Metabolic Pathways for Photobiological Hydrogen Production by Nitrogenase- and Hydrogenase-Containing Unicellular Cyanobacteria *Cyanothece*

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*Running title: Photobiological H2 production by Cyanothece*

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Background: *Cyanothece* produces H2 catalyzed by hydrogenase or nitrogenase.

Results: Photo-H2 is nitrogenase mediated (via PSI) with reductant originating from catabolism and ATP from photophosphorylation.

Conclusion: Forcing additional ATP production by inhibiting NDH-2 increases the photo-H2 production rate at the expense of dark-H2.

Significance: Pathways for accelerating generation of ATP and reductant are identified as targets for further optimization of H2 yield by Cyanothece.

SUMMARY

Current biotechnological interest in nitrogen-fixing cyanobacteria stems from their robust respiration and capacity to produce hydrogen. Here we quantify both dark- and light- induced H2 effluxes by *Cyanothece* sp. Miami BG 043511 and establish their respective origins. Dark, anoxic H2 production occurs via hydrogenase utilizing reductant from glycolytic catabolism of carbohydrate (autofermentation). Photo-H2 is shown to occur via nitrogenase and requires illumination of PSI, while production of O2 by co-illumination of PSII is inhibitory to nitrogenase above a threshold pO2. Carbohydrate also serves as the major source of reductant for the PSI pathway mediated via non-photochemical reduction of the plastoquinone pool by NADH dehydrogenases type-1 and type-2 (NDH-1, NDH-2). Redirection of this reductant flux exclusively through the proton-coupled NDH-1 by inhibition of NDH-2 with flavone increases the photo-H2 production rate by 2-fold (at the expense of the dark-H2 rate), due to production of additional ATP (via the proton gradient). Comparison of photobiological hydrogen rates, yields, and energy conversion efficiencies reveals opportunities for improvement.

The biotechnological potential of photobiological hydrogen production by cyanobacteria and algae to renewable fuel production has not yet been realized. However, experimental and theoretical investigations of the maximum attainable rates (1), advances in culturing methods (2-3), enzyme manipulation (4-5), and metabolic engineering in model strains (6-8) have provided insight into the fundamental mechanisms and revealed new opportunities and limitations.

Biological hydrogen production can be mediated by either of two broad classes of enzymes, the hydrogenases or the nitrogenases (8-10). Both classes are O2 sensitive, requiring anoxic environments to function maximally (11). Here we shall describe hydrogen metabolism by *Cyanothece*, a genus of unicellular, aerobic, nitrogen-fixing (diazotrophic) cyanobacteria.
Unlike the heterocyst-forming diazotrophic cyanobacteria (e.g. Nostoc and Anabaena spp.) that spatially separate oxygen-evolving photosynthesis from oxygen-sensitive nitrogen fixation (11), *Cyanothece* performs strictly regulated temporal separation of photosynthesis and nitrogen fixation (12). This temporal separation ensures that an intracellular anoxic environment conducive to nitrogenase activity is maintained. Deactivation of photosynthesis and an increased cellular respiration are thought to maintain this anoxia (12-14), regulated by an intrinsic circadian rhythm (15-16), as even non-growing (stationary phase) cells exhibit this cycling (15, 17). Specifically, is has been shown in *Cyanothece* ATCC 51142 that one of the 4 copies of psbA, the gene encoding the D1 subunit of Photosystem II, is highly up-regulated throughout the dark phase of the circadian cycle. This isoform of D1 (psbA4) varies significantly from the isoforms expressed during the light phase, especially at the C terminal residues involved in binding the Mn-cluster and allowing O2 evolution (18). It is hypothesized that expression of this isoform and incorporation of the translated D1 protein into the reaction center would lead to a PSII core incapable of evolving O2. Expression of this isoform during the dark phase would allow the cell to retain a viable PSII quaternary framework so that an active D1 can quickly be re-incorporated during the subsequent light phase. Illumination of *Cyanothece* while this inactive D1 were expressed could afford photobiological H2 production by enzymes normally sensitive to O2. A homolog of this isoform (psbA4) is found in the draft genome of the *Cyanothece* strain studied herein.

The enzymatic reduction of dinitrogen to ammonia in *Cyanothece* is catalyzed by a Mo-nitrogenase, the most common and most efficient of the nitrogenase classes. Mo-nitrogenase (nitrogenase hereafter) requires 16 ATP and 8 electrons per N2 fixed, with 2 of these electrons diverted to the obligate reduction of H+ to H2. The observed ratio of proton reduction to nitrogen reduction by this class of enzyme is at least 25% (from the reaction stoichiometry) and at maximum 100% (in the absence of N2, where the nitrogenase functions like an ATP-powered hydrogenase, requiring 4 ATP per H2 produced) (7-8). Of note, the alternative nitrogenases (V-nitrogenase and Fe-nitrogenase) found in some strains naturally favor a higher ratio of proton reduction to nitrogen reduction. *Cyanothece* species accumulate carbohydrate, primarily in the form of glycogen granules, during the day and subsequently degrade this carbohydrate at night to provide the energy and reductant for nitrogenase function and O2 respiration (19). This natural diurnal cycling, efficient conversion of intracellular carbohydrate to energy, efficient respiration and intracellular anoxia, and presence of both classes of hydrogen producing enzymes makes *Cyanothece* species among the best candidates for H2 production (20-21).

*Cyanothece* sp. Miami BG 043511 is a marine strain that has been reported to possess both the bidirectional hydrogenase (Class 3d, bidirectional NAD-linked H2-ase) and nitrogenase, but to lack the membrane bound respiratory or “uptake” hydrogenase (Class 2a, cyanobacterial uptake hydrogenase) (22), typically found expressed alongside nitrogenase (23). We have, however, identified the complete gene coding for the uptake hydrogenase in the strain and have observed its transcription under both photoautotrophic and auto-fermentative conditions (Skizim & Krishnan, in preparation). We can therefore assert that all three hydrogen metabolizing enzymes found in cyanobacteria are present in this strain.

NADH and reduced ferredoxin (FDx) are the direct substrates for the bidirectional hydrogenases and nitrogenases, respectively, as established by in vitro assays of enzymes from multiple microorganisms (24-25). The NADH-dependent reduction of H+ to H2 by hydrogenase is often cited as thermodynamically unfavorable, as the standard potential at pH 7 for NAD+/NADH is -320 mV, compared to -420 mV for H+/H2. However, standard conditions refer to 1 bar H2 pressure, conditions that are never found in biological cells. By contrast, the calculated thermodynamic redox potential for H+/H2 is nearly identical to that of NAD+/NADH in a 0.1% H2 atmosphere (1000 ppm, or 0.001 bar) (26). Consequently, pyridine nucleotides can serve as efficient reductant sources under biological conditions, especially when H2 backpressure is minimized. In addition to NADH and FDx, NADPH can be utilized indirectly, either by the reduction of NADH catalyzed by the pyridine nucleotide transhydrogenase (if expressed and
active), or by the reduction of the plastoquinone (PQ) pool via NAD(P)H dehydrogenase and subsequent reduction of ferredoxin through excitation of PSI (Scheme 1).

Cyanobacteria are known to possess two NAD(P)H dehydrogenase (NDH) enzymes to exchange reductant between NAD(P)H and the lipid soluble PQ pool (27-29). NDH type-1 is homologous to Complex I of the respiratory chain of mitochondria and bacteria (the NAD(P)H:quinone oxidoreductase). Interestingly however, of the 14 minimal subunits that form the complex in E. coli, only 11 are found in the genomes of cyanobacteria. The missing subunits (genes nuoE, nuoF, and nuoG) encode the NDH dehydrogenase module, which in E. coli functions as the energy input device, and consequently the mechanism by which cyanobacteria use NAD(P)H via this enzyme is still unknown (30). Experimental evidence has verified that NADPH and NADH can both be oxidized by the complex in purified cyanobacterial membranes (29) and a variety of mechanisms for electron donation to the complex have been supposed; this remains an open question (30). NDH type-1 (NDH-1) is capable of oxidizing both NADH and NADPH and (of importance to the present study) translocates (pumps) protons upon each reducing equivalent (NAD(P)H) exchanged. NDH type-2 is comprised of a single subunit, does not pump protons, and can only oxidize NADH.

The contribution of dark (autofermentative) hydrogen production arising from either hydrogenase- or nitrogenase-mediated pathways has been largely ignored in cyanobacteria. We show herein that hydrogen production in Cyanothece sp. Miami BG 043511 can be of the dark, autofermentative type (primarily via hydrogenase), as well as light-induced (via nitrogenase). By utilizing monochromatic excitation sources and detection of dissolved hydrogen, we monitor the kinetics of hydrogen evolution from both light- and dark-pathways within the same experimental incubation, thus allowing visualization of the independent responses of each pathway (light and dark) to applied stresses. We show that both intracellular reductant and ATP availability are limiting factors for maximal photo-H₂ production, and that by increasing reductant availability via dark anaerobic incubation, or by channeling the flow of reductant through one of the specific NADH dehydrogenases to increase ATP availability, we can substantially increase the rate of photo-H₂ production.

EXPERIMENTAL PROCEDURES

Growth of Culture. Cyanothece sp. Miami BG 043511 was obtained from the University of Hawaii Culture Collection, where it was maintained as Synechococcus sp. Miami BG 043511 (many Cyanothece species were originally misclassified as Synechococcus (31)). Cultures were grown in ASP2 medium (32) without combined inorganic nitrogen at 30°C under diurnal conditions (12 hr light, 12 hr dark) with a light intensity of ~30 µE m⁻² s⁻¹. Cultures were not bubbled with any gases nor shaken.

Dissolved H₂ Rate Electrode & LED Illumination System. The first generation of our home-built H₂ microcell is described elsewhere (33). Our second (current) generation electrochemical microcell is also a reverse Clark-type electrode for measuring dissolved H₂ concentration (see Supporting Information). This 2nd generation microcell has an increased sensitivity of ~2 x 10⁻⁹ Coulomb H₂, fast response time (100 ms), and micro-volume sample chamber (6.5 µL). During measurement, H₂ is constantly consumed by oxidation at a Pt/Ir electrode, allowing the instantaneous rate of H₂ production to be measured. The microcell responds linearly to H₂ and performs for several months using an oversized Ag/AgCl reference electrode (~0.5 g AgCl) to consume the H⁺ product of H₂ oxidation. In this article, H₂ production rate is presented as the current (nA) arising from the oxidation of dissolved H₂. H₂ yield was determined by integration with respect to time to yield electrical charge, which was converted to moles of H₂ by Faraday’s second law. The microcell is equipped with light emitting diodes (LEDs) at wavelengths of 670±10 and 735±10 nm (FWHM), for exciting both Photosystems (670 nm) or PSI only (735 nm), respectively. The PAR intensity (400-700 nm) of the 670 nm LED at the sample is 1,010 µE and only 2.8 µE for the 735 nm LED. As a result, minimal excitation of PSII from the spectral tail of the 735 nm LED occurs, as confirmed by electrochemical O₂ measurements in another Clark-type cell.
Culture Incubation. Stationary phase cells were taken from photoautotrophic growth conditions between hours 10 and 11 after the onset of the light cycle (circadian “dawn”), concentrated (40x) by centrifugation, re-suspended in fresh media, and placed in the 6.5 µl sample chamber of the electrochemical cell. When biochemical inhibitors were added to the cultures, this was done five minutes prior to concentration and incubation on the hydrogen rate electrode. Inhibitors stocks were made with water or DMSO as solvent, not ethanol (exogenous ethanol may serve as a reductant source). The cell was covered by a quartz disc and removed from light, where cellular respiration created anoxia within a matter of seconds. Dissolved H₂ was continuously consumed as described above. When pulsed illumination was supplied to the culture, it was done using repeated pulses of 10s duration, separated by 90s dark time, i.e. 10% duty cycle.

Turbidity and Dry Weight Measurements.

Cell density was measured as turbidity by absorbance at 730 nm. Optical densities were measured spectrophotometrically on a Thermo Scientific Evolution 60 UV-Vis. Dry weights of cells were taken by filtering cells through Whatman GF/C glass microfiber filters (1.4 µm pore size) and drying the filters in an 80°C oven for 24 hours.

RESULTS

Differential excitation and inhibition of Photosystems I and II. The selective excitation of PSI (denoted 735-photo-H₂) and of PSII + PSI together (denoted 670-photo-H₂) is shown in Figure 1. Cells were first subjected to 12 hours of complete darkness and anoxia prior to the 120 minutes of pulsed illumination shown. The sharp rise in H₂ oxidation current corresponds to the 10s of illumination during each 100s light-dark cycle (repeated 72x). When both Photosystems are excited by 670 nm light (Fig 1A), the H₂ production arising from each successive flash gradually decreases, completely ceasing after approximately 100 minutes. H₂ production also gradually decreases but more slowly both when near-infrared 735 nm light (Fig 1C) is used (which only excites PSI) and when PSII is chemically inhibited (Fig 1B) with DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea). DCMU acts as a quinone analog, which binds to the Q₈ pocket of PSII and blocks the transfer of electrons from Qₐ (Scheme 1). The decrease in the absence of O₂ production must therefore be due to the depletion of accessible intracellular reductant, which is common to all three conditions. The faster decay seen with light that excites both PSI + PSII is expected due to the O₂ sensitivity of nitrogenase, and increases throughout the duration of the photoperiod, consistent with an intracellular buildup of O₂ from PSII. DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) blocks the plastoquinone binding site on cytochrome b₅f and therefore prevents the flow of electrons into PSI. When cells were treated with DMBIB (Fig 1D) and illuminated with light, photo-induced H₂ production is completely eliminated, indicating that the photo-H₂ signal is completely dependent on electron transfer through PSI. This illustrates that photo-H₂ is entirely nitrogenase mediated, as we would still expect to see 670-photo-H₂ via PSII (steps 1→2→3→4b→7 in Fig 1E) in the presence of DBMIB if hydrogenase were mediating the process, as PQH₂ oxidation by reverse electron flow through the NADH dehydrogenases is likely to occur (34-35). Further, the fact that H₂ yield does not decrease when PSII is inhibited (in fact, the average yield increases 2.35x ± 0.75 with DCMU and 2.5x ± 1.3 with 735 nm illumination, based on triplicate measurements) indicates that reductant from intracellular catabolism (glycogen) rather than water serves as the immediate electron source for photo-H₂ in this strain (steps 8→4→3→5→6).

Interestingly, the fact that we do observe photo-H₂ when 670 nm light is used confirms that Cyanothece strains are capable of maintaining lower levels of intracellular O₂ during the dark phase of their circadian cycle. This may be due to higher levels of respiration by this strain (though this would ‘steal’ electrons that could reduce protons to H₂), or the incorporation of an inactive D1 protein into PSII during dark periods (as described in the introduction). Alternatively, the possibility also exists that the nitrogenase found in Cyanothece exhibits a higher tolerance for O₂ and that it is this quality which allows it to produce H₂ in response to visible light.

Increased dark pre-incubation leads to increased photo-H₂. Figure 2 illustrates the effect of the dark, anaerobic pre-incubation period on 735-photo-H₂. As above, cells were taken from
photoautotrophic growth and placed in the sample chamber of the electrochemical cell. Dark periods (autofermentation) of 0.75, 1.5, 4, 8, 12, and 16 hours were allowed before the cells were exposed to three hours of pulsed illumination with 735 nm light. Figure 2A shows the rate of H₂ production measured for each of these experiments, and Figure 2B illustrates the yield of 735-photo-H₂ produced (as picomoles H₂) during each three-hour illumination window. (The integral of the production (as picomoles H₂) when cells were treated with flavone was 16.4 µmol H₂ gDW⁻¹ h⁻¹, or 2-fold higher than the control rate. This increase occurs at the expense of a 2.5-fold lower dark-H₂ production rate. These effects are evident in Figure 3B, where the cumulative yield of H₂ (both dark- and light-) is plotted as a function of time. These data reveal that the carbohydrate pool, which serves as precursor to cellular reductant for conversion to H₂, is shared between the photo-H₂ and dark-H₂ pathways, as the increased photo-H₂ is coupled to a decline in the dark-H₂ rate, a redirection of reductant between the pathways.

We also point out the decrease in dark-H₂ production in Figure 3 common to treatment with both flavone and rotenone. Because the rate of glycolysis is regulated by the cellular energy charge (ATP availability) as well as the NADH/NAD⁺ poise, it is reasonable to expect inhibitors that affect proton pumping and equilibration of the intracellular reductant pools to have upstream effects on glycolysis. The overall decrease in dark-H₂ production common to both inhibitors may well be due to a down-regulation of glycolysis in response to increased NAD(P)H which cannot reduce the plastoquinone pool due to the action of these inhibitors. There also may be some net flux of reductant out of the PQ pool (PQH₂ to NADH) leading to dark-H₂ production in the native system. When NDH-2 is inhibited with flavone, this reaction would have to proceed via NDH-1 (requiring ATP) and become unfavorable. We believe this explains the observation of decreased dark-H₂ prior to the first illumination period with flavone treatment.

In flavone-treated cells, the light saturated rate of 735-photo-H₂ production starts to decline after 60 minutes of pulsed illumination and drops to 50% of the initial rate after approximately 2-3 hours. This decrease in rate is common to control cells without flavone treatment as well, and is presumably due to the depletion of substrate for nitrogenase (reductant, protons, or ATP). Interestingly, in a similar experiment composed of a 20 hour anoxic incubation of a 5 mL sample in sealed glass vials under continuous saturating white light illumination (1.81 mW cm⁻² s⁻¹), only 15.7% of the total intracellular carbohydrate was depleted over the 20 hour incubation (same experiment length as rate data presented here). Therefore, there exists significant room to improve the light saturated 735-photo-H₂ rate, if the dark catabolic rate and subsequent generation of NADH could be enhanced.

Inhibition of the NADH dehydrogenases (Types 1 and 2). Figure 3 illustrates the effect on hydrogen production rate (A) and yield (B) when catabolically derived reductant is funneled to PQ reduction through each of the specific NADH dehydrogenases found in the strain. Figure 3A shows the rate of hydrogen production of (i) control cells, where both enzymes are active, (ii) cells treated with 50 µM flavone, where only NDH-1 is active, and (iii) cells treated with 20 µM rotenone, where only NDH-2 is active. Each condition was subjected to a 20-hr incubation with four pulse trains of saturating light at 735 nm (Fig. S2). The average 735-photo-H₂ production rate when cells were treated with flavone was 16.4 µmol H₂ gDW⁻¹ h⁻¹, or 2-fold higher than the control rate. This increase occurs at the expense of a 2.5-fold lower dark-H₂ production rate. These
**Decreased capacity for ATP Phosphorylation.** Two well-documented protonophores were used to collapse the proton gradient and uncouple photophosphorylation from photosynthetic electron transfer. Both 2,4-dinitrophenol (DNP) and carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) are ionophores that shuttle protons across biological membranes and were used to collapse the proton motive force responsible for generation of ATP via the F1F0 complex (36). Additionally, N,N'-dicyclohexylcarbodiimide (DCCD) was employed to directly inhibit ATP synthesis by blocking the flow of protons through the F0 channel and subsequently prevent phosphorylation of ADP by the F1 complex (37). The disruption of ATP synthesis and its effect on cellular energy charge by each of these inhibitors is shown in Figure 4. Figure 4A illustrates the cumulative yield of H2 produced by the cells (both dark-H2 and photo-H2) as a function of time (the white areas indicate the four 60-minute pulsed illumination periods, as before). Both FCCP (5 µM) and DNP (50 µM) increase the dark-H2 rate. This increase is attributed to an increased rate of carbohydrate catabolism to compensate for the loss of ATP and collapse of the proton gradient caused by the protonophores. The bidirectional hydrogenase is the expected enzymatic outlet for this increase in dark-H2 via NADH oxidation. By contrast, both protonophores decrease the photo-H2 production rate; the rate approaches zero at the end of each 1 hr pulse train. This decrease is attributed to the loss of ATP production by photophosphorylation, which is essential for nitrogenase-dependent photo-H2 production. This decrease is illustrated as well in Figure 4B, in which the average photo-H2 rates during the 1 hr illumination periods for each of the four pulse trains are shown. All three inhibitors initially decrease the observable photo-H2 by more than 50% (illumination period #1). While FCCP and DNP retain their inhibition activity, the inhibition effect of DCCD decreases successively with each subsequent pulse train. Because DCCD is a cross-linker that physically binds to the F0 channel, we hypothesize that the cell can effectively sequester the DCCD by this binding activity. Therefore, by synthesizing more F0 channels, the cell can recover from this initial inhibition. Because DNP and FCCP do not bind to proteins like DCCD does, the cell cannot sequester them, and their capacity for inhibition remains unchanged with time.

**DISCUSSION**

By designing an experimental method that exposes *Cyanothece* to segments of both illumination and darkness during a single anoxic incubation, our electrochemical H2 sensor can observe (with high kinetic resolution) the response of both H2 producing enzymes to environmental and biochemical stresses and thereby allows the current mechanistic study. As such we are able to determine the relative contributions from hydrogenase and nitrogenase towards light- and dark- H2 production, identify shared pools for both processes, and indicate factors limiting the yield of each.

The complete elimination of photo-H2 production when DBMIB is added to the sample (inhibiting electron flow through Photosystem I) can be attributed to loss of ATP generation from cyclic electron flow (photophosphorylation) and/or elimination of the production of reduced ferredoxin, based on the known outcome under aerobic conditions (36). Both are obligate substrates for nitrogenase, and the complete elimination of photo-H2 under these conditions indicates that the sole enzyme responsible for photo-induced H2 production in *Cyanothece* is nitrogenase. Our data illustrate an increased capacity for photo-H2 production with increasing dark pre-incubation, independent of PSI-dependent water oxidation. This result indicates the tight coupling of photo-H2 to dark, anaerobic metabolism, where substrate builds up and becomes kinetically more accessible as anaerobic conditions progress. This increased availability of reductant translates into a greater photo-H2 production rate once the pulsed illumination commences. ATP levels fall during dark anoxia (38) and thus only accumulation of reductant can be responsible for this increase due to dark time.

**Scheme 1** presents a minimal diagram of reductant and ATP generation to guide interpretation and assignment of the pathways. Under dark anoxia, cells catabolize their glycogen and sugar reserves to produce glucose-6-phosphate (G6P), which enters either glycolysis or the oxidative pentose phosphate pathway to produce NADH or NADPH, respectively. Subsequent oxidation of pyruvate by pyruvate:ferredoxin...
oxidoreductase (PFOR) generates reduced ferredoxin in the dark (PFOR is the primary source of reduced ferredoxin during dark, anaerobic, and anabolic nitrogen fixation for unicellular nitrogen fixers (8)). NAD(P)H has a reducing potential of -320 mV, substantially less than that of ferredoxin at -415 mV. As the latter is the obligate electron donor for nitrogenase-dependent H₂ production, NAD(P)H can only convert into reduced ferredoxin through either (a) being energized by PSI and light, or (b) interconversion by ferredoxin:NADP⁺ oxidoreductase (FNR) in the dark, a thermodynamically uphill reaction that requires buildup of NAD(P)H. Our results demonstrate the predominance of the former pathway, where catabolically generated NAD(P)H exchanges hydride (reductant) with the PQ pool via either of two NAPDH dehydrogenases. This hydride is energized to the level of reduced ferredoxin and free proton (coupled to ATP generation) by photo-excitation of PSI and made available for nitrogenase. This sudden shift in equilibrium between NAD(P)H and reduced ferredoxin and ATP manifests as the sharp increase in hydrogen evolution observed upon illumination.

Hydride exchange from NAD(P)H to the plastoquinone pool can occur in cyanobacteria by either of two NADH dehydrogenases present in the organism: NDH type-1 and NDH type-2. As mentioned earlier, NDH-1 translocates a proton from the stroma to lumen as it reduces plastoquinone (Scheme 1) and thus contributes to the intracellular proton gradient. NDH-2 lacks this ability. The channeling of all hydride flux from NAD(P)H to PQ through NDH-1 (rather than both NDH-1 and NDH-2) would provide the most ATP generation per hydride equivalent exchanged (by the proton pumping of NDH-1) and best accommodate the high ATP demand of nitrogenase. In the case of cells treated with flavone, we indeed see this effect. The maximal sustained (2.5 hours) rate of hydrogen production in Cyanothece sp. Miami BG 043511 observed in these experiments was 16.4 µmol H₂ gDW⁻¹ h⁻¹ (15.8 ml H₂ L⁻¹ h⁻¹) with flavone. To ensure that the increase in 735-photo-H₂ was not due to an increase in the plastoquinone concentration (arising from regulatory effects of NDH-1 or NDH-2), we measured the PQ pool size and confirmed that it did not change following treatment with either flavone or rotenone (Fig. S3).

Our findings reveal that of the two factors limiting the rate of nitrogenase mediated 735-photo-H₂, reductant limitation is stronger than ATP limitation under most physiological conditions, as evidenced by the increase in 735-photo-H₂ production rates with increasing prior dark time where reductant accumulates (Fig. S4), and by the decrease in H₂ production rate as the illumination period exhausts the available pool. Only when cells have ample reductant available is an ATP limitation observed, leading to the observed increase in 735-photo-H₂ when reductant flux is forced through NDH-1. Further evidence of reductant limitation as the most severe limitation is that photo-H₂ production increases in the presence of exogenous reduced carbon (3.3-fold increase when 685 mM glycerol was added to cells). Our H₂ production rate of un-supplemented stationary phase cells is 1.64 µmol H₂ mg Chl⁻¹ h⁻¹. Based on carbohydrate degradation and hydrogen production yields after 20 hrs, our numbers correspond to a 24% energy conversion efficiency (ECE) of carbohydrate to hydrogen from the strain.

Higher rates have been reported by Borodin and colleagues (21) for the same strain grown in a photobioreactor where actively growing cells were held at a chlorophyll concentration of ~4 µg/mL (6.3 mL H₂ L⁻¹ h⁻¹, or calculated to 70 µmol H₂ mg Chl⁻¹ h⁻¹). More recently, extremely high rates of 373 µmol H₂ mg Chl⁻¹ h⁻¹ and 465 µmol H₂ mg Chl⁻¹ h⁻¹ (supplemented with 50 mM glycerol) have been reported for Cyanothece sp. ATCC 51142 (39). If confirmed, these would set a new benchmark, as they are significantly higher than the maximum rates published to date for both heterocystous diazotrophs (167.6 µmol H₂ mg Chl⁻¹ h⁻¹ for Anabaena variabilis sp. ATCC 29413 PK84 mutant) (40) and for strains which produce H₂ via the bidirectional hydrogenase only (3.1 µmol H₂ mg Chl⁻¹ h⁻¹ for Arthrospira maxima (41)). However, our own attempt to replicate the glycerol rate data using the ATCC 51142 strain have yet to confirm these results, and the reported rates exceed the maximum theoretical rate of nitrogenase mediated H₂ production of 40 µmol H₂ mg Chl⁻¹ h⁻¹ as calculated by Bothe, et al. based on maximal carbon fixation rates of roughly 100
μmol mg Chl⁻¹ hr⁻¹ and a C/N ratio of 6 in cyanobacteria (8).

It is interesting to note that while the addition of the protonophores decreases the yield of photo-H₂, they substantially increase the dark-H₂ production yield. This confirms that the two mechanisms of hydrogen production (light- and dark-) are catalyzed by different enzymes that respond differently to cellular energy stress. While the decreased photophosphorylation with membrane uncouplers in the light serves to limit nitrogenase of its key energy source (ATP), under dark conditions this disruption stimulates the cell to accelerate the rate of carbohydrate catabolism and thus increase ATP generation by substrate level phosphorylation (glycolysis) (Scheme 1, step 8b). Faster carbohydrate catabolism under anoxia increases the ratio of NADH:NAD⁺ within the cell, which the bidirectional hydrogenase serves to alleviate by reducing protons to H₂ and regenerating NAD⁺ (6). The analogous response of increased fermentative hydrogen production in the presence of FCCP was seen in Cyanothece sp. PCC 7822 (42) and in the non-photosynthetic bacterium E. coli when ATP levels were decreased by the introduction of futile ATPases that continually hydrolyze ATP (43).

The model in Scheme 1 presents multiple targets identified herein for improving H₂ production to better match the solar cycle and improve the H₂ yield. For instance, future improvements to cyanobacterial photo-H₂ production via nitrogenases can be anticipated by: 1) maximizing the total pool size of carbohydrate (e.g. by growth in high salt media) and hence NADH and NADPH turnover, 2) increasing the expression of NDH-1 thus increasing both ATP generation and reductant flux from carbohydrate catabolism to PSI, 3) engineering carbohydrate catabolism so that less glycolytic flux and more OPP flux occurs thereby producing more reductant for delivery to ferredoxin via PSI, 4) increasing the rate of nitrogenase mediated H₂ production by lowering its ATP demand, and 5) engineering nitrogenase so a larger percentage of electrons reduce H⁺ instead of N₂. In fact, genetic engineering of the Mo-nitrogenase from the non-photosynthetic bacterium Azotobacter vinelandii has shown that replacement of a single amino acid in the enzyme results in approximately 80% of the electrons being redirected to H₂ from N₂ (4).

Additionally, by disrupting the two genes encoding homocitrate synthase in Anabaena sp. PCC 7120, citrate can be incorporated into the FeMo cofactor of nitrogenase, thus favoring H₂ production in a N₂ atmosphere (5). Lastly, the knockout of anaerobic pathways which recycle NADH to NAD⁺ (i.e. lactate dehydrogenase, as has been done in Synechococcus sp. PCC 7002 (6) should increase the accessible intracellular reductant pool under anoxia. If coupled to the removal of the two hydrogenases within the cell, this approach could divert a substantially increased flux of reductant to nitrogenase for H₂ production. The recent development of a transformation system in Cyanothece sp. PCC 7822 with the use of ssDNA (44) may enable realization of the above mentioned genetic manipulations for further improving H₂ production by strains of this genus. These manipulations will undoubtedly help overcome the reductant and ATP limitations we observe from in vivo nitrogenase dependent H₂ production rates.

Additionally, from a biotechnological standpoint, minimizing O₂ production from the system has to be a future research focus as well, in order to realize the potential of photo-H₂ pathways. This could involve 1) the search for O₂ insensitive nitrogenases, 2) the development of a large scale optical filtering system such that only near-infrared light is transmitted to the bioreactor, and/or 3) the selective expression of an inactive D1 isoform to inactivate PSII dependent O₂ evolution under H₂ producing conditions.

In conclusion, Cyanothece strains are promising because of their ability to grow on minimal media without combined nitrogen, their robust energy metabolism during anoxia, their ability to maintain low-oxic intracellular conditions conducive to H₂ generating enzymes, and their strong coupling of carbohydrate catabolism to PQ reduction allowing rapid photo-H₂ production in response to illumination. While the net yield of H₂ from Cyanothece is not increased over dark fermentative levels by the addition of illumination periods (see control in Fig. 3B), the rate of H₂ production from the photo-pathway is significantly increased. Biotechnologically, this affords the most rapid conversion of intracellular reductant (which is shared between the dark- and photo- pathways) to H₂ (2-fold increase observed here). We imagine a
system where reductant could accumulate under anoxia (perhaps in a strain with the hydrogenase knocked out) and then be quickly extracted (by conversion to H$_2$) during an illumination period. The cycle could then be repeated.

Lastly, nitrogenase-mediated H$_2$ production is unique in being unidirectional and not inhibited by H$_2$ accumulation, a quality not found with the bidirectional hydrogenase. While significant challenges remain in developing the potential of aquatic microbes for biological hydrogen production, including overall energy conversion efficiencies and scalability concerns, we hope that through the use of the above targets, subsequent studies can further our understanding and bring us closer to realizing the biotechnological potential of nitrogenase-mediated H$_2$ production from Cyanothece.

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FOOTNOTES

*N.S., G.A., and C.D designed the research; N.S. performed the research; N.S., G.A., A.K., and C.D. analyzed the data; N.S. and C.D wrote the paper. The authors declare no competing financial interests.
Photobiological H$_2$ production by Cyanothece

FIGURE LEGENDS

FIGURE 1. Immediate source of reductant for photo-H$_2$ production in Cyanothece sp. Miami BG 043511 is glycogen. (A) Red light (λ = 670 nm) excites both PSII and PSI, while (C) near-infrared light (NIR, λ = 735 nm) excites PSI only and has no direct effect on PSII. (B) DCMU (10 µM) inhibits PSII by blocking reduction of Q$_B$. An increase in photo-H$_2$ is observed both when red light is supplied to the culture in the presence of DCMU (2.35x ± 0.75 (SD)) and when NIR light is used for illumination (2.49x ± 1.3 (SD)) due to diminished O$_2$ poisoning from elimination of PSII-dependent O$_2$ evolution. (D) DBMIB (40 µM) blocks transfer of electrons from reduced PQ to PSI which blocks the nitrogenase-mediated pathway (PSI dependent), but would not prevent H$_2$ase mediated photo-H$_2$ production via PSII (1→2→3→4b→7). The lack of photo-H$_2$ with this treatment indicates a photo-H$_2$ pathway entirely mediated by nitrogenase, while the low level dark-H$_2$ current is due to hydrogenase. (E) A minimal model for photo-H$_2$ production shows possible routes for electron flow to nitrogenase and hydrogenase with reductant originating from the reduced PQ pool (formed from H$_2$O via visible light and PSII) or glycogen (via NADH dehydrogenase). In both cases NIR light and PSI is required to transfer electrons to the level of reduced ferredoxin (substrate for nitrogenase) in order to observe photo-H$_2$. Hydrogenase mediated H$_2$ production may occur in the dark via NADH formed via carbohydrate catabolism (8b→7). H$_2$ electrode calibration: 100 nA = 14.3 µmol H$_2$ gDW$^{-1}$ h$^{-1}$.

FIGURE 2. Effect of dark, preincubation time (autofermentation, AF) on photo-H$_2$ capacity. Cells were taken from photoautotrophic growth and placed under dark, anaerobic conditions for lengths of time ranging from 0.75 to 16 hrs before the onset of a three hour period of pulsed illumination (735 nm LED, represented by the horizontal gray bars). Dissolved H$_2$ was measured as current arising from the oxidation of dissolved H$_2$. The rate (A) of hydrogen evolution and cumulative yield (B) of 735-photo-H$_2$ arising from the pulsed illumination window are shown. Error bars indicate 5% variability in H$_2$ yield. The horizontal black bars (AF time & hv) represent the time segments for the top trace only (16h dark).

FIGURE 3. The effect on H$_2$ production of selective inhibitors of the two NADH dehydrogenases. (A) The rate of dissolved H$_2$ production was measured continuously from cells where both NADH dehydrogenases were functional (CTRL, C), where only NDH Type-1 was active (50 µM flavone, F), and where only NDH Type-2 was active (20 µM rotenone, R). (B) The cumulative yield of dark- and photo-H$_2$ evolution is shown, with the four 1-hr photo-incubation periods (735-photo) indicated by vertical white rectangles. The light-saturated maximal rate of photo-H$_2$ production (extracted from the four photo-incubation periods) was observed when only NDH type-1 was active, and was quantified as 16.4 µmol H$_2$ gDW$^{-1}$ h$^{-1}$. This rate increase of 2-fold (compared to the control) was at the expense of a dark-H$_2$ production rate approximately 2.5-fold slower. This indicates a shared pool of reductant for both pathways (light- and dark-).

FIGURE 4. Effect of inhibiting membrane-coupled ATP generation on H$_2$ production. Both FCCP (5 µM) and DNP (50 µM) are protonophores (uncouplers of the proton gradient); DCCD (250 µM) blocks proton flow through the F$_0$ channel of ATPase. All three of these inhibitors prevent the potential energy stored in the proton motive force from being translated into chemical energy as ATP. The cumulative yields of H$_2$ production (A) and individual photo-H$_2$ production rates from the four pulsed illumination windows (B) are shown. While the cells treated with DNP and FCCP show inhibited photo-H$_2$ capacity, their dark-H$_2$ production rate is increased, consistent with increased glycolytic flux to compensate for stress introduced by the uncouplers.

SCHEME 1. Proposed model for coupling of carbohydrate catabolism to photo-H$_2$ production in Cyanothece where non-photochemical reduction of PQ by catabolically derived NAD(P)H is the primary source of reductant for PSI-dependent, nitrogenase-mediated photo-H$_2$. Sites of proton pumping coupled to ATP generation are denoted by blue arrows, and sites of inhibitor action are denoted with a red X. An
abbreviated scheme of carbohydrate catabolism showing the reduction of NADH by glycolysis and the reduction of NADPH by the oxidative pentose phosphate pathway (OPP) is shown in the inset at left. Stoichiometries are not included. Abbreviations: CHO – carbohydrates, G6P – glucose-6-phosphate, GAP – glyceraldehyde phosphate, PYR – pyruvate, Ac-CoA – acetyl coenzyme-A, QA& QB – PSII-bound plastoquinone molecules, PQ – the pool of membrane soluble plastoquinones, b6f – cytochrome b6f, FNR – ferredoxin:NADP⁺ oxidoreductase, N₂ase – nitrogenase, TH – pyridine nucleotide transhydrogenase, [NiFe]-H₂ase – bidirectional hydrogenase.
Photobiological $H_2$ production by Cyanothece

Figure 1

E. Electron Flow

$H_2O$ \[ \rightarrow \] PSII \[ 1 \] NDH-1,2 \[ 4a \] NADPH \[ 8a \] CHO

DCMU \[ 2 \] PQ \[ 3 \] NADH \[ 8b \]

DBMIB

$N_2ase$ \[ 6 \] FDx \[ \rightarrow \] PSI \[ \rightarrow \] $H_2ase$
Figure 2
Figure 3

Photobiological $H_2$ production by Cyanothece
Figure 4

A

\[ \mu \text{mol H}_2 / g \text{ CDW} \]

\begin{align*}
\text{time, hrs} & \\
0 & 6 & 12 & 18 & 24 & 30 & 36 & 48 & 60 \\
\end{align*}

- DNP
- FCCP
- CTRL
- DCCD

B

\[ \mu \text{mol photo-H}_2 / g \text{ CDW} / \text{hr} \]

|       | 1 | 2 | 3 | 4 |
|-------|---|---|---|---|
| CTRL  |   |   |   |   |
| DNP   |   |   |   |   |
| FCCP  |   |   |   |   |
| DCCD  |   |   |   |   |

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