

Review

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Acclimation of unicellular cyanobacteria to macronutrient deficiency: emergence of a complex network of cellular responses

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Cyanobacteria are equipped with numerous mechanisms that allow them to survive under conditions of nutrient starvation, some of which are unique to these organisms. This review surveys the molecular mechanisms underlying acclimation responses to nitrogen and phosphorus deprivation, with an emphasis on non-diazotrophic freshwater cyanobacteria. As documented for other micro-organisms, nutrient limitation of cyanobacteria elicits both general and specific responses. The general responses occur under any starvation condition and are the result of the stresses imposed by arrested anabolism. In contrast, the specific responses are acclimation processes that occur as a result of limitation for a particular nutrient; they lead to modification of metabolic and physiological routes to compensate for the restriction. First, the general acclimation processes are discussed, with an emphasis on modifications of the photosynthetic apparatus. The molecular mechanisms underlying specific responses to phosphorus and nitrogen-limitation are then outlined, and finally the cross-talk between pathways modulating specific and general responses is described.

Introduction

Cyanobacteria are among the most widely distributed micro-organisms in the biosphere and they play a dominant role in the global nitrogen and carbon cycles. Their metabolism is based on oxygenic photosynthesis, similar to that of eukaryotic algae and plants. This process provides ATP and reducing equivalents from the splitting of water, which enables the bacteria to assimilate simple inorganic nutrients for their anabolic demands. Due to their abundance and high metabolic activities, micro-organisms may deplete the environment of essential nutrients. A prominent example of an environmental effect caused by the metabolic activity of cyanobacteria is the depletion of carbon dioxide from the atmosphere during the course of evolution, and the concomitant accumulation of the 'waste product' of photosynthesis, oxygen.

Deprivation of essential nutrients is frequently the limiting factor in cyanobacterial cell growth, and the need to adapt to periods of nutrient limitation is a major source of selective pressure in diverse natural environments. Various bacteria respond to nutrient limitation by entering a morphogenetic programme resulting in spore formation. Although they do not sporulate, unicellular cyanobacteria undergo substantial changes in response to nutrient starvation and exhibit sophisticated strategies that allow survival

for long periods under stress conditions. This survival strategy, employing a 'stand-by' energy metabolism (see below), differs fundamentally from the dormant state of spores and akinetes (spore-like cells produced by some filamentous cyanobacteria) and requires a highly regulated switch of cellular activities. Research in the past decade has led to fundamental new insights into the molecular mechanisms of these responses.

Classically, acclimation responses to nutrient limitation are grouped into specific and general, or common, responses. The specific responses are the acclimation processes that occur as a result of limitation for a particular nutrient, whereas the general responses occur under any starvation condition. This review will mainly describe studies in the unicellular, non-nitrogen-fixing strains *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 (hereafter, *Synechococcus* and *Synechocystis*, respectively). First, we will describe the general acclimation processes, with an emphasis on modifications of the photosynthetic apparatus, as a fundamental aspect of cyanobacterial acclimation. Next, we will review the specific responses to limitation of the macronutrients phosphorus and nitrogen. As little substantial progress has been made in understanding the molecular mechanisms underlying responses to sulfur limitation, the reader is referred to the existing literature. Inorganic carbon availability is a major environmental factor affecting

growth of photosynthetic micro-organisms such as cyanobacteria; this subject is reviewed elsewhere (Kaplan & Reinhold, 1999; Badger & Price, 2003).

General acclimation responses

Certain general acclimation responses are exhibited by a large variety of micro-organisms (Hecker & Völker, 1998), including cyanobacteria. Commonly, starvation stress imposes significant metabolic changes manifested by increased catabolism and decreased anabolism. Growth arrest is among the general phenomena observed in all micro-organisms during starvation. It is likely that starvation triggers regulated arrest of the cell cycle in cyanobacteria, as reported for other micro-organisms; however, the underlying mechanism is obscure. In unicellular cyanobacteria, completion of cell division in the absence of further cell growth leads to disappearance of elongated pre-divisional cells and, during prolonged starvation, the subcellular structure changes considerably (see Fig. 1; Li & Sherman, 2002; Wanner *et al.*, 1986). The most obvious changes are degradation of the intracellular membranes, the thylakoids, and the accumulation of cellular inclusions (Allen, 1984). Under conditions of nitrogen and sulfur starvation, glycogen granules accumulate, and in various species, poly- β -hydroxybutyrate (PHB) inclusions may be

formed (Hai *et al.*, 2001) as storage products of fixed CO₂. In contrast, under conditions of phosphorus starvation, the nitrogen and energy storage compound cyanophycin (multi-L-arginyl-poly-L-aspartic acid) is synthesized in various cyanobacteria (Allen, 1984).

At the level of gene expression and protein synthesis, an overall reduction in the rate of protein synthesis is observed upon starvation (Aldehni *et al.*, 2003; Sauer *et al.*, 2001). However, the synthesis of certain proteins, which are required for the acclimation process, is strongly enhanced upon nutrient step-down. Even during prolonged starvation, several proteins continue to be produced at appreciable levels. A global proteomics approach has allowed the identification of proteins either induced or repressed under general stress conditions. One of the proteins induced under general stress was identified as a homologue of thioredoxin peroxidase (also designated peroxiredoxin) (Aldehni *et al.*, 2003). This enzyme was shown to be essential for growth under high photon flux (Perelman *et al.*, 2003). Induction of this protein during both sulfur and nitrogen starvation suggests that the trigger for expression may be oxidative stress, which occurs subsequent to nutrient starvation conditions.

An important aspect of survival under stress conditions is the protection of the genetic material. A *Synechococcus* homologue of the *Escherichia coli* Dps protein, a non-specific DNA binding protein essential for protection of bacterial DNA under stress (Almiron *et al.*, 1992; Martinez & Kolter, 1997; Wolf *et al.*, 1999), was identified and shown to accumulate in nutrient-limited cells (Pena *et al.*, 1995; Pena & Bullerjahn, 1995). The alternative sigma factor, SigE, of *Synechococcus* sp. strain PCC 7002 is involved in expression of Dps upon entry of this cyanobacterium into stationary phase (Gruber & Bryant, 1998). Further studies are required to elucidate the detailed regulation of Dps expression and to find out whether additional DNA-binding proteins with protective functions are present. Additional proteins that strongly accumulate in *Synechococcus* under conditions of nitrogen and sulfur starvation include the outer-membrane porins SomA/SomB (Sauer *et al.*, 2001; K. Forchhammer, unpublished), which might enhance the ability of the organisms to scavenge nutrients from their surroundings. Moreover, striking changes in cell physiology are related to the modification of the photosynthetic apparatus, as will be outlined below.

Modification of the photosynthetic apparatus

All photosynthetic organisms must tune their energy input, or excitation rate, to cellular metabolic capacity. During growth under nutrient-sufficient conditions, the reducing potential produced by the photosynthetic electron-transport chain is used for anabolic reactions. Nutrient limitation slows down the reoxidation of the final electron acceptors, and therefore electron transfer activity must be down-regulated (Grossman *et al.*, 1993; Schwarz & Grossman, 1998). The adjustment of the photosynthetic

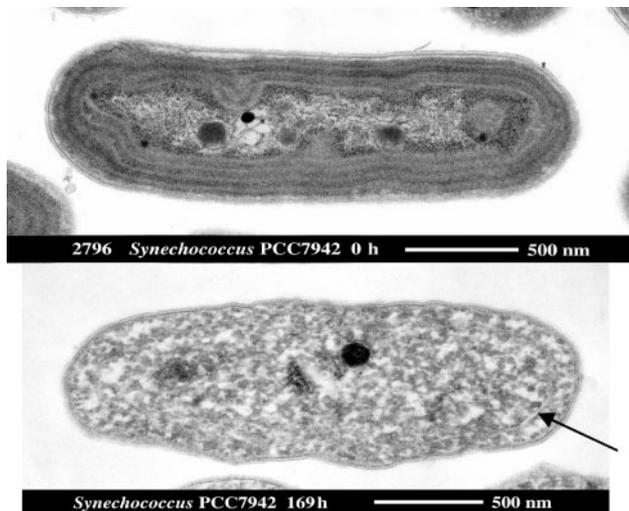


Fig. 1. Morphological changes resulting from nitrogen starvation. Transmission electron micrographs of *Synechococcus elongatus* PCC 7942 cells grown in nutrient-sufficient medium (top) or starved for nitrogen (bottom). Chlorosis results from incubation in combined nitrogen-free medium for 7 days as described by Sauer *et al.* (2001). Note the granular white structures in the cytoplasm in the chlorotic cell due to the accumulation of glycogen as well as the almost complete degradation of the intracellular membrane system, the thylakoids. The arrow points towards a remaining thylakoid structure in the chlorotic cell. Micrographs were prepared by G. Wanner (University of Munich).

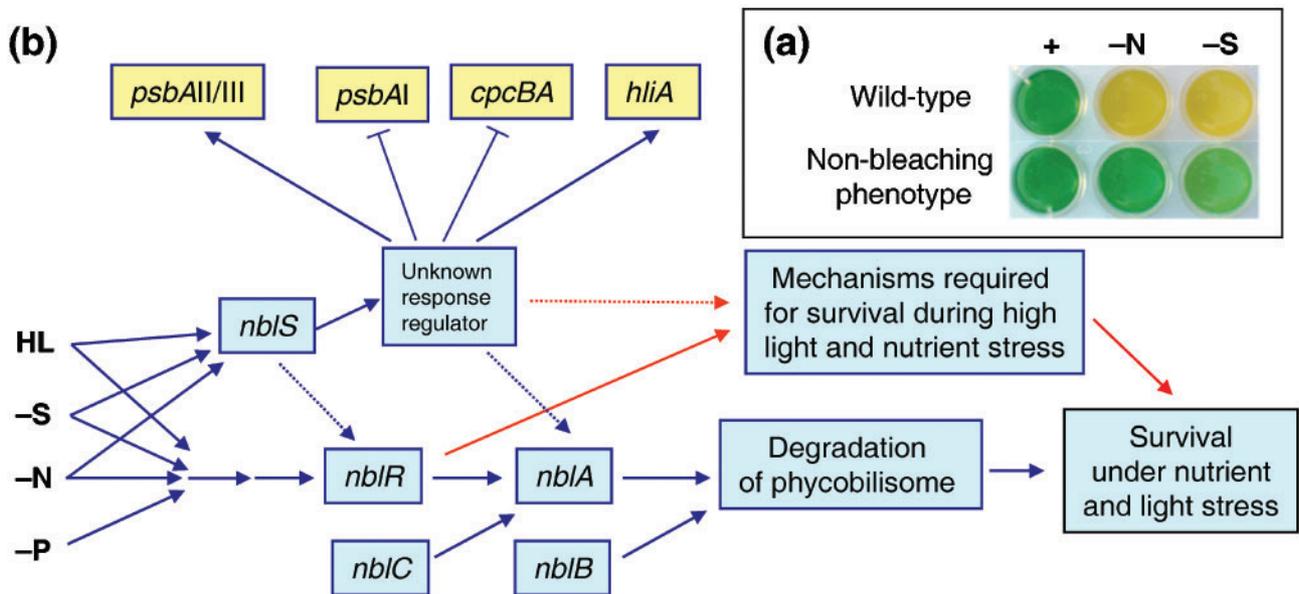


Fig. 2. General acclimation responses to nutrient stress in *Synechococcus elongatus*. (a) Cultures of wild-type *Synechococcus* exhibiting degradation of the light-harvesting pigments upon nitrogen (–N) and sulfur (–S) starvation, and a mutant demonstrating a non-bleaching phenotype. Cells were grown in nutrient-sufficient medium (+) or starved for 48 h. (b) The current model of the phycobilisome degradation pathway (*nbl* pathway). The signalling pathway is triggered by deprivation of sulfur (–S), nitrogen (–N) or phosphorus (–P), as well as by illumination with high light intensity (HL). Red arrows indicate modulation of acclimation responses that are crucial for survival in adverse environmental conditions. Dotted arrows indicate that the sensor kinase, NblS, may control *nblA* and additional genes through NblR or through an as-yet-unknown response regulator. Yellow shading indicates genes that are controlled by the NblS pathway specifically under high light intensity. *hli*, high light inducible gene; *cpcBA*, the operon encoding the a and b subunits of phycocyanin, the major phycobilisome pigment in *Synechococcus*; the *psbA* genes encode the D1 polypeptide of photosystem II. *PsbAI*, which is repressed under high light, encodes form I of D1, whereas *psbAII* and *III*, encoding form II of D1, are induced by high photon flux.

apparatus to nutrient-limiting conditions is a process that causes apparent changes; when cyanobacteria are maintained under conditions of starvation for an essential nutrient, they turn yellow (Fig. 2a). This process, termed chlorosis or bleaching, was described long ago (Allen & Smith, 1969) and has attracted manifold research activities. The common scheme of chlorosis is the degradation of photosynthetic pigments, in particular, the phycobiliproteins, which constitute the major light-harvesting antenna in cyanobacteria, as well as chlorophyll *a*, the pigment in the reaction centres and core antenna of photosystem (PS) I and PSII. The kinetics of pigment degradation is rather variable and depends on the specific nutrient that is absent as well as on other environmental conditions such as CO₂ supply, temperature and light intensity (Barker-Astrom *et al.*, 2005; Collier & Grossman, 1992; Görl *et al.*, 1998). Furthermore, different cyanobacterial strains may respond quite differently to various starvation conditions. For example, sulfur starvation causes rapid chlorosis in *Synechococcus* (Collier & Grossman, 1992), while *Synechocystis* does not degrade its phycobilisome in response to sulfur deprivation (Richaud *et al.*, 2001).

Characterization of *Synechococcus* cells that were subjected

to prolonged nitrogen starvation delineated a cascade of events that may represent a general acclimation process. In the first phase, a rapid degradation of phycobilisomes occurs (see details below). Next, the cells gradually degrade other proteins and pigments, and finally they become almost completely depigmented and enter a survival mode. These changes are reversible; following the addition of a combined nitrogen source, the cells return to vegetative growth within a few days. Determination of viability by plating suggests that only 5–10% of cells are capable of forming colonies on solid media following reintroduction of combined nitrogen. However, analysis of cell viability using vital stains revealed that almost all cells retain viability and are able to recover in liquid medium (Görl *et al.*, 1998). The discrepancy between the two methods for assessment of viability indicates that most of the population enters a viable but non-culturable (VBNC) state, a physiological state that is reflected in the failure of bacterial cells to form colonies (Desnues *et al.*, 2003).

Physiological analysis of long-term-starved cells that were maintained for more than 2 months in nitrogen-depleted medium revealed that the cells retained residual activities of PSI as well as PSII, amounting to approximately 0.1% of

the activity observed in growing cells (Sauer *et al.*, 2001). Net oxygen evolution and anabolic activities, however, were not detected under these conditions. Therefore, it was suggested that cells perform a pseudo-cyclic electron flow (water-to-water cycle) in which oxygen, generated by PSII activity, is directly reduced by PSI. Interestingly, *Synechocystis* was recently shown to possess type A flavoproteins, which may directly reduce oxygen to water using electrons from PSI, thus without producing the toxic superoxide radical and hydrogen peroxide associated with the previously known mechanisms for pseudo-cyclic electron flow (Helman *et al.*, 2003). This electron-transport chain may generate membrane potential to fuel residual cellular activities. *In vivo* protein labelling revealed that the apparently dormant cells slowly turn over a subset of their cellular proteins, in particular proteins involved in photosynthesis and redox homeostasis, whereas proteins participating in the translational machinery are hardly synthesized *de novo* (Sauer *et al.*, 2001). In summary, the cyanobacterial mode of survival appears to be based on constant low metabolism, in contrast to the fully dormant stage exhibited by bacterial spores.

Degradation of the phycobilisome

Modulation of the phycobilisome, the light-harvesting antenna typical of most cyanobacteria, upon nutrient depletion involves repression of synthesis of new pigments as well as active degradation. Starvation for phosphorus (Collier & Grossman, 1992), inorganic carbon (Barker-Astrom *et al.*, 2005) or iron (Singh & Sherman, 2000) results in partial loss of pigment. Nitrogen and sulfur starvation, on the other hand, elicit substantial pigment loss in *Synechococcus*, with nitrogen depletion inducing rapid and complete phycobilisome degradation (Collier & Grossman, 1992). The phycobilisome is an ultrastructure consisting of pigmented proteins (phycobiliproteins) as well as non-pigmented (linker) polypeptides (Glazer, 1985; Grossman *et al.*, 1993; MacColl, 1998). The rapid and complete degradation of this abundant complex, which may constitute up to 50% of soluble cellular protein, indicates the existence of highly effective degradation machinery.

To address the molecular mechanisms underlying the degradation process and its regulation, mutants were isolated that do not degrade their light-harvesting pigments under nutrient starvation. This phenotype was termed non-bleaching (Nbl) since the mutants appear blue-green when starved, rather than yellowish or bleached as do the wild-type cultures. Molecular analysis of non-bleaching mutants uncovered several genes essential for phycobilisome degradation. The pioneering work using this approach led to identification of *nblA*, a small gene encoding a 59 amino acid polypeptide, which is induced upon starvation (Collier & Grossman, 1994). Subsequently, genes encoding NblA-like peptides were identified and characterized in various cyanobacterial species (Baier *et al.*, 2001; de Alda *et al.*, 2004; Delumeau *et al.*, 2002; Luque *et al.*, 2003; Richaud *et al.*, 2001; Li & Sherman, 2002). In fact, examination of the available complete genomic sequences indicated the

existence of at least one *nblA* homologue in all organisms that possess a phycobilisome (cyanobacteria and red algae). NblA does not exhibit homology to any other proteins of known function, and its specific role in phycobilisome degradation is subject to speculation. Recent studies have suggested association of NblA with specific components of the phycobilisome (Luque *et al.*, 2003), but the functional significance of this association is yet to be established.

Under nitrogen starvation, certain filamentous cyanobacteria differentiate heterocysts, cells that have a specialized nitrogen fixation function. To enable the nitrogenase activity of heterocysts, oxygen evolution is depressed and the phycobilisomes are degraded as part of the down-regulation of their photosynthetic apparatus. NblA is essential for the specific phycobilisome degradation that occurs in these cells. The *Anabaena nblA* mutant, however, develops functional heterocysts, indicating that phycobilisome degradation is not essential for heterocyst development or nitrogen fixation (Baier *et al.*, 2004). An additional component required for the degradation process is NblB (Dolganov & Grossman, 1999). This protein exhibits homology to the chromophore-interacting region of phycocyanin lyases, enzymes that covalently attach a chromophore to the proteinaceous component of the pigment. Possibly, interaction of NblB with the chromophore is required for the disassembly of the phycobilisome, rendering it susceptible to protease action.

Pigment degradation must be tightly controlled either by environmental cues or by their physiological or biochemical consequences. Several regulatory components required for degradation have been identified. NblS (van Waasbergen *et al.*, 2002) and NblR (Schwarz & Grossman, 1998), are homologues of histidine kinase sensors and response regulators of two-component signal transduction pathways, respectively. Mutation in *nblS* or *nblR* results in severe impairment of phycobilisome degradation; however, the proposed function of the components encoded by these genes as a sensor-regulator pair still awaits proof. NblC, a recently identified component of the *nbl* pathway, is a homologue of eubacterial anti-sigma factors (R. Schwarz, unpublished). These three regulatory components are required for efficient transcription induction of *nblA*. Previous analysis of a *Synechococcus nblS* mutant (van Waasbergen *et al.*, 2002) as well as recent studies of a *nblS* homologue (also termed *dspA* or *hik33*) mutant in *Synechocystis* (Hsiao *et al.*, 2004; Tu *et al.*, 2004) indicated that the *nbl* pathway, which modulates pigment degradation during nutrient stress, interacts with a signal transduction chain critical for transcription regulation under high-light conditions (Fig. 2). This suggestion is supported by the pleiotropic effect of *nblR* mutations on cell survival during high light and nutrient stress (Schwarz & Grossman, 1998) (see legend of Fig. 2 for details). The signal that triggers the *nbl* pathway is yet to be identified. The presence of a PAS domain in NblS suggests that light or redox changes may serve as a trigger for this pathway (van Waasbergen *et al.*, 2002). An additional modulator of general acclimation

responses may be the protein encoded by *slr2031*, a homologue of eubacterial regulators of σ^B ; cultures of an *slr2031* mutant exhibit a lower proportion of cells capable of resuming growth following nitrogen or sulfur starvation (Huckauf *et al.*, 2000).

In summary, isolation and characterization of non-bleaching mutants has revealed several components of the phycobilisome degradation pathway. A novel screen employing fluorescence-activated cell sorting is expected to greatly facilitate the isolation of additional mutants and consequently genes involved in the pathway (Perelman *et al.*, 2004). Characterization of the available and newly isolated mutants will help to delineate the mechanism of disintegration and degradation of the light-harvesting antennae as well as the details of the signal transduction cascade.

Nutrient-specific molecular responses

Cyanobacteria are equipped with a suite of responses allowing them to cope with limiting concentrations of specific nutrients. The common theme of specific responses is the induction of efficient transport systems and acquisition of the limiting nutrient from a large variety of sources. These responses require sensitive signal perception and transduction systems to modulate cellular processes at various levels of regulation.

Acclimation to phosphorus limitation

When starved for phosphorus, *Synechococcus* cells significantly induce phosphate uptake: a 50-fold increase in V_{\max} for phosphate transport is observed upon phosphorus starvation (Grillo & Gibson, 1979). Furthermore, periplasmic alkaline phosphatases, for example PhoA (Ray *et al.*, 1991) and PhoV (Wagner *et al.*, 1995), release phosphate from various compounds, making it available for the transport systems. PhoA, an atypically large alkaline phosphatase, is strongly induced upon starvation (Ray *et al.*, 1991). This enzyme may provide the ability to scavenge phosphate from a large variety of substrates, as suggested by its *in vitro* activity. The responses to phosphorus limitation are governed by a sensor kinase and a response regulator comprising a two-component signal transduction pathway. Genome-wide transcription analysis of *Synechocystis* revealed a phosphate regulon (Pho regulon), three clusters of genes exhibiting substantial induction upon phosphate starvation (Suzuki *et al.*, 2004). These include two sets of genes encoding putative phosphate-specific transport systems (*pst1* and *pst2*), and *phoA* and *nucH*, which encode alkaline phosphatase and extracellular nuclease, respectively. An additional single gene encoding a periplasmic protein of unknown function, *urtA*, was found to be highly repressed upon phosphorus starvation. Importantly, the Pho regulon may extend beyond the genes described above (*ppa* and *ppx* may be included; see below); definition of the Pho regulon by Suzuki *et al.* (2004) is currently based on genes which exhibit more than sevenfold induction or repression following phosphorus starvation. Transcription analysis of

Synechocystis sensor (SphS) and regulator (SphR) mutants indicated exclusive regulation of the currently defined Pho regulon by these components. Examination of the promoter sequences of *Synechocystis* Pho regulon genes as well as gel mobility shift experiments identified a 'Pho box', a promoter sequence required for transcription activation by SphR (Suzuki *et al.*, 2004). The molecular mechanism underlying activation of SphS by phosphorus starvation and signal transduction to SphR is yet to be elucidated. The presence of a PAS domain in the C-terminal region of SphS suggests involvement of light or changes in redox state as possible signals (Suzuki *et al.*, 2004).

A study showing that complete genomic sequences may provide insight into ecological features came from analysis of the genomic region of *phoR* and *phoB*, encoding the sensor kinase and response regulators involved in acclimation to phosphorus limitation in two *Prochlorococcus* species (MED4 and MIT9313) (Scanlan & West, 2002). *Prochlorococcus* MIT9313, an ecotype acclimated to the low light intensity present in deep waters, carries a mutation introducing a stop codon into the coding region of *phoR*. Furthermore, this species possesses aberrant *ptrA*, a putative transcription regulator of phosphorus utilization. In contrast, the high-light-acclimated ecotype *Prochlorococcus* MED4 contains intact copies of *phoR* and *ptrA*. The mutations found in the *Prochlorococcus* MIT9313 may reflect the relatively high phosphorus concentrations found in deep waters, and therefore the dispensability of the components regulating the response to phosphate starvation.

Micro-organisms are able to accumulate inorganic phosphate in excess of their immediate requirements. Polyphosphates, linear polymers of tens to hundreds of phosphate units linked by high-energy phosphoanhydride bonds, serve as a phosphate reservoir (Kornberg *et al.*, 1999). Polyphosphate synthesis is dependent on Ppk (ATP-polyP phosphotransferase), whereas degradation of the polymer is achieved through the activity of Ppx (exophosphatase) and Ppa (inorganic pyrophosphorylase). The latter two enzymes are induced upon phosphorus starvation of *Synechocystis* (Gomez-Garcia *et al.*, 2003) and may be included in the Pho regulon. Ppa activity appears to be essential for survival: no fully segregated mutant in this gene has been isolated. *ppx* mutant strains exhibit aberrant growth as compared to the wild-type under phosphorus depletion but interestingly, during growth in replete medium as well. Aside from being a phosphate reservoir, polyphosphates have been assigned an elaborate series of functions including a regulatory role, and may function as an ATP substitute and divalent metal chelator (Kornberg *et al.*, 1999). Based on these multiple roles, the growth impairment of the *ppx* mutant of *Synechocystis* during phosphate-replete growth is not surprising. PolyP accumulation has been documented in a wide range of organisms under both favourable and stress conditions (Kornberg *et al.*, 1999). Interestingly, the Ppa homologue of *Synechococcus* is depressed during either nitrogen or sulfur starvation (Aldehni *et al.*, 2003).

Accumulation of polyP is consistent with this repression of its degrading enzyme, Ppa; however, the cellular function of polyP as a general stress effector is unknown.

A recent study indicated substantial changes in the lipid composition of photosynthetic membranes upon phosphorus limitation in cyanobacteria as well as in green algae and higher plants. This is reflected in substitution of some of the phosphatidylglycerol with sulfoquinovosyl-diacylglycerol (SQDG). Interestingly, a *Synechococcus* mutant impaired in SQDG synthesis becomes starved for phosphate sooner than the wild-type strain. This suggests that while maintaining the essential anionic composition of the photosynthetic membranes, the change in lipid composition allows some phosphate to be used for other cellular functions (Frentzen, 2004).

Acclimation to nitrogen deprivation

The global nitrogen control system. Generally, ammonium is the preferred nitrogen source of cyanobacteria; its utilization prevents the use of alternative nitrogen sources via a regulatory system referred to as 'global nitrogen control' (reviewed by Flores & Herrero, 1994; Herrero *et al.*, 2001). In the absence of usable combined nitrogen sources, diazotrophic strains are able to fix atmospheric N₂ and thereby circumvent nitrogen depletion. In contrast, non-diazotrophic strains face nitrogen starvation, a situation which ultimately blocks anabolic metabolism and causes nitrogen chlorosis. Recently, it became evident that the global nitrogen control system shares common components with the specific responses of cyanobacteria to combined-nitrogen depletion. Various aspects of global nitrogen control have been reviewed recently (Herrero *et al.*, 2001, 2004; Forchhammer 2004), and therefore will only be outlined as regards their relevance to the specific responses of non-diazotrophic cyanobacteria to nitrogen starvation. The various nitrogen compounds that serve as nutrients are first converted to ammonium intracellularly. Ammonium is then assimilated via the glutamine synthetase–glutamate synthase (GS-GOGAT) pathway by incorporation into the carbon skeleton of 2-oxoglutarate, resulting in the synthesis of glutamate. 2-Oxoglutarate synthesis by isocitrate dehydrogenase is the final step of the oxidative branch of the TCA cycle in cyanobacteria (Tandeau de Marsac & Lee, 1999), and its consumption via GOGAT is directly coupled to ammonium assimilation. Consequently, limitation of the GS-GOGAT cycle by ammonium depletion leads to accumulation of 2-oxoglutarate, which serves as an indicator of the cellular nitrogen status (Irmler *et al.*, 1997; Forchhammer, 1999; Muro-Pastor *et al.*, 2001). Two 2-oxoglutarate-responsive elements, P_{II} and NtcA, have been recognized so far in cyanobacteria. Although these proteins are generally present in cyanobacteria, they seem to affect gene expression differently in freshwater and marine cyanobacterial strains. The marine strains are adapted to very low ambient combined nitrogen concentrations and do not exhibit the tight repression in response to ammonium as

described for the freshwater *Synechococcus* or *Synechocystis* strains. Thus, the regulatory role of these proteins in the marine strains remains to be further elucidated and we refer the reader to recent publications concerning marine *Synechococcus* and *Prochlorococcus* strains (Lindell *et al.*, 2002; Zubkov *et al.*, 2003; Bird & Wyman, 2003).

The P_{II} signalling protein. The cyanobacterial P_{II} protein is a member of the large family of P_{II} signal transduction proteins, which have widespread roles in nitrogen control in bacteria, plants, and some archaea (for recent reviews see Arcondeguy *et al.*, 2001; Forchhammer, 2004). Similar to the P_{II} signalling protein in *E. coli* (Kamberov *et al.*, 1995), *Synechococcus* P_{II} binds ATP and 2-oxoglutarate in a cooperative manner (Forchhammer & Hedler, 1997) and was the first 2-oxoglutarate-responsive factor recognized in cyanobacteria. In the presence of increased 2-oxoglutarate levels, corresponding to nitrogen-limited conditions, P_{II} is phosphorylated at a seryl residue (Ser-49) (Forchhammer & Tandeau de Marsac, 1995a). P_{II}-P is dephosphorylated *in vitro* at low 2-oxoglutarate levels (Ruppert *et al.*, 2002), which correspond *in vivo* to conditions of nitrogen excess (ammonium supplementation) or limiting inorganic carbon supply (see Fig. 3). In general, P_{II} signal transduction is based on protein–protein interactions with receptor proteins, in which P_{II} modulates the activities of the target proteins. The protein–protein interactions are sensitive to the signal input state of P_{II} proteins, including their binding of effector molecules and, if applicable, their state of covalent modification. Recently, the key enzyme of the arginine synthesis pathway, *N*-acetylglutamate kinase (NAGK), was identified as the first receptor for P_{II} in a cyanobacterium (Heinrich *et al.*, 2004; Burillo *et al.*, 2004). Complex formation and catalytic activation by P_{II} of NAGK was shown to depend both on the phosphorylation state of P_{II} and on its binding of effector molecules (Heinrich *et al.*, 2004; Maheswaran *et al.*, 2004). Earlier studies suggested that P_{II} plays only a subordinate role in the regulation of nitrogen assimilation via global nitrogen control, since the response of several ammonium-depressed genes to the absence or presence of ammonium was not significantly altered in P_{II} mutants (Lee *et al.*, 2000). The contribution of P_{II} to global nitrogen control appeared to be limited to the regulation of ammonium/CO₂-dependent nitrate/nitrite uptake (Forchhammer & Tandeau de Marsac, 1995b; Lee *et al.*, 1998; Hisbergues *et al.*, 1999). However, recent results suggest that P_{II} is intimately involved in the response of cyanobacteria to nitrogen limitation (see below).

The global nitrogen control protein NtcA. A second 2-oxoglutarate-responsive protein is the global nitrogen control protein, NtcA. NtcA is a versatile DNA-binding protein, which acts as a transcriptional activator (and in some cases as a transcriptional repressor) of a large number of genes whose products are mostly involved in nitrogen metabolism (reviewed by Herrero *et al.*, 2001,

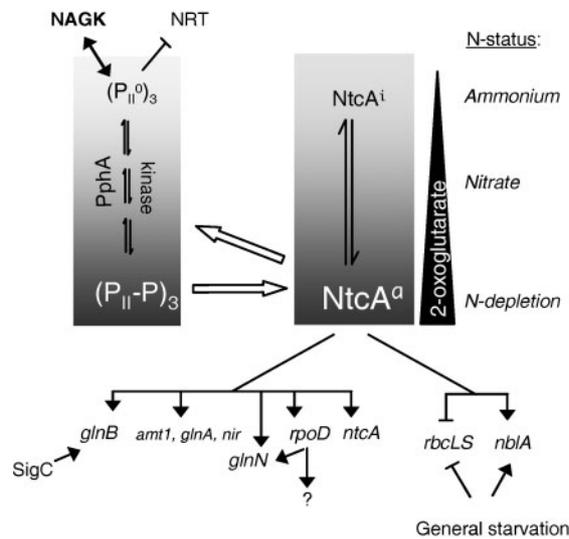


Fig. 3. Regulatory network between P_{II} , NtcA and the expression of nitrogen-regulated genes in nitrogen-starved non-diazotrophic cyanobacteria. This model integrates data obtained from *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942 and therefore illustrates a possible scenario of global nitrogen control rather than the exact pathway in a particular cyanobacterium. The thick arrows indicate the mutual interactions between P_{II} and NtcA at the level of protein function. The thin arrows indicate gene activation. The intracellular 2-oxoglutarate concentration under various nitrogen regimes (right) is symbolized by the width of the black arrowhead. The homotrimeric P_{II} protein may switch between the completely non-phosphorylated protein and phosphorylated forms containing one, two or three phosphorylated subunits (for clarity, only the fully phosphorylated form is shown). Phosphorylation is mediated by an as-yet-unknown kinase; dephosphorylation is catalysed by protein phosphatase PphA (Ruppert *et al.*, 2002). Non-phosphorylated P_{II} interacts with *N*-acetylglutamate kinase (NAGK) and negatively regulates the nitrate/nitrite permease (NRT) activity. Activated NtcA enhances transcription of *ntcA* and *glnB* as well as other classical NtcA promoters, and may switch from an inactive state (NtcAⁱ) to an activated state (NtcA^a). NtcA-dependent genes of the ammonium assimilation pathway include *amt1*, *glnA* and *nir* (Vazquez-Bermudez *et al.*, 2002a). Furthermore, NtcA stimulates expression of the alternative sigma factor gene *rpoD*, and of the alternative glutamine synthetase gene *glnN*, exhibiting a non-canonical NtcA-binding promoter sequence. RpoD might control the expression of other genes in addition to *glnN*. Some NtcA-dependent genes such as *nbIA* and *rbcLS* are under multiple nutrient/stress control, while NtcA affects the transcription of these genes only under conditions of nitrogen starvation. The repressive effect of activated NtcA on *rbcLS* expression is indicated by the interrupted line. For further details, see text.

2004) (see Fig. 3). NtcA is recognized as the major mediator of global nitrogen control at the level of gene expression (Luque *et al.*, 1994). An NtcA-deficient mutant is unable to activate genes whose expression is depressed by ammonium, and thus NtcA mutants are unable to grow

on nitrogen sources such as nitrate. The NtcA protein belongs to the CRP class of transcription factors and binds as a homodimer to a conserved palindromic sequence (GTA-N₈-TAC). DNA binding as well as transcriptional activity are directly stimulated by 2-oxoglutarate *in vitro* (Vazquez-Bermudez *et al.*, 2002b; Tanigawa *et al.*, 2002). Consistent with these *in vitro* properties, the *in vivo* activity of NtcA is subject to metabolic regulation, such that under conditions of nitrogen excess (low 2-oxoglutarate levels), the NtcA protein is inactive (Luque *et al.*, 2004) even if the *ntcA* gene is highly expressed from a strong promoter. Increased 2-oxoglutarate concentrations and the absence of ammonium lead to *in vivo* stimulation of NtcA activity (Vazquez-Bermudez *et al.*, 2003). The *ntcA* gene is autoregulated by NtcA: the greater the activity of NtcA, the more NtcA protein is produced, resulting in a strong increase of NtcA abundance under nitrogen-limited conditions (Luque *et al.*, 2004). It has been suggested that under those conditions, promoters with low-affinity NtcA-binding sites can be activated. Indeed, several genes have been identified which exhibit putative non-perfect NtcA-binding sites in the promoter region (Herrero *et al.*, 2004). One of the genes exhibiting a putative non-canonical NtcA site is *glnN*, encoding an alternative glutamine synthetase of type III (Reyes *et al.*, 1997). The *glnN* product helps *Synechococcus* cells recover more rapidly after a prolonged period of nitrogen chlorosis, although this gene product is not essential for survival (Sauer *et al.*, 2000). Differential affinity of NtcA towards various DNA-binding sites provides a simple mechanism to modulate gene expression in response to a gradual limitation of the nitrogen source. The high-affinity sites, such as the NtcA site in the *glnA* gene (encoding the housekeeping glutamine synthetase, GSI), require only low amounts of NtcA and are activated as soon as nitrogen sources 'poorer' than ammonium, e.g. nitrate, are utilized (Vazquez-Bermudez *et al.*, 2002c). In contrast, low-affinity sites are only activated when the abundance of NtcA is greatly increased. Recently, P_{II} was identified as a factor that is involved in strong activation of NtcA under conditions of nitrogen starvation.

Functional interaction of P_{II} and NtcA during nitrogen deprivation. Synthesis of GlnN following nitrogen deprivation is impaired not only in an NtcA-deficient mutant but also in a mutant deficient in P_{II} (Sauer *et al.*, 2000), suggesting a link between P_{II} and NtcA under conditions of nitrogen deprivation. To investigate this potential relationship, protein synthesis patterns of *Synechococcus* wild-type, P_{II} - and NtcA-deficient mutants were compared (Aldehni *et al.*, 2003). All proteins whose synthesis responded specifically to nitrogen deprivation (and did not respond to sulfur starvation), were shown to be under NtcA control, demonstrating the universal role of NtcA as the master-regulator of nitrogen-controlled gene expression. Surprisingly, however, the P_{II} -deficient mutant exhibits a similar phenotype. It is also unable to specifically respond to combined-nitrogen deprivation at the level of

altered protein expression patterns, confirming a functional relationship between P_{II} and NtcA.

Another class of proteins respond to both nitrogen and sulfur deprivation. Most of these are not affected in NtcA or P_{II} mutants; some, however, are indeed affected in the NtcA or P_{II} mutant background, but only during nitrogen deprivation. One of the NtcA/P_{II}-dependent so-called 'general starvation-repressed proteins' was identified as a subunit of RubisCO. Northern blot analysis revealed that the RubisCO (*rbcSL*) genes are in fact rapidly down-regulated following nitrogen deprivation in an NtcA/P_{II}-dependent manner. This suggests that some genes which respond to different kinds of nutrient stresses, such as *rbcLS*, are controlled by separate mechanisms under the various conditions, e.g. by NtcA during nitrogen depletion and by an as-yet-unidentified factor during sulfur starvation. In no case do the NtcA- and P_{II}-deficient mutants show an impaired response during sulfur deprivation, demonstrating their nitrogen specificity. The P_{II} requirement in activating NtcA-dependent gene expression following nitrogen deprivation was studied independently by Northern blot analysis on four different NtcA-dependent transcripts (Paz-Yepes *et al.*, 2003). Whereas in the wild-type, these transcripts accumulate rapidly after shifting cells from nitrate-supplemented to combined nitrogen-depleted medium, this transcriptional response is strongly impaired in a P_{II}-deficient mutant. A P_{II} Ser₄₉Ala mutant, which cannot be phosphorylated (Lee *et al.*, 2000), also shows impaired activation of NtcA (Paz-Yepes *et al.*, 2003), suggesting that the phosphorylated form of P_{II} preferentially stimulates NtcA-dependent transcription following nitrogen step-down.

The fine-tuning of NtcA activity by P_{II} was investigated by using *luxAB* reporter fusions to the *glnB* promoter, which is regulated by NtcA (Aldehni *et al.*, 2003). In a truncated version of the *glnB* promoter, in which the constitutive, σ^{70} -dependent start site 1 was deleted, retaining only the NtcA-dependent start site (*tsp2*), differential P_{II} dependency of reporter gene (luciferase) activity could be detected. In the presence of ammonium, *luxAB* expression was repressed to very low levels in both the wild-type and the P_{II}-deficient mutant. In the presence of nitrate, *luxAB* was moderately expressed in the wild-type, but considerably derepressed in the P_{II}-null mutant. When cells were shifted to nitrogen-depleted conditions, promoter activity increased rapidly to very high levels in the wild-type, whereas the P_{II} mutant failed to increase reporter activity further. Together, these observations suggest the following functional relationships between P_{II} and NtcA. (i) In the presence of nitrate, the P_{II}-deficient mutant exhibits significantly higher NtcA-dependent reporter gene activity than the wild-type, suggesting that P_{II} is inhibitory for NtcA under those conditions. This confirms older studies showing a partial derepression of nitrogen-regulated enzymes in the P_{II}-deficient background in the presence of nitrate (Forchhammer & Tandeau de Marsac, 1995b). (ii)

The high activation of NtcA following nitrogen step-down requires P_{II}. In addition, activation of NtcA by phosphorylated P_{II} occurs only in the absence of nitrate, suggesting that an additional factor is needed to distinguish between the presence of nitrate and the absence of any nitrogen source. To clarify these points, the mechanism of functional interaction between NtcA and P_{II} must be determined.

Reciprocally to the effect of P_{II} on NtcA activation, NtcA affects P_{II} under nitrogen-limited conditions. In the NtcA-deficient mutant, phosphorylation of the P_{II} protein is drastically affected: almost no phosphorylation occurs after nitrogen step-down (Lee *et al.*, 1999; Sauer *et al.*, 1999), suggesting that P_{II} kinase might be under NtcA control. Furthermore, transcription of the P_{II}-encoding *glnB* gene is strongly enhanced by activated NtcA under conditions of nitrogen deprivation (see above). These mutual interactions result in a positive feedback loop: phosphorylated P_{II} activates NtcA; in turn, activated NtcA augments the levels of the NtcA and P_{II} proteins as well as stimulating P_{II} phosphorylation (see Fig. 3).

Cross-talk between signalling pathways

Both specific and general acclimation responses have been demonstrated in cyanobacteria. However, categorizing the responses in this way, while conceptually relevant, may be an oversimplification from a mechanistic point of view. For example, a modulator of specific responses may also be involved in the control of an apparently general response. Support for such a scenario is provided by the aberrant phycobilisome degradation exhibited by the NtcA mutant, specifically during nitrogen starvation (Sauer *et al.*, 1999); however, when chlorosis is induced by sulfur starvation, NtcA mutants acclimate similarly to the wild-type. Furthermore, induction of the *nblA* gene following nitrogen step-down is partially impaired in an NtcA-deficient mutant (Luque *et al.*, 2001). This is consistent with the *nblA* gene containing a complex promoter with several transcriptional start points and multiple non-perfect NtcA binding sites. These data indicate that efficient transcriptional induction of *nblA* under nitrogen limitation requires NtcA in addition to the global nutrient-responsive element NblR. Furthermore, modulators of general responses may engage in cross-talk to induce specific responses. For example the histidine kinase NblS, which is required for modulation of *nblA*, is involved in transcriptional control of genes responding to changes in light intensity. Additionally, the alternative sigma factor, SigC, is involved in expression of the *glnB* gene during the stationary phase of growth (Asayama *et al.*, 2004). The cross-talk between signalling cascades may be even more elaborate than is currently believed, orchestrating coordination of the cellular responses under a variety of stress conditions.

Future prospects

Much knowledge has been accumulated regarding the cellular responses enabling cyanobacterial cells to cope with

nutrient starvation. Nevertheless, several topics still await experiments that would pave the way for detailed clarification of the mechanisms underlying these responses. As described, our current knowledge suggests mechanisms for sensing the cellular nitrogen status. However, the signals for sulfur and phosphorus status have not been elucidated, nor have the triggers for the general acclimation responses. Having in mind the common signalling pathway modulating degradation of the phycobilisome, one may similarly assume a common physiological or biochemical consequence of starvation for each specific nutrient that serves to induce the general pathway. To date, most of the studies concerning general acclimation responses have been focused on modulation of the photosynthetic apparatus. Clearly, additional aspects such as control of the cell cycle, DNA protection, modulation of membrane permeability, detoxification of reactive oxygen species, and others, may contribute to cell survival. Laboratory studies of starvation usually employ a rapid transition to nutrient-deficient conditions. This sort of protocol probably induces a set of responses that may not be identical to those induced when the cells experience gradual nutrient depletion. Tools for global analyses of gene expression have already been employed to investigate the responses of *Synechocystis* towards temperature, redox, light, salt, iron and carbon stresses and will certainly also broaden our understanding of N/P/S-starvation and help to further elucidate the complex network of specific and general responses.

Studies of heterotrophic eubacteria have highlighted phenomena related to stress physiology, which, although not yet documented for cyanobacteria, may be relevant to their acclimation strategy. Bacterial cultures at the so-called 'stationary phase' of growth were shown to be highly dynamic; clones having a growth advantage at the stationary phase (GASP) tend to take over the population (Finkel & Kolter, 1999). Another example is quorum sensing, the expression of genes under density-dependent control, mediated by bacterial pheromones and crucial for numerous stress-related bacterial responses (Bassler, 2002). Although quorum sensing has not been documented for cyanobacteria, this mechanism may present a novel aspect of their acclimation responses based on intra-species interaction and communal behaviour.

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