Role of the *Synechococcus* PCC 7942 nitrogen regulator protein PipX on NtcA controlled processes

Javier Espinosa [1], Karl Forchhammer[2], and Asunción Contreras[1]*

[1] División de Genética, Universidad de Alicante, Apartado 99, E-03080 Alicante, Spain

[2] Institut für Mikrobiologie und Molekularbiologie, Universität Giessen, Heinrich-Buff-Ring 26-32, D 35392 Giessen, Germany

*Corresponding author. Mailing address: División de Genética, Facultad de Ciencias, Universidad de Alicante, Apartado 99, E-03080 Alicante, Spain. Phone: 34 96 590 3957. Fax: 34 96 590 9569. E-mail: contrera@ua.es

ABSTRACT

The *Synechococcus* sp. PCC 7942 nitrogen regulator PipX interacts in a 2-oxoglutarate dependent manner with the global nitrogen transcription factor NtcA and the signal transduction protein P_{II}. *In vivo*, PipX is involved in the NtcA-dependent induction of *glnB* and *glnN* genes. To further investigate the extent to which PipX is involved in global nitrogen control, the effect of *pipX* inactivation on various nitrogen regulated processes was determined. The PipX deficient mutant was able to use nitrate as a nitrogen source and to efficiently inhibit the nitrate transport upon ammonium addition but showed reduced nitrate and nitrite reductase activities and a delay in the induction of nitrate utilization after transfer of cultures from ammoniun to nitrate containing media. In contrast to the wild-type, glutamine synthetase activity was not upregulated upon depletion of combined-nitrogen from cultures of the mutant strain. Inactivation of *pipX* impaired induction of *nblA* and delayed phycobilisome degradation, but did not affect recovery of nitrogen-deprived cultures. Taken together, the results obtained in this work indicate that PipX interacts with NtcA to facilitate efficient acclimation of cyanobacteria to conditions of nitrogen limitation.

INTRODUCTION

Cyanobacteria are phototrophic organisms that perform oxygenic photosynthesis. Autotrophic growth requires the constant assimilation of ammonium via the glutamine synthetase-glutamate synthase (GS-GOGAT) cycle (Muro-Pastor *et al.*, 2005), resulting in consumption of the carbon-skeleton of 2-oxoglutarate to yield glutamate. GS, which catalyses the incorporation of ammonium into glutamate to yield glutamine is the key enzyme of nitrogen assimilation. Two types of GS are produced by the non-diazotrophic cyanobacterium *Synechococcus sp.* PCC 7942 (hereafter called *Synechococcus*): GSI, a typical eubacterial GS encoded by *glnA*, and GSIII, encoded by *glnN*. The GSIII class is strongly induced in nitrogen-depleted cultures and appears to play a role in acclimation to conditions of nitrogen starvation (Sauer *et al.*, 2000). Due to the lack of 2-oxoglutarate dehydrogenase in cyanobacteria, synthesis of 2-oxoglutarate represents the final step in the oxidative branch of the TCA cycle and directly links 2-oxoglutarate levels to nitrogen assimilation (Herrero *et al.*, 2001). Therefore, the cellular 2-oxoglutarate concentration is an excellent indicator of the cell carbon to nitrogen balance. 2-oxoglutarate modulates the activity and/or binding properties of three key cyanobacterial nitrogen regulators: the signal transduction protein P_{II}, the transcriptional activator NtcA, and the regulatory factor PipX (Forchhammer, 2004; Espinosa *et al.*, 2006).

In cyanobacteria, multiple metabolic and developmental processes are induced by nitrogen starvation. NtcA, the global regulator for nitrogen control, activates genes involved in nitrogen assimilation, heterocyst differentiation and acclimation to nitrogen starvation (Herrero *et al.*, 2001; Luque *et al.*, 2001; Sauer *et al.*, 2000). NtcA belongs to the CAP/CRP (the catabolite activator protein or cyclic AMP receptor protein) family of transcriptional activators. 2-oxoglutarate stimulates complex formation between PipX

and NtcA (Espinosa *et al.*, 2006), binding of NtcA to target sites (Vazquez-Bermudez et al., 2002) and transcription activation *in vitro* (Tanigawa et al., 2002). NtcA plays a key role in cyanobacterial nitrogen assimilation, being required for the expression of multiple genes repressed by ammonium, the preferred nitrogen source (Herrero *et al.*, 2001). Genes or operons directly regulated by NtcA in *Synechococcus* include the *nirA* operon, encoding the *nirA-nrtABCD-narB* genes for nitrate assimilation, the *nirB* operon, encoding genes for maximum nitrate assimilation, the *glnA* and *glnN* genes for GSI and GSII, respectively, the *glnB* for P_{II}, the *nblA* gene required for phicobilisome degradation (Luque *et al.*, 2001) and the *ntcA* gene itself. In agreement with this, *Synechococcus* NtcA deficient mutants are highly pleiotropic, being unable to assimilate nitrate, upregulate GS (Vega-Palas *et al.*, 1990), or acclimate appropriately to nitrogen starvation (Sauer *et al.*, 1999).

The P_{II} signal transduction protein is one of the most conserved and widespread signal transduction proteins in nature and plays key roles in nitrogen assimilatory processes (Arcondeguy *et al.*, 2001; Ninfa & Jiang, 2005). Signal transduction by P_{II} (*glnB* product) in cyanobacteria displays unique features both with respect to covalent modification as well as to downstream signalling (Forchhammer, 2004). Physiological studies showed that P_{II} mediates the short term ammonium inhibition of nitrate transport (Lee *et al.*, 2000) and controls nitrate uptake in response to light availability (Kloft & Forchhammer, 2005). Furthermore, nitrate reductase in *Synechococcus* may be directly regulated by P_{II} (Takatani *et al.*, 2006). However, in these cases, no physical interaction with P_{II} was reported. Using yeast two-hybrid approaches, three cyanobacterial P_{II} receptors could recently be identified: The enzyme N-acetyl-glutamate kinase (NAGK) (Burillo *et al.*, 2004; Heinrich *et al.*, 2004) and the regulatory factor PipX (Burillo *et al.*, 2004; Espinosa *et al.*, 2006) in *Synechococcus*, and the non conserved membrane

protein PamA in *Synechocystis* sp. PCC 6803 (Osanai *et al.*, 2005). The physiological role of the P_{II} interaction with these protein receptors is only understood in the case of NAGK. The P_{II}-NAGK regulatory interaction has been conserved across domains of life during the evolution of oxygenic photosynthetic organisms (Burillo *et al.*, 2004; Chen *et al.*, 2006; Sugiyama *et al.*, 2004). P_{II} stimulates NAGK activity and relieves arginine inhibition to different extents in *Synechococcus* and *Arabidopsis thaliana* (Chen et al., 2006; Heinrich et al., 2004; Maheswaran et al., 2004).

Binding of PipX to P_{II} and NtcA is inversely affected by 2-oxoglutarate (Espinosa *et al.*, 2006). Complex formation between PipX and P_{II} was impaired by 2-oxoglutarate in the presence of ATP, while the binding of PipX to NtcA could only be observed in the presence of 2-oxoglutarate. *In vivo*, PipX activated NtcA-dependent promoters *glnB* and *glnN* under conditions of nitrogen deficiency, corresponding to high intracellular levels of 2-oxoglutarate, supporting a role for PipX in the activation of NtcA-dependent promoters under nitrogen starvation. In order to investigate the involvement of PipX on nitrogen assimilation and acclimation to nitrogen deficiency, we now analyze the impact of the genetic inactivation of *pipX* on previously characterized NtcA-controlled processes.

METHODS

Molecular genetic techniques and growth conditions

The strains and plasmids used in this work are listed in Table 1. Cloning procedures were carried out with *E. coli* DH5α, using standard techniques. *Synechococcus* strains were grown photoautotrophically at 30ºC while shaking under constant illumination (75 μE m^{-2} s^{-1}) provided by cool white fluorescent lights in BG11 medium (nitrate-containing), $BG11_0$ (no added nitrogen) or BG11-NH_4 ($BG11_0$ supplemented with 10 mM NH_4Cl, 10 mM Hepes/NaOH pH 7.8). For growth on plates, the medium was solidified by addition of 1% (w/v) agar. Plates were incubated at 30ºC under constant illumination. For initiation of nitrogen deprivation, mid exponential cultures (OD_{750nm} of 0,5) were harvested by centrifugation, washed twice with $BG11_0$ and finally resuspended in $BG11_0$. Whenever appropriate, cultures contained kanamycin (10 μg ml^{-1}) or chloramphenicol (5 μg ml^{-1}).

Construction of plasmids and *luxAB* reporter strains

To construct plasmid pUAGC103, a 206 bp fragment corresponding to the upstream region of the *nblA* gene was PCR amplified using primers 5′-GGCGCTGCCTGGGAAAGTCACGCC-3′ and 5′-GGAGCCTCCGGCACTGCAGATG-3′ and cloned into the neutral site II targeting vector pAM1580. Plasmids pRK2013 and pRL443 were used, respectively, as a helper and conjugative plasmid to transfer pUAGC103 into *Synechococcus* strains (Elhai *et al.*, 1997). Transformants were selected on chloramphenicol containing BG11 or BG11-NH_4 plates.

Enzymatic activities

Nitrate uptake activity was assayed by measuring nitrate depletion from the external medium as described previously (Lee *et al.*, 1998). BG11 cultures grown to mid

exponential phase were used to determine nitrate and nitrite reductase activities at 30 °C using dithionite-reduced methyl viologen as the electron donor. Nitrate reductase activity (Herrero *et al.*, 1985) was measured in permeabilized cells by including mixed alkyltrimethylammonium bromide (MTA) in the reaction mixture at a final concentration of 50 $\mu g \cdot ml^{-1}$. Nitrite reductase activity (Herrero & Guerrero, 1986) was also assayed in cells made permeable with MTA (250 $\mu g \cdot ml^{-1}$). Production (nitrate reductase) or disappearance (nitrite reductase) of nitrite during the reactions was measured as described previously (Snell & Snell, 1949). One unit of enzymatic activity corresponded, respectively, to 1 µmol of nitrite formed or disappeared per min.

Glutamine synthetase activity (transferase assay) from mid-exponential cultures was measured in permeabilized cells as described (Bender *et al.*, 1977).

Determination of chlorophyll *a*

Chlorophyll *a* concentration was determined from methanolic extracts as described (Mackinney, 1941).

Determination of luciferase activity

To determine bioluminescence, 1 ml of cultures was adjusted with fresh medium to an OD_{750nm} of 0.5, supplemented with decanal to a final concentration of 0.25 mM from a 50 mM stock solution made up in 10% dimethyl sulfoxide. Light emission was recorded in a Berthold LB9509 luminometer. Bioluminescence was recorded every 20 seconds for 10 minutes. Light emission increased to a maximum and then declined. Maximum luminescence at the peak, presented as RLU (Relative light Units) by the instrument, is the value used at each selected time point.

Determination of pigments contents spectrophotometrically

Wild type and $PipX^-$ strains were grown in BG11 or BG11-NH_4 until they reached mid exponential phase (OD_{750nm} 0.4-0.5) and then cultures were centrifuged, washed and

finally resuspended in $BG11_0$. A whole-cell absorbance spectrum was carried out in order to estimate pigment contents. 1ml of cultures was taken at indicated times, diluted with fresh medium to an optical density at 750nm of 0.5 and absorbance spectra (550-750nm) was recorded on a UV/Visible Ultrospec 3100 pro (Amersham). Pigment content was calculated based on absorbance maxima at 631nm for phycocyanin and 684nm for chlorophyll *a*.

RESULTS

Nitrate assimilation in the PipX$^-$ strain

The expression of genes required for nitrate assimilation in *Synechococcus* depends on NtcA. Therefore, NtcA-deficient strains do not grow on nitrate, have undetectable nitrite reductase activity and low levels of nitrate reductase (Vega-Palas *et al.*, 1990). In addition to the NtcA-dependent induction in response to the absence of ammonium, the *nirA* operon is also subjected to activation by the nitrite-dependent activator NtcB (Aichi *et al.*, 2004; Maeda *et al.*, 1998). The fact that the PipX$^-$ strain grows well in nitrate-containing medium, where it was originally selected, appeared at odds with our previous suggestions of PipX cooperating with NtcA-dependent activation of target promoters (Espinosa *et al.*, 2006). To investigate the involvement of PipX in the regulation of the nitrate assimilation genes, we analyzed the functions encoded by the *nirA* operon in a PipX-deficient mutant.

Nitrate and nitrite activities were assayed in cells from the wild type and PipX$^-$ strains adapted to nitrate. As shown in Fig. 1, nitrate and nitrite reductase activities were significantly reduced in the PipX$^-$ mutant, thus supporting the implication of PipX in positive regulation of the *narB* and *nirA* genes. Nitrate consumption by PipX$^-$ strains grown in nitrate-containing media was significantly slower than nitrate consumption by the wild-type strain (compare Fig. 2A and B), thus indicating that PipX deficiency has a negative effect on nitrate assimilation. As it is the case with wild-type cells, addition of ammonium to PipX$^-$ cells caused an immediate inhibition of nitrate utilization (Fig. 2 A and B) indicating that posttranslational regulation of nitrate assimilation by P_{II} does not require PipX.

Since ammonium-repressed cultures of *Synechococcus* can not utilize nitrate, the rate of nitrate assimilation after transfer of ammonium-grown cultures to medium

containing nitrate was compared between wild type and PipX$^-$ cultures. Nitrate consumption was determined three hours after shifting ammonium-repressed cultures to nitrate-containing medium. The NtcA$^-$ strain, which is unable to utilize nitrate, was included as a negative control. As shown in Fig. 2C, the PipX$^-$ culture removed nitrate from the medium at a rate significantly lower than the wild type strain, thus strongly suggesting that PipX has a role on the induction of the *nirA* operon.

Taken together, the results indicate that the *Synechococcus* PipX$^-$ mutant assimilates nitrate with less efficiency than its wild type counterpart. The defect is more pronounced in cultures previously grown with ammonium, suggesting that PipX is required for rapid acclimation to ammonium withdrawal. On the other hand, the finding that the PipX$^-$ mutant retains the ability to tune down nitrate uptake efficiently when the nitrate-adapted cultures are suddenly faced with ammonium, suggests that PipX is not required for the regulation of P_{II} functions under conditions of nitrogen sufficiency (low levels of 2-oxoglutarate).

Glutamine synthetase activity is positively regulated by PipX

Nitrogen assimilation by *Synechococcus* cells requires glutamine synthetase activity, provided mainly by GSI, and under nitrogen deficiency conditions, also by GSIII. To determine the impact of *pipX* inactivation on global glutamine synthetase activity, we compared the ability of wild type and PipX deficient strains of *Synechococcus* to induce glutamine synthetase activity upon transfer of cultures from ammonium or nitrate supplemented media to media lacking a combined nitrogen source. Comparison of wild type and PipX$^-$ strains indicated that PipX is required for appropriated induction of glutamine synthetase activity (Fig. 3). Glutamine synthetase levels were significantly lower in the mutant strain, an effect observed when the nitrogen source of cultures was either nitrate (compare time 0 in Fig. 3A) or ammonium

(compare time 0 in Fig. 3B). In addition, no induction of glutamine synthetase activity was observed in the PipX deficient strain when cultures were deprived of nitrogen, thus indicating an important deficiency of glutamine synthetase activity in the PipX$^-$ mutant. The basal levels of glutamine synthetase activity found in the PipX$^-$ strain were indistinguishable from those of the NtcA$^-$ strain grown with ammonium or in cells starved for nitrogen up to 2 days (data not shown).

Therefore, PipX exerts a positive role in the nitrogen regulation of the glutamine synthetase activity in *Synechococcus*. As it is the case with NtcA, PipX is required for both, basal levels of glutamine synthetase activity (as seen in ammonium-grown cultures) and for appropriated upregulation of glutamine synthetase under nitrogen limiting conditions.

PipX is involved in induction of chlorosis but not in the recovery of cultures from nitrogen starvation

When *Synechococcus* cultures are subjected to nutrient limitation and other stress conditions, they modify the composition of the photosynthetic machinery by degrading their light-harvesting antennae, the phycobilisomes (Schwarz & Forchhammer, 2005). The loss of phycobilisomes and reduction of the chlorophyll *a* content are responsible for the yellow appearance of the chlorotic cultures. This acclimation process, known as chlorosis or bleaching, requires strong expression of the *nblA* gene, which is subject to positive control by NtcA (Luque *et al.*, 2001) and NblR, a response regulator involved in general acclimation (Schwarz & Grossman, 1998). NblR-deficiency results in a strong *non bleaching* phenotype, but the NtcA deficient mutant is still able to enter chlorosis although it shows a delay in phycobilisome degradation and a faster reduction of the chlorophyll *a* content (Sauer *et al.*, 1999).

As shown in Fig. 4A, nitrogen deprived cultures of the PipX null mutant showed the same phenotype than the NtcA$^-$ strain, a weak and transient *non-bleaching* appearence. Pigment analysis revealed the same pattern of pigment degradation that have been reported for the NtcA strain under nitrogen starvation, a delay in phycocyanin degradation and a faster loss of chlorophyll a content (Fig. 4B-4D). As expected for a protein specifically involved in 2-oxoglutarate signaling, induction of chlorosis by sulfur starvation was not affected in the PipX$^-$ strain (data not shown).

NtcA is required for cell survival of chlorotic cultures, since NtcA$^-$ cells subjected to nitrogen starvation rapidly loose the ability to reinitiate growth on nitrogen containing media (Sauer *et al.*, 1999). To determine the involvement of PipX in cell survival after chlorosis, wild type, NtcA$^-$ and PipX$^-$mutant strains were tested for their ability to survive up to 9 days in nitrogen-depleted media. In contrast with the NtcA-strain, the ability of the PipX$^-$ strain to resume growth was very similar to that of the wild type strain (Fig. 5), indicating that PipX is not required for the NtcA-dependent survival of nitrogen starved cultures.

PipX activates the *nblA* gene under nitrogen deficiency

To confirm that the delayed phycocyanin degradation in cultures of the PipX$^-$ strain during nitrogen induced chlorosis was related to defects in NtcA-dependent activation of the *nblA* gene, promoter fusions to *luxAB* reporter genes were used to analyze the consequences of *pipX* inactivation on the nitrogen dependent induction of the *nblA* gene. The P_{nblA}::*luxAB* construct included the previously characterized regulatory region containing five transcription start sites, three NtcA binding sites, and putative NblR binding sites (Luque *et al.*, 2001). It also includes a recently reported putative light-responsive element (HLR1) involved in negative regulation (Kappell *et al.*, 2006).

Reporter expression was determined by bioluminiscence measurements from cultures of wild type, NtcA$^-$ and PipX$^-$ mutant derivatives grown with ammonium and shifted to nitrogen depleted medium. As shown in Fig. 6, the nitrogen starvation response was impaired in both the NtcA$^-$ and PipX$^-$ mutants but the extent of the impairment was different. Consistent with previous results using *luxAB* fusions to other NtcA dependent promoters (Espinosa *et al.*, 2006), the PipX contribution to *nblA* upregulation was very significant, but smaller than that of NtcA, indicating that PipX is also involved in the NtcA-dependent transcriptional induction of the *nblA* gene.

DISCUSSION

The results presented here provide insight into the role of PipX in nitrogen control. *Synechococcus* PipX$^-$ cells showed reduced activity of nitrogen assimilation enzymes (glutamine synthetase, nitrate reductase and nitrite reductase), retarded induction and slower rate of nitrate consumption, and, when subjected to nitrogen starvation, retarded phycobilisome degradation and a faster reduction of the chlorophyll content. Therefore, the PipX-deficient mutant shows a pleiotropic phenotype reminiscent of that of the NtcA$^-$ strains. For some traits the PipX$^-$ and NtcA$^-$ phenotypes were very similar, while for others the PipX$^-$ phenotype was less severe than the NtcA$^-$. Assays for glutamine synthetase activity (Fig. 3 and data not shown) and induction of chlorosis (Fig. 4A and data not shown) gave similar results with the two mutant strains. Nitrate assimilation, an ability lost in the NtcA$^-$ strain, was impaired but still operative in the PipX deficient mutant (Fig. 2). Given that, at promoters strictly dependent of NtcA for activation, PipX plays a positive but non essential role for transcription (Espinosa *et al.*, 2006), it is not surprising that PipX$^-$ mutants display a less severe or leaky NtcA$^-$ phenotype. The auxiliary role of PipX at NtcA dependent promoters can also be inferred from induction experiments with the P_{nblA}::*luxAB* reporter fusion (Fig. 6). The nitrogen-specific role of PipX in chlorosis strongly suggests that PipX participates in the NtcA-dependent activation of the *nblA* gene. The finding that PipX is not required for survival of cultures after nitrogen starvation (Fig. 5) also supports the view that PipX is involved in the early induction, but not in the long-time maintenance, of NtcA activity.

Taken together, the results presented here strongly suggest that, in addition to the previously analyzed *glnB* and *glnN* genes (Espinosa *et al.*, 2006) and the *nblA* promoter studied here (Fig. 6), operons or genes for nitrate assimilation (*nirA* and

probably *nirB*), glutamine synthesis (*glnA*) and additional genes for pigment regulation are also under PipX control. Thus, the relatively small but representative sample of NtcA-dependent genes analyzed so far at the level of transcriptional induction and/or protein activity, demonstrate the importance of PipX in global nitrogen regulation. The mechanism and molecular details involved in transcriptional activation by PipX at NtcA-dependent promoters is presently being addressed.

In vivo, PipX-NtcA and PipX-P_{II} complexes are likely to form, respectively, under high and under low intracellular levels of 2-oxoglutarate. Consistent with this, the genetic analyses performed so far show the implication of PipX in NtcA activation when the intracellular 2-oxoglutarate levels rise as a consequence of combined-nitrogen depletion from cultures. On the other hand, complex formation between PipX and the P_{II} protein should be expected when cultures are faced with ammonium. Since the P_{II}-dependent inhibition of the nitrate uptake exerted by ammonium remained functional in the PipX$^-$ strain (Fig. 2B), the PipX protein does not play a role in the P_{II}-mediated regulation of NRT. Considering that *Synechococcus* P_{II} proteins are very abundant, able to interact with different partners, and sense and adopt different conformations and modification status according to the nitrogen/carbon ratio, it seems more likely that PipX-P_{II} complexes serve to modulate PipX availability and/or activity. In other words, although the physiological significance of the PipX-P_{II} complexes remain to be established, the experimental evidences gathered so far favor the idea of PipX being a P_{II} target rather than a P_{II} regulator.

ACKNOWLEDGEMENTS

We thank are grateful to R. Blasco for technical help, I. Luque for constructing plasmid pUAGC103, the Ministerio de Educación y Ciencia for grants BMC2002-01156 and BFU2005-02231 and a predoctoral fellowship to J.E., to the Generalitat Valenciana for grant GV04B-525 and to the Deutsche Forschungsgemeinschaft for grant Fo195/4.

FIGURE LEGENDS

Figure 1

Nitrate and nitrite reductase activities from *Synechococcus* cells. Mean values and standard deviations from five independent assays are represented. Nitrate reductase (grey bars), nitrite reductase (white bars). Wild type (WT), PipX null mutant ($PipX^-$).

Figure 2

Nitrate uptake from *Synechococcus* strains. Nitrate taken up ($\mu moles.mg^{-1}$ Chl *a*) is plotted as a function of time. Uptake assays were performed on nitrate grown cells from (A) wild-type or (B) $PipX^-$ cultures incubated for 0–30 min in the presence of nitrate, with (open symbols) or without (closed symbols) addition of ammonium. Mean values and standard deviations from four independent assays are represented. (C) Induction of nitrate uptake from ammonium repressed cultures. Nitrate taken up was measured during 60 minutes on previously ammonium-grown cells, which were incubated in ammonium-free, nitrate supplemented BG11 medium for 3 hours. Mean values and standard deviations from 4 independent assays are represented. Wild type (WT), PipX null mutant ($PipX^-$), NtcA null mutant ($NtcA^-$).

Figure 3

Glutamine synthetase activity from *Synechococcus* strains. Cultures were grown in BG11 (A) and BG11-NH_4 (B), transferred to $BG11_0$ and assayed immediately (time 0) and at 16 and 24 hours. Glutamine synthetase (transferase) activity is given in Units (μmoles per minute) per mg of chlorophyll *a*. Mean values and the corresponding standard deviations from three independent experiments are reported. Wild type, (grey bars) $PipX^-$ (white bars)

Figure 4

Pigment degradation by *Synechococcus* strains. (A) Cultures of wild-type and mutant derivatives grown in BG11-NH_4 (N+) or incubated in $BG11_0$ (–N) for 48 and 96 h. (B) Absorbance spectra from wild type (orange) and $PipX^-$ (blue) strains in BG11-NH_4 (+) or incubated in $BG11_0$ (-) for 2, 4 and 10 days. The absorbance peak of phycocyanin (PC), and chlorophyll *a* (Chl) are indicated. Groups of spectra were shifted along the *y* axis for clarity. (C) Relative amount of phycocyanin as a function of time. (D) Relative amount of Chlorophyll *a* as a function of time. Wild type (WT); PipX null mutant ($PipX^-$).

Figure 5

Survival of *Synechococcus* strains after nitrogen starvation. Drops of cultures incubated for 4 to 9 days on $BG11_0$ medium were spotted onto BG11-NH_4 plates and incubated for 2 weeks. Wild type (WT), PipX null mutant ($PipX^-$), NtcA null mutant ($NtcA^-$).

Figure 6

Time course of P_{nblA}::*luxAB* induction in *Synechococcus* strains. Cells were grown in the presence of ammonia and when they reached mid exponential phase, they were shifted to combined-nitrogen free medium. After the initiation of nitrogen deprivation at time point 0, bioluminescence from the reporter strains was recorded. A representative time course experiment is shown from 3 independent experiments that yielded similar results. Wild type (WT), PipX null mutant ($PipX^-$), NtcA null mutant ($NtcA^-$).

BIBLIOGRAPHY

Aichi, M., Maeda, S., Ichikawa, K. & Omata, T. (2004). Nitrite-responsive activation of the nitrate assimilation operon in Cyanobacteria plays an essential role in up-regulation of nitrate assimilation activities under nitrate-limited growth conditions. *J Bacteriol* **186**, 3224-3229.

Andersson, C. R., Tsinoremas, N. F., Shelton, J., Lebedeva, N. V., Yarrow, J., Min, H. & Golden, S. S. (2000). Application of bioluminescence to the study of circadian rhythms in cyanobacteria. In *Methods in Enzymology*, pp. 527-542.

Arcondeguy, T., Jack, R. & Merrick, M. (2001). P(II) signal transduction proteins, pivotal players in microbial nitrogen control. *Microbiol Mol Biol Rev* **65**, 80-105.

Bender, R. A., Janssen, K. A., Resnick, A. D., Blumenberg, M., Foor, F. & Magasanik, B. (1977). Biochemical parameters of glutamine synthetase from *Klebsiella aerogenes*. *J Bacteriol* **129**, 1001-1009.

Burillo, S., Luque, I., Fuentes, I. & Contreras, A. (2004). Interactions between the nitrogen signal transduction protein P_{II} and N-acetyl glutamate kinase in organisms that perform oxygenic photosynthesis. *J Bacteriol* **186**, 3346-3354.

Chen, Y. M., Ferrar, T. S., Lohmeir-Vogel, E., Morrice, N., Mizuno, Y., Berenger, B., Ng, K. K., Muench, D. G. & Moorhead, G. B. (2006). The P_{II} signal transduction protein of *Arabidopsis thaliana* forms an arginine-regulated complex with plastid N-acetyl glutamate kinase. *J Biol Chem* **281**, 5726-5733.

Elhai, J., Vepritskiy, A., Muro-Pastor, A. M., Flores, E. & Wolk, C. P. (1997). Reduction of conjugal transfer efficiency by three restriction activities of *Anabaena* sp. strain PCC 7120. *J Bacteriol* **179**, 1998-2005.

Espinosa, J., Forchhammer, K., Burillo, S. & Contreras, A. (2006). Interaction network in cyanobacterial nitrogen regulation: PipX, a protein that interacts in a 2-oxoglutarate dependent manner with P_{II} and NtcA. *Mol Microbiol* **61**, 457-469.

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A* **76**, 1648-1652.

Forchhammer, K. (2004). Global carbon/nitrogen control by P_{II} signal transduction in cyanobacteria: from signals to targets. *FEMS Microbiol Rev* **28**, 319-333.

Hanahan, D. (1985). Techniques for transformation of *Escherichia coli*. In *DNA cloning*, pp. 109–135. Edited by e. Glover D. Oxford, UK: IRL Press.

Heinrich, A., Maheswaran, M., Ruppert, U. & Forchhammer, K. (2004). The *Synechococcus elongatus* P_{II} signal transduction protein controls arginine synthesis by complex formation with N-acetyl-L-glutamate kinase. *Mol Microbiol* **52**, 1303-1314.

Herrero, A. & Guerrero, M. G. (1986). Regulation of nitrite reductase in the cyanobacterium *Anacystis nidulans*. *J. Gen. Microbiol.* **132**, 2463–2468.

Herrero, A., Flores, E. & Guerrero, M. G. (1985). Regulation of nitrate reductase cellular levels in the cyanobacteria *Anabaena variabilis* and *Synechocystis* sp. *FEMS Microbiology Letters* **26**, 21–25.

Herrero, A., Muro-Pastor, A. M. & Flores, E. (2001). Nitrogen control in cyanobacteria. *J Bacteriol* **183**, 411-425.

Kappell, A. D., Bhaya, D. & van Waasbergen, L. G. (2006). Negative control of the high light-inducible *hliA* gene and implications for the activities of the NblS sensor kinase in the cyanobacterium *Synechococcus elongatus* strain PCC 7942. *Arch Microbiol*.

Kloft, N. & Forchhammer, K. (2005). Signal transduction protein P_{II} phosphatase PphA is required for light-dependent control of nitrate utilization in *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **187**, 6683-6690.

Lee, H. M., Flores, E., Herrero, A., Houmard, J. & Tandeau de Marsac, N. (1998). A role for the signal transduction protein P_{II} in the control of nitrate/nitrite uptake in a cyanobacterium. *FEBS Lett* **427**, 291-295.

Lee, H. M., Flores, E., Forchhammer, K., Herrero, A. & Tandeau De Marsac, N. (2000). Phosphorylation of the signal transducer P_{II} protein and an additional effector are required for the P_{II}-mediated regulation of nitrate and nitrite uptake in the Cyanobacterium *Synechococcus* sp. PCC 7942. *Eur J Biochem* **267**, 591-600.

Luque, I., Zabulon, G., Contreras, A. & Houmard, J. (2001). Convergence of two global transcriptional regulators on nitrogen induction of the stress-acclimation gene nblA in the cyanobacterium *Synechococcus* sp. PCC 7942. *Mol Microbiol* **41**, 937-947.

Mackinney, G. (1941). Absorption of light by chlorophyll solutions. *J. Biol. Chem.* **140**, 315-322.

Maeda, S., Kawaguchi, Y., Ohe, T. A. & Omata, T. (1998). cis-acting sequences required for NtcB-dependent, nitrite-responsive positive regulation of the nitrate assimilation operon in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J Bacteriol* **180**, 4080-4088.

Maheswaran, M., Urbanke, C. & Forchhammer, K. (2004). Complex formation and catalytic activation by the P_{II} signaling protein of N-acetyl-L-glutamate kinase from *Synechococcus elongatus* strain PCC 7942. *J Biol Chem* **279**, 55202-55210.

Muro-Pastor, M. I., Reyes, J. C. & Florencio, F. J. (2005). Ammonium assimilation in cyanobacteria. *Photosynth Res* **83**, 135-150.

Ninfa, A. J. & Jiang, P. (2005). P_{II} signal transduction proteins: sensors of alpha-ketoglutarate that regulate nitrogen metabolism. *Curr Opin Microbiol* **8**, 168-173.

Osanai, T., Sato, S., Tabata, S. & Tanaka, K. (2005). Identification of PamA as a P_{II}-binding membrane protein important in nitrogen-related and sugar-catabolic gene expression in *Synechocystis* sp. PCC 6803. *J Biol Chem* **280**, 34684-34690.

Sauer, J., Gorl, M. & Forchhammer, K. (1999). Nitrogen starvation in *Synechococcus* PCC 7942: involvement of glutamine synthetase and NtcA in phycobiliprotein degradation and survival. *Arch Microbiol* **172**, 247-255.

Sauer, J., Dirmeier, U. & Forchhammer, K. (2000). The *Synechococcus* strain PCC 7942 *glnN* product (glutamine synthetase III) helps recovery from prolonged nitrogen chlorosis. *J Bacteriol* **182**, 5615-5619.

Schwarz, R. & Grossman, A. R. (1998). A response regulator of cyanobacteria integrates diverse environmental signals and is critical for survival under extreme conditions. *Proc Natl Acad Sci U S A* **95**, 11008-11013.

Schwarz, R. & Forchhammer, K. (2005). Acclimation of unicellular cyanobacteria to macronutrient deficiency: emergence of a complex network of cellular responses. *Microbiology* **151**, 2503-2514.

Snell, F. D. & Snell, C. T. (1949). Colorimetric Methods of Analysis. pp. 804-805: Van Nostrand, New York.

Sugiyama, K., Hayakawa, T., Kudo, T., Ito, T. & Yamaya, T. (2004). Interaction of N-acetylglutamate kinase with a P_{II}-like protein in rice. *Plant Cell Physiol* **45**, 1768-1778.

Takatani, N., Kobayashi, M., Maeda, S. & Omata, T. (2006). Regulation of Nitrate Reductase by Non-Modifiable Derivatives of P_{II} in the Cells of *Synechococcus elongatus* Strain PCC 7942. *Plant Cell Physiol* **47**, 1182-1186.

Tanigawa, R., Shirokane, M., Maeda Si, S., Omata, T., Tanaka, K. & Takahashi, H. (2002). Transcriptional activation of NtcA-dependent promoters of *Synechococcus* sp. PCC 7942 by 2-oxoglutarate in vitro. *Proc Natl Acad Sci U S A* **99**, 4251-4255.

Vazquez-Bermudez, M. F., Herrero, A. & Flores, E. (2002). 2-Oxoglutarate increases the binding affinity of the NtcA (nitrogen control) transcription factor for the *Synechococcus glnA* promoter. *FEBS Lett* **512**, 71-74.

Vega-Palas, M. A., Madueno, F., Herrero, A. & Flores, E. (1990). Identification and cloning of a regulatory gene for nitrogen assimilation in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J Bacteriol* **172**, 643-647.

TABLE 1. Strains and plasmids.

Strain or Plasmid	Relevant characteristics	Source or reference
E. coli DH5α	F^- ϕ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1* λ^-	(Hanahan 1985)
Synechococcus sp. PCC7942	Wild-type *Synechococcus sp.* PCC 7942	Pasteur culture collection
Synechococcus SA591	$PipX^-$ (kanamycin resistance cartridge inserted into the *pipX* gene of strain PCC 7942)	(Espinosa *et al.* 2006)
Synechococcus MNtcA	$NtcA^-$ (kanamycin resistance cartridge inserted into the *ntcA* gene of strain PCC 7942	(Sauer *et al.* 1999)
Synechococcus NblR45	$PipX^-$ (kanamycin resistance cartridge inserted into the *nblR* gene of strain PCC 7942)	(Luque *et al.* 2001)
Synechococcus WT-C103	Cm^R derivative of strain PCC7942; P_{nblA}*::luxAB* inserted into chromosome neutral site	This work
Synechococcus SA591-C103	Cm^R derivative of strain SA591; P_{nblA}*::luxAB* inserted into chromosome neutral site	This work
Synechococcus MNtcA-C103	Cm^R derivative of strain MNtcA; P_{nblA}*::luxAB* inserted into chromosome neutral site	This work
pAM1580	Amp^r Cm^r derivative of pAM1573 plasmid carrying *luxAB* reporter genes.	(Andersson *et al.* 2000)
pRK2013	Km^r Helper plasmid	(Figurski and Helinski 1979)
pRL443	Amp^r Conjugal plasmid	(Elhai *et al.* 1997)
pUAGC103	pAM1580 derivative with P_{nblA}::*luxAB* fusion	This work

Fig.1

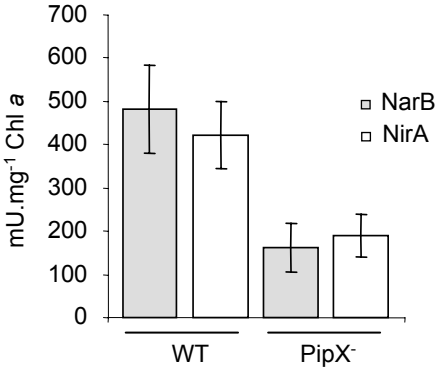

700
600
500
400
300
200
100
0
mU.mg-1 Chl a
NarB
NirA
WT
PipX-

Fig.2

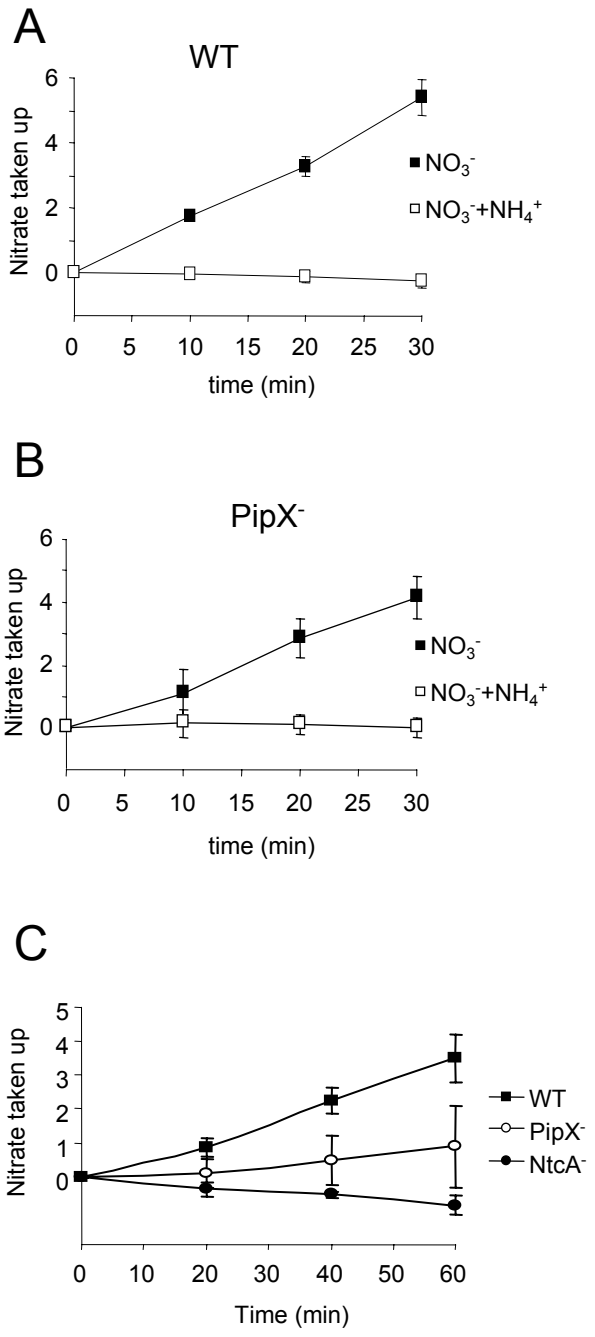

A
WT
Nitrate taken up
6
4
2
0
NO3-
NO3-+NH4+
0 5 10 15 20 25 30
time (min)
B
PipX-
Nitrate taken up
6
4
2
0
NO3-
NO3-+NH4+
0 5 10 15 20 25 30
time (min)
C
Nitrate taken up
5
4
3
2
1
0
WT
PipX-
NtcA-
0 10 20 30 40 50 60
Time (min)

Fig. 3

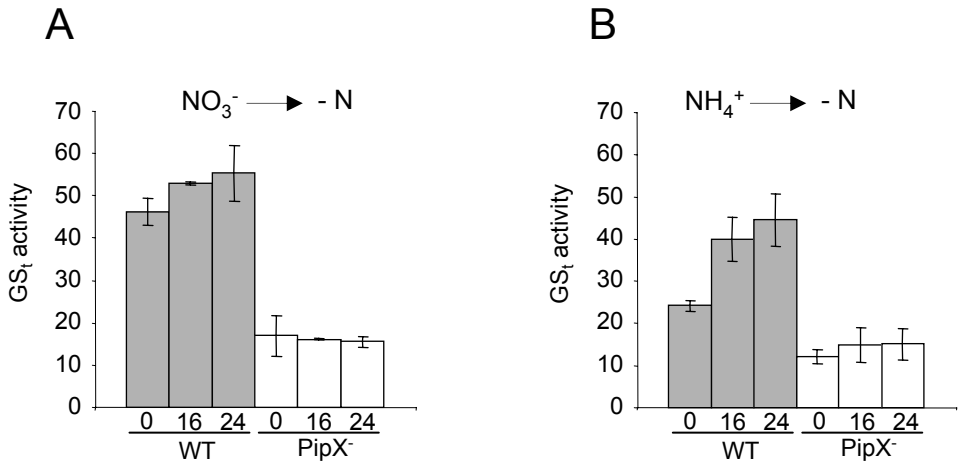

A
B
NO_3^- → - N
NH_4^+ → - N
GS_t activity
70
60
50
40
30
20
10
0
0 16 24 0 16 24
WT
PipX-

Fig.4

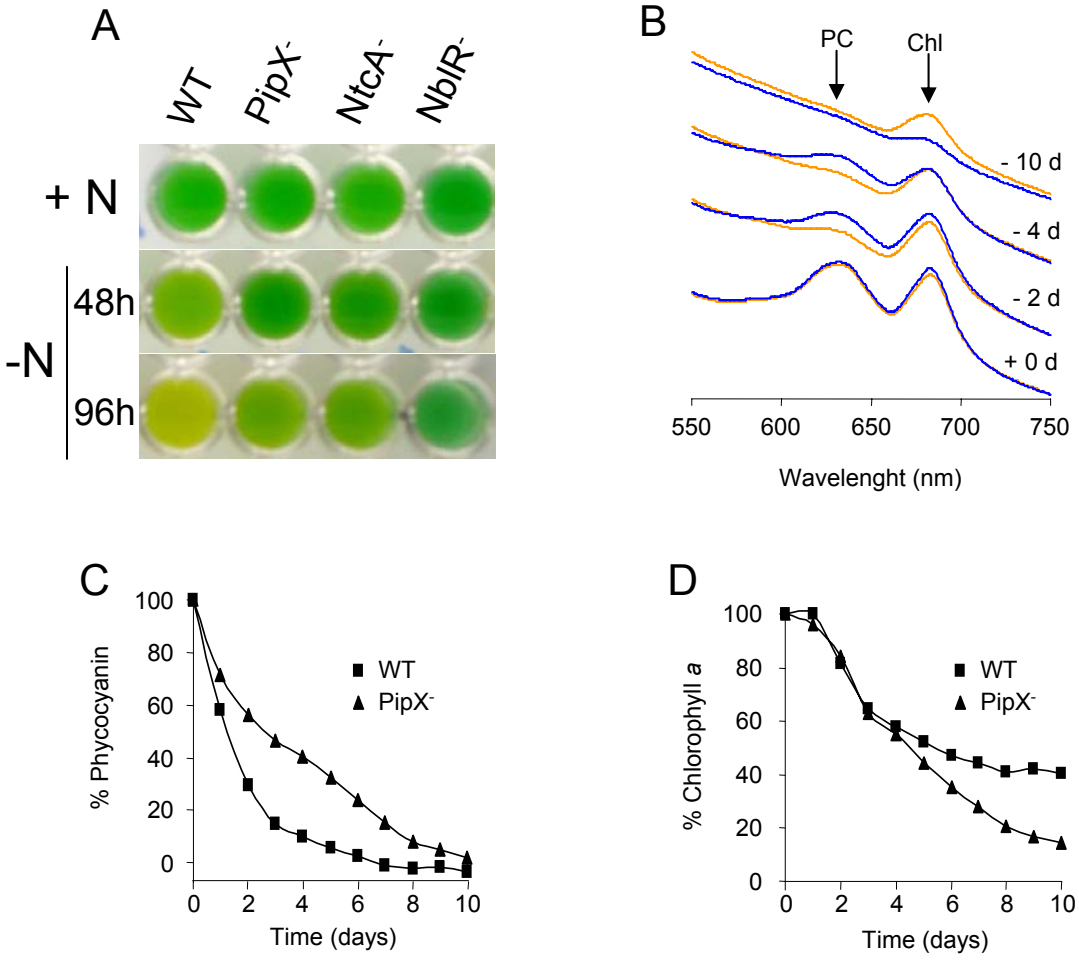

A
WT
PipX-
NtcA-
NblR-
+ N
-N
48h
96h
B
PC
Chl
- 10 d
- 4 d
- 2 d
+ 0 d
550 600 650 700 750
Wavelenght (nm)
C
% Phycocyanin
100
80
60
40
20
0
WT
PipX-
0 2 4 6 8 10
Time (days)
D
% Chlorophyll a
100
80
60
40
20
0
WT
PipX-
0 2 4 6 8 10
Time (days)

Fig.5

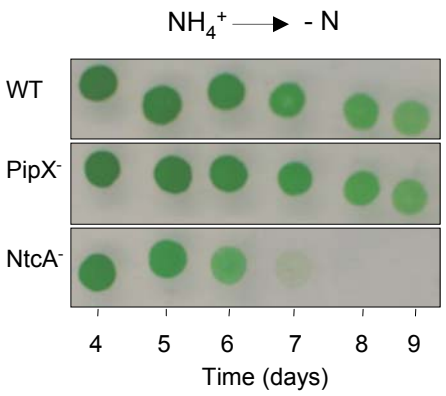
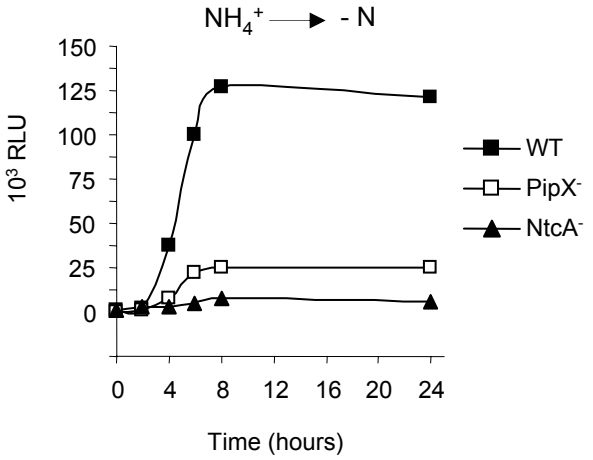

NH4+ ⟶ - N
WT
PipX-
NtcA-
4
5
6
7
8
9
Time (days)

Fig.6


NH4+ ⟶ - N
150
125
100
75
50
25
0
10^3 RLU
0
4
8
12
16
20
24
Time (hours)
WT
PipX-
NtcA-