The nitrogen interaction network in *Synechococcus* WH5701, a cyanobacterium with two PipX and two P_{II}-like proteins

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Nitrogen regulation involves the formation of different types of protein complexes between signal transducers and their transcriptional or metabolic targets. In oxygenic phototrophs, the signal integrator P_{II} activates the enzyme *N*-acetyl-L-glutamate kinase (NAGK) by complex formation. P_{II} also interacts with PipX, a protein with a tudor-like domain that mediates contacts with P_{II} and with the transcriptional regulator NtcA, to which it binds to increase its activity. Here, we use a combination of *in silico*, yeast two-hybrid and *in vitro* approaches to investigate the nitrogen regulation network of *Synechococcus* WH5701, a marine cyanobacterium with two P_{II} (GlnB_A and GlnB_B) and two PipX (PipX_I and PipX_II) proteins. Our results indicate that GlnB_A is functionally equivalent to the canonical P_{II} protein from *Synechococcus elongatus*. GlnB_A interacted with PipX and NAGK proteins and stimulated NAGK activity, counteracting arginine inhibition. GlnB_B had only a slight stimulatory effect on NAGK activity, but its potential to bind effectors and form heterotrimers in *Synechococcus* WH5701 indicates additional regulatory functions. PipX_II, and less evidently PipX_I, specifically interacted with GlnB_A and NtcA, supporting a role for both *Synechococcus* WH5701 PipX proteins in partner swapping with GlnB_A and NtcA.

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INTRODUCTION

Cyanobacteria are phototrophic organisms that perform oxygenic photosynthesis. Autotrophic growth requires the constant assimilation of ammonium via the GS-GOGAT 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 TCA 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 line with this, interactions of the nitrogen regulators P_{II} and NtcA are modulated by this metabolite.

The trimeric 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 (Fokina *et al.*, 2010a) 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 *Synechococcus 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 P_{II}–NAGK complex consists of a hexameric (trimer of a dimer) NAGK toroid with each of its two faces binding one P_{II} trimer along its threefold symmetry axis. In the complex, the T-loops of P_{II} are retracted and contact the N- and C-terminal domain of each enzyme subunit, favouring the catalytically active NAGK conformation (Fokina *et al.*, 2010b; Llácer *et al.*, 2007). In the PipX–P_{II} complexes, one P_{II} trimer with its T-loops in a vertically extended conformation sequesters three PipX molecules (Llácer *et al.*, 2010; Zhao *et al.*, 2010). ATP in concert with elevated 2-oxoglutarate levels prevents complex formation

Abbreviations: FC1/2, flow cell 1/2; NAGK, *N*-acetyl-L-glutamate kinase; SPR, surface plasmon resonance.

A supplementary table of primer sequences is available with the online version of this paper.

of P_{II} with either NAGK or PipX (Espinosa *et al.*, 2006; Maheswaran *et al.*, 2004), suggesting that PipX–P_{II} complexes could also have a function under nitrogen sufficient conditions. Genetic analyses indicate that PipX is toxic to *S. elongatus* in the absence of P_{II} (Espinosa *et al.*, 2009, 2010), but the molecular basis of this toxicity remains unknown.

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 (Vázquez-Bermúdez *et al.*, 2002), transcription activation *in vitro* (Tanigawa *et al.*, 2002) and complex formation between NtcA and PipX (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 in *S. elongatus* under nitrogen limitation (Espinosa *et al.*, 2006, 2007). The PipX–NtcA complexes consist of one active (2-oxoglutarate-bound) NtcA dimer and two PipX molecules. Each NtcA subunit binds one PipX molecule in such a way that it stabilizes the active NtcA conformation (Llácer *et al.*, 2010).

The presence of P_{II} and PipX paralogues in some cyanobacteria raises questions on their potential to increase the regulatory capabilities of cells subjected to complex and changing environments. In this context, we use *in silico*, yeast two-hybrid and *in vitro* approaches to investigate the nitrogen regulation network of *Synechococcus* WH5701, a marine cyanobacterium encoding two P_{II} and two PipX proteins that thrives in estuaries (Scanlan *et al.*, 2009). The aim was to elucidate interaction partners and particular functions of the P_{II} and PipX proteins encoded by the genome of *Synechococcus* WH5701.

METHODS

Strains and growth conditions. Strains and plasmids used in this work are listed in Table 1. Oligonucleotides are described in Supplementary Table S1 (available with the online version of this paper). Constructs were analysed by automated dideoxy DNA sequencing. All cloning procedures were carried out in *Escherichia coli* DH5 α using standard techniques.

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

Construction of plasmids. To obtain pUAGC781 and pUAGC786, the *ntcA* sequence (annotated as ORF WH5701_01395) was amplified by PCR with primers YTH-WHNTCA-F and YTH-WHNTCA-R from *Synechococcus* WH5701 genomic DNA and cloned into *EcoRI*–*Sall*-digested pGAD424 and pGBT9, respectively.

The *glnBA* (ORF WH5701_15331) and *glnBB* (ORF WH5701_11829) sequences were amplified by PCR using genomic DNA as template

with primer pairs YTH-WHGLNB1-F/YTH-WHGLNB1-R and YTH-WHGLNB2-F/YTH-WHGLNB2-R, respectively, and cloned into *SmaI*–*BamHI*-digested pGAD424 and pGBT9, yielding plasmids pUAGC782, pUAGC787 (GlnB_A:AD and GlnB_A:BD) and pUAGC783 and pUAGC788 (GlnB_B:AD and GlnB_B:BD).

To obtain pUAGC784 and pUAGC789, the *pipXI* (ORF WH5701_00105) sequence was amplified by PCR from genomic DNA with primers YTH-WHIPX1-F and YTH-WHIPX1-R and cloned into *EcoRI*–*Sall*-digested pGAD424 and pGBT9, respectively. Similarly, the *pipXII* (ORF WH5701_06246) sequence was amplified with primers YTH-WHIPX2L-F and YTH-WHIPX2L-R and cloned into *EcoRI*–*BamHI*-digested pGAD424 and pGBT9, resulting in plasmids pUAGC785 and pUAGC795.

To obtain pUAGC798 and pUAGC799, the *argB* (ORF WH5701_12044) sequence was amplified by PCR with primers YTH-WHNAGK-F and YTH-WHNAGK-R from genomic DNA of *Synechococcus* WH5701 and cloned into *Sall*–*BamHI*-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.

***In silico* analysis.** The sequences of P_{II}, PipX, NAGK and NtcA homologues from *Synechococcus* WH5701 were retrieved from the NCBI database. Routinely, alignments were carried out by using CLUSTAL_X 2 (Larkin *et al.*, 2007) with default settings. Crystal complex structures of NAGK–P_{II}, P_{II}–PipX and NtcA–PipX were obtained from the protein database (PDB) with accession nos 2V5H, 2XG8 and 2XKO, respectively. PyMOL 0.99rc6 (DeLano Scientific LLC) was used to represent interaction surfaces of PipX, P_{II} and NtcA.

Cloning, overexpression and purification of *Synechococcus*

WH5701 NAGK. To clone *argB* from *Synechococcus* sp. WH5701, the gene was amplified by PCR with chromosomal *Synechococcus* sp. WH5701 DNA and primers NAGKwh5701for and NAGKwh5701rev. The PCR product was first cloned into the A/T cloning site of cloning vector pGEM-T (Promega). The resulting plasmid was restricted with *NdeI* and *BamHI* to isolate the *argB*-encoding DNA fragment that was then cloned into the *NdeI*/*BamHI* site of pET-15b (Novagen), resulting in a gene product with an N-terminally fused His₆-tag. To overexpress the recombinant WH5701 *argB* gene for purification of NAGK (WH NAGK), the pET15b-WH5701*argB* vector was transformed into *E. coli* BL21(DE3). Overexpression and purification was carried out as described previously (Maheswaran *et al.*, 2004).

Cloning, overexpression and purification of *Synechococcus*

WH5701 GlnB_A and GlnB_B. WH5701 *glnBA* and *glnBB* genes were amplified by PCR from WH5701 genomic DNA using the following pairs of primers: GlnB1for and GlnB1rev for *glnBA* and GlnB2for and GlnB2rev for *glnBB*. The resulting PCR products were cloned into the *BsaI* site of pASK-IBA3, to yield *glnBA* or *glnBB* products with a C-terminally fused Strep-tag II peptide. The plasmids, pIBA3-GlnB1 and pIBA3-GlnB2, were transformed into *E. coli* strain BL21(DE3). Overexpression and Strep-tag II affinity purification of the recombinant proteins was carried out as described previously (Heinrich *et al.*, 2004).

NAGK activity assays. The activity of recombinant His₆-tagged WH NAGK was assayed with a coupled enzyme assay, coupling the ADP-generating NAGK reaction via pyruvate kinase and lactate dehydrogenase to the oxidation of NADH, which was recorded at 340 nm. The assay procedure has been described in detail previously (Beez *et al.*, 2009). One unit of NAGK catalyses the conversion of 1 μ mol *N*-acetyl-L-glutamate (NAG) min^{–1}.

Table 1. Strains used in this work

Strain or plasmid	Genotype or relevant characteristic	Source or reference
<i>E. coli</i> DH5 α	F ⁻ ϕ 80d <i>lacZ</i> Δ <i>M15</i> Δ(<i>lacZYA</i> – <i>argF</i>)U169 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>deoR thi-1 supE44 gyrA96 relA1 λ</i> ⁻	Hanahan (1985)
<i>E. coli</i> BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B</i> (r _B ⁻ m _B ⁻) λ[DE3 (<i>lacI lacUV5-T7</i> gene 1 <i>ind1 sam7 nin5</i>)]	Phillips <i>et al.</i> (1984)
<i>Saccharomyces cerevisiae</i> Y187	<i>MATα ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4Δ met⁻ gal80Δ URA::GAL1_{UAS}–GAL1_{TATA}–lacZ</i>	Harper <i>et al.</i> (1993)
<i>Saccharomyces cerevisiae</i> PJ696	<i>MATα ade2Δ trp1-901 leu2-3, 112 ura3-52 his3-200 cyh^r can^r gal4Δ gal80Δ met2⁻ GAL2::ADE2 GAL1::HIS3 GAL7:lacZ</i>	James <i>et al.</i> (1996)
<i>Synechococcus</i> sp. WH5701	Wild-type <i>Synechococcus</i> sp. WH5701	D. Scanlan (DNA source)
pGAD424	<i>LEU2</i> , <i>GAL4</i> (768–881)AD, Ap ^r	Bartel <i>et al.</i> (1993)
pGBT9	<i>TRP1</i> , <i>GAL4</i> (1–147)BD, Ap ^r	Bartel <i>et al.</i> (1993)
pUAGC6	<i>GAL4AD</i> : NtcA (<i>S. elongatus</i>)	Espinosa <i>et al.</i> (2010)
pUAGC8	<i>GAL4BD</i> : NtcA (<i>S. elongatus</i>)	Espinosa <i>et al.</i> (2010)
pUAGC11	<i>GAL4AD</i> : P _{II}	Burillo <i>et al.</i> (2004)
pUAGC12	<i>GAL4BD</i> : P _{II}	Burillo <i>et al.</i> (2004)
pUAGC61	<i>GAL4AD</i> : NAGK (<i>S. elongatus</i>)	Burillo <i>et al.</i> (2004)
pUAGC62	<i>GAL4BD</i> : NAGK (<i>S. elongatus</i>)	Burillo <i>et al.</i> (2004)
pUAGC471	<i>GAL4AD</i> : PipX	Espinosa <i>et al.</i> (2010)
pUAGC472	<i>GAL4BD</i> : PipX	Espinosa <i>et al.</i> (2010)
pUAGC781	<i>GAL4AD</i> : NtcA (<i>Synechococcus</i> sp. WH5701)	This work
pUAGC782	<i>GAL4AD</i> : GlnB _A	This work
pUAGC783	<i>GAL4AD</i> : GlnB _B	This work
pUAGC784	<i>GAL4AD</i> : PipX _I	This work
pUAGC785	<i>GAL4AD</i> : PipX _{II}	This work
pUAGC786	<i>GAL4BD</i> : NtcA (<i>Synechococcus</i> sp. WH5701)	This work
pUAGC787	<i>GAL4BD</i> : GlnB _A	This work
pUAGC788	<i>GAL4BD</i> : GlnB _B	This work
pUAGC789	<i>GAL4BD</i> : PipX _I	This work
pUAGC795	<i>GAL4BD</i> : PipX _{II}	This work
pUAGC798	<i>GAL4AD</i> : NAGK (<i>Synechococcus</i> sp. WH5701)	This work
pUAGC799	<i>GAL4BD</i> : NAGK (<i>Synechococcus</i> sp. WH5701)	This work
pASK-IBA3	Ap ^r , StrepTag cloning vector	IBA GmbH
pIBA3-GlnB1	pASK-IBA3: GlnB _A	This work
pIBA3-GlnB2	pASK-IBA3: GlnB _B	This work
pGEM-T	Ap ^r , cloning vector	Promega
pGEM-T-WH5701argB	pGEM-T derivative carrying <i>argB</i> gene	This work
pET-15b	Ap ^r , HisTag cloning vector	Novagen
pET15b-WH5701argB	pET-15b derivative with His ₆ –NAGK	This work

Surface plasmon resonance (SPR) experiments. SPR assays were performed using a BIAcore X biosensor system (GE Healthcare). Activation of the NTA-sensor chip with NiSO₄ was performed as described previously (Beez *et al.*, 2009). Recombinant His₆-tag WH NAGK was loaded on the surface of flow cell 2 (FC2) by injecting 40 nM protein solution in HBS buffer (10 mM Na-HEPES, pH 7.5, 150 mM NaCl and 0.005 % Nonidet P40) containing 0.5 mM arginine at a flow rate of 10 μl min⁻¹, until a binding signal of approximately 3600 resonance units (RU) had been obtained. To test the binding of GlnB proteins to the FC2 surface-bound NAGK, 25 μl of a 1 μM solution of GlnB_A or GlnB_B in HBS buffer was injected over flow cell 1 (FC1) and FC2 at a flow rate of 15 μl min⁻¹. The response difference between FC1 and FC2 was recorded (ΔRU). Following injection, the sensor chip was washed with HBS buffer to record complex dissociation. To regenerate the surface, NAGK was first removed with 20 μl 1M imidazole pH 7.5 and after washing with HBS buffer, new NAGK protein was bound as described above.

RESULTS AND DISCUSSION

Cyanobacteria with a second *pipX* gene also have a second and atypical *glnB* gene

The genomes from the cyanobacteria *Gloeobacter violaceus*, *Acaryochloris marina* and *Synechococcus* WH5701 were found to contain two *pipX* ORFs, while the genomes of other cyanobacteria contained just one. In all three cases, the two PipX predicted sequences were remarkably similar to each other, suggesting independent and relatively recent gene duplication events. The identity of these proteins to *S. elongatus* PipX ranged from 44 to 50 %.

Because PipX forms complexes with P_{II} and NtcA, and in *S. elongatus* P_{II} counteracts PipX toxicity (Espinosa *et al.*,

2009, 2010), we wondered if the presence of two *pipX* genes is associated with more than one gene copy for PipX interacting partners. In all three species, *G. violaceus*, *A. marina* and *Synechococcus* WH5701, a second *glnB*-like gene sequence was detected. Only one type of *ntcA* and *argB* sequence was present in these cyanobacteria.

It has been noted that cyanobacterial P_{II} proteins are monophyletic and that a minority of species encode two *glnB* genes (Sant'Anna *et al.*, 2009; Scanlan *et al.*, 2009). From each of the three genomes with two *pipX*-like and two *glnB*-like genes, one of the two predicted P_{II} amino acid sequences, referred to as GlnB_A, was closer to most cyanobacterial P_{II} proteins (83, 86 and 77 % identity was found between *S. elongatus* and *G. violaceus*, *A. marina* and *Synechococcus* WH5701, respectively). The other sequence, GlnB_B, was not so typical (57, 48 and 65 % identity, respectively, with *S. elongatus* P_{II}), strongly suggesting that GlnB_B proteins were not the result of recent duplication events.

Sequence comparisons suggest that both GlnB_A and GlnB_B proteins may be functional and have a role in carbon to nitrogen balance and energy signalling in these organisms. Most of the key residues conforming to the ATP or 2-oxoglutarate binding sites are present in both GlnB_A and GlnB_B. There is one K40R change in the 2-oxoglutarate binding site of *Gloeobacter* GlnB_B and two changes in *Acaryochloris* GlnB_B, one in the ATP binding site (R101Q) and one in the 2-oxoglutarate binding site (K40Q). Furthermore, most of the residues involved in intersubunit interactions are also conserved between *S. elongatus* P_{II} and GlnB_A or GlnB_B, the few exceptions being conservative changes or changes affecting regions of higher variability in cyanobacteria (Table 2 and data not shown). Thus, the conservation and similarity of trimerization surfaces between both types of proteins suggests the possibility of heterotrimer formation *in vivo*.

The differences between typical and atypical P_{II} proteins from these three cyanobacteria are particularly evident at the region expanding the T-loop, where most of the residues forming the cyanobacterial signature (Palinska

et al., 2002) are nested. Except for an I18V change present in *Synechococcus* WH5701, all 14 signature residues were present in the three GlnB_A proteins, while 7, 8 or up to 12 changes were present in their GlnB_B counterparts (Table 3), thus strongly supporting the idea that GlnB_A proteins are the orthologues of P_{II} and that functions involving T-loop interactions are likely to be defective in GlnB_B proteins. The R45-S49-E85 NAGK interaction signature (Llcer *et al.*, 2007) is part of the cyanobacterial P_{II} signature. Residue R45 is absent in all three GlnB_B proteins, with S49 and E85 absent in two of them, strongly suggesting that GlnB_A, but not GlnB_B proteins, would be involved in regulation of NAGK activity in these three cyanobacteria.

Structure-informed prediction of interacting partners in the nitrogen regulation network of *Synechococcus* WH5701

As a first approach to elucidate the regulatory connections that could take place in cyanobacteria encoding two P_{II} and two PipX proteins, we focused on one of these organisms, *Synechococcus* WH5701. Its two predicted *pipX* products were named as PipX_I and PipX_II. We disregarded the wrong start codon annotated in the database for PipX_II and used instead the previous AUG that seems the most likely start codon. The inclusion of 25 additional amino acids at the N terminus of PipX_II considerably increased its similarity to other PipX proteins, given that this region of the tudor domain is important for PipX-mediated interactions and is therefore conserved.

The information gained from the structurally characterized NAGK–P_{II}, PipX–P_{II} and PipX–NtcA complexes of *S. elongatus* was next used to find out the most likely interacting partners for *Synechococcus* WH5701. To facilitate comparison between interaction surfaces of homologue proteins, residues differing in *Synechococcus* WH5701 proteins are also shown in Fig. 1.

Only one conservative change (E15D) in the P_{II} surface interacting with NAGK was found in GlnB_A (the D15 residue is also found in GlnB_B) (Fig. 1a). This similarity

Table 2. Amino acid substitution residues involved in P_{II} intersubunit contacts present in GlnB_A and GlnB_B

Substitutions are indicated for each position (when present) in amino acid letter code. For simplicity, the nine residues that do not change are not shown.

	Intersubunit contacting residues													
	K2	K3	E5	K17	M28	V30	R34	V53	V93	D97	Q98	I100	I102	A111
GlnB_A WH5701									I	G	T			
GlnB_B WH5701						I				E	K	V		
GlnB_A <i>Gloeobacter</i>											R	V		
GlnB_B <i>Gloeobacter</i>	N			E			E	A		T		V	V	
GlnB_A <i>Acaryochloris</i>									I			V		
GlnB_B <i>Acaryochloris</i>	R	T	T		I	L	K	I		Q	K	V	V	L

Table 3. Amino acid substitution of residues that constitute the cyanobacterial P_{II} signature

Substitutions are indicated for each position (when present) in amino acid letter code. There were no substitutions in *Synechococcus* P_{II}, *Gloeobacter* GlnB_A or *Acaryochloris* GlnB_A.

	Cyanobacterial P _{II} signature													
	R9	I18	I/V26	S31	R34	Q42	R45	S49	T52	E54	Q57	L59	E85	S94
GlnB_A WH5701		V												
GlnB_B WH5701		T		T			T	A			N	V		T
GlnB_B <i>Gloeobacter</i>	Q	Q	Q		E	H	I	F	A	Q	A		S	L
GlnB_B <i>Acaryochloris</i>		L			K	S	Q		E		R		Q	

strongly argues in favour of very similar functions for *S. elongatus* P_{II} and GlnB_A. Three additional substitutions, F11V and two changes affecting two out of the three NAGK interaction signature residues (R45T and S49A), were present at GlnB_B, arguing against conservation of function. Thus, these sequence features and the similarity of GlnB_A and NAGK from *Synechococcus* WH5701 (WH NAGK) to their homologues in *S. elongatus* agrees with previous predictions of conservation of the P_{II}–NAGK interaction in organisms that perform oxygenic photosynthesis (Burillo *et al.*, 2004). In addition, the data also suggest the possibility of cross-regulation between heterologous proteins.

GlnB_A was more similar to P_{II} than GlnB_B with respect to the P_{II} surface interactions with PipX (Fig. 1b). Two conservative changes were found in GlnB_A, Y51F (a change also present in GlnB_B and other P_{II} proteins from cyanobacteria and plants) and D14E, whose proximity to D15 (E15 in *S. elongatus*) restored the Glu–Asp pair (Asp–Glu in *S. elongatus*). In addition to the above-mentioned F11V change, three other differences (E32D, Q57N and L59V) were found in GlnB_B. Although it is difficult to predict the impact of these differences on interactions with the PipX_I and PipX_II proteins, it seems reasonable to argue that GlnB_A would be a better binding partner than GlnB_B for (at least) *S. elongatus* PipX.

In agreement with the great similarity between the two *Synechococcus* WH5701 PipX proteins, most of the differences compared with *S. elongatus* PipX at the interaction surface of the tudor domain were found in both PipX_II and PipX_I (Fig. 1c–d), with only one conservative change (L14M) specific to PipX_I. Four substitutions were identical (L36M, P51V, G53P and F42L) and one was found in two versions (R54L/R54Y). Thus, the possibility that both PipX_II and PipX_I would be able to form complexes with GlnB_A could not be excluded at this stage. Given the similarity between the different proteins and the architecture of the P_{II}–PipX complexes, heterologous interactions also seem likely (P_{II}–PipX_II, P_{II}–PipX_I, GlnB_A–PipX).

Since the PipX regions interacting with P_{II} and NtcA overlap, the rationale just discussed for GlnB_A applies to putative PipX interactions with NtcA. Except for F42, which did not contact NtcA, all substitutions discussed for

PipX also affect residues involved in direct contacts with NtcA, a protein with 71 % identity to that of *S. elongatus*. Differences at two of the nine residues making direct contacts with PipX were found for *Synechococcus* WH5701 NtcA (WH NtcA), corresponding to the substitutions S218A and Q219K (Fig. 1e), which are also common in the cyanobacteria group. It seems reasonable to assume that this NtcA protein could interact with PipX_II and PipX_I.

Protein interactions involving nitrogen regulators from *Synechococcus* WH5701

Previously we used yeast two-hybrid approaches to infer the existence of P_{II}–NAGK, P_{II}–PipX and PipX–NtcA complexes in *S. elongatus* and to provide evidence of the specificity of the interactions between wild-type and mutant derivatives of the different proteins involved (Burillo *et al.*, 2004; Espinosa *et al.*, 2006, 2010; Llcer *et al.*, 2007, 2010). Here, we generated additional two-hybrid constructs to analyse interactions between the relevant proteins from *Synechococcus* WH5701. These include NAGK, NtcA, the two P_{II} (GlnB_A, GlnB_B) and the two PipX paralogues (PipX_II and PipX_I). Expression of reporters was determined in Y187/PJ696 diploids as described previously (Burillo *et al.*, 2004). In all cases, proteins were fused independently to both GAL4BD and GAL4AD domains. Fusion proteins from the model cyanobacterium *S. elongatus* were included in the analyses, allowing us to obtain information for heterologous recognition between *S. elongatus* and *Synechococcus* WH5701 proteins. A typical yeast two-hybrid interaction assay for the *Synechococcus* WH5701 proteins is shown in Fig. 2(a). Results from pairing of heterologous proteins, alongside already known interactions for *S. elongatus* proteins, are summarized in Fig. 2(b).

As predicted in the light of the extensive residue conservation between GlnB_A and P_{II}, particularly at the interacting surface, strong interaction signals were found between WH NAGK and GlnB_A. Consistent with the lack of key residues at GlnB_B, no interaction signal could be detected between WH NAGK and GlnB_B. GlnB_A, but not GlnB_B, interacted with all three PipX proteins, strongly suggesting that GlnB_B is not able to perform the two functions that have been characterized for P_{II} in

Fig. 1. Relevant residues in *Synechococcus* WH5701 proteins compared with those involved in P_{II}-NAGK, P_{II}-PipX and NtcA-PipX complexes from *S. elongatus*. (a) Surface representation of P_{II} in the P_{II}-NAGK complex (PDB file 2V5H) with the interaction surface in light brown. Substitutions on key interacting residues (for one P_{II} subunit) present in GlnB_A, GlnB_B or both, are indicated in yellow, red and green, respectively. (b) Surface representation of P_{II} in the P_{II}-PipX complex (PDB file 2XG8) with the interaction surface in blue. Substitutions are indicated as in (a). (c, d) Surface representation of PipX in the P_{II}-PipX (PDB file 2XKO) and NtcA-PipX (2XG8) complexes coloured in blue and green, respectively. Substitutions on key interacting residues present in PipX_{II}, PipX_I or both are indicated in orange, red and yellow, respectively. (e) Surface representation of the NtcA interaction surface, coloured green, in the NtcA-PipX complex (PDB file 2XKO). Key residues are numbered and substitutions on *Synechococcus* WH5701 NtcA are shown in yellow.

cyanobacteria, that is NAGK regulation and sequestration of PipX. All three PipX derivatives interacted with *S. elongatus* P_{II} and GlnB_A, and signals from some of the heterologous

pairs (such as P_{II}/PipX_{II}) were as strong as those between the *S. elongatus* (P_{II}/PipX) or *Synechococcus* WH5701 proteins (GlnB_A/PipX_{II}). Both GlnB_A and GlnB_B interacted with themselves, which agrees with their predicted trimeric structure, and with each other (also to *S. elongatus* P_{II}), which supports the possibility that they form heterotrimers *in vivo*. If that was the case, the presence of GlnB_B in *Synechococcus* WH5701 could interfere with the formation of complexes by GlnB_A. This would be reminiscent of the situation in other bacteria for which heterotrimer formation for P_{II} proteins (GlnK and GlnB in *E. coli*) have been demonstrated (Forchhammer *et al.*, 1999).

Interestingly, although the PipX proteins from *Synechococcus* WH5701 were closer to each other than to *S. elongatus* PipX (Fig. 1c, d), interaction signals with the NtcA proteins from each of the two organisms were clearly detected for *S. elongatus* PipX and PipX_{II}, but hardly for PipX_I. Since the only relevant difference between PipX_{II} and PipX_I at the interacting surface affects positions 14 and 54 (Fig. 1b–c), one or two of the Leu residues present in PipX_{II} (Y54 and M14 in PipX_I) may be responsible for the stronger interaction signals between PipX_{II} and NtcA. Differences at these residues could also explain the lower interaction signals of PipX_I with P_{II} and GlnB_A, compared with those of PipX_{II}.

Detection of signals between PipX_I and NtcA proteins (and here *Synechococcus* WH5701 and *S. elongatus* NtcA proteins were indistinguishable) required longer incubation times and was possible just for the BD:PipX_I/AD:NtcA pairs. However, signals were independently detected on both selective media and X-Gal indicator plates, and were significantly higher than those from the negative controls (Fig. 2b and data not shown). We are inclined to think that the extremely weak interaction signals observed for BD:PipX_I/AD:NtcA fusion proteins could be relevant, and therefore the possibility of complex formation between NtcA and PipX_I in *Synechococcus* WH5701 should not be excluded at present.

Taken together, the yeast two-hybrid data point to PipX_{II} as a true PipX orthologue involved in partner swapping with GlnB_A and NtcA, but do not necessarily exclude the PipX_I-NtcA interaction in *Synechococcus* WH5701. The weaker interaction signals mediated by PipX_I, in comparison with PipX_{II}, could be interpreted in evolutionary terms as a sign of divergence in which one of the duplicated proteins (PipX_I) would tend to lose some of its functions, in this case the ability to perform partner swapping with P_{II} and NtcA. It would be interesting to know whether this apparent loss of activity is accompanied by acquisition of new functions.

***In vitro* properties of NAGK–P_{II} complexes from *Synechococcus* WH5701 and activation of NAGK proteins by GlnB_A and GlnB_B**

Subsequent biochemical analysis of complexes involving *Synechococcus* WH5701 proteins was performed using

Fig. 2. Yeast two-hybrid interactions mediated by P_{II}, NAGK, PipX and NtcA from *Synechococcus* WH5701. (a) Representative interaction assay for *Synechococcus* WH5701 proteins. (b) Schematic representation of yeast two-hybrid interactions amongst nitrogen regulators from *S. elongatus* and *Synechococcus* WH5701 proteins (white and grey boxes, respectively). The number of arrowheads indicates the relative strength of the interaction as described by Burillo *et al.* (2004). Dashed lines indicate very weak signals (PipX_I–NtcA).

purified NAGK, GlnB_A and GlnB_B, essentially as described previously for the *S. elongatus* proteins (Heinrich *et al.*, 2004; Maheswaran *et al.*, 2004). The difficulty of obtaining soluble proteins for *Synechococcus* WH5701 NtcA, PipX_II and PipX_I precluded analysis of the corresponding complexes at this stage, and therefore we focused on the properties of the different P_{II}–NAGK complexes.

First, we used purified proteins to independently assay the physical interaction between NAGK and GlnB_A or GlnB_B by SPR spectroscopy analyses. As shown in Fig. 3, rapid complex formation on the WH NAGK surface was observed with GlnB_A, but no interaction could be detected with GlnB_B. Therefore, the results from this *in vitro* analysis closely matched those obtained with the yeast-two hybrid system.

Next we determined the enzymic activity of *Synechococcus* WH NAGK in the absence and presence of GlnB_A or GlnB_B proteins. As shown in Fig. 4(a), WH NAGK is very inactive in the absence of a P_{II} protein. The free enzyme does not show Michaelis–Menten kinetics, but instead a slow sigmoidal increase of activity with increasing substrate. Below 15 mM NAG, almost no activity could be detected; the activity could only be reasonably assayed at 50 mM NAG (data not shown). Addition of GlnB_A dramatically increased the activity of WH NAGK and resulted in a typical Michaelis–Menten kinetics with an apparent K_m for NAG of 1.5 ± 0.5 mM and a V_{max} of 7.3 ± 0.6 U mg⁻¹. Notably, addition of GlnB_B (but not BSA when used as a control; data not shown) also had a slight, but measurable, stimulatory effect on NAGK activity, resulting in a hyperbolic substrate saturation curve with an apparent K_m for NAG of 4.9 ± 1.2 mM and a V_{max} of 1.9 ± 0.2 U mg⁻¹.

As expected for a typical hexameric NAGK, the WH NAGK activity was subjected to non-competitive inhibition by arginine. In the absence of P_{II} proteins, the half-maximal inhibitory concentration (IC₅₀) was approximately 40 µM. Addition of GlnB_A reduced the inhibitory effect of arginine on enzyme activity by 40-fold, shifting the IC₅₀ to 1.6 mM (Fig. 4b). In contrast,

Fig. 3. BiaCore SPR analysis of complex formation of WH NAGK with GlnB_A or GlnB_B. The sensor chip was prepared by bringing approximately 3600 resonance units (RU) His₆-tagged WH NAGK to the surface of FC2 of an NTA-sensor chip, with FC1 serving as a control. To assay the interaction with GlnB proteins, 25 µl of a 1 µM GlnB_A (solid line) or GlnB_B (dashed line) solution were injected as analyte at a flow rate of 15 µl min⁻¹ over FC1 and FC2, and the response difference (ΔRU) between FC1 and FC2 was recorded. Following injection, the surfaces of FC1 and FC2 were washed with HBS buffer (see Methods).

GlnB_B has almost no effect on arginine inhibition (Fig. 4b, inset).

In summary, we have demonstrated that GlnB_A is able to significantly stimulate NAGK activity by changing its kinetic properties and decreasing the sensitivity to arginine inhibition. In addition to unravelling molecular details of the predicted interaction between WH NAGK and GlnB_A, we show that GlnB_B is still able to weakly stimulate WH NAGK *in vitro*. The latter result, which is at odds with sequence predictions and the negative results obtained with two solid techniques for protein–protein interactions (Figs 2 and 3), suggest that the GlnB_B–WH NAGK interaction responsible for the weak stimulation of NAGK activity

Fig. 4. Influence of GlnB_A and GlnB_B on WH5701 NAGK activity. (a) NAGK activity assays were performed using 3 μg recombinant WH NAGK in the absence (◆, dotted line) or presence of 1.2 μg GlnB_A (■, solid line) or GlnB_B (▼, dashed line) as described in Methods. The mean (±SD) values of triplicate assays are shown. (b) Inhibition of WH NAGK activity by arginine in the absence or presence of GlnB_A or GlnB_B. Coupled assays were performed in the presence of 50 mM NAG and 10 mM ATP together with increasing concentrations of arginine, as indicated. Assays using 3 μg NAGK and 1.2 μg GlnB_A or GlnB_B were performed as described in Methods. In the presence of GlnB_A (■, solid line) the half-maximal inhibitory concentration of arginine is approximately 1.6 mM, whereas the free WH NAGK (see inset, ◆ and dotted line) shows half maximal inhibition at approximately 40 μM arginine. Addition of GlnB_B (inset, ▲ and dashed line) has almost no effect on allosteric arginine inhibition.

observed *in vitro* is too weak and transient for detection by yeast two-hybrid or SPR assays. Whatever the case, the possibility of heterotrimerization also calls attention to the regulatory potential associated with the presence of two P_{II} proteins in *Synechococcus* WH5701.

Concluding remarks

This work brings attention to the regulatory potential of cyanobacteria such as *G. violaceus*, *A. marina* and

Synechococcus WH5701 encoding paralogues of P_{II} and PipX, two proteins involved in nitrogen signal transduction by protein–protein interactions. We have experimentally addressed basic questions on the nitrogen interaction network of *Synechococcus* WH5701, an euryhaline cyanobacterium encoding each two P_{II} (GlnB_A and GlnB_B) and PipX (PipX_II and PipX_I) proteins. Our results show that GlnB_A and PipX_II are functionally equivalent to P_{II} and PipX from the model cyanobacterium *S. elongatus*. PipX_II, and to a lesser extent PipX_I, specifically interacted with GlnB_A and NtcA, in agreement with the idea that at least PipX_II would mediate partner swapping between GlnB_A and NtcA according to the intracellular 2-oxoglutarate levels. GlnB_A significantly stimulates NAGK activity and antagonizes inhibition by arginine. Given the potential of GlnB_B to form heterotrimers with GlnB_A, it is tempting to propose that in *Synechococcus* WH5701, GlnB_B could act as a negative regulator to fine-tune the efficiency of GlnB_A. The slight activation of NAGK by GlnB_B could allow a fine-tuned activation of the enzyme depending on its degree of heterotrimerization with GlnB_A. Further work is required to understand the molecular details and particularly the possible contributions of this complicated nitrogen interaction network to the adaptations of *Synechococcus* WH5701 to the wide range of salinity and nutrient conditions arising in estuaries.

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