Annual Progress Report 2005/06

Metabolic Engineering of Hydrogen Production in Cyanobacterial Heterocysts

Overview

The overall objective of this research is to provide a platform for direct cyanobacterial hydrogen production. We are focusing on two key elements: i) developing a cyanobacterial system that is robust and allows hydrogen production from water in the presence of the stoichiometrically produced molecular oxygen, and ii) developing a system for efficient assembly and maturation of the Fe-Fe hydrogenase needed for hydrogen production in cyanobacteria. Both approaches are addressed in the report below.

1) Hydrogen Production in Cyanobacterial Heterocysts

Investigators

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Introduction

Hydrogen holds great promise as the energy carrier of choice for the fuel cell-based economy in coming years. Currently, the production of hydrogen relies mainly on the steam reforming of gasoline or natural gas. A major drawback of this process is that carbon dioxide, which adds to the atmospheric greenhouse gas budget, is produced as an end product. In order to mitigate global warming, the ideal source of hydrogen should be renewable and carbon-neutral. Our research project involves engineering photosynthetic bacteria to produce molecular hydrogen from water using sunlight. Our hope is to create a sustainable biological system to produce hydrogen with minimal input other than water and sunlight.

Goal

Our goal is to create a light-driven biological system to extract hydrogen from water. This involves creating an organism that can perform both oxygenic (oxygen-evolving) photosynthesis and hydrogen evolution. In this system, light energy is used to split water into oxygen and hydrogen during oxygenic photosynthesis. The hydrogen is then released as hydrogen gas through the action of an anaerobic (oxygen-sensitive) enzyme, hydrogenase. The inherent problem with such design is that hydrogenases are readily inactivated by oxygen from photosynthesis.

Solution: Spatial Separation of Photosynthesis and Hydrogen Evolution

Our solution entails separating these two incompatible biochemical pathways physically, in which photosynthesis and hydrogen evolution occur in two different cell types (Fig. 1). This design can be achieved by taking advantage of the unique adaptation found in some nitrogen-fixing filamentous cyanobacteria. When starved of fixed nitrogen, some filamentous cyanobacteria develop specialized cells called heterocysts. The low oxygen concentration in heterocysts allows nitrogenase (another anaerobic enzyme; nitrogenase reduces or fixes nitrogen gas to ammonia) to be expressed and function properly. The remaining cells (vegetative cells) are photosynthetic and can conserve light energy into reducing equivalents for the reduction of nitrogen gas in heterocysts.

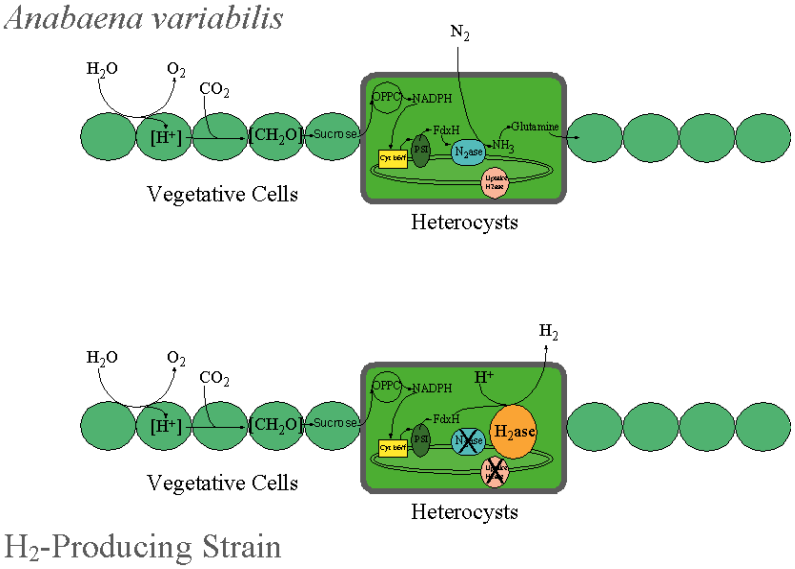

Figure 1; Schematic illustration of photosynthesis and nitrogen fixation in filamentous cyanobacteria such as *Anabaena* (top panel). Proposed scheme for the conversion of *Anabaena* to a hydrogen-producing strain (lower panel).

Progress

We have characterized the heterocyst protein expression system described in the last progress report. Several problems were identified. On-going research and future experiments are focused on addressing the problems.

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2) Development of a Molecular System for Efficient Production and Maturation of Fe-only Hydrogenases

Investigators
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Introduction

Shewanella oneidensis MR-1 is a facultative anaerobic γ- proteobacterium frequently found at many suboxic niches. [1-3, 4]. The microorganism utilizes a wide range of organic and inorganic compounds, metal ions like Fe(III), Mn(IV), Cr(VI) and radionuclides like U(VI) as terminal electron acceptors during anaerobic respiration and growth. [5-7]. The spectrum of *S. Oneidensis* electron donors usage is more limited, and includes some metabolic end products of primary fermenters, such as lactate and formate. [8, 9]. The use of hydrogen as electron donor by *Shewanella* strains was described in early reports. [10, 11, 12, 13, 14,15].

Analysis of the *S. oneidensis* genome reveals two putative hydrogenase gene clusters, *hydA* (SO3920-SO3926), as shown in Figure 3, and *hyaB* (SO2089-SO2099). [16]. Based on structural features, HydA is predicted to be a periplasmic [Fe-Fe] hydrogenase and HyaB a periplasmic [Ni-Fe] hydrogenase. The assembly of the [Fe-Fe] hydrogenase active site, the H-cluster, involves helper proteins for proper folding and maturation. *S. oneidensis* MR-1 *hydA* gene cluster contains accessory proteins, *hydE, hydF and hydG,* which are believed to be involved in the correct assembly and folding of the [Fe-Fe] hydrogenase according to sequence homology to *Chlamydomonas. reinhardtii* and to other several prokaryotes. [17]. Functions were suggested recently for HydE, HydG and HydF in the process of [Fe-Fe] maturation [18, 19-21], however, most of the process is unknown yet. The role and activity of the helper proteins in *S. oneidensis* MR-1 had not been established yet, in addition, the *hydA* gene cluster contains an additional hypothetical protein (*hydX*, SO3924) which function is totally unknown.

A better understanding of the molecular processes involved in maturation and modification of the *hydA* gene product is important for our cyanobacterial biohydrogen project described above. Since we have accumulated over the past years significant expertise in the biochemical and genetic analyses in *S. oneidensis* MR, we have chosen this microbe as a model microorganism for developing and understanding of [Fe-Fe] hydrogenase assembly, folding and maturation.

Results

Hydrogen production by S. oneidensis MR-1 under anaerobic conditions

When *S. oneidensis* cells were grown anaerobically in mineral medium (MM) [7] supplemented with either lactate or pyruvate as electron donor and fumarate as electron acceptor, we discovered the formation of molecular hydrogen. Figure 1 shows the correlation between medium composition, growth phase and hydrogen formation. Hydrogen production was observed only in the excess of electron donor and was not detected otherwise. Hydrogen production was observed when cells entered stationary phase. We concluded that the onset of hydrogen formation correlated with the depletion of electron acceptor (fumarate) and with cells entering stationary phase.

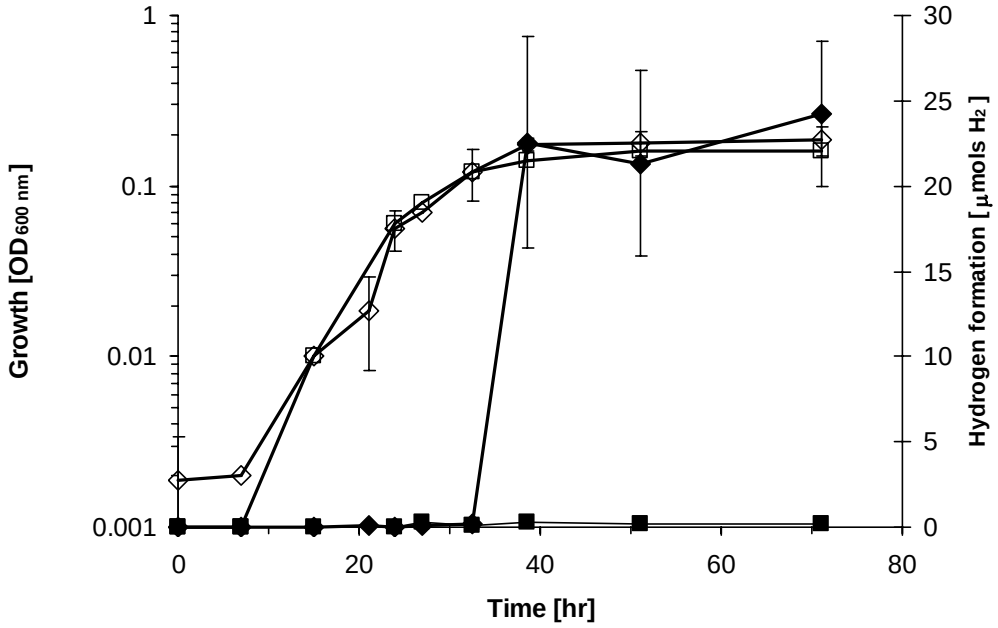

Figure 1. Hydrogen production in *S. oneidensis* is dependent on medium composition and growth phase.
B: Hydrogen formation during growth on MM amended with either balanced amounts of pyruvate and fumarate (squares) or in excess of pyruvate (diamonds). Open symbols: optical density (600 nm), closed symbols: H_2 formed (μmols).
Error bars represent standard deviation for at least triplicate cultures in all experiments.

Transcriptional analysis, organization and expression of hydA and hyaB genes

We examined the expression pattern of the two *S. oneidensis* hydrogenase genes, *hydA* and *hyaB,* in a series of reverse transcriptase (RT) PCR experiments. First we discovered that that both *hydA* and *hyaB* genes are transcribed only under anaerobic conditions. In order to study the correlation of *hydA* and *hyaB* transcription with growth, we performed a series of time-course RT-PCR assays on cDNA from anaerobically-grown cultures. Results demonstrate that both *hydA* and *hyaB* transcription starts during exponential growth phase (15-24 hours), before hydrogen production is detected.

Transcriptional organization of the *hydA* gene cluster was performed by PCR amplification with primers pairs design to amplify intergenic regions of *hydA, hydB, fdh, hydG, hydX, hydE* and *hydF* on cDNA from anaerobic culture. Results, shown in Fig. 2, demonstrate that *hydA, hydB, fdh, hydG, hydX, hydE* and *hydF* are expressed as polycistronic unit during growth under those conditions.

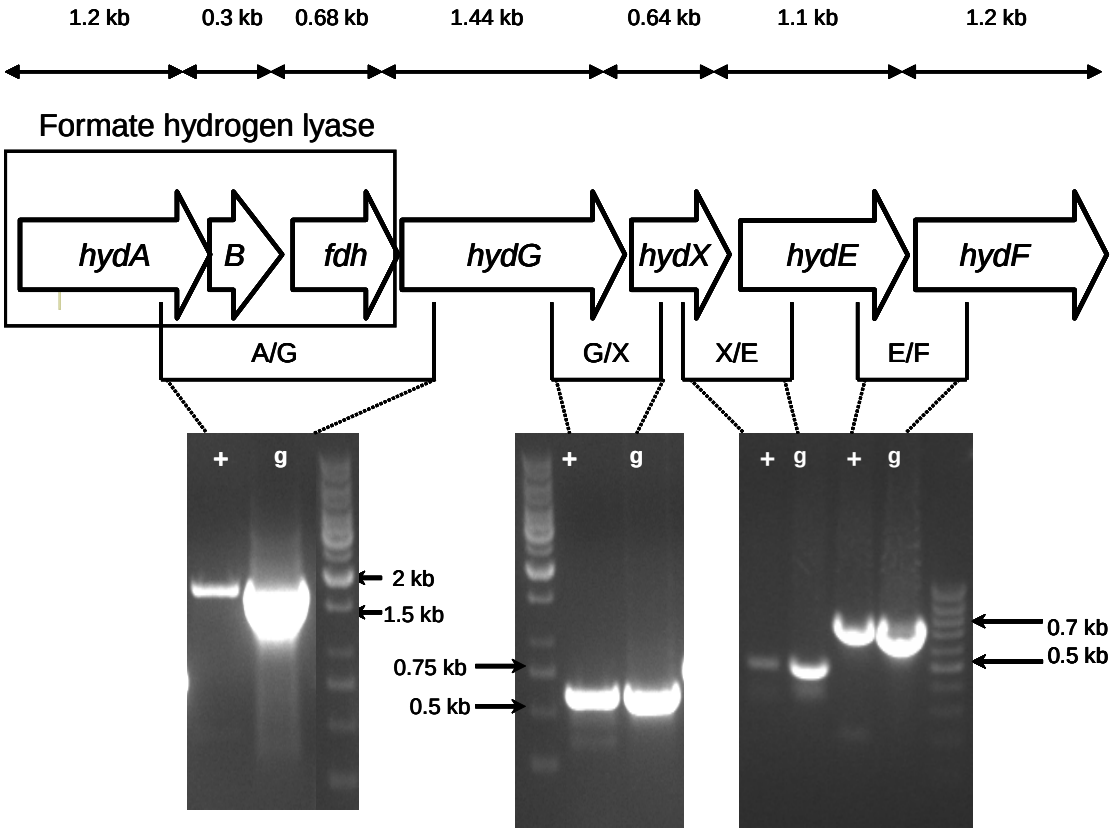

Figure 2. Physical map of the *hydA* gene locus in *S. oneidensis* MR-1 and RT-PCR analysis of *hydA*(SO3920) and the down stream genes on the *hydA* gene cluster (*hydB*: SO3921, *fdh*: SO3922; *hydG*:SO3923; *hydX*: SO3924; *hydE*:SO3925; *hydF*:SO3926) All RT assays were performed on 25 ng of cDNA that was made from RNA tested and verified as clean from DNA contamination by previous PCR analysis
g, genomic DNA as template; +, assays with cDNA from RNA with RT as template.

The role of HydA and HyaB in hydrogen production and consumption

In order to study HydA and HyaB role in hydrogen production and consumption, we constructed in-frame deletions of *hydA*, *hyaB* , and both *hydA* & *hyaB* and tested these strains in both *in-vivo* and cell suspension experiments.

In a set of *in-vivo* experiments all strains were grown anaerobically in MM supplemented with electron donor excess and were tested for growth and hydrogen production. Results shown in Fig. 3 demonstrate similar growth for all strains. Hydrogen production, however, was significantly different: highest hydrogen production was

obtained from wild-type cells and similarly from cells carrying only active HyaB hydrogenase (Δ*hydA*). Significantly lower amount of hydrogen was observed from cells carrying only active HydA (Δ*hyaB).* No hydrogen was detected from cells lacking both hydrogenases (Δ*hydA*Δ*hyaB)*, demonstrating that HydA and HyaB are necessary for hydrogen production and are the only hydrogenases in *S. oneidensis* MR-1. The data also suggests that HyaB is the kinetically dominant H_2 forming hydrogenase.

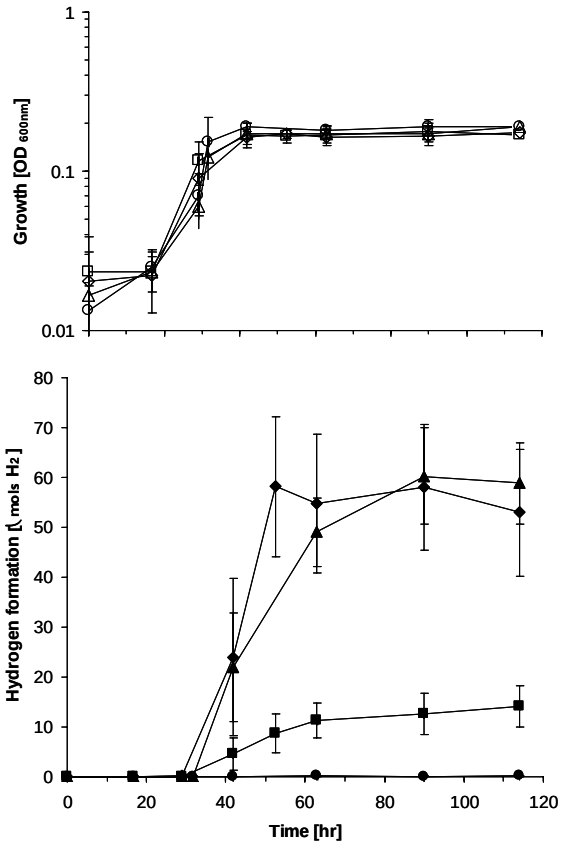

Figure 3. Comparison of growth (A, open symbols) and H_2 production (B, closed symbols) in *S. oneidensis* wild type (♦), cells carrying HyaB only (Δ*hydA* mutant, ▲), cells carrying HydA only (Δ*hyaB* mutant, ■) and cells lacking active H_2ase (Δ*hydA* Δ*hyaB* mutant ●)
Error bars represent standard deviation for at least triplicate cultures in all experiments.

. *In-frame deletion of hydA accessory genes***.**

In order to study the effect of the putative accessory genes in *hydA* operon on the [Fe-Fe] hydrogenase activity, we created in-frame deletions of either *hydG*, *hydE*, *hydF* or *hydX* genes in *S. oneidensis* MR-1 wild-type, and in the background of mutant strains carrying either active HydA (*ΔhyaB)* or HyaB (*ΔhydA)* hydrogenases We then characterized the phenotype of *ΔhydG* deletion strains by testing anaerobic growth and hydrogen evolution from cells grown with electron donor excess. We found that deletion of *hydG* and disruption of the HydG protein results in the complete loss of H_2 evolution

from HydA [Fe-Fe]-hydrogenase without affecting HyaB [Ni-Fe] hydrogenase function (Fig 4).

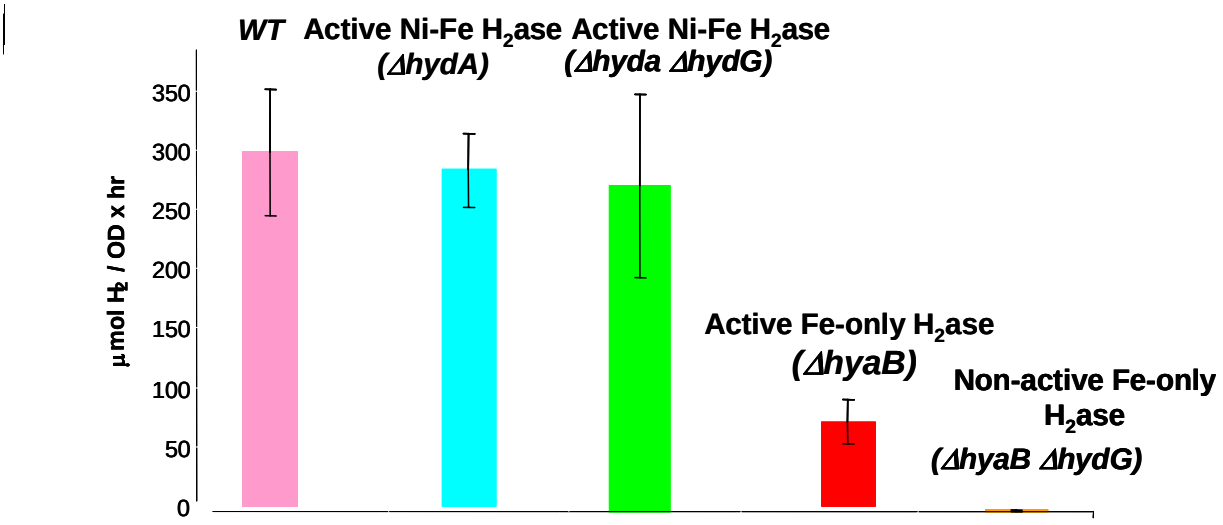

Figure 4. Hydrogen production of *ΔhydG* strains**.** Hydrogen measurements were taken from samples grown anaerobically in the excess of electron donor. Hydrogen measurements were normalized to cell biomass. Data is from 60 hrs. at start of stationary phase.

Future plans

We have developed an experimental platform to study the role and function of the putative accessory proteins HydG, HydE, HydF and HydX in the assembly, maturation and folding of [Fe-Fe] hydrogenase. The knowledge obtained from the *S. oneidensis* model system will be important for the engineering and optimization of the cyanobacterial hydrogen producing system.

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Publications

Meshulam-Simon, G., Choo, A. D. and Spormann, A. M. 2006. Hydrogen metabolism of *Shewanella oneidensis* MR-1. Appl. Envir. Microbiol., in preparation.

Meshulam-Simon, G, Choo, A. D. and Spormann, A. M. Hydrogen metabolism of *Shewanella oneidensis* MR-1. West Coast Bacterial Physiologists meeting at Asilomar, Pacific Grove, CA., December 2005

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