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DEVELOPMENT OF AN EFFICIENT ALGAL H_2-PRODUCTION SYSTEM

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Abstract

Two major problems facing the development of a commercial photobiological algal H_2-producing system are the low rates of H_2 evolution and the sensitivity of the H_2-evolving enzyme system to O_2, a by-product of the photosynthetic water-splitting process. The objective of our project is to generate O_2-tolerant mutants from the green alga *Chlamydomonas reinhardtii* that are high producers of H_2 for use in a photobiological water-splitting, H_2-producing system that is cost effective, renewable, scalable, and non-polluting.

We are currently employing a dual approach to address the O_2-sensitivity problem. The first approach, based on classical mutagenesis and selection procedures, depends on the ability of a mutagenized population of algal cells to survive under conditions that require them to either produce (H_2-production selection) or consume (photoreductive selection) H_2 in the presence of controlled amounts of O_2. The second approach, based on molecular genetic strategies, involves the cloning of the hydrogenase gene from *C. reinhardtii* and identification of expression factors required for optimal H_2-evolution activity. The latter approach will complement the first in our future goal of generating a commercial organism suitable for use in the private sector.

Our previous work established conditions for application of the H_2-production and photoreductive selective pressures, as well as for the quantitative determination of the rates and O_2 sensitivity of the H_2-producing system in selected organisms. This estimation is done by measuring the V_0 (initial rate of H_2 evolution in the absence of O_2) and the I_{50} for O_2 inactivation of H_2 evolution (the concentration of O_2 in the gas phase that inhibits the rate of the reaction to 50% of the V_0 value) in algal cells. Mutants with an increased O_2 I_{50} were obtained by application of the H_2-production

pressure to cell wall-less *C. reinhardtii* cells in the presence of increasing amounts of added O_2. However, those mutants were not stable and reverted to a WT phenotype. Our most important accomplishment last year was demonstrating the feasibility of a proprietary chemochromic sensor screening procedure for quickly identifying individual algal clones on agar plates that photoproduce H_2 (but only under anaerobic conditions).

Our accomplishments for the past year include: (a) improvement of the specificity of the photoreductive pressure by including atrazine in addition to DCMU in the selection medium to prevent the selection of mostly herbicide-resistant mutants; (b) improvement of the H_2-production selection by adding acetate to the induction medium and pre-exposing the algal cells to O_2 prior to selective pressure; (c) construction and testing of a glove-box system for screening algal mutants on agar plates in the presence of controlled amounts of O_2 (the current set-up allows us to screen 72 clones/day) using the chemochromic sensor; (d) isolation of mRNA from induced and non-induced algal cells to be used in the construction of a subtraction expression library; and (e) successful generation of antibodies against the algal hydrogenase. In our first round of selection/screening of about 2000 mutant algal clones, we isolated a stable *C. reinhardtii* clone that evolves H_2 at a rate 4 times (greater than 70% of maximum electron transport capacity of the organism) that of the WT. This represented an unanticipated breakthrough that underscores the fact that we have been able to successfully address one of the major problems facing algal H_2 production systems. This was possible due to the way we have been selecting and screening for mutants. However, this particular procedure may not select for mutants with more than about a 3 times increase in tolerance to oxygen. Improving the O_2-tolerance of our mutant strains demonstrated to be high producers of H_2 will be one of the focus points of our research during the next year. This will involve adjustments in our current selection and screening process, and a search for additional assays for O_2 sensitivity of the H_2-production pathway. Concomitantly, we will finish the construction of the subtractive library and will start to probe it with the hydrogenase antibody to attempt to identify transformants carrying hydrogenase cDNA.

Introduction

Photobiological H_2-production from w ter by green algae is catalyzed by a chloroplast stromal enzyme, the reversible hydrogenase, t at catalyzes both hydrogen production and uptake. The enzyme is induced by the anaerobic incubation of algal cells in the dark, but is inhibited by the presence of very low concentrations of O_2. The latter property, coupled with the low rates of H_2 evolution in WT cells and the low lig t saturation level of algal photosynthesis in general, has precluded the use of algae in applied H_2-producing systems.

Commercialization of a cost-effective, algal H_2-production system will ultimately depend on the availability of strains that produce H_2 directly from water under aerobic conditions at high rates. Previous work aimed at identifying O_2-tolerant, H_2-producing algal mutants was based on a photoreductive selection procedure involving the H_2-uptake activity of the reversible hydrogenase (McBride et al., 1977). We have used McBride's experimental conditions and applied the selection to WT *C. reinhardtii* cells. However, the resultant mixed population of cells exhibited only slightly

higher tolerance to O_2 (Ghirardi et al., 1997a) and initial rates of H_2 evolution in the absence of O_2 (i.e., the V_0) similar to those of the parental WT. These results suggested that the selective pressure was not very specific for O_2-tolerant organisms, but that desired mutants could be obtained if an effective secondary screening procedure could be identified.

We also developed a novel selective pressure to isolate O_2-tolerant, H_2-producing mutants of *C. reinhardtii*, which depends on the role of metronidazole (MNZ) in algal photosynthetic electron transport (Ghirardi et al., 1996). Experimental conditions for this second selective pressure include the addition of O_2 to inhibit O_2-sensitive hydrogenases. This should favor the selection of O_2-tolerant, H_2-producing clones. The effect of MNZ concentration, light intensity, activation state of the hydrogenase, and added O_2 concentration were studied, and parameters for application of the selective pressure were previously determined. An algal variant (D5) with increased O_2 I_{50} (the concentration of O_2 in the gas phase that inhibits the rate of the reaction to about 50% of the V_0) for H_2 evolution activity (0.98% O_2 compared to 0.30% O_2 for the parental cell wall-less cw15 strain) was isolated by application of H_2-production selective pressure in the presence of 2.8% added O_2 (Ghirardi et al., 1996, 1997b). Subsequently, an unstable mutant (IM6) with an even higher O_2 I_{50} (1.4% O_2) was isolated by application of the selective pressure to the D5 variant under 5% O_2 (Ghirardi et al., 1997a, Seibert et al., 1997).

The work done up until last year suggested that neither of the above two selective pressures was specific enough to generate stable O_2-tolerant, H_2-producing mutants at desirable levels. Furthermore, the measurement of an O_2 I_{50} for H_2 evolution to confirm the O_2 tolerance of clones surviving either of the two selective pressures required many time-consuming steps and had become a severely limiting factor for rapidly identifying useful mutants. It became clear that a new secondary screening procedure was required to speed up the mutant identification process. A very sensitive thin-film, membrane detector for H_2 was recently developed at the National Renewable Energy Laboratory, and it is based on the chemochromic properties of a WO_3/Pd film upon direct exposure to H_2 (Benson et al., 1996). This film was adapted in a proprietary manner (U.S. patent pending) for biological use, and its potential utility for detecting H_2 production by individual algal colonies grown on agar plates was demonstrated under anaerobic conditions (Ghirardi et al., 1997b). Progress using the selection/screening process will be discussed in this paper, and we will also describe some improvements made on the application of the two selection pressures in order to improve their selectivity.

Finally, in order to speed up the production of a commercial organism, it will be useful to more specifically mutate specific amino acid residues on the hydrogenase (the enzyme that releases molecular H_2) that are found to be involved in the O_2 sensitivity problem. Unfortunately, the algal hydrogenase gene has not been cloned yet, and does not seem to have sequence homology to prokaryote hydrogenases. Also, the nature of the residues involved in conferring O_2-sensitivity to hydrogenases are not known (however, see McTavish et al., 1995). As a consequence, it is not currently possible to improve the production rates and O_2 sensitivity of the hydrogenase enzyme by site-directed mutagenesis. Given the fact that the expression of the hydrogenase activity requires anaerobic induction, we have initiated a molecular biological approach to clone the algal hydrogenase gene, based on the construction of a subtraction expression library using induced and non-induced cells. This strategy depends on the assumption that the hydrogenase enzyme is

transcriptionally-regulated in *C. reinhardtii*, which should be the case, since the hydrogenase is a photosynthetic protein that is encoded by a nuclear gene (Taylor, 1989). Moreover, an expression library of subtracted genes between induced and non-induced cells can also be used to identify other proteins that may be required for the expression and stability of hydrogenase activity under anaerobic conditions. With the successful cloning of the algal WT hydrogenase gene, we will be able to compare its sequence to those of the mutants that we are currently generating by the classical genetics approach. This sequence comparison will serve as a guide for site-directed mutagenesis that should further stabilize the hydrogenase in the presence of O_2 and increase the rates of H_2 evolution.

Results and Discussion

Mutagenesis

Mutations in *C. reinhardtii* cells were induced by treatment with ethylmethane sulfonate (EMS) for different periods of time. EMS is an alkylating agent that chemically tautomerizes guanines or thymines and alters their base-pairing affinities and thus causes mutations. We chose to use EMS instead of nitrosoguanidine, as in previous experiments, to minimize the occurrence of multiple-linked mutations. We also shifted the research from the cw15 cell wall-less strain to a WT *C. reinhardtii* strain because the cw15 cells were too fragile to survive chemochromic screening on agar plates (see later). In order to maximize the frequency of a single-point mutation in each surviving cell, we

Table I. Effect of time of exposure to 5 μg/ml EMS on the number of surviving WT *C. reinhardtii* cells

Time of exposure (min)	Number of surviving cells	Percentage of survivors
0	3.50×10^7	100
5	2.77×10^7	79
10	2.07×10^7	59
15	1.72×10^7	49
20	1.19×10^7	34
25	1.02×10^7	29
30	7.70×10^6	22

attempted to achieve about 50% surviv l level with the mutagen treatment (Prof. Walter Niehaus, personal communication). Table I show the number of surviving WT *C. reinhardtii* cells following treatment with 5 μg/ml EMS for different periods of time (see also Fig. 1, Flynn et al., submitted). The number of surviving organisms was determined by counting the number of green surviving colonies on agar plates 2-3 weeks after mutagenesis. Three EMS-treatment populations yielding 66%, 56% and 42% surviving organisms were chosen for subsequent application of the two selective pressures described in the Introduction and below.

Photoreductive Selection

Wild-type populations of cells were submitted to photoreductive selection in the presence of 10% O_2 and 3 μM DCMU for 3 weeks. The Chl concentration of the culture decreased significantly during the first 2 weeks, but started increasing again on the third week (not shown). The resulting mixed population, PRS1, had an estimated O_2 I_{50} of 0.60% O_2, which is about 1.5 times as high as that of the WT population (Ghirardi et al., 1997; Flynn et al., submitted), and a V_0 similar to the WT. The relatively small increase in the O_2 I_{50} observed after photoreductive selection suggested that the selective pressure might not be specific for O_2-tolerant organisms. One of the possibilities was that DCMU-tolerant mutants were being co-selected by the procedure. This would explain the rapid recovery in Chl concentration of the culture if a herbicide-tolerant population of mutants took over the culture. The possibility of DCMU-tolerance was investigated by determining the effect of DCMU on the viability of both the WT and the PR3 (derived from the PRS1) populations. The two populations were replica-plated onto agar containing different amounts of DCMU. The plates were then incubated in a growth chamber for 3 weeks under photoautotrophic conditions and the surviving colonies were counted. Figure 1 clearly demonstrates that randomly-selected clones from the PR3 population (closed circles) contained a large number of organisms that exhibited an increased tolerance to DCMU when compared to the WT population (open triangles), as hypothesized (see also Flynn et al., submitted).

DCMU, an urea-type herbicide, inhibits O_2 evolution and electron transport through photosystem II. It works by binding to amino acid residues on the reducing side of the D1 reaction center protein. Another group of photosystem II herbicides, the triazines, shares some but not all binding residues with DCMU. We attempted to increase the specificity of photoreductive selection for O_2-tolerant organisms by applying the selective pressure in the presence of both DCMU and atrazine. This should eliminate herbicide-resistant mutants which survive photoreductive selection but do not exhibit O_2-tolerance to H_2 production. Given the lack of complete overlap between the binding residues for the two herbicides, few of the survivors (single-point mutants) are expected to have a double resistance phenotype. The two herbicides were shown to completely inhibit cell growth at less than 10 μM total herbicide concentration in WT cells (Flynn et al., submitted). Therefore, we chose to add 15 μM DCMU + 15 μM atrazine to subsequent photoreductive selection experiments to ensure complete inhibition of photoautotrophic growth, an absolute requirement for success of this selective procedure.

The application of photoreductive pressure to a mutagenized population of WT cells in the presence of the two herbicides resulted in a population of survivors, PR10, that did not show an increase in the Chl concentration after 4 weeks of selection (data not shown), demonstrating that very few clones survived the pressure. The O_2 I_{50} for H_2 evolution of selected clones from this population was about the same as that for the WT population, but this time the V_0 increased about two-fold over the estimated V_0 for the WT population (not shown). More importantly, very few of the survivors had increased resistance to DCMU, atrazine, or both herbicides combined (maximum resistance was to less than 5 μM atrazine or 5 μM DCMU), as verified by the effect of different doses of these herbicides on the growth of selected surviving clones on agar (not shown). Moreover, none of the herbicide-resistant surviving clones passed the chemochromic screening. The results are very

encouraging, since many of the surviving clones have much higher rates of H_2 evolution. However, since the selection did not yield mutants with large increases in O_2-tolerance, we will work next under higher O_2 partial pressures to attempt to obtain more desirable mutants. These should have not only higher V_0s but also higher O_2 I_{50}s.

H_2-Production Selection

In order to improve the specificity of the H_2-production selection for O_2-tolerant, H_2-producing mutants, we added 10 mM sodium acetate to the induction medium preceding the selection. Acetate is metabolized by *C. reinhardtii* and serves as a source of energy to promote new protein synthesis. We thus expected that this would result in an increased level of hydrogenase activity. Figure 2 shows that, indeed, the resistance of *C. reinhardtii* cells to MNZ increases when the induction of the enzyme is done in the presence of acetate. We also attempted to improved the specificity of this selection by introducing a 2-minute pre-exposure of the cells to O_2, before the MNZ treatment. The results, shown in Fig. 2 suggest that, as expected, the toxic effect of MNZ can be modulated by pre-inactivation of the O_2-sensitive hydrogenase by oxygen.

The specificity of the H_2-production selection procedure for O_2-tolerant, H_2-producing mutants was determined by analyzing the V_0 and O_2 I_{50} for H_2 evolution in a representative sample of survivors. These clones survived H_2-production selective pressures applied in the presence of either 2.8% or 5% O_2. These two parameters have been used as diagnostic tools in determining whether a survivor is an electron-transport, an antenna, or an O_2-tolerant, H_2-producing mutant (Flynn et al., submitted). Electron transport mutants should have lower rates of ferredoxin reduction than the WT and, thus, lower rates of H_2 evolution at saturating light intensity. Mutants with a smaller antenna complement should show higher rates of H_2 evolution (per Chl) than the WT at saturating light intensity, since the number of Chl molecules per reaction center is decreased in these mutants. Finally, O_2-tolerant mutants should have an increased O_2 I_{50}, but no significant changes in the V_0 are expected. Out of 24 random clones tested, 15 shows lower rates of H_2 evolution (suggesting that they are electron transport mutants), 6 had higher rates of H_2 evolution per Chl (suggesting that they are antenna mutants), and 3 showed increases in the O_2 I_{50} (characteristic of an O_2-tolerant mutant). It is clear that this type of selection pressure produces at least two different classes of mutants besides O_2-tolerant, H_2-producing clones. The effect of the selection on the kinetic parameters for H_2 evolution of a *mixed population of survivors*, was to slightly increase the O_2 I_{50} and double the V_0 (data not shown). This effect is not what was expected, given that only 6 out of 24 mutants were shown to be antenna mutants (and thus with higher rates of H_2 evolution), while the majority of the survivors (15 out of 24) were expected to exhibit lower rates of H_2 evolution. As was the case with the survivors from the photoreductive selection, it is apparent that this selection procedure requires a secondary screening process to rapidly identify specific desired mutants. These results may also suggest that our assay for O_2 I_{50} should be re-evaluated as a means of measuring O_2 sensitivity.

Secondary Screening

The chemochromic screening procedure is shown in Figure 3, which depicts the steps involved in preparing the selected clones (see also Fig. 4, Flynn et al., submitted). Clones that survived the

selective pressure were transferred to agar and arranged in a grid pattern using sterile tooth picks. The colonies were then allowed to grow on agar for 7-14 days, replica-plated using sterile velveteen cloth, and grown on plates for another 7-14 days. The agar plate was then covered by a piece of filter paper and the cells were anaerobically induced on the plates in the dark for 4 h. At the end of the induction period, the plates were transferred to an anaerobic chamber and preexposed to a controlled amount of O_2 for a fixed period of time. The chemochromic sensor film was applied and the plates were then exposed to light. The H_2 produced by active colonies leaves a blue dot on the film (Ghirardi et al., 1997a; Seibert et al., 1997) that is easily seen by eye. The sensitization reaction on the sensor film is reversible, and the dots fade after a few minutes of exposure of the film to O_2 (Ghirardi et al., 1997a).

The relative intensity of the dots on the chemochromic film (determined visually) was correlated with the capacity for H_2 evolution (V_0) by the individual clones. Two colonies that gave rise to, respectively, very intense and less intense dots on the chemochromic sensor, were grown in liquid medium, anaerobically induced, and then used for H_2-evolution measurements. Figure 4 shows the initial rates of H_2 evolution versus O_2 concentration for cells derived from the clone that originated the intense signal (D2) and from the one that originated a less intense signal (E4). The clear correlation between the estimated V_0 and intensity of the dot confirms that the film responds to the amount of H_2 produced by algal colonies (Flynn et al., submitted).

Chemochromic film screening was then applied to survivors from photoreductive and H_2-production selective pressures done at 5% O_2. A total of about 2,000 clones were screened at 3% O_2, and 32 clones were selected. Figure 5 shows the results from a subsequent screening of the 32 clones (now co-located on the same agar place), in the presence of 8% O_2 (Flynn et al., submitted). The first important outcome of this subsequent screening experiment is the demonstration that, in contrast to our results last year, **mutants that we have select this year are stable** and do not rapidly revert. The first panel in Fig. 5 represents the response of the film when the screen is not preceded by a pre-exposure of the cells to O_2. Most of the pre-selected clones produced some H_2 under these conditions, and generated blue spots on the sensor. The second panel shows the response of the film after a 2-min pre-exposure of the induced cells to 8% O_2. It is clear that pre-exposure to O_2 inactivates a larger number of colonies but still allows H_2 evolution by many of the clones to occur. The last panel shows the reversibility of the film sensitization reaction: the blue dots fade when the sensor is exposed to O_2 for several minutes. V_0 and O_2 I_{50} values for 5 of the 32 clones were determined and are shown in Table II, Columns 3 and 4. It is very encouraging that all of the screened clones that were tested exhibited significantly higher V_0s for H_2 evolution compared to the WT. Indeed, the highest rate reported in Table II, 454 μmoles H_2·mg Chl^{-1}·h^{-1}, is 4 times that of the WT and **by far the highest H_2-production rate reported in the literature for green algae**. It should be emphasized that we are not generating antenna mutants, since the Chl a/b ratio of these clones are unchanged from the WT. The time courses of H_2 evolution of the 24.g1 clone and WT cells are shown in Fig. 6. The cells used in the experiment were exposed to about 1.4% O_2 in the electrode chamber for 2

Table II. Kinetic parameters of selected algal clones

Mutant Isolate	Original selective pressure	V_0 (μmoles H_2·mg Chl^{-1}·h^{-1})	O_2 I_{50} for H_2 evolution (relative units)	Half-life of the rate of H_2 evolution (relative units)
9.c2	mz	245	1.01	0.86
6.b2	mz	224	0.81	1.74
9.b5	mz	226	1.19	2.84
24.g1	pr	454	1.01	2.00
23.i6	pr	370	0.82	1.26
WT	n.a.	111	1.00	1.00

min before the actinic light was turned on. The Y-axis shows the H_2 concentration in the electrode chamber during the experiment. It is evident that, under the experimental conditions of the experiment shown, the rates of H_2 evolution of the isolated clone are substantially higher than that of the WT.

The result described above represents a major break-through in our research, since the 24g.1 mutant demonstrates rates of H_2 evolution that correspond to at least 70% of the maximum electron transport capacity of algal cells (under aerobic conditions), as compared to a much lower value for WT cells. The increases in the estimated O_2 I_{50} observed with the clones in Table II were not consistent, which suggests that either the O_2 I_{50} for H_2 evolution is not be the best method of testing for O_2-tolerant H_2-production under the conditions we are using, that the two pressures are still not optimal for specific selection of O_2-tolerant organisms, or that the current screening procedure is only identifying high H_2-producing mutants but not O_2-tolerant ones. To address the first possibility, we used an alternative approach to estimate the O_2-tolerance of the H_2-producing capacity, based on the half-life of the H_2 evolution activity measured up to 45 s after the onset of illumination. Since illumination also induces O_2 evolution, it was reasoned that the hydrogenase activity in WT cells would decrease more rapidly in the light than the activity of O_2-tolerant clones. The initial rate of H_2 evolution upon illumination (time zero) and the rate after 40-45 s after onset of illumination were measured and a half-life was calculated based on a single exponential decay model. Table II (Column 5) indicates that the rate of H_2 evolution by WT *C. reinhardtii* decays more rapidly than that of most of the tested selected mutant clones (this is a preliminary result at this point and must be confirmed). This does indicate, though, that both selective pressures can identify mutants of *C. reinhardtii* with both higher H_2-production rates and increased O_2 resistance.

With additional rounds of mutagenesis and selection at higher O_2 concentrations, and by tuning both the selection and the screening procedures, we expect to identify much more O_2-tolerant mutants in addition to the high H_2-producers isolated so far.

Generation of antibodies

The hydrogenase enzyme of *C. reinhardtii* was purified by Happe and Naber (1993), and its N-terminus was sequenced up to 24 amino acid residues. The enzyme was shown to contain one subunit of 49 kDa, which was detected only in extracts from induced cells. We synthesized an oligopeptide containing this particular sequence of residues, coupled it to the Rabbit Serum Albumin (RSA) and injected it into rabbits to induce an immunogenic response. The immunized rabbits were bled and their sera tested against the coupled oligopeptide on a sheet of nitrocellulose. A positive reaction was observed with sera from two of the rabbits (not shown). The sera recognized two bands, with molecular weights of just over 66 kDa, and of about 100 kDa, corresponding to 1-2 and 14-15 molecules of oligopeptide per RSA molecule. Extracts from induced and non-induced cells were also challenged with the immune sera, which recognized two bands of about 48 and 49 kDa, present only in extracts from induced cells (not shown). These results suggest that the immunized sera do indeed recognize the algal hydrogenase in cell extracts. However, given the low yield of the reaction and the fact that the sera pick up two protein bands in denaturing gels (and not one band as reported by Happe and Naber [1993]), further work will be required before we can be absolutely sure that the protein bands that we observe correspond to the reversible hydrogenase enzyme.

Subtraction Library

Total RNA and mRNA were extracted from *C. reinhardtii* cells that had been induced for 0, 0.5, 1, and 4 hours, respectively. The quality of the total RNA was shown to be very high, based on the appearance of even the low molecular weight ribosomal RNA bands in an agarose gel, which are present only in a diffuse form when the RNA sample is partially degraded. The translational capacity of the isolated mRNA was determined with a wheat germ *in vitro* translation system. All mRNA isolates were able to direct the synthesis of high molecular weight proteins, which confirms their integrity. The mRNA from non-induced cells was photobiotinylated using the Subtractor kit from Invitrogen. The mRNA from induced cells is being reverse transcribed into cDNA molecules and will be used in the subsequent hybridization step with the photobiotinylated mRNA, followed by purification through an avidin column. This procedure will yield cDNA species that are unique to anaerobically-induced cells. Among them we expect to find the hydrogenase cDNA as well as cDNA encoding for other proteins that may be required for optimal H_2 production by the enzyme.

Conclusions and Future Work

The two selective pressures have been improved and are being used in combination with a secondary chemochromic screening procedure to identify O_2-tolerant mutants of *C. reinhardtii* that are unexpectedly high producers of H_2. We have successfully isolated mutants with greatly increased H_2-production capacity compared to the WT (corresponding to at least 70% of the maximum aerobic electron transport capacity of the organism). However, our current selection/screen design will have to be adjusted to select for much better O_2-tolerant mutants as well. One proposed change is to expose induced selected clones to higher O_2 partial pressures (or for longer periods of time), and then transfer the plates to the anaerobic glove-box for screening in the absence of added O_2. Also, the nature of our assay for O_2-tolerance based on an O_2 I_{50} might be re-evaluated at this point (we suspect technical problems in doing the assay) and perhaps replaced by an alternative procedure based on

the half-life of the initial rate of H_2 evolution, as shown in Table II. Future work will involve using our selection and screening tools to examine large numbers of new mutants in order to identify useful O_2-tolerant, high H_2-producing clones.

The antibodies that we generated by means of a synthetic oligopeptide have shown a positive reaction against protein bands in extracts from induced cells. In contrast to Happe's work (1993), two protein bands were recognized by the immune sera. It is possible that one of them represents a translationally-modified form of the enzyme, or that one of them is a precursor or degradation form of the other. We will confirm that the detected protein bands do indeed represent the hydrogenase enzyme by physiological correlating the activation/inactivation of enzyme activity with the immunological detection of the protein bands on Western blots. If this turns out to be the case, we will have in our hands a powerful tool to (1) perform physiological studies on the expression of the reversible hydrogenase in algal cells, and (2) use in the cloning of the hydrogenase gene by the subtractive library approach.

The subtracted cDNA will be inserted into an expression vector and used to transform *E. coli*. The transformants will be probed with the antibody, as described above, to identify the ones carrying the hydrogenase cDNA, or, alternatively, with a DNA probe. This DNA probe will be constructed based on a nucleotide sequence homologous to that encoding the 24 amino acid residues sequenced by Happe. Finally, the cDNA inserts of other transformants will be sequenced, and we will attempt to identify their potential expression factors in order to gain some understanding on the regulation of the hydrogenase activity under anaerobic conditions.

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Figure Legends

Figure 1. Percentage of the initial population of control WT *C. reinhardtii* (open triangles) and survivors from a photoreductive experiment (PR3) that are viable after 3 weeks on agar plates containing different concentrations of the herbicide DCMU.

Figure 2. Kinetics of population survival after metronidazole selection. A population of mixed *C. reinhardtii* cells derived from EMS treatment was anaerobically induced in the presence of 0 or 5 mM sodium acetate. Following induction but prior to illumination, the induced cells were either preexposed to 5% O_2 for two minutes in the dark or treated directly with 40 mM MNZ in the presence of 5% O_2 and 400 $\mu E \cdot m^{-2} \cdot s^{-1}$ for different periods of time. Aliquots were taken at each time point, the cells were washed and plated for colony counting.

Figure 3. Procedure for preparation of survivors from the two selective pressures for chemochromic screening. Clones that survived the selective pressure were transferred to an agar plate and arranged in a grid pattern using sterile tooth picks. The colonies were grown on agar for 7-14 days, replica-plated using sterile velveteen cloth, and allowed to grow on plates for another 7-14 days. The agar plate was then covered with a piece of filter paper, and the cells were anaerobically induced on the plates in the dark for 4 h. At the end of the induction period, the plates were transferred to an

anaerobic chamber and preexposed to a controlled amount of O_2 for a fixed period of time for screening.

Figure 4. Initial rates of H_2 evolution measured in the presence of different initial concentrations of O_2 in the assay medium. The V_0 was estimated from a single exponential decay fit to the curve. The D2 strain has a higher V_0 and generated a more intense spot in the chemochromic sensor film than the E4 strain.

Figure 5. Response of the chemochromic sensor to H_2 produced by anaerobically activated algal colonies on an agar plate illuminated for 5 min in the presence of 8% O_2. The first panel shows H_2 evolution by colonies that were not pre-exposed to O_2. In the second panel, algal colonies were pre-exposed to 8% O_2 for 2 min before illumination. The last panel indicates that the sensitization of the chemochromic film is reversible because the blue spots fade upon exposure of the sensitized film to O_2 for several minutes.

Figure 6. Change in H_2 concentration as a function of time, measured with WT (lower curve) and with the 24.g1 mutant clone (upper curve). This clone was identified by application of chemochromic screening under 8% O_2 to a population of survivors from a photoreductive selection done in the presence of 5% O_2 and 15 μM each of DCMU and atrazine. Anaerobically-induced cells were added to the electrode chamber, pre-set to about 1.4% O_2. The cells were then incubated in the dark for 2 min before the actinic light was turned on. After monitoring H_2 evolution for 1 min, the lights were turned off, again.

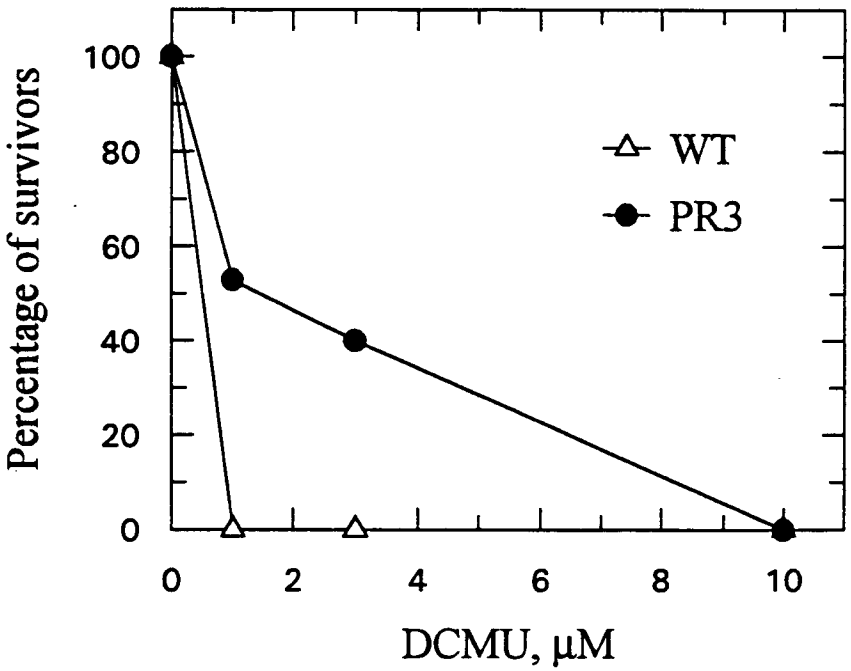

Percentage of survivors
100
80
60
40
20
0
0
2
4
6
8
10
DCMU, μM
WT
PR3

Figure 1

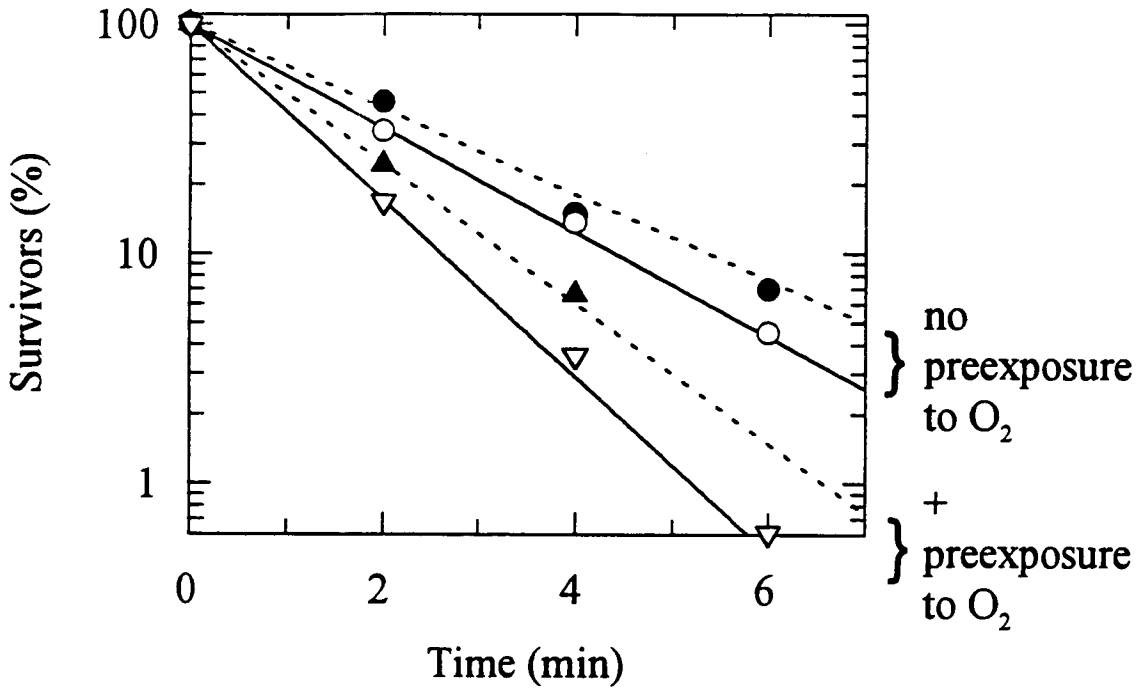

100
10
1
Survivors (%)
0
2
4
6
Time (min)
no preexposure to O_2
+ preexposure to O_2
+Acetate, - preexposure
- Acetate, - preexposure
+ Acetate, + preexposure
- Acetate, + preexposure

Figure 2

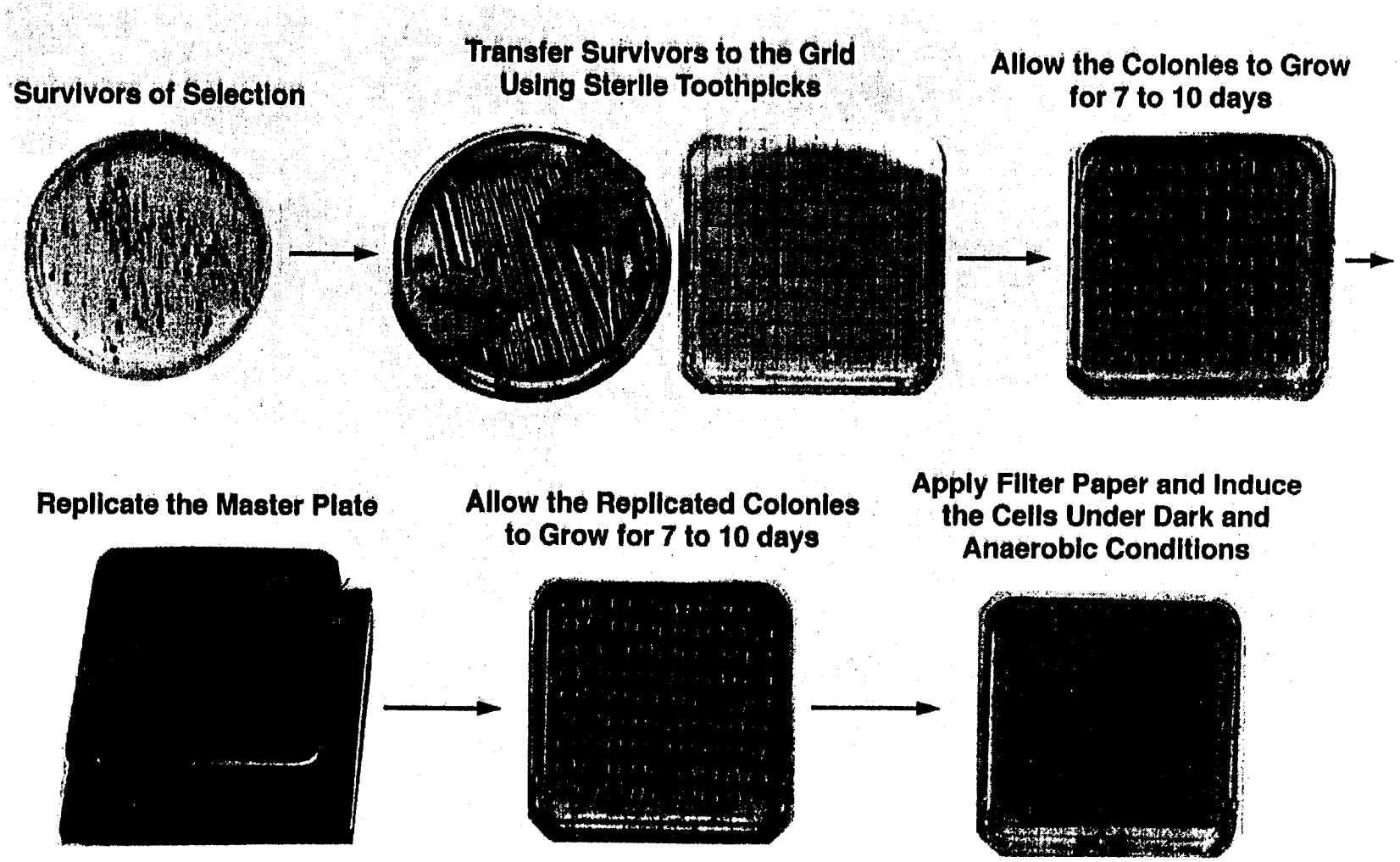

Survivors of Selection
Transfer Survivors to the Grid Using Sterile Toothpicks
Allow the Colonies to Grow for 7 to 10 days
Replicate the Master Plate
Allow the Replicated Colonies to Grow for 7 to 10 days
Apply Filter Paper and Induce the Cells Under Dark and Anaerobic Conditions

Figure 3

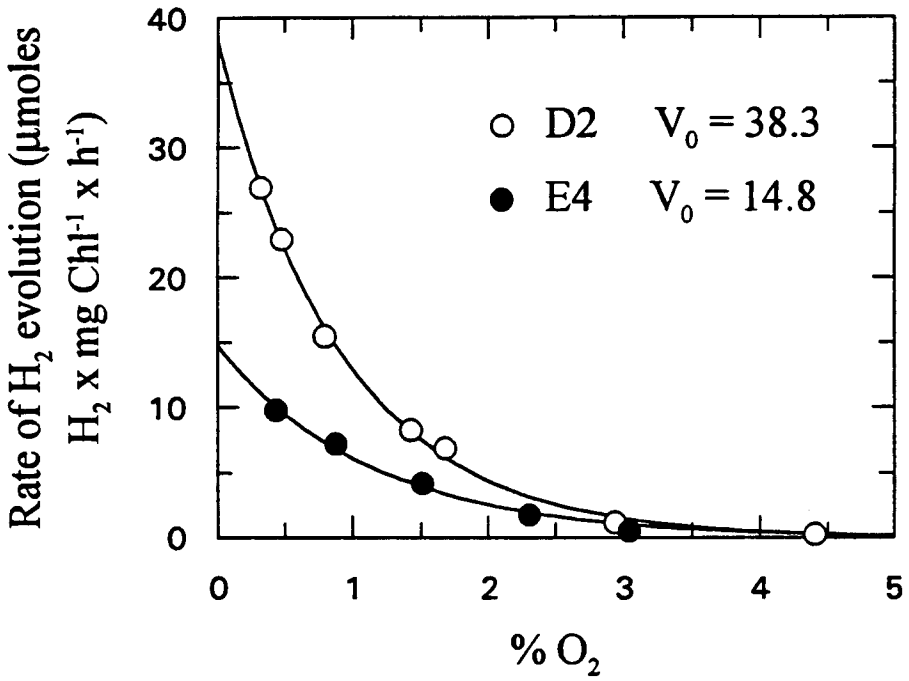
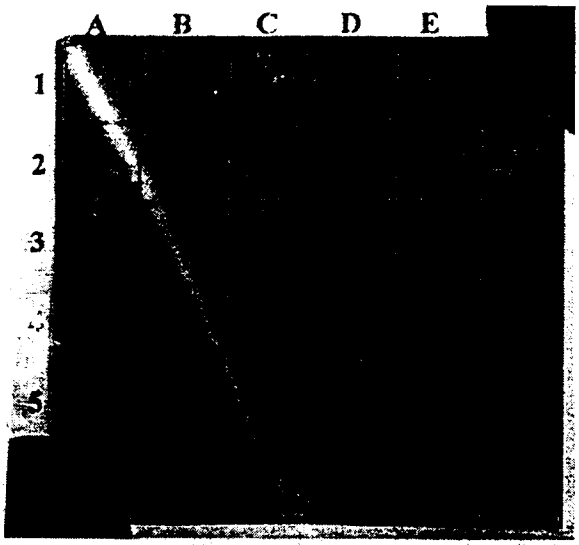
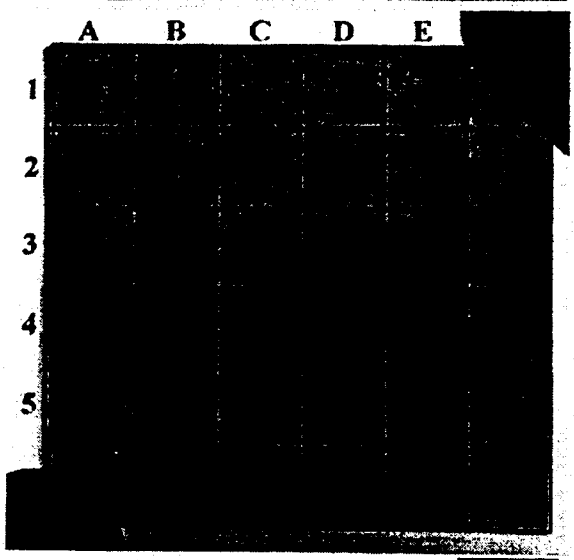

Rate of H_2 evolution (µmoles H_2 x mg Chl^{-1} x h^{-1})
40
30
20
10
0
0
1
2
3
4
5
% O_2
D2 V_0 = 38.3
E4 V_0 = 14.8

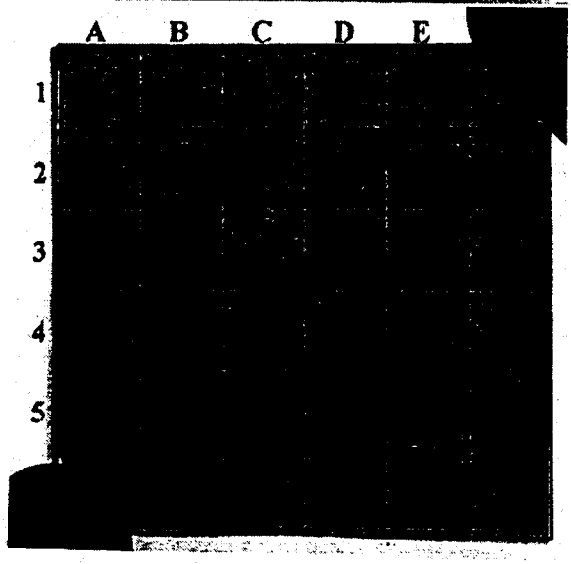
Figure 4



No O_2 pre-exposure



Two min. O_2 pre-exposure



Several min. after O_2 exposure

Figure 5

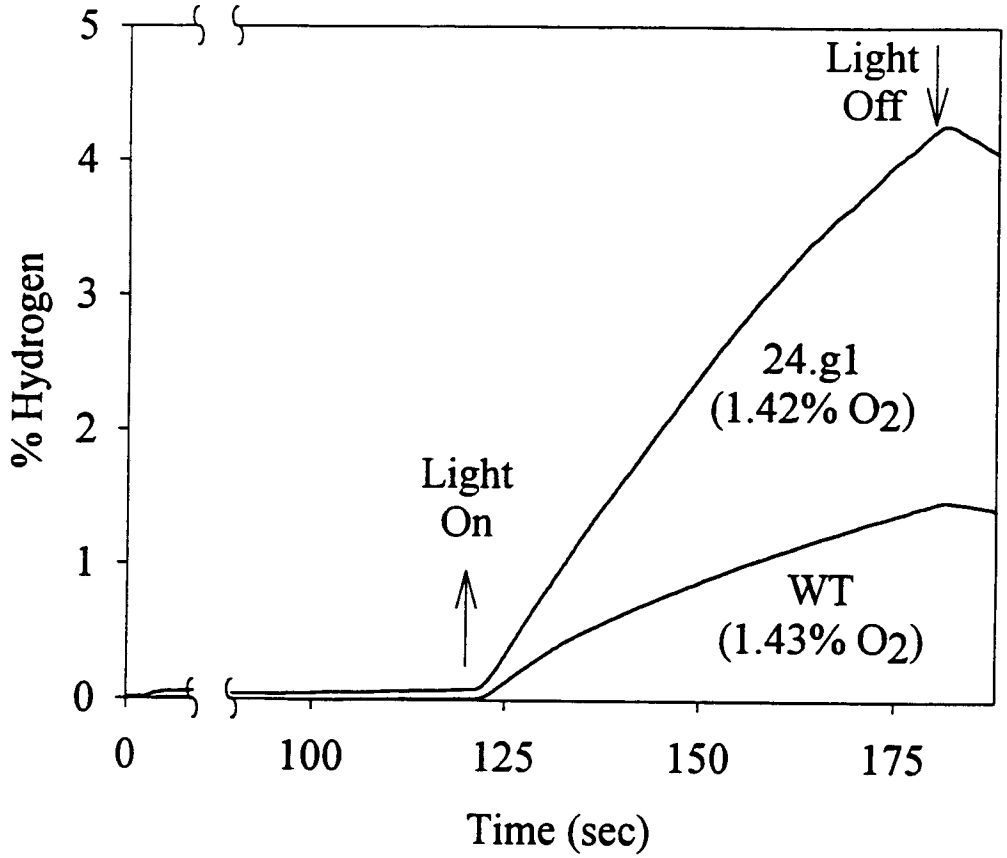

5
4
3
2
1
0
% Hydrogen
Light
Off
24.g1
(1.42% O2)
Light
On
WT
(1.43% O2)
0
100
125
150
175
Time (sec)

Figure 6