Increasing Hydrogen Photoproduction By Genetic Engineering

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Abstract

Reversible hydrogenases are enzymes which can reversibly produce molecular hydrogen from hydrogen ions. The overall objective of this project is to genetically transform certain high hydrogen producing strains of cyanobacteria with cloned reversible hydrogenase genes derived from well characterized cyanobacterial species. Within the past few years, several reversible hydrogenase gene clusters have been sequenced (*Anacystis nidulans, Synechocystis* PCC 6803, and *Anabaena variabilis*), and these sequences are available from GenBank. Based on the sequences, suitable PCR primers will be synthesized and used to amplify them. These will then be cloned into *E. coli* with the use of a commercially available cloning vector. Upon the successful cloning of the reversible hydrogenase gene clusters, these will be placed in a shuttle vector which can replicate in both *E. coli* and a given strain of cyanobacteria. The shuttle vector containing the reversible hydrogenase gene cluster will be introduced back into the cyanobacterial strain from which the cluster originated in the anticipation that the resulting higher copy number of the reversible hydrogenase genes will cause an increase in the reversible hydrogenase protein and in turn an increase in hydrogen production. The hydrogen production of the transformed cyanobacterial strain will be measured by gas chromatography. In addition, the amplified reversible hydrogenase gene cluster of a given strain will be introduced into the other cyanobacterial strains which contain a known cluster, and their hydrogen production measured. This will establish whether the reversible hydrogenase gene cluster of a particular strain can augment and complement the function of the gene cluster of a different strain in such a way as to increase the production of hydrogen. Finally, the cloned reversible hydrogenase gene clusters will be introduced into certain strains of nitrogen fixing cyanobacteria which already have a high rate of hydrogen production from their nitrogenase enzyme. The hydrogen production of

the genetically transformed cyanobacteria will be measured by gas chromatography in order to determine if the reversible hydrogenase can complement and supplement the function of nitrogenase in a manner which increases the production of hydrogen.

Introduction

One attractive method for the production of hydrogen gas is the conversion of solar energy to H_2 by algal cells. This process, often called photobiological H_2 production, occurs in eukaryotic algae, prokaryotic cyanobacteria, and photosynthetic bacteria. Cyanobacteria can grow on simple mineral salts with light as the energy source, CO_2 as their carbon source, and water as an electron source and reductant (Rao and Hall, 1996). We propose to genetically manipulate cyanobacteria to increase their production of hydrogen gas.

The cyanobacteria, unlike the green algae, have two sets of enzymes that generate hydrogen gas. The first one is nitrogenase and it is found in the heterocyst of heterocystous cyanobacteria when these are grown under nitrogen limiting conditions. As a product of fixing nitrogen gas to ammonia, H_2 is produced. However, the reaction has a high ATP requirement, and this in turn lowers the potential solar energy conversion efficiency to low levels (Benemann, 1996). Also, nitrogenase is highly sensitive to O_2. Despite this, our culture collection contains strains of nitrogen fixing cyanobacteria which have a high hydrogen producing capability and an increased resistance to O_2 (Kumazawa and Mitsui, 1989; Luo and Brand, 1997). These cyanobacteria make potentially excellent candidates for genetic manipulation in an attempt to further increase their hydrogen production.

The other hydrogen producing enzyme in cyanobacteria is hydrogenase and actually consists of two distinct enzymes in many species. The first is uptake hydrogenase, and it only has the ability to oxidize H_2. The enzyme is found in the membranes of heterocysts, where it transfers the electrons from H_2 to the reduction of O_2 via the respiratory chain in a reaction known as oxyhydrogen or Knallgas reaction. The concomitant generation of ATP can be used for further nitrogen fixation. The H_2 can also be used by the uptake hydrogenase to generate low potential reductants for N_2 fixation (Adams et al., 1981; Schmetterer, 1994). The other hydrogenase of cyanobacteria is reversible or bidirectional hydrogenase and as the name implies, it can either take up or produce H_2. Although its specific physiological function is not known, in *Anacystis nidulans* where it has been best studied, the reversible hydrogenase is associated with the cytoplasmic membrane and is assumed to function as an electron acceptor from both NADH and H_2 (references in Boison et al., 1998).

The reversible hydrogenase is a multimeric enzyme consisting of either four or five different subunits, apparently depending on the species (Fig. 1) [Schmitz et al., 1995; Boison et al., 1998]. In *Anabaena variabilis*, four genes (*hoxH, hoxY, hoxU,* and *hoxF*) have been isolated and sequenced (Schmitz et al., 1995). In *Anacystis nidulans* however, Boison et al. (1996, 1998) identified and sequenced a fifth gene called *hoxE* in addition to the four genes mentioned above. Interestingly, *hoxE* can not be detected in the genome of *Anabaena variabilis* by heterologous hybridization with a *hoxE* probe from *Anacystis nidulans* even though the other reversible hydrogenase subunits show a high homology

at the amino acid level (Boison et al., 1998).

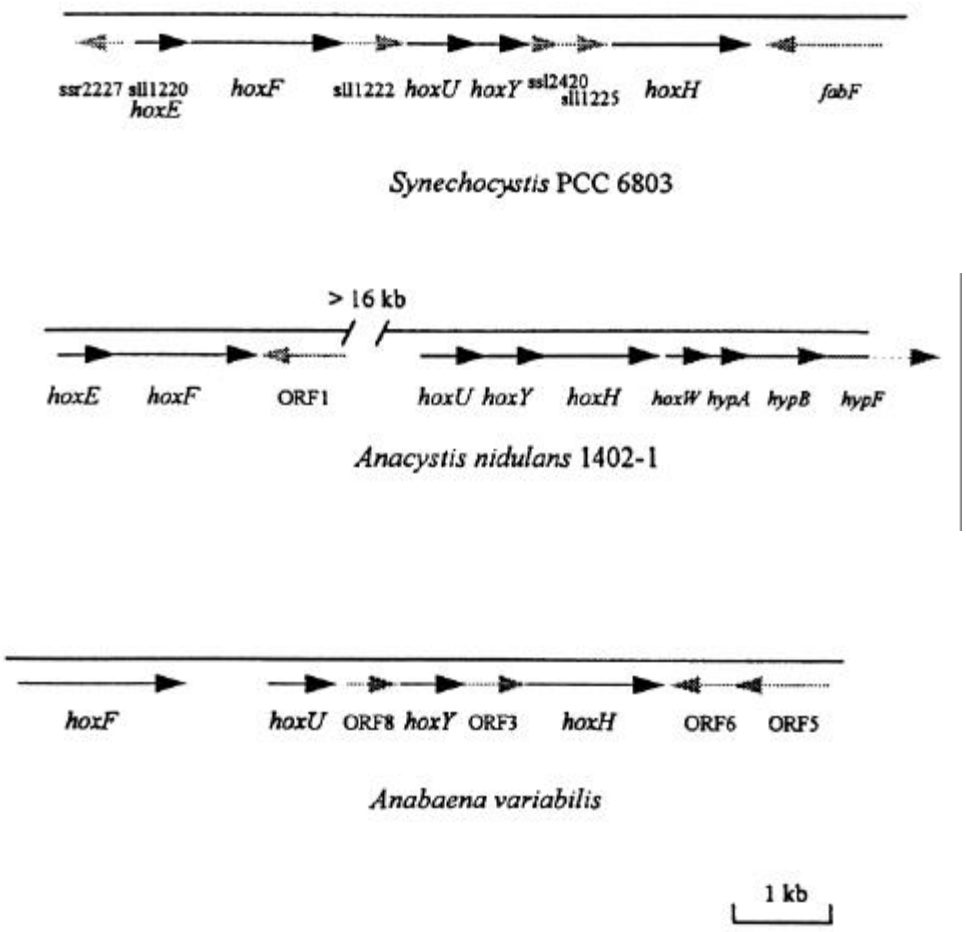

Figure 1. Gene clusters of the bidirectional hydrogenase of *Anacystis nidulans*, *Synechocystis* PCC 6803, and *Anabaene variabilis* (figure from Boison et al., 1998)

They mention that *hoxE* in addition to the four other genes has also been identified and sequenced in *Synechocystis* PCC6803. In *Anabaena variabilis* and *Synechocystis* PCC6803, the genes coding for the reversible hydrogenase all occur on the same segment of DNA. For *Anacystis nidulans* however, the genes *hoxE* and *hoxF* are located on a segment of DNA which is separated by over 16 kb of intervening DNA from the segment which contains the genes *hoxU*, *hoxY*, and *hoxH* (Boison et al., 1998). The sequenced DNA segment which contains the genes *hoxU*, *hoxY*, and *hoxH*, also contains the accessory genes *hoxW*, *hypA*, *hypB*, and *hypF*. The precise function of these genes are not known but they are believed to be involved in the maturation process of the hydrogenase. As one of our goals, we propose to amplify by PCR the segments which contain the reversible hydrogenase genes of the various species, and introduce these into various species of cyanobacteria through genetic transformation, including the high hydrogen producers in our collection, in order to increase their reversible hydrogenase gene copy number and thereby increase their hydrogen production.

Preliminary data

The various high H_2 photoproducers in the culture collection can serve as a standard by which the genetically transformed strains will be compared. The first steps in the goal of cloning the reversible hydrogenase genes have been undertaken. After attempting several unsuccessful methods, high molecular weight DNA was isolated from a variety of cyanobacteria of the genus *Anabaena* and *Synechococcus* by a modification of the method from van den Hondel described by Lambert and Carr (1982). The isolation of high molecular weight DNA is of the utmost importance since the reversible hydrogenase gene clusters reside on long fragments of DNA which in the case of *Anabaena variabilis* is 8.9 kb long (Fig. 1)[Schmitz et al., 1995]. The modified van den Hondel method also allows for the isolation of plasmid DNA. If a particular plasmid from a species is desired, the entire DNA of the cyanobacteria can be isolated, the recovered DNA electrophoresed on a gel, and the plasmid band extracted from the gel. In addition to isolating the desired DNA, the putative PCR primers which will be used to amplify the reversible hydrogenase gene clusters, are in the process of being identified with the use of the computer program OLIGO. These primers will then be used to initiate the PCR protocol.

Future Work

Our proposed future work is indicated in Table 1 below, along with the approximate time required for their completion:

Table 1. Proposed future work and times needed for their completion

tasks	time (months)
1)isolate DNA from *Anacystis nidulans, Synechocystis* PCC 6803, and *Anabaena variabilis*	3
2)PCR amplification of reversible hydrogenase gene clusters	3
3)cloning of gene clusters in *Escherichia coli*	3
4)preselection of clones in *Escherichia coli*	6
5)construction of shuttle vector for *Anacystis nidulans,Synechocystis* PCC 6803, and *Anabaena variabilis*	12
6)cloning of gene clusters in *Anacystis nidulans,Synechocystis* PCC 6803, and *Anabaena variabilis*	18
7)testing by gas chromatography of increased hydrogen producing clones of *Anacystis nidulans,Synechocystis* PCC 6803, and *Anabaena variabilis*	21
8)construction of shuttle vector for selected strains in culture collection	27
9)cloning of gene clusters in selected strains of culture collection	33
10)testing by gas chromatography of increased hydrogen producing clones of selected strains in culture collection	36
11)maintenance of culture collection	36

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