



UPPSALA
UNIVERSITET

Synthetic Biology for Renewable Energy

Engineering Hydrogen Production in Cyanobacteria



Sean M. Gibbons

Degree project in biology, Master of science (1 year), 2010
Examensarbete i biologi 15 hp till magisterexamen, 2010
Biology Education Centre, Uppsala University
Supervisors: Dr. Thorsten Heidorn and Professor Peter Lindblad

1. Summary

As the fossil fuel economy grows increasingly untenable, it becomes more and more important that humanity develops alternative energy solutions. Climate change is occurring at an alarming pace, disrupting the biosphere, precipitating international conflicts over finite resources and destabilizing the global economy. Synthetic biology provides scientists with a method for rapidly developing renewable energy via biological systems. Most synthetic biology approaches thus far have focused on reprocessing low value feedstock into high value products in a heterotrophic chassis (such as *E. coli*). For energy production, it is important to turn toward photosynthetic organisms and harness the abundant solar energy that falls on the planet. This project focused on developing synthetic hydrogen-producing genetic circuits in *Nostoc punctiforme*, a heterocystous cyanobacterium. The synthetic circuits developed were comprised of a codon optimized [Fe-Fe] hydrogenase (*hydA1/hydA2* from *Chlamydomonas reinhardtii*, or *hydA* from *Clostridium acetobutylicum*) under the control of a synthetic *lacI*-repressible P_{irc} promoter. Attempts were made to combine these synthetic hydrogenase circuits with the [Fe-Fe] hydrogenase maturation systems from *C. reinhardtii* and *Clo. acetobutylicum* (*hydEF/hydG*, and *hydE/hydF/hydG*, respectively), which have been reported to be necessary for [Fe-Fe] hydrogenases to become catalytically active. The expression of synthetic hydrogenases was confirmed through Western Blot analysis and hydrogenase activity was measured using a hydrogen electrode. Results indicate that the synthetic hydrogenases were transcribed and translated into protein, but they were catalytically inactive without of their respective maturation factors.

2. Introduction

2.1 The energy dilemma

The hydrocarbon economy is faltering as oil reserves dwindle worldwide (Hirsch, 2008). Commodity prices have begun to fluctuate drastically due to the uncertain cost of petroleum, which resulted in food riots around the world in 2008. With a steadily decreasing energy supply and the demands on energy systems continually growing, the planet is in dire straits economically, geopolitically and environmentally. In order to halt the advance of climate change, rescue the global economy and ensure nations' energy security, humanity must find a way to harness currently available (non-fossilized) energy. The largest source of energy on Earth, excluding the future potential for thermonuclear fusion reactors, is the sun. Human civilization consumes 15 TW annually while approximately 80,000 TW of solar energy fall on the Earth's surface each year (Makarieva et al., 2008). For hundreds of millions of years, this solar flux has been the driving force for life on Earth. It is estimated that photosynthesis absorbs and distributes seven times more energy to the biosphere (~100 TW) than is consumed anthropogenically each year (Makarieva et al., 2008). Photobiological (microalgal) production of energy-rich molecules, using inexhaustible sunlight and abundant water, could provide fuels to sustainably maintain human civilization. In light of recent scientific and technological advances, hydrogen production via cyanobacteria shows great promise for supplanting the consumption of fossilized hydrocarbons.

2.2 Cyanobacteria and the rise of oxygenic photosynthesis

Approximately four billion years ago, after the formation of Earth, the first signs of life began to appear, scratching out a lithotrophic existence in an oxygen-starved atmosphere (Towe, 1996; Cleaves et al., 2008). Anoxygenic photosynthesis probably developed very early in evolution (Pierson and Olson, 1989), harnessing the power of the sun, but the real metabolic revolution didn't occur for another billion or so years, when photosynthetic eubacteria evolved the ability to split water into electrons, protons and gaseous oxygen (Nisbet et al., 2007). This new metabolic mode, termed oxygenic photosynthesis, effectively gave living organisms access to an endless supply of electrons from water and became such a prolific process that it filled Earth's atmosphere with oxygen, fundamentally transforming the biogeochemistry of the planet (Wille et al., 2007). Moving electrons from chemically stable water molecules to high-energy carbon-carbon bonds spanned an immense reduction-oxidation (redox) gap, powered by a reliable stellar wind of solar electromagnetic radiation, which ultimately increased the size and scope of life and made the development of structurally complex eukaryotic (multicellular) organisms possible (Blankenship and Hartman, 1998). Almost all ecosystems on Earth today are directly or indirectly dependent on oxygenic photosynthesis.

The subjects of this Earth-transforming story, and the only organisms known to have evolved oxygenic photosynthesis, are the cyanobacteria (Nisbet et al., 2007). For a billion years, the electrons in water sat untouched by photosynthetic life because it was energetically unfeasible to oxidize water and reduce NADP^+ (an electron carrier needed for the reduction of inorganic carbon) in a single step. In an evolutionary leap, cyanobacteria pioneered the coupling of two photosynthetic reaction centers, P680 and

P700 (named for the wavelengths of light they optimally absorb), referred to as photosystem II (PSII) and photosystem I (PSI), respectively (Allen and Martin, 2007; Mimuro et al., 2008). Thus, the redox problem was resolved by first pumping low-energy electrons up the redox gradient to a temporary reservoir (the quinone pool/electron transfer chain), from which the second photosystem (PSI) could pull out excited electrons in order to transfer them to an even higher energy state, sufficient for the reduction of NADP^+ to $\text{NADPH} + \text{H}^+$.

The rising concentration of oxygen in the atmosphere presented new challenges to early life on Earth. Most species at the time were obligate anaerobes or microaerophiles, and only those organisms that had the ability to deal with highly reactive oxygen radicals and persist in an oxygen-enriched atmosphere would inherit the majority of Earth's habitats (Brioukhanov and Netrusov, 2007; Bendall et al., 2008). Cyanobacteria, in addition to overcoming the general toxicity of oxygen (a byproduct of their new metabolism), had to find a way to acquire nitrogen under aerobic conditions (fixing atmospheric nitrogen gas into biologically-available ammonia). The molybdenum-iron nitrogenase enzyme is highly conserved among diazotrophs (nitrogen-fixers) and is very sensitive to oxygen, which destroys the activity of the enzyme. Cyanobacteria got around this issue by separating oxygenic photosynthesis and nitrogen fixation, either spatially or temporally (Tsygankov, 2007). Some cyanobacteria only activate nitrogen fixation under dark anaerobic conditions, when PSII is unable to evolve oxygen. Other cyanobacteria, like filamentous *Nostoc punctiforme*, form a specialized cell type for nitrogen fixation, called a heterocyst. Heterocysts deactivate their PSII complexes, grow thickened cell walls, and exhibit higher intracellular respiration rates, which keep oxygen levels very low (Cardona, 2009). Vegetative cells provide the heterocysts with carbohydrates, while the heterocysts provide the vegetative cells with fixed nitrogen (Cardona, 2009). This report focuses on heterocystous cyanobacteria because they provide the possibility for stable hydrogen production under aerobic growth conditions.

2.3 Hydrogen metabolism in cyanobacteria

Nitrogenase is responsible for the following reaction: $\text{N}_2 + 8 \text{H}^+ + 8 \text{e}^- + 16 \text{ATP} \rightarrow 2 \text{NH}_3 + \text{H}_2 + 16 \text{ADP} + 16 \text{P}_i$ (Tikhonovich and Provorov, 2007). This process is very energy-intensive, requiring 8 moles of ATP for every mole of ammonia produced. In addition to ammonia, the nitrogenase enzyme also produces a molecule of hydrogen gas for every molecule of gaseous nitrogen that it fixes. Hydrogen production via nitrogenase is relatively inefficient, due to the large amounts of ATP required, but it can still be used to produce measurable amounts molecular hydrogen. One impediment to producing hydrogen in this way is the presence of uptake-hydrogenases (Schütz et al., 2004), which oxidize nitrogenase-produced hydrogen in order to minimize the energy loss from N-fixation by reclaiming ATP via the oxyhydrogen reaction, removing oxygen from the interior of the cell and providing reducing equivalents for other cellular processes (Tamagnini et al., 2007). Concordantly, uptake-hydrogenase enzyme activity has been shown to correlate with nitrogenase activity (Schütz et al., 2004). A *Nostoc* uptake-hydrogenase knockout mutant has been successfully constructed at Uppsala University and it shows a significantly higher hydrogen output than the wild-type (Lindberg et al., 2004).

Bidirectional hydrogenases, as their name implies, are capable of reversible catalysis of hydrogen formation or consumption ($2\text{H}^+ + 2\text{e}^- \rightleftharpoons \text{H}_2$). Unlike the uptake-hydrogenases, the activity of these enzymes is independent of nitrogenase activity (Schütz et al., 2004). These enzymes seem to act as putative escape valves for the excess reducing power that could build up during metabolism, but their exact function is still under debate (Tamagnini et al., 2007). Cyanobacterial bidirectional hydrogenases have a nickel-iron active site. These nickel-iron hydrogenases are more aerotolerant, but less productive than the iron-iron hydrogenases found in other anaerobic eubacteria and in eukaryotic green algae (Ghirardi et al., 2007). Both nickel-iron and iron-iron hydrogenases require maturation proteins in order to attain catalytic activity, but it has also been suggested that certain [Fe-Fe] hydrogenases do not require a maturation process (Asada et al., 2000; King et al., 2006). These maturation proteins are involved with the insertion of metal clusters into the active site of the hydrogenases (Fontecilla-Camps et al., 2009). Hydrogenases are much more efficient hydrogen producers than nitrogenases, and hold great promise for future biotechnological applications (Tamagnini et al., 2007).

The overall physiology of heterocystous cyanobacteria is highly complex. Advances in genomics, transcriptomics, proteomics and metabolomics are helping scientists develop a holistic view of hydrogen metabolism (Cardona, 2009). In terms of the obstacles facing photobiological hydrogen production, they can be broken down into a few basic issues (Tamagnini et al., 2007). The first main hurdle is that the most prolific hydrogen-producing enzymes, the [Fe-Fe] hydrogenases, are highly oxygen sensitive. For a photobioreactor to be cost-effective, it should produce hydrogen under atmospheric conditions. Some investigations have looked into finding or engineering oxygen-tolerant hydrogenases (Ghirardi et al., 2007). The difficulty with oxygen-tolerant hydrogenases is that their hydrogenase activity has so far been inversely proportional to their level of oxygen tolerance (as seen in [Ni-Fe] hydrogenases). In heterocystous cyanobacteria, this obstacle has been overcome by creating an anoxic microenvironment inside the heterocyst. Another complication arises due to the competition of different metabolic pathways for electrons from PSI. Most of the reducing power generated via oxygenic photosynthesis is diverted towards carbon and nitrogen fixation in *Nostoc punctiforme*. The goal is to better insulate energy-yielding pathways from competing metabolic processes (Agapakis et al., 2010). Dr. Matthias Rögner, from Ruhr-Universität in Bochum, estimated at a recent conference in Sigtuna, Sweden, that under optimal growth conditions ~75% of all electrons coming from PSI could potentially be diverted to hydrogen production without negatively impacting the organism. While metabolic engineering can be vastly complex, scientists can theoretically simplify this problem by catching the electrons at their source (PSI). PSI hands its electrons off to a ferredoxin, which then shuttles the electrons away to various pathways (Tamagnini et al., 2007). One idea is to physically link a ferredoxin ligand to a hydrogenase, in order to compete with the native ferredoxins and snatch electrons away from alternate pathways and transfer them directly to the hydrogenase (Agapakis et al., 2010).

2.4 The ideal hydrogen-producing organism

Nostoc punctiforme was isolated from a mutualistic association with a cycad (Costa and Lindblad, 2002). *Nostoc*, when living symbiotically, has a higher heterocyst frequency than it does as a free-living organism, and will devote much of its metabolism to the production and secretion of specific nitrogen-rich metabolites that are beneficial to its plant host (Enderlin and Meeks, 1983). Since *Nostoc* has evolved to mass-produce a specific metabolite when living symbiotically, it seems almost pre-programmed for fuel production. Heterocystous N-fixing cyanobacteria have minimal nutritional requirements, high photosynthetic efficiencies and can create anoxic microenvironments inside specialized cells that allow anaerobic processes to occur under aerobic conditions. All of these attributes are conducive to affordable bioreactor design for hydrogen production. If *Nostoc* hydrogen metabolism can be effectively engineered, using novel synthetic biology tools, the door to developing successful photobiological hydrogen-yielding technologies is opened.

2.5 Synthetic biology: an emerging field

An astounding result of recent genomic sequencing projects is that the length of a genome does not predict the morphological or physiological complexity of an organism. For example, length of the human genome is similar to that of the fruit fly (Mukherji and van Oudenaarden, 2009). Instead, it has been found that biological modularity can help explain the diversity of form and function in the natural world (Mukherji and van Oudenaarden, 2009). A limited subset of predictable biological “parts” can be assembled in various ways to produce molecular “devices”, which can be arranged into “systems” to carry out different functions. In this light, one can view a living cell as a combination of co-regulated genetic circuits, working in tandem. Another surprising discovery made recently shows the inherent resiliency of biological circuitry to rewiring. Isalan et al. (2008), in order to test the limits of perturbing regulatory networks in biological systems, found that randomly rewiring *Escherichia coli* transcriptional networks by synthetically altering transcription factor/promoter pairings only resulted in faulty growth in 5% of cases. This level of tolerance to random restructuring of biological networks is highly conducive to adaptation and evolvability of living systems by allowing large-scale alterations to be made to an organisms genome without significantly impeding its growth (Isalan et al., 2008; Mukherji and van Oudenaarden, 2009). Biological modularity and network resiliency provide powerful mechanisms for the rapid evolution of novel or optimized processes by reshuffling pre-existing genes/proteins (Isalan et al., 2008; Mukherji and van Oudenaarden, 2009; Picataggio, 2009).

As scientists’ understanding of biology grows in breadth and in depth, they come closer to the goal of being able to rationally design biological systems. Thanks to the enormous accumulation of whole-system biological data and the discovery of the modular nature of genetic and enzymatic elements during the past few decades, coupled with advances in *in silico* data analysis/modeling and rapid *in vitro* DNA synthesis technology, a new field, called synthetic biology, has emerged (Picataggio, 2009). Synthetic biology allows for the rational manipulation of microbial phenotypes by combining systems biology, bioinformatics, protein design and engineering. A web resource for explaining this new

and rapidly growing field can be found at <http://syntheticbiology.org/>. With a comprehensive understanding of the molecular regulation of gene expression and protein function, biologists can begin to assemble a toolbox of reliable promoters, repressors, activators, ribosomal binding sites, reporters, signaling devices and enzymes, that can be used to design metabolic circuits in a cellular chassis (an autonomous self-replicating framework, or superstructure, that acts as the platform for synthetic circuitry) (Picataggio, 2009). Standardization of genetic tools will streamline process engineering and expand our potential to quickly develop microbial systems for the production of renewable fuels and high-value molecules (Picataggio, 2009).

Synthetic biologists have already succeeded in characterizing thousands of biological parts with defined functions and performance parameters, which can be accessed openly at the wiki site <http://partsregistry.org/> maintained by the Massachusetts Institute of Technology (MIT), but they are not yet capable of engineering whole biological systems with the same precision and reliability that, say, electrical engineers are accustomed to. One of the main challenges is to identify the subset of genes that are absolutely necessary for the survival of a minimal genome - the smallest number of genes that allows for the replication of an organism in a particular environment (Cho et al., 1999). Until researchers can build a living cell from the ground up, they won't totally understand the limits of metabolic engineering. The immense potential for engineering crucial synthetic metabolic circuits has already been demonstrated by the work of Dr. Jay Keasling, who produced an important precursor to the anti-malarial drug artemisinin in *E. coli* (Hale et al., 2007). Keasling's work has cut the cost of artemisinin by ten fold and will provide many people in the third world with access to crucial malaria treatments, literally saving *millions* of lives.

E. coli is an ideal cellular chassis for molecular reprocessing of low-value substrates into high-value products, but in order to use synthetic biology to tackle humanity's energy needs, it is important that we move away from heterotrophic organisms and focus on developing a photosynthetic chassis that can harness the power of the sun. Approaching solar fuel production via direct synthesis from sunlight avoids the drawbacks of traditional fermentation-based methods whose biomass feedstocks often compete directly with food crops (Tenenbaum, 2008). Researchers at UC Davis have recently developed efficient synthetically-derived fuel (isobutyraldehyde, which can be converted to isobutanol) from cyanobacteria (Atsumi et al., 2009). This result is encouraging, and suggests that the field of photosynthetically-derived fuels will continue to grow. In the absence of a minimal genome, *Nostoc punctiforme* seems to be an ideal chassis for hydrogen production.

2.6 The development of synthetic biology tools for cyanobacteria

The creation of standard biological parts (promoters, ribosomal binding sites, repressors, activators, etc.) for cyanobacteria is simplified by the work already completed in *E. coli* and by the current open-source nature of synthetic biology resources, fostered by organizations like the BioBricks Foundation (<http://bbf.openwetware.org/>) and iGEM (<http://2009.igem.org>). With the current understanding of transcriptional and translational regulation in cyanobacteria, scientists can re-design genetic regulatory elements and

codon-optimised genes from distantly related organisms *in silico* and synthesize these constructs *in vitro* for expression in cyanobacteria. Collaborators in France have developed codon-optimized (for expression in *E. coli*, *Synechocystis*, *Nostoc* and *Anabaena*) synthetic [Fe-Fe] hydrogenase genes from *Chlamydomonas reinhardtii* and *Clostridium acetobutylicum* that have been linked to a synthetic ferredoxin ligand derived from a chlamydomonal ferredoxin (Jaramillo, A., 2009, École Polytechnique, Palaiseau, France, privileged information). Recently, researchers in the Department of Photochemistry and Molecular Science have characterized P_{trc} promoters, derived from the *lacUV5* promoter, ribosomal binding sites and an expression vector (pPMQAK1) that are broadly functional in *E. coli* and in cyanobacteria (Brosius et al., 1985; Huang et al., 2010). With these tools, it is now possible to express synthetic [Fe-Fe] hydrogenases and their respective maturation proteins in cyanobacteria.

2.6 Project aims

My work focused on synthetic *hydA1* and *hydA2*, from *C. reinhardtii*, and synthetic *hydA*, from *Clo. acetobutylicum*, along with their respective synthetic (codon-optimized) maturation systems (*hydEF* and *hydG* from *C. reinhardtii*, and *hydE*, *hydF* and *hydG* from *Clo. acetobutylicum*). My degree project was centered around the characterization of all available synthetic constructs in *Nostoc punctiforme* wild type and a *Nostoc punctiforme* NHM5 uptake-hydrogenase mutant (Lindberg et al., 2004). To test for the expression of synthetic hydrogenases in *Nostoc*, Western Blots using Hyd-specific polyclonal antibodies were used. To determine the activity level of the synthetic hydrogenases in *Nostoc* strains and in *E. coli*, hydrogen evolution in the presence of reduced methyl viologen was measured with a hydrogen electrode. The overarching goal of these experiments was to engineer and characterize hydrogen-evolving heterocystous cyanobacteria for pollution-free renewable energy production.

3. Results

3.1 Cloning of synthetic hydrogenase genes and maturation systems

Codon-optimised hydrogenase genes *hydA1* and *hydA2* from *C. reinhardtii*, and *hydA* from *Clo. acetobutylicum* were cloned with or without a linked synthetic ferredoxin-encoding gene and with or without the Lac repressor-regulated P_{trc1O} or P_{trc2O} promoter-operator. The maturation system genes from *C. reinhardtii* (*hydEF* and *hydG*) and *Clo. acetobutylicum* (*hydE*, *hydF*, and *hydG*) were also cloned with or without the P_{trc1O} or P_{trc2O} promoters regulating expression of the maturase genes. Attempts were made to combine hydrogenase constructs with maturation system constructs, but none proved successful. Only a fraction of the total possible construct combinations were successfully produced (due to cloning complications) and confirmed via sequencing. Constructs were named from their components, e.g. pSBmatca contains the cloned maturation system genes from *Clo. acetobutylicum* and pSBmatcr contains the cloned maturation system genes from *C. reinhardtii* in the synthetic biology vector pSB1AC3, pSBhydA contains the cloned *hydA* gene in pSB1AC3 and *ptrc1hydA1+fd* contains the cloned *hydA1* gene preceded by the P_{trc1O} promoter-operator and followed by the synthetic ferredoxin-encoding gene in the pPMQAK1 shuttle vector. Tables 4 and 5 in the materials and

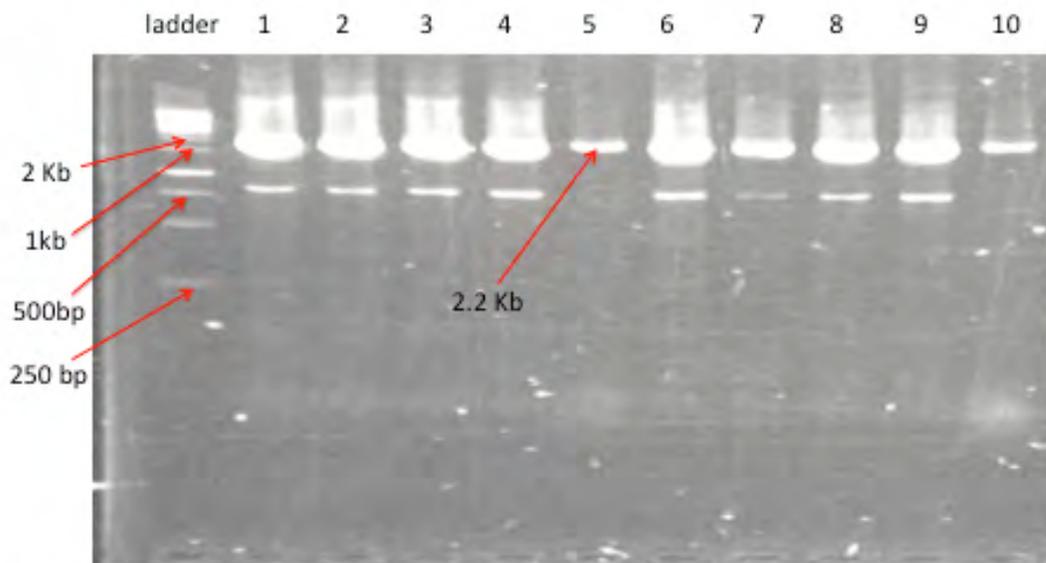


Figure 1. Agarose gel electrophoresis of colony PCR products, using primers VF2 and VR, for successful *hydA1* transformants of *Nostoc punctiforme*. Lanes 1, 2, 3, and 4 are *trc1hydA1+fd* transformants in *N. punctiforme*. Lane 5 is the positive control for *trc1hydA1+fd* in *E. coli*. Lanes 6, 7, 8, and 9 are *trc2hydA1+fd* transformants in *N. punctiforme*. Lane 10 is the positive control for *trc2hydA1+fd* in *E. coli*. The upper band corresponds to the hydrogenase gene (2.2Kb), while the lower band is likely due to non-specific priming.

methods section shows the completed plasmid constructs. All construction work was carried out in *E. coli* and inserts were verified by colony polymerase chain reaction and sequencing. All sequence-confirmed promoter/hydrogenase constructs were ligated into pPMQAK1 and transformed into *Nostoc punctiforme* and its NHM5 mutant using triparental mating. Figure 1 shows an image of an agarose gel of colony PCR reactions from successful *Nostoc* transformants carrying *ptrc1hydA1+fd* and *ptrc2hydA1+fd*.

3.2 Synthetic hydrogenase expression in cyanobacteria

Polyclonal antibodies against recombinant, full-length HydA2 were ordered from Agrisera (Vännäs, Sweden). These antibodies were certified for the detection of both HydA1 and HydA2 from *C. reinhardtii*. Proteins were extracted from wild type *Nostoc punctiforme* and its NHM5 mutant, as well as from strains carrying finished synthetic constructs. Protein extracts were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then analyzed by Western Blot. Figures 2 and 3 show the resulting blots. The blots show a band for the HydA2, but not for the HydA1.

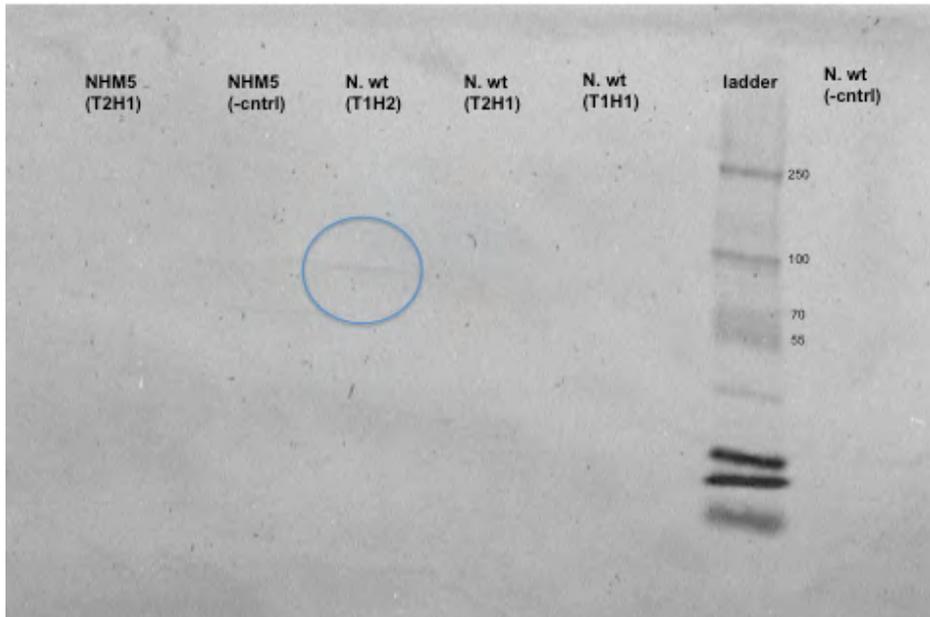


Figure 2. A Western Blot of protein extractions from *Nostoc punctiforme* ATCC 29133 wild type (wt) and its mutant strain NHM5 with and without synthetic hydrogenases. All constructs were carried by the pPMQAK1 vector and expressed in *Nostoc*. The primary polyclonal antibodies were made against HydA2, but should also recognize HydA1. T1H1 refers to *trc1hydA1*, and T1H2 refers to *trc2hydA2* (see table 1). A faint band is circled in the T1H2 lane. Wild type *Nostoc* and the NHM5 mutant without a construct were used as negative controls (-cntrl). Size markers on the ladder are in kilodaltons.

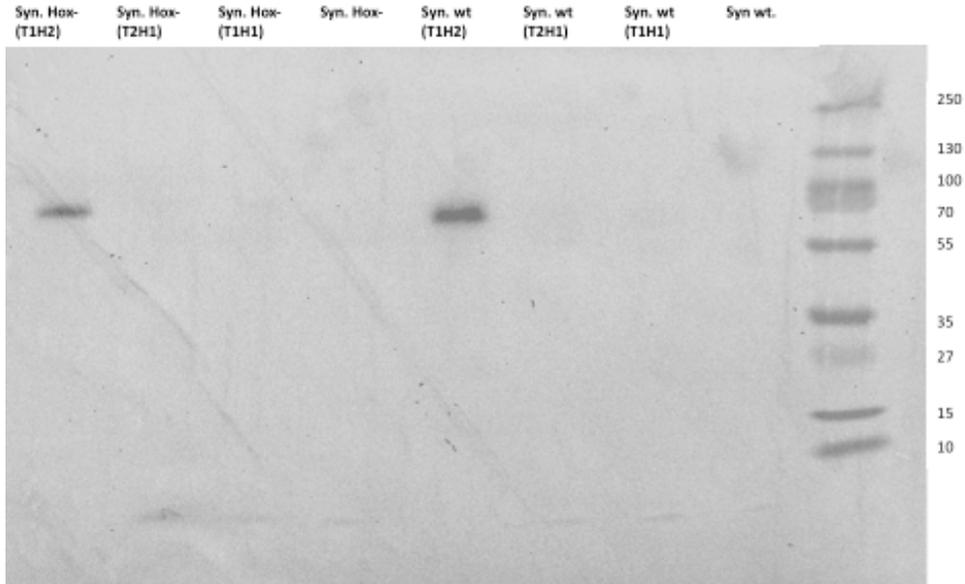


Figure 3. Western Blot of *Synechocystis sp.* PCC 6803 wild type and its Hox⁻ mutant with and without synthetic hydrogenases. All constructs were carried by the pPMQAK1 vector and expressed in *Synechocystis*. The primary polyclonal antibodies were made against HydA2, but should also recognize HydA1. T1H1 refers to *trc1hydA1*, and T1H2 refers to *trc1hydA2* (see Table 1). Hox⁻ is a mutant strain of *Synechocystis* that lacks its sole native hydrogenase, referred to as Hox. Wild type and Hox⁻ *Synechocystis* without a construct were used as negative controls. Protein extracts for *Synechocystis* were prepared by Thiyagarajan Gnanasekaran, whereas I ran the gel and prepared the blot. Size markers on the ladder are in kilodaltons.

3.3 Hydrogen production measurements

A Clark-type electrode was converted for hydrogen measurement. Clark-type electrodes consist of coupled platinum and silver electrodes, which are usually used to measure oxygen concentration, but can be converted for hydrogen measurement as well. Hydrogen-saturated water was used to calibrate the electrode (data not shown). Cyanobacterial and *E. coli* cells harboring the completed synthetic constructs (Table 1) in the pPMQAK1 vector were grown for two hours in the dark under anaerobic conditions (bubbling with argon). Whole-cell extracts containing methyl viologen and sodium dithionite were measured for hydrogen evolution. Reduced methyl viologen acts as an electron donor to the hydrogenase and sodium dithionite reduces methyl viologen. No difference in hydrogen evolution was detected between the wild type *E. coli* strain and the strains containing synthetic constructs (data not shown). Additionally, no hydrogen evolution was detected in any of the *Nostoc* strains (data not shown). As controls, hydrogen evolution was measured in *Synochocystis* and a *Synechocystis* Hox⁻ mutant (lacking the native [Ni-Fe] bidirectional *hox* hydrogenase gene). As expected, wild type *Synechocystis* showed significant hydrogen evolution due to its lone bidirectional hydrogenase (Hox), while the Hox⁻ mutant showed no measureable hydrogen evolution (Fig. 4).

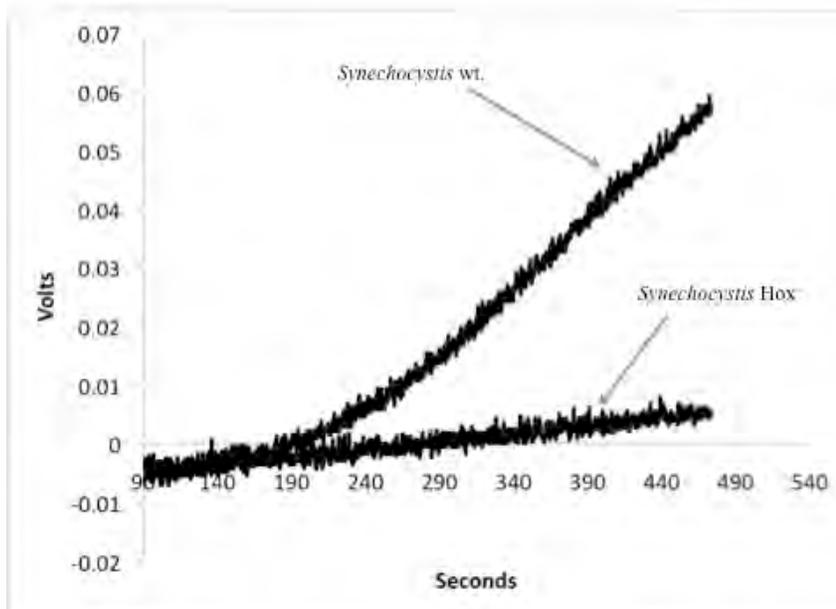


Figure 4. Hydrogen production by *Synechocystis* wild type and a Hox^- mutant, measured by the Clark-type electrode. Increasing voltage (y-axis) corresponds to rising hydrogen concentration in solution. The shallow slope of the Hox^- plot reflects a slight upward drift of the baseline.

4. Discussion

4.1 Manipulating synthetic constructs in cyanobacteria

Only a small percentage of the total possible construct combinations were completed. The cloning work was complicated due to the apparent toxicity of the P_{trc} promoters and/or the synthetic gene products (*i.e.* hydrogenase or maturase enzymes). Early on in the cloning work *E. coli* DH5- α cells were used, which do not contain the $lacI^q$ gene, which encodes the transcriptional repressor for the P_{trc} promoters. This meant that the synthetic genes were being expressed without regulation. This led to very low transformation efficiencies. Additionally, sequences of the plasmid DNA from successful transformants showed several mutations in the P_{trc} promoters. In order to resolve these cloning problems, the cloning strain was switched to *E. coli* NEB5- α , which is $recA^-$ and has the $lacI^q$ gene in its genome. This helped the cloning process considerably, but successful transformants were still difficult to obtain. I found that hydrogenase genes linked to the ferredoxin-encoding gene were more difficult to clone, especially *hydA* from *Clo. acetobutylicum*. This is encouraging, because it suggests that the synthetic ferredoxin exerts a physiological effect, perhaps by binding to elements of the electron transport chain. The maturation system genes were also extremely difficult to clone. This was probably due to their size (5-6 Kb) and other putative effects on cellular metabolism, providing strong selection pressure for promoter-deficient mutants. The largest cloning difficulty was in combining the hydrogenase constructs with their maturation systems. These combined constructs approach 9-10 Kb, in addition to the 10 Kb shuttle vector backbone. Ligations and transformations of these ~17 Kb plasmids have so far been unsuccessful. The possibility of transforming hydrogenase and maturation system genes into cyanobacteria on separate plasmids is currently being explored. Methods for inserting the maturation system genes directly into the cyanobacterial chromosome could also be pursued in future work, in order to circumvent the need for building such large constructs.

4.2 Successful hydrogenase expression

The results of the Western Blot for *Nostoc* wild type and the NHM5 mutant (Fig. 2) showed that, at least in the case of HydA2, the synthetic hydrogenase was expressed. Since HydA2 was the antigen used to produce the polyclonal antibodies used in this experiment, they were expected to have a higher affinity for HydA2 than for HydA1. The band in Figure 5 is very weak, suggesting that it might only be background and/or the protein extraction was not sufficiently concentrated, but the band for HydA2 is clearly distinguishable in the *Synechocystis* blot (Fig. 3). The blotting protocol will have to be optimized, perhaps by prolonging primary antibody incubation times or varying the temperature of incubation, in order to see if HydA1 is present. Future work will focus on loading more protein and altering the stringency of the antibody incubation steps. The next blot should also include a positive control for HydA1 and HydA2, consisting of a protein extract from anaerobically induced *C. reinhardtii*. I expect that the level of expression for the synthetic HydA1 and HydA2 should be similar, since they have the same promoters and ribosomal binding sites. It is possible that HydA1 is somehow targeted for degradation in the cell, in which case it will be necessary to do transcriptional

studies of the synthetic hydrogenases in order to determine if and how much HydA1 is transcribed.

4.3 Hydrogen production measurements

As expected, *Synechocystis* wild type showed significant hydrogen evolution, while the *Synechocystis* Hox⁻ mutant evolved no hydrogen (Fig. 4). *Nostoc punctiforme* wild type and the NHM5 mutant did not evolve hydrogen under dark/anaerobic conditions, and neither did any of the *Nostoc* strains with synthetic HydA1 or HydA2 (data not shown). This was expected, because prior investigations have shown that [Fe-Fe] hydrogenases require maturation before they exhibit catalytic activity (Bock et al., 2006; King et al., 2006). Measuring hydrogenase activity in *E. coli* was complicated by the presence of three native [Ni-Fe] hydrogenases (two uptake and one bi-directional), but no difference was observed between the wild type and construct-containing strains. Future work will focus on introducing the synthetic [Fe-Fe] maturation systems into cyanobacterial strains, either through direct insertion into the genome or via a shuttle vector with a different antibiotic resistance cassette from the hydrogenase constructs. I expect to observe hydrogen production once the maturation genes are in place, since this has already been demonstrated in *E. coli* (King et al., 2006; Agapakis et al., 2010).

4.4 Synthetic biology for renewable energy

The synthetic biology tools for cyanobacteria used in this study (Tables 2 and 3) were recently developed and tested in the Department of Photochemistry and Molecular Science at Uppsala University (Huang et al., 2010). These tools (vectors, promoters, ribosomal binding sites), which were not previously available/characterized, provide scientists with the ability to rapidly engineer and optimize valuable metabolic processes in photosynthetic bacteria. The work in this report was made possible by these new tools. My results have demonstrated that synthetic codon-optimized genes from eukaryote were successfully introduced into a bacterium, separated by eons of evolution, and accurately transcribed and translated into a peptide. My results also support the idea that synthetic [Fe-Fe] hydrogenases are not functional in the absence of their maturation factors (Agapakis et al., 2010). In subsequent work, I hope to show that these synthetic genes become catalytically active in the presence of synthetic chlamydomonal and/or clostridial hydrogenase maturation systems. If these highly productive, yet oxygen sensitive, [Fe-Fe] hydrogenases can be successfully expressed in cyanobacteria, then future work should concentrate on confining their expression to the anaerobic interior of heterocysts. It is my hope and my conviction that hydrogen production via heterocystous cyanobacteria will, in the near future, help assuage or even replace humanity's consumption of fossil fuels.

5. Materials and methods

5.1 Bacterial strains and growth media

Table 1 shows the bacterial strains used in this work. Glycerol stocks of all strains were stored at -80° C.

Table 1. Bacterial strains

Strain	Description	Source
<i>Escherichia coli</i> DH5- α	<i>F' endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K^- m_K^+), λ^-</i>	Invitrogen
<i>Escherichia coli</i> NEB5- α	<i>F' endA1 glnV44 thi-1 recA1⁻ relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K^- m_K^+), λ^-</i>	New England Biolabs
<i>Escherichia coli</i> HB101	F- <i>mcrB mrr hsdS20(rB- mB-) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(SmR) glnV44 λ^-</i>	Promega
<i>Nostoc punctiforme</i> ATCC 29133	wild type	ATCC
<i>Nostoc punctiforme</i> ATCC 29133 NHM5	<i>hupL⁻</i>	ATCC
<i>Synechocystis</i> sp. PCC 6803	wild type	PCC
<i>Synechocystis</i> sp. PCC 6803 Hox ⁻	<i>hox⁻</i>	PCC

ATCC = American Type Culture Collection, Manassas, Virginia

PCC = Pasteur Culture Collection, Paris, France

Cyanobacteria were grown on BG11 medium, composed of the following ingredients (per liter of deionized water): 1.5 g NaNO₃, 0.04 g K₂HPO₄, 0.075 g MgSO₄*7H₂O, 0.036 g CaCl₂*2H₂O, 0.006 g citric acid, 0.006 g ferric ammonium citrate, 0.001 g EDTA (disodium salt), 0.02 g NaCO₃, 1 mL trace metal mix, 10 g agar (if needed). The trace metal mix was prepared as follows (per liter of deionized water): 2.86 g H₃BO₃, 1.81 g MnCl₂*4H₂O, 0.222 g ZnSO₄*7H₂O, 0.39 g NaMoO₄*2H₂O, 0.079 g CuSO₄*5H₂O, 49.4 mg Co(NO₃)₂*6H₂O. BG11 medium was autoclaved at 120° C for 20 minutes. *E. coli* was grown in SOC and LB media. SOC medium was prepared as follows (per liter of deionized water): 20 g Bacto-tryptone, 5 g Bacto-yeast extract, 0.5 g NaCl, 0.186 g KCl and 10 g agar (if needed). SOC medium was autoclaved at 120 °C for 20 minutes. After SOC medium had cooled to 50 °C, 10 mL of a 2 M filter-sterilized glucose stock was added per liter of medium. LB medium was prepared by adding 10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, 10 g of NaCl, and 10 g agar (if needed) to 1 L of deionized

water. LB medium was autoclaved at 120 °C for 20 minutes. The pH for all media was around 7. Antibiotics were added to cooled media (~50 °C) after autoclaving (50 µg/mL kanamycin, 60 µg/mL chloramphenicol and 100 µg/mL ampicillin).

5.2 Plasmids

Table 2 shows the plasmids used in this work.

Table 2. Plasmids

Strain	Derived from	Description	Source
pPMQAK1	pAWG1.1 ^a	RSF1010 replicon, AmpR ^b , KanR ^b , BioBrick MSC ^c	Huang et al., 2010
pSB1A2	pUC19 ^d	High copy number, AmpR, BioBrick MSC ^c	Registry of Standard Biological Parts (http://partsregistry.org)
pSB1AC3	pUC19 ^d	High copy number plasmid, AmpR, CamR, BioBrick MSC ^c	Registry of Standard Biological Parts (http://partsregistry.org)
pSB1AK3	pUC19 ^d	High copy number plasmid, AmpR, KanR, BioBrick MSC ^c	Registry of Standard Biological Parts (http://partsregistry.org)
pRL493	pIC20H ^e	conjugal helper plasmid	Elhai et al. 1994

^a Oliveira and Lindblad, 2008

^b ampicillin and kanamycin resistance genes from pSB1AK3

^c BioBrick cloning site containing recognition sequences for EcoRI, XbaI, SpeI, and PstI

^d Yanisch-Perron et al., 1985

^e Marsh et al., 1984

5.3 Agarose gel electrophoresis

Gels were run in a 1X sodium borate buffer at 200 V on a 1% agarose in sodium borate buffer with thiazole orange (1X = 100µl thiazole orange L⁻¹). A 20X sodium borate buffer was made by combining 38.17 g of sodium borate decahydrate and 33 g of boric acid, bringing the total volume to 1 L with deionized water. A 1 Kb DNA ladder (Fermentas, Burlington, Ontario) was used to determine the size of the PCR products.

5.4 Colony polymerase chain reaction

Single colonies were picked from SOC + antibiotic plates (transformations of ligation reactions from the previous day), grown overnight at 37° C. Colonies were suspended in 10 µl of SOC medium, and 1 µl of this suspension was used as the template for polymerase chain reaction (PCR). The master mix recipe for 20 µl PCR reactions was as follows: 13.3 µl of nano-pure water, 2 µl of 10 X DreamTaq™ buffer (Fermentas), 2 µl of 2 mM dNTPs, 0.1 µl of DreamTaq™ polymerase (Fermentas), 0.8 µl of forward and reverse primers (10 mM) and 1 µl of template. BioBrick primers VF2 and VR, which flank the BioBrick restriction sites present in all synthetic biology vectors (pSB...), as

well as in pPMQAK1, were used for PCR (Table 3). The thermocycler program used to amplify the 2 Kb hydrogenase fragments was as follows: Step 1, 95 °C for 3 minutes; Step 2, 47 °C for 30 seconds; Step 3, 72 °C for 2.5 minutes; Step 4, 95 °C for 30 seconds; Step 5, Return to step 2 X 25; Step 6, 72 °C for 3 minutes; Step 7, 4 °C indefinitely. For PCR confirmation of maturation system inserts, Step 3 was run for 4.5 minutes.

Table 3. Primers

Name	Sequence (5' to 3')	Source
VF2 (BBa_G00100)	tgccacctgacgtctaagaa	Registry of Standard Biological Parts (http://partsregistry.org)
VR (BBa_G00101)	attaccgcctttgagtgagc	Registry of Standard Biological Parts (http://partsregistry.org)

5.5 Cloning work

Digestions and ligations

Synthetic hydrogenase genes and maturation systems were chemically synthesized *in vitro* with flanking BioBrick restriction enzymes sites (EcoRI, SpeI, XbaI, PstI) by collaborators in Paris.

Plasmid DNA was obtained from cell pellets using the GeneElute™ plasmid prep kit (Sigma Aldrich) and purified after digestion using the Nucleospin Extract II™ kit (Macherey-Nagel). For digestions, FastDigest™ restriction enzymes (Fermentas) were used. Digestions were carried out at 37 °C for 30 minutes. Digestion reactions were as follows: 1 µl FastDigest™ enzyme per microgram of DNA and appropriate volume of 10X FastDigest™ buffer.

Ligations were done with QuickLigase™ (New England Biolabs). Reactions were carried out at room temperature for 6 minutes. GENTle software (University of Cologne) was used to calculate the optimal concentrations of digested fragments, based on fragment and vector lengths. 1 µl of QuickLigase™ was added to 19 µl of the ligation reaction, consisting of 10 µl of 2X QuickLigase™ buffer and 9 µl digest.

BioBrick standard assembly

All of the cloning and shuttle vectors used in this report are BioBrick compatible (with defined prefix and suffix restriction sites), which means that standard BioBrick assembly methods can be used (Fig. 5). BioBrick vectors include a prefix and a suffix, surrounding the insertion site. The prefix has EcoRI and XbaI restriction sites. The suffix has SpeI and PstI restriction sites. These restriction sites are unique to the prefix and suffix, and are not found elsewhere on the vector. XbaI and SpeI have compatible sticky ends, when cut, and can thus be ligated together. When XbaI and SpeI are ligated together, they create a small scar region in the DNA sequence that can no longer be recognized by their respective restriction enzymes. These prefixes and suffixes allow for rapid and efficient cloning. A schematic of the three antibiotic (3A) assembly process is shown in Figure 5.

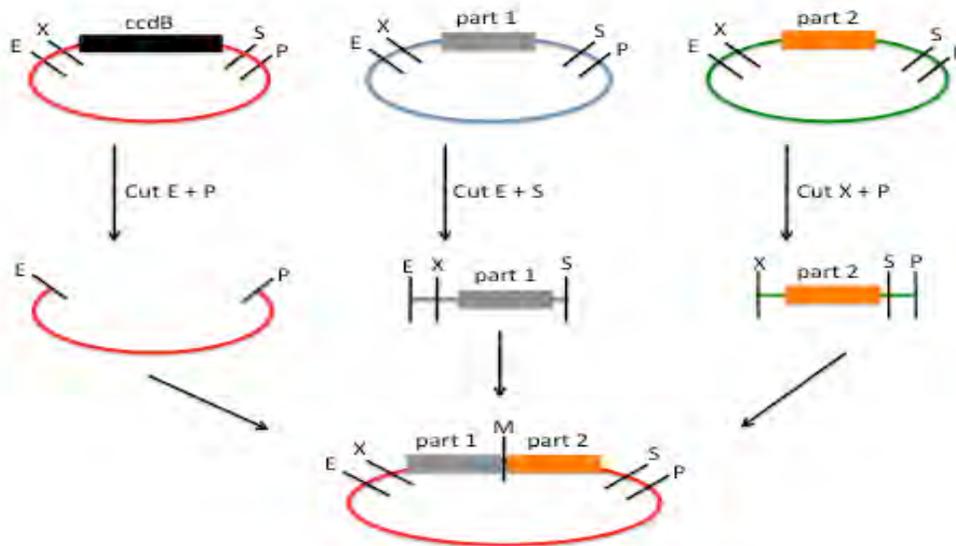


Figure 5. Three antibiotic (3A) assembly. The colors of the three arbitrary plasmid backbones indicate different antibiotic resistance cassettes (for instance, red = pSB1AC3, blue = pSB1A2, and green = pSB1AK3). E denotes EcoRI, X denotes XbaI, S denotes SpeI, and P denotes PstI. X and S sticky ends can be ligated together, but they produce a small scar region (M) that is no longer a functional restriction enzyme recognition site. The image shows a method by which Part 1 is placed upstream of Part 2 and ligated into the red plasmid in a single step. Digested fragments do not need to be separated via gel electrophoresis, because only the desired ligation will produce successful colonies when transformed. Since the red plasmid contains the *ccdB* death gene and has a different antibiotic resistance than the other two plasmids, ligations of Part 1, Part 2, and *ccdB* back into their parent plasmid are avoided because they will not promote growth on the “red” selective media when transformed into *ccdB*-susceptible *E. coli*. Additionally, the parent plasmids for Parts 1 and 2 could have the same antibiotic resistance cassette (e.g. chloramphenicol), as long as the final desired plasmid has a different antibiotic cassette (e.g. kanamycin). Part 2 could easily be placed in front to Part 1 by switching the digestion procedure so that Part 2 is cut by E+S and Part 1 by X+P. The prefix and suffix are regenerated after each construction, and the process can, therefore, be repeated indefinitely to add new parts to the construct. Primer VF2 binds 70 bp upstream of the EcoRI recognition site and primer VR binds 100 bp downstream of the PstI recognition site for all BioBrick vectors. http://openwetware.org/wiki/Synthetic_Biology:BioBricks

For cloning procedures, standard BioBrick high-copy number plasmids pSB1AC3, pSB1A2 and pSB1AK1 were used (www.partsregistry.org, Table 2). Since the BioBrick prefix and suffix are regenerated after each construction, the process is iterative and can be repeated many times. Parts can be combined in any order by selectively cutting the biobrick sites flanking them. Successful plasmid constructs produced in this work are shown in Tables 4 and 5. The recently developed, BioBrick compatible, broad host range shuttle vector pPMQAK1 was used for transferring constructs from *E. coli* to *Nostoc*

(Huang et al., 2010). For triparental mating, the conjugal HB101 *E. coli* strain containing pRL493 was used (Elhai et al., 1994).

Table 4. Plasmid constructs for cloning in *E. coli*

DNA cloned ^a	Cloning Vector ^a	Selection	Constructs
<i>P_{trc10}</i> (E, P) ^{b,c}	pSB1A2 (E, P)	Amp	pSB <i>trc1</i>
<i>P_{trc20}</i> (E, P) ^{b,c}	pSB1A2 (E, P)	Amp	pSB <i>trc2</i>
<i>hydA</i> (E, P) ^b	pSB1AK3 (E,P)	Kan	pSB <i>hydA</i>
<i>hydA1</i> (E, P) ^b	pSB1AK3 (E,P)	Kan	pSB <i>hydA1</i>
<i>hydA2</i> (E, P) ^b	pSB1AK3 (E,P)	Kan	pSB <i>hydA2</i>
<i>hydA</i> +fd ^d (E, P)	pSB1AK3 (E,P)	Kan	pSB <i>hydA</i> +fd
<i>hydA1</i> +fd ^d (E, P)	pSB1AK3 (E,P)	Kan	pSB <i>hydA1</i> +fd
<i>hydA2</i> +fd ^d (E, P)	pSB1AK3 (E,P)	Kan	pSB <i>hydA2</i> +fd
<i>ptrc1</i> (E, S) + <i>phydA</i> (X, P)	pSB1AC3 (E, P)	Cam	pSB <i>trc1hydA</i>
<i>ptrc2</i> (E, S) + <i>phydA</i> (X, P)	pSB1AC3 (E, P)	Cam	pSB <i>trc2hydA</i>
<i>ptrc1</i> (E, S) + <i>phydA1</i> (X, P)	pSB1AC3 (E, P)	Cam	pSB <i>trc1hydA1</i>
<i>ptrc2</i> (E, S) + <i>phydA1</i> (X, P)	pSB1AC3 (E, P)	Cam	pSB <i>trc2hydA1</i>
<i>ptrc1</i> (E, S) + <i>phydA1</i> +fd (X, P)	pSB1AC3 (E, P)	Cam	pSB <i>trc1hydA1</i> +fd
<i>ptrc2</i> (E, S) + <i>phydA1</i> +fd (X, P)	pSB1AC3 (E, P)	Cam	pSB <i>trc2hydA1</i> +fd
<i>ptrc1</i> (E, S) + <i>phydA2</i> (X, P)	pSB1AC3 (E, P)	Cam	pSB <i>trc1hydA2</i>
<i>ptrc1</i> (E, S) + <i>phydA2</i> +fd (X, P)	pSB1AC3 (E, P)	Cam	pSB <i>trc1hydA2</i> +fd
<i>matcr</i> (E, P) ^b	pSB1AK3 (E,P)	Kan	pSB <i>matcr</i>
<i>matca</i> (E, P) ^b	pSB1AK3 (E,P)	Kan	pSB <i>matca</i>
<i>ptrc2</i> (E, S) + <i>pmatcr</i> (X+P)	pSB1AC3 (E, P)	Cam	pSB <i>trc2matcr</i>
<i>ptrc1</i> (E, S) + <i>pmatca</i> (X+P)	pSB1AC3 (E, P)	Cam	pSB <i>trc1matca</i>

^a letters in parentheses indicate what restriction enzymes were used to cut DNA before ligation: X, XbaI; S, SpeI; E, EcoRI; P, PstI

^b all synthetic codon-optimized genes and promoters were designed *in silico* and synthesized chemically by the Jaramillo group at École Polytechnique in Palaiseau, France; the exact sequences of the hydrogenase genes are not reported (privileged information); all synthetic hydrogenases and maturation systems are preceded by the ribosomal binding site (RBS) BBa_B0034 developed as the standard synthetic biology RBS by Elowitz (efficiency = 1.0; Elowitz *et al.*, 2000), followed by the *rrnBT1*-derived (ribosomal RNA operon T1 terminator) termination site BBa_B0015 from *E. coli* (Uptain and Chamberlin, 1997), and flanked by BioBrick restriction sites (<http://partsregistry.org>)

^c the *trc* promoters are derived from the *tac* promoter by increasing the distance between the -35 and -10 regions of the promoter by 1 base pair and adding *lac* operators (Brosius *et al.*, 1985; Huang *et al.*, 2010)

^d the linked ferredoxin gene (fd) is a codon-optimized version of the *petF* ferredoxin from *C. reinhardtii* developed by the Jaramillo group at École Polytechnique in Palaiseau, France

Table 5. Transferring constructs from cloning plasmids to shuttle vector^a

Constructs	Shuttle Vector	Selection	Resulting constructs
pSB <i>trc1hydA</i> (E, P)	pPMQAK1 (E, P)	Kan	<i>ptrc1hydA</i>
pSB <i>trc2hydA</i> (E, P)	pPMQAK1 (E, P)	Kan	<i>ptrc2hydA</i>
pSB <i>trc1hydA1</i> (E, P)	pPMQAK1 (E, P)	Kan	<i>ptrc1hydA1</i>
pSB <i>trc2hydA1</i> (E, P)	pPMQAK1 (E, P)	Kan	<i>ptrc2hydA1</i>
pSB <i>trc1hydA1+fd</i> (E, P)	pPMQAK1 (E, P)	Kan	<i>ptrc1hydA1+fd</i>
pSB <i>trc2hydA1+fd</i> (E, P)	pPMQAK1 (E, P)	Kan	<i>ptrc2hydA1+fd</i>
pSB <i>trc1hydA2</i> (E, P)	pPMQAK1 (E, P)	Kan	<i>ptrc1hydA2</i>
pSB <i>trc1hydA2+fd</i> (E, P)	pPMQAK1 (E, P)	Kan	<i>ptrc1hydA2+fd</i>

^a The shuttle vector was used to express the hydrogenase genes in cyanobacteria; the shuttle vector constructs were grown in *E. coli* and then transferred to the cyanobacterial strains via triparental mating.

Cloning

For cloning of synthetic hydrogenases, the NEB5- α (New England Biolabs) strain of *E. coli* was used. Competent cells were prepared using the CCMB80 method from OpenWetWare (http://openwetware.org/wiki/TOP10_chemically_competent_cells) and stored at -80° C. CCMB80 was prepared as follows: 10 mM KOAc pH 7, 80 mM CaCl₂ * 2H₂O, 20 mM MnCl₂ * 4H₂O, 10 mM MgCl₂ * 6H₂O, 10% glycerol, adjust pH to 6.4, filter sterilize and store at 4 °C. Briefly, cells were grown to an absorbance of 0.3 at 600 nm, spun down and washed twice in CCMB80. Cells were then resuspended in CCMB80 and 100 μ l aliquots of the cell suspension were added to 1.5 mL tubes and stored in the freezer at -80° C. All cloning work was done with SOC medium (20 mM glucose). A 100 μ l portion of cells (one tube from the CCMB80 procedure) was thawed on ice for 10 minutes and then transformed with 10 μ l of a ligation reaction and left on ice for 30 minutes. Cells were then heat-shocked at 47 °C for 30 seconds and left on ice again for 5 minutes. 900 μ l of SOC was then added to 100 μ l of cells, which were incubated at 37 °C for 1 hour. Cells were then spun down for 10 minutes at 3000 g. 900 μ l of the supernatant was discarded and the cell pellet was resuspended in the remaining 100 μ l of SOC. The resulting cell suspension was then plated on SOC medium (using a sterilized bent glass rod) with the appropriate antibiotic(s) (kanamycin, chloramphenicol or ampicillin). Plates were then incubated overnight at 37 °C.

5.6 Triparental mating

Triparental mating was carried out as described previously (Cohen et al., 1994). In brief, the cargo strain (containing pPMQAK1 carrying the desired insert) was grown overnight in SOC medium, shaking (200 rpm) at 37 °C (with 50 μ g/mL kanamycin). The conjugal strain (HB101 pRL493, which facilitates the conjugal transfer of the shuttle vector from *E. coli* to the cyanobacterium) was grown in the same way as the cargo strain, but with 100 μ g/mL ampicillin (Elhai et al., 1994). *Nostoc* cells were grown for two weeks in

BG11 medium at ~ 30 microeinsteins $\text{m}^{-2} \text{s}^{-1}$ of light. Cells were spun down at 3000 g for 10 minutes and resuspended in one tenth the original volume of antibiotic-free medium. The cargo and conjugal strains were mixed and spun down again at 3000 g for 10 minutes and resuspended in fresh antibiotic-free medium. *Nostoc* cells were sonicated five times for 10 seconds using at output setting of 7 and a 1 second pulse. Sonicated cells were diluted to a desired concentration (for *Nostoc* a 1:1 dilution – one part medium and one part cell suspension - worked well). 200 μl of the *E. coli* mixture and 100 μl of the sonicated cyanobacterial cells (at the desired dilution) were mixed in a 1.5 mL tube and incubated at 30 °C in the light for 1.5 hours. After incubation, the mixture was transferred onto an antibiotic-free BG11 plate and incubated for three days at 30 °C at ~ 30 microeinsteins $\text{m}^{-2} \text{s}^{-1}$ of light. After three days, plates were washed with 300 μl of sterile BG11 medium and the supernatant was then transferred to an antibiotic-containing BG11 plate (see Table 1 for the list of antibiotic resistance cassettes on the different vectors). Cells were spread over the plates using sterile 1 mm glass beads.

5.7 Protein extraction

Cells were grown for three weeks in BG11 medium under a light intensity of ~ 30 microeinsteins $\text{m}^{-2} \text{s}^{-1}$ and then 30 mL of the culture was centrifuged at 3000 g for 15 minutes. Cells were resuspended in 700 μl of a protein extraction buffer (50 mM Tris-HCl pH 7.8, 0.1% Triton X-100, 2% sodium dodecyl sulfate, and 10 μl 2-mercaptoethanol). 200 μl of 0.1 mm (diameter) glass beads were added to each extraction (in a screw-cap tube). Cells were lysed using a bead-beater, shaken four times for 30 seconds at the top speed. Lysed extracts were incubated on ice for 1 minute. Extracts were then centrifuged at 20,800 g for 10 minutes. The supernatant was transferred to a fresh tube and the centrifugation step was repeated. Extracts were stored at 4 °C.

5.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Protein extracts were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A 12% acrylamide running gel was made as follows: 3.35 mL water, 2.5 mL 1.5 M Tris-HCl pH 8.8, 100 μl 10% (w/v) SDS, 4.0 mL acrylamide/bis (37.5:1, 30% stock, Bio-Rad), 100 μl 10% ammonium persulfate, and 10 μl TEMED. A layer of isopropanol was placed on top of the gel to prevent drying. The gel was allowed to polymerize for 45 minutes. The isopropanol was then removed and the gel was rinsed with water. A stacking gel was prepared as follows: 3.05 mL water, 1.25 mL 0.5M Tris-HCl pH 6.8, 50 μl 10% (w/v) SDS, 655 μl acrylamide/bis (37.5:1, 30% stock, Bio-Rad, Hercules, CA), 25 μl 10% ammonium persulfate, and 5 μl TEMED. The stacking gel was poured on top of the running gel, and a 10-well comb was inserted on top. Samples were mixed with a 4X sample buffer, prepared as follows: 3.8 mL water, 1.0 mL 0.5 M Tris-HCl pH 6.8, 0.8 mL glycerol, 1.6 mL 10% (w/v) SDS, 0.4 mL 2-mercaptoethanol, and 0.4 mL 1% (w/v) bromophenol blue. Before loading samples on the gel, the complete loading mixture was heated to 98 °C for 4 minutes. 10 μl of sample was added to each well. In one of the wells, 4 μl of a PageRuler™ Plus (Fermentas) prestained protein ladder was added. Gels were run at around 30 mA/gel at 100 Volts for 1.5 hours (or until loading dye reached the bottom of the gel). A 5X running buffer stock was

prepared by adding 9 g Tris base, 43.2 g glycine, and 3 g SDS to 600 mL of deionized water (Appel et al., 2000).

5.9 Western Blotting and protein staining

After proteins were separated by SDS-PAGE, they were transferred to an Amersham™ Hybond™ equilibrium potential for chloride (ECL) membrane (GE Healthcare, Waukesha, WI). A TE-22 transfer tank (GE Healthcare) was used for blotting. After running the gel, the stacking gel was removed and the running gel was placed in a transfer-buffer soaked sandwich consisting of a thick sponge, a gel-sized piece of blotting paper, the gel, the gel-sized ECL membrane, another piece of gel-sized blotting paper, and a thin sponge. The sandwich components were held in place by a plastic cage, which was inserted into the TE-22 transfer tank. The tank was then filled to the appropriate level with transfer buffer, which was prepared as follows: 3.03 g Trizma base, 14.4 g glycine, 800 mL deionized water and 200 mL methanol. The blotting was then run overnight at 17 V and 60 mA, with a magnetic stir bar spinning at a low speed in the bottom of the TE-22 tank. The membrane was then blocked for 2 hours in 1X Tween (0.1%) TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) with 5% (w/v) lowfat milk powder. After blocking, the membrane was probed with polyclonal rabbit anti-HydA2 antibodies (Agrisera) at a 1:5000 dilution in 1X Tween TBS (T-TBS) and shaken for one hour at room temperature. The membrane was then rinsed with fresh T-TBS for 10 minutes, and rinsed twice more in the same way. The membrane was then probed with a secondary anti-rabbit –IgG antibody conjugated to horseradish peroxidase (HRP, GE Healthcare), at a 1:5000 dilution in T-TBS for 1 hour at room temperature. The membrane was then dried with blotting paper and placed on a clean piece of plastic wrap. ECL Western Blot Analysis development solution was mixed and applied to the membrane, which was then incubated at room temperature for 1 minute. The membrane was then dried again with blotting paper and transferred facedown onto a fresh piece of plastic wrap, which was then folded around the membrane. To visualize the stain, a Chemidoc (Bio-Rad) instrument with Quantity One software (Bio-Rad) was used. A picture of the membrane with the pre-dyed ladder was taken using the “Epi White” setting and saved as a tiff file. The Chemidoc instrument was then set to “Chemiluminescence” mode, to image light being emitted from HRP. The exposure time was set to 30 minutes. The final image was saved and then superimposed over the picture of the ladder, using Quantity One software.

Duplicate gels were stained to ensure proper protein separation. After electrophoresis, the gels were fixed in a solution of 7% glacial acetic acid and 40% methanol (in deionized water) for 1 hour. Gels were then stained overnight at room temperature in a staining solution composed of 4 parts of a 1X working solution and 1 part methanol. The working solution was prepared as follows: 800 mL deionized water with Brilliant Blue G-colloidal concentrate (1 % w/v, Sigma Aldrich). Gels were then destained for 2 hours in deionized water (periodically emptying and refilling the water). Gels were wrapped in plastic and a picture was taken using the Epi White setting on the Chemidoc imager.

5.10 Preparation of hydrogen electrode

A CB1-D control box and a standard Hansatech S1 electrode disc (Hansatech, pictured in Fig. 6) were used for hydrogen measurements. For hydrogen measurements, the switch on the back of the control box was set to “HYDROGEN” and the platinum and silver electrodes were electroplated. The platinum electrode was cleaned before electroplating by applying a drop of Aqua Regia solution (4 parts H₂O, 3 parts 12 M HCl and 1 part 16 M HNO₃). The Aqua Regia solution was washed away with deionized water after 10 minutes and the electrode surface was then dried with a tissue. A custom-made electroplating device with a platinum-tipped lead was used to prepare the hydrogen electrode (with a setting for silver and platinum electroplating at 0.3 V). The electroplating device was switched to “platinum”. A drop (~100 µl) of 2 M H₂SO₄ was placed on the platinum electrode and the electroplating lead was inserted into the droplet (without touching the electrode). The current was switched on for 10 minutes. The platinum electrode was rinsed with deionized water and dried. A drop of a 2 % chloroplatinic acid solution (~100 µl) was placed onto the platinum electrode and the electroplating lead was placed into the droplet. The current was switched on for 40 minutes. The platinum electrode was gently rinsed with water and dried, so as not to disturb the layer of platinum black (a black-grey layer). The silver electrode was submerged in a 50% potassium chloride solution and the same electroplating protocol was followed (switched the current on for 20 minutes, and set the electroplating device to “silver”). The electrode apparatus was assembled as per manufacturer instructions (Hansatech, 1992). The electrode water bath was set to circulate at 25 °C. For equilibration of the freshly prepared electrode, 1 mL of deionized water was added to the electrode cell for 1.5 hours (or until a stable baseline was reached).

Hydrogen measurement

E. coli cells were inoculated in 6mL LB medium and grown aerobically overnight with shaking (200 rpm) at 37 °C and then pelleted at 3000 g at room temperature for 10 minutes and resuspended in 6 mL of fresh LB medium (with 50 µg/mL kanamycin). The cell suspension was then transferred to an airtight glass vial with a rubber septum. A long needle was attached to an argon gas source and inserted into the septum, and a short needle was inserted into the septum as well. The long needle bubbled argon into the growth medium and the shorter needle was above the level of the liquid, allowing gas to vent out of the tube. Tubes were bubbled in this way for 2 hours, before hydrogen evolution was measured.

Cyanobacterial cells were grown aerobically in BG11 medium (with appropriate antibiotics) for three weeks, shaking under a light intensity of ~30 microeinsteins m⁻² s⁻¹. 25 mL of cells were spun down in the same way as *E. coli* cells and resuspended in 6 mL of fresh BG11 medium (with either kanamycin, chloramphenicol or ampicillin, depending on the vector – see Table 2). Cell suspensions were transferred into airtight glass vials with a rubber septum. Cells were grown as described above, but in the dark (in airtight vials wrapped in aluminum-foil). After 2 hours, hydrogen production was measured.

For whole-cell hydrogen production measurements, 850 μl of culture was added to the electrode cell and allowed to find a baseline. When the instrument settled to a stable baseline, 50 μl of a 100 μM methyl viologen solution and 100 μl of a 100 μM dithionite solution were added to the culture (Oxelfelt et al., 1995). Hydrogen production was determined as a change in voltage (increasing voltage correlates with an increasing concentration of dissolved hydrogen).



Figure 6. The Hansatech S1 electrode disc is a Clark-type electrode (<http://www.hansatech-instruments.com>).

5.11 Sequencing

All sequencing reactions were sent to Macrogen (Seoul, Korea). Sequencing results were analyzed using Lasergene software (DNA Star, Madison, WI) and alignments were performed using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

5.12 Safety

All relevant safety requirements and protocols can be found in the Department of Photochemistry and Molecular Science (Fotomol) Safety Manual (<http://www.fotomol.uu.se/Internt/index.shtm>).

6. Acknowledgements

I would like to thank the Swedish Fulbright Commission and the U.S. Fulbright Program for funding my Fulbright graduate research fellowship in Sweden. I also want to thank Uppsala University and the Department of Photochemistry and Molecular Science (Fotomol) for supporting my thesis work at Ångström Laboratory. A special thank-you is due to Professor Peter Lindblad, my Fulbright supervisor, and Professor Stenbjörn Styring, who encouraged me to come and work at Fotomol. Much thanks, as well, is due to Dr. Thorsten Heidorn, my thesis supervisor, for supporting and directing my project work. I appreciate the support that I have received from all the members of the Microbial Chemistry group at Fotomol. Particularly, I would like to recognize the generous help and training that I received from fellow Master student Thiyagarajan Gnanasekaran, PhD candidates Daniel Camsund and Hsin-Ho Huang, and post-doctoral researchers Dr. Paulo Oliveira and Dr. Petra Kellers.

7. References

7.1 Citations

Agapakis, C., Ducat, D., Boyle, P., Wintermute, E., Way, J., and Silver, P. (2010) Insulation of a synthetic hydrogen metabolism circuit in bacteria. *Journal of Biological Engineering* **4**:3: doi:10.1186/1754-1611-4-3.

Allen, J.F., and Martin, W. (2007) Out of thin air. *Nature* **445**: 630-632.

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

Asada, Y., Koike, Y., Schnackenberg, J., Miyake, M., Uemura, I., and Miyake, J. (2000) Heterologous expression of clostridial hydrogenase in the cyanobacterium *Synechococcus* PCC7942. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* **1490**: 269-278.

Atsumi, S., Higashide, W., and Liao, J.C. (2009) Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat Biotech* **27**: 1177-1180.

Bendall, D.S., Howe, C.J., Nisbet, E.G., and Nisbet, R.E.R. (2008) Introduction. Photosynthetic and atmospheric evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363**: 2625-2628.

Blankenship, R.E., and Hartman, H. (1998) The origin and evolution of oxygenic photosynthesis. *Trends in Biochemical Sciences* **23**: 94-97.

Bock, A., King, P.W., Blokesch, M., and Posewitz, M.C. (2006) *Maturation of Hydrogenases*. San Diego, CA: Elsevier.

Brioukhanov, A., and Netrusov, A. (2007) Aerotolerance of strictly anaerobic microorganisms and factors of defense against oxidative stress: A review. *Applied Biochemistry and Microbiology* **43**: 567-582.

Brosius, J., Erfle, M., and Storella, S. (1985) Spacing of the -10 and -35 regions in the tac promoter: effect on its in vivo activity. *The Journal of Biological Chemistry* **260**: 3539-3541.

Cardona, T. (2009) The heterocysts of *Nostoc punctiforme*: from proteomics to energy transfer. *Doctoral Dissertation, Uppsala University*.

Cho, M.K., Magnus, D., Caplan, A.L., McGee, D., and Ethics of Genomics Group (1999) GENETICS: Ethical Considerations in Synthesizing a Minimal Genome. *Science* **286**: 2087-2090.

Cleaves, H., Chalmers, J., Lazcano, A., Miller, S., and Bada, J. (2008) A Reassessment of Prebiotic Organic Synthesis in Neutral Planetary Atmospheres. *Origins of Life and Evolution of Biospheres* **38**: 105-115.

Cohen, M.F., Wallis, J.G., Campbell, E.L., and Meeks, J.C. (1994) Transposon mutagenesis of *Nostoc* sp. strain ATCC 29133, a filamentous cyanobacterium with multiple cellular differentiation alternatives. *Microbiology* **140**: 3233-3240.

Costa, J.-L., and Lindblad, P. (2002) Cyanobacteria in Symbiosis with Cycads. In *Cyanobacteria in Symbiosis*, pp. 195-205. Netherlands: Springer

Elhai, J., Cai, Y., and Wolk, C.P. (1994) Conduction of pEC22, a plasmid coding for MR.EcoT22I, mediated by a resident Tn3-like transposon, Tn5396. *J Bacteriol* **176**: 5059-5067.

Elowitz, M., Leibler, S. (2000) A synthetic oscillatory network of transcriptional regulators. *Nature* **403**: 335-338.

Enderlin, C.S., and Meeks, J.C. (1983) Pure culture and reconstitution of the Anthoceros-*Nostoc* symbiotic association. *Planta* **158**: 157-165.

Fontecilla-Camps, J.C., Amara, P., Cavazza, C., Nicolet, Y., and Volbeda, A. (2009) Structure-function relationships of anaerobic gas-processing metalloenzymes. *Nature* **460**: 814-822.

Ghirardi, M.L., Posewitz, M.C., Maness, P.-C., Dubini, A., Yu, J., and Seibert, M. (2007) Hydrogenases and Hydrogen Photoproduction in Oxygenic Photosynthetic Organisms*. *Annual Review of Plant Biology* **58**: 71-91.

Hale, V., Keasling, J.D., Renninger, N., and Diagana, T.T. (2007) Microbially Derived Artemisinin: A Biotechnology Solution to the Global Problem of Access to Affordable Antimalarial Drugs. *Am J Trop Med Hyg* **77**: 198-202.

Hansatech (1992) Hydrogen Measurement Using Modified Oxygen Control Box CB1-D Notes for Users. In: Hansatech, pp. 1-12. Norfolk, England: Hansatech Instruments

Hirsch, R.L. (2008) Mitigation of maximum world oil production: Shortage scenarios. *Energy Policy* **36**: 881-889.

Huang, H.-H., Camsund, D., Lindblad, P., and Heidorn, T. (2010) Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. *Nucleic Acids Research*: doi:10.1093/nar/gkq1164.

Isalan, M., Lemerle, C., Michalodimitrakis, K., Horn, C., Beltrao, P., Raineri, E. et al. (2008) Evolvability and hierarchy in rewired bacterial gene networks. *Nature* **452**: 840-845.

King, P.W., Posewitz, M.C., Ghirardi, M.L., and Seibert, M. (2006) Functional Studies of [FeFe] Hydrogenase Maturation in an Escherichia coli Biosynthetic System. *J Bacteriol* **188**: 2163-2172.

Lindberg, P., Lindblad, P., and Cournac, L. (2004) Gas Exchange in the Filamentous Cyanobacterium Nostoc punctiforme Strain ATCC 29133 and Its Hydrogenase-Deficient Mutant Strain NHM5. *Appl Environ Microbiol* **70**: 2137-2145.

Makarieva, A.M., Gorshkov, V.G., and Li, B.-L. (2008) Energy budget of the biosphere and civilization: Rethinking environmental security of global renewable and non-renewable resources. *Ecological Complexity* **5**: 281-288.

Marsh, J.L., Erfle, M., and Wykes, E.J. (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. *Gene* **32**: 481-485.

Mimuro, M., Tomo, T., and Tsuchiya, T. (2008) Two unique cyanobacteria lead to a traceable approach of the first appearance of oxygenic photosynthesis. *Photosynthesis Research* **97**: 167-176.

Mukherji, S., and van Oudenaarden, A. (2009) Synthetic biology: understanding biological design from synthetic circuits. *Nat Rev Genet* **10**: 859-871.

Nisbet, E.G., Grassineau, N.V., Howe, C.J., Abell, P.I., Regelous, M., and Nisbet, R.E.R. (2007) The age of Rubisco: the evolution of oxygenic photosynthesis. *Geobiology* **5**: 311-335.

Oliveira, P., and Lindblad, P. (2008) An AbrB-Like Protein Regulates the Expression of the Bidirectional Hydrogenase in *Synechocystis* sp. Strain PCC 6803. *J Bacteriol* **190**: 1011-1019.

Oxelfelt, F., Tamagnini, P., Salema, R., and Lindblad, P. (1995) Hydrogen, uptake in *Nostoc* strain PCC 73102 : effects of nickel, hydrogen, carbon and nitrogen. *Plant physiology and biochemistry* **33**: 617-623.

Picataggio, S. (2009) Potential impact of synthetic biology on the development of microbial systems for the production of renewable fuels and chemicals. *Current Opinion in Biotechnology* **20**: 325-329.

Pierson, B.K., and Olson, J.M. (1989) *Evolution of photosynthesis in anoxygenic photosynthetic prokaryotes*. Washington, D.C.: American Society for Microbiology.

Schütz, K., Happe, T., Troshina, O., Lindblad, P., Leitão, E., Oliveira, P., and Tamagnini, P. (2004) Cyanobacterial H₂ production — a comparative analysis. *Planta* **218**: 350-359.

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

Tenenbaum, D.J. (2008) Food vs. Fuel: Diversion of Crops Could Cause More Hunger. *Environmental Health Perspectives* **116**: A224-A257.

Tikhonovich, I.A., and Provorov, N.A. (2007) Cooperation of plants and microorganisms: Getting closer to the genetic construction of sustainable agro-systems. *Biotechnology Journal* **2**: 833-848.

Towe, K.M. (1996) Environmental oxygen conditions during the origin and early evolution of life. *Advances in Space Research* **18**: 7-15.

Tsygankov, A. (2007) Nitrogen-fixing cyanobacteria: A review. *Applied Biochemistry and Microbiology* **43**: 250-259.

Uptain, S., Chamberlin, M. (1997) *Escherichia coli* RNA polymerase terminates transcription efficiently at rho-independent terminators on single-stranded DNA templates. *Proc Natl Acad Sci* **94**: 13548-13553.

Wille, M., Kramers, J.D., Nägler, T.F., Beukes, N.J., Schroder, S., Meisel, T., Lacassie, J.P., Voegelin, A.R. (2007) Evidence for a gradual rise of oxygen between 2.6 and 2.5 Ga from Mo isotopes and Re-PGE signatures in shales. *Geochimica et Cosmochimica Acta* **71**: 2417-2435.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUC19 vectors. *Gene* **33**: 103-119.