Immunolocalization of NblA, a protein involved in phycobilisome turnover, during heterocyst differentiation in cyanobacteria

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In unicellular non-diazotrophic cyanobacteria, NblA is a small polypeptide required for phycobilisome degradation during macronutrient limitation. In the filamentous N2-fixing *Tolypothrix* sp., a *nblA* gene (*nblA*) lies upstream of the *cpeBA* operon that encodes phycoerythrin apoproteins. Using a specific anti-NblA antibody it was found that in strains of *Tolypothrix* sp. NblA abundance increases under nitrogen-limiting conditions but the protein is also present in cells grown in nitrogen-replete medium. Gold immunolabelling experiments showed that, upon a nitrogen shift-down, NblA is preferentially located in the differentiated heterocysts, where O2 evolution has to be shut off for nitrogenase to operate. The results lead to the proposal that NblA is a necessary ‘cofactor’ but not the triggering factor that governs phycobilisome degradation in *Tolypothrix* sp.

INTRODUCTION

Most cyanobacteria collect light energy through supramolecular complexes attached to the surface of the thylakoid membranes, the phycobilisomes. They are composed of water-soluble pigmented phycobiliproteins (allophycocyanin, phycocyanin and sometimes phycoerythrin or phycoerythrocyanin) involved in light absorption, and non-pigmented linker polypeptides that are essential for the stability and assembly of the complex (Glazer, 1989). For optimization of growth, photosynthetic organisms must continuously and precisely tune light-harvesting and metabolic capacities; both the number and composition of the phycobilisomes are sensitive to environmental changes, in particular to light and nutrient availability (Grossman et al., 2001; Tandeau de Marsac & Houmard, 1993; Sauer et al., 2001).

NblA (nbl stands for non-bleaching) is a polypeptide of *M* ~ 7000 required for phycobilisome degradation in non-diazotrophic cyanobacteria (Baier et al., 2001; Collier & Grossman, 1994). In such cyanobacteria, phycobilisome degradation under nitrogen-limited conditions can provide an intracellular source of nitrogen, and reduce both photodamage and oxygen evolution (Collier et al., 1994; Ochoa de Alda et al., 1996; Sauer et al., 2001). In *Synechococcus* sp. strain PCC 7942 and *Synechocystis* sp. strain PCC 6803, high levels of *nblA* transcripts (Grossman et al., 2001; Luque et al., 2001; Richaud et al., 2001), as well as proteins (Baier et al., 2001) accumulate under macronutrient limitation. For filamentous diazotrophic cyanobacteria belonging to sections IV and V (Rippka & Herdman, 1992), the lack of a combined nitrogen source leads to differentiation of heterocysts (cells specialized for aerobic N2 fixation). Phycobiliprotein content within the heterocysts seems to vary with strain ecophysiology (Meeks & Ehlaï, 2002). In the heterocysts prepared from *Azolla* symbioses with either *Anabaena pinnata* or *Anabaena carolíniâ*, total phycobiliprotein complement does not seem to differ from that of the vegetative cells (Kaplan et al., 1986). In contrast, for free-living heterocyst-forming species phycobiliprotein degradation has been reported (Wood & Haselkorn, 1980). The rationale for such a degradation was that it participates in the turning down of photosystem II activity and O2 production within the heterocysts (Wolk, 1996). The much lower photosynthetic activity observed *in situ* for the cyanobacterial partner within the symbioses could be considered as an alternative strategy that has been selected by symbiotic species.

The filamentous cyanobacterium *Tolypothrix* sp. PCC 7601 has been widely used as a model strain, in particular because it can modulate its phycobiliprotein content according to the available light wavelengths, a phenomenon known as complementary chromatic adaptation (Grossman et al., 2001; Tandeau de Marsac & Houmard, 1993). Although this free-living strain is unable to form functional heterocysts, a spontaneous revertant of it, strain PCC 7601/1, can form fully differentiated heterocysts and fix N2 aerobically (Rippka & Herdman, 1992). We thus chose to use these...
strains to study the function of NblA in free-living filamentous heterocystous cyanobacteria. Complete genomic sequences revealed that in some strains there may exist up to four nblA gene copies. Based on sequence similarities, the *Tolypothrix* PCC 7601 nblA gene that we have studied was designated nblAI (Luque et al., 2003), and this name will be used throughout. Anti-NblAI antibodies were raised and used to probe extracts from cells grown under nitrogen-depleted and nitrogen-replete conditions. From the data obtained it is concluded that NblAI has a specific role in heterocyst physiology.

**METHODS**

**Culture and growth conditions.** *Tolypothrix* sp. PCC 7601 (also known as *Calothrix* sp. and *Fremyella diplosiphon* UTEX 481) and SF33 (a derivative of the latter; Cobley et al., 1993), are unable to form functional heterocysts. They were grown axenically in BG-11 liquid medium (nitrate-containing; Rippka, 1988) supplemented with 10 mM NaHCO₃ bubbled with wet air (10 l min⁻¹) enriched to 1 % (v/v) CO₂ and illuminated by cool-white fluorescent light (25 μmol m⁻² s⁻¹). *Tolypothrix* sp. PCC 7601/1 is a spontaneous revertant of PCC 7601 that forms heterocysts and fixes nitrogen aerobically (Rippka & Herdman, 1992). When it was grown in BG-11o (BG-11 lacking combined nitrogen) air agitation and NaHCO₃ were omitted. Cultures were agitated by shaking at 30 r.p.m. because air bubbling tends to break the filaments and, as a consequence, bleaching occurs in BG-11o for the cells no longer connected to the terminal heterocysts.

To achieve nitrogen starvation, exponentially growing cells (OD₇₅₀ 0.75) were harvested by centrifugation (5000 g, 10 min at room temperature), washed in an equal volume of BG-11 medium, recentrifuged and resuspended in BG-11 at the original cell concentration. Non-N₂-fixing cultures bleached within 1 day, as a result of a 50 % loss of phycocyanin, phycocyanin and allophycocyanin. In contrast, chlorophyll a and protein contents of the cultures remained constant during this time period (Guétat & Stanier, 1979).

Cyanobacterial cell mass was estimated by measuring the chlorophyll a concentration of the cultures. Chlorophyll a was determined in 90 % aceton extracts (Porra, 1991). Whole-cell spectra were recorded from 350 to 800 nm with an Amino DW-2 spectrophotometer.

**Production and purification of His₆-tagged NblAI.** The nblAI gene was PCR amplified as a 295 bp fragment using the following primers: forward [5'-CCAGGGCTCAGTGAATCCCAATTG-3'] and reverse [5'-TGGTAAGAGAAGAATGGCTGTCGACGTC-3'] (Ncol and BamHI sites underlined). After restriction by Ncol and BamHI, it was cloned into Ncol/BamHII-restricted pProEx-Hbt plasmid (Gibco-BRL). The ligation mixture was used to transform Escherichia coli DH5α cells, and selection was achieved on ampicillin plates. One transformant E. coli DH5α(pProNblAI) was selected for further use, and DNA sequencing confirmed the identity of the insert to the genomic sequence.

E. coli DH5α(pProNblAI) cells were grown at 37 °C to OD₅₉₅ 0.5 and induced for 5 h to produce His₆-NblAI, by adding 0.6 mM IPTG. After centrifugation, cell pellets were resuspended (1/10 initial volume) in 50 mM Tris/HCl pH 7.8, 1 mM DTT and 1 mM Pefabloc. The cell suspension was sonicated four times for 25 s, and cell debris removed by centrifugation at 17 000 g for 20 min. The supernatant was loaded on Ni-columns according to the manufacturer’s instructions, and the recombinant protein eluted with 250 mM imidazole in 50 mM sodium phosphate buffer pH 8 containing 300 mM NaCl. His₆-tagged NblAI was treated with the TEV protease to remove the His-tag. Proteins were analysed by LiDS-PAGE.

**Cell fractionation and PAGE.** Samples (1 ml) of cells from the late exponential phase (OD₅₉₅ 0.75), and from 1 day-bleached batch cultures exhibiting the same chlorophyll a concentration, were centrifuged at 2500 g for 15 min. Pellets were washed twice with buffer (30 mM Tris/HCl pH 8.3, 0.1 mM Pefabloc, 1 mM EDTA), and stored at −20 °C until use. For electrophoresis, pellets were resuspended in 150 μl loading buffer (30 mM Tris/HCl pH 6.8, 8 M urea, 10 %, v/v, glycerol, 5 % SDS, 5 % β-mercaptoethanol and 0.005 % bromophenol blue). Samples were boiled for 5 min and centrifuged at 15 000 g for 5 min prior to loading. Proteins (10 μl samples containing the same amount of chlorophyll) were separated using Tris-Tricine gels (Schagger & von Jagow, 1987) with a resolving gel (16-5 % T, 2-6 % C), a spacer gel (10 % T, 2-6 % C) and a stacking gel (8 % T, 2-6 % C). Electrophoresis was run at 30 V for 1 h, and then at 100 V. LiDS-PAGE was performed using the same Tris-Tricine buffer system at constant voltage (70 V) at 4 °C for 16 h.

**Polyclonal antibodies.** A 14 amino acid synthetic peptide corresponding to a less-conserved region of the *Tolypothrix* PCC 7601 NblAI, NH₂-EEQKNNQEQSQKC-COOH (a Cys residue was added at the carboxy-terminal end; Fig. 1), was produced by Syntem (Nîmes, France). The peptide was purified by HPLC and conjugated to maleimide-activated key-hem lipem haemocyanin via covalent linkage to the carboxy-terminal cysteine. Two rabbits were immunized with this conjugate, using a combination of Freund’s complete and incomplete adjuvants. One of these antisera (P4236) was used for the present studies, and IgGs from this serum were purified using the Avichrom kit (Sigma).

**Immunodetection.** For immunoblottings, proteins were transferred to PVDF membranes, using a semi-dry transfer system (Laurel, 1993). Immunoblot sensitivity was greatly enhanced by autoclaving wet PVDF filters after the protein transfer as described by Swedlow et al. (1986). Immunodetection was as follows. After blocking with 5 % non-fat dry milk proteins, in TTBS (20 mM Tris pH 7.5, 500 mM NaCl) containing 0.05 % Tween 20, for 30 min at room temperature, the membranes were incubated overnight at 4 °C with anti-NblAI purified IgGs as primary antibody (1/750 dilution in TTBS); then a goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad, 1/6000 dilution in TTBS) was used for 2 h at room temperature. Immunoreactive bands were detected using an ECL Western blotting detection kit (Amersham Biosciences). To ascertain the specificity of the antiserum for NblAI, the solution containing the purified IgGs (1/750 dilution in TTBS) was first incubated for 24 h at 4 °C with two PVDF membranes (5 x 10 cm) coated with E. coli DH5α total soluble proteins (2.5 mg). The supernatant corresponding to immunopurified IgGs was then incubated with the blot obtained after electrophoresis of the proteins to be analysed.

**Electron microscopy and immunogold labelling.** *Tolypothrix* sp. cells were centrifuged at room temperature and fixed at 4 °C for 60 min in glutaraldehyde (2 %, v/v) in 0-1 M sodium phosphate buffer pH 7-4 containing 0-4 M sucrose, and embedded in LR White medium-grade resin after dehydration. Immunolabelling reactions were performed as previously described (Lichtlé et al., 1992), using anti-NblAI antibodies at a 1/1000 dilution for 2 h, and gold-labelled goat anti-rabbit antibodies (10 and 15 nm gold particles, Bio Cell Gold Conjugates) at a 1/30 dilution for 60 min. Thin sections were examined with a JEOL CX2 electron microscope. Quantifications were performed on thin sections labelled with the 10 nm gold particles. For each strain and each culture conditions, two grids were independently labelled with 10 nm gold particles. At least 14 sections were analysed for each labelling condition and each
Vegetative cells, hormogonia and heterocysts, as well as a similar surface devoid of cells that represented background, were cut out from the pictures, and the corresponding surfaces weighed. For each sample, the number of gold particles was counted, and arbitrary units defined as the number of gold particles per g paper. Data were analysed using the SPSS comprehensive statistical software.

RESULTS AND DISCUSSION

Characterization of the *Tolypothrix* PCC 7601 *nblAI* gene

The *cpeBAYZ* cluster, encoding the phycoerythrin β and α subunits (CpeB and CpeA; Mazel *et al*., 1986) and the putative chromophore lyases (CpeY and CpeZ; Kahn *et al*., 1997), was found in the central part of an ~7 kb *EcoRI* fragment. By sequencing the upstream region, a *nblA* homologue (addition to accession number X04592) was found separated by 464 bp from the *cpeB* ATG translation initiation codon (Fig. 1A). A similar gene clustering is found in the genome of red algae and *Nostoc* sp. strains, which are also filamentous N₂-fixing cyanobacteria (http://www.jgi.doe.gov; unpublished results). Heterologous hybridizations performed under low-stringency conditions (30 % mismatching tolerated), and using the *Tolypothrix* PCC 7601 cloned *nblA* gene as a probe, allowed detection of a putative homologue in *Nostoc* MAC sp. PCC 8009, *Anabaena* sp. PCC 7120 and *Microcoleus* sp. PCC 7113 were restricted by *EcoRI*, and subjected to agarose gel electrophoresis before transfer to a nylon membrane. Hybridization was performed at 55°C. (C) Comparison of *NblA* sequences: *Tolypothrix* sp. PCC 7601 (*NblAI*-PCC7601, this work), *Nostoc punctiforme* PC-73102 (DOE Joint Genome Institute, http://www.jgi.doe.gov), *Synechocystis* PCC 6803 (http://www.kazusa.or.jp/cyano/) and *Synechococcus elongatus* PCC 7942 (NblAI-PCC7942, Collier & Grossman, 1994). The number of residues of each *NblA* protein is shown to the right of the alignment. Invariant (*) and conserved (;) amino acids residues are indicated below the alignment. The underlined sequence corresponds to that used to raise the anti-*NblAI* antibodies.
the *Tolypothrix* PCC 7601 polypeptide [60 % identity (89 % similarity)] is a member of the *Nostoc punctiforme* (PCC 73102) *nblA* multigene family (genome sequence available at http://www.jgi.doe.gov). Because of its high similarity with the *Nostoc punctiforme* *NblAI*, the *Tolypothrix* PCC 7601 gene product will hereafter be designated NblAI. It is only 35 % identical to the NblA of the unicellular *Synechococcus* PCC 7942 (Fig. 1C). The rather poor strict sequence conservation of the NblA polypeptides well agrees with the result of the hybridization experiment.

Expression of NblAI

To raise antibodies against NblAI, we chose a poorly conserved region of the protein so as to avoid, assuming that a *nblA* gene family may exist, possible cross-reactions with other NblA-like polypeptides. A synthetic peptide corresponding to the least-conserved region in the NblA protein family (Fig. 1B) was used to raise antibodies. BLAST searches for short nearly exact matches showed no significant overlap of the immunizing peptide with any known protein of the non-redundant database (release April 2003). The antibodies recognized the synthetic peptide, either free or conjugated to the key-hole limpet haemocyanin carrier used during the immunization process.

In parallel, the *E. coli* DH5α(pProNblAI) strain was engineered so as to overexpress a recombinant His<sub>6</sub>-NblAI protein. After induction with IPTG, the His<sub>6</sub>-tagged NblAI produced in *E. coli* can easily be visualized after Coomassie blue staining, both in crude extract (Fig. 2A, lane 1) and after purification by affinity chromatography on Ni-columns (Fig. 2A, lane 3). After proteolysis with the rTEV protease and passage through a Ni-column, untagged NblAI was recovered in the filtrate (Fig. 2A, lane 4). Immunodetection experiments were performed using anti-NblAI IgGs previously immunopurified by incubation with *E. coli* total proteins. The recombinant NblAI polypeptide, as well as its His-tag-less derivative (Fig. 2B, lanes 3 and 4) were the only polypeptides to be recognized by the antibodies.

The *Tolypothrix* sp. strains were analysed for their NblAI content, using the specific antibodies, after growth in nitrate-containing medium and following a shift to nitrogen-free medium. *Tolypothrix* sp. PCC 7601 and its SF33 mutant derivative that makes shorter filaments (Cobley *et al*., 1993) bleach if deprived of exogenous combined nitrogen source. In contrast, strain PCC 7601/1 is able to differentiate fully functional heterocysts, and thus grows under aerobic N<sub>2</sub>-fixing conditions. Antibodies recognized the same protein in all of the above-mentioned *Tolypothrix* sp. strains (Fig. 3A). Under the same conditions, no bands could be detected with the preimmune serum. The protein detected by the antibodies has an apparent molecular mass of ~15 kDa, i.e. about twice that expected for the NblAI polypeptide. In a recent study we have shown that this 15 kDa form – a dimer or a complex formed by NblAI and another small molecule – is always present, and that the ~7 kDa form does exist but it is only found at such a molecular mass when associated with phycobiliproteins. Such a situation only occurs when cells are grown under red light, conditions under which both the 15 and 7 kDa proteins coexist (Luque *et al*., 2003). The NblAI increase observed in the three *Tolypothrix* sp. strains (Fig. 3A) during nitrogen starvation confirmed the increase in NblAI content recently described for *Tolypothrix* sp. PCC 7601 (Luque *et al*., 2003). The increase in NblAI content is by a factor of 2·9 for *Tolypothrix* PCC 7601/1 and 4 for *Tolypothrix* PCC 7601. A more pronounced difference could have been expected since for *Tolypothrix* PCC 7601/1 there is only one terminal heterocyst per filament. However, it was not possible to grow the cells under the same conditions because with *Tolypothrix* PCC 7601/1 the filaments break if the cultures are bubbled. This breakage leads to bleaching for the cells detached from the heterocysts and to NblAI accumulation in each of these cells. Such a breakage was much less pronounced but probably not completely prevented by the gentle shaking we applied during growth.

In an attempt to quantify the amount of NblAI present in the cells, *Tolypothrix* sp. PCC 7601 total cell proteins were run in parallel with decreasing concentrations of His<sub>6</sub>-tagged NblAI protein (Fig. 3B). Quantification showed that

**Fig. 2.** LiDS-PAGE analysis of total proteins from *E. coli* cells carrying the His<sub>6</sub>-NblAI-producing recombinant plasmid after IPTG induction (lane 1) or before (lane 2), fraction eluted from the Ni-affinity column (lane 3), and filtrate from a second Ni-affinity column loaded with the rTEV-treated eluate of the first column (lane 4). Arrowheads on the right indicate the position of His<sub>6</sub>-NblAI and NblAI proteins. (A) Coomassie blue staining (~50 µg total proteins, 6 µg His<sub>6</sub>-NblAI and 1 µg NblAI). (B) Immunodetection with anti-NblAI, after transfer to PVDF membranes (~18 µg total proteins, 0·3 µg His<sub>6</sub>-NblAI and 0·3 µg NblAI).
there was about 250 ng of NblAI for ~40 μg of phycobiliproteins. After correction for the molecular masses (~5-9 MDa for a phycobilisome and ~15 kDa for dimeric NblAI), the cell would contain about 2 NblAI protein molecules per phycobilisome.

**Immunocytological detection of NblAI**

*Tolypothrix* sp. PCC 7601/1 grows as long filaments of vegetative cells in a nitrate-containing medium, but under N$_2$-fixing conditions it develops terminal heterocysts and terminal hormogonia spaced by about 200 vegetative cells. For each culture condition, two grids were independently labelled with 10 nm gold particles, and analysed. The results of the statistical analysis are shown in Table 1. The number of gold particles present in the *Tolypothrix* sp. PCC 7601/1 heterocysts is about six times that in vegetative cells, and this enhancement is specific to the heterocyst since we did not detect any significant NblAI accumulation within hormogonial cells (Table 1).

Upon nitrogen starvation *Tolypothrix* sp. PCC 7601 bleaches and cannot differentiate functional heterocysts nor fix N$_2$. However, under these conditions it can reduce acetylene if anaerobic conditions are provided, indicating that the nitrogenase itself is functional (Rippka & Herdman, 1992). In nitrogen-starved cells, an increase in the NblAI content was observed, in agreement with the protein blot (Fig. 3), and all the cells from a filament exhibited an even labelling. The NblAI content of *Tolypothrix* sp. PCC 7601 nitrogen-starved cells is similar to that found in *Tolypothrix* sp. PCC 7601/1 heterocysts (Table 1). On the electron micrographs shown in Fig. 4, it can be seen that the thylakoids of the nitrogen-starved cells (panel 2) are clearly disorganized compared to those of the vegetative cells (panel 1).

To produce pictures with more visible labelling, the immunodetection experiment was repeated with *Tolypothrix* sp. PCC 7601/1, but using 15 nm gold particles. Electron micrographs are shown in Fig. 4 (panels 3–7). In the vegetative cells (panels 3 and 7), the thylakoids exhibit a well-defined peripheral organization. In contrast, in the heterocysts, only shorter and less structured pieces of photosynthetic}

![Fig. 3.](image-url) **Table 1.** Quantification of the immunogold particles in ultrathin sections of *Tolypothrix* sp. PCC 7601 and PCC 7601/1 cells

Data correspond to measurements performed on two independent cultures for each of: (i) *Tolypothrix* sp. PCC 7601 grown with nitrate or after 1 day of nitrogen starvation; and (ii) *Tolypothrix* sp. PCC 7601/1 grown under N$_2$-fixing conditions. For each culture, two grids were independently labelled with 10 nm gold particles and, for each labelling condition and each sample, more than 14 sections were analysed.

<table>
<thead>
<tr>
<th><em>Tolypothrix</em> sp. strain</th>
<th>Cell type</th>
<th>Growth conditions</th>
<th>Labelling*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCC 7601/1</td>
<td>Vegetative</td>
<td>N-starved</td>
<td>12 ± 4</td>
</tr>
<tr>
<td></td>
<td>Heterocysts</td>
<td>N-starved</td>
<td>79 ± 13</td>
</tr>
<tr>
<td></td>
<td>Hormogonia</td>
<td>N-starved</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>PCC 7601</td>
<td>Vegetative</td>
<td>+ Nitrate</td>
<td>32 ± 8</td>
</tr>
<tr>
<td></td>
<td>Vegetative</td>
<td>N-starved</td>
<td>76 ± 10</td>
</tr>
</tbody>
</table>

*Number of gold particles counted within a given cell type after subtraction of the background (particles counted in equivalent surfaces free of cells). Results are shown as means ± SEM.
membrane spread all over the cytoplasm can be observed and inclusion bodies have disappeared (panels 4 and 6). The enhancement in the number of gold particles present in the Tolypothrix sp. PCC 7601/1 heterocysts as compared with that of vegetative cells is clearly visible. This enhancement was again very specific for the heterocyst, the NblAI content of the hormogonial cells (Fig. 4, panel 5) being similar to that observed in the vegetative cells.

**Conclusion**

The stimuli that lead to phycobilisome degradation may vary depending on the organism (Richaud et al., 2001). At present NblA proteins have been studied mainly in unicellular non-N₂-fixing cyanobacteria, for which phycobilisome degradation appears to be a very important adaptive mechanism that improves survival of the cyanobacteria under high light conditions and during nutrient limitation (Görl et al., 1998; Schwarz & Grossman, 1998; Sauer et al., 2001; van Waasbergen et al., 2002). For the heterocyst-forming strains, nitrogen starvation leads to the switching on of the N₂-fixation process and the shut-off of O₂ evolution which is necessary for the functioning of the nitrogenase. Our results show that, although nblAI expression is indeed enhanced by nitrogen starvation, NblAI is also present in cells grown under nitrogen-replete conditions. These data are in agreement with the hypothesis that NblAI is a necessary ‘cofactor’ recruited for phycobilisome degradation but not the triggering factor, unless a threshold concentration would be necessary (Luque et al., 2001). In filamentous heterocystous cyanobacteria, differential expression of nblAI could thus be part of the developmental cascade of events that accompanies heterocyst differentiation.

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