Diazocyte development in the marine diazotrophic cyanobacterium *Trichodesmium*

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The establishment of non-diazotrophic cultures of the filamentous marine cyanobacterium *Trichodesmium erythraeum* IMS101 enabled the first detailed investigation of the process leading to the development of its unique nitrogen-fixing cell type, the diazocyte. Trichome heterogeneity was apparent already within 3–8 h, while the differentiation of mature diazocytes, containing the nitrogenase enzyme, required 27 h after the removal of combined nitrogen. The distribution of ‘pro-diazocytes’ within the trichomes correlates with the localization of mature diazocytes, which suggests that pattern regulation is an early event during diazocyte development. The development was initially identified as changes in the subcellular ultrastructure, most notably the degradation of glycogen granules and gas vacuoles. These changes were preceded by the induced expression of the global nitrogen regulator *ntcA* at an early stage of combined nitrogen deprivation, followed by elevated expression of genes related to nitrogen metabolism and their corresponding proteins. The strongest induction (10-fold) was related to the transcription of the respiratory gene *coxB2*, apparent already at an early stage, which suggests an important role for respiration and the subsequent energy generation in the subcellular changes found, and in the creation of the reducing environment required for nitrogen fixation in diazocytes.

INTRODUCTION

Nitrogen-fixing cyanobacteria have developed various strategies to protect the oxygen-sensitive nitrogenase enzyme, the most well known being the development of heterocysts (Haselkorn, 2007). A different strategy is practised by the ecologically highly important marine cyanobacterial genus *Trichodesmium*, which unlike other filamentous non-heterocystous cyanobacteria, fixes nitrogen exclusively in the day/light period, although the genus does not differentiate heterocysts (Dugdale et al., 1961; Saino & Hattori, 1978; Capone et al., 1997). Instead, *Trichodesmium* develops a proportion of the vegetative cells into nitrogen-fixing diazocytes (Berman-Frank et al., 2001). The majority of existing reports have shown that both cultured and natural populations of *Trichodesmium* sequester the nitrogenase enzyme into diazocytes (Bergman & Carpenter, 1991; Fredriksson & Bergman, 1997; Berman-Frank et al., 2001; Orcutt et al., 2009), although data contradicting this also exist (Ohki, 2008). Diazocytes are morphologically distinguished from the flanking vegetative cells by their reduced number of storage compounds and the prominent gas vacuoles (Fredriksson & Bergman, 1997), giving them a more homogeneous and transparent appearance (Bryceson & Fay, 1981; El-Shehawy et al., 2003). Hence, some morphological and physiological characters of the diazocytes resemble those known to characterize heterocysts, while others do not. Unlike heterocystous species, *Trichodesmium* spp. have retained all components of the photosynthetic apparatus in the diazocytes and lack a heterocyst-type thickened external cell envelope (Fredriksson & Bergman, 1997). However, similar to heterocysts, higher levels of respiratory enzymes (CoxA; Bergman et al., 1993) and the primary ammonia-assimilating enzyme glutamine synthetase (GS) (GlnA; Carpenter et al., 1992) are found in diazocytes. Diazocytes seem to cope with the oxygen sensitivity of the nitrogenase enzyme by a temporal reduction in oxygenic photosynthesis during the peak in nitrogenase activity at midday, which co-occurs with higher respiratory and Mehler activities (Kana, 1993; Berman-Frank et al., 2001; Milligan et al., 2007; Kranz et al., 2009). The temporal downregulation of oxygenic photosynthesis is linked to quick rearrangements of the phycobilisome antennae associated with the photosystems (Küpper et al., 2004, 2009). The frequency of diazocytes fluctuates on a diurnal basis (5–35 %), correlating positively with the nitrogenase activity, both being at maximum at midday (Fredriksson & Bergman, 1995; Lin et al., 1998). This dynamic behaviour may be caused by the ability of diazocytes to divide and revert into non-fixing vegetative cells, unlike the situation for the permanently differentiated heterocysts.

As in all other nitrogen-fixing cyanobacteria, the nitrogen-fixation activity and the synthesis of the NifH protein in *Trichodesmium* are downregulated upon addition of higher concentrations of combined nitrogen (Ohki et al., 1991;
Mulholland et al., 2001; Holl & Montoya, 2005), while under lower or a shorter temporal supply of combined nitrogen, nitrogen fixation and nitrogen assimilation can co-occur (Holl & Montoya, 2005). *Trichodesmium* grown on a continuous supply of high concentrations of combined nitrogen has been shown to lack diazocytes (Sandh et al., 2011). In heterocystous cyanobacteria, the global nitrogen regulator NtcA is a key regulator of the expression of genes involved in nitrogen metabolism (Herrero et al., 2004). Removal of combined nitrogen leads to a rapid autoinduction of ntcA transcription followed by enhanced transcription of the heterocyst regulator hetR (Muro-Pastor et al., 2002; Zhang et al., 2006). The product, HetR, in turn induces genes involved in heterocyst development and maturation, such as proteins involved in heterocyst cell envelope formation (Black & Wolk, 1994), and in the creation of reducing conditions inside the heterocysts (Jones & Haselkorn, 2002). After 13–24 h, fully mature, approximately regularly spaced intercalary heterocysts are formed, later followed by the expression of nif genes and the assembly of a functional nitrogenase complex (Adams 2000; Ehira et al., 2003; Haselkorn, 2007). The hetR gene is also present in *Trichodesmium* (Janson et al., 1998), and its temporal expression pattern suggests a possible involvement in diazocyte differentiation (El-Shehawy et al., 2003; Sandh et al., 2009).

The occurrence of diazocytes and their involvement in nitrogen fixation in *Trichodesmium* is by now well established, as is their selective presence under nitrogen-depleted conditions (Sandh et al., 2011). However, the process that transforms selected photosynthetic vegetative *Trichodesmium* cells into sets of nitrogen-fixing diazocytes is still to be resolved. Here we set out to decipher the cellular and molecular events during the emergence of diazocytes (in time and space) upon combined nitrogen deprivation.

**METHODS**

**Growth conditions.** Cultures of *Trichodesmium erythraeum* IMS101 were grown in YBCII medium (Chen et al., 1996) with the addition of 100 μM potassium nitrate in 2 l polycarbonate (PC) flasks. The cultures were illuminated with 80 μmol photons m⁻² s⁻¹ during 12/12 h light/dark cycles with continuous aeration at 25–27 °C. Nitrate was supplemented as previously described (Sandh et al., 2011). After 14 days of growth with nitrate, three replicate cultures were filtered onto 5 μm PC filters (Whatman), 2 h after the onset of the light period, and were washed with 200 ml YBCII medium lacking nitrate before being resuspended in YBCII medium lacking nitrate. From these cultures, samples of 200–300 ml were taken at 0, 3, 8, 27 (1 day), 51 (3 days) and 75 h (6 days) after transfer to growth on atmospheric nitrogen gas, and morphological, physiological and molecular changes were examined at each time point as described below.

**Growth measurement.** Growth was monitored by following the variations in chlorophyll (Chl a) concentrations (Meeks & Castenholz, 1971) over time.

**Diazocytes, nitrogenase localization and cellular ultrastructure.** The appearance of diazocytes was analysed at each time point in the time series by light microscopy after staining with 5 % (v/v) acidic Lugol’s solution (Bryceson & Fay, 1981; Sandh et al., 2009), and their frequency (percentage of total filament length) and the average length of the zones were determined. At each time point, more than 1000 cells were counted in the three replicates. Ultrastructural changes, and the disappearance and reappearance of the diazocytes and the nitrogenase enzyme (NifH), were also followed using immuno-gold labelling and transmission electron microscopy (TEM), as described in Sandh et al. (2011).

**Nitrogenase activity.** The nitrogenase activity was determined using the acetylene reduction assay, as previously described (Sandh et al., 2011).

**Immunoblotting.** Immunoblotting was performed using the same procedure and antibodies as described in Sandh et al. (2011).

**Genomic DNA extraction.** Genomic DNA was extracted from *Trichodesmium* cells using a modified cetyltrimethylammonium bromide (CTAB)/phenol-based method (Wilson, 1998).

**RNA extraction and cDNA synthesis.** RNA was isolated from *Trichodesmium* cells using a commercial extraction kit (RNeasy Mini, Qiagen). After extraction, an additional DNase treatment was performed, followed by an RNA cleanup, performed according to the manufacturer’s protocol (Qiagen). Real-time RT-PCR analyses (see below) were performed with RNA as template, to test for and avoid possible DNA contamination. The concentration of RNA was determined using a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies), and 500 ng of RNA was used for cDNA synthesis using the qScript cDNA SuperMix (Quanta Biosciences).

**Real-time PCR.** Real-time PCR was carried out on an iCycler iQ (Bio-Rad) using the iQ SYBR Green Supermix (Bio-Rad). The PCR primers were as follows: coxB2R, GTCTTGATATTGGCTGCTCC; coxB2F, TCACATCAGCTTGTCTCCG; glnAR, TGAGAAGCATT-ACCACGAA; glnAB, CCCCAGATTAGGCGTCTCC; primers for nifH, ntcA and hetR were as in El-Shehawy et al. (2003) and for 16S rRNA as in Sandh et al. (2009). All primers are presented in the 5′–3′ mode. The real-time program was as follows: 95 °C for 5 min, 40 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. All reactions were ended with a melt curve to detect possible non-specific PCR products. The relative quantification of cDNA was performed using serial dilutions of a known concentration of genomic DNA from *T. erythraeum* IMS101 normalized to 16S rRNA. The efficiencies of all real-time reactions were 97 ± 2 %.

**RESULTS**

Following the removal of a combined nitrogen source from the medium, the growth of *Trichodesmium* initially slowed down but then gradually increased (Fig. 1a). This occurred shortly after the induction of the nitrogenase activity and within the first 27 h (Fig. 1b). The nitrogenase activity, in turn, was preceded by a gradual appearance of zones of lighter cells located in central areas of the *Trichodesmium* trichomes (Fig. 2a). These subsets of cells represented the nitrogenase-containing diazocytes, in accordance with previous findings (El-Shehawy et al., 2003). As seen in Fig. 2(a), no zones were found in trichomes grown on nitrate (time 0 h), while slightly lighter zones emerged, albeit faintly, already within 3–8 h into the diazotrophic...
phase (Fig. 2a, 8 h). These lighter zones were denoted ‘pro-
diazocytes’, as they were not as morphologically distinct as
the ‘mature’ diazocytes that appeared between 8 and 27 h
after the initiation of the nitrogen stepdown (Fig. 2a, 27 h).
After 27 h, distinct transparent zones were present in all
trichomes, independently of trichome length. In shorter
trichomes a zone was located in the central area of the
trichomes, while in longer trichomes multiple regularly
spaced zones, composed of 2–30 cells, were present along
the trichome (Fig. 2b). The diazocyte zones formed initially
were significantly longer (~25 %) and made up a larger
percentage of the total cell population than those
remaining after 3–6 days (15 %; Fig. 3).

TEM analyses revealed distinct changes in the cellular
ultrastructure of cells in the diazocyte zones (Fig. 4a),
corroborating recent studies (Sandh et al., 2009, 2011). The
most obvious adaptation was an extensive reduction in gas
vacuole size and number, combined with a more condensed
thylakoid network and, as evident from the Lugol staining,
a reduced carbohydrate storage. Immunogold-TEM localization
of NifH (5 nm gold particles) verified that the nitrogenase
label was limited to these lighter cells (Fig. 4b).

Next, the expression profiles of genes, and the corresponding
proteins, involved in nitrogen control and metabolism
were examined using real-time RT-PCR and immunoblot-
ting (Figs 5 and 6). The data obtained revealed significant
changes early on during nitrogen deprivation and hence
diazocyte development. For instance, the expression of the
gene encoding the global nitrogen regulator NtcA showed a
significant increase and peaked early on, already within
8 h, after the removal of combined nitrogen, and this
finding was mirrored at the NtcA protein levels (Fig. 5a, b).
Additionally, the synthesis of the Fe subunit of the
nitrogenase complex increased significantly at the same
time as the emergence of the mature diazocytes, i.e. after
27 h (Fig. 5d). This was also apparent at the transcriptional
(nifH) level (Fig. 5c). In addition, a low but transient
increase in nifH transcription was detected already after
3 h, which was followed by the appearance of low levels of
the NifH protein after 8 h (Fig. 5c, d), although the
nitrogenase activity was still undetectable (Fig. 1b).

The expression of the gene glnA (Tery_3834), encoding the
primary ammonia-assimilating enzyme in cyanobacteria,
GS, showed a more gradual increase during nitrogen
deployment and the development of the diazocytes, with a
significant change in the levels of the GS protein at the end
of the developmental period (Fig. 5e, f). Out of the two
copies of glnA present in the Trichodesmium genome
(http://www.jgi.doe.gov), the one studied here is more
closely related to the copy of glnA present in most
cyanobacteria (Tery_3834; 63–82 % sequence identity
using BLASTP). The transcription of hetR, encoding the
main regulator for heterocyst differentiation, showed no
significant change in expression profile upon removal of
nitrate (Fig. 6a). Finally, a strong differential expression
was detected in the transcription of coxB2 (Tery_1779),
encoding the respiratory cytochrome oxidase subunit II.
The expression was enhanced already within 3 h, and a
greater than 10-fold increase was apparent within 8 h of
the removal of nitrate. This was followed by a down-
regulation to a basal expression level, still about twofold
above the level found in nitrate-grown cultures (Fig. 6b).

**DISCUSSION**

We here present what is believed to be the first data on the
temporal structural development and patterning of

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**Fig. 1.** Growth and nitrogenase activity of cultures of *T. erythraeum* IMS101 following combined nitrogen stepdown. (a) Growth, measured as the concentration of Chl a per millilitre of culture at time 0, and at 3, 8, 27, 51 and 75 h following nitrate removal. (b) Nitrogenase activity, measured by the acetylene reduction technique at the same time points following nitrate removal.
diazocytes along the trichomes elicited by transfer of actively grown *Trichodesmium* cultures into a combined-nitrogen-depleted medium. Additionally, we present the first data on the expression of key genes and proteins involved in the developmental and metabolic functions of cyanobacteria. First, our findings verify and considerably extend those of Sandh et al. (2011) in demonstrating a positive correlation between the lack of combined nitrogen and the presence of diazocytes in *Trichodesmium*. Although the drastic removal of combined nitrogen employed here may not be common under natural conditions, the data still illustrate events that in time and space govern the development of diazocytes and their nitrogen-fixation physiology. Our data furthermore stress the existence of specific nitrogen-fixing cells in *Trichodesmium*, which has been questioned (Ohki, 2008), while another study has suggested that diazocytes occur only in older cultures (Orcutt et al., 2009).

The emergence of slightly lighter zones, ‘pro-diazocytes’, in central areas of trichomes already within 3–8 h of the removal of combined nitrogen (Fig. 2a), shows that the localization of the diazocyte zone is dictated early on during the developmental process. However, the subsequent ‘overproduction’ of potentially immature diazocytes (after 27 h) may be a response to the severe nitrogen depletion experienced as the end cells of the zone revert back to vegetative cells (day 3). The formation of a longer diazocyte zone may otherwise be a way to buffer the impact of their oxygen-producing vegetative neighbouring cells and reflect the fact that fully mature functional diazocytes require longer than 27 h to develop. In line with this assumption is the finding that the higher diazocyte frequency at 27 h is not reflected in the highest nitrogen-fixation activity. Indeed ‘pro-heterocysts’ may develop in heterocystous cyanobacteria that later redifferentiate into vegetative cells, allowing only one centrally located heterocyst to develop (Wilcox et al., 1975). The secretion of heterocyst pattern-regulating compounds, such as the PatS pentapeptide, is involved in the intercalary pattern formation (Yoon & Golden, 1998). As with heterocysts, regularly spaced diazocytic zones are formed in longer *Trichodesmium* trichomes (Fig. 2b), while one zone develops close to the centre in short trichomes (Fig. 2).

**Fig. 2.** Diazocyte formation after combined nitrogen stepdown. (a) Light micrographs of Lugol-stained trichomes of *T. erythraeum* IMS101 at fixed time intervals (0, 3, 8, 27, 51 and 75 h) after placement in combined-nitrogen-free medium. Note the development of the lighter cells in central areas of the trichomes, starting at 8 h (marked by a dashed bracket), with distinct ‘mature’ zones (marked by brackets) being seen from 27 h onwards. Bar, 25 μm. (b) Representative example of multiple diazocyte zones in longer trichomes. Lighter-stained diazocyte zones are marked by brackets. The micrograph was taken 75 h after removal of combined nitrogen. Bar, 100 μm.

**Fig. 3.** Induction of diazocytes after combined nitrogen stepdown. The frequency of diazocytes of the total cell populations (●) and mean diazocyte zone lengths (white bars) after the onset of nitrate deprivation. The diazocytes counted after 8 h represent ‘pro-diazocytes’ with slightly lighter Lugol staining, but are much darker than the mature zones present after 27 h.
The expression of ntcA/NtcA and glnA/GS during the development of diazocytes found here points to a similar role for NtcA in *Trichodesmium* to that in other cyanobacteria (Fig. 5; Herrero et al., 2004). The increased C:N ratio activates NtcA and leads to an autoinduction of its own expression (Fig. 5a, b), as well as the induction of genes involved in nitrogen regulation, e.g. assimilation of newly fixed nitrogen (glnA/GS; Fig. 5e, f). The role of NtcA in relation to nifH/NifH expression is not clear, as high levels of NtcA are not always linked to increased nifH expression, although NtcA binds to the promoter of the nif operon in other cyanobacteria (Post et al., 2011). NifH and GlnA have been shown to be present exclusively and at a higher concentration, respectively, in diazocytes (Carpenter et al., 1992). In heterocystous species, NtcA also induces the expression of the main heterocyst regulator HetR (Buikema & Haselkorn, 1991; Muro-Pastor et al., 2002), which in turn induces a cascade of events that eventually leads to the differentiation of mature heterocysts. The transcription of hetR in *Trichodesmium* showed no significant change following combined nitrogen depletion (Fig. 6a). However, hetR transcription is known to be repressed by ammonium in *Trichodesmium*, and a strong upshift in hetR transcription is evident at night time under nitrogen-fixing conditions (El-Shehawy et al., 2003; Sandh et al., 2009). A similar transient increase in expression in the dark may have been missed in our study, and further studies are needed to clarify the potential role of HetR and the cellular expression of hetR during diazocyte differentiation. The presence of hetR in the genomes of several other non-heterocystous diazotrophic cyanobacteria (Janson et al., 1998; Zhang et al., 2009) might also suggest a more general role in the regulation of nitrogen fixation than specifically for diazocyte development.

The strong initial induction and the 10-fold upshift in expression of the respiratory gene coxB2 after 8 h during diazocyte development (Fig. 6b) stress the importance of respiration in this process. Respiration is for instance most likely involved in the depletion of carbon storage (glycogen) and other changes in the subcellular ultrastructure identified. The coxB2 gene examined here resembles a subunit of the heterocyst-specific cytochrome oxidase, cox2 (Jones & Haselkorn, 2002). To this may be added the findings that an increased cytochrome oxidase level is found in mature diazocytes in natural *Trichodesmium* populations (Bergman et al., 1993), and that the dark respiration declines at the end of the light period (Kranz et al., 2009), the time period when samples with the highest levels were taken. The twofold increase in coxB2 transcription maintained under the diazotrophic conditions may be important in generating the reducing conditions needed for the de novo synthesis and activity of the nitrogenase enzyme (Kana, 1993; Milligan et al., 2007). These findings are also in line with the induced catalytic carbon physiology of nitrogen-fixing *Trichodesmium* cultures evidenced by proteome analysis (Sandh et al., 2011).

To summarize, our data are believed to constitute the first report of the stepwise and temporal development of the
Fig. 5. Expression of nitrogen-regulated genes and the synthesis of the corresponding proteins following nitrate deprivation in *T. erythraeum* IMS101. (a), (c) and (e) Real-time RT-PCR quantification of ntcA, nifH and glnA mRNA abundance, respectively, during diazocyte development following nitrate removal. The values were normalized to those of 16S rRNA levels. (b), (d) and (f) Protein levels of NtcA, NifH and GlnA (GS), respectively, during diazocyte development, given as pixel intensities after Western blot detection using a chemiluminescent detection system.

Fig. 6. Expression of *hetR* and *coxB2* following nitrate deprivation in *T. erythraeum* IMS101. Real-time RT-PCR quantification of *hetR* (a) and *coxB2* (b) mRNA abundance following nitrate removal. The values were normalized to those of 16S rRNA levels.
zones/groups of diazocytes along trichomes of *Trichodesmium*, as well as of the expression pattern of key molecular indicators, following combined nitrogen deprivation. We show that diazocyte pattern formation is decided on early during the developmental process. Moreover, there is a strong coupling between diazocyte development and basic cellular processes, such as ammonia assimilation and respiration, the latter being particularly pronounced in generating a reducing environment in the diazocytes (Bryceson & Fay, 1981), and/or in producing the energy needed for the development of the diazocytes and their nitrogen-fixation activity.

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