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High-Throughput Biosensor Discriminates Between Different Algal H_2-Photoproducing Strains

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ABSTRACT: A number of species of microalgae and cyanobacteria photosynthetically produce H_2 gas by coupling water oxidation with the reduction of protons to molecular hydrogen, generating renewable energy from sunlight and water. Photosynthetic H_2 production, however, is transitory, and there is considerable interest in increasing and extending it for commercial applications. Here we report a Petri-plate version of our previous, microplate-based assay that detects photosynthetic H_2 production by algae. The assay consists of an agar overlay of H_2-sensing *Rhodobacter capsulatus* bacteria carrying a green fluorescent protein that responds to H_2 produced by single algal colonies in the bottom agar layer. The assay distinguishes between algal strains that photoproduce H_2 at different levels under high light intensities, and it does so in a simple, inexpensive, and high-throughput manner. The assay will be useful for screening both natural populations and mutant libraries for strains having increased H_2 production, and useful for identifying various genetic factors that physiologically or genetically alter algal hydrogen production.

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KEYWORDS: *Rhodobacter capsulatus*; H_2 sensor; high-throughput screening; photobiohydrogen; *Chlamydomonas reinhardtii*; H_2 production

Introduction

There is great interest in the development of hydrogen as a renewable, sustainable fuel. Within this endeavor, the green alga *Chlamydomonas reinhardtii* has emerged as a model organism for investigating H_2 production, where H_2 is produced by three different pathways: dark fermentative degradation of carbon products, photofermentative starch degradation, and light-dependent water oxidation. The light-dependent pathways occur only as the organism transitions from a dark, anaerobic state into the light, perhaps in response to excess reductant (Happe et al., 2002). Dark anaerobic incubation poises the algal cultures in state 2, due to the accompanying starch degradation that maintains a reduced plastoquinone pool. Moreover, under these conditions, the carbon fixation pathway is inactivated. As the culture is illuminated, cyclic electron flow (CEF) and build-up of a proton gradient (Kruse et al., 2005; Tolleter et al., 2011) occur, and linear electron flow (LEF; Tolleter et al., 2011) is down-regulated. As the culture adapts to the light, the gradual accumulation of photosynthetic O_2 leads to inactivation of the O_2-sensitive hydrogenase (Ghirardi et al., 1997), photosynthetic reductant is again re-directed to CO_2 fixation into starch, and the ratio of CEF to LEF is re-adjusted to the prevailing light intensity. Challenges facing sustained photosynthetic H_2-production under solar intensities include: understanding and manipulating the regulation and induction of H_2 production (Toepel et al., 2013); preventing down-regulation of LEF by minimizing the induction of CEF and proton gradient accumulation under high-light conditions; avoiding the extreme sensitivity of H_2 metabolism to O_2, where O_2 is the obligatory by-product of photosynthetic water oxidation; avoiding saturation of photosynthesis at solar light intensity; and, selecting organisms and reactor designs optimized for industrial scale (Ghirardi et al., 2005; Kosourov and Seibert, 2009). Techniques used to sustain H_2 production have included algal cultivation under low sulfur (Melis et al., 2000) or low ammonia (Philipps et al., 2012) in order to limit photosystem II (PSII) O_2 production. These methods, however, inherently result in very low H_2-production rates. In order to identify mutants with increased H_2 production, various groups have focused on screening for phenotypes related to the challenges described above. In this indirect manner, the following mutants were recently identified: truncated antennae mutants showing increased light saturation level of photosynthesis in mass cultures (Kirst et al., 2012a,b; Polle et al.,

Conflicts of interest: M.W. works for GeneBiologics, LLC, which in part carried out this research.

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2003), mutants altered in, respectively, the ability to carry out state 1 to 2 transition (Kruse et al., 2005), the establishment of the proton gradient and/or CEF (Tolleter et al., 2011); and, mutants attenuated in the photosynthesis to respiration capacity ratios (Ruhle et al., 2008). However, the development of an efficient screening technique that directly detects H_2 photoproduction is critical to high-throughput identification of additional H_2-producing strains. One assay currently used for mid-throughput screening is based on the sensitization of a palladium/tungsten oxide film by H_2 (Seibert et al., 1998). This assay was successfully used to screen for hydrogenase (−) mutants of *C. reinhardtii* (Posewitz et al., 2004, 2005) and used to evolve and select the *E. coli hycE* subunit of the [NiFe]-hydrogenase 3 for increased in vivo activity (Maeda et al., 2008). The assay, however, cannot detect H_2 in the presence of O_2 and has a relatively low sensitivity. The lack of appropriately sensitive, high-throughput assays has hindered sampling large libraries, and no high-throughput assays have been described so far for use in screening natural populations for H_2-producing organisms.

Recently we described a H_2-sensing assay based on the HupSL H_2-sensing system of *Rhodobacter capsulatus* (Wecker et al., 2011) where emerald Green Fluorescent Protein (emGFP) was used as a reporter behind the *hupR* H_2-sensing promoter. When the sensing bacteria were grown in liquid medium in the presence of headspace H_2, the assay was capable of detecting H_2 at levels as low as those found in the terrestrial atmosphere. The H_2-sensing bacterial strain was used in a microtiter plate-based assay to detect dark fermentative H_2 production from *C. reinhardtii* during co-culture of the algae with the H_2-sensing *R. capsulatus*. We now report the use of the assay within agar overlays to analyze for photosynthetic H_2 production from algal colonies.

Materials and Methods

Growth of Algal Strains

Chlamydomonas strains were maintained either on Tris-Acetate-Phosphate (TAP) agar medium (Harris, 1989) or in shake flasks at 50 RPM, under 30 $\mu E\,m^{-2}\,s^{-1}$ cool, white fluorescent light. All *pgrl1* cultures were maintained on 10 μg/mL paromomycin (0.3 μg/mL for liquid cultures). For the overlay assay, Chlamydomonas strains were grown in 1.5 L Roux bottles bubbled with 2% CO_2 in air to culture densities of 16–20 μg/mL total chlorophyll, and applied to 1.2% TAP agar plates either via pipette, or plated in a 1:3 dilution series using a MC32 Spoke Inoculating Manifold (Dan-Kar Corp, Woburn, MA), loading approximately 2.2×10^4 CC124 cells and 1.12×10^5 *pgrl1* cells at the lowest dilution. Plates were then grown under 30 $\mu E\,m^{-2}\,s^{-1}$ fluorescent light. The *hydEF*, *hydG*, UTEX10, and SAG24.91 strains were received from the Matthew Posewitz lab, *tla2* was received from Anastasios Melis, and CC124 and *pgrl1* were received from Gilles Peltier. CC425 was obtained from the Chlamydomonas Culture Center. For photoautotrophic growth, algae were cultivated on tris–phophate buffer plates with HCl substituted for the acetic acid normally used in the TAP formula. Natural algal samples were collected from surface soil on the banks of a local creek (elevation 5,300 feet) in direct sunlight, mixed with stream water, filtered through a 30 μm filter and immediately plated onto 1.2% agar plates of modified Bold's medium (Andersen, 2005) with 50 mg/mL Kanamycin A. The plates were grown for 1 month under 30 $\mu E\,m^{-2}\,s^{-1}$ fluorescent light prior to overlay treatment for H_2 detection.

The *R. capsulatus* H_2-Sensing Agar Overlay

Plasmid pH_2emGFP was transferred and maintained in *R. capsulatus* in the RCVBN media having a final concentration of 0.15% NH_4Cl in order to limit nitrogenase-driven H_2-production (Wecker et al., 2011). Prior to use in the overlay, pH_2emGFP *R. capsulatus* was grown anaerobically under N_2 at 30 $\mu E\,m^{-2}\,s^{-1}$ incandescent light in liquid RCVBN to a cell density of 0.5 $OD_{660\,nm}$. Preliminary tests were carried out with bacterial cell densities between 0.1 and 1.0 $OD_{660\,nm}$ for the overlay. These tests showed essentially the same detection response when the culture was brought to an $OD_{660\,nm}$ equivalent of 0.5 prior to use. The makeup of the overlay was held constant since the intensity of the fluorescence signal and bacterial respiration of algal-produced O_2 are both dependent upon bacteria concentration. Ten milliliters of original 0.5 OD_{660nm} culture were used for each round petri plates overlay with a 3 mm overlay thickness, whereas rectangular plates required 15 mL of overlay. The bacterial culture was spun aerobically for 5 min at 4,000*g*, resuspended in 5 mL aerobic RCVBN and added to 5 mL of 50°C aerobic RCVBN in 1.4% Type A agar (Sigma, St. Louis, MO) immediately prior to plating. Algae plates to be overlaid were allowed to dry uncovered for 30 min so colonies would not lift off during pouring of the overlay, and then pre-incubated for 1 h at the indicated light level prior to adding the overlay. Plates were then overlaid with the above *R. capsulatus* H_2-sensing strain, exposed to the indicated light level for 16 h (unless otherwise indicated) using a 200 W Diamond LED light (Advanced LED, Bentonville, AR), and then imaged. Where DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) was used, the inhibitor was added to the overlay agar at 20 μM directly prior to pouring. The DCMU was found to be equally active at concentrations of between 4 and 200 μM DCMU.

Data Analysis and Processing of Overlay Images

Plates were imaged with a FluorChem Q imaging system (Cell Biosciences, Santa Clara, CA). Green fluorescence from GFP (which detects H_2) was measured with the EM-green CY2 filter, exposed for 3 s and developed at the following filter settings: Black 15,000 U; White 65,000 U; Gamma 0.5 U. Chlorophyll fluorescence was imaged with the EMSN CY5 filter, exposed for 250 ms and developed at the following filter settings: Black 1,000 U; White, 65,000 U; and a Gamma of 0.5 U. Quantification of images into relative H_2-sensor Fluorescence Units and Chlorophyll Fluorescence Units was carried out at the above exposure settings using a single

standard circular area with a diameter set to that of the largest colony size (approximately 4 mm). Four background values were taken from a clean area of each plate using the same standard area. The average of these four values was subtracted from experimental values.

Methyl Viologen Hydrogenase Assay on Whole Colonies

After overlay imaging, algal plates were transferred to a N_2 anaerobic glove box (Labmaster SP, MBraun, Inc., Stratham, NH). The most concentrated colonies were removed from under the overlay and transferred to 450 μL of reaction buffer (27.5 mM potassium phosphate, pH 6.9, 5.5 mM methyl viologen, 0.11% Triton X-100) in 7.5 mL serum vials. To each vial were added 50 μL of 100 mM sodium dithionite in 30 mM NaOH. The vials were then sealed, and incubated for 1 h at room temperature. After incubation, 300 μL of headspace gas were removed with a gas-tight syringe and H_2 levels were measured by gas chromatography (Agilent 7890A). Trials were carried out in biological triplicate. H_2 evolution levels measured using this assay gave a maximum of 300 nmol H_2 per colony. Since the actual amount of colony transferred to the MV reaction was difficult to assess, hydrogen production determined using this assay was qualitatively reported.

Results and Discussion

Development of the H_2-Sensing Overlay Assay for Detection of Fermentative H_2

When dark-incubated, H_2-producing *C. reinhardtii* D66 and CC425 colonies within an agar matrix were overlaid with H_2-sensing *R. capsulatus*, exposed to different O_2 concentrations for 10 h and then incubated in air for another 12 h, they showed a distinct halo of emGFP fluorescence in comparison to their respective non-H_2 producing mutants *hydG* and *hydEF*. Surprisingly, increasing O_2 exposure during the 10-h incubation period led to increased signal strength as opposed to inactivation of the hydrogenase (Supplementary Fig. S1); this may be due to the O_2 requirement for optimal synthesis of the emGFP reporter molecule (Heim et al., 1994). Nevertheless, these preliminary results indicated that the H_2 assay could be carried out using largely aerobic techniques; that is, anaerobically grown *R. capsulatus* can be applied aerobically as an overlay to the aerobically grown algae and the assay can be incubated aerobically on the benchtop. Thus, sufficiently anaerobic conditions are established within the algal colony during the assay incubation period such that a significant number of algal cells induce their H_2 production capabilities. We expect that this is largely due to exclusion of atmospheric O_2 by the agar overlay as well as respiration of photosynthetic O_2 by the algae and the *R. capsulatus*.

Discrimination of H_2-Photoproduction Capabilities Among Algal Strains

The overlay assay was conducted under illumination in order to determine whether it could assess the extent of H_2 photoproduction in algae previously characterized for this activity. Figure 1 depicts H_2 production by various algal strains at increasing levels of light intensity as measured by the H_2-sensor overlay. In these images, algal chlorophyll fluorescence is shown in red while the green halo indicates H_2 production as reported by the overlaid, H_2-sensing bacteria. Hydrogen production is seen in the wild type *C. reinhardtii* strains CC425, D66, and CC124, while the hydrogenase-less mutants *hydEF* (derived from CC425) and *hydG* (derived from D66) do not produce a signal. Hydrogen is produced by the wild type cultures either fermentatively in the dark, or photosynthetically, when exposed to light up to about 100 $\mu E\,m^{-2}\,s^{-1}$, with no observed signal at 300 $\mu E\,m^{-2}\,s^{-1}$ light and above (not shown). It is expected that H_2 production is inhibited at higher light levels in response to a combination of excess intracellular photosynthetic O_2 production leading to inactivation of the hydrogenase (Ghirardi et al., 1997) and/or down-regulation of linear electron flow by over-accumulation of a proton gradient generated by CEF during anaerobiosis-induced state 2 (Tolleter et al., 2011); this is addressed in the next section. The *C. reinhardtii* light harvesting antenna mutant *tla2* shown in Figure 1 produced H_2 at light intensities up to 300 $\mu E\,m^{-2}\,s^{-1}$, which is beyond the level at which the wild type produced measurable H_2. Since *tla2* colonies were plated using the same approximate number of cells as the wild type, these observations confirm its higher light-saturation property; it is known that the mutant contains approximately 20–25% of the total chlorophyll of the wild type on a per-cell basis, and 26% of the rate of photosynthetic O_2 production on a per-cell basis (Kirst et al., 2012a). Also included in Figure 1 are two *Chlamydomonas moewusii* algal strains, UTEX 10 and SAG 24.91 whose H_2-production capabilities versus wild type *C. reinhardtii* strains were previously studied under various assay conditions (Meuser et al., 2009). In the overlay assay, UTEX 10 showed a H_2-production profile similar to that of the wild type *C. reinhardtii* strains, whereas SAG 24.91 produced little observable H_2 in the dark, and produced H_2 only at levels under 30 $\mu E\,m^{-2}\,s^{-1}$ light. The overlay assay measures H_2 over long time periods in the presence of diminished levels of O_2 and, to this extent, it more closely resembles H_2 measurements taken from sulfur-deprived cells grown under continuous illumination, where SAG 24.91 was found to produce sixfold less H_2 than CC124 over 24 h (UTEX 10 was not tested; Meuser et al., 2009).

The Effects of CEF Mutation, DCMU and Acetate on Hydrogen Production

CEF may involve a number of competing pathways (Kramer and Evans, 2011), of which two are known to exist in *Chlamydomonas*, the PGRL (Proton Gradient Regulation Like) pathway (Tolleter et al., 2011) and the type II NADPH dehydrogenase pathway (Jans et al., 2008). The PGRL1 protein of *Arabidopsis* has recently been shown to act as a ferredoxin-plastoquinone reductase (Hertle et al., 2013); as such, it is proposed to accept electrons from ferredoxin

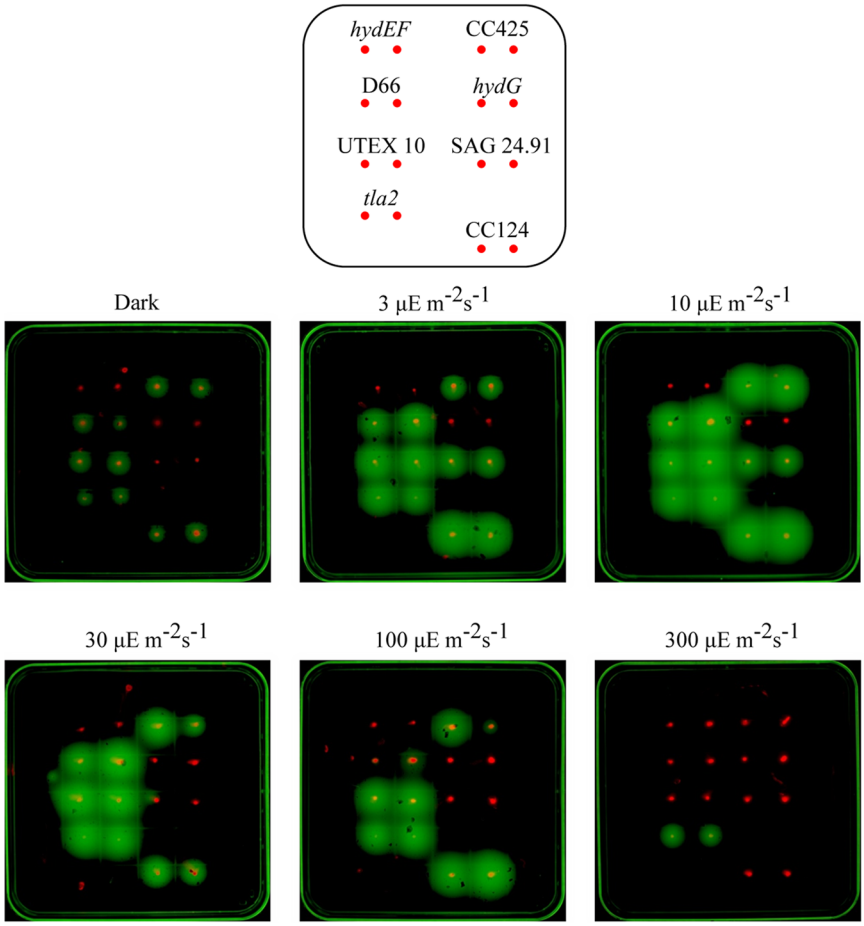

Figure 1. Discrimination of H_2-production levels by algal strains using the *R. capsulatus* emGFP overlay screening assay. Shown are composite images indicating H_2 production in green (Ex485/Em520 nm filter settings) and colony density in red, (Ex632/Em699 nm filter settings) as taken with a Fluorchem Q imaging system. Strains were transferred in duplicate to the agar plates in the indicated arrangement and grown aerobically for 2 days at 30 $\mu E\, m^{-2}\, s^{-1}$ prior to exposure for 1 h aerobically at the indicated light level. This was followed by overlay with emGFP *R. capsulatus*, and co-incubation aerobically at the indicated light levels for 16 h prior to imaging.

through its interaction with PGR5 and in turn reduce quinones and mediate NADPH-independent CEF. PGRL1 is clearly needed for establishment of a proton gradient under high light intensities (Tolleter et al., 2011). Dark, anaerobically induced *Chlamydomonas pgrl1* mutant cultures have been shown to produce more H_2 than the wild type when briefly exposed to 200 $\mu E\, m^{-2}\, s^{-1}$ light, but equal amounts of H_2 when tested under 50 $\mu E\, m^{-2}\, s^{-1}$ light (Tolleter et al., 2011). Figure 2 compares H_2 production of parental strain CC124 with the *pgrl1* mutant after a 16-h incubation under different light intensities. Both strains produced H_2 in the dark, and additional H_2 production was observed at low light intensities. Light levels above 100 $\mu E\, m^{-2}\, s^{-1}$ prevented H_2 production by the CC124 strain, whereas the *pgrl1* mutant produced some H_2 at even the highest tested light level (1,000 $\mu E\, m^{-2}\, s^{-1}$), confirming the previously described *pgrl1* phenotype. The overlay signal generally coincided with hydrogenase activity as measured by in vitro methyl viologen (MV) analysis of colonies taken from the overlaid plates. However, hydrogenase activity was not detectable using the MV assay on *pgrl1* colonies exposed to high light, and, while the overlay signal persisted out to 24 h illumination, activity detected using the MV assay diminished or disappeared at 12 and 24 h time points (see Table I and Fig. 2a). Since the MV assay confirmed the observed loss of H_2 production at high light levels for the CC124 strain, we conclude that loss in H_2 production is not due solely to redirection of reductant flow to CEF and down-regulation of LEF by the non-dissipated proton gradient; rather the hydrogenase is either being inactivated by O_2 generated through PSII, or the enzyme is otherwise being down-regulated. Detection of H_2 from both assays appeared only approximately 4–6 h after adding the overlay, possibly due to the algal hydrogenase being induced by anaerobiosis following overlay with the *Rhodobacter* cultures.

In the presence of the PSII inhibitor DCMU, the strength of the signal for H_2 production was similar to that seen in the absence of DCMU (compared in Fig. 2 and Supplementary Fig. S2). Typically, H_2 production in Chlamydomonas is reduced 80–90% in the presence of DCMU. This discrepancy

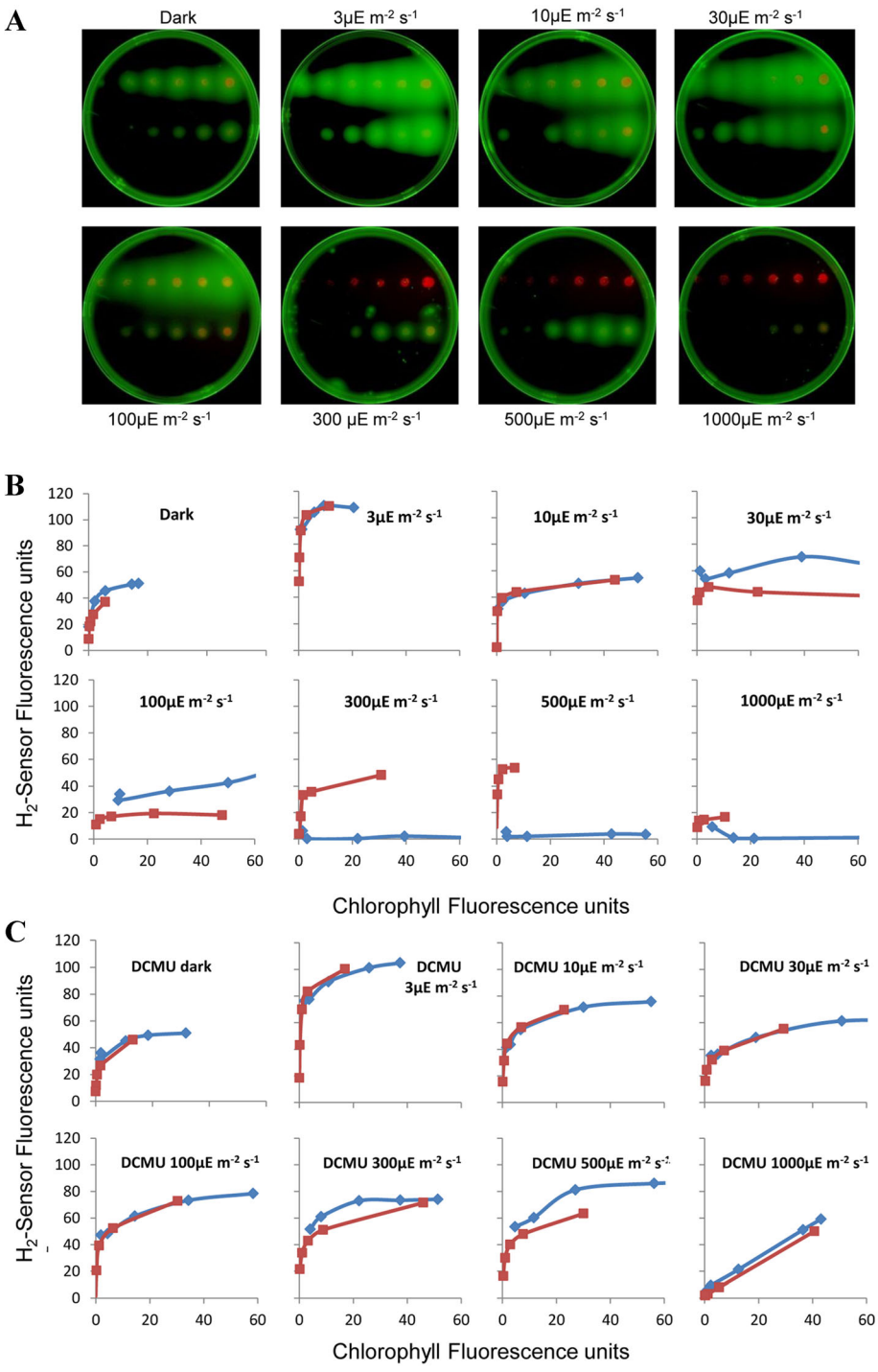

Figure 2. Comparison of H_2 production between the *pgrl1* mutant and its parental wild type. **A**: Wild type (top row on each plate) and the pgrl1 mutant (bottom row) algae were transferred from right to left onto TAP plates in a threefold dilution series and grown for 2 days at 30 $\mu E\ m^{-2}\ s^{-1}$ light prior to being overlaid with the H_2-sensing system, incubated for 16 h at the indicated light intensity and imaged. All plates were imaged at the same settings. **B**: Graphical analyses of the same assays. H_2-sensor Fluorescence units and the Chlorophyll Fluorescence units were determined as reported in the Materials and Methods Section. **C**: The same analysis as in plate B, but 20 μM DCMU was added to the overlay prior to plating.

with in-solution assays may either be due to poor linearity of the assay, or due to some physiological aspect of the Chlamydomonas cells when grown under the overlay. In addition, H_2 production in the presence of DCMU was not abolished at higher light levels and was essentially identical between the WT and *pgrl1* strains at each light level tested (Fig. 2c, and Supplementary Fig. S2). As mentioned earlier, it is important to note that there are two types of light-

Table I. A comparison of H_2 production in the overlay assay versus colony methyl viologen hydrogenase activity.

		Light intensity ($\mu E\, m^{-2}\, s^{-1}$)					
Strain	Assay	10	30	100	300	500	1,000
CC124	emGFP	++	++	++	−	−	−
	MV	++	++	++	−	−	−
pgrl1	emGFP	++	++	++	+	+	(+)
	MV	+	++	+	−	−	−

	$\mu E\, m^{-2}\, s^{-1}$		Time (h)				
Strain	Light	Assay	0	1	4.5	12	24
CC124	30	emGFP	−	−	+	++	++
		MV	−	−	+	++	+
	500	emGFP	−	−	−	−	−
		MV	−	−	−	−	−
Pgrl1	30	emGFP	−	−	−	++	++
		MV	−	−	−	++	−
	500	emGFP	−	−	−	++	++
		MV	−	−	−	−	−

dependent H_2 photoproduction, pathways, one that requires PSII and PSI activities and uses water as the oxidant, and a second that requires only PSI activity and uses reductant from starch degradation. It is possible that the latter is equally efficient in the wild type and *pgrl1* strains at all light levels, and that flux through this pathway is low enough to prevent O_2 accumulation or induction of state transitions at high light intensities. Indeed, limited H_2 photoproduction by sulfur-deprived cultures has been shown to occur in the presence of DCMU by both the wild type and the *pgrl1* mutant (Kosourov et al., 2003; Tolleter et al., 2011).

When the CC124 and *pgrl1* strains were grown photoautotrophically on plates lacking acetate, both strains lost the ability to produce H_2 at light levels greater than 10–30 $\mu E\, m^{-2}\, s^{-1}$ (Supplementary Fig. S3). Acetate increases the rate of mitochondrial respiration in *Chlamydomonas*, and is known to reduce its net rate of photosynthesis, with direct effects upon PSII (Roach et al., 2013). Hence, photoautotrophic conditions generate increased intracellular levels of O_2 under illumination and result in lower H_2 production, either because of higher O_2 inactivation of hydrogenase and/or onset of CEF at lower light intensities than when compared to mixotrophically grown cultures.

Use of the Overlay for Assaying H_2 Production in Microorganism Libraries

The curves seen in Figure 2b indicate that the H_2 production signal is not linear and that it saturates at fairly low cell density. This is not surprising in that the output signal reflects

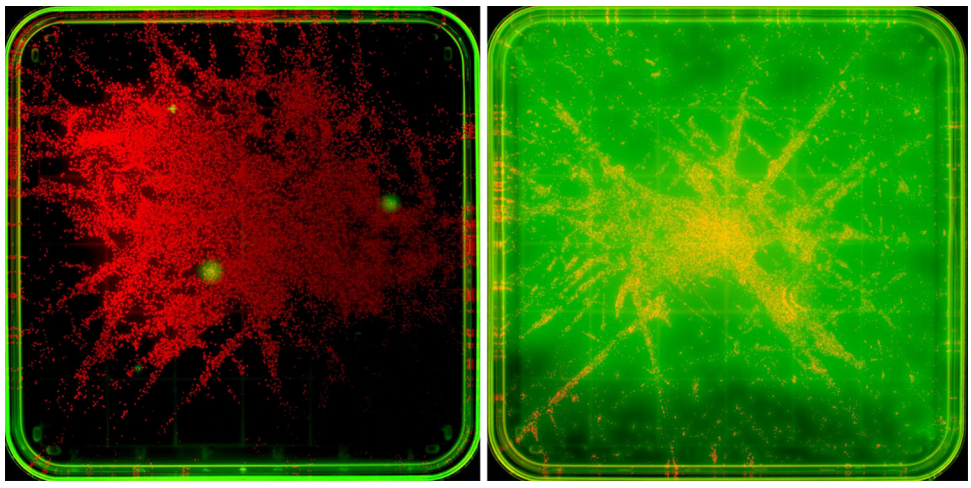

Figure 3. The detection of 1 in 10,000 H_2-producing colonies. The left plate shows an overlay response of H_2-positive, D66 *C. reinhardtii* colonies in a 10,000-fold excess of the hydrogenase-less *hydG* colonies. The right plate shows the opposite ratio, where H_2-producing colonies are in 10,000-fold excess. Plates were imaged at the same sensitivity settings for comparison.

an averaged level of H_2 production over the entire length of the assay, and is necessarily dependent upon a number of non-linear parameters, including: the *R. capsulatus* H_2-sensing system (comprised of hydrogenase HupUV that both consumes H_2 and transmits the state of its activity through a protein cascade of HupT, HupR and eventually the RNA polymerase); emGFP (which must be appropriately oxidized and which may degrade over time); photosynthesis and respiration levels of both the *Chlamydomonas* and *Rhodobacter* cells; variable nutrient requirements of the two species during the course of the assay; and, the complex nature of H_2 metabolism in *Chlamydomonas*. To determine the feasibility of using the H_2-sensing overlay for detection of H_2-overproduction mutants, wild type strain D66 was diluted into the *hydG* mutant strain and plated (Fig. 3). After 4 days of growth at 30 $\mu E\, m^{-2}\, s^{-1}$ light, the algae were overlaid with the sensor bacteria and incubated for 2 days at 30 $\mu E\, m^{-2}\, s^{-1}$ light. Remarkably, two to four wild type H_2-producing colonies were detected from a field of approximately 30,000 H_2-mutant colonies. Colonies that do not produce H_2 were not distinguishable from wild type colonies when the opposite dilution ratio was made, suggesting that the assay is less suitable for detection of loss-of-H_2-production phenotype. Positive H_2-producing colonies were easily recovered from under the overlay, and positively assayed for H_2 production (Supplementary Fig. S4). Furthermore, we carried out the assay at 300 $\mu E\, m^{-2}\, s^{-1}$ light on natural algal populations using locally-collected surface soil samples. The samples showed approximately five H_2-positive hits from a field of ~1,000 algal colonies (Fig. 4). Isolates are being made from these high-light H_2-producing colonies for further characterization.

We anticipate that this assay will be useful for detecting algal strains having an increased H_2-production phenotype due to a variety of possible biochemical alterations, such as increased hydrogenase tolerance to O_2, increased respiration, decreased CEF, increased proton gradient dissipation, and decreased photosynthetic O_2 production. The assay can be

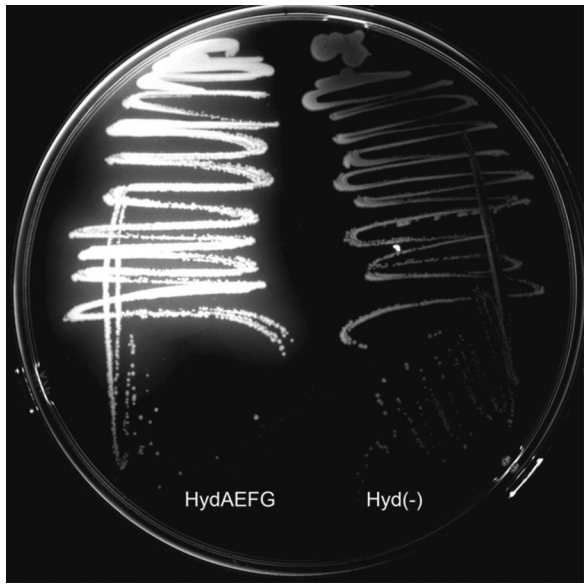

Figure 5. Detection of H_2 production by *E. coli* expressing the clostridial hydrogenase HydA. The strain on the right is background hydrogenase (−) strain MC4100 FTD147 (Redwood et al., 2008) into which was inserted a DE3 T7 polymerase induction system. The strain on the left is the same MC4100 FTD147 DE3 strain but carrying plasmids harboring genes for *C. acetobutylicum* HydA, HydE, HydF, and HydG as previously described (King et al., 2006). The strains were struck out on Luria Broth plates containing 100 μM Isopropyl β-D-1-thiogalactopyranoside, grown for 12 h, layered with the *R. capsulatus* emGFP H_2-sensor overlay and allowed to incubate for 16 h at 3 $\mu E\, m^{-2}\, s^{-1}$ light prior to imaging with CY2 filters on the FluorChem Q.

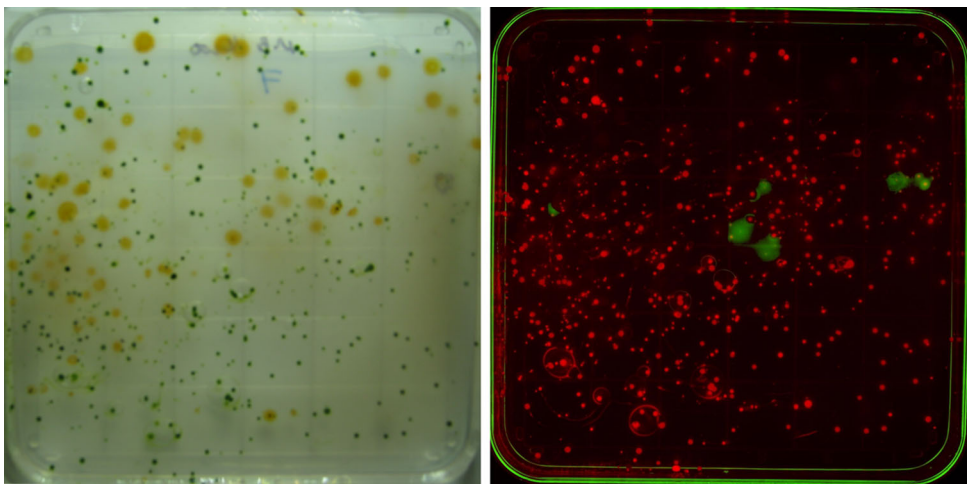

Figure 4. Isolation of algal strains from a surface soil sample assayed to produce H_2 at 300 $\mu E\, m^{-2}\, s^{-1}$ light. The plate imaged on the left was grown for 1 month on modified Bolds medium containing 50 mg/mL Kanamycin A, and represents a variety of microorganisms. It was then assayed for H_2 production (right image) after overnight exposure to 300 $\mu E\, m^{-2}\, s^{-1}$ light. The two colonies on the far right represent potential leads.

used for both fermentative and photosynthetic H_2 determinations, and it should be applicable for in vivo directed evolution of hydrogenases (Nagy et al., 2007; Stapleton and Swartz, 2010, 2011), for the screening of active in vivo hydrogenase fusion proteins such as hydrogenase-ferredoxin (Yacoby et al., 2011), and for driving increased H_2 yields in *E. coli* (Hu and Wood, 2010; Kim et al., 2011) and possibly cyanobacterial (Asada et al., 2000; Ducat et al., 2011) model systems. To this end, we have successfully detected H_2 produced by *E. coli* expressing the clostridial hydrogenase HydA on agar plates (see Fig. 5), and thus established the usefulness of the assay for organisms other than microalgae (King et al., 2006; Redwood et al., 2008).

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