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Diurnal expression of *hetR* and diazocyte development in the filamentous non-heterocystous cyanobacterium *Trichodesmium erythraeum*

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The marine non-heterocystous cyanobacterium *Trichodesmium* fixes atmospheric N_2 aerobically in light. *In situ* immunolocalization/light microscopy of NifH revealed that lighter, non-granulated cell regions observed correspond to the nitrogenase-containing diazocyte clusters in *Trichodesmium* IMS101. The number of diazocyte clusters per trichome varied from 0 to 4 depending on trichome length. The constant percentage of diazocytes (approx. 15 %) in cultured strains and five natural populations suggests a developmentally regulated differentiation process. Real-time RT-PCR showed that *ntcA*, encoding the global nitrogen regulator in cyanobacteria, and *hetR*, the key regulatory gene in heterocyst differentiation, are both constitutively expressed during a 12 h/12 h light/dark cycle. *hetR* in addition showed a distinct peak in the dark (close to midnight) while *nifH* expression commenced 6–8 h later. The expression of all three genes was negatively affected by addition of ammonia. Some early heterocyst differentiation genes were also identified in the genome of *Trichodesmium*. The data suggest that *hetR* and *ntcA* may be required for development and function of diazocytes in *Trichodesmium*.

INTRODUCTION

Trichodesmium spp. are marine filamentous cyanobacteria that lack the ability to form akinetes, heterocysts and hormogonia. Therefore, they are classified as undifferentiated filamentous cyanobacteria (Rippka *et al.*, 1979), but this has been questioned after the discovery of the nitrogenase-containing diazocytes (Bergman & Carpenter, 1991; Fredriksson & Bergman, 1997). Members of the genus *Trichodesmium* contribute significantly to global N_2 fixation (Capone *et al.*, 1997; Karl *et al.*, 2002). The N_2-fixing enzyme nitrogenase is highly sensitive to oxygen, but the genus *Trichodesmium* is capable of carrying out oxygenic photosynthesis and N_2 fixation in light, although it lacks the protective mechanisms provided by heterocysts. Some protective strategies operative in *Trichodesmium* spp. have been identified. These include locating the nitrogenase complex exclusively within the diazocytes, forming short stretches of cells (approx. 15 %) in the trichomes (Bergman & Carpenter, 1991; Janson *et al.*, 1994; Fredriksson & Bergman, 1995, 1997; Lin *et al.*, 1998), and a lowering of oxygenic photosynthesis around mid-day when N_2 fixation is at its maximum (Berman-Frank *et al.*, 2001).

In the heterocystous cyanobacterium *Anabaena* PCC 7120, differentiation of a vegetative cell into a heterocyst requires the activation of several genes in a cascade-like mechanism of transcriptional activations (Cai & Wolk, 1997). This differentiation process depends on both the global nitrogen regulator NtcA and the cell differentiation regulatory protein HetR (Wolk, 2000). *ntcA* encodes a transcriptional regulator that belongs to the CRP family of bacterial regulators (Herrero *et al.*, 2001), which activates the expression of genes involved in nitrogen assimilation upon ammonium withdrawal by binding to specific sites in their promoters. NtcA is required for development and function of mature heterocysts, and for the expression of nitrogen transport and assimilation systems as well as N_2 fixation (Herrero *et al.*, 2001). HetR is a key regulator of heterocyst differentiation (Buikema & Haselkorn, 1991) and is an autoregulated serine-type protease that can degrade itself and possibly other proteins (Zhou *et al.*, 1998). HetR shows enhanced induction within 1–2 h of nitrogen step-down, and within 3·5 h *hetR* expression is clearly localized to spaced cells, presumably developing pro-heterocysts (Black *et al.*, 1993). Induction of *hetR* upon nitrogen step-down depends on NtcA (Muro-Pastor *et al.*, 2002). The

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Abbreviation: L/D, light/dark.

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presence of *hetR* in filamentous non-heterocystous strains, including *Trichodesmium* (Janson *et al.*, 1998; Orcutt *et al.*, 2002; Schiefer *et al.*, 2002), and the requirement of *hetR* for akinete differentiation (Leganés *et al.*, 1994) suggest that *hetR* is not exclusively involved in heterocysts differentiation (see Wolk, 2000).

Like heterocysts, diazocytes are the exclusive carriers of nitrogenase and fix nitrogen aerobically in the light, and show morphological and physiological changes. These include a decrease in cyanophycin granules and synthesis of additional membranes (Fredriksson & Bergman, 1997), an increase in glutamine synthetase levels (Carpenter *et al.*, 1992), and an increase in cytochrome oxidase levels (Bergman *et al.*, 1993). Such changes suggest that differential gene expression may be required to generate diazocytes.

As cell differentiation is a fundamental biological process we initiated a study on identification of the molecular basis for the differentiation of the novel N_2-fixing cell type (diazocytes) recently discovered in *Trichodesmium* (Fredriksson & Bergman, 1997; Lin *et al.*, 1998; Berman-Frank *et al.*, 2001). For this purpose, we followed the occurrence and distribution pattern of diazocytes in trichomes and applied the sensitive real-time RT-PCR and SYBR Green I assay to monitor the expression of *ntcA*, *hetR* and *nifH* in cultures of *Trichodesmium* IMS101 grown under a 24 h light/dark regime, in the absence and presence of ammonia. A comparative search between the *Trichodesmium* IMS101 and *Anabaena* PCC 7120 genomes was also performed. Possible mechanisms involved in diazocyte development are discussed.

METHODS

Culture and growth conditions. The *Trichodesmium* strains examined are listed in Table 1. Cultures of *Trichodesmium* IMS101 (*T. erythraeum*) were maintained under a 12 h light/12 h dark regime (L/D cycle) in an amended seawater medium (Prufert-Bebout *et al.*, 1993) at 28 °C. The light intensity was 75 μmol m^{-2} s^{-1} and the cultures (600 ml) were aerated at a rate of approx. 200 ml min^{-1}. Under these conditions *Trichodesmium* IMS101 showed a doubling time of 25–28 h.

Natural populations of *Trichodesmium*, from the coastal waters of Zanzibar (Indian Ocean at the East Coast of Tanzania), were collected on several occasions between 1995 and 1997 from 125 ml net-towed surface samples (Table 1). Hand-picked colonies, identified as *T. erythraeum* (Lugomela *et al.*, 2002), were fixed in 1 % borax-buffered formalin and analysed by light microscopy.

Table 1. *T. erythraeum* strains examined and their origin

Strain/population	Origin/sampling site
T. erythraeum IMS101	Cultured isolate, non-axenic
T. erythraeum NC2*	Cultured isolate, axenic
T. erythraeum TR-1*	Cultured isolate, axenic
T. erythraeum XIII*	Cultured isolate, axenic
T. erythraeum†	Natural population, Indian Ocean

*Isloated by Dr. J. Waterbury (WHOI, Woods Hole, USA); NC2 identical to *T.* IMS101.
†Collected 2 May 1995 (130 trichomes); 20 May 1995 (100); 20 March 1996 (300); 10 April 1996 (102); 15 January 1997 (500).

***In situ* immunolocalization/light microscopy.** Trichomes were mounted on poly-L-lysine-coated glass slides (Sigma, product no. P0425), fixed and permeablized in 100 % ethanol overnight at −20 °C (Lin *et al.*, 1998). A rabbit anti-*Rhodospirillum rubrum* NifH antibody (100-fold dilution) was used as the primary antibody in conjunction with a fluorescein-conjugated secondary anti-rabbit antibody (200-fold dilution) (Molecular Probes, product no. A-11069). Trichomes were examined by light microscopy using an Olympus microscope fitted with U-MWU filters (BP330–385, BA420, DM400).

Acetylene reduction assay. Samples (2 ml) of *Trichodesmium* cultures were placed in 10 ml serum vials, 10 % (v/v) acetylene was injected and vials were incubated for 1 h under the same growth conditions as those given above. Head-space gas samples were withdrawn, and the ethylene production was measured using a Perkin-Elmer F11 gas chromatograph. Ethylene concentrations were calculated by reference to calibrated ethylene gas standards (Lundgren *et al.*, 2001).

Sampling and RNA extraction. At each sampling time during the 12 h/12 h L/D cycle, cells were collected into sterile plastic tubes and quickly filtered through 5 μm Millipore filters (Whatman, cat. no. 110613). The filters were immediately vortexed in RLT buffer containing *β*-mercaptoethanol (Qiagen, cat. no. 74104). The filters were discarded and the samples were stored at −80 °C. RNA was extracted using RNeasy Mini Extraction Kit (Qiagen, cat. no. 74104). Relative quantification (see below) of 16S rRNA (GenBank accession no. AF013030) was performed to verify that equal amounts of total mRNA were loaded in the real time RT-PCR runs (results presented in the figure legends).

PCR and cloning. Primer sequences used to amplify part of the sequences of *ntcA* (AF169961), *hetR* (AF091323) and *nifH* (U90952) from *Trichodesmium* IMS101 were designed accordingly (NCBI database): pntcAP, 5′-TGA TGA TTG AAA CCT TGG CA-3′; pntcAM, 5′-CTC TGC GAT TGC TTG ATG AG-3′; phetRP, 5′-TGA ACC CAA ACG GGT TAA AG-3′; phetRM, 5′-GCT TCA CTT AGA GGC ATC CG-3′; pnifHP, 5′-CGG TGG CAT TAA GTG TGT TG-3′; pnifHM, 5′-ACC TAA ACG GAC ACC ACC AG-3′.

Trichodesmium IMS101 DNA was extracted using the Genomic DNA isolation kit (Qiagen, buffer set cat. no. 19060, QIAprep spin cat. no. 27104) and PCR was carried using HotStar *Taq* polymerase (Qiagen, cat. no. 203203). PCR products with *ntcA*, *hetR* and *nifH* primers were cloned using the TOPO 2.1 TA cloning kit (Invitrogen, cat. no. 45-0641) and sequenced by MWG Biotech (Germany) to verify that the primers bound to the right targets. These primers were then used for the SYBR Green I assay below.

Real-time RT-PCR and SYBR Green I assay. The real-time PCR reactions were carried out in an iCycler iQ (Bio-Rad) using a QuantiTect SYBR Green RT-PCR kit (Qiagen, cat. no. 204243). The reaction mixture was prepared to contain 1× QuantiTect SYBR Green RT-PCR Master mix, 0·5 μl QuantiTect RT Mix, 0·5 μl fluorescein (1 μM; Bio-Rad, 170-8780), 0·5 μM of each primer, and RNase-free water to 50 μl. The RT-PCR programme was as follows: 50 °C for 30 min, 95 °C for 15 min, 40 cycles at 94 °C for 30 s, 54 °C (55 °C for *nifH*) for 30 s and 72 °C for 30 s. The fluorescence values were collected at 72 °C. The relative cDNA quantities of *ntcA*, *hetR* and *nifH*, and hence mRNA, were determined using serial dilutions of *Trichodesmium* IMS101 DNA (330 μg ml^{-1}) as a standard (at least four dilutions were included in each measurement). Following each run, a melt curve analysis step was performed according to the

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iCycler iQ manual to verify that the PCR products from the mRNA and DNA were the same, and that primer dimers were absent or if present that they were not stable at 72 °C and hence not incorporated into the datasets. PCR products were visualized on 2 % agarose gels stained with ethidium bromide.

Genome analysis. A comparative genomic analysis between the heterocystous *Anabaena* PCC 7120 (http://www.kazusa.or.jp/cyano/Anabaena/index.html) and *T. erythraeum* IMS101 (http://genome.jgi-psf.org/draft_microbes/trier/trier.home.html) was undertaken using BLAST.

Effects of PatS. The synthetic PatS-5 peptide (a generous gift from Dr J. W. Golden, Texas A & M, Texas, USA), known to prevent heterocyst development in *Anabaena* PCC 7120 (Yoon & Golden, 1998), was added to *Trichodesmium* IMS101 cultures at concentrations ranging from 1 to 10 μm. After 36 h, the occurrence of diazocytes was analysed using *in situ* immunolabelling of NifH and the trichomes were examined by light microscopy (as above).

RESULTS AND DISCUSSION

It has been proposed that the occasionally observed perceptibly lighter regions in *Trichodesmium* spp. may be sites for N_2 fixation (Carpenter & Price, 1976; Bryceson & Fay, 1981). Light microscopy analysis of cultured *Trichodesmium* IMS101 (*T. erythraeum*; Janson *et al.*, 1999) and trichomes from five natural populations of *T. erythraeum* (Table 1), collected in the Indian Ocean, revealed non- or less-granulated regions of cells within the trichomes with different optical properties making them appear as 'lighter' (Fig. 1). These regions were flanked by cells of a darker more granulated appearance. By using *in situ* immunolocalization/light microscopy of dinitrogenase reductase (NifH) we were able to demonstrate that these non-granulated or lighter regions corresponded to the regions of NifH-containing diazocytes (Fig. 2). Such diazocyte regions were present in all *Trichodesmium* strains examined here, whether axenic or not (Tables 1 and 2).

This finding allowed a light microscopy search for diazocyte regions in a large number of trichomes in five naturally growing *T. erythraeum* populations (Tables 1 and 2), the purpose being to document their occurrence and distribution along trichomes. The regions were intercalary, terminal, or occasionally at both locations in the trichomes

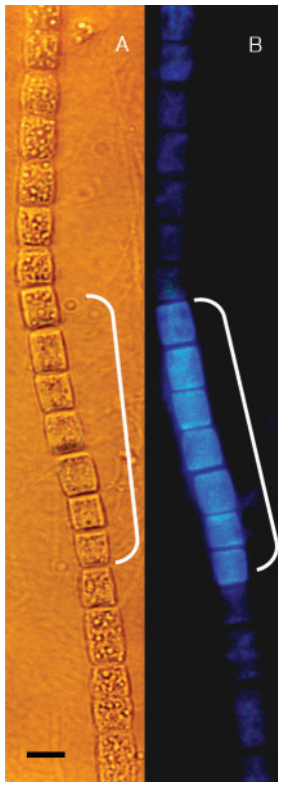


Fig. 2. Light micrographs depicting the coincident location of the non-granulated cells and the NifH-labelled cells, representing diazocytes, in one individual trichome of *Trichodesmium* IMS 101. (A) An intercalary region composed of a stretch of non-granulated cells (bracketed); (B) the same trichome labelled with an anti-NifH antibody and viewed using fluorescence microscopy. The presence of nitrogenase (diazocytes) is seen as blue fluorescence. Bar, 8 μm.

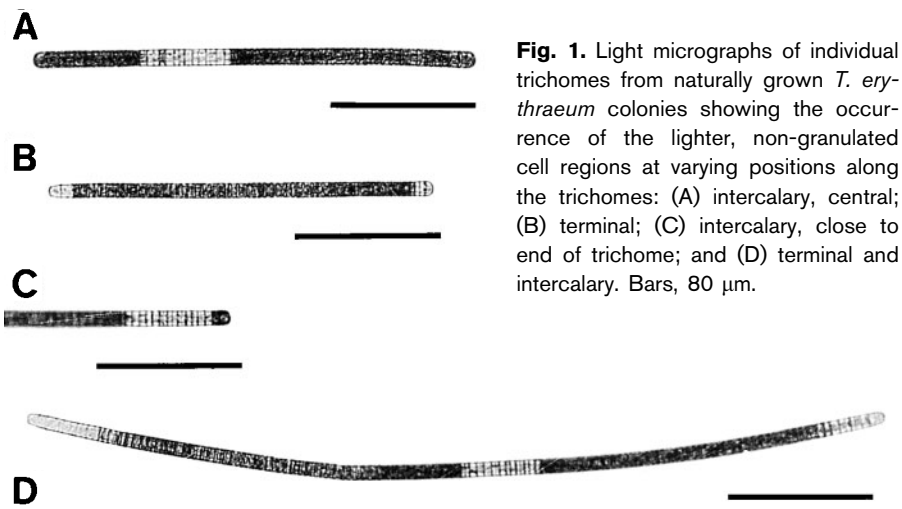


Fig. 1. Light micrographs of individual trichomes from naturally grown *T. erythraeum* colonies showing the occurrence of the lighter, non-granulated cell regions at varying positions along the trichomes: (A) intercalary, central; (B) terminal; (C) intercalary, close to end of trichome; and (D) terminal and intercalary. Bars, 80 μm.

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Table 2. Percentage of differentiated cells (non-granulated) and cells with NifH (diazocytes) in cultured and naturally grown *Trichodesmium* strains

Mean percentages are given and figures in parentheses denote number of cells counted.

	Cultivated isolates	Natural populations	References
Cell differentiation (non-granulated cells)	18 % (150)*	16 % (1130)†	This study
Diazocytes (NifH-labelled cells)	14 % (700)*	–	This study
	15 % (300)‡	–	Lin *et al.* (1998)
	–	14 % (1500)§‖	Fredriksson & Bergman (1995)
	–	10 % (25)¶	Janson *et al.* (1994)

*The three axenic *Trichodesmium strains* (Table 1).
†*T. erythraeum*, Indian Ocean.
‡The non-axenic *Trichodesmium* IMS101.
§*T. tenue*, *T. thiebautii*, *T. erythraeum*, the Caribbean Sea.
‖The diazocyte frequence ranged from 5 to 24 % depending on the time of day.
¶*T. contortum*, Caribbean Sea.

(Fig. 1A–D). The intercalary diazocyte regions were mostly positioned in central areas of the trichomes (Fig. 1A, D), and most trichomes had one or two regions (Fig. 3A). In cultured *Trichodesmium* IMS101 about 60 % of the trichomes had only one diazocyte region. Higher frequencies of regions (up to four regions) were present in the natural populations of *T. erythraeum*, often composed of longer trichomes (Fig. 3B). The latter is possibly due to the more favourable growth conditions in the field. Trichomes composed of fewer than about 40 cells seemed to lack diazocyte regions (Fig. 3B). Presumably, such non-diazocytous short trichomes are formed by fragmentation. The most fragile junction of *Trichodesmium* trichomes is that between a diazocyte and the nearby vegetative cell (authors' observations).

The proportion of differentiated, lighter (non-granulated) cells per trichome was strikingly similar (approx. 17 %) in both the cultured and the naturally grown populations (Table 2). This percentage also coincided with the frequency in diazocyte regions identified (approx. 14 %), irrespective of site of collection or *Trichodesmium* axenicity (Table 2). As the growth conditions for the populations examined here were radically different, we hypothesized that a developmental rather than an environmental mechanism underpins diazocyte formation in *Trichodesmium*. In heterocystous cyanobacteria such as *Nostoc* and *Anabaena* the percentage of heterocysts is also constant, peaking at 5–10 %, and *hetR* is a master gene for the heterocyst differentiation (Buikema & Haselkorn, 1991; Adams & Duggan, 1999). The role of *hetR* in some non-heterocystous

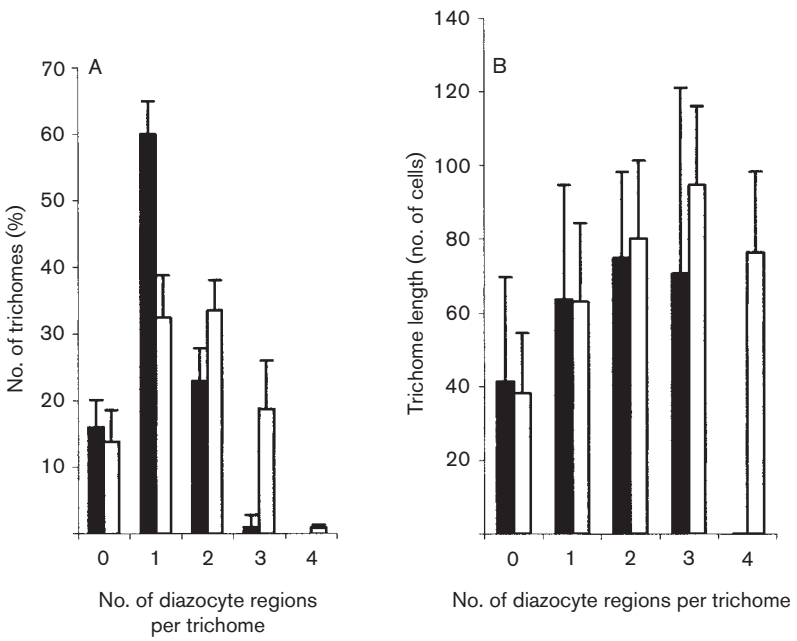


Fig. 3. Relationship between number of diazocyte regions per trichome and (A) the percentage of total trichomes counted, and (B) the number of cells per trichome. Black bars, *Trichodesmium* IMS101; white bars, naturally grown *T. erythraeum*.

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cyanobacteria, including *Trichodesmium* (Janson *et al.*, 1998, Orcutt *et al.*, 2002; Schiefer *et al.*, 2002), is still obscure.

In order to search for the role of *hetR* and its possible connection to diazocyte formation in *Trichodesmium* we made use of the real-time RT-PCR methodology and the SYBR Green I assay. This is a powerful technique for detecting and quantifying low mRNA expression levels (Gibson *et al.*, 1996; Heid *et al.*, 1996; Lie & Petropoulos, 1998). We first examined the expression of *hetR* and the global regulatory gene involved in nitrogen control and heterocyst differentiation, *ntcA* (Herrero *et al.*, 2001), in N_2-fixing cultures. As nitrogenase is confined to diazocytes, the expression of *nifH*, the gene encoding dinitrogenase reductase, was simultaneously followed. The specificity of the three primer pairs used was checked by amplification and cloning of the fragments generated, followed by sequencing and comparisons with already published cyanobacterial sequences using BLAST provided at the NCBI database site (http://www.ncbi.nlm.nih.gov/BLAST/). The characteristic T_m of the amplified fragments were easily distinguished from the T_m of non-specific products and primer dimers on a melt curve chart (data not shown). Measurements of fluorescence intensity were only performed at temperatures above the T_m of the primer dimers and below that of the specific PCR products. Product identification was performed by comparing the respective T_m on the melt curve charts and the respective molecular size on agarose gels.

The expression of *ntcA*, *hetR* and *nifH* in samples of *Trichodesmium* IMS101 was followed over 12 h/12 h L/D periods. As seen in Fig. 4, *ntcA* was constitutively expressed at moderate levels through the diurnal cycle. In contrast, *hetR* expression showed a distinct diurnal rhythmicity. It increased sharply from 20:00 h and reached a fourfold higher expression 2 h into the dark phase. Expression then decreased to a minimum just before 08:00 h in the morning, when the light was again switched on. The data also show that *hetR* is still expressed, albeit at low levels, under the more nitrogen-replete periods (daytime). The timing in the *hetR* expression pattern was persistent in all experiments ($n=10$), although expression levels at the peak varied between 3- and 10-fold.

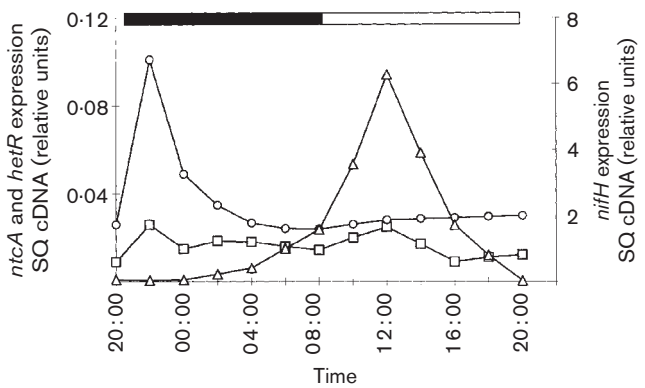


Fig. 4. Diurnal expression profiles of *ntcA* (□), *hetR* (○) and *nifH* (△) in *Trichodesmium* IMS101. Expression was assayed using real-time RT-PCR, during a 12/12 h L/D cycle. SQ, starting quantities. SQ 16 rRNA = 5·14 (SD ± 0·42, $n=9$). The black bar represents the 12 h dark period and the white bar the 12 h light period.

As expected, the peak in *nifH* expression occurred at midday (Fig. 4), about 2 h before the maximum nitrogenase activity was reached (measured as acetylene reduction). These findings corroborate those reported previously for *nifH* expression in *Trichodesmium* IMS101 using Northern blots (Chen *et al.*, 1998; Dominic *et al.*, 1998), immunolocalization of NifH (Fredriksson & Bergman, 1995) and acetylene reduction assays (Mulholland & Capone 1999). While NifH synthesis was below the detection level of Northern blots when the light was turned off (20:00), we were able to demonstrate by using the more sensitive real-time RT-PCR technique that initiation of *nifH* transcription commenced at about midnight and slowly increased until the light was again turned on at 08:00; it then increased rapidly (Fig. 4). This finding may explain the presence of the nitrogenase protein during the night (Zehr *et al.*, 1993; Fredriksson & Bergman, 1995; Chen *et al.*, 1998), and further confirms that the nitrogenase protein abundance is reflected in the *nifH* transcription pattern (Chen *et al.*, 1998).

To investigate the possible role of *ntcA* and *hetR* in relation to N_2 fixation and diazocyte formation a source of combined nitrogen was added to the N_2-fixing *Trichodesmium* IMS101 cultures (Fig. 5). When 100 μM NH_4Cl was added at 20:00 h ($n=4$), *ntcA* expression decreased and the upshift in *hetR* expression, seen under N_2-fixing conditions at 22:00 (Fig. 4), was diminished and followed by a slow continuous decrease. Likewise, *nifH* expression was negatively affected (Fig. 5), and no acetylene reduction activity was recorded. The upshift seen at the end of the light period (Fig. 5) was probably due to exhaustion of NH_4^+ from the growth medium. Higher concentrations of NH_4^+ were avoided as they may negatively affect *Trichodesmium* growth. A repression of *ntcA*, *hetR* and *nifH* expression and heterocyst differentiation by combined nitrogen is well known for heterocystous cyanobacteria (Herrero *et al.*, 2001; Adams & Duggan, 1999), and the expression of *ntcA* and *hetR* are mutually dependent on each other (Muro-Pastor *et al.*, 2002).

To get further insights into the possible genetic background to diazocyte development, a comparative genomic analysis between the heterocystous *Anabaena* PCC 7120 and *T. erythraeum* IMS101 was undertaken using BLAST. Genes involved in regulating early stages of heterocyst differentiation in *Anabaena* PCC 7120 (Wolk, 2000) were identified in *Trichodesmium* IMS101 while several others were not (Table 3). As expected, *ntcA* and *hetR* were

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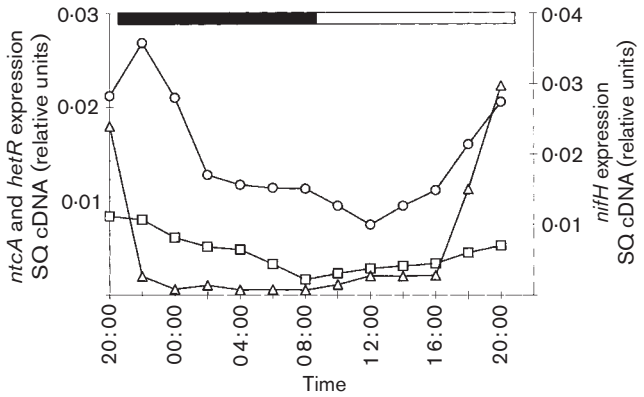


Fig. 5. Effects of adding combined nitrogen (NH_4^+) on diurnal expression profiles in *Trichodesmium* IMS101. Expression was assayed, as in Fig. 4, for *ntcA* (□), *hetR* (○) and *nifH* (△) after the addition of 100 μM NH_4Cl at 20:00 h ($n=4$). Note the difference in scale on the *y*-axes compared to Fig. 4. Upshifts in expression levels at the end of the light phase are due to exhaustion of (NH_4^+). SQ, starting quantities. SQ 16 rRNA = 2·17 (SD ± 0·45, $n=9$). The black bar represents the 12 h dark period and the white bar the 12 h light period.

identified and showed a high amino acid identity. Other genes identified were *hanA*, encoding a DNA-binding protein necessary for heterocyst initiation (Nagaraja & Haselkorn, 1994; Khudyakov & Wolk, 1996), and *devR*, a response regulator also essential for heterocyst formation (Campbell *et al.*, 1996). Among the genes known to control heterocyst pattern formation (individual heterocyst distribution at regular intervals), *patB* (a putative transcription regulator; Liang *et al.*, 1993) was found but not *patA* or *patS* (Liang *et al.*, 1992; Yoon & Golden, 1998, 2001). In heterocystous cyanobacteria, differentiation may be initiated in a cluster of cells (proheterocysts), but only one develops into a mature heterocyst, while the others regress to become vegetative cells (Wilcox *et al.*, 1973). PatS is presumed to regulate this resolution into individual heterocysts via lateral inhibition (Yoon & Golden 1998, 2001). Diazocytes in contrast always remain in groups of about 3–20 cells (Figs 1 and 2; Lin *et al.*, 1998). Additions of the inhibitory synthetic PatS-5 peptide to *Trichodesmium* IMS101, at concentrations known to prevent heterocyst development (Yoon & Golden, 1998, 2001), had no influence on the frequency or the distribution of the NifH-labelled diazocytes. This also supports the absence of *patS* in *Trichodesmium* IMS101. Not unexpectedly, genes involved in the synthesis of the thick heterocystous cell envelope, composed of polysaccharides and glycolipids (Wolk, 2000), were not identified in the genome of *Trichodesmium* (Table 3). In spite of fixing N_2 aerobically during the day, diazocytes lack the equivalent external cell-wall layers (Bergman & Carpenter, 1991; Fredriksson & Bergman, 1997).

Table 3. Gene homologies between *Trichodesmium* IMS101 and *Anabaena* sp. strain PCC 7120

Genes related to heterocyst differentiation were compared by using BLAST analyses of the genome of *Trichodesmium* IMS101 (http://genome.jgi-psf.org/draft_microbes/trier/trier.home.html) and the genome of the heterocystous *Anabaena* sp. strain PCC 7120 (http://www.kazusa.or.jp/cyano/Anabaena/index.html). It should be noted that the genome of *Trichodesmium* is not fully sequenced and analysed. Only amino acid identities above 57 % were considered. ND, No homology detected.

Gene	Amino acid identity (%)
ntcA	78
hetR	76
hanA	75
devR	75
devH	69
patB	58
hetP, *hetC*	ND
patA, *patS*	ND
devA, *devB*, *devC*	ND
hepK, *hepA*, *hepB*, *hepC*	ND
hglC, *hglD*, *hglE*, *hglK*	ND

Ever since the discovery in 1991 (Bergman & Carpenter, 1991) of the nitrogenase-containing cells (the diazocytes: Fredriksson & Bergman, 1997), no studies have been directed towards understanding the mechanisms related to diazocyte development and pattern formation in *Trichodesmium*. The data presented here, however, clarify some novel aspects, and probably extend to other cyanobacteria such as *Katagnymene* spp. which show a similar N_2-fixing behaviour (Lundgren *et al.*, 2001). First, we demonstrate that the morphologically distinct lighter stretches seen in certain *Trichodesmium* trichomes are the nitrogenase-containing diazocyte zones. Secondly, that individual trichomes may have multiple, regularly spaced diazocyte regions, the number and location of which is related to growth conditions and length of the trichomes. Thirdly, that a developmentally regulated diazocyte differentiation exists, as there is a constant mean frequency (approx. 15 %) of diazocytes under the highly varying external conditions. This in turn led us to argue that *hetR* may be an actor in diazocyte development. For instance, we were able to show a distinct diurnal rhythmicity in *hetR* expression in a cyanobacterium, while *ntcA* was more stably expressed during the 24 h growth cycle. The peak in *hetR* expression preceded the onset of *nifH* expression by the same time span (6–8 h) as that noted between *hetR* and *nifH* gene expression in heterocystous cyanobacteria, when grown at similar doubling times (for *Trichodesmium* 25–28 h under our conditions) (Fig. 4; Adams & Duggan, 1999; Yoon & Golden, 2001). In order to maintain a highly constant mean daytime frequency of diazocytes (present study; Fredriksson & Bergman, 1995; Lin *et al.*, 1998) new diazocytes must regularly develop as trichomes grow.

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Moreover, there is a diurnal rhythmicity in the frequency of cells with NifH (diazocytes) in natural *Trichodesmium* populations, with maximum frequencies being reached in the daytime (approx. 24 %) and a minimum (approx. 5 %) just before dawn (Fredriksson & Bergman, 1995). This suggests a diurnal redifferentiation of diazocytes (diazocytes are, unlike heterocysts, not permanently differentiated but able to divide; Fredriksson & Bergman, 1997), and that the time span for differentiation of new diazocytes is located between the peak in *hetR* and the onset of *nifH* expression (Fig. 4). *hetR* expression is considerably reduced (as is that of *ntcA* and *nifH*) in the presence of an external nitrogen source (Fig. 5), which is also typical for all other *hetR*-dependent heterocystous cyanobacteria (Adams & Duggan, 1999). The presence of some crucial early heterocyst differentiation genes in the genome of *Trichodesmium* also points to some shared cell differentiation mechanisms. However, to fully verify the linkage between *hetR* expression and diazocyte formation the construction of *Trichodesmium hetR* mutants is required, and such attempts are now under way.

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REFERENCES

Adams, D. G. & Duggan, P. S. (1999). Heterocyst and akinete differentiation in cyanobacteria. *New Phytol* **144**, 3–33.

Bergman, B. & Carpenter, E. J. (1991). Nitrogenase confined to randomly distributed trichomes in the marine cyanobacterium *Trichodesmium thiebautii*. *J Phycol* **27**, 158–165.

Bergman, B., Siddiqui, P. J. A., Carpenter, E. J. & Peschek, G. A. (1993). Cytochrome oxidase: subcellular distribution and relation to nitrogenase expression in the nonheterocystous marine cyanobacterium *Trichodesmium thiebautii*. *Appl Environ Microbiol* **59**, 3239–3244.

Berman-Frank, I., Lundgren, P., Chen, Y.-B., Küpper, H., Kolber, Z., Bergman, B. & Falkowski, P. (2001). Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*. *Science* **294**, 1534–1537.

Black, T. A., Cai, Y. & Wolk, C. P. (1993). Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. *Mol Microbiol* **9**, 77–84.

Bryceson, I. & Fay, P. (1981). Nitrogen fixation in *Oscillatoria* (*Trichodesmium*) in relation to bundle formation and trichome differentiation. *Mar Biol* **61**, 159–166.

Buikema, W. J. & Haselkorn, R. (1991). Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena* 7120. *Genes Dev* **5**, 321–330.

Cai, Y. & Wolk, C. P. (1997). *Anabaena* sp. strain PCC 7120 responds to nitrogen deprivation with a cascade-like sequence of transcriptional activations. *J Bacteriol* **179**, 267–271.

Campbell, E. L., Hagen, K. D., Cohen, M. F., Summers, M. L. & Meeks, J. C. (1996). The *devR* gene product is characteristic of receivers of two-component regulatory systems and is essential for heterocyst development in the filamentous cyanobacterium *Nostoc* sp. strain ATCC 29133. *J Bacteriol* **178**, 2037–2043.

Capone, D. G., Zehr, J. P., Paerl, H. W., Bergman, B. & Carpenter, E. J. (1997). *Trichodesmium*: a globally significant marine cyanobacterium. *Science* **276**, 1221–1229.

Carpenter, E. J. & Price, C. C. (1976). Marine *Oscillatoria* (*Trichodesmium*): explanation for aerobic nitrogen fixation without heterocysts. *Science* **191**, 1278–1280.

Carpenter, E. J., Bergman, B., Dawson, R., Siddiqui, P. J., Soderback, E. & Capone, D. G. (1992). Glutamine synthetase and nitrogen cycling in colonies of the marine diazotrophic cyanobacteria *Trichodesmium* spp. *Appl Environ Microbiol* **58**, 3122–3129.

Chen, Y.-B., Dominic, B., Mellon, M. T. & Zehr, J. P. (1998). Circadian rhythm of nitrogenase gene expression in the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium* sp. strain IMS 101. *J Bacteriol* **180**, 3598–3605.

Dominic, B., Chen, Y.-B. & Zehr, J. P. (1998). Cloning and transcriptional analysis of the *nifUHDK* genes of *Trichodesmium* sp. IMS 101 reveals stable *nifD*, *nifDK* and *nifK* transcripts. *Microbiology* **144**, 3359–3368.

Fredriksson, C. & Bergman, B. (1995). Nitrogenase varies diurnally in a subset of cells within colonies of the non-heterocystous cyanobacteria *Trichodesmium* spp. *Microbiology* **141**, 2471–2478.

Fredriksson, C. & Bergman, B. (1997). Ultrastructural characterization of cells specialized for nitrogen fixation in a non-heterocystous cyanobacterium, *Trichodesmium*. *Protoplasma* **197**, 76–85.

Gibson, U. E., Heid, C. A. & Williams, P. M. (1996). A novel method for real time quantitative RT-PCR. *Genome Res* **6**, 995–1001.

Heid, C. A., Stevens, J., Livak, K. J. & Williams, P. M. (1996). Real time quantitative PCR. *Genome Res* **6**, 986–994.

Herrero, A., Muro-Pastor, A. M. & Flores, E. (2001). Nitrogen control in cyanobacteria. *J Bacteriol* **183**, 411–425.

Janson, S., Carpenter, E. J. & Bergman, B. (1994). Compartmentalization of nitrogenase in a non-heterocystous cyanobacterium, *Trichodesmium contortum*. *FEMS Microbiol Lett* **118**, 9–14.

Janson, S., Matveyev, A. & Bergman, A. (1998). The presence and expression of *hetR* in the non-heterocystous cyanobacterium *Symploca* PCC 8002. *FEMS Microbiol Lett* **168**, 173–179.

Janson, S., Bergman, B., Carpenter, E. J., Giovannani, S. J. & Vergin, K. (1999). Genetic analysis of natural populations of the marine diazotrophic cyanbacterium *Trichodesmium*. *FEMS Microbiol Ecol* **30**, 57–65.

Karl, D., Michaels, A., Bergman, B. & 7 other authors (2002). Dinitrogen fixation in the world's oceans. *Biogeochem* **57/58**, 47–98.

Khudyakov, I. & Wolk, C. P. (1996). Evidence that *hanA* gene coding for HU protein is essential for heterocyst differentiation in, and cyanophage A-4(L) sensitivity of, *Anabaena* sp. strain PCC 7120. *J Bacteriol* **178**, 3572–3577.

Leganés, F., Fernández-Piñas, F. & Wolk, C. P. (1994). Two mutations that block heterocyst differentiation have different effects on akinete differentiation in *Nostoc ellipsosporum*. *Mol Microbiol* **12**, 679–684.

Liang, J., Scappino, L. & Haselkorn, R. (1992). The *patA* gene product, which contains a region similar to CheY of *Escherichia coli*, controls heterocyst pattern formation in the cyanobacterium *Anabaena* 7120. *Proc Natl Acad Sci U S A* **89**, 5655–5659.

Liang, J., Scappino, L. & Haselkorn, R. (1993). The *patB* gene product, required for growth of the cyanobacterium *Anabaena* spp. strain PCC 7120 under nitrogen-limiting conditions, contains ferredoxin and helix-turn-helix domains. *J Bacteriol* **175**, 1697–1704.

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Lie, Y. S. & Petropoulos, C. J. (1998). Advances in quantitative PCR technology: 5′ nuclease assays. *Curr Opin Biotechnol* **9**, 43–48.

Lin, S., Henze, S., Lundgren, P., Bergman, B. & Carpenter, E. J. (1998). Whole-cell immunolocalization of nitrogenase in marine diazotrophic cyanobacteria, *Trichodesmium* spp. *Appl Environ Microbiol* **64**, 3052–3064.

Lugomela, C., Lyimo, T., Bryceson, I., Semesi, A. K. & Bergman, B. (2002). *Trichodesmium* in coastal waters of Tanzania: diversity, seasonality, nitrogen and carbon fixation. *Hydrobiologia* **477**, 1–13.

Lundgren, P., Söderbäck, E., Carpenter, E. J. & Bergman, B. (2001). *Katagnymene*: characterization of a novel marine diazotroph. *J Phycol* **37**, 1052–1062.

Mulholland, M. & Capone, D. G. (1999). Nitrogen fixation, uptake and metabolism in natural and collected populations of *Trichodesmium* spp. *Mar Ecol Prog Ser* **188**, 33–49.

Muro-Pastor, A. M., Valladares, A., Flores, E. & Herrero, A. (2002). Mutual dependence of the expression of the cell differentiation regulatory protein HetR and the global nitrogen regulator NtcA during heterocyst development. *Mol Microbiol* **44**, 1377–1385.

Nagaraja, R. & Haselkorn, R. (1994). Protein HU from the cyanobacterium *Anabaena*. *Biochimie* **76**, 1082–1089.

Orcutt, K. M., Rasmussen, U., Webb, E. A., Waterbury, J. B., Gundersen, K. & Bergman, B. (2002). Characterization of *Trichodesmium* spp. by genetic techniques. *Appl Environ Microbiol* **68**, 2236–2245.

Prufert-Bebout, L. E., Pearl, H. W. & Lassen, C. (1993). Growth, nitrogen fixation and spectral attenuation in cultivated *Trichodesmium*. *Appl Environ Microbiol* **59**, 1350–1359.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol* **111**, 1–61.

Schiefer, W., Schutz, K., Hachtel, W. & Happe, T. (2002). Molecular cloning and characterization of *hetR* genes from filamentous cyanobacteria. *Biochim Biophys Acta* **1577**, 139–143.

Wilcox, M., Mitchison, G. J. & Smith, R. J. (1973). Pattern formation in the blue-green alga, *Anabaena*. I. Basic mechanisms. *J Cell Sci* **12**, 707–725.

Wolk, C. P. (2000). Heterocyst formation in *Anabaena*. In *Prokaryotic Development*, pp. 83–104. Edited by Y. V. Brun & L. J. Shimkets. Washington, DC: American Society for Microbiology.

Yoon, H.-S. & Golden, J. W. (1998). Heterocyst pattern formation controlled by a diffusible peptide. *Science* **282**, 935–938.

Yoon, H.-S. & Golden, J. W. (2001). PatS and products of nitrogen fixation control heterocyst pattern. *J Bacteriol* **183**, 2605–2613.

Zehr, J. P., Wyman, M., Miller, V., Duguay, L. & Capone, D. G. (1993). Modification of the Fe protein of nitrogenase in natural populations of *Trichodesmium thiebautii*. *Appl Environ Microbiol* **59**, 669–676.

Zhou, R., Wei, X., Jian, N., Li, H., Dong, Y., His, K.-L. & Zhao, J. (1998). Evidence that HetR protein is an unusual serine-type protease. *Proc Natl Acad Sci U S A* **95**, 4959–4963.

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