DEVELOPMENT OF AN EFFICIENT ALGAL H_2-PRODUCTION SYSTEM

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Abstract

The ultimate goal of our research is to generate *Chlamydomonas reinhardtii* mutants that are sufficiently tolerant to O_2 to produce H_2 under aerobic conditions. We have been addressing this goal by means of both classical genetic and molecular biology approaches. Two generations of algal mutants were produced by application of chemical mutagenesis, selection, and screening to a wild-type algal strain. The H_2 production activity of the mutants showed up to 9 times higher tolerance to O_2 when compared to the parental strain. However, since the algal hydrogenase gene sequence was not known, it was not possible to determine whether the chemical mutagenesis that gave rise to the O_2 tolerant phenotypes was due to a single-point mutation affecting the hydrogenase gene or whether it was due to a secondary mutation in another gene.

We have concomitantly pursued a molecular biology approach, in order to enhance the probability of ultimately obtaining a commercially-viable organism. Our purpose was to first clone the hydrogenase gene and then to use molecular genetic means to generate random mutations in the hydrogenase gene. The transformed genes would be used in the future to re-transform wild-type *C. reinhardtii,* and O_2-tolerance of the resulting transformants would be determined by chemochromic screening, as was done for the classical mutants.

Current results include the successful cloning and sequencing of not only the Fe-only reversible hydrogenase gene but also a second Fe-only hydrogenase gene in *C. reinhardtii*. Preliminary studies suggesting differences in the expression of the two genes are also reported. The cloning of the algal hydrogenase will also allow us to re-analyze our mutants obtained previously by the classical mutagenesis approach. This will determine the specific locus of each genotype and may help guide us in possible future site-directed mutagenesis approaches to improving O_2 tolerance

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Introduction

Mutant bacterial organisms containing hydrogenases that are able to operate at higher O_2 concentrations have been described (Gogotov 1986, McTavish *et al.* 1995, Maness *et al.* 1999), suggesting that the enzyme is amenable to manipulations that may affect its O_2 tolerance. These observations led us initially to investigate several classical genetic approaches to generate and isolate *C. reinhardtii* mutants that can produce H_2 in the presence of O_2. They involved using random chemical mutagenesis of the organism, followed by the application of selective pressures under gradually increasing O_2 concentrations. The two selective pressures (McBride *et al.* 1977; Ghirardi *et al.* 1996, 1997a, 1997b; Flynn *et al.* 1999) were based on the reversible activity of the algal hydrogenase, e.g., H_2-production and H_2-uptake. A chemochromic sensor was also developed to allow us to quickly screen the survivors of the selective pressures for H_2-producing clones. Using this combination of mutagenesis, selection and screening, we isolated two generations of H_2-production mutants, 76D4 and 141F2, with, respectively, 4 and 9 times higher tolerance to O_2 compared to the WT parental strain (Flynn *et al.*1999; Ghirardi *et al.*,1999; Seibert *et al.* 2000). We also isolated two generations of H_2-uptake mutants, 104G5 and 155G6, with, respectively, 2 and 3 times higher tolerance to O_2 (Ghirardi *et al.* 1999; Seibert *et al.* 2000). The latter were also more quickly reactivated upon removal of O_2 from the medium.

The classical genetic approach described above is a good choice if one is interested in isolating an O_2-tolerant, H_2-producing *organism*, independent of whether the mutation affects the hydrogenase gene or some other gene that confer the same phenotype in the organism. Indeed, genetic crosses involving our different O_2-tolerant mutants in the future will indicate whether more than one locus is involved in the isolated phenotypes. However, if one proposes to generate an O_2-tolerant *hydrogenase* (instead of an O_2-tolerant, H_2-producing *organism*), the preferable approach is random error-prone polymerase chain reaction (PCR) mutagenesis of the cloned gene. This technique will generate random mutants of the cloned hydrogenase gene, which then will be re-introduced back into the wild-type organism for expression.

The *C. reinhardtii* hydrogenase, an Fe-only enzyme has been isolated to purity by Happe and Naber (1993), who also sequenced 24 amino acid residues from the N-terminal portion of the enzyme. However, until recently, no further information on its amino acid or nucleotide sequence was available. Iron-only hydrogenases are a class of enzymes that catalyze either H_2 production or H_2 uptake. These enzymes have been found in anaerobic bacteria or in green algae, and contain two or three 4Fe4S centers and one 2Fe2S center (Adams 1990). The catalytic site of the enzyme consists of a 4Fe4S and a 2Fe2S and is called the H-cluster. Iron-only hydrogenases usually have high specific activity but are very easily inactivated by O_2 and CO. Two different approaches were used to clone the *C. reinhardtii* enzyme, using the polymerase chain reaction to amplify selected mRNA sequences following their conversion into cDNA by the reverse transcriptase (RT-PCR). In the first approach, degenerate primers were designed based on the N-terminal amino acid sequence of the enzyme (Happe and Naber 1993), and they were used in 5' rapid amplification of cDNA ends (RACE) reactions to partially amplify the corresponding mRNA (Ghirardi *et al.* 1998). Many clones

were obtained, but none succeeded in further amplification of the 3' end of the mRNA. In the end, we were unable to clone and sequence the complete gene by this approach. In this paper, we describe a second approach that turned out to be successful. It was based on the high degree of homology between bacterial and algal Fe-only hydrogenases, which was used to guide the design of gene-specific 5' and 3'-end primers used to amplify the catalytic region of the hydrogenase gene from a pool of algal mRNA. The amplified product was then used as a probe to isolate the complete gene from a cDNA algal library.

Materials and Methods

Cell growth, anaerobic induction, mRNA extraction, reverse transcription and RT-PCR

Chlamydomonas reinhardtii cell wall-less strain cw15 (currently identified as cc125) was grown in the light in 15 mM potassium phosphate, 10 mM NH_4Cl, 1 mM $MgSO_4$, 1.5 mM Na_3 citrate, 0.5 mM $CaCl_2$, 0.5 mM $FeCl_3$ and 1 x Hutner's trace elements (Harris 1989) to an OD_{750} of ~1. Harvested cells were anaerobically induced as previously described (Ghirardi *et al.* 1997) and immediately lysed using binding buffer (S.N.A.P kit, Invitrogen Inc.). The pooled total RNA was further enriched for poly-A^+ RNA using the FastTrack 2.0 kit by the same manufacturer. The resulting mRNA was reverse transcribed at 50^oC using poly-dT_{15} primers and random hexamers according to the Superscript II kit by Life Technologies Inc. An aliquot of the cDNA was PCR amplified in 30 mM Tricine, pH 8.4 (at 20 ^oC), 3.5 mM $MgCl_2$, 5 mM ß-mercapto-ethanol, 0.01% gelatin and 0.01% Igepal CA-630, 1.2 mM dNTPs, 1.5 µM primers with annealing at 58 ^oC for 30 cycles.

cDNA library screening and lambda phage plasmid excision

The amplified products were cloned onto the cloning vector pUC19. An amplification product generated from clone RC53_43 was non-isotopically labeled using digoxigenin-11-dUTP (Roche Molecular Biology) and used to screen a lambda ZAP II *C. reinhardtii* cDNA library (kindly provided by John Davies, Exelixis Inc.). Positive plaques were purified by replating and rescreening and the cDNA inserts retrieved as pBluescript SK(-) plasmids using the Rapid Excision kit by Stratagene Inc, with modifications. The cDNA clone containing the amplified product was designated Hyd A.

Expressed sequence tag (EST) amplification and cDNA library screening

Two specific primers, BE5P1 and BE3P1 were used to amplify an EST (shown to have a high degree of homology to other Fe-only hydrogenases in the GenBank database) from a sample of the cDNA library (see above), using the Failsafe PCR kit (Epicentre Inc.). Clone EST_26, which contained the amplified nucleotide sequence of the EST was used to generate digoxigenin-labelled probes for cDNA library screening. A strongly hybridizing clone, designated Hyd B, was obtained, retrieved in pBluescript SK (-) and shown to be derived from the EST.

DNA sequencing and assembly of Fe-only hydrogenase clones Hyd A and Hyd B

Cesium chloride density gradient purified plasmid DNA was submitted to the primer walk service at the Iowa State University Sequencing and DNA Synthesis Facility. Sequence reads were evaluated based on the chromatograms and assembled using the Wisconsin Package V. 10 software by Genetics Computer Group, Inc. Both strands of the DNA were sequenced independently.

Northern blot analysis

Five μg of total RNA was fractionated by electrophoresis on 1.2% formaldehyde agarose gels and transferred to Nytran N^+ membranes by capillary blotting. The nucleic acid was fixed by UV cross-linking and baking for 2 h at 80 ^oC. Membranes were prehybridized in 6 x SSC buffer, 10% dextran sulfate, 50% formamide, 1 x Denhardt's solution, 0.1% SDS and 100 μg/mL denatured salmon sperm DNA at 42^oC for ≥ 1 h. Denatured ^{32}P labelled probe was added and the blots were washed at medium stringency (0.5 x SSC, 0.5% SDS at 58^oC) after overnight hybridization. Membranes were exposed to storage phosphor screens (Kodak Inc.) and scanned with a STORM 860 PhosphorImager (Molecular Dynamics Inc.). Quantitation was performed with the ImageQuant software provided. Nytran membranes were stripped in hot 0.5% SDS and rehybridized as needed.

H_2-Evolution Assay

MOPS buffer (50 mM, pH 6.8) was added into a water-jacketed chamber (a 2.5 ml volume held at 25°C) and equipped with two Clark electrodes (YSI 5331, Yellow Springs, OH), one poised for the measurement of H_2 and the other for O_2 production (Ghirardi *et al.* 1997b). The O_2 concentration in the cuvette was set close to zero with Ar, and 200 μl of anaerobically induced cell suspension were injected into the buffer. The cell suspension was illuminated (320 μE m^{-2} s^{-1}, PAR incandescent light filtered through 1% $CuSO_4$) for three minutes. The initial rates of H_2 production were estimated from the initial slope of the curves.

Results and Discussion

RT-PCR amplification of the hydrogenase active site

A multiple sequence alignment of Fe-only hydrogenases from *Clostridium pasteurianum*, *Trichomonas vaginalis* and *Desulfovibrio vulgaris* was obtained and used as input to the CODEHOP algorithm (http://www.blocks. fhcrc.org/codehop.html) to generate potential primers for the PCR amplification reaction. The chosen primers, RC5 and RC3, had 64 and 32 degeneracies, respectively. An anaerobically induced sample of *C. reinhardtii* provided the source mRNA material for direct RT-PCR amplification. A double-band centered around 800 basepairs was evident in the lanes originating from induced mRNA but not in the controls (not shown). The band was excised and blunt-end cloned into the vector pUC19. One of the resulting clones, RC53_43 showed all four strictly conserved cysteines that are involved in binding the catalytic 4Fe-4S cluster bridged to a 2Fe-

2S cluster, also termed the H-cluster (the active site of the reversible hydrogenase enzyme). It contained a 43 amino acid insertion between H-cluster motif 2 and 3, similar to a 28 amino acid insertion at the same location in a putative Fe-only hydrogenase clone from *Scenedesmus obliquus* (Wunschiers *et al.* 2001). Figure 1 shows that the expression of this active site sequence is dependent upon anaerobiosis, supporting its possible role as a hydrogenase.

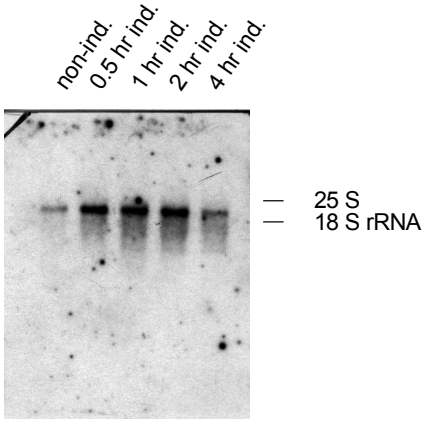

Figure 1. Northern blot of mRNA isolated from non-induced and anaerobically-induced *C. reinhardtii* cells and probed with RC53_43.

Retrieval of full-length Hyd A and Hyd B clones

The active site clone RC53_43 was used as a probe to retrieve a full-length cDNA clone from the cDNA library. From 30,000 lambda plaques plated, 40 hybridized to the active site probe. The lambda plaques were subject to an *in-vivo* excision procedure to obtain the cDNAs as pBluescript SK (-) plasmids. Restriction site analysis and DNA sequencing proved one of our clones, denoted Hyd A, to be 100% identical in the first 1,83 kb to an unpublished clone in GenBank, denoted hyd1 (Accession no. AF 289201). Hyd A was retrieved as a 2.45 kilobase cDNA exhibiting an open reading frame (ORF) encoding 498 amino acids. It also contains the previously reported 24 amino acid N-terminus (Happe and Naber 1993), preceded by a 56 residue leader peptide that directs the protein to the chloroplast stroma.

The deduced amino acid sequence of clone RC53_43 also revealed another close match in a BLAST search to an expressed sequence tag (EST) from *C. reinhardtii*. We designed primers for the amplification of this EST from the cDNA library. The resulting clone EST_26 was identical in nucleotide sequence to the EST, as revealed by DNA sequencing. It was also distinctively different from the active site clone RC53_43 in that it contained a unique restriction site not found in the latter. Clone EST_26 was used as probe to screen a cDNA library, which led to the retrieval of a full-length clone, Hyd B. The Hyd B cDNA is slightly larger in size than Hyd A and is predicted to encode a 505 residue enzyme. An online service (http://www.cbs.dtu.dk/services/ChloroP-1.0.html) predicted the presence of a chloroplast sequence with a cleavage site after 16 amino acids from the start of the ORF.

Table 1 shows that a comparison of our newly isolated clones to Hydrogenase I from *Clostridium pasteurianum* (Meyer and Gagnon 1991) and Hyd A from *Scenedesmus obliquus* (Florin *et al.* 2001), as expected, places them in closer relationship with the latter (Accession no. AJ271546, Table 1). *Scenedesmus* and *Chlamydomonas* Fe-only hydrogenases share a number of characteristic features only found in the green algal enzymes: a short (*Scenedesmus*) to intermediate (*Chlamydomonas*) insertion in-between H-cluster motifs 2 and 3 and the lack of the more distal 4Fe-4S clusters normally found in all bacterial and protozoan Fe-only hydrogenases (Cammack 1999). The biological implications of these unique features are not known at present.

Table 1. Comparison of amino acid sequences of different cloned Fe-only hydrogenases.

C. reinhardtii **hydrogenase clone**	***C. pasteurianum*** **Cp 1H**	***S. obliquus*** **Hyd A**
Hyd A	47 % similar, 40 % identical	69 % similar, 63 % identical
Hyd B	48 % similar, 42 % identical	71 % similar, 63 % identical

Analysis of Hyd A and Hyd B mRNA levels upon anaerobic induction

The expression of the two hydrogenase clones was studied by Northern blot analyses, and compared to the expression of the enzyme activity by incubation under anaerobic conditions. Figure 2 (left panel) shows that a small amount of Hyd A mRNA is detected under aerobic conditions (0 h) and that it increases about 4-fold during a 4-h anaerobic incubation period. These data are in agreement with measurements of light-dependent hydrogenase activity (Fig. 2, right panel). The levels of Hyd B mRNA, on the other hand, seem to change little during the anaerobic induction treatment, and do not correlate with enzyme activity. The results suggest that Hyd A encodes the chloroplast reversible Fe-only hydrogenase linked to photosynthetic electron transport through ferredoxin. The nature of the hydrogenase encoded by Hyd B is not known at present. It is possibly a second chlorolast protein, and its physiological role will be the focus of future research.

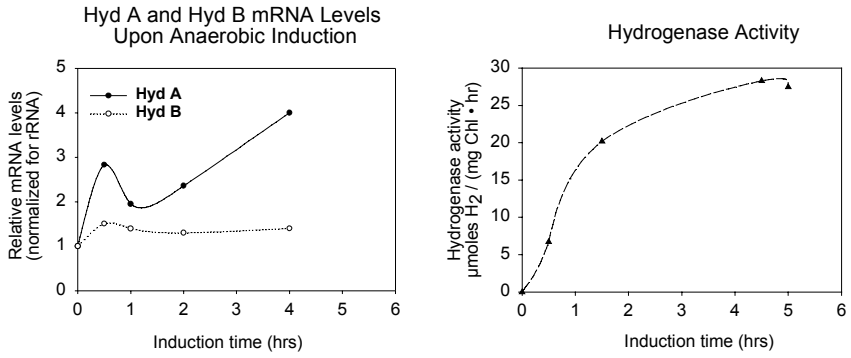

Figure 2. Quantitation of mRNA levels of Hyd A and Hyd B upon anaerobic induction of *C. reinhardtii* cells (A) and overall hydrogenase activity as judged by amperometric measurement of H_2 evolution (B).

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