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Phototrophic hydrogen production from a clostridial [FeFe] hydrogenase expressed in the heterocysts of the cyanobacterium *Nostoc* PCC 7120

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Abstract

The conversion of solar energy into hydrogen represents a highly attractive strategy for the production of renewable energies. Photosynthetic microorganisms have the ability to produce H_2 from sunlight but several obstacles must be overcome before obtaining a sustainable and efficient H_2 production system. Cyanobacteria harbor [NiFe] hydrogenases required for the consumption of H_2. As a result, their H_2 production rates are low, which makes them not suitable for a high yield production. On the other hand, [FeFe] enzymes originating from anaerobic organisms such as *Clostridium* exhibit much higher H_2 production activities, but their sensitivity to O_2 inhibition impairs their use in photosynthetic organisms. To reach such a goal, it is therefore important to protect the hydrogenase from O_2. The diazotrophic filamentous cyanobacteria protect their nitrogenases from O_2 by differentiating micro-oxic cells called heterocysts. Producing [FeFe] hydrogenase in the heterocyst is an attractive strategy to take advantage of their potential in a photosynthetic microorganism. Here, we present a biological engineering approach for producing an active [FeFe] hydrogenase (HydA) from *Clostridium acetobutylicum* in the heterocysts of the filamentous cyanobacterium *Nostoc* PCC7120. To further decrease the O_2 amount inside the heterocyst, the GlbN cyanoglobin from *Nostoc commune* was coproduced with HydA in the heterocyst. The engineered strain produced 400 μmol-H_2 per mg Chlorophyll *a*, which represents 20-fold the amount produced by the wild type strain. This result is a clear demonstration that it is possible to associate oxygenic photosynthesis with H_2 production by an O_2-sensitive hydrogenase.

Keywords *Clostridium acetobutylicum* · Cyanobacteria · Cyanoglobin · Heterocyst · Hydrogen · *Nostoc*

Introduction

The world's energy requirements are increasing continuously, while the use of fossil fuels is responsible for major climate changes. It has therefore become necessary to develop renewable non-polluting energy sources to meet both the short- and long-term needs. H_2 constitutes a good environmentally friendly biofuel because it has a high energy-to-weight ratio and because water is the only by-product released during its combustion. However, hydrogen is mostly produced by steam reforming of methane, a very efficient process, but which produces one molecule of CO_2 for three molecules of H_2 (Fonseca and Assaf 2004). The use of oxygenic photoautotrophs (cyanobacteria and microalgae) to obtain H_2 seems to constitute the most promising biofuel production strategy, since the energy required for this process is provided by solar light. However, several limitations have to be overcome before photosynthetic microorganisms can be used to develop robust H_2 production processes. The main obstacles here are the poor efficiency of the enzymes catalyzing H_2 production under phototrophic conditions and their sensitivity to O_2 inhibition (Gutekunst et al. 2014; Hemschemeier et al. 2008).

Nitrogenases and hydrogenases (H_2ases) are the two main classes of enzymes that catalyze the production of

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H_2 in cyanobacteria. H_2ases are metalloproteins able to catalyze the reversible reduction of H^+ into H_2 without ATP (for a recent review, see Peters et al. 2015). These latter enzymes are usually subdivided into three independent phylogenetic classes: [Fe] H_2ases, [FeFe] H_2ases, and [NiFe] H_2ases. All the cyanobacterial H_2ases are [NiFe] enzymes, which have been subdivided into two groups: the bidirectional [NiFe] H_2ases (Hox), which are able to produce or oxidize H_2, and the uptake H_2ases, which consume the H_2 produced by nitrogenases to limit the loss of energy (Houchins and Burris 1981; Sellstedt and Lindblad 1990; Tamagnini et al. 2007). On the other hand, nitrogenases produce H_2 as a by-product during the nitrogen reduction process. They have a relatively low turnover and require large amounts of ATP (Noar et al. 2015).

Many heterotrophic anaerobic microorganisms such as *Clostridium* harbor [FeFe] H_2ases involved in the production of large amount of H_2 to release the reducing power from reduced cofactors during fermentation (Chen et al. 2001). [FeFe] H_2ases from anaerobic bacteria are the most efficient H_2 producing enzymes known to exist so far; they are therefore thought to be the most promising catalysts for H_2 production (Vignais and Billoud 2007). The maturation process of these enzymes has been found to involve only three proteins, HydE, HydF, and HydG (King et al. 2006; Posewitz et al. 2004), making possible the heterologous production of an active enzyme. However, the appealing perspective of using [FeFe] H_2ases in phototrophic organisms is unfortunately not easily feasible because of the fast and irreversible inactivation of these enzymes in the presence of low amount of O_2. The challenge of using cyanobacterial strains to produce [FeFe] H_2ases consists in finding means to separate the concomitant productions of O_2 and H_2.

To solve this problem, the nitrogen fixation process in filamentous cyanobacteria might provide inspiration, because nitrogenases are also sensitive to O_2 inactivation. As a result, diazotrophic cyanobacteria separate the processes of photosynthesis and nitrogen fixation either in time or in space. The filamentous strains differentiate heterocysts, which are specialized micro-oxic cells that fix N_2 whenever combined nitrogen sources become limited (for a recent review, see Muro-Pastor and Hess 2012). The nitrogenase is specifically produced in the heterocysts, where it is reduced by electrons originating from ferredoxin (Elhai and Wolk 1990; Lockau et al. 1978). Hijacking the heterocyst to produce [FeFe] H_2ase is an attractive strategy which has been applied with success in the case of the [FeFe] enzyme of the facultative anaerobe bacterium *Shewanella oneidensis* (Gärtner et al. 2012), and which is appealing for HydA enzyme of *Clostridium acetobutylicum*.

In this study, the *hydA* and *hydEFG* genes encoding [FeFe] H_2ase and the proteins involved in its maturation, respectively, were obtained from the anaerobic bacterium *Clostridium acetobutylicum* and expressed in the heterocysts of the filamentous diazotrophic cyanobacterium *Nostoc* PCC 7120 (which will be called *Nostoc* from now on). To further decrease the O_2 concentration inside the heterocysts, an O_2-scavenger, the *glbN* gene encoding the cyanoglobin of *N. commune* (Potts et al. 1992) was cloned in *Nostoc* in addition to the *hydAEFG* operon. The recombinant strain was able to produce significant levels of H_2. The results obtained here show that this engineered *Nostoc* strain is able to successfully synthesize an active [FeFe] H_2ase and to produce H_2 under phototrophic conditions.

Methods and materials

Growth conditions

Nostoc sp. PCC 7120 and its derivatives were grown in BG11 medium at 30 °C in air under continuous illumination (40 μE m^{-2} s^{-1}). Cultures of recombinant strains were supplemented with neomycin (50 μg ml^{-1}). Heterocyst formation was induced by transferring the cultures (OD 750 = 0.8) to BG110 (BG11 without sodium nitrate) by filtering them. The growth was maintained for 4 days. The presence of heterocysts was confirmed by microscopy.

Construction of the recombinant vectors

The synthesis of the *hydAEFG* synthetic operon was performed by Genecust (http://www.genecust.com/fr). After the process of synthesis, the whole operon was sequenced to check that no mutations were present. The pRL25T plasmid was used to clone the operon. The promoter regions of *patB* (500 bp) and *nifH* (700 bp) were amplified by PCR from *Nostoc* genomic DNA and cloned into the BglII and *Eco*RI restriction sites of the pRL25T plasmid. The lower primers contained a multicloning site (MCS). In the second step, the *hydAEFG* synthetic operon was cloned in the resulting plasmids into the *Apa*I and *Sca*I restriction sites of the MCS. The promoter of NsiR (71 bp) (Muro-Pastor 2014) was synthesized (by Genecust). The GeneBank accession number of the synthetic operon is: BankIt2082731 Synthetic MG870198.

The *glbN* gene from *Nostoc commune* (GeneBank accession number: M92437.1) was synthesized by Gencust and fused to the promoter of the *patB* gene by sequence and ligation independent cloning (Jeong et al. 2012). The amplified product was cloned into the *Kpn*I and *Bam*HI sites of the pRL25T plasmid harboring the *nifH-hydAEFG* operon.

All the PCR primers used in this study are listed in Table 1.

Table 1 List of the primers used in this study

Name	Sequence (5′-3′)	Experiment
hydA RT fw	GCATGTAATTGTGGCAATGG	Semiquantitative RT-PCR analysis
hydA RT rev	ATTTGTTGTGGGCTTTTTGC	
*hydE*RT fw	AAACGTTACCGTCTGGAACC	
*hydE*RT rev	TGGCAAACCAATAAACAA	
*hydF*RT fw	CAAAGCAATGGAACTGCTA	
*hydF*RT rev	TCATTTTCTGGTGCCAAGC	
*hydG*RT fw	AATGACGCTGGTATTGGAACC	
*hydG*RT rev	AACGGCAATTCGAATAAGG	
rnpB RT fw	AGAAGGGCTGAGACGAAACA	
rnpB RT rev	ACCACCGCCCTCTTACTAAC	
pnifH fw	TATAAGATCTGGTGCATTAGTTTGTACTTG	Cloning of the *nifH* promoter
pnifH rev	TATAGAATTCGAGCTCGTCGACCCGGGAT CCATCGATGGGCCCCATTGTTCTCTTTTCCTGCAATTG	
ppatB fw	TATAAGATCTGTCTTTAAATATACATGGTTTGGG	Cloning of the *patB* promoter
ppatB rev	TATAGAATTCGAGCTCGTCGACCCGGGATCCAT CGATGGGCCCCATATAACTTTCTTCCCACCC	
glbN fw	CAAACGCATTGCCACAGACA	Quantitative RT-PCR analysis
glbN rev	ATCTAGCGCAGCTTTGGTGT	

H_2 production assays

Cells were grown as described for heterocyst induction, under light. A 40-ml volume of cell culture was harvested and concentrated about threefold, yielding 10 μg Chl*a* ml^{-1} (μg chlorophyll/ml of culture). Concentrated cultures (12 ml) were transferred to Hungate tubes (leaving a 4.4-ml head space volume). The vials were sparged and filled with Argon (Ar), and the samples were maintained under illumination (60 μmol photons m^{-2} s^{-1}). When indicated, 12 μl of 2 mM DCMU was added to each vial. One hundred microliters of headspace gas was removed periodically using a gastight syringe and injected into a gas chromatography system (Agilent 7820) equipped with a thermal conductivity detector and a HP-plot molsieve 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. The H_2 production is expressed as micromols of H_2 per milligram of chlorophyll *a*.

RT-PCR

Quantitative and semiquantitative RT-PCR analyses were performed on RNA samples isolated from cultures grown in BG11 or BG110 media. RNA was extracted as described previously (Xu et al. 2003). Chromosomal DNA was removed by treating RNA preparations (50 μl) with 1 μl of DNase (Ambion at 2 U/μl) for 1 h at 37 °C. DNase treatment was checked by RT-PCR, omitting the reverse transcription step. One microgram of total RNA was subjected to RT-PCR with SuperScript One-Step RT-PCR (Invitrogen). The standard program was 5 min at 94 °C, followed by 35 cycles of 40 s at 94 °C, 45 s at 50 °C and 45 s at 72 °C, and a final 5 min at 72 °C. The primers used are listed in Table 1.

Mass spectrometry analyses

Nostoc cells grown in BG110 were broken mechanically using a mini-BeadBeater 1 (Biospec) in 25 mM TRIS, 75 mM NaCl pH 7.5 in the presence of protease inhibitors (ProteaseArrest, GBiosciences). Cell extract was ultracentrifuged for 30 min at 110,000*g*. Proteins in the supernatant were separated by SDS PAGE. After staining with Coomassie Blue, the bands in the gel corresponding to the theoretical molecular mass of the heterologous proteins were cut out and analyzed for identification by LC-MSMS on a Q-Exactive plus mass spectrometer coupled to a nano liquid chromatography (Thermo Fisher Villebon sur Yvette, France) as previously described (Boughanemi et al. 2016). For protein identification, spectra were processed using the Proteome Discoverer software program (Thermo Fisher Scientific, version 2.1.0.81). The following parameters were used: *Nostoc* PCC7120 (GI TaxID = 103690) and *Clostridium acetobutylicum* (GI TaxID = 1488) extracted from NCBI; enzyme: trypsin; dynamic modification: oxidation/+ 15.995 Da (Met); static modification: carbamidomethyl/+ 57.021 Da (Cys); mass values: monoisotopic; precursor mass tolerance: ± 10 ppm; fragment mass tolerance: ± 0.02 Da; and missed cleavages: 2. Proteins were defined as identified whenever two unique "rank 1" peptides passed the high confidence filter.

Results

Design and characterization of the recombinant *Nostoc* strain expressing *hydAEFG* in the heterocyst

In *Clostridium acetobutylicum*, the genes encoding the [FeFe] H_2 ase HydA and the accessory proteins HydE, HydF, and HydG are located separately in the genome (Nolling et al. 2001). To achieve the coordinated production of these proteins in the heterocysts of *Nostoc*, a synthetic biology approach was used to express the four *hyd* genes from the same artificial operon. The *hydAEFG* open reading frames (ORFs) were optimized based on the codon usage of *Nostoc* (Kaneko et al. 2001). The ribosome binding site (RBS) of the *petE* gene from *Nostoc*, which is known to be a moderately efficient RBS (Lentini et al. 2013; Vellanoweth and Rabinowitz 1992), was inserted 8 bp upstream of the initiation codons of *hydE*, *hydF*, *and hydG*. In artificial operons, it has been established that distances between cistrons ranging between 0 and 50 bp do not introduce any bias in the levels of expression of the genes (Lentini et al. 2013). We therefore set the distance between each *hyd* cistrons (A-E, E-F, F-G) at 25 bp. The transcriptional terminator of the *rrnB* gene of *Escherichia coli* was chosen to terminate the transcription of the operon since it has been found to be functional in cyanobacteria (Wang et al. 2012). To express the *hydAEFG* genes specifically in the heterocysts, their transcription was placed under the control the 700 bp-long promoter of the *nifH* gene which is a heterocyst-specific gene encoding nitrogenase reductase under N_2-regime (Elhai and Wolk 1990; Golden et al. 1991; Ungerer et al. 2010). The resulting *nifH*'-'*hydAEFG* operon was cloned in the pRL25T replicative plasmid and introduced into *Nostoc*, yielding the Nhyd1 recombinant strain. The growth and morphology of the Nhyd1 strain were similar to that of the wild type in the presence of nitrate or N_2 (Fig. 1a, b).

To test whether the *hydAEFG* genes were properly expressed, total RNA and proteins were extracted from

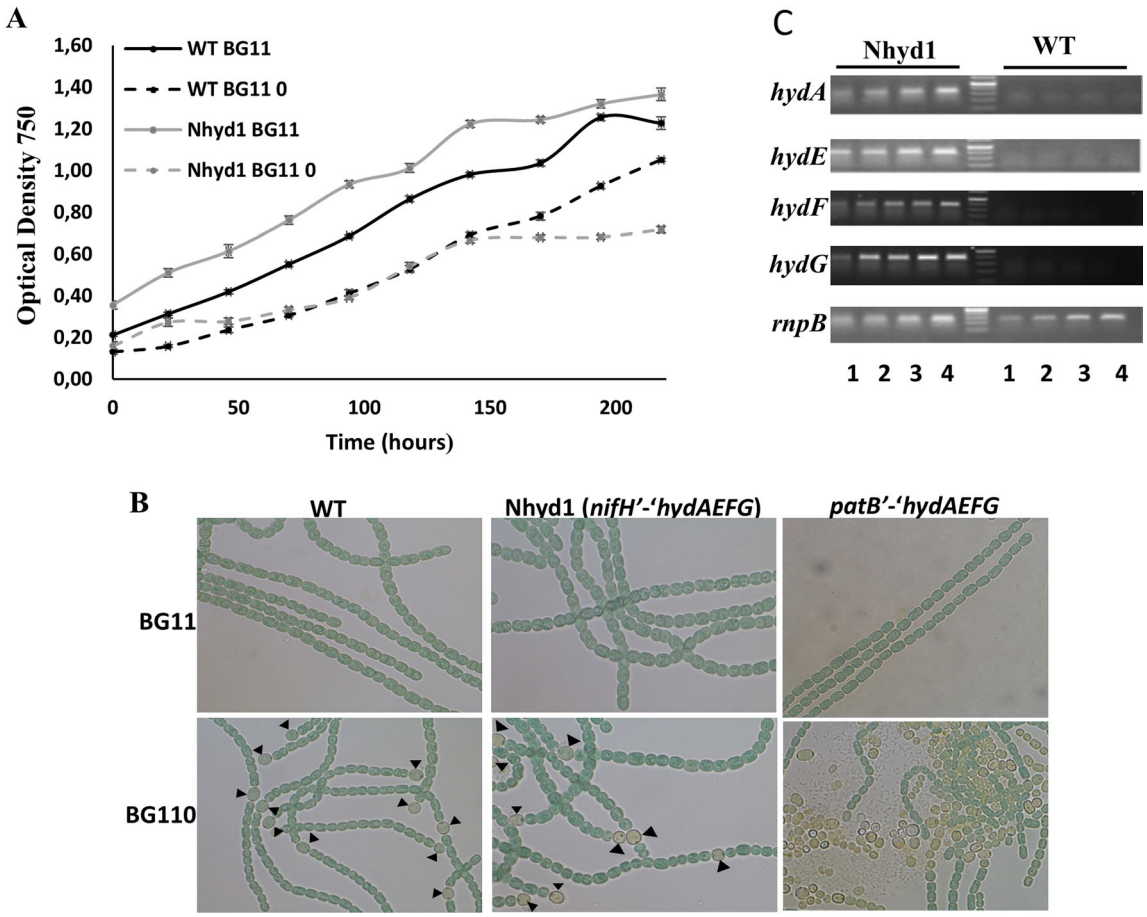

Fig. 1 Characterization of the Nhyd1 strain. **a** Growth curve of *Nostoc* strains grown in either BG11 (nitrate-containing medium) or BG110 (nitrate-free medium). **b** Microscope images of *Nostoc* strains grown in either BG11 or BG110**.** Heterocysts are indicated by black arrows. **c** Expression of the *hydAEFG* operon under the control of the *nifH* promoter in *Nostoc*: semiquantitative RT-PCR analysis of *hydAEFG* gene transcription. RNAs were collected from the Nhyd1 recombinant strain or the wild type strain 24 h after the onset of the nitrogen depletion step. Samples were collected at various times during the exponential phase of the PCR (1: cycle 26; 2: cycle 28, 3: cycle 30, 4: cycle 35). All RT-PCR experiments were performed in triplicate, and similar results were consistently obtained. Expression of the *rnpB* gene was used as the control assay

the Nhyd1 strain 24 h after the nitrogen step-down. Semiquantitative RT-PCR analysis indicated that the four genes were actually transcribed in the recombinant strain (Fig. 1c). LC-MSMS mass spectrometry analysis confirmed that HydA, HydE, HydF, and HydG were actually present in the soluble fraction of the protein extract obtained from the Nhyd1 strain grown under N_2 regime (Table 2), but not in the protein extract obtained from the Nhyd1 strain grown in the presence of nitrate. All in all, these results show that the artificial *nifH*'-'*hydAEFG* operon was correctly expressed in *Nostoc* grown under N_2 -regime. We asked if expressing the *hydAEFG* operon at different times of the differentiation process would have any impact on the H_2 production, and it was found that the *nifH* promoter was the best candidate. Expressing the *hydAEFG* operon under the early promoter Nsir1 was toxic to the strain. The *patB* gene promoter, which is expressed at an intermediate time after the onset of the nitrogen step-down (13–18 h later) allowed the highest level of H_2 production, but the strain was unhealthy and fragmented (Fig. 1b).

In vivo H_2 production assays

The mean level of H_2 production (160 μmol-H_2 mg-Chla^{-1}) obtained with the recombinant *Nostoc* strain Nhyd1 (*nifH*'-'*hydAEFG*) was in average ninefold higher than the WT level (Fig. 2b). H_2 production was observed only when the cells were shifted to nitrate-free medium, which is consistent with the fact that the transcription of *hydAEFG* was under the control of the heterocyst-specific promoter *nifH* (Golden et al. 1991). The amount of H_2 produced was dependent on light intensity used for the growth, with the highest activity recorded at a light intensity of 60 μEm^{-2} (compare Fig. 2b, c). It is important to note that the hydrogenase activity measured required the inhibition of the electron transfer between PSII and the plastoquinones by DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea, Sigma), (compare Fig. 2a, b). Unlike nitrogenase, HydA was therefore not active under phototrophic conditions in the Nhyd1 strain.

Table 2 Mass spectrometry identification of Hyd proteins in extracts of *Nostoc* Nhyd1 strain grown under a N_2 regime

Protein	GeneBank accession number	Unique peptides[a]	% coverage[b]
HydA	AAB03723	13	19.7
HydE	AAK79598	18	57
HydF	AAK79617	33	79.8
HydG	AAK79324	12	27.5

The results presented are representative of four independent experiments

[a] Number of unique peptides detected

[b] Protein sequence coverage by matching peptides

Effects of cyanoglobin on H_2 production

One possible explanation for the fact that HydA activity required PSII inhibition might be that certain quantity of O_2 generated by PSII activity in the vegetative cells diffuses in the heterocysts and inhibits HydA. It was therefore postulated that increasing the consumption of O_2 in the heterocysts prior to *hydA* induction might allow H_2 production under phototrophic conditions. To test this hypothesis, the *glbN* gene of *Nostoc commune* (Potts et al. 1992) was expressed in Nhyd1 under the control of the *patB* promoter, yielding the Nhyd2 strain. The *patB* promoter is transcribed specifically in the heterocyst around 13–18 h after the onset of nitrogen starvation (Jones et al. 2003). The *glbN* gene encodes a cyanoglobin that has been found to bind to O_2 with a high affinity (Thorsteinsson et al. 1996). The qRT-PCR data presented in Fig. 3a indicate that the transcription of the *glbN* gene in the recombinant strain was induced 18 h after the nitrate depletion step. Mass spectrometry analysis showed that the GlbN protein was present in the protein extract of the recombinant strain after 18 h of growth in BG110. GlbN was recovered with 50% of coverage, and a number of unique peptides of 5. The growth and the morphology of the Nhyd2 strain were similar to that of the wild type (Fig. 1, Fig. 3b). Interestingly, Nhyd2 strain produced large amounts of H_2 under the conditions promoting the activity of PSII (i.e., in the absence of DCMU). This finding is in agreement with the latter hypothesis, since the production in the heterocyst of a protein capable of binding to O_2 allows H_2 production during photosynthesis. The average level of H_2 produced by the Nhyd2 strain was 400 μmol-H_2 mg-Chla^{-1}, which to our knowledge is the highest amount of H_2 produced so far by a *Nostoc* strain under a phototrophic regime (Fig. 3c). It is worth noting that the *Nostoc* recombinant strain described above produced H_2 only under an Argon atmosphere. However, when cyanoglobin was produced, a weaker H_2 production was also recorded in the presence of air (Fig. 3d).

Discussion

The use of heterocysts of filamentous diazotrophic cyanobacteria for H_2 production from HydA of *C. acetobutylicum* has been in the mind of scientists from years. In the study from Gärtner et al. (2012), the periplasmic [FeFe] H_2ase of *Shewanella oneidensis* MR-1 has been produced in the heterocysts of *Nostoc PCC 7120*; and *in situ* H_2 production has been measured in the recombinant strain, which indicated that the enzyme was active in this cell type (Gärtner et al. 2012). On the other hand, the heterologous

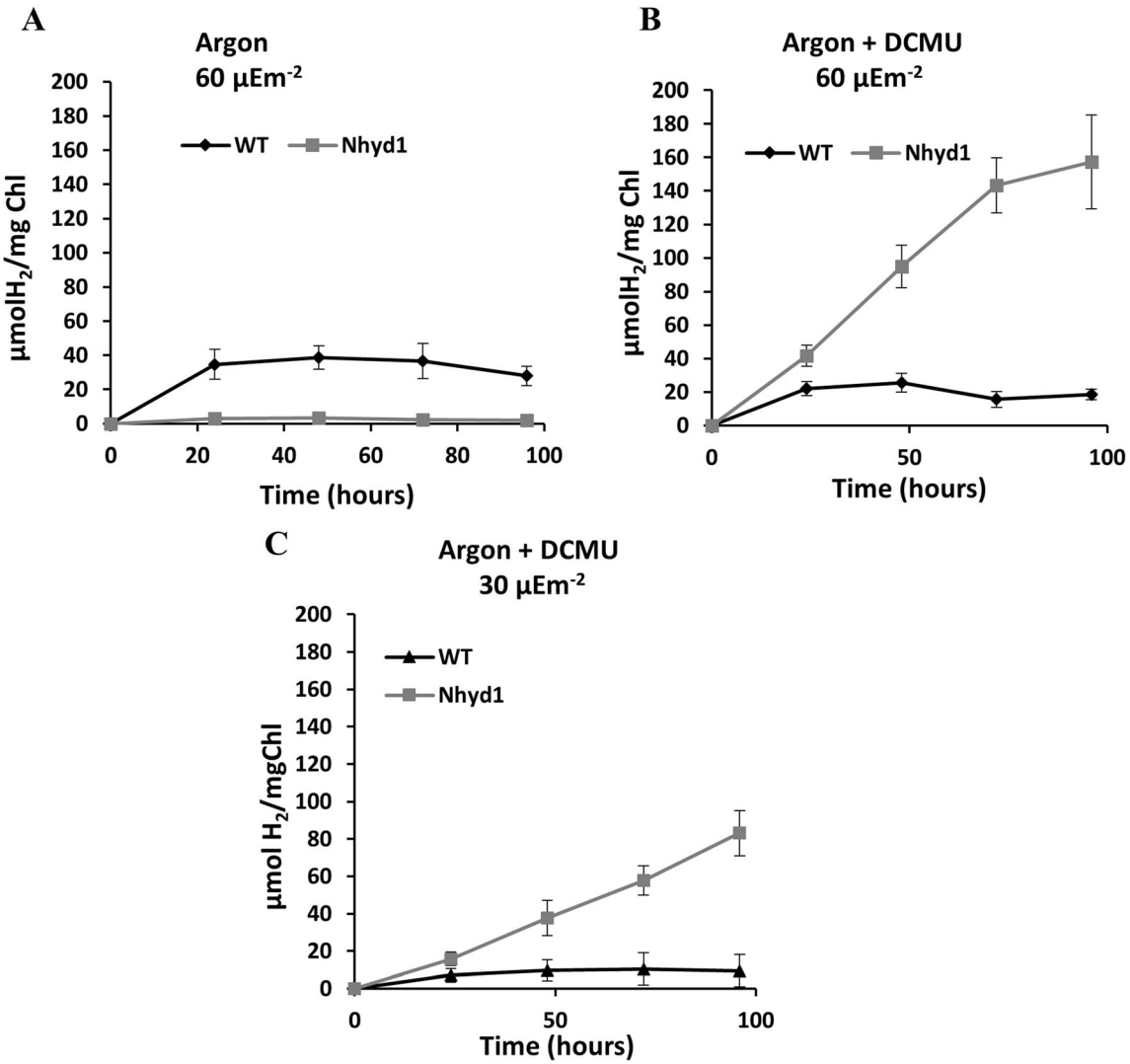

Fig. 2 H_2 production kinetics by the wild type and the Nhyd1 *Nostoc* strains. Time zero indicates the onset of the nitrogen depletion. The cell cultures were maintained under continuous illumination at 60 μEm^{-2} (**a**, **b**) or 30 μEm^{-2} (**c**). Cultures were maintained under Argon in the presence (**b**, **c**) or absence of DCMU (**a**). Means ± SEM ($n = 7$)

expression of the [FeFe] H_2ase HydA of *C. acetobutylicum* in the unicellular non-nitrogen-fixing cyanobacterium *Synechococcus elongatus* PCC 7942 has been found to produce H_2 at the rate of 2.8 µmol-H_2 mg-Chl$a^{-1}H^{-1}$. However, due to its O_2-sensitivity, the activity of this enzyme has been monitored under anaerobic conditions along with the inhibition of PSII activity (Ducat et al. 2011). Taken together, these two studies have demonstrated that HydA can be expressed in a photosynthetic organism and that the redox machinery at work in the heterocyst can supply a heterologous H_2ase with electrons. It was therefore reasonable to assume that the production of an active HydA in the heterocyst is possible. In the present study, we established that when HydA was produced in the heterocysts of *Nostoc*, a substantial amount of H_2 was produced (160 µmol-H_2 mg-Chla^{-1}). The enzyme, however, was active only when the photosystem II was inhibited. We concluded that the micro-oxic conditions present in the heterocysts might not be optimal for promoting HydA activity. In addition to the "respiration protection" system which reduces the O_2 concentration in the heterocysts, it has been proposed that the nitrogenase displays a specific protection mechanism consisting in the reduction of O_2 by the nitrogenase iron protein, generating hydrogen peroxide which is subsequently reduced by a peroxidase (Thorneley and Ashby 1989). Later, the rubrerythrin RbrA has been proposed to be the peroxidase involved in the auto-protection mechanism of nitrogenase (Zhao et al. 2007). Since no similar auto-protective mechanism has been reported for HydA, it is therefore plausible that it would be more sensitive to O_2 inhibition in the heterocyst than nitrogenase. We therefore hypothesized that decreasing the amount of O_2 in the heterocysts would provide an additional protection. We actually demonstrated here that the expression of the cyanoglobin encoding gene from *Nostoc commune* in the heterocysts prior to the

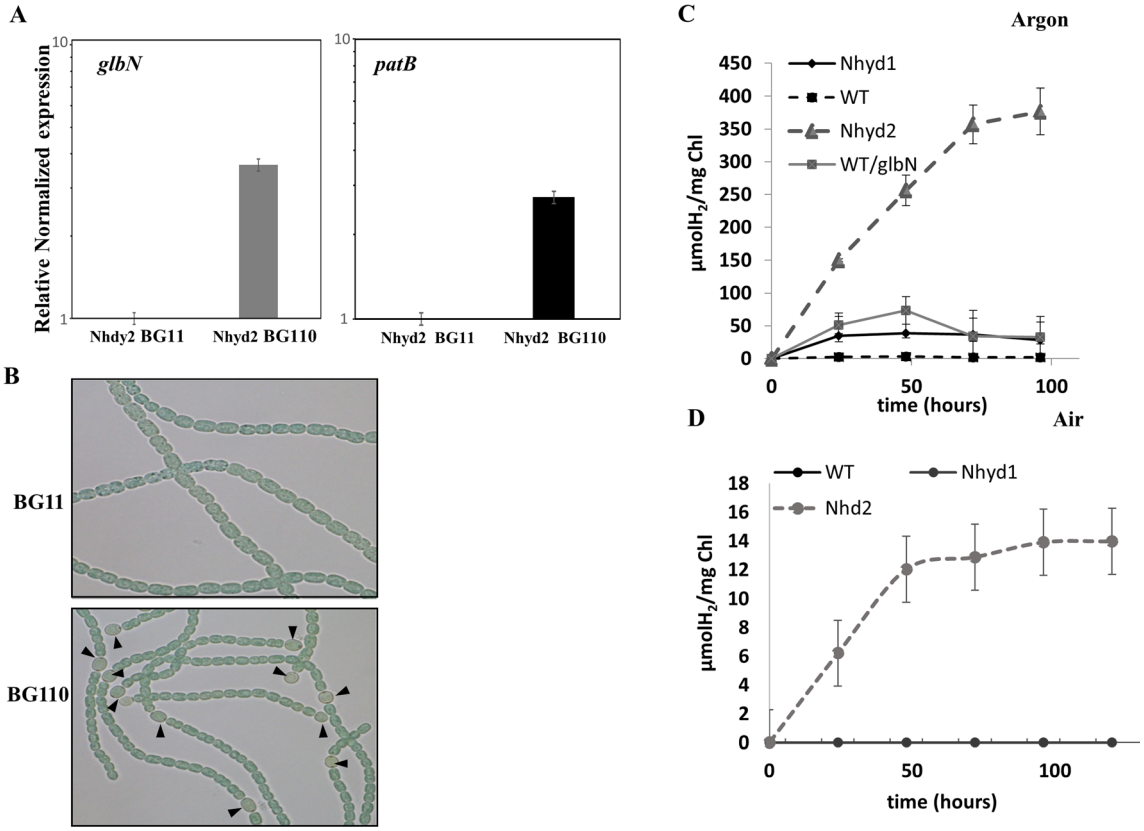

Fig. 3 Cyanoglobin production and effects on *Nostoc*. **a** qRT-PCR analysis of the *glbN* transcripts in the recombinant strain *Nhyd2*; values were normalized to the *rnpB* transcript, and the values obtained in the BG11 (nitrate-containing medium) condition were set to 1. In the BG110 (BG11 without nitrate) condition, mRNAs were extracted 18 h after the onset of the nitrogen depletion step. qRT-PCR analysis of the *patB* transcripts served to check the induction of the *patB* promoter. Each sample was measured in triplicate, and error bars give the standard deviations. **b** Microscope images of *Nhyd2* strain grown in either BG11 or BG110 medium. Heterocysts are indicated by black arrows. **c** Effects of *glbN* expression under *patB* promoter on H_2 production in Argon by the following strains: wild type, Nhyd1 (*nifH*'-'*hydAEFG*), Nhyd2 (*nifH*'-'*hydAEFG*/*patB*'-'*glbN*), WT/*glbN*. Time zero is the onset of the nitrogen depletion step. The cell cultures were maintained under continuous illumination at 60 μEm^{-2}. Means ± SEM (n=7). **d** Effects of *glbN* expression under *patB* promoter on H_2 production in Air by the following strains: wild type, Nhyd1 (*nifH*'-'*hydAEFG*), Nhyd2 (*nifH*'-'*hydAEFG*/*patB*'-'*glbN*), WT/*glbN*. Time zero is the onset of the nitrogen depletion step. The cell cultures were maintained under continuous illumination at 60 μEm^{-2}. Means ± SEM ($n = 7$)

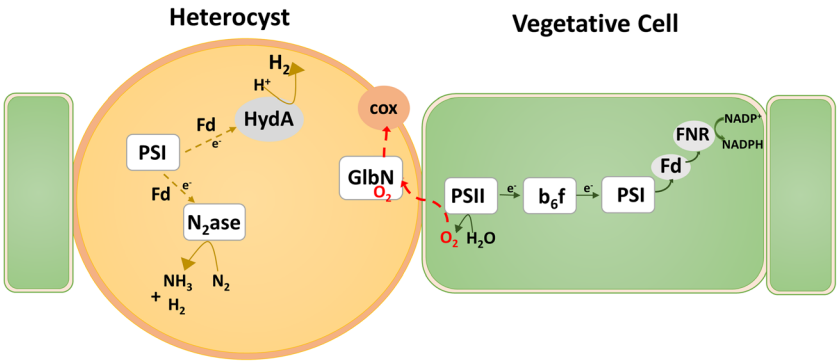

Fig. 4 Hypothetical model of H_2 production by HydA in the Nhyd2 *Nostoc* strain. The vegetative cells perform photosynthesis while nitrogen fixation occurs in the heterocyst. HydA uses electrons transferred by a heterocyst-ferredoxin to produce H_2. The decrease of O_2 concentration through the respiration activity of the heterocyst is not indicated. The O_2 generated by the photosynthetic activity of the vegetative cells, which might reach the heterocyst, is sequestrated by the cyanoglobin GlbN protein and then transferred to the cytochrome *c* oxidase. PSI photosystem I, PSII photosystem II, Fd ferredoxin, FNR ferredoxin, NADP oxidoreductase, N_2ase nitrogenase, cox cytochrome *c* oxidase, b_6f b_6f complex

expression of the *hydAEFG* operon enabled H_2 production and photosynthesis to occur simultaneously. The cyanoglobin GlbN in *Nostoc commune* is thought to protect the nitrogenase from oxidation, because GlbN has been shown to bind reversibly to O_2 with a high affinity. It has been postulated that the cytochrome oxidase complex might be a partner of cyanoglobin in the O_2 scavenging processes in the heterocyst of *Nostoc commune* (Hill et al. 1996; Thorsteinsson et al. 1996). Since *Nostoc* PCC7120 also produces cytochrome *c* oxidase in the heterocyst (Valladares et al. 2003), it is possible that the GlbN protein transfers O_2 to this complex which would regenerate its apo form, maintaining O_2 consumption and therefore HydA activity. Under argon, the Nhyd2 recombinant strain expressing *glbN* produces approximately 400 μmol-H_2 mg-Chla^{-1}, which is more than 20-fold higher than the rate of H_2 production in the wild type strain under the same experimental conditions. The production of cyanoglobin also promoted H_2-production under aerobic conditions. Although it is weak, this activity shows that the O_2 level was successfully lowered in the heterocysts. Figure 4 shows a schematic representation of the function of HydA in the heterocyst of *Nostoc* expressing the *glbN* gene.

The choice of the promoter used for the transcription of *hydAEFG* genes turned out to be a crucial factor for H_2 production. Expressing the *hydAEFG* operon from a late-phase promoter (the *nifH* gene) was found in the present study to be the best compromise between growth and stable production. Since heterocysts of cyanobacteria are attractive means to produce O_2-sensitive enzymes other than H_2ases under aerobic conditions, exploring several heterocyst-specific promoters may be a strategy worth testing in future studies.

The finding that HydA is able to reduce protons into H_2 under phototrophic conditions indicates that it is able to accept electrons from the endogenous electron donors of *Nostoc*. In the heterocyst, the ferredoxin FdxH is considered as the natural electron donor to nitrogenase (Razquin et al. 1994). At this stage of our study, we do not know which of FdxH or PetF ferredoxins serve as the electron donor to HydA. The production of ferredoxin from *C. acetobutylicum* in the Nhyd2 strain might optimize H_2 production in this recombinant strain.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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