Filling Knowledge Gaps in Biological Networks: integrating global approaches to understand H_2 metabolism in Chlamydomonas reinhardtii

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Abstract

The green alga *Chlamydomonas reinhardtii* (Chlamydomonas) has numerous genes encoding enzymes that function in fermentative pathways. Among these genes, are the [FeFe]-hydrogenases, pyruvate formate lyase, pyruvate ferredoxin oxidoreductase, acetate kinase, and phosphotransacetylase. We have systematically undertaken a series of targeted mutagenesis approaches to disrupt each of these key genes and 'omics' techniques to characterize alterations in metabolic flux. Funds from DE-FG02-07ER64423 were specifically leveraged to generate mutants with disruptions in the genes encoding the [FeFe]-hydrogenases HYDA1 and HYDA2, pyruvate formate lyase (PFL1), and in bifunctional alcohol/aldehyde alcohol dehydrogenase (ADH1). Additionally funds were used to conduct global transcript profiling experiments of wildtype Chlamydomonas cells, as well as of the *hydEF-1* mutant, which is unable to make H_2 due to a lesion in the [FeFe]-hydrogenase biosynthetic pathway. In the wildtype cells, formate, acetate and ethanol are the dominant fermentation products with traces of CO_2 and H_2 also being produced. In the *hydEF-1* mutant, succinate production is increased to offset the loss of protons as a terminal electron acceptor. In the *pfl-1* mutant, lactate offsets the loss of formate production, and in the *adh1-1* mutant glycerol is made instead of ethanol. To further probe the system, we generated a double mutant (*pfl1-1 adh1*) that is unable to synthesize both formate and ethanol. This strain, like the *pfl1* mutants, secreted lactate, but also exhibited a significant increase in the levels of extracellular glycerol, acetate, and intracellular reduced sugars, and a decline in dark, fermentative H_2 production. Whereas wild-type Chlamydomonas fermentation primarily produces formate and ethanol, the double mutant performs a complete rerouting of the glycolytic carbon to lactate and glycerol. Lastly, transcriptome data have been analysed for both the wildtype and *hydEF-1*, that correlate with our observed fermentative metabolites. Intriguingly, over half of the most differentially regulated genes are of unknown function.

Project description

Chlamydomonas reinhardtii is a model system for the study of eukaryotic photosynthesis and the generation of biofuels. Our laboratory is focused on understanding the mechanisms of H_2 photoproduction, and elucidating the metabolic underpinnings facilitating hydrogenase activity. It is known that *Chlamydomonas* cultures when acclimated to dark, anoxic conditions are capable of producing H_2. During anoxia, fermentative pathways are activated leading to the formation of products which include formate, acetate, ethanol, and H_2. Our earlier studies using microarray analysis demonstrated the transcriptional regulation of fermentative pathways during anoxic acclimation.

The overall goal of our research is to create a metabolic network model designed around ongoing experimental work in the eukaryotic green alga *Chlamydomonas reinhardtii*. This information will subsequently be leveraged in the rational optimization of phototrophic microorganisms for renewable bioenergy applications. The proposed research will provide a fundamental understanding of essential metabolic pathways in photosynthetic green algae and enable the rational engineering and optimization of those pathways. The research at CSM, in collaboration with the Carnegie Institute of Washington at Stanford University, is focused on the characterization of wildtype and mutant strains of *C. reinhardtii* using high throughput 'omics' based approaches to support computational modeling studies at the National Renewable Energy Laboratory (NREL). We are focusing our current studies on anaerobic acclimation in wildtype cells, and on the examination of mutants that are (a) unable to produce H_2 due to a disruption of the [FeFe]-hydrogenase maturation protein *HYDEF* (*hydEF-1* mutant), (b) lacking one of the two hydrogenases *HYDA2* (*hydA2* mutant), and (c) additional mutants that have been generated in fermentation pathways including: alcohol dehydrogenase (*adh1 mutant*), malic enzyme 4 (*mme4 mutant*), and pyruvate formate lyase (*pfl1 mutant*). These strains will be used to examine changes in the proteome, metabolome and transcriptome in *C. reinhardtii* during anaerobic acclimation. These studies will provide insights into metabolic adaptation strategies in mutants with specific anoxic pathways disrupted, and contribute to the development of a more informed model of algal metabolism.

Publications

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in *Chlamydomonas reinhardtii* mutants lacking PFL1 and both PFL1 and ADH1. *Plant Cell* **24**, 692-707.

Technical Progress Highlights:

Anaerobic Acclimation in *Chlamydomonas reinhardtii* - Both prokaryotic and eukaryotic photosynthetic microbes experience conditions of anoxia, especially during the night when photosynthetic activity ceases. In Chlamydomonas reinhardtii, dark anoxia is characterized by the activation of an extensive set of fermentation pathways that act in concert to provide cellular energy, while limiting the accumulation of potentially toxic fermentative products. Metabolite analyses, quantitative PCR, and high density Chlamydomonas DNA microarrays were used to monitor changes in metabolite accumulation and gene expression during acclimation of the cells to anoxia. Elevated levels of transcripts encoding proteins associated with the production of H_2, organic acids, and ethanol were observed in congruence with the accumulation of fermentation products. The levels of over 500 transcripts increased significantly during acclimation of the cells to anoxic conditions. Among these were transcripts encoding transcription/translation regulators, prolyl hydroxylases, hybrid cluster proteins, proteases, transhydrogenase, catalase, and several putative proteins of unknown function. Overall, this study uses metabolite, genomic, and transcriptome data to provide genome-wide insights into the regulation of the complex metabolic networks utilized by Chlamydomonas under the anaerobic conditions associated with H_2 production.

Pathway Flexibility When Hydrogenase Activity is Disrupted - 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 CO_2 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 O_2. 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.

H_2-Fermentative Metabolism in other algae - Several species of green algae use [FeFe]-hydrogenases to oxidize and/or produce H_2 during anoxia. To further define unique aspects of algal hydrogenase activity, the well-studied anaerobic metabolisms of *Chlamydomonas reinhardtii* were compared with four strains of Chlamydomonas moewusii and a *Lobochlamys culleus* strain. In vivo and in vitro hydrogenase activity, starch accumulation/degradation, and anaerobic end product secretion were analyzed. The *C. moewusii* strains showed the most rapid induction of hydrogenase activity, congruent with high rates of starch catabolism, and anoxic metabolite accumulation. Intriguingly, we observed significant differences in morphology and hydrogenase activity in the *C. moewusii* strains examined, likely the result of long-term adaptation and/or genetic drift during culture maintenance. Of the *C. moewusii* strains examined, SAG 24.91 showed the highest in vitro hydrogenase activity. However, SAG 24.91 produced little H_2 under conditions of sulfur limitation, which is likely a consequence of its inability to utilize exogenous acetate. In *L. culleus*, hydrogenase activity was minimal unless pulsed light was used to induce significant H_2 photoproduction. Overall, our results demonstrate that unique anaerobic acclimation strategies have evolved in distinct green algae, resulting in differential levels of hydrogenase activity and species-specific patterns of NADH reoxidation during anoxia.

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

Disruption of PFL1 and ADH1 - *Chlamydomonas reinhardtii*, a unicellular green alga, often experiences hypoxic/anoxic soil conditions that activate fermentation metabolism. We isolated three Chlamydomonas mutants disrupted for the pyruvate formate lyase (*PFL1*) gene; the encoded PFL1 protein catalyzes a major fermentative pathway in wild-type Chlamydomonas cells. When the *pfl1* mutants were subjected to dark fermentative conditions, they displayed an increased flux of pyruvate to lactate, elevated pyruvate decarboxylation, ethanol accumulation, diminished pyruvate oxidation by pyruvate ferredoxin oxidoreductase, and lowered H_2 production. The *pfl1-1* mutant also accumulated high intracellular levels of lactate, succinate, alanine, malate, and fumarate. To further probe the system, we generated a double mutant (*pfl1-1*

adh1) that is unable to synthesize both formate and ethanol. This strain, like the pfl1 mutants, secreted lactate, but it also exhibited a significant increase in the levels of extracellular glycerol, acetate, and intracellular reduced sugars and a decrease in dark, fermentative H_2 production. Whereas wild-type Chlamydomonas fermentation primarily produces formate and ethanol, the double mutant reroutes glycolytic carbon to lactate and glycerol. Although the metabolic adjustments observed in the mutants facilitate NADH reoxidation and sustained glycolysis under dark, anoxic conditions, the observed changes could not have been predicted given our current knowledge of the regulation of fermentation metabolism.