 h/18 h L/D regimen, fluctuated arrhythmically under subsequent L/L conditions.

The circadian rhythms in *Synechococcus* RF-1 at the biochemical level

In a diurnal L/D regimen, *Synechococcus* RF-1 fixes nitrogen at a high rate but almost exclusively during the dark periods. Studying the time course of nitrogenase synthesis by using chloramphenicol and nitrate revealed that re-initiation of nitrogenase synthesis was required for nitrogen fixation to take place during the dark period of each cycle (Huang & Chow, 1988a). Northern blot analysis with the *nif* gene as a DNA probe demonstrated that *Synechococcus* RF-1 synthesizes nitrogenase-specific mRNA continuously in the arrhythmic L/L culture. The synthesis of the nitrogenase mRNA is cyclic and occurs exclusively during the dark periods when the L/L culture is entrained by a diurnal 12 h L/12 h D regimen (Huang & Chow, 1990). The circadian rhythmic *nif* gene expression, which corresponds in time to the nitrogenase activity, persists after the culture is transferred from a diurnal L/D regimen to continuous light (Huang & Chow, 1990). These results indicate that the nitrogenase activity rhythm in *Synechococcus* RF-1 is controlled at the transcription level.

It is known that the *nif* gene in *Synechococcus* RF-1 is repressed in the presence of nitrate (Huang & Chow, 1988a). Therefore, a culture of *Synechococcus* RF-1 growing in nitrate-containing medium does not have nitrogenase activity. When a culture growing in a nitrate-containing medium is exposed to a diurnal L/D regimen, a circadian nitrogenase activity rhythm manifests itself after the culture is transferred to a nitrate-free medium and is incubated in constant illumination (Huang & Chou, 1991). The results indicate that the circadian nitrogen-fixing rhythm of *Synechococcus* RF-1 can be induced while the *nif* gene is repressed. Therefore, the induction of this rhythm in *Synechococcus* RF-1 is independent of the expression of the rhythm.

The circadian rhythms in *Synechococcus* RF-1 can be observed by examining the synthesis rate of polypeptides (Huang *et al.*, 1994). When a L/L culture, growing in nitrate-containing medium, is labelled with [³⁵S]methionine after being exposed to a diurnal L/D regimen, the protein synthesis rate, examined by SDS-PAGE and autoradiographic techniques, revealed that more than ten polypeptides other than nitrogenase, were synthesized at a circadian oscillating rate under free-running conditions (Huang *et al.*, 1994). Ten of the polypeptides were identified as having relative molecular masses of 65, 61, 58, 38, 36, 33, 24, 23, 20 and 18 kDa. When the L/L culture was entrained by environmental factors other than L/D regimens, such as a raised or lowered temperature regimen, the polypeptides synthesized under the new circadian-clock control were identical to those synthesized previously after entrainment by a diurnal L/D regimen. The pattern of phase relationships among those polypeptides with circadian synthesis rates was also similar in the different cases. These observations suggest that induction by a L/D or a temperature regimen started the same oscillator, possibly the only one operative in *Synechococcus* RF-1. The results also indicate that there are fixed phase relationships among the clock-controlled polypeptides, although the phase of the synthesis rhythm for each polypeptide is different when entrained by different stimuli (Huang *et al.*, 1994).

Circadian rhythm mutants

Genetic analysis is a powerful tool in the elucidation of biological control mechanisms. Mutants of *Synechococcus* RF-1 have been induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Some mutants appear to be abnormal only in their nitrogenase activity rhythm, while others, such as mutants CR-1 and CR-2, have simultaneously lost the ability to establish the nitrogenase activity and leucine-uptake rhythms when exposed to a diurnal L/D regimen (Huang *et al.*, 1993). Analysis of the protein synthesis rates in these mutants revealed that the 58 kDa polypeptide is synthesized arrhythmically by mutant CR-1 under conditions in which the wild-type can be induced to synthesize it rhythmically. No polypeptide in mutant CR-2 can be induced to synthesize rhythmically by exposure to a L/D regimen (T.-C. Huang, unpublished data).

Circadian rhythms in prokaryotes other than *Synechococcus* RF-1

Synechococcus BG43511 is a fast-growing nitrogen-fixing marine cyanobacterium. The cell division cycles of a culture of this organism can be synchronized by a dark preconditioning treatment (Mitsui *et al.*, 1986). Cell division, nitrogenase activity and photosynthesis of the preconditioned culture were found to show circadian rhythmicity with a period of about 24 h after being entrained by a 12 h L/12 h D regimen. Other marine isolates identified as *Cyanothece* BH63 and BH68 (ATCC 51142) which are morphologically and physiologically similar to *Synechococcus* BG43511 have been isolated from intertidal sands in Sherman's laboratory (Reddy *et al.*, 1993). When the cultures of *Cyanothece* ATCC 51142 are maintained under diurnal L/D cycles, nitrogenase activity occurs in the dark. The nitrogenase activity rhythm persists after the culture is transferred to L/L. An oscillating behaviour of carbohydrate granule formation of strain ATCC 51142 was reported recently (Schneegurt *et al.*, 1994).

Synechococcus WH7803 is a red marine cyanobacterium isolated by L. Brand in 1978 (Sweeney & Borgese, 1989). This culture does not fix nitrogen. It was reported that there is a circadian rhythm in the cell division of the cyanobacterium (Sweeney & Borgese, 1989). In order to establish whether the circadian cell division rhythm is linked to the generation time of the culture, the culture was grown under conditions in which the generation time is longer than 24 h. Because the circadian cell division rhythm persisted for at least four cycles in L/L at a constant temperature ranging from 16 to 22 °C, it was deduced that the cell division rhythm is not linked to the generation time of the culture.

In the process of studying light-inducible gene expression in *Synechococcus* pcc 7942, Schaefer & Golden (1989) observed that the *psbA* gene which encodes D1, a major protein component of photosystem II, expresses itself rhythmically. A reporter plasmid containing the *psb* gene promoter and the bacterial luciferase gene (*luxAB*) from *Vibrio harveyi* was constructed (Kondo *et al.*, 1993). The transformed cyanobacterial strain (AMC149) containing the reporter *lux* gene was shown to exhibit a circadian rhythmicity in bioluminescence (Kondo *et al.*, 1993). Improvements in the technique for administering the reporter enzyme's substrate (decanal) and a highly sensitive photon-counting camera allow monitoring of the bioluminescence of single colonies (Kondo & Ishiura, 1994).

Outlook

The characteristics of the circadian rhythm of prokaryotes, such as its persistence under constant conditions, its phase-resetting by exposure to altered L/D cycles or by a pulse of low temperature and the temperature compensation of its free-running period, are

in general similar to those of eukaryotes. These findings may indicate that the circadian rhythms of prokaryotes and eukaryotes are controlled by similar, perhaps identical, mechanisms. Therefore, the prokaryotic system will, in all probability, play an important part in the unravelling of the fundamental aspects of biological clocks. For example, the recent discovery that the synthetic rate of many polypeptides can be induced to vary rhythmically in *Synechococcus* RF-1 (Huang *et al.*, 1994) provides a system for the cloning and characterization of the clock control genes. The establishment of the oscillating luciferase reporter system in *Synechococcus* 7942 (Kondo *et al.*, 1993), a strain that is extensively used for genetic analysis, provides an efficient system for studying a circadian rhythm by means of a genetic approach.

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