Synthesis and proteolytic degradation of nitrogenase in cultures of the unicellular cyanobacterium *Gloeotheca* strain ATCC 27152

John P. H. Reade,^{1,2†} Lisa J. Dougherty,¹ Lyndon J. Rogers² and John R. Gallon¹

Author for correspondence: John R. Gallon. Tel: +44 1792 295376. Fax: +44 1792 295447.
e-mail: j.r.gallon@swansea.ac.uk

¹ Biochemistry Research Group, School of Biological Sciences, University of Wales Swansea, Swansea SA2 8PP, UK

² Institute of Biological Sciences, University College of Wales, Sir George Stapledon Building, Aberystwyth SY23 3DD, UK

In cultures of the unicellular cyanobacterium *Gloeotheca* sp. ATCC 27152 growing under alternating 12 h light and 12 h darkness, nitrogenase activity appears as cultures enter the dark phase. Synthesis of both component proteins of nitrogenase commences immediately prior to the appearance of activity and continues until about 8 h into the period of darkness. The two components (Fe-protein and MoFe-protein) are synthesized in a molar ratio of about 3:1. Degradation of the nitrogenase proteins starts as early as 4 h into the dark period and increases markedly as cultures enter the light phase. As a result, both nitrogenase proteins are completely absent from cultures during most of the light phase. In contrast, all of the other proteins investigated appeared to be present throughout the cycle of alternating light and darkness. Degradation of nitrogenase depends upon protein synthesis during the last 6 h of darkness and is prevented by addition of protease inhibitors. Two proteins, of *M*, 47 000 and 29 000, are specifically synthesized during this period and it is possible that they have a role in nitrogenase degradation. Proteolytic activity of extracts of *Gloeotheca*, measured as the ability to degrade azocasein, increased markedly during the early part of the light period, but this increase did not depend on protein synthesis. This activity does not therefore correspond to that specifically involved in nitrogenase catabolism, though it may act on initial breakdown products generated by a nitrogenase-specific degradative system. A phycobiliprotein appears to act as a temporary store of the degradation products of nitrogenase.

Keywords: nitrogenase synthesis and degradation, unicellular cyanobacteria, *Gloeotheca*, N₂ fixation, proteolytic enzymes

INTRODUCTION

Gloeotheca is a unicellular cyanobacterium that can fix N₂ in air despite the fact that O₂ is a potent inhibitor of nitrogenase (Fay, 1992; Gallon, 1992). Moreover, like

other cyanobacteria, *Gloeotheca* generates O₂ as a result of its photosynthetic activity. When grown in air under alternating 12 h light and 12 h darkness, batch cultures of *Gloeotheca* fix N₂ only during the dark period, thereby effecting a temporal separation between oxygenic photosynthesis and N₂ fixation (Gallon, 1981). This behaviour reflects the appearance and disappearance of the Fe-protein of nitrogenase (Du & Gallon, 1993).

The temporal separation of photosynthesis and N₂ fixation is found in many other non-heterocystous cyanobacteria (reviewed by Bergman *et al.*, 1997). In

[†] **Present address:** Crop and Environmental Research Centre, Harper Adams University College, Newport, Shropshire TF10 8NB, UK.

Abbreviations: DRAG, dinitrogenase reductase-activating glycohydrolase; DRAT, dinitrogenase reductase ADP-ribosyl transferase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

contrast, heterocystous cyanobacteria grown under alternating light and darkness fix N_2 either during the period of illumination or both in the light and in the dark (Khamees *et al.*, 1987; Mullineaux *et al.*, 1981). In these organisms, oxygenic photosynthesis (in vegetative cells) and N_2 fixation (in heterocysts) are spatially separated.

Among cyanobacteria, fluctuations in nitrogenase activity are not always reflected in the appearance and disappearance of the Fe-protein of nitrogenase. Though this is the case in *Gloeotheca* (Du & Gallon, 1993) and in another unicellular cyanobacterium *Cyanothece*, formerly called *Synechococcus* (Campbell *et al.*, 1994; Chow & Tabita, 1994; Colón-López *et al.*, 1997), the filamentous non-heterocystous cyanobacterium *Oscillatoria limosa* behaves differently. In this organism, the Fe-protein can be detected more or less throughout a diurnal illumination cycle but a putatively inactive form predominates during the light period, when no nitrogenase activity can be detected, and is converted to an assumed active form prior to the appearance of activity (Villbrandt *et al.*, 1992). A similar correlation between the onset of N_2 fixation and the appearance of an assumed active form of the Fe-protein exists in the unusual non-heterocystous cyanobacterium *Trichodesmium* (Chen *et al.*, 1998; Ohki *et al.*, 1992; Zehr *et al.*, 1993), which fixes N_2 during the day, and in the heterocystous cyanobacterium *Anabaena variabilis* (Ernst *et al.*, 1990).

Active nitrogenase has a second component, the MoFe-protein. However, in *O. limosa* and *Trichodesmium*, the MoFe-protein of nitrogenase can be detected throughout a cycle of alternating light and darkness, though the intracellular concentration of this protein is generally greater during the period of N_2 fixation (Villbrandt *et al.*, 1992; Zehr *et al.*, 1993). The lack of nitrogenase activity during a specific phase of the illumination cycle therefore appears to be exerted predominantly by disappearance or inactivation of the Fe-protein. In contrast, in *Cyanothece*, the MoFe-protein disappears from cultures during the light phase of a cycle of alternating light and darkness (Colón-López *et al.*, 1997). In *Gloeotheca*, synthesis of the MoFe-protein occurs only during the latter part of the light period and the first half of the dark period (Gallon *et al.*, 1988), but it is not known whether this protein persists in cells during the period in which no nitrogenase activity can be detected.

Protein turnover reflects a balance between two components: synthesis and degradation. In *Gloeotheca*, synthesis of one of the components of nitrogenase, the MoFe-protein, has already been studied (Gallon *et al.*, 1988; Maryan *et al.*, 1986). However, there have been no parallel studies on the synthesis of the Fe-protein, though it is known that this protein appears in cultures towards the end of the light phase of a cycle of alternating 12 h light and 12 h darkness (Du & Gallon, 1993). Although the disappearance of the Fe-protein at the end of the

dark phase (Du & Gallon, 1993) implies that it is degraded, nothing is known about the nature of this process. We have, for example, found endopeptidase and aminopeptidase activities in extracts of *Gloeotheca* (F. W. Wagner, D. L. Gleaves & J. R. Gallon, unpublished observations), but these have not specifically been linked to turnover of nitrogenase.

Studies on the degradation of radiolabelled nitrogenase and measurements of proteolytic activity have therefore been undertaken in cultures of *Gloeotheca* growing under alternating 12 h light and 12 h darkness to investigate whether the decline in N_2 fixation that occurs in this organism towards the end of the dark period can be explained in terms of nitrogenase degradation.

METHODS

Growth of cultures. A capsule-free strain of *Gloeotheca* sp. (Du & Gallon, 1993) derived from strain ATCC 27152, was grown at 25 °C in 15 l batch cultures in modified ASM-1 medium (Gallon *et al.*, 1978). Cultures were bubbled with air at 0.3 l min⁻¹ (l culture)⁻¹ and illumination was provided as an alternating cycle of 12 h light (30 µmol photons m⁻² s⁻¹ over the waveband 400–700 nm) and 12 h darkness. Experiments were performed during the phase of exponential growth, 6–10 d after inoculation.

N_2 fixation. Nitrogenase (EC 1.18.6.1) activity was measured using the acetylene reduction technique as described by Gallon *et al.* (1993).

Synthesis and degradation of nitrogenase. Nitrogenase synthesis was measured as incorporation of radioactivity into the enzyme from Na₂³⁵SO₄ (Gallon *et al.*, 1988; Maryan *et al.*, 1986). A sample (100 ml) was removed from the stock culture, centrifuged at 7000 g for 10 min and resuspended in 100 ml of ASM-1 medium (Gallon *et al.*, 1978) lacking MgSO₄. Then, Na₂³⁵SO₄ (3.7 MBq; Amersham) was added and the sample incubated for 4 h at 25 °C under identical conditions of illumination to those prevailing in the stock culture. At the end of the incubation period, proteins were extracted as described by Du & Gallon (1993) except that, instead of using the French press, cells were broken by sonication for 5 min (given as 30 s bursts separated by 2 min) at 0 °C.

Degradation of nitrogenase was measured by sampling 8 l of the stock culture at the beginning of the dark period. After centrifugation at 7000 g for 10 min, the cells from this sample were resuspended in 800 ml SO₄²⁻-free ASM-1 medium and incubated in the dark for 4 h at 25 °C with 29.6 MBq Na₂³⁵SO₄. During this period, ³⁵S was incorporated into the nitrogenase proteins. The sample was then centrifuged at 7000 g for 10 min and the radiolabelled cells resuspended in 8 l complete ASM-1 lacking radioisotope. This culture was then incubated at 25 °C under illumination identical to that received by the stock culture. Samples (1 l) were removed every 4 h and proteins extracted as described by Du & Gallon (1993), except that cell breakage was accomplished by 5 min sonication (30 s bursts separated by 2 min) at 0 °C. Total radioactivity in 10 µl of the broken cell preparation was then estimated by scintillation counting using a ¹⁴C internal standard.

SDS-PAGE. Samples from a single experiment, each containing an identical amount of protein (normally about 25 µg), were applied to the wells of a 10% (w/v) polyacrylamide gel overlaid with a 5% (w/v) stacking gel. Electrophoresis was carried out at 200 V in a Bio-Rad Mini-PROTEAN II electrophoresis system until the bromophenol blue had migrated to the bottom of the gel.

Autoradiography. After SDS-PAGE, the gel was placed on a section of Whatman 3MM chromatography paper cut to the same size as the gel and dried under vacuum for 35 min at 80 °C in a Bio-Rad model 224 Gel Drier. The dried gel was incubated in the dark with Hyper film β -max (Amersham) for 72 h at -72 °C. The film was then developed and fixed. A mixture of 14 C-labelled proteins, subjected to SDS-PAGE in parallel with the experimental samples, served to estimate the M_r values of radioactive proteins in the *Gloeotheca* extracts.

Western blot analysis. After separation by SDS-PAGE, proteins were transferred to nitrocellulose membrane using a Bio-Rad Mini Trans-Blot Cell. Proteins were visualized by immunospecific Western blotting as described by Stal & Bergman (1990), using polyclonal antisera raised to the following proteins: the MoFe-protein and the Fe-protein of nitrogenase, dinitrogenase reductase-activating glycohydrolase (DRAG) and dinitrogenase reductase ADP-ribosyl transferase (DRAT) from *Rhodospirillum rubrum* (all obtained from Dr P. Ludden, University of Wisconsin, Madison, WI, USA), the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) from *Synechococcus* strain PCC 7942 (Dr S. Gutteridge, DuPont Central Research and Development, DE, USA) and C-phycoerythrin from *Phormidium persicinum* (Dr R. MacColl, New York State Department of Health, NY, USA). The M_r values of these proteins were estimated from the behaviour of a mixture of proteins of known M_r (106 000, 80 000, 49 500, 32 500, 27 500 and 18 500) subjected to SDS-PAGE in parallel with the *Gloeotheca* extracts and visualized on the nitrocellulose membranes by staining with 0.1% (w/v) Ponceau S in 0.17 M acetic acid.

Proteolytic activity. Proteolytic activity was measured with azocasein as a substrate, using a modification of the method of Plantner (1991). A sample of culture (2 l) was harvested by centrifugation at 7000 g for 15 min. The pelleted cells were resuspended in 2 ml 50 mM phosphate buffer, pH 7.4 and broken by 5 min sonication (30 s bursts separated by 2 min) at 0 °C. To 0.45 ml of this broken-cell extract was added 0.75 ml azocasein (10 mg ml⁻¹ in sodium phosphate buffer). After incubation at 30 °C for 1 h, the reaction was terminated by addition of 0.175 ml 10% (w/v) trichloroacetic acid. The reaction mixture was then centrifuged at 3000 g, after which 0.8 ml of the supernatant was added to 60 µl 10 M NaOH. The absorbance of this solution was then measured at 440 nm against a zero-time blank. One unit of proteolytic activity is defined as catalysing the liberation of sufficient trichloroacetic-acid-soluble material to give an increase in A_{440} of 1 after incubation for 1 h at 30 °C. A control incubation, in which broken-cell extract was substituted by 0.45 ml phosphate buffer, demonstrated that non-enzymic breakdown of azocasein was insignificant.

Measurement of protein. The protein content of extracts of *Gloeotheca* was measured as described by Bailey (1962). In the case of samples subjected to SDS-PAGE (Du & Gallon, 1993), 10 µl of sample was withdrawn for measurement of protein

Fig. 1. Nitrogenase activity and the intracellular concentrations of various proteins in cultures of *Gloeotheca* sp. ATCC 27152 growing under alternating 12 h light (L) and 12 h darkness (D). Nitrogenase activity (a) was measured as acetylene reduction during the 60 min period following the sampling time indicated. Intracellular proteins are shown as immunospecific Western blots after SDS-PAGE of extracts of *Gloeotheca*, developed using antiserum to (b) the MoFe-protein of nitrogenase, (c) the Fe-protein of nitrogenase, (d) DRAT, (e) DRAG, (f) the large subunit of Rubisco or (g) C-phycoerythrin.

prior to addition of 2-mercaptoethanol and bromophenol blue, which interfere with the assay.

RESULTS

Turnover of nitrogenase and other proteins in *Gloeotheca*

Batch cultures of *Gloeotheca* grown under alternating 12 h light (30 µmol m⁻² s⁻¹) and 12 h darkness showed patterns of nitrogenase activity similar to those reported previously (Du & Gallon, 1993; Gallon *et al.*, 1988), with N₂ fixation confined almost entirely to the dark period (Fig. 1a). N₂ fixation was maximal 4–6 h into the dark (D4–6). (The shorthand notation adopted here uses the prefix 'L' to refer to measurements made, or samples taken, during the light phase, and 'D' to refer to measurements made during the period of darkness. Thus D1 refers to the situation in a culture 1 h after the onset of darkness, whilst L2 applies to a culture 2 h into the light phase. Cultures enter the light phase at L0 and enter the dark phase at D0.)

The MoFe-protein of nitrogenase (M_r 60 300) could be detected immunologically throughout the dark period (Fig. 1b) but rapidly disappeared once illumination commenced and did not reappear until cultures entered

Fig. 2. Synthesis of proteins in cultures of *Gloeotheca* growing under alternating 12 h light (L) and 12 h darkness (D). Cultures were incubated with $^{35}\text{SO}_4^{2-}$ for 4 h during the period indicated, after which extracts (each containing 25 μg protein) were subjected to SDS-PAGE and autoradiography. The bands on the left hand side (headed M_r) illustrate the mobilities of radioactively labelled proteins of M_r (top to bottom) 106000, 80000, 32500, 27500 and 18500.

the following dark phase. The Fe-protein of nitrogenase (M_r 38500 and 40000) behaved similarly (Fig. 1c), though this protein could be detected earlier than the MoFe-protein, at L10 rather than at D0, and persisted rather longer into the following light period. This behaviour was consistent with that reported previously for the Fe-protein (Du & Gallon, 1993). The larger, 'modified' (Du & Gallon, 1993) form of the Fe-protein appeared first (as the upper band in Fig. 1c), but disappeared by about D4. Thereafter, the smaller form of the Fe-protein (lower band) predominated, though the larger form reappeared as cultures entered the light phase.

Unlike the protein components of nitrogenase (Fig. 1b, c), all of the other proteins investigated could be detected throughout the period of alternating light and darkness (Fig. 1d–g). DRAT and DRAG are enzymes responsible for the reversible modification of the Fe-protein of nitrogenase from *R. rubrum* (Ludden *et al.*, 1988) and other bacteria. Antigenically similar proteins have been reported in *Gloeotheca* (Du *et al.*, 1994), though there is no evidence that these proteins act on nitrogenase in this organism. Antiserum to DRAT reacted with two proteins in extracts of *Gloeotheca* subjected to SDS-PAGE. A strong reaction was seen with a protein of M_r 69000 and a weaker reaction with a protein of M_r 60000 (Fig. 1d). A single protein, of M_r 63000, reacted with antiserum to DRAG (Fig. 1e). Antiserum to the large subunit of Rubisco, the enzyme that catalyses photosynthetic CO_2 fixation in plants and cyanobacteria, reacted with a single protein of M_r 50000 in extracts of *Gloeotheca* (Fig. 1f). Two proteins, of M_r 20000 and 17000, reacted though only weakly, with antiserum raised against the accessory photosynthetic pigment C-phycoerythrin (Fig. 1g). These corresponded, respectively, to the β and α subunits of this protein (Mazel *et al.*, 1986; Rumbeli *et al.*, 1987). Spectroscopic analysis of the phycobiliprotein pigments showed that their overall

Fig. 3. Degradation of radiolabelled proteins in cultures of *Gloeotheca* growing under alternating 12 h light (L) and 12 h darkness (D). Cultures were incubated with $^{35}\text{SO}_4^{2-}$ during the first 4 h of darkness (D0–D4), after which the radioactive substrate was removed. Samples were removed at D4 and at subsequent 4 h intervals as shown at the head of each gel track. Extracts were then subjected to SDS-PAGE and autoradiography (a). The tracks headed M_r show the mobilities of radioactively labelled proteins of M_r (top to bottom) 66000, 45000, 36000, 29000, 23000, 20000 and 14000. For comparative purposes (b), a Western blot showing the Fe-protein of nitrogenase in each sample is shown below the corresponding gel track.

intracellular concentration did not fluctuate substantially during growth of *Gloeotheca* under alternating light and darkness, though the concentrations of phycoerythrin were lowest during the early part of the dark phase (data not shown).

Protein synthesis

As revealed by autoradiography of SDS-PAGE gels (Fig. 2), radioactivity from $^{35}\text{SO}_4^{2-}$ was incorporated predominantly into three proteins during the first 4 h of the dark period (D0–4). These proteins had M_r values of 60000, 39000 and 20000, respectively. During the period D4–8, incorporation of radioactivity into the two larger proteins appeared to be slightly less than during D0–4, whilst that into the smallest protein was greater. In addition, radioactivity was now observed in proteins of M_r 47000 and 29000. During the final 4 h of darkness (D8–L0), incorporation of radioactivity into the protein of M_r 39000 had markedly declined whilst virtually no incorporation into the protein of M_r 60000 was observed. Significant radioactivity was incorporated into the proteins of M_r 47000, 29000 and 20000. For the first 4 h after cultures entered the light period (L0–L4) incorporation of radioactivity from $^{35}\text{SO}_4^{2-}$ was largely confined to the protein of M_r 20000, though some incorporation into the proteins of M_r 47000 and 29000 continued. Thereafter, however, many proteins became radioactively labelled.

Densitometry scans of the autoradiographs of the SDS-PAGE gels showed that, during the period D0–4, 34% of the total radioactivity incorporated into protein was found in the protein of M_r 60000 whilst 37% was found in the protein of M_r 39000. Most of the remaining radioactivity (20%) was found in the protein of M_r 20000. These figures were confirmed by scintillation counting of appropriate gel slices.

Fig. 4. Degradation of azocasein by extracts prepared from cultures of *Gloeotheca* growing under alternating 12 h light (L) and 12 h darkness (D). At D6 (6 h into the dark period; see arrow), 0.1 mg chloramphenicol ml⁻¹ was added to one culture (■). The control culture (●) received no chloramphenicol.

Protein degradation

The proteins of M_r 60000 and 39000, radioactively labelled during the first 4 h of darkness, had almost completely disappeared by the start of the following light period at L0 (Fig. 3a). Densitometry of autoradiographs suggested that breakdown of these proteins started between D4 and D8. In contrast, as radioactivity in these proteins steadily declined during the last 8 h of darkness and for 4 h into the succeeding light period, it accumulated in a protein of M_r 17000 (compare D4, D8, L0 and L4). After L8, radioactivity also appeared in a number of other proteins.

Proteolytic activity in *Gloeotheca*

In cultures of *Gloeotheca* grown under alternating light and darkness, proteolytic activity, measured as the ability of extracts to degrade the artificial substrate azocasein, varied between 0.046 and 0.616 units (mg protein)⁻¹. Activity was very low between D3 and D9 but thereafter increased, reaching a maximum between L3 and L6 (Fig. 4).

Addition of 0.1 mg chloramphenicol ml⁻¹ to cultures of *Gloeotheca* at D6 completely abolished protein synthesis by D8 (data not shown). It has previously been shown that this treatment markedly decreased the decline in nitrogenase activity normally seen during the last 6 h of the dark phase and also allowed immunologically identifiable Fe-protein of nitrogenase to persist well into the following light phase (Du & Gallon, 1996). Addition of chloramphenicol at D6 did not, however, prevent the subsequent increase in the ability of extracts of *Gloeotheca* to degrade azocasein (Fig. 4).

Addition of 1 μ M pepstatin A, an inhibitor of aspartic proteases, at D6 had no more than a slight stimulatory effect on nitrogenase activity during the remainder of the dark phase and did not allow activity to extend into the following light period (Fig. 5a). Nevertheless, degradation of the Fe-protein was markedly decreased fol-

Fig. 5. The effect of 1 μ M pepstatin A on nitrogenase activity (a) and synthesis of the Fe-protein of nitrogenase (b) in cultures of *Gloeotheca* growing under alternating light (L) and darkness (D). Acetylene reduction for the period D6–L7 (a) is shown in the presence (●) and absence (○) of 1 μ M pepstatin A, added at D6. The presence of the Fe-protein in Western blots after SDS-PAGE of extracts of cultures receiving pepstatin A is shown for each sample point (b). In the absence of pepstatin A, the Fe-protein in cultures of *Gloeotheca* behaved as shown in Fig. 1(c).

lowing addition of pepstatin A (compare Fig. 5b with Fig. 1c). In the presence of pepstatin A, immunologically detectable Fe-protein persisted in cells of *Gloeotheca* until at least L7.

Similar effects were seen following addition of other inhibitors of proteolysis at D6. These included 1 μ M PMSF or 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF), both inhibitors of serine proteases, and 1 mM Na₂EDTA, which inhibits metalloproteases. In this last case, the inhibitor was added in the presence or absence of 5 mM CaCl₂, which prevents the EDTA-mediated inhibition of nitrogenase activity in *Gloeotheca* (Gallon & Hamadi, 1984; Hamadi & Gallon, 1981). Except when EDTA was added alone (in which case no activity was seen), these additions had little effect on nitrogenase activity, which had usually completely disappeared by L1. However, in every case, degradation of the Fe-protein was markedly decreased so that the protein remained detectable in cell extracts until at least L5. In contrast to these findings, addition of 10 μ M leupeptin, an inhibitor of thiol proteases, did not prevent nitrogenase degradation when added at D6. This may indicate that thiol proteases are not involved in nitrogenase breakdown though it is also possible that leupeptin, which at neutral pH carries a positive charge, may not penetrate the cells of *Gloeotheca* very effectively.

DISCUSSION

As reported previously (Du & Gallon, 1993; Gallon *et al.*, 1988), the appearance of the Fe-protein of nitrogenase at L10 (Fig. 1c) is closely followed by detectable nitrogenase activity (Fig. 1a) in cultures of *Gloeotheca* growing under alternating light and darkness. Although the Western blot suggests that synthesis of the MoFe-protein, which appears at D0 (Fig. 1b), lags slightly behind that of the Fe-protein, this may simply reflect the lower sensitivity of the antiserum to this protein. It seems more likely that synthesis of the two proteins of nitrogenase is coordinately regulated in cultures of *Gloeotheca*. On the other hand, none of the other proteins investigated showed such substantial fluctuations in intracellular concentration, so it seems that the appearance and disappearance of the protein components of nitrogenase in cultures grown under alternating light and darkness is unusual among *Gloeotheca* proteins. Although it appears that the intracellular concentrations of the DRAT-like proteins were greatest during the period of illumination (Fig. 1d), whilst those of the DRAG-like protein were greatest during the early part of the dark period (Fig. 1e), it is not known whether the conditions of blotting allow any quantitative deductions to be made. Moreover, these fluctuations have not been consistently observed. As a consequence, no significance is ascribed to the variations seen in Fig. 1(d and e). The poor reaction between extracts of *Gloeotheca* and antibodies raised against phycoerythrin in *P. persicinum* (Fig. 1g) may result from structural differences in this protein between the two organisms.

Maryan *et al.* (1986) showed that radioactivity from $^{35}\text{SO}_4^{2-}$ could be incorporated into the MoFe-protein of *Gloeotheca* nitrogenase which appeared on SDS-PAGE as two bands of M_r 60000 and 55000 representing, respectively, the β and α subunits of this protein. The conditions of electrophoresis used in this study were selected to identify proteins over a wide range of M_r values and were apparently unable to resolve these two polypeptides. This has also been observed in other systems (Kennedy *et al.*, 1976). The radioactive band with an M_r of 60000, (Figs 2, 3a), whose position on the gels coincides exactly with that of the MoFe-protein as determined by Western blotting, probably therefore includes both the α and β subunits of the MoFe-protein. The radioactive band at M_r 39000 (Figs 2, 3a) corresponded with the protein that reacted with antiserum to the Fe-protein of nitrogenase and can therefore be assigned to the Fe-protein of nitrogenase. However, the two forms visible in Western blots (Fig. 1c) could not be distinguished in the autoradiographs (Figs 2, 3a). In our experience, this is not an uncommon occurrence and probably reflects the poorer resolving power of autoradiography over that of Western blotting.

De novo synthesis of both nitrogenase proteins was largely confined to the first 8 h of the dark period (D0–8). This confirms the findings of Gallon *et al.* (1988) for the MoFe-protein and is in marked contrast to general protein synthesis, most of which takes place

during the last 8 h of the light phase (L4–D0). The distinct period of synthesis of both nitrogenase proteins coincides with the phase of rising nitrogenase activity.

During the period of maximum synthesis of the nitrogenase proteins (D0–D4), 37% of the total radioactivity incorporated into protein was found in the band that, on SDS-PAGE, corresponded to the Fe-protein of nitrogenase, whilst 34% was found in the band that corresponded to the MoFe-protein. The amino acid sequences of the nitrogenase proteins from *Gloeotheca* have not been determined, but sequences for the nitrogenase proteins of the similar unicellular cyanobacterium *Cyanothece* (*Synechococcus*) RF-1 (Huang & Chow, 1988) are available from GenBank (accession no. U22146). These show that, excluding the initiating methionine residue (which is usually removed during post-translational processing), the polypeptide chain of the Fe-protein of nitrogenase contains 15 residues (cysteine or methionine) that contain sulphur. Similarly the α and β polypeptide chains of the MoFe-protein of nitrogenase contain, respectively, 22 and 19 sulphur-containing amino acid residues. Assuming that the mature polypeptide chains of the Fe-protein of *Gloeotheca* nitrogenase (a dimer) contain 30 atoms of S per molecule whilst the polypeptide chains of the MoFe-protein (an $\alpha_2\beta_2$ tetramer) contain 82 atoms of S per molecule, then the relative incorporation of radioactivity from $^{35}\text{SO}_4^{2-}$ into these proteins implies that they are synthesized in the ratio of 3 mol Fe-protein per mol MoFe-protein. This is consistent with the observation that several N_2 -fixing bacteria contain an excess of Fe-protein over MoFe-protein and also implies that *Gloeotheca* contains sufficient Fe-protein to allow the MoFe-protein to function as two catalytically independent halves, each with a binding site for Fe-protein (see, for example, Smith & Eady, 1992).

Only one protein, of M_r 20000, was identified as being synthesized throughout the diurnal illumination cycle. This protein is tentatively identified as a subunit of a phycobiliprotein, based on its M_r , which was within the range (17000–22000) reported for these proteins (Sidler, 1994). The electrophoretic behaviour of this radioactive protein also correlates well with that of a *Gloeotheca* polypeptide that gave a weak antigenic reaction with antibodies to C-phycoerythrin (Fig. 1g) and is probably the β subunit of that protein, as well as with a pigmented area that was easily visible in SDS-PAGE gels.

Degradation of the nitrogenase proteins starts between D4 and D8 (Fig. 3a). It should be noted, however, that synthesis of nitrogenase continues during the dark period, in parallel with this degradation. For example, by L0, virtually all of the ^{35}S nitrogenase that had been synthesized during the period D0–4 had disappeared (Fig. 3a). Nevertheless, immunologically identifiable Fe-protein (Figs 3b, 1c) and MoFe-protein (Fig. 1b) could easily be detected on Western blots. Non-radioactive nitrogenase had therefore clearly been synthesized after $^{35}\text{SO}_4^{2-}$ had been removed from the culture at D4 and had not been completely degraded by L0. These findings

imply that, for at least part of the dark period, synthesis and degradation of nitrogenase occur simultaneously in *Gloeothecce*.

Like the protein of M_r 20000 that was synthesized throughout the illumination cycle (Fig. 2), the protein of M_r 17000 that accumulated as nitrogenase was being degraded (Fig. 3a) may be a subunit of a phycobiliprotein. For example, it also corresponded to a polypeptide (probably the α subunit) that reacted with antiserum to C-phycoerythrin. However, because of distortions that occurred when the gels were dried prior to autoradiography, it is not possible at this stage to state categorically that the protein of M_r 17000 is distinct from the protein of M_r 20000 that was readily labelled during incubation with $^{35}\text{SO}_4^{2-}$. Just as autoradiography did not resolve either the α and β subunits of the MoFe-protein of nitrogenase or the two forms of the Fe-protein, this procedure may not be able to distinguish satisfactorily between the α and β subunits of the various phycobiliproteins that are synthesized by *Gloeothecce*. Hence, in Figs 2 and 3(a), radiolabelled phycobiliproteins appear as a diffuse smear rather than as a series of separate bands. Wyman *et al.* (1985, 1986) have proposed that phycoerythrin may act as a reserve of nitrogen in the marine unicellular cyanobacterium *Synechococcus* sp. WH7803 (DC2), but there is no evidence that phycobiliproteins represent a temporary store of newly fixed nitrogen in *Gloeothecce* (Gallon *et al.*, 1990). This does not, of course, preclude the possibility that one or more of these proteins acts as a reservoir of amino acids released by catabolism of nitrogenase, as is implied by the accumulation of radioactivity in the protein of M_r 17000 as ^{35}S disappears from nitrogenase.

Although degradation of both proteins of nitrogenase occurred during the period D8–L4, in neither case could any immunologically identifiable breakdown product be seen (Fig. 1; Du & Gallon, 1993). This contrasts with the situation *in vitro* (Dougherty *et al.*, 1996; Gallon *et al.*, 1995) but implies that, *in vivo*, the initial stages of degradation are followed by a rapid and complete breakdown of nitrogenase. During the period L4–8, neither the Fe-protein nor the MoFe-protein of nitrogenase could be detected in extracts of *Gloeothecce*. In this respect, *Gloeothecce* resembles *Cyanothece* sp. strain ATCC 51142 (Colón-López *et al.*, 1997) but differs from all of the other non-heterocystous cyanobacteria so far examined, none of which show complete turnover of nitrogenase when grown under alternating light and darkness. In all of these cyanobacteria, both the MoFe-protein and the Fe-protein of nitrogenase could be detected in cells throughout the illumination cycle (Chen *et al.*, 1998; Ohki *et al.*, 1992; Villbrandt *et al.*, 1992; Zehr *et al.*, 1993).

Degradation of nitrogenase appears to commence between D4 and D8 (Fig. 3a), but is particularly active when cells enter the light phase (Fig. 1). This degradation is dependent upon protein synthesis, since it is markedly inhibited by addition of chloramphenicol at D6 (Du &

Gallon, 1996). It also depends upon the activity of proteolytic enzymes. The simplest explanation for these observations would be that one or more component of a nitrogenase-specific proteolytic system is synthesized during the latter stages of the dark period. The observation, reported here, that a range of protease inhibitors interfere with this degradation suggests that aspartic, serine and metalloproteases (but not necessarily thiol proteases) may all be involved in degradation of the Fe-protein, possibly acting as a cascade of closely associated enzymes. Previous work has shown that this proteolytic system shows considerable specificity towards the *Gloeothecce* enzyme. For example, extracts that rapidly degraded the Fe-protein of *Gloeothecce* nitrogenase were unable to degrade the equivalent protein from *Klebsiella pneumoniae* (Gallon *et al.*, 1995), though they could degrade the Fe-protein from *R. rubrum* (L. J. Dougherty & J. R. Gallon, unpublished observations).

During the last 8 h of the dark period (D4–L0) and the early part of the following light period (L0–L4), two proteins, of M_r 47000 and 29000, were specifically synthesized by cultures of *Gloeothecce*. The appearance of these proteins coincided with the breakdown of nitrogenase, so it is tempting to speculate that they may be involved in nitrogenase catabolism. However, there is no direct evidence for this. On the other hand, the proteins of M_r 47000 and 29000 do not appear to be components of the azocasein-degrading system whose stimulation after D9 was independent of protein synthesis. Similarly, the enzymes that degrade azocasein do not correspond to the proteolytic system specifically involved in nitrogenase breakdown, since azocasein degradation was stimulated even in the presence of chloramphenicol and does not, apparently, show a high degree of substrate specificity. The azocasein-degrading enzymes may, however, catalyse the further breakdown of initial degradation products generated by one or more nitrogenase-specific protease whose synthesis commences after D6.

Even when nitrogenase degradation was prevented by, for example, addition of chloramphenicol (Du & Gallon, 1996) or inhibitors of proteases (Fig. 5b), nitrogenase activity declined during the latter part of the dark period. Loss of activity is not, therefore, simply a consequence of nitrogenase breakdown. Rather, it appears that N_2 fixation declines as a consequence of a decreased breakdown of glucan reserves that would, in turn, limit the supply of ATP and reductant for nitrogenase synthesis and activity, the supply of carbon skeletons for assimilation of newly fixed nitrogen and the rate of respiration, which declines in parallel with the fall in nitrogenase activity (Gallon, 1992; Gallon *et al.*, 1988). Since respiratory consumption of O_2 is an important mechanism by which *Gloeothecce* limits the inhibitory effect of O_2 on N_2 fixation, the observed decline in glucan-supported respiration may exacerbate O_2 -inactivation of nitrogenase. This dependence of nitrogenase activity on glucan breakdown also explains

why, even in untreated cultures of *Gloeotheca*, nitrogenase activity declines after D6 despite the fact that nitrogenase proteins are present in the cells until L2 (Fig. 1).

As well as inhibiting nitrogenase activity and, at least transiently, nitrogenase synthesis (Maryan *et al.*, 1986), it is possible that O_2 might stimulate degradation of nitrogenase in *Gloeotheca*. If so, nitrogenase breakdown during the latter part of the dark period could increase as a result of the decline in respiratory O_2 consumption that occurs at the same time. Such an effect of O_2 could also explain the rapid disappearance of nitrogenase that occurs once cultures of *Gloeotheca* enter the light phase and commence photosynthesis (Fig. 1b, c). In support of a link between O_2 and nitrogenase breakdown, extracts of *Gloeotheca* degraded nitrogenase more rapidly when incubated in air than in the absence of O_2 (Gallon *et al.*, 1995). However, based on studies with *A. variabilis* and *Cyanotheca* sp. strain ATCC 51142, it has been further suggested that O_2 -damaged nitrogenase is preferentially targeted for proteolysis (Colón-López *et al.*, 1997; Durner *et al.*, 1996). Though this is an attractive hypothesis, it does not appear to be the case in *Gloeotheca*. For example, nitrogenase that had been inactivated by transient exposure to O_2 was no more rapidly degraded *in vitro* in the absence of O_2 than was active nitrogenase, which had not been exposed to O_2 (Gallon *et al.*, 1995). It therefore appears likely that the nitrogenase-specific proteolytic system in extracts of *Gloeotheca* is stimulated directly by O_2 rather than by O_2 -inactivation of its substrate. On the other hand, proteolytic degradation of nitrogenase *in vitro* was also stimulated by ATP or by the presence of reducing conditions (Dougherty *et al.*, 1996), and it could be increases in these rather than in O_2 that explain the stimulation of nitrogenase breakdown that occurs when cultures of *Gloeotheca* enter the light period.

In cultures of a number of cyanobacteria, including unicellular strains, the diurnal pattern of N_2 fixation seen under alternating light and darkness reflects an endogenous circadian rhythm. For example, this pattern persists after cultures are transferred from alternating light and darkness to constant illumination (Chen *et al.*, 1998; Colón-López *et al.*, 1997; Mitsui *et al.*, 1986; Mitsui & Suda, 1995; Stal & Krumbein, 1985). This is not, however, the case in *Gloeotheca* (Gallon & Chaplin, 1988). In this cyanobacterium, the perfect diurnal pattern of N_2 fixation seen under alternating light and darkness is lost following removal of the period of darkness, reverting within 24 h to the pattern seen in cultures maintained under constant illumination, in which peaks of nitrogenase activity are separated by about 40 h (Mullineaux *et al.*, 1981). Thus, in *Gloeotheca*, the endogenous rhythm in N_2 fixation is not circadian. The diurnal pattern of N_2 fixation seen under alternating 12 h light and 12 h darkness therefore appears to be imposed by the illumination regime (Gallon *et al.*, 1988). Thus, whilst it is possible that the fluctuations in nitrogenase synthesis and degradation

reported here reflect an endogenous circadian rhythm in *Gloeotheca*, there is no evidence to support this view. The decline in nitrogenase activity, the inhibition of nitrogenase synthesis and the onset of degradation, all of which occur at around D6, coincide with a decreased rate of glucan breakdown and an increase in the intracellular ratio of ADP to ATP (Gallon *et al.*, 1988). This is probably a consequence of depletion of intracellular glucan reserves after several hours of darkness. The accompanying decline in respiratory activity (Gallon, 1992) would lead to an increase in intracellular O_2 . Singly or in combination, these factors could affect the rate of N_2 fixation by acting on nitrogenase synthesis, inactivation and degradation without involving any endogenous circadian rhythm. The recommencement of nitrogenase synthesis after a period of darkness requires at least 4 h of illumination (Gallon *et al.*, 1988), but there is a further lag of about 6 h before the nitrogenase proteins reappear. This may reflect a need to replenish reserves that have become depleted in the dark.

In *Cyanotheca* (*Synechococcus*) strain BG 43511, the diurnal pattern of N_2 fixation under alternating light and darkness has further been explained in terms of synchronous cell division, with nitrogenase activity confined to a specific phase in the cell cycle (Mitsui *et al.*, 1986). This cyanobacterium has a doubling time close to 24 h under these conditions. In contrast, *Gloeotheca* has a minimum doubling time of about 50 h when grown diazotrophically under alternating 12 h light and 12 h darkness (Gallon *et al.*, 1988). It therefore seems most unlikely that the fluctuations in nitrogenase activity, synthesis and degradation seen in *Gloeotheca* could be related to events regulated by the cell cycle.

ACKNOWLEDGEMENTS

We gratefully acknowledge the support of the AFRC, now BBSRC, (UK) who funded this research and the award of an EPSRC studentship to L. J. D. We also thank Drs S. Gutteridge, P. Ludden and R. MacColl for their generous gifts of antisera.

REFERENCES

- Bailey, J. L. (1962). Estimation of protein: Folin-Giocalteau reagent. In *Techniques in Protein Chemistry*, p. 293–294. Edited by J. L. Bailey. Amsterdam: Elsevier.
- Bergman, B., Gallon, J. R., Rai, A. N. & Stal, L. J. (1997). N_2 fixation by non-heterocystous cyanobacteria. *FEMS Microbiol Rev* **19**, 139–185.
- Campbell, C., Takeyama, H. & Mitsui, A. (1994). Cyclic synthesis of messenger RNA from nitrogenase *nifH* gene in synchronous cultures of marine unicellular cyanobacterium, *Synechococcus* sp. strain Miami BG043511. *J Mar Biotechnol* **2**, 39–43.
- 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.
- Chow, T. J. & Tabita, F. R. (1994). Reciprocal light-dark transcrip-

- tional control of *nif* and *rbc* expression and light-dependent posttranslational control of nitrogenase activity in *Synechococcus* sp. strain RF-1. *J Bacteriol* 176, 6281–6285.
- Colón-López, M. S., Sherman, D. & Sherman, L. A. (1997). Transcriptional and translational regulation of nitrogenase in light-dark- and continuous-light-grown cultures of the unicellular cyanobacterium *Gloeotheca* sp. strain ATCC 51142. *J Bacteriol* 179, 4319–4327.
- Dougherty, L. J., Brown, E. G. & Gallon, J. R. (1996). Nitrogenase-specific proteolytic activity in the unicellular cyanobacterium *Gloeotheca*. *Biochem Soc Trans* 24, 401S.
- Du, C. & Gallon, J. R. (1993). Modification of the Fe protein of the nitrogenase of *Gloeotheca* (Nägeli) sp. ATCC 27152 during growth under alternating light and darkness. *New Phytol* 125, 121–129.
- Du, C. & Gallon, J. R. (1996). Degradation and modification of Fe-protein of nitrogenase in cultures of *Gloeotheca* grown under alternating light and darkness. *Physiol Mol Biol Plants* 2, 87–94.
- Du, C., Reade, J. P. H., Rogers, L. J. & Gallon, J. R. (1994). Dinitrogenase reductase ADP-ribosyl transferase and dinitrogenase reductase activating glycohydrolase in *Gloeotheca*. *Biochem Soc Trans* 22, 332S.
- Durner, J. I. B., Knörzer, O. C. & Böger, P. (1996). Proteolytic degradation of dinitrogenase reductase from *Anabaena variabilis* (ATCC 29413) as a consequence of ATP depletion and impact of oxygen. *J Bacteriol* 178, 606–610.
- Ernst, A., Liu, Y.-D., Reich, S. & Böger, P. (1990). Diurnal nitrogenase modification in the cyanobacterium *Anabaena variabilis*. *Bot Acta* 103, 183–189.
- Fay, P. (1992). Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiol Rev* 56, 340–373.
- Gallon, J. R. (1981). The oxygen sensitivity of nitrogenase: a problem for biochemists and microorganisms. *Trends Biochem Sci* 6, 19–23.
- Gallon, J. R. (1992). Reconciling the incompatible: N₂ fixation and O₂. (Tansley review no. 44). *New Phytol* 122, 571–609.
- Gallon, J. R. & Chaplin, A. E. (1988). Nitrogen fixation. In *The Biochemistry of Algae and Cyanobacteria*, pp. 147–173. Edited by L. J. Rogers & J. R. Gallon. Oxford: Oxford University Press.
- Gallon, J. R. & Hamadi, A. F. (1984). Studies on the effects of oxygen on acetylene reduction (nitrogen fixation) in *Gloeotheca* sp. ATCC 27152. *J Gen Microbiol* 130, 495–503.
- Gallon, J. R., Ul-Haque, M. I. & Chaplin, A. E. (1978). Fluoroacetate metabolism in *Gloeocapsa* sp. LB795 and its relationship to acetylene reduction (nitrogen fixation). *J Gen Microbiol* 106, 329–336.
- Gallon, J. R., Perry, S. M., Rajab, T. M. A., Flayeh, K. A. M., Yunes, J. S. & Chaplin, A. E. (1988). Metabolic changes associated with the diurnal pattern of N₂ fixation in *Gloeotheca*. *J Gen Microbiol* 134, 3079–3087.
- Gallon, J. R., Perry, S. M. & Flynn, K. J. (1990). The immediate fate of N₂ fixed by *Gloeotheca* growing under alternating light and darkness. In *Nitrogen Fixation: Achievements and Objectives*, p. 534. Edited by P. M. Gresshoff, L. E. Roth, G. Stacey & W. E. Newton. New York: Chapman & Hall.
- Gallon, J. R., Pederson, D. M. & Smith, G. D. (1993). The effect of temperature on the sensitivity of nitrogenase to oxygen in the cyanobacteria *Anabaena cylindrica* (Lemmermann) and *Gloeotheca* (Nägeli). *New Phytol* 124, 251–257.
- Gallon, J. R., Reade, J. P. H., Dougherty, L. J., Pederson, D. M. & Rogers, L. J. (1995). Degradation of nitrogenase in *Gloeotheca*. In *Nitrogen Fixation: Fundamentals and Applications*, p. 230. Edited by I. A. Tikhonovich, N. A. Provorov, V. I. Romanov & W. E. Newton. Dordrecht: Kluwer.
- Hamadi, A. F. & Gallon, J. R. (1981). Calcium ions, oxygen and acetylene reduction (nitrogen fixation) in the unicellular cyanobacterium *Gloeocapsa* sp. 1430/3. *J Gen Microbiol* 125, 391–398.
- Huang, T.-C. & Chow, T.-J. (1988). Comparative studies of some nitrogen-fixing unicellular cyanobacteria isolated from rice fields. *J Gen Microbiol* 134, 3089–3097.
- Kennedy, C., Eady, R. R., Kondorosi, E. & Rekosh, D. K. (1976). The molybdenum-iron protein of *Klebsiella pneumoniae* nitrogenase: evidence for non-identical subunits from peptide 'mapping'. *Biochem J* 155, 383–389.
- Khamees, H. S., Gallon, J. R. & Chaplin, A. E. (1987). The pattern of acetylene reduction by cyanobacteria grown under alternating light and darkness. *Br Phycol J* 22, 55–60.
- Ludden, P. W., Roberts, G. P., Lowery, R. G. & 9 other authors (1988). Regulation of nitrogenase activity by reversible ADP-ribosylation of dinitrogenase reductase. In *Nitrogen Fixation: Hundred Years After*, pp. 157–162. Edited by H. Bothe, F. J. de Bruijn & W. E. Newton. Stuttgart: Fischer.
- Maryan, P. S., Eady, R. R., Chaplin, A. E. & Gallon, J. R. (1986). Nitrogen fixation by the unicellular cyanobacterium *Gloeotheca*. Nitrogenase synthesis is only transiently repressed by O₂. *FEMS Microbiol Lett* 34, 251–255.
- Mazel, D., Gugliemi, G., Houmard, J., Sidler, W., Bryant, D. A. & Tandeau de Marsac, N. (1986). Green light induces transcription of the phycoerythrin operon in the cyanobacterium *Calothrix* 7601. *Nucleic Acids Res* 14, 8279–8290.
- Mitsui, A. & Suda, S. (1995). Alternative and cyclic appearance of H₂ and O₂ photoproduction activities under nongrowing conditions in an aerobic nitrogen-fixing unicellular cyanobacterium *Synechococcus* sp. *Curr Microbiol* 30, 1–6.
- Mitsui, A., Kumazawa, S., Takahashi, A., Ikemoto, H., Cao, S. & Arai, T. (1986). Strategy by which nitrogen-fixing unicellular cyanobacteria grow photoautotrophically. *Nature* 323, 720–722.
- Mullineaux, P. M., Gallon, J. R. & Chaplin, A. E. (1981). Acetylene reduction (nitrogen fixation) by cyanobacteria grown under alternating light-dark cycles. *FEMS Microbiol Lett* 10, 245–247.
- Ohki, K., Zehr, J. P. & Fujita, Y. (1992). Regulation of nitrogenase activity in relation to the light-dark regime in the filamentous non-heterocystous cyanobacterium *Trichodesmium* sp. NIBB 1067. *J Gen Microbiol* 138, 2679–2685.
- Plantner, J. J. (1991). A microassay for proteolytic activity. *Anal Biochem* 195, 129–131.
- Rümbeli, R., Suter, F., Wirth, M., Sidler, W. & Zuber, H. (1987). Gamma-N-methyl asparagine in phycobiliproteins from the cyanobacteria *Mastigocladus laminosus* and *Calothrix*. *FEBS Lett* 221, 1–2.
- Sidler, W. A. (1994). Phycobilisome and phycobiliprotein structure. In *The Molecular Biology of Cyanobacteria*, pp. 139–216. Edited by D. A. Bryant. Dordrecht: Kluwer.
- Smith, B. E. & Eady, R. R. (1992). Metalloclusters of the nitrogenases. *Eur J Biochem* 205, 1–15.
- Stal, L. J. & Bergman, B. (1990). Immunological characterization of nitrogenase in the filamentous cyanobacterium *Oscillatoria limosa*. *Planta* 182, 287–291.
- Stal, L. J. & Krumbein, W. E. (1985). Nitrogenase activity in the non-heterocystous cyanobacterium *Oscillatoria* sp. grown under alternating light-dark cycles. *Arch Microbiol* 143, 67–71.
- Villbrandt, M., Stal, L. J., Bergman, B. & Krumbein, W. E. (1992). Immunolocalization and Western blot analysis of nitrogenase in

Oscillatoria limosa during a light-dark cycle. *Bot Acta* **105**, 90–96.

Wyman, M., Gregory, R. P. F. & Carr, N. G. (1985). Novel role for phycoerythrin in a marine cyanobacterium, *Synechococcus* strain DC2. *Science* **230**, 818–820.

Wyman, M., Gregory, R. P. F. & Carr, N. G. (1986). Role of phycoerythrin in marine picoplankton *Synechococcus* spp. – response. *Science* **234**, 1423–1424.

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.

.....
Received 10 August 1998; revised 16 February 1999; accepted
26 March 1999.