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

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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 PII. 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 decreased nitrate and nitrite reductase activities and a delay in the induction of nitrate utilization after transfer of cultures from ammonium- 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-depleted cultures. Taken together, the results 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. Glutamine synthetase, which catalyses the incorporation of ammonium into glutamate to yield glutamine is the key enzyme of nitrogen assimilation. Two types of glutamine synthetase are produced by the non-diazotrophic cyanobacterium *Synechococcus* sp. PCC 7942 (hereafter called *Synechococcus*): GSI, a typical eubacterial glutamine synthetase 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 PII, 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 GSIII, respectively, the *glnB* for PII, the *nblA* gene required for phycobilisome 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 glutamine synthetase (Vega-Palas et al., 1990), or acclimate appropriately to nitrogen starvation (Sauer et al., 1999).
The PII 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 PII (gln product) in cyanobacteria shows unique features with respect to both covalent modification and downstream signalling (Forchhammer, 2004). Physiological studies showed that PII 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 PII (Takatan et al., 2006). However, in these cases, no physical interaction with PII was reported. Using yeast two-hybrid approaches, three cyanobacterial PII receptors could recently be identified: the enzyme N-acetylglutamate kinase (NAGK) (Burillo et al., 2004; Heinrich et al., 2004) and the regulatory factor PipX (Burillo et al., 2006; 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 PII interaction with these protein receptors is only understood in the case of NAGK. The PII-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). PII 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 PII and NtcA is inversely affected by 2-oxoglutarate (Espinosa et al., 2006). Complex formation between PipX and PII 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 in nitrogen assimilation and acclimation to nitrogen deficiency, we now analyse 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 Escherichia coli DH5α, using standard techniques. Synechococcus strains were grown photoautotrophically at 30°C while shaking under constant illumination (75 μM m⁻² s⁻¹) provided by cool white fluorescent lights in BG11 medium (nitrate-containing), BG11a (no added nitrogen) or BG11-NH₄ (BG11a supplemented with 10 mM NH₄Cl, 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 30°C under constant illumination. For initiation of nitrogen deprivation, mid-exponential cultures (OD₇₅₀ 0.5) were harvested by centrifugation, washed twice with BG11, and finally resuspended in BG11a. 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 pUA101, a 206 bp fragment corresponding to the upstream region of the nblA gene was PCR amplified using primers 5'-GGCGCTGGTGGAAAGTCACGCC-3' and 5'-GAA-GCCTCCGCACTGCAGATG-3'. (1999). Construction of the two reporter plasmids pUA101-NLS and pUA101-2-NS was done by the procedure of Datsenko & Wanner (2000). The 2-NS plasmid was constructed by introducing a twitching reporter system (Al-Babili & Choule, 1997) into the PII gene of Synechococcus. The luxAB genes were cloned into the neutral site of the Synechococcus chromosome (Lee et al., 2000). The luxAB genes were introduced into the Synechococcus chromosome by the use of a non-invasive conjugal transfer system (Al-Babili et al., 1995). The luxAB reporter strain was constructed by the growth of the strain in the presence of 1% (w/v) agar. Plates were incubated at 30°C under constant illumination. For initiation of nitrogen deprivation, mid-exponential cultures (OD₇₅₀ 0.5) were harvested by centrifugation, washed twice with BG11, and finally resuspended in BG11a. Whenever appropriate, cultures contained kanamycin (10 μg ml⁻¹) or chloramphenicol (5 μg ml⁻¹).

Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>E. coli DH5α</td>
<td>F⁻ 480lacZAM15A(lacZYA–argF)U169 endA1 recA1 hsdR17(tk⁻ mK⁺) deoR thi-1 supE44 gyrA96 relA1 λ⁻</td>
<td>Hanahan (1985)</td>
</tr>
<tr>
<td>Synechococcus sp. PCC 7942</td>
<td>Wild-type Synechococcus sp. PCC 7942</td>
<td>Pasteur culture collection</td>
</tr>
<tr>
<td>Synechococcus SA591</td>
<td>PipX⁻ (kanamycin resistance cartridge inserted into the pipX gene of strain PCC 7942)</td>
<td>Espinosa et al. (2006)</td>
</tr>
<tr>
<td>Synechococcus MNtcA</td>
<td>NtcA⁻ (kanamycin resistance cartridge inserted into the ntcA gene of strain PCC 7942)</td>
<td>Sauer et al. (1999)</td>
</tr>
<tr>
<td>Synechococcus NblR45</td>
<td>NblR⁻ (kanamycin resistance cartridge inserted into the nblR gene of strain PCC 7942)</td>
<td>Luque et al. (2001)</td>
</tr>
<tr>
<td>Synechococcus WT-C103</td>
<td>Cm⁻ derivative of strain PCC 7942; PₐnbA::luxAB inserted into chromosome neutral site</td>
<td>This work</td>
</tr>
<tr>
<td>Synechococcus SA591-C103</td>
<td>Cm⁻ derivative of strain SA591; PₐnbA::luxAB inserted into chromosome neutral site</td>
<td>This work</td>
</tr>
<tr>
<td>Synechococcus MNtcA-C103</td>
<td>Cm⁻ derivative of strain MNtcA; PₐnbA::luxAB inserted into chromosome neutral site</td>
<td>This work</td>
</tr>
<tr>
<td>pAM1580</td>
<td>Amp⁻ Cm⁻ derivative of pAM1573 plasmid carrying luxAB reporter genes</td>
<td>Andersson et al. (2000)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km⁻ helper plasmid</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pRL443</td>
<td>Amp⁻ conjugal plasmid</td>
<td>Elhai et al. (1997)</td>
</tr>
<tr>
<td>pUA101</td>
<td>pAM1580 derivative with PₐnbA::luxAB fusion</td>
<td>This work</td>
</tr>
</tbody>
</table>
targeting vector pAM1580. Plasmids pRK2013 and pRL443 were used as a helper and conjugal plasmid, respectively, to transfer pUAGC103 into *Synechococcus* strains (Elhai et al., 1997). Transformants were selected on chloramphenicol-containing BG11 or BG11-NH₄ 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 μg ml⁻¹. Nitrite reductase activity (Herrero & Guerrero, 1986) was also assayed in cells made permeable with MTA (250 μg 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 nitrate formed or disappeared per min.

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

**Determination of chlorophyll a.** Chlorophyll a concentration was determined from methanolic extracts as described by Mackinney (1941).

**Determination of luciferase activity.** To determine bioluminescence, 1 ml of cultures was adjusted with fresh medium to an OD₇₅₀ of 0.5, supplemented with decanal to a final concentration of 0.5 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 s for 10 min. 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 pigment contents spectrophotometrically.** Wild-type and PipX⁻ strains were grown in BG11 or BG11-NH₄ until they reached mid-exponential phase (OD₇₅₀ 0.4–0.5) and then cultures were centrifuged, washed and finally resuspended in BG11. A whole-cell absorbance spectrum was carried out in order to estimate pigment contents. One millilitre of culture was taken at the indicated times, diluted with fresh medium to an OD₇₅₀ of 0.5 and absorbance spectra (550–750 nm) was recorded on a UV/Visible Ultrospec 3100 pro (Amersham). Pigment content was calculated based on absorbance maxima at 631 nm for phycocyanin and 684 nm 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, and 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 subject 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 analysed 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, b), thus indicating that PipX deficiency has a negative effect on nitrate assimilation. As is the case with wild-type cells, addition of ammonium to PipX⁻ cells caused an immediate inhibition of nitrate utilization (Fig. 2a, b), indicating that post-translational regulation of nitrate assimilation by PII 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 3 h 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. 2(c), 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<sup>−</sup> 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 PII 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<sup>−</sup> 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<sup>−</sup> mutant. The basal levels of glutamine synthetase activity found in the PipX<sup>−</sup> strain were indistinguishable from those of the NtcA<sup>−</sup> strain grown with ammonium or in cells starved of nitrogen for 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 is the case with NtcA, PipX is required both for basal levels of glutamine synthetase activity (as seen in ammonium-grown cultures) and for appropriate 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 <i>a</i> 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 <i>et al.</i>, 2001) and NbrL, a response regulator involved in general acclimation (Schwarz & Grossman, 1998). NbrL 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 <i>a</i> content (Sauer <i>et al.</i>, 1999).

As shown in Fig. 4(a), nitrogen-deprived cultures of the PipX<sup>−</sup> null mutant showed the same phenotype as the NtcA<sup>−</sup>
strain, a weak and transient non-bleaching appearance. Pigment analysis revealed the same pattern of pigment degradation that has been reported for the NtcA strain under nitrogen starvation, a delay in phycocyanin degradation and a faster loss of chlorophyll a content (Fig. 4b–d). As expected for a protein specifically involved in 2-oxoglutarate
signalling, 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 lose 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 for 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 analyse 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 NbR-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 bioluminescence 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 contribution of PipX 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 decreased 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 on 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 analysed $glnB$ 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 analysed so far at the level of 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 under high and low intracellular levels of 2-oxoglutarate, respectively. 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 depletion of combined nitrogen 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 remains to be established, the experimental evidence gathered so far favours the idea of PipX being a P$_{II}$ target rather than a P$_{II}$ regulator.

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