PipX, the coactivator of NtcA, is a global regulator in cyanobacteria

Javier Espinosa[a], Francisco Rodríguez-Mateos[b], Paloma Salinas[a], Val F. Lanza[c], Ray Dixon[d], Fernando de la Cruz[c], and Asuncion Contreras[a,1]

[a]División de Genética and [b]Departamento de Matemática Aplicada, Universidad de Alicante, 03080 Alicante, Spain; [c]Departamento de Biología Molecular, Facultad de Medicina, Universidad de Cantabria and Instituto de Biomedicina y Biotecnología de Cantabria, 39011 Santander, Spain; and [d]Department of Molecular Microbiology, John Innes Centre, Norwich NR4 7UH, United Kingdom

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To modulate the expression of genes involved in nitrogen assimilation, the cyanobacterial P_{II}-interacting protein X (PipX) interacts with the global transcriptional regulator NtcA and the signal transduction protein P_{II}, a protein found in all three domains of life as an integrator of signals of the nitrogen and carbon balance. PipX can form alternate complexes with NtcA and P_{II}, and these interactions are stimulated and inhibited, respectively, by 2-oxoglutarate, providing a mechanistic link between P_{II} signaling and NtcA-regulated gene expression. Here, we demonstrate that PipX is involved in a much wider interaction network. The effect of *pipX* alleles on transcript levels was studied by RNA sequencing of *S. elongatus* strains grown in the presence of either nitrate or ammonium, followed by multivariate analyses of relevant mutant/control comparisons. As a result of this process, 222 genes were classified into six coherent groups of differentially regulated genes, two of which, containing either NtcA-activated or NtcA-repressed genes, provided further insights into the function of NtcA–PipX complexes. The remaining four groups suggest the involvement of PipX in at least three NtcA-independent regulatory pathways. Our results pave the way to uncover new regulatory interactions and mechanisms in the control of gene expression in cyanobacteria.

nitrogen regulation | transcription | translation | photosynthesis

Cyanobacteria are phototrophic organisms that perform oxygenic photosynthesis. Autotrophic growth requires the constant assimilation of ammonium via the glutamine synthetase–glutamate synthase cycle, resulting in consumption of the 2-oxoglutarate (2-OG) (1, 2) that accumulates during nitrogen starvation, making this metabolite an excellent indicator of the intracellular carbon-to-nitrogen balance (3, 4). The 2-OG, the signal of nitrogen deficiency, modulates the activity and/or binding properties of three key cyanobacterial nitrogen regulators: the signal transduction protein P_{II}; the transcriptional activator NtcA; and PipX, a regulatory factor that can interact with either NtcA or P_{II}.

The homotrimeric P_{II} protein, one of the most conserved and widespread signal transduction proteins in nature, plays key roles in nitrogen assimilatory processes (5). P_{II} contains three binding sites (one per subunit) for 2-OG and ATP (6, 7), and it regulates the activity of *N*-acetyl-glutamate-kinase (NAGK), a key enzyme for biosynthesis of arginine, by direct protein–protein interactions (3, 8, 9). The 2-OG stimulates binding of NtcA to target sites (10), transcription activation in vitro (11), and complex formation between NtcA and PipX (12). The interaction between PipX and NtcA is known to be relevant under nitrogen limitation for activation of NtcA-dependent genes in *Synechococcus elongatus* and *Anabaena* sp. PCC 7120 (hereafter, *Anabaena*) (12–14). The NtcA–PipX complex consists of one active (2-OG–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 and probably helps recruit RNA polymerase without providing extra DNA contacts (15, 16). The tudor-like domain of PipX provides the contacts for both NtcA–PipX and P_{II}–PipX interactions. When nitrogen is abundant, intracellular levels of 2-OG are low and sequestration of PipX by P_{II} decreases NtcA–PipX complex formation. A summary of the interactions involving NtcA, PipX, or P_{II} is shown in Fig. 1.

The PipX partner-swapping model predicts that, at least under the physiological range of 2-OG levels, *pipX* mutations specifically impairing PipX–P_{II} complexes would favor formation of NtcA–PipX complexes. Crystal structures of PipX–P_{II} complexes, surface plasmon resonance, P_{II}-stimulated NAGK activity assays, and yeast two-hybrid analysis established the importance of PipX residues Y32 and E4 for interactions with P_{II} proteins and of Y32 for interactions with NtcA (15, 17). Reporter and transcript analyses indicated that both Y32A and E4A mutations had stimulatory effects on the NtcA-activated genes *glnB*, *glnN*, and *nblA* but did not address differences between the in vivo action of $PipX^{E4A}$ and $PipX^{Y32A}$, two proteins with different biochemical properties. Here, we show that the in vivo properties of $PipX^{E4A}$ and $PipX^{Y32A}$ are indeed very different and that these differences affect both NtcA-dependent and NtcA-independent genes.

The 2-OG–dependent partner swapping of PipX between P_{II} and NtcA provides a mechanistic link between P_{II} signaling and NtcA-regulated gene expression but does not exclude the possibility that PipX, either by itself or bound to P_{II}, could participate in additional protein–protein interactions influencing gene expression. To address the question of whether PipX affects *S. elongatus* gene expression in an NtcA-independent manner, we compared transcript profiles of *pipX* mutants in cultures grown with either ammonium or nitrate. In these conditions, and to a

Significance

P_{II}, a signal transduction protein involved in nitrogen control in bacteria and plants, and NtcA, the transcriptional nitrogen regulator of cyanobacteria, can form complexes with P_{II} interacting protein X (PipX). We demonstrate by a combination of genetic, transcriptomic, and multivariate analyses that PipX is involved in a much wider interaction network affecting nitrogen assimilation, translation, and photosynthesis. Two groups of genes differentially regulated by *pipX* provided further insights into the function of NtcA–PipX complexes and an improved definition of the consensus NtcA binding motif. The other four groups suggested the involvement of PipX in NtcA-independent regulatory pathways. Our results pave the way to uncover new regulatory interactions and mechanisms in the control of gene expression in cyanobacteria.

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[1]To whom correspondence should be addressed. E-mail: contrera@ua.es.

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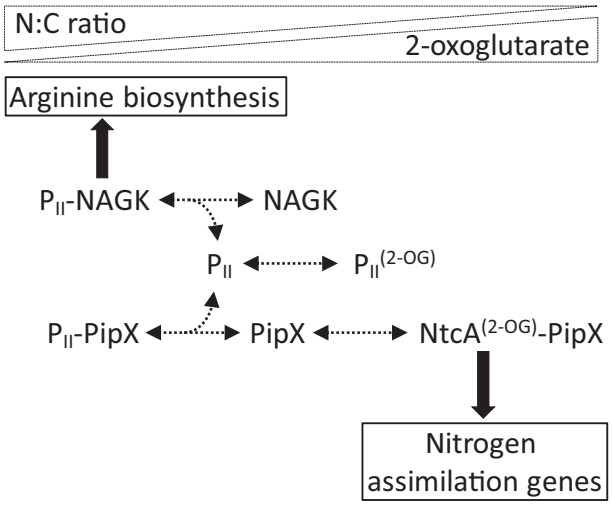

Fig. 1. PipX swapping model and the nitrogen interaction network. The functions and interactions mediated by PipX according to the 2-OG levels are schematized.

greater extent in ammonium, the levels of 2-OG are relatively low, NtcA is mainly inactive, and null *pipX* mutations are not expected to have a significant impact on the NtcA regulon (13). We reasoned that the $pipX^{E4A}$ and $pipX^{Y32A}$ mutations provide a means to bypass the need to use *ntcA* mutants and/or conditions of nitrogen deprivation to identify NtcA target genes. Most relevant to this work, the combined analyses of *pipX* mutant strains enabled us to identify additional regulatory targets of PipX in a context in which we could further characterize the NtcA regulon.

Results and Discussion

Global Phenotypic Impact of *pipX* Mutations Under Nitrogen-Rich Conditions. The involvement of PipX in gene expression and its potential roles in global regulation other than nitrogen control by NtcA were investigated in *S. elongatus* strains grown in either nitrate or ammonium and carrying either a null (Δ*pipX*) or point mutation ($pipX^{Y32A}$ or $pipX^{E4A}$) derivative. Under these nitrogen regimes, particularly in ammonium, the concentration of 2-OG is expected to be relatively low and transcriptional regulation by NtcA–PipX complexes would not have widespread importance in WT cells. Because the $pipX^{Y32A}$ or $pipX^{E4A}$ allele is associated with a resistance marker (C.S3 cassette) that decreases *pipX* gene expression (18), a WT derivative with the same insertion in the identical position (strain CS3X) was required to perform isogenic mutant/control comparisons. Therefore, two strains were used as WT controls: WT *S. elongatus* for mutant strain SA591, which carries the Δ*pipX* allele, and CS3X for strains $CS3X^{E4A}$ and $CS3X^{Y32A}$, which carry the $pipX^{Y32A}$ and $pipX^{E4A}$ alleles, respectively (*SI Appendix*, Table S1). A total of six global transcriptome comparisons were carried out, considering three mutant/control pairs for each of the two nitrogen regimes: ammonium and nitrate.

Scatter plots of $\log_2$ fold changes of *pipX* mutants vs. their respective controls are represented in Fig. 2. For each mutant/control comparison, subsets of genes that are up-regulated or down-regulated more than fourfold in the mutants only in nitrate, only in ammonium, or in both conditions are highlighted. Interestingly, many genes were differentially regulated in the Δ*pipX* mutant, and for each condition, there were more genes up-regulated than down-regulated in the absence of an active *pipX* allele, suggesting that PipX participates more frequently in negative regulation than in positive regulation under the experimental conditions tested (Fig. 2*A*). Whereas the global impact of $pipX^{Y32A}$ on gene expression seemed roughly similar to the effect of the Δ*pipX* allele, the effect of $pipX^{E4A}$ was restricted to a smaller number of genes and, importantly, most of those genes were up-regulated in both nitrate and ammonium (Fig. 2 *B* and *C*).

The results supported a role for PipX in both negative and positive regulation of multiple target genes in *S. elongatus* under conditions of nitrogen sufficiency and demonstrate that the mutations $pipX^{Y32A}$ and $pipX^{E4A}$ have very different effects on gene expression. To gain deeper insights into the functions of PipX in vivo while adding robustness to the analysis, we next analyzed combined information from all 10 transcript datasets.

Multivariate Analysis of *S. elongatus* Transcripts. To identify groups of genes with discrete expression patterns defined by the Δ*pipX*, $pipX^{E4A}$, or $pipX^{Y32A}$ alleles, we performed multivariate analysis with standardized residuals from linear regressions of data (log-transformed) from mutant vs. control strains (both $CS3X^{E4A}$ and $CS3X^{Y32A}$ vs. CS3X and SA591 vs. WT) cultured in the presence of either ammonium or nitrate. First, we demonstrated that only a small proportion of the transcriptome responded to the nitrogen source and/or to *pipX* alleles. A total of 1,663 genes with

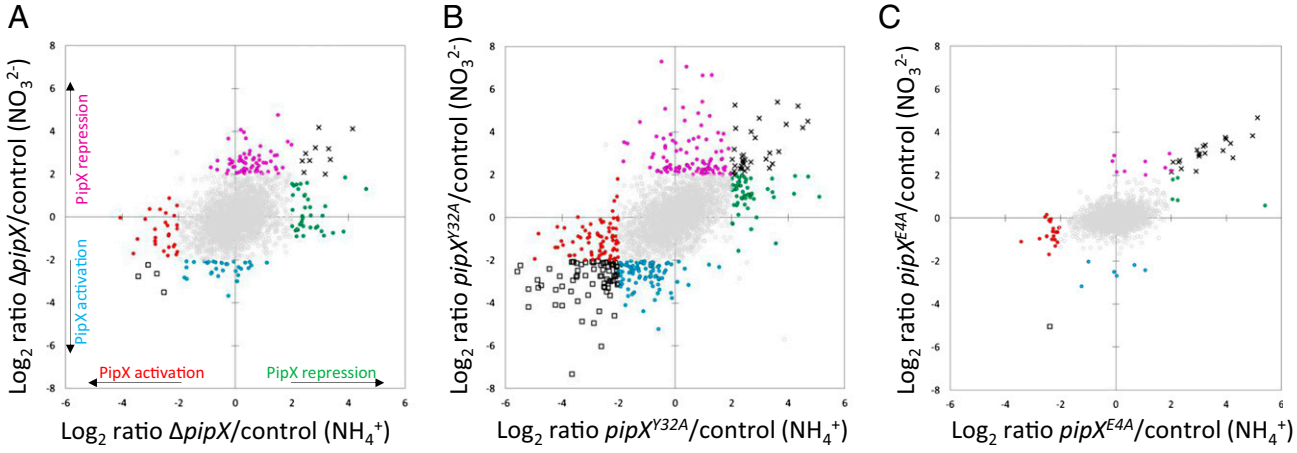

Fig. 2. Effect of *pipX* mutations on nitrate and ammonium transcriptomes. The mutant/control $\log_2$ ratios in nitrate vs. ammonium are represented as a scatter plot. (*A*) Δ*pipX*/WT with indication of the inferred PipX functions. (*B*) $CS3X^{Y32A}$/CS3X. (*C*) $CS3X^{E4A}$/CS3X. Genes shown in colors and in gray represent above and below, respectively, the cutoff for a $\log_2$ ratio >2 (absolute values). Positive values in nitrate (purple), ammonium (green), or both conditions (x) and negative values in nitrate (blue), ammonium (red), or both conditions (□) are shown.

residuals lower than 1.5 were considered as nonresponsive in any of the six mutant/control comparisons, and the distributions of their residuals were fitted well by truncated normal distributions (gray dots in *SI Appendix*, Fig. S1 and Table S3). For the ammonium and nitrate conditions, 1,958 and 2,052 genes, respectively, were nonresponsive in any of the three mutant/control comparisons. From the group of genes with residuals greater than 1.5, only those with residuals exceeding a threshold value of 2.5 for at least one of the six variables were selected as being differentially regulated. The resulting 282 genes, after excluding *pipX* itself, were individually analyzed to discard those with reads mapping mainly to the noncoding strand (Dataset S1), bringing the number down to 257 genes.

To explore the existence of different expression profiles within the 257 differentially regulated genes further, principal component analysis (PCA) of residuals was used to extract the first two principal components, accounting for about 70% of the total variance. The plot of the data for these two components (Fig. 3*A*) suggested the classification of the 257 genes into four main groups (classes 1, 2, 3, and 4), defined by using *k*-means cluster analysis. As shown in Fig. 3*A* (*Inset*), changes in expression for Δ*pipX* in both ammonium and nitrate and for $pipX^{Y32A}$ mainly in ammonium were associated with the first axis (PC1). In contrast, changes for $pipX^{E4A}$ in both ammonium and nitrate and, to a lesser extent, for $pipX^{Y32A}$ in nitrate, were associated with the second component (PC2).

The relatively large sizes of the groups and the wide distribution of the dataset, particularly in classes 2 and 3, prompted us to use additional classification criteria to obtain better-defined groups with smaller numbers of similarly regulated genes. This was done by defining an additional four groups according to independent clustering by hierarchical Ward's minimum variance and fuzzy c-means methods. Only those genes that were coherently grouped into the same class with the three clustering methods were retained. The cluster dendrogram from Ward's method was then cut to produce six coherent groups comprising a total of 222 genes.

As a result of this classification process, the four original groups became six groups. The 35 genes that fell outside these groups (Fig. 3*A*, gray dots, and Dataset S2) included the genes with the lowest expression levels (among the 257 differentially regulated genes), as well as genes with rather unique expression patterns. Only class 4 (43 genes) was left untouched, although class 1 lost some members (32 genes remained) and classes 2 and 3 (losing 10 and 17 genes, respectively) were each split into two new classes: 2.1 (37 genes), 2.2 (43 genes), 3.1 (18 genes), and 3.2 (49 genes) (Datasets S3–S8). Differences between the new classes originating from a common class, that is, 2.1 vs. 2.2 and 3.1 vs. 3.2, were largely related to the third principal component (Fig. 3*B*, PC3 axis), which accounted for an additional 13% of the total variance and was mainly correlated with differences in expression provided by Δ*pipX* and $pipX^{Y32A}$ alleles, with the ammonium conditions providing the greatest differences in these mutant control comparisons. Hereafter, each of the six classes was treated and analyzed as a distinct class.

NtcA-Activated Genes and NtcA Binding Motifs in *S. elongatus*. The distinctive feature of class 4 transcripts was their up-regulation in $CS3X^{E4A}$ in both nitrate and ammonium and in $CS3X^{Y32A}$ only in nitrate (Figs. 3*A* and 4*A*). Notably, a rather small, but still significant, down-regulation in the Δ*pipX* strain was observed in nitrate. Class 4 comprised most of the paradigmatic nitrogen assimilation genes or operons from *S. elongatus*, that is, gene targets known or predicted to be activated by NtcA–PipX complexes under conditions of nitrogen deficiency (*SI Appendix*, Table S2). They include *ntcA* itself, genes encoding the key glutamine synthetase enzyme *glnA*, ammonium transporters *amtB* and *amt1*, components of the nitrate transport and assimilation system *nirA*/*nrtABCD*/*narB*, and components of the cyanate transport and assimilation system *cynABD* and *cynS*. The transcriptional response in all five genetic backgrounds and two nitrogen regimes is illustrated in Fig. 4*B* for two paradigmatic NtcA target genes: *ntcA*, which is autogenously regulated, and *nirA*, which encodes nitrite reductase. Both genes were similarly affected by the mutant alleles Δ*pipX*, $pipX^{Y32A}$, and $pipX^{E4A}$ under each of the nitrogen regimes tested.

Class 4 expression patterns suggest that WT PipX can weakly activate some NtcA target genes in nitrate but not in ammonium-grown cells. In contrast, $PipX^{Y32A}$ and, to a greater extent, $PipX^{E4A}$ appear to be competent to interact with NtcA and coactivate target promoters at low concentrations of 2-OG. Our

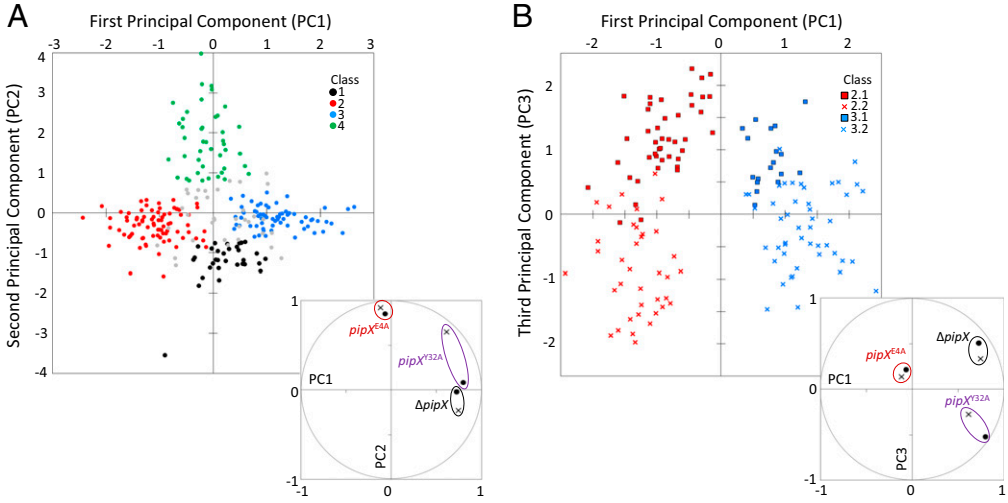

Fig. 3. Multivariate analysis and clustering of differentially expressed genes. (*A*) Scores for the two first principal components in the PCA of standardized residuals from mutant/control comparisons for the 257 genes with residuals larger than 2.5 in at least one comparison. Classified and nonclassified genes are colored and gray, respectively. (*Inset*) Scatter of mutant/control comparisons plotted as the correlation coefficients between them and the first two principal components in the unit circle. Nitrate (x) and ammonium (●) are shown. (*B*) Same as in *A*, but the first and third principal components in the PCA are represented only for genes classified originally in classes 2 and 3. Different symbols and colors identify genes from the final classes (2.1, 2.2, 3.1, and 3.2). (*Inset*) Same as in *A*, but PC2 is replaced by PC3.

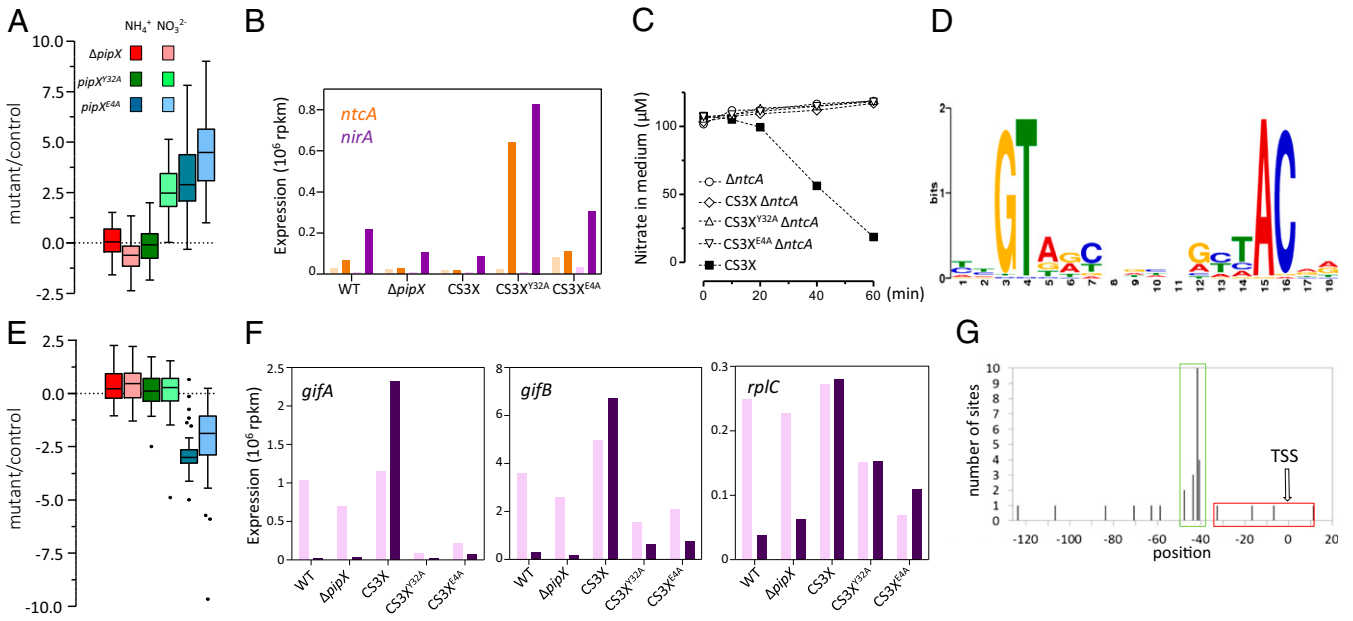

Fig. 4. Gain-of-function mutations $pipX^{E4A}$ and $pipX^{Y32A}$ target NtcA-regulated genes. (*A*) Box plot representation (median, box from first to third quartile and Tukey whiskers) of the within-group variance of mutant/control comparisons in class 4. (*B*) Expression pattern of the *ntcA* and *nirA* genes in ammonium (light-colored bars) and nitrate (dark-colored bars). (*C*) Effect of *ntcA* inactivation on nitrate uptake. Nitrate was added at time 0 to cell suspensions containing 20 μg of chlorophyll *a* per milliliter. A representative experiment is shown. (*D*) WebLogo based on 30 NtcA binding motifs identified with MEME upstream of sequences from class 4 genes. (*E*) Box plot representation of class 1 expression patterns. Outliers are represented by dots. (*F*) Expression patterns of class 1 genes *gifA*, *gifB*, and *rplC*. Light and dark bars are used for ammonium and nitrate, respectively. (*G*) Positioning of 29 NtcA boxes (*SI Appendix*, Table S2) relative to the TSS with activation (green) and repression (red) functions (details are provided in *SI Appendix*, Fig. S3 *A* and *B*).

interpretation of the effects of $pipX^{Y32A}$ and $pipX^{E4A}$ alleles on the expression of NtcA gene targets is that the lower affinity of $PipX^{E4A}$ or $PipX^{Y32A}$ (compared with PipX) for P_{II} would result in increased concentration of the mutant NtcA–PipX complexes, and consequent activation of NtcA targets in nitrate-grown cells. In addition, the lower affinity of $PipX^{Y32A}$ (compared with $PipX^{E4A}$) for NtcA would account for the nitrogen regulation observed in $CS3X^{Y32A}$ (i.e., the differences between nitrate and ammonium cultures). This result suggests that the mutant protein $PipX^{Y32A}$, despite its reduced affinity for P_{II}, is still engaged in partner swapping between NtcA and P_{II}. Strong support for the idea that the two mutant proteins still interact with P_{II} in vivo comes from the finding that the toxicity conferred by $pipX^{Y32A}$ and $pipX^{E4A}$ alleles is counteracted by P_{II} (17, 19).

To provide functional evidence that the mutant proteins $PipX^{Y32A}$ and $PipX^{E4A}$ exerted their effect on class 4 genes by interacting with NtcA rather than an NtcA-independent mechanism, we tested two functions that require NtcA in *S. elongatus*: growth on nitrate as a nitrogen source and nitrate transport. The prediction was that the *ntcA* null allele should be epistatic to the $pipX^{Y32A}$ and $pipX^{E4A}$ alleles.

When we attempted to inactivate *ntcA* by homologous recombination with the *ntcA::aphII* null allele, kanamycin-resistant clones carrying the inactive allele were obtained for CS3X, $CS3X^{E4A}$, or $CS3X^{Y32A}$ when transformants were selected on plates containing ammonium but not on nitrate. Furthermore, the ammonium-selected clones failed to grow on nitrate and to transport it (Fig. 4*C*). This therefore implies that the increased expression of the nitrate transport and assimilation systems in the $CS3X^{E4A}$ and $CS3X^{Y32A}$ strains requires NtcA. Thus, the results support the activation of class 4 by mutant NtcA–PipX complexes.

To provide additional evidence that the $PipX^{Y32A}$ and $PipX^{E4A}$ exerted their effect on class 4 genes by interacting with NtcA, and to gain further insights into the NtcA regulon in *S. elongatus*, we looked for NtcA binding motifs in promoter regions. The canonical NtcA-activated promoter is composed of an NtcA binding box, traditionally described with the consensus $GTAN_8TAC$, centered at ~41.5 nt upstream from the transcription start site (TSS), and separated 22–23 nt from a −10 box conforming to the consensus TAN_3T (20). In addition to this orthodox promoter structure, which matches that of the *Escherichia coli* class II Crp-dependent promoters, NtcA can activate from positions further upstream or from sequences not matching the reported consensus (21).

NtcA binding motifs were found in a few genes or operons experimentally characterized in *S. elongatus* (22–24) and in others predicted to be controlled by NtcA in the cyanobacterium *S. elongatus* PCC 6301 (25), which is almost identical to *S. elongatus*. Remarkably, NtcA binding motifs were found in association with 29 of the 30 transcription units in class 4 (Fig. 5 and *SI Appendix*, Table S2), a result indicating that class 4 contains almost exclusively operons directly activated by NtcA.

The extended NtcA binding consensus derived from the in silico analysis of class 4 genes (Fig. 4*D*) included the experimentally characterized binding site at the *glnN* promoter, previously referred to as atypical (23, 26). The high levels of transcripts found in strain $CS3X^{Y32A}$ in nitrate and in strain $CS3X^{E4A}$ in both ammonium and nitrate enabled us to map putative TSSs roughly from the RNA-sequencing read data (*SI Appendix*, Table S2 and Fig. S2). This, together with the predicted −10 elements, helped position the putative NtcA boxes. According to our analysis, most NtcA boxes appear to be centered at the canonical −41.5 position (Fig. 4*G* and *SI Appendix*, Fig. S3*A*).

Taking into account both the presence and positions of NtcA boxes and the expression patterns, only two class 4 genes were atypical: *Synpcc7942_1363* (the only gene in this class without recognizable NtcA boxes) and tRNA-Phe. Although direct regulation and binding in the absence of predictable NtcA binding sites has recently been suggested (27, 28), the rather attenuated NtcA-dependent pattern of *Synpcc7942_1363* could be explained

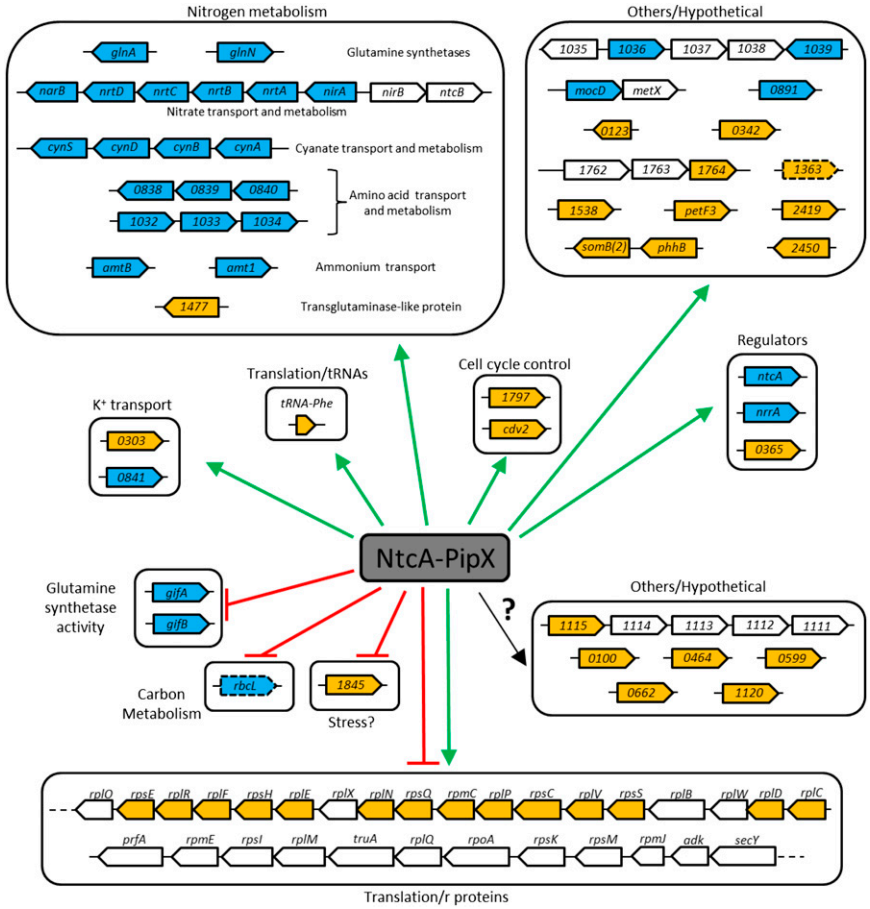

Fig. 5. Genomic organization of the *S. elongatus* NtcA–PipX regulon. Transcriptional units identified from the multivariate analysis as targets of NtcA–PipX complexes are shown in separate panels according to functional categories. ORFs and their orientation on the chromosome are shown as wide arrows in blue or orange, respectively, when there is or is not experimental or in silico evidence (22–25). Dashed arrows indicate no recognizable NtcA boxes. Green arrows and red blunt lines refer to activation and repression roles, respectively, inferred for NtcA–PipX. The question mark refers to genes with NtcA boxes of unknown function.

by indirect activation. On the other hand, tRNA-Phe was up-regulated in strains CS3X^{E4A} and CS3X^{Y32A} in nitrate and its promoter contains an NtcA binding site. However, in contrast to all other class 4 genes, which exhibit higher expression in nitrate, tRNA-Phe was induced in ammonium (Dataset S9). A precedent for a rather atypical regulation of a translation-related factor by NtcA is *gltX*, encoding the glutamyl-tRNA synthetase, which is expressed in both nitrate and ammonium in an NtcA-dependent manner (29).

In summary, transcriptomic analysis from a panel of five *S. elongatus* strains and two nitrogen conditions, followed by multivariate analyses, resulted in the identification of a coherent group of genes activated by the NtcA–PipX complex. These assignments, when considered alongside previously known NtcA target genes, identified new members of the NtcA regulon. In silico analysis allowed us to expand the consensus NtcA binding motif and to map NtcA boxes in promoter regions. The results provide further insights into the 2-OG–dependent partner swapping of PipX between NtcA and P_{II}, as well as the properties of the PipX^{Y32A} and PipX^{E4A} proteins.

NtcA-Repressed Genes. As happens with other members of the CRP family of transcriptional activators (30), NtcA can also mediate repression when its binding sites overlap the RNA polymerase binding region located between −40 and +20 nt relative to the TSS. Paradigms of this type of control are the *Synechocystis* sp. PCC 6803 genes *gifA* and *gifB*, encoding the glutamine synthetase-inactivating factors IF7 and IF17 (31).

If repression of target genes is also favored by mutations increasing NtcA–PipX complex formation, the prediction is that *pipX* mutations would have the opposite effect on NtcA-repressed and NtcA-activated transcripts. As shown in Fig. 3*A*, classes 1 and 4 occupy opposite positions along the PC2 axis, suggesting that NtcA may negatively control class 1 genes. Furthermore, the box plot of class 1 (Fig. 4*E*) can be regarded as a roughly inverted version of the class 4 box plot, with the main difference being exhibited by the *pipX*^{Y32A} allele.

The extended consensus from Fig. 4*D* was then used to search for NtcA boxes outside class 4, for which the complete set of 257 differentially regulated genes was used. Only 10 new hits (associated with nine genes) were found, four of which corresponded to three class 1 genes and included *gifA* and *gifB*, with NtcA boxes located at −32.5 and −16.5, respectively (*SI Appendix*, Fig. S3*B* and Table S2). Their expression pattern in WT *S. elongatus* (Fig. 4*F*) is consistent with the regulatory importance of IF7 and IF17 in the nitrogen metabolism of cyanobacteria (32, 33). Although PipX does not seem to be required for nitrate repression, the *pipX*^{E4A} and *pipX*^{Y32A} alleles confer repression in nitrate and, to a lesser extent, also in ammonium. Neither *gifA* or *gifB* showed ammonium induction in strain CS3X, a phenomenon that remains to be investigated.

Interestingly, 16 ribosomal genes were found in class 1. Two NtcA binding sites were found upstream of the coding region of *rplC*, the first gene of the main ribosome cluster. The site centered at −41.5, suggesting activation, is at odds with the inclusion of this gene in class 1. However, the second NtcA site at +10.5 is easier to reconcile with the negative effect of the gain-of-function alleles on transcript levels (Fig. 4 *E* and *F*). It is worth noting that the ribosomal genes that make a major contribution to the class 1 box plot were not down-regulated by CS3X^{Y32A} in nitrate. Taken together, these results suggest complex NtcA-dependent regulation at the ribosomal gene cluster and an intriguing connection between the nitrogen signaling system and the gene expression machinery.

No recognizable NtcA boxes were found at the remaining 16 genes found in class 1. These include *rbcL*, reported to be NtcA-repressed in *Anabaena* (34). Here, it is tempting to propose the involvement of the two orphan response regulators from class 4: NrrA, reported to be part of the NtcA regulatory cascade in *Anabaena* (35, 36), and the *Synpcc7942_0365* gene product, either or both of which may account for indirect NtcA-dependent repression of some class 1 genes.

A simple interpretation of the results presented so far on classes 4 and 1 (Fig. 4 and *SI Appendix*, Fig. S3 and Table S2) is that whereas positive regulation tends to be directly exerted by NtcA, negative regulation would tend to be exerted indirectly, via an NtcA-activated repressor.

NtcA Targets Outside Classes 1 and 4. The only gene outside class 1 matching functional and structural criteria for NtcA repression was *Synpcc7942_1845* from class 2.1 (*SI Appendix*, Fig. S4). Its expression differed significantly from the representative pattern of class 2.1 (Fig. 6). As expected for NtcA-repressed genes, it was down-regulated in CS3X^{E4A} and, to a greater extent, in CS3X^{Y32A}. It also contains an NtcA binding site centered at −2.5 (*SI Appendix*, Fig. S3*B*). However, the expression pattern of *Synpcc7942_1845* differed between the two control strains, being higher in the CS3X background, a result suggesting that additional regulatory mechanisms may prevent transcript accumulation in ammonium in the WT. Recent data indicate that *Synpcc7942_1845* acts as a general stress protein in *S. elongatus* (37), suggesting that it may be subjected to multiple regulatory controls.

Only six genes, belonging to class 2.2 (one gene), class 3.2 (three genes), and the group of nonclassified genes (two genes), had NtcA boxes for which we could not infer a particular function. Their atypical expression patterns may be due to the presence of cryptic NtcA sites or to the confluence of additional regulatory systems.

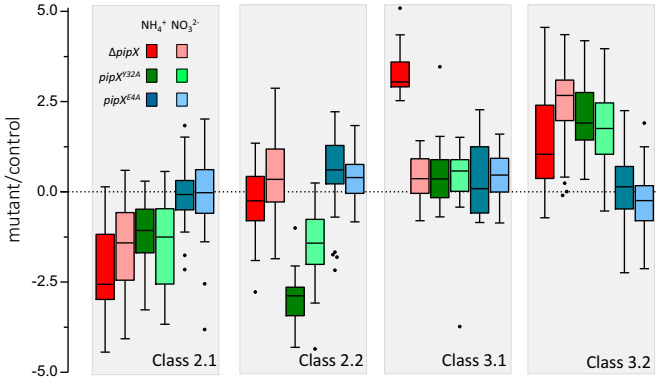

Fig. 6. Box plot representation of the within-group variance of the mutant/control comparisons in classes 2.1, 2.2, 3.1, and 3.2. Details are as described in Fig. 4*A*.

Recent transcriptomic and ChIP studies of the *Anabaena* NtcA regulon allowed the identification of large numbers of NtcA targets in this heterocyst-forming cyanobacterium (28, 38). In this context, it is worth noting that our multivariate analysis did not aim to provide a comprehensive study of NtcA targets but, instead, was designed to identify genes with paradigmatic and simple regulation by PipX, which may be representative of interactions with NtcA or with other transcriptional regulators. In this context, several NtcA target genes previously characterized in *S. elongatus* did not score above the cutoff levels. Notably, *glnB*, also expressed from a strong NtcA-independent promoter (39), *gltX*, subjected to both positive and negative regulation by NtcA (29) and *nblA*, controlled by several additional regulators (40–42), were not detected in our analysis.

NtcA-Independent Expression Patterns and Functions of PipX Regulons. The effects of mutations on the expression patterns of classes 2.1, 2.2, 3.1, and 3.2 are illustrated in Figs. 3*B* and 6. The distinctive feature of class 2.1 transcripts was their down-regulation by Δ*pipX* and *pipX*^{Y32A} alleles in both nitrate and ammonium. Exactly the opposite effect was found for class 3.2 genes: up-regulation by Δ*pipX* and *pipX*^{Y32A} alleles in both nitrate and ammonium. Class 2.2 was characterized by significant down-regulation by *pipX*^{Y32A}, especially in ammonium, and class 3.1 was characterized by up-regulation by Δ*pipX* in ammonium.

Most of the mutant/control changes detected in our analysis were similar in nitrate and ammonium cultures. The finding that the Δ*pipX* allele affected class 2.1 and class 3.2 genes similarly in nitrate and ammonium cultures suggests that PipX plays the same role in both nitrogen-rich conditions. On the other hand, only Δ*pipX* in class 3.1 and *pipX*^{Y32A} in class 4 affected gene expression specifically in one condition. Although the molecular basis of PipX regulation of class 3.1 genes remains elusive, the 2-OG–dependent partner swapping of PipX between NtcA and P_{II} (12) provides the background to interpret the class 4 expression pattern, further indicating that 2-OG was limiting for NtcA–PipX^{Y32A} complex formation but not for NtcA–PipX^{E4A} complex formation in our ammonium cultures.

The effect of Δ*pipX*, *pipX*^{E4A}, and *pipX*^{Y32A} alleles on the transcript levels of classes 2.1, 2.2, 3.1, and 3.2 cannot be reconciled with the involvement of NtcA–PipX complexes in the regulation of their corresponding genes. In particular, the drastic impact of *pipX* inactivation at classes 2.1, 3.1 (specifically in ammonium), and 3.2 and the lack of effect of the alleles *pipX*^{E4A} (in classes 2.2 and 3.1) and *pipX*^{Y32A} (in class 3.1) do not support the involvement of NtcA. Furthermore, with very few exceptions (in classes 2.2 and 3.2; *SI Appendix*, Table S2), NtcA boxes were absent from the genes or transcription units involved. Because both the response to the Δ*pipX*, *pipX*^{E4A}, and *pipX*^{Y32A} alleles and the promoter structure (discussed above) argued against the involvement of NtcA–PipX complexes in regulation of class 2.1, 2.2, 3.1, and 3.2 genes, our working hypothesis is that each of these four groups constitutes regulons influenced by PipX in an NtcA-independent manner.

To investigate further the internal coherence of the groups of genes obtained by multivariate analyses, which was based on the ability of *S. elongatus* gene transcripts to respond similar to *pipX* alleles, we followed the cluster of orthologous genes (COG) classification system to assign functions within groups.

The distribution of both COG categories and genes of unknown function differed greatly across the six groups (*SI Appendix*, Figs. S5 and S6). Genes of unknown function made similar contributions to the complete *S. elongatus* genome (*ca.* 37%) and to the 222 genes included in the six groups analyzed here (*ca.* 34%) but were especially abundant in class 2.1 (24 of 37 genes) and rare in class 1 (four of 32 genes). Translation was

overrepresented (14% vs. 7% in the complete *S. elongatus* genome), but ribosomal proteins were found exclusively in class 1 (18 of 32 genes) and tRNAs were found almost exclusively in class 3.2 (12 of 13 genes), thus revealing a strong connection between PipX and translation. The second most abundant category was energy production and conversion, which was particularly abundant in class 3.1 (9 of 18 genes were photosynthesis-related genes). Inorganic ion transport and metabolism and amino acid transport and metabolism were found almost exclusively in class 4 (21 of 43 genes in total), in close agreement with their role in nitrogen assimilation. Carbohydrate transport and metabolism was relatively well represented in class 2.2 (5 of 43 genes).

PipX Modulon, a Working Model. The results presented in this work provided important insights into the *S. elongatus* NtcA regulon while revealing unexpected functions of PipX (Fig. 7). The finding that the six groups of genes that emerged from the multivariate analysis showed very good internal coherence gave credit to the hypothesis that PipX is involved in processes other than coactivation/corepression of NtcA targets, which are mainly related to nitrogen assimilation (class 4 and some class 1 genes). In this context, the finding that the double-mutant *ntcA pipX* is less viable than the *ntcA* single mutant, as inferred by failure to segregate the Δ*pipX* allele in the *ntcA* null mutant (*SI Appendix*, Fig. S7), supports the involvement of PipX in NtcA-independent functions.

The finding that regulation by PipX could be observed in both or just one of the nitrogen conditions used (ammonium in class 3.1) supports the idea that signals other than 2-OG affect PipX interactions. The identification as members of the PipX modulon of highly expressed genes for ribosomal proteins (class 1), tRNAs (class 3.2), and photosynthesis (class 3.1) further suggests that NtcA-independent regulons participate in the adaptation of the cyanobacterial machineries for translation and photosynthesis to nutrient or other environmental changes. The emerging picture is that of PipX as a multifunctional protein involved in fine-tuning of different gene expression programs in response to different signals.

The effect of *pipX* inactivation on transcript levels indicated a positive role for PipX in class 2.1 and a negative role in classes 3.1 and 3.2. On the other hand, the impact of $pipX^{Y32A}$ on class 2.2 transcripts indicated that PipX has the potential to act as a negative regulator, probably under environmental conditions not tested in this work. How PipX exerts the different roles inferred here is still a matter of speculation. It may function by interacting with transcriptional, signal transduction, or even posttranscriptional regulators.

To account for the six groups of genes identified here with common expression patterns, we propose the involvement of PipX in a minimum of four types of regulatory complexes, of which only one (NtcA–PipX complex) is presently characterized.

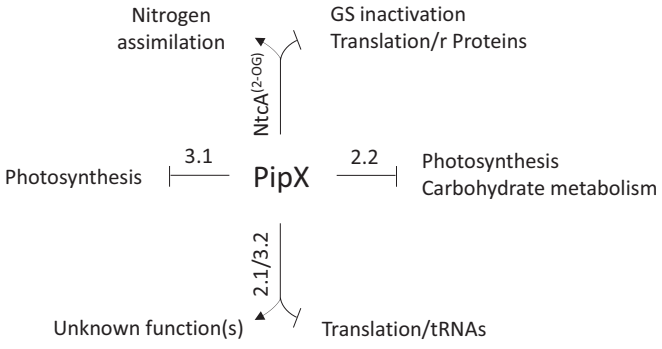

Fig. 7. PipX regulatory functions inferred from this work. Positive regulation and negative regulation are depicted by arrowheads and blunt lines, respectively.

The rather symmetrical effects of *pipX* mutations in the expression patterns of classes 1 and 4, reflecting the involvement of NtcA–PipX complexes in both groups, have a parallel in classes 2.1 and 3.2, which gave remarkably symmetrical box plots; thus, it is tempting to speculate that the same regulatory complex could be involved in activating and repressing these genes. Different complexes would be involved in repression of class 2.2 and 3.1 genes.

The intracellular availability of PipX and its specific interactions in particular environmental conditions or genetic backgrounds are probably determined by a network of interactions involving NtcA, P_{II}, their binding proteins and effectors (Fig. 1), and perhaps P_{II}-modifying enzymes (43, 44). This complexity makes it difficult to infer a potential role for P_{II} in the proposed PipX complexes. Notably, P_{II} proteins acting in complexes with DNA-binding transcriptional regulators have been reported in other systems (45–47).

The crucial impairment of binding to P_{II} caused by the PipX substitution Y32A argues against the involvement of P_{II} in the regulation of class 2.2 genes, where the $pipX^{Y32A}$ allele causes gain of function (as a repressor). Direct involvement of the tudor-like domain, and therefore of P_{II}, also appears unlikely in class 3.1 regulatory complexes, because $pipX^{Y32A}$ and $pipX^{E4A}$ alleles had no effect on the repressive function of PipX; here, it is tempting to propose a role for the C-terminal α-helices of PipX in repression. Finally, although the (complete) loss of function conferred by $pipX^{Y32A}$ would support the involvement of the tudor-like domain in regulation of class 2.1 and 3.2 genes, the silent effect of the $pipX^{E4A}$ allele argues against direct involvement of P_{II}.

Whatever mechanisms are involved, our results suggest that PipX participates in at least three novel regulatory scenarios, different from the known NtcA–PipX complexes, to modulate gene expression.

Materials and Methods

Strains, Growth Conditions, and Nitrate Transport Assays. *S. elongatus* strains and plasmids are listed in *SI Appendix*, Table S1. Growth, genetic manipulations and nitrate uptake assays were as described (13, 17).

Preparation and RNA Analysis. For RNA preparations, 100-mL cultures of each strain were grown in BG11 or $BG11^{A}$ until a OD_{750} nm of ∼0.9 was attained. RNA was purified with a Qiagen RNeasy Protect Bacteria Mini Kit and on-column RNase-free DNase I digestion. Samples were assayed for RNA integrity using an Agilent 2100 Bioanalyzer and quantified with a Qubit fluorometer (Life Technologies). Removal of 16S and 23S rRNA from total RNA was performed using a MicrobExpress Bacterial mRNA Purification Kit (Ambion) or treatment with a Ribo-Zero Magnetic Kit (Epicenter). RNA samples were divided into multiple aliquots of ≤5 μg of RNA, and separately enriched mRNA samples were pooled, run on the 2100 Bioanalzyer to confirm reduction of 16S and 23S rRNA before preparation of cDNA fragment libraries with a ScriptSeq v2 RNA-Seq Library Preparation Kit, and sequenced (Illumina HiSeq2000; Macrogene).

Computational Methods. Ten datasets of unique mappable reads covered each nucleotide strand specifically for an average of ∼120 to 500 times for the chromosome and 1.5 to 190 times for the plasmid. Read alignments were performed using Bowtie2 (48) against an *S. elongatus* reference chromosome and endogenous plasmid (GenBank accession nos. CP000100 and CP000101, respectively). Gene expression, represented as reads per kilobase, was determined by Samtools (49), the Artemis Genome Browser (Wellcome Trust Sanger Institute) (50), and homemade Perl scripts. The data were normalized by quantiles (51). Statistical analysis was performed using the DESeq package (52) and R software (www.r-project.org/). Normalized reads per kilobase per million data are provided in Dataset S9.

NtcA motifs were first identified with MEME (53) (150 nt upstream of the TSSs or, when unpredicted, of initiation codons and a background consisting of a fourth-order Markov model of the entire genome) and were used to search for palindromic motifs between 6 and 20 bp. FIMO was used (54) to identify NtcA boxes outside class 4. The position-specific probability matrix for the motif was derived from the 30 matches provided by MEME. Searches were performed in sequences comprising 250 nt upstream and 50 nt

downstream of the TSSs or 250 nt upstream of the initiation codon. The hits were filtered using two criteria: *P* value <0.0001 and q-value <0.7.

A COG list was downloaded from Cyanobase (http://genome.microbedb.jp/cyanobase/SYNPCC7942), with manual assignation where relevant.

Multivariate analyses were carried out with SPSS (IBM) and R. PCA was applied on the correlation matrix, with varimax rotation of the first two principal components. Package cluster (55) was used for fuzzy c-means clustering (56).

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