INTRODUCTION

Survival and proliferation of bacteria in natural habitats depends on the ability to rapidly acclimatize to changes in the environment, which requires sensitive signal perception and signal transduction systems. Genomic sequences of bacteria, and in particular of cyanobacteria, have revealed a remarkably large number of genes which code for homologues of eukaryotic-type signalling proteins, such as protein-serine/threonine and protein-tyrosine kinases as well as various types of protein phosphatases (Ponting et al., 1996), but only a few of them have a known physiological function (reviewed by Bork et al., 1996), but only a few. Among the protein phosphatases, members of the protein phosphatase 2C (PP2C) family are particularly abundant (Bork et al., 1996), but only a few of them have a known physiological function (reviewed by Shi, 2004). Recently, the function of a novel PP2C member could be identified in the cyanobacterium Synechocystis PCC 6803. The enzyme, termed PphA, catalyses the dephosphorylation of the phosphorylated signal transduction protein PII (Irmler & Forchhammer, 2001).

Abbreviations: C, inorganic carbon; GS, glutamine synthetase; IF, GS inactivating factor; MSX, L-methionine-DL-sulfoximine; pNPP, p-nitrophenyl phosphate; PP2C, protein phosphatase 2C.

The phosphorylated signal transduction protein PII (PII-P) in the cyanobacterium Synechocystis sp. strain PCC 6803 is dephosphorylated by PphA, a protein phosphatase of the 2C family (PP2C). In this study, the physiological conditions of PII-P dephosphorylation were investigated with respect to the in vivo specificity of PII-P towards PphA and the cellular abundance of PphA in cells growing under different nitrogen regimes. Furthermore, the consequences of impaired PII-P dephosphorylation with respect to short-term inhibition of glutamine synthetase (GS) were studied. With a contribution of approximately 15% of total Mn2+-dependent p-nitrophenyl phosphate hydrolysis activity, PphA has only a minor impact on the total PP2C activity in Synechocystis extracts. Nevertheless, residual PII-P dephosphorylation in PphA-deficient cells could only be observed after prolonged incubation in the presence of ammonium. The abundance of PphA correlates with the phosphorylation state of PII under nitrogen-replete conditions and is specifically enhanced by nitrite. Regulation of pphA expression operates at the post-transcriptional level. In the presence of nitrate/nitrite, PphA is present in molar excess over PII-P, enabling the cells to rapidly dephosphorylate PII-P in response to changing environmental conditions. A PphA-deficient mutant is not impaired in short-term inhibition of GS activity following ammonium treatment. Down-regulation of GS occurs by induction of gfl genes (encoding GS inactivating factors 7 and 17), which is controlled by NtcA-mediated gene repression. Thus, impaired PII-P dephosphorylation does not affect ammonium-prompted inactivation of NtcA.
cyanobacteria: the non-phosphorylated form of PII forms a tight complex with N-acetylglutamate kinase (NAGK), the key enzyme of arginine biosynthesis (Heinrich et al., 2004; Burillo et al., 2004). PII stimulates NAGK activity by an order of magnitude, and thus subjects arginine synthesis to global C/N control through the dependence on the phosphorylation state of PII (Heinrich et al., 2004; Maheswaran et al., 2004).

Insights into the mechanism of the PII phosphorylation/dephosphorylation cycle in *Synechococcus elongatus* could be obtained by biochemical and physiological studies (reviewed by Forchhammer, 1999, 2004). The *S. elongatus* PII protein binds the effector molecules 2-oxoglutarate and ATP in a synergistic manner (Forchhammer & Hedler, 1997). In the ATP and 2-oxoglutarate bound state, PII is phosphorylated by an ATP-dependent kinase activity (Forchhammer & Tandeau de Marsac, 1995a). Dephosphorylation of PII-P is catalysed by the PP2C homologue PphA. *In vitro*, PphA reactivity towards PII-P was strongly affected by the addition of ATP, ADP and 2-oxoglutarate. It was suggested that these effector molecules modulate the molecular recognition of PII-P by PphA through binding to PII (Ruppert et al., 2002). Inhibition of PII-P dephosphorylation by ATP was strongly enhanced by 2-oxoglutarate and to a lower extent by oxaloacetate. In the presence of physiological levels of the effector molecules, PII-P dephosphorylation mainly responded to the 2-oxoglutarate levels (Forchhammer et al., 2004).

Despite the detailed knowledge about *in vitro* PII dephosphorylation by PphA, the physiological context of PII dephosphorylation has been poorly investigated. Only the short-term response of PII-P dephosphorylation has been reported in a PII-deficient mutant of *Synechocystis* PCC 6803 (MPphA) (Irmler & Forchhammer, 2001; Forchhammer et al., 2004). Within a period of 30 min, the mutant was unable to dephosphorylate PII-P in response to various external stimuli, suggesting that PphA was the major PII-P phosphatase in these cells. However, the contribution of PphA to total phosphatase activity in *Synechocystis* cells was not known, nor has the long-term acclimatization of the PII-P phosphorylation state to various C/N conditions been investigated in the PII-deficient mutant. Also, the abundance and cellular localization of PphA under various growth conditions was not known. The present investigation was performed to clarify these points, which are crucial to understanding the *in vivo* specificity of PII-PphA recognition and to gain deeper insights into the *in vivo* conditions of PPIA-mediated PII dephosphorylation in *Synechocystis* PCC 6803.

**METHODS**

**Culture conditions.** *Synechocystis* sp. PCC 6803T (Grigorieva & Shestakov, 1982) and the derived PII-deficient mutant MPphA (pphAKan; Irmler & Forchhammer, 2001) were grown in liquid BG11 medium (Rippka, 1988) supplemented with 5 mM NaHCO₃. Either 17-6 mM NaNO₃ (BG11°) or 5 mM NH₄Cl (BG11°N) buffered with 20 mM HEPES/NaOH pH 7-5 was used as nitrogen source. The cultures were incubated photoautotrophically at an illumination of 40 μmol photons m⁻² s⁻¹ at 25 °C and were constantly shaken at 150 r.p.m. The mutant strain was maintained with kanamycin (30 μg ml⁻¹). For low-carbon conditions, mid-exponential-phase cultures were shifted to flasks filled to the top with bicarbonate-free BG11° medium and incubated without stirring at an irradiance of 20 μmol photons m⁻² s⁻¹. Growth of the cultures was monitored by measuring the OD₅₇₅.

**Determination of the modification state of PII.** The phosphorylation state of PPIA in *in vivo* and *in vitro* was analysed by non-denaturing PAGE followed by immunoblot analysis of PPIA as described by Forchhammer & Tandeau de Marsac (1994). In the figures, PII, PII1, PII2 and PII3 represent isoforms of trimeric PII carrying no, one, two or three phosphorylated subunits, respectively.

**Enzyme assays**

*ρ*-Nitrophenyl phosphate (pNPP) phosphatase activity. Phosphatase activity with pNPP as substrate was assayed in cell-free extracts according to Mackintosh (1993). Cultures of *Synechocystis* PCC 6803 wild-type and MPphA were grown in BG11° medium to an OD₇₅₀ of 0.8. The cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris/HCl pH 7-4, 2 mM MgCl₂, 50 mM KCl, 0-5 mM EDTA and 1 mM benzamidine) and broken by sonication. Cell debris and insoluble material was removed by two consecutive centrifugation steps (10 min at 10 000 g and 1 h at 100 000 g). The final supernatants were dialysed against a buffer without divalent cations (20 mM Tris/HCl pH 7-5, 50 mM NaCl, 1 mM DTT and 0-5 mM EDTA). In a 1 ml standard assay, 250 μg protein of the supernatants was reacted with 3 mM pNPP in a buffer (20 mM Tris/HCl pH 8-3, 10 mM NaCl and 0-2 mM DTT) in the presence or absence of 0-4 mM MnCl₂. The increase in A₄₀₀ was recorded in an Ultrospec 3000 spectrophotometer (Amersham-Pharmacia) against a blank reaction in which pNPP was omitted.

**In situ glutamine synthetase (GS) assay.** GS activity was determined by the formation of γ-glutamylhydroxamate (transferase assay) as described previously (Forchhammer & Tandeau de Marsac, 1995b).

**In vitro** PII dephosphorylation in cell extracts. Cultures of the wild-type and MPphA strain of *Synechocystis* PCC 6803, grown in liquid BG11° medium to mid-exponential phase, were treated with 0-5 mM DON (6-diazoo-5-oxo-1-norleucine) for 1 h prior to harvest, to maximize the phosphorylation state of PII. Extracts of these cells were prepared in a Tris/HCl pH 7-4, 4 mM EDTA buffer by grinding with glass beads in a RiboLyser (Hybaid) apparatus as described by Heinrich et al. (2004). After removal of small effector molecules through a protein-desalting spin column (Pierce) the extracts were concentrated by ultrafiltration using a 5 kDa cut-off membrane (Nanosept, Pall Life Science). The extracts (approx. 200 μg protein) were denatured in a denaturing PAGE followed by immunoblot analysis.

**PphA expression under different growth conditions.** To analyse nitrogen-dependent expression of PphA in *Synechocystis* sp. PCC 6803, a pre-culture was grown in BG11° or in modified BG11° medium, in which molybdenum was replaced by tungsten (4:8 μM), as indicated. At the mid-exponential phase of growth, the cells were harvested by centrifugation and washed in medium free of combined nitrogen (BG11°N). The cells were resuspended in BG11° or in modified BG11° medium (containing tungsten) to an OD₅₇₅ of approx.
0.4 and distributed to culture flasks. NH₄Cl (5 mM final concentration), NaNO₃ or NaNO₂ (concentrations as indicated) were added, the cultures were incubated for 24 h as described above and then samples were removed for Ppha analysis. For the analysis of Ppha expression in the presence of NaNO₂ and MSX (L-methionine-DL-sulfoximine), BG110-grown cells were washed in BG110 medium and resuspended in nitrogen-free medium to an OD₇₅₀ of 0.5. One half of the culture was treated with MSX (0.2 mM final concentration) as a control, while the other half was treated with MSX and NaNO₂ (10 mM final concentration). Samples were removed after 4 h and 8 h incubation.

To determine Ppha abundance in the various samples as described above, the cells from a sample equivalent to 1 ml with an OD₇₅₀ of 1 were harvested by centrifugation. Cell pellets were resuspended in 80 µl SDS sample buffer (75 mM Tris/HCl pH 6.8, 100 mM DTT, 70 mM SDS, 10%, w/v, glycerol and bromophenol blue) and were lysed by heating for 5 min at 95°C. After removing the cell debris by centrifugation, 20 µl aliquots were subjected to electrophoresis in a 12.5% SDS-polyacrylamide gel. Following electrophoresis, Ppha was revealed by immunoblot analysis, using polyclonal antibodies (Pineda, Berlin) raised against purified Ppha (Irmler & Forchhammer, 2001). The amount of Ppha was quantified using a Bio-Rad Fluor-S Image software (Bio-Rad).

RNA isolation, Northern blot hybridization and RT-PCR analysis.
For analysis of ppha mRNA abundance under different growth conditions, wild-type cells were grown as described above and 50 ml aliquots were removed from the cultures for RNA extraction. The samples were rapidly chilled on ice, centrifuged, and the cells were stored at −80°C. For analysis of ggf mRNA abundances, wild-type and MPpha cells were grown in BG110 medium to an OD₇₅₀ of 0.7. A 50 ml sample was removed from each culture for RNA analysis at time zero and then the cultures were treated with NH₄Cl (5 mM final concentration). At 15 min after ammonium addition, a second 50 ml sample was removed. RNA was isolated by using the RiboPure-Bacteria kit (Ambion) according to the manufacturer’s description; 5 µg and 15 µg of total RNA was used for Northern blot analysis of the ggf (IF7, IF17) and ppha genes, respectively, according to the method of Sambrook et al. (1989). RNA probes were denatured by treatment with formamide. The samples were loaded and separated in a 1.5% or 1.2% (w/v) agarose gel containing formaldehyde and were transferred to nylon membrane (Roth-Nylon plus, Roth). DNA probes internal to IF7 (GS inactivating factor 7), IF17 (GS inactivating factor 17) and rnpB (RNase P subunit B) were obtained by PCR amplification (Table 1). The ppha probe was a DNA fragment corresponding to nucleotides 317 to 765 of the ppha (sll1771) coding region generated by restriction of plasmid pT7-7ppha (Irmler & Forchhammer, 2001) with KpnI and Smal.

The DNA probes were labelled with [³²P]dCTP (Amersham-Pharmacia) by using the Megaprime DNA labelling kit (Amersham-Pharmacia). The hybridization signals were visualized by exposing the membrane to a phosphorimager screen (K-Imaging Screen, Bio-Rad) and the data were recorded in a phosphorimager (Molecular Imager FX, Bio-Rad). Quantification was performed using the Bio-Rad Quantity One software.

The RT-PCR analysis of ppha mRNA abundance under different growth conditions was performed by using the Qiagen OneStep RT-PCR kit (Qiagen) according to the manufacturer’s description. The primers used are shown in Table 1. PCR products were separated on a 2% (w/v) agarose gel. Quantification was performed using the Bio-Rad Quantity One software.

RESULTS AND DISCUSSION

Characterization of pNPP phosphatase activity in a Ppha-deficient mutant of Synechocystis sp. PCC 6803

The Synechocystis sp. PCC 6803 genome contains eight putative PP2C homologues and several other putative phosphatases (Kaneko et al., 1996; Zhang et al., 1998; Shi, 2004). To estimate the contribution of Ppha to the total phosphatase activity in Synechocystis PCC 6803, we used pNPP, a broad range substrate for various phosphatases (Mackintosh, 1993), to determine total phosphatase activity in cell-free extracts from wild-type and Ppha-deficient cells (Table 2). As a typical member of the PP2C family, Ppha requires Mg²⁺ or Mn²⁺ ions for catalytic activity; reactivity towards the artificial substrate pNPP is only observed in the presence of Mn²⁺ ions at pH values above 8 (Ruppert et al., 2002). Therefore, we determined pNPP hydrolysis activity in cell-free extracts at pH 8.3 in the presence or absence of 0.4 mM MnCl₂. In the absence of divalent cations, pNPP hydrolysis was almost identical in extracts from wild-type and MPpha cells (Table 2), indicating the activity of phosphatases not belonging to the 2C family. In the presence of Mn²⁺ ions, pNPP hydrolysis increased strongly due to the contribution of PP2C phosphatases. Under these assay conditions, extracts from wild-type cells displayed approximately 15% higher Mn²⁺ specific activity than extracts from MPpha cells, revealing

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Table 1. Primers used for RT-PCR analysis or to generate probes for RNA–DNA hybridization

<table>
<thead>
<tr>
<th>Probe</th>
<th>Primer*</th>
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<tbody>
<tr>
<td>IF7</td>
<td>F: 5’-CCGGACCAACCCATGATTTACG-3’&lt;br&gt; R: 5’-AGGTTGTGCGCCGAAACTGGG-3’</td>
</tr>
<tr>
<td>IF17</td>
<td>F: 5’-GCTCCCAAGCAGAATTTCCT-3’&lt;br&gt; R: 5’-CACGCCCTTGACTGGCGATA-3’</td>
</tr>
<tr>
<td>rnpB</td>
<td>F: 5’-GTGACAGAGAATGTGAGTAG-3’&lt;br&gt; R: 5’-GGCAGGAAAGAGAAAAGAATTT-3’</td>
</tr>
<tr>
<td>ppha</td>
<td>F: 5’-AGACGGATCCCGGCTGATTG-3’&lt;br&gt; R: 5’-TCCGAAGTGATACCCTCCGATT-3’</td>
</tr>
</tbody>
</table>

*F, forward; R, reverse.

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Table 2. Reactivity of cell extracts from the wild-type (WT) and the Ppha-deficient mutant (MPpha) towards pNPP

<table>
<thead>
<tr>
<th>Buffer conditions</th>
<th>WT</th>
<th>MPpha</th>
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<tbody>
<tr>
<td>Divalent cation-free</td>
<td>0.7 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>0.4 mM MnCl₂</td>
<td>5.9 ± 1.3</td>
<td>5.2 ± 1.2</td>
</tr>
<tr>
<td>Mn-specific activity</td>
<td>5.2 ± 1.5</td>
<td>4.4 ± 1.3</td>
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Activities are given in nmol pNPP hydrolysed min⁻¹ (mg protein)⁻¹. Means ± SD from three independent experiments are given.
the contribution of PphA to the Mn^{2+}-specific pNPP hydrolysis activity.

**P_{II}-P dephosphorylation in PphA-deficient cells**

The PphA-deficient mutant is characterized by its inability to rapidly dephosphorylate P_{II}-P in response to various stimuli, such as addition of ammonium, CO₂ shortage, inhibition of CO₂ fixation or any treatments which inhibit photosynthetic electron flow (Irmler & Forchhammer, 2001; Forchhammer et al., 2004). Whereas in wild-type cells P_{II}-P is rapidly dephosphorylated within minutes after an appropriate treatment like the addition of 5 mM ammonium, P_{II} remains phosphorylated in MPphA cells, indicating that PphA is the only specific P_{II}-P phosphatase in *Synechocystis* PCC 6803. In contrast to the impaired fast P_{II}-P dephosphorylation, we observed that 1 day after exposure to ammonium, P_{II} accumulated in its non-phosphorylated form in MPphA cells (Fig. 1A). When, however, cells were maintained in the presence of nitrate under C₃-limiting conditions (non-aerated, standing culture) the phosphorylation state of P_{II} remained high in MPphA cells whereas P_{II} was almost completely dephosphorylated in the wild-type (Fig. 1B). Compared to ammonium-treated cells, this is an intriguing difference. Complete P_{II} dephosphorylation in wild-type cells indicates that the 2-oxoglutarate level, which was shown to be the major signal for P_{II} dephosphorylation (Forchhammer et al., 2004), is low under both conditions, in agreement with measurements of cellular 2-oxoglutarate levels (Muro-Pastor et al., 2001). A possible explanation could be a lower activity of P_{II} kinase activity in ammonium-grown cells, which only becomes evident in the PphA-deficient background. Indeed, it has been reported that P_{II} kinase activity is impaired in a NtcA-deficient mutant of *S. elongatus* (Lee et al., 1999; Sauer et al., 1999), indicating that this activity is under nitrogen control. This is further supported by *in vitro* measurements of P_{II} kinase activity in *S. elongatus* extracts: in extracts from ammonium-grown cells, kinase activity was much lower than in extracts from nitrate-grown cells (H. Dierks, Diplomarbeit, Lehrstuhl für Mikrobiologie der LMU München, and K. Forchhammer, unpublished data).

Accumulation of non-phosphorylated P_{II} in ammonium-treated MPphA cells could be caused by a residual P_{II}-P dephosphorylation activity catalysed by other cellular phosphatases or may result from degradation of P_{II}-P concomitantly with *de novo* synthesis of non-phosphorylated P_{II}. To distinguish between these possibilities, the long-term effect of ammonium was analysed in the presence of the protein synthesis inhibitor chloramphenicol, to inhibit *de novo* protein synthesis (Fig. 1C). Following the addition of antibiotic and ammonium, P_{II} was completely dephosphorylated in the wild-type after 2-5 h, whereas only a minor change in the phosphorylation status of P_{II} occurred in MPphA during this time period, as expected. However, during prolonged incubation of the PphA-deficient mutant, P_{II}-P was completely substituted by non-phosphorylated P_{II}. The concomitant arrest of cell growth revealed that chloramphenicol was biologically active and *de novo* protein synthesis was in fact inhibited. Therefore, the apparent P_{II}-P dephosphorylation cannot be attributed to protein turnover, but rather results from a slow, PphA-independent, P_{II}-P dephosphorylation activity catalysed by phosphatases which are already present in nitrate-grown cells.

To gain further insight into the residual P_{II}-P dephosphorylating activity, crude extracts from wild-type and PphA-deficient cells were tested for their ability to dephosphorylate endogenous P_{II}-P protein. As shown in Fig. 2, P_{II}-P was rapidly dephosphorylated in wild-type extracts in a Mg^{2+}-dependent manner, whereas no dephosphorylation was detected in the absence of divalent cations. By contrast, Mg^{2+}-dependent dephosphorylation of P_{II} was almost completely absent in MPphA extracts. Although PphA contributes only a minor proportion of total PP2C activity (see above), *in vitro* and *in vivo* dephosphorylation of P_{II}-P

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Fig. 1. Dephosphorylation of P_{II}-P in wild-type (WT) and PphA-deficient (MPphA) *Synechocystis* PCC 6803 cells. (A) *In vivo* dephosphorylation of P_{II}-P upon addition of 5 mM NH₄Cl to nitrate-growing cells. The phosphorylation state of P_{II} was analysed before addition (0 h) and after different times as indicated. (B) Response to C₃ limitation: cells were incubated under C₃-limiting conditions (see Methods) for 48 h and the phosphorylation state of P_{II} was analysed. (C) Ammonium-promoted *in vivo* P_{II}-P dephosphorylation in the presence of 30 μg chloramphenicol ml⁻¹.
is strongly impaired in the PphA-deficient mutant, implying that PII-P is a poor substrate for the other protein phosphatases. The mechanistic basis of its specificity towards PphA deserves further investigation.

**Nitrogen-source-dependent accumulation of PphA**

To reveal the cellular localization of PphA, extracts of *Synechocystis* PCC 6803 cells were fractionated into soluble and particulate (membrane-containing) fractions, and the presence of PphA was analysed by immunoblotting. The analysis was performed with nitrate-grown cells and with cells that had been treated with 5 mM ammonium for 15 min. As shown in Fig. 3, under both conditions, PphA was only recovered in the soluble fraction, with no traces detectable in the particulate fraction, confirming the assumption from primary structure analysis that PphA is a soluble, cytoplasmic protein. The abundance of PphA in extracts from cells that were grown in standard BG11N medium was estimated by densitometric quantification of the PphA reactive band compared to PphA standards. Approximately 40–50 ng PphA was detected in 15 μg total cellular protein, corresponding to about 0.3% of total cellular protein (data not shown). To elucidate whether PphA is differentially expressed under conditions corresponding to different degrees of PII phosphorylation, extracts from cells which had been grown with different nitrogen supplies were subjected to immunoblot analysis (Fig. 4). In fact, the amount of PphA varied considerably under those conditions. In cells that werestarved for combined nitrogen or were grown in the presence of ammonium (each for 24 h), only low levels of PphA could be detected. The cellular PphA abundance was higher with increasing nitrate or nitrite concentrations in the medium. Maximal levels were obtained with approximately 10 mM nitrate or 5 mM nitrite (Fig. 4A). To reveal whether the reduced amount of PphA in ammonium-grown cultures was due to ammonium repression or was caused by the absence of nitrate/nitrite, cells grown in nitrate-supplemented medium were either challenged with 5 mM ammonium (without removal of nitrate, Fig. 4B) or shifted to nitrate-free, ammonium-supplemented medium (Fig. 4C). The amount of PphA rapidly decreased in the culture in which nitrate was substituted by ammonium, whereas the addition of ammonium in the presence of nitrate did not affect the abundance of PphA. This indicated that the removal of nitrate rather

![Fig. 2. In vitro dephosphorylation of P II-P in cell-free extracts prepared from wild-type (WT) and PphA-deficient (MPphA) *Synechocystis* PCC 6803. Extracts were prepared from cells in which P II was maximally phosphorylated (lanes 0) as described in Methods. Dephosphorylation by endogenous phosphatases was assayed in a buffer containing either none or 20 mM MgCl₂, as indicated. After 30 and 120 min incubation at 37 °C, 5 μg total protein was subjected to non-denaturing PAGE and the phosphorylation state of P II was analysed by immunoblotting.](image1)

![Fig. 3. Cellular localization of PphA in *Synechocystis* PCC 6803. Analysis was performed with nitrate-grown cells and with cells that had been treated with 5 mM ammonium for 15 min. PphA was detected by immunoblotting following SDS-PAGE of samples of cell-free extract (ce), supernatant of 100 000 g centrifugation (S) and pellet of 100 000 g centrifugation (P).](image2)

![Fig. 4. Abundance of PphA in *Synechocystis* PCC 6803 cells under different growth conditions. Cell-free extracts were prepared and analysed by immunoblotting. (A) Cells were incubated for 24 h in the absence of combined nitrogen source (–), or were grown in the presence of 5 mM NH₄Cl or in the presence of 2, 5 or 10 mM nitrate or nitrite, as indicated. Nitrate, even at 10 mM concentration, was not toxic to the cells. (B) Addition of ammonium to nitrate-utilizing cells. Cells grown in BG11N were challenged with 5 mM NH₄Cl, and after different times, as indicated, aliquots were removed and analysed. (C) Shift from nitrate- to ammonium-supplemented medium. After different times, as indicated, aliquots were removed for the determination of PphA abundance.](image3)
than the addition of ammonium led to reduced PphA levels. The differential accumulation of PphA at various concentrations of nitrate and nitrite was intriguing and raised the question of a specific effect of these molecules. To clarify this point, nitrate reductase was poisoned by growing cells in a medium in which molybdenum was replaced by tungsten. Under this condition, the cells expressed a non-active nitrate reductase. After verification of the absence of nitrate reductase activity in these cells, the response of PphA accumulation to the nitrogen source was re-examined (Fig. 5A). In the absence of functional nitrate reductase activity, nitrate could not enhance the PphA level, whereas the positive effect of nitrite was not impaired. This implied that nitrite (an intermediate of nitrate reduction) was directly involved in the control of the PphA abundance. To distinguish between an anabolic effect and a direct impact of nitrite on PphA accumulation, an experiment was performed in the presence of the GS inhibitor MSX. After shifting cells to nitrogen-depleted medium containing MSX, the culture was divided into two and nitrite was added to one of the samples. Aliquots were withdrawn and PphA abundance was analysed (Fig. 5b). PphA levels increased in the presence of nitrite whereas they decreased in its absence, suggesting that nitrite assimilation is not required to promote PphA accumulation. This strongly suggests a direct effect of nitrite on the level of PphA.

The nitrite dependence of PphA accumulation raised the question of whether the nitrite-specific gene activator NtcB (Aichi et al., 2001) was involved in this response. However, almost identical levels of PphA were detected in a NtcB-deficient mutant compared to the wild-type under the various nitrogen conditions (data not shown), implying that pphA expression is not under NtcB control. To reveal whether the abundance of PphA is regulated at the transcriptional level, the amount of pphA mRNA was examined by Northern blot analysis (Fig. 6A). As noted previously (Irmler & Forchhammer, 2001), pphA mRNA appears as a rather unstable transcript, since a broad band with considerable amount of degradation was observed with no apparent difference between preparations from different nitrogen regimes. Hence quantitative RT-PCR was carried out to clarify this expression pattern (Fig. 6B, C). Based on cycles required for exponential PCR amplification of pphA (Fig. 6B), RNA preparations from cells grown under various nitrogen regimes were amplified with 22 cycles. Regardless of the nitrogen conditions, the abundance of the pphA transcript was not significantly altered, suggesting a low constitutive expression of pphA (Fig. 6C). The apparent lack of transcriptional control agrees with the NtcB independence of PphA, suggesting that its accumulation is

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**Fig. 5.** Effect of nitrite on the accumulation of PphA in *Synechocystis* PCC 6803. Cell-free extracts were prepared and PphA was revealed by immunoblotting. (A) Cells were incubated for 24 h in the absence of combined nitrogen source (−N) or were grown in the presence of 5 mM NH₄Cl or in the presence of 20 or 10 mM nitrate or nitrite, as indicated. In the medium, molybdenum was replaced with tungsten, so that the cells expressed a non-active nitrate reductase. (B) Nitrate-grown cells (NO₃⁻) were transferred to a medium containing 0-2 mM MSX (inhibitor of GS) in the presence or absence of nitrite (10 mM) as described in Methods. The abundance of PphA was analysed at the times indicated.

**Fig. 6.** Northern blot analysis and RT-PCR analysis of pphA transcripts from *Synechocystis* PCC 6803 under different growth conditions. The cells from which RNA was prepared were first grown in nitrate-supplemented medium and then transferred to medium supplemented with ammonium, nitrite, nitrate or no nitrogen source (−N) for 24 h. (A) Northern blot analysis of pphA expression. The mRNA level of the constitutively expressed *rnpB* gene was used as a loading and transfer efficiency control. The experiment was repeated three times; one representative experiment is shown. (B) Determination of RT-PCR cycles required for pphA amplification prior to saturation. Amplification is exponential between 21 and 23 cycles. RNA from nitrite-grown cells was used. (C) RT-PCR analysis. pphA was amplified to 22 cycles. The product of the constitutively expressed *rnpB* gene was used as control.
rather controlled post-transcriptionally. Possible explanations are nitrite-dependent control at the level of pphA translation or PphA protein stability; this awaits further investigation.

Whatever the molecular mechanism for pphA expression, PphA abundance seems to be optimized to allow rapid dephosphorylation of PII-P in nitrate- and nitrite-grown cells. In the presence of ammonium, PII is barely phosphorylated, due to the low PII kinase activity. Under these conditions, low amounts of PphA are sufficient to maintain PII in the dephosphorylated state. In nitrate- or nitrite-grown cells, however, the amount of PphA increases as PII is phosphorylated. The elevated levels of PphA might be required to rapidly dephosphorylate PII-P under appropriate conditions. Considering the size and the estimated abundance of PphA (Mᵣ 28472; approx. 0.3% of total protein) and of PII (Mᵣ of monomer 12397; 0.1–0.2% of soluble protein, Forchhammer & Tandeau de Marsac, 1994; K. Forchhammer, unpublished), PphA seems to be in nearly stoichiometric amounts or slightly in excess over its substrate PII-P. In vitro studies suggest that, as long as the cellular concentrations of ATP and 2-oxoglutarate are high, PphA is not able to dephosphorylate PII-P (Ruppert et al., 2002). When, however, the cellular 2-oxoglutarate level decreases, PphA can rapidly dephosphorylate PII-P without the need for multiple substrate turnover. This enables the cells to rapidly respond to changing 2-oxoglutarate concentrations.

**Effect of PphA deficiency on short-term inhibition of GS**

The PphA-deficient strain was not impaired in acclimatizing to different nitrogen sources, as far as cell growth was concerned. Since it was shown recently that NtcA-dependent gene expression depends on PII-P dephosphorylation under conditions of nitrogen deprivation (Aldehni et al., 2003; Paz-Yepez et al., 2003), we wanted to investigate whether impaired PII-P dephosphorylation affects an NtcA-dependent response. In *Synechocystis* PCC 6803, GS activity is down-regulated by the inactivating factors IF7 and IF17, whose expression depends on NtcA (Garcia-Dominguez et al., 2000). In the absence of ammonium, active NtcA represses transcription of the IF-encoding *gif* genes. The addition of ammonium leads to inactivation of NtcA (Herrero et al., 2001), and thereby to the loss of *gif* repression. Synthesis of IF7/IF17 then depresses GS activity. Down-regulation of GS activity can therefore be used as a means to determine *in vivo* inactivation of NtcA following ammonium addition, a strategy which has been used successfully in previous studies (Muro-Pastor et al., 2001). To investigate whether the PphA-deficient mutant was able to rapidly acclimatize to ammonium with respect to the regulation of GS activity, nitrate-grown cells of the wild-type and MPphA strain were challenged with 5 mM NH₄Cl, and after different times, aliquots were removed and the *in situ* GS (transferase) activity was determined. As shown in Fig. 7(A), GS activity declined in the mutant even faster than in the wild-type, indicating that the lack of PphA (and thereby the lack of PII-P dephosphorylation) does not affect the rapid *in vivo* inactivation of GS. The mechanistic basis of the accelerated GS inactivation in MPphA requires further investigation. To confirm that GS inactivation was indeed due to ammonium-prompted *gif* gene expression, the levels of *gifA* and *gifB* mRNA from cells that were challenged with ammonium was analysed by Northern blotting (Fig. 7B). The same expression pattern as reported in previous studies of ammonium-treated *Synechocystis* wild-type cells was observed. The transcript of the tightly controlled *gifB* gene increased strongly and that of *gifA* partially (Muro-Pastor et al., 2001) in both the wild-type and MPphA strains, confirming that *gif* induction is independent of PII-P dephosphorylation.

It was previously known that NtcA-dependent gene expression is down-regulated in PII-P-deficient mutants acclimatized to ammonium (Lee et al., 2000; Aldehni et al., 2003; Paz-Yepez et al., 2003). This investigation shows, in addition, that the presence of PII-P does not impair the response of NtcA to ammonium, at least as far as *gif* gene repression is concerned. This conclusion is corroborated by the observation that in PII S49D/E mutants, which potentially mimic phosphorylated PII-P, NtcA-dependent gene expression still responds to the nitrogen status (Paz-Yepez et al., 2003).

![Fig. 7. GS activity and *gif* expression in *Synechocystis* PCC 6803 wild-type and MPphA cells. (A) Short-term response of GS (transferase) in wild-type (●) and MPphA-deficient cells (○) to the addition of 5 mM ammonium. GS activity prior to the addition of ammonium was taken as 100%. (B) Northern blot analysis of the expression of *gifA* and *gifB* (encoding IF7 and IF17, respectively) in wild-type and the mutant MPphA in nitrate-grown cells and 15 min after addition of ammonium (5 mM).](http://mic.sgmjournals.org)
Previous in vitro studies showed direct regulation of NtcA by 2-oxoglutarate levels (Vazquez-Bermudez et al., 2002; Tanigawa et al., 2002). Whether this sufficiently explains the rapid NtcA response towards ammonium remains to be demonstrated; however, PII dephosphorylation seems not to be involved in this process.

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