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NblC, a novel component required for pigment degradation during starvation in *Synechococcus* PCC 7942

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Summary

Adjustment of photosynthetic light harvesting to ambient conditions is essential to allow efficient energy capturing and to prevent surplus excitation and the cellular damage resulting from it. Degradation of the cyanobacterial light harvesting complex, the phycobilisome, is a general acclimation response occurring under various stress conditions. This study identifies a novel component, NblC, which mediates phycobilisome degradation under nitrogen, sulphur and phosphorus starvation. Our study indicates the requirement of NblC for efficient expression of *nblA*, an essential component of the degradation pathway; accumulation of *nblA* transcripts upon nutrient starvation was impaired in the NblC-mutant. Furthermore, expression of NblC under the control of a foreign promoter resulted in accumulation of *nbl*A transcripts and degradation of the light harvesting complex. Transcription of *nblC* is induced upon nutrient starvation, suggesting the requirement of elevated levels of NblC under these conditions. Importantly, NblC could not exert its positive effect on *nbl*A expression in the absence of the response regulator NblR. Sequence alignment suggests kinase motifs as well as homology of NblC to anti-sigma factors. Accordingly, we suggest a mode of action for this newly identified modulator, which provides new insights into regulation of gene expression in response to environmental stimuli.

Introduction

All photosynthetic organisms possess accessory pigment complexes that allow for efficient absorption of the light energy required for the synthesis of ATP and reducing power. Excess absorbed light, however, may damage the photosynthetic apparatus as well as other cellular components (Foyer *et al*., 1994; Asada, 1999; Niyogi, 1999). Therefore, it is critical that light harvesting machinery be adjusted to the metabolic capacity of the cells. Obviously, surplus excitation may occur under high photon flux, though cells may also experience over excitation when light intensity is relatively low, for example under conditions that slow down anabolism (low temperature and nutrient limitation). Hence, photosynthetic organisms must tune their light harvesting capacity to a large variety of ambient conditions.

The major light harvesting antennae in cyanobacteria and red algae, the phycobilisome, is a cytoplasmic macromolecular complex that is anchored to the photosynthetic membranes (Glazer, 1985; Grossman *et al*., 1993; MacColl, 1998). This complex is composed of various polypeptide subunits, which bind open tetrapyrol as chromophores, as well as non-pigmented polypeptides. The phycobilisome is a highly abundant complex and under low light intensity may comprise up to 50% of soluble cellular protein. Certain environmental stimuli, for example nitrogen starvation, trigger complete and rapid degradation of this ultra-structure (Allen and Smith, 1969; Collier and Grossman, 1992). Thus, the cells possess a highly potent and tightly regulated degradation machinery, which upon activation results in the rapid turnover of the components of the light harvesting complex.

A genetic approach was previously used to evaluate the mechanisms underlying pigment degradation in *Synechococcus* sp. PCC 7942 (also termed *Synechococcus elongatus*, hereafter *Synechococcus*). Mutants were isolated, which unlike the wild-type strain, do not degrade their light harvesting pigments under nutrient starvation (Collier and Grossman, 1994; Schwarz and Grossman, 1998; Dolganov and Grossman, 1999; van Waasbergen *et al*., 2002). This phenotype was termed non-bleaching (nbl), since upon starvation; the mutants appear blue-green rather than yellowish or bleached like the wild-type cultures. Molecular analysis of the mutants isolated so far enabled the identification of four components essential for modulation of the light harvesting antennae. NblA and NblB are thought to be specifically involved in the degradation process (Collier and Grossman, 1994; Dolganov and Grossman, 1999), whereas NblS and NblR have a regulatory

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role (Schwarz and Grossman, 1998; van Waasbergen *et al*., 2002). Based on sequence homology, the latter two components were classified as the sensor-kinase and response regulator of a two-component signal transduction pathway, which modulates transcription of *nblA* as well as that of additional genes. Transcription of *nblA* is strongly induced upon nutrient starvation (Collier and Grossman, 1994; Baier *et al*., 2001; 2004; Luque *et al*., 2003). The protein encoded by this gene does not exhibit homology to any protein of known function and its role in the degradation process is still obscure; however, all phycobilisome-containing organisms examined so far possess at least one *nblA* homologue.

To further delineate this process of phycobilisome breakdown, we recently isolated a large number of non-bleaching mutants using fluorescence-activated cell sorting (FACS) (Perelman *et al*., 2004). This study describes the analysis of one such mutant, which resulted in identification of NblC, a novel component of the degradation pathway, which exhibits homology to anti-sigma factors. The data presented demonstrate the requirement of NblC for efficient accumulation of *nbl*A transcripts upon starvation. Characterization of the NblC-mutant as well as wild type and mutant strains expressing NblC by a foreign promoter provides new insights into modulation of gene expression in response to environmental stress.

Results

Identification of nblC*, a novel gene required for phycobilisome degradation during nutrient starvation*

To elucidate the mechanism underlying phycobilisome degradation, we recently isolated mutants that exhibited aberrant modulation of their pigments during nutrient starvation. Mutagenized cell populations starved for either sulphur or nitrogen were screened by FACS, and cells maintaining relatively high fluorescence were sorted and plated (Perelman *et al*., 2004). Non-bleaching strains identified in this manner were screened by polymerase chain reaction (PCR) using primers to the known genes comprising the *nbl*-pathway (*nblA*, *nblB*, *nblR* and *nblS*). The mutants were created by random transposon insertion and therefore, an inactivated gene was expected to yield a PCR fragment of higher molecular weight than its wild-type homologue. Figure 1A shows that mutant M104 possessed unaltered copies of the previously identified genes of the *nbl*-pathway, as PCR products of identical size were produced from genomic DNA of the wild type and the mutant.

The genomic region of M104 bearing the transposon was cloned and the nucleotide sequence flanking the transposon was determined. The transposon was found to be inserted into the coding region of a gene designated

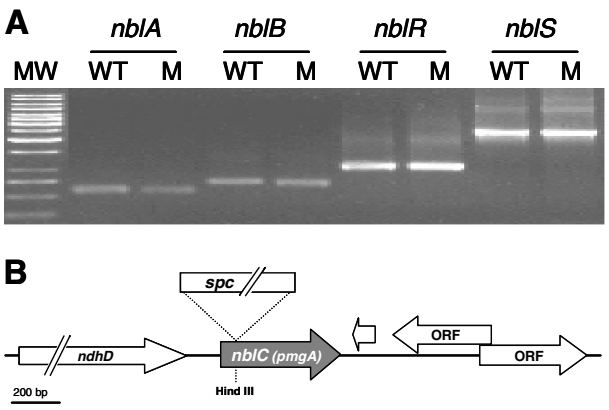

Fig. 1. Identification of novel gene of the *nbl*-pathway.
A. Analysis by PCR of the genes previously shown to be required for phycobilisome degradation (*nblA*, *nblB*, *nblR* and *nblS*), in the wild type (WT) and mutant M104 (M). MW, molecular weight markers.
B. Physical map of the genomic region near the transposon insertion point in mutant M104. The bold arrow indicates *nblC*, the gene interrupted in M104. *ndhD* – the ORF upstream of *nblC*, which exhibits homology to a subunit of NADH dehydrogenase. The ORFs shown represent hypothetical proteins. HindIII, site used for directed inactivation of *nblC*. spc, spectinomycin cassette.

nblC. Direct inactivation of *nblC* by insertion of a spectinomycin-resistance cassette (Fig. 1B) resulted in a mutant, NblCΩ, which exhibited a non-bleaching phenotype under nutrient starvation. Cultures of this mutant retained some of the blue-green colour during nitrogen and sulphur starvation (Fig. 2A), similarly to mutant M104 (not shown) and in contrast to wild-type cultures, which exhibited the yellowish colour typical of phycobilisome degradation (Fig. 2A). Inactivation of *nblC* resulted in a moderate non-bleaching phenotype as shown by comparison to the *nblR*-mutant, NblRΩ (Fig. 2A), a previously isolated mutant impaired in a response regulator essential for the degradation of phycobilisomes (Schwarz and Grossman, 1998). Absorbance spectra of the different strains (Fig. 2B) indicated various levels of phycocyanin, the major pigment of the light harvesting complex, in agreement with the bulk appearance of the starved cell cultures. Following 48 h starvation, the wild-type cells retained 10–15% of the phycocyanin level present during nutrient replete growth, NblCΩ retained 25%, whereas NblRΩ, maintained 50–60% of the pigment level of unstarved culture (Fig. 2C).

Pigment analyses of NblCΩ suggest a role for this gene in the modulation of pigment levels during starvation. It was nevertheless possible that the phenotype of the insertionally inactivated mutants resulted from an effect of the transposon or the spectinomycin-resistance cassette on adjacent genes. To examine this possibility, a DNA fragment (about 1.2 kb), bearing the coding region of *nblC* and its putative promoter region, was inserted into a neutral site within the genome of NblCΩ. The

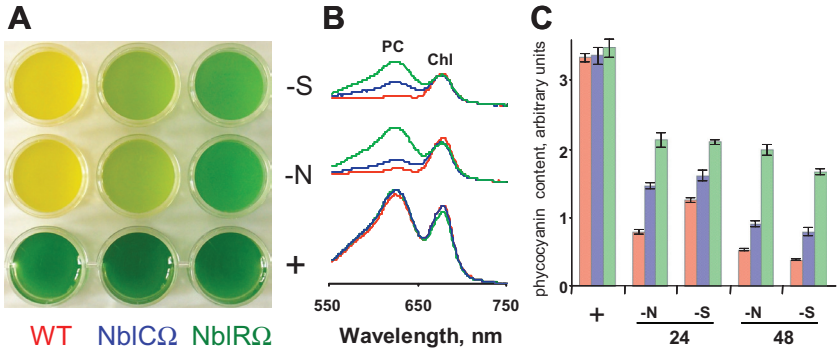

Fig. 2. Phenotype of the newly isolated non-bleaching mutant, NblCΩ. Cultures (A), and absorbance spectra (B) of wild type (WT), NblCΩ and NblRΩ grown in complete medium (+) or following nitrogen (–N) or sulphur (–S) starvation for 48 h. (C) Calculated amount of phycocyanin based on absorbance maxima at 620 nm (normalized to OD_{750}) during replete growth and following 24 and 48 h of starvation. Absorbance maxima of phycocyanin (PC), the major pigment of the phycobilisome, and chlorophyll a (Chl) are indicated. Absorbance spectra were taken for cultures diluted to OD_{750} of 0.01. Groups of spectra were shifted along the *y* axis for clarity. Colours in (B) and (C) represent the different strains as indicated in (A). $P < 0.03$ using the Mann–Whitney non-parametric test (relevant to phycocyain level during starvation in the different strains. PC levels in strains grown under nutrient sufficient conditions were not significantly different). Data shown are from a single representative experiment out of three. In each experiment, every culture was starved in two separate tubes and two spectral measurements were performed for each culture.

resulting transformant exhibited pigment degradation under nutrient starvation that was similar to the wild-type strain (not shown). These data confirm that NblC is required for phycobilisome degradation during nutrient starvation.

NblC is homologous to anti-sigma factors of RNA polymerase

To gain insight into possible functions of NblC, we used BLAST and CLUSTALW to detect homologies to known proteins (Fig. 3). NblC is homologous to bacterial anti-sigma factors, specifically to SpoIIAB, the sporulation-related anti-sigma factor of various *Bacillus* species and its homologues, as well as to RsbW (regulator of sigma B), the regulator of general acclimation responses of *B. subtilis* (Haldenwang, 1995). NblC exhibits 27% amino acid identity and 46% similarity to SpoIIAB from *B. stearothermophilus* and 26% identity and 50% similarity to RsbW from *B. subtilis*. Notably, the homology found between NblC and the *Bacillus* anti-sigma factors, SpoIIAB and RsbW, is comparable with the homology shared between the latter two regulators (30% identity and 53% similarity). Interestingly, SpoIIAB has been recently affiliated to a large group of functionally diverse proteins, the GHKL superfamily, which includes gyrases, histidine kinase sensors, heat shock proteins, and DNA repair enzymes (Campbell *et al.*, 2002). Proteins belonging to this superfamily exhibit an unconventional Bergerat ATP-binding fold (Bergerat *et al.*, 1997). Notably, NblC possesses the GHKL motifs (indicated by horizontal lines in Fig. 3). Furthermore, crystal structure analysis of SpoIIAB of *B. stearothermophilus* (Campbell *et al.*, 2002) identified the amino acid residues important for ATP binding and possibly for kinase function (Fig. 3, see legend for details). Interestingly, there is considerable amino acid similarity in the positions involved in ATP binding in SpoIIAB and comparable sites in NblC. In fact, out of the 10 amino acids implicated in ATP binding, six are identical between the two proteins and two exhibit conservative changes (Fig. 3, see legend for details). In summary, existence of the Bergerat motifs and the substantial identity to SpoIIAB in positions implicated in ATP binding, suggest that NblC may use ATPase activity to exert its function (see *Discussion*).

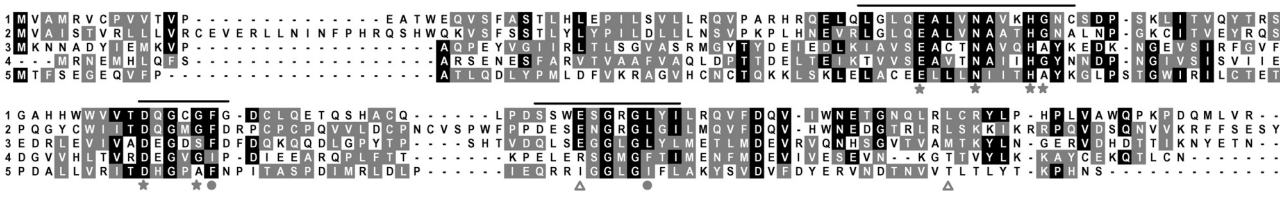

Fig. 3. Sequence homology between NblC and anti-sigma factors. Amino acid alignment of NblC of *Synechococcus elongatus* (1), PmgA of *Synechocystis* PCC 6803 (2), RsbW of *Bacillus subtilis* (3), SpoIIAB of *Bacillus stearothermophilus* (4) and SpoIIAB-like protein from *Chlamydia trachomatis* L2 (5). Horizontal lines indicate Bergerat ATP binding motifs. Amino acids assigned to ATP binding in SpoIIAB are indicated by asterisks, circles or triangles. The former two indicate identical or similar amino acids respectively, to the NblC sequence, whereas the triangles indicate non-conservative changes in the particular position.

Aside from the indicated eubacterial proteins, NblC exhibits homology (49% and 62% identity and similarity) to PmgA (photomixotrophic growth) of *Synechocystis* 6803 (Fig. 3) and its homologues from various cyanobacteria (not shown). PmgA was shown to be required for acclimation of the photosynthetic apparatus to high light intensity (Hihara and Ikeuchi, 1997; Hihara *et al*., 1998); however, the molecular basis of this process has not been elucidated.

NblC is required for modulation of expression of nblA

nblA, which is significantly induced during nutrient limitation, encodes an essential component of the phycobilisome degradation process (Collier and Grossman, 1994; Baier *et al*., 2001; Li and Sherman, 2002; Luque *et al*., 2003; Baier *et al*., 2004). Therefore, we followed expression of *nblA* in NblCΩ to evaluate whether the impaired pigment degradation exhibited by this mutant results from aberrant expression of *nblA*. Northern analysis (Fig. 4A) indicated reduced abundance of *nblA* transcripts in NblCΩ during nitrogen and sulphur starvation; quantitation of the signal indicated that NblCΩ exhibited only 50% of the level of *nblA* transcripts compared with wild type. NblRΩ is more severely impaired in *nblA* expression than NblCΩ; it exhibits 80% reduction in the level of *nblA* transcripts compared with wild type. Taken together; the Northern analysis suggests that the non-bleaching phenotype of NblCΩ originates from aberrant accumulation of *nblA* transcripts during nutrient starvation.

It was interesting to also evaluate the effect of inactivation of *nblC* on expression of *nblA* during growth in the presence of sufficient levels of nutrients. Northern analysis using a DNA probe is not sensitive enough to detect the low levels of *nblA* transcripts present under these conditions (Fig. 4A). An RNA probe (riboprobe), which provides high sensitivity, indicated two- to threefold elevated content of *nblA* transcripts in NblCΩ compared with the wild-type strain (Fig. 4B).

NblC is induced during both nitrogen and sulphur starvation

Nitrogen and sulphur starvation of wild-type cultures resulted in an increased abundance of *nblC* transcripts; up to fivefold increase was observed with nitrogen starvation resulting in faster stimulation of transcript accumulation (Fig. 5). Aside from the major induced transcripts, around 0.5 kb, faint bands around 1.2 and 2 kb appear to be induced by starvation. The latter bands suggest multicistronic transcripts originating from the genomic region around *nblC*, the nature of which is yet to be established. In conclusion, induction of *nblC* transcripts during starvation together with impaired *nblA* expression in NblCΩ suggests a role for NblC in modulation of *nblA* during nutrient deprivation.

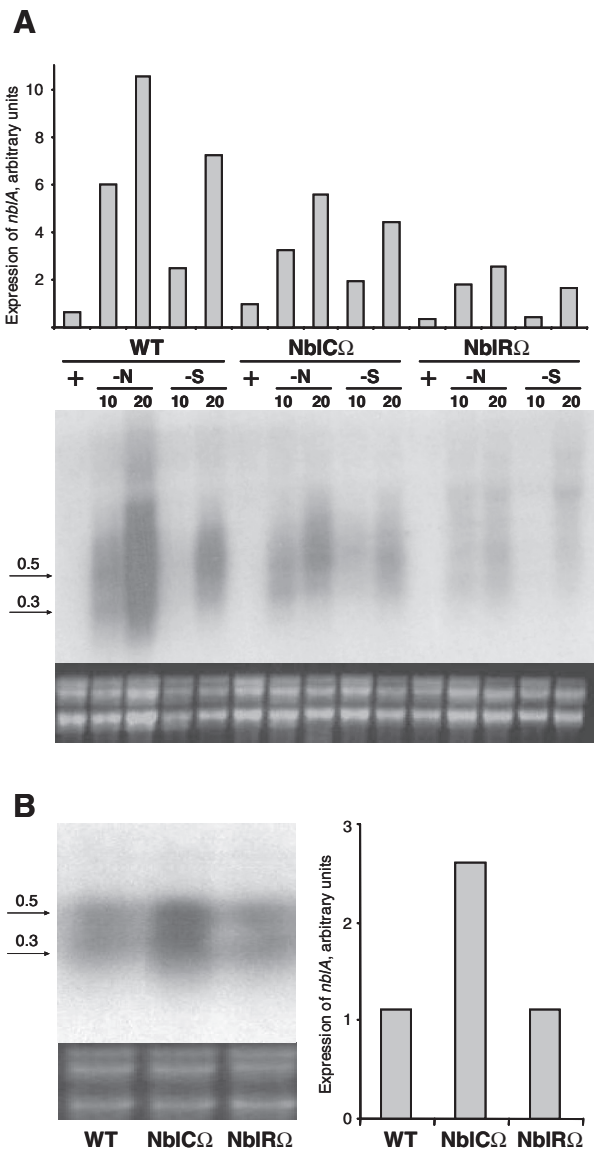

Fig. 4. NblC modulates expression of nblA.
A. Northern analysis using dsDNA probe of *nblA* transcripts in wild type (WT), NblCΩ and NblRΩ grown in complete medium (+) or following nitrogen (–N) or sulphur (–S) starvation for the indicated time (h).
B. Northern analysis of RNA from nutrient-replete cultures using a riboprobe for *nbl*A. Data shown are from a single representative experiment out of three.

NblC is not required for repression of cpcBA *during nutrient limitation*

The level of phycocyanin at any time point following the onset of starvation is determined by the relative rates of pigment degradation versus new pigment production.

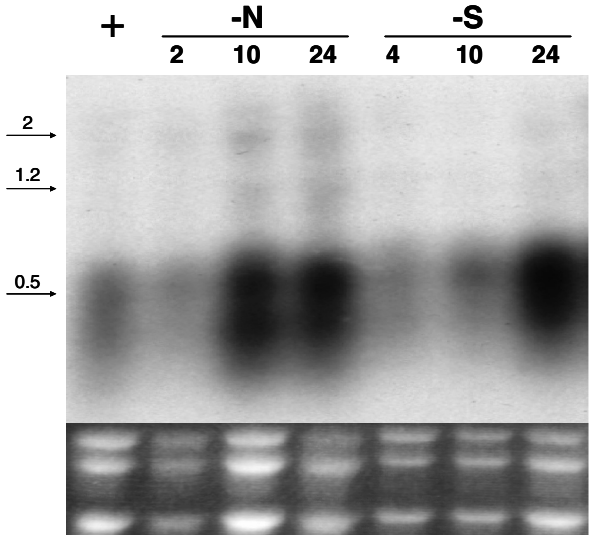

Fig. 5. NblC is induced during both nitrogen and sulphur starvation. Northern analysis of *nblC* transcripts in wild-type cells grown in complete medium (+) or following nitrogen (–N) or sulphur (–S) starvation for the indicated time (h). Data shown are from a single representative experiment out of three.

Northern analysis of *cpcBA*, the operon encoding the α and β subunits composing the apo-protein of phycocyanin, indicates normal suppression of this operon during nitrogen and sulphur starvation in NblCΩ (Fig. 6). Therefore, the higher level of phycocyanin in starved NblCΩ cells (Fig. 2) is apparently due solely to impaired degradation.

NblCΩ is not defective in induction of the specific nutrient genes, glnN *and* rhdA

Characterization of the NblC-mutant suggests a role for this gene product in the regulation of phycobilisome degradation. This process is classified as a general acclimation response as it occurs during sulphur, nitrogen, and phosphorus starvation (Collier and Grossman, 1992). To investigate possible involvement of this newly identified modulator in nutrient-specific responses, we compared induction of *rhdA*, which encodes a thiosulphate : cyanide sulphurtransferase-like protein (Laudenbach *et al.*, 1991), induced upon sulphur starvation, and *glnN*, which, encodes a type III glutamine synthetase induced upon nitrogen starvation (Sauer *et al.*, 2000), between the wild type and NblCΩ. Northern analysis indicated similar induction of these genes in the wild type and the mutant (Fig. 7). Normal induction of these specific responses in the mutant suggest that at least some of the nutrient-specific responses are not regulated by NblC. Importantly, proper induction of nutrient-specific genes in the mutant rules out the possibility that the observed lower abundance of *nblA* transcripts (Fig. 4A) reflects secondary effects of *nblC*-inactivation on the transcription apparatus.

Ectopic expression of NblC and NblR

To further investigate the role of NblC, as well as that of the previously described effector of phycobilisome degradation, NblR, we expressed these proteins under the control of the promoter of *phoA* (the gene encoding alkaline phosphatase), which is highly expressed upon phosphorous starvation (Ray *et al.*, 1991). We chose this starvation-responsive promoter because it was reasonable to assume the presence of the cellular cues required for activation of the components of the phycobilisome degradation pathway under the inducing conditions.

In the wild-type strain, *nbl*A expression is induced (fourfold) by phosphorus starvation (Fig. 8A). Ectopic expression of NblC and NblR by the alkaline phosphatase promoter resulted in further increase in *nbl*A expression

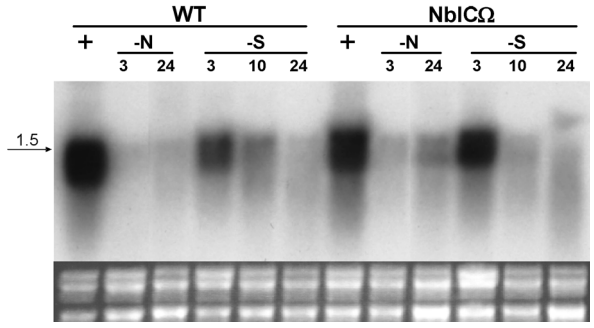

Fig. 6. NblC is not required for repression of *cpcBA* during nutrient limitation. Northern analysis of *cpcBA*, the operon encoding subunits of phycocyanin, the major pigment of the light harvesting antennae, in wild type (WT) and NblCΩ grown in complete medium (+) or following nitrogen (–N) or sulphur (–S) starvation for the indicated time (h). Data shown are from a single representative experiment out of three.

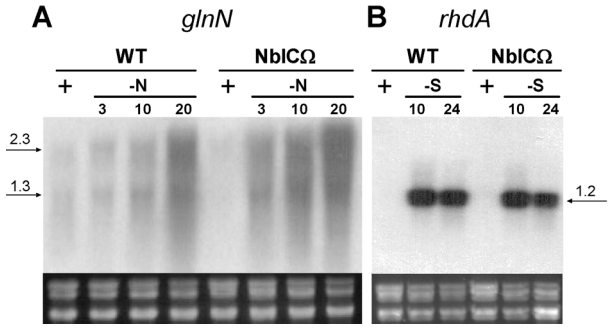

Fig. 7. Proper induction of *glnN* and *rhdA*, encoding nutrient-specific functions, in NblCΩ. Northern analysis of genes specifically induced by nitrogen and sulphur starvation (*glnN* and *rhdA* respectively) in wild type (WT) and NblCΩ grown in complete medium (+) or following nitrogen (–N) or sulphur (–S) starvation for the indicated time (h). Data shown are from a single representative experiment out of three.

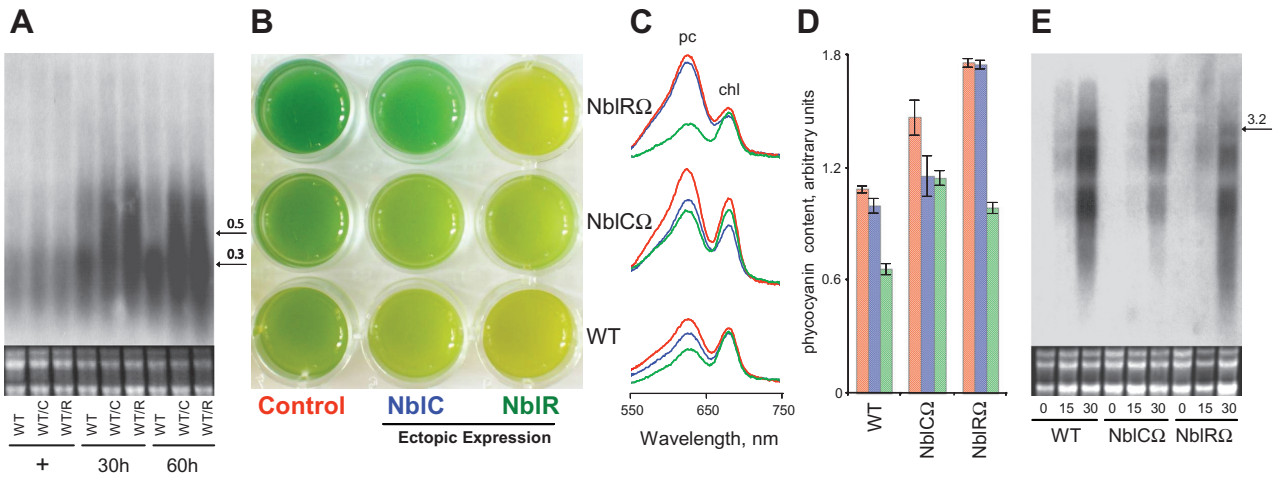

Fig. 8. Effect of ectopic expression of NblC and NblR on *nblA* expression and phycobilisome degradation. Northern analysis of *nblA* using riboprobe (A) of non-starved cultures (+) or following 30 or 60 h phosphorus starvation. Strains analysed were wild type (WT) or this strain transformed with NblC or NblR fused to *phoA*-promoter (WT/C and WT/R respectively). Cultures (B), absorbance spectra (C) and calculated amount of phycocyanin (D) following starvation for phosphorus for 11 days of wild type (WT), NblCΩ and NblRΩ, either as parental strains (control) or transformed with NblC or NblR under the control of the *phoA* promoter. Experimental details are as described in legend to Fig. 2. Colours in (C) and (D) represent the different genotypes as following: red (control) – parental strains (wild type, NblCΩ and NblRΩ), blue and green – ectopic expression of NlbC and NblR respectively, in each of the parental strains. Phycocyanin levels were significantly different ($P < 0.03$ using the Mann–Whitney non-parametric test), except for two sets of comparisons: (1) NblCΩ expressing NblC or NblR (2) NblRΩ compared with this mutant expressing NblC. (E) Northern analysis of *phoA* of non-starved cells (0) or cultures starved for phosphorus for 15 and 30 h.

(40% and 85% respectively, Fig. 8A, 60 h starvation). For as yet unknown reasons, the effect of the ectopic expression on pigmentation was observed only following longer starvation time (Fig. 8B–D).

It is established that phosphorous limitation results in partial phycobilisome degradation (Collier and Grossman, 1992). Expression of NblC by the *phoA*-promoter, however, resulted in further loss of phycobilisomes as suggested by the colour of the cultures (Fig. 8B), absorbance spectra (Fig. 8C) and phycocyanin level (Fig. 8D). NblC expression in the wild-type strain caused 10% reduction in phycocyanin level compared with the control (Fig. 8D). This relatively small effect was nevertheless statistically significant and reproducible. Additionally, production of NblR by the *phoA*-promoter supported phycobilisome degradation to a greater extent than the effect of NblC expression; 40% reduction in phycocyanin level were observed, compared with the control strain (Fig. 8D). As shown, both of the ectopically expressed modulators stimulated transcription of *nblA* (Fig. 8A) indicating that degradation of phycobilisome mediated by ectopic expression of NblC and NblR employs the *nbl*-degradation pathway, through a mechanism similar to the natural degradation occurring during sulphur and nitrogen starvation (Collier and Grossman, 1994).

To delineate the relative positions of the regulatory components of the phycobilisome degradation pathway and examine their ability to complement each other's function, we expressed NblC and NblR in the NblCΩ and NblRΩ mutants. As the *pho*A promoter was used to drive the ectopic expression, we examined the transcripts abundance of the native *pho*A in the mutants as compared with the wild-type strain (Fig. 8E). *pho*A transcripts level increased 20- to 30-fold in response to phosphate starvation of the mutants whereas wild-type cells exhibited 40-fold induction (Fig. 8E). In spite of these quantitative differences, the substantial induction of *pho*A-promoter in the mutants allowed the use of this promoter to drive the ectopic expression.

Expression of NblC or NblR in NblCΩ results in 20% reduction in phycocyanin content, compared with the control strain (NblC-mutant). Despite this considerable effect of the ectopically expressed modulators in NblCΩ, phycocyanin level is higher compared with the wild-type strain expressing NblC or NblR (Fig. 8D). While analysing these data one has to bear in mind that in wild-type cells, *nblA* is induced by phosphorus starvation (Collier and Grossman, 1994; Fig. 8A). Furthermore, inactivation of NblC and NblR impairs phycobilisome degradation during phosphorus starvation (Fig. 8B and D). Therefore, the higher level of phycocyanin in NblCΩ expressing NblC or NblR (as well as in NblRΩ expressing NblR) may be explained by aberrant induction of the native phosphorus-limitation-responsive pathway. Namely, ectopic expression in the genetic background of wild type, adds to the effect of the native pathway, whereas phycobilisome degradation in the mutants relies substantially on the ectopic expression.

Importantly, expression of NblC in NblRΩ did not enable any pigment loss (Fig. 8B–D), suggesting that NblR is crucial for *nblA* expression and elevated levels of NblC cannot compensate for its absence.

In summary, increased abundance of *nblA* transcripts consequent to expression of NblC may suggest that this modulator exerts a positive effect on transcription. NblC, however, is incapable of inducing *nblA* in the absence of NblR (see *Discussion*).

Discussion

Modulation of gene expression by NblC

This study identified a component, designated NblC, required for efficient expression of *nblA* and consequently for the degradation of the cyanobacterial light harvesting complex during nutrient limitation. Several lines of evidence clearly indicate that NblC is required for efficient upregulation of *nblA*. Firstly, NblCΩ, the mutant impaired in *nblC*, exhibits reduced level of *nblA* transcripts during nutrient starvation, compared with the wild-type strain (Fig. 4A). In addition, expression of *nblC* by a foreign promoter stimulates accumulation of *nblA* transcripts upon promoter induction (Fig. 8A). Furthermore, increased expression of *nblC* during sulphur or nitrogen starvation (Fig. 5) is consistent with the requirement for elevated levels of NblC for upregulation of *nblA* expression.

In addition to its role in modulation of *nblA* expression under nutrient limitation, NblC is required for partial repression of *nblA* expression during nutrient replete growth as suggested by Northern analysis (Fig. 4B). Despite the elevated level of *nblA* transcripts exhibited by NblCΩ under nutrient sufficiency, the mutant does not exhibit partial phycobilisome degradation under these conditions. This may suggest additional steps of regulation (e.g. activation of the components of the degradation pathway by physiological changes imposed by starvation), aside from the upregulation of *nblA* expression, as well as possible modulation of the assembly of the pigment complex.

Induction of *nblA* and the resulting pigment degradation are classified as general acclimation responses, namely those observed upon deprivation of any of a number of nutrients; therefore, we assign a role for NblC as a modulator of common responses to nutrient limitation in *Synechococcus*. Repression of *cpcBA*, the operon encoding the subunits of phycocyanin, is a general response not affected by the impairment of *nblC*. Thus, a combination of pathways serves to control the general acclimation responses in *Synechococcus* and apparently, NblC takes part in some of them.

The two nutrient-specific genes examined in this study, *glnN* and *rhdA*, were induced in NblCΩ to the same extent as in wild-type cells (Fig. 7). A more global analysis of gene expression in the wild type and NblCΩ is required, however, to evaluate whether the role of NblC is confined to modulation of general acclimation responses or it also takes part in regulation of certain specific responses.

Notably, expression of NblC and NblR by *pho*A-promoter in NblCΩ results in reduced chlorophyll levels. Possible implications of these modulators on chlorophyll levels are currently being studied.

Possible mechanism for NblC function

Amino acid alignment (Fig. 3) indicated homology between NblC and bacterial anti-sigma factors. Specifically NblC shows homology to the sporulation-related anti-sigma factor of several *Bacillus* species, SpoIIAB and its homologues, and to RsbW, an anti-sigma factor involved in modulation of general acclimation responses (Haldenwang, 1995). In addition to the overall homology, NblC is characterized by GHKL motifs typical of a diverse group of ATPases (Bergerat *et al*., 1997) and exhibits a high degree of identity to SpoIIAB residues implicated in ATP binding and hydrolysis (Fig. 3). Based on this homology it may be speculated that NblC functions as a kinase and activates an effector required for expression of *nblA* during starvation.

NtcA, a transcription factor exerting global nitrogen control, was shown to be required for efficient transcription activation of *nblA* during nitrogen starvation (Luque *et al*., 2001). Hence, one should not exclude possible interactions between NtcA and NblC, which are required for *nblA* activation, specifically during nitrogen starvation.

Having in mind the overall sequence homology of NblC to SpoIIAB and SpoIIAB-like sequences, an alternative working hypothesis, which assigns to NblC a role of an anti-sigma factor, is proposed. During replete-growth an as yet unidentified sigma factor (sigma X) allows for low level of transcription of *nblA*. NblC interacts with this factor competing with its binding to the RNA-polymerase core, thereby resulting in partial repression of the *nblA*-promoter. Such a mechanism is consistent with the elevated level of *nblA* transcripts observed in non-starved NblCΩ as compared with the wild-type strain (Fig. 4B). This mechanism may provide 'fine-tuning' of *nblA* transcription and consequently of the amount of the pigment antennae in response to changes in ambient conditions (light intensity, temperature, etc.), to avoid excess excitation of the photosynthetic apparatus during growth in medium with sufficient level of nutrients. The precise role of NblC under such replete conditions is currently being examined.

Upon nutrient starvation, the level of NblC may increase, as suggested by the elevated level of *nblC* transcripts (Fig. 5). This may lead to efficient masking of the

cognate sigma factor and allows for substantial induction of *nblA* by NblR; the latter was previously shown to be essential for transcription induction of *nblA* during nutrient starvation (Schwarz and Grossman, 1998). Sequestration of sigma X by NblC presumably minimizes the interaction of the sigma factor with the promoter region of *nblA*, thereby allowing for efficient binding of NblR.

The model described is also consistent with results of ectopic expression of NblR in NblCΩ and NblC in NblRΩ. Expression of NblR in NblCΩ leads to increased pigment degradation as compared with the parental mutant strain (Fig. 8B–D). According to the model, elevated levels of NblR compensate for the lack of sequestration of sigma X. Furthermore, ectopic expression of NblC in NblRΩ does not result in any pigment degradation (Fig. 8B–D); NblR is essential for *nblA* induction and NblC, although having a positive outcome on expression of *nblA* during nutrient starvation cannot exert its effect on the absence of NblR.

Although still hypothetical, the plausible function of NblC as an anti-sigma factor provides new insights into modulation of promoter selectivity in cyanobacteria in response to environmental stimuli. To further delineate the function of NblC, we are currently identifying cellular components interacting with this novel modulator of phycobilisome degradation.

Experimental procedures

Strains, culture conditions and isolation of non-bleaching mutants

Synechococcus sp. PCC 7942 (hereafter, *Synechococcus*) and all strains resulting from molecular manipulations of this wild-type were cultured as previously described (Schwarz and Grossman, 1998).

Non-bleaching mutants were isolated as described elsewhere (Perelman *et al*., 2004). Briefly, *Synechococcus* cells were mutagenized by transformation with a transposon-based inactivation library. The library was obtained by *in vitro* transposition of a genomic library using EZ::TN™ KAN-2 insertion kit (Epicentre). Following growth of transformants in liquid growth medium in the presence of 25 μg ml^{-1} kanamycin, the cultures were starved for either sulphur or nitrogen and cells exhibiting relatively high fluorescence were selected by FACS and plated. A second screen for non-bleaching phenotypes was performed by re-streaking colonies on solid medium devoid of added sulphur or nitrogen source. Genomic DNA of non-bleaching strains was analysed by PCR to identify clones that possessed correct copies of the known genes of the *nbl*-pathway. The following sets of primers were used to detect *nblA*, *nblB*, *nblR* and *nblS* respectively: ACCCGAGGGGGATCTGTG and ATCGCTG GGTCAACAACG; ATGACTCCGGCGGAAATA and CTTCTA GACCATCGCCCC; CTGCGGAGTGCTATTCAG and GAATC GGGAACCCATGAC; GTTGCTGTAATCGCCTCA and CTA GAATGGCACGATGCA. A particular mutant, M104, was further analysed.

Cloning of the genomic fragment bearing the transposon

Identification of the transposon insertion site in mutant M104 was performed by ligation of genomic-PstI fragments to Bluescript KS and selection of the transformed *Escherichia coli* (DH5α) in the presence of kanamycin; only the desired clones were resistant to the antibiotic due to nptII included in the transposon. The sequence of the genomic regions neighbouring the transposon was determined using the transposon-specific primers provided with the EZ::TN™ insertion kit.

Insertional inactivation of the gene impaired in M104, nblC

A DNA fragment containing *nblC* and its flanking regions was obtained by PCR on genomic DNA of *Synechococcus* using the primers TTGACGCCGATGTATCTG and TAGCCACAA TACCTGCTG. This fragment was cloned into pGEM® T Easy vector (Promega) and a spectinomycin-resistance cassette (Prentki and Krisch, 1984) was inserted into the HindIII site (see Fig. 1B). The resulting construct was transformed into *Synechococcus*, and clones resistant to spectinomycin were selected. PCR on genomic DNA of several transformants confirmed double homologous recombination and complete chromosome segregation (not shown). One of these clones, NblCΩ, was selected for further analysis.

Cloning of nblC *in a neutral site*

A 2 kb pstI fragment bearing the genomic region immediately downstream of rbcLS, the operon encoding the large and small subunits of Rubisco, served as a neutral site for insertion. Insertion of a kanamycin-resistance cassette (containing nptII derived from Tn5, Beck *et al*., 1982) into the HincII site within this genomic fragment resulted in a construct, pNS1-Km, which was used to replace the native 2 kb pstI fragment in *Synechococcus*. Cyanobacterial clones resulting from transformation with this construct exhibited similar characteristics to wild-type cells in terms of growth in complete medium and degradation of phycobilisome as well as survival during nutrient starvation (not shown). The unique EcoRV site in pNS1-Km, upstream to nptII, the gene conferring kanamycin resistance, was used to produce a 'T-vector' from pNS1-Km. The plasmid was digested with EcoRV and then treated with *Taq*- polymerase (Fermentas) in the presence of 0.2 mM dTTP. The resulting 'T-vector' was used to clone a PCR product containing the coding region of *nblC* and 468 bp upstream of the start codon, bearing the presumed promoter region, yielding pES17. Transformation of NblCΩ with pES17 resulted in replacement of the genomic region bearing the neutral site with the homologous fragment containing *nblC* and the kanamycin-resistance cassette. PCR on genomic DNA of several transformants confirmed double homologous recombination and complete chromosome segregation (not shown). One of these clones, NblCΩ-ES17, was selected for further analysis.

Construction of fusions to phoA *promoter*

The coding regions of NblC and NblR were fused to a DNA

fragment bearing the promoter region of *phoA*, encoding alkaline phosphatase. This fragment was previously used to drive expression of *nblA* under phosphate starvation (Collier and Grossman, 1994). Fusion constructs were made by a two-step PCR process. The primers used for construction of the fusion products were as follows:

5′-promoter primer: TTCAGCTCAATTATTCATC
3′-promoter primer (5′sequence of *nblR* is underlined):
CGAGGCTGGCGCGATCATCGTCTTGATTACTGAAG
3′-promoter primer (5′sequence of *nblC* is underlined):
CACTGGGCAGACCCTCATCGTCTTGATTACTGAAG
3′-*nblR* primer: CTGCGGAGTGCTATTCAG
3′-*nblC* primer: TAGCCACAATACCTGCTG

The 3′-promoter-primers resulted in the production of promoter-PCR products, which contain a short region overlapping the 5′region of either NblR or NblC. A second PCR-step included a promoter-PCR product which served as a 'mega primer' for a template containing the relevant ORF (NblR or NblC); annealing was based on the short overlap between the promoter-PCR product and the template. Addition of the 5′-promoter primer and the 3′-ORF primer to the second PCR-step allowed effective amplification of the combined template.

Each of fusion products was cloned into a shuttle vector and introduced into the wild type, NblCΩ and the NblRΩ.

Nucleic acid isolation, Northern analysis and spectral measurements

RNA isolation was performed as previously described (Tu *et al*., 2004). Riboprobe for *nblA* was made by *in vitro* transcription using MEGAscript T7 (Ambion).

dsDNA probes for *nblA* and *nblC* for Northern hybridization were obtained by PCR using the primers mentioned above for analysis of *nblA* in non-bleaching mutants and for inactivation of *nblC* respectively. Additional fragments used as probes were made by PCR using primers as follows:

TGCTTTCACCAAGGTGGTGG and GCAACTGCAGCAGC AGCTTT for *cpcB*
CATCAATGGCTCGGGCAA and TCTTTGAGCACCGGCAGA for *glnN*
GCCAAAAGGCGTTCCTAG and CGGGACCAGTTTCAACCG for *rhdA* and
CGCAATTTGCACCAGATGGC and AGACCCAGCGGCTTG ATATC for *phoA*.

To quantify transcript abundance, scanned autoradiographs were subjected to image analysis and signals were normalized to rRNA.

Absorbance spectra of cell suspensions were measured by Cary 100 spectrophotometer equipped with a Labsphere DRA-CA-30I diffuse reflectance accessory. Phycocyanin quantitation was based on absorbance maxima at 620 nm. Each culture was starved in two separate tubes and two spectral measurements were performed for every starved culture.

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