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Hydrogen Production in the Cyanobacterium *Synechocystis* sp. PCC 6803 with Engineered Subunit of the Bidirectional H_2-ase

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Abstract

Hydrogenase (H_2-ase) enzyme holds great promise as a bio-generator for bio-solar hydrogen (H_2) production. Consequently, an oxygen-tolerant H_2-ase is needed in a photosynthetic organism. In this work, a mutant strain of *Synechocystis* sp. PCC 6803 with modified H_2-ase analyzed under various physiological conditions. The growth rate was higher than that of wt strain and cellular capacity to fix carbon was increased, as shown by higher glycogen accumulation. Oxygen evolution by mutant strain in chemostats was higher than by wt cells over a range of pH levels. The mutant displayed significantly higher hydrogen (H_2) production than wt cells, especially at high pH. Examinations of electron flow pathways in the presence of various inhibitors indicated that the genetically modified H_2-ase apparently behaves similarly to the wt with respect to its electron source. Remarkably, it was consistently observed H_2 production under continuous light conditions, in the presence of oxygen (O_2), under many circumstances in both chemostat and batch tests. H_2 production in the light was improved under alkaline pH in mutant strain than wt. The data suggest that the genetically modified hydrogenase (H_2ase) is a functionally active. Several lines of evidence suggest that O_2 may be important in draining electrons from the donor side of photosystem I (PSI) in turn increases the linear electron flow and thereby helping to feed the H_2ase activity. In conclusion, the bidirectional H_2ase in *Synechocystis* may play a critical role in cell physiology not only under anoxic conditions but also under O_2-evolving activity.

Keywords: *Cyanobacteria, Hydrogen, Hydrogenase, Protein engineering.*

1. Introduction

Biosolar hydrogen (H_2), produced via direct biophotolysis of water by cyanobacteria or green algae, is attractive as a renewable energy carrier (Ananyev et al., 2008; Antal et al., 2006; Benemann, 2000). Cyanobacteria are a large, important, and promising group of autotrophic bacteria for hydrogen (H_2) production. They perform oxygenic photosynthesis using water as an electron donor and are found in almost every ecological niche. They have evolved many means for adapting to the varying environmental conditions they face every day, and which in turn impact their quantum efficiency (and utility for H_2 production). Among these, *Synechocystis* sp. PCC 6803, the first cyanobacterial species with a sequenced genome (Kaneko et al., 1996), can grow under photoautotrophic, photomixotrophic, and heterotrophic growth conditions, and can produce H_2 by direct and/or indirect biophotolysis (Dutta et al., 2005; Ghirardi et al., 2000 and 2007; Levin et al., 2004; Kruse et al., 2005; Sakurai and Masulawa, 2007). In *Synechocystis* sp. PCC 6803, H_2 can be produced by a bidirectional Ni-Fe hydrogenase (Appel et al., 2000; Dutta et al., 2005; Ghirardi et al., 2000), a heteropentameric complex with a diaphorase moiety comprised of three subunits (HoxE, HoxF and HoxU) and with a hydrogenase moiety comprised of a small subunit (HoxY) and a large subunit (HoxH) containing the active site (Albracht, 1994; Appel and Schulz, 1996; Burgdorf et al., 2005). The bidirectional Ni-Fe hydrogenase enzyme is sensitive to O_2 (Appel and Schulz, 1996; Madamwar et al., 2000) with H_2 production only reported under anoxic conditions, and is NAD(P)H dependent (Vignais and Colbeau, 2004 and 2007). M55, an NDH-1 deficient mutant, has been shown to produce more H_2 than wild type (wt) cells (Cournac et al., 2004), perhaps because of decreased oxygen evolution associated with lower photosystem II (PSII) activity and/or greater NAD(P)H availability due to the nonfunctional NDH-1 complex.

Synechocystis sp. PCC 6803 can make H_2 by means of its Ni-Fe hydrogenase (H_2ase) enzyme, but the role of this enzyme in the cell's overall metabolism is not totally clear. It has been suggested that the Ni-Fe H_2ase acts as an electron valve during photosynthesis in *Synechocystis* sp. PCC 6803, based on observations that a ΔhoxH mutant was impaired in the oxidation of PSI, had higher fluorescence of PSII, and had different transcript levels of the photosynthetic genes, *psbA*, *psaA* and *petB*, when compared with wild type (wt) cells (Appel et al., 2000). The enzyme has also been proposed to play a role in fermentation, functioning as a mediator in the release of excess reductant under anaerobic conditions (Stal et al., 1997; Troshina et al., 2000). The Ni-Fe H_2ase in *Synechocystis*

sp. PCC 6803 is insensitive to light, reversibly inactivated by O_2, can be quickly reactivated by NADH or NADPH (Cournac et al., 2004) and is constitutively expressed in the presence of O_2 (Appel et al., 2000). It is proposed to play a role mainly under anaerobic or microaerobic conditions and at the onset of exposure to light, before the enzyme is inactivated by photosynthetic O_2.

In *Synechocystis* sp. PCC 6803, H_2 production has been reported only under anoxic conditions (Appel and Schulz, 1996), purportedly because of O_2 sensitivity of the Ni-Fe H_2ase (Appel and Schulz, 1996, Madamwar et al., 2000). Therefore, one urgent task for biosolar H_2 production by cyanobacteria has been to decrease O_2 sensitivity of the Ni-Fe H_2ase. Within the diverse group of bidirectional Ni-Fe H_2ase enzymes, there are known to be six sets of highly conserved amino acid sequences, identified as regions L0 to L5 (Long et al., 2007). The Cx_2Cx_3H conserved residues present in the L3 region, arginine residue in the L4 region, and the cysteine residues (Cx_2Cx_2H) in the L5 region of the C-terminus are directly involved in coordination of the active site of the Ni-Fe H_2ase. Ni-Fe H_2ases have been found in a wide variety of bacteria, with considerable variation in the sizes of the subunits (Vignais and Colbeau, 2004 and 2007). In this work, a dramatic deletion in the C-terminus region of the HoxH subunit of the bidirectional hydrogenase was generated by inserting an antibiotic resistance cassette. A fully segregated mutant harboring the truncated HoxH subunit was evident by PCR.

2. Materials and Methods

2.1. Strain and Growth Conditions.

The *Synechocystis* sp. PCC 6803 wt, ndh1B-deficient mutant strain M55, and truncated-HoxH engineered mutant were grown in BG-11 medium supplemented with 100 μg of kanamycin ml^{-1} where necessary, 80 mM sodium bicarbonate, and 35 mM HEPES buffer under constant illumination of approximately 50 μE m^{-2} s^{-1} at 30ºC. The cells were harvested in mid log growth. Cell numbers and chlorophyll were estimated spectrophotometrically (Li and Sherman, 2002).*2.2. Deletion of the c-terminal of hoxH subunit of the bidirectional hydrogenase.*

The construction of the plasmid was as follow: the *hoxH* gene from *Synechocystis* sp. PCC 6803, including 300 bp downstream, was amplified by PCR using the forward primer (5'ATGTCTAAAACCATTGTTATCGATCCCGTTACCCG3') and the reverse primer (5' ACCGGTTTAATCCCGCTGGATGGA 3'). PCR product was cloned into the pGEM-T vector (Promega, San Luis, CA). *Sma* I was used to open the plasmid at 1130 bp after the start of the *hoxH* gene, yielding blunt ends that were treated with shrimp alkaline phosphatase. *Eco*R I was used to cut a kanamycin resistance cassette from the pUC-4 KIXX plasmid (Pharmacia, Uppsala, Sweden), the ends were blunted with DNA polymerase I (Klenow fragment), and the cassette was ligated with the opened *hoxH*-containing plasmid and cloned into *E. coli*. A plasmid containing the disrupted *hoxH* gene construct was used to transform *Synechocystis*. Segregation was achieved under ascending concentration of kanamycin, homoploidy was confirmed by PCR and the amplified gene was sequenced to confirm the location of the kanamycin cassette insertion (bp 1130) in the hoxH gene. Transformants were selected on BG-11 agar plates containing 50 mg kanamycin ml–1. Correct segregation of the transformants was checked by PCR.

*2.3. Type 1 NADPH-dehydrogenase strain of Synechocystis sp. PCC 6803 (M55).*The M55 strain used in this study is the same strain created by Teruo Ogawa. This strain is defected in the *ndhB* gene of the NDH-1 complex, M55 strain was used in this study due to its unique hydrogen production capabilities (Cournac et al., 2004).

2.4. High Throughput Screening Assay Measurements.

H_2 production from each culture was analyzed under a variety of conditions using a high throughput screening assay in 96-well microtiter plate format (Schrader et al., 2008). An aqueous solution containing the color indicator and catalyst overlays the H_2-producing culture, H_2 evolved by the cells diffuses up through the H_2-permeable barrier into the overlying solution, reacts with the catalyst and color indicator, and causes a visually detectable color change. In brief, H_2-producing cultures placed in the wells of a lower 96-well microtiter plate (1 mL; Nunc, Rochester, NY) with the indicator solution above it in a 96-well, membrane-bottom plate (0.2 μm PTFE filter plates, Pall, Ann Arbor, MI). The two plates sealed together and well-to-well crosstalk prevented with a molded silicone plate sealer (BioTech Solutions, Vineland, NJ) and a butyl rubber gasket, both perforated above each well. The top of the upper plate is sealed with an adhesive microtiter plate sealing mat (USA Scientific Titer Tops, Ocala, FL), butyl rubber gasket, and microtiter plate lid, thick pieces of Plexiglas clamped all together using 12 pieces of all-thread with locknuts on the bottom and wing nuts on the top.

Cells were grown and harvested as stated above, then re-suspended in the appropriate media. Optical density

measurement of cells was corrected by cell number due to the observed cell number variability among the strains used in this study; therefore, 10^9cells/well was consistently used from all strains regardless of their optical density measurement. Growth measurements of all strains after assay incubation time were almost insignificant. It seems likely that high cell density coupled with stagnant incubation growth conditions in the high through put screening assay almost arrested the growth. Although, the screening assay growth conditions is not near to physiological growth conditions for these strains, this conditions is favorable for hydrogen production.

2.5. Bioreactor Analysis.

Sterilized bioreactors (Applikon, Schiedam, The Netherlands) containing a liquid volume of 2 liters and total volume of 3 liters were inoculated with the respective cyanobacterial culture at an OD_{730} of 0.1. The reactors contained BG-11 medium, pH 7.5, supplemented with 100 μg ml^{-1} kanamycin where necessary, 80 mM sodium bicarbonate and 35 mM HEPES. They were continuously stirred at 700 rpm, illuminated with 175 μE m^{-2} s^{-1} PAR, and held at 30°C. The pH was controlled with 1 M HCl. Once the cultures reached an OD_{730} of 0.6, BG-11 medium, supplemented as stated above, was fed and wasted at a rate such as to maintain the OD_{730} of 0.6. The dissolved O_2 level was continuously monitored and the gas in the headspace was measured regularly for H_2, CO_2 and N_2 by a gas chromatograph (GC).

2.6. GC analysis of H_2.

In separate GC vial assays, cells were concentrated to $4x10^{10}$ cells mL^{-1} and 0.5 mL was transferred into a 4 mL, screw-top, glass GC vial. The vials were placed in a hypoxic glove bag with a nitrogen atmosphere (0.1% O_2, Coy Laboratory Products, Grass Lake, Michigan, USA) for 3 hours. Each vial was capped with a PTFE lined septa screw cap, inverted, and incubated under continuous light of 50 μE m^{-2} s^{-1} on a rotary shaker table at 30°C. Dark-treated samples were covered with aluminum foil and placed under exactly the same condition. H_2 buildup in the headspace of the GC vials was measured via 100 μL injections of headspace into a GC (Series 6890N, Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a thermal conductivity detector, using an airtight, glass syringe (VICI Valco Instruments Inc., Houston, TX, USA). The GC column was GS-CARBONPLOT 30 m x 0.32 mm x 3.0 mm (Cat. No. 113-3133, Agilent Technologies Inc., Santa Clara, CA, USA), the carrier gas was argon, the oven temperature was 35°C, and the detector temperature was 150°C. The column flow rate was 1.6 mL min^{-1}. An H_2 standard curve was used to calculate moles of H_2 from the reported peak areas.

2.7. Inhibitor tests.

Metabolic and electron carrier inhibitors were applied to the cultures of wt, M55, and engineered strains at the following concentrations in 96-well plates: 10 mM malonic acid (a succinate dehydrogenase inhibitor); 10 μM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea; blocks electron transfer from Photosystem II to cyt b6/f); 20 μM DBMIB (dibromomethylisopropyl benzoquinone; cyt b6/f inhibitor), 1 mM PCP (pentachlorophenol; cyt *bd* inhibitor); and 1 mM KCN (inhibits all terminal oxidases). All inhibitors were mixed with the cells in the 96-well, high-throughput screening assay plates, which were placed in a hypoxic glove bag with a nitrogen atmosphere (0.1% O_2, Coy Laboratory Products, Grass Lake, Michigan, USA) for 3 hours, then closed and incubated at 30°C with light/dark cycles of 8 min light/52 min dark for five days.

3. RESULTS

3.1. Segregation of the mutant.

In Fig. 1, a fully segregated mutant is created; the kanamycin cassette was inserted in the middle of L4, thereby terminating transcription of the remainder of the enzyme, including L5. The truncated HoxH subunit in the engineered mutant retains the Cx_2Cx_3H conserved residues present in the L3 region. However, the truncation removed both the arginine residue in the L4 region and the cysteine residues (Cx_2Cx_2H) in the L5 region of the C-terminus. These residues are proposed to form and directly involved in the active site of the Ni-Fe bidirectional hydrogenase (Fig. 2). Therefore, the truncated HoxH subunit introduces dramatic changes in two of the conserved regions, necessitating a critical reconfiguration of the suggested structure of the active site to maintain an active enzyme in this mutant.

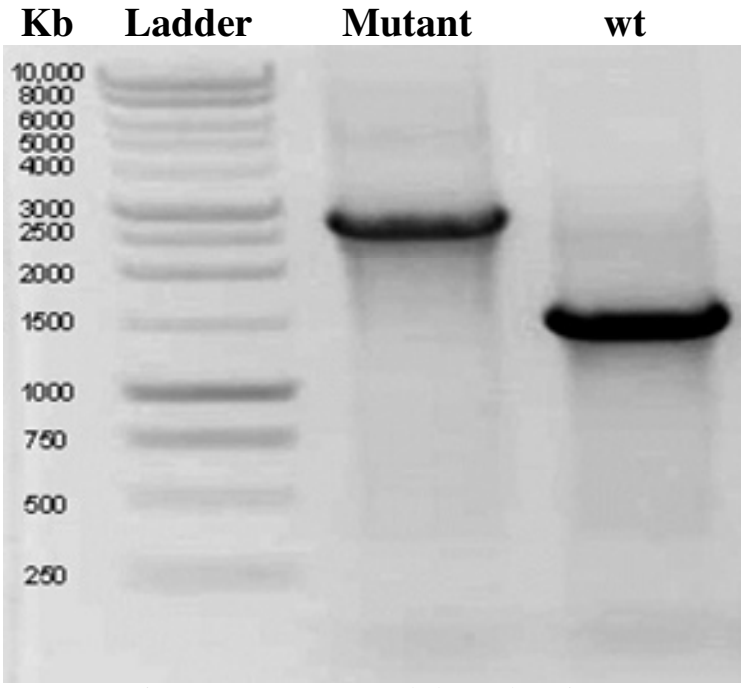

Figure 1. DNA agarose gel electrophoresis.

Image shows a fully segregated mutant compared to wild type hoxH genes, the same primers were used to amplify hoxH regions in mutant strain and wild type strain.

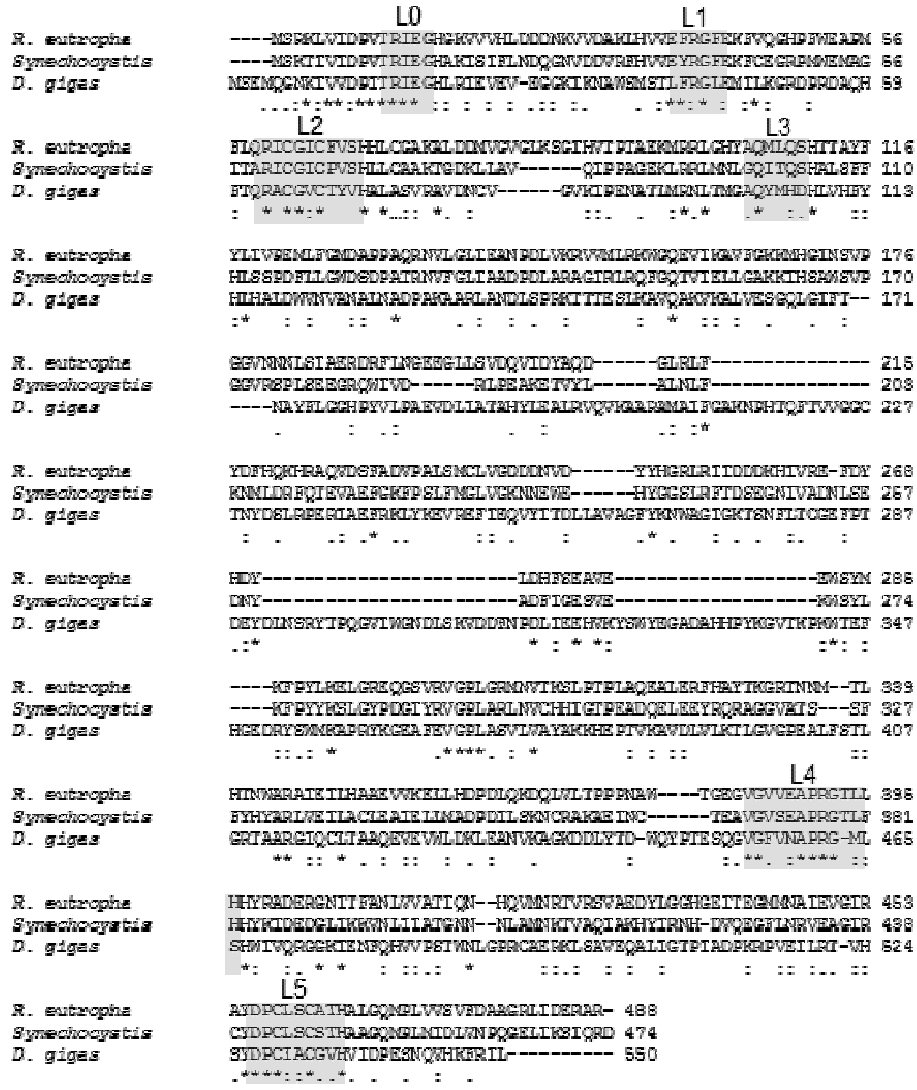

Figure 2. Amino acid sequence alignment.

Amino acid sequence comparison of the HoxH subunits in *Ralstonia eutropha, Synechocystis* sp. PCC 6803, and *Desulfovibrio gigas* (structurally characterized). The six highly conserved regions, L0-L5, are shaded.

3.2. Bioinformatics search.

The HoxH subunit has been reported to range from 471 aa to 507 aa, in comparison with the 374 aa of the truncated HoxH subunit in engineered strain. However, bioinformatic search has so far uncovered two interesting HoxH subunit open reading frames (RFs) smaller than the previously reported range (Fig. 3), a large H_2ase subunit from *Gloeocapsa alpicola* str. CALU 743 (Accession: AAO85440) and a HoxH subunit of *Anabaena siamensis* TISTR 8012 (Accession: AAN65267). Furthermore, in the purple sulfur bacterium, *Allochromatium vinosum*, which has two Ni-Fe H_2ases, one soluble and one membrane bound (19), hoxH subunit of the soluble hydrogenase is only 349 aa (Accession: AAX89152), provides an additional non-cyanobacterial sequence with shorter hoxH subunit similar to the truncated hoxH subunit of engineered strain. Indeed, the high amino acid residues identity between the engineered hoxH subunit and that naturally exist in *Gloeocapsa alpicola* str. CALU 743 and *Anabaena siamensis* TISTR 8012 (Fig. 3) suggests that two regions (L4 and L5) have no role in the active site as shown in Fig. 2.

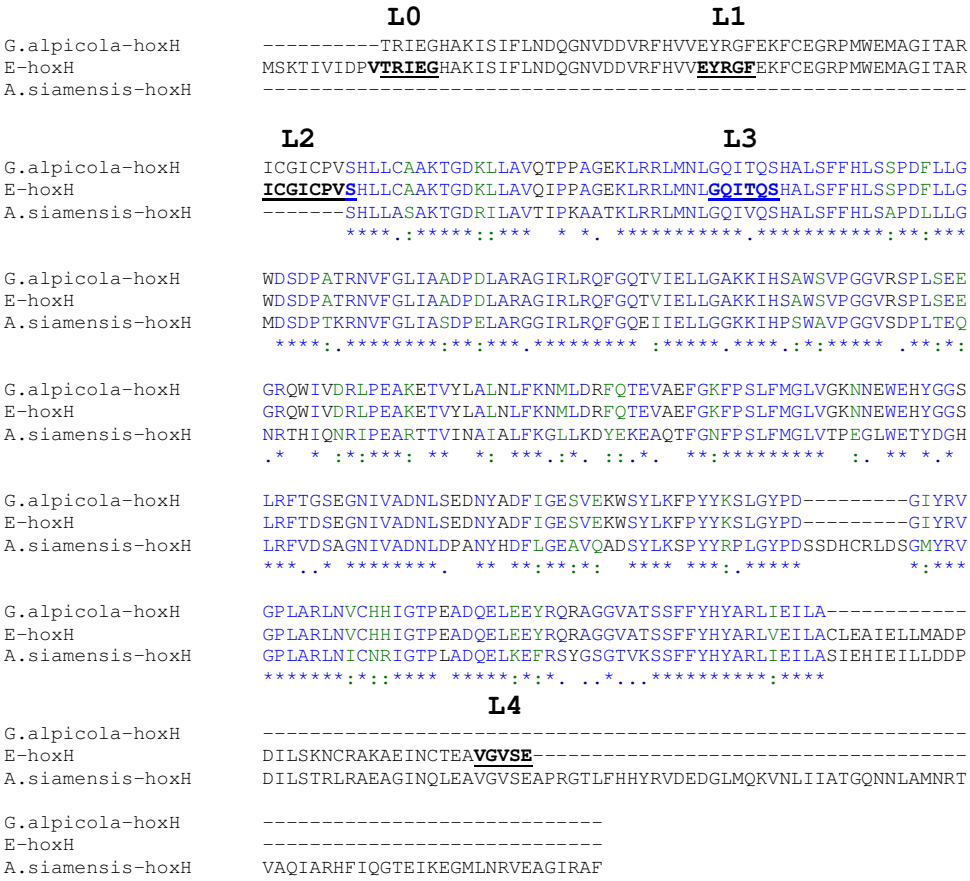

Figure 3. Amino acid sequence alignment.

G. alpicola HoxH (Accession: AAO85440), the truncated engineered hoxH subunit (E-hoxH), and the HoxH subunit of *A. siamensis* (Accession: AAN65267). Underline bolded amino acid residues in hoxH subunit of engineered strain show the conserved motifs (L0 – L5) compared to the structurally characterized proteins of *D. gigas* and *R. eutropha* (Fig. 2).

3.3. Growth and O_2 Evolution.

The growth of engineered strain was analyzed under different conditions to characterize its phenotype and to study the impact of the engineered subunit on the overall physiology of this strain (Table 1). Surprisingly, under regular light conditions (50 µE m^{-2} s^{-1}) engineered mutant showed a significantly higher growth rate than wt cells, especially under photoautotrophic conditions. However, engineered mutant grew slower at high light intensity (125 µE m^{-2} s^{-1}) than at regular light (50 µE m^{-2} s^{-1}). This may indicate that mutant's light conversion capacity is higher than that of the wt and that engineered mutant is more efficient in its carbon fixation capacity. However, under high light intensity engineered mutant's capacity for carbon fixation and/or its ability to cope with photoinhibition may have been significantly decreased, as compared to wt cells. To analyze this observation further, O_2 evolution by engineered mutant and wt cells grown in chemostats were monitored. The engineered mutant shows slightly greater O_2 evolution than wt at the preferred pH level (7.5), but much greater O_2 evolution when the pH was raised to 9.0 (with O_2 concentrations in both chemostats reaching levels of about three times atmospheric concentrations). This suggests that engineered mutant is more photosynthetically active than wt regardless of the pH. Also, higher O_2 evolution in engineered mutant not only supported higher growth rates but also led to storage of more glycogen in the cell, which was evident by higher accumulation of glycogen granules (discussed in more detail below).

Table 1. Growth rate expressed in doubling time (hour).

	50 µE m^{-2} s^{-1}		125 µE m^{-2} s^{-1}	
	Mixotrophic	Autotrophic	Mixotrophic	Autotrophic
Wild type strain	10.6 (0.34)	6.5 (0.071)	9.3 (0.083)	5.5 (0.051)
Engineered^{hoxH} strain	6.5 (0.036)	6.1 (0.043)	8.8 (0.118)	7.5 (0.212)

Doubling time recorded for wild type and the engineered hoxH subunit (E^{hoxH}) strain of *Synechocystis* sp. PCC 6803 under different growth conditions. Standard deviation values of the analyzed samples are in parenthesis.

3.4. Source of hydrogen in the engineered strain.

The presence of hydrogen in the truncated NiFe-H_2ase of engineered strain raises the question; whether H_2 source in engineered mutant is catalyzed by the bidirectional hydrogenase or there is an unknown hydrogen metabolizing enzyme(s) in present in *Synechocystis* sp. PCC 6803 that has/have not been described. Therefore, a mutant with N-terminal deletion in the hoxH gene (sll1226) will knock out the hoxH subunit protein and ensure non-functional bidirectional hydrogenase; it is unexpected to have a bidirectional hydrogenase without its active site containing subunit (Eckert et al., 2012; Pinto et al., 2012). This mutant provides a definite genetic evidence for the engineered mutant questionable hydrogen production. Indeed, hydrogen production in wt, engineered mutant and ΔhoxH strains (Fig. 4) clearly shows that engineered mutant produces significantly higher hydrogen compared to wt strain. However, hydrogen in ΔhoxH stain was observed in very low amount. It may be reasonable to say that hydrogen production profile in engineered mutant is similar to wt, where it follows the presence of abundant carbon source (glucose/glycogen). In ΔhoxH strain, hydrogen observed under autotrophic growth conditions in limited quantities (Fig. 4), but no hydrogen was observed in the presence of glucose. This data suggest that hydrogen production in engineered mutant is predominantly catalyzed by the bidirectional hydrogenase. The ΔhoxH strain provides conclusive genetic evidence that engineered mutant possess a functional bidirectional hydrogenase.

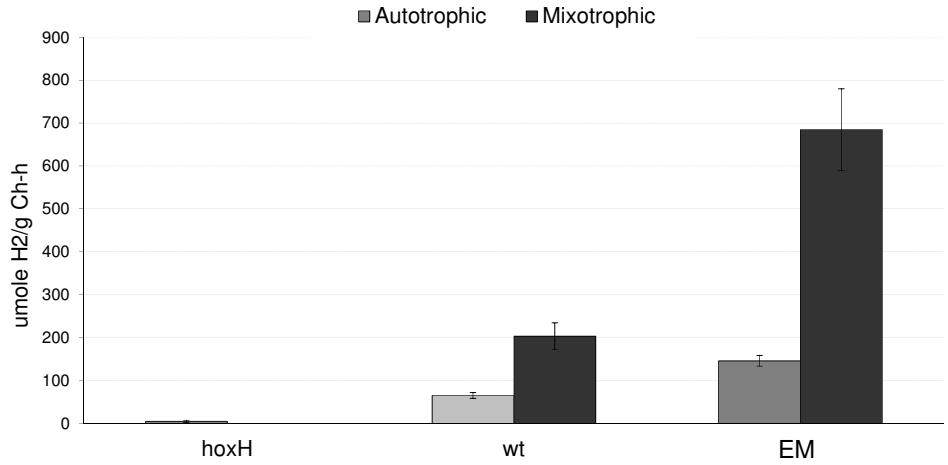

Figure 4. Hydrogen production by *Synechocystis* sp. PCC 6803 strains.

Compariosn of hydrogen production between wt, ΔhoxH and engineered mutant (EM), grown under different nutritional conditions, autotrophically (grey) and mixotrophically (black) with 5mM glucose under continuous white light of 50 uE m^{-2} s^{-1}, cells collected and incubated in 4-ml GC-vials for hydrogen measurements. In mixotrophic GC-sample, glucose was also added to the vials. pH 8 was maintained by 10mM TES buffer in BG11 medium. Error bars represent one standard deviation among triplicate samples.

3.5. Light and H_2 production.

In engineered mutant, a slight amount of H_2 production was observed under dark conditions, although essentially no H_2 was produced by either wt or M55. Increasing light exposure significantly increased H_2 production by engineered mutant and M55 but wt cells did not respond nearly so dramatically. Surprisingly, under continuous light exposure M55 produced H_2 at rates equivalent to those observed under light/dark cycling conditions and H_2 production by engineered mutant increased. The ability of engineered mutant to produce H_2 for five days under full dark conditions was unique, and may have been due to high levels of glycogen and/or polyhydroxyalkanoate carbon reserves. Electron photomicrographs showed clear evidence that engineered mutant accumulated significantly more glycogen granules than wt cells (data not shown), and glycogen measurements in bioreactor experiments with engineered mutant and M55 showed large amounts of glycogen in engineered mutant and none in M55 (data not shown). The presence of large amounts of carbon reserves can support H_2 production by indirect biophotolysis (Kruse et al., 2005). It is also possible that the truncation of HoxH in engineered mutant eliminated or greatly reduced its H_2 uptake capability, whereas H_2 uptake likely remained functional in wt and M55.

Increasing the nitrate concentration double the level in BG-11 media, did not affect H_2 production by wt; it may have caused H_2 production by both engineered mutant and M55 to decrease. A decrease in H_2 production could have been associated with a toxic effect and/or high electron consumption to reduce nitrate to ammonia, the usable form of nitrogen (8 moles of NADPH required per mole of nitrate reduced to ammonia). Indeed, redirecting the electron supply of the nitrate assimilation pathway increased H_2 production (Baebprasert et al., 2011; Osanai et al., 2013).

3.6. pH effect in Bioreactors.

Bioreactors were inoculated with wt or engineered mutant and placed under constant illumination. The amounts of H_2 in the headspace of the bioreactors over time were measured by injecting headspace samples into a GC. An identical control reactor was set up containing media only, with no cells. Over 8 days, no H_2 was measured in the control reactor headspace, indicating that the H_2 was produced biologically. At near-neutral pH (7.5) the wt cells produced slightly more H_2. However, when the pH was raised to 9.0, H_2 production by wt cells decreased substantially, perhaps due to a transitory shock effect, while that by engineered mutant was less adversely affected, consistent with observations from GC-vials experiments.

3.7. pH effect in GC vials.

To further analyze pH effects on engineered mutant, H_2 production was analyzed in GC vial batch experiments to compare it with bioreactor mode. Because of the direct effect of proton availability on electron flow and thereby hydrogen production, pH is expected to play a critical role in H_2 production. Therefore, H_2 production measured at three different pH levels: acidic (pH = 5), neutral (pH = 7), and alkaline (pH = 9) for wt and engineered mutant (Fig. 5). Engineered mutant showed a 26-fold increase in H_2 production when pH changed from either acidic or neutral to alkaline (pH 9), while H_2 production in wt changed only 5-fold (Fig. 5). These data are consistent with observations in the bioreactor experiments, confirming that pH effects were comparable in both batch and chemostat culture conditions.

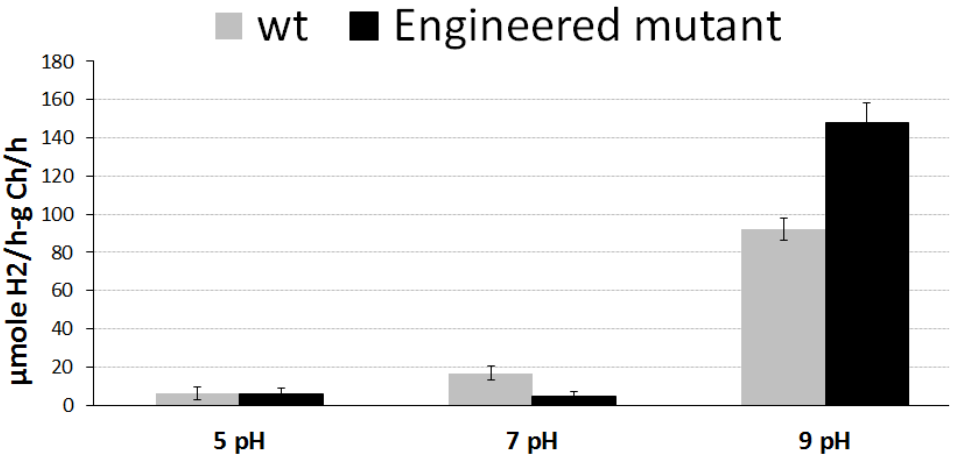

Figure 5. Effects of pH on hydrogen production.

Synechocystis sp. PCC 6803 wt and EM (engineered mutant) strains incubated in 4ml GC vials with 8 min light, 52 min dark. Error bars represent one standard deviation among triplicate samples.

3.8. Metabolic inhibitors and H_2 production.

The use of respiratory inhibitors to impede specific electron flow pathways, individually or in combination, can help to analyze interactions between the H_2ase and its source(s) of electrons. Therefore, the effects of several metabolic inhibitors on electron availability and electron flow pathways for H_2 production by wt, M55, and engineered mutant cells were examined. Figure 6 shows that inhibitor treated engineered mutant produced more H_2 than wt cells did in many cases and that the inhibitor response profiles of engineered mutant differed from those of wt and M55 cells in some ways. For example, addition of KCN caused H_2 production to increase greatly for all three strains, with engineered mutant showing the largest increase (Fig. 6A). This observation suggests that the engineered mutant H_2ase is similar to the wt (and M55) H_2ase regarding its source of electrons. However, H_2 production by engineered mutant decreased slightly when electron flow through succinate dehydrogenase (SDH) to the PQ pool was inhibited by malonic acid addition, while H_2 production by wt increased slightly and M55 H_2 production was not affected significantly (Fig. 6A). This could suggest greater dependence by the engineered mutant H_2ase on electron flow through SDH. All strains showed low levels of H_2 production in the presence of DCMU and DBMIB, indicating that H_2 production is greatly dependent on the flow of electrons through PSI for all three strains. Interestingly, Pentachlorophenol (PCP; Cyd inhibitor) did not increase H_2 production as expected. However, PCP in the presence of KCN and malonic acid increased H_2 production when compared to DCMU/KCN/M and DBMIB/KCN/M effects (Fig. 6B).

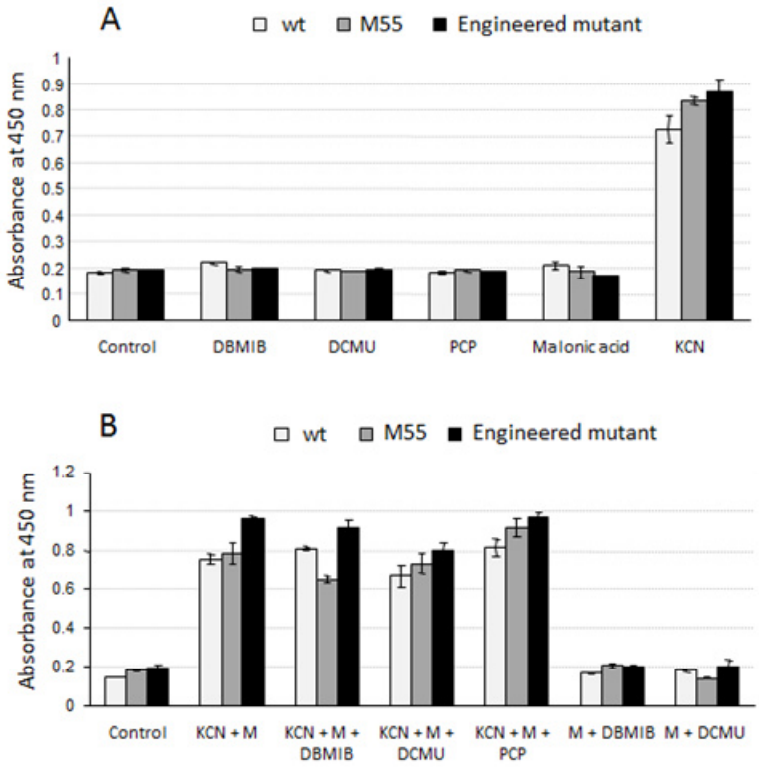

Figure 6. Effects of selected metabolic inhibitors on hydrogen production.

Compariosn between wt, M55, and engineered strains, inhibitors concentration were 10 mM malonic acid as a succinate dehydrogenase inhibitor; 10µM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea; as a photosystem II inhibitor); 20 µM DBMIB (dibromomethylisopropyl benzoquinone; as a cytochrome b6/f complex inhibitor); 1mM PCP (pentachlorophenol; as a quinol oxidase (cytochrome *bd*) inhibitor); 1 mM KCN as a cytochrome oxidase inhibitor.

4. Discussion

Under photoautotrophic conditions, photosynthesis is the main source of electrons for the electron transport chain and thus the Ni-Fe H_2ase. In spite of the fact that O_2 is known to inhibit the Ni-Fe H_2ase (Appel and Schulz, 1996; Cournac et al., 2004), both wt and engineered mutant showed consistent H_2 production under continuous light with measurable amounts of oxygen. Engineered mutant typically produced more H_2 than wt cells, especially at high pH, which correlated with higher O_2 concentrations accumulated in the bioreactor headspace. Steady state levels of CO_2 in the engineered mutant and wt bioreactors were identical (data not shown), indicating that their respective rates of respiration stayed the same over the course of experiment. Taken together, these data imply that higher O_2 evolution in engineered mutant is due to increased PSII activity and a greater supply of electrons, which may raise glycogen accumulation and in turn increase H_2 production by increasing electron flow through PSI from both direct and indirect biophotolysis pathways (Kruse et al., 2005). Interestingly, H_2 evolution by some algal strains under full illumination was reported by Skjanes et al. (2008), but the authors speculated that it was due to microanaerobic conditions in biofilms within their reactors. Our data provide strong evidence that H_2 can be produced under full illumination and in the presence of oxygen accumulated in the head space with no evidence of biofilm formation. Furthermore, the chemostat culture mode in the bioreactor provides optimum, controlled conditions for the cells to grow and stay photosynthetically active. These data are fully supported with H_2-ase characteristics of *Gloeocapsa alpicola* str. CALU 743. In this species hoxH subunite is over 65% identical to the engineered mutant. Furthermore, hydrogen production increased considerably under aerobic growth conditions without increase in the transcript level of the hoxH subunit (Sheremetieva et al., 2002). These observations add conclusive evidences that the engineered hoxH subunit is functionally active and is O_2-insenstive.

Engineered mutant, with the new chimera of the Ni-Fe H_2ase, clearly is directly or indirectly more adapted to higher pH than wt, and this feature allows the strain to produce more H_2 under high pH conditions. This directly correlates with the dramatic change in the HoxH subunit and partially explains the higher rates of H_2 production from engineered mutant observed in bioreactors and GC-vials (Fig. 5). At high pH, a hydroxyl group in the active site may provide O_2-tolerance and help keep the active site in the ready form (active H_2ase) (Long et al.,

2007), which has higher affinity for H_2 than for O_2 (George et al., 2004). This may provide a possible explanation for higher H_2 production in alkaline BG-11 medium.

Perhaps the truncated structure of the H_2ase in engineered mutant makes possible a mechanism to cope with the high pH levels that favor greater H_2 production. This has been shown in another enzyme, carbonic anhydrase, a carbon-concentrating enzyme that catalyzes the reversible hydration of carbon dioxide to form bicarbonate, a reaction required for many functions, including carbon assimilation and pH homeostasis (Raven, 2006). However, cellular pH homeostasis is a complex phenomenon intersecting with several other biological and bioenergetic processes, like protonation and deprotonation of many functionally critical molecules in the cell (e.g. tyrosine in PSII and phytochrome). Therefore, it is hard rule out the presence of a general pH effect that directly and/or indirectly improved hydrogen production in different strains of *Synechocystis* sp. PCC 6803. However, high pH provides an increasing advantage for hydrogen production in engineered mutant compared to wt *Synechocystis* sp. PCC 6803. Recently, hydrogen production in *Arthrospira maxima*, Ni-Fe H_2ase containing filamentous cyanobacteria, was seen to be improved significantly at pH 10.2 (Ananyev et al., 2008).

Surprisingly, the large, general increase in H_2 production in the presence of the cyt oxidase inhibitor (Fig. 6), KCN, is consistent with H_2 production observed under full light in the bioreactor. This result can be explained partially by O_2-controlled redistribution of electron fluxes between photosynthetic and respiratory chains (Berry et al., 2002). Terminal oxidases compete with the acceptor side of PSI for electrons from the plastoquinone pool ($PQH_2 \rightarrow$ cyd $\rightarrow O_2$) and cyt c_6 (cyt $c_6 \rightarrow$ cyt $aa_3 \rightarrow O_2$), thus decreasing electron availability for the reduction of PSI ($PQH_2 \rightarrow$ *bf* $\rightarrow$ Pc/cyt $c_6 \rightarrow$ PSI) (Fig. 7). In the presence of DCMU, *Synechocystis* sp. PCC 6803 retains electron flow to PSI, as well as functioning of respiratory electron transport chains donating electrons to the plastoquinone pool, e.g. through the NAD(P)H-dehydrogenase (NDH-1) complex (NADPH $\rightarrow$ NDH-1 $\rightarrow$ PQ$\rightarrow$ *bf* $\rightarrow$ Pc/cyt $c_6\rightarrow$ PSI) (Benemann, 2000). These data suggest that linear electron transport efficiency depends on the presence of O_2 in the cell, which is in line with previous reports (Berry et al., 2002; Howitt et al., 2001). Therefore, under O_2-deficient conditions, the efflux of electrons from PSI becomes the rate-limiting step (Benemann, 2000; Howitt et al., 2001).

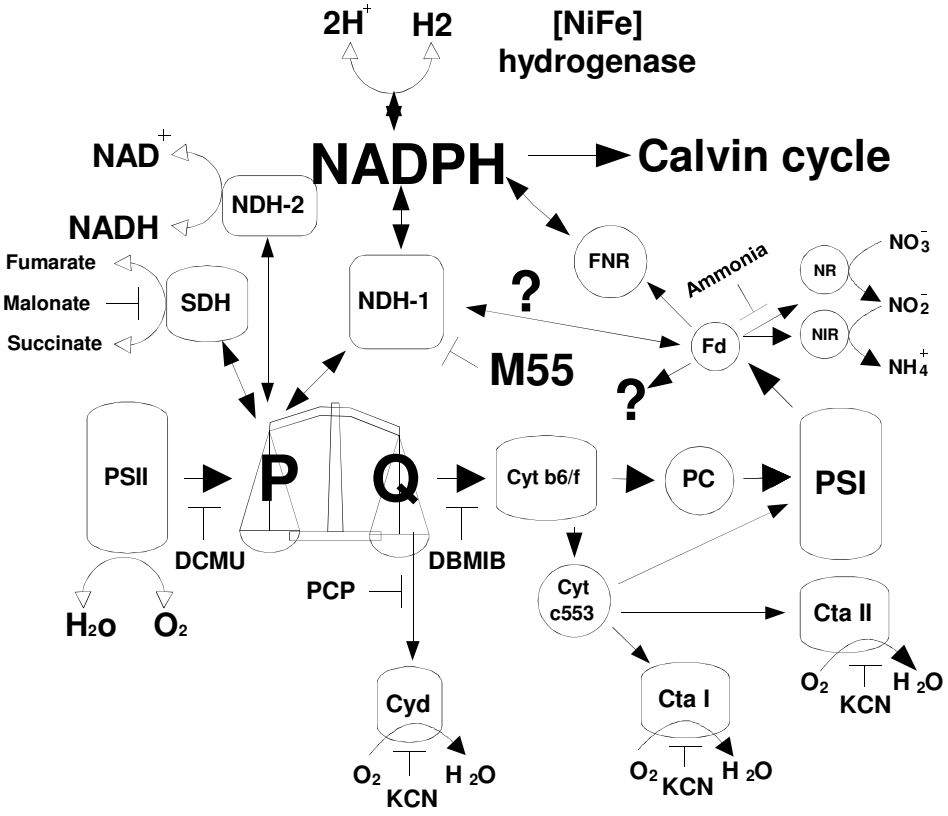

Figure 7. Photosynthetic and respiratory electron complexes in *Synechocystis* sp. PCC 6803.

The photosynthetic and respiratory electron carrier complexes that are known to interact with and/or in the thylakoid membrane of *Synechocystis* sp. PCC 6803 (adapted from Gutthann et al. 2007). Arrows indicate possible interactions between redox complexes. Abbreviations are PSII: photosystem II; PSI: photosystem I; Cyt b6/f: cytochrome b6/f complex; PQ: plastoquinone; Fd: ferredoxin; FNR: ferredoxin:NADP reductase, PC: plastocyanin, Cyt. c553: cytochrome *c*553, NR: nitrate reductase, NiR: nitrite reductase, cyt *bd*: quinol oxidase,

CtaI: cytochrome *c* oxidase and CtaII: alternative cytochrome *c* oxidase; NDH-1: NADPH-dehydrogenase (complex I); SDH: succinate dehydrogenase; NDH-2: type 2 NADH-dehydrogenase; Malonate (succinate dehydrogenase inhibitor); DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea; PSII inhibitor); DBMIB (dibromomethylisopropyl benzoquinone; b6/f inhibitor); PCP (pentachlorophenol; cyt *bd* inhibitor); KCN (potassium cyanide; cyt oxidase inhibitor).

The data in this study suggest a more complex role of O_2 in H_2 production than has been described previously. First, it is well known that O_2 can inactivate the Ni-Fe H_2ase by reversibly binding at the active site. Also, however, it has been shown that a sufficiently high presence of reducing power (NADPH) may overcome the inhibitory effect of O_2 on the Ni-Fe H_2ase, at least at low O_2 concentrations (Cournac et al., 2004). In our experiments, KCN alone or in combination with other inhibitors (DCMU, DBMIB, PCP and malonate) was able to increase H_2 production over levels observed in untreated cells (Fig. 6B). This data suggest that, under these conditions, respiration was the major electron sink, that interrupting this electron consuming pathway and redirecting the electrons was a successful strategy to increase H_2 production, and that the truncated HoxH H_2ase is coupled to the same electron donor as the native Ni-Fe H_2ase in wt cells.

In fact, the orchestrated regulation of cell metabolism implies that engineered mutant would have to coordinate many processes (carbon assimilation and pH homeostasis) in order to grow faster, evolve more O_2, and/or adapt to a high pH environment, as observed in our experiments. The observations with engineered mutant suggest that the bidirectional H_2ase may serve as more than an electron valve, as suggested by Appel et al. (2000). Flux of energy indicates that the cells benefit metabolically by producing some H_2 under full light conditions. Indeed, the data presented in this paper consistently show that H_2 production may be a constant output component of photosynthetic activity in *Synechocystis* sp. PCC 6803. Whether this postulate may be generalized to other cyanobacterial species is unknown. These experiments provide several lines of evidence that H_2 is sustainably produced under full light in chemostat culture (bioreactor), batch culture (GC vials) and the high throughput screening assay. However, data presented here suggested that hydrogen production in the engineered mutant is directly related to the truncated chimera of the Ni-Fe H_2ase regardless of any possible contribution by other, unknown hydrogen production pathway(s).

5. CONCLUSION

In this work, data provide evidence for an active, rationally engineered Ni-Fe H_2ase based on the truncation of the native HoxH subunit. Interestingly, this new H_2ase chimera altered the physiology of *Synechocystis* sp. PCC 6803 so that cells grew faster under regular light conditions, accumulated more glycogen, and evolved more H_2. The mutant strain showed a unique phenotype under a variety of growth conditions, with pH being a critical factor affecting H_2 production. This new chimera of the Ni-Fe H_2ase was catalytically more robust at high pH, with 26-fold more H_2 produced at pH 9 than at pH 5: 147 μmol H_2/g Chl/h versus 5.7 μmol H_2/g Chl/h, respectively. Finally, observations with the engineered mutant phenotype strongly suggest an important role of H_2 production in the lives of *Synechocystis* sp. PCC 6803 and perhaps other cyanobacterial species.

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