# Supporting Information

## Ermakova et al. 10.1073/pnas.1407327111

### SI Materials and Methods

**Construction of *Anabaena flv* Mutants.** The upstream regions of *all0177* and *all0178* genes were amplified by PCR with Phusion high-fidelity DNA polymerase (Thermo Scientific) using primers 1 and 2 for *all0177* and primers 3 and 4 for *all0178* (Table S1), followed by restriction with PstI and XbaI. The downstream regions were amplified by PCR with primers 5 and 6 for *all0177* and primers 7 and 8 for *all0178*, followed by restriction with BamHI and SalI. Corresponding pairs of fragments were ligated together with the SalI-PstI fragment carrying *sacB* gene from pRL271 and the XbaI-BamHI fragment containing kanamycin/neomycin resistance cassette from pRL448. Resulting plasmids were transferred into *Anabaena* by triparental mating (1). Double recombinants were selected based on resistance of clones to neomycin and sucrose, and full segregation of the mutants was verified by PCR (Fig. S1). The obtained Δ*flv1B* mutant lacked 1,545 bp of the *flv1B* gene coding sequence. The Δ*flv3B* mutant lacked 588 bp of the *flv3B* coding sequence starting from the translation start codon.

For construction of the Δ*flv1B/3B* double mutant, the BamHI-XbaI region of the plasmid for *all0177* described above was replaced with the spectinomycin/streptomycin resistance cassette obtained from pEYFP-His6-SpR (2) by using primers 9 and 10. Afterward this plasmid was transferred into Δ*flv3B* and followed by selection with sucrose, neomycin, and spectinomycin. Segregation of the mutants was verified by PCR (Fig. S1).

**Isolation of Heterocysts.** Heterocysts were isolated by using a protocol based on lysozyme treatment to destroy the vegetative cells in the presence of a high osmolarity buffer to protect the isolated heterocysts, according to Ow et al. (3). For activity measurements, isolated heterocysts were immediately suspended in 50 mM Hepes-NaOH with 400 mM of sucrose to reach the Chl *a* concentration of 15 $\mu g \cdot ml^{-1}$. For protein isolation, the harvested heterocysts were stored at −80 °C. To confirm purity of heterocysts, we monitored the absence of active PS II centers from vegetative cells by Chl *a* fluorescence by using DUAL-PAM-100 (Walz).

**Nitrogenase Activity.** Nitrogenase activity was determined by acetylene reduction assay (4). The cultures were incubated for 4 d in $N_2$-fixing conditions. Samples preparation and measurements were performed as described in Leino et al (5)., but with gas mixture composed of 20.95% $O_2$, 0.039% $CO_2$, and 79.01% Ar, from which 10% was replaced with acetylene.

**Immunodetection.** Total cell extracts from $N_2$-fixing filaments were obtained by breaking cells in TS2 0.75 W Cell Disruptor (one shot machine, Constant Systems Ltd) by applying three shots of 40 $kg/in^2$ with cooling on ice in between. Sample buffer composition, conditions of electrophoresis, and immunodetection has been described in Zhang et al. (6). Anti-NifH antibody was purchased from Agrisera.

**Differential Gel Electrophoresis.** The isoelectrofocusing of proteins, second-dimension SDS/PAGE, image acquisition, and data analysis were performed as described (7). Filaments or isolated heterocysts were suspended in 5 mL of ice-cold solution comprising 8 M urea and 2 M thiourea. The cells were broken in TS2 0.75 W Cell Disruptor as described above. After this, 4% CHAPS was added to finalize the solubilization, followed by the purification of total protein extract by 2-D Clean-Up Kit (GE Healthcare). The purified protein extract was suspended in 30 mM Tris·HCl (pH 8.8), 8 M urea, 2 M thiourea, and CHAPS and labeled by using CyDye differential gel electrophoresis (DIGE) Fluor minimal dyes (GE Healthcare) according to producer's protocol. The identification of proteins was done according to ref. 8. Based on the ratio of NifK/RbcL, representing protein amount of the nitrogenase subunit NifK (expressed only in heterocysts) calculated over the protein amount of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase RbcL (presented only in vegetative cells), our heterocyst preparations contained 6–7 heterocysts per one vegetative cell (Table S2).


1. Elhai J, Wolk CP (1988) Conjugal transfer of DNA to cyanobacteria. *Methods Enzymol* 167:747–754.
2. Birungi M, et al. (2010) Possibilities of subunit localization with fluorescent protein tags and electron microscopy examplified by a cyanobacterial NDH-1 study. *Biochim Biophys Acta* 1797(9):1681–1686.
3. Ow SY, et al. (2008) Quantitative shotgun proteomics of enriched heterocysts from Nostoc sp. PCC 7120 using 8-plex isobaric peptide tags. *J Proteome Res* 7(4):1615–1628.
4. Dilworth MJ (1966) Acetylene reduction by nitrogen-fixing preparations from Clostridium pasteurianum. *Biochim Biophys Acta* 127(2):285–294.
5. Leino H, et al. (2014) Characterization of ten highly $H_2$ producing cyanobacteria isolated from the Baltic Sea and Finnish lakes. *Int J Hydrogen Energy*, 10.1016/j.ijhydene.2014.03.171.
6. Zhang P, Allahverdiyeva Y, Eisenhut M, Aro EM (2009) Flavodiiron proteins in oxygenic photosynthetic organisms: photoprotection of photosystem II by Flv2 and Flv4 in Synechocystis sp. PCC 6803. *PLoS ONE* 4(4):e5331.
7. Carmel D, Battchikova N, Holmström M, Mulo P, Aro EM (2013) Knock-out of low $CO_2$-induced slr0006 gene in Synechocystis sp. PCC 6803: Consequences on growth and proteome. *Photosynthesis Research for Food, Fuel and the Future. Advanced Topics in Science and Technology in China*, 654–658.
8. Battchikova N, Zhang P, Rudd S, Ogawa T, Aro EM (2005) Identification of NdhL and Ssl1690 (NdhO) in NDH-1L and NDH-1M complexes of Synechocystis sp. PCC 6803. *J Biol Chem* 280(4):2587–2595.
9. Mella-Herrera RA, Neunuebel MR, Kumar K, Saha SK, Golden JW (2011) The sigE gene is required for normal expression of heterocyst-specific genes in Anabaena sp. strain PCC 7120. *J Bacteriol* 193(8):1823–1832.

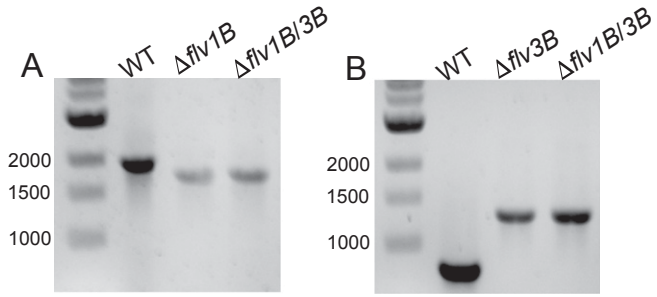


**Fig. S1.** Verification of *flv* mutants by PCR. (*A*) PCR with primers 11 and 12 (Table S1) for *all0177* and DNA isolated from the WT and mutants. Expected length of PCR products is 2,070 bp for WT, 1,657 bp for Δ*flv1B*, and 1,775 bp for Δ*flv1B/3B*. (*B*) PCR with primers 13 and 14 (Table S1) for *all0178* and DNA isolated from WT and the mutants. Expected length of PCR products is 848 bp for WT and 1,485 bp for mutants.

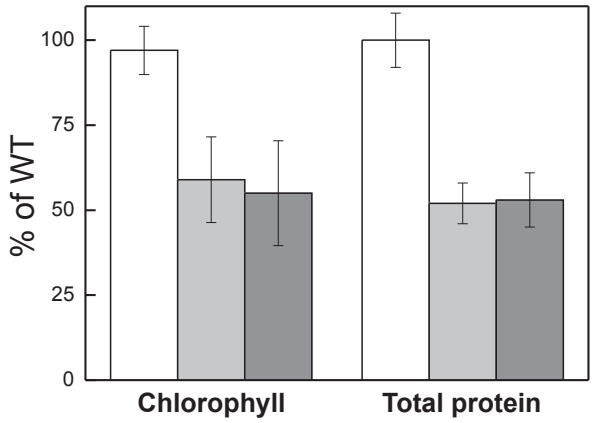


**Fig. S2.** The relative content of Chl *a* and total protein in mutant strains over the $OD_{750}$ value in percent of the WT values after 4 d in $N_2$-fixing conditions. white bars, Δ*flv1B*; light gray bars, Δ*flv3B*; dark gray bars, Δ*flv1B/3B*.

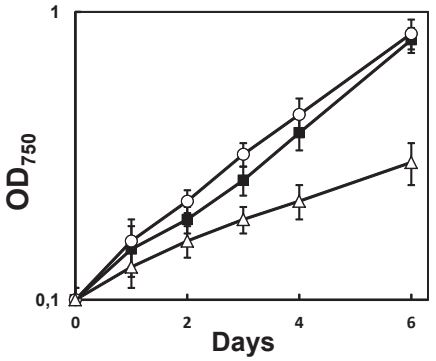


**Fig. S3.** The growth of *Anabaena* WT and mutant strains in $N_2$-fixing conditions under fluctuating light (20 μmol photons·$m^{-2}$·$s^{-1}$ for 5 min and 500 μmol photons·$m^{-2}$·$s^{-1}$ for 30 s). ■, WT, ○, Δ*flv1B*, △, Δ*flv3B*. The growth rate of the Δ*flv3B* (0.19 ± 0.04, $P < 0.001$) was approximately 54% of the WT rate (0.35 ± 0.05).

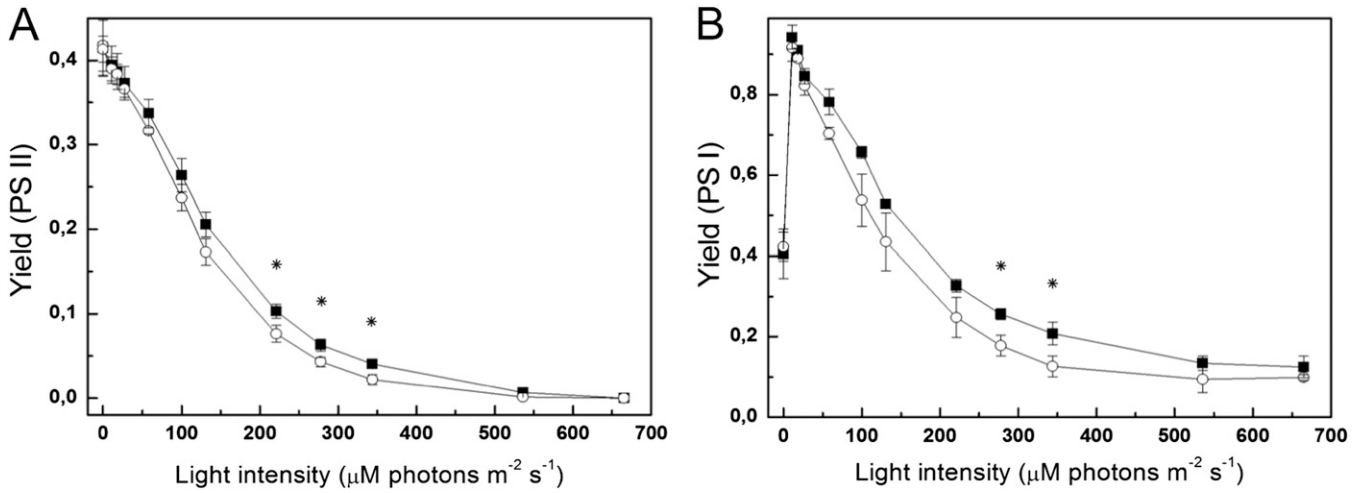


**Fig. S4.** Light response of the PS II yield (*A*) and PS I yield (*B*) recorded from the WT (■) and Δ*flv3B* (○) mutant strain. Mean ± SD, $n = 3$, asterisks indicate statistically significant differences between the WT and the mutant (*$P < 0.05$).

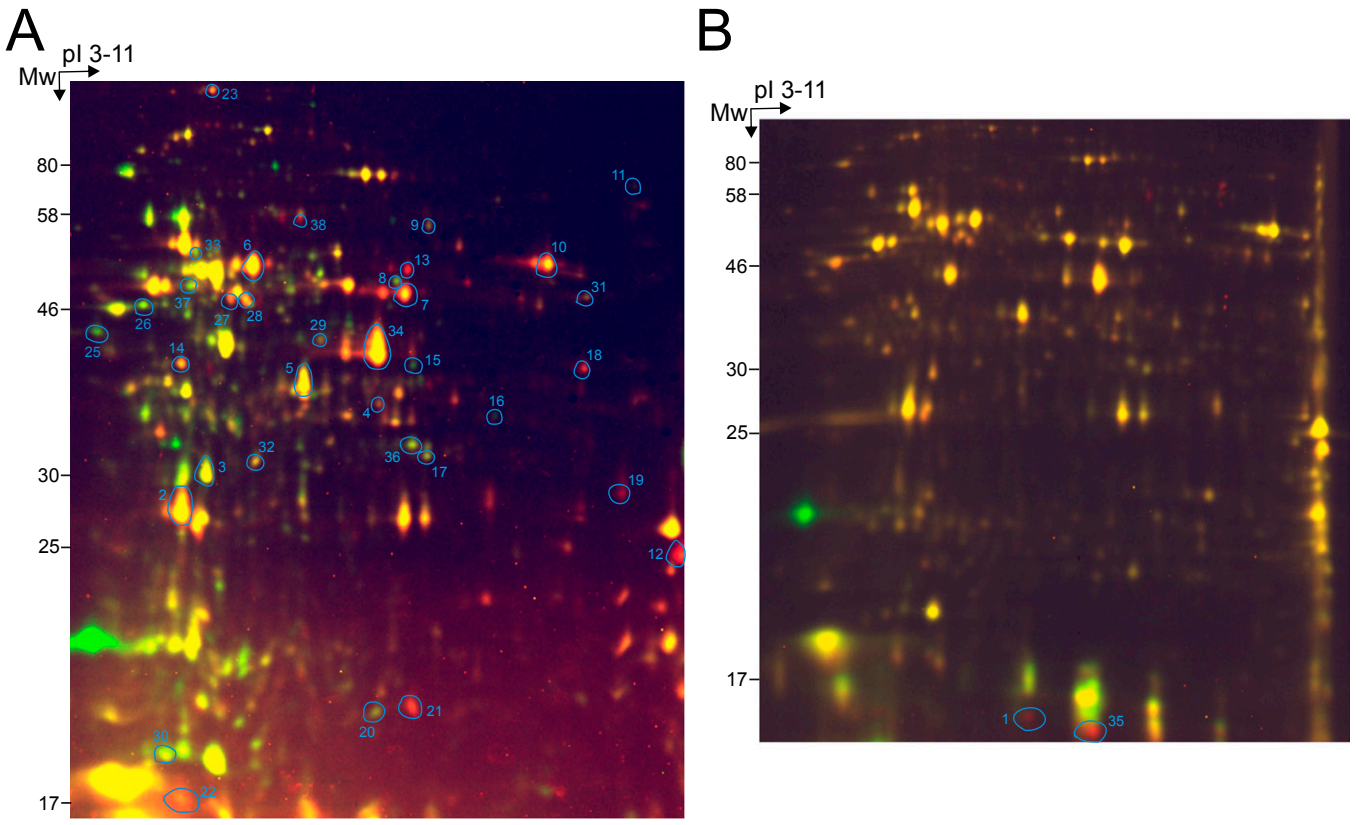


**Fig. S5.** Two-dimensional profile of proteomes of the WT and the mutant strains Δ*flv3B* (*A*) and Δ*flv1B* (*B*). The overlay mages of Cy3-control and Cy5-mutant samples from heterocyst-enriched fractions. Red spots correspond to proteins that are more abundant in the WT, green spots to proteins that are more abundant in the mutant strain, and yellow spots to proteins that are equally abundant in both strains. Numbered spots correspond to those were identified with MS/MS (Tables S3 and S4).

**Table S1. Oligonucleotides used for the construction (1–8), verification (9–12) of *flv* mutants, and in quantitative real-time PCR (13–48)**

| No. | Primer name | Sequence |
|---|---|---|
| 1 | all0177-L-Pst-Fw | CCACTGACTGcagaccatagcggtttggtggaagatg |
| 2 | all0177-L-Xba-Rev | CCACTGATCtagattttgcccagtctttaattgattgga |
| 3 | all0178-L-Pst-Fw | CCACTGACTGcagcactaactacctacgactaactact |
| 4 | all0178-L-Xba-Rev | CCACTGATCTAgattcgtctccgagcgcaaataaacag |
| 5 | all0177-R-Bam-Fw | CTACTGAGGAtccgaaggcaatcatctggaatacatga |
| 6 | all0177-R-Sal-Rev | CCACTGAGTCgacgttgttaaatattcagacctcagcat |
| 7 | all0178-R-Bam-Fw | CTACTGAGGATccgaagttatctatacttgtgatgctttt |
| 8 | all0178-R-Sal-Rev | CCACTGAGTCgactggggattaggtatagaaactctct |
| 9 | XbaI+Sp-Fw | TCTAGAaggacagaaatgcctcgacttc |
| 10 | BamH+Sp-Rev | GGATCCcctgatagtttggctgtgagca |
| 11 | Δflv1B_s | GGGGTGAGAACCCAAACC |
| 12 | Δflv1B_as | TCAACCGAGCATTCCACATA |
| 13 | Δflv3B_s | TGCTTTTTGTTACGGTAATCTTGCC |
| 14 | Δflv3B_as | GCATCCCAAAAGCATCACAAGTATA |
| 15 | nifH1_S | TTCTACAAACCCCTCACAGC |
| 16 | nifH1_AS | GATTTACCGATACCGCCTTT |
| 17 | nifH2_S | ACAAATCCTTATCTACGCAACC |
| 18 | nifH2_AS | ATAGCTGCCAAGGTGTTTTG |
| 19 | psaB1_S | CGCGCTCTCAGCATTACT |
| 20 | psaB1_AS | GTTCTGTTTTTACCCAGCACTT |
| 21 | psbAI_S | CAATCCATCATCGACTCACA |
| 22 | psbAI_AS | GTTGATAGCAGGAGCGGTTA |
| 23 | rbcL_S | TGGTACTTGGACGACCGTAT |
| 24 | rbcL_AS | GATGGAGCCTTCTTCAAACA |
| 25 | lox_S | GCGCAGTCTTTGAACGTATT |
| 26 | lox_AS | TAGTTCGCCTTCCGTATGAG |
| 27 | Mn-cat_S | AGGTTTTCCTTGGAATGGTC |
| 28 | Mn-cat_AS | CATGGTATCACGAGCAATCA |
| 29 | coxA1_S | CATGACACAAGCTCAGTTGC |
| 30 | coxA1_AS | GTAGTTCTGTCCGCACCAAG |
| 31 | coxA3_S | CACTGCCTACAATCAAGCATC |
| 32 | coxA3_AS | GAGTTGAACGCCTTCTATGG |
| 33 | fdxH_S | GGCAAAGTTGTTGAAGGTGA |
| 34 | fdxH_AS | TGAATTAAGCAAGGTACGGTTC |
| 35 | apcA_S | AATCGGTGTTCGTGAAATGT |
| 36 | apcA_AS | CCTACTGCAACGCACCTACT |
| 37 | pecA_S | CAGCAACACTGAAATGCAAG |
| 38 | pecA_AS | CCTTCTGGAGTGGAAGCATA |
| 39 | rbrA_S | ATTTCGTGAAACAGCAGAGC |
| 40 | rbrA_AS | CGTAGGTTTCGCCTTCAATA |
| 41 | sodB_S | GCTCATGGACAAAAAGCACT |
| 42 | sodB_AS | CCCTCATCTGACTGGACAAC |
| 43 | glpX_S | GGGTAAGGGCGAAAAGAATA |
| 44 | glpX_AS | AGCATTTGGTTGGGTACAAA |
| 45 | rpoA_S* | CAACTCTCTGTACGGGCCTA |
| 46 | rpoA_AS* | GCTTCTTTCTTGGGGTAAGG |
| 47 | flv3A_S | GAGGAACTCACCGGACGTT |
| 48 | flv3A_AS | GCTGCACCCAAATCTACAAC |
| 49 | fdaI_S | AGCCACAGGTCAGAGTTACG |
| 50 | fdaI_AS | AAAGTCGTTTTTGCCAGATG |

**rpoA* was used as a reference gene (1).

1. Mella-Herrera RA, Neunuebel MR, Kumar K, Saha SK, Golden JW (2011) The sigE gene is required for normal expression of heterocyst-specific genes in Anabaena sp. strain PCC 7120. *J Bacteriol* 193(8):1823–1832.

**Table S2. Amount of heterocysts per vegetative cell in preparations**

| Strain | RbcL/NifK (relative amount) | | Heterocysts:vegetative cells in filaments | Heterocysts:vegetative cells in preparations |
|---|---|---|---|---|
| | Filaments | Heterocyst-encriched | | |
| WT | 2.74 ± 0.14 | 0.39 ± 0.04 | 1:10 | 7:1 |
| Δ*flv1B* | 2.61 ± 0.26 | 0.41 ± 0.04 | 1:10 | 6:1 |
| Δ*flv3B* | 2.70 ± 0.15 | 0.38 ± 0.05 | 1:10 | 7:1 |

Amount of heterocysts and vegetative cells was calculated on micrographs for whole filaments, and by the ratio of RbcL/NifK from DIGE in heterocyst preparations.

**Table S3. Relative changes in protein amounts in the Δ*flv3B* mutant compared with the WT**

| Accession no. | Gene name | Protein name | Spot no. | Δ*flv3B* vs. WT (filaments) | Δ*flv3B* vs. WT (heterocysts) |
|---|---|---|---|---|---|
| *alr0524* | *pecA* | Phycoerythrocyanin (PEC) alpha chain | 1 | N/A | N/A |
| *all1455* | *nifH* | Nitrogenase iron protein 1 | 2 | 0.5 ± 0.0 | 0.5 ± 0.1 |
| *all2563* | *tal* | Transaldolase | 3 | 1.5 ± 0.1 | 1.5 ± 0.0 |
| *alr1744* | — | Sulfolipid biosynthesis protein | 4 | 0.6 ± 0.0 | — |
| *all4563* | *fda II* | Fructose bisphosphate aldolase class II | 5 | 0.7 ± 0.1 | 0.7 ± 0.0 |
| *all1440* | *nifK* | Nitrogenase molybdenum-iron protein beta chain | 6 | 0.7 ± 0.0 | 0.6 ± 0.0 |
| *alr1524* | *rbcL* | Ribulose bisphosphate carboxylase large chain | 7 | 0.6 ± 0.1 | 0.7 ± 0.0 |
| *all1872* | — | All1872 protein | 8 | 2.1 ± 0.2 | 2.4 ± 0.2 |
| *all0177* | *flv1B* | Putative diflavin flavoprotein A 5 | 9 | 3.2 ± 0.4 | 3.3 ± 0.5 |
| *all1454* | *nifD* | Nitrogenase molybdenum-iron protein alpha chain | 10 | 0.6 ± 0.1 | 0.5 ± 0.1 |
| *all2038* | — | All2038 protein | 11 | 3.8 ± 0.7 | 4.6 ± 0.4 |
| *alr0525* | *pecC* | Phycobilisome linker polypeptide. PEC-associated. rod | 12 | N/A | N/A |
| *all0687* | *hupL* | [NiFe] uptake hydrogenase large subunit | 13 | — | 0.3 ± 0.0 |
| *alr1041* | *glpX* | D-fructose 1.6-bisphosphatase class 2/sedoheptulose 1.7-bisphosphatase | 14 | 0.7 ± 0.0 | 0.7 ± 0.1 |
| *alr2355* | *ald* | Alanine dehydrogenase | 15 | — | 3. 5 ± 0.4 |
| *all3735* | *fda I* | Fructose bisphosphate aldolase class I | 16 | 2.2 ± 0.1 | 1.9 ± 0.1 |
| *alr5285* | *accA* | Ac-CoA carboxylase carboxyl transferase subunit alpha | 17 | 1.5 ± 0.2 | 1.3 ± 0.0 |
| *alr0128* | *chlP* | Geranylgeranyl hydrogenase | 18 | 0.8 ± 0.1 | 0.7 ± 0.1 |
| *all1743* | *por* | Protochlorophyllide oxidoreductase | 19 | 0.6 ± 0.0 | 0.4 ± 0.0 |
| *all4287* | — | Peptidyl-propil cis-trance isomerase B | 20 | 1.4 ± 0.1 | 2.6 ± 0.1 |
| *all1472* | — | All1472 protein | 21 | — | 0.4 ± 0.1 |
| *all2453* | *petC1* | Cyt b6f iron-sulfur subunit 1 | 22 | 0.6 ± 0.1 | 0.5 ± 0.1 |
| *all1267* | *acnB* | Aconitate hydratase 2 | 23 | 0.8 ± 0.2 | 0.7 ± 0.0 |
| *all2567* | — | Probable phosphoketolase 2 | 24 | 2.1 ± 0.4 | — |
| *alr0834* | — | Porin. major outer membrane protein | 25 | 1.6 ± 0.1 | 2.3 ± 0.3 |
| *all4499* | — | All4499 protein | 26 | 1.4 ± 0.1 | 2.1 ± 0.4 |
| *all0737* | *trxB* | Thioredoxin reductase (NADPH) | 27 | 0.6 ± 0.1 | 0.4 ± 0.0 |
| *all0737* | *trxB* | Thioredoxin reductase (NADPH) | 28 | 0.6 ± 0.1 | 0.5 ± 0.0 |
| *all4645* | *glgC* | Glucose-1-phosphate adenyltransferase | 29 | 0.5 ± 0.2 | — |
| *all4196* | *adk1* | Adenylate kinase 1 | 30 | 2.0 ± 0.1 | 2.2 ± 0.5 |
| *all1939* | — | Processing proteinase | 31 | 1.1 ± 0.1 | 1.4 ± 0.0 |
| *all2315* | *ilvC* | Ketol-acid reductoisomerase | 32 | 0.7 ± 0.1 | 1.0 ± 0.0 |
| *all1663* | *murE* | UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-2.6-diaminopimelate ligase | 33 | 1.6 ± 0.1 | 4.2 ± 0.8 |
| *alr5275* | — | 6-P-gluconate dehydrogenase. decarboxylating | 34 | 0.8 ± 0.1 | 0.5 ± 0.1 |
| *alr0529* | *cpcA* | C-phycocyanin alpha chain | 35 | 0.6 ± 0.0 | — |
| *all5062* | *gap2* | Glyceraldehyde-3-phosphate dehydrogenase 2 | 36 | 0.6 ± 0.0 | — |
| *all3556* | — | Succinate-semialdehyde dehydrogenase | 37 | 1.0 ± 0.1 | 1.8 ± 0.3 |
| *all0178* | *flv3B* | Putative diflavin flavoprotein A 2 | 38 | — | N/A |

Proteins were identified by MS/MS from DIGE performed with protein samples isolated from whole $N_2$-fixing filaments and from heterocyst-enriched fraction of WT and Δ*flv3B* strain. Spot no. corresponds to the numbering of proteins in Fig. S5. Mean ± SD, $n = 3$. N/A, not applicable.

**Table S4. Relative changes in protein amounts in the Δ*flv1B* mutant compared with the WT**

| Accession no. | Gene name | Protein name | Spot no. | Δ*flv1B* vs. WT (filaments) | Δ*flv1B* vs. WT (heterocysts) |
|---|---|---|---|---|---|
| *alr0524* | *pecA* | Phycoerythrocyanin (PEC) alpha chain | 1 | N/A | N/A |
| *all1455* | *nifH* | Nitrogenase iron protein 1 | 2 | 0.8 ± 0.3 | 0.8 ± 0.2 |
| *all2563* | *tal* | Transaldolase | 3 | 0.8 ± 0.1 | — |
| *all4563* | *fda II* | Fructose bisphosphate aldolase class II | 5 | 1.3 ± 0.1 | — |
| *all1440* | *nifK* | Nitrogenase molybdenum-iron protein beta chain | 6 | 0.9 ± 0.1 | 1.0 ± 0.1 |
| *alr1524* | *rbcL* | Ribulose bisphosphate carboxylase large chain | 7 | 1.0 ± 0.0 | — |
| *all0177* | *flv1B* | Putative diflavin flavoprotein A 5 | 9 | — | N/A |
| *all1454* | *nifD* | Nitrogenase molybdenum-iron protein alpha chain | 10 | 1.0 ± 0.1 | 1.0 ± 0.1 |
| *all0687* | *hupL* | [NiFe] uptake hydrogenase large subunit | 13 | 0.8 ± 0.1 | 0.7 ± 0.1 |
| *all3735* | *fda I* | Fructose bisphosphate aldolase class I | 16 | 1.5 ± 0.0 | — |
| *all0737* | *trxB* | Thioredoxin reductase (NADPH) | 27 | 1.0 ± 0.0 | 0.6 ± 0.0 |
| *all0737* | *trxB* | Thioredoxin reductase (NADPH) | 28 | 1.0 ± 0.1 | 0.6 ± 0.1 |
| *alr5275* | — | 6-P-gluconate dehydrogenase. decarboxylating | 34 | 0.7 ± 0.1 | 0.7 ± 0.1 |
| *all5062* | *gap2* | Glyceraldehyde-3-phosphate dehydrogenase 2 | 36 | 1.6 ± 0.2 | — |

Proteins were identified by MS/MS from DIGE performed with protein samples isolated either from whole filaments or heterocyst-enriched fraction of WT and Δ*flv1B* strain. Spot no. corresponds to the numeration of proteins on the Fig. S5. Mean ± SD, $n = 3$. N/A, not applicable.