Genetic disruption of both *Chlamydomonas reinhardtii* [FeFe]-hydrogenases: Insight into the role of HYDA2 in H2 production

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Meuser JE, D'Adamo S, Jinkerson RE, Mus F, Yang W, Ghirardi ML, Seibert M, Grossman AR, Posewitz MC, "Genetic disruption of both Chlamydomonas reinhardtii [FeFe]-hydrogenases: Insight into the role of HYDA2 in H2 production," Biochemical and Biophysical Research Communications, January 2012 417(2):704–709

Genetic disruption of both Chlamydomonas reinhardtii [FeFe]-hydrogenases: Insight into the role of HYDA2 in H_2 production

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Chlamydomonas reinhardtii (Chlamydomonas throughout) encodes two [FeFe]-hydrogenases, designated HYDA1 and HYDA2. While HYDA1 is considered the dominant hydrogenase, the role of HYDA2 is unclear. To study the individual functions of each hydrogenase and provide a platform for future bioengineering, we isolated the Chlamydomonas hydA1-1, hydA2-1 single mutants and the hydA1-1 hydA2-1 double mutant. A reverse genetic screen was used to identify a mutant with an insertion in HYDA2, followed by mutagenesis of the hydA2-1 strain coupled with a H_2 chemosensor phenotypic screen to isolate the hydA1-1 hydA2-1 mutant. Genetic crosses of the hydA1-1 hydA2-1 mutant to wild-type cells allowed us to also isolate the single hydA1-1 mutant. Fermentative, photosynthetic, and in vitro hydrogenase activ-ities were assayed in each of the mutant genotypes. Surprisingly, analyses of the hydA1-1 and hydA2-1 single mutants, as well as the HYDA1 and HYDA2 rescued hydA1-1 hydA2-1 mutant demonstrated that both hydrogenases are able to catalyze H_2 production from either fermentative or photosynthetic path-ways. The physiology of both mutant and complemented strains indicate that the contribution of HYDA2 to H_2 photoproduction is approximately 25% that of HYDA1, which corresponds to similarly low levels of in vitro hydrogenase activity measured in the hydA1-1 mutant. Interestingly, enhanced in vitro and fer-mentative H_2 production activities were observed in the hydA1-1 hydA2-1 strain complemented with HYDA1, while maximal H_2-photoproduction rates did not exceed those of wild-type cells.

Introduction

Diverse bacteria and microbial eukaryotes, including many green algae, encode [FeFe]-hydrogenase (HYDA) enzymes that cat-alyze the reversible reduction of protons to H_2 [1,2]. Anaerobic H_2 production in green algae (s.s. Chlorophyta), such as Chlamydo-monas reinhardtii (Chlamydomonas throughout), can be generally classified into three distinct pathways: a low-production, fermen-tative pathway linked to carbohydrate catabolism, and two distinct photosystem-linked pathways [photosystem II (PSII)-dependent and PSII-independent] [3]. PSII-dependent H_2 photoproduction is limited by inactivation of the native algal hydrogenases by O_2 [4,5]. The O2 sensitivity of algal hydrogenases is likely a regulatory feature that helps to direct electron flow for the greatest competi-tive benefit to the alga in the natural environment. Hydrogenases likely serve as an ''electron valve'' when H2 photoproduction rates are highest during the transition from anoxia to aerobic photosynthesis [6]. However, sustained aerobic H_2 photoproduction repre-sents an energetically wasteful process. If the natural regulation of H_2 production in green algae, including O_2 sensitivity and com-petition for electrons with other pathways, can be reengineered, the high quantum efficiencies by which algae can theoretically oxi-dize water to H_2 [7] could be leveraged to produce a carbon-free fuel more efficiently than can be achieved using existing technol-ogy for carbon-based biofuel production by vascular plants.

Hydrogen metabolism in Chlamydomonas is catalyzed by two [FeFe]-hydrogenase (HYDA) paralogs, HYDA1 and HYDA2 [8,9]. Examination of relative enzyme activities by gene-silencing tech-niques indicate that HYDA1 catalyzes the majority of the in vitro hydrogenase activity, but revealed no evidence for a substantive role of HYDA2 in algal H_2 production [10]. Interestingly, all green

algae for which hydrogenase activity has been characterized contain at least two enzyme isoforms, with recent phylogenetic analysis suggesting that gene duplication events occurred independently in each alga [1]. This finding indicates that there has been selective pressure for duplicating and maintaining two copies of the hydrogenase, which may have evolved to have distinct functions.

We have undertaken a project focused on eliminating the activity of both HYDA1 and HYDA2 in Chlamydomonas by insertional mutagenesis to study the role of the two hydrogenases in algal metabolism and to establish a platform for the heterologous expression of hydrogenases in a system that is not complicated by native enzyme activity.

2. Materials and methods

2.1. Algal cultures and growth conditions

C. reinhardtii strain CC124 (*nit*$^-$, *mt*$^-$) was obtained from the Chlamydomonas Genetic Center (http://www.chlamy.org/). *C. reinhardtii* strain D66 (*nit*$^-$, *cw15*, and *mt*$^+$) [11] was obtained from Steven Pollock and used for mutant generation. For the physiological experiments, axenic algal cultures (100 mL) were inoculated at 10^5 cells/mL in 250 mL Erlenmeyer flasks capped with silicone sponge enclosures (Chemglass, Vineland, NJ, USA), grown in Tris–Acetate–Phosphate (TAP) medium [12], pH 7.2, and shaken at 120 rpm under constant fluorescent irradiance (24 h photoperiod at 80 μmol photon m^{-2} s^{-1} PAR by GE Ecolux 6500k T5 bulbs). Cells were harvested at 48 h post-inoculation during mid-logarithmic growth (16–24 μg Chl/mL) for measuring in vitro and H_2 photoproduction activities, and at 60 h post-inoculation (24–33 μg Chl/mL) for dark, fermentative H_2 evolution measurements.

2.2. Chlorophyll determination and cell count

Chlorophylls were extracted in 95% ethanol, cell debris pelleted by centrifugation (9000*g* for 5 min), and total Chl spectrophotometrically quantified [12]. Cell numbers were determined using a Beckman Z2 Coulter Counter (Beckman Coulter, Brea, CA, USA).

2.3. Mutant generation, screening, and isolation

D66 was first transformed with a 1.7 kb PCR-fragment, amplified from the pSL72 plasmid [13] using RIM-F2 (5′-ACCAATCGTCACACGAGC-3′) and RIM-R2 (5′-CTTTCCATCGGCCCAGCAAC-3′) primers and containing the *AphVIII* gene under the control of the *PSAD* promoter (Fig. 1A) Electroporation was performed using a modification of the procedure reported by Shimogawara et al. [13]. Briefly, cells were collected by centrifugation at 3000 *g* for 5 min, resuspended in TAP medium supplemented with 40 mM sucrose (TAP + sucrose) to a final cell density of 1–4 × 10^8 cells/mL, and 250 μL of the suspension was placed into a disposable 4-mm gap electroporation cuvette (Bio-Rad, Hercules, CA, USA). Marker gene DNA was resolved by electrophoresis in 1% agarose gels, excised, and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Purified marker gene DNA (100 ng) was added to the cell suspension in the electroporation cuvette and incubated at 4 °C for 10 min. An exponential electric pulse of 0.8 kV at a capacitance of 25 μF was applied to the sample using the Gene Pulser II (Bio-Rad) electroporation apparatus. Following electroporation, cells were transferred to 10 mL of fresh liquid TAP medium and incubated for 16–18 h at 23 °C under dim light. The cells were then centrifuged (3000*g* for 5 min), resuspended in 1 mL of TAP medium, spread onto selective TAP medium (5 μg/mL paromomycin, 1.2% agar), and incubated for 1–2 weeks (24 h photoperiod at 60 μmol photon m^{-2} s^{-1} PAR). Single colonies were picked and transferred to 200 μL of liquid TAP medium in individual wells of 96-well microtiter plates. Transformant screening for marker gene insertion within the *HYDA2* gene was as described previously [14,15] using primers (Supplementary Table 1) designed with Primer 3 software [16].

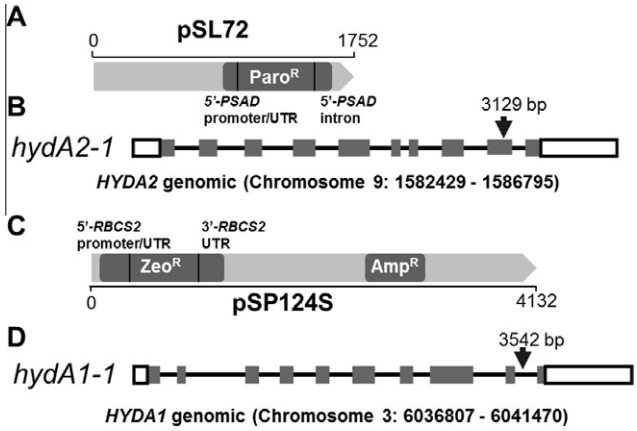

Fig. 1. (A) pSL72 vector used for the generation of the *hydA2-1* mutant. (B) *hydA2-1* gene showing the site of gene disruption (arrow), which is in exon 9. (C) pSP124S vector used for generation of the *hydA1-1 hydA2-1* mutant, and (D) *hydA1-1* gene showing the site of gene disruption (arrow). Grey boxes represent exons, black lines represent introns, and white boxes represent 5′-, 3′-UTRs.

2.4. Phenotypic screening for the hydA1-1/hydA2-1 mutant

To isolate the *hydA1-1 hydA2-1* mutant, a secondary screen was performed. The pSP124S plasmid (Fig. 1C), which confers Zeo^R, was purified using the Qiagen Plasmid Mini Kit (Qiagen), Swa1-linearized and concentrated to approximately 1 μg/μL using the QIAquick MinElute Gel Extraction Kit (Qiagen). The *hydA2-1* mutant cells were grown to 10–25 μg Chl/mL, 50 mL of cells were pelleted by centrifugation (3160 *g* for 5 min) and resuspended in 100 μL of sterile TAP + sucrose and 1 μg of plasmid DNA. The resulting 250 μL volume was transferred to a 4-mM gap electroporation cuvette (Eppendorf, Hauppauge, NY, USA) and incubated on ice for 15 min prior to electroporation using an ECM 630 electroporator (BTX Genetronics, San Diego, CA, USA) with settings of 0.8 kV, 1575 Ω and 25 μF. Immediately following electroporation, cells were transferred to 10 mL of TAP + sucrose and placed in dim light (20 μmol photon m^{-2} s^{-1} PAR) on an orbital shaker for 16–18 h. The cells were then centrifuged (3160 *g* for 5 min), resuspended in 1 mL of TAP + sucrose medium, and plated on selective TAP medium (3.3 μg/mL Zeocin, 1.2% agar). Following drying in a sterile hood, the plates were placed under constant illumination (50 μmol photon m^{-2} s^{-1} PAR) for 1–2 weeks until individual colonies were transferred onto fresh plates. Colonies were allowed to grow for 1–2 weeks under constant illumination (20 μmol photons m^{-2} s^{-1} PAR) before screening for H_2-photoproduction activity. The screen involved physical vapor deposition of PTFE/Pd/WO_3 onto glass plates to produce chemochromic H_2 sensors [5,17,18]. The colonies were excluded from light within velvet bags and anaerobically-adapted by filling and evacuating the air lock of an anaerobic chamber (COY Laboratory Products Inc., Grass Lake, MI, USA) nine times with ultra-high-purity (UHP) N_2 gas. All pressurized gases were obtained from General Air (Denver, CO, USA). The colonies were stored under a slightly positive pressure in UHP N_2 for 2–4 h before the H_2 photoproduction assay. The assay involved chemochromic sensors atop autoclaved #2 filter paper (Whatman Inc.) placed directly in contact with the colonies

immediately before exposure to 380 μmol photon m^{-2} s^{-1} PAR incandescent light for 3 min. Colonies negative for H_2-photoproduction were recovered, including the *hydA1-1 hydA2-1* mutant.

The original *hydA1-1 hydA2-1* mutant was backcrossed four times [19] to Chlamydomonas strains CC124 and D66, always selecting for both $Paro^R$ and Zeo^R. From the final cross, progenies were selected for (a) Zeo^R, (b) $Paro^R$, or (c) both $Paro^R$ and Zeo^R, which represent the *hydA1-1*, *hydA2-1*, and *hydA1-1 hydA2-1* mutants, respectively. PCR was used to define the mating type of the progeny and confirm sexual crossing using previously described primers [20] and, after the progeny of the backcrossed strains were selected on the basis of antibiotic resistance, to demonstrate that each strain had the expected disruption phenotype. Two *mt*-progeny from the final backcross that exhibited no antibiotic resistance and no insertions in *HYDA* genes (based on PCR) were selected as WT control strains. The mutant phenotypes described were from two selected progeny of each genotype (WT, *hydA1-1*, *hydA2-1*, and *hydA1-1 hydA2-1*). For each of these progeny, data from 4 independent experiments were averaged (n = 8 in total).

2.5. Genomic isolation and gene identification

Genomic DNA was isolated using the phenol:chloroform technique of Newman et al. [21], and DNA upstream of the pSP124S insertion site in *HYDA1* was amplified by genome walking (Genome-Walker Universal Kit, Clontech, Mountain View, CA, USA). A fragment from a PvuII-digested genomic DNA library was used as a template to amplify a product with the sequence for the AP1 (5′-GTAATACGACTCACTATAGGGC-3′) and nested AP3 (5′-CGTGGTCGACGGCCCGGG-3′) adapter primers, and the Ble3F (5′-GCGAAGTCGTCCTCCACGAAGTC-3′) and nested Ble4F (5′-GCCGGTCGGTCCAGAACTCG-3′) primers, which were designed to specifically anneal to the Zeo^R gene (*ble*) [22]. The downstream region flanking the site of DNA insertion (from 3004 to 3749 bp of *HYDA1*) was mapped by PCR methods on genomic DNA isolated from the *hydA1-1 hydA2-1* mutant, using *HYDA1* gene-specific primers (below) that anneal to different regions of the full-length genomic sequence. DNA regions flanking the pSP124S insertion site were precisely determined using a *HYDA1* specific primer (3′R3: 5′-GGAGAAGGACGAGAAGAAGTGAGG-3′) and a pSP124S specific primer (GSP1-R: 5′-CAAAATCAACGGAGGATCGTTACA-3′) to amplify across the site at which the marker gene inserted. For primary and secondary PCRs on DNA fragments of the PvuII-digested library, we used AP1 and AP3 nested primers, each with a single *HYDA1* gene-specific primer (3′F2: 5′-AAGAAGCTGATCACCAAGATGCAG-3′). All of the PCRs were performed using the KOD Hot-Start Mastermix (EMD Chemical, Gibbstown, NJ, USA), and the products generated were sequenced (Davis Sequencing, Davis, CA, USA).

2.6. Complementation

BAC clones containing the *HYDA1* and *HYDA2* full-length genomic sequences (including 5′ and 3′ predicted UTRs) were obtained from the Clemson University Genetics Center. The *HYDA2* gene, including the 5′ and 3′ predicted UTRs, was excised from the BAC clone (1G24) with ZraI and then cloned into the pUC19 plasmid (New England Biolabs, Ipswich, MA, USA); the *AphVII* gene, which confers Hygromycin B resistance (Hyg^R), was isolated from pHyg3 [23] (generously provided by Patrice Hamel) by HindIII digestion and subcloned into the pUC19:*HYDA2* plasmid, yielding the pUC19:*HYDA2*:*AphVII* plasmid pSD1928. This plasmid was linearized with ScaI, the DNA was dephosphorylated with Antarctic Phosphatase (New England Biolabs), and the fragment was resolved by agarose gel electrophoresis and purified from the gel using the QIAquick DNA Gel Extraction Kit (Qiagen). Transformation into the *hydA1-1 hydA2-1* mutant was performed by electroporation as described above. Transformants were selected on TAP plates containing 10 μg/mL of Hygromycin B (Sigma–Aldrich, St. Louis, MO, USA).

For introduction of *HYDA1* into the *hydA1-1 hydA2-1* mutant, a BAC clone (34H3) was digested with NotI, and the fragment containing the full-length genomic *HYDA1* sequence (including 5′ and 3′ predicted UTRs) was cloned into pSP124S at the NotI site. The *ble* gene was then excised from the plasmid with HindIII and replaced with the *AphVII* gene such that the plasmid confers Hyg^R. This plasmid was then digested with XbaI, the DNA ends dephosphorylated, the linearized plasmid resolved by electrophoresis (1.2% agarose gel) and purified from the agarose with the QIAquick Gel Extraction Kit (Qiagen). Electroporation was performed as described above, and the transformants were selected on TAP plates containing 10 μg/mL of Hygromycin B.

2.7. Anaerobic induction

Liquid cell cultures (20–50 mL) were concentrated by centrifugation (3716 *g* for 10 min) and resuspended in 0.1 volume of anaerobic induction buffer [AIB (50 mM potassium phosphate, pH 7.2; 3 mM $MgCl_2$)]. Concentrated cells (2–5 mL) were transferred to aluminum foil-covered, butyl rubber septa-sealed, 13-mL glass serum vials and purged vigorously with UHP Ar for 30 min to establish anoxia. Following purging, the cells were incubated with no agitation at room temperature (25 °C) for the times indicated. All transfers of cells, solutions, and gases were performed using UHP Ar-flushed, gas-tight syringes (Hamilton Company, Reno, NV, USA).

2.8. H_2 photoproduction

Maximal in vivo H_2-photoproduction rates were determined using a Pt–Ag/AgCl polarographic electrode system (ALGI, Golden, CO, USA) equipped with a temperature-controlled, water-jacketed 1-mL glass cell and a commercially-available YSI 5331 electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) poised at +0.6 V. One hundred microliters of 10-fold concentrated, anaerobically-acclimated cells were diluted in 900 μL of UHP Ar-purged, 50 mM 3-morpholinopropanesulfonic acid (MOPS), pH 6.9. As previously described [24], a 30 s dark: 90 s light: 30 s dark regime using saturating LED (Luxeon III Star, Lumileds, San Jose, CA, USA) irradiance of 2000 μmol photon m^{-2} s^{-1} PAR was used to determine maximal H_2-photoproduction rates. The electrode was calibrated before each measurement using a 50 mM MOPS solution purged with UHP 5% H_2 (Ar balance) and then UHP Ar to determine the H_2 response and baseline, respectively.

2.9. In vitro hydrogenase activity

MV assays for hydrogenase activity were performed at 4 and 24 h post-Ar-purging by transferring 100 μL of the anaerobically-adapted cells to a 1.2 mL solution of Ar-purged sodium dithionite-reduced MV [1 mL of MV solution (10 mM MV; 50 mM potassium phosphate, pH 6.9; 0.2% Triton X-100) plus 200 μL of freshly-prepared 100 mM sodium dithionite in 30 mM NaOH (all obtained from Sigma–Aldrich)] in sealed, Ar-purged, 13-mL vials. Reactions were incubated at 37 °C in a shaking (160 cycles min^{-1}) water bath (Boekel Grant ORS200) for 2–10 min before 100 μL of headspace gas was assayed by gas chromatography as previously described [5].

2.10. Dark hydrogenase activity

Following periods of dark, anaerobic acclimation, 0.2 mL of headspace gas was removed using an UHP Ar-purged Hamilton

gas-tight syringe (Hamilton Company) and analyzed by gas chromatography.

3. Results

Fig. 2A shows in vitro methyl viologen mediated hydrogenase activity for each of the mutants and the WT control. These data provide direct insights into the levels of active enzyme present. Low levels of in vitro activity (<25%) were observed in strains containing only HYDA2, while activities similar to that of WT were catalyzed in the mutant with only HYDA1. Only 0.1–0.5% of maximal WT hydrogenase activity (Fig. 2A–C) was observed in the *hydA1-1 hydA2-1* mutant, activity that is not observed in either the *hydEF-1* [18] or *hydG-1* mutants [24], which are devoid of the required hydrogenase maturation enzymes. The remaining activity may be the consequence of low levels of HYDA1, as the *HYDA1* gene contains an insertion that is confined to an intron. Hydrogenase activity can be restored to the levels observed in the single mutant strains if either *HYDA1* or *HYDA2* are expressed ectopically in the double mutant, as shown in Fig. 3.

As shown in Fig. 2B, each HYDA isoform contributes to H_2 photoproduction at levels that paralleled in vitro activity measurements (Fig. 2A). While HYDA2 clearly contributes to H_2 photoproduction, WT levels of production, or greater, are only observed in double mutant strains rescued with the *HYDA1* gene (Fig. 3); these rates of production are never attained in strains that only harbor *HYDA2*. Despite the higher than WT level of in vitro activities observed in the double mutant rescued by introduction of *HYDA1* (Fig. 3), these strains show maximal in vivo H_2-photoproduction rates that are similar to the WT, support for the conclusion that the hydrogenase enzyme level may not be the primary factor limiting in vivo H_2 photoproduction [25].

Total dark, anaerobic H_2 production in the hydrogenase mutants did not parallel the patterns of in vitro activity (compare Fig. 2C with A). Instead, the levels of dark, fermentative H_2 that were produced by both *hydA1-1* and *hydA2-1* were similar to that of WT, indicating that the very low levels of H_2 generated by dark, anaerobic Chlamydomonas cells can be catalyzed by the native activity of either hydrogenase paralog. Furthermore, the natural fermentative H_2 flux is sufficiently low such that even in the *hydA1-1 hydA2-1* mutant approximately 10% of WT dark H_2 production is observed (Fig. 2C). Conversely, the *hydA1-1 hydA2-1*::*HYDA1* rescued strain (Fig. 3) showed enhanced fermentative H_2 production; however, the production levels were still low with respect to the molar levels of the primary fermentative products (formate, acetate, and ethanol) [5]. Similarly, the low WT levels of dark, fermentative H_2 production could be restored by *HYDA2* expression in the *hydA1-1 hydA2-1* mutant alone (Fig. 3). Thus, when expressed using its native promoter, HYDA2 is insufficient to drive maximal H_2 photoproduction; however, it is sufficient to oxidize all of the reductant trafficked to H_2 production during dark fermentation.

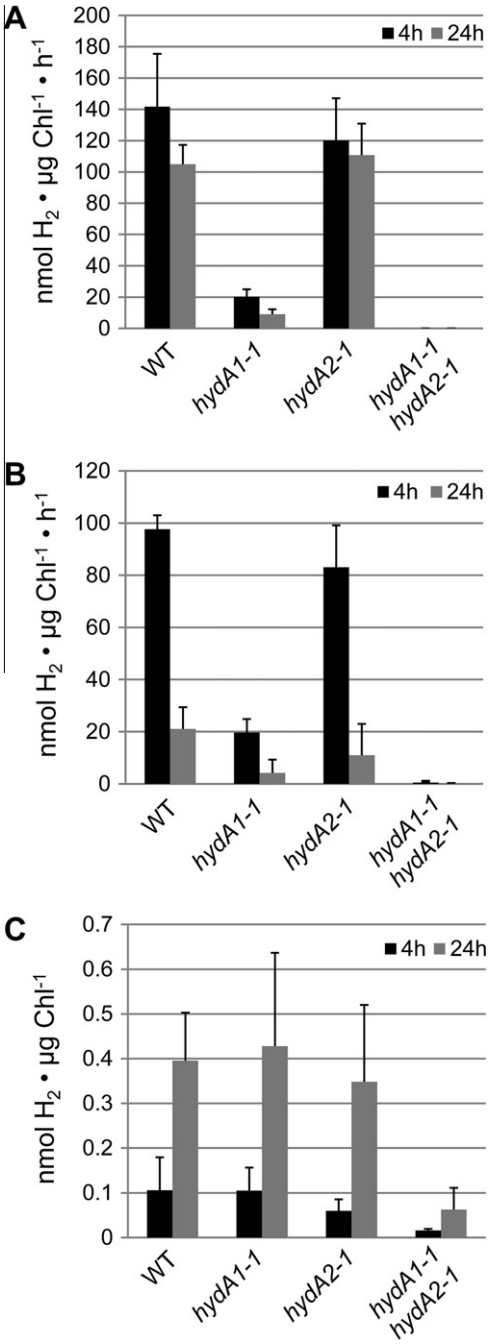

Fig. 2. Hydrogenase activities in the WT, single hydrogenase mutants (*hydA1-1* and *hydA2-1*), and the *hydA1-1 hydA2-1* mutant following 4 and 24 h of anoxia. (A) MV-mediated, in vitro HYDA activities. (B) Maximal initial H_2 photoproduction rates. (C) Total dark, anaerobic H_2 accumulation.

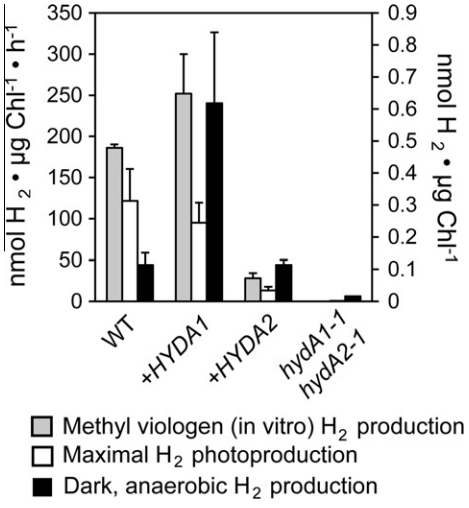

Fig. 3. Hydrogenase activities of WT cells, the *hydA1-1 hydA2-1* mutant and the double mutant complemented with HYDA1 and HYDA2 following 4 h of anaerobiosis. Dark, anaerobic production activities (black columns) are reported on the right axis, with MV-activity (gray columns) and maximum H_2 photoproduction (white columns) on the left axis.

4. Discussion

While an efficient biological system that directly converts solar flux to H_2 is theoretically feasible, it may not exist in nature because there may be little selective pressure favoring such a pathway. While the high maximal H_2-photoproduction rates observed in Chlamydomonas may be related to characteristics of the truncated hydrogenases that are specifically present in Chlorophycean algae, the extreme O_2 sensitivity of these small (~48 kD) hydrogenases is likely a function of these enzymes that is necessary to prevent the wasteful loss of cellular reductant. Although the occurrence of algal H_2 photoproduction suggests the possibility of exploiting such a system for energy production, the high initial rates of H_2 synthesis are not sustained because of O_2 inhibition and competition among diverse metabolic pathways, including photosynthetic carbon fixation. Towards the goal of generating a system for sustainable H_2 production, our work provides a genetic background suitable for approaches that utilize heterologous expression of non-native and engineered hydrogenases in a Chlamydomonas strain free of the complications associated with the presence of endogenous hydrogenase activity.

The principal aim of the present study was to generate and characterize null mutants in each *HYDA* gene of Chlamydomonas in order to evaluate the relative contributions of HYDA1 and HYDA2 in Chlamydomonas H_2 production. The function of HYDA2 in Chlamydomonas H_2 metabolism has been enigmatic, with no clear physiological role revealed by either amiRNA analysis [10], or biochemical approaches [26]. Although *HYDA2* transcript levels accumulate over 50-fold in response to anoxia (~20% of the increase observed for the HYDA1 transcript) [27], its contribution and role(s) in Chlamydomonas H_2 production was not known prior to this analysis. This study is the first to unequivocally demonstrate that HYDA2 accepts reductant from dark fermentation and the photosynthetic electron transport chain, however; only native HYDA1 activity can sustain maximal rates of H_2 photoproduction (Fig. 2B).

Our results demonstrate that HYDA1 and HYDA2 can independently catalyze both light and dark H_2 production. The HYDA1 enzyme can sustain near WT levels of H_2 photoproduction and, when inserted in the double mutant background, can allow for elevated H_2 synthesis relative to WT cells under dark anaerobic conditions; this latter finding suggests that elevated HYDA1 levels allow the enzyme to more effectively compete for reductant during dark fermentation. Interestingly, while the transgenic insertion of the native *HYDA1* gene resulted in increased in vitro activities (Fig. 3), in vivo H_2-photoproduction levels were not enhanced above that of the WT control, an indication that WT HYDA1 level/activity in Chlamydomonas may be natively matched to meet maximal reductant flow from the photosynthetic electron transport chain.

In summary, the combined activity data from the *hydA* mutants and complemented strains strongly support two major conclusions: (a) both HYDA enzymes are capable of coupling to fermentative and H_2-photoproduction activities and (b) HYDA1 appears to be the physiologically dominant and more catalytically active H_2-producing enzyme. Additionally, a Chlamydomonas genetic background is now available for interrogating numerous questions related to hydrogenase activities, their interactions with other proteins in the cell, as well as for the development of novel algal systems that exhibit elevated levels of H_2 production.

Acknowledgments

The authors of this work gratefully acknowledge the US AFOSR Grants FA9550-05-1-0365 and FA9550-11-1-0211, NASA Grant NNG05GL52H, NSF Grants 0824469 and 0951094 and the US DOE Office of Science Contract No. DE-AC3699G010337. We also acknowledge technical assistance from Alex Trujillo, Devin Karns, Randor Radakovits and Edward Dempsey.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.002.

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