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Mutations at *pipX* Suppress Lethality of P_{II}-Deficient Mutants of *Synechococcus elongatus* PCC 7942[▽]

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The P_{II} proteins are found in all three domains of life as key integrators of signals reflecting the balance of nitrogen and carbon. Genetic inactivation of P_{II} proteins is typically associated with severe growth defects or death. However, the molecular basis of these defects depends on the specific functions of the proteins with which P_{II} proteins interact to regulate nitrogen metabolism in different organisms. In *Synechococcus elongatus* PCC 7942, where P_{II} forms complexes with the NtcA coactivator PipX, attempts to engineer P_{II}-deficient strains failed in a wild-type background but were successful in *pipX* null mutants. Consistent with the idea that P_{II} is essential to counteract the activity of PipX, four different spontaneous mutations in the *pipX* gene were found in cultures in which *glnB* had been genetically inactivated.

Cyanobacteria are phototrophic organisms that perform oxygenic photosynthesis. Autotrophic growth requires the constant assimilation of ammonium via the coordinated action of glutamine synthetase (GS) and glutamate synthase, also known as the GS-GOGAT cycle, resulting in consumption of 2-oxoglutarate (34). Due to the lack of 2-oxoglutarate dehydrogenase in cyanobacteria, synthesis of 2-oxoglutarate represents the final step in the oxidative branch of the trichloroacetic acid cycle and directly links 2-oxoglutarate levels to nitrogen assimilation (35). Thus, 2-oxoglutarate accumulates during nitrogen starvation, making this metabolite an excellent indicator of the intracellular carbon-nitrogen balance (12, 25).

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 (20, 30, 41). 2-Oxoglutarate, the signal of nitrogen deficiency, stimulates binding of NtcA to target sites (45), transcription activation in vitro (44), and complex formation between the global nitrogen regulator NtcA and its coactivator factor PipX, a regulatory protein conserved in cyanobacteria (5, 9). The interaction between PipX and NtcA is known to be relevant for maximal activation of NtcA-dependent genes under nitrogen limitation (9, 10). PipX-deficient cultures of *Synechococcus elongatus* PCC 7942 showed reduced activity of nitrogen assimilation enzymes, retarded nitrogen induction, a slower rate of nitrate consumption, and when subjected to nitrogen starvation, retarded phycobilisome degradation and faster reduction of the chlorophyll content (10).

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 (28). 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 in reference 13). In cyanobacteria, several proteins are known to form complexes with P_{II}. The first two P_{II} receptors were identified in *S. 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 (5, 6, 42), and the regulatory factor PipX (5, 9). The nonconserved membrane protein PamA was identified as a P_{II} receptor in *Synechocystis* sp. strain PCC 6803 (37). Structural and functional details are only known for the P_{II}-NAGK complex (29). 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 (23, 27), nitrate reductase (43), and the control of inorganic carbon transport (21).

P_{II} proteins bind 2-oxoglutarate and ATP synergistically. In *S. elongatus* and *Synechocystis* sp. strain PCC 6803, the T loop is phosphorylated at a seryl residue (S49) located at the apex of the solvent-exposed T loop (12). The phosphorylation status of P_{II} correlates with 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 (9, 31), suggesting that PipX-P_{II} complexes could also have a function under nitrogen-sufficient conditions. In this context, previous analyses indicated that PipX is not required for P_{II}-dependent functions like ammonium inhibition of nitrate transport (10) or stimulation of NAGK activity (8). However, we show here that P_{II} does, indeed, affect PipX functions. Our results indicate that P_{II} is essential under standard growth conditions in *S. elongatus* and that the *pipX* gene is a target of suppressor mutations in P_{II}-deficient cultures.

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MATERIALS AND METHODS

Strains and growth conditions. The strains and plasmids used in this work are listed in Table 1. Constructs and genomic mutations were analyzed by automated

TABLE 1. Strains and plasmids used in this work[a]

Strain or plasmid	Genotype or relevant characteristic(s)	Source or reference
Strains		
E. coli DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1* λ$^-$	16
Synechococcus sp. strain PCC 7942	Wild-type strain	Pasteur Culture Collection
Synechococcus sp. strain MP2	P_{II}^- (*glnB*::CK2) Km^r	14
Synechococcus sp. strain MP2-A	*glnB*::CK2 P_{II}^{S49A} Sm^r Km^r	27
Synechococcus sp. strain MP2-E	*glnB*::CK2 P_{II}^{S49E} Sm^r Km^r	27
Synechococcus sp. strain GlnBK	*glnB*/*glnB*::CK2 Km^r	This work
Synechococcus sp. strain GlnBS(+)	*glnB*/*glnB*::CS3(+) Sm^r	This work
Synechococcus sp. strain GlnBS(−)	*glnB*::CS3(−) Sm^r	This work
Synechococcus sp. strain GlnBS(−)$^{T\ loop\ +\ 7}$	*glnB*::CS3(−)/*glnB 133_134insATAAAGCTTATCGATACCGTC* Sm^r	This work
Synechococcus sp. strain SA410	PipX(Con) [Φ(CK1-*pipX*)] Km^r	9
Synechococcus sp. strain SA591	PipX$^-$ (*pipX*::CK1) Km^r	9
Synechococcus sp. strain SA410-GlnBS(−)	PipX(Con) *glnB*/*glnB*::CS3(−) Sm^r Km^r	This work
Synechococcus sp. strain SA591-GlnBS(−)	PipX$^-$ *glnB*::CS3(−) Sm^r Km^r	This work
Synechococcus sp. strain SA410-GlnBS(+)	PipX(Con) *glnB*/*glnB*::CS3(+) Sm^r Km^r	This work
Synechococcus sp. strain SA591-GlnBS(+)	PipX$^-$ *glnB*::CS3(+) Sm^r Km^r	This work
Plasmids		
pBluescriptII SK(+)	Cloning vector, Ap^r	Stratagene
pPM128	*glnB*::CK2, Km^r	14
pUAGC59	pBluescript SK(+) with 1.8 kb from *pipX* region, Ap^r	9
pUAGC453	pBluescriptII SK(+) with CS3, Ap^r Sm^r	40
pUAGC700	pBluescriptII SK(+) with 1.3-kb *glnB* genomic region, Ap^r	This work
pUAGC701	pUAGC700 with CS3 into *glnB* [*glnB*::CS3(−)], Ap^r Sm^r	This work
pUAGC702	Same as pUAGC701, with CS3 in opposite orientation [*glnB*::CS3(+)], Ap^r Sm^r	This work

[a] Note that some strains are heteroallelic for *glnB* and therefore unstable (see text for details).

dideoxy DNA sequencing. All cloning procedures were carried out with *Escherichia coli* DH5α by using standard techniques.

S. elongatus 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. The media used were BG11 ($BG11_0$ plus 17.5 mM $NaNO_3$ and 10 mM HEPES/NaOH, pH 7.8) or BG11-NH_4 ($BG11_0$ plus 10 mM NH_4Cl and 10 mM HEPES/NaOH, pH 7.8). For growth on plates, the medium was solidified by addition of 1% (wt/vol) agar. Plates were incubated at 30°C under constant illumination. The transformation procedure was essentially as described previously (15). Cells were incubated for 48 h at 30°C under constant illumination on nitrocellulose filters (Millipore), and transformants were selected on kanamycin-, chloramphenicol-, or streptomycin-containing BG11 plates. The antibiotic concentrations used were 10 μg ml^{-1} (kanamycin), 5 μg ml^{-1} (chloramphenicol), and 2 μg ml^{-1} (streptomycin).

Construction of plasmids. To construct pUAGC700, a 1.3-kb genomic DNA fragment including *glnB* was PCR amplified with primers 5′ GTCTAGAATTC CTTCCTGTTTGTGATG 3′ and 5′ GGACGGGATCCCTACCACCGCCTTT 3′, cut with EcoRI and BamHI, and cloned into pBluescript SK(+). A HincII-EcoRV fragment containing the CS3 cassette from pUAGC453 was then cloned into the AfeI site of pUAGC700, rendering plasmids pUAGC701 and pUAGC702.

PCR verification of the correct integration of resistance cassettes in *S. elongatus* was carried out with primers 5′ ACAAAACGGTTTACCAGCAT 3′ (CS3-2F) or 5′ CAACAAAGCTCTCATCAACC 3′ (CK2-F) and 5′ AACTGCAGT CGACGCTGACTTAGATTGCGTCG 3′ (GlnB-1R). Detection of *glnB* alleles by PCR were carried out with primers 5′ GGCTTAAGGAGAATTCCCTTGA AGAAG 3′ (GlnB-1F) and GlnB-1R.

RT-PCR analysis. For analysis of *pipX* mRNA abundance under nitrate growth conditions, cells were grown under standard conditions until they reached an optical density at 750 nm of 0.5. Fifty-milliliter aliquots were removed from the cultures for RNA extraction. The samples were rapidly chilled on ice and centrifuged, and pellets were stored at −80°C. Total RNA was isolated by the hot phenol method. Reverse transcription (RT)-PCR analysis of *pipX* mRNA was performed with 0.5 μg of total *S. elongatus* RNA that was retrotranscribed in a total volume of 30 μl with RevertAid H Minus Moloney murine leukemia virus reverse transcriptase (Fermentas) and primers 5′ CTGCCTCTGAATTCCTAG CTGGCTACAG 3′ (for *pipX*) and 5′ GGGGGTACCTTGATTCAGAC 3′ (for *sipA*). Ten microliters of the retrotranscription reaction mixture was subjected to 30 PCR cycles with primers 5′ GAGAATTCGCTTCCGAGAACTACC 3′ and 5′ CTGCCTCTGAATTCCTAGCTGGCTACAG 3′ and NETZYME DNA polymerase. Twenty-four PCR cycles with primers 5′ ACCCGGATCCCTCTA TGGATTTTG 3′ and 5′ GGGGGTACCTTGATTCAGAC 3′ were used for *sipA*, which was used as a 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.

RESULTS

The *glnB* gene is essential in *S. elongatus*. Inactivation of the *glnB* gene of *S. elongatus* was attempted by routinely used procedures of allelic replacement with two different cassettes providing kanamycin (CK2) or streptomycin (CS3) resistance. The three constructs used to inactivate *glnB* are schematically represented in Fig. 1A. Plasmid pPM128, previously used to generate *glnB* null strain MP2 (14), was used as a source of the *glnB*::CK2 allele. Two other constructs, each one carrying the CS3 cassette in a different orientation in the AfeI site of the *glnB* gene, were also produced, *glnB*::CS3(+) and *glnB*::CS3(−). In all three cases, viable antibiotic-resistant transformants were obtained and subsequent PCR amplifications produced bands matching the expected sizes of cassettes CK2 (307 bp), CS3(−) (265 bp), and CS3(+) (254 bp), thus confirming their presence in the appropriated location within the *S. elongatus glnB* gene (Fig. 1B, lanes K, S+, and S−).

Due to the presence of multiple chromosome copies in cyanobacteria (3), gene inactivation in *S. elongatus* requires verification that allelic replacement has been completed. In this

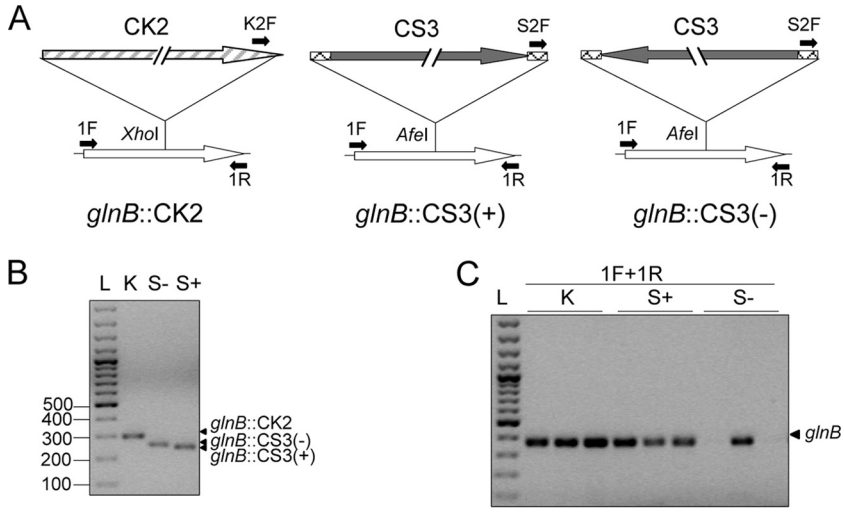

FIG. 1. Genetic inactivation of *glnB*. (A) Schematic representation of *glnB* null alleles. Relevant restriction sites in *glnB* (open arrows) are indicated. Positions of primers used to verify allele replacement are indicated by black arrows. (B) PCR verification of the correct integration of resistance cassettes with primers CS3-2F (S2F) or CK2-F (K2F) and GlnB-1R (1R) in a representative clone after transformation of *S. elongatus* with *glnB*::CK2 (lane K), *glnB*::CS3(−) (lane S−), and *glnB*::CS3(+) (lane S+). Lane L, size marker (GeneRuler 100-bp Plus DNA Ladder from Fermentas). PCR products corresponding to specific alleles are indicated at the right, and relevant marker sizes in base pairs are shown at the left. (C) Detection of *glnB* alleles by PCR with primers GlnB-1F (1F) and GlnB-1R (1R) in three independent clones after four consecutive transfers onto selective media. Other details are the same as in panel B.

context, the persistence of wild-type alleles after transfers to new media under selective conditions indicates that the targeted gene is essential. That was the case with the three types of clones generated here, since after several consecutive transfers onto selective plates they remained heteroallelic for *glnB* or gave rise to gene rearrangements (see below). As shown in Fig. 1C, wild-type *glnB* alleles were clearly observed by PCR in all of the independent *glnB*::CK2 and *glnB*::CS3(+) transformants analyzed (lanes K and S+ and data not shown). These two types of clones retained wild-type *glnB* alleles after at least six transfers of colonies onto selective media. In contrast, some of the *glnB*::CS3(−) transformants apparently lost wild-type *glnB* alleles after the fourth transfer onto streptomycin-containing media (Fig. 1C, lanes S−, and data not shown). The viability of these apparently segregated GlnBS(−) clones was compromised, since they failed to grow when transferred from solid to liquid media (data not shown). However, additional subculturing of these *glnB*::CS3(−) transformants on plates was possible and allowed the detection of a longer PCR product (Fig. 2A). Sequence analysis of this unexpected PCR product revealed the presence of a 21-bp insertion into the AfeI site of the *glnB* gene, corresponding to the ends of the CS3 cassette (allele *glnB133_134insATAAAGCTTATCGATACCGTC*, [hereafter, *glnB133_134ins*]). The rearrangement was probably generated by recombination between homologous sequences at the two junctions of the CS3 cassette with the *glnB* gene and resulted in seven extra amino acids between residues E44 and R45 (Fig. 2B), located at the flexible T loop of P_{II}. In contrast to their predecessor GlnBS(−) clones, derivatives in which the *glnB133_134ins* allele was detected (strain GlnBS(−)$^{T\ loop\ +\ 7}$) could be cultivated in liquid media. Detection of this spontaneously generated rearrangement of the *glnB*::CS3(−) allele suggested that the protein product, designated $P_{II}^{T\ loop\ +\ 7}$, conferred a selective advantage on *S. elongatus* cultures deficient in P_{II}.

In summary, attempts to generate *S. elongatus* P_{II}-deficient cells with three different null alleles, *glnB*::CK2, *glnB*::CS3(+), and *glnB*::CS3(−), failed. The alleles or allelic pairs detected by PCR in particular cultures from transformant clones were *glnB*/*glnB*::CK2, *glnB*/*glnB*::CS3(+), *glnB*/*glnB*::CS3(−), *glnB*::CS3(−), and *glnB*::CS3(−)/*glnB133_134ins*. Since cultures in which *glnB*::CS3(−) was apparently in homozygosis were invariably classified as *glnB*::CS3(−)/*glnB133_134ins* in the next PCR test, it seems likely that viable cells in those cultures had already acquired the *glnB133_134ins* allele. Taken together, the results indicated that *glnB* is essential for culture growth, at least under our laboratory conditions.

***glnB* null mutants carry mutations in *pipX*.** On several occasions, we noticed that our P_{II}-deficient cultures (MP2 strains) carried secondary mutations affecting the *pipX* coding sequence. A point mutation (allele *pipX160C>T*, which encodes the substitution R54C) was found fortuitously while cloning *glnB* sequences for plasmid construction. Since PCR amplification of genomic DNA, followed by direct sequencing, confirmed that the *pipX160C>T* mutation was present in the MP2 strain used for DNA amplification, we obtained a "new" culture of the MP2 strain (a gift from K. Forchhammer) to perform further analysis. Although single-PCR amplification and subsequent sequencing demonstrated the presence of the wild-type *pipX* allele in this strain, after performing RT-PCR assays, we became aware that this strain also carried sequences with a 22-bp deletion in *pipX* (allele *pipX25_46del*) causing a frameshift after position N7. Subsequent PCR analysis confirmed that both alleles, *pipX25_46del* and wild-type *pipX*, were present in this MP2 strain that we had received from K. Forchhammer's laboratory. Furthermore, a strain with the same or-

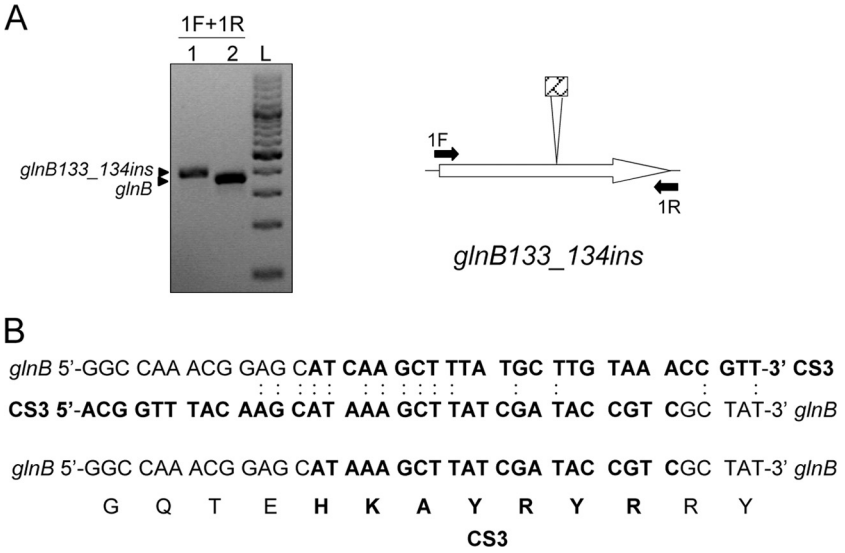

FIG. 2. The spontaneously generated *glnB* rearrangement. (A) Schematic representation of the *glnB133_134ins* allele and detection of *glnB* and *glnB133_134ins* alleles with primers GlnB-1R (1R) and GlnB-1F (1F). Wild type, lane 2; GlnBS(−)$^{\Gamma\ loop\ +7}$, lane 1. Relevant alleles are indicated at the left. Other details are as in Fig. 1. (B) Alignment of DNA sequences at the *glnB*-CS3 and CS3-*glnB* junctions from the *glnB*::CS3(−) allele and sequences present after the rearrangement. Predicted amino acids are shown in one-letter code. Nucleotides and predicted amino acids derived from the CS3 cassette are in bold.

igin that had already been genetically manipulated in another laboratory (a gift from F. Fernández-Piñas) was also found to be heteroallelic (*pipX*/*pipX25_46del*) after PCR analysis.

Sequence analysis of *pipX* from strains MP2-E and MP2-A, which both carry the same *glnB*::CK2 insertion, revealed a distinct *pipX* point mutation in each strain, allele *pipX194T>A*, which encodes substitution L65Q (in strain MP2-E), and a single-base deletion upstream of *pipX* (*pipX-92delT*) (in strain MP2-A). Since several independent PCR amplifications and subsequent sequence analyses gave identical results, these strains were apparently homoallelic at the time of the analysis. The presence of two distinct mutations in strains with the same origin suggested that the parental strain (MP2) might be heteroallelic for *pipX*. Further evidence for the genetic heterogeneity of *pipX* alleles in P_{II}-deficient strains was also obtained retrospectively. In particular, three different *pipX* alleles (*pipX*, *pipX194T>A*, and *pipX25_46del*) were detected in strains derived from the same MP2 culture in our laboratory after selection for different genetic traits. Table 2 summarizes the mutant alleles selected in the different MP2 derivatives analyzed.

Figure 3 illustrates the information on the locations and predicted impacts on the amino acid sequence of the spontaneous mutations found at the *pipX* open reading frame or the *pipX* promoter region. The location of *pipX-92delT*, just upstream of the *pipX* open reading frame, suggested that it could be a promoter-down mutation. To obtain additional evidence of the impact of the *pipX-92delT* mutation on *pipX* expression, we compared *pipX* mRNA levels in strains carrying the wild-type or mutant allele by RT-PCR. As shown in Fig. 4, *pipX* mRNA levels were significantly reduced in the *pipX-92delT* (represented as $X^{P\downarrow}$) mutant, while transcripts from unrelated controls were not altered, supporting the idea that the *pipX-92delT* mutation diminished the transcription of *pipX*. The effects of the R54C and L65Q mutations on PipX interactions will be analyzed elsewhere. Importantly, none of the *pipX* changes were found in wild-type *S. elongatus* strains, suggesting that the four mutations detected were bona fide suppressor mutations and that loss-of-function mutations at *pipX* alleviate the P_{II} deficiency phenotype.

TABLE 2. Spontaneous mutations found at *pipX* in strains carrying *glnB*::CK2 alleles in homozygosis

Mutation[a]	Strain origin	P_{II} protein expressed
pipX25_46del	MP2	None
pipX160C>T	MP2	None
pipX194T>A	MP2	None
pipX-92delT	MP2-A	P_{II}^{S49A}
pipX194T>A	MP2-E	P_{II}^{S49E}

[a] The mutation position is the distance, in nucleotides, from the start codon to the mutation site.

Inactivation by *pipX* suppresses the lethality associated with P_{II} deficiency. The finding of different putative suppressor mutations at *pipX* suggested that the lethality phenotype associated with P_{II} deficiency was due to an excess of active PipX protein, harmful to *S. elongatus* cells unless counteracted by P_{II}. If that were the case, inactivation of *glnB* alleles would be facilitated by elimination of PipX. To test this idea, we separately introduced the *glnB*::CS3(+) and *glnB*::CS3(−) alleles into a PipX$^-$ strain (SA591) in which the CK1 cassette replaced part of the *pipX* coding sequence. As an additional control, the *glnB*::CS3(+) and *glnB*::CS3(−) alleles were introduced in parallel into kanamycin-resistant strain SA410 [PipX-(Con)], where the *pipX* gene is transcribed from a constitutive promoter present in the CK1 cassette. The only phenotypic features noticed in the PipX(Con) strain were a small increase in the nitrogen induction of NtcA-dependent promoters (10)

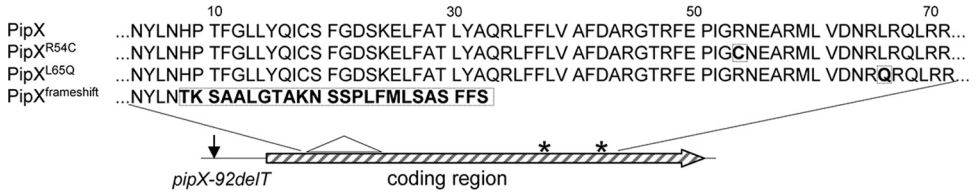

FIG. 3. Spontaneous mutations at *pipX*. (A) Comparison of the predicted amino acid sequences at the relevant regions of PipX, shown in one-letter code. Amino acids that differ from the wild type are in bold and boxed. The asterisks and triangle on the *pipX* genomic map indicate, respectively, point mutations and a deletion at mutant alleles. The vertical arrow indicates the site of the *pipX-92delT* mutation.

and a level of PipX protein twice that of the wild type (data not shown).

PCR analyses confirmed the correct integration of the CS3(+) and CS3(−) cassettes within *glnB* for each of the two strains used (Fig. 5A). After several transfers onto selective media, wild-type *glnB* alleles were observed in the PCR analysis of transformants from PipX(Con) (Fig. 5B, lanes $X^{C}S-$ and $X^{C}S+$). Thus, as previously observed with the wild-type strain (Fig. 1C), the presence of active *pipX* alleles prevented homozygosis of the *glnB*::CS3(+) and *glnB*::CS3(−) alleles. In contrast, homozygosis of both the *glnB*::CS3(+) and *glnB*::CS3(−) alleles was easily achieved in the $PipX^{-}$ background, as shown by the absence of *glnB* alleles in these $PipX^{-}$ recombinants (Fig. 5B, lanes $X^{-}S-$ and $X^{-}S+$). These results confirmed that the presence of functional *pipX* genes interfered with the inactivation of *glnB*, implying that an intact *glnB* gene is required for viability only in the presence of an active *pipX* gene.

DISCUSSION

Genetic inactivation of the genes that encode P_{II} proteins in many microorganisms leads to severe growth defects or death (2, 7, 32, 33, 48). In cyanobacteria, growth defects and death associated with P_{II} deficiency have also been reported, but depending on the model system, particular laboratory strains, or culture conditions, the *glnB* gene was considered essential or not essential. In *S. elongatus* PCC 7942 and *Synechocystis* sp. strain PCC 6803, *glnB* null mutants have been reported (14, 21, 24). In *Nostoc punctiforme* PCC 73102, *glnB* null mutants could not be obtained and the *glnB* gene was reported to be essential (17). In *Anabaena* sp. strain PCC 7120, previous attempts to inactivate *glnB* were unsuccessful (26). However, that report was followed by two others in which the obtention of *glnB* null mutants was described (38, 49). In the most recent of these, the authors showed that successful inactivation of *glnB* could only be achieved when the expression pattern of downstream genes of unknown function was altered (38). In the present work, we show the importance of P_{II} proteins for viability in the model system of *S. elongatus*, a cyanobacterium where successful inactivation of *glnB* was previously reported. Attention is called to the occurrence of suppressor mutations at the *pipX* gene of P_{II}-deficient strains.

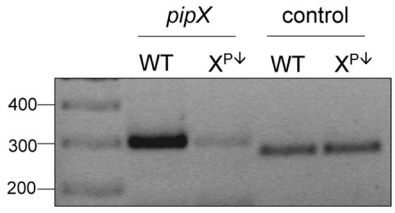

FIG. 4. Effect of *pipX-92delT* mutation on *pipX* transcript levels. RT-PCR amplification of *pipX* and *sipA* (used as a loading control) in the wild type (WT) and MP2-A ($X^{P\downarrow}$). A representative experiment from one of two independent RNA extractions is shown. The values on the left are molecular sizes in base pairs.

The metabolic basis of the defect of *glnB* null mutants in the studied microorganisms appears to be diverse. In *E. coli*, *glnB* null mutants show elevated activity of the nitrogen response regulator NtrC that results in increased activity of the Nac protein which, in turn, represses *serA* to levels insufficient for normal growth (4). This regulatory cascade is not even conserved in other enterobacteria (36). In *Rhodospirillum rubrum*, the other model system for which the metabolic defect is known, the poor-growth phenotype is due to an excess of GS activity (47). In *S. elongatus*, the gene that encodes GS (*glnA*) is activated by NtcA and GS activity is associated with P_{II} deficiency in *S. elongatus*. However, none of the MP2 strains analyzed here carried mutations in the *glnA* or *ntcA* gene. Since mutations inactivating the *ntcA* gene have been reported (46), the impaired phenotype of *S. elongatus glnB* null mutants appears to be related to an unknown PipX activity, thus raising questions of whether *glnB* null mutants obtained in other cyanobacteria contain compensatory changes at *pipX* and whether previous inactivation of *pipX* would facilitate recovery of *glnB* null mutants in species that have been so far recalcitrant to *glnB* inactivation.

Previous studies with P_{II}-deficient strains carrying the *glnB*::CK2 allele (strains MP2) suggested that P_{II} is required to stimulate NtcA activity under conditions of nitrogen deprivation (11, 39) and has an inhibitory role with nitrate as a nitrogen source (1, 11). Structural determination of the NAGK-P_{II} complexes (29) provided a rationale for the negative impact that mutations at Ser49, the phosphorylatable residue of P_{II}, have in interactions with NAGK (5, 18, 29). Although no differences among P_{II}, P_{II}^{S49A}, and P_{II}^{S49E} were found regarding interactions with PipX (5) or in vitro interactions with the effectors 2-oxoglutarate and ATP (27), Ser49 has nevertheless been implicated in the regulation of NtcA activity. Two studies carried out with MP2 strains claimed that substitution of P_{II}^{S49A}, but not P_{II}^{S49E}, for P_{II} prevented the induction of NtcA-dependent genes (27, 39). Since the authors assumed that mutations S49A and S49E may mimic, respectively, the nonphosphorylated and phosphorylated states of P_{II}, the inference was that P_{II} phosphorylation was important for NtcA function (27, 39). Since we cannot safely exclude the possibility

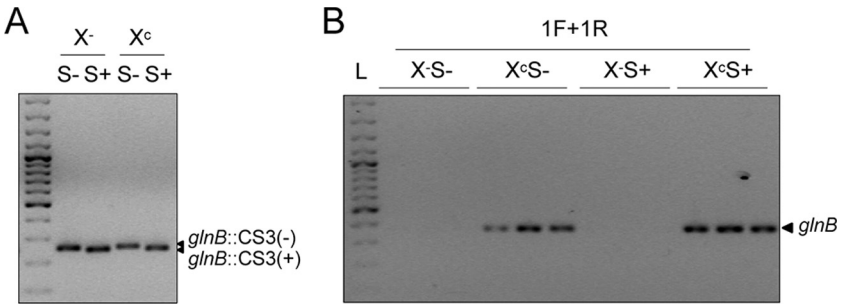

FIG. 5. Genetic inactivation of *glnB* in *pipX* mutants. (A) PCR verification of the correct integration of resistance cassettes in a representative clone after transformation of *pipX* (lanes X^-) and *pipX*(Con) (lanes X^C) mutant strains with *glnB*::CS3(−) (lanes S−) and *glnB*::CS3(+) (lanes S+). (B) Segregation of *glnB* alleles verified by PCR in three independent clones of *pipX* and *pipX*(Con) mutant strains transformed with *glnB*::CS3(−) and *glnB*::CS3(+) after four consecutive transfers onto selective media. See Fig. 1B for other details and lane labeling.

that the *pipX* mutations detected in strains MP2-A and MP2-E (Table 2) were already present in the particular cultures used then, inferences about NtcA-dependent activities in MP2 derivatives should be made with caution. Isogenic strains are needed to reevaluate the effect of P_{II} point mutations on NtcA function.

The effect of 2-oxoglutarate on PipX-P_{II} and PipX-NtcA complexes suggests that these protein associations are physiologically relevant at low and high C/N ratios, respectively (9), but little is known about the physiological role of PipX-P_{II} complexes. As a starting point to get insights into the function of PipX-P_{II} complexes, we contemplated three simple scenarios that are not necessarily incompatible: (i) PipX regulates P_{II} functions, (ii) P_{II} regulates PipX functions, and (iii) the complex itself has its own functions. The possibility that PipX-P_{II} complexes bind to additional proteins, fed by recent reports of ternary complexes in which P_{II} proteins are involved (19, 22), cannot be excluded at present. On the other hand, we did explore whether PipX is required for the P_{II}-dependent inhibition of nitrate transport by ammonium (10), stimulation of NAGK activity (8), or P_{II} phosphorylation (data not shown) and obtained negative responses in all of the cases examined. However, the results presented here strongly support a role for P_{II} in counteracting unknown but toxic functions of PipX in *S. elongatus*.

The features and occurrence of the spontaneous mutations at *pipX* suggest that P_{II} deficiency is best suppressed by partial rather than complete loss-of-function mutations at *pipX*, and some gene dosage (or specific activity) of *pipX* may improve the fitness of MP2 strains. This would explain the genetic heterogeneity noticed in *pipX* alleles. The coexistence of wild-type and mutant *pipX* alleles in several P_{II}-deficient cultures, and particularly the apparent stability of the allelic combination *pipX*/*pipX25_46del*, may be an adaptive mechanism for P_{II}-deficient cultures of *S. elongatus*, raising questions about the extent of genetic heterogeneity in cyanobacteria.

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