Diazotrophy under continuous light in a marine unicellular diazotrophic cyanobacterium, *Gloeotheca* sp. 68DGA

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Nitrogenase is extremely sensitive to molecular oxygen (O₂), and unicellular diazotrophic cyanobacteria separate nitrogen (N₂)-fixation and photosynthesis to protect nitrogenase from O₂ produced by photosynthesis. When grown under 12 h light/12 h dark cycles (LD), the marine unicellular diazotrophic cyanobacterium *Gloeotheca* sp. 68DGA expressed the nitrogenase protein and its activity (acetylene reduction activity) only during the dark phase. However, this strain was able to grow diazotrophically under continuous light (CL). To determine whether nitrogenase synthesis and N₂-fixation are temporally separated from photosynthesis in the *Gloeotheca* cells that have fully acclimated to CL, the proportion of cells containing nitrogenase (the Fe-protein of nitrogenase) in the culture was measured using an immunocytochemical technique. Cells were grown in a continuous-culture device to maintain constant cell density. Under LD, the cells showed diurnal oscillation of nitrogenase activity, photosynthesis, respiration and the expression and the abundance of the Fe-protein. The oscillation was gradually reduced after the transfer of the cells to CL, and was lost after 23–25 days of cultivation under CL. In CL-acclimated cultures, the Fe-protein was always detected in about 94 % of the cells, although the nitrogenase activity was about one-third of the maximum activity in LD-acclimated cultures. These results suggest that synthesis of nitrogenase proceeds without diurnal oscillation in the CL-acclimated cells of *Gloeotheca* sp. 68DGA. As the respiration rate in CL-acclimated culture was as high as the maximum rate observed in LD-acclimated culture, O₂-uptake mechanism(s) may have been upregulated to maintain low intracellular pO₂.

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INTRODUCTION

Diazotrophic cyanobacteria are the only oxygenic phototrophs among the nitrogen (N₂)-fixing organisms. As nitrogenase (EC 1.18.6.1), the enzyme complex (the Fe-protein, and α - and β -subunits of the MoFe-protein) that catalyses N₂-fixation, is extremely labile to molecular oxygen (O₂) (Postgate, 1998), cyanobacteria have developed many strategies to separate N₂-fixation and photosynthesis spatially or temporally (Bergman *et al.*, 1997; Fay, 1992; Gallon, 1992; Wolk, 1999). Most unicellular diazotrophic cyanobacteria fix N₂ during the

dark phase of light/dark cycles or in the night under atmospheric conditions (Gallon & Chaplin, 1988; Huang & Chow, 1986; Mitsui *et al.*, 1986; Reddy *et al.*, 1993; Taniuchi & Ohki, 2007). However, some diazotrophic unicellular cyanobacteria fix N₂ during the light phase of 12 h light/12 h dark cycles (LD) even though photosynthetic O₂-evolution occurs. For example, *Gloeotheca* sp. PCC6909 fixed N₂ preferentially during the light phase of LD when it was maintained in continuous culture (Ortega-Calvo & Stal, 1991). We previously found that N₂-fixation of *Gloeotheca* sp. 68DGA, which normally is observed only in the dark phase under LD, proceeded after the dark-to-light transition when the assimilation of ammonia was inhibited by the addition of L-methionine sulfoximine (Taniuchi & Ohki, 2007). Furthermore, many unicellular cyanobacteria are able to grow diazotrophically under continuous light (CL) (Colón-López *et al.*, 1997; Grobbelaar *et al.*, 1986; Ohki *et al.*, 2008; Taniuchi &

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Abbreviations: CL, continuous light; DAB, 3,3'-diaminobenzidine tetrahydrochloride; LD, 12 h light/12 h dark cycles; SHAM, salicylhydroxamic acid.

Two supplementary figures are available with the online version of this paper.

Ohki, 2007). These observations suggest that the diurnal separation of N_2 -fixation and photosynthesis may not be essential for diazotrophic growth of unicellular cyanobacteria: the cells may fix N_2 while evolving O_2 . If this is the case, studies on N_2 -fixation by unicellular cyanobacteria under CL will provide useful information for understanding the mechanism(s) for protection of nitrogenase from O_2 , although this light condition never exists in natural environments. However, it is also possible that the diurnal separation is maintained under CL by the circadian clock, as is typically observed after the transition from LD to CL, enabling effective N_2 -fixation in the absence of O_2 -evolution. Even if no synchronicity is observed in the whole culture after prolonged cultivation of the cells under CL, individual cells may separate N_2 -fixation and photosynthesis diurnally. We cannot distinguish between the two possibilities as long as the nitrogenase activity is measured with cell suspensions: it is necessary to distinguish N_2 -fixing cells from non- N_2 -fixing cells in a culture.

In many unicellular diazotrophic cyanobacteria, including *Gloeotheca* sp. 68DGA, used in this study, nitrogenase undergoes a daily cycle of synthesis and degradation, disappearing during the light phase under LD (Chow & Tabita, 1994; Colón-López *et al.*, 1997; Taniuchi & Ohki, 2007). If N_2 -fixation and photosynthesis are separated diurnally within individual cells under CL, a considerable number of cells in the culture would not have nitrogenase. We have established an immunocytochemical method to detect nitrogenase in individual cells of cyanobacteria (Taniuchi *et al.*, 2008). In this study, immunodetection of nitrogenase in individual cells of *Gloeotheca* sp. 68DGA was carried out during the acclimation processes between LD and CL. As acclimation took several generations (cf. Taniuchi & Ohki, 2007), *Gloeotheca* sp. 68DGA was grown in a continuous-culture device to maintain constant cell density. We measured abundance of the nitrogenase-containing cells and changes in nitrogenase activity, photosynthesis and respiration in cultures that were acclimated to either LD or CL, and during the transition phase between LD and CL.

METHODS

Strain and culture conditions. *Gloeotheca* sp. 68DGA was the same strain as used in our previous studies (Ohki *et al.*, 2008; Taniuchi & Ohki, 2007). Culture conditions were also the same as those described previously (Taniuchi & Ohki, 2007). Seed cultures were maintained under 12 h light/12 h dark cycles (LD) or under continuous light (CL) with air-bubbling.

A chemostat-type continuous-culture device equipped with a cylindrical culture vessel (500 ml) was used for all experiments (Ikeya *et al.*, 1997). The cells from the seed culture were inoculated into the culture vessel of the continuous-culture device filled with fresh medium. After the cell density in the vessel reached $2.0\text{--}2.5 \times 10^6$ cells ml^{-1} , fresh medium was introduced at a dilution rate of 0.016 h^{-1} (for LD) or 0.011 h^{-1} (for CL) to maintain constant cell density. Growth rates were determined by counting the cell number using a bacteria-counting chamber under a light microscope, and the means $\pm$ SD of five separate counts are presented.

Nitrogenase activity. Nitrogenase activity was measured using the acetylene reduction method as described previously (Ohki & Fujita, 1988).

Photosynthesis and respiration. O_2 -evolution and respiration were measured with a Clark-type O_2 electrode (Hansatech Oxygraph; Hansatech Instruments) as described previously (Taniuchi & Ohki, 2007).

Nitrogenase detection. Conditions for SDS-PAGE and Western blot analysis were the same as those described previously (Taniuchi & Ohki, 2007). For immunocytochemical detection of nitrogenase, cells were fixed in paraformaldehyde and preserved in methanol at -30°C until use. The fixed cells were treated with DMSO (for permeabilization) and non-immune rabbit serum (for blocking), and then incubated with polyclonal antibody generated against the recombinant Fe-protein of nitrogenase (NifH) from *Trichodesmium* sp. NIBB1067 (Ohki, 2008). The immunoreaction was visualized with horseradish-peroxidase-conjugated secondary antibody and chromogenic substrate, 3,3'-diaminobenzidine tetrachloride (DAB) in the presence of H_2O_2 . Detailed conditions for the immunocytochemical analysis are available in Taniuchi *et al.* (2008). The percentage of the immunostained cells in the culture was determined by triplicate counting of 200 cells under the light microscope.

Transcriptional analysis by RT-PCR. Total RNA was extracted from equal numbers of cells ($\sim 3.0 \times 10^8$ cells) using the Rneasy Plant Mini kit (Qiagen) and preserved at -80°C . The RNA preparations were treated with RNase-free DNase I [DNase (RT Grade) for Heat Stop, Wako] to eliminate possible genomic DNA contamination. The first-strand cDNAs were synthesized from total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was carried out with the primer pair HFF (5'-ACACCAAAGCACAAACCACCA-3')/HRR (5'-GCTTTTCCTTCACGGATAGGCAT-3') to amplify the 322 bp of *nifH* (Ohki *et al.*, 2008). The 449 bp of *rnpB* fragment was used as an internal control for the RT-PCR analysis with the primer pair *rnpB*-F (5'-TGAGGAAA-GTCCGGGCT-3')/*rnpB*-R (5'-TAAGCCGGGTCTGTTC-3') (Vioque, 1997). The PCR conditions were as follows: 24 or 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Negative control reactions were carried out without reverse transcriptase.

RESULTS

Transition from LD to CL

Changes of abundance and activity (measured as acetylene reduction) of nitrogenase, the capacity for net O_2 -evolution (apparent photosynthetic activity) and respiration of *Gloeotheca* sp. 68DGA during the transition from LD to CL are summarized in Fig. 1. In agreement with our previous results (Taniuchi & Ohki, 2007), nitrogenase activity was restricted to the dark phase and its peak appeared around the middle of the dark phase in LD (Fig. 1a, 0–24 h). The mean of the maximum nitrogenase activity [$27.6 \pm 2.1 \times 10^{-16}$ (mol C_2H_4) h^{-1} cell $^{-1}$] under LD was the same as that of the cells in the exponential growth phase of a batch culture (Taniuchi & Ohki, 2007). The capacities for net O_2 -evolution and respiration were inversely correlated: O_2 -evolution peaked in the middle of the light phase, while respiration peaked during the middle of the dark phase (Fig. 1b, 0–24 h). Immunoreactive bands corresponding to the Fe-protein (apparent molecular

Fig. 1. Changes in nitrogenase activity (acetylene reduction activity), net O_2 -evolution, respiration and the abundance of nitrogenase (the Fe-protein) and the percentage of immunostained cells following transfer of *Gloeotheca* sp. 68DGA cells from LD to CL. Cells were grown in a continuous-culture device in a medium without combined nitrogen to maintain cell density at around 2×10^6 cells ml^{-1} . The cells were cultured for several generations under LD (LD-acclimated cells) and then transferred to CL (indicated by the arrowheads at 24 h). The black and grey shading in the upper bar indicate the dark phase of LD and the subjective dark phase (the period corresponding to the dark phase in LD) of CL, respectively. (a) Nitrogenase activity measured by acetylene reduction. The mean and standard deviation of the results from triplicate measurements are shown for each time point. (b) Net O_2 -evolution (open circles) and respiration (closed circles) measured with an O_2 electrode. Means of the results from duplicate measurements are presented. (c) Western blot analysis of the Fe-protein. Soluble fractions extracted from about 2.5×10^7 cells were subjected to SDS-PAGE. The Fe-protein was detected using an anti-Fe-protein antibody. The immunostained bands of 41 and 37 kDa that correspond to the Fe-protein are indicated by an arrow. (d) Proportion of nitrogenase-containing cells in the culture, determined by immunostaining of the cells as described in Methods. The mean $\pm$ SD of the results from triplicate counts of 200 cells is shown for each time point. See Fig. S1 for microscopic images of the immunostained cells.

masses of 41 and 37 kDa on SDS-PAGE, Figs 1c and 2c, indicated by an arrow) were detected 2 h prior to the appearance of nitrogenase activity, and disappeared at the end of the dark phase (Fig. 1c, 0–24 h). In addition, faint immunoreactive protein bands, with an apparent molecular mass of ~ 27 kDa, were often observed when large amounts of the 41 and 37 kDa proteins were present. About 85% of the cells were immunostained at the beginning of the dark phase of LD (Fig. 1d, 0 h). The proportion of immunostained cells increased to about 97% after entering the dark phase and then rapidly decreased to about 60% at the end of the dark phase. It continued to decline in the light phase to reach a minimum near the middle of the light phase. Although the staining was faint (Fig. 1d, 12–24 h, and Supplementary Fig. S1,

available with the online version of this paper), about 30% of the cells remained immunostained at this stage. The number of immunostained cells increased in the latter half of the light phase to reach about 80% at the end of the light phase. Hereafter, we refer to the cells that were cultured under LD as ‘LD-acclimated cells’.

When LD-acclimated cells were transferred to CL (Fig. 1, from 24 h), nitrogenase activity could not be detected during the first subjective dark phase (the period corresponding to the dark phase of LD, Fig. 1, 24–36 h). However, nitrogenase activity reappeared before starting the second subjective dark phase (Fig. 1a, 46 h) as we have observed previously using batch culture (Taniuchi & Ohki, 2007). Nitrogenase activity did increase towards the end of the first subjective light phase

(the period corresponds to the light phase of LD, Fig. 1, 46 h), and peaked near the middle of the second subjective dark phase (Fig. 1a, 54 h), then decreased rapidly to zero 2 h after starting the second subjective light phase (Fig. 1a, 62 h). Net O₂-evolution declined during the first subjective dark phase but was always greater than zero (Fig. 1b, 24–36 h). O₂-evolution substantially increased during the first half of the first subjective light phase (Fig. 1b, from 36 h), and then decreased to zero during the second subjective dark phase (Fig. 1b, 48–60 h). The diurnal oscillation in respiration was maintained after transfer to CL (Fig. 1b, after 24 h). The Fe-protein was present during the first to second subjective dark phase even though its activity was not detected (Fig. 1a vs Fig. 1c, 24–42 h). The Fe-protein disappeared before the culture entered the third subjective light phase (Fig. 1c, 60–72 h). The proportion of immunostained cells remained high (>87%) between the beginning of the first subjective dark phase and the middle of the second subjective dark phase (Fig. 1d, 24–54 h) and then decreased to about 60% near the middle of the second subjective light phase (Fig. 1d, 66 h). The number of the immunostained cells oscillated diurnally for several generations after the second subjective light phase (Fig. 1d, from 72 h). After prolonged incubation under CL, nitrogenase activity did not decrease to zero (Fig. 1a, from 360 h), but it still oscillated between 5 and 15 × 10⁻¹⁶ (mol C₂H₄) h⁻¹ cell⁻¹, with the peak being observed during the subjective dark phase.

Acclimation to CL

When the cells were cultured under CL for a prolonged period (more than nine generations, 23 days), the diurnal

oscillation of nitrogenase activity, net O₂-evolution, respiration and the abundance of the Fe-protein were no longer observed (Fig. 2, 1296–1464 h). Instead, nitrogenase activity, net O₂-evolution and respiration remained constant at around 10.0 ± 0.61 × 10⁻¹⁶ (mol C₂H₄) h⁻¹ cell⁻¹, 6.9 ± 0.28 × 10⁻¹⁵ (mol O₂) h⁻¹ cell⁻¹ and -5.8 ± 0.22 × 10⁻¹⁵ (mol O₂) h⁻¹ cell⁻¹, respectively (mean between 1296 and 1322 h). Also, the level of the Fe-protein was constant (Fig. 2c). The majority of cells (93.8 ± 5.4%) were positively immunostained between 1296 and 1322 h (see also Supplementary Fig. S2). Hereafter, we refer to the cells that were fully acclimated to CL as 'CL-acclimated cells'.

Transition from CL to LD

The diurnal rhythm of nitrogenase activity resumed very rapidly after transfer of CL-acclimated cells to LD (Fig. 2a, from 1464–1476 h). Nitrogenase activity initially decreased during the first dark phase (Fig. 2a, 1476 h). However, the nocturnal increase in nitrogenase activity was restored during the second dark phase (Fig. 2a, from 1488 h). Maximum nitrogenase activity during the second dark phase [28.4 ± 1.9 × 10⁻¹⁶ (mol C₂H₄) h⁻¹ cell⁻¹] was comparable to that of LD-acclimated cells.

Transcriptional analysis of *nifH* in the cells grown under LD and CL

LD-acclimated cells showed diurnal oscillation of expression of *nifH*, the gene encoding the Fe-protein of nitrogenase (Fig. 3a). The transcript level increased during

Fig. 2. Nitrogenase activity (acetylene reduction activity), net O₂-evolution, respiration, the abundance of nitrogenase (the Fe-protein) and proportion of immunostained cells in CL-acclimated cells and the changes thereto after transfer of the culture to LD. The time of transfer is indicated by the arrowheads (at 1464 h). Experimental conditions were the same as those in Fig. 1. See Fig. S2 for microscopic images of the immunostained cells.

Fig. 3. RT-PCR analysis of the *nifH* gene transcript. The cells were collected at 3 h intervals from a culture grown under LD (a) or CL (b). Cells grown with combined nitrogen (NaNO_3) were used as a negative control (c). Total RNA extracted from $\sim 3 \times 10^8$ cells was used as a template.

the first half of the dark phase (Fig. 3a, 3 and 6 h) and decreased gradually thereafter. The *nifH* transcript was almost undetectable in the middle of the light phase (Fig. 3a, 18 and 21 h), and then appeared at the end of the light phase (Fig. 3a, 24 h). In contrast, CL-acclimated cells expressed *nifH* constitutively, although the expression level was slightly lower than the maximum level in LD-acclimated cells (Fig. 3a, 3 and 6 h vs Fig. 3b). The expression level of *rnpB* used as an internal control was almost the same in LD- and CL-acclimated cells. The *nifH* transcript could not be detected when total RNA extracted from the cells grown with combined nitrogen (NaNO_3) was used as a template for RT-PCR analysis (Fig. 3c).

DISCUSSION

When cells of *Gloeotheca* sp. 68DGA were fully acclimated to CL, they fixed N_2 (reduced acetylene) constantly at a rate corresponding to one-third of the maximum rate of LD-acclimated cells (Fig. 1a, 0–12 h vs Fig. 2a, 1296–1322 h). This level of N_2 -fixation activity was similar to that calculated by integrating and averaging the oscillating N_2 -fixation activity in LD-acclimated cells (37 % of the maximum), suggesting the possibility that individual CL-acclimated cells expressed N_2 -fixation activity diurnally in a non-synchronous manner. Whole-cell immunodetection of nitrogenase, however, enabled us to exclude this possibility. As the proportion of nitrogenase-containing *Gloeotheca* sp. 68DGA cells in LD-acclimated cultures oscillates between 97 and 30 %, the proportion of immunostained cells in a CL culture would be at most 65 %, if the culture consisted of non-synchronously

oscillating cells. However, the proportion of immunostained cells in CL-acclimated cultures was far greater (94 % on average, Fig. 2d), indicating that virtually all the cells express nitrogenase and maintain enzyme activity constitutively in CL-acclimated culture. As the anti-Fe-protein antibody used in this study reacted with proteins smaller than 35 kDa that were probably the degradation products of the Fe-protein (Dougherty *et al.*, 1996), as well as with the Fe-protein (Figs 1c, 2c), the proportion of the Fe-protein-containing cells would be overestimated. However, the calculations mentioned above are basically not changed because the overestimation of the Fe-protein-containing cells may occur to a similar extent in CL-acclimated culture and in LD-acclimated culture; the amount of the Fe-protein (41 and 37 kDa, indicated by an arrow in Fig. 1c and Fig. 2c) in CL-acclimated culture was the same as that during the dark phase of LD-acclimated culture, and the immunostained bands smaller than 35 kDa were present in both CL- and LD-acclimated cultures (Fig. 1c, 4–8 h vs Fig. 2c, 1440–1464 h). We thus conclude that nitrogenase synthesis and probably N_2 -fixation proceed without diurnal oscillation in the CL-acclimated cells of *Gloeotheca* sp. 68DGA. Direct detection of N_2 uptake in individual cells (e.g. with a high-resolution nanometre-scale secondary-ion mass spectrometer, cf. Popa *et al.*, 2007) is necessary to confirm the constitutive N_2 -fixation in CL-acclimated cultures. As the level of nitrogenase activity in CL-acclimated cells was low, it seems that the accumulation of intracellular fixed N (or the ratio of N to C in the cells) was insufficient to downregulate nitrogenase activity (cf. Taniuchi & Ohki, 2007), forcing the cells to fix N_2 .

When LD-acclimated cells were transferred to CL, nitrogenase activity was not detected during the first subjective dark phase, although the nitrogenase was present (Fig. 1a). We previously showed that nitrogenase activity in the first subjective dark phase was restored when photosynthetic O_2 -evolution was inhibited by addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea or by reducing the light intensity (Taniuchi & Ohki, 2007). Therefore, the inhibition of nitrogenase activity observed during the first subjective dark phase is to be ascribed to the inhibitory effect of O_2 . After full acclimation of the cells to CL, nitrogenase activity remained constant (Fig. 2a). As the net O_2 -evolution was higher in this phase than that during the first subjective dark phase (Fig. 1b, 24–36 h, vs Fig. 2b, open circles), the CL-acclimated cells seemed to have developed O_2 -tolerance. However, the pO_2 that resulted in 50 % inhibition of nitrogenase activity was the same (~ 0.3 pO_2) in both LD- and CL-acclimated cells (data not shown).

The high rate of respiration (O_2 -uptake) observed in CL-acclimated cells (Fig. 1b vs Fig. 2b, closed circles) may be a mechanism(s) to reduce intracellular pO_2 . Respiratory protection of nitrogenase has been proposed in unicellular diazotrophic cyanobacteria (Maryan *et al.*, 1986; Shieh & Chang, 1992). Recently, Weng & Shieh (2004)

demonstrated a KCN-resistant and salicylhydroxamic acid (SHAM; inhibitor for plant mitochondria-type alternative oxidase)-sensitive O_2 photoreduction pathway in *Synechococcus* sp. RF1. The electrons generated by photosystem II may be transferred from cytochrome b_6f complex to O_2 through ferredoxin. Nitrogenase activity of *Synechococcus* sp. RF1 decreased when this pathway was blocked by SHAM. Contributions of the Mehler reaction have been proposed for reducing the intracellular pO_2 in *Trichodesmium thiebautii*, a non-heterocystous cyanobacterium that fixes N_2 preferentially during the daytime (Kana, 1993). During the Mehler reaction, the electrons produced by photosynthesis are transferred to O_2 to form superoxide. A single-step reduction of superoxide to H_2O by A-type flavoproteins was demonstrated in *Synechocystis* sp. PCC6803 (Helman *et al.*, 2003). Homologous genes encoding A-type flavoproteins were found in several cyanobacteria including *Trichodesmium* spp. In N_2 -fixing *Trichodesmium* colonies, about 75 % of the O_2 produced by photosynthesis was consumed by light-dependent O_2 reduction, most likely via the Mehler reaction (Milligan *et al.*, 2007). Similar mechanism(s) may be employed in *Gloeotheca* sp. 68DGA.

The diurnal oscillation of nitrogenase activity was rapidly recovered after CL-acclimated cells were transferred to LD (Fig. 2, after 1488 h). An endogenous rhythm that regulates the onset of the diurnal oscillation in N_2 -fixation (cf. Taniuchi & Ohki, 2007) appears to be reset by the insertion of a single dark phase (Fig. 2, 1464–1476 h). The down-regulation of nitrogenase activity during the latter half of the second dark phase (Fig. 2, 1494–1500 h, cf. Taniuchi & Ohki, 2007) seems to indicate that the level of fixed N_2 increased to a high enough level during the first half of the second dark phase.

In conclusion, diurnal oscillation of nitrogenase synthesis and probably of N_2 -fixation and photosynthesis is not necessary for the diazotrophic growth of *Gloeotheca* sp. 68DGA. Our results suggest that nitrogenase synthesis proceeds without diurnal oscillation in CL-acclimated cultures. Development of O_2 -uptake mechanism(s) to maintain low intracellular pO_2 under CL has been suggested. The rapid recovery of diurnal oscillation observed upon transfer from CL to LD suggests that N_2 -fixation in *Gloeotheca* sp. 68DGA is primarily regulated by the endogenous rhythm, but is modulated in response to intracellular and environmental factors, e.g. the amount of fixed N_2 and the changes in pO_2 .

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