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Genetic Engineering of Cyanobacteria to Enhance Biohydrogen Production from Sunlight and Water

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Abstract To mitigate global warming caused by burning fossil fuels, a renewable energy source available in large quantity is urgently required. We are proposing large-scale photobiological H_2 production by mariculture-raised cyanobacteria where the microbes capture part of the huge amount of solar energy received on earth's surface and use water as the source of electrons to reduce protons. The H_2 production system is based on photosynthetic and nitrogenase activities of cyanobacteria, using uptake hydrogenase mutants that can accumulate H_2 for extended periods even in the presence of evolved O_2. This review summarizes our efforts to improve the rate of photobiological H_2 production through genetic engineering. The challenges yet to be overcome to further increase the conversion efficiency of solar energy to H_2 also are discussed.

Keywords Cyanobacteria · Hydrogen · Hydrogenase · Nitrogenase · Photobiological H_2 production

INTRODUCTION

Photobiological production of H_2 by cyanobacteria and eukaryotic microalgae that use H_2O as the electron donor has the potential to produce renewable clean energy on a scale sufficient to meet much of the world energy demand (Ghirardi et al. 2007, 2009; Sakurai and Masukawa 2007; Tamagnini et al. 2007; Bothe et al. 2010; Ghirardi and Mohanty 2010). In cyanobacteria, H_2 gas is generated by either hydrogenase or nitrogenase (Tamagnini et al. 2002, 2007). Both enzymes are sensitive to inactivation by O_2. In contrast to hydrogenase that catalyzes the reversible reduction of protons to H_2, nitrogenase catalyzes the unidirectional production of H_2 as an obligatory side reaction during the fixation of N_2:

$$N_2 + 8e^- + 8\,H^+ + 16\,ATP \rightarrow H_2 + 2\,NH_3 + 16\,(ADP + P_i) \quad (1)$$

In the absence of N_2 (e.g., under Ar), all electrons are allocated to H_2 production:

$$2e^- + 2\,H^+ + 4\,ATP \rightarrow H_2 + 4(ADP + P_i) \quad (2)$$

Although the reaction of nitrogenase requires a large investment of ATP (2 ATP per e^-), the reaction is practically irreversible, thus allowing H_2 to be accumulated as high as 20–30% of the total atmosphere, even with simultaneous O_2 evolution (Sakurai and Masukawa 2007; Yoshino et al. 2007). Nitrogenase is restricted to bacteria and archaea, and nitrogen-fixing oxygenic phototrophs are limited to a subset of the cyanobacteria. Heterocyst-forming cyanobacteria are able to reconcile the two incompatible processes of O_2-sensitive nitrogenase and of oxygenic photosynthesis by undergoing differentiation in which about 5–10% of the vegetative cells become heterocysts that provide a microaerobic environment, allowing nitrogenase to function in an aerobic environment (Wolk et al. 1994; Berman-Frank et al. 2003) (Fig. 1). Because of their ability to generate energy by oxygenic photosynthesis while forming a separate space for anaerobic reactions, we are taking advantage of heterocyst-forming cyanobacteria to improve nitrogenase-based H_2 production through gene engineering. Such improvements will allow the development of large-scale photobiological H_2 production on the sea surface.

INACTIVATION OF UPTAKE HYDROGENASE (HUP)

One of the major obstacles to efficient solar energy conversion to H_2 is the presence of hydrogenases that reabsorb

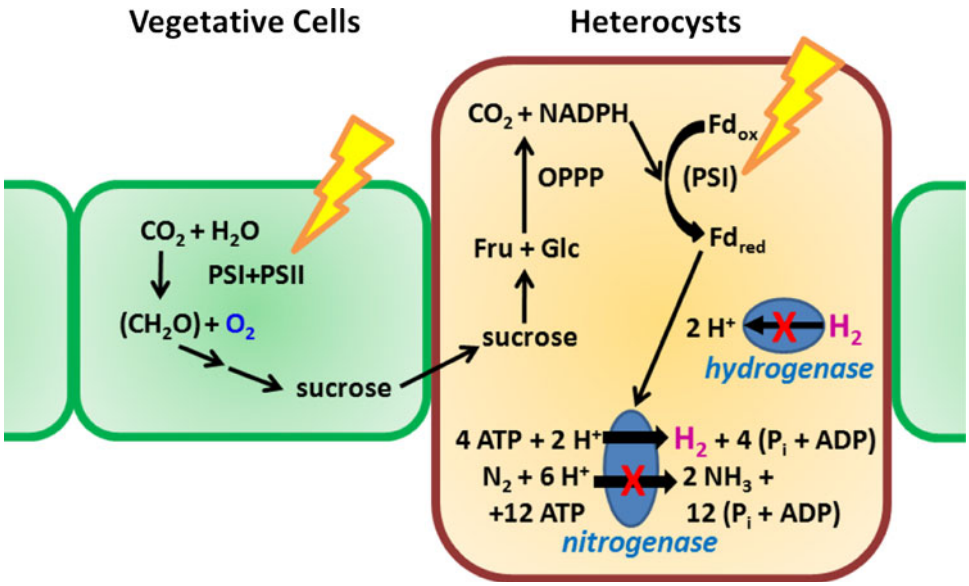

Fig. 1 Photobiological H_2 production by heterocyst-forming cyanobacteria. Vegetative cells of cyanobacteria produce carbohydrates (CH_2O) by oxygenic photosynthesis (the Calvin cycle). Under nitrogen-limited conditions, a subset of cells forms developmentally specialized heterocysts that do not produce O_2 because they lack photosystem II activity. Heterocysts are surrounded by peptidoglycan, glycolipid, and polysaccharide layers, which restrict the entry of O_2, and a small amount of O_2 that diffuses into heterocysts is removed by respiration; thus, the inside of heterocysts is kept microoxic. The microoxic environment protects the O_2-labile nitrogenase from inactivation by O_2, and the enzyme is utilized for the unidirectional H_2 production. Electrons required for nitrogenase reaction depend on sucrose provided by vegetative cells. Sucrose is degraded by the metabolic pathway involving the oxidative pentose phosphate pathway (OPPP), generating NADPH used for the reduction of ferredoxin (Fd) by photosystem I (PSI) and for the formation of ATP by oxidative phosphorylation. ATP is also formed by photophosphorylation involving photosynthetic electron transport system and PSI. In wild-type cells, H_2 produced is absorbed by the Hup. By inactivating the gene encoding Hup, the mutant cells can accumulate H_2 even in the presence of O_2 produced by photosynthesis. By creating selective mutations in the gene encoding nitrogenase, the mutated nitrogenases direct the electron flux through the enzyme selectively toward proton reduction in the presence of N_2

the H_2 produced by nitrogenases, especially in the presence of O_2 (Tamagnini et al. 2002, 2007; Sakurai and Masukawa 2007). Two distinct types of hydrogenases are known in cyanobacteria: the Hup and the bidirectional hydrogenase (Hox). Hup catalyzes a virtually unidirectional uptake of H_2, and Hox catalyzes both uptake and production of this gas. Many heterocystous cyanobacteria contain both Hup and Hox, although a few have only Hup (Tamagnini et al. 2007; Masukawa et al. 2009). *Anabaena* sp. PCC 7120 has been chosen as a model strain to improve nitrogenase-based H_2 production (Masukawa et al. 2002a) because it is amenable to genetic engineering (Elhai and Wolk 1988) and its complete genomic sequence is available (Kaneko et al. 2001). As this organism contains both types of hydrogenases, each individual and both hydrogenases were inactivated by targeted gene disruption, producing two single mutants, ΔHup and ΔHox, and a double mutant, ΔHupΔHox (Masukawa et al. 2002a). Elimination of Hup activity resulted in a 4- to 7-fold increase in the rates of H_2 production in an Ar atmosphere compared with wild-type cells, while the effects of inactivation of Hox activity on H_2 production were not evident under the conditions tested. Hup-disrupted mutants also were shown to be effective in enhancing H_2 production by several other *Anabaena* and *Nostoc* strains of cyanobacteria (Happe et al. 2000; Lindberg et al. 2002; Schütz et al. 2004; Carrasco et al. 2005; Yoshino et al. 2007).

A promising approach to further improve photobiological H_2 production in the presence of O_2 is to initially select parental strains with high nitrogenase activity and inactivate their Hup activities. Out of 13 heterocystous strains tested, *Nostoc* sp. strain PCC 7422 exhibited the highest nitrogenase activity as measured by the acetylene reduction assay (Yoshino et al. 2007). After determining the nucleotide sequences of Hup-encoding genes of *Nostoc* sp. PCC 7422, the Hup-minus mutant (ΔHup) was constructed by insertional disruption of *hupL*. When the ΔHup mutant cultures were grown in an initial headspace gas of Ar + 5% CO_2 under continuous illumination, they accumulated H_2 up to 20–30% (v/v), concomitant with oxygen evolution. The presence of 20% O_2 in the initial headspace gas of the ΔHup cultures inhibited H_2 accumulation by $<$20%, suggesting a low susceptibility of the nitrogenase of this mutant to O_2. A high conversion efficiency of light energy to H_2 of 1.8% versus total solar radiation (averaged over 6 days) was obtained for the ΔHup mutant at an

incident light energy of 70 μmol photons m^{-2} s^{-1} of photosynthetically active radiation. Under laboratory (optimal) conditions, efficiencies exceeding 1% versus total solar radiation are sometimes reported (Kumazawa and Mitsui 1994; Sakurai and Masukawa 2007); however, these high efficiencies are only attained at low light intensities of about one twenty-fifth of full sunlight received on the equator, and the efficiencies greatly decline with increasing light intensities as shown in the PCC 7120 ΔHup mutant (Masukawa et al. 2002b). Under full sunlight, the highest efficiencies reported were about 0.1% (Tsygankov et al. 2002). Possible strategies to overcome the problem of low light saturation in cyanobacteria include decreasing the number of light-harvesting antenna (Melis 2009; Kosourov et al. 2011) and/or reaction center concentrations by targeted mutagenesis and selection of wild-type strains with better tolerance to high light intensity.

MODIFICATION OF THE CATALYTIC ACTIVE CENTER OF NITROGENASE

According to Eqs. 1 and 2, one expects to be able to increase the H_2 production activity of nitrogenase by decreasing the electron allocation to N_2 fixation. Although replacement of N_2 by Ar is effective for increasing H_2 production, this approach increases the operational cost for large-scale generation of H_2. Mutagenesis of nitrogenase offers an alternative mechanism to redirect electron flow and overcome this N_2 competition.

The well-characterized molybdenum-containing nitrogenase consists of an Fe protein (dinitrogenase reductase) and a MoFe protein (dinitrogenase). The Fe protein contains a single [4Fe–4S] cluster and supplies electrons from reduced ferredoxin or flavodoxin to the MoFe protein. The latter contains two unique metal clusters, the [8Fe–7S] P-cluster and the [1Mo–7Fe–9S–1X–homocitrate] FeMo cofactor, where the FeMo-co is believed to be the active site that binds and reduces substrates. Homocitrate is required for efficient nitrogen fixation. The crystal structure of the purified MoFe protein from the homocitrate synthase gene (*nifV*) disruption mutant of *Klebsiella pneumoniae* revealed an altered enzyme that contains citrate, instead of homocitrate, in its FeMo-co (Mayer et al. 2002). Citrate-containing nitrogenase was shown to catalyze the reduction of N_2 poorly, but this enzyme was able to reduce protons effectively in an N_2 atmosphere. The cyanobacterium *Anabaena* sp. PCC 7120 has two homocitrate synthase genes, *nifV1* and *nifV2*, in its chromosome (Kaneko et al. 2001). With the ΔHup strain as the parental strain, two single gene disruption mutants, ΔHupΔNifV1 and ΔHupΔNifV2, and a double gene disruption mutant, ΔHupΔNifV1ΔNifV2, were constructed (Masukawa et al. 2007). N_2-fixing growth rates of the two *nifV* single mutants and the double mutant were decreased moderately and severely, respectively, compared with those of the parental ΔHup strain. For the ΔHupΔNifV1 cells, both the rate of H_2 production and the heterocyst frequency were sustained at higher levels than those for the parental ΔHup strain, leading to significantly increased rates of H_2 production by the former culture compared with those by the latter culture in the presence of N_2. Although the presence of N_2 inhibited H_2 production by the ΔHupΔNifV1ΔNifV2 mutant less strongly than that by the parental ΔHup strain and the other *nifV* mutants, H_2 production activity of the former mutant was low. With *Anabaena* sp. PCC 7120, the inactivation of *nifV1* has proven effective in improving H_2 production in the presence of N_2.

Not only the FeMo-co itself but also the amino acid residues in the vicinity of the FeMo-co are important in substrate reduction. Substitutions of selected amino acids in the vicinity of the FeMo-co active site within *Azotobacter vinelandii* nitrogenase were shown to eliminate or greatly diminish N_2 fixation while, in some cases, allowing for effective proton reduction (Seefeldt et al. 2009). Therefore, certain amino acid exchanges near FeMo-co in cyanobacterial nitrogenase might produce variant MoFe proteins that redirect the electron flux through the enzyme preferentially to proton reduction, producing more H_2 in the presence of N_2 in an aerobic environment. Based on the crystal structure of *A. vinelandii* MoFe protein (Einsle et al. 2002), portions of 19 amino acid residues, all highly conserved, are predicted to reside within 5 Å of FeMo-co. Out of this set, six residues (Q193, H197, Y236, R284, S285, and F388) in the NifD subunit of *Anabaena* MoFe protein (equivalent to residues Q191, H195, Y229, R277, S278, and F381 in *A. vinelandii*) were targeted for mutagenesis in an attempt to direct electron flow selectively toward proton reduction in the presence of N_2. Each of the selected six residues was replaced by nonpolar, polar, or charged residues by using a parental *Anabaena* strain with ΔNif and ΔHup mutations; in total, 49 NifD variants were constructed (Masukawa et al. 2010). Several variants examined in an N_2 atmosphere significantly increased their in vivo rates of H_2 production, approximating rates equivalent to those in an Ar atmosphere when measured on a chlorophyll *a* basis, and these cultures accumulated high levels of H_2 compared to the reference strains including the ΔHup strain. The R284H culture exhibited the most dramatically increased levels of accumulated H_2 compared to the reference strain cultures when grown under N_2. The H_2 accumulation by this mutant under N_2 after 1 week was 87% of that observed for the reference strains under Ar. This variant has the potential of being used as the parental strain for further engineering of *Anabaena* in efforts to attain even greater levels of photobiological H_2 production.

CONCLUDING COMMENTS

We have demonstrated several promising strategies for enhancing photobiological production of H_2 in an aerobic, nitrogen-containing environment. More than 1% conversion efficiencies of light energy to H_2 have been attained with the ΔHup mutant under laboratory conditions. To further increase the conversion efficiency under outdoor conditions, there are many challenges to be overcome. These hurdles involve the factors limiting light utilization efficiency at high light intensity, inhibitory effects of fixed nitrogen on nitrogenase activity, the low turnover rate of nitrogenase (6.4 s^{-1}), etc. By combining several effective improvements through genetic engineering, high-H_2-producing cyanobacterial strains suitable for large-scale production could be created.

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