DEVELOPMENT OF AN EFFICIENT ALGAL H_2-PRODUCTION SYSTEM

Maria L. Ghirardi, Timothy Flynn, Marc Forestier and Michael Seibert
National Renewable Energy Laboratory
Golden, CO 80401

Abstract

The direct photoevolution of H_2 from water by green algae is a transient phenomenon, due to the rapid inactivation of the reversible hydrogenase (the enzyme catalyzing the reduction of protons to H_2) by O_2, a by-product of photosynthesis. Moreover, the expression of the algal H_2 production activity requires prolonged anaerobic treatment of the cells in order to induce the enzyme. We are addressing the O_2-sensitivity problem of algal hydrogenase by means of both classical genetics and molecular biology approaches. The ultimate goal of our research is to generate a *Chlamydomonas reinhardtii* mutant that is sufficiently tolerant to O_2 to produce H_2 under aerobic conditions. The availability of such mutant will permit the development of a commercial photobiological H_2-production system that is cost effective, renewable, scalable and non-polluting.

The classical mutagenesis/selection approach that we have developed to obtain such a desirable mutant takes advantage of the reversible activity of the algal hydrogenase. We have designed two selective pressures that require mutagenized algal cells to survive by either metabolizing (photoreductive selective pressure) or evolving (H_2-evolution selective pressure) H_2 in the presence of O_2 concentrations that inactivate the wild-type (WT) enzyme. Given the generally low specificity of the two selective pressures, the surviving organisms are subsequently subjected to a positive screen using a chemochromic sensor that detects H_2 evolved by the algae. Clones that are found to exhibit high H_2-evolution activity in the presence of O_2 are characterized in more detail using biochemical assays. The strategy currently employed consists of re-mutagenizing, re-selecting and re-screening first generation mutants under higher selective stringency in order to accumulate single-point mutations, and thus, to further increase the O_2 tolerance of the organism.

Results for the past year include further optimization of the two selective pressures; the adoption of a new assay for characterizing the O_2 tolerance of selected clones; the isolation of a clone, 104G5 with 14 times higher tolerance to O_2 than the WT (obtained by the application of one round of photoreductive selective pressure); and the isolation of first and second generation mutants with, respectively, 3-4 and 10 times higher tolerance to O_2 than the WT (by application of two rounds of the H_2-evolution selective pressure).

In order to enhance our probability of ultimately obtaining a commercially-viable organism, we have also been pursuing a molecular biology approach, which is synergistic with the classical genetic strategy described above. We intend to (a) clone the hydrogenase gene and use site-directed mutagenesis (based on information gathered from mutations generated by the classical approach that affect the O_2 tolerance of the hydrogenase) to further increase the O_2 tolerance of the enzyme and (b) identify other proteins whose presence may be require for optimal algal H_2 evolution. Two techniques are currently being used to achieve these goals, mainly RT-PCR (which allows the amplification of a specific DNA sequence out of a population of isolated mRNA) and the construction of a subtractive library, which will contain mRNAs representing proteins that are expressed only upon anaerobic induction of the cells. This is on-going work, and current year advances consisted of the production of the subtracted DNA from induced minus uninduced cells corresponding to 1 and 4 h of anaerobic treatment.

Preliminary results with the University of California, Berkeley on the development of a two-phase algal H_2-production system will not be presented here, but have suggested the scientific feasibility of employing indirect biophotolysis to develop a commercial algal H_2-production system as a mid-term solution until an O_2-tolerant organism becomes available.

Introduction

Green algae such as *Chlamydomonas reinhardtii* can photoproduce H_2 from water in a reaction catalyzed by the reversible hydrogenase enzyme (Gaffron and Rubin 1942). In the light, electrons released by the oxidation of water molecules are transferred through photosystems II and I to the low redox potential carrier ferredoxin. Normally, reduced ferredoxin supplies electrons to the CO_2 fixation pathway. However, following an anaerobic treatment in the dark, algal cells induce the reversible hydrogenase (Ghirardi et al 1997b), an enzyme that can combine photosynthetically-generated electrons and protons to generate H_2 gas. The hydrogenase pathway competes with CO_2 fixation for electrons from reduced ferredoxin. This competition is short-lived, though, due to the prompt deactivation of hydrogenase by O_2 that is concomitantly released by photosynthetic water oxidation (Schulz 1996). The O_2-sensitivity trait of the hydrogenase has precluded the use of green algae in a direct biophotolysis H_2-production system (Benemann 1996).

The occurrence of reversible hydrogenase enzymes is not restricted to algae. They are present in many anaerobic microorganisms, in photosynthetic bacteria, and in cyanobacteria (Adams 1990, Wu and Mandrand 1993, Albracht 1994). The physiological roles and biochemical characteristics of these hydrogenases are variable. However, in all instances the enzyme is ultimately inactivated by O_2. However,

mutant organisms containing hydrogenases that are able to operate at higher O_2 concentrations have been described (Gogotov 1986, McTavish et al. 1995, Weaver et al. 1999), suggesting that the enzyme is amenable to manipulations that may affect its O_2 tolerance. These observations led us to investigate several approaches to generate and isolate *C. reinhardtii* mutants that can produce H_2 in the presence of O_2.

We originally proposed to use random mutagenesis, followed by employing selective pressures under gradually increasing O_2 concentrations, to isolate the desired phenotype. Two selective pressures were designed (Ghirardi et al. 1996, 1997, 1997b), based on the reversible activity of the algal hydrogenase, e.g. H_2-production and H_2-uptake. Under H_2-production selective pressure, algal cells are required to survive a short treatment with metronidazole (MZ), a drug that competes with the hydrogenase for electrons from photosynthetically-reduced ferredoxin. Reduced MZ generates a radical that, in the presence of O_2, produces superoxide radicals and H_2O_2, both of which are toxic to the algae. The selective pressure is applied in the presence of O_2 levels that are known to deactivate the WT hydrogenases. In organisms that have a hydrogenase that is active following exposure to O_2, some of the electrons from reduced ferredoxin can be used for H_2 production instead of MZ reduction and decreased toxicity can be observed (Ghirardi et al. 1996, 1997, 1997b). Similarly, the photoreductive pressure requires the algal cells to survive in an atmosphere of CO_2, H_2, and controlled concentrations of O_2, in the presence of the herbicides 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) and atrazine. These herbicides block photosynthetic O_2 evolution and electron flow on the reducing side of photosystem II and prevent electrons from water from reaching the hydrogenase enzyme. The surviving organisms grow by fixing CO_2 with electrons obtained from the oxidation of H_2, catalyzed by an O_2-tolerant hydrogenase, and ATP generated by cyclic electron transport around photosystem I (Ghirardi et al. 1997, Flynn et al. submitted).

However, it quickly became apparent that the selective pressures were not specific enough and yielded a mixed population of survivors. A chemochromic sensor was then developed in collaboration with D. Benson, NREL, to allow us to quickly screen the survivors of the selective pressures for H_2-producing clones. The screening was based on the ability of a multilayer, thin film device containing WO_3 and Pd to change color (from transparent to blue) upon exposure to nanomoles of H_2. The usefulness of the film in detecting H_2 evolved by algal colonies on agar plates was demonstrated previously (Ghirardi et al. 1998; Seibert et al. 1998; Flynn et al., submitted). We will discuss a procedure that was developed to detect H_2 production by O_2-tolerant algal mutants using the chemochromic sensor, and present evidence that the combination of mutagenesis, selection and screening steps successfully results in the isolation of clones with increased tolerance to O_2.

The use of random mutagenesis to generate *C. reinhardtii* mutants, followed by selection for the desired phenotype has been successfully used in biochemical research for many years. This technique is employed when the gene that encodes a particular protein has not been cloned, precluding the use of site-directed mutagenesis. The algal hydrogenase 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, the DNA sequence of the gene encoding the hydrogenase enzyme in *C. reinhardtii* has not been determined, and site-directed mutagenesis is not possible at present. We have explored two techniques to clone the algal hydrogenase gene. The first one, RT-PCR (reverse transcriptase polymerase chain reaction) represents a short-cut approach that, if successful, could be accomplished in a relatively short period of time. The second technique, subtractive hybridization, is a longer-term approach, and relies on the assumption that

the hydrogenase is transcriptionally regulated, as are the majority of the photosynthetic genes in *C. reinhardtii*. Progress on both techniques will be reported.

Materials and Methods

Cell Growth

Wild-type (WT) *C. reinhardtii* ($137c^+$) was a gift from Prof. S. Dutcher, University of Colorado, Boulder. Algal cells were grown photoautotrophically in basal salts (BS), a modification of Sueoka's high salt medium (Harris 1989) that includes citrate to prevent salt precipitation. This formulation contains the following salts: 10 mM NH_4Cl, 1 mM $MgSO_4$, 7.5 mM KH_2PO_4, 7.5 mM K_2HPO_4, 1.5 mM Na_3-citrate, 0.5 mM $CaCl_2$, 20 μM $FeCl_3$, and 1/2 x Hutner's trace elements. This medium can be solidified with 1.5% w/v agar and amended with 0.5 g/l yeast extract (Difco) for plates, and may be supplemented with 10 mM sodium acetate depending on the experiment. Liquid cultures were grown under continuous cool white fluorescent lamp illumination (70 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR) at 25°C and agitated on a shaker. Cells were harvested by centrifugation at 2000 x g for 10 min and resuspended in liquid BS medium.

Mutagenesis

Mid-log phase cultures were harvested and resuspended in liquid BS to yield a 10 ml suspension of $7x10^6$ cells/ml. Ethylmethane sulfonate (EMS) was added to a final concentration of 5 μl/ml (46 mM), and the cells were incubated with gentle agitation for various periods of time. At the end of the incubation period the cells were washed and resuspended in 50 ml of the same medium lacking EMS. For the 5-bromouracil (5BU) mutagenesis, 550 ml of liquid BS medium were inoculated with 20 ml of mid log phase culture to give an initial cell density of 4.9×10^4 cells/ml. The culture was grown overnight under fluorescent illumination (70 $\mu E \cdot m^{-2} \cdot s^{-1}$) and then sparged with 2% CO_2 (50 ml/min). A filter-sterilized stock solution of 5-bromouracil (dissolved in BS) was then added to the culture to a final concentration of 1 mM. The culture was incubated under the same conditions for another 48 h, at which point the cells were harvested, washed, and resuspended in 50 ml of BS medium. Liquid cultures from either the EMS or 5BU mutagenesis were grown in the light as above for at least 7 days before being submitted to the selective pressures.

Photoreductive Selection (PR) Procedure

Liquid cultures of mutagenized algal cells (250 ml, 2.8×10^5 cells/ml) in BS were treated with 15 μM each of DCMU and atrazine, and the flasks were placed in anaerobic jars. The gas phase contained 16.5% H_2, 2% CO_2, 5-20% O_2, balanced with Ar. The cultures were grown for a couple of weeks with stirring and illuminated with fluorescent light (70 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR). At the end of the selection period, the cells were washed with BS medium and revived in liquid BS medium plus 10 mM sodium acetate.

H_2-Production Selection (MZ) Procedure

A suspension of anaerobically-induced algal cells was mixed with an anaerobic MZ-Na azide solution to

final concentrations of 40 mM MZ and 1 mM sodium azide at 2.8 x 10^6 cells/ml. While maintaining darkness, O_2 was added to 5% in the gas phase, and the mixtures were shaken vigorously for 4 min. Immediately following the O_2 treatment, the cultures were exposed for 6 min to light (320 $\mu E \cdot m^{-2} \cdot h^{-1}$ PAR) filtered through a solution of 1% $CuSO_4$ with mixing. At the end of the selection period, the cells were washed with BS medium and either resuspended in the same medium or plated for cell counting.

Chemochromic screening

Individual colonies surviving mutagenesis and selection were transferred to square petri dishes that can easily accommodate an 8 x 8 colony matrix and a square chemochromic sensor. Following a 7-14 day growth period, the agar plates were made anaerobic overnight to induce the algal hydrogenase and then preexposed to 21% O_2 for different periods of time in the dark to deactivate the WT phenotype. The plates were immediately transferred to an anaerobic glove box, the sensor applied, and the colonies were illuminated for 3 minutes to photoevolve H_2. At the end of the illumination period, the sensors were analyzed for the location of blue dots, corresponding to the algal colonies that still evolved H_2 following the O_2 pretreatment. The identified clones were then transferred from the original plate to liquid BS + 10 mM acetate, and were cultivated for further characterization.

H_2-Evolution Assay

Mid-log phase algal cultures were harvested and resuspended in 20 ml phosphate buffer (Ghirardi et al. 1997b), supplemented with 15 mM glucose and 0.5% v/v ethanol, and were then made anaerobic with Ar bubbling. Concurrently, 2 ml of an enzymatic O_2-scrubbing system (Packer and Cullingford 1978) that consisted of 1mg/ml glucose oxidase and 27,720 units/ml catalase was dispensed into dialysis tubing (6-8 kD MW cutoff) and made anaerobic as above. The dialysis bags were added to the cell suspensions and the vials were sealed, covered with aluminum foil, and incubated at room temperature for 4 h. Following this induction treatment, the cell suspensions were kept at 4 °C overnight. The assay reaction consisted of exposing the cells to various levels of O_2 for two minutes, reestablishing anaerobiosis, and adding reduced methyl viologen to serve as the electron donor to the hydrogenase. The reaction mixtures were incubated in the dark for 15 minutes at 30°C in a shaking water bath, and the reaction was stopped by adding trichloroacetic acid. The presence of H_2 was detected by gas chromatography.

Results and Discussion

Classical Mutagenesis

Random mutagenesis of WT *C. reinhardtii* cells was done with either ethylmethane sulfonate (EMS) or 5-bromouracil (5BU), in order to generate different types of mutants. EMS alkylates adenine and guanine and gives rise to tautomeric shifts, allowing adenine to pair with cytosine and guanine to pair with thymine (Klug and Cummings 1983). As a result, A-T↔G-C transition mutations are created. Bromouracil is an analog of thymine that, instead of pairing with adenine, pairs with guanine, causing A-T↔G-C transition mutations (Klug and Cummings 1983). The frequency of mutants among survivors increases with mutagen dose, but so does the damage to the genetic background (Bos 1987). Therefore, killing rates of

less than 60% were chosen to minimize damage to the remainder of the genome, at the expenses of high mutation frequency. The problem of decreased mutant frequencies among the survivors is normally solved by employing effective selection procedures.

Populations of algal cells treated with either of the two mutagens for different periods of time were then submitted to either the PR or the MZ selective pressure, in the presence of controlled amounts of O_2. Figure 1 summarizes the fate of each mutagenized population. Cells mutagenized with EMS

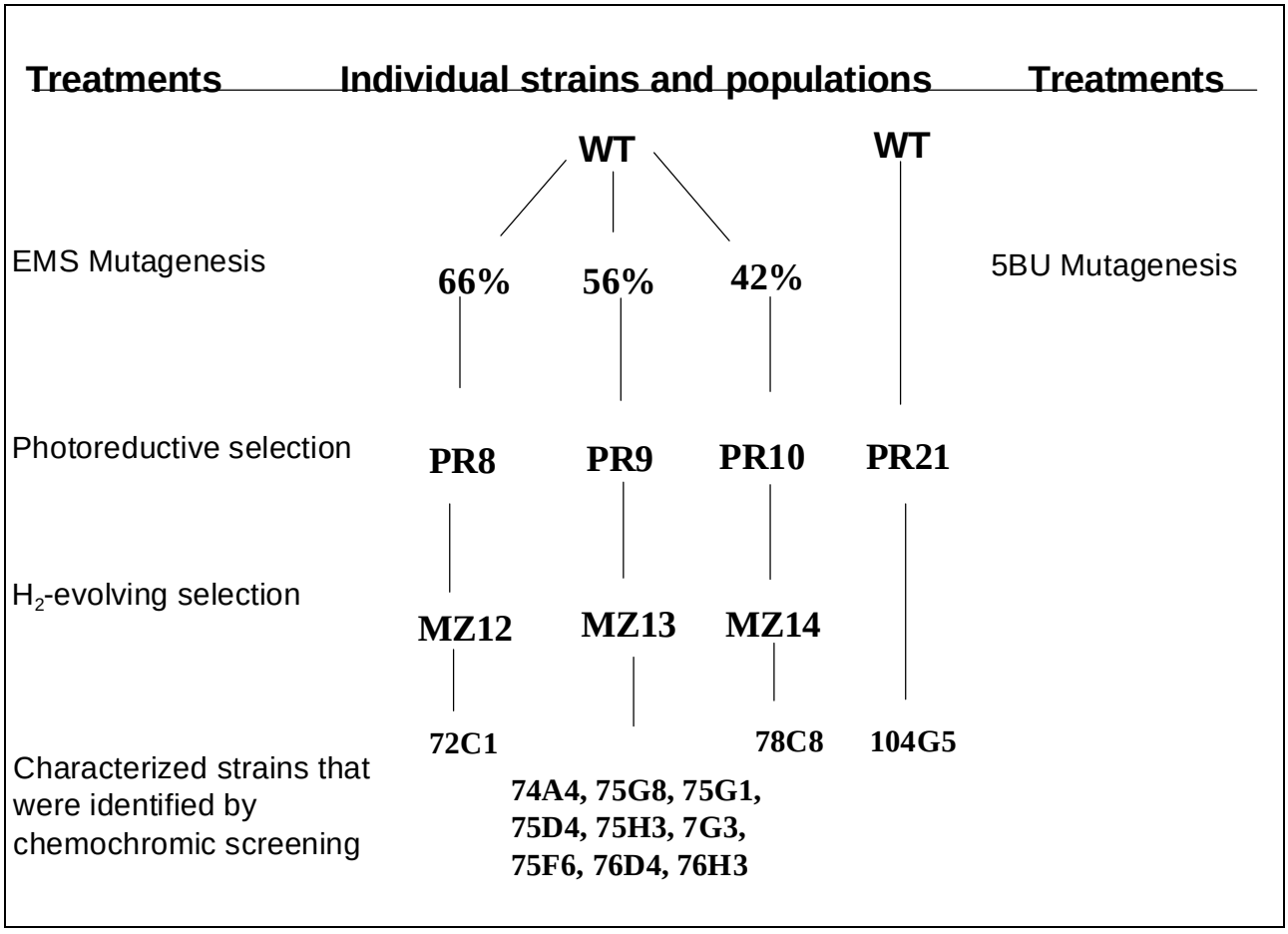

Figure 1. Treatment history of the various strains.

were initially submitted to the PR selective pressure in the presence of 5% O_2. The survivors from this selective pressure, populations PR8, PR9, and PR10 were grown for 7-14 days, plated, and chemochromically screened following deactivation with atmospheric levels of O_2 for 2 min. Clones that produced the best signal during the screening procedure were characterized in a more detailed manner, using a polarographic method for determination of O_2 tolerance. None of the selected clones had a significant increase in O_2 tolerance, although the 24g1 mutant and others showed up to 4 times higher rates of H_2 evolution, compared to the WT control (Ghirardi et al. 1998). The lack of improvement in O_2-tolerance in this first round of mutants forced us to re-assess the PR selection protocol. We determined

that the initial O_2 concentration of 5% O_2 set in the anaerobic jar was significantly decreased by cellular respiration of the cultures during the application of the selective pressure. This problem was solved in subsequent experiments by replacing the gas mixture daily until the culture became chlorotic, indicating that the majority of the cells were dead.

The PR8, PR9 and PR10 populations, which contained mutants with higher rates of H_2 evolution (see above), were subsequently submitted to the MZ selective pressure following deactivation by 5% O_2. The surviving populations (less than 3% of the initial cell density), MZ12, MZ13 and MZ14, were resuspended in liquid medium and plated to yield single colonies. Two hundred and forty clones from each of the three populations were screened as above, and selected clones were further assayed to determine their O_2 tolerance relative to WT cells.

In addition, one population of WT cells was mutagenized with 5-bromouracil (see Fig. 1, right), which was done by adding the mutagen to actively dividing cells for a total incubation time of 2 days. This long exposure may have allowed more than a single mutation to occur. The resulting population was submitted to the PR selection in the presence of 20% O_2. Gases were exchanged in the anaerobic jar on a daily basis. The survivors from this PR selection were screened, and the best clones were also assayed for O_2 tolerance.

In the past, measurement of the O_2 tolerance of the clones had been done by determining an O_2 I_{50} for H_2 evolution, that is, the concentration of O_2 added to the gas phase that inhibited the rate of algal H_2 evolution by half of the value measured in the absence of added O_2 (Ghirardi et al. 1996, 1997, 1997b, 1998; Seibert et al. 1998). Rates of H_2 evolution were measured polarographically with a Clark-type electrode, poised for the detection of H_2. Anaerobically-induced algal cells, resuspended in the presence of an O_2-scrubbing system (to insure maintenance of anaerobiosis) were treated with O_2 in the dark for 2 min and illuminated. The rate of H_2 produced during the first 5 min of illumination was used to calculate an initial rate of H_2 evolution by the algal cells. There was a major problem with this procedure. The presence of the O_2-scrubbing enzymatic system in the cell suspension was very effective in keeping the culture anaerobic by quickly consuming the O_2 added to the gas phase during the dark inactivation of the enzyme, resulting in variable concentrations of O_2 throughout that period. Thus, the O_2 I_{50}s previously obtained, may have been overestimated. In order to eliminate this problem, a new assay procedure to estimate O_2 tolerance of selected clones was adopted and optimized. The new procedure involved physically separating the algal cell suspension from the O_2-scrubbing enzymatic system during the dark anaerobic incubation period, and then transferring only the cells to a separate vial where O_2 deactivation and H_2-evolution activity measurements were done (see Materials and Methods section). To deactivate WT hydrogenases, a controlled amount of O_2 was added to the anaerobic cell suspension in the sealed vial and incubated under vigorous mixing for 2 min. Anaerobic conditions were then rapidly re-established, and methyl viologen (reduced by addition of sodium dithionite) was added to serve as the electron donor to the hydrogenase. The mixture was incubated in the dark for 15 min at 30°C, and the reaction was stopped by the addition of trichloroacetic acid. The amount of H_2 produced was then detected by gas chromatography.

Table 1 summarizes the characteristics of the indicated selected clones from each experiment. The survivors derived from the MZ13 selection showed the best response to the chemochromic screening

Table 1. Characteristics of selected strains.

Original population	Strain	V_o (μmoles H_2/(mg Chl x h)	% of V_0	O_2 I_{50}
WT	-	39	0.26	0.22
MZ12	72C1	81	14	-
MZ13	76D4	78	18	0.82
	76H3	72	35	0.96
	74A4	64	15	-
	75G8	50	26	-
	75G1	82	17	-
	75D4	88	18	-
	75H3	67	27	-
	75G3	73	29	-
MZ14	78C8	64	9	-
PR21	104G5	82	59	2.8
76D4	141F2 (2nd MZ treatment)	88	42	2.0

(possibly because better mutants had been generated by the EMS mutagenesis step), and thus contributed the most clones to the assay. The parameters used to initially characterize the O_2 tolerance of the mutants included the maximum rate of H_2 evolution measured without any exposure to O_2 (V_0) and the amount of H_2-evolution activity remaining after an exposure to 2% O_2 for 2 min (% of V_0). The % of V_0 parameter was used to roughly compare the relative O_2-tolerance of the mutant clones to the WT strain. Four strains were more fully characterized by titrating the H_2-evolution activity following deactivation of the enzyme with increasing levels of O_2 for 2 min. The O_2 I_{50} was then estimated by fitting the data to a single exponential decay function. Figure 2 shows O_2 titration curves for, respectively, WT, 76D4 and 104G5 clones. The estimated O_2 I_{50}s are shown in Table 1.

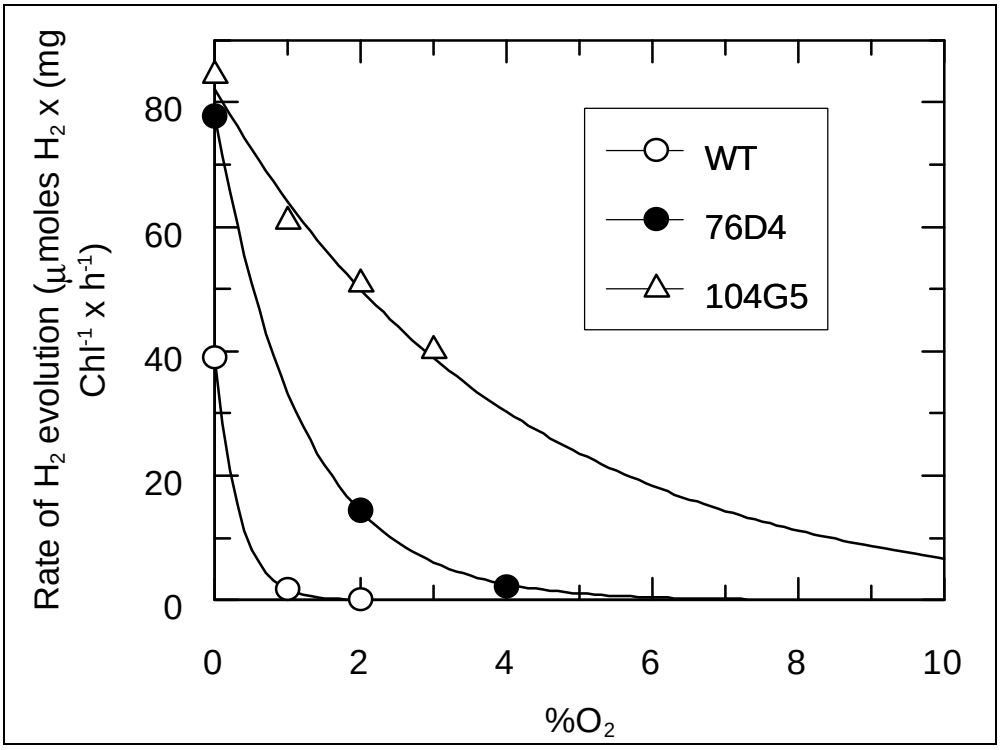

Figure 2. O_2 titration of the rates of H_2 evolution by selected clones

Further inspection of Table 1 reveals that all of the mutants identified by the screening assay were improved with respect to V_0 and O_2-tolerance, compared to their parental WT strain. The V_0s were increased in all of the mutants, with a 2.3-fold increase observed in clone 75D4. The O_2 I_{50}s were increased by 3.7-4.4-fold in clones 76D4 and 76H3. The least improved mutant, 78C8 had only a 9% increase in O_2-tolerance compared to the WT strain, which may represent the minimum phenotype for surviving the conditions used in this H_2-evolving selection experiment. The 76D4 clone was remutagenized with EMS (61% survival), reselected using a second MZ procedure with a selective pressure of 40% O_2 in the dark for 5 min, and finally screened, following deactivation with 100% O_2 for 5 min. Table 1 shows that one of the resulting clones, 141F2 had over a 2-fold increase in I_{50} compared to its parent (76D4) and almost a 10-fold improvement compared to the grandparent WT strain. The 104G5 clone was our best isolate, and had an O_2 I_{50} about 14 times higher than the WT strain. It is important to point out that this strain was isolated after a single photoreductive selection, and verifies that the modification of the PR procedure have been successful.

Given the range of increased O_2-tolerance measured with the different clones in Table 1, one could argue that there is more than one genotype that gives rise to the O_2-tolerant phenotype. The following three obvious possibilities exist: (a) different amino acid substitutions at a single critical residue, (b) random substitutions distributed throughout the O_2-sensitive domain, or (c) mutations on genes other than the

hydrogenase, causing a decrease in intracellular O_2 concentration, such as through increased rates of respiration or decrease cell membrane permeability to atmospheric O_2. It is noteworthy mentioning that two rounds of mutagenesis/selection/screening yielded an organism with increasingly higher O_2 tolerance, supporting either of the last two possibilities discussed above.

Molecular Biology

RT-PCR

The N-terminal portion of algal hydrogenase sequenced by Happe and Naber (1993) does not show homology to other hydrogenases nor to any proteins in the available databases. However, using the known *C. reinhardtii* genome bias towards GC (Rochaix 1995), we designed 2 degenerate nested DNA primers and used them in two subsequent rounds to amplify mRNA isolated from algal cells that had been induced for at least 1h, using the 5' RACE (Rapid Amplification of cDNA Ends) technique. Figure 3 shows the strategy used to specifically amplify the algal hydrogenase. The first

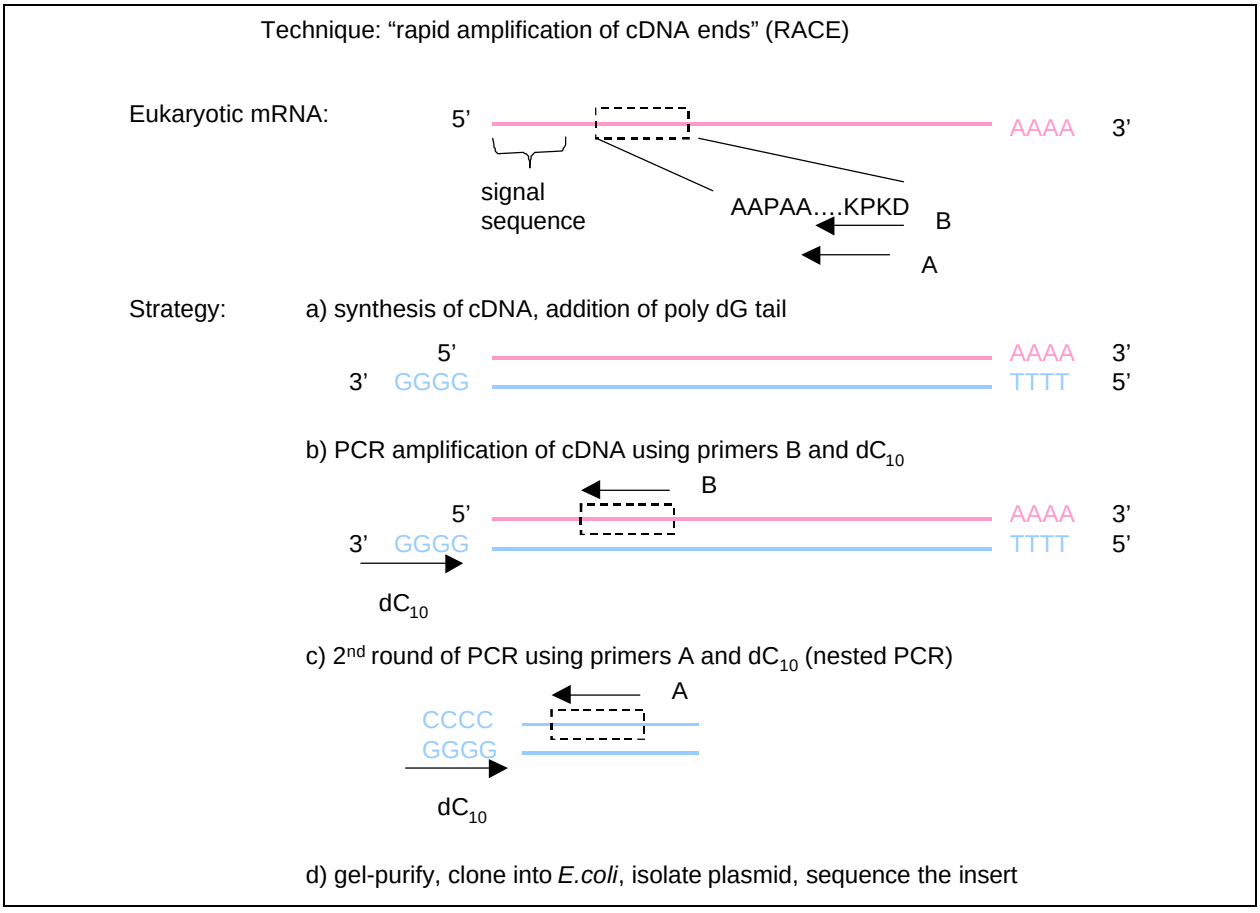

Figure 3. Strategy for RT-PCR amplification of the algal reversible hydrogenase

amplification (using the B primer) yielded a product about 350 bp long, which was not obtained upon amplification of mRNA extracted from uninduced algal cells (Ghirardi et al. 1999). This band was subjected to a second round of amplification (using the A primer), resulting in another 300-350 bp long band. The second product was cut from the gel and is presently being cloned into *E. coli* for further amplification and sequencing.

Subtractive Hybridization

Subtractive hybrization is one of the techniques available for the identification of genes that are only expressed under anaerobic treatment of algal cells (Kozian and Kirschbaum 1999). Figure 4

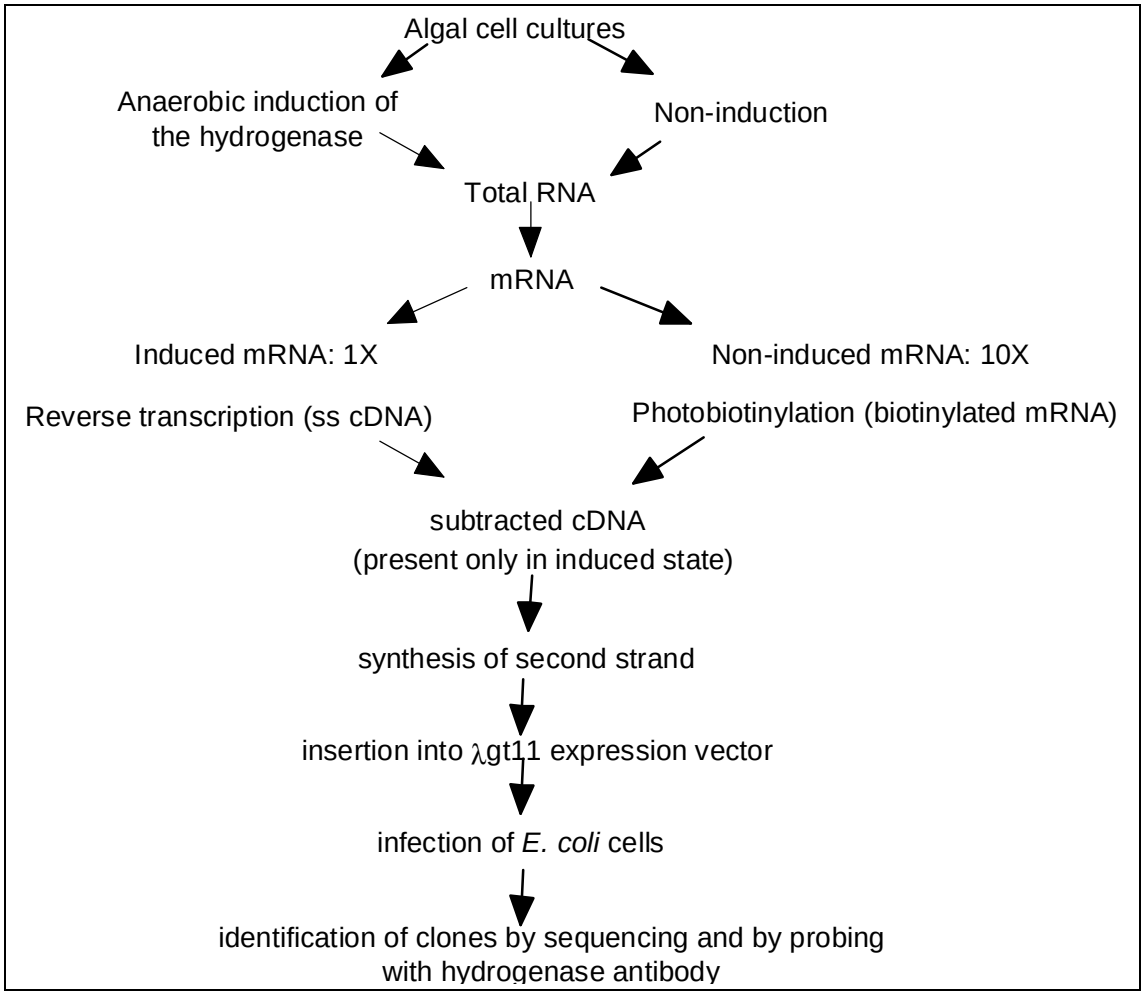

Figure 4. Construction of a subtractive expression library

summarizes the steps involved for obtaining the subtracted mRNA, and in the subsequent construction of a subractive expression library in *E. coli*. We extracted, on the average, 40 μg of total RNA per each 100 μg Chl of induced or non-induced algal cells. The respective mRNA samples were purified by means of an oligo dT cellulose column with an average yield of 1.4% of the total RNA. The photobiotinylation of

the non-induced mRNA resulted in the binding of 6-15 molecules of biotin per nucleotide, and the reverse transcription of the induced mRNA into cDNA (using the Not I poly dT primer) had a yield of about 30%. The subtractive hybridization was done with the Invitrogen Subtractor**7** kit, with modifications, and yielded 15% of the initial induced cDNA. There were no significant difference in yield between 1-h and 4-h induced samples. In order to clone the subracted cDNA, we initially added a poly dG tail to it and used the reverse transcriptase-catalyzed reaction to synthesize a second strand (Land et al. 1981), using an Eco RI dC primer. The double-stranded subtracted cDNA is presently ready to be digested with Eco RI and Not I restriction enzymes, and inserted into the λgt11 expression vector; the vector will be used to subsequently infect *E. coli*. Identification of the subtracted cDNA species will be done by probing with the hydrogenase antibody (generated in collaboration with Professor Melis last year) and sequencing of clones for homology with known algal proteins.

At this point it is not possible to opt for only one of the above-described technique to clone the algal hydrogenase. They both have advantages and disadvantages. RT-PCR is the technique of choice if one is able to design primers that are specific for the hydrogenase. The design of primers that we are using was based on the published N-terminal sequence of the hydrogenase, assuming that the sequence is correct. In the past, we were not successful in specifically amplifying the algal hydrogenase, using another set of degenerate primers. However, we are currently using a pair of optimized primers (Ghirardi et al. 1999), and, if not successful, will abandon the PCR approach. The subtractive hybridization approach is much more time-consuming and complex. However, it has the advantage of producing not only the hydrogenase gene but also genes that encode proteins that are expressed upon anaerobic treatment. Some of those proteins may be required for optimal expression of the hydrogenase activity.

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