NblA is essential for phycobilisome degradation in *Anabaena* sp. strain PCC 7120 but not for development of functional heterocysts

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Phycobilisomes (PBS) are the major light-harvesting complexes of cyanobacteria. These usually blue-coloured multiprotein assemblies are rapidly degraded when the organisms are starved for combined nitrogen. This proteolytic process causes a colour change of the cyanobacterial cells from blue-green to yellow-green (‘bleaching’). As is well documented for the unicellular, non-diazotrophic cyanobacteria *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803, a gene termed *nblA* plays a key role in PBS degradation. Filamentous, diazotrophic cyanobacteria like *Anabaena* adapt to nitrogen deprivation by differentiation of N₂-fixing heterocysts. However, during the first hours after nitrogen deprivation all cells degrade their PBS. When heterocysts mature and nitrogenase becomes active, vegetative cells resynthesize their light-harvesting complexes while in heterocysts the phycobiliprotein content remains very low. Expression and function of *nblA* in *Anabaena* sp. PCC 7120 was investigated. This strain has two *nblA* homologous genes, one on the chromosome (*nblA*) and one on plasmid delta (*nblA*-p).

Northern blot analysis indicated that only the chromosomal *nblA* gene is up-regulated upon nitrogen starvation. Mutants with interrupted *nblA* and *nblA*-p genes, respectively, grew on N₂ and developed functional heterocysts. Mutant Δ*nblA*-p behaved like the wild-type. However, mutant Δ*nblA* was unable to degrade its PBS, which was most obvious in non-bleaching heterocysts. The results show that NblA, encoded by the chromosomal *nblA* gene, is required for PBS degradation in *Anabaena* but is not essential for heterocyst differentiation.

**INTRODUCTION**

Cyanobacteria show a worldwide distribution, inhabiting nearly all illuminated ecosystems. They have developed a high adaptability to changing environmental conditions like nutrient availability, light or temperature (Bhaya *et al*., 2000). Combined nitrogen is one limiting factor in aquatic environments and cyanobacteria have developed different mechanisms to adapt to this nutrient stress. Diazotrophic cyanobacteria withstand nitrogen deprivation by fixing molecular nitrogen, while non-diazotrophic strains differentiate into so-called resting cells that are able to survive prolonged periods of nutrient stress (Görl *et al*., 1998; Li & Sherman, 2002). One of the responses exhibited by all cyanobacteria when they are starved for nitrogen is the degradation of phycobilisomes (PBS). PBS are the major light-harvesting complexes of the photosynthetic apparatus of cyanobacteria and red algae. They are large multi-protein complexes that can constitute up to 50% of the total cellular protein. PBS are composed of the pigmented phycobiliproteins, which together with chlorophyll a give cyanobacteria their typical blue-green colour, and of non-pigmented linker polypeptides (MacColl, 1998). Degradation of PBS is thought to reduce absorption of excess light energy under the stress situation and to provide substrates for protein synthesis required for the acclimation process.

When non-diazotrophic cyanobacteria like *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus* 7942) or *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) are starved for nitrogen, they completely degrade their PBS within 24–48 h (Collier & Grossman, 1992; Li & Sherman, 2002). This loss of phycobiliproteins is visible as a colour...
change of the cyanobacterial cells from blue-green to yellow-green, a phenomenon named nitrogen chlorosis (Boresch, 1910; Allen & Smith, 1969). Such bleaching is less obvious in diazotrophic cyanobacteria because PBS degradation occurs only transiently in these strains. Diazotrophic, filamentous cyanobacteria such as Anabaena adapt to nitrogen deprivation by differentiating heterocysts, cells that are specialized for fixation of N\textsubscript{2} in an aerobic environment (Wolk et al., 1994). In a filament, approximately every tenth vegetative cell undergoes this differentiation process. During the first hours after nitrogen step-down, PBS degradation appears to start in all vegetative cells (Bradley & Carr, 1976) but manifests itself only in heterocysts where the PBS content is low to essentially zero (Wolk & Simon, 1969; Thomas, 1970). However, mature heterocysts may regain phycobiliproteins later on (Thomas, 1972; Peterson et al., 1981a).

The proteolytic degradation of PBS proceeds in an ordered manner (Yamanaka & Glazer, 1980; Collier & Grossman, 1992). Several attempts have been made to identify the proteases involved in PBS degradation. Proteolytic activities have been shown to be capable of degrading phycobiliproteins in vitro, but the roles of these proteases in vivo remain unclear (Foulds & Carr, 1977; Wood & Haselkorn, 1979; Boussiba & Richmond, 1980; Lockau et al., 1988; Maldener et al., 1991; Nanni et al., 2001). The protein that seems to trigger PBS degradation during nitrogen starvation is NblA. Its gene nbla was first identified in Synechococcus 7942 by complementation of mutants with a non-bleaching (nbl) phenotype (Collier & Grossman, 1994). In Synechococcus 7942, gene nbla is expressed at a very low level in nutrient-replete medium, but is highly up-regulated under nitrogen and sulfur starvation and, to a lesser extent, during phoshorus starvation. NblA shows no similarity to proteins with known function. The molecular mechanism by which NblA triggers degradation of PBS is not clear. Collier & Grossman (1994) suggested that NblA may activate a PBS-degrading protease. Alternatively, NblA could tag or disrupt the PBS, rendering it susceptible to degradation. As recently shown, NblA binds to phycobiliproteins, preferentially to the z-subunits of phycocyanin, phycoerythrin or phycoerythrocyanin (Luque et al., 2003; our unpublished results). In the databases several genes from cyanobacteria and red algae encoding NblA homologues are found. The predicted size of the proteins ranges over 54–65 amino acids, corresponding to about 6.9–7.5 kDa. In Synechocystis 6803, two nbla homologues are present (Kaneko et al., 1996), both of which are required for PBS degradation (Baier et al., 2001). In contrast to Synechococcus 7942, induction of nbla expression in Synechocystis 6803 occurs only upon nitrogen starvation and not upon sulfur starvation (Richaud et al., 2001; Li & Sherman, 2002). In the genome database of Anabaena sp. PCC 7120 (hereafter Anabaena 7120), two nbla homologous ORFs exist: ORF asr4517 on the chromosome (named nbla) and ORF asr8504 on plasmid Delta (named nbla-p) (Kaneko et al., 2001). Here we present results on expression and function of nbla in this filamentous, diazotrophic cyanobacterium.

**METHODS**

**Strains and growth conditions.** Anabaena (Nostoc) 7120 and its derived mutants as well as Anabaena variabilis ATCC 29413 (= Nostoc sp. PCC 7937) (hereafter Anabaena 29413) were grown at 28°C under constant illumination (cool-white fluorescent lamps, mean light intensity 50 µmol photon m\textsuperscript{–2} s\textsuperscript{–1}) in BG11 medium which contains 17-6 mM NaNO\textsubscript{3} as nitrogen source, or in BG11\textsubscript{p} which lacks sodium nitrate (Rippka, 1988). Liquid cultures were bubbled with 2% (v/v) CO\textsubscript{2} in air. For growth on plates, the medium was solidified with separately autoclaved 0.6% (w/v) agar (Difco). Mutants were grown in the presence of 150 µg neomycin ml\textsuperscript{–1}.

For nitrogen starvation experiments, cyanobacterial cultures exponentially growing in BG11 medium were collected by filtration through a nylon net filter (11 µm pore size; Millipore), washed twice with BG11\textsubscript{p}, resuspended in this medium and incubated further under growth conditions. During step-down experiments with the homozigous mutants Δnbla and Δnbla-p, neomycin was omitted.

Chlorophyll content was estimated from methanicol extracts according to Tandeau de Marsac & Houmard (1988). Whole-cell absorbance spectra from 550 to 750 nm were recorded on a Beckman DU 640 spectrophotometer and corrected for cell scattering at 750 nm. The phycocyanin to chlorophyll ratio was estimated from whole-cell spectra. Phycocyanin and chlorophyll was calculated from corrected absorbance at 625 and 678 nm, respectively, as described by Myers et al. (1980). The values were not corrected for the small absorbance of phycocerythrocyanin at 625 nm.

Strains of Escherichia coli were grown under standard conditions. When appropriate, antibiotics were added to the medium to final concentrations of 50 µg ampicillin ml\textsuperscript{–1}, 50 µg kanamycin sulfate ml\textsuperscript{–1} or 25 µg chloramphenicol ml\textsuperscript{–1}.

**Inactivation of nbla and nbla-p.** For interposon inactivation, nbla and nbla-p containing DNA and flanking regions were amplified by PCR, creating restriction sites for cloning (XhoI and SacI) and for insertion of the antibiotic resistance cassette (XbaI). For details of construction see Fig. 1. A 2194 bp fragment containing the nbla-p region and a 1118 bp fragment containing the nbla region were ligated into pBluescript (XhoI/Sacl). The C.K3 cassette, derived as an XbaI fragment from plasmid pRL448 (Elhai & Wolk, 1988a), was ligated into the created XbaI sites, replacing a 100 bp fragment of nbla-p and a 60 bp fragment of nbla, respectively. These constructs were cloned into suicide plasmid pRL271 (Black et al., 1993), resulting in pBB3 (nbla-p) and pBB7 (nbla). Transfer of plasmids between Anabaena and E. coli was achieved by conjugation using strain J53 bearing RP4 and cargo strain HB101, bearing helper plasmid pRL528, in triparental matings (Elhai & Wolk, 1988b). Selection of double recombinants was performed using sacB as positive selection marker (Cai & Wolk, 1990).

**Acetylene reduction assays.** Eight millilitres of culture was placed in a 33 ml tube sealed with a rubber stopper and shaken at 28°C in the light in the presence of 10% (v/v) acetylene in air. After 1 h, the reaction was stopped by addition of 0.6 ml of 40% (w/v) trichloroacetic acid. Ethylene in the gas phase was measured using analytical gas chromatography (Perkin Elmer Auto System, flame-ionization detector) equipped with a GS GasPro column (20 m x 0.32 mm; J&W Scientific).

**RNA isolation and Northern blot analysis.** Aliquots of 50 ml of culture were quickly harvested by centrifugation at 4°C. Cell pellets were rapidly frozen and stored at −20°C until processed. For RNA preparation, cell pellets were homogenized by grinding in liquid nitrogen. RNA was prepared with the TRIZOL Reagent according to the instructions of the supplier (Gibco BRL Life Technologies).
Fig. 1. Constructs for interposon inactivation of nblA and nblA-p. (a) The nblA-p encoding region was amplified by PCR with primers #1.1 [5’-GGCTTACTTAGCTAATCTCGAQAGTGC-3’ (Xhol site inserted)], #1.2 [5’-GCTAAATTCTGT-TCTAGAGATATGTCATAGG-3’ (XbaI site inserted)], #1.3 [5’-GCAGATGATGATTCTAGAAACGAC-3’ (XbaI site inserted)] and #1.4 [5’-CTGAAAGCAGCTTCTCTGTAG-3’ (SacI site inserted)] and the C.K3 cassette was inserted in the created XbaI site. For details see Methods. (b) The nblA encoding region was amplified by PCR with primers #2.1 [5’-CGTCCCAAAAGATGGCTCATATTC-3’ (SacI site inserted)], #2.2 [5’-CATGGCTCATATCTAGAATGTTTGAG-3’ (XbaI site inserted)], #2.3 [5’-CCGTGAAGCCACATATGCTAGC-3’ (XbaI site inserted)] and #2.4 [5’-GGCTGGACTGCTCTCGATGTAC-3’ (XhoI site inserted)] and the C.K3 cassette was inserted in the created XbaI site. For details see Methods. Restriction sites in primers are underlined.

About 6 μg RNA per lane was separated by electrophoresis and transferred to Nylon membranes according to standard methods (Sambrook et al., 1989). Blots were hybridized with 32P-labelled DNA probes specific for nblA and nblA-p from Anabaena 7120 and nblA from Anabaena 29413, respectively. Signal intensities were quantified using Quantity one 4.1.1 Software (Bio-Rad).

Preparation of crude extracts, SDS-PAGE and immunoblotting. Aliquots of 50 ml of culture were harvested by centrifugation at 4 °C. Cell pellets were homogenized in extracting buffer [250 mM Tris, pH 7.5, 25 mM EDTA, 30 % (v/v) sucrose, 5 mM DTT, 1 % (v/v) protease inhibitor mix (containing 100 mM PMSF and 250 mM p-aminobenzamidine in DMSO)] by vortexing with glass beads (150–210 μm; Sigma). The supernatant of two consecutive centrifugations (10 min at 15,000 g) served as crude extract. Heterocysts were isolated as described by Peterson & Wolk (1978), but under aerobic conditions. Isolated heterocysts were homogenized in extracting buffer lacking protease inhibitor mix by grinding in liquid nitrogen. After grinding, 1 % (v/v) protease inhibitor mix was added to the cell extract followed by centrifugation as described above. Protein was estimated according the Bio-Rad protein assay with BSA as a standard. SDS-PAGE was performed in slab gels containing 12 % (w/v) acrylamide; bisacrylamide (29:1) in the buffer system of Laemmli (1970). For Western blots, proteins were transferred electrophoretically to nitrocellulose membranes. Subunits of photosystem II were detected by antiseras raised against proteins D1 and D2 from oat, protein PsbO from oat and protein CP47 from tobacco (Exss-Sonne et al., 2000). Antigen–antibody complexes were visualized with peroxidase conjugated anti-rabbit IgG from goat (Sigma) and developed with the ECL Western blot detection kit (Amersham Pharmacia).

Microscopy. Micrographs were taken on a Leitz microscope equipped with a × 40 objective lens, with phase-contrast and fluorescence optics. Heterocyst frequencies were determined by inspection of ~1000 cells per sample.

Immunocytochemistry. Cultures of Anabaena 7120 and mutant ΔnblA were starved for nitrogen for 24 h as described above. Subsequently, cells were harvested by centrifugation and the pellets rinsed three times with EM buffer (50 mM KH2PO4/Na2HPO4, pH 7). Fixation of cells was performed with 2.5 % (w/v) glutaraldehyde in EM buffer for 1.5 h. After washing three times with EM buffer, cells were dehydrated in a graded ethanol series (15, 30, 45, 60, 75, 90 %, v/v, ethanol in distilled water, 20 min for each dilution) and finally twice in 100 % ethanol for 1 h. After dehydration, the samples were embedded stepwise in LR White resin (The London Resin Co.) infiltrated with 50 % (v/v) LR White/ethanol for 30 min, 66 % (v/v) LR White/ethanol for 14 h and three times for 1 h in 100 % LR White. The last three steps were performed under vacuum. Subsequently, the samples were transferred to resin-filled gelatin capsules and left to polymerise for 24 h at 35 °C. Preparation of ultrathin sections and immunocytochemical treatment were performed as described by Michel et al. (1998).
Specific antisera against phycocyanin from *Aphanothece halophytica* (Sigma) were raised in rabbit (Michel *et al.*, 2003). The antiserum dilution was 1 : 30. As the second antiserum, a gold-coupled anti-rabbit IgG (British BioCell International) was used (dilution 1 : 30). The size of the immunogold particles was 15 nm.

**RESULTS**

**Expression of nblA in Anabaena**

In the genome sequence of *Anabaena* 7120 two *nblA* homologous genes are found: ORF asr4517 on the chromosome (*nblA*) and ORF asr8504 on plasmid Delta (*nblA*-p) (Kaneko *et al.*, 2001). Gene *nblA* encodes a polypeptide (NblA) of 65 aa with a predicted molecular mass of 7542 Da, *nblA*-p encodes a putative polypeptide (NblA-p) of 61 aa with a predicted molecular mass of 7397 Da. NblA and NblA-p sequences are similar to each other (66 % identity) and to NblA polypeptides from the unicellular cyanobacteria *Synechococcus* 7942 and *Synechocystis* 6803, the filamentous *Nostoc punctiforme* and *Tolypothrix* sp. PCC 7601, and to polypeptides encoded by ORFs *ycf18* of red algae. The highest similarity was found to the NblA sequence of *Nostoc punctiforme* (93 % identity with NblA and 64 % identity with NblA-p of *Anabaena* 7120), whereas the identity to the other NblA sequences ranges over 22 to 38 %.

In the closely related cyanobacterium *Anabaena* 29413 we detected only one *nblA* gene using PCR technique with degenerated primers and screening of a genomic DNA library (Jacob, 2002). Its deduced amino acid sequence is 100 % identical to NblA from *Anabaena* 7120, their DNA sequences show 96 % identity (Jacob, 2002; cf. draft sequence of the *Anabaena* 29413 genome, http://genome.jgi-psf.org/draft_microbes/anava/). The nucleotide sequence of *nblA* from *Anabaena* 29413 is available from the GenBank-EMBL database under accession number AJ 504665. No homologue of the plasmid-encoded gene was found in this strain (Jacob, 2002).

![Fig. 2. Northern blot analysis of nblA expression in Anabaena 7120 and Anabaena 29413 after nitrogen step-down. Cultures exponentially growing in medium containing nitrate were transferred to medium lacking combined nitrogen and were incubated further under growth conditions. RNA was isolated from cells harvested before (+N) and at various times (1, 3, 6, 24 h) after nitrogen deprivation, and were hybridized with DNA probes specific for nblA and nblA-p of Anabaena 7120 (triangles) or for nblA of Anabaena 29413 (circles), respectively. The intensities of the bands (corrected for the 16S rRNA signal intensities) are plotted in relative units.](image-url)
Expression of nblA genes was analysed following nitrogen deprivation. Cultures, grown to exponential phase in medium containing nitrate, were transferred to medium lacking fixed nitrogen. Before step-down and at various times thereafter, cells were harvested and RNA was prepared. In Northern blot analyses, transcripts of 500–900 nt were detected with nblA-specific DNA probes in Anabaena 7120 as well as in Anabaena 29413. In both strains the level of nblA transcripts increased about fivefold within the first hour after nitrogen step-down, the shortest time point measured, then decreased and remained at the 24 h level (Fig. 2) under nitrogen-depleted conditions (data not shown). With a DNA probe specific for nblA-p no transcripts could be detected, indicating that the plasmid-encoded gene of Anabaena 7120 is not expressed under these conditions (Fig. 2).

**Inactivation of the chromosomal nblA gene leads to a non-bleaching phenotype**

Mutants of Anabaena 7120 with disrupted genes nblA (ΔnblA) or nblA-p (ΔnblA-p), respectively, were generated by insertional mutagenesis (for details see Methods and Fig. 1). According to Southern blot analysis the mutants were homozygous. When grown in nitrogen-replete medium, the growth rates of both mutants were indistinguishable from that of the wild-type (not shown). When starved for nitrogen, mutant ΔnblA developed a ‘non-bleaching’ phenotype while mutant ΔnblA-p behaved like the wild-type. In both wild-type and mutant ΔnblA-p, degradation of phycobiliproteins became visible about 24 h after nitrogen step-down by a colour change from blue-green to yellow-green. Mutant ΔnblA, however, stayed blue-green. This difference can be seen in the whole-cell absorbance spectra shown in Fig. 3. The phycocyanin absorbance peak at ~625 nm decreased after nitrogen step-down in wild-type and mutant ΔnblA-p but remained high in mutant ΔnblA. Two days after nitrogen step-down, the phycocyanin to chlorophyll ratio remained at 90% of the initial value in mutant ΔnblA, while in mutant ΔnblA-p and in wild-type the phycocyanin to chlorophyll ratio decreased to 67 and 70%, respectively (Table 1).

In diazotrophic filamentous cyanobacteria, the bleaching upon nitrogen starvation is most obvious in heterocysts. This can be seen microscopically as a loss of phycobiliprotein fluorescence. Both mutants ΔnblA and ΔnblA-p differentiated functional heterocysts. Heterocysts of mutant ΔnblA, however, retained their phycobiliproteins and consequently showed intense red fluorescence, while heterocysts of wild-type and mutant ΔnblA-p showed a strong bleaching phenotype (Fig. 4). This difference in the phycobiliprotein contents in heterocysts of mutant ΔnblA and of the wild-type was confirmed by immunocytochemical analysis of the phycocyanin content of heterocysts and vegetative cells, using the immunogold technique (Fig. 5). The electron micrographs show that heterocysts of mutant ΔnblA contained much more phycocyanin than wild-type heterocysts. The differences in the phycocyanin content of vegetative cells were less obvious, as predicted from the

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<th>Wild-type</th>
<th>ΔnblA</th>
<th>ΔnblA-p</th>
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<tr>
<td>+N</td>
<td>100</td>
<td>100</td>
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<tr>
<td>$t_{24}$</td>
<td>86±3</td>
<td>95±4</td>
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<td>$t_{48}$</td>
<td>70±1</td>
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Table 1. Phycocyanin (PC) to chlorophyll (Chl) ratios of Anabaena 7120 wild-type and its mutants ΔnblA and ΔnblA-p before (+N) and 24 and 48 h after transfer to nitrogen-free medium

The values are means of at least three separate experiments and were calculated as a percentage of the initial value after transfer to nitrogen-free medium. The 100% values for wild-type, ΔnblA and ΔnblA-p were 1·1±0·4, 1·1±0·3 and 1·0±0·2, respectively.

![Figure 3](http://mic.sgmjournals.org)  
**Fig. 3.** Whole-cell absorbance spectra of Anabaena 7120 and its mutants ΔnblA and ΔnblA-p immediately ($t_0$) and 48 h ($t_{48}$) after transfer to nitrogen-free medium. The spectra were normalized to 1 at 678 nm.
modest changes in the phycocyanin to chlorophyll ratios (Table 1). Similar results were obtained with an anti-allophycocyanin antiserum (not shown).

**NblA is not essential for differentiation of functional heterocysts**

Inactivation of nblA had no significant effect on function of heterocysts and, consequently, on viability under nitrogen-limiting conditions. The growth behaviour of the ΔnblA mutant on N₂ was indistinguishable from that of the wild-type. The non-bleaching heterocysts of this mutant developed unimpaired nitrogenase activity (Fig. 6). Heterocyst frequencies of ΔnblA and of the wild-type were similar, 11.1 ± 0.6% in the mutant and 10.5 ± 0.3% in the wild-type.

In vegetative cells, most PBS are physically and functionally connected to photosystem II (reviewed by Sidler, 1994). Heterocysts, which normally contain low amounts of PBS,
are thought to lack active, O₂-evolving photosystem II (see Wolk et al., 1994). To find out whether the presence of phycobiliproteins in heterocysts of mutant ΔnblA affects the presence or composition of photosystem II in these cells, we analysed some photosystem II subunits, namely D1 and D2 (core proteins of the reaction centre), CP47 (a chlorophyll-binding protein) and PsbO (a stabilizing protein of the oxygen-evolving system) (for review see Bricker & Ghanotakis, 1996), by Western blot analysis of extracts of vegetative cells and of isolated heterocysts (Fig. 7). Using specific antisera, subunits D2, CP47 and PsbO were detected in cells of filaments grown on nitrate, and in vegetative cells and heterocysts from N₂-grown cultures. Subunit D1, which together with subunit D2 forms the core of the reaction centre of photosystem II, was not detectable in heterocysts isolated from either wild-type or mutant ΔnblA.

**DISCUSSION**

The requirement of NblA for PBS degradation under conditions of nitrogen deficiency is well-documented for the unicellular, non-diazotrophic cyanobacteria *Synechococcus*
We have investigated expression and function of NbLA in Anabaena 7120, a filamentous diazotrophic strain, to determine its role in heterocyst development. In the genome sequence of Anabaena 7120 two NbLA genes are found, NbLa and NbLA-p. The chromosomal NbLA gene was expressed (Fig. 2). Even after nitrogen step-down, expression of the plasmid-encoded NbLA-p could not be detected, neither in the wild-type (Fig. 2) nor in the ∆NbLA mutant (data not shown). Furthermore, mutant ∆NbLA-p degraded its PBS like the wild-type (Fig. 3 and Table 1). Hence, NbLA-p may be expressed only under very specific conditions or may be a silent gene. The latter presumption is supported by the finding that the closely related cyanobacterium Anabaena 29413 appears to possess just one NbLa gene. Anabaena 29413 seems to have no plasmids (Houmard & Tandeau de Marsac, 1988; see also http://genome.jgi-psf.org/draft_microbes/anava/) whereas Anabaena 7120 contains six plasmids (Kaneko et al., 2001).

Analysis of transcript accumulation of NbLA following nitrogen step-down (Fig. 2) showed an expression pattern qualitatively similar to that found for expression of NbLa genes in Synechococcus 7942 (Collier & Grossman, 1994), Synechocystis 6803 (Baier et al., 2001; Richaud et al., 2001; Li & Sherman, 2002) and Tolypothrix PCC 7601 (Luque et al., 2003). Maximally increased transcript levels of NbLA were detected 1 h after nitrogen deprivation in the two Anabaena strains investigated (Fig. 2), corroborating that expression of NbLA is one of the early responses to nitrogen starvation also in heterocyst-forming cyanobacteria. However, the increase in NbLA transcript levels in the Anabaena strains was only about fivefold whereas in the unicellular, non-diazotrophic strains Synechococcus 7942 and Synechocystis 6803, NbLA transcript levels increase up to 50-fold upon nitrogen step-down (Collier & Grossman, 1994; Baier et al., 2001; Richaud et al., 2001; Li & Sherman, 2002). A similar high increase of NbLA transcript accumulation was reported for Tolypothrix sp. PCC 7601, a filamentous cyanobacterium that is unable to fix N₂ (Luque et al., 2003). These obvious quantitative differences in NbLA expression by non-diazotrophic cyanobacteria and by the N₂-fixing Anabaena strains are in agreement with a lower extent of overall PBS degradation in Anabaena 7120 (this work) and Anabaena 29413 (Maldener et al., 1991).

Inactivation of the chromosomal NbLA gene of Anabaena 7120 reduced the extent of degradation of phycobiliproteins during nitrogen starvation (Fig. 3 and Table 1). In contrast to unicellular, non-diazotrophic cyanobacteria which usually degrade their phycobiliproteins nearly completely within 1–2 days of nitrogen deprivation, the PBS in vegetative cells of filamentous, diazotrophic species are only transiently and, in most cases, only partly degraded after step-down (see Grossman et al., 1994 for an overview). The pigments are resynthesized when heterocysts differentiate and nitrogenase becomes active. PBS degradation proceeds...
to near completion only in the developing heterocysts. Hence, the consequences of the \textit{nblA} mutation in \textit{Anabaena} are most obvious in this cell type (Figs 4 and 5). This is confirmed by the immunolabelling experiments of de Alda \textit{et al.} (2004) which showed that NblA is preferentially located in the heterocysts of \textit{Tolypothrix} sp. PCC 7601/1 (\textit{Tolypothrix} sp. PCC 7601/1 is a spontaneous revertant of \textit{Tolypothrix} sp. PCC 7601 that regained the ability to form functional heterocysts). The molecular mechanism which allows these ammonia-generating cells to maintain an elevated level of NblA and, consequently, a lower level of PBS is unknown.

The inability of mutant \textit{ΔnblA} to degrade PBS had no gross effects on heterocyst differentiation and function. Mutant \textit{ΔnblA} formed heterocysts with a frequency and nitrogenase activity (Fig. 6) like those of the wild-type. Thus, \textit{nblA} is certainly not involved in heterocyst differentiation. It is known that heterocysts of \textit{Anabaena azollae} and of \textit{Nostoc} species retain (or regain) phycobiliproteins when these cyanobacteria live in symbiosis with their plant partners (Braun-Howland & Nierzwicki-Bauer, 1990; Kaplan \textit{et al.}, 1986). Since, for example, the heterocyst frequency of \textit{Anabaena azollae} as a cyanobiont is about threefold higher than in the free-living state, the symbiosis is certainly a special situation (see Meeks & Elhai, 2002, for detailed information). Also detectable in the heterocysts of symbiotic \textit{Anabaena azollae} was the photosystem II core subunit D1 (Braun-Howland & Nierzwicki-Bauer, 1990) which is essential for activity of the photosystem. Of the four photosystem II proteins we have analysed (Fig. 7), the D1 subunit was the only one that was specifically absent from heterocysts, both in the wild-type and the \textit{ΔnblA} mutant of \textit{Anabaena} 7120. Hence, PBS degradation is not mandatory for turning down photosystem II activity within heterocysts, as has been suggested (Wolk, 1996). Our studies do not provide clear information on the location of phycobiliproteins in heterocysts but the distribution of phycocyanin (Fig. 5) and allophycocyanin (unpublished results) within the cells suggests binding to thylakoid membranes. Although heterocysts appear to lack a functional photosystem II, their phycobiliproteins may nevertheless be organized in PBS and function in light harvesting. Evidence in support of this assumption is provided by previous studies which could demonstrate efficient excitation energy transfer from phyco- biliproteins to photosystem I in isolated heterocysts from wild-type \textit{Anabaena variabilis} (Peterson \textit{et al.}, 1981b) and in a \textit{Synechocystis} mutant lacking photosystem II ( Mullineaux, 1992). In the cyanobacterium \textit{Spirulina platensis}, PBS transfer most of the absorbed light energy to photosystem I rather than to photosystem II (Rakhimberdieva \textit{et al.}, 2001).

Besides their function in light harvesting, the phycobiliproteins and, because of its high abundance and rapid degradation, in particular phycocyanin, represent an intracellular store of nitrogen (Allen & Smith, 1969; Boussiba & Richmond, 1980, Yamanaka & Glazer, 1980; Grossman \textit{et al.}, 1994). During nitrogen starvation, degradation of PBS is thought to provide amino acids for the protein synthesis required for the acclimation process. However, under the conditions tested in this study mutant \textit{ΔnblA} switched to nitrogen fixation like the wild-type although only a rather small loss of phycobiliproteins was observed in this mutant following nitrogen step-down (Fig. 3 and Table 1). Such an NblA-independent degradation of PBS was also described for \textit{ΔnblA} mutants of \textit{Synechococcus} 7942 (Collier & Grossman, 1994) and \textit{Synechocystis} 6803 (Li & Sherman, 2002). Besides phycobiliproteins, various other proteins (Fleming & Haselkorn, 1974) as well as cyanophycin, a non-ribosomally synthesized polypeptide consisting of arginine and aspartic acid (Simon, 1971), may be used as nitrogen reserves in \textit{Anabaena}. Similarly, inactivation of \textit{nblA} in \textit{Synechococcus} 7942 caused no dramatic effect on cell viability during nutrient limitation (Collier & Grossman, 1994) and \textit{ΔnblA} mutants of \textit{Synechocystis} 6803 entered the non-dividing, dormant state like the wild-type, although somewhat faster (Li & Sherman, 2002). However, degradation of PBS, albeit not essential for adaptation to nitrogen deficiency, may be of advantage for cyanobacteria in their natural environments where various environmental factors, like light intensity, temperature and nutrient availability, may change frequently.

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