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George Weir graduated from UW Oshkosh in August 2009 with a degree in microbiology. In 2006 he participated in the NSF-REU summer research program at UW Oshkosh where he became interested in biotechnology and genetics. He was accepted to the biology/microbiology graduate program at UW Oshkosh where he will continue to work with Dr. Kallas on photosynthesis and gene expression profiling in cyanobacteria.

Kraig Short graduated from UW Oshkosh in December 2008 with a degree in microbiology. His research under Dr. Kallas began in spring 2008 as part of a biotechnology class. Biofuels have always been of interest to him, and his undergraduate research has shown him the numerous ways to explore energy sources. Kraig plans to attend graduate school at UW Oshkosh in spring 2010.

Dr. Toivo Kallas is a professor of microbial genetics and biotechnology and director of the Proteomics and Functional Genomics Core Facility in the Department of Biology & Microbiology. He received his Ph.D. from the University of Oregon and postdoctoral training from the Institut Pasteur and University of California Berkeley. His research concerns photosynthetic energy conversion and adaptive mechanisms of microalgae.

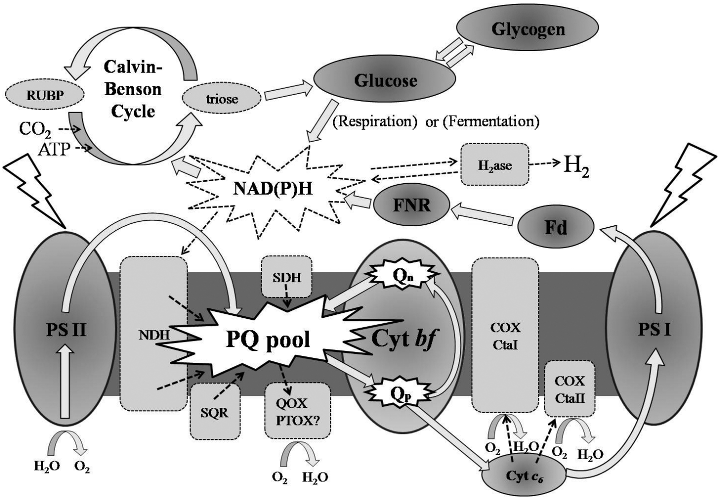
Abstract

In photosynthesis, microalgae such as cyanobacteria capture enormous amounts of solar energy and convert carbon dioxide (CO_2) into biopolymers that support life on earth and hold great potential for production of carbon-neutral biofuels. The photosynthetic process involves electron transfer reactions mediated by reaction center and cytochrome *bf* protein complexes. The *bf* complex also functions in regulation of photosynthesis by mechanisms that are not fully understood. We used the marine cyanobacterium *Synechococcus* PCC 7002 and mutants with genetically altered cytochrome *bf* complexes to investigate the role of this complex in regulation of adaptive gene expression responses. Native and mutant *Synechococcus* cultures were grown in CO_2-limited photosynthetic conditions, RNA molecules were extracted, and fluorescently labeled copies of these were hybridized to whole-genome microarrays (“gene chips”) to detect gene expression levels. Numerous genes were differentially expressed in the cytochrome *bf* mutants, illustrating the importance of the *bf* complex for gene regulation and adaptation. The research contributes to understanding the regulation of electron transport in globally important microalgae and to potential biofuels applications of these organisms.

Introduction

Cyanobacteria, once known as blue-green algae (Greek = κυανός [kyanós] meaning blue), account for ~50% of oceanic photosynthesis and overall ~25% globally (Partensky, Hess, & Vaulot, 1999). They have persisted on earth for ~3.5 billion years and are responsible for supplying the earth's atmosphere with oxygen (O_2). Cyanobacteria convert atmospheric carbon dioxide (CO_2) into energy-rich biomass and produce oxygen as a byproduct in photosynthesis, requiring only simple inorganic nutrients (CO_2, nitrogen, phosphorus, sulfur, and trace nutrients) for growth and survival. They are the evolutionary ancestors of plant chloroplasts and thus established photosynthesis as a novel and globally important mechanism for energy conversion that supports the abundant and vast biodiversity we see on our planet today (Grey, 1989).

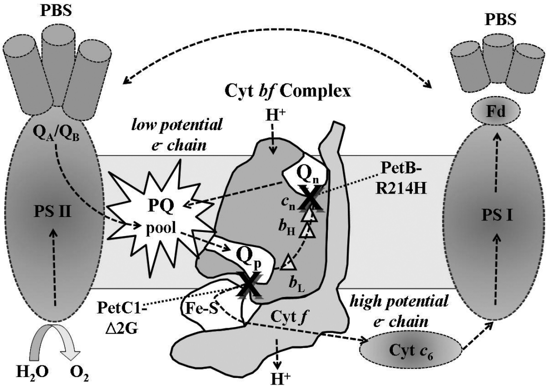
Figure 1
Cytochrome* bf *Complex at the Crossroads of Photosynthesis and Respiration



Note. Photosystems II and I (PS II and PS I), plastoquinone pool (PQ pool), cytochrome *bf* complex (Cyt *bf*), the two plastoquinone binding sites of the *bf* complex (Q_n and Q_p), NAD(P)H dehydrogenase (NDH), succinate dehydrogenase (SDH), sulfide-quinone oxidoreductase (SQR), quinol oxidase (QOX), cytochrome oxidase (COX), cytochrome c_6 (Cyt c_6), ferredoxin (Fd), ferredoxin-NAD(P)H oxidoreductase (FNR), bidirectional hydrogenase (H_2ase), hydrogen (H_2), sunlight (lightning bolts), carbon dioxide (CO_2), adenosine tri-phosphate (ATP), ribulose bisphosphate (RUBP), triose phosphates or glyceraldehyde-3-phosphate (triose). The dark region depicts the thylakoid membrane, and thick, light arrows indicate major electron transfer pathways.

The photosynthetic apparatus consists of photosystem II (PS II), photosystem I (PS I), and cytochrome (Cyt) *bf* protein complexes located within thylakoid membranes (see Figure 1). The light-harvesting phycobiliprotein (PBS) complexes (see Figure 2) capture sunlight, causing electrons to flow between the two photosystems via the plastoquinone pool (PQ pool) and Cyt *bf* complex (Blankenship, 2002). The electron transport chain between PS II and PS I consists of a series of oxidation/reduction (loss or gain of electrons) reactions, or "redox" reactions. Through such redox reactions, the Cyt *bf* complex generates a transmembrane gradient of hydrogen ions (H+ or protons) that drives adenosine triphosphate (ATP) synthesis to supply chemical energy for cellular processes (Kallas, 1994). Two electron transfer pathways occur within the cytochrome *bf* complex. Briefly, oxidation (loss of electrons), of plastoquinol from the PQ pool results in electron flow into both the high- and low-potential chains (see Figure 2). Mutations that impede electron transfer in either the high- or low-potential chains slow the turnover of the *bf* complex (Yan & Cramer, 2003; Nelson, Finazzi, Wang, Middleton-Zarka, Whitmarsh, & Kallas, 2005). However, impaired electron flow in the low-potential chain, as in our *Synechococcus* PetB-R214H mutant (Nelson et al.), results in electron transfer to O_2 and production of damaging oxygen radicals (Horn, 2005).

Figure 2
Electron Transfer Pathways and Mutations of the Cytochrome* bf *Complex



Note. Rieske iron-sulfur protein (Fe-S), plastoquinol oxidation site (Q_p), plastoquinone reduction site (Q_n), cytochrome *f* (Cyt *f*), b_L, b_H, and c_n hemes, phycobiliprotein light-harvesting proteins (PBS), ferredoxin (Fd), plastoquinone pool (PQ pool), plastoquinone sites of PS II (Q_A/Q_B). Dashed arrows indicate the Cyt *bf* high- and low-potential electron transfer pathways. PetC-Δ2G and PetB-R214H show the locations of these mutations in these pathways, and the black Xs show the sites of electron blockage. Arching dashed arrow illustrates the redistribution of PBS light-harvesting complexes mediated by redox-sensing and signaling by the Cyt *bf* complex.

The cytochrome *bf* complex also senses the redox level of electron transport and signals the redistribution of light-harvesting PBS proteins to adjust electron flow between PS II and PS I (Wollman, 2001) as illustrated in Figure 2. This regulation allows cyanobacteria to adjust their metabolism to changing environments. Moreover, the *bf* complex has been implicated in redox signaling of longer-term, adaptive gene expression events (Allen, 2004). In living organisms, the genetic information in deoxyribonucleic acid (DNA) is transcribed into messenger ribonucleic acid (mRNA) and then translated into the proteins that perform most biological functions. Therefore, knowledge of transcription (gene expression) provides ways to understand and manipulate biological processes. The electron transport pathways in photosynthesis that allow cyanobacteria to adapt to different environments are poorly understood. Such knowledge will be important for understanding the intrinsic biology of cyanobacteria and for applications such as the development of hydrogen or triacyglycerol (biodiesel) biofuels production strategies. We used high-density microarrays ("gene chips") and strains of the marine cyanobacterium *Synechococcus* PCC 7002 (Van Baalen, 1962) with genetically altered cytochrome *bf* complexes to investigate the hypothesis that the low- and high-potential chains of this complex play specific roles in signaling changes in gene expression. Mutants PetB-R214H (Nelson et al., 2005) and PetC1-Δ2G (Yan & Cramer, 2003) have impaired electron flow in the low- and high-potential chains, respectively. We show that perturbations of the cytochrome *bf* complex profoundly alter the expression levels of numerous genes.

Methods

Cyanobacterial Strains and Culture Conditions

Strains of the unicellular, marine cyanobacterium *Synechococcus* 7002 were grown as described by Nelson et al. (2005). Mutant PetB-R214H has a mutation in the cytochrome *bf* low potential chain causing slower turnover of the *bf* complex, slower growth (Nelson et al.), and overproduction of oxygen radicals (Horn, 2005). Mutant PetC1-Δ2G (Yan & Cramer, 2003) has a mutation in the *bf* high-potential chain, also causing slower turnover of the complex, only somewhat slower growth relative to the wild type, and no increase in oxygen radical production.

RNA Isolation, Purification, and Quantification

RNA molecules were extracted by a hot phenol method as in Brudler et al. (2003) with modifications. The method relies on rapid cell harvest and quenching of metabolism followed by extractions with phenol and chloroform, phase separations, and alcohol precipitation to remove contaminating proteins. DNA was removed from the resulting "crude RNA" preparation by means of Ambion® Turbo DNA-free reagents. RNAs were quantified by UV absorbance measurements at 260 nm (A_{260}) according to the formula 1.0 $A_{260} = 33\ \mu g$ RNA/mL.

Fluorescent Labeling and Dye Incorporation

Complementary DNA (cDNA) copies of RNAs were synthesized by reverse transcription (RT) from random primers and labeled with fluorescent tags by ChipShot™ Indirect Labeling reagents (Promega, Madison, WI). The RT reaction incorporated an aminoallyl uridine-5'-triphosphate (UTP) into the cDNA which was then covalently linked to Cy3 fluorescent dye. The frequencies of dye incorporation (FOI) and quantities of recovered cDNA were calculated by means of extinction coefficients for Cy3 dye at 550 nm and single-stranded DNA at 260 nm according to the manufacturer's instructions. FOI ranged from ~14–29 pmol dye per ng cDNA.

High-Density Oligonucleotide Microarrays and cDNA Hybridizations

Custom, 4-plex microarrays of *Synechococcus* 7002 were purchased from

NimbleGen®. These microarrays are synthesized by a massively parallel, "on-chip," DNA synthesis process that employs nanomirror arrays and light-activated catalysis (Singh-Gasson et al., 1999). Each 4-plex slide contains four microarrays (each 1 x 0.5 cm). Each microarray contains ~72,000, 60-mer oligonucleotide probes (short segments of *Synechococcus* DNA) with seven probes for most genes repeated three times on each array. In addition, each array contains ~6,000 high-density probes for upstream, untranslated (UTR) regions of ~100 selected genes. These UTR probes were designed to map RNA transcription start sites and regulatory regions of genes of interest.

In gene expression microarrays, labeled cDNA copies of mRNAs bind through complementary base pairing to corresponding, single-stranded probe DNAs on the microarray. The intensity of the fluorescent signal on each spot of the microarray is proportional to the amount of cDNA bound and therefore to the amount of mRNA in the sample and to the expression level of a particular gene. Microarray hybridizations were performed at the UW Madison Gene Expression Center. A four-chambered mixer was glued onto the 4-plex array slide to cover and separate each of the four microarray compartments on the slide. Labeled cDNAs (~800–1600 ng) from *Synechococcus* wild type, PetB-R214H, or PetC1-Δ2G mutants and alignment oligonucleotides in a final volume of 8.0 μL of hybridization buffer were applied to each array and incubated at 42°C. After ~16 hours of incubation, the mixing chamber was removed and unbound cDNAs were removed through a series of washes.

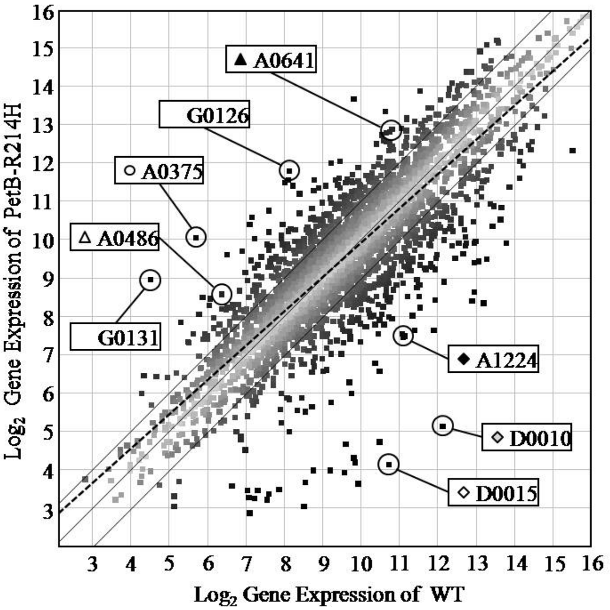
Microarray Scanning and Software

Following washes and drying, the microarray slide was immediately scanned at 5 μm resolution in an Axon GenePix™ 4000B scanner with 532 nm excitation light and Cy3 fluorescence emission detected at ~570 nm. Initial data processing was done with NimbleScan™ software. The four individual microarrays were aligned to each other by means of signals from the alignment oligonucleotides. The mean signal intensities of the four arrays were normalized via a Robust Multichip Average (RMA) algorithm (Irizarry et al., 2003) in the NimbleScan™ software. Data files containing normalized, mean expression levels for each of the ~3,000 genes of *Synechococcus* from the wild type and mutant datasets were exported for further analysis in ArrayStar® v3.0 software. ArrayStar® is a user-friendly microarray analysis software package that allows gene expression data to be linked to known or putative gene functions and has remarkable visualization graphics for data analysis.

Results

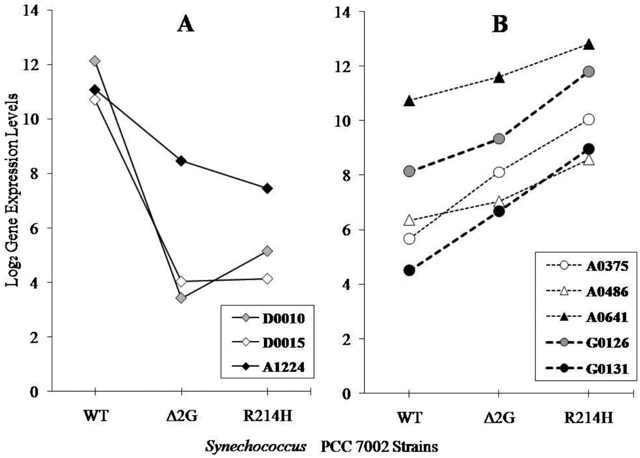
To investigate the impacts of the cytochrome *bf* complex mutations on gene expression, the *Synechococcus* PetB-R214H low-potential chain and PetC1-Δ2G high-potential chain mutant cultures and a wild type (control) were grown in CO_2-limited, photosynthetic conditions to mid-exponential phase (O.D.$_{750nm}$ ~0.5) and harvested for RNA extractions. Labeled cDNA copies of RNAs were hybridized to the NimbleGen® 4-plex microarrays as described in the Methods section. Nearly 800 genes in the PetB-R214H mutant and ~400 genes in the PetC1-Δ2G mutant showed 2-fold or greater difference in expression relative to the control (see Figure 3). More than 60 genes showed 8-fold or greater differences. Eight genes that may be of particular interest with respect to redox regulation or signaling are highlighted in Table 1 and Figures 3 and 4. Three of these genes (A0375, G0126, G0131) were up-regulated extensively in the PetB-R214H mutant and less so in PetC1-Δ2G (displayed in Figures 3 and 4, and Table 1 as circles). Two of these genes (A0486, A0641) were moderately up-regulated in both PetB-R214H and PetC1-Δ2G (displayed in Figures 3 and 4, and Table 1 as triangles). The final three genes (D0010, D0015, A1224) were significantly down-regulated in both PetB-R214H and PetC1-Δ2G (displayed in Figures 3 and 4, and Table 1 as diamonds). These data demonstrate that large numbers of genes were differentially expressed in response to perturbations either of the cytochrome *bf* complex low- and/or high-potential electron transport chains.

Figure 3
Scatter Plot of Gene Expression Levels in* Synechococcus *PCC 7002 PetB-R214H vs. Wild Type



Note. The *x*- and *y*-axes represent $\log_2$ gene expression values in the wild type and PetB-R214H cytochrome *bf* low-potential chain mutant, respectively. Each point represents 1 of the ~3,000 genes in the *Synechococcus* genome. Points outside of the solid lines show genes over- or under-expressed by ≥ 2-fold in PetB-R214H relative to the wild type control. The dotted line represents the best-fit line to all of the data points. Circles indicate selected genes described further in Table 1 and Figure 4 (the same gene symbols are used throughout).

Figure 4
Expression Levels of Selected Genes of Potential Interest



Note. The *y*-axis represents $\log_2$ gene expression values of selected genes in *Synechococcus* PCC 7002 wild type (WT) and the PetC1-Δ2G (Δ2G), and PetB-R214H (R214H) mutants. **Panel A**: Each line represents one gene (D0010, grey diamond; D0015, white diamond; A1224, black diamond) that was significantly down-regulated compared to the wild type. **Panel B**: Each line represents one gene (A0375, white circle; A0486, grey circle; A0641, black circle; G0126, white triangle; G0131, black triangle) that was significantly up-regulated compared to the wild type.

Table 1
Selected Genes Up- or Down-Regulated in Cytochrome **bf** *Mutants*

Gene symbol	Seq ID*	Fold change (+ or –)		Gene name**	Gene function
		R214H vs. WT	Δ2G vs. WT		
○	A0375	+ 21	+4	*cytM*	Cytochrome c_M
●	G0126	+13	+2	N/A	Hypothetical protein
●	G0131	+22	+4	N/A	Hypothetical protein
△	A0486	+5	+2	*rpaB*	Two-component response regulator (*ycf27*)
▲	A0641	+4	+2	*ompR*	Transcriptional regulator protein, LuxR family
◇	D0015	– 94	–101	N/A	Conserved hypothetical protein containing helix-turn-helix domain
◆	D0010	–126	–414	N/A	CobW/P47K family protein
◆	A1224	–12	–6	N/A	Response regulator receiver domain protein

*Seq IDs: abbreviated gene sequence identifiers (e.g., A0375 for SYNPCC7002_A0375) from the National Center for Biotechnology Information Web site (www.ncbi.nlm.nih.gov).

**Gene name, N/A: not available or unknown.

Discussion and Conclusions

Cyanobacteria flourish in diverse environments such as those of nutrient-deprived oceans, of extreme high or low light intensity, extreme temperatures, under desiccation, or in dense, anaerobic mats (Johnson et al., 2006). Each environment requires adjustments in metabolism and gene expression activities. In plant and algal chloroplasts, the cytochrome *bf* complex signals the redox-dependent redistribution of light-harvesting proteins between the photosystems (Wollman, 2001) and has been implicated in redox-regulation of gene expression (Allen, 2004). However, many aspects of the signaling mechanisms and the role of the *bf* complex in gene regulation remain unclear. In the current study, mutants of the marine coastal cyanobacterium *Synechococcus* PCC 7002 with impaired electron flow in the cytochrome *bf* low- (PetB-R214H; Nelson et al., 2005) and high-potential chains (PetC1-Δ2G; Yan & Cramer, 2003) were used to investigate the impact of these domains on global gene expression. Data presented here show that perturbation of electron transfer in the *bf* complex altered the expression of numerous genes (see Figures 3 and 4, and Table 1). These data support the hypothesis that the *bf* complex and particularly its low-potential domain play important roles in redox-signaling of gene expression.

The PQ pool and cytochrome *bf* complex occupy central positions at the intersection of photosynthetic and respiratory electron transfer chains in cyanobacteria (see Figure 1). The PQ pool becomes reduced through the activity of PS II during photosynthesis and by NAD(P)H (NDH) and succinate dehydrogenases (SDH) during darkness (Kallas, 1994). Because cytochrome oxidases (COX; see Figure 1) of cyanobacteria have low activities, the PQ pool becomes reduced during dark incubation. Under these conditions, NAD(P)H levels rise and anaerobic fermentation pathways may become active, including that for hydrogen production. Detailed understanding of electron flow through these pathways and the regulatory events mediated by the PQ pool and cytochrome *bf* complex will be important for understanding the intrinsic biology of cyanobacteria and for development of biofuels applications (Hu et al., 2008).

Our preliminary high-density microarray gene expression data show that mutations in the cytochrome *bf* complex altered the expression of hundreds of genes, including many that respond uniquely to perturbations of the cytochrome *bf* low- or high-potential electron transfer chains. Nearly 800 genes in the PetB-R214H mutant and more than 400 in PetC1-Δ2G showed 2-fold or greater differences in expression relative to the wild type control. Sixty genes in PetB-R214H and 34 in PetC1-Δ2G showed 8-fold or greater differences. We selected eight of these as examples of genes that may be targets or components of redox signaling based on the current data (see Table 1 and Figure 4). Three of these genes were up-regulated in both mutants but substantially more so in PetB-R214H. Gene A0375 (see white circles in Figures 3 and 4, and Table 1) encodes a cryptic cytochrome (Cyt c_M) protein and is widely distributed among cyanobacteria (Bialek et al., 2008). Cyt c_M donates electrons to a cyanobacterial respiratory cytochrome oxidase in vitro, but otherwise its function is unknown (Bernroitner et al., 2009). G0131 and G0126 encode hypothetical, membrane-spanning proteins. G0131 (see black circles in Figures 3 and 4, and Table 1) and G0126 (see grey circles in Figures 3 and 4, and Table 1) specify 21.5 kDa and 26 kDa proteins, respectively. The functions of these are completely unknown, but membrane proteins have possible roles in energy transduction and/or signaling.

Genes A0641 (see black triangles in Figures 3 and 4, and Table 1) and A0486 (see white triangles in Figures 3 and 4, and Table 1) were moderately up-regulated in both mutants. A0641 is related to known, LuxR family transcriptional regulators. A homolog of this gene, PedR in the cyanobacterium *Synechocystis* PCC 6803, may sense redox potential or electron flux on the acceptor site of PS I (Nakamura & Hihara, 2006). Gene A0486 is related to a 2-component response regulator (RpaB) involved in up-regulation of PS I genes at low light intensity in *Synechocystis* (Seino, Takahashi, & Hihara, 2009). Analysis of the *Synechococcus* 7002 A0641 and A0486 genes could help address whether signaling related to slowed Cyt *bf* turnover (occurring in both PetB-R214H and PetC1-Δ2G mutants) may be mediated in part by the redox state of acceptors "downstream" of PS I.

Genes D0010, D0015, and A1224 were dramatically down-regulated in both the low- (PetB-R214H) and high-potential chain (PetC1-Δ2G) mutants. D0010 (see grey diamonds in Figures 3 and 4, and Table 1) encodes a 41.6 kDa CobW/P47 protein involved in cobalamin (vitamin B_{12}) synthesis. *Synechococcus* 7002 requires vitamin B_{12} for growth. The steep down-regulation of this gene in both mutants suggests that its expression is regulated by increased reduction of the PQ pool or increased oxidation of carriers downstream of PS I, which should occur in both mutants. Gene D0015 (see white diamonds in Figures 3 and 4, and Table 1) encodes a 17 kDa hypothetical protein that has a helix-turn-helix domain and is thereby a putative transcriptional regulator. A1224 (see black diamonds in Figures 3 and 4, and Table 1) encodes a 15.4 kDa response-regulator receiver domain protein and is thus also a putative component of a redox signal transduction chain.

Overall, large numbers of genes in the cyanobacterium *Synechococcus* PCC 7002 were differentially expressed in response to mutations in the cytochrome *bf* complex low- or high-potential chains. These data support the hypothesis that many genes are regulated in response to the redox state of the cytochrome *bf* low-potential chain, the high-potential chain, or to overall slowdown of the complex. Differentially regulated genes include ones for several hypothetical proteins as well as putative sensor or response regulator proteins that may have roles in redox sensing and signaling. Data presented here establish the basis for detailed investigations of the impacts of electron transfer mutations and environmental conditions on global gene expression in *Synechococcus* PCC 7002, a marine cyanobacterium that efficiently converts solar radiation and inorganic carbon into biomass. Further studies will involve replicates of the conditions tested here to gain statistically robust data as well as tests of additional mutants and culture conditions to gain insight into mechanism of adaptation and potential biofuels applications. Genes identified in these studies will be potential targets for future mutagenesis and functional studies aimed at determining the roles of specific proteins.

Acknowledgements

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