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ORIGINAL RESEARCH

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Deletion of Flv3A facilitates long-term H_2 photoproduction in diazotrophic Anabaena sp. PCC 7120

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Abstract

Molecular hydrogen (H_2) is a promising energy carrier, and its production by photosynthetic microorganisms holds substantial potential for advancing renewable energy generation. The nitrogenase-mediated H_2 production using heterocyst-forming cyanobacteria represents a promising approach, as the process utilizes light energy and photosynthetic reductants while being naturally protected from O_2-rich environments by its restriction to microoxic heterocyst cells. We investigated the impact of deleting the vegetative cell-specific flavodiiron protein, Flv3A, on the long-term H_2 photoproduction of the model heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120. The H_2 photoproduction response was evaluated under varying atmospheric conditions, with or without N_2 and O_2, and compared to the Δ*hupL* mutant, which is deficient in the large subunit of uptake hydrogenase, and the Δ*hupL*/*flv3A* double mutant. Unlike the Δ*hupL* mutant, H_2 photoproduction in Δ*flv3A* is not enhanced by increased nitrogenase activity or high accumulation of sugars in cells. Our results suggest that the absence of the vegetative cell-localized Flv3A positively affects H_2 photoproduction in heterocysts by simultaneously downregulating *hupL* expression and enhancing the O_2 tolerance of nitrogenase *via* a yet unexplored mechanism. These findings advance our understanding of nitrogenase-driven H_2 production and provide a new strategy to address key limitations in long-term photobiological H_2 production.

1 | INTRODUCTION

Under the severe environmental threat posed by burning fossil fuels, it is essential to develop renewable and carbon-neutral substitutes for fossil resources. Molecular hydrogen (H_2), a clean and versatile energy carrier, is one of the most appealing options projected to play a key role in the future global energy system (Song et al., 2022). In this context, photobiological H_2 production by cyanobacteria and green algae is of great interest as it exploits nature's most abundant renewable resources, like water and sunlight. Cyanobacterial H_2 photoproduction mainly relies on two O_2-sensitive enzymes: bidirectional hydrogenase (Hox) and nitrogenase. Hox is a heteropentameric enzyme, which catalyzes the reversible reduction of protons to H_2 depending on the redox status of the cell (Cournac et al., 2004; Tamagnini et al., 2007). H_2 production mediated by Hox has been usually described under dark anaerobic conditions (Troshina et al., 2002; Carrieri et al., 2011), for very short periods during dark-to-light transitions (Appel et al., 2000) or upon a sudden increase in light intensity (Burgstaller et al., 2022). Nitrogenase is a multiprotein enzyme complex that catalyzes the unidirectional production of H_2 as an inevitable by-product of the N_2 fixation (Bothe et al., 2010). Filamentous heterocystous cyanobacteria have evolved the remarkable ability to protect N_2 fixation from an O_2-rich environment by constraining the process to specialized cells, the so-called heterocysts. The micro-oxic condition inside

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heterocysts enables high nitrogenase activity under atmospheric levels of O_2 and concomitantly with O_2-evolving photosynthesis in neighboring vegetative cells (Flores et al., 2015). The heterocystous cyanobacterium *Anabaena* sp. PCC 7120 (hereafter *Anabaena*) expressing the molybdenum nitrogenase catalyzes a high energy- and electron-intensive process where N_2 and protons are simultaneously reduced to ammonium and H_2, respectively, according to equation 1 (Bothe et al., 2010):

$$\text{Under air}: N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \qquad (1)$$

$$\text{In the absence of } N_2: 2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4P_i \qquad (2)$$

In the absence of N_2, nitrogenase allocates all electrons towards the production of H_2, lowering the ATP requirement from 16 (in the air) to 4 ATP per H_2 produced, according to equation 2 (Sagir and Hallenbeck, 2019).

A major metabolic bottleneck for efficient H_2 production by nitrogenase is the rapid recycling of H_2 by the uptake hydrogenase (Hup), encoded by *hupSL*. The oxidation of H_2 by Hup provides electrons to the heterocyst metabolism and decreases O_2 partial pressure around nitrogenase *via* the oxyhydrogen reaction (Tamagnini et al., 2007). To this day, the inactivation of Hup through genetic engineering represents the most effective approach for enhancing the H_2 photoproduction yield in numerous *Anabaena* strains (Mikheeva et al., 1995; Happe et al., 2000; Masukawa et al., 2002; Yoshino et al., 2007; Nyberg et al., 2015). It is noteworthy that the inactivation of Hup significantly alters the overall metabolic equilibrium in the filaments, impacting oxygen scavenging capacity and energy metabolism in heterocysts, as well as carbon metabolism in vegetative cells (Ekman et al., 2011; Kourpa et al., 2019), pointing out a regulatory network between the different cell types. Recently, we provided evidence for a novel regulatory network between H_2 metabolism in the heterocysts and oxygenic photosynthesis in the vegetative cells of diazotrophic *Anabaena* (Santana-Sánchez et al., 2023). Cyanobacteria, among other oxygenic photoautotrophs, have evolved the flavodiiron proteins (FDPs) to protect the photosynthetic apparatus in response to environmental fluctuations in light (Allahverdiyeva et al., 2015). FDPs function as efficient electron sinks, directing excess electrons from the photosynthetic electron chain to O_2 *via* the Mehler-like reaction (Helman et al., 2003; Allahverdiyeva et al., 2011). *Anabaena* possesses six FDPs. Alongside *flv2* and *flv4*, whose localization remains unknown, *Anabaena* possesses two copies of genes encoding Flv1 and Flv3 proteins. Flv1A and Flv3A are highly similar to their homologs in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) and are expressed in vegetative cells (Ermakova et al., 2013). Both Flv1A and Flv3A are required to perform the Mehler-like reaction during the dark-to-light transition. Furthermore, they have a crucial role under fluctuating light intensities, suggesting a functional analogy to their homologous in *Synechocystis* (Allahverdiyeva et al., 2013). However, distinct functional roles were observed under ambient CO_2 levels, where Flv3A is capable of mediating a moderate O_2 photoreduction, independently of Flv1A (Santana-Sánchez et al., 2019). Flv1B and Flv3B are exclusively localized in heterocysts (Ermakova et al., 2013). The Flv3B homooligomer was shown to mediate the Mehler-like reaction, providing the micro-oxic environment required for nitrogenase activity (Ermakova et al., 2014). Overexpression of Flv3B contributes to the maintenance of micro-oxic conditions inside the heterocysts, resulting in increased H_2 production, which was attributed to Hox hydrogenase activity (Roumezi et al., 2020). The function of Flv1B remains unknown.

Interestingly, the deletion of *flv3A*, but not *flv1A*, resulted in enhanced short-term H_2 photoproduction under oxic and micro-oxic conditions (Santana-Sánchez et al., 2023). This enhancement was attributed to nitrogenase activity as evidenced by the increased H_2 photoproduction in Δ*flv3A* under N_2-free atmosphere, when the nitrogenase-mediated H_2 production is boosted by the exclusive allocation of electrons to the reduction of protons. The transcript analysis performed by the authors revealed that the increased H_2 yield in Δ*flv3A* mutant can be attributed to a strong repression of *hupL via* a yet unknown regulatory mechanism.

In this work, we demonstrated that the *Anabaena* Δ*flv3A* mutant can sustain long-term H_2 photoproduction. Unlike the Δ*hupL* mutant, H_2 photoproduction in Δ*flv3A* is not enhanced by increased nitrogenase activity or high accumulation of sugars in cells. Our results suggest that the absence of the vegetative cell-localized Flv3A positively affects H_2 photoproduction in heterocysts by simultaneously downregulating *hupL* expression and enhancing the O_2 tolerance of nitrogenase *via* a yet unexplored mechanism.

2 | MATERIALS AND METHODS

2.1 | Strains and culture conditions

The *Anabaena* sp. PCC 7120 obtained from the Pasteur Culture Collection was used as the wild-type (WT). The Δ*flv1A* and Δ*flv3A* mutants (Allahverdiyeva et al., 2013) and the Δ*hupL* mutant (Masukawa et al., 2002) have been described previously. For constructing the Δ*hupL*/*flv3A* double mutant, the plasmid for *flv3A* deletion with a neomycin/kanamycin resistance cassette was transformed into Δ*hupL*, followed by selection with sucrose, neomycin, and spectinomycin as described by Cai and Wolk (1990). Segregation of the mutant was verified by polymerase chain reaction (PCR) (Figure S1; Table S1).

Culture stocks were maintained in Z8 medium (Kotai, 1972) without combined nitrogen sources (Z8x) under continuous illumination of 30 μmol photons m^{-2} s^{-1} and 100 rpm shaking at 22°C. The growth media of Δ*flv1A* and Δ*flv3A* mutants were supplemented with 40 μg ml^{-1} neomycin, Δ*hupL*/*flv3A* was additionally supplemented with 40 μg mL^{-1} spectinomycin, while Δ*hupL* was supplemented only with 40 μg mL^{-1} spectinomycin. For pre-experimental cultivation, the filaments were harvested at the late logarithmic phase (OD_{750} of around 1.0–1.2), inoculated at $OD_{750} = 0.1$ in 350 mL of fresh Z8x medium without antibiotics, and transferred in 500 mL Erlenmeyer flasks. Pre-cultures were grown at 26°C with continuous air bubbling under continuous illumination of 50 μmol photons m^{-2} s^{-1} (cool-daylight fluorescence lamps, Lumilux T8 15W/865; Osram) for 8 days.

2.2 | H_2 photoproduction experiments

Pre-cultures were harvested at the late logarithmic phase ($OD_{750} = 0.8–1.0$), inoculated at 5 μg mL^{-1} Chlorophyll *a* (Chl *a*) in 10 mL of fresh Z8x and transferred into 70-ml gas-tight vials sealed with butyl rubber stoppers. The headspace of vials was flushed with air, argon (Ar) or Ar + 21% O_2 for 20 min and then supplemented with 1% CO_2. The vials were placed horizontally under continuous overhead illumination of 120 μmol photons m^{-2} s^{-1} (cool-white fluorescent lamps, Philips Master TL-D T8 15 W/ 840), 80 rpm shaking at 26°C for 3–6 days. All experiments were repeated several times with at least three vials for each condition.

2.3 | H_2 and O_2 determination

The H_2 and O_2 contents were determined every 24 h, as described previously (Leino et al., 2012). Briefly, 150 μL gas sample from the headspace of vials was injected into a gas chromatograph (GC) (Clarus 500; PerkinElmer, Inc.) equipped with a thermal conductivity detector and a molecular sieve 5A column (60/80 mesh). The instrument was calibrated with the 0.5% H_2 in argon standard (AGA) and air. The H_2 and O_2 specific yields were calculated based on the initial Chl *a* content in suspensions.

2.4 | Chl *a* and glycogen determination

Chl *a* was extracted by resuspending a pellet of centrifuged cells (1 mL, 9400 x *g*) in 90% methanol and determined spectrophotometrically at 665 nm by applying 12.7 L mg^{-1} cm^{-1} extinction coefficient (Ritchie 2006). Glycogen content was determined according to Ernst et al. (1984) with some modifications (Murukesan et al., 2019). Briefly, the pellet of centrifuged cells (1 mL, 9400 x*g*) was resuspended in 100% methanol, and the methanol supernatant was discarded after centrifugation. Residual pellets were washed once with 0.1 M Na-acetate buffer (pH 4.8) and resuspended in the same buffer (1 mL total volume) before heating at 120°C for 20 min to solubilize glycogen. After this, samples were cooled down to room temperature, and volumes were adjusted back to 1 mL using the same buffer. Aliquots of 100 μL of the Na-acetate buffer containing 4 units of amyloglucosidase (1011-51G-F, Sigma) were added to the mixtures and treated in a water bath for 14 h at 55°C. 100 μL of the treated samples were used for the glucose determination with a Glucose Hexokinase kit (DiaSys Diagnostic Systems). The glycogen content was expressed in glucose equivalents (180.2 g mol^{-1}) per Chl *a* content.

2.5 | Nitrogenase activity

The acetylene reduction assay was used to determine nitrogenase activity as described previously (Leino et al., 2014). Sealed vials containing the H_2-producing cultures were flushed with Ar, supplemented with 10% acetylene, and returned back to the same condition where H_2 production was performed for 20 h. A 100 μL gas sample from the headspace was diluted in 5 mL vials containing air. The reasons for the sample dilution are explained in Volgusheva et al. (2019). Then, 30 μL of the diluted gas was used for ethylene determination, which was carried out by GC (Clarus® 580, PerkinElmer, Inc.) equipped with Carboxen®-1010 PLOT capillary column and FID detector. Acetylene reduction activity was calculated based on the Chl *a* content.

2.6 | Hydrogenase activity

Hydrogenase activity was measured as reported by King et al. (2006) with slight modifications. Reactions were prepared anaerobically by flushing the vials with argon for 15 minutes. The reaction mixture included 10 mM sodium dithionite, 0.2% (w/v) Triton X-100, and 10 mM methyl viologen in 50 mM potassium phosphate buffer (pH 7.0). Following five days of H_2 production, 1 mL of micro-oxic cell suspension was added to 1 mL of reaction mixture. Samples were incubated in a water bath at 30°C, and H_2 evolution was quantified by gas chromatography after 15, 30, and 45 minutes.

2.7 | Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple range test for mean comparisons. Statistical analysis was performed with SPSS software (version 16.0), and differences were considered significant at $p < 0.05$.

3 | RESULTS

In previous research, we demonstrated through short-term experiments that diazotrophic *Anabaena* filaments deficient in the vegetative cell-specific Flv3A, but not Flv1A, exhibited enhanced H_2 photoproduction under air, air +1% CO_2, and especially argon atmospheres. (Santana-Sánchez et al., 2023). To evaluate whether this phenotype can be sustained over a long-term process, we monitored H_2 photoproduction by *Anabaena* WT, Δ*flv1A* and Δ*flv3A* filaments for up to 144 h. The Δ*hupL* mutant was used as a reference strain, which has a high H_2 production yield due to the absence of H_2 recycling pathway in heterocysts (Masukawa et al., 2002; Leino et al., 2012). We compared H_2 production under three different conditions: (1) under air, when H_2 is primarily produced as a by-product of the N_2 fixation reaction, (2) under oxic conditions in the absence of N_2, when nitrogenase allocates all electrons to H_2 evolution, and (3) under micro-oxic conditions without N_2, when the inhibitory effects of O_2

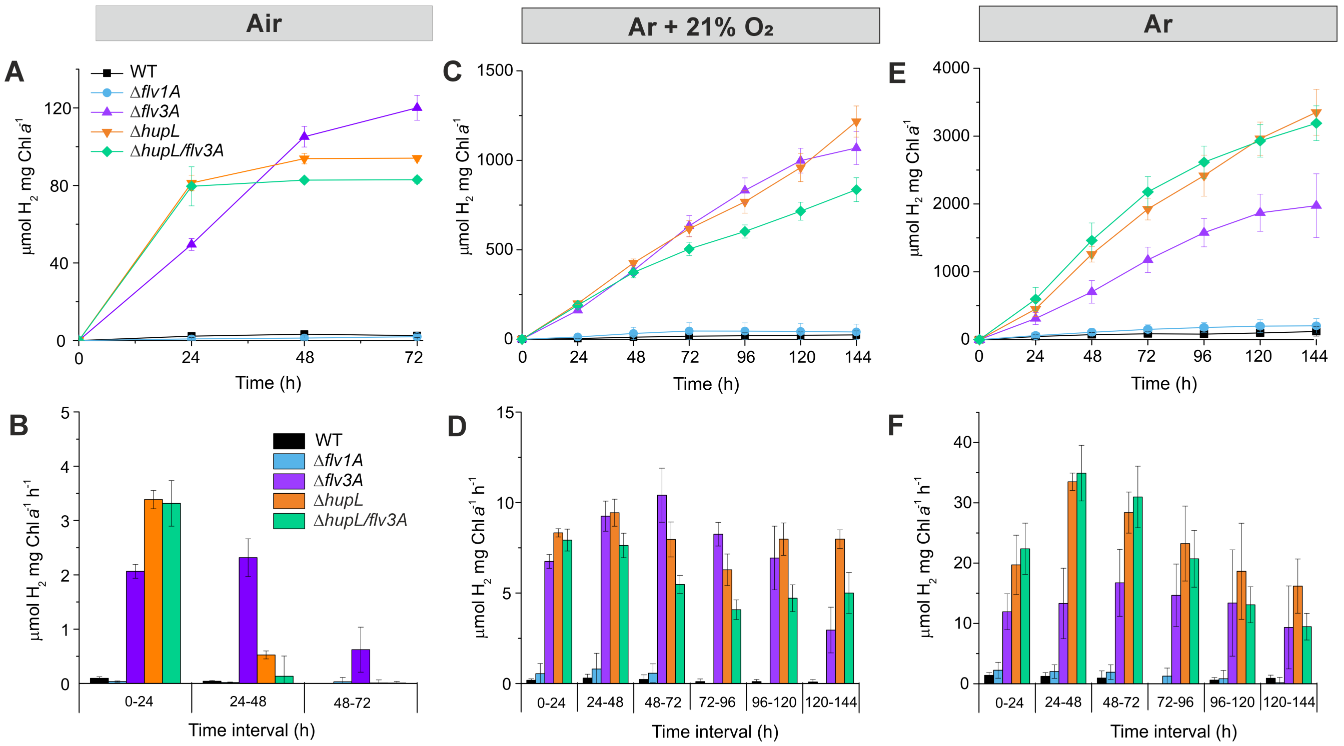

FIGURE 1 H_2 photoproduction in *Anabaena* WT and mutants under different atmospheres. H_2 photoproduction yields (A, C, E) and rates (B, D, F) under air, oxic (Ar + 21% O_2) and micro-oxic (Ar) conditions supplemented with 1% CO_2. Error bars represent the standard deviation between 5 independent biological replicates (3 technical replicates in each).

to nitrogenase are minimized. In all conditions, the cultures were supplemented with 1% CO_2 to avoid C_i limitation. As expected, the WT filaments, which possess the efficient H_2 recycling pathway, demonstrated negligible H_2 yields under air, oxic and micro-oxic conditions (Figure 1A, C, E). The Δ*flv1A* strain showed a similar trend, consistent with previous work (Santana-Sánchez et al., 2023).

3.1 | Long-term H_2 photoproduction in Δ*flv3A*, Δ*hupL* and Δ*hupL/flv3A* strains under air atmosphere (air +1% CO_2)

Under an atmosphere of air supplemented with 1% CO_2, the Δ*flv3A* filaments reached a maximum H_2 photoproduction rate of around 2 µmol H_2 (mg Chl *a*) $^{-1}$ h^{-1}, which was maintained for nearly 48 h before declining by 74% on the third day of incubation (Figure 1B). The Δ*hupL* mutant reached a 1.5-fold higher maximum H_2 photoproduction rate during the first 24 h, which sharply declined and eventually the filaments stopped producing H_2 by day 3 (Figure 1B), thus resulting in 1.3-fold higher H_2 yield at day 3 in the Δ*flv3A* mutant relative to Δ*hupL* (Figure 1A). These results suggest that, despite the absence of HupL in Δ*flv3A*, similar to Δ*hupL*, the lack of Flv3A may have an additional positive impact on the H_2 photoproduction during prolonged incubation under air. This observation prompted us to investigate whether deleting *flv3A* could enhance the long-term H_2 photoproduction yield in the Δ*hupL* mutant background by constructing the double knock-out mutant Δ*hupL/flv3A* (Figure S1). Unfortunately, the inactivation of *flv3A* in the Δ*hupL* mutant did not have a clear impact on long-term H_2 photoproduction under air. The Δ*hupL/flv3A* mutant demonstrated a lower H_2 photoproduction yield after 72 h compared to Δ*flv3A*, displaying H_2 photoproduction kinetics similar to the Δ*hupL* filaments (Figure 1A, B).

3.2 | Long-term H_2 photoproduction in Δ*flv3A*, Δ*hupL* and Δ*hupL/flv3A* strains under oxic conditions in the absence of N_2 (Argon + 21% O_2 + 1% CO_2)

Next, we examined whether the suppression of N_2 fixation (in the absence of N_2) would enhance the H_2 photoproduction in Δ*flv3A* in the long-term process. Under oxic conditions without N_2 (Ar + 21% O_2 + 1% CO_2 atmosphere), the H_2 yield after 72 h significantly increased by 5.3-, 6.5-, and 6-fold in Δ*flv3A*, Δ*hupL* and Δ*hupL/flv3A* mutants, respectively, compared to those observed under air +1% CO_2 atmosphere (Figure 1C). The Δ*flv3A* and Δ*hupL* mutants demonstrated similar yields of H_2 photoproduction over 144 h. However, the inactivation of Flv3A in the Δ*hupL* background resulted in reduced H_2 yield from 72 h onwards. The Δ*hupL* and Δ*flv3A* filaments reached 2.8- and 4.5-fold higher maximum H_2 photoproduction rates than those under air +1% CO_2 atmosphere, respectively (Figure 1D, B). The Δ*hupL/flv3A* filaments demonstrated a 2.4-fold higher H_2 photoproduction rate at 24 h, compared to that under air +1% CO_2 condition, followed by a rapid decline.

3.3 | Long-term H_2 photoproduction in Δ*flv3A*, Δ*hupL* and Δ*hupL/flv3A* strains under micro-oxic conditions in the absence of N_2

Although the absence of N_2 improved the long-term H_2 photoproduction under oxic conditions, the rate of H_2 photoproduction remained limited, likely due to the inhibitory effect of O_2 on the nitrogenase enzyme. To assess this hypothesis, we monitored long-term H_2 photoproduction under N_2 deprivation and micro-oxic conditions (Ar + 1% CO_2). As shown in Figure 1E, in the absence of both N_2 and O_2, the H_2 photoproduction improved to different extents in all H_2-producing strains, compared to their performance under oxic conditions without N_2 (Figure 1C). The Δ*flv3A* mutant demonstrated ~2-fold higher H_2 yield (compared to the oxic conditions) over the long-term process but yielded 40% lower H_2 than Δ*hupL* by the end of the experiment. Importantly, H_2 photoproduction in micro-oxic Δ*flv3A* filaments reached a peak rate of 10 μmol H_2 (mg Chl *a*)$^{-1}$ h^{-1} during the first 24 h (Figure 1F), considerably faster than under oxic (Ar + 21% O_2 + 1% CO_2) conditions, where this rate was only reached on the third day of the experiment (Figure 1D, 48–72 h). Moreover, the high rate of H_2 production by micro-oxic Δ*flv3A* filaments was sustained throughout the whole incubation period (Figure 1F, 0–144 h). The Δ*hupL* mutant demonstrated ~3-fold higher H_2 yield compared to oxic conditions from 48 h onwards. The strongest effect of O_2 limitation in Δ*hupL/flv3A* filaments was deducted from the observation that this strain exhibited a ~ 4-fold increase in the H_2 yield compared to the oxic conditions and achieved similar extent and kinetics of H_2 photoproduction than the Δ*hupL* mutant (Figure 1E). Both Δ*hupL* and Δ*hupL/flv3A* mutants demonstrated a rapid increase in H_2 photoproduction rate during the first 48 h, reaching a maximum level ~ 3.5-fold higher than that observed under oxic conditions without N_2, followed by a gradual decrease (Figure 1F).

The distinct changes in the performance of the H_2-producing mutants could be explained by the different net O_2 accumulation during the long-term process. Both Δ*flv3A* and Δ*hupL* under Ar + 1% CO_2 atmosphere demonstrated higher O_2 yield than WT over 144 h, with a more pronounced increase observed in the Δ*hupL* (Figure 2A). These results suggest that the gradual decay of the H_2 photoproduction rate (Figure 1F) observed in the mutants deficient in HupL is caused by the inhibitory concentrations of O_2 reached somewhere after 48–72 h. Indeed, the measurement of nitrogenase activity in the Δ*hupL* mutant during prolonged micro-oxic conditions demonstrated a strong induction of the nitrogenase activity during the first 72 h, followed by gradual decay. In contrast, the nitrogenase activity in the Δ*flv3A* mutant was similar to WT and showed limited induction during the first 50 h. Overall the Δ*hupL* mutant sustained higher nitrogenase activity throughout the experiments compared to WT (Figure 2B), which, besides the lack of uptake hydrogenase, contributes to the higher H_2 production observed in this mutant.

3.4 | Evaluation of Hox hydrogenase contribution to long-term H_2 photoproduction by Δ*flv3A* filaments under microoxic conditions

We previously suggested that H_2 photoproduction in the Δ*flv3A* mutant, as observed in short-term experiments, is predominantly mediated by nitrogenase (Santana-Sánchez et al., 2023). This conclusion was supported by the enhanced H_2 photoproduction in Δ*flv3A* under N_2-free conditions, where the absence of N_2 eliminates its fixation, allowing nitrogenase to allocate all available electrons exclusively to H_2 production. Our earlier investigation into the potential contribution of Hox to H_2 evolution in Δ*flv3A* revealed a strong transcriptional downregulation of *hoxH*, suggesting negligible Hox activity in diazotrophic Δ*flv3A* filaments (Santana-Sánchez et al., 2023).

In this study, we further examined whether Hox hydrogenase plays a role in sustaining long-term H_2 photoproduction in Δ*flv3A* mutant. To evaluate this, we assayed Hox hydrogenase activity in

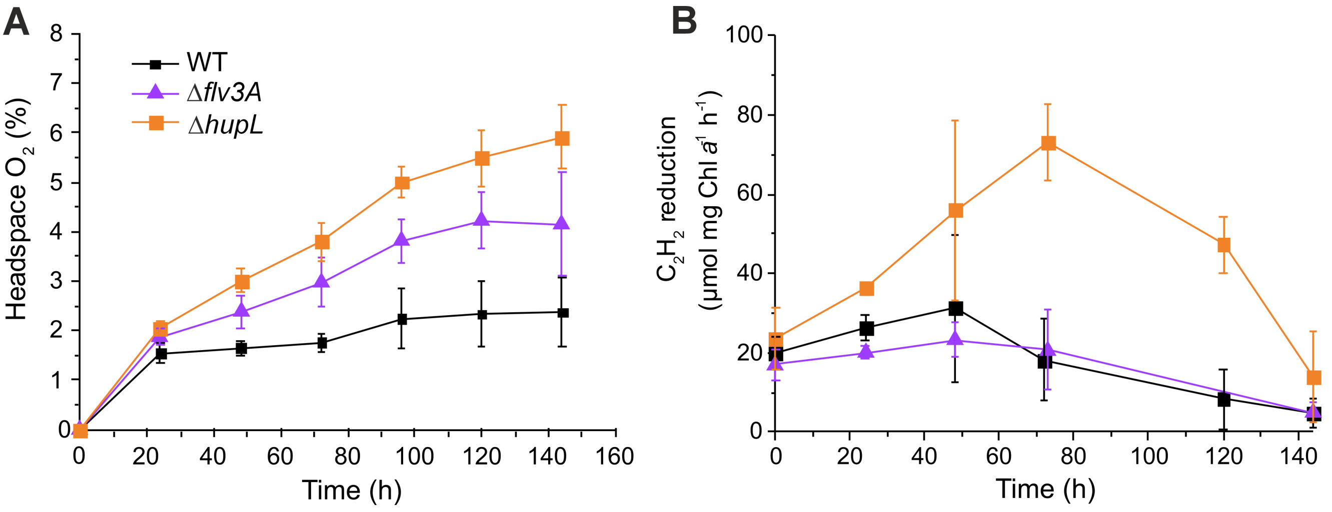

FIGURE 2 Long-term O_2 accumulation in the headspace (A) and nitrogenase activity (B) of cultures under Ar + 1% CO_2 atmosphere. Data represents an average of five independent experiments (with three vials in each) expressed as data ± standard deviations.

micro-oxic Δ*flv3A* filaments after 5 days of cultivation under two conditions: argon and nitrogen atmospheres. The results showed no significant differences in Hox hydrogenase activity between the two samples (Figure 3A), while H_2 photoproduction was completely diminished in the presence of N_2 (Figure 3B). These findings exclude a significant role for Hox in sustaining long-term H_2 photoproduction by diazotrophic Δ*flv3A* filaments under micro-oxic conditions.

3.5 | Accumulation of glycogen during the long-term H_2 photoproduction in Δ*flv3A*, Δ*hupL* and Δ*hupL*/*flv3A* strains

An important factor driving the nitrogenase-mediated H_2 production in diazotrophic *Anabaena* is the supply of sugars to heterocysts, which are synthesized by vegetative cells (Mariscal and Flores, 2010). Thus, the amounts of sugars stored in cells and their consumption may determine the efficiency of H_2 production in the long-term process. To examine whether the H_2 photoproduction observed in the *Anabaena* strains was affected by differential carbon storage, we monitored the net glycogen content in the filaments during the long-term process.

Under oxic conditions without N_2, there was no marked difference in the glycogen accumulation of the Δ*hupL* and Δ*flv3A* mutants compared to WT (Figure 4A). The Δ*hupL*/*flv3A* mutant maintained significantly lower levels of glycogen compared to the other strains. Under this condition, we did not observe a clear correlation between glycogen accumulation and H_2 yield, likely due to different factors limiting the transport and metabolism of glycogen across the diazotrophic filaments.

In the absence of O_2 and N_2 (Ar atmosphere), the mutants deficient in Flv3A displayed similar glycogen content to WT (Figure 4B). On the other hand, Δ*hupL* demonstrated significantly higher accumulation of glycogen compared to all the strains (Figure 4B). Interestingly, after 24 h of incubation, glycogen accumulation in Δ*hupL* mutant was about double compared to Δ*flv3A*, which correlated with ∼2 times higher yield and rate of H_2 photoproduction observed in Δ*hupL* in comparison to Δ*flv3A* (Figure 1E, F).

4 | DISCUSSION

The engineering of efficient H_2-producing cell factories based on oxygenic photosynthetic microorganisms is greatly challenged by the O_2 sensitivity of key enzymes involved in the process (Loo and Koo, 2019). Heterocyst-forming cyanobacteria are considered promising candidates for photobiological H_2 production since the maintenance of microoxic conditions within heterocyst cells enables high nitrogenase activity, even under atmospheric levels of O_2. Recently, we have described increased oxic and micro-oxic H_2 photoproduction in diazotrophic *Anabaena* filaments deficient in the vegetative cell-specific Flv3A during short-term experiments (Santana-Sánchez et al., 2023). The cause of increased H_2 yield in Δ*flv3A* was investigated by transcript and protein analysis, which demonstrated the strong repression of the uptake hydrogenase (Hup) in heterocysts *via* a yet unknown mechanism. To further explore the potential of the *Anabaena* Δ*flv3A* mutant for sustained H_2 photoproduction, we monitored its long-term H_2 photoproduction performance in comparison to the WT strain, as well as the Δ*hupL* and Δ*hupL*/*flv3A* mutants, under oxic and micro-oxic conditions.

In our previous study using short-term experiments, we provided evidence that H_2 photoproduction in Δ*flv3A* is mainly driven by nitrogenase, with no indication of Hox hydrogenase contribution due to its strong transcriptional repression (Santana-Sánchez et al., 2023). Here, we further reinforced this conclusion by demonstrating that long-term H_2 photoproduction in Δ*flv3A* is strongly dependent on the nitrogenase capacity to allocate electrons either to H_2 production (Ar +1% CO_2) or N_2 fixation (N_2 + 1% CO_2), regardless of the Hox hydrogenase

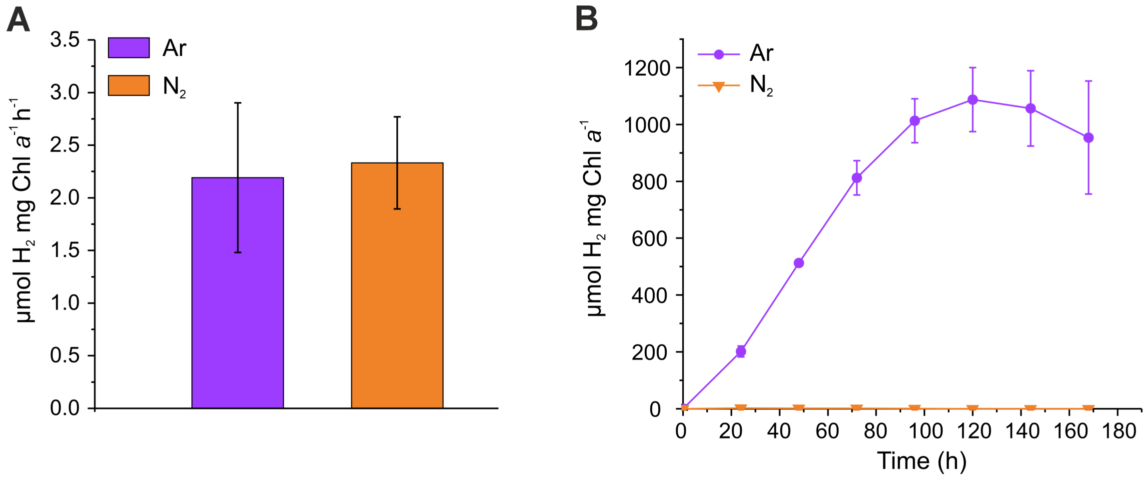

FIGURE 3 Long-term H_2 photoproduction and hydrogenase activity of Δ*flv3A* mutant under Ar and N_2 atmosphere. Hydrogenase activity on the fifth day of incubation (A) and H_2 photoproduction yields over 7 days (B). The error bars represent the standard deviation between 2-3 independent biological replicates.

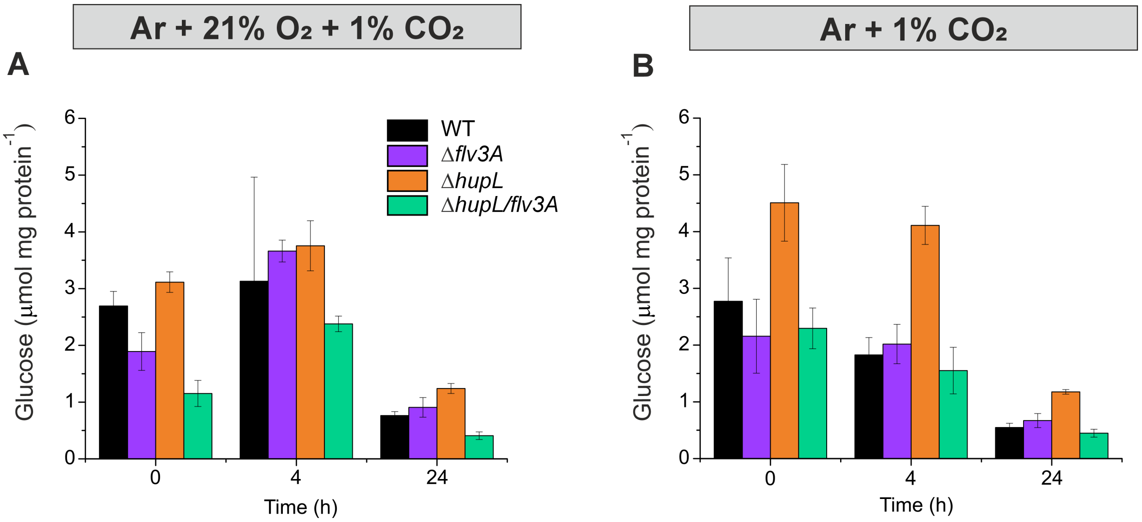

FIGURE 4 Changes of glycogen content during H_2 production. Glycogen content under (A) oxic (Ar + 21% O_2) and (B) micro-oxic (Ar) conditions supplemented with 1% CO_2. Glycogen contents are expressed in glucose equivalents per mg of protein. Data are presented as data ± standard deviations ($n = 3$).

activity (Figure 3). Previously, diazotrophic Δ*flv3A* filaments grown under air +1% CO_2 showed a H_2 photoproduction rate of ~6 µmol H_2 (mg Chl *a*)$^{-1}$ h^{-1} during 5 min of illumination with 500 µmol photons m^{-2} s^{-1} (Santana-Sánchez et al., 2023). The lower H_2 productivities (~2 µmol H_2 (mg Chl *a*)$^{-1}$ h^{-1}) presented here under similar HC conditions (Figure 1B) might be due to the lower light intensity applied to the cultures (120 µmol photons m^{-2} s^{-1}), as irradiance was shown to strongly affect the nitrogenase-mediated H_2 production (Allahverdiyeva et al., 2010; Marques et al., 2011).

The sole deletion of *flv3A* in diazotrophic *Anabaena* resulted in about a 1.3-fold increase in the H_2 photoproduction yield after three days of incubation under air +1% CO_2 atmosphere in comparison to the Δ*hupL* (Figure 1A). The analysis of the H_2 photoproduction kinetics under those conditions revealed that rather than reaching a high rate followed by a sharp decline, as observed in Δ*hupL* and Δ*hupL/flv3A*, the Δ*flv3A* filaments were able to sustain H_2 photoproduction during the first 2 days of incubation (Figure 1B). These results suggest that, besides the repression of *hupL*, the deletion of *flv3A* may have further metabolic consequences favoring a moderate and prolonged H_2 photoproduction mediated by nitrogenase under air conditions.

In the absence of N_2, nitrogenase allocates all electrons to the production of H_2, thus functioning as an ATP-powered hydrogenase, which requires only 4 ATP per H_2 produced (Bothe et al., 2010). Previous studies have used the production of H_2 in the absence of N_2 as a measure of total electron flow to nitrogenase, also known as the total *in vivo* nitrogenase activity (Schubert and Evans, 1976; Wilson et al., 2021). In this study, we show that when abolishing N_2 fixation under oxic conditions (Ar +21% O_2), the total nitrogenase activity in the Δ*flv3A* mutant catalyzed a significant increase of the H_2 photoproduction rate over a period of 3 days (achieving a peak 4.5-fold higher than that observed under air supplemented with 1% CO_2), before gradually declining (Figure 1D). In contrast, redirecting electrons from N_2 fixation to H_2 photoproduction in the Δ*hupL* mutant resulted in a maximum H_2 photoproduction rate that was only 2.8-fold higher than that under air (+1% CO_2). This rate then remained relatively stable throughout the long-term experiments. The distinct positive effects of removing N_2 from the atmosphere on the H_2 photoproduction in the Δ*flv3A* and Δ*hupL* mutants might be related to differences in nitrogenase efficiency in allocating electrons to either N_2 fixation or H_2 production. Yet, the factors regulating this electron allocation are not fully understood and seem to vary in response to stress conditions such as high light and CO_2 deprivation (Lindberg et al., 2004).

Our results indicate that excess O_2 affects the H_2 photoproduction of Δ*hupL* and Δ*flv3A* differently. While minimization of the inhibitory effect of O_2 (under Ar) did not further improve the maximum rate of H_2 photoproduction exhibited by Δ*flv3A* filaments compared to oxic conditions (Ar +21% O_2), the Δ*hupL* mutant showed a 3.5-fold increase (Figure 1D, F). Analysis of the nitrogenase activity by acetylene reduction assay under micro-oxic conditions (Figure 2B) revealed that the significant enhancement of the H_2 photoproduction in Δ*hupL* mutant (Figure 1F) could be attributed to the strong induction of nitrogenase activity, as both processes follow similar kinetics over the long-term experiments. It is possible that the nitrogenase activity of Δ*hupL* was enhanced by the increased supply of sugars from vegetative cells containing high levels of glycogen (Figure 4B). Yet a direct correlation between glycogen content and reductant availability for nitrogenase still needs to be clarified to support this hypothesis.

Micro-oxic cultures of both Δ*flv3A* and Δ*hupL* mutants accumulated photosynthetically produced O_2, compared to WT filaments (Figure 2A). This phenotype can be partially explained by the loss of Hup-dependent O_2 consumption g activity in both mutants (Bothe et al., 2010). Although previous short-term experiments under oxic conditions showed no significant changes in net photosynthetic O_2 production in Δ*flv3A* or Δ*hupL* (Santana-Sánchez et al., 2023; Lindberg et al., 2004), we cannot discard the possibility of adjustments in the

O_2 exchange during the long-term micro-oxic conditions tested in this study. Under this condition, the Δ*hupL* strain showed a strong decline in H_2 photoproduction after 48 h, likely due to O_2 inhibition of nitrogenase (Figure 2B). This is in line with previous studies showing high O_2 sensitivity of the Δ*hupL* filaments under similar micro-oxic conditions supplemented with 6% CO_2 (Kosourov et al., 2014). Interestingly, despite the strong repression of *hupL*, the Δ*flv3A* mutant was able to maintain a high rate of H_2 photoproduction during long-term incubation under micro-oxic conditions (Figure 1F) without altering nitrogenase activity (Figure 2B) or glycogen content (Figure 4B). These results suggest that the inactivation of Flv3A reduces the adverse effect of O_2 on H_2 photoproduction mediated by nitrogenase, likely *via* a yet-uncharacterized regulatory mechanism.

Despite quite efficient H_2 photoproduction by Δ*flv3A* mutant under oxic environment (Ar +21% O_2), it took almost 72 h for this strain to reach the rate observed in micro-oxic filaments already on the first day. This data could suggest that under oxic conditions, Δ*flv3A* prioritizes lowering the O_2 concentration inside heterocysts by diverting reducing equivalents obtained from the oxidation of sugars (*via* the OPP pathway) towards, for instance, respiratory terminal oxidases and Flv3B-mediated Mehler-like reaction (Magnuson, 2019; Ermakova et al., 2014). This adjustment in the distribution of reductants might not necessarily be accompanied by an increased accumulation of sugars (Figure 4A). The induction of respiratory-derived energy supply to nitrogenase might explain the gradual increase of the H_2 photoproduction rate under oxic conditions without N_2. Nevertheless, this metabolic adjustment in Δ*flv3A* could not be sustained longer than 72 h, likely due to another limiting factor, such as ATP availability (Lindberg et al., 2004). In contrast, Δ*hupL* strains were able to readily supply enough energy and reductants to reach the maximum activity of nitrogenase during the first 48 h under oxic conditions in the absence of N_2 (Figure 1D). Under this condition, the sole deletion of *hupL* allows a constant flow of reducing power to sustain a relatively stable nitrogenase activity during the long-term process (Figure 1D). Interestingly, the Flv3A-mediated metabolic processes in vegetative cells seem to play an important role in this phenotype, as the deletion of *flv3A* in the Δ*hupL* background significantly diminished the H_2 photoproduction yield observed in Δ*hupL* (Figure 1D).

5 | CONCLUSION

Overall, this study examines the impact of deleting the vegetative cell-specific Flv3A on long-term H_2 photoproduction under oxic and micro-oxic conditions. Our results suggest that the absence of a Flv3A positively impacts H_2 photoproduction by reducing the negative effect of O_2, while simultaneously downregulating *hupL* expression *via* an unknown regulatory mechanism. In contrast to Δ*hupL*, H_2 photoproduction in Δ*flv3A* did not benefit from increased nitrogenase activity or higher accumulation of sugars in the form of glycogen. Interestingly, beyond its contribution to the Mehler-like reaction, Flv3A may play an important role in creating optimal conditions to sustain H_2 photoproduction of Δ*hupL* filaments under oxic conditions without N_2. Further analysis of the metabolic and structural consequences of *flv3A* deletion is necessary to identify both the strengths and bottlenecks that could guide the development of future photobiological H_2 production strategies.

AUTHOR CONTRIBUTIONS

Meilin He: Methodology, investigation, validation, writing – original draft. Anita Santana-Sánchez: Investigation, writing – original draft, review and editing, visualization. Gábor Szilveszter Tóth: Investigation, writing – review and editing, visualization. Maria Ermakova: Methodology, investigation, writing – review and editing. Darius Collard: investigation. Sergey Kosourov: Methodology, investigation, supervision, writing – review and editing. Yagut Allahverdiyeva: Conceptualization, funding acquisition, supervision, writing – review and editing.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Allahverdiyeva Y., Ermakova M., Eisenhut M., Zhang P., Richaud P., Hagemann M., Cournac L., Aro E.-M. (2011) Interplay between flavodiiron proteins and photorespiration in *Synechocystis* sp. PCC 6803. *Journal of Biological Chemistry*, 286(27): 24007–24014.

Allahverdiyeva Y., Mustila H., Ermakova M., Bersanini L., Richaud P., Ajlani G., Battchikova N., Cournac L., Aro E.M. (2013) Flavodiiron proteins Flv1 and Flv3 enable cyanobacterial growth and photosynthesis under fluctuating light. *Proceedings of the National Academy of Sciences of the United States of America*, 110(10): 4111–4116.

Allahverdiyeva, Y., Isojärvi, J., Zhang, P., Aro, E.M. (2015) Cyanobacterial oxygenic photosynthesis is protected by flavodiiron proteins. *Life*, 5(1), pp.716–743.

Allahverdiyeva, Y., Leino, H., Saari, L., Fewer, D.P., Shunmugam, S., Sivonen, K., Aro, E.M. (2010) Screening for biohydrogen production by cyanobacteria isolated from the Baltic Sea and Finnish lakes. *International journal of hydrogen energy*, 35(3), pp.1117–1127.

Appel, J., Phunpruch, S., Steinmüller, K., Schulz, R. (2000) The bidirectional hydrogenase of Synechocystis sp. PCC 6803 works as an electron valve during photosynthesis. *Archives of microbiology*, 173(5), pp.333–338.

Bothe H., Schmitz O., Yates M.G., Newton W.E. (2010) Nitrogen fixation and hydrogen metabolism in cyanobacteria. *Microbiology and Molecular Biology Reviews*, 74(4): 529–551.

Burgstaller, H., Wang, Y., Caliebe, J., Hueren, V., Appel, J., Boehm, M., Leitzke, S., Theune, M., King, P.W., Gutekunst, K. (2022) Synechocystis sp. PCC 6803 Requires the Bidirectional Hydrogenase to Metabolize Glucose and Arginine Under Oxic Conditions. *Frontiers in Microbiology*, 13(NREL/JA-2700-82418).

Cai, Y. P., Wolk, C. P. (1990) Use of a conditionally lethal gene in Anabaena sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. *Journal of bacteriology*, 172: 3138–3145.

Carrieri, D., Wawrousek, K., Eckert, C., Yu, J., Maness, P.C. (2011) The role of the bidirectional hydrogenase in cyanobacteria. *Bioresource technology*, 102(18), pp.8368–8377.

Cournac L., Guedeney G., Peltier G., Vignais P.M. (2004) Sustained photoevolution of molecular hydrogen in a mutant of *Synechocystis* sp. strain PCC 6803 deficient in the type I NADPH-dehydrogenase complex. *Journal of Bacteriology*, 186(6): 1737–1746.

Ekman M., Ow S.Y., Holmqvist M., Zhang X., van Wagenen J., Wright P.C., Stensjo K. (2011) Metabolic adaptations in a H$_2$ producing heterocyst-forming cyanobacterium: potentials and implications for biological engineering. *Journal of Proteome Research*, 10(4): 1772–1784.

Ermakova M., Battchikova N., Allahverdiyeva Y., Aro E.M., 2013. Novel heterocyst-specific flavodiiron proteins in Anabaena sp. PCC 7120. *FEBS Letters*, 587(1): 82–87.

Ermakova M., Battchikova N., Richaud P., Leino H., Kosourov S., Isojarvi J., Peltier G., Flores E., Cournac L., Allahverdiyeva Y., Aro E.M. (2014) Heterocyst-specific flavodiiron protein Flv3B enables oxic diazotrophic growth of the filamentous cyanobacterium *Anabaena* sp. PCC 7120. *Proceedings of the National Academy of Sciences of the United States of America*, 111(30): 11205–11210.

Ernst, A., Kirschenlohr, H., Diez, J., Böger P. (1984) Glycogen content and nitrogenase activity in *Anabaena variabilis*. *Archives of Microbiology*, 140: 120–125.

Flores, E., López-Lozano, A., Herrero, A. (2015) Nitrogen fixation in the oxygenic phototrophic prokaryotes (cyanobacteria): the fight against oxygen. *Biological nitrogen fixation*, pp.879–890.

Happe T., Schütz K., Böhme H. (2000) Transcriptional and Mutational Analysis of the Uptake Hydrogenase of the Filamentous Cyanobacterium *Anabaena variabilis* ATCC 29413. *Journal of Bacteriology*, 182(6): 1624–1631.

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

King P.W., Posewitz M.C., Ghirardi M.L., Seibert M. (2006) Functional Studies of [FeFe] Hydrogenase Maturation in an Escherichia coli Biosynthetic System. *Journal of Bacteriology*, 188(6): pp. 2163–2172.

Kosourov S., Leino H., Murukesan G., Lynch F., Sivonen K., Tsygankov A.A., Aro E.M., Allahverdiyeva Y. (2014) Hydrogen photoproduction by immobilized N$_2$-fixing cyanobacteria: understanding the role of the uptake hydrogenase in the long-term process. *Applied and Environmental Microbiology*, 80(18): 5807–5817.

Kótai J. (1972) *Instructions for preparation of modified nutrient solution Z8 for algae*. publication B-11/69. Norwegian Institute for Water Research, Oslo, Norway.

Kourpa, K., Manarolaki, E., Lyratzakis, A., Strataki, V., Rupprecht, F., Langer, J.D., Tsiotis, G. (2019) Proteome analysis of enriched heterocysts from two hydrogenase mutants from Anabaena sp. PCC 7120. *Proteomics*, 19(19), p.1800332.

Leino H, Shunmugam S, Isojärvi J, Oliveira P, Mulo P, Saari L, Battchikova N, Sivonen K, Lindblad P, Aro E-M, Allahverdiyeva Y. (2014) Characterization of ten H2 producing cyanobacteria isolated from the Baltic Sea and Finnish lakes. *International Journal of Hydrogen Energy*, 39:8983–8991.

Leino, H., Kosourov, S.N., Saari, L., Sivonen, K., Tsygankov, A.A., Aro, E.M., Allahverdiyeva, Y. (2012) Extended H2 photoproduction by N2-fixing cyanobacteria immobilized in thin alginate films. *International journal of hydrogen energy*, 37(1), pp.151–161.

Lindberg, P., Lindblad, P., Cournac, L. (2004) Gas exchange in the filamentous cyanobacterium Nostoc punctiforme strain ATCC 29133 and its hydrogenase-deficient mutant strain NHM5. *Applied and Environmental Microbiology*, 70(4), pp.2137–2145.

Lu, Y., Koo, J. (2019) O2 sensitivity and H2 production activity of hydrogenases—A review. *Biotechnology and Bioengineering*, 116(11), pp.3124–3135.

Magnuson, A., 2019. Heterocyst thylakoid bioenergetics. *Life*, 9(1), p.13.

Mariscal, V., Flores, E. (2010) Multicellularity in a heterocyst-forming cyanobacterium: pathways for intercellular communication. In *Recent Advances in Phototrophic Prokaryotes*, pp. 123–135. Springer, New York, NY.

Marques, A.E., Barbosa, A.T., Jotta, J., Coelho, M.C., Tamagnini, P., Gouveia, L. (2011) Biohydrogen production by Anabaena sp. PCC 7120 wild-type and mutants under different conditions: light, nickel, propane, carbon dioxide and nitrogen. *Biomass and Bioenergy*, 35(10), pp.4426–4434.

Masukawa H., Mochimaru M., Sakurai H. (2002) 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. *Applied Microbiology and Biotechnology*, 58(5): 618–624.

Mikheeva L.E., Schmitz O., Shestakov S.V., Bothe H. (1995) Mutantsof the cyanobacterium Anabaena variabilis altered in hydrogenase activities. *Z. Naturforsch*, 50c:505–510.

Murukesan, G., Lynch, F., Allahverdiyeva, Y., Kosourov, S. (2019) Acclimation responses of immobilized N$_2$-fixing heterocystous cyanobacteria to long-term H$_2$ photoproduction conditions: carbon allocation, oxidative stress and carotenoid production. *Journal of Applied Phycology*, 31: 131–143.

Nyberg, M., Heidorn, T., Lindblad, P. (2015) Hydrogen production by the engineered cyanobacterial strain Nostoc PCC 7120 ΔhupW examined in a flat panel photobioreactor system. *Journal of Biotechnology*, 215, pp.35–43.

Ritchie, R.J. (2006) Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. *Photosynthesis Research*, 89: 27–41.

Roumezi B., Avilan L., Risoul V., Brugna M., Rabouille S., Latifi A. (2020) Overproduction of the Flv3B flavodiiron, enhances the photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Nostoc* PCC 7120. *Microbial Cell Factories*, 19(65): 1–10.

Sağır, E., Hallenbeck, P. C., 2019. Photofermentative Hydrogen Production, Editor(s): Ashok Pandey, S. Venkata Mohan, Jo-Shu Chang, Patrick C. Hallenbeck, Christian Larroche. In *Biomass, Biofuels, Biochemicals, Biohydrogen* (Second Edition). Elsevier, pp. 141–157, ISBN 9780444642035.

Santana-Sánchez, A., Nikkanen, L., Werner, E., Tóth, G., Ermakova, M., Kosourov, S., Walter, J., He, M., Aro, E.-M., Allahverdiyeva, Y. (2023) Flv3A facilitates O2 photoreduction and affects H2 photoproduction independently of Flv1A in diazotrophic *Anabaena* filaments. *New Phytologist*. 237: 126–139.

Santana-Sánchez, A., Solymosi, D., Mustila, H., Bersanini, L., Aro, E. M., Allahverdiyeva, Y. (2019) Flavodiiron proteins 1–to-4 function in versatile combinations in O$_2$ photoreduction in cyanobacteria. *Elife*. 8, e45766.

Schubert, K.R., Evans, H.J. (1976). Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. *Proceedings of the National Academy of Sciences*, 73(4), pp.1207–1211.

Song, H., Luo, S., Huang, H., Deng, B., Ye, J. (2022). Solar-Driven Hydrogen Production: Recent Advances, Challenges, and Future Perspectives. *ACS Energy Letters*, 7(3), pp.1043–1065.

Tamagnini P., Leitão E., Oliveira P., Ferreira D., Pinto F., Harris D.J., Heidorn T., Lindblad P. (2007). Cyanobacterial hydrogenases: diversity, regulation and applications. *FEMS microbiology reviews*, 31(6): 692–720.

Troshina, O., Serebryakova, L., Sheremetieva, M., Lindblad, P. (2002). Production of H2 by the unicellular cyanobacterium Gloeocapsa alpicola CALU 743 during fermentation. *International Journal of Hydrogen Energy*, 27(11–12), pp.1283–1289.

Volgusheva, A., Kosourov, S., Lynch, F., Allahverdiyeva, Y. (2019) Immobilized heterocysts as microbial factories for sustainable nitrogen fixation. *Journal of Biotechnology*, 306: 100016.

Wilson, S.T., Caffin, M., White, A.E., Karl, D.M. (2021) Evaluation of argon-induced hydrogen production as a method to measure nitrogen fixation by cyanobacteria. *Journal of Phycology*, 57(3), pp.863–873.

Yoshino F., Ikeda H., Masukawa H., Sakurai H. (2007) High photobiological hydrogen production activity of a Nostoc sp. PCC 7422 uptake hydrogenase-deficient mutant with high nitrogenase activity. *Marine Biotechnology (NY)*, 9(1): 101–112.

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