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 O_2 tolerant to produce H_2 under aerobic conditions. We have been addressing this goal by means of both classical genetics and molecular biology approaches.

The classical mutagenesis/selection approach that we have developed to obtain such mutants 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 (H_2-uptake selective pressure) or evolving (H_2-production selective pressure) H_2 in the presence of O_2 concentrations that inactivate the wild-type (WT) enzyme. 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 and second 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.

Current year's results include (i) the isolation of a second-generation H_2-uptake mutant with an O_2 I_{50} over 5 times higher than the WT strain, and (ii) the observation that the H_2-evolution activity of H_2-uptake mutants is more easily re-activated upon removal of O_2 and subsequent addition of reductant to the assay medium than that of the WT strain or of the H_2-production mutants.

In order to enhance the 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. Our purpose is to first clone the hydrogenase gene and then to use site-directed mutagenesis to further increase the O_2 tolerance of the enzyme. The types of site-directed mutants that we will attempt to generate will be based on sequence information gathered from the O_2-tolerant mutants described above. Two techniques are currently being used to clone the gene, namely RT-PCR (which allows for the amplification of a specific DNA sequence out of a population of expressed sequences) and insertional mutagenesis (which consists of interrupting genes by introduction of a tagged plasmid, followed by screening for mutants unable to evolve H_2; the tag allows identification and sequencing of the interrupted gene).

Current results include: (i) identification of two partial clones by RT-PCR, using primers based either on the published N-terminal sequence of the algal hydrogenase or on regions of conserved homology between a variety of Fe-only hydrogenases and (ii) isolation of 4 insertional mutants with reduce H_2 evolution activity but WT rates of photosynthesis and respiration.

Introduction

The ability of green algae such as *Chlamydomonas reinhardtii* to photoproduce H_2 from water depends on the activity of 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, to cyclic electron transport, and to a variety of secondary pathways. However, following an anaerobic treatment in the dark, algal cells induce the reversible hydrogenase (Ghirardi et al., 1997b), an enzyme that can re-combine photosynthetically-generated electrons and protons to evolve H_2 gas. The hydrogenase competes with the other ferredoxin-dependent pathways for photosynthetic electrons. This competition is normally 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 problem associated with the hydrogenase has precluded the use of green algae in a direct biophotolysis H_2-production system (Benemann, 1996).

Mutant 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. Figure 1 is a summary of these approaches. They involved using random mutagenesis, followed by 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. Due to the lack of specificity of the selective pressures, 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 (see later for explanation) to O_2 compared to the WT

parental strain (Flynn et al.,1999; Ghirardi et al., 1999; Seibert et al., in press). We also isolated a H_2-uptake mutant, 104G5, with 13 times higher tolerance to O_2 (Ghirardi et al., 1999; Seibert et al., in press). These results have confirmed the validity of the designed approach for the isolation of the desired phenotype.

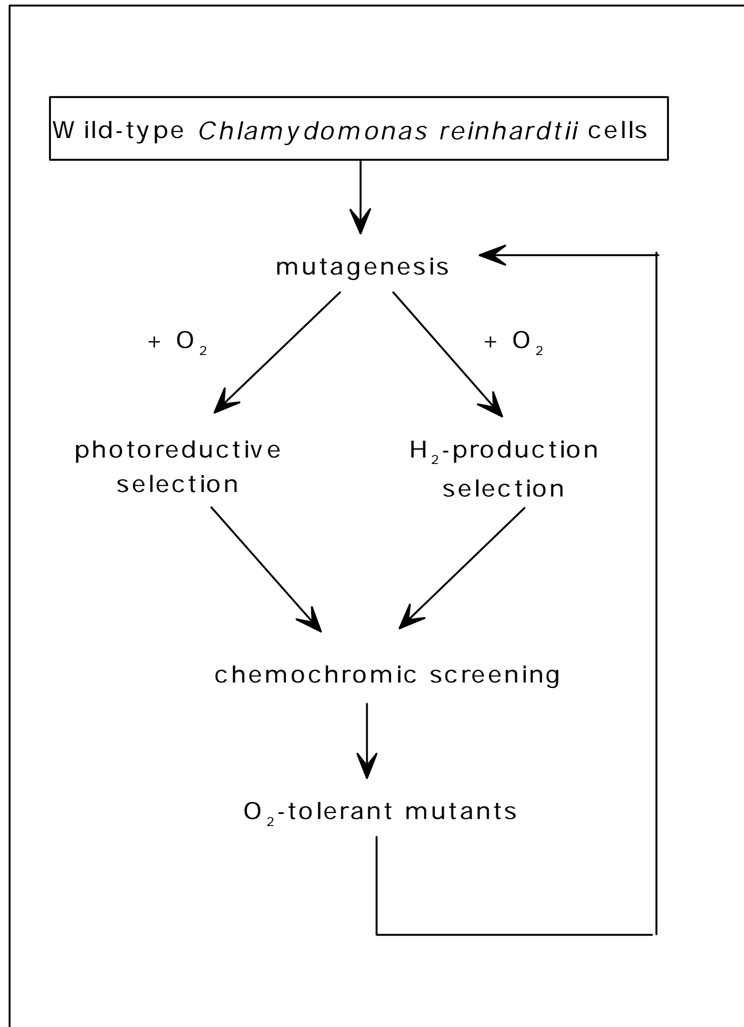

Figure 1. Strategy for the generation of O_2-tolerant algal mutants that produce H_2 under aerobic conditions.

The classical genetics approach described above is the best 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 confers the same phenotype to 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 ideal approach is 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, at this point, the DNA sequence of the gene encoding the hydrogenase enzyme in *C. reinhardtii* has not been reported, and site-directed mutagenesis is not possible at present. We are currently exploring two techniques to clone the algal hydrogenase gene. The first one, RT-PCR (reverse transcriptase polymerase chain reaction), is based on the specific amplification of nucleic acid sequences upon introduction of primers that hybridize to the desired gene. The second technique utilizes insertional mutagenesis, followed by screening for mutants that have lost the capacity to photoevolve H_2. This procedure depends on the insertion of a tagged plasmid in the *Chlamydomonas* genome, which interrupts genes at random. Using the chemochromic sensor, we can pick out those clones in which the plasmid interrupts genes required for photoevolution of H_2. Using the tag in the plasmid, one can then recover sequences of the interrupted gene near the plasmid insertion site and use this information to further sequence the gene. Our expectation is that either of the techniques will lead us to cloning the hydrogenase gene, which in turn will allow us to use molecular biology techniques to specifically target the hydrogenase gene for changes in O_2-tolerance.

Materials and Methods

Cell Growth

Wild-type (WT) *C. reinhardtii* (137c$^+$) was a gift from Prof. S. Dutcher, Washington University, St. Louis, MO. Algal cells were grown photoautotrophically in basal salts (BS) (Flynn et al., 1999), a modification of Sueoka's high salt medium (Harris, 1989) that includes citrate to prevent salt precipitation during autoclaving. 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

Liquid BS medium (550 ml) was inoculated with 20 ml of mid-log phase cells to give an initial density of 4.9 x 10^4 cells/ml. The culture was grown overnight under cool white fluorescent illumination (70 $\mu E \cdot m^{-2} \cdot s^{-1}$) and then sparged with 2% CO_2 in air (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 72 h, at which point the cells were harvested, washed, and resuspended in 50 ml of BS medium. Liquid cultures from 5BU mutagenesis were grown in the light as above for at least 7 days before submitting them to H_2-uptake selective pressure.

H_2-Uptake Selection Procedure

Liquid cultures of mutagenized algal cells (250 ml, 2.8 x 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, 30% O_2, and 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.

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 (Seibert et al., in press). Following a 7-14 day growth period, the agar plates were made to go anaerobic overnight, to order to induce the algal hydrogenase and then pre-exposed to 21% O_2 for different periods of time in the dark to deactivate any remaining WT phenotype. The plates were immediately transferred to an anaerobic glove box, the sensor applied, and the colonies 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 Assays

Anaerobic Induction

Mid-log phase algal cells were harvested to give 200 µg/ml Chl in phosphate buffer (Ghirardi et al., 1997b) supplemented with 15 mM glucose and 0.5% v/v ethanol. Concurrently, an enzymatic oxygen-scrubbing system (Packer and Cullingford, 1978) that consisted of 1mg/ml glucose oxidase (Sigma, St. Louis, MO) and 27720 units/ml catalase (Sigma, St. Louis, MO) was prepared, and 2 ml of the enzymatic mixture was dispensed into dialysis tubing (6-8 kD MW cutoff). In an anaerobic glove box, the dialysis bags were added to the cell suspensions in small vials, and the vials were sealed, covered with aluminum foil to ensure darkness, and incubated at room temperature for 4 h. Following this induction treatment, the cell suspensions were kept at 4 °C overnight and assayed for H_2-evolution activity using either the light-Clark electrode or the dark methyl viologen/gas chromatography assay.

Light – Clark Electrode assay (L-CE)

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 to the desired initial concentration by bubbling with Ar, 200 µl of anaerobically induced cell suspension was injected into the buffer, and the cells were exposed to the pre-set O_2

concentrations for two minutes in the dark. Some respiratory oxygen consumption did take place in the dark, so that the O_2 concentration was not absolutely constant over time. Following O_2 deactivation, the cell suspension was illuminated (320 $\mu E\ m^{-2}\ s^{-1}$, PAR incandescent light filtered through 1% $CuSO_4$) for three minutes. The initial rates of H_2 production were plotted as a function of initial O_2 concentration.

Methyl Viologen-Gas Chromatography assay (MV-GC)

The assay reaction consisted of exposing the anaerobically-induced cells to various levels of O_2 for two minutes, re-establishing 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. The reaction was then stopped by the addition of trichloroacetic acid, and the presence of H_2 was detected by gas chromatography.

Results and Discussion

In the past, we used ethyl-methane sulfonate (EMS) to generate random mutants of *C. reinhardtii*, from which we isolated first (76D4) and second generation (141F2) H_2-production mutants (Ghirardi et al., 1999; Flynn et al., 1999; Seibert et al., in press). We also used 5-bromouracyl (5-BU) to generate another population of mutants, from which the first generation H_2-uptake 104G5 mutant was isolated (Ghirardi et al., 1999; Seibert et al., in press). These results are shown in Figure 2.

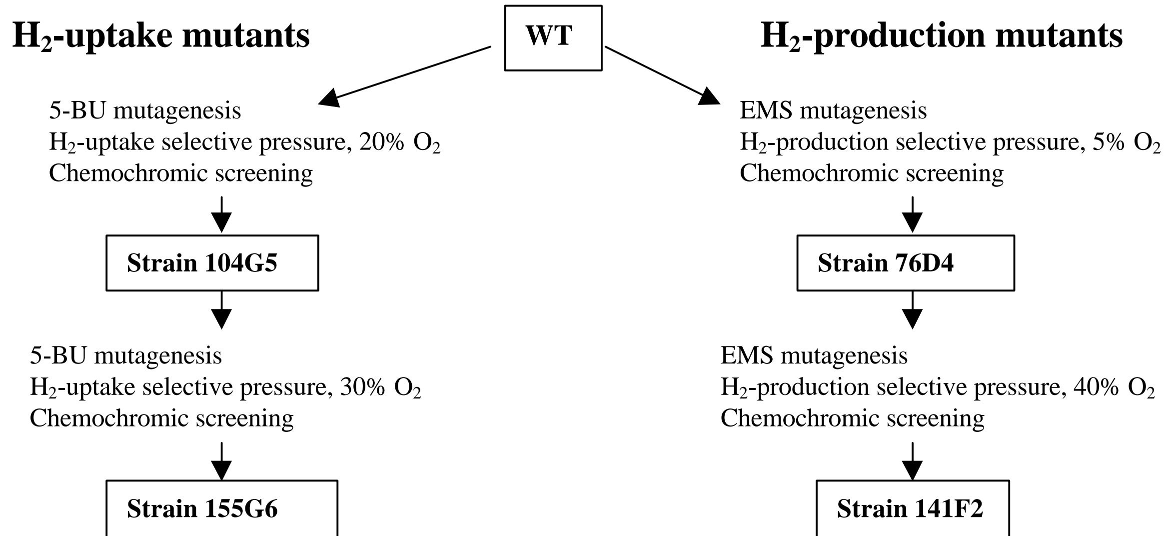

Figure 2. Histories of the different algal strains used in our past and current work.

In order to further improve the O_2-tolerance of the 104G5 mutant, we re-mutagenized it with 5-BU and re-submitted the resulting mutant population to H_2-uptake selective pressure. Selection was done

in the presence of 30% O_2, conditions that are more stringent than those used to isolate the 104G5 mutant (20% O_2). The survivors from this selective pressure were then chemochromically screened, following inactivation by O_2. Clones that produced the best signal during the screening procedure were characterized in a more detailed manner as described below. The best mutant was strain 155G6, as indicated in Fig.2.

Measurements of the O_2 tolerance of all of the isolated clones was 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 inactivation by O_2 (Ghirardi et al. 1996, 1997, 1997b, 1998; Seibert et al. 1998). Rates of H_2 evolution were measured by either of two techniques: (i) polarographically with a Clark-type electrode, poised for the detection of light-induced H_2 evolution (L-CE) or (ii) by gas chromatography, using dark-reduced, methyl-viologen-induced H_2 evolution (MV-GC). In the past, the validity of data obtained from the L-CE measurement assay had been questioned, due to the consumption of the added O_2 by the O_2-scrubbing enzymes present in the sample suspension. Since then, we have modified the assay by separating the O_2-scrubbing system from the sample by using a dialysis membrane (see Anaerobic Induction in the Materials and Methods Section). The two assays are described in detail in the Materials and Methods Section. Figure 3 shows the O_2 I_{50}s, of the different mutants that we have isolated thus far, determined by both of the assays.

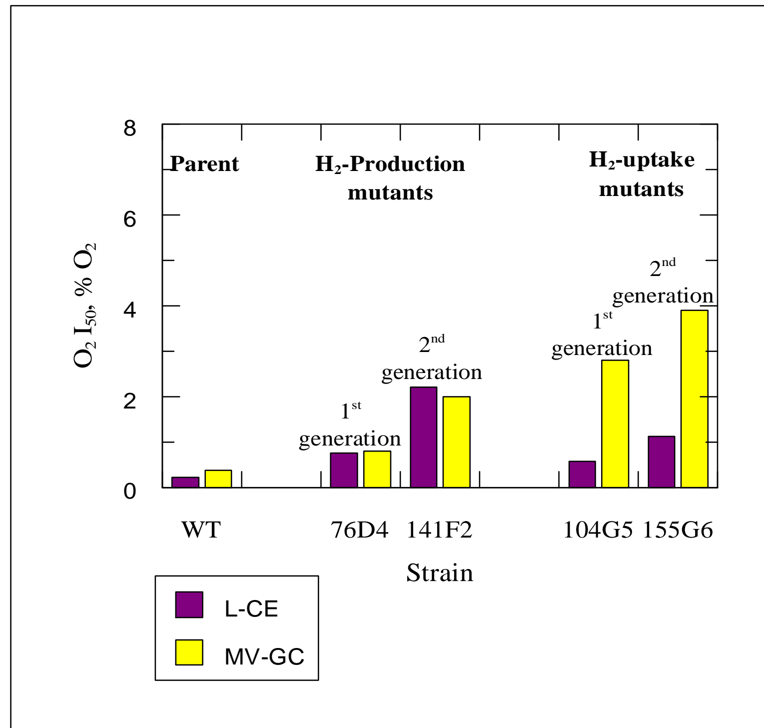

Figure 3 . Estimates of O_2 I_{50}s determined for each of the 4 isolated O_2-tolerant algal mutants. The O_2 I_{50}s were determined by two types of assays, as indicated.

It is clear from Fig. 3 that the two assays give different O_2 I_{50} values for the H_2-uptake mutants, but same values for the WT strain and H_2-production mutants. Moreover, the segregation of the O_2-scavenging enzymes in a dialysis bag does not seem to have changed the estimated O_2 I_{50} values (Ghirardi et al., 1999; Flynn et al., 1999; Seibert et al., in press), suggesting that the inactivation of the enzyme by O_2 must be a process that occurs as soon as the enzyme comes in contact with O_2. Given the different nature of the two assays (see above), it appears that the H_2-uptake mutants, besides their tolerance to some O_2, are also able to reactivate their O_2-inhibited hydrogenase enzyme once the O_2 is removed and a reductant is added (as is done in the MV/GC assay). This trait may have given the cells an advantage during the application of the H_2-uptake selective pressure, if the initial dark starvation period was not long enough to deprive the cells of all their internal metabolic reserves. In this case, when the mixture of $H_2/CO_2/O_2$ is added, the cells will initially respire the added O_2 and only then will shift to a H_2 consumption mode. This situation would be repeated each time the gases are replaced, until the cells run out of substrate for respiration. Evidently, mutants with a hydrogenase that is reactivated more quickly in the presence of a reductant, following consumption of added O_2, will start metabolizing H_2/CO_2 for their metabolism more rapidly (and thus outgrow the competitors).

Conclusions

Based on their responses to the removal of O_2 and addition of reductants to the assay, we conclude, at least on a preliminary basis, that the observed O_2-tolerant phenotypes of the H_2-production mutants were probably caused by mutations affecting the hydrogenase enzyme only, while the phenotypes of the O_2-uptake mutants could be explained by either a hydrogenase mutation or mutations affecting other genes. For example, it is possible that the H_2-uptake mutations involve the activation of protective mechanisms that shield the WT hydrogenase from O_2, such as changes in membrane permeability to O_2. Also, it is conceivable that some of the O_2-tolerance phenotypes are due to an increase in the activity of the chloroplast O_2-scavenging system. We find, however, that it is not necessary to invoke mutations in other genes to explain the phenotype of all of our mutants. These phenotypes could all be due to single point mutations in the hydrogenase gene, that improve its tolerance to O_2, since previous work (McTavish et al., 1995) demonstrates that single amino acid changes can improve the O_2 tolerance of hydrogenases from non-photosynthetic organisms. Future work will be necessary to clarify this point.

Acknowledgements

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