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Effects of spontaneous mutations in PipX functions and regulatory complexes on the cyanobacterium *Synechococcus elongatus* strain PCC 7942

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In *Synechococcus elongatus* sp. PCC 7942, PipX forms complexes with P_{II}, a protein found in all three domains of life as an integrator of signals of the nitrogen and carbon balance, and with the cyanobacterial nitrogen regulator NtcA. We recently showed that previous inactivation of *pipX* facilitates subsequent inactivation of the *glnB* gene. Here, we show that the three spontaneous *pipX* point mutations *pipX-92delT*, *pipX160C>T* and *pipX194T>A,* initially found in different *glnB* strains, are indeed suppressor mutations. When these mutations were reconstructed in the wild-type background, the *glnB* gene could be efficiently inactivated. Furthermore, the point mutations have different effects on PipX levels, coactivation of NtcA-dependent genes and protein–protein interactions. Further support for an *in vivo* role of PipX–P_{II} complexes is provided by interaction analysis with the *in vivo*-generated $P_{II}^{T\text{-}loop+7}$ protein, a P_{II} derivative unable to interact with its regulatory target *N*-acetyl-L-glutamate kinase, but which retains the ability to bind to PipX. The implications of these results are discussed.

INTRODUCTION

Cyanobacteria are phototrophic organisms that perform oxygenic photosynthesis. Autotrophic growth requires the constant assimilation of ammonium via the glutamine synthetase–glutamine oxoglutarate aminotransferase-glutamate cycle, resulting in consumption of 2-oxoglutarate (Muro-Pastor *et al.*, 2005). Due to the lack of 2-oxoglutarate dehydrogenase in cyanobacteria, synthesis of 2-oxoglutarate represents the final step in the oxidative branch of the tricarboxylic acid cycle and directly links 2-oxoglutarate levels to nitrogen assimilation (Muro-Pastor *et al.*, 2001). Thus, 2-oxoglutarate accumulates during nitrogen starvation, making this metabolite an excellent indicator of the intracellular carbon to nitrogen balance (Forchhammer, 2004; Laurent *et al.*, 2005).

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). 2-Oxoglutarate, the signal of nitrogen deficiency, stimulates binding of NtcA to target sites (Vazquez-Bermudez *et al.*, 2002), transcription activation *in vitro* (Tanigawa *et al.*, 2002) and complex formation between NtcA and PipX, a regulatory protein conserved in cyanobacteria (Burillo *et al.*, 2004; Espinosa *et al.*, 2006). The interaction between PipX and NtcA is known to be relevant for maximal activation of NtcA-dependent genes under nitrogen limitation (Espinosa *et al.*, 2007, 2006). PipX-deficient cultures of *Synechococcus elongatus* sp. PCC 7942 (hereafter *S. elongatus*) showed reduced activity of nitrogen assimilation enzymes, retarded induction and a slower rate of nitrate consumption and, when subjected to nitrogen starvation, retarded phycobilisome degradation and a faster reduction of the chlorophyll content (Espinosa *et al.*, 2007).

The homotrimeric P_{II} protein is one of the most conserved and widespread signal transduction proteins in nature and plays key roles in nitrogen assimilatory processes (Leigh & Dodsworth, 2007). P_{II} proteins contain three binding sites (one per subunit) for 2-oxoglutarate and ATP and their primary function is to regulate, by direct protein–protein interactions, the activity of proteins implicated in nitrogen metabolism (reviewed by Forchhammer, 2008). In cyanobacteria, several proteins are known to form complexes with P_{II}. The first two P_{II} receptors were identified in *S.*

Abbreviation: NAGK, *N*-acetyl-L-glutamate kinase.

Two supplementary figures, showing conservation involving amino acid residues 54 and 65 on PipX proteins and yeast two-hybrid interactions involving P_{II} and PipX derivatives, are available with the online version of this paper.

elongatus: the enzyme *N*-acetyl-L-glutamate kinase (NAGK), a P_{II} target conserved across domains of life during the evolution of oxygenic photosynthetic organisms (Burillo *et al.*, 2004; Chen *et al.*, 2006; Sugiyama *et al.*, 2004), and the regulatory factor PipX (Burillo *et al.*, 2004; Espinosa *et al.*, 2006). The non-conserved membrane protein PamA has been identified as a P_{II} receptor in *Synechocystis* sp. PCC 6803 (Osanai *et al.*, 2005). Structural and functional details are only known for the P_{II}–NAGK complex (Llacer *et al.*, 2007). This complex consists of two polar P_{II} trimers sandwiching one ring-like hexameric NAGK, with the flexible T-loop, a key element for regulatory interactions, adopting a novel compact shape. Other P_{II} functions for which direct protein–protein interactions have not been reported yet include the control of nitrate transport (Kloft & Forchhammer, 2005; Lee *et al.*, 2000) – nitrate reductase (Takatani *et al.*, 2006) – and the control of inorganic carbon transport (Hisbergues *et al.*, 1999).

P_{II} proteins bind 2-oxoglutarate and ATP synergistically. In *S. elongatus* and *Synechocystis* sp. PCC 6803, the T-loop is phosphorylated at a seryl residue (S49), located at the apex of the solvent-exposed T-loop (Forchhammer, 2004). The phosphorylation status of P_{II} correlates with the 2-oxoglutarate levels, both being maximal during nitrogen starvation. ATP in concert with elevated 2-oxoglutarate levels prevents complex formation of P_{II} with either NAGK or PipX (Espinosa *et al.*, 2006; Maheswaran *et al.*, 2004), suggesting that PipX–P_{II} complexes also have a function under nitrogen-sufficient conditions. PipX does not seem to be required for P_{II}-dependent functions like the ammonium inhibition of nitrate transport (Espinosa *et al.*, 2007) or the stimulation of NAGK activity (Espinosa *et al.*, 2008).

Recently, we have shown that P_{II} was essential under standard growth conditions in *S. elongatus* and that PipX was involved in the phenomenon. Here, we show that all three spontaneous point mutations at *pipX* that we identified in different *glnB* strains were indeed suppressor mutations contributing to survival in P_{II}-deficient backgrounds and that the spontaneously generated protein $P_{II}^{\text{T-loop}+7}$ retains the ability to interact with PipX and not with its regulatory target NAGK. The effects of mutations *pipX-92delT*, *pipX160C>T* and *pipX194T>A* on PipX levels, coactivation of NtcA-dependent genes and protein–protein interactions are reported.

METHODS

Strains and growth conditions. Strains, plasmids and oligonucleotides used in this work are listed in Tables 1 and 2. Constructs and genomic mutations were analysed by automated dideoxy DNA sequencing. All cloning procedures were carried out in *Escherichia coli* DH5α and GM119 using standard techniques. *Synechococcus* strains were routinely grown photoautotrophically at 30 °C while shaking under constant illumination (40 μmol photons m^{-2} s^{-1}) provided by cool white fluorescent lights. Media used were blue-green algae medium $BG11_0$ (no added nitrogen), BG11 ($BG11_0$ plus 17.5 mM $NaNO_3$ and 10 mM HEPES/NaOH pH 7.8) and $BG11_A$ ($BG11_0$ plus 5 mM NH_4Cl and 5 mM HEPES/NaOH pH 7.8). For growth on plates, the medium was solidified by addition of 1 % (w/v) agar. Plates were routinely incubated at 30 °C under constant illumination. *S. elongatus* strains were transformed essentially as described by Golden & Sherman (1984). Whenever used, antibiotic concentrations for *S. elongatus* were 10 μg kanamycin ml^{-1}, 5 μg streptomycin ml^{-1} and 5 μg chloramphenicol ml^{-1}.

Yeast culture and transformation procedures were performed as described by Ausubel (1999). Interaction signals between pairs of fusion proteins were determined using the three reporters present in PJ696/Y187 diploids as previously described (Burillo *et al.*, 2004).

Construction of plasmids and strains. Plasmid pUAGC59 was used as template for QuickChange Mutagenesis in combination with either primers PipX-L65Q-F/PipX-L65Q-R (*pipX194T>A* change) or PipX-R54C-F/PipX-R54C-R (*pipX160C>T* change), resulting in plasmids pUAGC387 and pUAGC388, respectively. To obtain plasmid pUAGC389, the *pipX-92delT* sequence from MP2-A (Espinosa *et al.*, 2009) was amplified by PCR with primers Syn2060-1F and PipX-6R, digested with *Nru*I and *Sal*I, and the corresponding fragment was used to replace the *Nru*I/*Sal*I fragment of pUAGC59. A C.S3 cassette from pUAGC453 was cloned into the Klenow-treated *Nhe*I site of plasmids pUAGC387, pUAGC388 and pUAGC389, giving pUAGC390, pUAGC391 and pUAGC392, respectively. Stable transformation of *S. elongatus* with pUAGC387, pUAGC388 and pUAGC389 was confirmed by PCR with primers PipX-6R and CS3-2F. The presence of *pipX* wild-type or mutant alleles was checked by sequencing analysis. Subsequently, plasmids pFAM2 and pFAM84W were independently transformed into control and *pipX* derivatives. Correct integration of reporter P_{glnB}::*luxAB* or P_{glnN}::*luxAB* fusions into the neutral site NSII was confirmed by PCR with primers NSII-1F and NSII-1R.

To obtain plasmids that carry ΦC.K1-$pipX^{R54C}$ and ΦC.K1-$pipX^{L65Q}$, an *Xho*I–*Cla*I fragment excised from digested pUAGC388 and pUAGC387 was cloned into *Xho*I–*Cla*I-digested pUAGC410, resulting in plasmids pUAGC681 and pUAGC682, respectively. After *S. elongatus* clones transformed with pUAGC681 and pUAGC682 were selected with kanamycin and the presence of *pipX* mutant alleles was verified by sequencing analysis, these mutants and the strain SA410 were subsequently transformed with pUAGC702 (Espinosa *et al.*, 2009) bearing *glnB*::C.S3(+). Transformants were selected on kanamycin- and streptomycin-containing BG11 plates. To obtain pUAGC613, C.K1 from pRL161 was extracted by *Hin*cII digestion and cloned into *Bam*HI/*Pst*I-digested pUAGC623 and Klenow filled.

Correct integration of the resistance cassette C.K1 into *S. elongatus* was verified by PCR with primers CK1-2F and PipX-3X-1R. Mutations were verified by automated dideoxy DNA sequencing. Detection of *glnB* alleles by PCR was carried out with either GlnB-1F or CS3-2F as a forward primer and GlnB-1R as a reverse primer.

To obtain pUAGC471, *pipX* sequences were amplified by PCR from genomic DNA with primers PipX-OV-2F and PipX-3X-1R, the product was cut with *Eco*RI and ligated to *Eco*RI-digested pGAD424 and pGBT9, giving plasmids pUAGC471 (GAL4AD:PipX) and pUAGC472 (GAL4BD:PipX), respectively. The *pipX160C>T* allele ($PipX^{R54C}$) was amplified by PCR from a MP2 derivative (Espinosa *et al.*, 2009) using primers PipX-OV-2F and PipX-3X-1R and cloned into pGEX-3X vector giving plasmid pUAGC406. *pipX160C>T* and *pipX194T>A* sequences were amplified by PCR with primers PipX-OV-2F and PipX-3X-1R from pUAGC406 and genomic sequences, respectively, and cloned into the *Eco*RI site of pGAD424 and pGBT9, giving plasmids pUAGC497 (GAL4AD:$PipX^{R54C}$), pUAGC498 (GAL4BD:$PipX^{R54C}$), pUAGC371 (GAL4AD:$PipX^{L65Q}$)

and pUAGC372 (GAL4BD: PipX^{L65Q}). The *glnB133-134ins* allele was amplified by PCR with primers GlnB-1F and GlnB-1R from the GlnBS(−)$^{T\text{-}loop+7}$ strain (Espinosa *et al.*, 2009) and introduced into yeast two-hybrid plasmids by recombination cloning (Kolonin *et al.*, 2000). To this end, *Xho*I-digested pUAGC11 and *Bgl*II-digested pUAGC12 were co-transformed with the PCR product into yeast strains PJ696 and Y187, respectively, giving plasmids pUAGC373 (GAL4AD : $P_{II}^{T\text{-}loop+7}$) and pUAGC374 (GAL4BD : $P_{II}^{T\text{-}loop+7}$). To obtain pUAGC6 and pUAGC8, the *ntcA* sequence was amplified by PCR from genomic DNA with primers NtcA-YTH-1F and NtcA-1R and cloned into *Eco*RI–*Sal*I-digested pGAD424 and pGBT9, respectively. All pGAD derivatives were sequenced using ACTAseq plus GAD-REV primers, and GBT-1F plus GBT-2R primers for pGBT derivatives.

RT-PCR analysis. To analyse the abundance of *pipX* mRNA under nitrate growth conditions, cells were grown under standard conditions until they reached OD_{750} 0.5. Aliquots (50 ml) were removed from the cultures for RNA extraction. The samples were rapidly chilled on ice and centrifuged, and the pellets were stored at −80 °C. Total RNA was isolated using the hot phenol method. RT-PCR analysis of *pipX* mRNA was performed using 0.5 µg total *Synechococcus* RNA that was retrotranscribed in a total volume of 30 µl with the RevertAid H Minus M-MuLV reverse transcriptase (Fermentas) using oligonucleotide PipX-3X-1R (for *pipX*) and Sip1-BTH-R (for *sipA*) as primers. A 10 µl sample of the retrotranscription reaction was subjected to 30 PCR cycles with primers PipX-OV-2F and PipX-3X-1R using NETZYME DNA polymerase. A total of 24 PCR cycles with primers Sip1-BTH-F and Sip1-BTH-R were used for *sipA*, used as loading control. For each pair of primers, a parallel reaction was carried out without reverse transcriptase as a control for DNA contamination of RNA preparations.

Determination of luciferase activity. Bioluminescence measurements were determined, essentially, as described previously (Espinosa *et al.*, 2007). Light emission was recorded using a Perkin Elmer Victor 3 microplate luminometer.

Immunoblot analysis of PipX. H_6-PipX was overexpressed in XL1-Blue *E. coli* cells as described previously (Espinosa *et al.*, 2006). Purified protein was sent to Pineda antibodies service in order to produce a PipX antiserum. After 90 days of rabbit immunization, antiserum raised against H_6-PipX was obtained.

To isolate proteins from *S. elongatus*, whole-cell protein extracts were prepared from 10 ml cells growth to mid-exponential phase (OD_{750} around 0.5). Cell pellets were lysed and the supernatant fraction was collected. Soluble protein concentrations were determined by Bradford reagent. Equal amounts of protein (60 µg) from each whole-cell extract sample were separated in a linear gradient (5–20 %) SDS-PAGE for immunoblotting. Proteins were transferred to 0.1 µm PVDF membranes (GE Healthcare) by semi-dry blot transfer, according to the manufacturer's instructions. To verify equal loading and transfer of proteins onto PVDF membranes, staining with Fast Green FCF dye was carried out after blotting.

Polyclonal rabbit antiserum raised against PipX was used at a 1 : 5000 dilution and PipX was detected using horseradish-peroxidase-conjugated anti-rabbit at dilution 1 : 10 000. Detection was carried out using an ECL Plus Western blotting detection kit (GE Healthcare) and scanning in a Typhoon 9410 fluorescence imaging system (GE Healthcare).

Multiple alignment and sequence comparison. Forty-seven PipX homologues obtained from the National Center for Biotechnology Information (NCBI) database were aligned using CLUSTAL_X (Larkin *et al.*, 2007) with default settings. RefSeq numbers are: YP_172742.1, *Synechococcus elongatus* PCC 6301; YP_001865314.1, *Nostoc punctiforme* PCC 73102; NP_484529.1, *Nostoc* sp. PCC 7120; ZP_01632661.1, *Nodularia spumigena* CCY9414; CAO89102.1, *Microcystis aeruginosa* PCC 7806; ZP_02976743.1, *Cyanothece* sp. PCC 7424; YP_001804130.1, *Cyanothece* sp. ATCC 51142; ZP_00514538.1, *Crocosphaera watsonii* WH 8501; NP_001035873.1, *Synechocystis* sp. PCC 6803; ZP_01624711.1, *Lyngbya* sp. PCC 8106; YP_001519639.1, *Acaryochloris marina* MBIC11017; ZP_01726412.1, *Cyanothece* sp. CCY0110; YP_001735159.1, *Synechococcus* sp. PCC 7002; ZP_02943302.1, *Cyanothece* sp. PCC 8801; NP_682399.1, *Thermosynechococcus elongatus* BP-1; YP_001660607.1, *Microcystis aeruginosa* NIES-843; NP_894038.1, *Prochlorococcus marinus* str. MIT 9313; YP_001018149.1, *Prochlorococcus marinus* str. MIT 9303; YP_476982.1, *Synechococcus* sp. JA-2-3B'a(2-13); YP_382322.1, *Synechococcus* sp. CC9605; YP_729816.1, *Synechococcus* sp. CC9311; YP_376658.1, *Synechococcus* sp. CC9902; NP_896752.1, *Synechococcus* sp. WH 8102; ZP_01086530.1, *Synechococcus* sp. WH 5701; YP_001224293.1, *Synechococcus* sp. WH 7803; ZP_01081458.1, *Synechococcus* sp. RS9917; YP_724299.1, *Trichodesmium erythraeum* IMS101; ZP_01472290.1, *Synechococcus* sp. RS9916; YP_001550275.1, *Prochlorococcus marinus* str. MIT 9211; YP_475449.1, *Synechococcus* sp. JA-3-3Ab; YP_001226724.1, *Synechococcus* sp. RCC307; NP_923420.1, *Gloeobacter violaceus* PCC 7421; ABE10750.1, uncultured *Prochlorococcus marinus* clone ASNC1092; ABE10884.1, uncultured *Prochlorococcus marinus* clone ASNC2259; YP_001517213.1, *Acaryochloris marina* MBIC11017; NP_874784.1, *Prochlorococcus marinus* subsp. *marinus* str. CCMP1375; YP_001090637.1, *Prochlorococcus marinus* str. MIT 9301; YP_292919.1, *Prochlorococcus marinus* str. NATL2A; YP_001010771.1, *Prochlorococcus marinus* str. MIT 9515; NP_892511.1, *Prochlorococcus marinus* subsp. *pastoris* str. CCMP1986; YP_001483672.1, *Prochlorococcus marinus* str. MIT 9215; ZP_01124790.1, *Synechococcus* sp. WH 7805; NP_925339.1, *Gloeobacter violaceus* PCC 7421; YP_396886.1, *Prochlorococcus marinus* str. MIT 9312; ZP_01085880.1, *Synechococcus* sp. WH 5701; YP_001014274.1, *Prochlorococcus marinus* str. NATL1A; ABE11268.1, uncultured *Prochlorococcus marinus* clone HF10-88F10). This alignment was used to generate a sequence logo, created with WebLogo (http://weblogo.berkeley.edu//logo.cgi) (Crooks *et al.*, 2004).

RESULTS AND DISCUSSION

Point mutations R54C and L65Q or a lower level of PipX suffice to overcome lethality in P_{II}-deficient backgrounds

Spontaneous mutations in the *pipX* gene were found in P_{II}-deficient and P_{II}-null derivative strains (Espinosa *et al.*, 2009). In addition to one internal deletion, which is likely to cause a complete loss-of-function, we found three point mutations. *pipX-92delT*, located upstream of the coding region, is likely to decrease expression of the *pipX* gene, while the effect of mutations *pipX160C>T* and *pipX194T>A* encoding PipX^{R54C} and PipX^{L65Q}, respectively, could not be directly anticipated. To obtain indications of the possible effect of the R54C and L65Q substitutions on PipX functions, we analysed PipX sequences available at the NCBI database. A consensus sequence derived from a multiple alignment of the more relevant C-terminal sequences of PipX is shown in Supplementary Fig. S1, available with the online version of this paper. Residues R54 and L65 do not belong to any

4 **Table 1.** Strains used in this study

Strain or plasmid	Genotype or relevant characteristics	Source or reference
E. coli DH5α	F^- ϕ80*dlacZ*ΔM15Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17* (r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1* λ^-	Hanahan (1985)
S. cerevisiae Y187	MATα *ura3-52 his3-200 ade2-101 trp1-901 leu2-3, 112 gal4Δ met*$^-$ *gal80Δ URA*::$GAL1_{UAS}$-$GAL1_{TATA}$-*lacZ*	Harper *et al.* (1993)
S. cerevisiae PJ696	MATa *ade2Δ trp1-901 leu2-3,112 ura3-52 his3-200 cyh*^{r} *can*^{r} *gal4Δ gal80Δ met2*$^-$ *GAL2*::*ADE2 GAL1*::*HIS3* GAL7:*lacZ*	James *et al.* (1996)
Synechococcus sp. PCC7942	Wild-type *Synechococcus* sp. PCC 7942	Pasteur culture collection
Synechococcus SA591	$PipX^-$, *pipX*::C.K1(−), Km^r	Espinosa *et al.* (2006)
Synechococcus SA410	PipX(Con) [Φ(C.K1–*pipX*)], Km^r	Espinosa *et al.* (2006)
Synechococcus $CK1X^{R54C}$	$PipX^{R54C}$(Con) [Φ(C.K1(+)– *pipX160C*>*T*)], Km^r	This work
Synechococcus $CK1X^{L65Q}$	$PipX^{L65Q}$(Con) [Φ(C.K1(+)–*pipX194T*>*A*)], Km^r	This work
Synechococcus SA410-GlnBS(+)	PipX(Con), *glnB*/*glnB*::C.S3(+), Km^r Sm^r	Espinosa *et al.* (2009)
Synechococcus $CK1X^{R54C}$-GlnBS(+)	$PipX^{R54C}$(Con), *glnB*::C.S3(+), Km^r Sm^r	This work
Synechococcus $CK1X^{L65Q}$-GlnBS(+)	$PipX^{L65Q}$(Con), *glnB*::C.S3(+), Km^r Sm^r	This work
Synechococcus SA591-FAM2	$PipX^-$, P_{glnB}::*luxAB*, Km^r Cm^r	Espinosa *et al.* (2006)
Synechococcus SA591-FAM84W	$PipX^-$, P_{glnN}::*luxAB*, Km^r Cm^r	Espinosa *et al.* (2006)
Synechococcus CS3X	Φ(C.S3(−)–*pipX*), Sm^r	This work
Synechococcus $CS3X^{P\downarrow}$	Φ(C.S3(−)–*pipX-92delT*), Sm^r	This work
Synechococcus $CS3X^{R54C}$	$PipX^{R54C}$ [Φ(C.S3(−)–*pipX160C*>*T*)], Sm^r	This work
Synechococcus $CS3X^{L65Q}$	$PipX^{L65Q}$[Φ(C.S3(−)–*pipX194T*>*A*)], Sm^r	This work
Synechococcus CS3X-FAM2	Φ(C.S3(−)–*pipX*), P_{glnB}::*luxAB*, Sm^r Cm^r	This work
Synechococcus $CS3X^{P\downarrow}$-FAM2	Φ(C.S3(−)–*pipX-92delT*), P_{glnB}::*luxAB*, Sm^r Cm^r	This work
Synechococcus $CS3X^{R54C}$-FAM2	$PipX^{R54C}$, P_{glnB}::*luxAB*, Sm^r Cm^r	This work
Synechococcus $CS3X^{L65Q}$-FAM2	$PipX^{L65Q}$, P_{glnB}::*luxAB*, Sm^r Cm^r	This work
Synechococcus CS3X-FAM84W	Φ(C.S3(−)–*pipX*), P_{glnN}::*luxAB*, Sm^r Cm^r	This work
Synechococcus $CS3X^{P\downarrow}$-FAM84W	Φ(C.S3(−)–*pipX-92delT*), P_{glnN}::*luxAB*, Sm^r Cm^r	This work
Synechococcus $CS3X^{R54C}$-FAM84W	$PipX^{R54C}$, P_{glnN}::*luxAB*, Sm^r Cm^r	This work
Synechococcus $CS3X^{L65Q}$-FAM84W	$PipX^{L65Q}$, P_{glnN}::*luxAB*, Sm^r Cm^r	This work
Synechococcus WTK	C.K1 into Neutral site I, Km^r	This work
pBluescriptII SK(+)	Cloning vector, Ap^r	Stratagene
pGAD424	*LEU2*, GAL4(768–881)AD, Ap^r	Bartel *et al.* (1993)
pGBT9	*TRP1*, GAL4(1–147)BD, Ap^r	Bartel *et al.* (1993)
pUAGC11	GAL4AD : P_{II}	Burillo *et al.* (2004)
pUAGC12	GAL4BD : P_{II}	Burillo *et al.* (2004)
pUAGC6	GAL4AD : NtcA	This work
pUAGC8	GAL4BD : NtcA	This work
pUAGC406	GST : $PipX^{R54C}$, Ap^r	This work
pUAGC702	C.S3(+) into *glnB*. Ap^r Sm^r	Espinosa *et al.* (2009)
pUAGC471	GAL4AD : PipX	This work
pUAGC472	GAL4BD : PipX	This work
pUAGC497	GAL4AD : $PipX^{R54C}$	This work
pUAGC498	GAL4BD : $PipX^{R54C}$	This work
pUAGC371	GAL4AD : $PipX^{L65Q}$	This work
pUAGC372	GAL4BD : $PipX^{L65Q}$	This work
pUAGC373	GAL4AD : $P_{II}^{T\text{-loop}+7}$	This work
pUAGC374	GAL4BD : $P_{II}^{T\text{-loop}+7}$	This work
pUAGC410	Φ(C.K1(+)–*pipX*), Ap^r Km^r	Espinosa *et al.* (2006)
pUAGC393	Φ(C.S3(−)–*pipX*), Ap^r Sm^r	This work
pUAGC390	Φ(C.S3(−)–*pipX194T*>*A*), Ap^r Sm^r	This work
pUAGC391	Φ(C.S3(−)–*pipX160C*>*T*), Ap^r Sm^r	This work
pUAGC392	Φ(C.S3(−)–*pipX-92delT*), Ap^r Sm^r	This work
pUAGC59	pBluescript SK(+) with 1.8 kb from *pipX* region, Ap^r	Espinosa *et al.* (2006)
pUAGC387	*pipX194T*>*A*, Ap^r	This work
pUAGC388	*pipX160C*>*T*, Ap^r	This work
pUAGC681	Φ(C.K1(+)–*pipX160C*>*T*), Ap^r Km^r	This work

Table 1. cont.

Strain or plasmid	Genotype or relevant characteristics	Source or reference
pUAGC682	Φ(C.K1(+)–*pipX194T>A*), Ap^r Km^r	This work
pFAM2	*glnB*::*luxAB*, Ap^r Cm^r	Aldehni *et al.* (2003)
pFAM84W	*glnN*::*luxAB*, Ap^r Cm^r	Aldehni & Forchhammer (2006)
pUAGC613	C.K1 into NSI, Ap^r Km^r	This work
pRL161	C.K1, Ap^r Km^r	Elhai & Wolk (1988)
pUAGC623	pBluescriptII SK(+) with NSIA and NSIB, Ap^r	Salinas *et al.* (2007)

of the invariant positions in PipX, but both are moderately conserved. Position 54 is occupied in most PipX orthologues by either Arg or Tyr. Position 65 is always occupied by a hydrophobic residue, with Leu being the most frequent amino acid. Thus, the non-conservative nature of the amino acid substitution and the relative conservation of Arg54 and Leu65 strongly suggested that mutations R54C and L65Q could alter PipX properties.

To confirm that the spontaneous point mutations found in P_{II}-deficient backgrounds were indeed suppressing lethality, mutations *pipX-92delT*, *pipX160C>T* and *pipX194T>A* were introduced at their original chromosomal location, by allelic replacement, into a wild-type background. To this end, the streptomycin resistance cassette C.S3 was used as a linkage marker and placed 121 bp upstream (from the initial ATG) of the corresponding *pipX* alleles (Fig. 1a). To exclude polar effects and minimize possible artefacts due to the presence of the C.S3 cassette, a streptomycin-resistant control strain retaining the wild-type *pipX* allele (CS3X) was generated in parallel to mutant strains ($CS3X^{P\downarrow}$, $CS3X^{R54C}$ and $CS3X^{L65Q}$). Homozygosity for C.S3 alleles was promptly achieved and it was confirmed that the presence of the streptomycin resistance cassette C.S3 did not confer significant phenotypic differences to the wild-type *S. elongatus* strain under standard or stress conditions (data not shown). RT-PCR analyses of $CS3X^{P\downarrow}$ and the control strain showed that there were lower transcript levels in the mutant (Fig. 1b), thus confirming that the negative effect on *pipX* transcript levels observed in the MP2-A strain (Espinosa *et al.*, 2009) was indeed caused by the *pipX-92delT* change.

To determine the effect of point mutations on the levels and/or stability of PipX in *S. elongatus*, cultures of strains CS3X, $CS3X^{P\downarrow}$, $CS3X^{R54C}$ and $CS3X^{L65Q}$ were obtained

Table 2. Oligonucleotides used in this study

Name	Sequence (5′–3′)
CS3-2F	ACAAAACGGTTTACCAGCAT
CK1-2F	GGGGATCTCATGCTGGAG
ACTAseq	AGGGATGTTTAATACCACTAC
GAD-REV	GTTGAAGTGAACTTGCGG
GBT-1F	GTGGAGACTGATATGCC
GBT-2R	CCTACAGGAAAGAGTTACTC
GlnB-1F	GGCTTAAGGAGAATTCCCTTGAAGAAG
GlnB-1R	AACTGCAGTCGACGCTGACTTAGATTGCGTCG
NSII-1F	AGGTTGTCCTTGGCGCAGCG
NSII-1R	AGCGGATTTTGCATCACGAAGC
Syn2060-1F	GGCCTGCCGAAGGAGCAGTGGTACG
PipX-6R	GACTTGCTGCAGCTGG
PipX-OV-2F	GAGAATTCGCTTCCGAGAACTACC
PipX-3X-1R	CTGCCTCTGAATTCCTAGCTGGCTACAG
PipX-L65Q-F	GGTCGACAACCGTCAGCGCCAGCTGCGCCGAGATGC
PipX-L65Q-R	GCATCTCGGCGCAGCTGGCGCTGACGGTTGTCGACC
PipX-R54C-F	GCTTTGAGCCAATCGGTTGTAATGAAGCGCGGATGTTGG
PipX-R54C-R	CCAACATCCGCGCTTCATTACAACCGATTGGCTCAAAGC
NtcA-YTH-1F	CACGGAATTCATGCTGGCCAACG
NtcA-1R	GCTGGCCAACGAGAATTCTCTGCTG
Sip1-BTH-F	ACCCGGATCCCTCTATGGATTTTG
Sip1-BTH-R	GGGGGTACCTTGATTCAGAC

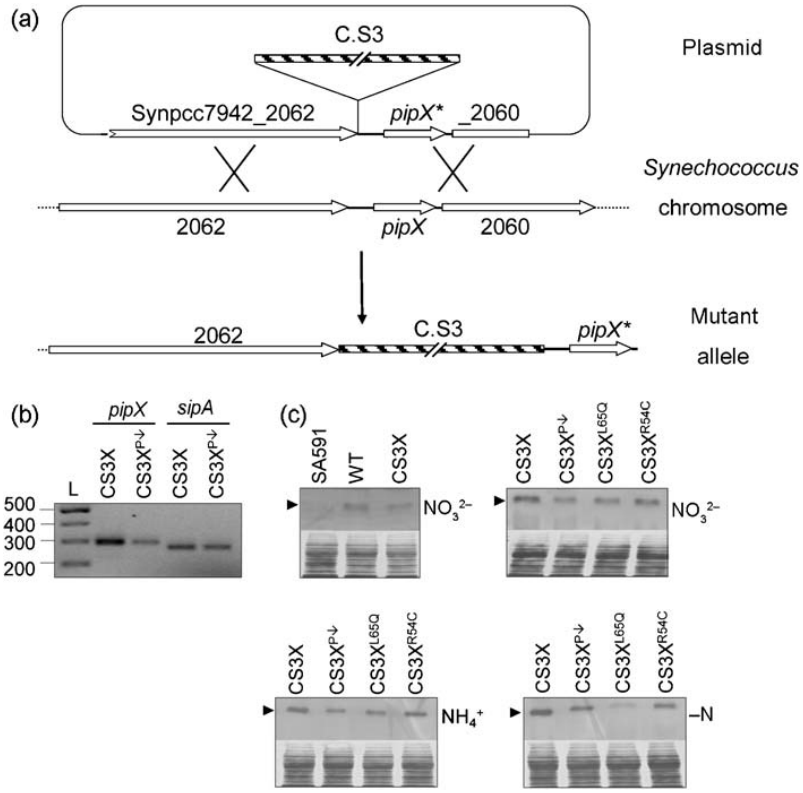

Fig. 1. Effects of point mutations on *pipX* transcription and PipX accumulation. (a) Strategy used to construct strains CS3X, CS3X$^{P\downarrow}$, CS3X^{R54C} and CS3X^{L65Q} carrying ΦC.S3–*pipX*, ΦC.S3–*pipX-92delT*, ΦC.S3–*pipX*^{R54C}and ΦC.S3–*pipX*^{L65Q}, respectively. Mutant *pipX* alleles linked to C.S3 are collectively designated here *pipX**. The position of C.S3 in relation to the *pipX* gene and flanking genomic regions is shown. (b) Effect of *pipX-92delT* mutation on *pipX* transcript levels. Amplification of *pipX* and *sipA* (used as a loading control) by RT-PCR in the CS3X and CS3X$^{P\downarrow}$ strains. A representative experiment from two independent RNA extractions is shown. Molecular sizes (bp) are given on the left. (c) Effect of *pipX* mutations on PipX protein accumulation levels. Immunodetection was performed from cells grown in the presence of nitrate (NO_3^{2-}), ammonium (NH_4^+) and in a medium lacking combined nitrogen for 24 h (–N). Immunodetected PipX is indicated by an arrowhead. The protein loading control for each lane is shown. Strains used were wild-type (WT), PipX-null mutant (strain SA591) and CS3X derivatives.

under different nitrogen regimes (ammonium, nitrate and 24 h after nitrogen depletion) and analysed by Western blots (Fig. 1c). In close agreement with the RT-PCR data, reduced protein levels were observed in strain CS3X$^{P\downarrow}$, the differences from the control strain were small but significant under all tested conditions. On the other hand, no significant differences were observed between CS3X^{R54C} and the control strain CS3X, while the level of protein in CS3X^{L65Q} was significantly reduced under nitrogen-deprivation conditions. In summary, two of the three spontaneous point mutations affected PipX levels in at least one of the environmental conditions analysed.

Because cyanobacteria contain multiple chromosome copies, the persistence, in the course of genetic inactivation trials, of wild-type alleles under selective conditions indicates that the targeted gene is essential. In this context, we showed that, while *glnB* was readily inactivated in *pipX*-null mutants, the presence of *pipX* alleles prevented homozygosis of cassette-inactivated alleles of *glnB* in the two *S. elongatus* strains used as wild-type controls. In the control strain SA410, carrying the marker fusion Φ(C.K1–*pipX*), transcription of the *pipX* gene takes place from a constitutive promoter present in the C.K1 cassette and results in twice the level of PipX protein, irrespective of whether cultures were grown with nitrate or ammonium, or supplemented with kanamycin (Fig. 2a).

The levels of PipX protein were slightly but consistently lower in strains carrying the Φ(C.S3-*pipX*) allele (compare WT and CS3X in Fig. 1c). Interestingly, the small decrease

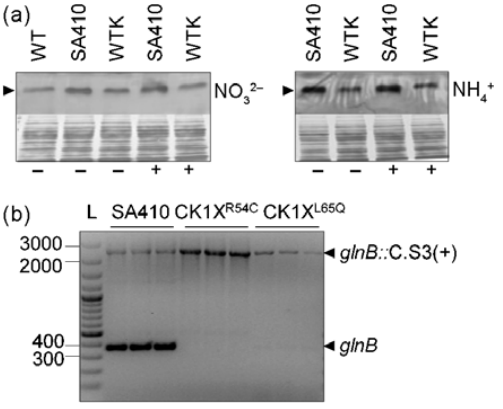

Fig. 2. Genetic inactivation of *glnB* in *pipX* mutants. (a) *In vivo* overexpression of PipX mediated by the C.K1 promoter. Immunodetection of PipX in strains grown to mid-exponential phase in the presence of either nitrate (NO_3^{2-}) or ammonia (NH_4^+) in the presence (+) or absence (–) of kanamycin. Immunodetected PipX is indicted by an arrowhead. The protein loading control for each lane is shown. Strains: wild-type (WT), SA410 (ΦC.K1–*pipX*) and WTK (C.K1 inserted into Neutral site I). (b) Segregation of *glnB* alleles, verified by PCR, with primers GlnB-1F and GlnB-1R in three independent clones of strains carrying ΦC.K1–*pipX* (SA410), ΦC.K1–*pipX*^{R54C} (CK1X^{R54C}) and ΦC.K1–*pipX*^{L65Q} (CK1X^{L65Q}) after transformation with *glnB*::C.S3(+) and three consecutive transfers onto selective media. Lane L, size marker (GeneRuler 100 bp plus DNA ladder, Fermentas). PCR products corresponding to specific alleles are indicated to the right, and relevant marker sizes (bp) are shown to the left.

in PipX levels caused by the upstream insertion of the C.S3 facilitated rapid *glnB* inactivation in strain CS3X (data not shown). This supports the notion that a relatively small decrease in *pipX* gene dosage was enough to suppress lethality, as could be inferred by the occurrence of the *pipX-92delT* change in strain MP2A (Espinosa *et al.*, 2009) and the present results with CS3X $^{P\downarrow}$ (Fig. 1c).

To investigate the effects of mutations R54C and L65Q on PipX function, we turned to strain SA410, in which the levels of PipX do interfere with inactivation of *glnB*, to construct isogenic strains encoding the mutant proteins PipX^{R54C} or PipX^{L65Q}. Cultures from SA410 and mutant derivatives CK1X^{R54C} and CK1X^{L65Q} were then transformed in parallel with allele *glnB*::C.S3(+) and analysed by PCR as described previously (Espinosa *et al.*, 2009). As expected, wild-type *glnB* alleles persisted in all independent transformant clones from the control strain bearing Φ(C.K1–*pipX*) (Fig. 2b, lanes SA410). In contrast, homozygosis for *glnB*::C.S3(+) was easily achieved in both Φ(C.K1–*pipX*^{R54C}) and Φ(C.K1–*pipX*^{L65Q}) backgrounds, since only the longer *glnB*::C.S3(+) alleles could be amplified from the different transformants tested (Fig. 2b, lanes CK1X^{R54C} and CK1X^{L65Q}). Therefore, each one of the original spontaneous point mutations (*pipX160C>T* and *pipX194T>A*) suffice to suppress the lethality associated with *glnB* inactivation.

Effect of *pipX* mutations on NtcA-dependent activation of reporter genes

The effects of the same three spontaneous point mutations on the function of PipX as a co-activator of NtcAwere analysed in strains containing the NtcA-dependent promoter derivatives P$_{glnB}$::*luxAB* and P$_{glnN}$::*luxAB*. Each of the two gene fusions carries a single NtcA-dependent promoter with distinct nitrogen-dependent induction. In these constructs, reporter expression is strictly dependent on NtcA but exhibits different induction profiles (Aldehni & Forchhammer, 2006; Aldehni *et al.*, 2003). Reporter expression was determined by bioluminescence measurements from control and *pipX*-mutant-derivative strains, grown to mid-exponential phase in the presence of ammonium or nitrate and after cultures were shifted from ammonium-containing to nitrogen-depleted medium. Results for mutant activity levels are presented relative to their appropriate wild-type control (Fig. 3).

When CS3X and its derivatives were compared, results indicated that the three point mutants were affected in activation of P$_{glnB}$::*luxAB* and P$_{glnN}$::*luxAB* reporters, the defects being more evident after nitrogen depletion. However, none of these were as impaired as the *pipX* null mutant control. Taking into account the reduced protein levels shown in the Western blots by strain CS3X$^{P\downarrow}$ under all conditions tested, its reduced level of activity could be attributable to lower levels of protein rather than to a specific defect in NtcA coactivation. The same would apply to the strain expressing PipX^{L65Q}, that under nitrogen limitation showed lower levels of PipX. In contrast, the lower activity of strain CS3X^{R54C} did not correlate with reduced protein levels, suggesting that PipX^{R54C} is specifically impaired in NtcA coactivation. To take into account possible artefacts related to gene dosage differences, the same experiments were performed with strain SA410 and mutant derivatives. In close agreement with results obtained from CS3X derivatives, strains expressing mutant proteins PipX^{L65Q} and PipX^{R54C} were both distinctly affected in activation of P$_{glnB}$::*luxAB* and

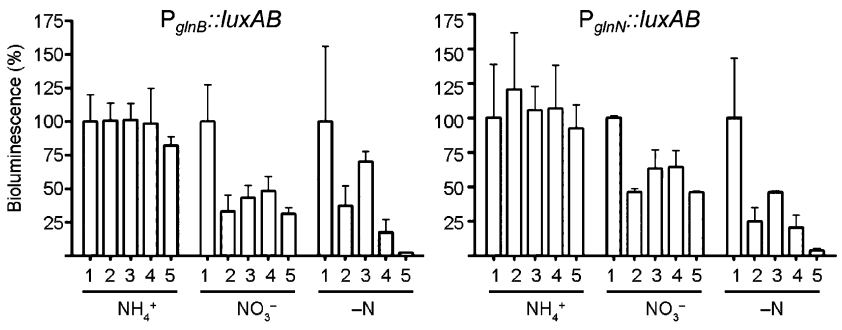

Fig. 3. Effect of *pipX* mutations on P$_{glnB}$::*luxAB* and P$_{glnN}$::*luxAB* expression. Bioluminescence of mutant strains, expressed as percentages of *glnB* (left) and *glnN* (right) promoter activities, were determined in cells grown in the presence of ammonium or nitrate and 24 h after cultures were shifted from ammonium-containing to nitrogen-depleted medium, taking as a reference (100 %) the activity of the control strain CS3X. Mean values (± SD) from at least three independent experiments performed in duplicate are plotted. Strains: CS3X, 1; CS3X$^{P\downarrow}$, 2; CS3X^{R54C}, 3; CS3X^{L65Q}, 4; SA591, 5. Relative activities [RLU ml^{-1} OD$_{750}^{-1}$ (10 s)$^{-1}$] of the control strain grown in ammonia or nitrate and after cultures were shifted from ammonium-containing to nitrogen-free media were 618 ± 124, 62 292 ± 17 059, 964 170 ± 543 051, respectively, for P$_{glnB}$::*luxAB* and 1095 ± 425, 4384 ± 70, 118 195 ± 51 184, respectively, for P$_{glnN}$::*luxAB*.

3 P_{glnN}::*luxAB* reporters. Again, during nitrogen depletion, but not in nitrate-containing media, $PipX^{L65Q}$ was a less effective coactivator than $PipX^{R54C}$ (data not shown)

Complex formation by the mutant proteins $PipX^{L65Q}$, $PipX^{R54C}$ and $P_{II}^{T\text{-loop}+7}$

We next tested the ability of the two mutant proteins $PipX^{R54C}$ and $PipX^{L65Q}$ to interact with NtcA and P_{II} in the yeast two-hybrid system, previously used to provide evidence of the specificity of interactions mediated by PipX (Espinosa *et al.*, 2006). Here, we generated additional wild-type and mutant constructs and also included the PII derivative $P_{II}^{T\text{-loop}+7}$ (Espinosa *et al.*, 2009), a mutant protein that would allow us to explore the effect of a drastic disruption of the T-loop of P_{II} in interactions with PipX, in these analyses. Expression of reporters was determined in Y187/PJ696 diploids as described previously (Burillo *et al.*, 2004). In all cases, mutant and control proteins were fused independently to both GAL4BD and GAL4AD domains. The results of the yeast two-hybrid analysis, shown in Supplementary Fig. S2, available with the online version of this paper, are schematically summarized in Fig. 4.

The $PipX^{R54C}$ protein was affected in yeast two-hybrid interactions with NtcA, but not with P_{II}, suggesting that residue R54 may interact with NtcA to assist with transcriptional activation of NtcA-dependent promoters. On the other hand, the $PipX^{L65Q}$ protein was not affected in its ability to interact with P_{II} or NtcA in the yeast two-hybrid system. Although the *in vivo* defect of $PipX^{L65Q}$ may be just due to decreased stability in *S. elongatus* (see above), other functional defects cannot be excluded at present.

The presence of seven extra residues at the T-loop of $P_{II}^{T\text{-loop}+7}$ did impair interactions with NAGK in the yeast two-hybrid system; this was anticipated, since the T-loop forms one of the main interaction surfaces in the NAGK–P_{II} complex (Llacer *et al.*, 2007). Interestingly, the $P_{II}^{T\text{-loop}+7}$ protein, in spite of the anomalous and longer T-loop, was still able to interact with both P_{II} and PipX protein derivatives, implying that the T-loop does not play a relevant role in formation of PipX–P_{II} complexes. The mutant protein $P_{II}^{T\text{-loop}+7}$, fortuitously detected in *S. elongatus* in the course of a previous study, could confer a selective advantage in a P_{II}-deficient background (Espinosa *et al.*, 2009). If that was the case, the results obtained in the interaction analysis could indicate that complex formation between P_{II} and PipX, but not between P_{II} and NAGK, is important to prevent lethality associated with P_{II} deficiency in *S. elongatus*.

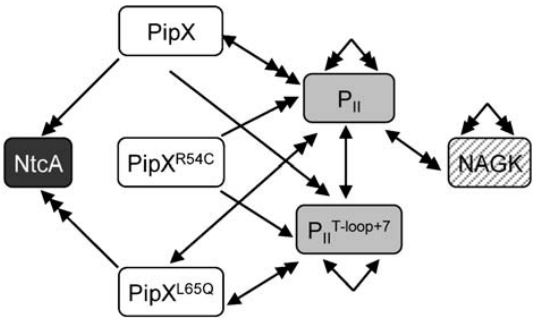

Fig. 4. Yeast two-hybrid interactions involving NtcA, P_{II} and PipX mutant derivatives. Schematic representation of interacting proteins is shown. The number of arrowheads indicates the relative strength of the interaction as described previously (Burillo *et al.*, 2004).

Point mutations R54C and L65Q and suppression of PipX lethality in P_{II}-deficient backgrounds

It is now clear that a small reduction in PipX levels suffices to overcome the toxic effect of PipX in *S. elongatus*. Direct evidence is provided by the fact that in strain CS3X, with lower level of *pipX* expression than the wild-type control, *glnB* is readily inactivated. The finding in P_{II}-deficient strains (Espinosa *et al.*, 2009) of mutations that reduce the level of *pipX* gene products (Fig. 1b) is another indication. However, the question regarding whether the other two known suppressor mutations work in the same way remains. That is, are $PipX^{R54C}$ and $PipX^{L65Q}$ just less active proteins with no specific defects or are they affected in specific functions? In this context, we have shown that the two mutant proteins have different properties. Although none of them was affected in P_{II} binding, and therefore they retained this specific function, both of them were somehow limited in their ability to activate NtcA. However, the molecular basis of this defect appeared different. $PipX^{R54C}$, but not $PipX^{L65Q}$, was impaired in yeast two-hybrid interactions with NtcA, providing a rationale for the reduced NtcA-dependent activation of reporters. On the other hand, $PipX^{L65Q}$, but not $PipX^{R54C}$, appeared unstable under nitrogen limitation (Fig. 1c), and thus a correlation was found between the levels of $PipX^{L65Q}$ and its ability to activate NtcA-dependent reporters.

It should be noted that relatively high 2-oxoglutarate levels are required for binding of PipX to NtcA (Espinosa *et al.*, 2009), arguing against the theory that complex formation between NtcA and PipX plays a major role in the toxic effect observed in P_{II} deficient nitrogen-containing cultures. Although assays for NtcA binding and NtcA activation with the mutant proteins $PipX^{L65Q}$ and $PipX^{R54C}$ showed differences between $PipX^{R54C}$ and $PipX^{L65Q}$, they did not provide conclusive evidence concerning or excluding a role in toxicity of the NtcA–PipX complexes. Therefore, in light of these results, we cannot yet exclude a role for NtcA–PipX complexes in toxicity. However, we were unable to obtain genetic evidence supporting the implication of NtcA in toxicity (Espinosa *et al.*, 2009) and thus we are inclined to think that PipX toxicity may be related to its binding to an as-yet unknown partner at the relatively low 2-oxoglutarate levels typical of nitrogen-rich cultures. It is tempting to speculate

that $PipX^{L65Q}$ and $PipX^{R54C}$ are both specifically affected in that currently unknown interaction.

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