

ISOLATION OF *CHLAMYDOMONAS* MUTANTS WITH IMPROVED OXYGEN-TOLERANCE

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ABSTRACT

The photoproduction of H₂ from water by anaerobically-induced algae is catalyzed by a bidirectional hydrogenase, an enzyme that is rapidly deactivated by exposure to low levels of O₂. We have developed two selective pressures, with which a mutant's survival depends on hydrogenase activity in the presence of O₂, and a chemochromic screening method to quickly identify and isolate desirable O₂-tolerant hydrogenase mutants. The clones that still produced H₂ after exposure to O₂ were further characterized by gas chromatography for maximal H₂-production rate and tolerance to O₂. The best mutant obtained by a single round of mutagenesis/selection/screening maintained up to 35% of its maximal H₂-production rate (measured with no exposure to O₂) following deactivation of the enzyme by 2% O₂ for 2 minutes, a condition that almost totally inactivates wild-type (WT) H₂ production. A mutant that underwent a second round of mutagenesis and selection exhibited 49% of its maximal activity following the deactivation treatment. This represents a 10-fold improvement over the WT strain and suggests that even better mutants will be forthcoming.

INTRODUCTION

Photobiological H₂-production by green algae is catalyzed by the reversible hydrogenase (1,2), a chloroplast stromal enzyme (3) that catalyzes both H₂ production and H₂ uptake in the organism. This nuclear-encoded protein (4) is induced by anaerobic incubation of algal cells in the dark but is inhibited by the presence of very low concentrations of O₂ (5). This problem has precluded the application of algae up to this point in applied H₂-producing systems.

Future development of a cost-effective, commercial H₂-production system using green algae will depend on the availability of strains that produce H₂ directly from water under aerobic conditions (6). One of our approaches to generate O₂-tolerant, H₂-producing algal mutants was based on a selection pressure involving the H₂-uptake activity of the reversible hydrogenase (7). Hydrogen uptake (or photoreductive) selection is applied to a population of mutagenized *Chlamydomonas reinhardtii* cells in an atmosphere of H₂, CO₂, and controlled concentrations of O₂, as well as in the presence of the herbicides 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) and atrazine. These herbicides blocks photosynthetic O₂ evolution and electron flow at the reducing side of photosystem II and prevent electrons from water from reaching the hydrogenase enzyme. The surviving organisms grow by fixing CO₂ with electrons obtained from the oxidation of H₂ catalyzed by the O₂-tolerant hydrogenase, and ATP generated by cyclic electron transport around photosystem I (5).

Since photoreductive selection only exploits the H₂ oxidation activity of the hydrogenase, we have also developed a new selective pressure designed to exploit the H₂-evolving function of the enzyme (8). This selection depends on the fact that, in the absence of CO₂, metronidazole (MNZ) will compete with the hydrogenase at the level of ferredoxin for electrons derived from the photosynthetic electron transport chain (9). Reduced MNZ generates a radical that is reoxidized by O₂ with the concomitant formation of superoxide radicals and H₂O₂, both of which are toxic to the algae. If the hydrogenase is still active following exposure to O₂, then some of the electrons from reduced ferredoxin can be used for H₂ production instead of MNZ reduction, and decreased toxicity can be observed (8).

The traditional assays (Clark electrode or gas chromatograph analysis) used to determine a clone's O₂-tolerance require many time-consuming steps and are a severely limiting factor for rapidly identifying useful mutants (10). This problem was solved by the development of a screening assay using a thin-film, multilayer chemochromic sensor (11) that permits the evaluation of hundreds of mutant clones in a single day. This sensor, when held in close contact to anaerobically induced algal colonies that can evolve H₂ after exposure to O₂, produces an easily visualized blue spot. These spots correspond to colonies that were able to withstand O₂ deactivation treatment, and allow for the rapid identification of desirable mutants clones (10). The combination of random mutagenesis, selection, and screening has yielded H₂-producing *Chlamydomonas* mutants with significantly improved O₂-tolerance.

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 (12) that includes citrate to prevent salt precipitation. This formulation contains the following salts: 10 mM NH₄Cl, 1 mM MgSO₄, 7.5 mM KH₂PO₄, 7.5 mM K₂HPO₄, 1.5 mM Na₃-citrate, 0.5 mM CaCl₂, 20 μM FeCl₃, and 1/2 x Hutner's trace elements (12). 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 ($70 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR) at 25°C and agitated on a shaker. Cells were harvested by centrifugation at $2000 \times g$ for 10 min and resuspended in liquid BS medium.

Mutagenesis: Mid-log phase cultures were harvested and resuspended in liquid BS to yield a 10^8 ml suspension of 7×10^6 cells/ml. Ethylmethane sulfonate (EMS) was added to a final concentration of $5 \mu\text{l/ml}$ (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. Liquid cultures 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 $15 \mu\text{M}$ 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% O_2 , balanced with Ar. The cultures were grown for a couple of weeks with stirring and illuminated with fluorescent light ($70 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ 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 MNZ-Na azide solution to final concentrations of 40 mM MNZ and 1 mM sodium azide and 2.8×10^6 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 ($320 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{h}^{-1}$ 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 8×8 colony matrix and the 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 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 phosphate buffer (8) 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 (13) that consisted of 1mg/ml glucose oxidase and 27720 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 consists 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 reactions 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

Figure 1 shows the dose response curve when WT cells were treated with the mutagen EMS (46 mM for various periods of time). The survivors from the 10, 15 and 20 minute cultures (corresponding to 66, 56, and 42% survival) were harvested (see methods) and used in future experiments.

Figure 2 shows the subsequent treatment histories of the various populations. Each of the three polygenic mutant populations were initially subjected to the PR selection, and individual surviving clones were subjected to the chemochromic screening. Screening and preliminary characterization of representative clones obtained from the populations PR8, PR9, PR10, failed to yield mutants with significantly improved O_2 -tolerance. The insufficient enrichment of desirable mutants by this particular experiment will be discussed later. The populations surviving the PR selection were maintained in liquid BS plus acetate and were then subjected to the MZ procedure. Figure 3 shows the killing kinetics of the MZ selections, where less than 3% of the initial population density survived in each case. The apparent decreased killing rate of the MZ14 population was probably caused by incomplete mixing of the gas and liquid phase during the dark deactivation treatment. Following resuspension in liquid BS, the resulting cultures were diluted and spread onto agar to obtain individual colonies derived from single cell clones.

Two hundred and forty clones from each of the three populations that survived the MZ selection pressure were isolated for the chemochromic screening assay. The colonies on agar plates were exposed to 21% O_2 for various periods of time for a maximum of 10 minutes before screening.

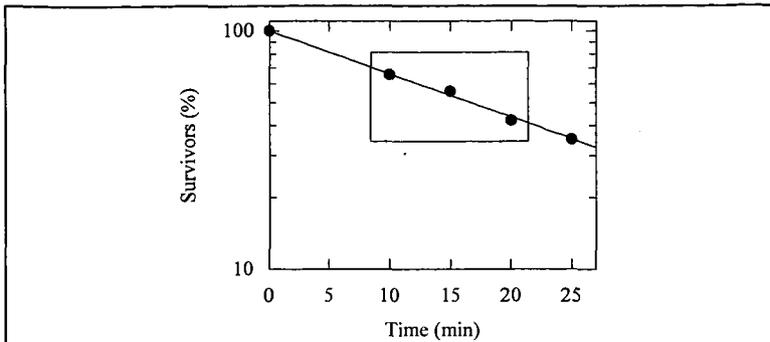


Figure 1: Ethylmethane sulfonate (EMS) mutagenesis of WT *C. reinhardtii* using 5 μ l/ml (46mM) EMS and a 10ml suspension of 7x10⁶ cells/ml. The harvested populations are indicated.

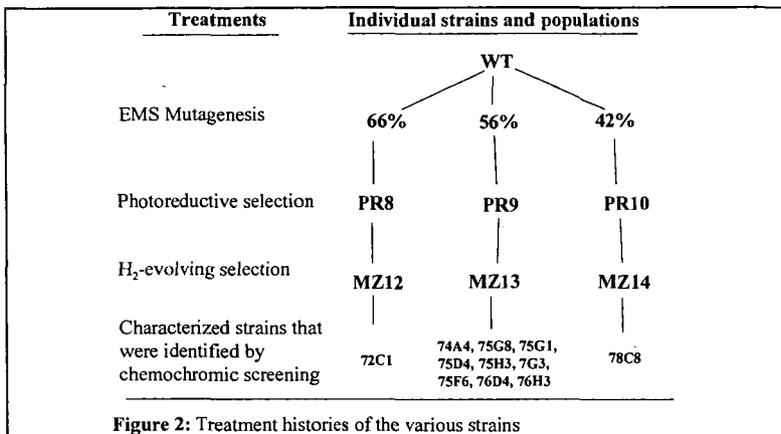


Figure 2: Treatment histories of the various strains

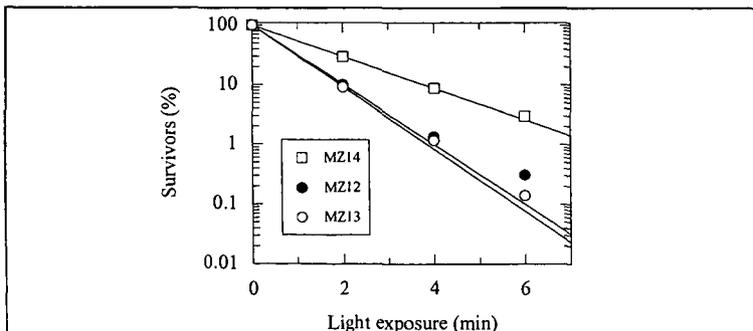


Figure 3: Metronidazole selection of the photoreductive selection survivors (PR8, PR9, and PR10), giving rise to MZ12, MZ13, and MZ14 respectively. The anaerobically induced cells were preexposed for 4 minutes to 5% O₂ before illumination.

The clones that produced the most H₂ under these conditions were further characterized using the methyl viologen assay. Table 1 summarizes the characteristics of the indicated selected clones. The parameters used to initially characterize the mutants included the maximum rate of H₂ evolution measured without any exposure to O₂ (V₀), and the amount of remaining H₂-evolution activity following an exposure to 2% O₂ for two minutes (% of V₀). The % of V₀ parameter was used to roughly compare the relative O₂-tolerance of the mutants compared to the WT strain. Four strains were more fully characterized by titrating the H₂-evolving activity following deactivation of the enzyme with increasing levels of O₂ for 2 minutes. The O₂ I₅₀ was estimated by fitting the data to a single exponential decay function. Inspection of Table 1 reveals that all of the mutants identified by the screening assay are improved with respect to V₀ and O₂-tolerance compared to their parental WT strain. The V₀'s were increased in all of the mutants, with a 2.3-fold increase in strain 75D4. The I₅₀'s, were increased by 3.7- and 4.4-fold in the strains 76D4 and 76H3. The least improved strain, 78C8, had only a 9% increase in O₂-tolerance compared to the WT strain, and may represent the minimum phenotype for surviving the conditions used in this H₂-evolving selection experiment.

The strain 76D4 was remutagenized with EMS (61% survival), selected using the MZ procedure with a selective pressure of 40% O₂ in the dark for 5 minutes, and finally screened following deactivation with 100% O₂ for 5 minutes. A resulting clone, 141F2, had over a 2-fold increase in I₅₀ compared to its parent (76D4) and almost a 10-fold improvement compared to the grandparent WT strain.

MZ population ^A	Strain	V ₀ ^B (μmoles H ₂ /(mg Chl x h))	% of V ₀ ^C	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%	-----
MZ14	78C8	64	9%	-----
	141F2	86	49%	2.04

Table 1: Characteristics of selected strains. A: see Fig. 2. B: maximum rate of H₂ evolution without any exposure to O₂. C: rate of H₂ evolution following deactivation by pre-exposure of the cells to 2% O₂ for 2 minutes (expressed as % of V₀). D: The O₂ concentration (in %) that reduces V₀ by 1/2 (2 minute exposure).

DISCUSSION

Mutagenesis of *C. reinhardtii* cells was induced by exposure to EMS, an agent that alkylates the keto groups of guanines and thymine. This causes anomalous base-pairing upon the replication of DNA (14) and ultimately results in transition mutation (G≡C becomes A=T). The frequency of mutants among survivors increases with mutagen dose, but so does the damage to the genetic background (15). Therefore, killing rates of less than 60% were chosen to minimize damage to the remainder of the genome. The problem of decreased mutant frequencies among the survivors is normally solved by employing effective selection procedures.

However, upon finding little improvement in O₂-tolerance in the populations obtained from the first photoreductive selection, it appeared that either the hydrogenase was not mutated, or the conditions employed were not sufficiently specific for the enrichment of desirable O₂-tolerant mutants. Since improved mutants could be generated by MZ selection (Table 1), but were not recovered from the original PR populations, the PR procedure clearly needs improvement. We have reexamined the initial PR selection protocol (see Materials and Methods section) and have determined that the initial O₂ concentration in the anaerobic jar could have been significantly decreased by cellular respiration of the cultures during the application of the selective treatment. This could and apparently did reduce the effectiveness of the selective pressure. This problem was solved by replacing the gas mixture daily until the culture became chlorotic indicating that the

majority of the cells are dead. Preliminary results obtained from this modification appear promising and will be published elsewhere.

The results of this paper clearly demonstrate the utility of the chemochromic screening technique. It has not only allowed us to rapidly identify mutants with desired phenotypes but also permitted the rapid assessment of the effectiveness of the selection procedures themselves. It is also encouraging that all of the strains that were identified with the chemochromic sensor were improved compared to their parental strain upon further characterization.

The mutants identified with the chemochromic sensor were chosen for further characterization based on the intensity of the color change following an exposure to various pretreatment doses of O₂. The clones that demonstrated the best potential, based on the intensity of the color change, were obtained from the MZ13 population, and were most heavily sampled. Given the range of increased O₂-tolerance (% of V₀, Table 1) detected among the 10 sampled first round mutants, one could argue that there is more than one genotype that gives rise to the O₂-tolerant phenotype. Three obvious possibilities exist: (a) different amino acid substitutions at a single critical residue, (b) random substitutions distributed throughout the O₂-sensitive domain, or (c) mutations of genes other than the hydrogenase that cause a decrease in intracellular O₂ concentration, such as through increased rates of respiration.

The ultimate goal of our research is to create an organism that photo-oxidizes water to H₂ under aerobic conditions using solar energy. Our current results are encouraging, and validate the use of classical mutagenesis/selection to obtain the desirable organisms.

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