Nitrogenase quantity varies diurnally in a subset of cells within colonies of the non-heterocystous cyanobacteria *Trichodesmium* spp.

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***Trichodesmium* is a marine filamentous cyanobacterium with the exceptional ability to fix atmospheric nitrogen during the day without differentiating the specialized oxygen-protective cells known as heterocysts. The localization of the Fe-protein of nitrogenase (dinitrogenase reductase) was examined in cross-sectioned colonies of three species: *Trichodesmium thiebautii*, *T. tenue* and *T. erythraeum*, using immunogold/transmission electron microscopy and immunofluorescence/light microscopy. The enzyme was confined to a limited subset of cells (on average 14%) in the sections, randomly distributed within the colonies in all three species. The frequency of nitrogenase-containing cells varied on a diurnal basis, being highest and comparatively constant during the day, the period of active nitrogen fixation. The percentage of nitrogenase-labelled cells decreased in the evening and approached zero just before dawn. After sunrise a period of rapid synthesis of nitrogenase followed. Although the frequency of nitrogenase-containing cells was constant during the day, the relative concentration of nitrogenase within the labelled cells clearly increased until noon and then successively decreased in the afternoon/night. The data imply that in three species of the non-heterocystous cyanobacterium *Trichodesmium* there are a limited number of cells, or possibly whole trichomes (filaments), which are specialized for nitrogen fixation. Most likely the same set of cells is active throughout the day, with the variation in activity being due to a variation in the quantity of nitrogenase within each cell. Comparisons between nitrogenase-containing *Trichodesmium* cells and heterocysts are discussed.**

Keywords: *Trichodesmium*, non-heterocystous cyanobacteria, nitrogenase, immunolocalization, plankton

INTRODUCTION

The colony-forming cyanobacterium *Trichodesmium* occurs over a vast area of the tropical and subtropical oceans. As a major contributor of both fixed nitrogen and fixed carbon, the genus *Trichodesmium* has great ecological importance for primary production in these oligotrophic waters (Carpenter, 1983; Carpenter & Romans, 1991). Besides fixing nitrogen, which is an extremely oxygen-sensitive process, diazotrophic cyanobacteria also perform oxygenic photosynthesis. To manage both processes they need to protect nitrogenase not only from oxygen in the

surrounding environment, but also from the oxygen being evolved endogenously (Fay, 1992; Gallon, 1992). In the course of evolution different strategies have developed to deal with this anomaly. The simplest strategy is to avoid oxic environments and to fix nitrogen only under micro-oxic conditions, as is the case in *Plectonema* (Weare & Benemann, 1974), whereas the most refined solution appears to be confinement of nitrogen fixation to specialized anaerobic cells known as heterocysts. This occurs in genera such as *Anabaena* and *Nostoc* (Haselkorn, 1978; Haselkorn & Buikema, 1992). Heterocysts lack the oxygen-evolving mechanism associated with photosystem II and synthesize additional cell wall layers lowering oxygen penetration. Yet other cyanobacteria separate the two processes in time and fix nitrogen only during dark,

Abbreviations: LM, light microscopy; TEM, transmission electron microscopy.

non-photosynthetic periods (Fay, 1992; Gallon, 1992). This is typical of the filamentous *Oscillatoria limosa* (Stal & Heyer, 1987).

Since *Trichodesmium* is a non-heterocystous cyanobacterium, its ability to fix nitrogen during the day, in parallel with photosynthetic activity in a highly oxic environment, is extraordinary (Bergman *et al.*, 1994). Experiments have in fact shown that nitrogen fixation in *Trichodesmium* is light-dependent (Ohki & Fujita, 1988). Maximal nitrogenase activities are reached at noon, when photosynthesis is most active, while there is no nitrogen fixation during dark hours (Saino & Hattori, 1978). The mechanism behind this exceptional behaviour is still unknown. It has been questioned whether *Trichodesmium* was the site of nitrogen fixation, or if the bacteria seen as more or less permanent constituents of the interior of natural *Trichodesmium* colonies were responsible for the measured activity. To identify the actual site of nitrogen fixation, immunolocalization of nitrogenase in *Trichodesmium* colonies has been performed in independent studies. Paerl *et al.* (1989) showed that in natural populations of *Trichodesmium* all cells within a colony contained equal amounts of nitrogenase, but later Bergman & Carpenter (1991) found that nitrogenase was confined to a limited number of cells or trichomes randomly distributed within the *Trichodesmium thiebautii* colonies. Although the exact location of nitrogenase was not agreed upon, it was clear that nitrogenase was almost exclusively confined to *Trichodesmium* and not to the associated bacteria. Some additional nitrogenase was subsequently detected in one thin filamentous cyanobacterium, occurring within the *Trichodesmium* colonies (Siddiqui *et al.*, 1992a). However, on a colony basis the quantity of nitrogenase in this putative cyanobacterium was insignificant.

To resolve the discrepancy regarding the occurrence of nitrogenase in *Trichodesmium*, an extended study of nitrogenase localization was performed. A large number of colonies representing three different *Trichodesmium* species were collected at regular intervals in a diel cycle and examined. An immunofluorescence technique was developed and used in conjunction with light microscopy (LM) to identify any variations in and between colonies over the diel cycle. Immunogold localization and transmission electron microscopy (TEM) were used as a complement to the immunofluorescence technique and in particular to ascertain whether the subcellular localization pattern previously found in *T. thiebautii* (Bergman & Carpenter, 1991) was valid for other colony-forming *Trichodesmium* species.

METHODS

Sample preparation. Colonies of *Trichodesmium* spp. were collected from the upper 20 m in the east Caribbean Sea in September 1992. A plankton net of 1 m diameter, 250 µm-mesh Nitex, was used for towing. The colonies were sampled in a diurnal cycle, every 2 h during the day and every 3 h during the night. Fusiform colonies were picked randomly with a plastic loop and isolated in filtered sea water (FSW). The colonies were immediately embedded in 2% (w/v) agar, transferred into

Fig 1. Colonies of more or less parallel *Trichodesmium* trichomes cross-sectioned in the central area of the colony, giving sections composed of cells representing different trichomes.

freshly prepared 3% (w/v) paraformaldehyde (TAAB Laboratories Equipment), and incubated for 1 h. They were then washed in FSW and dehydrated in an ethanol series. The samples were brought to the laboratory in 70% (v/v) ethanol, where they were further dehydrated and finally embedded in LR-White (London Resin Co.).

For localization of nitrogenase by immunofluorescence and immunogold the colonies were cross-sectioned in the centre (Fig. 1) using an ultramicrotome (Ultratome V; LKB). Thick sections (1 µm) were mounted on clean slides for LM examinations, while ultrathin sections were mounted on gold grids (300 mesh) for TEM examinations. At least four colonies per time point were examined, giving a total of 43 colonies included in the study.

Immunogold labelling. The grids were placed on drops of the blocking solution 5% (w/v) bovine serum albumin (BSA) for 10 min, followed by washing in 10 mM phosphate-buffered saline pH 7.4 (PBS) for 20 min. Incubation with the primary antibody against the small subunit, i.e. the Fe-protein, of nitrogenase (from *Rhodospirillum rubrum*; raised in rabbit; by courtesy of Dr Stefan Nordlund, Stockholm University) diluted 1:100 in 1% (w/v) BSA, was carried out at room temperature for 2 h. The sections were washed with PBS, incubated with the secondary antibody, goat anti-rabbit IgG conjugated with 10 nm gold particles (Amersham), diluted 1:20 with 1% (w/v) BSA, and washed in PBS and MQ water (Millipore). The sections were observed in a Zeiss EM 10A transmission electron microscope (Carl Zeiss) at 60 kV accelerating voltage. The primary antibody reacts monospecifically with a *Trichodesmium* protein of 38 kDa (Bergman & Carpenter, 1991) and the control for the secondary antibody showed that it does not label unspecifically.

Immunofluorescence labelling. Thick sections (1 µm) were blocked for 15 min with 5% (w/v) BSA and washed in PBS for 20 min. The primary antibody against the Fe-protein of nitrogenase was placed on sections at a 1:100 dilution in 1% (w/v) BSA. Incubations were carried out in a humid chamber for 1 h at room temperature. The sections were then washed with PBS and incubated in the secondary antibody, biotinylated goat anti-rabbit IgG (Amersham) diluted 1:500 in PBS, for 1 h. After washing with PBS, the sections were incubated in a Texas Red-streptavidin complex (Amersham) at a 1:100 dilution in PBS for 15 min, washed in PBS and mounted with 80% (v/v) glycerol in PBS. The sections were observed with a Nikon Optiphot fluorescence microscope, equipped with a Texas Red filter (maximum excitation 596 nm, maximum emitted light 600–615 nm). Several sections of each sample were checked to ascertain that the labelling result was consistent. In control experiments of both the biotinylated goat anti-rabbit antibody

and the Texas Red-streptavidin complex, no unspecific labelling was detected.

Speciation. *Trichodesmium* species were classified primarily according to cell dimensions and the cellular distribution of gas vacuoles (Janson *et al.*, 1995) in sections viewed by light microscopy (Fig. 2).

RESULTS

The majority of colonies collected at random in the east Caribbean Sea were identified as *T. thiebautii* and *T. tenue*, with a predominance of the former; only a few colonies of *T. erythraeum* were present. Thus, three species of *Trichodesmium* were examined in this study. Using LM and TEM the species were readily separated and identified (Fig. 2). Typically, *T. thiebautii* cells showed the largest diameter and many rather small gas vacuoles were spread irregularly over the cell area. In the thin trichomes of *T. tenue*, usually one to a few major gas vacuole(s) occupied a large part of the central cell area, while in cells of *T. erythraeum* the gas vacuole formed a cylindrical tube at the cell periphery. Each colony was composed of one *Trichodesmium* species and not a mixture (Fig. 2a–c).

When immunogold-labelled, using an antibody raised against the small subunit (Fe-protein) of the nitrogenase complex, and examined by TEM, all three species showed the same overall labelling pattern. Cross-sectioned colonies of *T. thiebautii*, *T. tenue* and *T. erythraeum* collected at noon had a small number of heavily labelled cells amongst a majority of unlabelled cells. Fig. 3 shows representative labelled and unlabelled neighbouring cells from within cross-sectioned colonies of the three *Trichodesmium* species. The nitrogenase label was restricted to the cytoplasm and spread throughout the cell, up to the cell wall, in all three species. There was no difference in thickness of the cell wall between labelled and unlabelled cells.

To determine the overall distribution and frequency of nitrogenase-labelled cells within colonies, an immunofluorescence labelling technique using thick sections and LM was employed. In contrast to immunogold labelling/TEM, this procedure allowed investigation of whole cross-sectioned colonies, often consisting of hundreds of more or less parallel trichomes. For comparative purposes all sections were taken from the central part of the colonies (Fig. 1), where the largest number of trichomes was found. The colony size ranged from about 30 to nearly 200 parallel trichomes. The cells containing nitro-

Fig 2. Toluidine-blue-stained cross-sectioned colonies of the three major fusiform *Trichodesmium* species occurring in the Caribbean. The colonies were classified primarily according to cell size and gas vacuole (arrows) arrangement: (a) *T. thiebautii*, with the largest cell diameter among the three, and several small gas vacuoles scattered over the cells; (b) *T. tenue*, with one or a few pronounced gas vacuole(s) per cell and a high number of trichomes in each colony; (c) *T. erythraeum*, with a distinct cylindrical gas vacuole 'tube' in the cell periphery and a cell diameter similar to that of *T. tenue*. Bar, 5 µm.

Fig 3. Immunogold localization/TEM of dinitrogenase reductase (Fe-protein) in cells of *Trichodesmium* colonies. Note the distinct differences in labelling between neighbouring cells in the three species: nitrogenase-containing (to the left) and unlabelled (to the right) respectively, and the uniform distribution of the nitrogenase label within each cell. (a) *T. thiebautii*, (b) *T. tenue*, (c) *T. erythraeum*. Bars, 1 µm. Abbreviations: gv, gas vacuole; v, vacuole-like structure.

Fig 4. Immunofluorescence/LM in *T. tenue* colonies labelled for dinitrogenase reductase (Fe-protein). Note the random distribution of nitrogenase-labelled cells within the colonies and the variation in intensity of label (fluorescence) at different time points (a) 06:10, (b) 08:00, (c) 12:00, (d) 14:00, (e) 19:30. Bar, 10 μ m.

Fig 5. Frequency of nitrogenase-containing cells in cross-sectioned *Trichodesmium* colonies. The frequency is given per section and was determined from immunofluorescence-labelled sections. The diagram shows the mean from four to five *Trichodesmium* colonies ($\pm$ SE) at each time point.

genase were always randomly distributed among the non-labelled cells and were to be found equally in trichomes at the periphery of the colonies as in central areas (Fig. 4). However, the relative intensity of nitrogenase label in individual nitrogenase-containing cells varied throughout the diurnal cycle. Label intensities were low in the morning, increased successively to a maximum in the middle of the day and decreased in the afternoon (Fig. 4). Both *T. tenue* (Fig. 4) and *T. thiebautii* (data not shown) demonstrated the same range of variation and diurnal pattern in nitrogenase label intensity.

The average frequency of nitrogenase-containing cells in four to five colonies per time point over the diel cycle is shown in Fig. 5. The frequency of nitrogenase-containing cells in the two most common species, *T. thiebautii* and *T. tenue*, showed a similar range of variation, from 0 to 24 %, even though the number of trichomes within each colony was much larger in *T. tenue* than in *T. thiebautii*. In the early morning, the number of nitrogenase-containing cells was low, on average about 5 % of the cells, and several colonies were devoid of nitrogenase label. After sunrise (about 06.00), the proportion of nitrogenase-labelled cells drastically increased up to a mean value of 14 % of the total cell number at around 08.00. The frequency then remained at approximately this level until sunset (about 18.00). When tested by the Kruskal-Wallis nonparametric rangsum test (Conover, 1980), no significant differences ($\alpha = 0.05$) were detected between the day-time samples, but analysis of data from the complete diel cycle showed significant differences between night and day. The highest frequency of labelled cells, approximately 24 %, was detected in two colonies sampled at 10.00 and 12.00, respectively, while in the evening, the frequency slowly decreased, reaching almost zero at late night/early morning.

DISCUSSION

The data presented clearly demonstrate that three naturally occurring *Trichodesmium* species, *T. thiebautii*, *T. tenue* and *T. erythraeum*, exhibit a restricted pattern of nitrogenase labelling, localized to a limited number of cells within each section of a colony. At most a quarter, and on average only 14 %, of the cells in a cross-sectioned colony contained nitrogenase. Colonies lacking nitrogenase were detected at dawn, but on no occasion did we detect nitrogenase label in all cells, although many colonies were examined. These results are in agreement with a previous localization study by Bergman & Carpenter (1991), but are converse to the labelling patterns obtained by Paerl *et al.* (1989), in which all *Trichodesmium* cells were claimed to be equally labelled for nitrogenase. A limited number of nitrogenase-containing cells has also been detected in *Trichodesmium* colonies collected at various time points and locations in the Caribbean (Siddiqui *et al.*, 1992b), and more recently in a species occurring as individual trichomes (Janson *et al.*, 1994). Both the antibody raised against the Fe-protein in *Rhodospirillum rubrum* (the present study) and a homologous antibody, raised against the Fe-protein of *T. thiebautii* (Siddiqui *et al.*, 1992b) gave similar labelling results. Together, such data favour the hypothesis that synthesis of nitrogenase is restricted to specialized cells and it is proposed that this restriction is a universal phenomenon among natural populations of *Trichodesmium* spp.

Whether these nitrogenase-containing cells represent whole trichomes, or a limited number of specialized cells within individual trichomes, is unclear. In *T. contortum*, occurring as large individual trichomes, nitrogenase was recently found to be restricted to a subset of about 15 % of the cells in each trichome (Janson *et al.*, 1994). The nitrogenase-containing cells were consecutively arranged and located in central parts of the trichomes. The *Trichodesmium* species examined here may also restrict the synthesis of nitrogenase to a subset of cells within each trichome. If colonies are composed of such trichomes, cross-sectioning would give the same random pattern of labelled cells as colonies in which, as previously suggested, only certain trichomes contained nitrogenase (Bergman & Carpenter, 1991). At present none of these possibilities can be excluded.

The frequency of nitrogenase-containing and tentatively nitrogen-fixing cells remained surprisingly constant in cross-sectioned colonies over the active period (daytime). In contrast, the relative quantity of nitrogenase in the labelled cells varied and correlated positively with nitrogenase activities in *Trichodesmium* spp. in which nitrogen fixation occurs during the daytime only, with a peak in activity around noon (Saino & Hattori, 1978; Bryceson & Fay, 1981; Carpenter, 1983; Capone *et al.*, 1990; Zehr *et al.*, 1993). Accordingly, the relative intensity of fluorescence in nitrogenase labelled cells increased from almost zero at 06.00 to a maximum at 12.00, and decreased during the afternoon. Hence, the variation in nitrogen fixation activity seen over the day is not due to variations

in the number of active cells, but primarily due to variations in nitrogenase synthesis within the individual nitrogenase-containing cells. Such results correlate positively with those previously obtained on nitrogenase abundance using immunoblot analyses of natural samples of *Trichodesmium* taken at different time-points (Capone *et al.*, 1990; Zehr, 1992). Regulation of nitrogenase activity may also be mediated by post-translational modification of the Fe-protein of the nitrogenase complex (Ohki *et al.*, 1992; Zehr *et al.*, 1993). However, it is not possible to discriminate between the modified and the unmodified forms with the immunological techniques used in our experiments.

Since the frequency of nitrogenase-containing cells is relatively constant, while label intensity varies, it is tempting to speculate that nitrogen fixation is restricted to a certain subset of cells predisposed for nitrogen fixation. If so, cell differentiation would be expected in *Trichodesmium*. The nitrogenase-harboring cells do not have any obvious heterocyst features, such as a thickened cell wall, which might serve as a protection against the surrounding oxygen. *Trichodesmium* spp. do, however, show a 'heterocystous behaviour', with nitrogen fixation occurring only during the daytime and with a peak activity at noon (Saino & Hattori, 1978). A crucial question is therefore how these *Trichodesmium* cells, apparently specialized for nitrogen fixation, manage to protect their nitrogenase against oxygen. As a random distribution of nitrogenase-containing cells within the sections was evident in all three *Trichodesmium* species, our data support the rejection (Carpenter *et al.*, 1990) of an earlier hypothesis of the presence of a central micro-oxic region for nitrogen fixation within the colony (Carpenter & Price, 1976). Nor does the uniform distribution of nitrogenase within the labelled cells (Fig. 3) indicate any spatial separation between nitrogenase and the photosynthetic thylakoid areas, as suggested for trichomes of a cultivated *Trichodesmium* sp. (Ohki *et al.*, 1991), or indeed from the surrounding oxygen, as nitrogenase label was equally intense close to the cell walls (Fig. 3). Although it is still possible that photosystem II is non-functional, the photoharvesting pigments of photosystem II, phycoerythrin and phycocyanin, which are often absent in heterocysts (Haselkorn, 1978), are present in the nitrogenase-producing cells of *Trichodesmium* (Siddiqui *et al.*, 1992b). There is the possibility that the nitrogenase of *Trichodesmium* exhibits an unusual oxygen resistance, although the *nif* gene sequences examined do not support such a hypothesis (Zehr & McReynolds, 1989; G. Sroga, U. Landegren & B. Bergman, unpublished results). Oxygen-protective mechanisms suggested so far include respiratory protection, indicated by a positive correlation between levels of the respiratory enzyme cytochrome oxidase and nitrogenase (Bergman *et al.*, 1993), and a rapid oxygen cycling caused by the Mehler reaction (Kana, 1993). Increased oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase, the key enzyme in carbon fixation, has also been suggested (Siddiqui *et al.*, 1992b).

Taken together, our data conclusively demonstrate that

the genus *Trichodesmium*, although non-heterocystous, exhibits a segregation of nitrogenase to a specific, possibly hitherto unknown cell type. Continued characterization of these specialized cells is now needed to reveal mechanisms for the exceptional nitrogen fixation behaviour in *Trichodesmium*.

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