Role of Hybrid Cluster Protein 4 in Anaerobic Metabolism in *Chlamydomonas reinhardtii*

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I would like to thank Dr. Clay Carter for his tireless support and guidance throughout this project and his willingness to pursue the goals of others.

Dedication

This thesis is dedicated to my parents Charles and Judy Olson, who have supported everything I have done and fostered my love of learning.

Abstract

The unicellular green algae *Chlamydomonas reinhardtii* (*C. reinhardtii*) has long been studied for its unique fermentation pathways and is recently being considered as a candidate organism in biofuel production. Fermentation in *C. reinhardtii* is facilitated by a network of three predominant pathways producing four major by products: formate, ethanol, acetate and hydrogen. Recent microarray studies have identified many previously unknown genes highly up-regulated during anaerobiosis, and new tools for the targeted gene disruption make reverse genetics possible in *C. reinhardtii* for the first time. For example, hybrid cluster protein 4 (HCP4) is one of the most highly up-regulated genes during anaerobiosis in *C. reinhardtii*, displaying a nearly 1,600 fold increase upon anoxia. Hybrid cluster proteins have long been studied for their unique spectroscopic properties, yet their biological functions remain unclear. In this study HCP4 was knocked down using artificial microRNAs, followed by extensive phenotypic analyses. This study shows that knockdown of HCP4 affects the regulation of many key fermentative genes as well as metabolic flux and nitrogen uptake.

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List of abbreviations

C. reinhardtii -- *Chlamydomonas reinhardtii*

PFL -- pyruvate formate lyase

PDC -- pyruvate decarboxylase

PFR -- pyruvate ferredoxin oxidoreductase

LDH -- lactate dehydrogenase

HYD -- hydrogenase

ADH -- alcohol dehydrogenase

PAT1/PAT2 -- phosphoacetyl transferase

ACK1/ACK2 -- acetate kinase

NR -- nitrate reductase

NiR -- nitrite reductase

GS/GOGAT -- glutamine sythetase/glutamate synthase cycle

GOGAT -- glutamine oxogluterate amidotransferase

TAP -- Tris acetate phosphate media

amiRNA -- artifical micro RNA

hcp4 -- transgenic *C. reinhardtii* containing amiRNA targeting HCP4

NREL -- National Renewable Energy Laboratory

HCP -- hybrid cluster protein

HCP4 -- hybrid cluster protein 4

CAIP -- calf intestinal alkaline phosphatase

General Introduction

Chlamydomonas reinhardtii and other microalgae have been the focus of global research into biofuel production for decades (*1*). Despite this effort, the pathways involved in the diverse fermentation metabolism of *C. reinhardtii* are not fully understood. A further understanding of the interactions between the various fermentation pathways active in anaerobic *C. reinhardtii* will yield more precise targets in the effort to engineer better microalgae strains for biofuel production. The purpose of this study is to use *C. reinhardtii* as a model system to investigate the interactions of various fermentation pathways in darkness-induced anaerobiosis. A further understanding of regulatory networks coordinating metabolic flux in *C. reinhardtii* is paramount in developing informed metabolic engineering strategies to boost biofuel production.

C. reinhardtii is a predominantly soil dwelling microalgae found globally (Harris, 2009). The genus Chlamydomonas belongs to the Chlamydomonadaceae family in the order Volvacales (Harris, 2007). Individual species of Chlamydmonas are defined by their body shape and size, but their structure (twin flagella, chloroplast, and pyrenoid) remain constant in the genus. *C. reinhardtii* has long been used as a model system for studying photosynthesis, nutrient deprivation, flagellar function, and H_2 production (*2*). Interest in *C. reinhardtii* as model organism for biofuel production has been renewed in recent years due to: 1) the discovery of its ability to perform anaerobiosis in the light, 2) its rapid growth rates compared to terrestrial plants, and 3) development of "omics" based approaches to elucidating metabolic pathways, including the development of genetic manipulation techniques which can be used for the optimization of metabolic processes (*3, 4*).

The generation of stable mutants in *C. reinhardtii* has traditionally been achieved by random genomic integration (*4*). This approach is cumbersome and requires the screening of thousands of mutants using suitable phenotypic criteria or extensive DNA analysis. Recently, tools have been developed that enable targeted gene disruption through the use of artificial microRNAs (amiRNAs) (*5, 6*).

It is apparent that *C. reinhardtii* experiences periods of anoxia in nature, and has evolved a diverse set of metabolic pathways to deal with this. In the lab, anoxia can be induced by placing sealed cultures in the dark, sparging oxygen from cultures (e.g. bubbling N_2), or by placing cells in sulfur free media and growing them in light (S is required for photosynthetic O_2 evolution). Under the latter conditions, O_2 uptake via respiration overcomes O_2 production via photosynthesis leading to anoxia. As illustrated in Figure 1, *C. reinhardtii* is unique among eukaryotes in that it contains four enzymes used in pyruvate fermentation, including: pyruvate formate lyase (PFL), pyruvate ferredoxin oxidoreductase (PFR), lactate dehydrogenase (LDH), and pyruvate decarboxylase (PDC).

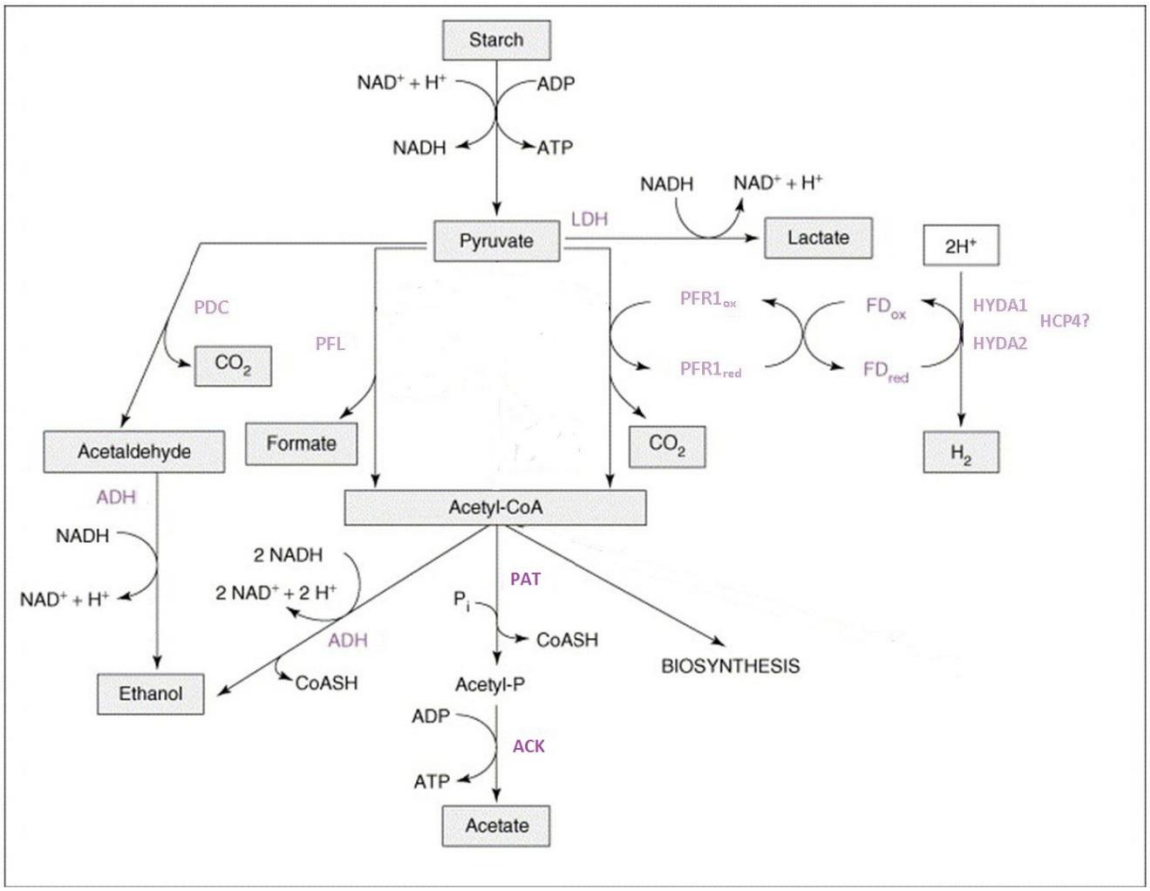

Figure 1. **Fermentation pathways of *Chlamydomonas reinhardtii.*** Following glycolysis pyruvate is further broken down to Acetyl-CoA by pyruvate formate lyase (PFL) and pyruvate ferredoxin oxidoreductase (PFR). Acetaldehyde is formed by pyruvate decarboxylase (PDC) from pyruvate. Hydrogenase (HYD) oxidizes reduced ferredoxin to form H_2 gas. In this model HCP4 is hypothesized to oxidize reduced ferredoxin. Ethanol is formed from acetaldehyde and acetyl-CoA via Alcohol dehydrogenase (ADH). Acetate is formed from Acetyl-CoA via phosphoacetyl transferase (PAT) and acetate kinase (ACK). Lactate is formed via Lactate dehydrognenase (LDH) Modified from (*7*).

C. reinhardtii expresses multiple enzymes usually associated with strict anaerobes such as [FeFe] hydrogenase and the [FeFe] hydrogenase maturation proteins rarely found in eukaryotes (*2, 8*). This extraordinary collection of fermentation pathways makes *C. reinhardtii* especially well adapted to anaerobiosis, and is able to produce multiple biofuels such as triacylglycerols, ethanol and hydrogen (*9*). These fermentation products along with the ability of *C. reinhardtii* to grow quickly to very high biomass densities in environments that will not compete with food stocks makes *C. reinhardtii* an excellent candidate for the development of biofuels.

There are currently many public and private labs working to make *C. reinhardtii* a commercially viable biofuel producing organism. For example, downregulation of the Tla1 gene, which has been shown to influence chlorophyll antenna size in *C. reinhardtii,* is currently being investigated as a possible way to grow *C. reinhardtii* to higher densities without the use of increased exogenous carbon (Tetali, Mitra et al. 2007; Mitra and Melis 2010). Oxygen sensitivity of hydrogenase remains a substantial hurdle in the commercialization of hydrogen gas production in *C. reinhardtii*, but may be overcome using mutational techniques and modeling of other more O_2 tolerant hydrogenases (Flynn, Ghirardi et al. ; Tosatto, Toppo et al. 2008). With tools being developed only recently for targeted gene disruption in *C. reinhardtii*, relatively few mutants have been generated targeting fermentation pathways. A mutation in the hydrogenase maturation gene HYDEF showed increased succinate production and increased expression of a malate forming enzyme MME4, during darkness induced anaerobiosis (Dubini, Mus et al. 2009). Cultures with a mutation in the central fermentative enzyme PFL showed no formate production, as expected, and had increased ethanol, D-lactate, CO_2 and hydrogen production in darkness induced anaerobiosis (Philipps, Krawietz et al. 2011). Interestingly the PFL mutant showed lower HYD protein and transcript, levels possibly indicating further regulation of these pathways than at the transcriptional level (Philipps, Krawietz et al. 2011).

Fermentation pathways

Fermentation in *C. reinhardtii* follows glycolysis by the breakdown of pyruvate. Six fermentation products are observed during darkness induced fermentation, H_2, CO_2, acetate, ethanol, formate, and glycerol (*10, 11*). However, the main products in darkness induced fermentation (anaerobiosis) are formate, acetate, and ethanol in a 2:1:1 ratio with H_2 and CO_2 given off as minor byproducts (*11, 12*). A fermentation ratio of 2:2:1 of the respective metabolites has also been reported (*2, 10*). This discrepancy could be due to culture conditions and strains of algae used in the studies (*8*).

The breakdown of pyruvate by PFL and PFR leads to the formation of acetyl-coenzyme A (acetyl-CoA) (*2, 8*). Along with acetyl-CoA production, pyruvate breakdown via PFL produces formate. An alternative method of producing formate in *C. reinhardtii* cells exists via CO_2 reductase activity, but Ohta (1987) demonstrated that PFL is the predominant producer of formate in *C. reinhardtii* cells by inhibiting PFL and showing a decrease in formate production. PFL is extremely rare in eukaryotes and has recently been localized to the mitochondria and chloroplast in *C. reinhardtii* (*13, 14*). There are conflicting data regarding whether PFL is induced during the onset of anaerobiosis. PFL transcripts are shown to increase during the onset of anaerobiosis in sulfur deprived and darkness induced anaerobiosis, but protein abundance did not change in chloroplast or mitochondria during the onset of anerobiosis (*2, 13, 15*). It is possible PFL is regulated via post-translational activation rather than increased transcript abundance. The breakdown of pyruvate via PFR results in the reduction of ferredoxin and release of CO_2 and has been localized to the chloroplast (*8, 13*). In other microbes PFR is responsible for the donation of electrons to ferredoxin and the resulting release of H_2 via hydrogenase enzymes (*2*). Acetyl-CoA produced by PFL and PFR can be converted into acetate

via the phosphoacetyl transferase (PAT1/PAT2) and acetate kinase (ACK1/ACK2) which yields ATP (*2, 8*). PAT2/ACK1 have been localized to the chloroplast while PAT1/ACK2 have been localized to the mitochondria (*13*). Alternatively, acetyl-CoA produced by PFL/PFR can be reduced to ethanol via alcohol dehydrogenase (ADH) while oxidizing two NADH molecules (Fig. 1) (*2, 16*).

Ethanol is also formed via PDC in the cytosol from the breakdown of pyruvate to acetylaldehyde and the further reduction of acetylaldehyde to ethanol by ADH oxidizing one NADH molecule in the process (*2, 8*). Recent data, however, shows that in *C. reinhardtii* ADH is localized almost exclusively in the chloroplast while PDC is not found in the mitochondria or chloroplast, suggesting that ADH primarily reduces acetyl-CoA derived from PFL and PFR rather than PDC (*13*). It is believed that the need to balance ATP production, NADH oxidation and limit the acidification of the environment and the accumulation of toxic byproducts lead to the dynamic response *C. reinhardtii* exhibits during anoxia (*2, 8*).

Hydrogen production in green algae was first demonstrated in 1942 by Hans Gaffron (*17*). Hydrogen production is facilitated by reversible [FeFe] hydrogenases of which there are two isoforms (HYDA1 and HYDA2) bound to the photosynthetic apparatus by ferredoxin. Hydrogen production takes place in a strictly anaerobic environment, as hydrogenase transcription and enzyme stability is severely compromised in the presence of oxygen (≈3% O_2) (*18*).

Algal hydrogenases show high similarity to hydrogenases found in strict anaerobes, fungi and protists (*19*). Algal hydrogenase is directly reduced by ferredoxin unlike other hydrogenases that rely on putative electron relays comprised of FeS clusters, either [2Fe2S] or [4Fe-4S] (*19*). Hydrogenase requires the maturation proteins HYDEF and HYDG, which are believed to synthesize the di-(thiomethyl)amine bridging ligand, or synthesize the CN and CO ligands (*19*).

Algal hydrogneases are nuclear encoded and translocated to the chloroplast upon anaerobiosis; immunoblot assays have not shown hydrogenase in aerobically growing cells suggesting de novo synthesis of hydrogenase upon anaerobiosis (*20*).

The precise transcriptional regulation of hydrogenase has not been elucidated but it is known that anaerobiosis is required for expression of all hydrogenase genes and hydrogenase maturation proteins in *C. reinhardtii*. Upon darkness induced anaerobiosis hydrognase expression is induced 100 fold, though starchless mutants show attenuated hydrogenase expression suggesting other transcriptional regulators other than O_2 (*2, 20, 21*).

Three hydrogen production pathways have been identified in *C. reinhardtii*, two photoproduction pathways (also termed biophotolysis) and one dark fermentation pathway. Biophotolysis pathways utilize electron flow through the photosynthetic apparatus. The first photoproduction pathway contains both photosystems (PSI and PSII) and is termed direct biophotolysis. This pathway was first demonstrated by Melis in 2000 by removing sulfate from the growth medium of *C. reinhardtii* (*3*). The removal of sulfate causes the inhibition of synthesis/repair of the D1/32 kD reaction center of PSII thus resulting in decreased H_2O oxidation and O_2 evolution. This causes sealed cultures of *C.reinhardtii* to become anaerobic in light within 24 hours of growth in sulfur deprived media (*19*). Electrons derived from residual light induced oxidation of water at PSII are transported to PSI where they are excited and donated to ferredoxin. Hydrogenase then oxidizes the reduced ferredoxin and forms H_2 (*8*). Direct biophotolysis is estimated to contribute 50 – 90% of the total electron flux produced during H_2 production in the light since the addition of the PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) caused an 80% drop in H_2 production in sealed sulfate deprived cultures (*22*). Direct biophotolysis is theorized to produce H_2 and O_2 in a 2:1 ratio (*23*). Thus

cellular respiration must be active to clear the oxygen from the culture before it reaches levels that inhibit hydrogenase. The second photoproduction pathway termed indirect biophotolysis utilizes the catabolism of starch to reduce the plastoquinone pool via NAD(P)H-plastoquinone oxido-reductase thereby facilitating photosynthetic electron flow while PSII is inhibited (*24*). Electrons entering the plastoquinone pool are transferred to the PSI reaction center where they are excited and donated to ferredoxin which is oxidized by hydrogenase to produce H_2.

The dark fermentative H_2 production pathway is coupled to starch catabolism (*10, 11*). This pathway most resembles hydrogen production pathways in anaerobic bacteria and amitochondriate eukaryokes. In this pathway the oxidation of pyruvate is directly coupled to the reduction of ferredoxin by PFR while producing acetyl-CoA and CO_2. Hydrogenase then oxidizes the reduced ferredoxin forming H_2. Upon darkness-induced anaerobiosis PFR shows a dramatic increase in transcript abundance, over 1,500 fold increase two hours post anaerobiosis (*2*). This is significantly higher than PFL, PDC or Hyd. If reduced ferredoxin was being oxidized exclusively by hydrogenase this large increase in transcript abundance would lead to high H_2 output but paradoxically darkness induced fermentation does not produce large amounts of H_2. Thus it is possible that hydrogenase is not the only enzyme oxidizing reduced ferredoxin during anaerobiosis and disruption of other proteins oxidizing ferredoxin may be a viable way of increasing H_2 output. Hydrogen production *in C. reinhardtii* is modulated by a variety of factors including CO_2 concentration, acetate, nitrate and nitrite. Hydrogen production is enhanced when CO_2 or acetate is present, which likely stems from the increased reduced organic compounds or accumulation of starch (*11, 25*). Aparicio (1985) showed a close relationship between nitrate and nitrite reduction and hydrogen production in *C. reinhardtii*. H_2 production in the dark was inhibited by the addition of nitrate or nitrite, H_2 production only resumed after all of the nitrite or nitrate was converted to ammonia. It is not fully understood how nitrate or

nitrite inhibit H_2 production but it is hypothesized that the action of nitrite reductase activity oxidizes ferredoxin in competition with hydrogenase (*8, 26*).

Hybrid Cluster Protein 4

The low H_2 output observed during darkness induced anaerobiosis leads to questions regarding limiting steps or competing pathways with hydrogenase. It has been shown that concurrent with the onset of anaerobiosis the Hybrid cluster protein 4 (HCP4) displays an over 1,500 fold increase in expression, making it one of the most highly up regulated genes during darkness induced anaerobiosis (*2*). A similar microarray study has shown that HCP4 is only moderately upregulated during sulfur deprived anaerobiosis in light (*15*). Similar to hydrogenase, HCP4 is an iron sulfur protein containing two subunits, a [4Fe-4S]2+/1+ or [2Fe-2S]2+/1+ and [4Fe-2S-2O], the so called "hybrid cluster" (For image see supplementary Figure 1) (*27*). The binding motif of [4Fe-4S] and [2Fe-2S] observed in HCP4 shows unique spacing of conserved cysteines making it similar to HCPs found in strict anaerobes (*2*). Although this protein has been studied extensively on a structural basis, its physiological role is not fully understood. HCP was found to be induced by hydrogen peroxide in *E. coli*, and is believed to play a role in oxidative stress defense (*27*). In *E. coli* HCP also shows up-regulation upon addition of nitrate or nitrite, and purified HCP protein displays hydroxylamine reductase activity, reducing hydroxylamine to ammonia (*28*). Hydroxylamine production was shown to require ferredoxin in *C. pasteurianum* (*29*) thus it has been proposed that in *C. reinhardtii* HCP4 could oxidize a reduced ferredoxin thereby directly competing with hydrogenase for electrons (*2*). Furthermore, two isoforms of HCP have shown structural similarity of carbon monoxide dehydrogenase which is able to reduce CO_2 (*30*).

Nitrogen metabolism

In *C. reinhardtii*, nitrate and ammonium are the predominant sources for nitrogen assimilation. Ammonium is preferentially up taken, but in the absence of ammonia, nitrate and to a smaller extent nitrite are utilized. Nitrate is converted into nitrite in the cytoplasm by the enzyme nitrate reductase (NR) and is further transported into the chloroplast. In the chloroplast nitrite is converted to ammonium via the ferredoxin dependent nitrite reductase (NiR) and is incorporated into L-glutamate via the glutamine sythetase/glutamate synthase cycle (GS/GOGAT) (*31, 32*).

Nitrate and ammonium are transported into the cell by specific transporters. *C. reinhardtii* more resembles the higher plant *Arabidopsis thaliana* in terms of nitrate transporters than it does the lower algae *Ostreococcus,* as *C. reinhardtii* contains three gene families of nitrate transporters NRT1, NRT2, and NAR1. Where Arabidopsis contains all three families, *Ostreococcus* only contains one (*8*). Once in the cytoplasm nitrate is reduced to nitrite by NR using NADH as an electron donor. *C. reinhardtii* NR shows high similiarity to cyanobacterial NR which uses ferredoxin as an electron donor (*33*). Light/dark cycles have been found to regulate NR activity with nitrate and nitrite uptake being highest in the light and lowest in the dark (*34*). Similarly it was found that a reduced plastoquinone pool enhances expression of NR in *C. reinhardtii* (*35*). Molybdenum was found to be an essential cofactor in NR. Molybdenum cofactor (Moco) along with FAD and heme b_{557} comprise NR prosthetic groups (*31*). The molybdenum carrier protein MCP1 involved in transport of molybdenum to NR is located in the vicinity of HCP4 and HCP1 genes in the *C. reinhardtii* genome, falling 268kb and 237kb away respectively. Following conversion of nitrate to nitrite by NR in the cytoplasm, nitrite is transported to the chloroplast by NAR1 nitrite transporters. NAR1 is a member of the formate nitrite transporter family which

is found in archea and eubacteria (*36*). It is hypothesized that NAR1 transporters limit the amount of nitrite that enters the chloroplast so that availability of carbon limits ammonium uptake in the GS/GOGAT cycle (*36*). Once in the chloroplast nitrite is reduced to ammonia via a six electron step reduction catalyzed by NiR using reduced Ferredoxin as an electron donor. NiR is solely regulated at the transcriptional level. NiR is shows similar regulation to NR as high nitrate or nitrite and low ammonia will increase transcription of NiR while high ammonia levels will decrease transcription (*8*).

Ammonium is preferentially uptaken over nitrate when available in media. Ammonia is transported to the chloroplast via two transporters families; low affinity and high capacity (LATS) and high affinity and low capacity (HATS). These gene families contain eight ammonium transporter genes (AMT) which display a marked diversity of regulation further emphasizing the need for efficient nitrogen uptake at range of environmental situations (*37*).

Nitrogen uptake is coupled to carbon metabolism in the cytosol and chloroplast stroma via GS/GOGAT in which glutamine is formed from glutamate and ammonium. In the cytosol glutamine synthetase (GS1; GLN1) forms glutamate at the cost of one ATP. GLN1 is down-regulated during darkness or when incubated in ammonium containing media (*38*). A second copy of GS (GS2; GLN2) is found in the chloroplast and is constitutively expressed (*39*). *C. reinhardtii* contains two glutamine oxoglutarate amidotransferase (GOGAT) enzymes that catalyze the reductive transfer of an amide group from glutamine to α-ketogluterate to form two glutamates; Fd-GOGAT and NADH-GOGAT. Fd-GOGAT requires a reduced ferredoxin and is upregulated in light unlike NADH-GOGAT (*38*). Both enzymes are hypothesized to be located in the chloroplast but their different regulatory patterns suggest different functions. Fd-GOGAT in concert with the chloroplast located GLN2 is hypothesized to be involved in the reassimilation of

photorespitory NH_4, while NADH-GOGAT and the cytosolic GLN1 is believed to be primarily used in the assimilation of exogeneous nitrogen (*8*).

Taken together it is clear that *C. reinhardtii* has as a diverse set of enzymes and pathways that are utilized in the uptake and assimilation of nitrogen. Somewhat suprisingly the uptake of nitrogen has not been widely studied during anaerobic conditions. Ferredoxin plays a large part in nitrogen metabolism at the NiR, Fd-GOGAT, and possibly the NR stages, and it is known that darkness inhibited electron flow around PSI and PSII will slow nitrate, nitrite and ammonia uptake (*40*). However the interplay between anaerobic energy production and anaerobic nitrogen metabolism has not been elucidated. HCP4 may be associated with anaerobic nitrogen metabolism based on the high up regulation of HCP4 during anaerobiosis and demonstrated hydroxylamine reductase activity of related proteins, as well as its hypothesized ability to oxidize a reduced ferredoxin.

Previous work has mapped the general fermentation pathways in *C. reinhardtii*. However it is not known how these pathways interact with each other to balance fermentative energy production with the production of potentially harmful byproducts. Recent microarray and proteomics studies have identified the several likely players and associated metabolic pathways, as well as new candidate genes of unknown function that are up-regulated during anaerobiosis. Of particular significance, HCP4 is one of the most highly up-regulated genes in darkness-induced anaerobiosis, yet its function remains unknown (*2*). This study aims to uncover the interaction of fermentation pathways by monitoring gene transcript levels as well as fermentative byproducts while knocking down expression of HCP4 via microRNA vector. This understanding will further illustrate the flexibility of the anaerobic response in *C. reinhardtii* as well as guide future studies in optimizing metabolic pathways for the production of biofuels

MATERIALS AND METHODS

Algae strains and growth conditions

Chlamydomonas reinhardtii type cc425 arg2 cw15 sr-u-2-60 mt+ was used in this study as wild type and background strain for transformations. All cultures were grown mixotrophically in Tris Acetate Phosphate (TAP) media (*8*) under 12 hr day / 12 hr night cycles on a shaking plate. During growth, wild type strains were supplemented with 100 µg/ml arginine. Cultures were illuminated with a photosynthetic photon flux of 150 µmol m^{-2} s^{-1} and temperature of 23°C.

Generation of amiRNA vectors

Vectors pChlami2 and pChlami3int were obtained from the *Chlamydomonas* resource center (University of Minnesota) and prepared according to Molnar et al. (*5*). amiRNA inserts were generated for HCP4 using WMD (web based microRNA designer) version 3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Home;project=stdwmd). HCP4 (XM_001694402) mRNA sequence was used to generate an appropriate amiRNA insert. Ninety nucleotide long oligonucleotides (i.e. desired inserts) were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Inserts were resuspended to a final concentration of 100 µM. To anneal the insert oligos, 10 µl of forward and reverse insert oligos were mixed with 20 µl 2X annealing buffer (20mM tris pH 8.0, 2mM EDTA, 100mM NaCl). The mixture was boiled for five minutes and gradually cooled overnight. The double stranded insert was purified using Qiagen PCR clean up kit (Qiagen, Venlo, Netherlands). The insert was phosphorylated with using Promega T4 Polynucleotide Kinase (Promega Corp., Madison, WI, USA). The vectors pChlami2 and pChlami3int were digested with SpeI and and dephosphorylated with calf intestinal alkaline phosphatase (CAIP). The dephosphorylated vectors were purified using Qiagen PCR clean up kit. The phosphorylated insert was then cloned into the vectors using Promega T4 DNA ligase

(Promega Corp., Madison, WI, USA). Mach1 *E. coli* were transformed by electroporation with the vectors and plated on 150 µg/ml ampicillin LB agar plates.

Individual *E. coli* colonies were picked and grown in LB broth at 37°C and DNA was extracted via Qiagen miniprep kit (Qiagen, Venlo, Netherlands). PCR reactions were performed to locate transformed colonies containing the vector and insert in the correct orientation using primers AmiRNAprec$_{for}$ (5'-GGTGTTGGGTCGGTGTTTTTG-3') and Spacer$_{rev}$ (5'-TAGCGCTGATCACCACCACCC-3') were used with Promega GoTaq Green Master Mix according to the manufacturer's instruction (Promega Corp., Madison, WI, USA). Candidate constructs containing the insert in the correct orientation were sequenced at the University of Michigan Sequencing Core (Ann Arbor, Michigan).

***C. reinhardtii* Transformation**

C. reinhardtii strain cc425 was transformed with vector pChlami2 containing the HCP4 amiRNA construct using a modified Kindle's glass bead method (*41*). Wild type cc425 cells were re-suspended to a density of 1x10^6 cells/ml. 300 µl of cells, 100 µl 20% polyethylene glycol (PEG), 2 µg HCP4 amiRNA vector, and 300 µg glass beads (.5mm diameter) were added to a 1.5 microcentrifuge tube and vortexed on high for 30 seconds. 150 µl of cells were plated on a 1.5% TAP agar plates and incubated. Single colonies growing on TAP were picked and the sequence verified at the University of Michigan sequencing core (Ann Arbor, Michigan).

Anaerobiosis acclimation assay

Liquid cultures were initiated by spiking 500 ml of TAP with equal amounts of wild type and HCP4 knockdown (*hcp4*) cells. Cultures were grown for four days under 12 hour light dark

cycles, illuminated with a photosynthetic photon flux of 150 µmol m^{-2} s^{-1} and temperature of 23°C. Following incubation cells were counted via hemacytometer and 160x10^6 cells were pelleted and washed with 25 ml HEPES buffer (28mM, pH 7.5). Pellets were then resuspended in 40ml HEPES buffer to a final concentration of 4x10^6 cells/ml in a 50ml conical tube and incubated in the light for 1 hour. Following incubation, cell vitality was assayed by noting swimming cells in each culture. The tubes were wrapped in foil and parafilm loosely applied to the tops. N_2 gas was bubbled through the cultures and light excluded by placing a foil covered box over the cultures. Dissolved oxygen was assayed using a Clark-type electrode following 10 minutes of N_2 bubbling to ensure anaerobiosis. As illustrated in Figure 2, subsamples were collected from the cultures at T0: following 50 minutes incubation in light, T1: after the initiation of anaerobiosis (following 10 minutes N_2 bubbling in dark), T2: 0.5 hours post anaerobiosis, T3: 1 hour post anaerobiosis, T4: 3 hours post anaerobiosis, T5: 5 hours past anaerobiosis.

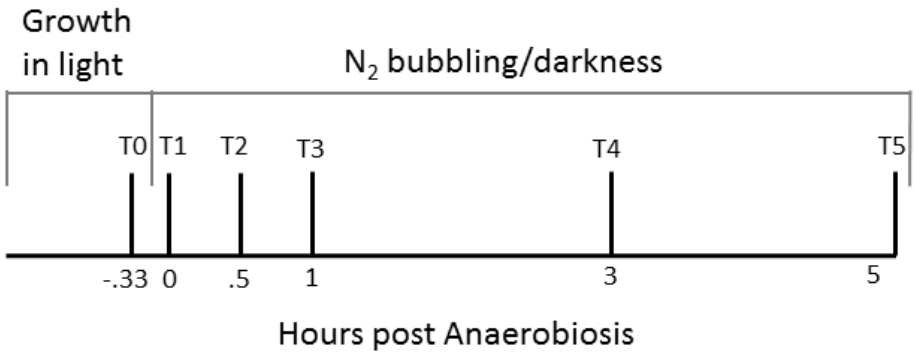

Figure 2. **Time points of subsamples taken from anaerobic cultures.** Samples were taken from anaerobic cultures 0.33 hours before induction of anaerobiosis, directly after anaerobiosis, 0.5 hours post anaerobiosis, one hour after anaerobiosis, three hours after anaerobiosis, and five hours after anaerobiosis. Anaerobiosis was initiated after 10 minutes of N_2 bubbling.

Trizol was used to extract RNA from the frozen pellets (Invitrogen, San Diego, California). Pellets were resuspended in 1ml trizol by pipetting. Samples were then incubated at room temp for 5 minutes. 200 µl of chloroform were added and tubes shaken by hand for 15 seconds then incubated at room temp for 2 minutes. Samples were spun at 11,000 g for two minutes at 4°C. The upper aqueous phase was placed in a new tube, 0.5 ml of isopropyl alcohol was added and the tube was incubated at room temperature for 10 minutes. Samples were then centrifuged at 11,000 g for 10 minutes at 4°C. The supernatant was removed and replaced with 1ml of 75% ethanol and mixed gently by hand. The mixture was centrifuged at 7000 g for five minutes at 4°C. The supernatant was removed and let air dry for five minutes. The RNA pellet was resuspended in 40 µl RNase-free H_2O and incubated at 55°C for 10 minutes. RNA was quantified and integrity was verified by running 500 µg of RNA in a 2% agarose gel.

Reverse transcription was carried out using a Qiagen Quantitect Reverse Transcription kit and 500 µg RNA according to manufacturer's instructions (Qiagen, Venlo, Netherlands). Real time PCR was performed using Rotor-gene SYBR Green PCR Kit (Qiagen, Venlo, Netherlands) and Corbett Research RG-3000 thermocycler (Qiagen, Venlo, Netherlands). One µl of CDNA was used for each reaction. Previously published primers were used to amplify 100-200 nucleotide regions of the following genes; Rack1, PDC, HYD, PFL, PFR, and HCP4 (supplemental table 1) (*2*). Cycling parameters contained a melting step at 95°C for 10 minutes followed by a 65 cycles of a 95°C (10 sec) melting step followed by a 60°C (15 sec) annealing/elongation step. Data were acquired on the FAM/Sybrgreen channel during the annealing/elongation step. A 10 minute step at 72°C ended the cycle. Relative expression was calculated using the comparative Ct Method (Applied Biosystems). The average cycle threshold (Ct) was calculated from triplicate measurements and two biological replicates. The Rack1 gene was used as the constitutive control.

Organic metabolite assays

The metabolites formate, ethanol and acetate were measured using kits (formate; 10979732035, acetate; 10148261035, ethanol; 10176290035) from Boehringer Mannheim / r-biopharm, Darmstadt, Germany. These enzymatic assays measure sample dependent production of NADH. NADH was measured at 340nm for the ethanol and acetate assays using a Beckman DU 650 spectrophotometer (Beckman Coulter, Brea, CA). NADH was measured in the formate assay using a Nanodrop ND-1000 spectrophotometer (Thermo scientific, Waltham, MA). Supernatant fractions from each timepoint were used as samples and HEPES buffer used as the blank. Manufacturer instructions were followed with slight modifications. The ethanol reaction volume was reduced to 525 µl and 100 µl sample volume was used in each reaction. The formate reaction volume was reduced to 61 µl and 40 µl sample volume was used. The Acetate reaction volume was reduced to 537 µl using a sample volume of 100 µl. Results are reported as the average of quadruplicate samples for formate and ethanol and triplicate samples of acetate at each timepoint.

Nitrogen uptake assays

Uptake of nitrate or ammonium was measured over a 24 hour period. Liquid cultures were initiated by spiking 500ml of TAP liquid with equal amounts of wild type and *hcp4* cells. Cultures were grown for four days under 12 hour light dark cycles, illuminated with a photosynthetic photon flux of 150 µmol m^{-2} s^{-1} and temperature of 23°C. Following incubation the cells density was measured using a hemocytometer and $160x10^6$ cells were pelleted and washed with ammonium solution (12mM Na-acetate, 28mM HEPES, 10mM NH_4Cl) or nitrate solution (12mM Na-acetate, 28mM HEPES, 10mM KNO_3). Cells were then resuspended in 40 ml of their respective ammonia or nitrate solutions at $4x10^6$ cells/ml. Cells were bubbled with N_2 in the

dark for ten minutes then capped and placed in the dark. Two ml subsamples were taken from the ammonium uptake experiment every two hours for 12 hours. Two ml subsamples were taken every two hours in the nitrate experiment for 12 hours and then every 4 hours until 24 hours had elapsed. After being taken samples were immediately centrifuged and separated into pellet and supernatant fractions.

RNA was extracted from the four hour timepoint of the ammonia and nitrate uptake samples and HCP4 knockdown was confirmed as described earlier.

Ammonium and nitrate remaining in solution were assayed using kits (ammonium; 11112732035, nitrate; 10905658035) from Boehringer Mannheim / r-biopharm, Darmstadt, Germany. Manufacturer's directions were followed with slight modifications. Reaction volume for the ammonia and nitrate assays were reduced to 503.3 µl, and 508.33µl respectively. NADH was measured spectroscopically using a nanodrop 2000c (Thermo scientific, Waltham, MA).

Results

Previous microarray analysis confirmed that HCP4 is one of the most highly up-regulated genes during anaerobiosis (*2*). To examine the effects of HCP4 knockdown in *C. reinhardtii* fermentation a pChlamiRNA2 vector containing an amiRNA-encoding insert targeting HCP4 was produced. As shown in Figure 3 the amiRNA was designed to bind 21 nucleotides in the 3' UTR of the HCP4 gene.

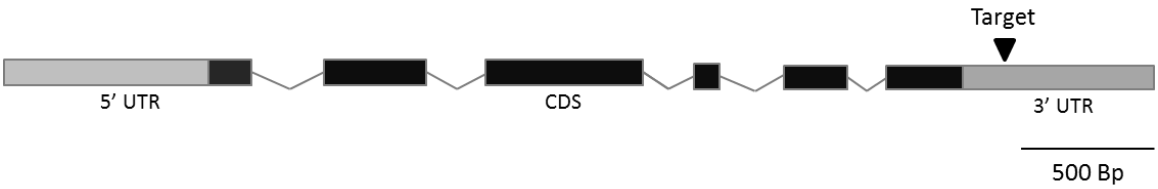

Figure 3. **amiRNA targeting HCP4.** HCP4 (XM_001694402) was targeted in the 3' UTR for degradation using a pChlamiRNA 2 vector (Molnar et al. 2009).

Gene expression profile

To confirm knockdown of HCP4, wild type cc425 and the transgenic mutant for HCP4 (*hcp4)* were grown in TAP media, washed and resuspended in 10mM HEPES buffer and subjected to five hours of anaerobiosis in the dark while being bubbled with a constant stream of N_2 gas. Subsamples were taken -0.33, 0, 0.5, 1, 3, and 5 hours post anaerobiosis as seen in Figure 2. mRNA was extracted from timepoints -0.33, 0, 1, 3, and 5. cDNA was synthesized and real-time PCR was performed. Figure 4 shows the relative transcript abundance of HCP4 in *hcp4* compared to cc425.

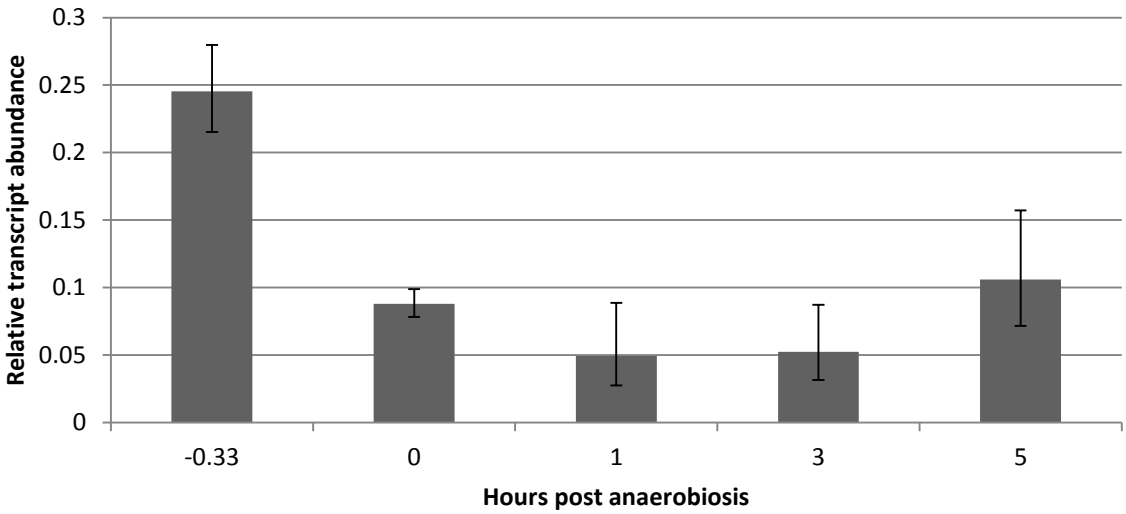

Figure 4. **The effect of amiRNA vector targeting HCP4 on transcript levels.** HCP4 mRNA levels were reduced in *hcp4* compared to wild type throughout the course of anaerobiosis. mRNA was extracted at the time points and cDNA levels measured in wild type and *hcp4*. HCP4 transcript levels were normalized using Rack1 as a control gene.

As shown in Figure 4 transcript levels of HCP4 were significantly knocked down in *hcp4* at all timepoints. Knockdown amounts range from 0.24 relative transcript abundance (4-fold knockdown) at 0.33 hours pre anaerobiosis to 0.05 relative transcript abundance (20-fold knockdown) at one hour post anaerobiosis. Once knockdown of HCP4 was confirmed the expression of other genes central to fermentation pathways were investigated to elucidate the effects of HCP4 knockdown. Relative transcript abundance was measured using cDNA synthesized from three and five hours post anaerobiosis. As shown in Figure 5, *hcp4* displayed altered gene transcription levels compared to wild type.

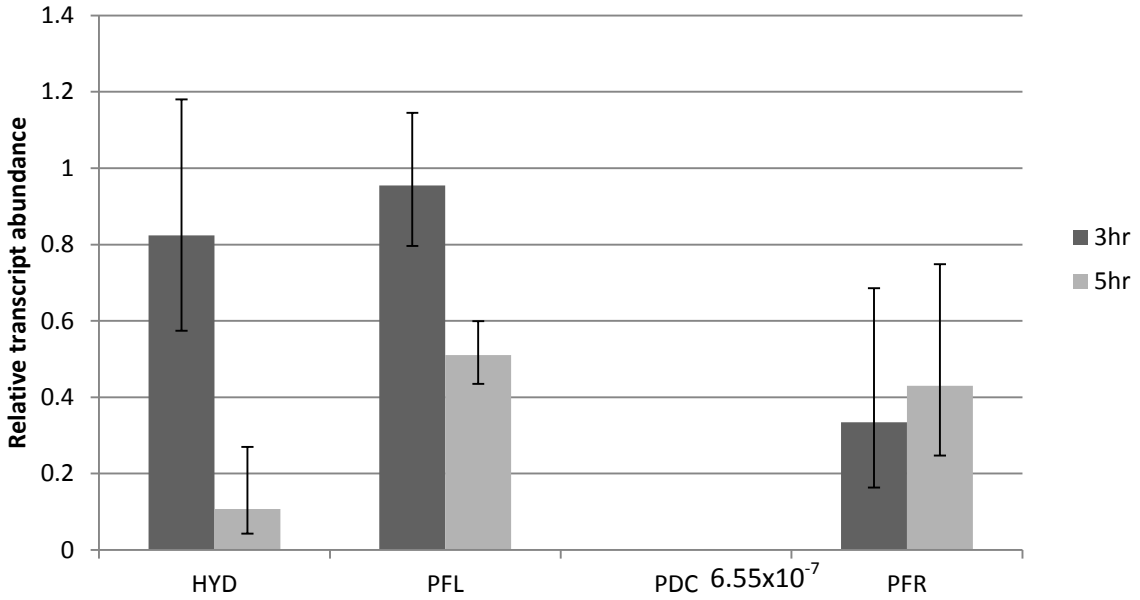

Figure 5. **The effect of HCP4 knockdown on major fermentation pathway genes**. Relative HYD, PFL, PDC, and PFR mRNA transcript levels were measured at three and five hours post anaerobiosis time points and compared to wild type levels. PDC and PFR showed significant downregulation after three hours of anaerobiosis. All transcripts showed significant downregulation after five hours of anaerobiosis.

Hydrogenase showed a marked decrease in expression between the three and five hour timepoints. At three hours post anaerobiosis HYD displayed a slight decrease in expression but was not statistically significant. At five hours, however, HYD showed a 0.1 relative transcript abundance corresponding to a 10 fold decrease in expression. Similarily, PFL was not significantly downregulated at three hours post anaerobiosis, but at five hours showed a significant drop in relative transcript abundance, dropping to 0.51 relative transcript abundance corresponding to a 2-fold decrease in expression. PFR expression was decreased at both the three and five hour time points, displaying 0.33 and 0.44 relative transcript abundance or a 3-fold and 2.27-fold decrease in expression respectively. PDC levels showed negligible expression

in the three and five hour time points. Relative transcript abundance for PDC ranged from $6.55x10^{-7}$ and $6.87x10^{-7}$ at three and five hour time points respectively.

Metabolite assays

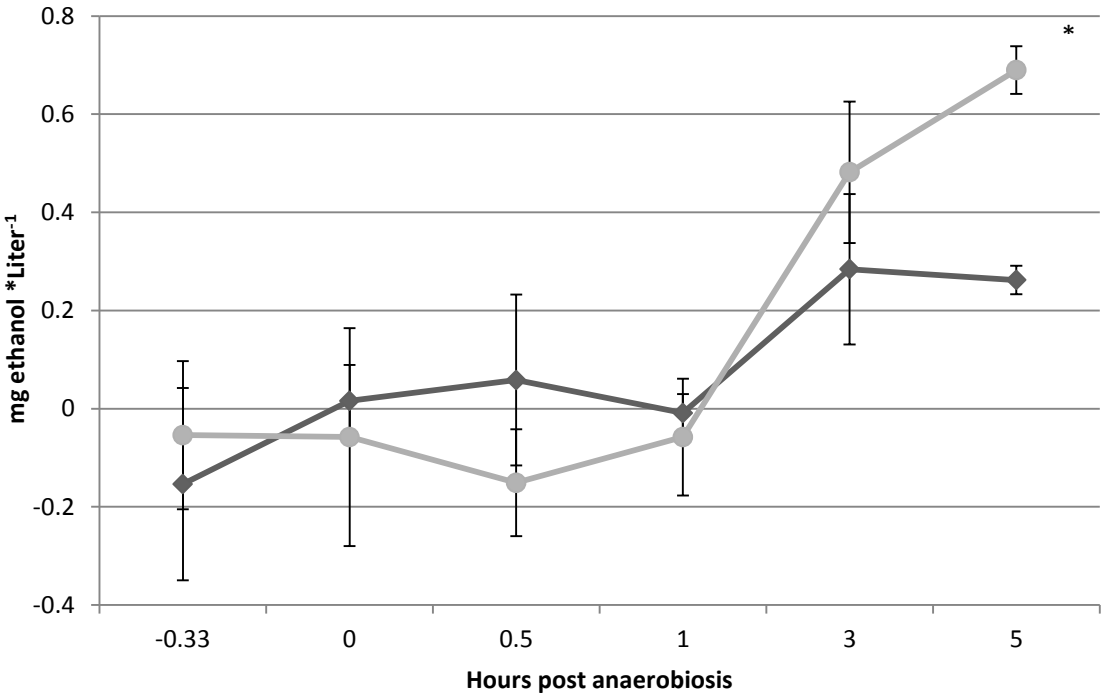

Figure 6. **Ethanol accumulation during darkness induced anaerobiosis.** Wild type strain cc425 (◆) and *hcp4* (●). Cultures were resuspended in 10 mM HEPES solution at -1 hour post anaerobiosis and constantly bubbled with N_2 in the dark starting at 0 hours post anaerobiosis. Significant changes are indicated with an * (t-test, $p < 0.5$).

Following gene expression studies the byproducts of these major fermentation pathways were analyzed. Metabolites ethanol, formate and acetate were measured using enzymatic UV-spec assays at all timepoints taken during the course of anaerobiosis. Production of ethanol was not significantly different between wild type and *hcp4* lines until the five hours post anaerobiosis.

At three hours post anaerobiosis wild type and *hcp4* showed measureable excreted ethanol at 0.28 and 0.48 mg*ml^{-1} respectively, yet these values were not statistically significant from each other. At five hours wild type and *hcp4* production significantly diverge as *hcp4* displays a 2.63 fold increase in excreted ethanol. Significant difference was calculated at five hours by a two tailed Student's unpaired t-test with equal variance (p=0.0002)

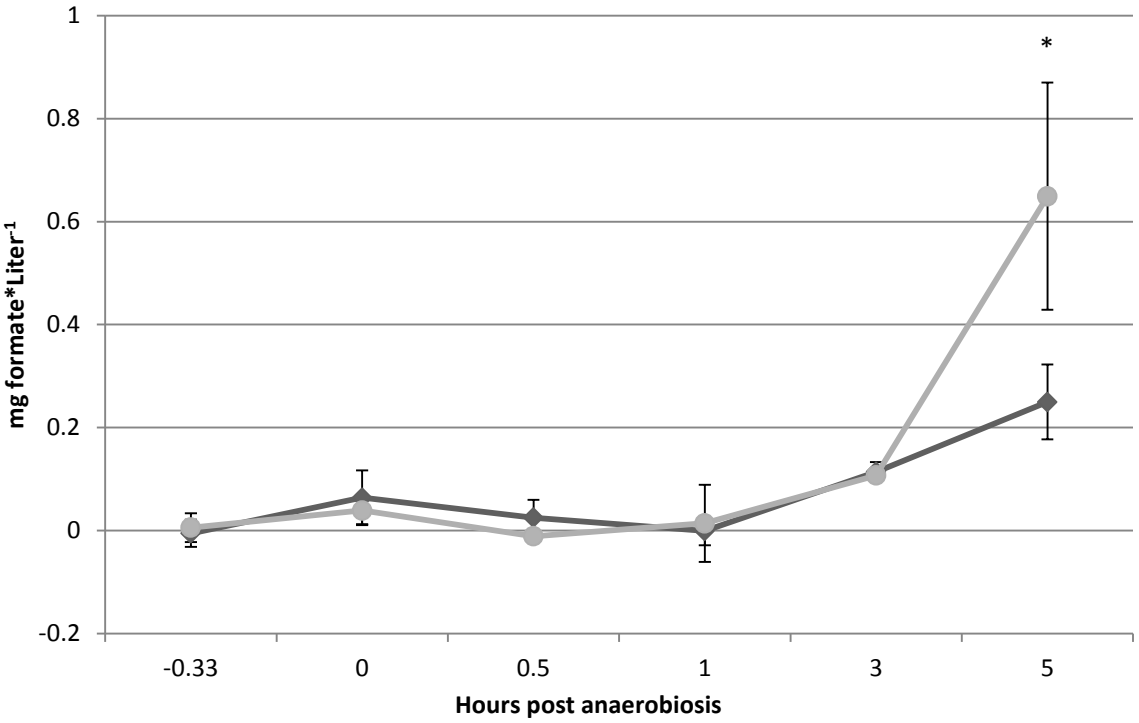

Figure 7. **Formate accumulation during darkness induced anaerobiosis.** Wild type strain cc425 (◆) and *hcp4* (●). Cultures were resuspended in 10 mM HEPES solution at -1 hour post anaerobiosis, and constantly bubbled with N_2 in the dark starting at 0 hours post anaerobiosis. Significant changes are indicated with an * (t-test, p <0.5).

Production of formate was not detectable until three hours post anaerobiosis. At three hours levels of formate excreted were not significantly different between wild type and *hcp4* which

were 0.11 and 0.1 mg formate*liter^{-1} respectively. At five hours post anaerobiosis production of formate significantly diverged between wild type and *hcp4.* At five hours wild type accumulated 0.25 mg formate*liter^{-1} and *hcp4* accumulated 0.65 mg formate*liter^{-1}. Thus *hcp4* had a 2.6 fold increase in formate accumulation at 5 hours post anaerobiosis. Significant difference was calculated at 5 hours by a two tailed Students unpaired t-test with equal variance (p=0.045).

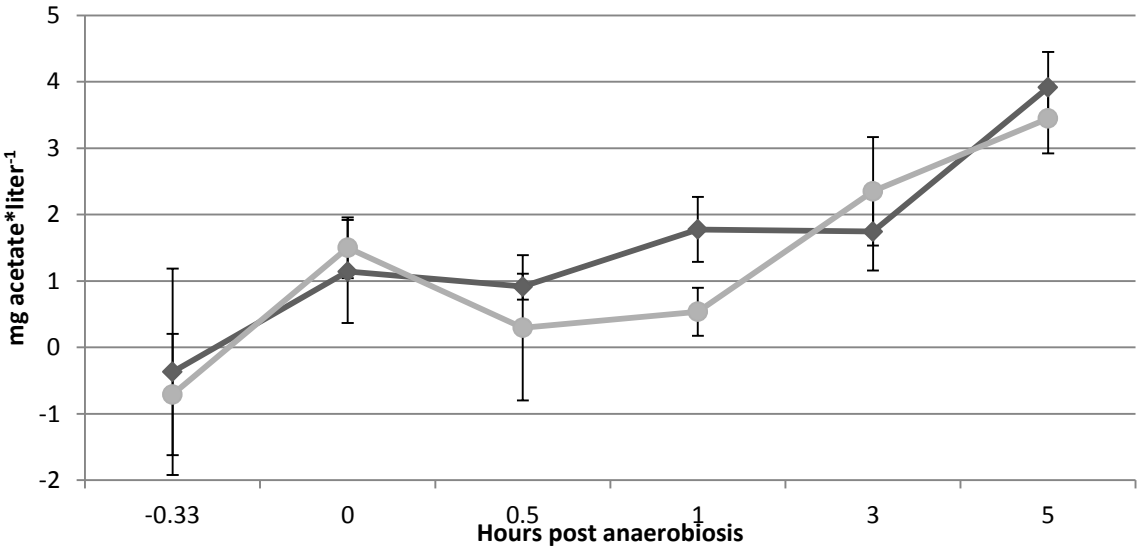

Figure 8. **Acetate accumulation during darkness induced anaerobiosis.** Wild type strain cc425 (◆) and *hcp4* (●). Cultures were resuspended in 10 mM HEPES solution at -1 hour post anaerobiosis and constantly bubbled with N_2 in the dark starting at 0 hours post anaerobiosis.

Acetate accumulation during anaerobiosis was not significantly different at any time point between wild type and *hcp4.* Both cultures showed acetate accumulation at zero hours post anaerobiosis. Acetate accumulation progressively increased in both cultures until 5 hours post anaerobiosis when wild type and *hcp4* accumulated 3.9 and 3.4 mg acetate*liter^{-1} respectively.

Table 1. **Metabolite totals from 3 and 5 hours post anaerobiosis.**

	Strain	Formate (mg)	Acetate (mg)	Ethanol (mg)	molar Ratio (formate:acetate:ethanol)
3 Hour	cc425	0.114	1.743	0.284	1:12:2.5
	hcp4	0.107	2.351	0.482	1:16:4.2
5 Hour	cc425	0.25	3.915	0.262	1:12:1
	hcp4	0.65	3.447	0.69	1:4:1

Metabolites accumulating in the media were analyzed using UV-spec assays. Total mg of each metabolite is reported and the ratio of each metabolite is provided at 3 and 5 hour post anaerobiosis timepoints.

As reported in Table 1, at three hours post anaerobiosis wild type accumulated the metabolites formate, acetate and ethanol at a 1:12:2.5 ratio. At the same timepoint *hcp4* accumulated these metabolites at a 1:16:4.2 ratio. At five hours post anaerobiosis wild type accumulated the metabolites formate, acetate and ethanol at a ratio of 1:12:1, where *hcp4* accumulated these metabolites at a ratio of 1:4:1.

Nitrogen uptake assays

Hybrid cluster proteins are up-regulated in *E. coli* upon the addition of nitrate or nitrite. Similarily, the only known physiological function of HCP proteins is that of hydroxylamine reductase in *E.coli*. Based on this information nitrate and ammonium uptake was assayed in wild type and *hcp4* strains under anaerobic conditions.

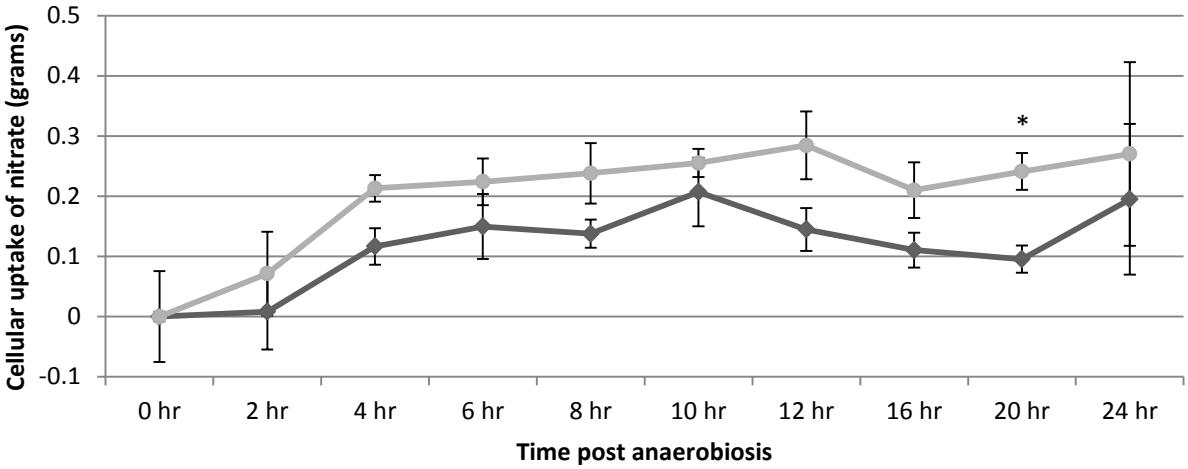

Figure 9. **Nitrate uptake during anaerobiosis.** Nitrate uptake was measured for 24 hours during anaerobiosis. Wild type strain cc425 (◆) and *hcp4* (●). Cultures were resuspended in 10 mM HEPES, 10 mM nitrate, 12 mM acetate solution. Cells were bubbled with N_2 for 10 minutes and kept sealed in the dark for 24 hours. Subsamples were taken every two hours and nitrate remaining in solution measured. Significant changes are indicated with an * (t-test, $p < 0.5$).

Nitrate uptake in wild type and *hcp4* were similar, but significant differences were measured in one out of ten timepoints. As shown in Figure 9 there is a general difference in the amount of nitrate uptaken during anaerobiosis, but only at 20 hours post anaerobiosis was there significantly more nitrate uptake than wild type cells. Four hours post anaerobiosis marked the first divergence in nitrate uptake with wild type and *hcp4* cells having uptaken 0.12 and 0.22 grams of nitrate respectively. 12 hours post anaerobiosis marks the greatest divergence in nitrate uptake with wild type and *hcp4* having uptaken 0.14 and 0.28 grams of nitrate respectively. The trend in this data shows that cc425 nitrate uptake peaked at 10 hours post anaerobiosis while *hcp4* peaked at 12 hours. Significant difference was calculated at 20 hours post anaerobiosis by a two tailed Student's unpaired t-test with equal variance ($p=0.046$).

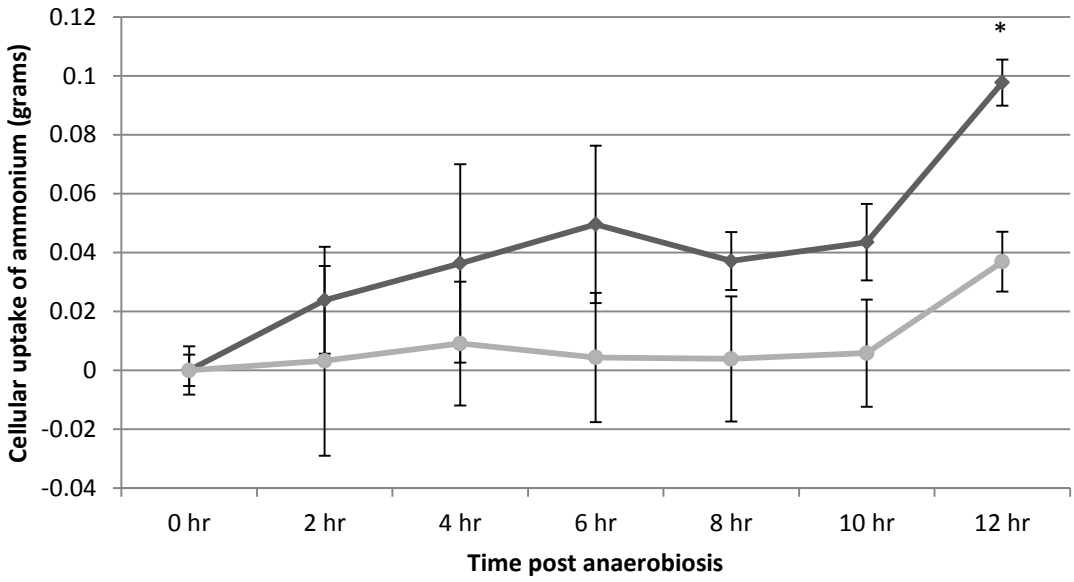

Figure 10. **Ammonium uptake during anaerobiosis**. Ammonium uptake was measured for 12 hours during anaerobiosis. Wild type strain cc425 (◆) and hcp4 (●). Cultures were resuspended in 10 mM HEPES, 10 mM ammonium, 12 mM acetate solution. Cells were bubbled with N_2 for 10 minutes and kept sealed in the dark for 12 hours. Subsamples were taken every two hours and ammonium remaining in solution measured. Significant changes are indicated with an * (t-test, $p < 0.5$).

Ammonium uptake was measured for 12 hours under anaerobic conditions. The assay was stopped at 12 hours due to low viability of the cells in the later stages of anaerobiosis. Wild type and *hcp4* ammonium uptake diverge at 8, 10, and 12 hour timepoints. At 8 hours post anaerobiosis ammonium uptake in wild type and *hcp4* were 0.03 and 0.003 grams respectively. At 12 hours post anaerobiosis ammonium uptake in wild type and *hcp4* were 0.1 and 0.06 grams of ammonium respectively. The general trend in figure 10 shows relatively constant ammonium uptake in wild type cells. Ammonium uptake in *hcp4* did not take place until 12 hours post

anaerobiosis. Significant difference was calculated at 12 hours post anaerobiosis by a two tailed Students unpaired t-test with equal variance (p=0.012).

Discussion

C. reinhardtii has a uniquely diverse set of metabolic pathways that enable it to cope with a multitude of environmental situations including extended periods of anaerobiosis. Recent metabolic and genetic studies have uncovered known and unknown genes, pathways, and proteins that are up-regulated or activated during anaerobiosis (*2, 7, 13*). The multiple pathways facilitating the continuation of glycolysis during anaerobiosis make *C. reinhardtii* fermentation one of the most diverse in the plant world and have been shown to be plastic in nature (*42*). Being one of the most highly up-regulated genes during anaerobiosis in *C. reinhardtii,* HCP4 proves to be an interesting candidate for study as its physiological role in organisms has not been precisely determined, yet is activated during O_2 deprivation in many prokaryotes. This study shows that knockdown of HCP4 impacts multiple fermentation pathways which respond by changing gene transcription as well as metabolite flux and nitrogen uptake.

It was shown that HCP4 transcription in *hcp4* was decreased consistently during anaerobiosis. The -0.33 hour timepoint showed a 4 fold knockdown while one hour later post-anaerobiosis a 20-fold knockdown was achieved. This increase in knockdown levels across timepoints in *hcp4* compared to wildtype is most likely due to the rapid induction of HCP4 upon anaerobiosis as shown in Mus et al. 2007. The upregulation of HCP4 in wild type with the concurrent knockdown of HCP4 in *hcp4* will produce greater disparity in transcript levels the more upregulation there is. Following induction of anaerobiosis, knockdown rates of HCP4 are

consistent, although the highest knockdown rate was achieved one hour post anaerobiosis. This trend in knockdown levels may be due to accumulation of a small amount of mRNA escaping post transcriptional gene silencing via the amiRNA. Knockdown of HCP4 shows dramatic differences in expression of fermentation pathways. It is shown in Figure 5 that HYD and PFL transcripts were dramatically reduced after five hours of anaerobiosis but show no significant change in expression after three hours of anaerobiosis. PFR, which links pyruvate metabolism to ferredoxin, was shown to be consistently knocked down at 3 and 5 hours post anaerobiosis. PDC however were also dramatically knocked down at all timepoints. PDC transcript levels were measured at earlier timepoints -0.33 and 1 hour, and was similarly knocked down (data not shown). Taken together a general depression in fermentation pathway gene expression is noted.

Metabolite assays also showed a significant difference between wild type and *hcp4*. It is shown in Figures 6 and 7 that accumulation of ethanol and formate in the media began at three hours post anaerobiosis. Significant differences in ethanol and formate accumulation between wild type and *hcp4* appear at five hours post anaerobiosis. At three hours post anaerobiosis *hcp4* shows a ratio of ethanol to formate production of 4.2:1, neither metabolite show significantly different accumulation between *hcp4* and wildtype at this time. This 4.2:1 ratio of ethanol to formate production perhaps indicates that acetyl-CoA is being produced by both PFL and PFR and is being converted to acetate as well as ethanol. It is possible that PFR activity is still present even with the reduced transcript levels witnessed at this time point which contributes acetyl-CoA that is favorably converted to acetate. Interestingly, formate and ethanol accumulation are both roughly 2.6-fold higher in *hcp4* than wild type at five hours post anaerobiosis, displaying a 1:1 ratio of production. Similarily, ethanol and formate production in wild-type occur at a roughly 2:1 ratio at three hours post anaerobiosis and a ratio of 1:1 at five

hours post anaerobiosis. PFL facilitates the breakdown of pyruvate into formate and acetyl-CoA in a 1:1 ratio. If we assume that the down-regulation of PFR shown in *hcp4* effectively blocks the breakdown of pyruvate by this pathway, and the more than anticipated acetate accumulation during fermentation in the media is due to excretion of acetate accumulated during mixotrophic growth, then these data suggests that the ethanol produced in *C. reinhardtii* at five hours post anaerobiosis is facilitated exclusively by PFL in both wild type and *hcp4.* Conversely, at three hours post anaerobiosis the ratio of ethanol to formate suggest that PFR is actively converting pyruvate into acetyl-CoA while reducing ferredoxin or acetyl-CoA produced by PFL is being converted to acetate. This shift in metabolic flux happens precisely when metabolites ethanol and formate start to accumulate in the media. It seems possible that the accumulation of metabolites may act as a regulator during anaerobiosis, possibly being detected directly, or by secondary signaling molecules such as pH or oxidative stress. Taken together, a general model of the response of fermentation pathway gene expression and metabolic flux is presented in figure 11. At five hours post anaerobiosis there is a general decrease in fermentation gene transcription with a hypothesized increase in electron flux through the PFL and ADH pathways.

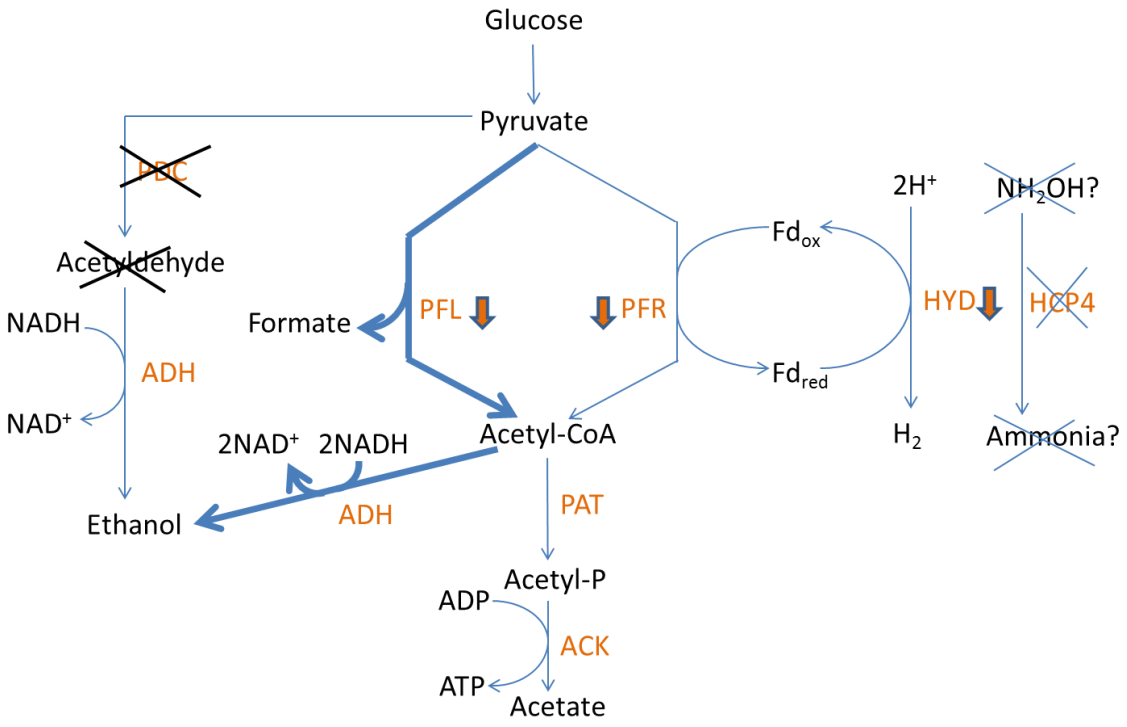

Figure 11. **Altered fermentation pathways upon HCP4 knockdown**. It was shown that in *hcp4* at five hours post anaerobiosis PDC, PFL, PFR, and HYD gene transcripts were lower than wild type. It is hypothesized that at five hours post anaerobiosis greater electron flux through PFL and ADH lead to greater formate and ethanol production in *hcp4*.

One of the most startling findings of this study was the drastic down-regulation of PDC. PDC is a cytoplasmic protein that is an integral component in yeast alcoholic fermentation. It has been shown that PDC does not participate highly in *C. reinhardtii* fermentation, but its activity and gene expression are both increased during anaerobiosis (*2, 42*). In yeast, PDC shows optimal activity at a pH 6.0 but only a maximal decrease of 7% was shown when resuspended at a pH of 7.4 which is close to the pH of this experiment (7.5) (*43*). PDC in maize is induced upon anaerobiosis only when cellular pH dropped from 7.4 to 6.8 (*44*). Expression data in *C.*

reinhardtii shows PDC up-regulation takes place in media at pH 7.0 and pH 7.3 (*2, 15*). Thus extracellular pH does not appear to similarly regulate PDC expression in *C. reinhardtii*. It is possible however that intracellular pH levels drop during anaerobiosis causing PDC to be activated. It is possible that an intracellular drop in pH is inhibited in *hcp*4 thereby inhibiting PDC transcription. A potential mechanism of HCP4 in controlling intracellular pH is currently unknown.

Acetate accumulation in wild type and *hcp4* were not significantly different in any timepoint and levels in both strains increased steadily throughout timepoints. Acetate levels were significantly higher than ethanol and formate which conflicts with previously published data stating the ratio of formate:acetate:ethanol should be roughly 2:1:1 or 2:2:1 (*10, 11*). While the data presented here offer a much different ratio of metabolic byproducts, differences in experimental procedures may account for this. In the Gfeller et al. and Ohta et al. studies cells were grown autotrophically using CO_2 as a carbon source. In this study however, acetate was used as a carbon source during mixotrophic growth. Thus when washed and resuspended in HEPES buffer before anaerobiosis, the cells likely contained high internal stores of acetate which may have been excreted during anaerobiosis.

HCP-family proteins have been implicated in nitrogen metabolism by studies in *E.coli* that show HCP up-regulation upon addition of nitrate or nitrite and also that purified HCP has hydroxylamine reductase activity (*28*). Hydroxylamine reductase catalyzes the reversible conversion of hydroxylamine to ammonia using ferredoxin as an electron donor (*29*). HCP4 in *C. reinhardtii* shows high amino acid sequence similarity with the *E.coli* HCP (which has demonstrated hydroxalamine reductase activity), with 41.7% identity and 59.1% similarity shown between the HCP4 and HCP from *E. coli* using Smith-Waterman local alignment (data not

shown). This study also shows a potential effect of HCP4 on nitrogen uptake during anaerobiosis. In this study *hcp4* showed increased nitrate uptake and decreased ammonia uptake compared to wild type. The effects of HCP4 knockdown were relatively weak in both cases, one out 10 timepoints in the nitrate uptake study showed significant difference, while one out of seven showed significant difference in the ammonium study. It was hypothesized by Aparacio (1985) that nitrite reductase requires ferredoxin to convert nitrite to ammonium. The data presented here shows nitrate uptake being enhanced which may indicate HCP4 is also competing with nitrite reductase for electrons from ferredoxin. It is interesting that significant changes in ammonium uptake were not present in *hcp4* until 12 hours post anaerobiosis. This lag in ammonium uptake may indicate that HCP4 has a primary role responsible for ammonium uptake in *C. reinhardtii* but secondary ammonium uptake mechanisms exist to cope with extensive nitrogen stress.

Taken together these data produce a preliminary model of HCP4's role in the cell during darkness-induced anaerobiosis. Taken from the high sequence similarity of HCP4 in *C. reinhardtii* to HCP in *E. coli,* and the increase in nitrate uptake when HCP4 is knocked down, these data support the hypothesis that HCP4 is oxidizing a reduced ferredoxin. With this working hypothesis, the other data collected in this experiment can be analyzed and a preliminary model produced. The knockdown of HCP4 causes PFR to be downregulated due to the reduced electron flow out of ferredoxin. Whether or not HCP4 competition for reduced ferredoxin has an impact on H_2 output is unknown. In this model HCP4 acts as a "release valve" for electrons from ferredoxin. A decreased reduction of pyruvate due to the depressed transcription of PFR may lead to a decrease of acetyl-CoA, which in turn would cause a decrease in NAD^+ and ATP production through the PAT/ACK or ADH pathways. This loss in NAD^+ and/or ATP production would be compensated for by the accumulation of PFL transcripts and an

increased production of formate and acetyl-CoA. The decrease in PFL transcripts at five hours post anaerobiosis could be explained by the increase in formate accumulation acting as a negative feedback to PFL gene transcription. PFL proteins present up to that point are still active. This hypothetical model displays the functional compensation of fermentation pathways in *C. reinhardtii* similarly shown in other studies (*42, 45*).

Future Directions

While it is clear that HCP4 knockdown affects the transcript levels of central fermentation pathways as well as the metabolite production in *C. reinhardtii,* further work is needed to fully characterize HCP4's role in anaerobic metabolism. Transcript abundance changes drastically upon knockdown of HCP4 but previous studies have shown that PFL and HYD are regulated not only at the transcriptional level but also are differentially activated post transcriptionally (*42, 45*). Further investigation of protein levels in wild type and *hcp4* would further guide understanding of the plasticity of these fermentation pathways. Quantifying H_2 production in *C. reinhardtii* in *hcp4* and wild type would further confirm or deny the hypothesis that HCP4 competes with HYD for electrons from ferredoxin, as well as the extent this mutation has in increasing the viability of H_2 production via *C. reinhardtii* at a commercial level. This assay could be performed by the relatively simple tungsten oxide/platinum screen developed by the National Renewable Energy Laboratory (NREL). This screen allows for hydrogen production to be qualitatively assayed and has been used as a screen in various forward genetic experiments (*42, 46*). Unfortunately the manufacturing process for this screen is patented and can only be obtained from the NREL, and is only produced at select times. Following this experiment a more global examination of metabolites (including quantification of H_2 production using mass-spec

and total starch breakdown) would generate the data needed to pinpoint HCP4's position in *C. reinhardtii* fermentation, as well as the potential HCP4 mutants have for increasing the production of other biofuels production. Furthermore, in vitro experiments with isolated ferredoxin and HCP4 could examine their interactions further, such as seen in Wolfe et al 2002 (*28*). The drastic downregulation of PDC was an unexpected and interesting finding. Although PDC and hydrogenase seem to have similar expression patterns (being upregulated during oxidative stress and anaerobiosis) their direct link is unclear. PDC expression is shown in other organisms to be dependent on intracellular pH. In this experiment cultures were heavily buffered at pH 7.5. It would be interesting to see whether intracellular or extracelluar pH changes upon knockdown of HCP4 in a less heavily buffered media. While HCP4 is most highly upregulated during anaerobiosis, it is also up-regulated during sulfur deprivation induced anaerobiosis. Sulfur deprivation induced anaerobiosis creates much more H_2 gas than darkness induced anaerobiosis, and if the hypothesis that HCP oxidizes reduced ferredoxin is supported than the increase in H_2 output could be drastically increased in these cultures. Therefore investigation of *hcp4* must be examined in these conditions as well to examine *hcp*4's potential impact on the economic feasibility of H_2 production in *C. reinhardtii*.

Significance of Study

This study demonstrates the *C. reinhardtii* fermentation metabolism is not only phylogenetically diverse and functionally plastic, but demonstrates that reverse genetic techniques can be applied to increase biofuel production. It was shown that down-regulating HCP4 causes a 2.6 fold increase in ethanol production and supports the hypothesis that HCP4 oxidizes a reduced ferredoxin. *C. reinhardtii* is considered a top candidate in the future production of biofuels such as ethanol, triacylglycerol, and H_2 gas. Extrapolating the data from this experiment into a

commercial setting assuming diurnal light/dark aerobic/anaerobic cycles, 500L pilot scale bioreactor, and five hours of anaerobiosis every darkness cycle, ethanol production in wild type *C. reinhardtii* would produce 3.275 Kg of ethanol daily, where *hcp4* would produce 8.625 Kg ethanol daily. This estimated commercial benefit is based on conservative estimates of cell density and bioreactor size based on scale up predictions by Melis et al 2001 (*47*). It is shown here that HCP4 plays an important role in anaerobic fermentation in *C. reinhardtii*. The increased ethanol and potentially increased H_2 production rates in this mutant may have sizeable benefits to mass production of biofuels in *C. reinhardtii*. Its potential role in nitrogen metabolism may also be an important avenue of future investigation.

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Supplemental material

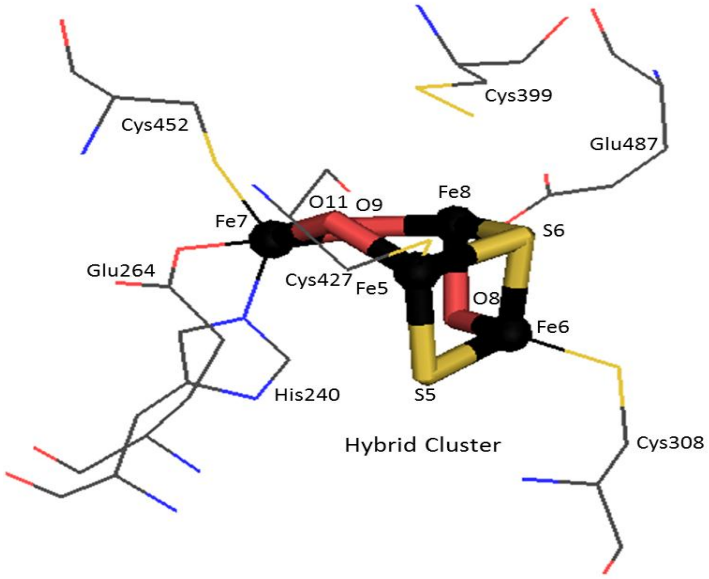
Supplemental table 1. **Primers used in RTPCR.**

Name	Protein ID	Sequence	Tm
Rack1	105734	Forward 5'-CTTCTCGCCCATGACCAC-3'	55.9°C
		Reverse 5'-CCCACCAGGTTGTTCTTCAG-3'	55.8°C
HYD1	183963	Forward 5'-GTCTATTCGCGGCAGCTC-3'	56.1°C
		Reverse 5'-TGCTGGACATGACTCAAAGG-3'	55°C
PFR1	122198	Forward 5'-GTCCGACGTGTCCTTCATCT-3'	56.6°C
		Reverse 5'-ACGGACATGACGTTGTTGAA-3'	54.7°C
PFL1	146801	Forward 5'-ATGTACGCGAACACCATGAA-3'	54.6°C
		Reverse 5'-GTCACCTGGGCGTACTTGAT-3'	54.7°C
PDC1	127786	Forward 5'-TACTCCACTGCCGGCTACTC-3'	58.6°C
		Reverse 5'-AGAGCCATGCGCTTGTAGAT-3'	56.6°C
HCP4	148255	Forward 5'-CCATGATGTGCTACCAGTGC-3'	56.2°C
		Reverse 5'-CCATGATGTGCTACCAGTGC-3'	54.1°C

Supplemental table 2. **amiRNA insert targeting HCP4.**

Forward 5’-
CTAGTGAGAGTAGTACGTTTAAAATATCTCGCTGATCGGCACCATGGGGGTGGTGGTGATCAGCGCTAT
ATTATAAACGTACTACTCTCG-3’

Reverse 5’-
CTAGCGAGAGTAGTACGTTTATAATATAGCGCTGATCACCACCACCCCCATGGTGCCGATCAGCGAGATA
TTTTAAACGTACTACTCTCA -3’



Supplemental Figure 1. **Image of HCP Cluster in Desulfovibrio desulfuricans.** Image is the "Hybrid Cluster" with coordinating amino acid residues. Atoms are colored: Fe black., S, Yellow., O, red. Image generated using Pymol software version 1.4.

Appendix

Along with presented data, progress has been made in other directions to uncover the links between fermentation pathways in *C. reinhardtii.* amiRNA vectors were assembled targeting two loci in the transcripts of PFL (Acession: AJ620191), HYD (Acession: AY055755) and one target for PDC (Accession XM_001703478) and one additional loci in HCP4. These amiRNAs were constructed in pChlami2 and pChlami3int. pChlami3int uses an enhanced promoter and improved selection marker (AphVIII) that confers broad spectrum antibiotic resistance. Various antibiotics were tested to find a reliable selection agent, but only paromomycin proved reliable in tests. *C. reinhardtii* transformed with PFL amiRNAs in pChlami2 was confirmed via sequencing but transcript levels during anaerobiosis did not indicate knockdown of PFL. pChlamiRNA3int vectors containing amiRNAs targeting PFL, HYD, HCP were sequenced and subsequently transformed into *C. reinhardtii*. After subjecting five strains of each transformant to the anaerobic induction protocol, only one potential PFL knockdown was identified. Dual transformation of C. reinhardtii using pChlami2 and pChlami3int vectors containing amiRNAs targeting a combination of PFL, PDC, HYD, and HCP4 were attempted, but viability of these lines were low and did not sustain extended periods of antibiotic selection. Transformation *C. reinhardtii* using agrobacterium using the protocol of Kumar et al was extensively tested but yielded no transformants (*48*). Sulfur free anaerobiosis induction was attempted in 1.9L Biostat A-Plus bioreactors but gas accumulation and cell survival proved unreliable.