Overproduction of the Flv3B flavodiiron, enhances the photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Nostoc* PCC 7120

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Abstract

**Background:** The ability of some photosynthetic microorganisms, particularly cyanobacteria and microalgae, to produce hydrogen (H\textsubscript{2}) is a promising alternative for renewable, clean-energy production. However, the most recent, related studies point out that much improvement is needed for sustainable cyanobacterial-based H\textsubscript{2} production to become economically viable. In this study, we investigated the impact of induced O\textsubscript{2}-consumption on H\textsubscript{2} photoproduction yields in the heterocyte-forming, N\textsubscript{2}-fixing cyanobacterium *Nostoc* PCC7120.

**Results:** The *flv3B* gene, encoding a flavodiiron protein naturally expressed in *Nostoc* heterocytes, was overexpressed. Under aerobic and phototrophic growth conditions, the recombinant strain displayed a significantly higher H\textsubscript{2} production than the wild type. Nitrogenase activity assays indicated that *flv3B* overexpression did not enhance the nitrogen fixation rates. Interestingly, the transcription of the *hox* genes, encoding the NiFe Hox hydrogenase, was significantly elevated, as shown by the quantitative RT-PCR analyses.

**Conclusion:** We conclude that the overproduced Flv3B protein might have enhanced O\textsubscript{2}-consumption, thus creating conditions inducing *hox* genes and facilitating H\textsubscript{2} production. The present study clearly demonstrates the potential to use metabolic engineered cyanobacteria for photosynthesis driven H\textsubscript{2} production.

**Background**

Development of renewable fuel as a clean alternative to fossil fuels is nowadays strongly needed. Besides solar energy, which represents the most abundant renewable energy, hydrogen (H\textsubscript{2}) is regarded as an attractive option for its high energy content and null
ecological impact: its combustion only releases water as a byproduct. In this regard, growing autotrophic, photosynthetic organisms (cyanobacteria and algae) to yield H₂ with minimized energy supply is a very promising alternative to fossil fuels.

In cyanobacteria, H₂ is produced by two different enzymes: hydrogenase and nitrogenase. In diazotrophic strains, H₂ is formed as a by-product of N₂ fixation activity performed by the nitrogenase. However, the nitrogenase is often associated to an uptake hydrogenase, encoded by the hup genes that catalyze the oxidation of H₂ into protons; the amount of H₂ produced during nitrogen fixation is thus rather limited [1]. The second type of enzymes producing H₂ are hydrogenases (H₂ases). Bidirectional NiFe H₂ases (called Hox), which catalyze both H₂ oxidation and proton reduction, are largely distributed across the cyanobacterial phylum [2, 3]. They form a heteropentamer with a H₂ase part (HoxYH) and a diaphorase part (HoxEFU). The physiological function of Hox hydrogenases in cyanobacteria is not well understood but they may serve as electron valve during photosynthesis in the unicellular cyanobacterium Synechocystis sp. PCC 6803 [4]. The expression of hox genes is induced in dark and/or anaerobic conditions [5] and is under the control of the regulators LexA and two members of the AbrB family (antibiotic resistance protein B) [6-8]. The sensitivity of cyanobacterial bidirectional H₂ases to oxygen (O₂) and the fact that their activity occurs in the dark or under anaerobic conditions are the major obstacles to obtaining efficient solar driven production of H₂ in cyanobacteria. Several strategies have so far been adopted to overcome the limits of the natural H₂-evolving mechanisms in cyanobacteria (for a review see [9]).

During photosynthesis, O₂ can be reduced to water through an enzymatic process involving flavodiiron proteins (Flvs) [10]. In cyanobacteria, Flvs catalyze the reduction of
$O_2$ into water using NADPH as an electron donor [11] and play a critical role during growth under fluctuating light regimes [12]. The filamentous heterocyte-forming cyanobacterium *Anabaena/Nostoc* PCC7120 (hereafter *Nostoc*) produces four Flv-s proteins in the vegetative cells (Flv1A, Flv2, Flv3A, and Flv4) and two Flv-s (Flv1B and Flv3B) specific to the heterocyte [13]. The Flv3B protein mediates light-induced $O_2$-uptake in the heterocyte, which benefits nitrogenase activity by providing a protection mechanism against oxidation [14]. In addition, the *Dflv3B* mutant displayed a broad effect on gene expression, which indicates that a regulation process links gene transcription to $O_2$ level in the heterocyte [14].

We recently reported that decreasing the $O_2$ level inside the heterocyte by producing the cyanoglobin GlbN allowed it to host an active FeFe $H_2$ase from *Clostridium acetobutylicum*. The recombinant strain displayed a significant $H_2$-production yield under phototrophic conditions [15]. These data suggest that engineering approaches increasing the anaerobiosis inside the heterocyte can be highly profitable for the activity of $O_2$-sensitive enzymes. To go further, we investigate here the impact of an overproduction of the flavodiiron Flv3B protein on the production of $H_2$ in *Nostoc*. We demonstrate that the recombinant strain produces on average 10-fold more $H_2$ than the parental strain and that the expression of the *hox* genes is induced in this genetic background.

**Results**

**Construction and characterization of a *Nostoc* recombinant strain overexpressing the *flv3B* gene**

In a transcriptomic study using an RNAseq approach, the transcription of *flv3B* (all0178) gene was induced 12 hours after nitrogen starvation [16]. In order to specifically overexpress the *flv3B* gene in the heterocytes without competing with the natural
promoter of this gene, we decided to place it under the control of a heterocyte-specific promoter whose transcription is induced at the same time than *flv3B*. For this, we analyzed the transcription of *flv3B* throughout the differentiation process by quantitative RT-PCR. We also concomitantly monitored the transcription of the *patB* gene, known to be expressed after the initiation of heterocytes development [17]. *flv3B* and *patB* genes showed very similar transcription profile (Figure 1). Both genes were induced 18 hours after nitrogen stepdown and their transcription increased through the development program (compare Figure 1A and 1B). The *patB* promoter was therefore chosen to drive *flv3B* overexpression in *Nostoc*, and the resultant recombinant strain was named WT/patB-*flv3B*. As a first step in the characterization of this strain, we checked the overexpression of *flv3B* in response to nitrogen starvation. We first carried out quantitative RT-PCR analyses and expressed the amount of *flv3B* transcripts in the recombinant strain relatively to their amount in the wild type. Results reveal a more than 10-fold increase in *flv3B* gene expression in the recombinant strain, also starting much sooner after nitrate depletion, indicating that *flv3B* gene was strongly overexpressed (Figure 1C). Because Flv3B from *Nostoc* and FlvB from *Chlamydomonas reinhardtii* amino acid sequences present 51% identity (Figure S1), we hypothesized that antibodies produced against FlvB from *C. reinhardtii* [18] could cross-react with Flv3B and hence could be used to analyze the amount of Flv3B protein in *Nostoc*. Since Flv1B from *Nostoc* displays 30% identity with FlvB from *C. reinhardtii*, the anti-FlvB antibodies could also cross-react with this protein. However, as only the *flv3B* gene was overexpressed, we assumed that FlvB antibodies could help assessing Flv3B overproduction. In the western blot analyses, the amount of RbcL protein served to check that equal amounts of proteins were loaded in each condition [19]. Data on Figure 1D show that a protein of the expected size of Flv3B (64 kDa) was detected only in BG11 medium (without nitrate), which is in agreement with
flv3B gene being specific to the heterocyte [13]. Moreover, this protein accumulated at a higher level in the WT/patB-flv3B strain. Altogether, these results indicate that the flv3B gene was overexpressed in the recombinant strain. The WT/patB-flv3B strain showed similar growth efficiency than the wild type under both nitrogen replete and deplete conditions (Figure 2A, Table 1), and both strains differentiated heterocytes equally well (Figure 2B). The frequency of heterocytes along the filament was similar between the two strains, with 12 vegetative cells on average in between two heterocytes (Figure 2C). Given that the overexpression of flv3B did not impair the growth ability of the strain, we proceeded with an analysis of its impact on H2-production.

**flv3B overexpression in the heterocyte powers H2-production**

The sensitivity of H2ases and nitrogenase to O2 is an important limitation to H2-photoproduction. By promoting O2 consumption in the heterocyte, the Flv3B protein is ought to protect enzymes evolving H2. To test this hypothesis, the wild type and the WT/patB-flv3B strains were first grown exponentially under aerobic conditions in nitrate replete medium. H2-production yield was then measured and compared after cells were transferred to nitrate-depleted medium. The recombinant strain produced 10 to 30-fold more H2 than the wild type under the same conditions (Figure 3A). H2 production increased with the experienced light irradiance, with the highest yield obtained under 60 µE.m-2. Flv3B overproduction is thus an efficient way to enhance H2 photoproduction in Nostoc.

**The presence of the uptake H2ase is required for a maximal H2 production**

Since the uptake H2ase consumes the H2 produced by the nitrogenase in the heterocyte and since its deletion enhanced H2 production [20], we investigated whether a deletion of
hupL gene, encoding the large subunit of the uptake $H_2$ase would show a cumulative effect with Flv3B overproduction. For this purpose, a deletion of hupL was constructed and the resultant strain transformed with the patB-flv3B containing plasmid. The deletion of hupL gene in an otherwise wild type background increased the $H_2$ production level, which is in agreement with data published previously [20] (Figure 3B). However, the absence of a further enhanced $H_2$ production following the overproduction of Flv3B in the DhupL strain was unexpected. Intriguingly, the DhupL/patB-flv3B strain produced 3.5-fold less $H_2$ than the WT/patB-flv3B strain (Figure 3B).

**Flv3B overproduction does not stimulate nitrogenase activity**

The deletion of the flv3B gene was shown to result in a decrease in both the amount of nitrogenase subunits and nitrogenase activity [14]. Therefore, the increased $H_2$ production in the flv3B overproducing strain could be a consequence of an increase in the activity of the nitrogenase. To test this hypothesis, we monitored nitrogenase activity in exponentially growing cultures after their transfer to a medium devoid of combined nitrogen. Results demonstrated that the overproduction of Flv3B protein did not enhance nitrogenase activity (Table 1). Therefore, the effect of Flv3B on $H_2$ production is unlikely to result from nitrogenase activity.

**Flv3B overproduction induces the expression of the bidirectional $H_2$ase encoding genes**

Since the only other enzyme able to produce $H_2$ in cyanobacteria is the bidirectional Hox $H_2$ase, we analyzed whether an induced expression of hox genes then results from the overproduction of Flv3B. The hoxH and hoxY genes encoding the $H_2$ase subunits as well as the hoxE,F,U genes encoding the diaphorase subunits belong to two separate operons [21]. To evaluate the expression of these operons, the transcription of two genes from
each operon (hoxH,Y and hoxE,F) was comparatively monitored in the wild type and the recombinant strains. Quantitative RT-PCR analysis was used to evaluate the transcription of these four genes after transfer of the strains into nitrogen deplete conditions to induce flv3B expression. The transcription of the four hox genes was weak in the wild type strain (Figure 4 A,B; Figure 5 A,B), which is in agreement with the fact that the hox genes are not expressed under aerobic conditions [21]. However, in the WT/patB-flv3B strain, 18 hours after nitrogen step down, the hoxE,F, H and Y transcripts level were on average 10-fold higher than in the wild type (Figure 4 C,D and Figure 5 C,D). The expression of the two hox operons encoding the H$_2$ase and diaphorase proteins is therefore induced in the strain overexpressing the flv3B gene under the heterocyte specific promoter patB. Consequently, the effect of flv3B overexpression on H$_2$ production may be mediated by the induction of hox genes.

Discussion

In this work we show that overexpression of flv3B gene from a promoter specific to the heterocyte enhanced the production of H$_2$ in aerobic cultures of Nostoc. So far, the only conditions in which H$_2$-evolution had been recorded in aerobically grown Nostoc were the use of mutants lacking the HupL subunit of the uptake H$_2$ase or the last step of the maturation system of this H$_2$ase [20] [22]. H$_2$ evolution mediated by Flv3B overproduction presents the advantage of sustaining the protective effect of the uptake H$_2$ase on the nitrogenase. By studying the phenotype of a Dflv3B mutant of Nostoc, Ermakova et. al [14] showed that Flv3B protected nitrogenase through light-induced O$_2$ consumption inside the heterocytes. The effect of Flv3B overproduction evidenced in our work could therefore result from a stimulated nitrogenase activity. But the recombinant strain displayed similar nitrogenase
activity as the wild type (Table 1), proof that another mechanism operates to enhance H₂ production.

In *C. reinhardtii*, the existence of intracellular microoxic niches in the chloroplast preserve FeFe-hydrogenase activity and support continuous H₂ production during growth in aerobic cultures [23]. The same authors suggested that Flv3 proteins were involved in this process [23]. A similar mechanism may be proposed to explain the effect of the Flv3B protein overproduction on H₂ evolution, in which the decrease in O₂ concentration in the heterocyte would reinforce the anaerobiosis in this cell type, thus promoting H₂ase synthesis and/or activity. We studied the transcription of hox genes encoding the bidirectional H₂ase as their induction is known to be concomitant to high H₂ase activity [21]. Data in Figures 4 and 5 indicate that flv3B overproduction led to a substantial induction of hoxE,F,H,Y genes expression that can explain the H₂ production measured in this strain. The LexA transcriptional factor was proposed to regulate hox genes transcription in *Nostoc* [21]. In the unicellular cyanobacterium *Synechocystis* PCC6803, LexA was suggested to act as a transducer of the intracellular redox state, rather than of the SOS response as in *E. coli* [24]. Based on this information, we suggest that an increased O₂-uptake driven by Flv3B overproduction can modify the redox state in the heterocytes, resulting in the observed induction of hox genes transcription.

Surprisingly, and contrary to what happens in the wild type background, the lack of the uptake H₂ase in the WT/patB/flv3B strain led to a decrease in H₂ production (Figure 3B). As the H₂ases are bidirectional enzymes, a possible interpretation of this result is that the Hup enzyme is responsible of the H₂ production observed in this recombinant strain.

However, this is rather unlikely since it was demonstrated that the Hup H₂ase is not able
to produce H$_2$ at any significant rate, and is considered to react only in the uptake direction [1, 25]. Through the oxidation of H$_2$, the Hup H$_2$ase provides electrons to the photosynthesis and respiratory processes [1] (Figure 6). Since the Hox H$_2$ase was suggested to use ferredoxin as reducing partner rather than NAD(P)H as previously admitted (reviewed in [9]), this enzyme may benefit from the electrons generated by the Hup H$_2$ase through regeneration of the reduced ferredoxin pool (Figure 6). This could explain the negative impact of the hupL deletion on the H$_2$-production yield in the WT/patB-flv3B strain (Figure 6). Our data show that metabolic engineering approaches are particularly relevant in the use of photosynthetic bacteria for biofuel production.

**Conclusion**

In the present study, the flv3B gene was specifically overexpressed in the heterocyte of Nostoc under the control of the patB promoter. The overproduction of the Flv3B flavodiiron enhanced the H$_2$ production yield by a factor of ten on average, which is not to be attributed to the nitrogenase since no increase in the nitrogenase activity was observed. The transcription of the hox genes was induced in the recombinant strain expressing the flv3B gene, suggesting that the additional H$_2$ produced relates to the activity of the bidirectional H$_2$ase. Facilitating the consumption of O$_2$ inside the heterocyte thus appears as a relevant step towards the design of an optimized Nostoc strain for H$_2$ production. This paves the way to further improvement to achieve sustainable production of H$_2$ by air-grown cyanobacteria.

**Methods**

**Growth conditions and heterocytes induction**

Cyanobacterial strains were grown in BG11 medium (nitrate replete) at 30 °C under
continuous illumination (30 µE m⁻² s⁻¹). Cultures of recombinant strains were supplemented with neomycin (50 µg mL⁻¹). Heterocyte formation was induced by transferring the exponentially growing cultures (OD 750 = 0.8) to BG11₀ (BG11 devoid of sodium nitrate) by filtration (0.2 µm pore size filters, Sigma) and resuspension of cells into the nitrate-free medium. The growth was maintained for 4 days. The presence of heterocytes was confirmed by light microscopy and their distribution within filaments was rated visually by counting the number of vegetative cells between two heterocytes. At least 400 total vegetative cells were counted for each strain.

In the H₂ production experiments, the strains were grown under continuous illumination of 20 µE m⁻² s⁻¹ or 60 µE m⁻² s⁻¹.

**Construction of plasmids and strains**

To construct the Flv3B overproducing strain, the promoter region of patB (all2512, 500 bp upstream the start codon) was amplified by PCR from *Nostoc* sp. PCC 7120 genomic DNA using the *ppatB* forward and *ppatB* reverse primers (Table 3). The *ppatB* reverse primer contained a multiple cloning site (ApaI, Clal, BamHI, Sall, Scal, EcoRI). The amplified promoter was cloned into BglII and EcoRI restriction sites of the pRL25T plasmid [26], yielding the pRL25T-*patB* plasmid. The open reading frame of *flv3B* gene was amplified using the *flv3B* forward and reverse primers (Table 3), and cloned into the ApaI and Scal restriction sites of the pRL*patB*. The recombinant plasmid (pRL25T-*patB-flv3B*) was analyzed by sequencing (Millegen). Conjugation of *Nostoc* was performed as described in reference [27]. Briefly, *E. coli* strains (bearing the replicative pRL25T-*patB-flv3B* and the RP-4 conjugative plasmid) grown to exponential growth phase, were mixed to an exponentially grown *Nostoc* culture. The mixture was plated on BG11 plates and Neomycin was added 24 hours later for plasmid selection. Plasmid extraction was used to analyze
the obtained recombinant clones.

Deletion of the *hupL* gene, yielding the *DhetL* strain, was obtained by homologous recombination replacing the *hupL3*’ gene (all0687C) with the gene encoding the spectinomycin/streptomycin resistance (Sp/Sm cassette hereafter). For this purpose, the upstream and downstream 1500 bp flanking the *hupL3*’ gene were amplified from *Nostoc* genomic DNA using the all0678 forward/ all0678 reverse and the Strp-all0678 forward/ Strp-all0678 forward, respectively; The Sp/Sm cassette was amplified using the Strp forward/Strp reverse primers (Table 3), using the pBAD42 plasmid (Addgen) as template. Gibson’s assembly technique (New-England Biolabs) was applied to insert the three resulting fragments into the suicide pRL271 vector linearized by SpeI. The resulting recombinant plasmid was conjugated into *Nostoc* as described above. The initial conjugants were selected by screening for resistance to 5 μg/mL of Sm, and the resulting cells were then grown on BG11 plates containing 5% sucrose to select double recombinants. Genomic DNA of the recombinant cells were analyzed by PCR.

The strains and plasmids used in this study are listed in Table 2.

**RNA Preparation and Reverse Transcription**

RNAs were prepared using the Qiagen RNA extraction kit (Qiagen) following the manufacturer instructions. An extra TURBO DNase (Invitrogen) digestion step was undergone to eliminate the contaminating DNA. The RNA quality was assessed by tape station system (Agilent). RNAs were quantified spectrophotometrically at 260 nm (NanoDrop 1000; Thermo Fisher Scientific). For cDNA synthesis, 1 μg total RNA and 0.5 μg random primers (Promega) were used with the GoScript™ Reverse transcriptase (Promega) according to the manufacturer instructions.

**Quantitative Real-Time-PCR for Transcriptional Analyses**
Quantitative real-time PCR (qPCR) analyses were performed on a CFX96 Real-Time System (Bio-Rad). The reaction volume was 15 μL and the final concentration of each primer was 0.5 μM. The qPCR cycling parameters were 95°C for 2 min, followed by 45 cycles of 95°C for 5 s, 55°C for 60 s. A final melting curve from 65°C to 95°C was added to determine the specificity of the amplification. To determine the amplification kinetics of each product, the fluorescence derived from the incorporation of BRYT Green® Dye into the double-stranded PCR products was measured at the end of each cycle using the GoTaq® qPCR Master Mix 2X Kit (Promega). The results were analysed using Bio-Rad CFX Maestro software, version 1.1 (Bio-Rad, France). The \( rnpB \) gene was used as a reference for normalization. A technical duplicate was performed for each point. The amplification efficiencies of each primer pairs were 80 to 100%. All of the primer pairs used for qPCR are reported in Table 3.

**Western blot analysis**

Proteins (75 μg) extracted from cyanobacterial strains were fractionated by performing SDS-PAGE 12%, and transferred to nitrocellulose membranes before being revealed with specific polyclonal antibodies. Immune complexes were detected with anti-rabbit peroxidase-conjugated secondary antibodies (Promega) and enhanced chemoluminescence reagents (Pierce). Anti-FlvB antibodies, developed against the FlvB protein of \( C. reinhardtii \) [18], were used at a 1: 1000 dilution. Anti-Rbcl antibodies (Agrisera) were used a 1: 5000 dilution.

**H\(_2\) production assays**

*Nostoc* wild type strain and its derivatives were grown as described above for heterocyte induction. Chlorophyll \( a \) concentration was quantified according to the following method: 1 mL of culture was centrifuged (5 min, 6700 g, 4°C), the pellet was resuspended in 1 mL of
cold methanol and incubated at 4°C for 30 minutes under shaking. Cells were then harvested (5 min, 6700 g, 4°C) and absorbance of the supernatant was measured at 665 nm and 720 nm. The chlorophyll a concentration was calculated according to the formula: [Chl a] = 12,9447 (A_{665}-A_{720}) and expressed in µg of Chla/mL of culture [28]. A 40-mL volume of cell culture was then harvested (5 min, 6700 g, 4°C) and cells were resuspended in sterile nitrate-depleted medium yielding a concentration of 10 µg Chl a mL^{-1}. 12 mL of this cell suspension were transferred to Hungate tubes (leaving a 4.4-mL head space volume). The vials were sparged with Ar (Ar), and the samples were maintained under illumination (20 or 60 µmol photons m^{-2} s^{-1}) for 96 hours. 100 µL of headspace gas was removed every 12 hours using a gastight syringe and injected into a gas chromatography system (Agilent 7820) equipped with a thermal conductivity detector and a HP-plot Molesieve capillary column (30 m, 0.53 mm, 25 µm), using argon as the carrier gas, at a flow rate of 4.2 mL/min, an oven temperature of 30 °C and a detector temperature of 150 °C. H₂ was quantified according to a standard calibration curve. H₂ production rate was expressed as mol of H₂ produced per mg of Chlorophyll.

**Nitrogenase activity**

An on-line acetylene reduction assay [29] was used to measure nitrogenase activity. Briefly, cyanobacterial strains were grown in batch cultures under light/dark cycles of 12 hours/12 hours. Nitrogenase activity was monitored for 20 hours. Before the onset of nitrogenase activity, *Nostoc* cultures were transferred to a GF/F filter (Whatman, 47 mm) and placed in a custom-made, light and temperature-controlled gas flow-through incubator connected to the gas chromatograph. Acetylene represented 10% of the gas mixture and the total gas flow rate was 1 l h^{-1}. Ethylene production was measured every 10 min by gas chromatography using an Agilent 7890 equipped with an auto-injector and a
photoionization detector.

References

1. Houchins JP, Burris RH: Light and dark reactions of the uptake hydrogenase in *anabaena 7120*. *Plant Physiol* 1981, 68:712-716.

2. Puggioni V, Tempel S, Latifi A: Distribution of Hydrogenases in Cyanobacteria: A Phylum-Wide Genomic Survey. *Front Genet* 2016, 7:223.

3. Tamagnini P, Leitao E, Oliveira P, Ferreira D, Pinto F, Harris DJ, Heidorn T, Lindblad P: Cyanobacterial hydrogenases: diversity, regulation and applications.*FEMS Microbiol Rev* 2007, 31:692-720.

4. Cournac L, Guedeney G, Peltier G, Vignais PM: Sustained photoevolution of molecular hydrogen in a mutant of *Synechocystis* sp. strain PCC 6803 deficient in the type I NADPH-dehydrogenase complex. *J Bacteriol* 2004, 186:1737-1746.

5. Kiss E, Kos PB, Vass I: Transcriptional regulation of the bidirectional hydrogenase in the cyanobacterium *Synechocystis 6803*. *J Biotechnol* 2009, 142:31-37.

6. Dutheil J, Saenkham P, Sakr S, Leplat C, Ortega-Ramos M, Bottin H, Cournac L, Cassier-Chauvat C, Chauvat F: The AbrB2 autorepressor, expressed from an atypical promoter, represses the hydrogenase operon to regulate hydrogen production in *Synechocystis* strain PCC6803. *J Bacteriol* 2012, 194:5423-5433.

7. Gutekunst K, Phunpruch S, Schwarz C, Schuchardt S, Schulz-Friedrich R, Appel J: LexA regulates the bidirectional hydrogenase in the cyanobacterium *Synechocystis* sp. PCC 6803 as a transcription activator. *Mol Microbiol* 2005, 58:810-823.

8. Oliveira P, Lindblad P: LexA, a transcription regulator binding in the promoter
region of the bidirectional hydrogenase in the cyanobacterium *Synechocystis* sp. PCC 6803. *FEBS Microbiol Lett* 2005, **251**:59-66.

9. Khanna N, Lindblad P: *Cyanobacterial hydrogenases and hydrogen metabolism revisited: recent progress and future prospects.* *Int J Mol Sci* 2015, **16**:10537-10561.

10. Allahverdiyeva Y, Suorsa M, Tikkanen M, Aro EM: *Photoprotection of photosystems in fluctuating light intensities.* *J Exp Bot* 2015, **66**:2427-2436.

11. Helman Y, Tchernov D, Reinhold L, Shibata M, Ogawa T, Schwarz R, Ohad I, Kaplan A: *Genes encoding A-type flavoproteins are essential for photoreduction of O₂ in cyanobacteria.* *Curr Biol* 2003, **13**:230-235.

12. Allahverdiyeva Y, Mustila H, Ermakova M, Bersanini L, Richaud P, Ajlani G, Battchikova N, Cournac L, Aro EM: *Flavodiiron proteins Flv1 and Flv3 enable cyanobacterial growth and photosynthesis under fluctuating light.* *Proc Natl Acad Sci U S A* 2013, **110**:4111-4116.

13. Ermakova M, Battchikova N, Allahverdiyeva Y, Aro EM: *Novel heterocyst-specific flavodiiron proteins in Anabaena sp. PCC 7120.* *FEBS Lett* 2013, **587**:82-87.

14. Ermakova M, Battchikova N, Richaud P, Leino H, Kosourov S, Isojarvi J, Peltier G, Flores E, Cournac L, Allahverdiyeva Y, Aro EM: *Heterocyst-specific flavodiiron protein Flv3B enables oxic diazotrophic growth of the filamentous cyanobacterium Anabaena sp. PCC 7120.* *Proc Natl Acad Sci U S A* 2014, **111**:11205-11210.

15. Avilan L, Roumezi B, Risoul V, Bernard CS, Kpebe A, Belhadjhassine M, Rousset M, Brugna M, Latifi A: *Phototrophic hydrogen production from a clostridial [FeFe] hydrogenase expressed in the heterocysts of the cyanobacterium Nostoc PCC 7120.* *Appl Microbiol Biotechnol* 2018, **102**:5775-5783.
16. Mitschke J, Vioque A, Haas F, Hess WR, Muro-Pastor AM: Dynamics of transcriptional start site selection during nitrogen stress-induced cell differentiation in *Anabaena* sp. PCC7120. *Proc Natl Acad Sci U S A* 2011, **108**:20130-20135.

17. Jones KM, Buikema WJ, Haselkorn R: Heterocyte-specific expression of *patB*, a gene required for nitrogen fixation in *Anabaena* sp. strain PCC 7120. *J Bacteriol* 2003, **185**:2306-2314.

18. Chaux F, Burlacot A, Mekhalfi M, Auroy P, Blangy S, Richaud P, Peltier G: Flavodiiron Proteins Promote Fast and Transient O$_2$ Photoreduction in *Chlamydomonas*. *Plant Physiol* 2017, **174**:1825-1836.

19. Nierzwicki-Bauer SA, Curtis SE, Haselkorn R: Cotranscription of genes encoding the small and large subunits of ribulose-1,5-bisphosphate carboxylase in the cyanobacterium *Anabaena* 7120. *Proc Natl Acad Sci U S A* 1984, **81**:5961-5965.

20. Masukawa H, Mochimaru M, Sakurai H: Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120. *Appl Microbiol Biotechnol* 2002, **58**:618-624.

21. Sjoholm J, Oliveira P, Lindblad P: Transcription and regulation of the bidirectional hydrogenase in the cyanobacterium *Nostoc* sp. strain PCC 7120. *Appl Environ Microbiol* 2007, **73**:5435-5446.

22. Nyberg M, Heidorn T, Lindblad P: Hydrogen production by the engineered cyanobacterial strain *Nostoc* PCC 7120 DeltahupW examined in a flat panel photobioreactor system. *J Biotechnol* 2015, **215**:35-43.

23. Liran O, Semyatich R, Milrad Y, Eilenberg H, Weiner I, Yacoby I: Microoxic Niches within the Thylakoid Stroma of Air-Grown *Chlamydomonas reinhardtii* Protect
[FeFe]-Hydrogenase and Support Hydrogen Production under Fully Aerobic Environment. *Plant Physiol* 2016, **172**:264-271.

24. Patterson-Fortin LM, Colvin KR, Owttrim GW: A LexA-related protein regulates redox-sensitive expression of the cyanobacterial RNA helicase, crhR. *Nucleic Acids Res* 2006, **34**:3446-3454.

25. Houchins JP, Burris RH: Comparative characterization of two distinct hydrogenases from *Anabaena* sp. strain 7120. *J Bacteriol* 1981, **146**:215-221.

26. Yang Y, Huang XZ, Wang L, Risoul V, Zhang CC, Chen WL: Phenotypic variation caused by variation in the relative copy number of pDU1-based plasmids expressing the GAF domain of Pkn41 or Pkn42 in *Anabaena* sp. PCC 7120. *Res Microbiol* 2013, **164**:127-135.

27. Cai YP, Wolk CP: Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. *J Bacteriol* 1990, **172**:3138-3145.

28. Ritchie RJ: Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. *Photosynth Res* 2006, **89**:27-41.

29. Dron A, Rabouille S, Claquin P, Talec A, Raimbault V, Sciandra A: Photoperiod length paces the temporal orchestration of cell cycle and carbon-nitrogen metabolism in *Crocosphaera watsonii*. *Environ Microbiol* 2013, **15**:3292-3304.

30. Wolk CP, Cai Y, Cardemil L, Flores E, Hohn B, Murry M, Schmetterer G, Schrautemeier B, Wilson R: Isolation and complementation of mutants of *Anabaena* sp. strain PCC 7120 unable to grow aerobically on dinitrogen. *J Bacteriol* 1988, **170**:1239-1244.

Tables

Table 1
| Measure                                      | Wild type | WT/patB-flv3B |
|---------------------------------------------|-----------|---------------|
| Exponential growth rate per day (BG110)     | 0.155     | 0.155         |
| Chl a content (mg Chl a/mL)                 | 4.49      | 8.9           |
| Nitrogenase activity (nmol N₂ /mg Chl a /h) | 17.3      | 11.2          |
| sd on nitrogenase activity                  | 0.001     | 0.00025       |

Two independent cultures of each strain were grown as explained in the Material and Methods section. For each strain, the nitrogenase activity values presented in this table were registered at T=4 hours of the light phase. Chl a: chlorophyll a; sd: standard deviation

Table 2: List of the bacterial strains and the plasmids used in this study.

| Strain name         | Description/ Antibiotic resistance                                      | Origin                  |
|---------------------|------------------------------------------------------------------------|-------------------------|
| Wild type           | *Nostoc/Anabaena PCC 7120 wild type strain**                           | Pasteur Cyanobacterial Collection |
| WT/patB-flv3B       | *Nostoc containing the pRL25T-patB-flv3B plasmid/ (NeoR)*             | This study              |
| ΔhupL               | *Nostoc deletion mutant of the hupL gene (Sp/SmR)*                      | This study              |
| ΔhupL/patB-flv3B    | ΔhupL mutant containing the pRL25T-patB-flv3B / (Sp/SmR and NeoR)*     | This study              |

| Plasmid name        | Description/ Antibiotic resistance                                      | Origin                  |
|---------------------|------------------------------------------------------------------------|-------------------------|
| pRL25T              | Replication vector derived from the pRL25C cosmid (NeoR)               | [26], [30]              |
| pRL25T-patB-flv3B   | pRL25T harboring the flv3B gene under the control of the patB promoter (NeoR) | This study              |

Table 3: sequence of the primers used in this study
| Name     | Sequence (5'->3') | Experiment               |
|----------|-------------------|--------------------------|
| rnpB forward | TCGTGAGGATAGTGCCACAG | Quantitative RT-PCR analysis |
| rnpB reverse | GGAAGTTTCTTCCCCAGTCC |                          |
| flv3B RT forward | TTTGGTTGAAGATGTGCTGC |                          |
| flv3B RT reverse | GCCAATGTAAGTTAGGCGCA |                          |
| patB forward | AGGGGCATGTAAGTGGAA |                          |
| patB reverse | TTGACTGCTCGACTGTAAGCA |                          |
| hoxE forward | GCGTCACCAGTATCGAAG |                          |
| hoxE reverse | TGGGGCGCTAGGGAAAATAA |                          |
| hoxF forward | ACCCGGCTGAATCTGGTTTA |                          |
| hoxF reverse |                          |                          |
| hoxH forward |                          |                          |
| hoxH reverse |                          |                          |
| hoxY forward |                          |                          |
| hoxY reverse |                          |                          |
| ppatB forward | TATAAGATCTGCTTTATAATACATAGTGTGGG | Cloning of patB promoter |
| ppatB reverse | TATAGAATTCCGAGTCGTCGACCCGGGATCCATCGATGGGCCAATCAGCA |                          |
| flv3B forward | TAT CCCGGG ATG GTA TCG ATG TCT ACG ACC |                          |
| flv3B reverse | TAT AGTACT TTA GTA ATA GTT GCC TAC TTT TCG |                          |
| Primer Set       | Forward Sequence                             | Reverse Sequence                             | Construction of the huf mutant                  |
|------------------|----------------------------------------------|----------------------------------------------|------------------------------------------------|
| Strp forward     | AATTCCCCTGCTCGCGCAGG                         | TACCATGTTAGCTAAAGCGCCCTCGT                   |                                                 |
| Strp reverse     | AGCTTAGTAAAGCCCTCGT                         |                                              |                                                 |
| all0678 forward  | TACCATGTTAGCTAAAGCGCCCTCGT                   | AGCTTAGTAAAGCGCCCTCGT                       |                                                 |
| all0678 reverse  | AGCTTAGTAAAGCGCCCTCGT                       | TACCATGTTAGCTAAAGCGCCCTCGT                   |                                                 |
| Strp-all0678 forward | TACCATGTTAGCTAAAGCGCCCTCGT                   | AGCTTAGTAAAGCGCCCTCGT                       |                                                 |
| Strp-all0678 reverse | AGCTTAGTAAAGCGCCCTCGT                       | TACCATGTTAGCTAAAGCGCCCTCGT                   |                                                 |

Figures

![flv3B transcripts level (WT)](image)

1. **flv3B transcripts level (WT)**
2. **patB transcripts level (WT)**
3. **flv3B transcripts level (WT/patB-flv3B)**
4. **WT** + Nitrate, - Nitrate  WT/patB-flv3B + Nitrate, - Nitrate
   - Anti-FlvB
   - -64 kDa
   - Anti-RbcL
   - -52 kDa
Flv3B overproduction analysis A, B, C: Quantitative RT-PCR analysis of flv3B (A, C) and patB (B, D) gene transcription. RNA were collected from the wild type (A, B) or the WT/patB-flv3B (C) strain at four different times (7, 18, 24 and 48 hours) after the onset of nitrogen depletion. Each sample was measured in triplicate and the standard deviation is indicated by error bars. Values were normalized to the rnpB transcript, relatively to the value obtained for the wild type strain, which was set to 1. D: Immunoblot analysis of the amount of Flv3B protein (upper panel) in the wild type and WT/patB-flv3B strains, carried out using antibodies produced against FlvB from Chlamydomonas reinhardtii [18]. Immunoanalysis of RbcL protein amount was carried out as a loading control (lower panel). The condition (+ Nitrate) stands for cultures performed in nitrate-containing medium, and the condition (- Nitrate) indicates cultures grown in nitrate-free medium.
Characterization of Nostoc strain overexpressing the flv3B gene

A: Growth curve of Nostoc strains grown in either nitrate-containing medium or nitrate free
medium. For each curve, three independent cultures were performed. The growth was assessed during twelve days by measuring the optical density at 750 nm. The standard deviation is indicated by error bars. B: Light microscope images of Nostoc strains grown in nitrate-containing medium or nitrate-free medium. For the last conditions, images were acquired 24 hours after nitrogen starvation. Heterocytes are indicated by black arrows. C: Heterocyte pattern formation in the wild type and the WT/patB-flv3B strain. Strains were grown in BG11 (nitrate-containing medium) to an OD750 of 0.4 and induced to form heterocytes by transfer to BG-110 medium (nitrate-free medium). Vegetative cells and heterocytes were scored microscopically 24 hours after nitrogen starvation. The data shown are representative of three independent experiments.
**Figure 3**

H2 production kinetics A: wild type or WT/patB-fvl3B were grown in nitrate-containing medium until OD 750nm = 0.8. Heterocyte formation was induced by transferring the strains to a nitrate-free medium during 24 hours. The strains were then incubated under light intensities of either 20μE/m2 or 60μE/m2, and H2 production was assessed by chromatography as explained in the methods section during four days. The values represent Means ±SEM (n=8). B: wild type, WT/patB-fvl3B, ΔhupL or ΔhupL/patB-fvl3B strains were grown under light intensities of 60μE/m2. Heterocyte formation and H2-production were respectively induced and performed as described above. The values represent Means ±SEM (n=8).
Figure 4

hoxY, H genes transcription analysis Quantitative RT-PCR analysis of hoxY and
hoxH gene transcription. RNA were collected form wild type (A, B) or WT/patB-fvl3B (C, D) at different times after the onset of the nitrogen depletion step. Each sample was measured in triplicate and the standard deviation is indicated by error bars. Values were normalized to the rnpB transcript.
Figure 5

hoxE, F genes transcription analysis. Quantitative RT-PCR analysis of hoxE and hoxF gene transcription. RNA were collected from wild type (A, B) or WT/patB-fvl3B (C, D) at different times after the onset of the nitrogen depletion step. Each sample was measured in triplicate and the standard deviation is indicated by error bars. Values were normalized to the rnpB transcript.
Figure 6

Hypothetical model of H2 production in Nostoc strain overproducing Flv3B

Nitrogen fixation occurring in the heterocyte produces H2 which is recycled by the Hup H2ase. Overexpression of the flv3B gene increases the uptake of O2 reinforcing the microoxie inside the heterocyte. The induction of hox genes transcription leads to H2 production. Fdred: reduced ferredoxin; Fdox: oxidized ferredoxin. Dashed lines stand for indirect effect.

Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

Fig S1.jpg