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Transcriptional effects of the signal transduction protein P_{II} (*glnB* gene product) on NtcA-dependent genes in *Synechococcus* sp. PCC 7942

Javier Paz-Yepes, Enrique Flores*, Antonia Herrero

Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC-Universidad de Sevilla, Avda. Américo Vespucio s/n, 41092 Sevilla, Spain

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Abstract **P_{II} proteins signal the cellular nitrogen status in numerous bacteria, and in cyanobacteria P_{II} is subjected to serine phosphorylation when the cells experience a high C to N balance. In the unicellular cyanobacterium *Synechococcus* sp. PCC 7942, the P_{II} protein (*glnB* gene product) is known to mediate the ammonium-dependent inhibition of nitrate and nitrite uptake. The analysis of gene expression through RNA/DNA hybridization indicated that a P_{II}-null mutant was also impaired in the induction of NtcA-dependent, nitrogen assimilation genes *amt1* (ammonium permease), *glnA* (glutamine synthetase) and *nir* (nitrite reductase), as well as of the N-control gene *ntcA*, mainly under nitrogen deprivation. This gene expression phenotype of the *glnB* mutant could be complemented by wild-type P_{II} protein or by modified P_{II} proteins that cannot be phosphorylated and mimic either the phosphorylated ($GlnB^{S49D}$ and $GlnB^{S49E}$) or unphosphorylated ($GlnB^{S49A}$) form of P_{II}. However, strains carrying the $GlnB^{S49D}$ and $GlnB^{S49E}$ mutant proteins exhibited higher levels of expression of nitrogen-regulated genes than the strains carrying the wild-type P_{II} or the $GlnB^{S49A}$ protein.**

Key words: Cyanobacteria; GlnB; Nitrogen assimilation genes; Nitrogen control

1. Introduction

P_{II} signal transduction proteins (*glnB* or *glnK* gene products) are widespread in prokaryotes where they act as sensors of the cellular nitrogen status through their binding of 2-oxoglutarate (the carbon skeleton into which N is incorporated) in the presence of ATP (for recent reviews, see [1,2]). The P_{II} proteins appear to have a remarkably wide spectrum of targets among assimilatory nitrogen metabolism proteins regulating gene transcription as well as enzyme activity [2]. In the best characterized system, *Escherichia coli* GlnB participates in the adenylylation-dependent inactivation of glutamine synthetase as well as in the regulation of expression of the *glnA* gene mediated by the NtrB–NtrC two-component regulatory system [1,2]. On the other hand, the second P_{II} paralog GlnK has recently been shown to behave as an inhibitor of the *E. coli* ammonium permease, AmtB [3]. The activity of the P_{II} proteins is influenced by their covalent modification, which takes place under low nitrogen conditions and, in the enterobacteria, consists of uridylylation of a Tyr residue [1,2].

In the O_2-producing photosynthetic bacteria, the cyanobacteria, a P_{II} protein of the GlnB type [2] was originally identified in *Synechococcus* sp. PCC 6301 [4]. Although P_{II} has been found in a number of cyanobacteria (e.g., *Synechococcus* sp. PCC 7942 [5], *Synechocystis* sp. PCC 6803 [6,7], *Nostoc punctiforme* [8]), it has been better characterized in the unicellular strain PCC 7942 [9–12]. Under low nitrogen (or, rather, low N/C balance) conditions, *Synechococcus* P_{II} is phosphorylated at Ser^{49}, and phosphorylation of one, two or the three subunits of the P_{II} trimer takes place in response to increasing N deficiency [9,10]. *Synechococcus* P_{II} synergistically binds 2-oxoglutarate and ATP so that liganded P_{II} appears to be the substrate of a kinase and unliganded P_{II}-P of a phosphatase (for details, see [11,12]). The P_{II}-P phosphatase from *Synechocystis* sp. PCC 6803 has recently been characterized [13,14]. In *Synechococcus* sp. PCC 7942, the P_{II} protein is essential for the ammonium-promoted post-translational inhibition of the ABC-type nitrate–nitrite permease [15]. An investigation using *Synechococcus* strains carrying mutated versions of P_{II} ($GlnB^{S49A}$, which mimics the unphosphorylated form of the protein, and $GlnB^{S49D}$ and $GlnB^{S49E}$, which would mimic the phosphorylated form) has indicated that the unphosphorylated form of P_{II} is always inhibitory for nitrate uptake, whereas the phosphorylated form can be inhibitory or non-inhibitory depending on the incubation conditions of the cells and probably reflecting an effect of 2-oxoglutarate on P_{II} [16]. In contrast to this well documented effect on the regulation of the activity of a permease, no transcriptional role could be inferred for P_{II} in *Synechococcus* sp. PCC 7942, since a P_{II}-null mutant (strain MP2) grows on nitrate as well as on ammonium and its levels of *nir* operon transcripts are similar to those of the wild type for both nitrate- and ammonium-grown cells [15]. It should be noted, however, that low *nir* operon transcript levels have been reported for the strains carrying the $GlnB^{S49D}$ and $GlnB^{S49E}$ mutations that, nonetheless, exhibit normal nitrate uptake activities [16]. Because of the frequent involvement of P_{II} proteins in systems that regulate transcription of nitrogen assimilation genes, in this work we pursued an analysis of the possible involvement of P_{II} in regulation of the expression of nitrogen assimilation genes in *Synechococcus* sp. PCC 7942.

Nitrogen-regulated genes in *Synechococcus* sp. PCC 7942 include, among others, the *nir* operon encoding nitrate assimilation proteins, in which the *nir* gene encoding nitrite reductase is the first gene, *glnA* encoding glutamine synthetase, *amt1* encoding a (methyl)ammonium permease, and *ntcA*

*Corresponding author. Fax: (34)-954-460065.
E-mail address: flores@cica.es (E. Flores).

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[17]. NtcA is an autoregulatory transcription factor that activates expression of nitrogen assimilation genes when the cyanobacterial cells are incubated in the absence of ammonium [18]. NtcA belongs to the CAP family of bacterial transcriptional regulators [19] and binds to defined sites in the promoters of the regulated genes ([20], for a review see [17]). The *nir* operon and the *amt1*, *glnA*, and *ntcA* genes are transcribed from NtcA-activated promoters [17,20,21]. NtcA appears to respond to the cellular N status of the cell, and the activities of in vitro DNA binding and transcription activation by NtcA are stimulated by 2-oxoglutarate [22,23]. In cyanobacteria, 2-oxoglutarate has the main metabolic role of serving as a substrate for the incorporation of ammonium [24] and reflects the C to N balance of the cell [25]. However, the mechanism by which 2-oxoglutarate influences NtcA activity is not yet known, and additional mechanisms of N control by 2-oxoglutarate in cyanobacteria are possible.

2. Materials and methods

2.1. Organisms and growth conditions

Synechococcus sp. PCC 7942 was grown axenically in the light (85 μE m^{-2} s^{-1}) at 30°C in medium $BG11_0C$ (BG11 medium [26] lacking $NaNO_3$ and supplemented with 0.84 g $NaHCO_3$ l^{-1}) supplemented with 8 mM NH_4Cl and 16 mM TES–NaOH buffer (pH 7.5). For mutants, the medium was supplemented with 10 μg Km ml^{-1} (strain MP2) or with 10 μg Km ml^{-1} and 2 μg Sm ml^{-1} (strains MP2S, MP2A, MP2D, and MP2E). The cultures were bubbled with a mixture of CO_2 (1% v/v) and air. At the mid-exponential phase of growth (2 μg of chlorophyll *a* ml^{-1}), the cells were harvested at room temperature, washed twice with $BG11_0C$, resuspended in BG11C or $BG11_0C$, and incubated under culture conditions with CO_2-enriched air (as above) for the times indicated for each experiment. The concentration of chlorophyll *a* of the cultures was determined in methanolic extracts of the cells [27].

2.2. RNA isolation and analysis

RNA was isolated from 40-ml samples of the cultures as previously described [6], and the obtained preparation was treated with RNase-free DNase (Roche) at 0.3 U μl^{-1}. For Northern blots, 20 μg of RNA were loaded per lane and electrophoresed in 1% agarose denaturing formaldehyde gels. Hybridization with the probes described below was done at 65°C in 5×SSPE (1×SSPE is 0.18 M NaCl, 10 mM sodium phosphate and 1 mM EDTA, pH 7.4), 5×Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 100 μg herring sperm DNA ml^{-1}. Filters were washed twice for 10 min each at 65°C with 2×SSPE and 0.1% SDS and once for 15 min at 65°C with 1×SSPE and 0.1% SDS. Alternatively, some hybridizations were performed at 65°C in 1 mM EDTA, 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS, and the filters were washed at 65°C with, successively, 2×SSC (1×SSC is 150 mM NaCl and 15 mM sodium citrate dihydrate) and 0.1% SDS, 1×SSC and 0.1% SDS, and 0.5×SSC and 0.1% SDS. Similar results were obtained with both hybridization protocols. Radioactive areas in Northern blots were visualized and quantified with a Cyclone storage phosphor system (Packard). Quantification was performed using windows covering all hybridization signals for each sample. The data obtained were then normalized using the *rnpB* signals (see below). For each gene, the data for the different samples in a filter were compared assigning a value of 1 to the data of the sample giving the strongest hybridization signals. This normalization in turn allowed comparison of the data from different filters.

2.3. DNA probes

DNA probes for *Synechococcus* sp. PCC 7942 genes were generated by PCR using as templates plasmids carrying the cloned genes (except for *psbA1*, which was amplified from genomic DNA) and oligonucleotide primers that produced the following gene fragments: *amt1* (from bp 13 to bp 345 with respect to the start of the coding sequence), *glnA* (from bp 37 to bp 392), *nir* (from bp 12 to bp 348), *ntcA* (from bp 25 to bp 374), and *psbA1* (from bp 111 to bp 997). As control of RNA loading and transfer efficiency, the filters were reprobed with a 0.57-kb *Xho*I–*Pst*I fragment that contains the RNase P RNA gene (*rnpB*) from *Synechococcus* sp. PCC 7942 [28]. Probes were labeled with a DNA labeling kit (Ready to Go, Amersham Pharmacia Biotech) and [α-^{32}P]dCTP.

3. Results

3.1. Short term P_{II} effects

Because the phosphorylation degree of P_{II} in *Synechococcus* sp. PCC 7942 has been shown to respond rapidly (within minutes) to changes in the incubation conditions of the cells [9–12], induction of nitrogen assimilation genes was tested in short term experiments in a P_{II}-null mutant, strain MP2 (*glnB*::Km^r), and the wild-type strain PCC 7942. RNA was isolated from ammonium-grown cells and from cells subjected to induction in media supplemented with nitrate as the nitrogen source or containing no source of nitrogen. These RNA preparations were hybridized with probes of the N-regulated, NtcA-dependent genes *amt1*, *glnA*, *nir*, and *ntcA*. For comparison, expression of the *psbA1* gene encoding a photosystem II D1 polypeptide [29] that appears not to be under N regulation in *Synechococcus* sp. PCC 7942 [30] was also analyzed, and hybridization with a probe of the *rnpB* (ribonuclease P RNA) gene was used as an RNA loading and transfer control. The results obtained are shown in Fig. 1. In the wild-type strain, as previously described, the *amt1* and *glnA* genes generated monocistronic transcripts [20,21], the *ntcA* gene, which is 666 bp long, generated transcripts of 0.8 kb and larger [20], and the *nir* probe detected a range of RNA molecules likely resulting from degradation of the *nir* operon transcript that

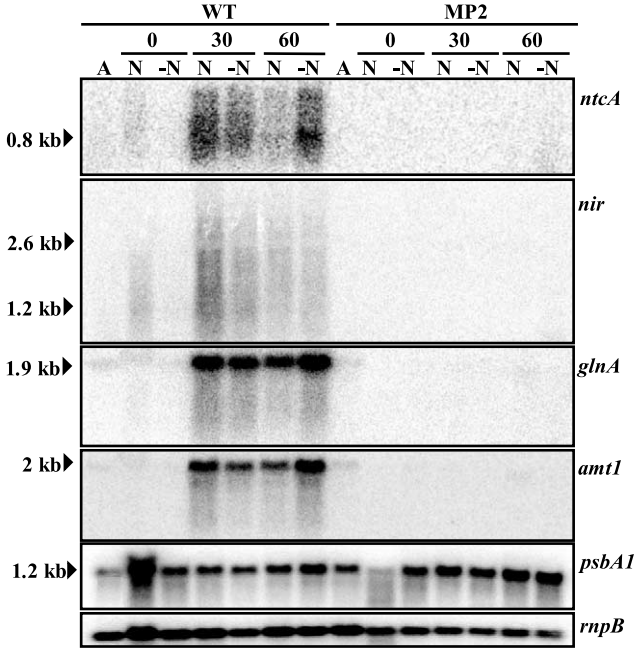

Fig. 1. Analysis of short term induction of N-regulated genes and *psbA1* in *Synechococcus* sp. strains PCC 7942 (WT) and MP2 (*glnB*::Km^r). RNA was isolated from ammonium-grown cells (A) or from ammonium-grown cells incubated for the indicated times (in minutes) in medium lacking any added nitrogen source (−N) or supplemented with 17.6 mM $NaNO_3$ (N). RNA preparations were then subjected to electrophoresis and hybridized with probes of the indicated genes. This experiment was performed four times with independent RNA preparations, and the results of a representative experiment are shown. Some transcript sizes are indicated on the left.

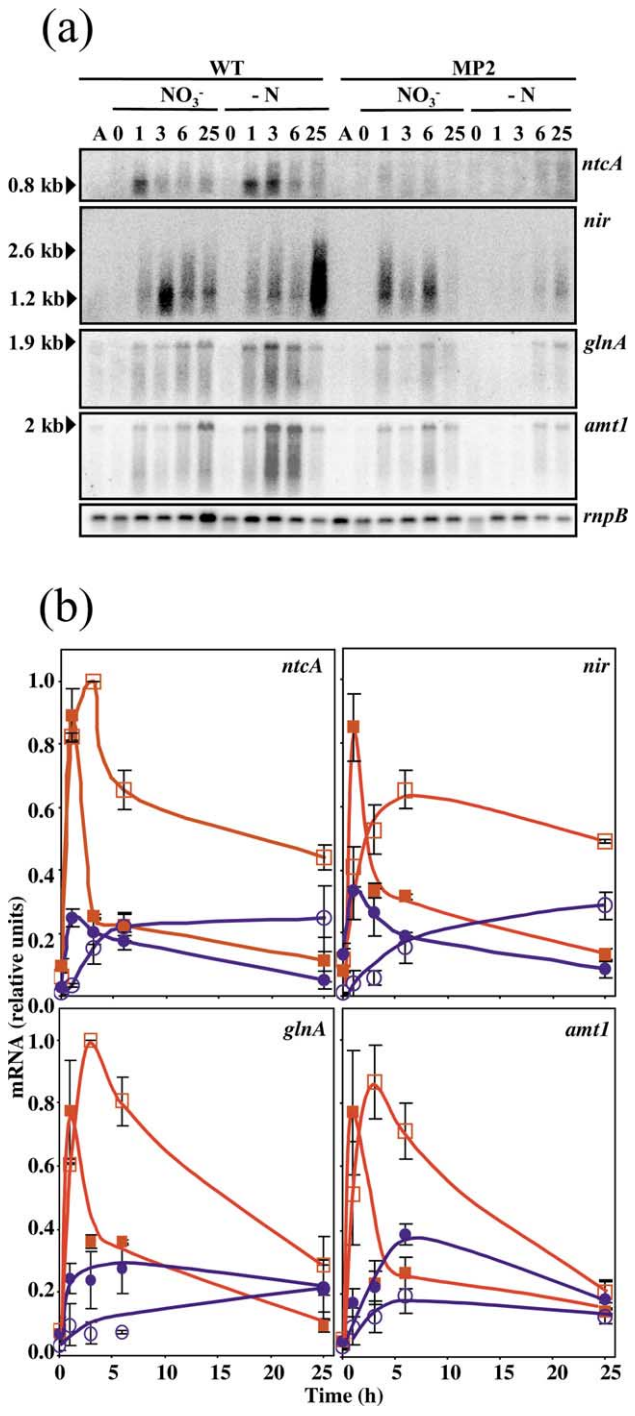

Fig. 2. Analysis of long term induction of nitrogen-regulated genes in *Synechococcus* sp. strains PCC 7942 and MP2 (*glnB*::Km^r). RNA was isolated from ammonium-grown cells (A) or from ammonium-grown cells incubated for the indicated times (in hours) in medium lacking any nitrogen source (−N) or supplemented with 17.6 mM $NaNO_3$ (N). RNA preparations were then subjected to electrophoresis and hybridized with probes of the indicated genes. This experiment was performed four times with independent RNA preparations. The results of a representative experiment are shown in (a), where some transcript sizes are indicated on the left, and relative transcript levels are presented in (b) as the mean of the data from the different hybridizations that were performed for each gene (standard deviations indicated by vertical bars). Wild type (red): nitrate (filled squares), −N (open squares); MP2 (blue): nitrate (filled circles), −N (open circles).

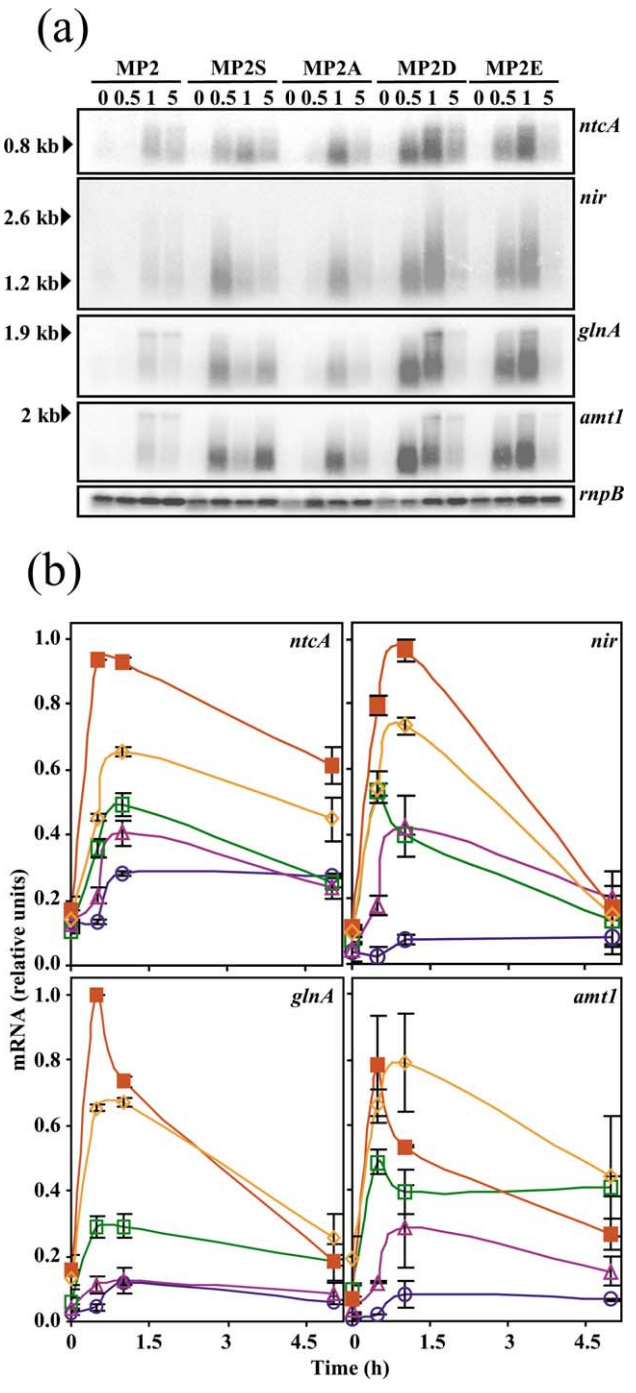

Fig. 3. Analysis of expression of N-regulated genes in *Synechococcus* sp. MP2 (*glnB*::Km^r) and derivative strains carrying in a heterologous genome location a *glnB* gene encoding wild-type P_{II} (MP2S), $GlnB^{S49A}$ (MP2A), $GlnB^{S49D}$ (MP2D) or $GlnB^{S49E}$ (MP2E). RNA was isolated from ammonium-grown cells incubated for the indicated times (in hours) in medium lacking any nitrogen source. RNA preparations were then subjected to electrophoresis and hybridized with probes of the indicated genes. This experiment was performed four times with independent RNA preparations. The results of a representative experiment are shown in (a), where some transcript sizes are indicated on the left, and relative transcript levels are presented in (b) as the mean of the data from the different hybridizations that were performed for each gene (standard deviations indicated by vertical bars). MP2 (circles, blue); MP2S (open squares, green); MP2A (triangles, purple); MP2D (filled squares, red); MP2E (diamonds, orange).

would be of about 9 kb [20,31]. Induction of genes in the wild type was already observed after 30 min of incubation in the absence of ammonium (with or without nitrate), but no strong induction of any of the N-regulated genes was observed in the MP2 mutant (Fig. 1). As an example, quantification of the hybridization signals from all the experiments performed with the *amt1* gene indicated expression levels in the MP2 mutant of about 20%, at 30 min of incubation, and of about 25%, at 60 min, of those obtained for the wild type. In contrast, a high level expression of *psbA1* was observed for both strains PCC 7942 and MP2 in the presence or absence of nitrate (Fig. 1).

Because MP2 cells adapted to grow on nitrate express the *nir* operon normally [15], we investigated expression of the N-regulated genes in long term induction experiments. In nitrate-containing medium, in the first hour of incubation, a burst of expression of the N-regulated genes took place in the wild type that was not observed, or was much diminished, in the MP2 mutant (Fig. 2). However, after 3 h of incubation, similar or not notably different expression levels were observed for the two strains. In contrast, in the absence of combined nitrogen, transcript levels for the different genes were much lower in the MP2 mutant than in the wild type for at least the first 6 h of incubation, with a difference between the two strains being still evident after 25 h (Fig. 2). These results indicated that inactivation of *glnB* affected expression of N-regulated genes mainly under N deprivation.

3.2. Response of P_{II} Ser^{49} mutants towards nitrogen step-down

Synechococcus strains MP2S, MP2A, MP2D and MP2E are derivatives of mutant MP2 that carry a *glnB* gene encoding wild-type P_{II}, $GlnB^{S49A}$, $GlnB^{S49D}$ or $GlnB^{S49E}$, respectively, inserted in a genome location different from the natural *glnB* site [16]. RNA was isolated from cells of cultures of these strains subjected to an ammonium to −N transition, and these RNA preparations were hybridized with probes of the *ntcA*, *nir*, *glnA*, *amt1*, and *rnpB* genes (Fig. 3). A relatively low level of expression of the N-regulated genes was observed in strain MP2 in the 5-h induction period, but inclusion of the wild-type *glnB* gene allowed the observation of higher levels of expression, specially at 30 and 60 min, for the different tested genes (see strain MP2S in Fig. 3). Complementation of the MP2 mutant phenotype of impaired expression of N-regulated genes was also obtained with the $GlnB^{S49D}$ and $GlnB^{S49E}$ mutant proteins. Indeed, transcript levels in strains MP2D and MP2E were higher than in strain MP2S for the whole incubation period in the case of the *ntcA* gene, or at least for the first hour of incubation in the case of *amt1*, *glnA* and *nir*. Complementation was also observed with the $GlnB^{S49A}$ protein, but transcript levels in strain MP2A were in general lower than in strain MP2S, specially in the shorter incubation periods, being close to those of mutant MP2 in the case of the *glnA* gene (Fig. 3). The mutant forms of P_{II} expressed in strains MP2A, MP2D and MP2E cannot be phosphorylated [16], but whereas $GlnB^{S49D}$ and $GlnB^{S49E}$ would mimic a phosphorylated form of P_{II}, $GlnB^{S49A}$ would represent a fixed unphosphorylated form.

4. Discussion

Results presented above indicate that the P_{II} protein is required in *Synechococcus* sp. PCC 7942 for acclimation of ammonium-grown cells to N step-down, which consists of induction of genes that would improve incorporation of low levels of ammonium (*amt1* and *glnA*) or permit assimilation of alternative nitrogen sources like nitrate or nitrite (the *nir* operon). Expression of these genes is known to require activation of their promoters by the cyanobacterial global N-control transcription factor NtcA [17], which in *Synechococcus* sp. PCC 7942 also activates expression of its own gene [20]. As shown in this work, expression of *ntcA* is also impaired in the P_{II}-null mutant MP2. In contrast to these N-regulated genes, expression of the photosystem II gene *psbA1* is not affected in the MP2 mutant, emphasizing the specificity of the P_{II} effects on nitrogen assimilation genes. Consistent with the fact that MP2 can grow using nitrate as a nitrogen source [10], similar expression levels of the regulated genes were observed in the MP2 mutant and the wild type in longer periods of incubation of the cells in the presence of nitrate (Fig. 2; see also [15]). However, in media lacking any source of combined nitrogen, expression of the N-regulated genes in the MP2 mutant is in general lower than in the wild type, even after a prolonged incubation of the cells. Hence, the transcriptional effect of P_{II} in *Synechococcus* sp. PCC 7942 appears to be primarily concerned with the response and acclimation of cells to N step-down, including the period of adaptation to nitrate-containing medium. Consistently, accumulation of glutamine synthetase III, the product of the *glnN* gene that is only expressed under conditions of nitrogen deprivation in *Synechococcus* sp. PCC 7942, has been shown to be impaired under nitrogen deficiency in strain MP2 [32].

Because the P_{II} effect takes place on NtcA-dependent genes, P_{II} could function facilitating or enhancing the response of NtcA. The mechanism of P_{II} action in transcriptional regulation in *Synechococcus* sp. PCC 7942 is unknown and, given the very different reported ways of action of P_{II} in various biological systems [2], different mechanisms are possible, from a direct effect on NtcA to an indirect effect via an intermediary protein factor. The fact that the $GlnB^{S49A}$ protein is, at least partially, effective in promoting gene expression is reminiscent, for instance, of the GlnK effect on NifL in *Klebsiella pneumoniae*. Under N-limiting conditions, GlnK relieves, independently of its uridylylation status, NifL inhibition of the *nif* gene transcriptional activator NifA [33]. However, the strong induction of N-regulated genes observed in strains MP2D and MP2E suggests a role of phosphorylation in determining the positive effect of P_{II} on transcription of the NtcA-dependent genes. In *Synechococcus* sp. PCC 7942, the trimeric P_{II} protein is known to occur in four different forms depending on its number of phosphorylated subunits, from none to three [9,10], and reaching a high degree of phosphorylation requires an about 1-h incubation period in the absence of nitrogen [10]. Strains MP2D and MP2E carry proteins $GlnB^{S49D}$ and $GlnB^{S49E}$, respectively, which would mimic fully phosphorylated P_{II} proteins. It is possible that the strong induction of N-regulated genes observed in strains MP2D and MP2E reflects the presence in the cells of such phosphorylated-like proteins from the start of the N step-down. Because, however, induction requires removal of ammonium, some factor(s) other than P_{II} phosphorylation must affect NtcA-dependent transcription under nitrogen deprivation. In this context, it is of interest that both P_{II} and NtcA appear to respond to 2-oxoglutarate and could therefore synergistically respond to this C to N balance signal. Expression of NtcA-dependent genes in nitrate-containing medium in strain MP2 indicates,

however, that the NtcA function does not necessarily require P_{II}.

The *glnB* gene is transcribed from two promoters in *Synechococcus* sp. PCC 7942, a constitutive promoter that ensures the presence of P_{II} protein in ammonium-grown cells and an inducible, NtcA-dependent promoter that determines the production of enhanced levels of P_{II} under low N conditions [34]. The positive effect of P_{II} on NtcA-activated transcription under N deprivation described in this work additionally implies positive autoregulation of the *glnB* gene.

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