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

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## Supplementary information

# 1. Methods

**Computational methods.** To carry out multivariate analysis of all three mutant vs control comparisons (6 variables = 3 mutants/control x 2 nitrogen culture conditions) the standardized residuals from linear regressions of (log-transformed) data for mutant vs control strains were calculated. Genes with residuals lower than 1.5 for all comparisons were considered as non-responding genes, (Fig. S1, gray dots) and truncated normal distributions were fitted to the distributions of their residuals (Table S3). A total of 282 genes, with residuals exceeding a threshold value of 2.5 for at least one variable, were considered as responding genes and selected for further analysis (the probability to be in this group would be lower than 0.005, with less than 13 genes expected by chance). Artemis Genome Browser was used to exclude genes with reads mapping mainly to the non-coding strand. The resulting 257 genes were subjected to different statistical analysis carried out with SPSS and R software's. First, the residuals of the 257 genes for the 6 comparisons were subjected to principal component analysis (PCA) and Varimax rotation was used to enhance the interpretability of the first two principal components. Classification into four main groups was obtained using k-means cluster analysis (1). Additional independent classifications into four groups were obtained by hierarchical Ward's minimum variance clustering and fuzzy c-means clustering, carried out using function fanny in package cluster (2). Genes coherently grouped with the three clustering methods (222 genes) were selected, and 6 groups were obtained by cutting the cluster dendrogram from Ward's method (classes 1, 2.1, 2.2, 3.1, 3.2 and 4).

**Determination and positioning of NtcA binding sites.** To determine the Transcription start site (TSS), reads were pooled and the position where the greatest number of reads began was considered the most likely TSS (Fig. S2). The resulting TSS differ to those determined by primer extension on 1 nt (for *ntcA*, *amt1* and *nirA*) 6 nt (for *glnA*) and 8 nt (for *glnN*). NtcA motifs present upstream Class 4 genes were identified with MEME (3). 150 nt upstream of the TSSs or, when unpredicted, of initiation codons for each gene and a background consisting of a fourth-order Markov model of the entire genome was used to search for palindromic motifs between 6 bp and 20 bp. The first statistically significant motif found among members of group 4 (*E*-value $1.3e^{-009}$) corresponded to a previously reported NtcA binding site. 30 out of 31 sequences presented one NtcA site according to MEME. To identify NtcA sites outside of class 4, FIMO was used (4). The position-specific probability matrix (PSPM) for the motif was derived from the 30 matches provided by MEME:

| Position | A | C | G | T |
|---|---|---|---|---|
| **1** | 0.166667 | 0.366667 | 0.083333 | 0.383333 |
| **2** | 0.166667 | 0.333333 | 0.133333 | 0.366667 |
| **3** | 0.000000 | 0.016667 | 0.966667 | 0.016667 |
| **4** | 0.000000 | 0.016667 | 0.000000 | 0.983333 |
| **5** | 0.616667 | 0.050000 | 0.183333 | 0.150000 |
| **6** | 0.383333 | 0.050000 | 0.416667 | 0.150000 |
| **7** | 0.083333 | 0.583333 | 0.100000 | 0.233333 |
| **8** | 0.333333 | 0.250000 | 0.183333 | 0.233333 |
| **9** | 0.283332 | 0.099999 | 0.349999 | 0.266665 |
| **10** | 0.266666 | 0.350000 | 0.100000 | 0.283333 |
| **11** | 0.233332 | 0.183332 | 0.249999 | 0.333332 |
| **12** | 0.233332 | 0.099999 | 0.583332 | 0.083332 |
| **13** | 0.149999 | 0.416666 | 0.049999 | 0.383332 |
| **14** | 0.149999 | 0.183332 | 0.049999 | 0.616666 |
| **15** | 0.983332 | 0.000000 | 0.016666 | 0.000000 |
| **16** | 0.016666 | 0.966666 | 0.016666 | 0.000000 |
| **17** | 0.366666 | 0.133332 | 0.333332 | 0.166666 |
| **18** | 0.383332 | 0.083332 | 0.366666 | 0.166666 |

Possible -10 elements were searched at positions 6-8 nucleotides upstream of determined TSS. Positioning of putative NtcA binding sites and -10 elements is shown in Figs. S3A-B.

**Functional classification of genes within the PipX modulon.** The COG list downloaded from Cyanobase on 30$^{th}$ (October of 2013) was manually re-annotated when relevant (see Dataset S1, Tables S1-8). Heat maps of functional groups amongst the different groups of genes were generated with the gplots library of R (Fig. S5). Correlations between list of genes and COGs were represented using linkage maps plotted with Circos (5) (Fig. S6).

**Inactivation of *pipX* in an *ntcA* null background.** *S. elongatus* WT and the *ntcA* null strain CS37 (*ntcA::Cm*$^{R}$, (6)) were transformed in parallel with plasmid pUAGC59.1 (7). Transformant clones selected on BG11$^{A}$ plates containing kanamycin were subjected to PCR analysis to verify segregation of *pipX* alleles (Fig. S7).

## 2. Supplementary Figures

**Figure S1. Analysis of regulated and non-regulated genes obtained after mutant/control comparisons.** The value of the residuals (ZRE) with highest dispersion for the mutant/control comparisons, grown in nitrate (*x* axis) or ammonia (*y*-axis), was calculated for each gene and represented as a scatterplot. In gray are depicted non-responsive genes with a ZRE<1.5 for all 6 comparisons (Δ*pipX*/WT, CS3X$^{Y32A}$/CS3X and CS3X$^{E4A}$/CS3X in nitrate or ammonia). In purple and red are, respectively, the genes considered as non-responsive in ammonia and nitrate (ZRE<1.5 in either nitrate or ammonia for all 3 mutant/control comparisons). In green are depicted genes with ZRE>1.5. Note that this group contain the 282 genes considered as being differentially regulated (ZRE>2.5).

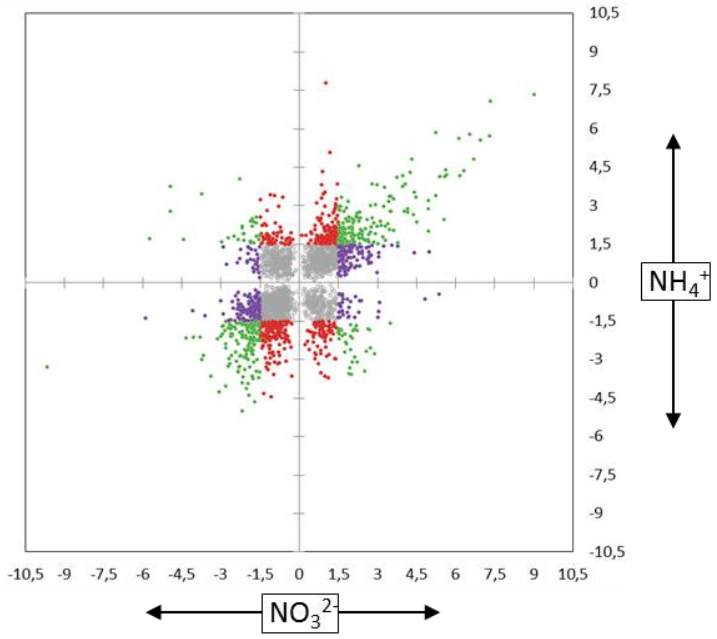


**Figure S2. Determination of the possible transcription start site (TSS) of *ntcA* (*Synpcc7942_0127*).** The image corresponds to Artemis software loaded with the *S. elongatus* chromosome (GenBank reference CP000100) and the BAM files of the CS3X$^{E4A}$ and CS3X$^{Y32A}$ strains corresponding to the nitrogen conditions indicated. The graphical view corresponds to coverage by strand of the entire *ntcA* genomic region. The nucleotide sequence of the *ntcA* promoter is shown below. The TSS, in this example -108 nt, was determined according to the position where reads began relative to the ATG initiation codon (+1).

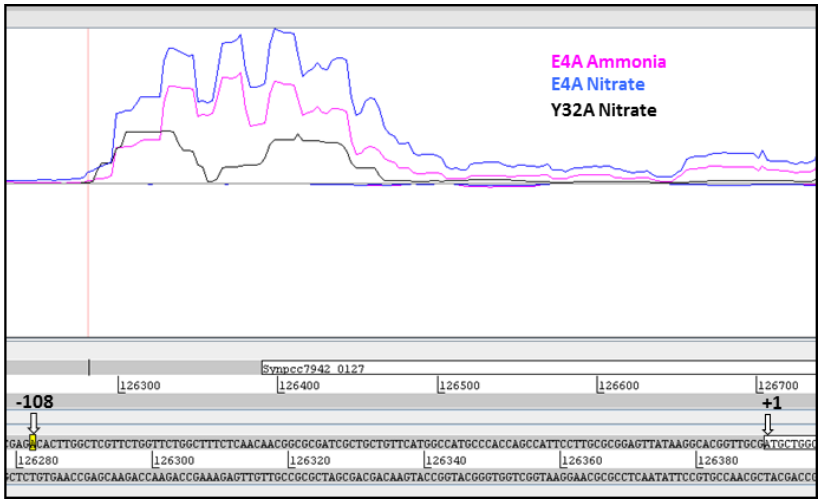


**Figure S3. Promoter sequences of genes with NtcA sites at activation (green boxed) or repression (red boxed) compatible positions.** A) Promoters of genes found in class 4 with NtcA boxes at canonical positions and -10 elements highlighted in green and blue, respectively. Boxed or underlined nucleotides refer, respectively, to TSS reported by primer extension (8-10) or inferred from this work. Numbers refer to *Synpcc7942* locus tags. B) Promoter of three class 1 (*gifA*, *gifB* and *rplC*) and one class 2.1 (locus tag *1845*) genes with their NtcA repressor sites and -10 elements (when recognizable) highlighted in red and blue, respectively. A putative NtcA box at activation position in *rplC* is highlighted in green. Initiation codons are in bold. Note that the NtcA site in *gifA* overlaps the -10 element.

A

| | | | | |
|---|---|---|---|---|
| *nirA* | GTT | GTAGTTTCTGTTAC | CAATTGCGAATCGAGAACTGCC | TAATCT GCCGAGTATGCAAGCTGCTT |
| *amt1* | ACT | GTTACATCGATTAC | AAAACAACCTTGAGTCTCGCTG | AATGCT TACAGAGATCTCACAAGGAT |
| *ntcA* | AAA | GTAGCAGTTGCTAC | AAGCAGCAGCTAGGCTAGGCCG | TACGGT AACGAGACACTTGGCTCGTT |
| *glnA* | TAT | GTATCAGCTGTTAC | AAAAGTGCCGTTTCGGGCTACC | TAGGAT GAAAGCGGTCAGCAATGCTT |
| *glnN* | TCT | GTATCTTTTCTAGC | GATCGAGCTGGTCACCATTGAG | TACGAT CAATTGACTAGCTTTTTTGG |
| *0840* | TTA | GTAGTCACCGTTAC | AGTCATTCCTTAGAACTTGTTT | TAGTTA ATTGGTAGCTGTTTACACCA |
| *1036* | ACA | GTAGCTACAGCTAC | GAATTGAAAGCTGGTGCCAGCC | TAGCTT GGATGGTTAAGCACTTTCAG |
| *1538* | CTC | GTATCAAGGGTAAC | GGTTCTTATTGGTTGACTTTAA | TACTTT AAAATTCTGACTCACCTCTC |
| *cynA* | GTT | GTAACGACGGCTAC | ATTTTGACCCTGGGGTTACTAC | TACCAT TCGCCCTTAACTGAGGAAAG |
| *amtB* | AAA | GTAGCAAAAGTTAC | GTATATCACCAGTCTGCCTAGCC | AGAGTT GTGAGATCTCCGAACCTTC |
| *1797* | AGT | GTGGTGGCGGTAAC | AGTTCTGAGCTAGACAGGGCCGT | TAAAGT AGCGCCAACTTGTGATGGC |
| *mocD* | CAG | GTAGCGATCGCTAC | AGCAGCAACAAAGATTGACACGA | TTGGTT AGAAAGAGACCTTGGGCGA |
| *0342* | AAA | GTGGTGTATGCGAC | AAATGAAGTGCTGCATCTCTTGC | TAAGAC TGTGGCAAAAGCCTTCGAA |
| *1032* | GCC | GTATCCCGAACTAC | AGAAGTGGACTCTGAGCGATTTC | TATAGT CCTTTCATGACATTTATGG |
| *nrrA* | CTC | GTAAAGGCGAATAC | AGAAGCCACAATGGACAGCTTGC | TAGGTT AAAGTCACAACTCCCAAGA |
| *phhB* | TCC | GTAGCAAAAAGCAC | GAGATTACTCGTCTCAAGTCGC | TACTTT TAAATGCACCTCGTGTTGAC |
| *1039* | TTC | GTAGCCTTGCTTAC | ACTTTGCCGATCTGGCCTCTTC | TAGGAT GCCAAAGCTGTGGCTTAGCC |

B

| | | | | |
|---|---|---|---|---|
| *gifA* | | TCC | GTAGCATTTGCTAC | AGTTTAAGGGCGAGGGTAATCGTTGGCCCAGTTTATCTGGG..$N_{70}$..**ATG** |
| *gifB* | | ACG | GTAGCAATTTATAC | AGAAAAATATTGCTAGATTTAAATCAAGCCTAGTTTTGCTT..$N_{16}$..**ATG** |
| 1845 | | | | TTT GTATCCGTTGCTAC CAAAGGTGGCCC **ATG** |
| *rplC* | ACC | GTATCGGTAGACAC | GATTCAGCAAAATGGTGTATTGT | TTATAT TTGTCGCTCT GTTGCGGTAAGCAC GAC..$N_{270}$..**GTG** |

**Figure S4. Expression patterns of Class 2.1 locus tag *1845*.** Light and dark bars are used for ammonium and nitrate.

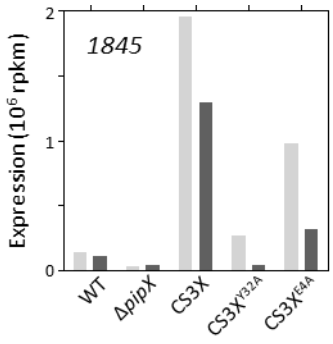



**Figure S5. COG functions within the PipX modulon.** A heatmap plot of gene distribution according to COG categories in the genome and the 6 classes defined here. The heat scale is the frequency of ORFs assigned to each individual COG category listed to the right.

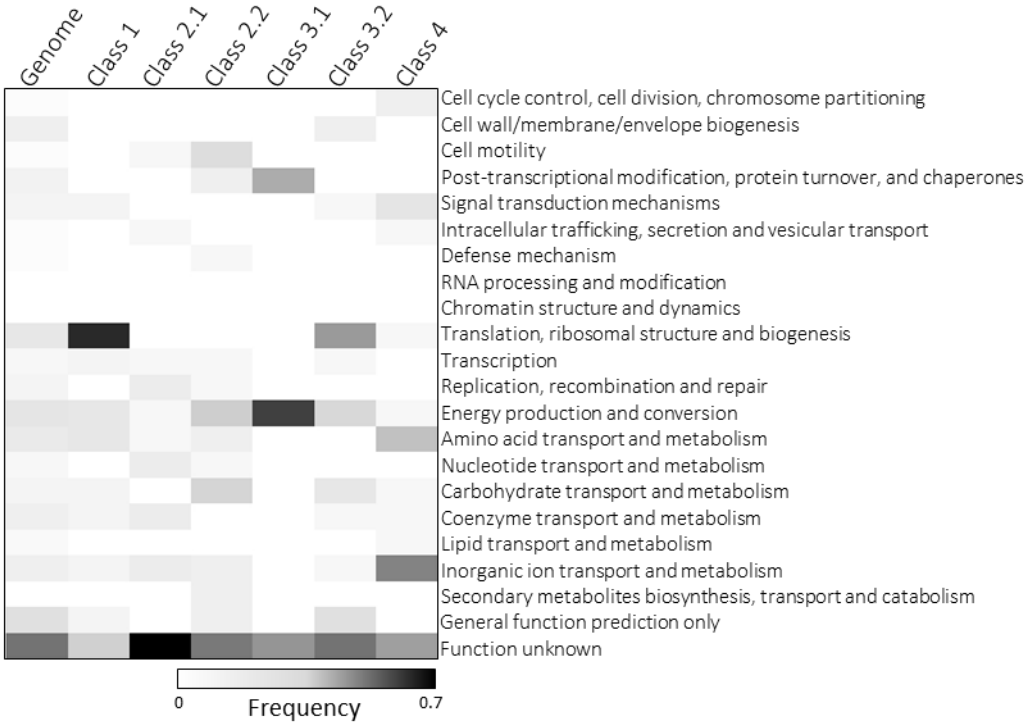


**Figure S6. Functional categories in the 6 groups defined by multivariate analysis.** Multiple maps connecting genes in the six groups with COGs categories are shown. Listed genes are named according to Synpcc7942 locus tag ID. COGs are depicted in different colors. C, Energy production and conversion; D, Cell cycle control, cell division and chromosome partitioning; E, Amino acid transport and metabolism; F, Nucleotide transport and metabolism; G, Carbohydrate transport and metabolism; H, Coenzyme transport and metabolism; I, Lipid transport and metabolism; J, Translation, ribosomal structure and biogenesis; K, Transcription; L, Replication, recombination and repair; M, Cell wall/membrane/envelope biogenesis; N, Cell motility; O, Post-translational modification, protein turnover, chaperones; P, Inorganic ion transport and metabolism; Q, Secondary metabolites biosynthesis, transport and catabolism; R, General function; T, Signal transduction mechanisms; U, Intracellular trafficking, secretion and vesicular transport; V, Defense mechanism; S, Function unknown.

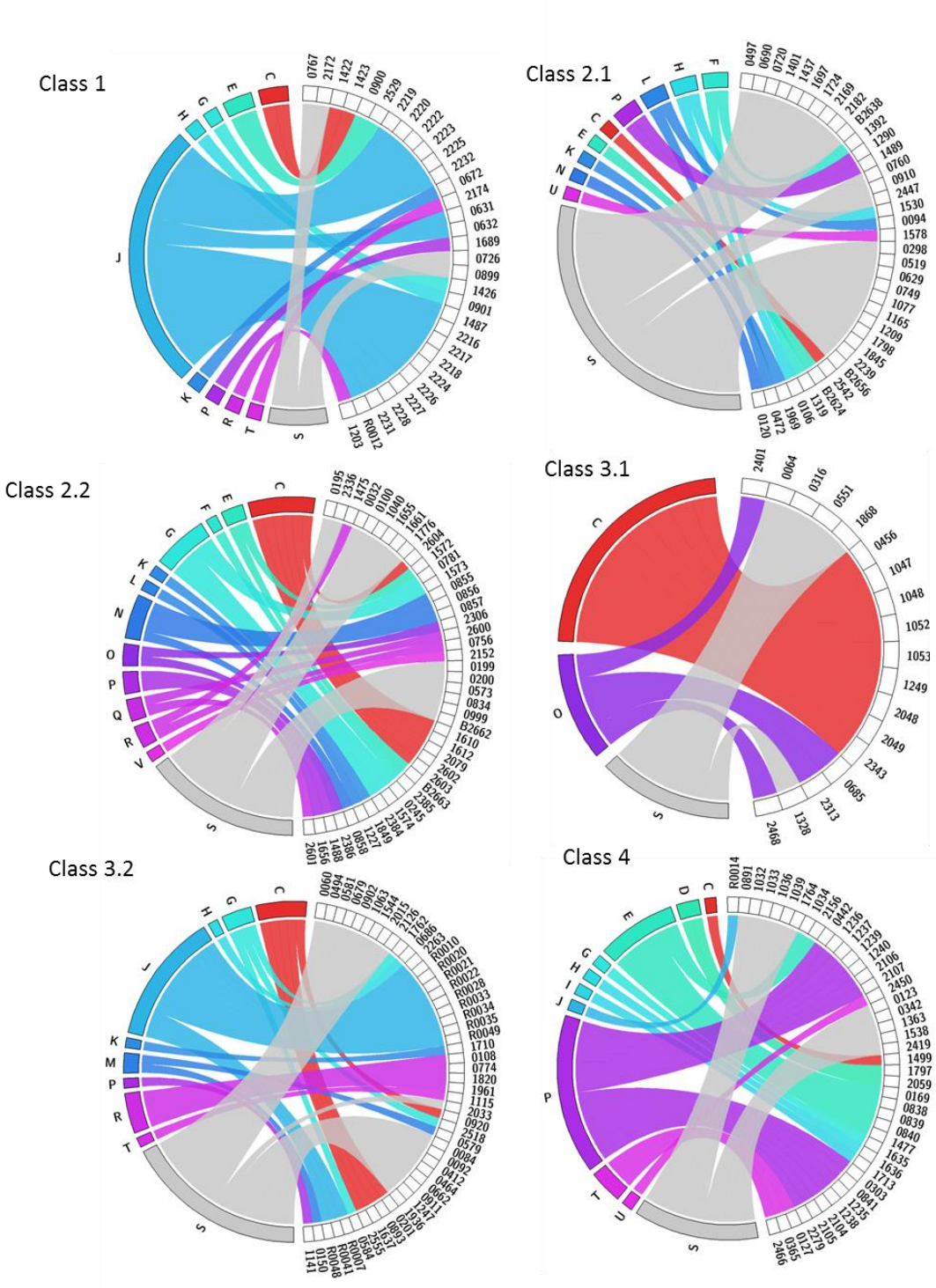
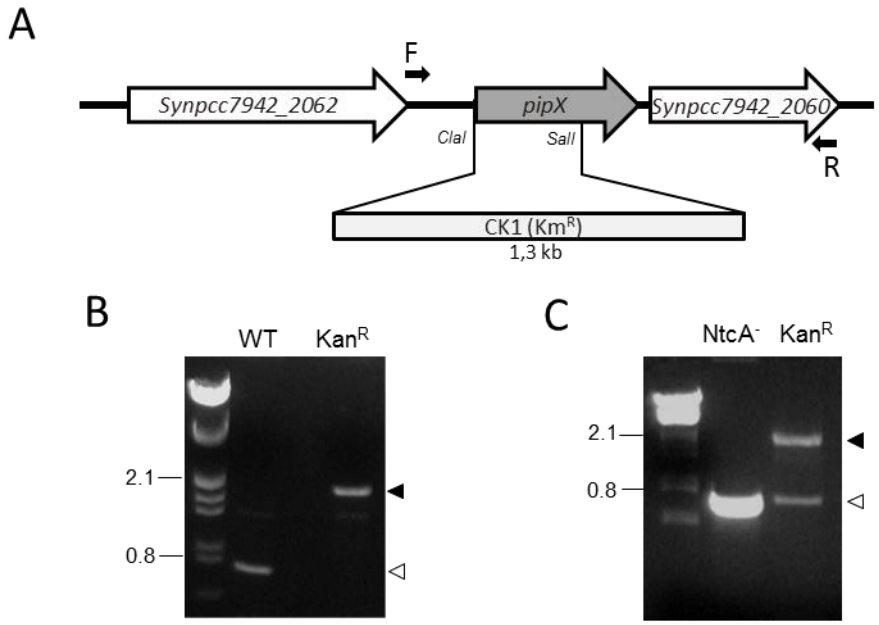


**Figure S7. Segregation of *pipX* inactivation allele in the *ntcA* null strain.** (A) Schematic representation of the *pipX::CK1* allele generated by insertion of the kanamycin-resistance cassette C.K1 with indication of relevant restriction sites and positions of primers (black arrows) used to verify allele replacement. (B) PCR verification of *pipX* alleles with primers PipX-126F (F) and PipX-5R (R) in *S. elongatus* (WT) and a representative kanamycin-resistant clone transformed with plasmid pUAG59.1 ($Kan^R$). (C). Idem in CS37 ($NtcA^-$). PCR products corresponding to *pipX* wild-type and *pipX::CK1* alleles are indicated as white or black arrowheads, respectively. Size markers (λ *Hind*III *Eco*RI) with indication of relevant band sizes (in Kilobases) are shown at the left.



## 3. Supplementary Tables

**Table S1. Strains and Plasmids used in this work.**

| Strain/Plasmid | Genotype or relevant characteristics | Reference |
|---|---|---|
| *S. elongatus* | Wild-type *Synechococcus elongatus* PCC 7942 | Pasteur Culture Collection |
| SA591 | (Δ*pipX*) PipX$^-$, (*pipX::C.K1*), Km$^r$ | (7) |
| CS3X | PipX, ϕ(*C.S3-pipX*), Sm$^r$ | (11) |
| CS3X$^{E4A}$ | PipX$^{E4A}$, ϕ(*C.S3-pipX$^{E4A}$*), Sm$^r$ | (12) |
| CS3X$^{Y32A}$ | PipX$^{Y32A}$, ϕ(*C.S3-pipX$^{Y32A}$*), Sm$^r$ | (12) |
| MNtcA | NtcA$^-$, (*ntcA::aphII*), Km$^r$ | (13) |
| CS3X-MNtcA | PipX NtcA$^-$, ϕ(*C.S3-pipX*), (*ntcA::aphII*), Sm$^r$ Km$^r$ | This work |
| CS3X$^{E4A}$-MNtcA | PipX$^{E4A}$ NtcA$^-$, ϕ(*C.S3-pipX$^{E4A}$*), (*ntcA::aphII*), Sm$^r$ Km$^r$ | This work |
| CS3X$^{Y32A}$-MNtcA | PipX$^{Y32A}$ NtcA$^-$, ϕ(*C.S3-pipX$^{Y32A}$*), (*ntcA::aphII*), Sm$^r$ Km$^r$ | This work |
| | | |
| pUAGC59.1 | (*pipX::C.K1*), Ap$^r$ Km$^r$ | (7) |
| pUAGC393 | ϕ(*C.S3-pipX*), Ap$^r$ Sm$^r$ | (11) |
| pUAGC375 | ϕ(*C.S3-pipX$^{E4A}$*), Ap$^r$ Sm$^r$ | (12) |
| pUAGC380 | ϕ(*C.S3-pipX$^{Y32A}$*), Ap$^r$ Sm$^r$ | (12) |
| pNTCA-KAN | (*ntcA::aphII*), Km$^r$ | (13) |

# Table S2. NtcA target genes in *S. elongatus*

| Transcription unit | Gene name | Published TSS | Predicted TSS (this work) | NtcA site | NtcA site position |
|---|---|---|---|---|---|
| *Synpcc7942_0123* | | n.d | n.d. | GTGGCGAAGAGTAC | n.d. |
| *Synpcc7942_0127* | *ntcA* | -108 (*-109*) | -108 | GTAGCAGTTGCTAC | (*-40.5*) |
| *Synpcc7942_0169* | *glnN* | -30 (*-22*) | -30 | GTATCTTTTCTAGC | (*-41.5*) |
| *Synpcc7942_0303* | | n.d | +51 | GTGATGAATGGCAC | -106.5 |
| *Synpcc7942_0342* | | -82 | -45[(a)] | GTGGTGTATGCGAC | -41.5 |
| *Synpcc7942_0365* | | -51 | n.d. | GTAATCTTTGTTAG | n.d. |
| *Synpcc7942_0442* | *amt1* | -102 (*-103*) | -102 | GTTACATCGATTAC | (*-40.5*) |
| *Synpcc7942_0840-0838* | | n.d. | -44 | GTAGTCACCGTTAC | -41.5 |
| *Synpcc7942_0841* | | n.d. | +26[(a)] | GTCGCGATTGATAC | -70.5 |
| *Synpcc7942_0891* | | -34 | -16[(a)] | GTAACTGGATACAC | -58.5 |
| *Synpcc7942_1032-1034* | | -25 | -26 | GTATCCCGAACTAC | -41.5 |
| *Synpcc7942_1036* | | -23 | -24 | GTAGCTACAGCTAC | -41.5 |
| *Synpcc7942_1039* | | -11 | -51[(a)] | GTAGCCTTGCTTAC | -47.5 |
| *Synpcc7942_1240-1235* | *nirA nrtA nrtB nrtC nrtD narB* | -56 (*-31*) | -30 | GTAGTTTCTGTTAC | (*-41.5*) |
| *Synpcc7942_1363* | | -105 | n.d. | *Not found* | n.d. |
| *Synpcc7942_1477* | | 0 | n.d. | GTAACATCTGACAC | n.d. |
| *Synpcc7942_1499* | *petF3* | -61 | -64 | GTGATTACCCCTAC | -123.5 |
| *Synpcc7942_1538* | | n.d. | -20 | GTATCAAGGGTAAC | -41.5 |
| *Synpcc7942_1635* | *somB(2)* | -63 | n.d | GTTGACTAGGCGAC | n.d. |
| *Synpcc7942_1636* | *phhB* | -53 | -25[(a)] | GTAGCAAAAAGCAC | -41.5 |
| *Synpcc7942_1713* | *mocD* | -39 | -40 | GTAGCGATCGCTAC | -41.5 |
| *Synpcc7942_1764* | | 0 | n.d. | GTAACAGAGACAAC | n.d. |
| *Synpcc7942_1797* | | -31 | -31 | GTGGTGGCGGTAAC | -47.5 |
| *Synpcc7942_2059* | *cdv2* (*sepF*) | -84 | n.d. | GTTTGTGCTATTAC | n.d. |
| *Synpcc7942_2107-2104* | *cynA cynB cynD cynS* | -16 | -16 | GTAACGACGGCTAC | -43.5 |
| *Synpcc7942_2156* | *glnA* | 0* (*-147*) | -141 | GTATCAGCTGTTAC | (*-40.5*) |
| *Synpcc7942_2279* | *amtB* | -72 | -36 | GTAGCAAAAGTTAC | -43.5 |
| *Synpcc7942_2419* | | n.d. | n.d. | GTTGCCCCTTCTAA | n.d. |
| *Synpcc7942_2450* | *gspD* | -70 | n.d. | GTGAGTGAATTTAC | n.d. |
| *Synpcc7942_2466* | *nrrA* | -23 | -23 | GTAAAGGCGAATAC | -43.5 |
| *Synpcc7942_R0014* | tRNA-Phe | n.d. | n.d. | GTTGCTCCTCTCAC | n.d. |
| *Synpcc7942_2529* | *gifA* | -102 | -102 | GTAGCATTTGCTAC | -16.5 |
| *Synpcc7942_0900* | *gifB* | -32 | -32 | GTAGCAATTTATAC | -32.5 |
| *Synpcc7942_2232-2203* | *rplC…prfA* | -67* | -292 | GTATCGGTAGACAC | -41.5 |
| | | | | GTTGCGGTAAGCAC | +11.5 |
| *Synpcc7942_1845* | | -24 | -13[(a)] | GTATCCGTTGCTAC | -6.5 |
| *Synpcc7942_0100* | | -63 | -25[(a)] | GTAGCCTAAGTCAC | -40.5 |
| *Synpcc7942_1115-1111* | | -174 | n.d. | GTAATCATTATTAC | n.d. |
| *Synpcc7942_0662* | | -59 | -59 | GTGGAAACCGTTAC | -62.5 |
| *Synpcc7942_0464* | | 0 | n.d. | GTAGCCACAGTCAC | n.d. |
| *Synpcc7942_0599* | | -29 | -29 | GTTGCAGTGGCAAC | -83.5 |
| *Synpcc7942_1120* | | -19 | n.d. | GTGGATGTCGTTAC | n.d. |

NtcA boxes in differentially regulated genes. The majority of the genes listed belong to Class 4, except for: *gifA*, *gifB* and the operon *Synpcc7942_2232-2203* (belonging to Class 1), *Synpcc7942_1845* (Class 2.1), *Synpcc7942_0100* (Class 2.2), *Synpcc7942_0662*, *_0464* and the operon *_1115-1111* (Class 3.2) and *Synpcc7942_0599* and *_1120* (original Class 3). Sequence of NtcA sites, identified using either MEME or FIMO (underlined genes), correspond to strand +. Transcription start sites (TSS) are relative to the initiation codon. When available, TSS obtained previously by RNAseq (14) or primer extension (in brackets and italics) is indicated in the column labelled as published TSS. The predicted TSS obtained in this work (see details in methods and supplementary Fig. 1) are also provided (n.d. means not determined and [(a)] the provided TSS could not be determined in our wild-type datasets). The position of the NtcA sites is relative to the TSS either determined by primer extension or obtained in this work. The asterisk (*) represents discrepancies with the first gene of the transcription unit reported by (14) (the TSS is relative to the start codon of ORFs *Synpcc7942_2157* and *_2233*).

**Table S3: Fitting of truncated normal distributions to the residuals of mutant/control comparisons for core genes.**

| | Δ*pipX* | | *pipX*$^{Y32A}$ | | *pipX*$^{E4A}$ | |
|---|---|---|---|---|---|---|
| | ammonium | nitrate | ammonium | nitrate | ammonium | nitrate |
| $\hat{\mu}$ | -0.0686 | -0.1099 | 0.0565 | -0.0453 | -0.0657 | -0.0703 |
| $\hat{\sigma}$ | 0.7956 | 0.7771 | 0.6749 | 0.7325 | 0.7142 | 0.5919 |
| *D* | 0.0133 | 0.0099 | 0.0114 | 0.0192 | 0.0187 | 0.0204 |
| *p* | 0.7456 | 0.9970 | 0.9826 | 0.5725 | 0.6043 | 0.4912 |

Estimated values of the parameters mean ( $\hat{\mu}$ ) and standard deviation ( $\hat{\sigma}$ ) by maximum likelihood (15)) and Kolmogorov-Smirnov goodness-of-fit statistics (*D*) and corresponding *p*-values. Parameters were estimated from the data, therefore, *p*-values are approximate.

## 4. Supplementary Material References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