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# Temporal Variability in Nitrogenase Gene Expression in Natural Populations of the Marine Cyanobacterium *Trichodesmium thiebautii*

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**We report a distinct diel periodicity in the abundance of *nifH* (dinitrogenase reductase) mRNA in natural populations of the nonheterocystous marine cyanobacterium *Trichodesmium thiebautii*. Our observations show that in addition to translational and posttranslational controls, *Trichodesmium* nitrogenase expression is also regulated at the transcriptional and/or posttranscriptional level.**

The nitrogen-fixing planktonic cyanobacterium *Trichodesmium* is the single most important biological source of new nitrogen in the tropical and subtropical oceans (3, 6, 12). It is unique among nonheterocystous diazotrophic cyanobacteria in that nitrogenase activity is confined to the daylight hours; i.e., there is no apparent temporal or spatial separation of nitrogen fixation from photosynthesis (2, 17). Just how *Trichodesmium* prevents inactivation of the oxygen-labile nitrogenase enzyme by either ambient or photosynthetically generated oxygen is not understood but may well involve several complementary protective mechanisms (4).

The development of nitrogen-fixing potential in natural *Trichodesmium* populations is highly regulated and requires the de novo synthesis of nitrogenase at the beginning of each diel cycle (2, 19). Controls over nitrogenase expression also operate at the posttranslational level and involve the activation and deactivation of the enzyme modulated through temporal changes in the modification state of the Fe protein (14, 19, 21). During the daylight hours, nitrogenase is normally present in the unmodified (active) form but undergoes modification to the deactivated form during the late afternoon (see Fig. 2a). Whereas activation of the enzyme is a light-dependent process, modification of the Fe protein just before nightfall is not and occurs even in *Trichodesmium* colonies held in continuous light beyond the normal onset of darkness (21). By comparison, less is known about the environmental regulation of nitrogenase transcription since methodological and practical difficulties encountered when working with natural populations have, to date, precluded such studies.

The purpose of the present study was to investigate the temporal relation between the abundance of transcripts originating from the *nif* structural gene operon and the diel variability in nitrogen fixation in natural populations of *Trichodesmium* spp. Our primary aims were to establish whether variation in transcript levels might influence the diurnal pattern of nitrogenase synthesis and activity in this organism and to identify the environmental cues (e.g., light) that might regulate *nif* mRNA abundance.

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Our observations were made during a 3-week period in August-September 1991 at oligotrophic stations located adjacent to the Bahama Islands. Samples were collected from near-surface waters with a 1-m-diameter, 202-μm nylon mesh plankton net deployed at a ship speed of 1 knot from the University of Miami research vessel (R/V) *Columbus Iselin*. Individual colonies of *Trichodesmium thiebautii* were isolated from the net sample with a Pasteur pipette or a plastic inoculating loop and resuspended in fresh Whatman GF/F-filtered surface seawater. Isolated colonies were then used for measurements of nitrogenase activity by acetylene reduction (2) or processed further for the extraction of total RNA.

RNA was isolated from 100 to 200 colonies at each time point within 15 min of collection by a hot acid-phenol extraction procedure (21). Northern (RNA) dot blots of samples containing up to 5 μg of total RNA were probed at high stringency as described previously (21) with the *Eco*RI-*Hin*dIII fragment of pTr359 (20) encoding a 359-bp gene internal region of *nifH* from *T. thiebautii*. Probe DNA was labelled with digoxigenin-dUTP by random priming as described in the manufacturer's recommendations (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and hybrids were detected with alkaline phosphatase-conjugated anti-digoxigenin and the chemiluminescent substrate AMPPD [3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane] (Tropix Inc., Bedford, Mass.). Blots exposed to autoradiography film were quantified by densitometry and corrected for minor variations (generally, <10%) in RNA loading by stripping and reprobing with the rRNA operon of *Synechococcus* sp. strain PCC 6301 (10).

Preliminary observations (Fig. 1) revealed that transcripts originating from the *nif* operon were a far more abundant (>50-fold) component of *Trichodesmium* RNA samples taken during the early part of the day (0800) than in those obtained in the late afternoon (1700). To establish how temporal variability in transcript levels correlated with the daily pattern of nitrogenase synthesis and nitrogen fixation, we monitored changes in the abundance of *nifH* mRNA during the course of four subsequent diel cycles. Although samples were collected at intervals over a period of approximately 2 weeks, we found striking similarities between data sets in the temporal pattern of transcript abundance and the diurnal expression of nitrogenase activity (Fig. 2b to e).

During each series of observations, *nif* mRNA first accumu-

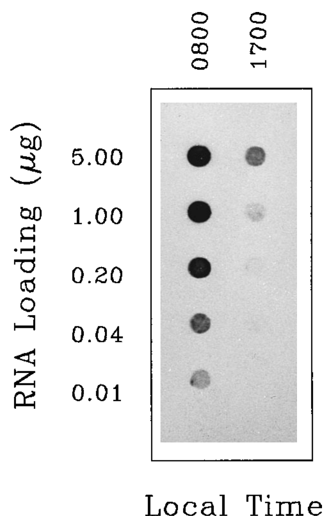


FIG. 1. Northern dot blot of *Trichodesmium* RNA samples obtained at 0800 and 1700 on 31 August 1991 probed with the *nifH* gene of *T. thiebautii* (see Materials and Methods). RNA loadings refer to the amounts of each RNA sample immobilized on the membrane.

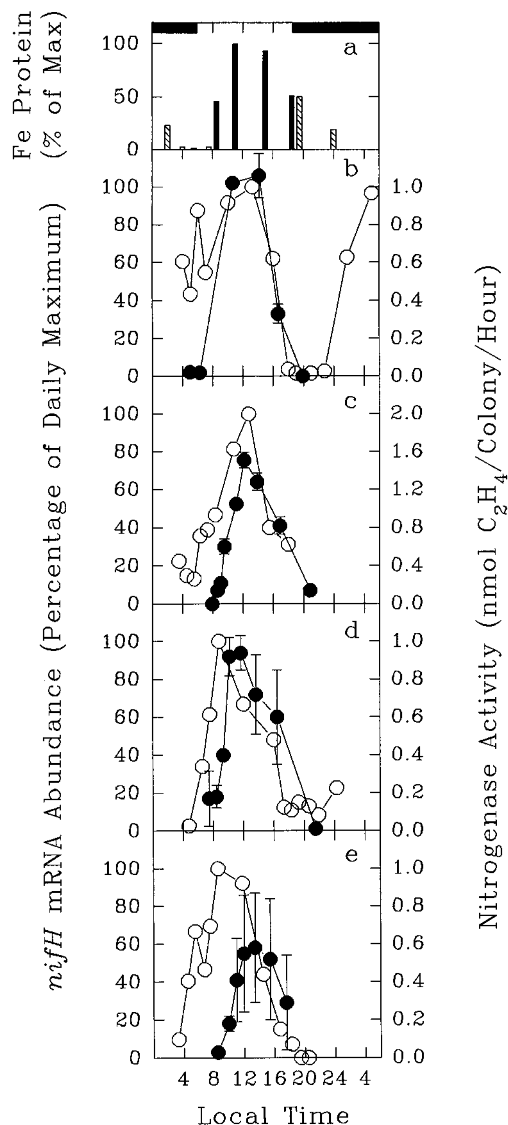


FIG. 2. (A) Diel variability in the relative abundance of the Fe protein of nitrogenase plotted as a percentage of the midday maximum with primary data from Capone et al. (2) and Zehr et al. (21). The hatched bars represent time points when 50% or more of the Fe protein was in the modified (higher-molecular-weight) form. (B to E) Temporal variability in expression of *nifH* (open symbols) and nitrogenase activity (closed symbols) in *T. thiebautii* sampled on 1 (B), 6 (C), 9 (D), and 14 (E) September 1991. mRNA measurements were normalized to the maximum signal recorded on each day of observation. Nitrogenase activity in nanomoles of $C_2H_4$ produced per colony per hour is expressed as the mean ± standard error ($n = 3$). Periods of darkness during each diel cycle are indicated by solid blocks on the upper ordinate of panel a.

lated some hours before sunrise and rapidly increased in abundance thereafter. This was a somewhat surprising observation since we had anticipated a closer temporal coupling between changes in *nif* mRNA abundance and translation of the nitrogenase enzyme. The diel pattern of nitrogenase protein synthesis in *Trichodesmium thiebautii* is well established (2, 21) (see Fig. 2a) and is characterized by the initial accumulation of both subunits just after daybreak. Evidently, although transcripts from the *nif* operon are present during the latter half of the night, translation and/or the stable accumulation of nitrogenase appears to be light dependent. To some extent at least, it is likely that this dependency may reflect a general biosynthetic requirement of *Trichodesmium* colonies exposed to prolonged darkness for ATP and reductant from photosynthesis. During the daylight hours, conditions which either promote or depress the accumulation of *nif* transcripts have parallel effects on the net synthesis of nitrogenase (18, 21).

Transcripts from the *nif* operon reached a maximum between midmorning and midday but declined subsequently during the afternoon so that by dusk, *nif* mRNA was close to or below the limit of detection. As observed previously (2, 17), nitrogen fixation was largely confined to the daylight hours: nitrogenase activity extended from about 1 to 2 h after daybreak until nightfall (Fig. 2b to e). Maxima in *nifH* mRNA abundance and nitrogenase activity were either coincident or separated by as much as 2 to 3 h. Peak nitrogenase activity was recorded at around midday and varied by a factor of approximately two- to threefold between sampling dates. Daily integrated rates of nitrogen fixation also varied by the same order (range, ~4 to 9 nmol of $C_2H_4$ per colony per day) but, nevertheless, were within the range recorded previously for natural populations of *T. thiebautii* (2, 5).

The decline in *nif* mRNA during the afternoon is coincident with the buildup in cell glutamine and glutamate pools (1) and also occurs in *Trichodesmium* colonies maintained in artificial light beyond the normal onset of darkness (18). Therefore, the decline in transcript levels appears to be regulated under ambient conditions by factors other than the natural diel variability in the light regime. Similarly, the accumulation of *nif* mRNA some hours before sunrise also militates against the specific involvement of light in the control and regulation of nitrogenase expression at this level. These observations suggest that endogenous signals rather than exogenous cues such as the availability of light regulate the net accumulation of *nif* mRNA in *Trichodesmium*.

The cyclical appearance of transcripts in *Trichodesmium* is reminiscent of the diel oscillations in *nif* mRNA levels observed in *Synechococcus* sp. strain RF-1, a unicellular diazotrophic cyanobacterium in which nitrogenase expression is under circadian control (8, 11). Although our data do not exclude the possibility that a circadian oscillator may regulate nitrogenase

expression at the transcriptional level, the diel pattern of nitrogen fixation in *Trichodesmium thiebautii* does not have the property of a circadian rhythm (15). Intriguingly, a free-running rhythm in photosynthetic activity has been observed in *Trichodesmium thiebautii*, which suggests that at least some cell processes in this organism may be under circadian control (16).

Our observations provide clear evidence that in addition to translational and posttranslational controls (2, 21), *Trichodesmium* nitrogenase expression is also regulated at the transcriptional and/or posttranscriptional levels. Our data do not allow us to distinguish between these latter regulatory mechanisms since we do not know whether the turnover rate of *nif* mRNA varies during the course of the diel cycle. The magnitude of the difference in *nif* mRNA abundance between morning and evening samples, however, is strongly indicative of specific controls operating at the transcriptional level. Establishing whether a circadian oscillator and/or a nitrogen regulatory signal transduction system such as those described in *Anabaena* spp. and other diazotrophs (7, 9, 13) is involved in activation of the *nif* operon, will depend upon future studies of the genetic basis of nitrogen fixation and assimilation in *Trichodesmium* spp.

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