Flexibility in Anaerobic Metabolism as Revealed in a Mutant of Chlamydomonas reinhardtii Lacking Hydrogenase Activity

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The green alga Chlamydomonas reinhardtii has a network of fermentation pathways that become active when cells acclimate to anoxia. Hydrogenase activity is an important component of this metabolism, and we have compared metabolic and regulatory responses that accompany anaerobiosis in wild-type C. reinhardtii cells and a null mutant strain for the HYDEF gene (hydEF-1 mutant), which encodes an [FeFe] hydrogenase maturation protein. This mutant has no hydrogenase activity and exhibits elevated accumulation of succinate and diminished production of CO₂ relative to the parental strain during dark, anaerobic metabolism. In the absence of hydrogenase activity, increased succinate accumulation suggests that the cells activate alternative pathways for pyruvate metabolism, which contribute to NAD(P)H reoxidation, and continued glycolysis and fermentation in the absence of O₂. Fermentative succinate production potentially proceeds via the formation of malate, and increases in the abundance of mRNAs encoding two malate-forming enzymes, pyruvate carboxylase and malic enzyme, are observed in the mutant relative to the parental strain following transfer of cells from oxic to anoxic conditions. Although C. reinhardtii has a single gene encoding pyruvate carboxylase, it has six genes encoding putative malic enzymes. Only one of the malic enzyme genes, MME4, shows a dramatic increase in expression (mRNA abundance) in the hydEF-1 mutant during anaerobiosis. Furthermore, there are marked increases in transcripts encoding fumarase and fumarate reductase, enzymes putatively required to convert malate to succinate. These results illustrate the marked metabolic flexibility of C. reinhardtii and contribute to the development of an informed model of anaerobic metabolism in this and potentially other algae.

Chlamydomonas reinhardtii is a unicellular, soil-dwelling, photosynthetic green alga that has a diversity of fermentation pathways, inferred from the full genome sequence (1, 2). It uses these pathways for ATP production during anoxia, catabolizing starch and other intracellular carbon substrates into the predominant fermentation products formate, acetate, ethanol, CO₂, and molecular hydrogen (H₂) in what is classified as heterofermentation (2–7). These metabolic pathways would be active primarily at night when high rates of respiration and the absence of photosynthetic O₂ evolution cause the rapid establishment of anoxia (8), especially in soil environments with high concentrations of microbes. Moreover, C. reinhardtii can balance the use of the tricarboxylic acid cycle with fermentation when the rate of respiratory O₂ consumption exceeds the rate of photosynthetic O₂ evolution (3, 4, 9). The catabolism of intracellular carbon stores during anoxic acclimation is a key component of C. reinhardtii metabolism as this alga does not appear to effectively assimilate extracellular sugars. Acquiring a better understanding of cellular metabolisms in algae such as C. reinhardtii under various conditions will facilitate the development of physiological models that predict metabolic circuits and the interactions among these circuits. Additionally, the secretion of fermentative metabolites by C. reinhardtii (and other algae) likely has significant impacts on the population dynamics of microbial consortia in environments inhabited by C. reinhardtii. There is also the potential to leverage the unique metabolic flexibility of C. reinhardtii for the production of valuable metabolites such as H₂, organic acids, and ethanol as renewable bioenergy carriers (4, 10, 11).

Although studies of C. reinhardtii metabolism have already significantly advanced our understanding of cellular responses to anoxia (5–7, 12), additional research efforts are required to gain further insights into the proteins involved in adaptation and acclimation of the cells to anaerobiosis, the modulation of cellular metabolite levels under these conditions, and the accurate localization of fermentation pathways and proteins to specific subcellular compartments. The availability of the C. reinhardtii genome sequence, combined with high throughput "omics"-based approaches, will be critical in this effort. Moreover, the use of specific mutant strains can help establish the foundation for a more comprehensive understanding of how cells adjust metabolite fluxes when specific reactions are blocked.

A number of studies have demonstrated that C. reinhardtii can rapidly acclimate to anaerobiosis by shifting to fermentative metabolisms (5–7, 12–14). The catabolism of pyruvate to acetyl-CoA in C. reinhardtii may proceed via fermentation pathways that use either PFL1 (pyruvate formate-lyase) (5, 6, 12, 15) or PFR1 (pyruvate ferredoxin oxido-reductase) (5, 6, 12).
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The acetyl-CoA is further metabolized to ethanol and/or acetate, and the reduced ferredoxin generated by PFR1 activity is putatively oxidized by a number of redox proteins, including one or both of the two \textit{C. reinhardtii} [FeFe] hydrogenases (HYDA1 and HYDA2) (16, 17), which are localized to the chloroplast stroma (18, 19). A recently isolated \textit{C. reinhardtii} mutant, \textit{hydEF-1}, is devoid of hydrogenase activity (20, 21) because of disruption of an [FeFe] hydrogenase maturase, which is required for proper enzyme assembly (20–24); consequently, neither of the hydrogenases are active in the mutant strain.

In this study we use molecular and physiological approaches to examine dark, anoxic acclimation of the \textit{C. reinhardtii} \textit{hydEF-1} mutant. Interestingly, the mutant secretes much higher levels of succinate during anoxia than the parental strain. Furthermore, transcripts encoding PYC1 (pyruvate carboxylase) and MME4 (a malic enzyme) increase significantly in the mutant relative to the parental strain during anaerobiosis. Both of these proteins have the ability to carboxylate pyruvate, providing precursors for the fermentative production of succinate. Additionally, transcripts encoding fumarase (FUM1 and FUM2) and FMR1 (fumarate reductase), enzymes that catalyze the final steps of succinate synthesis, are elevated in the \textit{hydEF-1} strain. Utilization of these pathways for succinate synthesis would result in NADH reoxidation, which would sustain additional cycles of glycolysis and explain both the relative increase in succinate and decrease in CO₂ production during anaerobiosis in the mutant relative to the parental strain.

Microarray studies were also performed and reveal that several transcripts encoding proteins associated with cellular redox functions and other aspects of anaerobic metabolism are differentially regulated in the mutant relative to the parental strain. These findings are discussed with respect to the identification of potential new fermentation pathways and the flexibility of whole-cell metabolism in \textit{C. reinhardtii} under dynamic environmental conditions.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—\textit{C. reinhardtii} CC-425 (cw15, sr- u- 60, arg7-8, mt"") and \textit{hydEF-1} mutant (derived from CC-425) cells were grown in Tris/acetate/phosphate medium (TAP)² (pH 7.2), supplemented with 200 mg·liter⁻¹ arginine for CC-425. Algal cultures were maintained at 25 °C, vigorously bubbled with air enriched with 3% CO₂, stirred using a magnetic stir bar, and illuminated with continuous light of 80 μmol photon m⁻² s⁻¹ photosynthetically active radiation at the surface of 1-liter Roux culture bottles (255 × 55 × 120 mm), in which cell densities ranged from 1 × 10⁸ to 3 × 10⁹ cells/ml of culture.

**Chlorophyll Measurements**—Chlorophyll \textit{a} and \textit{b} content were determined spectrophotometrically in 95% ethanol (25).

²The abbreviations used are: TAP, Tris/acetate/phosphate medium; qPCR, quantified by reverse transcriptase real time PCR; HPLC, high pressure liquid chromatography; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PCK, phosphoenolpyruvate carboxylase; MDH, malate dehydrogenase; FUM, fumarase; FMR, fumarate reductase; MME, malic enzyme.

Anaerobic Induction of Liquid Cell Suspensions—\textit{C. reinhardtii} cultures were grown on TAP medium to ~16–24 μg·ml⁻¹ total chlorophyll, centrifuged (500 ml of cells) at 3,000 × g for 1 min, and the cell pellet resuspended in one-tenth volume (50 ml) of anaerobic induction buffer containing 50 mM potassium phosphate (pH 7.2) and 3 mM MgCl₂ (26). The cells were then transferred to a sealed anaerobic vial in the dark and flushed with argon for 30 min. For measuring H₂ and O₂ production rates, 200 μl of cell cultures were placed in a Clark-type oxygen electrode assay chamber as described previously (6, 20). Clark-type oxygen electrodes were used simultaneously to measure light-induced H₂ and O₂ production rates. To measure fermentative H₂ production, 400 μl of head space gas was withdrawn from the sealed anaerobic vials and analyzed by gas chromatography (Hewlett Packard 5890 series II) using a Supelco column (60/80 mol sieve 5Å 6 feet × ⅛ inch) coupled to a TCD detector. H₂ remaining in solution was quantified using the Clark-type oxygen electrode described above without illumination of the sample chamber.

**CO₂ Measurement**—CO₂ levels were below detection limits in the serum vial head space of anaerobically acclimated cells. Therefore, following anaerobic induction, 1 ml of anoxic cells was transferred in a gas-tight syringe to a sealed vial into which 1 ml of 1 M HCl was added. The acidified cell suspension was vigorously shaken to liberate CO₂, which was quantified by gas chromatography (Hewlett Packard 5890 series II) using a Supelco column (80/100 PORAPAK N 6 feet × ⅛ inch × 2.1 mm) coupled to a TCD detector.

**Metabolite Analysis**—Organic acid analysis was performed by liquid chromatography (Hewlett Packard Series 1050 HPLC) using an Aminex HPX-87H (300 × 7.8 mm) ion exchange column. Dark-adapted cells were collected at the indicated time points and centrifuged (10,000 × g for 1 min), and the supernatant was transferred to a new vial and frozen in liquid N₂ for subsequent analysis. Samples were thawed, centrifuged, and filtered prior to HPLC analysis. One hundred μl of cell culture supernatant was injected onto the column and eluted using 8 mM filtered sulfuric acid (J. T. Baker, Inc.) as the mobile phase at a flow rate of 0.5 ml·min⁻¹ at 45 °C. Retention peaks were recorded using Agilent ChemStation software, and quantifications were performed by comparisons with known amounts of standard for each of the organic acids.

Ethanol was measured using a YSI 2700 SELECT electrochemical probe (YSI Inc., Yellow Springs, OH). Identical supernatants were used for metabolite and ethanol analysis; 10 μl of the supernatant was required for ethanol analysis.

**Extraction of RNA**—Total RNA was isolated using the Plant RNA reagent, as described by the manufacturer (Invitrogen). Approximately 40 μg of isolated RNA was treated with 5 units of RNase-free DNase (Ambion, Austin, TX) for 30 min at room temperature. The Qiagen RNaseasy MinElute kit (Qiagen, Valencia, CA) was used to purify total RNA. The A₂₆₀ of the eluted RNA was measured, and 4 μg of purified RNA was reserved to prepare labeled samples for microarray analysis.

**Reverse Transcriptase Real Time PCR (qPCR)**—The abundance of specific transcripts in total mRNA from each sample was quantified by reverse transcriptase real time PCR, designated qPCR, using the Engine Opticon system (Bio-Rad). First
strand cDNA synthesis was primed on purified total RNA using specific primers for each C. reinhardtii gene of interest. The specific primers (at 0.5 μM each; supplied by IDT) were annealed to 250 ng of RNA-free total RNA and extended for 1 h at 55 °C using 200 units of the reverse transcriptase Superscript III (Invitrogen). Four μl of the single-stranded cDNA from the reverse transcriptase reaction (final volume, 20 μl) was used as the template for real-time PCR amplifications, which were performed using the DyNAmo HS SYBR green reverse transcription-PCR kit according to the manufacturer’s instructions (Finzynies, Woburn, MA). Specific primers (0.3 μM) used for amplification were designed to generate amplicons of 100–200 nucleotides. Amplifications were performed using a Bio-Rad iCycler iQ detection system and the following cycling parameters: an initial single step at 95 °C for 10 min (denaturation) followed by 40 cycles of (a) 94 °C for 30 s (denaturation), (b) 56 °C for 45 s (annealing), and (c) 72 °C for 30 s (elongation). Following the 40 cycles, a final elongation/termination step was performed at 72 °C for 10 min. Primers used for qPCR are shown in supplemental Table 1, and were designed using Primer3 software (available online). The relative expression ratio of a target gene was calculated based on the 2^−ΔΔCT method (27), using the average cycle threshold (Ct) calculated from triplicate measurements. Relative expression ratios from three independent experiments (different experimental replicates) are reported. The level of accumulation of the RACK1 transcript was used as a normalization control because it shows near constant expression under the conditions used in these experiments.

Microarray Fabrication—Microarrays were fabricated at the Stanford Functional Genomics Facility at Stanford University, as described previously (6).

Labeling and Purification of Reverse-transcribed cDNAs—Labeling and purification of reverse-transcribed cDNAs were performed as described previously (28). Briefly, 4 μg of purified cDNA was adjusted to 4 μl with sterile MilliQ-treated water. One μl of oligo(dT)-(V) (2 μg·μl⁻¹) was heated for 10 min at 70 °C and quickly chilled on ice. The following reagents were added sequentially to the reaction mixture: 2 μl of 5× superscript buffer; 1 μl of 0.1 mM dithiothreitol; 0.2 μl of 50× dNTPs (5 mM dATP, dCTP, and dGTP and 10 mM dTTP); 1 μl of Cy3- or Cy5-dUTP; and 0.8 μl of Superscript III (200 units·μl⁻¹). The final reaction volume was 10 μl. After allowing the reaction to proceed at 42 °C for 2 h, an additional aliquot of 0.5 μl of Superscript III was added, and the reaction was continued for an additional 1 h at 50 °C. The reaction was stopped by the addition of 0.5 μl of 500 mM EDTA and 0.5 μl of 500 mM NaOH, and the solution was incubated at 70 °C for 10 min. Neutralization of the reaction mixture was achieved by adding 0.5 μl of 500 mM HCl. The QIAquick PCR purification kit (Qiagen, Valencia, CA) was used to purify the labeled cDNA as described previously (6).

Hybridization to the Oligonucleotide Array—Pre-hybridization and hybridization of fluorescent-labeled probes and washing of the arrays were as described previously (6, 28). Detailed and updated versions of the protocols used for RNA labeling, slide pre-hybridization, hybridization, and washing can be downloaded from the Chlamydomonas Center website.

### RESULTS

Metabolite Analyses—The C. reinhardtii hydEF-1 mutant lacks hydrogenase activity because of disruption of the HYDEF gene, which encodes a hydrogenase mature (20). This mutant was used to investigate the physiological responses of C. reinhardtii in the absence of H₂ metabolism during anaerobic acclimation. The fermentative products that accumulated in the medium during anoxia in both cultures mutant and parental control are shown in Table 1. Four significant differences observed between mutant and parental cells were as follows: (a) the absence of H₂ production in the mutant relative to the parental strain; (b) a marked difference in accumulation of CO₂ between the two strains; (c) a slight reduction in the rate of accumulation of formate, acetate, and ethanol in the mutant relative to the parental strain; and (d) elevated production of succinate, only in the mutant. Rescue of the hydEF-1 mutant phenotype and restoration of the fermentation profile (along with H₂ and CO₂ production) observed in the parental strain was achieved by introduction of a functional copy of the HYDEF gene (data not shown). The complemented strain was not used as a reference in the microarray experiments as integration of

| Time | CO₂ | H₂ | Formic acid | Succinic acid | Acetic acid | Ethanol |
|------|-----|----|-------------|---------------|-------------|--------|
| h    | mmol/liter |     |             |               |             |        |
| 0.5  | 0.05 ± 0.09 | 0.02 ± 0.01 | 0.5 ± 0.2 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.2 ± 0.2 |
| 2    | 0.14 ± 0.02 | 0.03 ± 0.01 | 1.3 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.7 ± 0.1 |
| 4    | 0.64 ± 0.08 | 0.06 ± 0.01 | 2.2 ± 0.1 | 0.1 ± 0.1 | 1.8 ± 0.3 | 1.1 ± 0.2 |
| 24   | ND²  | ND  | 4.8 ± 0.2 | 0.1 ± 0.1 | 4.3 ± 0.3 | 1.9 ± 0.1 |

² BD means below detection.
³ ND means not determined.
the introduced HYDEF gene into the genome could disrupt another cellular function.

Succinate is not detected in significant amounts in the medium of the parental culture but does accumulate in the mutant culture, particularly after longer periods (4 and 24 h) of anaerobiosis. CO2 levels do not increase significantly during acclimation to anaerobiosis in the mutant, whereas in parental cells CO2 levels steadily rise over the acclimation period. To account for succinate production and the near constant CO2 level in the mutant, we identified genes on the draft genome encoding enzymes associated with fermentative succinate production. These encoded enzymes were placed into metabolic pathways (Fig. 1) leading to the fermentative production of succinate, which requires the carboxylation of a three-carbon substrate and is consistent with diminished CO2 accumulation in the mutant relative to the parental strain. The proteins associated with the succinate pathways depicted in Fig. 1 are given in Table 2. Because hydrogenase activity is absent in the mutant, pyruvate flux via the pyruvate ferredoxin oxidoreductase (PFR1) pathway is anticipated to be altered because hydrogenase can no longer participate in PFR1/ferredoxin reoxidation. However, it should be noted that additional metabolic pathways could potentially oxidize ferredoxin, such as sulfite reductases or NAD(P)H-ferredoxin oxidoreductases, and metabolic flux could still proceed via PFR1 provided ferredoxin was reoxidized by alternative pathways. Under our experimental conditions the parental strain accumulates 10-fold more CO2 than H2 (on a molar basis) indicating that other pathways are involved in the process under our experimental conditions (Table 1). If all of the reduced ferredoxin was oxidized by hydrogenase, this stoichiometry (CO2/H2) should be 1:1. Relative to the parental strain, the increased succinate accumulation observed in the mutant would consume both the reducing equivalents and nearly all of the CO2 generated by pyruvate
ferrodoxin oxidoreductase activity. In *C. reinhardtii*, there are several potential pathways that could lead to the production of succinate. These include the following: (a) pyruvate carboxylation by pyruvate carboxylase to yield oxaloacetate; (b) pyruvate carboxylation by malic enzyme (MME) to yield malate; and (c) phosphoenolpyruvate (PEP) carboxylation by either phosphoenolpyruvate carboxykinase (PEPC) or PEP carboxykinase (PCK) to yield oxaloacetate, which can then be reduced to malate by malate dehydrogenase (MDH). The conversion of malate to succinate requires fumarase (FUM) and fumarate reductase (FMR) activities (Fig. 1).

**Gene Expression in the Succinate Pathway**—Pyruvate is the central fermentation metabolite in *Chlamydomonas*, and altered metabolism of pyruvate could lead to succinate synthesis. To explore the possibility that genes associated with pyruvate metabolism are differentially regulated in the hydEF-1 mutant, we used qPCR to monitor the abundance of transcripts encoding enzymes potentially involved in metabolizing pyruvate during anaerobic acclimation of the mutant and parental strain (Fig. 1). As shown in Fig. 2, transcripts encoding PFL1 and PFR1 increase significantly in both the mutant and parental strains following the imposition of anaerobic conditions. However, the PFR1 transcript increases more in the parental strain than in the mutant, whereas the PFL1 transcript increases more in the mutant than in the parental strain. This suggests a potential readjustment of the flux of metabolites through the PFL1 and PFR1 pathways during fermentation metabolism. A slight increase in the ratio of formate produced relative to the other secreted metabolites is observed in the mutant strain; however, the total amount of formate secreted is diminished in the mutant relative to the parental strain (Table 1). This finding may reflect decreased fermentative rates in the mutant or competition from the succinate pathway, which becomes active in the mutant (see below).

Although the transcripts level of encoding PFR1 are reduced in the mutant, flux through this pathway may still be required to generate CO₂ for the carboxylation reactions leading to succinate production. This would require ferrodoxin to be reoxidized by cellular pathways other than the [FeFe] hydrogenases (e.g. formation of NAD(P)H and/or reduction of sulfite). The fermentative metabolite data indicate that succinate production in the mutant attained a level that is 60–70% of the level that would be produced if all of the CO₂ generated during anaerobic acclimation of the parental strain were used for the synthesis of succinate. These data are suggestive of reduced metabolic flux through the pyruvate ferrodoxin oxidoreductase pathway in the mutant; however, pyruvate ferrodoxin oxidoreductase activity (at attenuated levels) may still serve to generate the reducing equivalents and CO₂ used to convert the three-carbon metabolites pyruvate and/or phosphoenolpyruvate to four-carbon fermentation products.

Transcripts encoding PEPC1, PYC1, and MME4 increase significantly only in the hydEF-1 mutant during anaerobiosis (Fig. 2 and supplemental Fig. 1), which suggests that PEPC1 and/or pyruvate carboxylation via PYC1 and/or MME4 may stimulate succinate formation in this strain. Although PFL1, PFR1, PCK1, and PYC1 are encoded by single genes and PEPC by two genes, there are six genes encoding putative malic enzymes. However, only the level of the MME4 transcript increases dramatically (some of the other transcripts increase to a much lesser extent) during anaerobiosis in the mutant strain, with only a small increase in the transcript level in the parental strain (note the differences in the scale for the qPCR results in Fig. 2). These results suggest that MME4 may be predominantly responsible for MME-dependent pyruvate carboxylation during anaerobiosis in the mutant strain. The levels of transcripts encoding PEPC2 and PCK1, enzymes that could also be involved in succinate formation, are not significantly different in the mutant and parental strains (data not shown). Overall, these results suggest that in the absence of active [FeFe] hydrogenases, fermentation metabolites are rerouted away from pyruvate catabolism toward the carboxylation of pyruvate (and perhaps PEP) through PYC1 and MME4 (and perhaps

| Enzyme assigned name | Annotation | Protein ID |
|----------------------|------------|------------|
| FUM1 | Fumarase | 195953 |
| FUM2 | Fumarase | 196655 |
| Fumarate reductase | Fumarate reductase/succinate dehydrogenase flavoprotein | 145357 |
| MDH1 NAD-dependent | Lactate/malate dehydrogenase | 137163 |
| MDH2 NAD-dependent | Malate dehydrogenase | 126023 |
| MDH3 NAD-dependent | Malate dehydrogenase | 158129 |
| MDH4 NAD-dependent | Malate dehydrogenase | 60444 |
| MDH5 NADP-dependent | Malate dehydrogenase | 192083 |
| MME1 NAD-dependent | Malic enzyme | 196833 |
| MME2 NADP-dependent | Malic enzyme | 147722 |
| MME3 NADP-dependent | Malic enzyme | 196832 |
| MME4 NADP-dependent | Malic enzyme | 196831 |
| MME5 NADP-dependent | Malic enzyme | 196351 |
| MME6 NADP-dependent | Malic enzyme | 126820 |
| PEPC1 isoform 1 | Phosphoenolpyruvate carboxylase | 80312 |
| PEPC2 isoform 2 | Phosphoenolpyruvate carboxylase | 182821 |
| PYK1 | Pyruvate carboxylase | 112730 |
| PYK2 | Pyruvate kinase | 156854 |
| PYK3a, b, c one gene | Pyruvate kinase variants a, b, c | 196261, 107530, 122254 |
| PYK4a, b, c one gene | Pyruvate kinase variants a, b, c | 149896, 119280, 04490 |
| PYK5 | Pyruvate kinase | 118203 |
| PYK6-5/ 6-3 incomplete model but same gene | Pyruvate kinase | 196892, 181547 |
PEPC1). Succinate formation is proposed to proceed via the pathways shown in Fig. 1. This is in accord with the findings that transcripts encoding one of the two C. reinhardtii fumarases (FUM1) and the fumarate reductase increase specifically in the mutant but not in the parental strain during anoxia (Fig. 3). Transcripts encoding the MDH enzymes increase to a small extent (particularly MDH2) in response to anaerobiosis, but these increases are similar in the mutant and parental strains (Fig. 3). Together, the qPCR data indicate that the three pathways shown in Fig. 1 for succinate production increase in the hydEF-1 mutant relative to the parental strain during anaerobiosis.

Attempts to demonstrate in vitro succinate formation using cellular extracts of parental and mutant cells were inconclusive. Succinate production was higher in hydEF-1 extracts relative to the parental strain (data not shown) when supplemented with pyruvate, bicarbonate, and NADH. However, the results were not statistically unequivocal, and we are refining the assay to optimize both cell breakage and the specific reaction conditions.

Microarray Analyses—Microarray analyses (28, 29) were performed to examine genome-wide differences between the parental strain and the hydEF-1 mutant during dark, anaerobic acclimation. The previous array data (6) for acclimation of the parental CC-425 strain to dark, anoxic conditions were compared with the current array results obtained for the hydEF-1 mutant. The relative levels of ~10,000 different transcripts (28) were analyzed at various times following exposure of cells to dark, anaerobic conditions. Transcript levels were filtered to capture those for which there was an elevation greater than 1 or a diminution of less than 0.5-fold in relative abundance at 0.5, 2, or 4 h following the imposition of anaerobic conditions. Genes encoding putative fermentative/metabolism proteins with transcripts that accumulated to over 3.0-fold following transfer of mutant cells to anoxic conditions (relative to aerobic control values) are presented in Table 3.

The microarray data (Table 3 and supplemental Table 2) indicate that the majority of metabolic pathways activated during dark, anoxic acclimation in the parental strain (6) are also activated in the mutant. These include transcripts encoding the [FeFe] hydrogenase maturase (HYDG), sulfite reductase, hybrid cluster protein, amylase, and NADH transhydrogenase. Several transcripts encoding enzymes involved in amino acid synthesis and catabolism are also observed to increase, which is consistent with the turnover of proteins involved in aerobic metabolism and the synthesis of proteins required for fermentation metabolism. Increased accumulation of transcripts associated with lipid metabolism was also observed, suggesting that lipid catabolism or reorganization occurs during acclimation to anoxia. Additionally, transcripts encoding fermentative enzymes, such as ACK1 (acetate kinase), PAT2 (phosphate acetyltransferase), pyruvate dehydrogenase kinase, pyruvate decarboxylase, HYDA1, and HYDA2 are observed to increase in both the parental control (6) and the mutant, but only at levels lower than 3-fold in the mutant, and therefore these genes do not appear in Table 3. There are also several transcripts encoding proteins of both known and unknown functions that were observed to increase in the mutant during anoxic acclimation but were not detected in previous microar-
ray analyses of anoxia in the parental strain (6). Included among these are several transcripts encoding components of complex I, sulfate adenylyltransferase, Rieske FeS protein, and cytochrome P450.

A second analysis of the microarray data compared transcript abundance in the \textit{hydEF-1} mutant and the parental strain after 4 h of exposure to anaerobic conditions. Table 4 shows the 15 most up-regulated and 10 most significantly down-regulated transcripts encoding putative proteins associated with defined metabolic processes. Among the most significantly up-regulated genes are those encoding nitrate reductase and the hydrogenase maturase HYDG (20). The increase in HYDG is likely a cellular response that reflects the inability of the cell to make an active hydrogenase enzyme. Alternatively, it may be a consequence of the insertional mutation in the adjacent gene, \textit{HYDEF} (20). Nitrate reduction represents an alternative terminal electron acceptor to proton reduction and may be increased in the mutant during anoxia as a mechanism to oxidize reduced ferredoxin that is generated by PFR1. Likewise, sulfate activation and reduction would be a way of eliminating intracellular reducing equivalents. As shown in Table 4, transcripts encoding sulfate adenylyltransferase, which catalyzes the first step of both the assimilatory and dissimilatory sulfate reduction pathways, accumulates to significantly higher levels in the mutant relative to the parental control. The conversion of sulfite to sulfide is catalyzed by sulfite reductase, whose transcripts increase during anoxia in both the mutant and parental strains. Additional fermentation transcripts encoding proteins associated with NADH oxidation/reduction and ATP synthesis/transport are differentially regulated, which is consistent with hydrogenase activity influencing the relative levels of NADH and ATP during anoxia. Interestingly, components of both mitochondrial complex I and cytochrome \textit{b}_{6}f are differentially up-regulated in the mutant, suggesting that the redox status of these electron transport chains has been altered in the \textit{hydEF-1} mutant.

Several genes, encoding some MMEs, FMR1, and PCK1, were not represented on the array.

**DISCUSSION**

The flexibility of algal metabolism is of interest for developing an informed understanding of the following: (a) metabolite exchange in natural microbial communities; (b) the role of unicellular algae in global carbon cycling; and (c) strategies for engineering phototrophic microorganisms for renewable energy production (11, 30–33). The \textit{C. reinhardtii} genome sequence has provided gene models for populating previously documented metabolic pathways, revealed a diversity of predicted metabolisms, provided insights into the evolution of the green algal lineage, and indicated that parallel processes may occur in multiple cellular compartments (5, 6). An intriguing aspect of algal physiology is the ways in which these organisms acclimate to anoxia (8, 9, 34, 35), which likely occurs as a consequence of microbial respiration at night in environments that are not well oxygenated (e.g. soil environment from which \textit{C. reinhardtii} was originally isolated). Several research efforts are currently focused on developing a more comprehensive understanding of anoxic metabolism in this alga to support future efforts to generate \textit{H}_{2}, organic acids, and/or ethanol. Hydrogenase activity in \textit{C. reinhardtii} has been the focus of extensive research because it cat-
### TABLE 3

Transcripts associated with metabolism/fermentation showing at least a ≥3.0-fold change in abundance in at least one time point following the transfer of *hydEF-1* mutant cells from light, aerobic conditions to dark, anaerobic conditions. Also shown is the identification number of the oligonucleotide, the protein ID, and the associated annotation from the JGI browser. Shaded transcripts are similarly upregulated in the parental strain.

| Oligo ID | Protein ID | Annotation | 0.5h mean fold change | t-test | 2h mean fold change | t-test | 4h mean fold change | t-test |
|----------|------------|------------|-----------------------|-------|---------------------|-------|---------------------|-------|
| 132.A    | 19626      | HYDYG, hydrogenase assembly factor | 3.2 | 1.22E+01 | 9.3 | 1.08E+01 | 8.0 | 7.50E+02 | 0.0 |
| 202.A    | 83066      | P451H, enolase | 2.8 | 3.21E+02 | 2.8 | 2.31E+02 | 3.1 | 3.83E+02 | 0.0 |
| 9407.E   | 19772      | MMO2, NADP-dependent malate dehydrogenase | 2.0 | 1.02E+02 | 4.2 | 1.29E+01 | 10.0 | 2.26E+01 | 0.0 |
| 962.C    | 11814      | Keto-acid reductase | 5.5 | 2.95E+02 | 2.0 | 5.00E+02 | 11.0 | 4.33E+02 | 0.0 |
| 1998.C   | 15764      | DUR, urea carboxylase | 1.6 | 3.36E+01 | 3.1 | 2.09E+01 | 2.1 | 2.26E+01 | 0.0 |
| 1185.C   | 15926      | End of acetyl-CoA carboxylase/biotin deacetylase | 2.1 | 1.03E+02 | 3.4 | 1.41E+02 | 2.7 | 2.87E+02 | 0.0 |
| 9432.E   | 16510      | Lysine deacetylase-like protein | 3.3 | 1.32E+02 | 10.0 | 8.80E+02 | 5.5 | 3.14E+02 | 0.0 |
| 2622.C   | 146556     | Multicopper oxidase, type 1 | 2.8 | 2.31E+01 | 5.5 | 1.91E+01 | 9.0 | 3.61E+01 | 0.0 |
| 6074.E   | 19233      | Sulfite reductase | 3.9 | 1.96E+01 | 4.2 | 7.80E+01 | 3.3 | 2.17E+01 | 0.0 |
| 144.A    | 184661     | NIT1, nitrate reductase | 4.2 | 2.40E+01 | 6.2 | 2.32E+01 | 4.5 | 2.68E+01 | 0.0 |
| 468.A    | 154650     | ATS1, sulfate adenyl transferase | 2.9 | 7.01E-01 | 2.3 | 1.12E+00 | 4.7 | 2.72E+00 | 0.0 |
| 1054.C   | 191051     | PAT2, phosphate acetyl transferase | 3.6 | 2.03E+01 | 2.2 | 1.87E+01 | 2.0 | 3.12E+00 | 0.0 |
| 9835.E   | 180155     | HSD1, putative protein | 4.5 | 2.01E+01 | 2.0 | 3.05E+01 | 3.3 | 3.85E+01 | 0.0 |
| 9552.E   | 157360     | Esterase/lipase | 2.5 | 1.68E+01 | 2.7 | 2.73E+01 | 4.0 | 3.13E+00 | 0.0 |
| 1265.C   | 129982     | ACK1, acetyl kinase | 2.2 | 1.37E+01 | 4.2 | 1.52E+01 | 4.9 | 1.03E+01 | 0.0 |
| 9744.E   | 154478     | Glutamate 5-kinase | 4.4 | 8.97E+01 | 5.7 | 1.25E+02 | 5.2 | 2.43E+01 | 0.0 |
| 615.C    | 109930     | Tyrosine-protein kinase | 4.0 | 2.41E+01 | 4.0 | 2.41E+01 | 7.1 | 2.64E+00 | 0.0 |
| 192.A    | 196944     | VPS34, Phosphatidylinositol 3-kinase | 2.5 | 3.19E+01 | 3.2 | 1.09E+01 | 3.6 | 3.52E+00 | 0.0 |
| 1209.C   | 196261     | PK3, Pyruvate kinase | 3.0 | 1.37E+01 | 4.7 | 1.03E+01 | 3.0 | 4.88E-01 | 0.0 |
| 4361.E   | 196383     | PK2, phosphoglycerate kinase | 4.1 | 1.81E+01 | 4.2 | 2.11E+01 | 4.4 | 1.40E+00 | 0.0 |
| 31.A     | 129128     | RPI1, ribose non-sugar protein, ubiquinol-cytochrome c reductase | 2.5 | 1.06E+01 | 4.6 | 1.34E+01 | 2.2 | 2.04E+00 | 0.0 |

* a Identification number of the oligonucleotide sequence used to make the array element is shown.  
* b Identification number of protein generated from the corresponding gene model (JGI *C. reinhardtii* version 3.0) is shown.  
* c Change in transcript abundance at 0.5, 2, and 4 h after transferring cells to dark, anaerobic conditions relative to time 0 (just prior to transfer) is shown.  
* d Average of three experimental replicates for time point 0.5 h, and four experimental replicates for time points 2 and 4 h. Two technical repetitions were done for each experimental repetition (each slide contained each array element in duplicate).  
* e Student’s *t*-test is shown.
analyzes the production of the renewable energy carrier H₂ (4, 33, 36–41). To further study the role of hydrogenases in algal physiology and to define the metabolic adjustments induced by the loss of hydrogenase activity, physiological responses observed during anoxic acclimation of a C. reinhardtii mutant lacking hydrogenase activity were examined relative to the parental strain. Interestingly, rather than causing an increased metabolic flux into alternative pathways operating at significant levels in the parental strain, activation of new metabolic pathways leading to succinate accumulation were observed. The ability of C. reinhardtii to switch to a pathway that is minimally active in the parental strain continues to demonstrate the metabolic versatility of this alga and its ability to alter its metabolism in response to environmental change.

### Succinate Accumulation

The increased production of succinate, the absence of H₂ production, and the decreased evolution of CO₂ were the most significant metabolic differences observed in the hydEF-1 mutant relative to the parental strain during dark, anoxic acclimation. In the mutant, decreased CO₂ evolution and increased abundance of transcripts encoding proteins able to carboxylate pyruvate, congruent with succinate production, suggest that in the absence of hydrogenase activity, pyruvate is carboxylated to generate either malate and/or oxaloacetate. These metabolites are putatively converted into succinate as depicted in Fig. 1. Both malic enzyme and pyruvate carboxylase use CO₂ as a substrate to carboxylate pyruvate, and the activity of either or both may be responsible for the nearly steady state levels of CO₂ observed in the mutant. Transcripts encoding both of these enzymes increase significantly in the mutant strain during anoxic acclimation.

### Table 4

The 15 most highly up-regulated and 10 most significantly down-regulated transcripts associated with metabolism in the hydEF-1 mutant relative to the CC-425 parental strain after 4 h of dark, anaerobic acclimation

| Oligo ID | Protein ID | Annotation                                                                 | Fold change in transcript abundance |
|----------|------------|-----------------------------------------------------------------------------|-------------------------------------|
| 216.A    | 59411      | NUDS5, NADH:ubiquinone oxidoreductase subunit                               | 34                                  |
| 163.A    | 76602      | ATP1, mitochondrial F1F0 ATP synthase α subunit                              | 21                                  |
| 9165.E   | 158121     | Putative mitochondria malonyl-CoA deacetylase                                | 18                                  |
| 92026.E  | 135699     | Aldehyde dehydrogenase                                                       | 15                                  |
| 468.A    | 183942     | ATSL, sulfate adenylyltransferase                                            | 14                                  |
| 94111.F  | 743        | Tetrahydrofolate, dehydrogenase cyclohydroxylase                            | 13                                  |
| 404.A    | 1851971    | PETF, cytochrome B6-F complex subunit O                                     | 13                                  |
| 202.A    | 83064      | PGH1 enolase                                                                | 13                                  |
| 5154.C   | 188027     | ADP/ATP carrier protein                                                      | 13                                  |
| 226.A    | 168551     | MAS, malate synthase                                                        | 13                                  |
| 3762.C*  | 112511     | AMYB2, β-amylase                                                            | 13                                  |
| 358.A    | 184661     | NIT1, nitrate reductase                                                      | 13                                  |
| 2622.C   | 146556     | Multicopper oxidase type 1                                                  | 13                                  |
| 132.A    | 196226     | HYDG, [FeFe]-hydrogenase maturase                                            | 13                                  |
| 6449.C   | 186369     | PIS1, phosphatidylinositol synthase                                          | 13                                  |

| Identification number of the oligonucleotide sequence used to make the array element is shown. |
| Identification number of protein generated from the corresponding gene model (JGI C. reinhardtii version 3.0) is shown. |
| Change in transcript abundance at 4 h after transferring cells to dark, anaerobic conditions relative to time 0 h (just prior to transfer) is shown. |
| Average of three experimental repetitions (two technical repetitions for each experimental repetition) is shown. |
| Student’s t test is shown. |
| Genes differentially regulated in the hydEF-1 mutant versus CC-425 in standard culture conditions (TAP, light, aerobic conditions); 428.A (0 h, 5.21; t test, 2.79E-02); 3762.C (0 h, 7.45; t test, 5.52E-02) are shown. |
have been reported (42–44). Overexpression of the gene (scfA) encoding the malic enzyme in *Escherichia coli* leads to succinate accumulation in a mutant background with elevated pyruvate levels resulting from disruptions of the genes encoding both lactate dehydrogenase and PFL (43, 44). Of the *C. reinhardtii* malic enzymes, ScfA is most similar to MME4.

Pyruvate carboxylation can also proceed via pyruvate carboxylase to form oxaloacetate, and transcripts encoding PYC1 increase during anoxia in the mutant. However, the transcripts of five putative MDH enzymes, required for the conversion of oxaloacetate into malate and subsequently succinate, do not increase dramatically in the mutant relative to the parental strain. Furthermore, the level of the PYC1 transcript increases ~20-fold, whereas the MME4 transcript increases in abundance by over 500-fold in the mutant. Based on the qPCR *Ct* values for the six MME genes, MME4 transcripts are least abundant under oxic conditions in both the parental strain and the *hydEF-1* mutant. The relatively strong induction of MME4 compared with PYC1 and MDH transcripts suggests that MME4 may be responsible for the majority of increased metabolic flux toward succinate production. An alternative pathway to succinate via PEPC1 may be modestly activated, based on transcript levels. In addition to PEPC, the conversion of phosphoenolpyruvate to oxaloacetate could also potentially proceed via PCK (43); however, this pathway does not appear to contribute to fermentative succinate formation in *E. coli* (45), and we do not observe significant differential regulation of the PCK1 transcript in *C. reinhardtii* (data not shown). Transcripts encoding fumarase and fumarate reductase, required for the conversion of malate to succinate, increase significantly only in the mutant during anoxic acclimation, which is consistent with CO2 production coming from pyruvate metabolism. Secretion of medium-derived acetate by cells at the onset of anaerobiosis is consistent with high levels of this organic acid at the 0.5-h time point (Table 1) relative to other fermentative metabolites. If the majority of acetate in the medium at 0.5 h is not derived from starch degradation, then the fermentation stoichiometry more closely approximates the formate/acetate/ethanol stoichiometry of 2:1:1 reported by others (7, 13). However, even if we adjust our calculations and assume that most of the acetate at 0.5 h is medium-derived, the sum of acetate and ethanol still remains slightly in excess of the detected levels of formate. This finding is consistent with CO2 production coming from pyruvate decarboxylation which, if mediated by PFR1, could contribute to the production of fermentative H2 in the parental strain.

The pathways leading to succinate production require CO2 input, which likely originates from the following: (a) PFR1; (b) PDC; (c) pyruvate dehydrogenase during anoxic acclimation when cells are metabolizing residual O2; or (d) reserves of bicarbonate that accumulate within cells during aerobic culturing. It is likely that PFR1 is still active to an extent and that CO2 produced by this pathway is used to carboxylate pyruvate or PEP. Ferredoxin could then potentially be used to generate NAD(P)H for succinate production. Together, the data suggest that hydrogenase activity does not appear to be exclusively responsible for ferredoxin oxidation in the parental strain, its absence in cells that have acclimated to dark, anoxic conditions causes the activation of metabolic pathways leading to succinate synthesis. As the amount of succinate produced in the mutant exceeds the H2 produced by the parental strain by 5–10-fold, the rerouting of metabolites does not simply compensate at a stoichiometric level for the loss of the electron valve activity of the hydrogenase. Regulatory processes must become
active that generate a new cellular homeostasis. The regulators that control this process have not been identified, although it is conceivable that the loss of hydrogenase activity results in increased cellular reductant that signals changes in gene expression via reduced NAD(P) or redox sensors such as thioredoxins (47, 48).

Transcriptome—The microarray data are consistent with the development of fermentation metabolism during dark anaerobiosis in both the mutant and parental strains. Transcripts encoding the hydrogenase maturation factor HYDG, and starch and pyruvate-catabolizing enzymes, including amylases, acetate kinase, pyruvate kinase, and phosphate acetyltransferase, all increase in the mutant and parental strains. Moreover, transcripts for pyruvate dehydrogenase kinase, which phosphorylates and inhibits pyruvate dehydrogenase, also increases in the mutant relative to the parental strain. The pyruvate dehydrogenase complex oxidizes pyruvate-forming NADH, acetyl-CoA, and CO₂ during aerobic metabolism, and inhibition of pyruvate dehydrogenase redirects pyruvate metabolism into fermentation pathways.

Dark anaerobiosis also elicits an increase in the abundance of several transcripts encoding enzymes involved in amino acid catabolism or synthesis, such as lysine decarboxylase, proline dehydrogenase, glutamate kinase, threonine synthase, cystathionine β-lyase, and asparagine synthase. This is likely a function of proteome reorganization as enzymes used in aerobic metabolism are degraded, and fermentation enzymes are synthesized. Transcripts also increase for cysteine desulfurase, an enzyme that mobilizes sulfide for the formation of FeS clusters that would be required by several fermentative FeS proteins, including ADH1, PFLA1, and PFR1.

The microarray data also indicate that dark anaerobiosis leads to increases in transcripts encoding subunits of the mitochondrial NADH-oxidizing complex I, including NIOAO8, which appears to be the most differentially up-regulated gene in the mutant relative to the parental strain after 4 h of anoxic acclimation. Other significant differences between the mutant and the parental strain include increases in levels of transcripts in the mutant for nitrate reductase (NIT1) and the sulfate adenosyltransferase. ATS1 activates sulfate for subsequent reduction. Transcripts also increase for cysteine desulfurase, an enzyme that mobilizes sulfide for the formation of FeS clusters that would be required by several fermentative FeS proteins including ADH1, PFLA1, and PFR1.

In summary, the fermentative metabolism of C. reinhardtii has been shown to readily accommodate the loss of hydrogenase activity by synthesizing succinate, which would contribute to the reoxidation of NADH and sustain glycolysis for the production of ATP during anaerobiosis. Activation of this pathway, which is minimally active in the parental strain (based on transcript and metabolite analyses), further illustrates the metabolic flexibility of C. reinhardtii, an alga that appears to be well adapted to withstand a variety of environmental challenges, including anoxia. It is possible that the ability to synthesize succinate in the absence of hydrogenase activity reflects an adaptation used in natural settings in which fermentation metabolism is elicited under conditions in which
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hydrogenase activity is inhibited (e.g. brief exposures to gases such as CO or O₂, which are likely not strictly excluded in certain environments). The transcriptome data indicate that the loss of hydrogenase activity results in the increased expression of several transcripts associated with a variety of cellular redox processes. Some of these processes have been associated with aerobic respiration, indicating that there are still several aspects of anoxic acclimation that are not fully understood and warrant further investigation. The metabolic capacity of *C. reinhardtii*, particularly during anaerobiosis, is extremely versatile and complicated. This alga readily responds to variations in culturing and assay conditions in ways that we are just beginning to elucidate. The availability of the *C. reinhardtii* genome sequence, high throughput omics-based approaches, and mutants disrupted in a variety of cellular process will provide the foundation to further examine and understand the metabolic flexibility of this fascinating phototroph.

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