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Chapter 8

Cyanobacteria and photosynthetic bacteria: metabolic engineering of hydrogen production

Dipankar Ghosh &
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Photobiological hydrogen production are light driven processes that either use water as a source of electrons cyanobacteria, or organic acids, during photofermentative hydrogen production by purple nonsulfur photosynthetic bacteria. Hydrogen evolution in these organisms is largely catalyzed by nitrogenase, with ATP being required. Although hydrogen is also produced by dark fermentation, photobiological hydrogen production has the advantage that it is driven by solar energy conversion. On the other hand, photobiological hydrogen production also suffers from practical limitations: sensitivity of hydrogenase to oxygen, hydrogen consumption driven by respiration, competition for electrons with carbon dioxide fixation, saturation of hydrogen production at low light intensities, as well as an incomplete understanding of the metabolic networks involved in hydrogen metabolism and the unavailability of genetic and molecular tools for improvement in many species. Here we summarize the use of metabolic engineering to increase photobiological hydrogen production.

Introduction

Metabolic engineering can be applied for the improvement of photobiological hydrogen production. In cyanobacteria, captured solar energy can be used to produce hydrogen by two major pathways. In indirect biophotolysis, photosynthesis driven CO_2 fixation leads to the accumulation of endogenous carbon reserves, such as glycogen. Subsequently, the stored macromolecular reserves provide electrons (NADH, NADPH and FADH₂) for hydrogen production that can be either light independent or light dependent. As well, water splitting can be directly coupled to the production of renewable hydrogen in a process called biophotolysis.

In photofermentation, carried out by the purple nonsulfur bacteria, electrons derived from organic acids are by different of membrane bound complexes (Rnf and/or FixABC) which use the energy from the energized membrane to carry out the thermodynamically unfavorable reduction of ferredoxin by NADH or NADPH. Reduced ferredoxin transfers electrons to nitrogenase which evolves hydrogen from protons in the absence of other reducible substrates. The photosynthetically derived electrochemical gradient helps to supply the ATP required by nitrogenase.

Thus, there are several light dependent hydrogen producing microbial platforms, either photoautotrophs or photoheterotrophs, including cyanobacteria and anaerobic photosynthetic bacteria (Gram negative nonsulfur purple bacteria). The metabolic engineering of these processes is complicated since the metabolic pathways are quite different. In fact, the hydrogen producing capacities of these groups differ greatly in several respects: carbon source or source of reducing energy, hydrogen producing enzymes with variable sensitivities and light harvesting pigments and photosystems, etc. Hydrogen production by cyanobacteria, photoautotrophs, is attractive since they derive their carbon requirements from atmospheric CO_2 and the electrons used are derived from water. However, the photofermentative hydrogen production approach has several advantages including: high theoretical conversion yields, no oxygen generation, the capacity to use a wide spectrum of light and the ability to use organic substrates, organic acids, sugars, and glycerol derived from wastes. However, this process also suffers from several drawbacks, including low light conversion efficiencies, low volumetric rates of hydrogen production and having several competing electron consuming pathways [1-3]. Here we review recent efforts in the application of metabolic engineering to the improvement of hydrogen production by cyanobacteria and photosynthetic bacteria.

Metabolic engineering of cyanobacterial hydrogen production

The availability of genome sequences and the natural transformation capabilities of *Synechocystis* sp. and *Synechococcus* sp. make these strains model cyanobacterial systems for metabolic engineering for the improvement of hydrogen production. Cyanobacteria are a physiologically and evolutionarily diverse group of prokaryotic oxygenic photoautotrophs which are tolerant to widely variable environmental conditions, including: pH, temperature and salinity.

Relevant metabolic pathways

Although cyanobacteria are strictly dependent upon light for growth, under anaerobic dark conditions many are capable of carrying out fermentation and producing hydrogen as a by-product of the dark anaerobic catabolism of photosynthetically derived energy storage substances (e.g., glycogen). Under these conditions, glycolysis and/or the pentose phosphate pathway results in the formation of pyruvate and reductant, with the consequent production of lactate, ethanol, acetate, formate, carbon dioxide and molecular hydrogen. In the light, or under aerobic conditions, electron flow through photosynthesis or the respiratory electron transport chain mediates the reduction of NADP^+ to NADPH or the reduction of hydrogenase. The TCA cycle is the central node directly affecting metabolic flux and electron balance. Although the TCA cycle had long been thought to be incomplete in these organisms, a recent study identified genes encoding a novel 2-oxoglutarate decarboxylase (SynPCC7002_A2770) and succinic semialdehyde dehydrogenase (SynPCC7002_A2771), thus completing the TCA cycle [4].

Although different nitrogen assimilation pathways are available, in most cases nitrogen assimilation will consume electrons that could in principle be used for hydrogen production. In particular, nitrate assimilation competes with hydrogen production for electrons, and hydrogen production has already been shown to increase in *Synechocystis* sp. under nitrate limitation [5]. Nitrate is transported into the cell by an active transport system and reduced to nitrite by nitrate reductase (encoded by *narB*). Nitrite reductase (*nirA*) reduces nitrite to ammonium. These two steps require two electrons and six electrons consecutively for complete reduction of nitrate to ammonium ions [6]. Therefore, nitrogen assimilation is a suitable target for metabolic engineering. Metabolic engineering of the cyanobacterium *Synechocystis* to knockout nitrate assimilation gave a 140-fold increase in hydrogen evolution [7].

Cyanobacterial diversity is vast allowing growth under different environmental conditions with strategies for tolerating adverse situations such as anaerobic or microaerobic environments. The major pathways for NADPH

regeneration after oxidation of carbon storage compounds (such as glycogen or sugars) to pyruvate have been assembled at the Joint Genome Institute's Integrated Microbial Genomes (IMG) database [8] where 58 cyanobacterial genome sequences are stored along with a notation of the presence or absence of fermentative enzymes along with fermentative pathway maps for products.

Cyanobacterial hydrogen producing enzymes [NiFe] hydrogenases

Uptake hydrogenase is another potential obstacle to high levels of hydrogen production by nitrogen fixing cyanobacteria. Group-A cytoplasmic [NiFe] hydrogenase (HupSL) is located in cyanobacterial heterocysts. Heterocysts are a differentiated cell, specialized for nitrogen fixation, where cells achieve microaerobic conditions by down regulating oxygenic photosynthesis, necessary for the proper functioning of oxygen sensitive nitrogenase. [NiFe] hydrogenases couple hydrogen oxidation with the aerobic respiratory chain, thus helping to maintain the intracellular anaerobic conditions necessary for biohydrogen production. This group of hydrogenases has dual functionality: providing nitrogenase with protection against oxygen inactivation, and recycling the hydrogen which is produced as an obligate side product of nitrogen fixation. Thus, uptake [NiFe]-hydrogenase is a target for metabolic engineering to increase hydrogen production by these organisms since it potentially oxidizes evolved hydrogen.

In addition, many cyanobacteria possess a Group B [NiFe] hydrogenase, the so-called bidirectional hydrogenase, potentially active under anaerobic conditions. The bidirectional hydrogenases (*hoxEFUYHW*) are soluble or loosely membrane bound [NiFe] hydrogenases found in both nitrogen fixing and non-nitrogen fixing cyanobacteria. The bidirectional hydrogenase has been partially purified and characterized in *A. variabilis* and found to differ from uptake hydrogenases from both a physical and catalytic standpoint [1,9]. Biohydrogen evolution in cyanobacteria has been improved through bio-engineering to redirect electron flux toward the bidirectional hydrogenase and away from competing electron requiring metabolic networks [9,10]. The bidirectional hydrogenase can be inhibited by a buildup in hydrogen partial pressure and therefore constant sparging with an inert gas can increase overall hydrogen production. These cyanobacterial [NiFe] hydrogenases are matured by the widespread [NiFe] hydrogenase maturation system, a set of genes (*hypABCDEF*) encoding chaperone proteins and proteins involved in Ni incorporation and CO/CN synthesis [10].

Among the several avenues for improvements in hydrogen production, one approach is the heterologous expression of hydrogenases with intrinsically

higher hydrogen production rates. [NiFe] hydrogenases might be successfully matured by the endogenous maturation system described above, but it might be thought to be problematic to express [FeFe] hydrogenases since these organisms are not known to contain the relevant maturation system consisting of HydE, HydF and HydG. Nevertheless, it has been reported that the [FeFe] hydrogenase (*hydA*) from *Clostridium pasteurianum* was successfully expressed and active in the cyanobacterium *Synechococcus elongates* without maturation protein co-expression [11]. Heterologous expression of the [Fe-Fe] hydrogenase and associated maturation proteins from *Shewanella* under the control of a heterocyst-specific promoter allowed formation of active hydrogenase suggesting that this could potentially provide a way to increase hydrogen production in this organism [12]. Since over expression of *hetR* or *patS* or *hetN* inactivation can improve heterocyst frequency, a combination of these approaches might be beneficial. However, further work is needed to increase rates and yields with heterologous [Fe-Fe]-hydrogenase expression.

Nitrogenase

Nitrogenase, a protein complex consisting of dinitrogenase (Mo-Fe protein) and dinitrogenase reductase (Fe protein), is the major enzymatic platform for cyanobacterial hydrogen production. Nitrogen reduction to ammonia by nitrogenase requires metabolic energy as ATP. Dinitrogenase reductase (NifH) specifically transfers electrons from the external electron donor ferredoxin or flavodoxin to dinitrogenase (NifD and NifK) which evolves hydrogen. Cyanobacterial nitrogenase, as with all other nitrogenases, is highly sensitive to oxygen, and thus requires one or several defense mechanism against the adverse effects of oxygen. Some heterocystous cyanobacteria, such as *Anabaena variabilis*, possesses a number of nitrogenases including: Mo-nitrogenases (*nif1* and *nif2*), and the alternative nitrogenases, V-containing nitrogenase (*vnfDGK*) and Fe-containing nitrogenases (*anf* gene) exist in heterocystous cyanobacteria [10].

Hydrogen production & metabolic engineering in photofermentative bacteria

Potential photofermentative biohydrogen producing bacteria include purple sulfur and purple nonsulfur bacteria: *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum*. Purple sulfur bacteria are obligate anaerobic autotrophs which utilize H_2 , H_2S and elemental sulfur, whereas the nonsulfur purple photosynthetic bacteria (PSB) do not use sulfur and are capable of growing aerobically on organic substrates in the absence of light. Hydrogen production in these organisms is associated with photofermentative metabolism of organic substances in absence of significant growth but with a nearly stoichiometric release of H_2

due to nitrogenase activity and the presence of a highly active anaerobic citric acid cycle [13]. Nitrogenase has the dual activities of nitrogen reduction as well as ATP-dependent H_2 evolution.

Enzymes of hydrogen metabolism

Four enzymes have been implicated in H_2 metabolism in the PSB: nitrogenase, uptake hydrogenase, fermentative [NiFe] hydrogenase and the CO-dehydrogenase/hydrogenase system. Nitrogenase is a unidirectional enzyme due to its hydrolysis of ATP. In almost all PSB, the majority of hydrogen production is by nitrogenase in the absence of fixed nitrogen, and this hydrogen production is therefore ATP dependent. It functions either in light, or in dark under anoxic or microaerobic environments. However, a few purple nonsulfur bacteria, such as *Rhodospirillum rubrum* and *Rhodocyclus gelatinosus* are capable of the co-expression of CO-dehydrogenase and hydrogenase which together catalyze hydrogen production from CO [1]. Uptake hydrogenase (*hupSL*) is membrane-bound enzyme which physiologically carries out H_2 oxidation. Fermentative [NiFe] hydrogenase functions during dark anaerobic fermentation in a system that can be considered analogous to the formate:hydrogen lyase system of enteric bacteria. Nitrogen fixing bacteria predominantly encode only molybdenum nitrogenase (Mo nitrogenase). However, some, including *Rhodobacter capsulatus* and *Rhodospirillum rubrum* additionally encode alternative nitrogenases, Fe-nitrogenase or V-nitrogenase, which fix nitrogen fixation under molybdenum limiting environments [14]. For example, *R. palustris* CGA009 possesses three different nitrogenases having different transition metal-containing cofactors at their active sites [14].

Improvements in hydrogen production through metabolic engineering

A number of approaches have been used in attempts to increase hydrogen production by PSB [2]. For example, under some conditions, light capture and conversion might be limiting overall hydrogen production. Thus, a 2.7-fold decrease in core antenna (LH1) content and a 1.6-fold increase in peripheral antennal (LH2) content lead to 50% more hydrogen production in *R. sphaeroides* [15]. The uptake hydrogenase might decrease net hydrogen production due to its hydrogen oxidizing activities, and a number of studies have shown improvement in hydrogen yields when this enzyme system is inactivated [16,17]. Normally, the highly regulated CBB pathway (Calvin–Benson–Bassham cycle for CO_2 -fixation) is used to assimilate atmospheric CO_2 , the sole source of cellular carbon during photo and chemoautotrophic growth. However, in many PSB, the CBB pathway also appears to play a major role in the maintenance of intracellular redox homeostasis under photoheterotrophic growth conditions by allowing CO_2 to serve as an electron

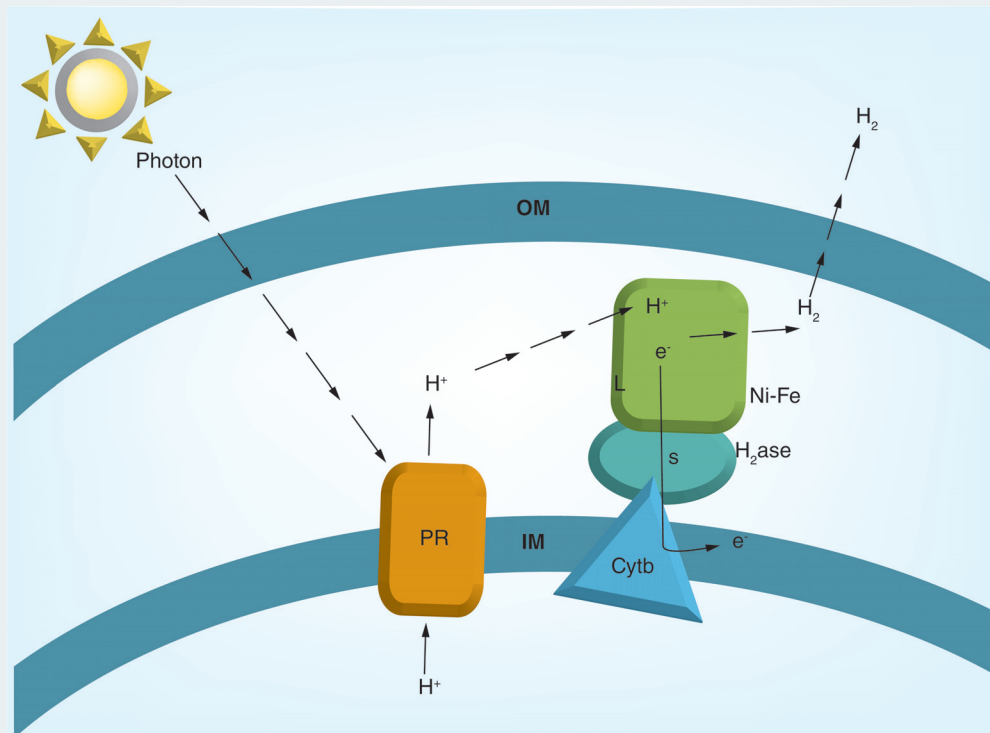
sink. Thus, mutations in this pathway, including RuBisCO and phosphoribulokinase (Prk) enzymes, fail to grow photoheterotrophically in the presence of ammonium unless an exogenous electron acceptor such as DMSO is provided, and pseudo-revertants can be obtained that express nitrogenase under these conditions [18,19], leading to greater hydrogen production [20].

Another potential route to improving hydrogen production could be by increasing the expression of nitrogenase. Nitrogenase expression is strongly regulated at the transcriptional and post-transcriptional levels in response to presence of fixed nitrogen. Two PII proteins, GlnB and GlnK, are key players in these processes since they are involved in the regulation of DraT and DraG, enzymes of the nitrogenase covalent modification regulatory system, and are also involved in regulation the activity of NifA, a *nif* specific transcriptional regulator [21]. These systems are key targets since H_2 production becomes negligible at moderate to high NH_4^+ concentrations with complete repression of nitrogenase expression. Thus, a $\Delta glnB$ - $\Delta glnK$ double mutant of *R. capsulatus* allows nitrogenase synthesis of in the presence 20 mM NH_4^+ with higher hydrogen production than that of the wild-type strain [21]. Similarly, elimination of glutamine synthase prevents NH_4^+ assimilation, leading to the production of substantial amounts of H_2 at relatively high NH_4^+ concentrations (15 to 40 mM) [22].

Hydrogen production in the PSB can also be improved by interfering with uptake hydrogenase (Hup) activity. Thus, mutants of *R. sphaeroides*, either lacking uptake hydrogenase, HupSL or its positive regulator, HupR, produce higher amounts of H_2 [23-25]. In a like manner, synthesis of poly- β -hydroxybutyrate, a major carbon storage material (PHB), competes with nitrogenase for electrons and is therefore a suitable target for metabolic engineering. Reduction of PHB synthesis appears to be an efficient method for increasing H_2 yield in PHB-synthesizing PSB [2,25].

Metabolic engineering to develop photosynthetic *Escherichia coli* for hydrogen production

The conversion of solar energy to biofuels by photosynthesis is seen as an ideal solution for addressing energy deficiency and fossil fuel dependence. Photosynthesis requires highly complex photosystems composed of numerous membrane-bound protein complexes and photosynthetic pigments, including chlorophyll. Therefore, engineering novel photosynthetic microorganisms is a very formidable challenge. However, another biological system capable of converting light energy into chemical energy, the rhodopsins, might be adapted to providing nonphotosynthetic organisms with the capacity to convert solar energy into hydrogen. Recently, a new type of rhodopsin, called proteorhodopsin, has been discovered to be widely

Figure 8.1 Function of proteorhodopsin for photobiological hydrogen production in *Escherichia coli*.

Proteorhodopsin captures light energy and transports protons across the membrane to [NiFe] hydrogenase which reduces proton to hydrogen by the addition of an electron in the periplasmic space. The electron is transferred through Fe-S clusters in the small subunit of [NiFe] hydrogenase and Cytb in the inner membrane.

Cytb: B-type cytochrome encoded by *hyaC* in *E. coli*; IM: Inner membrane; L: Large subunit of [NiFe] hydrogenase; OM: Outer membrane; PR: Proteorhodopsin; S: Small subunit of [NiFe] hydrogenase.

distributed in nature [26,27]. Proteorhodopsin (PR) is a retinal-binding bacterial integral membrane protein that functions as a light-driven proton pump. *Escherichia coli* does not have an intrinsic ability to absorb light energy, but can be made to capture light energy and convert it to hydrogen through the co-expression of proteorhodopsin, the [NiFe]-hydrogenase from *Hydrogenovibrio marinus*, and a heterologous biosynthetic operon for retinal synthesis [28]. The conversion efficiency of light energy to H_2 was approximately 3.4%. How this process functions on a molecular level is depicted in **Figure 8.1**. Proteorhodopsin transports protons across the membrane by absorption of light energy. Protons are transferred to the

active site of the large subunit of [NiFe]-hydrogenase (L) in the periplasm and reduced to H_2 by the addition of an electron, which is transferred thorough [Fe-S] clusters in the small subunit of [NiFe]-hydrogenase (S) and b-type cytochrome (Cytb, encoded by *hyaC* in *E. coli*) in the inner membrane. These results demonstrate that *E. coli* can be converted to a light-powered cell factory for biohydrogen production. Further experiments are required to improve rates and yields.

Challenges & opportunities in the metabolic engineering microbes of hydrogen production

There is no industrial biohydrogen production at present. It is clear from what has already been discussed here, as well as elsewhere [1,2,29], that some genetic manipulation of existing organisms will be necessary to make them suitable for an industrial process based on biological hydrogen production. This is because existing metabolic pathways do not permit either sufficient yields or rates. A number of promising alterations have already been produced and others can be envisaged. With the introduction of new and novel techniques, including CRISPR and Cas9 or Cre and lox, metabolic engineering strategies can now possibly be extended to a variety of promising hydrogen producers.

However, at the industrial level and additional concern is the stability of genetically engineered microorganisms. Any significant rate of reversion during industrial production would be problematic. Therefore, an additional criterion for genetic engineering of organisms for the large scale industrial production of hydrogen would be to ensure a high level of genetic stability. This could be achieved in a number of ways. For plasmid borne genes, a system that creates 'plasmid addiction' could be adopted. In one variation of this strategy, an essential gene function is placed on the plasmid that carries the metabolic gene of interest and removed from the bacterial chromosome. Thus, only cells that maintain the desired plasmid will survive. Alternatively, a natural addiction module consisting of a toxin and antitoxin can be adopted. This also removes the necessity to maintain the plasmid through costly antibiotic pressure. Another strategy is to irreversibly modify the chromosome, thus preventing reversion. A number of avenues are available for carrying out this strategy. Using the newer strategies of recombineering, markerless deletions can be carried out to remove unwanted gene function. Since the genetic material is missing, this cannot be reversed. Similarly, using these types of techniques to introduce foreign DNA into the host chromosome should create strains with a relatively high level of intrinsic genetic stability.

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Summary

- One of the major factors in H₂ production by cyanobacteria is the intrinsic low activity of nitrogenase. Improvements might be sought through expression of oxygen-tolerant [Ni-Fe] hydrogenase or through the heterocyst specific expression of highly active [Fe-Fe] hydrogenase.
- In photofermentative hydrogen production by photosynthetic bacteria, production rates and yields have been improved by applying metabolic engineering to the Calvin–Benson–Bassham pathway and eliminating uptake hydrogenases (*hup*).
- Metabolic engineering in dark fermentative *Escherichia coli* by co-expressing proteorhodopsin and [NiFe] hydrogenase has conferred the ability to capture photons on this microorganism for photobiological hydrogen production.

Key terms

Metabolic network:	a complete cascade of metabolic and physiological processes which governs substrate utilization and product formation by microbial cells. These networks consist of metabolic chemical reactions, metabolic pathways, regulatory interactions and enzyme systems.
Biophotolysis:	the use of solar energy to drive plant type photosynthesis, splitting water to produce oxygen and high energy electrons which are used to reduce protons to molecular hydrogen.
Photofermentation:	the light-driven decomposition of organic compounds by photosynthetic bacteria.

CO₂ fixation: the conversion of carbon dioxide to organic compounds (starch, glycogen) by microorganisms.

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