Workshop Program

11th Workshop on Cyanobacteria
Washington University in St. Louis
August 7 – 11, 2013

Hosted by:

I-CARES
Welcome to the 11th Workshop on Cyanobacteria! We are very pleased with the great level of interest in this year’s Workshop and with the opportunity to cohost a joint session with the 2013 Light Harvesting Satellite Meeting.

**Organizing Committee:**
Himadri Pakrasi
Robert Blankenship
Beronda Montgomery
Louis Sherman
Teresa Thiel

**Local Organizing Committee:**
Gayle Geren
Chad Henry
Kathryn Woerheide

**Workshop Program Contents**

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2. Oral Presentation Abstracts
3. Poster Presentation Abstracts
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**Workshop Sponsors:**
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<td>5:30-6:30</td>
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<tr>
<td>7:00-7:10</td>
<td>Introduction: Himadri Pakrasi Welcome: Holden Thorp, Provost, Washington University²</td>
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<td>7:10-7:45</td>
<td><strong>Plenary I:</strong> Robert Haselkorn, The University of Chicago: <em>HetR Structure and Metabolism in Anabaena Heterocyst Differentiation</em> (Introduction by Himadri Pakrasi, Washington University)²</td>
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<tr>
<td>7:45-8:00</td>
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<td>8:30-9:50</td>
<td><strong>Genomics, Bioinformatics, and Diversity</strong> (Chair: Rajeev Aurora, Saint Louis University)²</td>
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<tr>
<td>8:30-8:50</td>
<td>Oded Beja, Technion: <em>Viral-inspired photosynthesis?</em></td>
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<td>8:50-9:10</td>
<td>Christian Beck, Humboldt University Berlin: <em>A daily temporal program for rhythmic expression of protein-coding and non-coding genes in response to light and dark in Synechocystis sp. PCC 6803</em></td>
</tr>
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<td>9:10-9:30</td>
<td>Linda Vuorijoki, University of Turku: <em>Establishment of high-precision, wide dynamic range proteomics for high-throughput protein quantification in cyanobacterium Synechocystis sp. PCC 6803</em></td>
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<td>9:30-9:50</td>
<td>Wei-Jun Qian, Pacific Northwest National Laboratory: <em>Broad Light-dependent Redox Regulation on Protein Thiols in Cyanobacteria Revealed by Quantitative Site-Specific Proteomics Profiling</em></td>
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<td>9:50-10:15</td>
<td>Break</td>
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<td>10:15-10:50</td>
<td><strong>Plenary II:</strong> Jonathan Zehr, UC Santa Cruz: <em>Nitrogen-fixing cyanobacterial associations in the sea</em> (Introduction by James Golden, UC San Diego)²</td>
</tr>
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<td>11:00-12:00</td>
<td><strong>Bioengineering in Cyanobacteria I</strong> (Chair: Wim Vermaas, Arizona State University)²</td>
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<td>11:00-11:20</td>
<td>Daniel Ducat, Michigan State University: <em>Regulation of Photosynthesis via Metabolic Sink Capacity in Sucrose-Secreting Cyanobacteria &amp; Engineered Microbial Autotroph/Heterotroph Consortia</em></td>
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<td>Hélder Miranda, Uppsala University: <em>Optimizing cyanobacterial biofuel production</em></td>
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<td>11:40-12:00</td>
<td>Gaozhong Shen, The Pennsylvania State University: <em>Pathway engineering for production and characterization of carotenoids in cyanobacterium Synechococcus sp. PCC 7002: canthaxanthin synthesis, assembly and function</em></td>
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<td>Lunch⁴</td>
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<td>1:30-3:45</td>
<td>Free Time</td>
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<td>3:45-4:00</td>
<td>Group Photo³</td>
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<td>4:00-6:00</td>
<td>Poster Session³</td>
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<td>Hualing Mi, Chinese Academy of Sciences: <em>Biochemical properties of NADPH dehydrogenase complexes from cyanobacteria</em></td>
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<td>Ipsita Dutta, Arizona State University: <em>Biohydrogen production from Synechocystis sp. PCC6803: significance of the diaphorase moiety in hydrogenase formation</em></td>
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<td>Break</td>
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<td><strong>Photosynthetic and Respiratory Metabolism IV</strong> (Chair: Tae Seok Moon, Washington University)²</td>
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<td>4:15-4:35</td>
<td>Rajib Saha, The Pennsylvania State University: <em>Comparative Genome-Scale Modeling of the Metabolic Potential of Cyanobacteria Cyanothece sp. ATCC 51142 and Synechocystis sp. PCC 6803</em></td>
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<td>Ralf Steuer, Humboldt University Berlin: <em>Computational models for cyanobacterial metabolism: Systemic properties of phototrophic growth</em></td>
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<td>5:15-6:30</td>
<td>Transfer to Missouri Botanical Garden</td>
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<td>6:30-10:00</td>
<td>Workshop Dinner at Missouri Botanical Garden</td>
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Saturday, August 10, 2013

7:00-8:15 AM  Breakfast

8:30-9:05  Plenary IV: Masahiko Ikeuchi, The University of Tokyo: *Color tuning in cyanobacterial life* (Introduction by David Kehoe, Indiana University)

9:05-10:25  Stress Responses I (Chair: Beronda Montgomery, Michigan State University)

9:05-9:25  LaDonna Jones, Indiana University: *A sulfur-depleted form of phycocyanin gives Fremyella diplosiphon a fitness advantage in low sulfur conditions*

9:25-9:45  Sara B. Pereira, Instituto de Biologia Molecular e Celular: *Cyanobacterial extracellular polymeric substances (EPS): production, export and interactions with metal cations*

9:45-10:05  Yvonne Zilliges, Freie Universität Berlin: *The role of glycogen and PHB metabolism in stress responses*

10:05-10:25  Christiane Funk, Umeå University: *Importance of Deg/HtrA proteases in Synechocystis sp. PCC 6803*

10:25-10:50  Break

10:50-11:50  Stress Responses II (Chair: Beronda Montgomery, Michigan State University)

10:50-11:10  Nir Keren, Hebrew University of Jerusalem: *Coordinated Transporter Activity Shapes High Affinity Iron Acquisition in Cyanobacteria*

11:10-11:30  Luis López Maury, Institute of Plant Biochemistry and Photosynthesis: *Genome wide copper responses in Synechocystis sp. PCC 6803*

11:30-11:50  Hans Matthijs, University of Amsterdam: *Hydrogen peroxide as a biocide for selective suppression of harmful cyanobacteria*

11:50-1:30 PM  Lunch

1:30-3:00  Poster Discussion I (Led by Julian Eaton-Rye, University of Otago and Himadri Pakrasi, Washington University)

3:00-3:15  Break

3:15-4:30  Poster Discussion II (Ferran Garcia-Pichel, Arizona State University and Peter Lindblad, Uppsala University)

4:30-6:00  Free Time / Poster Viewing

6:00-7:30  Dinner with Light Harvesting Satellite Meeting Participants

7:40-9:05  Joint Session w/ Light Harvesting Satellite Meeting

7:40-7:45  Introduction to Joint Session: Robert Blankenship, Washington University

7:45-8:05  Jack Chidgey, University of Sheffield: *A cyanobacterial chlorophyll synthase-HliD-YCF39-YidC complex links chlorophyll and protein biosynthesis*

8:05-8:25  Diana Kirilovsky, Institute of Biology and Technology Saclay: *Photoprotection by thermal dissipation of excess absorbed energy: The orange carotenoid protein (OCP) and the fluorescence recovery protein (FRP)*

8:25-8:45  Michelle Liberton, Washington University: *Probing the Consequences of Antenna Truncation in Cyanobacteria*

8:45-9:05  Noam Adir, Technion: *X-ray crystallographic and cryo-TEM structures of the phycobilisome photosynthetic antenna complex indicate functional plasticity*

9:05  Refreshments & Poster Viewing
**Sunday, August 11, 2013**

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<td><strong>Cell Development and Differentiation I</strong> (Chair: Jack Meeks, UC Davis)</td>
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<td>8:30-8:50</td>
<td>Loralyn Cozy, University of Hawaii: <em>Regulation of cell division by a caspase-like protein in Anabaena PCC 7120</em></td>
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<td>Jessie James II, University of Missouri-Saint Louis: <em>Transcriptional Regulation of the nifB1 and nifB2 genes</em></td>
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<td><strong>Cell Development and Differentiation II</strong> (Chair: Teresa Thiel, University of Missouri-St. Louis)</td>
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<td>9:50-10:10</td>
<td>Alicia Muro-Pastor, CSIC: <em>Non-coding RNAs in Anabaena sp. PCC 7120</em></td>
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<td>10:10-10:30</td>
<td>Andrian Gutu, Harvard University: <em>Concerted action of two clock-controlled histidine kinases times the activation of circadian factor RpaA</em></td>
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<td><strong>10:40-11:15</strong></td>
<td><strong>Plenary V</strong>; Susan Golden, UC San Diego: <em>How Cyanobacteria Tell Time</em> (Introduction by Louis Sherman, Purdue University)</td>
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<td><strong>11:15-12:00</strong></td>
<td><strong>Awards, Closing, and Next Meeting</strong></td>
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<td>12:00 PM</td>
<td>Meeting Adjourn</td>
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**Workshop Sponsors:**

1. Location: College Hall
2. Location: May Auditorium, Simon Hall
3. Location: The Gargoyle and Schoenberg Gallery, Mallinckrodt Center
4. Location: Multiple locations, see local information dining options
5. Location: Edison Theater, Mallinckrodt Center
6. Light Harvesting Meeting Poster Session Location: Rettner Gallery, Laboratory Sciences
Talk Abstracts in Program Order

**Plenary**
1. Robert Haselkorn, The University of Chicago: *HetR Structure and Metabolism in Anabaena Heterocyst Differentiation*
2. Oded Beja, Technion: *Viral-inspired photosynthesis?*
3. Christian Beck, Humboldt University Berlin: *A daily temporal program for rhythmic expression of protein-coding and non-coding genes in response to light and dark in Synechocystis sp. PCC 6803*
4. Linda Vuorijoki, University of Turku: *Establishment of high-precision, wide dynamic range proteomics for high-throughput protein quantification in cyanobacterium Synechocystis sp. PCC 6803*
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Plenary
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Stress Responses
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Cell Development and Differentiation
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44. Xudong Xu, Chinese Academy of Sciences: Coordination by master regulator of submaster and patterning factor genes in control of heterocyst differentiation
45. Jessie James II, University of Missouri-Saint Louis: Transcriptional Regulation of the nifB1 and nifB2 genes
46. Alicia Muro-Pastor, CSIC: Non-coding RNAs in Anabaena sp. PCC 7120
47. Andrian Gutu, Harvard University: Concerted action of two clock-controlled histidine kinases times the activation of circadian factor RpaA

Plenary
HetR Structure and Metabolism in *Anabaena* Heterocyst Differentiation

Haselkorn, R., Ke, S., Nasser, A.O., Gornicki, P., Kim, Y.-C., Joachimiak, A., Joachimiak, G., Callahan, S., Yi, Z.

Department of Molecular Genetics & Cell Biology, The University of Chicago; Department of Biochemistry and Molecular Biology, The University of Chicago; Structural Biology Center, Argonne National Laboratory; Department of Microbiology, The University of Hawaii; Institute of Hydrobiology, Wuhan, China

Specialized cells for nitrogen fixation, heterocysts, differentiate at regular intervals along filaments of *Anabaena* under conditions of nitrogen starvation. The initial signal for nitrogen need is the buildup of 2-oxoglutarate, which binds to NtcA, which, with PII, activates transcription of numerous genes. Among these is *nrrA*, whose product in turn activates transcription of the *hetR* gene. The HetR protein, whose ectopic expression can bypass the foregoing cascade, binds to several promoter regions and activates their downstream genes. HetR is both sufficient and necessary for heterocyst differentiation. The steady-state level of HetR is determined by transcription/translation and by stability, the latter regulated by two peptides synthesized in heterocysts and diffused along the filament. The early pattern of heterocyst placement is determined by PatS, a 19-amino acid protein that is probably processed to yield a 5- or 6-amino acid peptide, (E)RGSGR, that binds to HetR. Peptide binding interferes with DNA binding and exposes HetR to protease, leading to its degradation. The pattern is maintained by another protein, HetN, which contains in its interior the sequence RGSGR, shown to be necessary for its activity in controlling HetR function.

HetR is a dimer with four domains. Two N-termini rich in lysine, arginine and an important glutamate interact to form a helix-turn-helix responsible for most of the contacts with the palindromic target in *Anabaena* DNA. DNase footprinting shows that precisely 30 base pairs of the target DNA are protected by HetR, in agreement with the X-ray structure determination. Moreover, mutagenesis of individual residues in HetR, indicated as important binding partners, result in altered migration of protein/DNA complexes and inability of the altered HetR to complement deletion mutants in *Anabaena*. The structure of the HetR/DNA complex has many unusual features, perhaps the most striking of which is the interaction of a glutamate residue that forms hydrogen bonds with three successive cytosines in each arm of the palindrome. This interaction explains the extreme conservation of three GC pairs in the relevant palindromes of all filamentous, nitrogen-fixing cyanobacteria sequenced to date, along with the glutamate residue in every known HetR protein.
Viral-inspired photosynthesis?

Svetlana Fridman, Debbie Lindell & Oded Beja

Faculty of Biology, Technion- Israel Institute of Technology, Haifa 32000, Israel

Cyanobacteria play a key role in marine photosynthesis, which contributes to the global carbon cycle and to the world oxygen supply. Genes encoding PSII reaction centre proteins are found in many cyanophage genomes, and it was suggested that the horizontal transfer of these genes might be involved in increasing phage fitness. Recently, based on metagenomics, we reported evidence for the existence of phages carrying PSI gene cassettes. Moreover, using a combination of different marine metagenomic datasets and a unique cross-comparison between them, some phages were predicted to contain both PSII and PSI genes as well as NAD(P)H dehydrogenase genes.

Using phage concentrates from the Pacific Ocean, we have now isolated, with Prochlorococcus as the host, a cyanophage that contains a PSI gene cassette (psaC, A, B, K, E, D and a modified, fused psaJF gene), 2 PSII genes (psbA & psbD) and 2 NAD(P)H dehydrogenase genes (ndhi & ndhP). The presence of PSII and PSI genes in the same phage in combination with electron transfer proteins like NAD(P)H dehydrogenase strongly suggests they play a role in photosynthetic electron flow in the cyanobacterial host during infection. We therefore suggest that, depending on the physiological condition of the infected cyanobacterial host, the virus may use different options to maximize survival. That is, viral encoded PSI may alternate between functioning with PSII in linear electron transfer and contributing to the production of both NADPH and ATP or functioning independently of PSII in a cyclic mode via the NAD(P)H dehydrogenase complex and thus producing only ATP.
A daily temporal program for rhythmic expression of protein-coding and non-coding genes in response to light and dark in *Synechocystis* sp. PCC 6803

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**Background**

Many organisms, including photosynthetic cyanobacteria, harbor a circadian clock, a mechanism driving sustained gene expression oscillations with a period of roughly 24 hours, to adapt to day and night [1]. The core circadian clock of cyanobacteria consists of only three proteins, encoded by the genes *kaiA*, *kaiB*, and *kaiC* [2]. *Synechocystis* contains multiple *kaiB* and *kaiC* gene copies in addition to the standard *kaiABC* cluster. It has been unknown so far to which extent these might modify a robust circadian timing.

**Results**

We investigated *Synechocystis* high-density microarray data of three high-resolution time-series experiments with alternating light/dark rhythm, transition to continuous light, and transition to continuous darkness. Using 'least oscillating set' normalization and a clustering approach we found a daily temporal program for rhythmic expression of protein-coding and non-coding genes under light/dark conditions. Surprisingly, we observed a tremendous fluctuation in total RNA amounts, caused by an over-accumulation of larger, mainly ribosomal, RNA species during the night, and their sudden drop at subjective dawn. All rhythms, however, damped out rapidly under continuous conditions. We further discuss important examples for small RNAs exhibiting daily cycles and report cases of correlated and anti-correlated accumulation between *cis*-encoded antisense transcripts and their putative targets, including the *kaiABC* mRNAs.

**Conclusions**

The cyanobacterium *Synechocystis* shows a daily temporal program for rhythmic gene expression that is lost under continuous light or darkness. By integrating multiple Kai timing systems and small RNA regulators, circadian conditionality might be achieved resulting in damped cellular rhythms under conditions where a robust endogenous rhythmicity might be not advantageous.

Establishment of high-precision, wide dynamic range proteomics for high-throughput protein quantification in cyanobacterium Synechocystis sp. PCC 6803

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The precise quantification of proteins over a wide dynamic range, afforded by the recently developed Selected Reaction Monitoring (SRM) proteomics technique¹, has not yet been utilized for cyanobacteria. Earlier proteome quantification techniques commonly fail to reliably quantify low abundant proteins in complex sample mixtures at the expense of highly expressed proteins such as phycobiliproteins, rubisco subunits etc. In an untargeted shotgun approach, the MS/MS spectra from the precursor ion fragmentation can also have unspecific signals arising from co-eluting matrix ions of similar mass-to-charge ratios. This results in errors in quantification of certain peptides even between similar samples. Besides the unspecific signals caused by the deficient selectivity, the MS/MS spectra is retrieved only from the most intense precursor ions, rendering low abundant proteins undetectable.

In an effort towards enabling high-precision SRM quantification of proteins of interest, we are developing sensitive assays for approximately 100 protein targets of importance for the metabolism of Synechocystis sp. PCC 6803. This SRM-assay will allow accurate quantification of proteins using validated transitions derived from unique peptides treated as protein surrogates. The specificity and sensitivity of the method relies on the application of a triple quadruple (QQQ) mass spectrometer where first (Q1) and third (Q3) quadruples act as mass filters for precursor ion and its fragments, respectively. To implement the SRM method in Synechocystis sp. PCC 6803, we first carried out shotgun proteomics analysis which resulted in the identification of more than 1800 proteins in single extracts of S. 6803 (approx. 55% coverage of the whole proteome). This information was then used to build the SRM-assays with the Skyline software², which together with retention time information, allowed us to create a multiplexed quantitative method for 100 targets. Where possible, we chose 3 unique peptides per protein, one precursor ion per peptide and 3-5 fragment ions for each precursor ion. The collision energies were optimized for each transition to improve the detection limit. A label free approach is used as it provides relative quantification of proteins instead of the more expensive, absolute quantification obtained with AQUA heavy labeled peptides.

The establishment and application of high-precision targeted proteomics for cyanobacteria is of great significance for acquiring accurate information-rich network information. The method is expected to enhance the reliability, throughput and accuracy of protein quantification in cyanobacteria allowing an eventual replacement of traditional single-target immunodetection-based protein quantification. When combined with other advanced methods that probe the complete system³-⁴, there is an opportunity to expand our information base and close the gap between cyanobacteria and more extensively studied model organisms such as Saccharomyces cerevisiae and Arabidopsis thaliana.

Broad Light-dependent Redox Regulation on Protein Thiols in Cyanobacteria Revealed by Quantitative Site-Specific Proteomics Profiling

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Photosynthetic cyanobacteria have been demonstrated to be the hosts for the production of biofuel from solar energy and carbon dioxide. During photosynthesis, light has been known to modulate the redox state of the photosynthetic electron transport chain. Redox regulation through cysteine oxidation modulates the activation or inactivation of enzymes linked to photosynthesis systems I and II, thus regulating various target processes. In this study, we developed a novel proteomic approach for selective enrichment of oxidized thiols with quantitative isobaric labeling to quantify the extent of oxidation on individual thiols, as well as the redox dynamics of the thiol proteome under different conditions (light, dark, and photosystem II inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea)) in cyanobacteria Synechocystis sp. PCC6803.

In this study, the cells were cultured under continuous light, or shifted to darkness for 2 h, or exposed to DCMU for 2 h in light before harvesting. Cells were lysed and pelleted using 10% TCA. Free thiols were initially blocked with N-ethylmaleimide (NEM), and excess NEM was precipitated by cold acetone. The reversible oxidized cysteines (Cys) were reduced by DTT, and enrichment of Cys-proteins was carried out by using Thiopropyl Sepharose resin. Following on-bead tryptic digestion and on-resin isobaric labeling for relative quantification using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

The proteomics results show consistent increases in the levels of cysteine thiol oxidation in the dark compared to the light condition. DCMU inhibition leads to further increase in the level of oxidation. In total, redox changes were observed in ~2600 peptides from ~1000 proteins, indicating the broad light-dependent redox regulation in this organism. Moreover, the level of thiol oxidation for many important proteins (such as thioredoxin, photosystems I and II reaction center protein, and oxidoreductases) and many other types of enzymes changed in response to light, dark, or the DCMU treatment. These results are consistent with earlier findings that specific electron carriers on the photosynthetic electron transport chain are increasingly oxidized during the dark cycle than under light, and that the chemical disruption of the electron flow alters the redox state of the electron carriers. The observation of dynamic changes of redox states on individual cysteine residues provides important functional information as to their roles in redox regulation in photosynthesis and metabolism. In addition to establishing a new approach for quantifying redox dynamics, this work also provides novel information important to understanding the redox biology in the photosynthesis of cyanobacteria.
Nitrogen-fixing cyanobacterial associations in the sea

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Nitrogen fixation is an important source of fixed nitrogen in the open ocean. There are a number of planktonic symbioses between cyanobacteria and unicellular eukaryotic algae. Some cyanobacteria are symbionts with marine diatoms and range in their association from epibionts to endosymbions that live within the host frustule. Although the symbionts are closely related they have large differences in genomes, with internal symbionts having deletions in nitrogen assimilation and metabolism pathways, including ammonium transporters.

Unicellular uncultivated cyanobacteria were discovered initially from gene sequences, and ultimately characterized by metagenomic sequencing of flow cytometry sorted cells. These unusual uncultivated cyanobacteria lack PSII, Rubisco, the TCA cycle and other core metabolic pathways. A second strain, UCYNA-2, has recently been also concentrated by flow cytometry, and its genome is highly similar to that of UCYNA-1. This strain appears to be a coastal strain with a closely related, but different host.

Many questions yet remain and these symbioses are intriguing systems since they are formed between cyanobacteria and unicellular photosynthetic eukaryotes. These systems undoubtedly hold clues for the understanding the basis and evolution of nitrogen fixing systems, and may be useful for biotechnological applications.
Regulation of Photosynthesis via Metabolic Sink Capacity in Sucrose-Secreting Cyanobacteria & Engineered Microbial Autotroph/Heterotroph Consortia

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Appropriate topic area for presentation/poster: Bioengineering in Cyanobacteria

Abstract:

We have previously demonstrated efficient production of sucrose in \textit{Synechococcus elongatus} PCC 7942 through the heterologous expression of a symporter of protons and sucrose (cscB). These strains can tunably export up to 80\% of the carbon they fix as sucrose and can maintain production secretion continuously over long periods of time (> weeks). While exporting sucrose, these engineered cyanobacteria increase overall biomass production, exhibit higher Photosystem II activity, increase carbon fixation rates and exhibit greater cellular chlorophyll concentration. We hypothesize that enhanced photosynthetic activity may result from an increase in metabolic sink capacity for photosynthesis, resulting in regulatory feedback and transcriptional control over photosynthesis-related genes. Indeed, we observe increases in the mRNA levels of most subunits of Photosystem II 48 hours after the induction of sucrose secretion pathways. Here, we discuss ongoing efforts in the lab to identify the regulatory factors responsible for sensing and responding to sucrose export, as well as efforts to demonstrate generality of this feedback through the identification of novel sucrose transporters that exhibit similar efficiencies of sucrose efflux in \textit{S. elongatus}.

The overall rate of sucrose production from engineered cyanobacteria is high enough to represent a viable alternative to sugar synthesis from sugarcane, and holds the promise of being non-competitive with terrestrial crop species for arable landmass. However, recovery of dilute carbohydrates from large volumes of cyanobacterial growth media may represent an economic bottleneck in the scale-up of sugar-secreting strains. We describe our preliminary efforts to directly utilize this alternative feedstock source through the design of synthetic autotroph-heterotroph microbial consortia, including the model heterotrophic species \textit{Escherichia coli} and \textit{Saccharomyces cerevisiae}.
Global warming and the rising cost of fossil fuels are the two major concerns of present day. The future of our civilization depends on the development of alternative energy sources for a sustainable industrial society. Biofuels are neutral regarding CO₂ emission and pose to be an attractive alternative to fossil fuels. The first generation biofuels rely on carbohydrate sources from land-based feed stocks, competing with food crops in land usage and in the long run can cause a greater environmental cost, promoting deforestation, soil erosion and loss of biodiversity. Cyanobacteria are photosynthetic microorganisms and can be advantageous for biofuel production as they present a higher photosynthetic efficiency compared to plants and have simpler nutrient requirements. The developments in the field of genetics and synthetic biology opened many possibilities regarding genetic manipulation of cyanobacteria for direct conversion of CO₂ into biofuel using the phosphoryltransfer potential and reducing power created in the photosynthetic process. In order to optimize the production of high value chemicals one has to consider the many different metabolic processes occurring in the cyanobacterial cell and understand the energy and carbon flow associated with them. This presentation will address different approaches in the attempt to channel these flows for an optimal biofuel production.
Pathway engineering for production and characterization of carotenoids in cyanobacterium Synechococcus sp. PCC 7002: canthaxanthin synthesis, assembly and function

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Carotenoids play essential functions in photosynthetic light harvesting and photoprotection. Evolution of genes and pathways for carotenogenesis led to carotenoid diversity in different cyanobacteria, mostly related to their beneficial functions under different growth environments. Pathway engineering in cyanobacteria has begun to provide tools for studies of carotenogenesis. Application of such methods has also led to the development of valuable biotechnological approaches for overproduction of different carotenoids in the photosynthetic cells with either ketolated or hydroxylated β-carotene derivatives for commercial applications. Canthaxanthin has been shown to play important roles when cells were grown under the light or oxidative stress conditions because of its enhanced antioxidant activity and other beneficial effects. Utilizing the established pAQ1Ex gene overexpression system, the β-carotene ketolase (crtW) gene from Brevundimonas sp. was overexpressed in Synechococcus sp. PCC 7002. This resulted in the accumulation of canthaxanthin and also increased amounts of other carotenoids, such as echinenone and cryptoxanthin and significantly reduced levels of β-carotene. Based on low temperature fluorescence emission measurements, the exchange of β-carotene to canthaxanthin led to lower PS II levels in cells but had no obvious effect on PS I levels. HPLC analysis of the pigment extracts from purified PS I trimers confirmed that the newly synthesized canthaxanthin is assembled into the PS I reaction center complexes. Although levels of PS II were somewhat lower in cells, the canthaxanthin-producing strain exhibited a growth rate similar to that of wild type, especially under high-irradiance conditions. Compared to the wild type, the canthaxanthin-producing strain accumulated more cell mass because the strain grew to higher OD in the stationary growth phase, a useful property for biotechnological applications.
Analysis of Bacterial Microcompartment Domain-Containing Proteins for Assembly, Chimeric Incorporation, and Selective Permeability in Carboxysomes.

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ABSTRACT

The carboxysome is an essential part of the CO₂ Concentrating Mechanism (CCM) that cyanobacteria have evolved to ensure effective CO₂ fixation in ambient CO₂. It encapsulates RubisCO with carbonic anhydrase in a protein shell with an apparently icosahedral shape. The carboxysome is also an intriguing example of a proteinaceous macromolecular self-assembled structure, other than virus capsids. Previous studies elucidated that, in both alpha- or beta-subtypes of carboxysome, proteins contain a single bacterial microcompartment domain (BMC domain; pfam00936), CsoS1s or CcmKs, respectively, are the predominant building blocks for such self-assembled structures. These single BMC containing proteins form hexamers with a central pore, presumably to provide selective permeability, at the 6-fold axis, and they self-assemble into layers that are proposed to constitute the facets of a carboxysome shell. An interesting observation is that in all cases multiple copies of single BMC containing genes which share high similarities to each other co-occur within a genome. Whether or not these copies are equally crucial for the carboxysome assembly and/or function is an open question. Furthermore, despite the fact that CsoS1s and CcmKs belong to two distinct clades on the phylogenetic tree of the BMC domain, they share enough structural features that forming a hybrid shell is theoretically possible. To address these two questions we solved crystal structures of multiple shell proteins from both the alpha- and beta-carboxysome: CcmK2 and CcmK4 from the *Synechococcus elongatus* PCC 7942 (*Syn7942*) and CsoS1 from *Prochlorococcus marinus* str. MIT9313, performed a series of complementation experiments by utilizing a carboxysome-less mutant of *Syn7942* as background, and identified subcellular location of individual BMC protein by *in vivo* fluorescent labeling. The ultrastructural and physiological characterization of varied resulting strains and the interesting structural features revealed from three new crystal structures of both CsoS1 and CcmKs will be discussed. Given that the carboxysome and its architecturally-related bacterial organelles are promising targets for applications in synthetic biology, metabolic engineering, and therapeutics delivery, our results will facilitate the establishing of general guidelines for designing synthetic BMCs using building blocks from varied sources.
Photobiological Ethylene Production in Synechocystis 6803

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Ethylene is used in the production of plastics such as polyethylene, polystyrene, PET, and PVC as well as textiles such as polyester. It can also be polymerized into gasoline, diesel, or jet fuels, or hydrated to produce ethanol. Consequently, ethylene is the most widely produced organic feedstock globally. It is currently produced exclusively from fossil fuels, and its production is the largest CO$_2$- emitting process in the chemical industry. In this study, we demonstrate the feasibility of using the cyanobacterium Synechocystis sp. PCC 6803 to photosynthetically produce ethylene from CO$_2$. The efe gene encoding an ethylene-forming enzyme from Pseudomonas syringae pv. Phaseolicola was expressed in Synechocystis sp. PCC 6803, leading to continuous ethylene production. The same ethylene production rate was sustained across four successive sub-cultures without apparent loss of ethylene-forming ability. Up to 5.5% of the fixed carbon was directed to ethylene synthesis, surpassing the published carbon-partition rate into TCA cycle. Factors limiting ethylene production, including efe expression levels, light intensity and nutrient status, were identified and alleviated, resulting in a peak production rate of 24,600 µL L$^{-1}$ h$^{-1}$ (30,800 µg L$^{-1}$ h$^{-1}$, 1100 µmol L$^{-1}$ h$^{-1}$, or 739 mg L$^{-1}$ day$^{-1}$), which is higher than that reported for other algae biofuels and chemicals. Additionally, consistent productivity was maintained in a photobioreactor for 30 consecutive days from a single culture. Ethylene is gaseous so it does not require cell harvesting and oil extraction. It is not toxic to the host or a food source for contaminating microbes. Ethylene is recovered from gas streams containing CO$_2$ and O$_2$ using technology currently employed by the chemical industry. Consequently, bio-ethylene does not suffer from many of the technical difficulties that are associated with the production of other biofuels, making bio-ethylene an attractive alternative to petroleum and an effective way to reduce CO$_2$ emissions. Finally, our preliminary technoeconomic analysis suggests that if scaled, this production rate would lead to photobiological ethylene production below the current market price of ethylene. This study suggests that Synechocystis, expressing the modified efe gene has potential to be an efficient biological catalyst for the uptake and conversion of CO$_2$ to ethylene.


Production of isoprenoids in *Synechocystis* PCC 6803

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In recent years, there has been an upsurge of interest in the research and development of sustainable alternatives to fossil fuels. Using photosynthetic microorganisms, like cyanobacteria, as production systems for such alternatives is advantageous, since they can achieve direct conversion of carbon dioxide from the atmosphere into the desired product, using sunlight as the energy source.

Isoprenoids, or terpenoids, is a large family of compounds, including carotenoids, tocopherol, phytol, sterols and hormones. There are two biosynthetic pathways leading to the formation of isoprenoids; the mevalonic acid pathway, which operates in the cytosol of eukaryotes and in archaea; and the methyl-erythritol-4-phosphate (MEP) pathway, which is of prokaryotic origin and also present in plant plastids [1]. The MEP pathway converts pyruvate and glyceraldehyde-3-phosphate in a series of steps to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). IPP and DMAPP are the building blocks for the subsequent formation of all isoprenoids. In an earlier study, The unicellular cyanobacterium *Synechocystis* PCC 6803 was modified to produce the volatile five-carbon compound isoprene from DMAPP, a reaction catalysed by the enzyme isoprene synthase [2]. In the project presented here, we are interested in the possibilities for producing other potentially useful isoprenoids, as well as in further investigating the isoprenoid biosynthesis in cyanobacteria. *Synechocystis* PCC 6803 is a suitable model organism for such studies, due to the ease with which it can be genetically modified.

Squalene is a naturally occurring 30-carbon isoprenoid, which has commercial use in cosmetics and in vaccines. If it could be produced sustainably on a large scale, it could also be used instead of petroleum as a raw material for fuels and as feedstock for the chemical industry. In some bacteria, squalene is produced as an intermediate metabolite in the formation of hopanoids, a class of penta-cyclic isoprenoid molecules. *Synechocystis* PCC 6803 possesses a gene, *slr2089*, predicted to encode squalene hopene cyclase (Shc), an enzyme converting squalene into hopene, which is then further modified to form hopanoids.

By inactivation of *slr2089* (*shc*), we explored the possibility for squalene production by cyanobacteria, as well as the potential effects of squalene accumulation and role of hopanoids in cyanobacteria (see also abstract by B. Pattanaik) [3]. The inactivation lead to an accumulation of squalene in the cells. Effects of light intensity and growth stage on squalene accumulation were investigated. We did not observe any significant growth deficiency in the *shc* knock-out strain compared to the wild type *Synechocystis* PCC 6803, under normal growth conditions or under high light conditions, suggesting that hopanoids produced by the action of Shc are not crucial for growth or light induced stress tolerance under the conditions tested.

References
[2] Lindberg et al., 2010, Metab Eng 12, 70.
Harnessing the photosynthetic metabolisms of cyanobacteria allows for the direct conversion of CO₂ and light energy to products of interest. While cyanobacteria have the potential to produce high value and commodity chemicals, production at industrial titers will require increases in product tolerance and predictable control of gene expression. In particular, our group is focused on producing the organic acids 3-hydroxypropionate (3-HP) and 2-hydroxypropionate (lactate) in the model cyanobacterium *Synechococcus* sp. PCC 7002. 3-HP is a Department of Energy target molecule for production from biomass and can be used to produce acrylate, a bulk commodity chemical used in the production of paints, coatings, and plastics. Lactate is used as a food additive and can be used to produce biodegradable plastics. A genetic screen was used to identify mutants with increased organic acid tolerance, a novel counter selection system was developed using organic acid sensitivity, and synthetic biology tools were used to control gene expression and explore end-product transport. A genetic screen was used to identify mutants of PCC 7002 that had an increased tolerance to acrylate and 3-hydroxypropionate. These mutants were determined to have loss of function mutations in the gene *acsA*, which is annotated as an acetyl-CoA ligase. AcsA was shown to have activity towards acrylate and 3-hydroxypropionate. Deletion of *acsA* resulted in a 2,800-fold and 26-fold increase in tolerance to acrylate and 3-hydroxypropionate, respectively. The resulting strain had a 3-HP tolerance of 260mM (>26g/L), which could be used as a host strain for industrial 3-HP production. The observed connection between organic acid sensitivity and presence of a functional *acsA* led to the development of a counter selection method based on acrylate sensitivity. Recombination into the *acsA* locus and selection on medium containing acrylate resulted in the construction of homozygous mutants without the use of antibiotic resistance markers. Additionally, *acsA* was used to introduce genes elsewhere on the chromosome via counter selection. The application of this counter selection system allowed us to rapidly construct and test new synthetic biology tools for gene expression in PCC 7002. These tools were used to express malonyl-CoA reductase, an enzyme that converts malonyl-CoA to 3-HP, under IPTG induction in PCC 7002. Additionally, these tools were used to express lactate dehydrogenase along with organic acids transporters in PCC 7002. Currently, work is being done to improve our expression tools to maximize production and identify efficient transporters of 3-HP and lactate.
The unicellular diazotrophic cyanobacteria of the genus *Cyanothece* demonstrate oscillations in nitrogenase activity and H₂ production when grown under 12h light-12h dark cycles. We established that *Cyanothece* sp. PCC 7822 allows for the construction of knock-out mutants and our objective was to improve the growth characteristics of this strain and to identify the nature of the intracellular storage granules. We report on the physiological and morphological effects of reduction in nitrate and phosphate concentrations in BG-11 media on this strain. We developed a series of BG-11-derived growth media and monitored batch culture growth, nitrogenase activity and nitrogenase-mediated hydrogen production, culture composition, and intracellular storage content. Reduction in nitrate concentrations from 17.6 to 4.41 mM and phosphate concentrations from 0.23 to 0.06 mM improved growth characteristics such as cell size and uniformity, and enhanced the rate of cell division. Cells grown in this low NP BG-11 were less complex, a parameter that related to the composition of the intracellular storage granules. Cells grown in low NP BG-11 had less polyphosphate, fewer polyhydroxybutyrate ad cyanophycin granules, thus allowing many smaller granules to become evident. Biochemical analysis and transmission electron microscopy using a histocytochemical staining technique demonstrated that these small granules contained glycogen. The glycogen levels and the number of granules per cell correlated nicely with a 2.3 to 3.3-fold change from the minimum at the end of the dark period to the maximum at end of the light period. The differences in granule morphology and related enzymes between *Cyanothece* ATCC 51142 and *Cyanothece* PCC 7822 provide insights into the formation of large starch-like granules in some cyanobacteria. Research supported by a grant from US DOE Genomics:GTL program.
Metabolic flux analysis of unicellular cyanobacteria under nitrogen fixing and non-fixing mixotrophic condition

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Cyanobacteria are photosynthetic prokaryotes explored for the production of liquid as well as gaseous biofuels. *Cyanothece* sp. ATCC 51142 is a nitrogen fixing cyanobacterium, widely studied for its nitrogenase-dependent hydrogen production. Biomass yield and hydrogen production by *Cyanothece* 51142 is reportedly enhanced under mixotrophic conditions [1, 2]. A comparative study of the underlying metabolism under nitrogen-fixing and non-fixing condition will provide better understanding and will facilitate metabolic engineering for commercial exploitation of *Cyanothece* as biofuel producer. We have undertaken steady-state $^{13}$C metabolic flux analysis ($^{13}$C-MFA) of *Cyanothece* 51142, under nitrate sufficient and deficient mixotrophic conditions with $^{13}$C-labeled glycerol and $^{12}$C-labeled CO$_2$. Competitive utilization of the two carbon substrates was reflected in the fractional labeling patterns of proteinogenic amino acids, which was used to estimate the rates of reactions in the central carbon metabolism. The flux analysis suggested greater role of oxidative pentose phosphate pathway and TCA cycle in nitrate deficient cultures, as compared to the predominance of CO$_2$ fixation pathways in nitrate sufficient condition. These observations could be complemented with measured concentrations of reducing equivalents, intermediates such as 2-oxoglutarate, and storage metabolites like glycogen, in addition to published flux balance analysis predictions [3]. Thorough in-depth understanding of underlying metabolism will be the key towards developing *Cyanothece* as a platform for biofuel production.

References:


Carboxysomes are giant proteinaceous assemblies that serve as simple organelles for CO$_2$ fixation in cyanobacteria and some chemoautotrophs. The carboxysome is the founding member of a diverse class of metabolic organelles referred to collectively as bacterial microcompartments (MCP’s). The outer shells of metabolically distinct MCP’s share a related structure, being assembled from the same family of shell proteins. Structural studies on the proteins that form the shells of MCP’s have provided numerous insights into their architecture, evolution, and the mechanisms of molecular transport that support their metabolic functions. Biochemical and protein engineering studies have illuminated the mechanisms of molecular recognition that allow the lumenal enzymes to be directed to the inner surface of the protein shell, opening routes to engineering designer compartments. Comparative genomic studies have helped identify relatively unexplored types of microcompartments, while also highlighting certain genomic patterns associated with different microcompartment subtypes, including the carboxysome. Recent studies will be reported on proteins that are closely associated genetically with the alpha-type carboxysome, but whose functions have yet to be delineated.
Redox changes accompanying carbon deprivation in cyanobacterium 
*Synechocystis* sp. PCC 6803

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Oxygenic photosynthesis by cyanobacteria and plants is a primary source of CO₂ fixation in the biome. Cyanobacteria employ a system of carbon transporters to bring inorganic carbon (Ci) into the cell. These transporters are part of the carbon concentrating mechanism (CCM) that saturates the active site of Rubisco with CO₂ and out-competes the energetically wasteful oxygenase reaction. While some of these Ci transporters are constitutively expressed, others are induced when a cell experiences a lower carbon environment. This research explores the metabolic changes that accompany carbon limitation in order to further understand changes in cellular physiology and explore a model of metabolite control of CCM induction. Using chlorophyll fluorescence sampling of cells experiencing carbon limitation, changes in the redox state of the plastoquinone pool can be observed. It has long been known that Ci-limitation leads to an over-reduced plastoquinone pool. (Miller and Canvin 1989) However, the present experimental design allows for comparison of chlorophyll fluorescence and blue-green fluorescence (NADPH) simultaneously, allowing an examination of the light and dark photosynthetic reactions. Upon switching from carbon replete to carbon limited environment, intracellular concentrations of NADP⁺ are lowered. This is seen using in vivo blue-green fluorescence as well as through biochemical analysis of cell extracts. It is hypothesized that a carbon-limited Calvin cycle slows the utilization of NADPH causing an increase in intracellular NADP⁺. Carbonic anhydrase inhibitor ethoxyzolamide (EZ) was used to simulate carbon deprivation and resulted in a large increase in blue-green fluorescence during actinic light exposure. Biochemical analysis shows nearly complete reduction of the NADP pool. These findings point to a potential use of EZ as a tool in analyzing NADPH using fluorometry. These results provide evidence for a model of cyanobacterial sensing intracellular metabolites in order to alter gene transcription. Previous evidence has shown that the LysR-type transcriptional regulator CcmR (NdhR) has oxidized NADP and α-ketoglutarate as cofactor molecules. (Daley et al. 2012) This research elucidates a number of physiological changes that accompany carbon limitation and can be used to create a model of carbon limitation lowering internal NADP⁺ concentrations. This change is sensed by CcmR, leading to a de-repression of CCM genes and an increase in carbon transporter expression. These results can be important for future research into cell and systems biology and may provide preliminary evidence for metabolite control of CCM induction. Future experiments will be aimed towards biochemical analysis of CcmR interaction with its oxidized NADP ligand and its effect on transcription. Additionally, further understanding of NADPH fluorescence transients, which are still not well characterized, will be explored.

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From genome-scale modeling to multi-scale kinetic model of carbon metabolism for cyanobacteria

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In recent times cyanobacteria became increasingly important for applied research, which is reflected by the growing attention towards these organisms within the scientific community. Computational biology already plays a key role in understanding of cellular processes in cyanobacteria but studies analyzing the kinetics of metabolic regulation are missing. The main reason for this lack is related to the principal limitation of the so far employed methodology, the genome-scale modeling. This approach neglects the dynamics of metabolic regulation or the existence of isoenzymes, and suffers from errors in incomplete gene annotation. Therefore, the vast majority of exiting models for cyanobacterial metabolism cannot deal with the suboptimal metabolism under changing environmental conditions.

In order to advance our understanding of the metabolic regulation in cyanobacteria and to decipher the trade-off between optimal cell growth and metabolic regulation, we developed a multi-scale kinetic model of carbon metabolism for \textit{Synechococcus elongatus} PCC 7942. This model is constrained by metabolic and transcriptomic data, ATP/ADP ratio, CO\textsubscript{2} level and growth rate.

This model will be used to answer the following questions: 1) Are there differences in Calvin-Benson cycle regulation between cyanobacteria and higher plants, 2) Why is photorespiration essential for cyanobacteria (only for glycine and serine biosynthesis or are there more essential functions), 3) what are the costs and benefits of glycolate shunt, 4) Which regulatory role play isoenzymes during the shift to different carbon resources under different environmental conditions, and 5) Whether or not it is possible to develop a constraint-based estimation of absolute concentrations from relative metabolic concentrations.

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Insights into the function of type 2 NAD(P)H dehydrogenases in Synechocystis sp. PCC 6803

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Membrane bound bacterial pyridine nucleotide dehydrogenases, which are involved in electron transfer, can be divided into two classes: type 1 and type 2 NAD(P)H dehydrogenases (NDH-1 and NDH-2) [1]. NDH-2s are enzymes that contain a flavin cofactor but do not generate proton gradient across membranes like NDH-1 enzymes. NDH-2s are usually single polypeptides with molecular masses around 50 kDa and without any energy-transducing site [2]. These enzymes catalyze a two-step transfer of electrons from NAD(P)H to quinones. The primary structure of NDH-2 enzyme usually contains two GXGXXG motifs within β-sheet-α-helix-β-sheet structures for binding NAD(P)H and flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) [3].

There are three genes encoding NDH-2s in Synechocystis sp. PCC 6803: ndbA (slr0851), ndbB (slr1743), and ndbC (slr1484) [4], where NADH- and FAD-binding motifs are conserved [5]. NdbC protein forms also two transmembrane helices unlike NdbA and NdbB.

Even though NDH-2s are found from many different organisms, in many cases, the specific role of NDH-2s is unclear. Our results demonstrate that ΔndbA mutant grows more slowly than wild type (WT) and other Δndb-mutants in high CO2-conditions (3%) but there is no difference concerning growth rates in standard low CO2 growth conditions. The defect in the growth rate of ΔndbA in high CO2-conditions is mostly caused by problems in efficient energy transduction to PSII because of large number of detached phycobilisomes.

ΔndbC mutant grows similarly to WT in high CO2-conditions but in darkness the electron transfer chain of this mutant is more reduced compared to WT. This indicates that NdbC protein is most probably related to respiration. NdbC protein might also interact with NDH-1 complex because the M55-mutant missing NdhB-subunit does not express at all NdbC protein. In addition to this, mutants missing NdhD1/D2-subunits express NdbC distinctly less compared to WT.

It is becoming more and more evident that all three Ndb-proteins serve a different function. This is supported by observation that NdbB protein is not important under conditions studied here unlike NdbA and NdbC. Hence these enzymes are essential to be investigated in order to comprehensively understand the electron transfer properties in the thylakoid membrane of Synechocystis sp. PCC 6803.

Biochemical properties of NADPH dehydrogenase complexes from cyanobacteria

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Although NAD(P)H dehydrogenase complex subunit constituents and physiological functions have been reported in plants and cyanobacteria, the biochemical properties of the complexes are not clear. In this work, we obtained three NDH complexes with molecular weight of 170, 300 and 500 kDa respectively purified from the cyanobacterium *Thermosynechococcus elongatus* and *Synechocystis* PCC 6803. We studied biochemical properties of the complexes. The NDH complexes oxidized NADPH but not NADH. Furthermore, ferredoxin (Fd) and ferredoxin-NADP⁺ oxidoreductase (FNR) were co-eluted, implying the electron donation from NADPH to NDH via the interaction with FNR. The complexes had an optimal pH around 8.0 and a higher affinity for the plastoquinone (PQ) analogues, suggesting that PQ is a suitable electron acceptor for the NDH. Furthermore, the activity is competitively inhibited by rotenone, suggesting that it possesses a quinone binding site, similar to mitochondria complex I. Using a highly purified NDH of 500 kDa without any NADPH dehydrogenase activity, we demonstrated the interaction between Fd and the NDH complex.
Biohydrogen production from *Synechocystis sp.* PCC6803: significance of the diaphorase moiety in hydrogenase formation

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The bidirectional hydrogenase in *Synechocystis sp.* PCC6803 is a pentameric enzyme that catalyzes the reaction between protons and electrons to form hydrogen (\(2H^+ + 2e^- = H_2\)). Since the reaction is a reversible one, the directionality of the enzyme (i.e. whether it will produce or consume \(H_2\)) depends upon its substrate identity and concentration. The hydrogenase in *Synechocystis* consists of two moieties, the hydrogenase moiety and the diaphorase moiety. While the hydrogenase moiety is responsible for bringing the protons (\(H^+\)) from the surrounding to the catalytic center of the enzyme, the diaphorase moiety brings in the electrons from its natural redox partner NAD(P)H. Having NADPH as the electron donor makes the reaction equilibrium shifted towards \(H_2\) oxidation rather than \(H^+\) reduction. However due to the presence of a NAD(P) binding domain in the HoxF subunit of the Diaphorase, NAD(P)H is the inherent redox partner of the enzyme. In order to find out how significant the diaphorase moiety is for the assembly and functionality of the enzyme, the genes encoding the diaphorase moiety were deleted and the genes encoding the hydrogenase moiety were overexpressed. The expressions of the genes were confirmed by real time PCR (RT-PCR). The partially overexpressed enzyme showed hydrogen production in presence of reduced methyl viologen both in vivo and in vitro, indicating that the enzyme can be assembled and functional even without the presence of diaphorase moiety. Since NAD(P)H binding domain was removed from the enzyme, new path now can be opened to find a more suitable electron donor for the enzyme.
Biohydrogen production from photosynthetic organisms is a desirable biotechnological prospect because the inexhaustible solar energy supply is coupled to the generation of a clean biofuel. Cyanobacteria are considered good models for biohydrogen production because they are simple organisms with a demonstrable ability to produce H$_2$ under certain physiological conditions. However, they also present low H$_2$ production yields, a facile reversal of reaction towards H$_2$ consumption and high enzyme sensitivity to O$_2$, the very byproduct of their oxygenic photosynthetic metabolism. Therefore, we undertook a survey, to find a cyanobacterial strain in Nature that is inherently better at producing H$_2$. We studied 36 newly isolated cyanobacterial strains from terrestrial, freshwater and marine intertidal settings for the presence of bidirectional hydrogenases, and characterized the standard H$_2$-evolving assay in the presence of excess reductant. *Lyngbya aestuarii* and *Microcoleus chthonoplastes*, obtained from intertidal marine microbial mats, displayed higher rates, did not reverse the direction of the reaction, and, importantly reached much higher concentrations of H$_2$ at steady state compared to the rest of the strains. Because of their ecological origin in environments that become quickly anoxic in the dark, we hypothesized that this capacity may have evolved to serve a role in the fermentation of the photosynthate. In fact, when forced to ferment, these cyanobacteria displayed similarly improved characteristics in H$_2$ production compared to standard strains. Among them, *L. aestuarii* strain BL J displayed the fastest specific rates and attained the highest steady state H$_2$ concentrations during fermentation of photosynthate, which proceeded via a mixed-acid fermentation pathway to yield acetate, ethanol, lactate, H$_2$, CO$_2$ and pyruvate. Contrary to expectations, the H$_2$ yield per mole of glucose was only average compared to other cyanobacteria. Nevertheless, the high specific rates and H$_2$ concentrations coupled with the lack of reversibility of the enzyme, at the expense of internal, photosynthetically generated reductants, makes *L. aestuarii* strain BL J and/or its enzymes, a potentially feasible platform for large-scale H$_2$ production.
Phycobilisome Structure and association with photosystem II based on 3D reconstruction using single particle electron microscopy

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Phycobilisomes (PBS) are protein-pigment super complexes in cyanobacteria and red algae. They harvest light and transfer energy to photosystems efficiently. Most PBS are composed of two major parts: peripheral rods and cores. While phycobiliproteins are chromophore-containing proteins responsible for light absorption, linker proteins play very important roles in rod and core formation, in attachment of rods to the core and association of PBS to thylakoid membranes. Although structures of some individual phycobiliproteins are solved, the structure the entire PBS complex remains to be solved. Here, we report the structure of entire PBS from Anabaena sp. PCC 7120, by 3D reconstruction using single particle electron microscopy. The PBS has a five cylinder core and 8 peripheral rods. We constructed various mutants lacking cpcG genes and demonstrated which individual cpcG gene is responsible for attachment of different rods to the core. Available crystal structures of PBPs trimers and linker proteins were docked onto the density map, building up an intact near-atomic model of the large PBS complex. We also obtained PBS-PSII complex particles and it is shown that the extruding trimer of the core with the terminal emitter at the bottom of PBS is tightly associated with a hole which is on the stromal side of PSII complex. We will describe the global distribution of chromophores in entire PBS and discuss its implication on light energy transfer pathway within PBS and to PSII.

**Keywords:** Cyanobacteria, Phycobilisome structure, Single particle electron microscopy, Photosystem II

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Phycobilisomes Feed Both Photosystems in One Megacomplex in 
*Synechocystis* sp. PCC 6803, a cyanobacterium

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Abstract:
Photosynthesis starts with the light energy from being absorbed by light harvesting antenna complex (LHC) and ends up invested in energy-rich sugars for life and in the extant fossil fuels for modern civilization. In cyanobacteria and red algae, phycobilisomes (PBS) absorb energy inaccessible to chlorophyll molecules from solar spectrum and transmit it to chlorophyll \(a\) in the reaction centers where photochemistry occurs. Although spatial orientations of chromophores in phycobilisomes and chlorophylls in reaction centers are essential for the efficient energy transfer, the exact interactions of the phycobilisome subunits with reaction centers are as yet unclear. Here, we have isolated a megacomplex composed of a phycobilisome with its energy acceptors, i.e., Photosystem I (PSI) and Photosystem II (PSII) after in vivo chemical cross-linking reactions. Spectroscopic analyses indicated that upon excitation, the phycobilisome transfers its energy to both PSI and PSII through close association of ApcD and PsaA, and ApcE and CP43, respectively. Ultrafast time-resolved fluorescence spectroscopy elucidated the detailed kinetic energy transfer. Our study has identified such structural and functional module. Thus, not only assembly and dissociation of PBS-PSI-PSII will regulate the phycobilisome-reaction centers’ energy distribution and energy balance of two photosystems, but also fine tuning of the interactions of three complexes within the megacomplex, will elegantly adjust the mode of photosynthetic electron flow adapting to the ecophysiological conditions.
Flavodiiron proteins Flv4 and Flv2 co-operate with light-harvesting antenna to enhance PSII photoprotection in Synechocystis sp. PCC6803

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Oxygenic photosynthesis was born with cyanobacteria, the ancestors of plant chloroplasts. The highly oxidizing chemistry of water splitting required concomitant evolution of efficient photoprotection mechanisms to keep the components of the photosynthetic machinery functional despite strong oxidative stress. Only recently, the role of A-type flavodiiron proteins (FDPs) in this context has been appreciated. FDPs are enzymes involved in electron transfer widely present in anaerobic bacteria but the appearance of oxygenic photosynthesis in cyanobacteria concomitantly induced unique modifications in FDPs. In Synechocystis there are four FDP genes: flv1 (sll1521), flv2 (sll0219), flv3 (sll0550) and flv4 (sll0217). In particular, Flv2 and Flv4 are encoded by an operon together with another small protein, Sll0218, and their expression is tightly related to the air level CO₂ conditions. The Flv2 and Flv4 proteins form a heterodimer which is localized in cytoplasm but also has a high affinity to membrane in the presence of divalent cations. Sll0218 resides in the thylakoid membrane in association with yet unidentified high molecular mass protein complex.

Here we investigated the effects of overexpression of the flv4-2 operon (flv4-2/OE) in Synechocystis and the relationship of the operon-encoded proteins with phycobilisomes (PBS). Overexpression of the flv4-2 operon resulted in an improved photochemistry of PSII. The flv4-2/OE mutant was more resistant to photoinhibition and exhibited a more oxidized state of the plastoquinone pool when compared with the control strain, with a consistent reduced production of singlet oxygen. Further, flash induced fluorescence decay and oxygen evolution measurements performed with different electron acceptors provided evidence that the operon functions in an alternative electron transfer pathway from PSII and alleviates excitation pressure by channeling up to 30% of electrons from PSII to Flv2/Flv4 heterodimer. Moreover regular PBS are required for a stable expression of the Flv4, Sll0218 and Flv2 proteins, suggesting that a non-distorted energy transfer from PBS to PSII is required for the flv4-2 operon photoprotection mechanism.

In conclusion, Flv2/Flv4 mediated electron transfer acts as a safety valve for PSII, in condition of high excitation pressure (such as air level CO₂ conditions and high light intensities) and requires a correct energy transfer from PBS to the PSII reaction centres.

Role of cyclic di-GMP signaling in light-dependent responses in cyanobacteria.

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Microorganisms use a variety of metabolites and signaling molecules to respond to external signals. Phenotypic changes can be accomplished by second messengers, nucleotide-based molecules that often amplify first messenger signals and rapidly elicit biochemical or physiological changes in a cell. The most common second messenger in bacteria is perhaps cyclic dimeric GMP (cyclic di-GMP or c-di-GMP). Levels of c-di-GMP play critical roles in regulating several cellular processes, among which are biofilm formation and cellular motility. Cyclic di-GMP signaling systems have been mainly characterized in pathogenic bacteria; however, these systems remain largely unexplored in cyanobacteria. In cyanobacteria, many putative c-di-GMP synthesis or degradation domains are found in genes that also harbor light-responsive signal input domains, suggesting that light is an important signal for altering c-di-GMP homeostasis. Indeed, such domains are the second most common output domain in photoreceptors – only outnumbered by a histidine kinase output domain. Cyanobacteria differ from other bacteria regarding the number and type of photoreceptor domains associated with c-di-GMP domains. Bioinformatics analyses of available genomes have established that the presence of c-di-GMP modulating domains reflects environmental characteristics and that these domains are extensively spread in the genomes of cyanobacteria. We are conducting functional explorations into the photoregulation and regulatory roles of c-di-GMP homeostasis in two cyanobacteria, \textit{Fremyella diplosiphon} and \textit{Synechocystis sp.} PCC 6803. These two organisms displayed different intracellular c-di-GMP concentrations under blue, green, and red light. We suggest that c-di-GMP could be associated with physiological adaptations attributed to different light quality such as biofilm formation and morphology changes. Our research is exploring the importance of environmental light fluctuations on the functions of c-di-GMP modulating-domain proteins linked to photoreceptor domains and the intracellular photoregulation of c-di-GMP levels in cyanobacteria.
Mapping photoautotrophic metabolism of cyanobacteria by isotopically nonstationary $^{13}$C flux analysis

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The development of fuel substitutes from renewable resources has become an urgent need as a result of current global energy and environmental problems. Recent studies have demonstrated the feasibility of converting energy from sunlight and carbon from CO$_2$ directly into biofuels using photosynthetic microorganisms. Despite the advances made in cyanobacterial biofuels production, the productivities achieved by cyanobacterial fermentations are currently too low for industrial feasibility and few tools are available that specifically address the challenges of redirecting and enhancing metabolic flux in photosynthetic microbes.

The ability to perform quantitative studies using isotope tracers and metabolic flux analysis (MFA) is critical for accurately assessing in vivo regulation of photoautotrophic metabolism, as well as crucial to identifying pathways that will maximize carbon flux from Calvin cycle intermediates into biofuel-producing pathways. Although $^{13}$C is the preferred isotope tracer for mapping central carbon metabolism in heterotrophic organisms, photoautotrophs assimilate carbon solely from CO$_2$ and therefore produce a uniform steady-state $^{13}$C-labeling pattern that is insensitive to fluxes. However, transient measurements of isotope incorporation following a step change from labeled to unlabeled CO$_2$ can be used to map carbon fluxes under autotrophic growth conditions. This involves quantification of intracellular metabolic fluxes based upon computational analysis of dynamic isotope labeling trajectories, which relies on the technique of isotopically nonstationary MFA (INST-MFA). Our group has developed experimental protocols and computational tools that enable INST-MFA to be applied to study autotrophic networks of realistic size and complexity.

To establish proof of concept, we recently applied $^{13}$C INST-MFA to quantify intracellular fluxes in the cyanobacterium *Synechocystis* sp. PCC 6803, a model photosynthetic organism, under photoautotrophic conditions (1). This involved using both GC-MS and LC-MS/MS to quantify the trajectories of metabolite labeling that result from introduction of $^{13}$C-labeled bicarbonate. Comparison of the INST-MFA flux map to theoretical values predicted by a linear programming (LP) method revealed inefficiencies in photosynthesis due to oxidative pentose phosphate pathway and malic enzyme activity, despite negligible photorespiration. Our ongoing work is focused on applying the INST-MFA approach to several engineered strains of *Synechococcus elongatus* PCC 7942 to enable the systematic improvement of cyanobacterial production of isobutyraldehyde (IBA, a direct precursor of isobutanol). Quantification of photosynthetic carbon fluxes in cyanobacteria will pinpoint pathway bottlenecks that can be subsequently removed in further rounds of metabolic engineering, thus leading to maximal IBA productivity by redirecting flux into IBA-producing pathways.

Comparative Genome-Scale Modeling of the Metabolic Potential of Cyanobacteria *Cyanothece* sp. ATCC 51142 and *Synechocystis* sp. PCC 6803#

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A number of trail-blazing metabolic models of cyanobacterial systems have been developed over the past decade. While these models have offered valuable insights into cyanobacterial biology, they remain incomplete in many ways: 1) They do not cover the full range of essential metabolism, 2) The descriptions of biomass used in these models are incomplete and/or derived from measurements of unrelated strains, and 3) The metabolic reactions contained within these models are not directly associated with genes and proteins and are often not elementally and charge-balanced. In addition, the similarities and differences between cyanobacterial metabolisms have not been addressed through comparative modeling.

Here, we assemble, update and compare genome-scale models (*iCyt773* and *iSyn731*) for two phylogenetically related cyanobacterial species, namely *Cyanothece* sp. ATCC 51142 and *Synechocystis* sp. PCC 6803. While *Cyanothece* 51142 is a potent unicellular diazotroph that produces large amounts of H₂ photosynthetically, *Synechocystis* 6803 is the first sequenced photosynthetic organism and is a workhorse organism for cyanobacterial synthetic biology. In our models, all reactions are elementally and charge balanced and localized into four different intracellular compartments (i.e., periplasm, cytosol, carboxysome and thylakoid lumen) and biomass descriptions are derived based on experimental measurements of these two strains. We have added new reactions absent from earlier models (266 and 322, respectively) that span most metabolic pathways with an emphasis on lipid biosynthesis. All thermodynamically infeasible loops are identified and eliminated from both models.

Comparisons of model predictions against gene essentiality data reveal a specificity of 0.94 (94/100) and a sensitivity of 1 (19/19) for the *Synechocystis* *iSyn731* model. The diurnal rhythm of *Cyanothece* 51142 metabolism is modeled by constructing separate (light/dark) biomass equations and introducing regulatory restrictions over light and dark phases. Specific metabolic pathway differences between the two cyanobacteria alluding to different bio-production potentials are reflected in both models. Additionally, we have applied these models to identify targets for metabolic engineering of *Synechocystis* 6803 to overproduce biofuel molecules including isoprene and n-alkanes.
Mapping cyanobacterial metabolism using metabolomics

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Cyanobacteria play an important role in global carbon cycling as primary producers in diverse environments. We have previously used untargeted mass spectrometry-based metabolite profiling to identify a number of unexpected metabolites and metabolic capabilities (uptake or release of metabolites) in a model unicellular cyanobacterium *Synechococcus* sp. PCC 7002. These metabolites and metabolic capabilities were classified as unexpected because their presence was not predicted from the genome’s annotation or, in multiple cases, also not accounted for in databases of metabolism (MetaCyc or KEGG) [1,2]. In order to link these metabolic capabilities to specific genes, we performed metabolite profiling on ten different strains of cyanobacteria which genomes were sequenced as part of the CyanoGEBA project [3]. We detected the presence of metabolites of interest (e.g. histidine betaine and derivatives, diverse oligosaccharides) only in subsets of analyzed cyanobacteria. These results along with available genome sequences serve as the basis for the identification of corresponding candidate biosynthetic genes. In addition to metabolite profiling we also performed stable isotope probing experiments to measure the degree of turnover of intracellular metabolites. We found that some metabolites are turned over in correlation with biomass growth while others are turned over extensively over shorter periods of time. These results provide an overall view of the carbon flow in the cell as well as point to suitable starting points for engineering heterologous pathways for biotechnological applications.

Computational models of cyanobacterial metabolism: Systemic properties of phototrophic growth.

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Cyanobacteria are the only known prokaryotes capable of oxygenic photosynthesis. Due to their capability to utilize atmospheric CO₂ for the synthesis of organic carbon compounds, the manifold biotechnological applications of cyanobacteria are at the forefront of current global challenges. The domestication of phototrophic microorganisms, such as cyanobacteria, to benefit humankind therefore remains one of the grand challenges of the 21st century. One step towards such a domestication of cyanobacteria are integrated experimental and computational approaches to understand the functional properties of phototrophic growth. The focus of this contribution is to describe the construction of computational models of cyanobacteria and the analysis of such models using kinetic and constraint-based methods. Specifically, we follow a modular approach to integrate the diverse cellular processes, such as the photosynthetic light reactions, cellular metabolism, the circadian clock, and carbon sequestration, into a coherent minimal cell model of phototrophic growth. The minimal cell model is supplemented with a model of carbon fluxes in a laboratory scale photobioreactor that allows to determine all CO₂ related exchange rates of a cyanobacterial culture. Taken together, our approach allows to elucidate the internal mechanisms of carbon fixation, and will be instrumental in future applications of cyanobacterial biotechnology.


Color tuning in cyanobacterial life
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Cyanobacteria respond to various light environments to optimize their phototrophic life. There are many photoreceptors so far characterized in cyanobacteria and even much more putative photoreceptor genes in the rapidly growing genome database. Cyanobacteriochrome is a large subgroup of such photoreceptors in cyanobacteria. Cyanobacteriochrome binds a linear tetrapyrrole chromophore in its specific GAF domain, which undergoes reversible photoconversion. It is somehow related to phytochrome but exhibits much more diversity in spectral properties and signaling pathways. Cyanobacteriochrome has so far been found in the cyanobacterial phylum. However, they are major photoreceptors in many species and involved in various photoresponses such as chromatic acclimation of photosynthetic pigments, phototactic motility and biofilm formation. Recent progress in biochemistry revealed diverse subtypes of cyanobacteriochrome in terms of spectral and photochemical properties. Structural studies revealed key features in the chromophore and apoprotein in this group. Functional and biochemical studies revealed downstream signaling that is further connected to various photoresponses. Summarizing these, I will discuss molecular relevance of color tuning of cyanobacteriochromes to the cyanobacterial life.
A sulfur-depleted form of phycocyanin gives *Fremyella diplosiphon* a fitness advantage in low sulfur conditions

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*Fremyella diplosiphon* is a freshwater, filamentous cyanobacterium that has an intriguing response to changes in sulfur concentrations in its environment. At sulfate levels below 150 uM and when grown in red light, *F. diplosiphon* carries out a "sulfur sparing" response by replacing the sulfur-rich phycobiliprotein isoforms phycocyanin1 (PC1) and PC2 with PC3, which possesses less sulfur containing amino acids. Phycobiliproteins are found in a light harvesting complex called the phycobilisome. Phycobilisomes are attached to the cytoplasmic side of thylakoid membranes capturing and channeling light energy to the photosynthetic reaction centers. Different phycobiliproteins best absorb different wavelengths of light, with the phycocyanins absorbing red light well and phycoerythrin absorbing green light well. Changing from a PC1/PC2 to a PC3-containing phycobilisome is a significant undertaking because phycobilisomes are the most abundant protein structure in the cell. The average PC1 and PC2 containing phycobilisomes have around 1,000 sulfur containing amino acids more than PC3-containing phycobilisomes. By remodeling one of the most abundant protein structures in the cell, there is a significant savings of sulfur containing amino acids that can be used for other cellular processes.

It has not been previously investigated whether the sulfur sparing response gives *F. diplosiphon* a growth advantage in sulfur-limited conditions. I performed a competition experiment between wild-type and mutant cells incapable of undergoing the sulfur sparing response (that is, they continue to act as if they are in high sulfur conditions when they become sulfur-limited) and determined the ratio of wild-type to mutant cells when grown in sulfur-replete and sulfur-deficient conditions. By day 20 in sulfur-deficient media in red light, wild-type cells comprised 92% of a population that was initially 45% wild type and 55% mutant. In sulfur-replete growing conditions over the same time period, however, the percentages of wild-type and mutant cells were virtually unchanged. Therefore, the capacity to produce PC3-containing phycobilisomes in low sulfur conditions provides *F. diplosiphon* with a strong fitness advantage in red light. In wild-type cells grown in green light and low sulfate, PE and PC3 both accumulate in the phycobilisomes, even though PC3 only absorbs red light well. This is because PC3 abundance, unlike PC2, does not decrease during growth in green light. I have also performed a competition experiment between wild-type and mutant cells in green light. In green light and low sulfate conditions, PC3 does not provide wild-type cells with the same fitness advantage as it does in low sulfate conditions and red light.
Cyanobacterial extracellular polymeric substances (EPS): production, export and interactions with metal cations

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Many cyanobacteria produce extracellular polymeric substances (EPS), mainly composed of polysaccharides that can remain associated to the cell or be released into the surrounding environment (RPS-released polysaccharides). The particular characteristics of these EPS, such as the presence of two different uronic acids, sulphate groups and high number of different monosaccharides (up to 13) makes them very promising for biotechnological applications. The polymer’s overall negative charge is particularly promising for heavy metals bioremediation [1,2]. Nevertheless, the efficient use of EPS-producing cyanobacteria (or their isolated polymers) in the removal of heavy metals from contaminated waters depends on the comprehensive knowledge on the pathways utilized for their synthesis and export, and on the interactions between the EPS-producing cyanobacteria and the metal ions.

To provide a first insight on the genes encoding proteins involved in EPS assembly and export (EPS-related genes), an in silico analysis of cyanobacterial genome sequences was performed. The results revealed that the EPS-related genes appear in multiple copies scattered throughout the genomes, either isolated or in small clusters [1]. Subsequently, a phylogenomic analysis was undertaken to elucidate the evolution of the cyanobacterial EPS assembly and export genetic machinery. The data gathered strongly supports that, in cyanobacteria, these process proceeds via a Wzy-dependent mechanism. In addition, it allowed the reconstruction of the evolutionary history of the cyanobacterial polysaccharide copolymerase (PCP; e.g. Wzc) and outer membrane polysaccharide export (OPX; e.g. Wza) protein families, revealing specific losses, HGT events and numerous paralogous duplications [3].

Bearing in mind the future implementation of metal removal systems using cyanobacterial EPS, the physiological/environmental conditions that promote polymer production by the marine N₂-fixing Cyanothecesp. CCY 0110 were evaluated. Furthermore, the produced RPS were characterized using a multidisciplinary approach. This strain was shown to a highly efficient EPS producer, releasing a polymer with suitable characteristics for biotechnological applications [4].

In parallel, the effects of different metals/metals concentrations in the growth/survival of Cyanothecesp. PCC 6909 wild type and its sheathless mutant were assessed. The affinity of various culture fractions for different metal(s) in mono- and multi-metal systems was also assessed. These studies unveiled different responses of these phenotypes to metal exposure, reflecting the specific metal requirements of the cells and the metal-binding efficiencies of their EPS [5]. In all cases, the RPS were the most efficient fraction in the metal-adsorption. These polymers can be easily separated from the cultures and immobilized or confined for biotechnological applications.

All microorganisms synthesize different kinds of polymeric inclusions to cope with starvation conditions temporally present in the environment. Most of them are massively accumulated when nutrients are limited, and are rapidly degraded under favorable conditions. The role of polymeric inclusions as a dynamic internal nutrient reservoir has been demonstrated for a variety of microorganisms. However, their metabolic role in the stringent control of microbial stress responses is yet insufficiently understood.

Our current study focuses on the metabolic roles of the polymeric carbon compounds glycogen and poly-β-hydroxybutyrate (PHB), in the cyanobacterial stringent response to nitrogen starvation. Here, one of the major acclimation responses is the degradation of the phycobilisome complex, a process referred as nitrogen chlorosis. This bleaching process is inhibited in cells impaired in glycogen biosynthesis and is modulated by the addition of organic carbon compounds that affect glycogen or PHB biosynthesis as well. By combining molecular and biochemical characteristics of various mutants impaired in glycogen and PHB synthesis in the non-diazotrophic cyanobacterium *Synechocystis* sp. PCC 6803 with systemic genetic and metabolic information allow to address the following features: (i) the metabolic background of nitrogen chlorosis, (ii) the metabolic roles of glycogen and PHB metabolism in the process of nitrogen chlorosis, (iii) interrelations in the biosyntheses of the two carbon polymers, and (iv) how cells adapt metabolism to specific physiological states.
Importance of Deg/HtrA proteases in *Synechocystis* sp. PCC 6803

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In the natural environment photosynthetic organisms are exposed to tremendous changes in light intensities and temperatures. Under these conditions, formation of reactive oxygen species and subsequent damage to proteins is increased. To cope with this problem plants and cyanobacteria contain a variety of chaperones and proteases that monitor proper folding and function of proteins. Particularly interesting candidates are enzymes of the multifunctional Deg family of ATP-independent serine endopeptidases. The three Deg proteases of the cyanobacterium *Synechocystis* sp. PCC 6803, called HtrA (high temperature requirement A), HhoA (HtrA homologue A) and HhoB (HtrA homologue B), are important for survival under high light and temperature stresses and might have redundant physiological functions.

We characterized recombinant HtrA, HhoA and HhoB *in vitro* and showed that all three proteases can degrade unfolded model substrates, but differ with respect to cleavage sites, temperature and pH optima. All three proteases form different homo-oligomeric complexes with and without substrate, implying mechanistic differences in comparison with each other and with the well-studied *Escherichia coli* orthologues DegP and DegS. Deletion of the PDZ domain decreased, but did not abolish, the proteolytic activity of all three proteases, and prevented substrate-induced formation of complexes higher than trimers by HtrA and HhoA.

In *vivo* these proteases seem to be involved in redox-regulated proteolysis. Deg proteases are able to degrade the extrinsic Photosystem II protein PsbO in a redox-dependent manner. PsbO contains two conserved cysteine residues, which form a disulfide bridge between its N-terminal loop and a beta-1 strand. It is known to be stable in its oxidized form, but after reduction PsbO becomes a substrate for Deg proteases *in vitro* and *in vivo*. Assembly to Photosystem II protects PsbO from degradation.

Proteomic and metabolomic studies comparing wild type cells with the Δdeg mutant, depleted of all three Deg proteases, revealed additional substrates and functions of the three Deg proteases. These results are discussed in light of our localization studies, detecting the Deg proteases both in the thylakoid- and the periplasma-membrane fraction.


Iron bioavailability limits biological activity in many aquatic and terrestrial environments. Within a single organism, multiple iron transporters may contribute to iron acquisition. Here, we present a functional characterization of a cyanobacterial iron transport pathway that utilizes concerted transporter activities. Cyanobacteria are significant contributors to global primary productivity with high iron demands. Certain cyanobacterial species employ a siderophore mediated uptake strategy; however many strains possess neither siderophore biosynthesis nor siderophore transport genes. The unicellular, planktonic cyanobacterium, Synechocystis sp. PCC 6803, employs an alternative to siderophore based uptake - reduction of Fe(III) species prior to transport through the plasma membrane. In this study, we combine short term radioactive iron uptake and reduction assays and a range of disruption mutants to generate a working model for iron reduction and uptake in Synechocystis sp. PCC 6803. We found that the Fe(II) transporter, FeoB, is the major iron transporter in Synechocystis sp. PCC 6803. In addition, we uncovered a link between a respiratory terminal oxidase (ARTO) and iron reduction - suggesting a coupling between these two electron transfer reactions. Furthermore, quantitative RNA transcript analysis identified a function for subunits of the Fe(III) transporter, FutABC in modulating reductive iron uptake. Collectively, our results provide a molecular basis for a tightly coordinated, high-affinity iron transport system.
Genome wide copper responses in *Synechocystis* sp. PCC 6803.

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Copper resistance in *Synechocystis* is mediated by the CopRS (hik31-rre34) two-component system. This two-component regulates its own expression and expression of a RND metal transport system (CopBAC) in response to copper in the media [1]. The system is also able to respond to changes in internal copper pools when plastocyanin is degraded in response to photosynthetic electron transport block [1, 2]. Furthermore CopS is able to bind copper directly with high affinity and is partially localized to thylakoid membranes where it could detect copper released from plastocyanin. Here we have used microarrays to interrogate the global responses to copper additions at non-toxic (0.3 µM) and toxic concentrations (3 µM) of copper in WT and in a mutant in the *copR* gene. Addition of the non-toxic copper concentration stimulated the metabolism and induced the switch in the use from cytochrome c₆ to plastocyanin. In contrast, high copper addition induced a stress response and affected the metabolism of several other metals. This included repression of ribosomal, photosynthetic and central metabolism genes and induction of chaperones and antioxidant enzymes genes. Finally CopRS seems to control only the expression of the *copMRS* and *copBAC* operons as all other genes that were differentially expressed by copper seemed to be unaffected in copR strains. These results will be discussed in relation to copper, and other metals metabolism in *Synechocystis*.


Hydrogen peroxide as a biocide for selective suppression of harmful cyanobacteria

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Abundant growth of harmful cyanobacteria in the environment is a world-wide problem. Using progress in photosynthesis research as a clue, a method has been developed for selective suppression of cyanobacteria with hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). Applications have been successful in a number of natural recreation lakes, where a single treatment with homogenously distributed H\textsubscript{2}O\textsubscript{2} prohibited blooming of cyanobacteria during the season\textsuperscript{1}. The method is based on the surprising observation that cyanobacteria with surplus availability of electrons at the stromal side of photosystem I use a pseudo Mehler type reaction that makes H\textsubscript{2}O and not H\textsubscript{2}O\textsubscript{2}\textsuperscript{2,3}. The latter is normally produced in plant and green algal chloroplasts during high light and/or nutrient limitation stress. In designing of the method the reasoning has been that lack of earlier exposure to H\textsubscript{2}O\textsubscript{2} makes expression of anti-oxidative stress enzymes less required in cyanobacteria than in green algae. Hence, cyanobacteria when suddenly exposed to H\textsubscript{2}O\textsubscript{2} will be sensitive, whereas green algae and diatoms are not\textsuperscript{4}. The method offers an environmentally friendly procedure against nuisance cyanobacteria at affordable costs.

References:
A cyanobacterial chlorophyll synthase-HliD-Ycf39-YidC complex links chlorophyll and protein biosynthesis

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The chlorophyll-synthase (ChlG) enzyme catalyses the addition of a phytol tail to the chlorophyll precursor chlorophyllide yielding chlorophyll, which is then incorporated into the photosystem complexes situated within the photosynthetic membranes of the cell. Whilst chlorophyll synthesis and thylakoid biogenesis are relatively well understood, the mechanism of chlorophyll delivery from one to the other remains unclear. This has been approached by using FLAG-tagged ChlG as a bait protein for pulldown experiments in the cyanobacterium Synechocystis PCC 6803. An enzymatically active complex was retrieved comprising ChlG and the light-inducible protein HliD, which associates with the Ycf39 protein, a putative assembly factor for PSII, and with the Alb3/YidC insertase. HliD is a small pigment binding protein which is likely to have a photoprotective role whilst Ycf39 and YidC have been linked with thylakoid formation. These findings implicate ChlG in chlorophyll ‘handover’ during thylakoid biogenesis and provide evidence for a link between chlorophyll biosynthesis and photosynthetic membrane assembly.
Probing the Consequences of Antenna Truncation in Cyanobacteria

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Photosynthetic antenna systems, such as those found in cyanobacteria, function in the process of converting sunlight into cellular fuel. In the cyanobacterium Synechocystis sp. PCC 6803, light harvesting is accomplished by a combination of membrane intrinsic pigment-proteins and large extrinsic phycobilisome complexes. Phycobilisomes associate with the cytoplasmic surface of thylakoid membranes, so that thylakoid layers are typically separated by a space sufficient for a double row of phycobilisomes. Earlier studies have shown that modulation of phycobilisome antenna size results in changes to the membrane spacing (1). We used a combination of approaches to explore the consequences of antenna modification in terms of physiology as well as membrane morphology and dynamics in wild-type Synechocystis 6803 and a series of mutants with varying degrees of phycobilisome truncation. Our work using transmission electron microscopy (TEM), hyperspectral confocal fluorescence microscopy (HCFM), and small angle neutron scattering (SANS) has revealed the adaptive strategies that these cells employ to compensate for antenna reduction. We found that as the phycobilisome antenna was truncated, large-scale changes in thylakoid morphology were observed, accompanied by increased physical segregation of the two photosystems (2). Repeating distances between thylakoid membranes measured by SANS were correlated with TEM data, and corresponded to the degree of phycobilisome truncation (3). We found that cyanobacterial membranes have a high degree of structural flexibility, and that changes in the membrane system in response to illumination are rapid and reversible. These results highlight the dynamic nature of the intracellular membrane system in cyanobacteria cells and suggest an adaptive strategy that allows cells to compensate for decreased light harvesting capability.


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The Bio-SANS instrument is a resource of the Center for Structural Molecular Biology at Oak Ridge National Laboratory that is supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research Project ERKP291. Bio-SANS is located at the Oak Ridge National Laboratory’s High Flux Isotope Reactor. The neutron source is sponsored by the Scientific User Facilities Division, Office of Basic Energy Sciences, U.S. Department of Energy.
Regulation of cell division by a caspase-like protein in Anabaena PCC 7120

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Cysteine-dependent aspartate-directed proteases, or caspases, are a diverse family of enzymes, best studied in relation to eukaryotic programmed cell death, although they have been implicated in a range of non-apoptotic functions [1]. Related metacaspases have been identified in bacteria, notably those that undergo complex development [2]. The heterocystous cyanobacterium, Anabaena PCC 7120 possesses a member of the caspase hemoglobinase fold (CHF) subfamily called HetF (Alr3546). Deletion of the hetF coding region, or mutation of the histidine/cysteine catalytic diad renders Anabaena unable to form heterocysts [3] [4]. In addition, cells appear enlarged and elongated. The inability of a hetF mutant to produce heterocysts has been ascribed to regulation of HetR protein accumulation. However, the cell division phenotype, which affects all cells in the population, has not been investigated.

To examine the role of HetF in vegetative cell physiology, the location and distribution of the nucleoid was observed by DAPI staining. Compared to the nucleoid of wild-type cells, which is compact and lobed, the nucleoid of the hetF mutant was diffusely distributed suggesting that elongation may be due to the inability of the cytokinetic Z-ring to constrict over the chromosome. Next, localization of HetF relative to the nucleoid was determined. Fusion of YFP to the C-terminus of HetF produced functional complementation of heterocyst formation and cell size, however, fluorescence was not observed. Upon closer examination of the HetF amino acid sequence, a caspase I cleavage motif was detected. Fusion of YFP to HetF immediately upstream of the putative cleavage site resulted in fluorescence that co-localized with the DAPI stained nucleoid and complementation of a hetF mutant. Taken together, these data suggest that HetF may be cleaved into an active form either autocatalytically or by another protease, and may target one or more proteins associated with the nucleoid to affect cell division. This work could give insights into the evolution of caspases and their roles in functions other than programmed cell death.


Coordination by master regulator of submaster and patterning factor genes in control of heterocyst differentiation

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In Anabaena/Nostoc species, single N₂-fixing heterocysts are semi-regularly intermitted with multiple CO₂-fixing vegetative cells. Formation of such a pattern largely depends on an activator/inhibitor system. HetR, a transcription factor, has been proposed to be the master regulator of heterocyst differentiation, and the diffusible RGSGR-containing peptide has been shown to inactivate HetR in vitro and induce its degradation in cells. How HetR coordinates cell differentiation and lateral inhibition remains to be clarified.

There are two types of binding sites for HetR: with (I) or without (II) the conserved recognition sequence. Among type-I binding sites tested in vitro, HetR strongly preferred those upstream of hetP and hetZ, the two submaster genes with overlapping functions. In a hetR mutant, co-expression of hetP and hetZ from PntcA restored heterocyst formation at terminal positions. Overexpression of hetR bypassed the hetP or hetZ mutation but not hetPhetZ double mutation. HetZ also showed interaction with HetR in yeast two-hybrid and pull-down assays and moderately inhibited HetR-promoted transcription of hetZ, hetP and patA. Like hetZ and hetP, patA is directly regulated by HetR via a type-I binding site, but it is required for differentiation of intercalary heterocysts. Upstream of patS and hetN, genes encoding RGSGR-containing inhibitors, there are multiple type-II HetR-binding sites. To these regions, HetZ and HetP showed binding activities. In the presence of hetR, hetZ more strongly promoted the expression of patS and hetN than hetP. After N-stepdown, HetR directly activates the expression of hetP, hetZ and patA, then in coordination with HetZ and HetP, activates the expression of RGSGR-containing inhibitor genes. The timing of and balance between the expression of these genes may be required for the self immunity of differentiating cells.
Anabaena variabilis is a photosynthetic, diazotrophic cyanobacterium with great potential in biofuel production and synthetic biology. *A. variabilis* makes two distinct Mo-nitrogenases: the Nif1 nitrogenase is expressed under aerobic growth conditions exclusively in specialized nitrogen-fixing cells called heterocysts, while the Nif2 nitrogenase is expressed in vegetative cells only under anaerobic conditions. The proteins necessary for synthesis and assembly of the Nif1 and Nif2 nitrogenases are encoded in two separate operons, which are transcribed from the promoters upstream of the first gene of the operon, *nifB1* (P_{nifB1}) and *nifB2* (P_{nifB2}), respectively. We analyzed the sequences upstream of the transcription start site of *nifB1* by creating P_{nifB1}-lacZ and P_{nifB1}-gfp transcriptional fusions. The sequence, TGAGTACA, is repeated three times in the region upstream of the transcription start sites of both P_{nifB1} and P_{nifB2}. Two of these motifs were found to be necessary for transcription of P_{nifB1}-lacZ. Two hybrid promoters were constructed and cloned upstream of lacZ and gfp to assay cell-type specific expression. The first hybrid promoter contained upstream sequences of P_{nifB2} fused to the transcription start site region of P_{nifB1}. The other hybrid promoter contained upstream sequences of P_{nifB1} fused to the transcription start site region of P_{nifB2}. Data on the latter is being collected, but expression analysis of the first hybrid promoter indicated that the P_{nifB2}.P_{nifB1} fusion promoter was able to direct heterocyst-specific transcription of the reporter genes. One explanation of these results is that cell-type specific expression of the Nif1 system is due to a heterocyst-specific sigma factor, while transcriptional activation of the *nif1* and *nif2* genes may be the result of a common activator protein that acts on the region upstream of the transcription start site.
Non-coding RNAs in *Anabaena* sp. PCC 7120

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Global approaches to the analysis of bacterial transcriptomes are unveiling a great deal of non-coding transcription, producing both antisense transcripts and small, potentially regulatory RNAs. Small regulatory RNAs (sRNAs) constitute a class of molecules involved in virtually every bacterial response to changes in environmental conditions. We are interested in the cyanobacterial responses to nitrogen stress, that in heterocystous strains include the differentiation of a specialized cell type. By using a modified RNA-Seq procedure we have previously described the transcriptome of *Anabaena (Nostoc)* sp. PCC7120 (both in the wild-type and a hetR strain unable to differentiate heterocysts) and identified transcriptional start sites (TSS) throughout the genome [1]. The dynamics of use of each TSS in response to nitrogen deficiency allows us to separate two categories of transcriptional responses; those taking place both in the wild-type and the hetR strain (most of them involving NtcA-activated promoters) and those that are exclusive of the wild-type strain (that define the HetR regulon). We are currently identifying small RNA molecules that could be involved in adaptation to nitrogen stress, including heterocyst differentiation. We are selecting TSS that are not associated to coding regions but exhibit dramatic changes in transcription when the cells are subjected to nitrogen step-down. We are further characterizing several sRNAs that seem to be conserved in several cyanobacterial species. Some of these sRNAs appear exclusively in genomes of heterocystous strains (essentially in every heterocystous strain included in the recently published phylogenetic tree [2]), whereas others show a wider distribution.


Concerted action of two clock-controlled histidine kinases times the activation of circadian factor RpaA

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Circadian clocks are endogenous self-sustained molecular oscillators that coordinate the organism’s physiology with the day-night cycle. In the cyanobacterium *Synechococcus elongatus* PCC 7942, the core pacemaker consists of a three-protein clock: KaiA, KaiB and KaiC that interact to generate circadian oscillations of KaiC phosphorylation and which is essential for producing oscillatory patterns of gene expression. The majority of genes are under circadian control in *S. elongatus*. However the precise mechanism by which the temporal information from the core oscillator is transduced to control gene expression is poorly understood. Here we investigated how the circadian transcription factor RpaA is regulated by the KaiABC clock. We find that two histidine kinases, CikA and SasA, interpret different states of the KaiABC clock and both directly regulate the activity of RpaA. SasA and CikA are activated by the KaiABC clock at distinct times of the day and regulate RpaA in opposite ways – one increases its activity by phosphorylating it and the other one decreases it, through dephosphorylation. This sequential action of two opposing enzymes produces an oscillation of RpaA activity that is offset from that of the KaiABC core clock. Such a mechanism of generating offset oscillations of clock output pathways is potentially used in other circadian systems and could also be important for a better timekeeping in spite of cellular and environmental fluctuations.
How Cyanobacteria Tell Time

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Cells of diverse organisms, from cyanobacteria to humans, execute temporal programs of physiological processes that are driven by circadian oscillators. For example, the *Synechococcus elongatus* circadian clock regulates global patterns of gene expression, the timing of cell division, and compaction of the chromosome. Our research uses *S. elongatus* as a model to understand how a cell keeps track of time, executes activities according to a temporal program, and synchronizes the internal clock with the external solar cycle. The components of the circadian oscillator are known (proteins KaiA, KaiB, and KaiC), their structures have been solved, and the circadian rhythm in phosphorylation of KaiC can be reconstituted in vitro. Our recent work has focused on the mechanisms that coordinate the clock with metabolism to keep circadian rhythms aligned properly with the solar day. The photosynthetic machinery plays a major role in this process through light-dependent modulation of the redox state of the quinone pool and the ratio of ATP/ADP. The oscillator protein KaiA binds oxidized quinones, and this interaction blocks the KaiC-stimulating activity of KaiA. The Kai oscillator can be reset in vitro and in vivo by pulses of oxidized quinone. In wild-type cells the quinone pool rapidly oxidizes temporarily when cells go into the dark, and acutely reduces when cells transition back into the light. These abrupt changes in quinone oxidation state, which can act as indicators of darkness onset, contrast with a gradual decrease in ATP/ADP ratio that falls continuously over the course of hours until the onset of light. Changes in the ATP/ADP ratio can also reset the oscillator, acting through KaiC. Thus, these two metabolic measures of photosynthetic activity can act in concert to signal both the onset and duration of darkness to the cyanobacterial clock. We are also investigating the biochemical states of KaiC that stimulate the major output pathway that controls gene expression. This work has shown that the default gene expression status, in the absence of the circadian oscillator, is on for the class I promoter P_{kaiBC} and off for the class II promoter P_{purF}, and that a specific phospho-state of KaiC affects both class I and class II promoter expression, but with opposite outcomes regarding stimulation and repression.
Poster Presentations
(alphabetical order)

1. Olalekan Aremu, University of Wisconsin - Oshkosh: Metabolic Engineering and Culture Strategies for Enhanced Isoprenoid Production in *Synechococcus* sp. PCC 7002 cyanobacteria

2. Bruce W. Arey, Pacific Northwest National Laboratory: High resolution electron and ion microscopy of photosynthetic complexes

3. Juliana Artier, Oklahoma State University: Understanding Cyclic Electron Transport In Different Mutants Of *Synechocystis* PCC 6803

4. Rajeev Aurora, St. Louis University: Deep sequencing of the sinus microbiome reveals an abundant levels of Cyanobacterial species

5. Seth D. Axen, U.S. Department of Energy-Joint Genome Institute: Evidence for the widespread distribution of the CRISPR-Cas system in the Phylum *Cyanobacteria*

6. Han Bao, Oklahoma State University: Second sphere ligands of the Mn cluster control the reactivity of water oxidation

7. Fiona K. Bentley, Colorado School of Mines: Redirection of photosynthate in *Synechococcus* sp. PCC 7002 for enhanced limonene biosynthesis

8. Ângela Brito, IBMC, Universidade do Porto: Prediction of bioactive compounds from cyanobacterial strains isolated from the Portuguese coast

9. Andrea W.U. Busch, Michigan State University: Stress adaptation and response to fluctuating environments – the role of putative outer membrane protein TspO of the cyanobacterium *Fremyella diplosiphon*

10. Robert H. Calderon, University of California, Berkeley: A Conserved Rubredoxin in the Thylakoid Membrane Functions in the Accumulation of Photosystem II

11. Jeffrey C. Cameron, University of California, Berkeley: Biogenesis of a Bacterial Organelle

12. Daniel Canniffe, University of Sheffield: Elucidating the routes of C8-vinyl reduction in (bacterio)chlorophyll biosynthesis

13. Kangming Chen, South Dakota State University: *Ava_4785*, an Intramembrane Metalloprotease, Is Required for Cold Acclimation in *Anabaena variabilis*

14. Dan Cheng, University of Arkansas at Little Rock: Critical Roles of PfsR in iron homeostasis of *Synechocystis* PCC 6803
15. Soumana Daddy, University of Arkansas at Little Rock: Inducible Carotenoid Binding Protein Complex From *Synechocystis* 6803

16. Spencer Diamond, University of California, San Diego: Circadian and Diurnal Control of Metabolism in *Synechococcus elongatus* PCC 7942

17. Dennis Dienst, Algenol Biofuels Germany GmbH: Transcriptomic response of *Synechocystis* sp. PCC 6803 to long-term ethanol production

18. Fitzpatrick S. Duncan, Australian National University, Canberra: Defining the Upper Thermal Limits of Oxygencic Photosynthesis: in *Thermosynechococcus elongates*

19. Julian J. Eaton-Rye, University of Otago: Deletion of PsbT in *Synechocystis* sp. PCC 6803 Results in Increased Turnover of D1 and Causes Q_{A} Oxidation To Be Blocked by Formate and Dimethyl-p-Benzoinone

20. Maria Ermakova, University of Turku: Flavodiiron proteins are distributed spatially to protect both photosynthesis and nitrogen fixation in filamentous heterocystous cyanobacteria

21. Daniela Ferreira, Arizona State University: Biosynthetic pathway of the cyanobacterial sunscreen scytonemin in *Nostoc punctiforme* ATCC 29113

22. Fei Gan, The Pennsylvania State University: Evolution and biogenesis of photosystem I in the siderophilic cyanobacterium *Leptolyngbya* sp. JSC1

23. Ferran Garcia-Pichel, Arizona State University: Global-warming induces a replacement of keystone cyanobacteria in Western North American soils

24. Gen Enomoto, University of Tokyo: Three cyanobacteriochromes synthesize and degrade c-di-GMP in *Thermosynechococcus*

25. Jaimie Gibbons, South Dakota State University: Identification of Two Genes Required for Long-Chain Hydrocarbon Production in Cyanobacterium *Anabaena* sp. PCC 7120

26. James W. Golden, University of California San Diego: Improved genetic tools for cyanobacteria

27. Sarah Griffith, Purdue University: The effects of light-dark cycles on the metabolism of *Cyanothece* sp. ATCC 51142 and *Cyanothece* sp. ATCC 7822

28. Liping Gu, South Dakota State University: Conversion of CO_{2} and H_{2}O into Fuel Alcohols by Engineered Cyanobacteria

29. Charles Halfmann, South Dakota State University: Genetic Engineering of the Filamentous Cyanobacterium *Anabaena* 7120 for the Photosynthetic Production of Limonene

30. Qingfang He, University of Arkansas at Little Rock: Cyanobacterial platform for production of plant secondary metabolites

31. Andrew Hitchcock, University of Southampton: A biophotovoltaic approach to understanding extracellular electron transport by *Synechocystis* sp. PCC6803
32. Sarah L. Hollingshead, University of Sheffield: Unraveling the mysteries of Mg-Protoporphyin IX Monomethylester (oxidative) Cyclase, the third committed enzyme in chlorophyll biosynthesis

33. Harvey J.M. Hou, Alabama State University: Effects of BP Oil Spill and Dispersant on Photosynthesis and Metabolism of Cyanobacteria

34. Kaori Inoue-Sakamoto, Kanazawa Institute of Technology: Water stress protein A (WspA) in cyanobacterial *Nostoc* species

35. John Irudayaraj, Indian Institute of Technology Bombay: An assessment of the capability of *Synechococcus* sp PCC 7002 metabolic network for the production of Bio-chemicals

36. Jon M. Jacobs, Pacific Northwest National Laboratory: Extending Proteomics into Cyanobacteria Characterization

37. Toivo Kallas, University of Wisconsin-Oshkosh: Characterization of 4(1H)-quinolone (4-1HQ) and 2-nonyl-1-hydroxy-4(1H)-quinolone (NQNO) as inhibitors of photosynthetic electron transfer and signaling

38. Rebecca Knight, The University of Texas, Austin: Production of 2′-Hydroxy 2-Myxol Fucoside in Response to pH, Blue Light Intensity, and Temperature in the Thermophilic Cyanobacterium, *Thermosynechococcus* BP-1

39. Jagdeesh Kottapalli, Washington University in St. Louis: Converting *E. coli* into a Nitrogen Bio-Fertilizer Using a Cyanobacterial *nif* Cluster: an iGEM project

40. Christina M. Kronfel, University of New Orleans: Characterization of the role of CpeF in phycoerythrin biosynthesis in *Fremyella diplosiphon* strain UTEX 481

41. Randy Lacey, University of Tennessee, Knoxville: Ethylene Affects the Phototactic Response in Cyanobacteria

42. Jacob J. Lamb, Norwegian University of Science and Technology: Pili-mediated electron donation to iron oxides in *Synechocystis* sp. PCC 6803

43. Yaqiong Li, University of Sydney: The growth of *Halomicronema hongdechloris* - a filamentous cyanobacterium containing chlorophyll *f* - under different light conditions

44. Peter Lindblad, Uppsala University: Design, engineering, and construction of photosynthetic microbial cell factories for renewable solar fuel production

45. Amy T. Ma, University of California San Diego: Proteomic Response of *Anabaena* sp. PCC 7120 to Grazing by Natural Amoebal Isolate HGG1

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47. Andrew L. Markley, University of Wisconsin – Madison: Fine tuning gene expression in *Synechococcus* sp. PCC 7002 and application in generating sustainable cyanobacterial photosynthetic biorefineries
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49. Rita Mota, IBMC, Universidade do Porto: Interactions between extracellular polymeric substances (EPS) produced by *Cyanothece* sp. CCY 0110 and heavy metals


51. Henna Mustila, University of Turku: Flavodiiron proteins Flv1 and Flv3 are essential for cyanobacterial growth under fluctuating light

52. Aparna Nagarajan, Washington University in St. Louis: Effects of antenna truncation on productivity and Photosystem II function

53. Matthew E. Nelson, University of Wisconsin – Oshkosh: Metabolic Engineering and Culture Strategies for Enhanced Isoprenoid Bioproduct-Biofuels Production in *Synechococcus* sp. PCC 7002 cyanobacteria

54. Anne Neuenfeldt, Algenol Biofuels Germany GmbH: Direct Coupling of Photosynthesis to Ethanol Production in Cyanobacteria

55. Adam Nguyen, University of New Orleans: Characterization of the Putative Bilin Lyase CpeT from *Fremyella diplosiphon* (UTEX 481)

56. Amelia Y. Nguyen, Washington University in St. Louis: Presence of a functional low-molecular weight thiol pool within the thylakoid lumen in the cyanobacterium *Synechocystis* sp. PCC 6803


58. John W.K. Oliver, University of California, Davis: Conversion of CO2 to Chemicals in Cyanobacteria

59. A. Omairi-Nasser, The University of Chicago: A gene regulation mechanism that allows synthesis of two ferredoxin:NADP oxidoreductase isoforms from a single gene in the cyanobacterium *Synechocystis* sp. PCC 6803

60. Georg W. Owttrim, University of Alberta: Temperature stress and RNA helicase expression: Multiple levels of (auto)regulation

61. Bagmi Pattanaik, Uppsala University: Characterization of a squalene hopene cyclase deficient strain of *Synechocystis* PCC6803


63. Charulata B. Prasannan, Indian Institute of Technology Bombay: Quantitative metabolite analysis for a cyanobacterial strain *Synechococcus* sp. PCC 7002

64. Brenda Pratte, University of Missouri – St. Louis: Transcriptional Regulation of Nitrogenase Genes
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65. Yeyan Qiu, South Dakota State University: Proteomic Study on Akinetes and Heterocysts in *Anabaena cylindrica*

66. Johnna L. Roose, Louisiana State University: The functional significance of the CyanoQ N-terminal lipid modification

67. Anne M. Ruffing, Sandia National Laboratories: *Synechococcus* sp. PCC 7002: A Superior Host for Converting Carbon Dioxide into Free Fatty Acids

68. Toshio Sakamoto, Kanazawa University: Glycosylated mycosporine-like amino acids and the genotypes in the terrestrial cyanobacterium *Nostoc commune*

69. Hidehiro Sakurai, Kanagawa University: Genetic Improvements and the Gas Compositions for Sustained Nitrogenase-based Photobiological H₂ Production by Heterocystous Cyanobacteria

70. Gustaf Sandh, Uppsala University: Revisiting the cell-type specific proteomes of N₂-fixing filaments of the heterocyst forming cyanobacterium *Nostoc punctiforme* ATCC29133 using ICAT labeling

71. Joseph E. Sanfilippo, Indiana University: Blue-green chromatic acclimation in marine *Synechococcus* is controlled by two putative AraC-class transcription factors

72. Kenneth Sauer, University of California, Berkeley: A Cyanobacterial Phylogenetic Tree Using PSI and PSII Protein Sequences

73. Georg Schmetterer, University of Vienna: Fructose is an antibiotic or a growth substrate for *Synechocystis* sp. PCC6803 depending on the pathway it is taken up into the cells

74. Tiago Toscano Selão, Nanyang Technological University: Biogenesis of the thylakoid membrane in *Synechocystis* sp. PCC6803

75. Ding-Ji Shi, Shanghai Ocean University: Improving *Anabaena* sp. Strain PCC 7120 for biodiesel production by down-regulation of *pepC* gene expression

76. Ryan Simkovsky, University of California San Diego: Novel O-antigen Synthesis Gene Mutants That Confer Resistance to Grazing in a Model Cyanobacterium-Amoeba System

77. Richard F. Simmerman, The University of Tennessee, Knoxville: Engineering Coupling of PSI to Metal-oxide Nanomaterials for Photovoltaic Applications

78. Shailendra P. Singh, Michigan State University: Light quality dependent alteration of reactive oxygen species (ROS) levels plays a role in the photoregulatory mechanism controlling cellular morphogenesis during complementary chromatic adaption (CCA) in *Fremyella diplosiphon*

79. Sujata V. Sohoni, Indian Institute of Technology Bombay: Fine tuning n-butanol production pathway in *Synechococcus spp* using Promoter Engineering

80. Karin Stensjö, Uppsala University: Cellular and Functional Specificity among Ferritin-like proteins in *Nostoc punctiforme*
81. Jerilyn A. Timlin, Sandia National Laboratories: Chemical Imaging of Cyanobacteria: A Picture is Worth a Thousand Words

82. O.N. Tiwari, Institute of Bioresources and Sustainable Development: Conservation and molecular characterization of freshwater cyanobacterial Germplasm of IBSD, Imphal, Manipur falling under Indo-Burma biodiversity hotspot

83. Esa Tyystjärvi, University of Turku: Temperature dependence of photoinhibition of PSII

84. Taina Tyystjärvi, University of Turku: The omega subunit of the RNA polymerase directs transcription efficiency in cyanobacteria

85. Arul M. Varman, Washington University in St. Louis: Metabolic engineering of Synechocystis sp. strain PCC 6803 for bio-production

86. Wim Vermaas, Arizona State University: The sll1951 Gene Encodes the Surface Layer Protein of Synechocystis sp. strain PCC 6803

87. Pramod P. Wangikar, Indian Institute of Technology Bombay: Gene regulatory interactions and ultradian rhythms in global transcription of Cyanothece sp 51142 under continuous light

88. Annegret Wilde, Justus-Liebig University Giessen: Light induced movement of cyanobacterial cells

89. Steven C. Wilson, University of California, Berkeley: Understanding Carboxysomes through Synthetic Biology

90. Lisa B. Wiltbank, Indiana University: Repression of phycoerythrin expression by a novel blue-green photoreceptor in the red-green chromatically acclimating cyanobacterium Fremyella diplosiphon

91. Aaron T. Wright, Pacific Northwest National Laboratory: Live cell characterization of temporal redox dynamics by chemical profiling in the photoautotroph Synechococcus sp. PCC 7002

92. Wei Xiao, Oklahoma State University: Construction of a recipient strain of Synechococcus sp. PCC 7002

93. Aki Yoneda, Washington University in St. Louis: Introduction of Acaryochloris marina genomic libraries into Synechocystis sp. PCC 6803 and a FACS-based screening strategy for chlorophyll d-producing Synechocystis cells

94. Ho-Sung Yoon, Kyungpook National University: Isolation and description of a globally distributed cryosphere cyanobacterium Oscillatoria sp. KNUA009 from Antarctica

95. Le You, Washington University in St. Louis: In Vivo Quantification of Flux Through A Cyanobacterial TCA Cycle

96. Jianping Yu, Purdue University: Analysis of metabolic fluxes under N-starvation in Synechocystis sp. PCC6803

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100. Lifang Zhang, Nanyang Technological University: Deletion of *Synechocystis* sp. PCC6803 Leader Peptidase LepB1 Affects Photosynthetic Complexes and Respiration

101. Pengpeng Zhang, Louisiana State University: The functional assembly of phycocyanin is involved in multiple electron transfer pathways regulating photosynthesis in *Synechocystis* sp. PCC 6803

102. Shuyi Zhang, The Pennsylvania State University: Vpp1 is required for the biogenesis of Photosystem I rather than thylakoid membranes in *Synechococcus* sp. PCC 7002

103. Xiaohui Zhang, Purdue University: Characterization of an uptake hydrogenase deletion mutant strain of *Cyanothece* sp. PCC 7822

104. Huilan Zhu, South Dakota State University: Genetic Transformation of *Anabaena cylindrica* ATCC 29414
Metabolic Engineering and Culture Strategies for Enhanced Isoprenoid Production in *Synechococcus* sp. PCC 7002 cyanobacteria

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Because of costs, climate change, and political uncertainty associated with fossil fuels, the development of carbon-neutral bioproducts and biofuels is a national economic and security imperative. Cyanobacteria efficiently capture enormous amounts of solar energy and convert atmospheric carbon dioxide (CO₂) into carbon polymers. We have introduced ‘codon-optimized’ isoprene synthase and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway genes into fast-growing, *Synechococcus* sp. PCC 7002 cyanobacteria and obtained production of isoprene (a C₅H₈ precursor for synthetic rubber and high-density, liquid aviation fuels) at rates promising for commercial development. Toward further improvement, we have inactivated the *glgA1* and *glgA2* genes for glycogen synthesis, a major competing carbon pathway. We are also pursuing down-regulation of light-harvesting capacity for increased cell density as well as modifications for regulated production of isoprene and other isoprenoid hydrocarbons. Inactivation of glycogen synthesis has initially resulted in only a modest increase in isoprene, suggesting that carbon is being diverted to soluble sugars as recently demonstrated by Xu et al. (2013 *Metabolic Engineering* 16, 56). Thus further modifications may be required to funnel additional carbon into the MEP pathway. We find that *Synechococcus* PCC 7002 grows well under 100% CO₂, and culture strategies are being developed to maximize CO₂ inputs. Results of the genetic engineering and culture work will be presented. U.S. patent 20110039323 (A.E. Wiberley, E.L. Singsaas, and T.D. Sharkey) has been issued for isoprene production work and a provisional patent application (T120019US) has been filed for inventions specific to cyanobacteria.

**Carbon partitioning in cyanobacteria.** Carbon is stored largely as glycogen in reactions catalyzed by glycogen synthases GlgA1 and GlgA2. Only a small fraction of carbon typically flows into the MEP pathway. Adapted from Lindberg et al., 2010 *Metabolic Engineering* 12, 70.
High resolution electron and ion microscopy of photosynthetic complexes

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High resolution imaging enables characterization of molecular machines such as phycobilisome antenna and photosynthetic complexes. Using synthetic biology approaches, *Synechocystis* sp. PCC 6803 strains with progressively truncated phycobilisome antenna complexes have been generated and studied using high resolution imaging [1]. We are investigating the topology of isolated thylakoid membranes from wild type and mutant strains with or without attached phycobilisomes. The transmission electron microscopy technique of negative staining is being used for high resolution characterization of isolated phycobilisomes and protein complexes in membranes in 2d projections, as well as the 3d surface topology by the helium ion microscope. The Zeiss Orion helium ion microscope (HeIM) is a new type of scanning microscope that uses helium ions for surface imaging and analysis. Its functionality is similar to a scanning electron microscope, but it uses a focused beam of helium ions instead of electrons. Since helium ions can be focused into a smaller probe size and provide a much smaller interaction volume at the sample surface compared to electrons, the helium ion microscope generates higher resolution images with better material contrast and improved depth of focus. Therefore, the helium ion microscope offers a significant advantage over traditional SEM technology. PNNL has become the first US national lab to acquire a ZEISS ORION PLUS helium-ion microscope. One of the Department of Energy’s (DOE’s) ten national laboratories, managed by DOE’s Office of Science, PNNL offers an open, collaborative environment for scientific discovery to researchers around the world. EMSL, the Environmental Molecular Sciences Laboratory, is a national scientific user facility sponsored by the Department of Energy’s Office of Science, Biological and Environmental Research program that is located at Pacific Northwest National Laboratory. EMSL’s technical experts and suite of custom and advanced instruments are unmatched. Its integrated computational and experimental capabilities enable researchers to realize fundamental scientific insights and create new technologies.

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Understanding Cyclic Electron Transport In Different Mutants Of *Synechocystis* PCC 6803

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Cyanobacteria have to adapt and survive to several adverse situations. Changes in the metabolism and gene expression are necessary to increase the selective fitness. Under low carbon (LC) conditions, cyanobacteria rely on the CO₂ concentration mechanism (CCM) to overcome the low rates of inorganic carbon (Ci) provided to Rubisco. CcmR, a LysR-type transcriptional regulator, is known to control its own expression and down-regulate the expression of Ci transporters under normal conditions (1). However, in LC conditions, the cell internally senses changes in [α-ketoglutarate] and [NADP⁺] that act as co-repressors of CcmR (2). The increased concentration of Ci enables its fixation on the Calvin cycle (CBB). We are studying the integration of these processes with photosynthetic electron transport producing ATP and NADPH. In our study we are analyzing knock out mutants of genes that might have a role in these processes. The photosynthetic electron transport can be linear (LET), involving an ending reaction with ferredoxin:NADP oxidoreductase (FNR) producing NADPH and ATP. Or it can be cyclic (CET), where the electron flow involves the NDH-1 complex and leads mainly to production of ATP. We are interested in understand what triggers the switch between LET and CET, and its regulation. Different subunits of FNR are supposed to be involved with LET and CET (3, 4). Both large (FNRₗ) and small (FNRₛ) subunits are present in *Synechocystis* sp. strain PCC6803 wild type (WT). However, FNRₛ is predominant under stress conditions such as N- starvation, high light (3) and LC (4). Two mutants of FNR have specific characteristics. FSI only present FNRₛ and is supposed to have mainly the cyclic photosynthetic reactions, while MI6 have FNRₗ, predominating the linear photosynthetic pathway. We studied the changes in chlorophyll fluorescence and P700 oxidation using Dual-PAM (pulse amplitude modulated fluorometry and absorbance spectroscopy) comparing WT and the mutants FS1, MI6 and ΔccmR. We noticed different patterns on samples when centrifuged from the non-centrifuged. The results showed interesting patterns and will be here presented.

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Deep sequencing of the sinus microbiome reveals an abundant levels of Cyanobacterial species

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To gain further insights into the etiology of chronic rhinosinusitis (CRS), a persistent inflammation of the nasal and paranasal mucosa, we analyzed the microbiome of patients with CRS. A lavage of middle meatus, the first mucosal surface encountered in the inhaled breath, was performed on 30 CRS patients and 12 healthy controls. Bacterial 16S rRNA genes were amplified from genomic DNA isolated from the lavage, and the amplicons were analyzed by deep sequencing. The most abundant bacterial species present, with 43% of the 800,000 sequence-reads belonged to the phylum Cyanobacteria. We identified 127 different uncultured species (at the 16S rRNA sequence or operational taxonomic units) in the lavage samples. No statistical difference in the abundance or species of Cyanobacteria was observed between CRS patients and the healthy controls. To confirm the presence of Cyanobacteria in the lavage samples the samples were analyzed by flow-cytometry to detect presence of phycoerythrin (PE) and allophycocyanin (APC). Our results indicate readily detectable presence of PE and APC double-positive cells that are quite large (~5 μM). Cyanobacteria are common in marine and aquatic environments. Consistent with their need to capture light for photosynthesis, Cyanobacteria are typically found close to the surface of the water column. Therefore, it is intriguing why these Cyanobacteria colonize a relatively dark environment like the middle meatus of the sinus. We will also present the results of the comparison of the sinus microbiome to other sites of human microbiome, where Cyanobacteria have been identified.
Evidence for the widespread distribution of the CRISPR-Cas system in the Phylum Cyanobacteria

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The CRISPR-Cas (clustered regularly interspaced short palindromic repeats, CRISPR associated genes) is a highly adaptable defense system present in a wide variety of bacteria across the tree of life. The system provides protection against infection of the cell by foreign DNA. While previous surveys have indicated that a small number of cyanobacteria contain the CRISPR-Cas system, a complete survey including the cyanobacterial genomes recently sequenced under the CyanoGEBA project had not yet been performed. We analyzed 126 cyanobacterial genomes and, surprisingly, found CRISPR-Cas in the majority except the marine subclade (\textit{Synechococcus} and \textit{Prochlorococcus}), in which cyanophages are a known force shaping their evolution. Multiple observations of CRISPR loci in the absence of \textit{cas1/cas2} genes may represent an early stage of losing a CRISPR-Cas locus. Our findings reveal the widespread distribution of the CRISPR-Cas system in the phylum \textit{Cyanobacteria} and provide a first step to systematically understanding CRISPR-Cas systems in cyanobacteria.
Second sphere ligands of the Mn cluster control the reactivity of water oxidation

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The active site of water oxidation in Photosystem II (PSII) is a Mn₄CaO₅ cluster that is located in a cavity between the two PSII subunits. In addition to the Mn₄CaO₅ and its amino acid ligands, water molecules filled in the cavity and the second sphere of amino acid ligands to the Mn₄CaO₅ also participate in poorly understood hydrogen bond networks that may modulate the function of the catalytic center. This cavity also serve as portions of the tunnels that lead from the Mn₄CaO₅ to the luminal surface of PSII, allowing for the exchange for the substrates and products of the water oxidation reaction.

Residue Val185 in the D1 subunit of PSII locate on the vicinity of O5 atom of Mn₄CaO₅ and face the broad channel as it passes the Mn₄CaO₅. Mutations of this residue were produced in Synechocystis sp PCC 6803, with the intention that the substitute residue would extend into the cavity to disturb the H-bond networks and tunnels (Fig.1). Three mutants were characterized by using O₂ polarography and fluorescence to determine their abilities to evolve oxygen and to undergo light driven charge separation. Of these the asparagine substitution produced the most interesting phenotype. While still able to evolve oxygen, it does so less efficiently than wild type PSII. The combination of a long lag phase prior to the onset of O₂ release and significantly decreased rate of O₂ release are observed in this mutant, consist with the result obtained from the D1-D61N mutant [1]. Temperature dependence of the rate constant of O₂ evolution was investigated by the time-resolved O₂ polarography. The Arrhenius plot for the wild type exhibit a straight line, consist with previous studies [2]. However, the activation energy in the mutant is only 22 kJ/mol as compared to 45 kJ/mol in Wild type. Correspondingly, the frequency factor of the mutant is five orders of magnitude lower than the Wild type. The phenotype of V185N mutant may be due to the perturbation of hydrogen bond networks and/or inefficient proton and water motion prior to the O₂ evolution.


Figure 1. Structure of Mn cluster and the key groups of surrounding hydrogen bond network.
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Cyanobacteria are emerging as promising production organisms for next generation biofuels and commodity chemicals. The ease of genetic manipulation and the ability of the cyanobacterial cell to act as a single bioprocessing unit by simultaneously harvesting and storing solar energy in the form of a wide variety of chemicals (including fatty acids, alkanes, alkenes, alcohols and terpenoids) are of considerable industrial significance. However, the major limitation of cyanobacteria as a production organism are the low yields of targeted product accumulation, due mainly to inefficiencies in solar energy conversion and the highly-regulated partitioning of photosynthate between metabolic pathways and associated carbon sinks. Here, we investigate the effect of blocking glycogen accumulation in the marine cyanobacterium, *Synechococcus* sp. PCC 7002, to see how this affects carbon partitioning between other major competing metabolic pathways of industrial significance, including the terpenoid biosynthetic pathway. Upon the onset of nitrogen deprivation, glycogen biosynthesis is up-regulated and becomes the major cellular carbon sink. However, the *glgC* strain, in which ADP-glucose pyrophosphorylase has been inactivated and is unable to synthesize glycogen, was found to secrete substantial amounts of organic acids instead of accumulating glycogen when deprived of nitrogen. Pyruvate, succinate and acetate were identified among the secreted products; however, ketoisocaproate was the most abundant secreted organic acid, which is indirectly synthesized from pyruvate and is the immediate precursor to leucine. To investigate carbon partitioning toward the terpenoid biosynthetic pathway under such conditions, we inactivated *glgC* in a limonene-producing strain of *Synechococcus* sp. PCC 7002. Limonene is a valuable commodity chemical used in the flavor and fragrance industry, and has potential to be developed for use as a biofuel and a renewable synthetic polymer. The limonene synthase gene from *Mentha spicata* was codon optimized for *Synechococcus* sp. PCC 7002 and introduced to the chromosomal DNA via double homologous recombination so that limonene could function as a reporter for carbon flux through the terpenoid pathway. Strategies for drawing on the pools of secreted acids in the *glgC* mutant upon nitrogen deprivation to increase carbon partitioning toward the terpenoid pathway for increased yields of limonene will be discussed.
Cyanobacteria are known to be a prolific source of bioactive compounds and some of these products are toxic to a wide array of organisms, including higher animals and humans. However, cyanobacteria can also produce secondary metabolites with promising therapeutic applications such as anticancer, antibiotic and anti-inflammatory activities. In a previous work, several cyanobacteria strains were isolated from intertidal zones on the Portuguese coast and characterized using a polyphasic approach [1]. In this study, a preliminary screening indicated that these cyanobacteria did not have the genes encoding proteins involved in the production of conventional cyanotoxins produced by strains from fresh and brackish waters. However, earlier it was shown that extracts of marine *Synechocystis* and *Synechococcus* were toxic to invertebrates, with crude extracts causing stronger effects than partially purified ones, suggesting the presence of different compounds with different chemical structures and possible synergistic effect(s) [2]. To further evaluate the potential of our isolates to produce bioactive compounds, a PCR screening for the presence of genes encoding non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS), targeting the adenylation (A) and ketosynthase (KS) domains respectively, was performed. DNA fragments were obtained for more than 80% of the strains tested, and the results revealed that PKS are more ubiquitous than NRPS genes. The sequences obtained were used to an *in silico* prediction of the compounds that can be produced by each cyanobacterial strain. The predicted compounds displayed a wide range of potential biological activities, being antibiotic and anticancer the more representative. To understand if the genes were transcribed under routine laboratory conditions, RT-PCR analyses were performed for selected strains. Overall, the results presented here will provide a useful insight for further metabolomic studies.


Stress adaptation and response to fluctuating environments – the role of putative outer membrane protein TspO of the cyanobacterium *Fremyella diplosiphon*

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*Fremyella diplosiphon* is a filamentous cyanobacterium undergoing complementary chromatic adaptation (CCA) [1]. During this process the light-harvesting antennae are restructured depending on the external light-quality. This process is transcriptionally controlled by the green/red photoreceptor RcaE that has also been implicated in control of morphology, iron acclimation and chlorophyll abundance [2-4]. CCA requires biosynthesis of new tetrapyrrole molecules that act as chromophores in light-harvesting. During the same process, as a part of changing the prevalent phycobiliproteins in the phycobilisomes, tetrapyrrole chromophores would also have to be degraded, recycled or exported, a process largely unknown. Here, we investigate the potential role(s) of a putative outer membrane protein, homologous to the tryptophan rich outer membrane transporter (TspO) protein found in eukaryotes and prokaryotes, in tetrapyrrole metabolism during CCA. The transcript of the gene encoding TspO from *F. diplosiphon* (*Fd*TspO) is upregulated under green light [5]. Differential regulation of *Fd*TspO due to light-quality is abolished in a mutant lacking functional RcaE under iron-depleted conditions [6]. The role of *Fd*TspO in photosynthetic pigment metabolism, stress-adaptation and nutrient acclimation processes is investigated. Our preliminary results suggest that *Fd*TspO is a predicted outer membrane protein capable of binding tetrapyrroles and that it is involved in stress-regulated processes in the cyanobacterial cell.


A CONSERVED RUBREDOXIN IN THE THYLAKOID MEMBRANE FUNCTIONS IN THE ACCUMULATION OF PHOTOSYSTEM II

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We have isolated a mutant of the unicellular green alga Chlamydomonas reinhardtii that has no detectable photosystem II (PSII) activity, whereas other components of the photosynthetic electron transport chain were still functional. This defect was shown to be due specifically to the absence of a gene, RBD1, encoding a thylakoid membrane-bound iron-sulfur protein known as a rubredoxin. Using the model cyanobacterium Synechocystis sp. PCC 6803, we have created a knockout in the orthologous gene and identified a similar PSII-specific phenotype. We have also characterized a mutant line of the model plant Arabidopsis thaliana lacking this rubredoxin, and its PSII-specific phenotype provides further support for the hypothesis that this rubredoxin has a conserved role in PSII-containing organisms, in contrast to previous reports indicating its role was in the assembly of photosystem I (PSI). We have found that PSII subunits are rapidly degraded in the Chlamydomonas mutant lacking this rubredoxin, so we hoped to accumulate PSII assembly intermediates by creating a double mutant which lacks RBD1 and has an inactive form of the chloroplast protease FtsH. We have found that not only does the inactivation of FtsH permit accumulation of PSII subunits in the rbd1 mutant, but that these subunits assemble into a highly photosensitive PSII complex capable of low levels of oxygen evolution. Experiments are underway to determine the structural and functional characteristics of the PSII from the double mutant and to uncover the precise role of this rubredoxin in the assembly or stability of PSII. Given that cyanobacterial, algal, and plant model organisms each have their own specific benefits and drawbacks, our work is an example of the advantages that can be gained by characterizing photosynthesis-associated genes in parallel model systems. (Supported in part by the Philomathia Foundation and the Gordon and Betty Moore Foundation)
Biogenesis of a bacterial organelle

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The carboxysome is a protein-based organelle required for carbon fixation in cyanobacteria, keystone organisms in the global carbon cycle. Structurally, the carboxysome is composed of thousands of protein subunits including hexameric and pentameric proteins that form a shell to encapsulate the enzymes ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and carbonic anhydrase (CA). Although many of the individual protein components of the carboxysome have been investigated, the mechanism for carboxysome biogenesis has remained elusive. Here we describe the sequence of events in carboxysome assembly in the cyanobacterium *Synechococcus elongatus* PCC7942. Controlled carboxysome biogenesis was accomplished by replacing the native five-gene ccm operon with an inducible version of the native ccm operon or variants systematically lacking each gene. Fluorescently labeled carboxysome proteins functioned as markers to track the assembly process in real-time. We identified the stages of carboxysome assembly and the requisite gene products necessary for progression through each. Our results demonstrate that, unlike membrane bound organelles of eukaryotes, in carboxysomes the interior of the compartment, the procarboxysome, is formed first at a distinct site within the cell. Subsequently, shell proteins assemble to encapsulate the procarboxysome. Assembly concludes with budding and distribution of functional carboxysomes within the cell. We propose that the principles of carboxysome assembly that we have uncovered extend to diverse bacterial microcompartments.
Elucidating the routes of C8-vinyl reduction in (bacterio)chlorophyll biosynthesis

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Most of the chlorophylls and bacteriochlorophylls utilised for light harvesting by phototrophic organisms carry an ethyl group at the C8 position of the molecule. This group is the product of the reduction of a vinyl group, catalysed by a C8-vinyl reductase, on a precursor of the mature pigment, historically believed to be protochlorophyllide [1,2]. Two unrelated classes of C8-vinyl reductase are known to exist, BciA and BciB [3-5]. Assays performed in vitro with recombinant enzymes have revealed that several chlorins carrying a vinyl group at the C8 position can act as substrates, including C8-vinyl chlorophyll [6-8]. However, in vivo data obtained from strains lacking an active C8-vinyl reductase indicate that BciA and BciB have individual preferential substrates on which they catalyse the C8-vinyl reduction.

Ava_4785, an Intramembrane Metalloprotease, Is Required for Cold Acclimation in *Anabaena variabilis*

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Cells respond to their environment by transducing signals across their membranes to elicit transcriptional responses. One mechanism by which information is transduced across the membrane involves regulated intramembrane proteolysis (RIP) of a transmembrane protein by an intramembrane metalloprotease, releasing an active form of either a membrane-anchored transcription factor (MTF) or a membrane-tethered signaling protein (MSP) in response to an extracellular or intracellular signal. The fact that RIP-mediated signal transduction does not require de novo protein synthesis allows the signal to be transmitted in no time. Another unique feature of RIP is that polytopic membrane proteases with catalytic-site motifs embedded within transmembrane segments cleave the transmembrane helix of a protein substrate inside the lipid bilayer, a hydrophobic environment that is distinct from the aqueous environment required for conventional proteolytic enzymes. Thus, intramembrane metalloproteases can accomplish many important membrane-involved tasks beyond the capability of their soluble cousins.

In this study, by inactivating five previously reported intramembrane metalloprotease genes in *Anabaena variabilis* ATCC 29413 [1], *ava_4785* was identified to be a novel gene required for cold acclimation. The *ava_4785* knock-out mutant showed a phenotype that is incapable of survival at both 4°C and freezing temperature after cold acclimation treatment from 30°C to 4°C, with 3-day acclimation sequentially at 25°C, 15°C, 10°C and 4°C, then back to 30°C directly; while same treated wild-type strain can still survive. Furthermore, the *ava_4785* promoter activity was monitored by a transcriptionally fused promoter-less green fluorescence protein (GFP). Strong GFP fluorescence signals were observed under fluorescent microscope after 1 h shift from 30°C to 4°C (with a 3h pre-treatment interval at 15°C), indicating that expression of *ava_4785* is indeed regulated by cold signal. Thus we concluded, for the first time, that an intramembrane metalloprotease coded by *ava_4785* is required for cold acclimation in *Anabaena variabilis*.

Critical Roles of PfsR in iron homeostasis of *Synechocystis* PCC 6803

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Iron is an essential cofactor in a multitude of cellular processes. Although iron is abundant on earth, its availability is normally limited because of its low solubility in aerobic ecosystems. Cyanobacteria have evolved complex regulatory networks to tightly control iron homeostasis, ensuring the essential function of iron yet avoiding cellular damage. We have previously discovered a regulatory protein PfsR (photosynthesis, Fe homeostasis and stress-response regulator), which plays an important role in stress response of *Synechocystis* PCC 6803 [1]. In this study, we investigated in depth the function of PfsR in iron homeostasis. The *pfsR* deletion mutant showed stronger tolerance to iron starvation and accumulated chlorophyll a, carotenoid and phycocyanin to a significantly higher level than wild-type under iron limitation conditions. Western Blot results, consistent with 77K fluorescence emission data, revealed that the *pfsR* mutant assembled more photosystem I and photosystem II compared with wild-type after iron step-down. In addition, the measurements of oxygen evolution capacity and yields of chlorophyll fluorescence indicated that both photosystem I and photosystem II activities were obviously higher in *pfsR* mutant than in wild-type cells under iron-starved conditions. Moreover, inactivation of PfsR led to up-regulation of *bfr* genes (encoding bacterioferritin) but down-regulation of *ho* genes (encoding heme oxygenase), especially upon iron deprivation. From the predicted sequence, PfsR is expected to have DNA-binding activity. The electrophoretic mobility shift assay in this study showed that PfsR exhibits affinity for its own promoter but not for promoters of *bfr* or *ho* genes. Therefore, PfsR is autoregulatory and regulates the expression of *bfr* and *ho* genes through indirect means. On the basis of these results, we conclude that PfsR is a DNA-binding regulatory protein plays a critical role in iron homeostasis.

Inducible Carotenoid Binding Protein Complex From *Synechocystis*6803

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Photosynthetic organisms provide food and oxygen that are essential for life on earth by harvesting solar energy and converting it into chemical energy. However, Changes in global environment might lead to stress conditions in photosynthetic organisms and limit the efficiency of photosynthesis. The damages can be largely contributed to oxygen-dependent destruction of photosynthetic apparatus and other cellular components. To survive, photosynthetic organisms have evolved several protective processes. Cyanobacteria, like other photosynthetic organism, protect themselves from light-induced stress by dissipating excess absorbed energy as heat. It is well known that in cyanobacteria carotenoid plays an essential role in photo protective mechanisms. In this study we have isolated a novel high light and iron stress inducible carotenoid-binding protein complex from the thylakoid membranes of *Synechocystis PCC 6803* cells that are exposed to high light intensity and long term iron deprivation. Pigment of the complex, were extracted in acetone—methanol (7/2) volume and separated by HPLC. The majority of the pigments in the complex are myxoxanthophyll and zeaxanthin, with a neglectable amount of Chl a and β carotene. We hypothesize that the complex may protects thylakoid membranes from extensive photooxidative damages, iron stress by direct or indirect scavenging of reactive oxygen species and most likely involve in state transition. Experiments are underway to determine the protein composition.
Circadian and Diurnal Control of Metabolism in *Synechococcus elongatus* PCC 7942

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The cyanobacterium *Synechococcus elongatus* PCC 7942 possesses a circadian clock with well-characterized functional components that drives robust 24-h rhythms of gene expression. It has been demonstrated that the circadian clock provides *S. elongatus* with a competitive growth advantage under diurnal growth conditions. However, the mechanism by which the clock improves fitness under these conditions is unknown. Our work attempts to deconstruct how signals from the external environment integrate with outputs of the circadian clock to regulate gene expression and metabolism. By growing *S. elongatus* under turbidostatic culture conditions in constant light, we have been able to demonstrate that the circadian clock drives a cycle of accumulation and degradation of the storage carbohydrate glycogen (with a roughly 24-h period) that is lost in mutants lacking circadian rhythms. Circadian mutants grown under diurnal light conditions also accumulate glycogen rhythmically, but display premature accumulation of glycogen that may negatively affect their metabolism during dark periods. Therefore, while diurnal cycles can drive degradation of storage carbohydrates through mass action, circadian rhythms may prime and adjust the timing of storage carbohydrate degradation to optimize diurnal metabolism within the expected environmental conditions.

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Transcriptomic response of *Synechocystis* sp. PCC6803 to long-term ethanol production


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Engineering cyanobacteria to forced fuel producers is supposed to considerably interfere with overall cell homeostasis, which in turn might counteract productivity and sustainability of the process. Therefore, in-depth characterization of the cellular response upon long-term production is inevitable for targeted improvement of a desired strain. Microarray analyses of an ethanol producing hybrid of *Synechocystis* PCC6803 revealed an unexpectedly restricted response when grown in batch cultures under highly-controlled conditions.

The response included a discoordinated *cpc*-operon expression as well as a successive increase of the recently identified small regulatory RNA SyR1 (*Synechocystis* ncRNA 1), which had previously been shown to influence cellular pigment composition.

The impact of a *syR1* inactivation mutant on ethanol production in *Synechocystis* PCC6803 was analyzed by variably designed cultivation experiments.
Defining the Upper Thermal Limits of Oxygenic Photosynthesis: in *Thermosynechococcus elongatus*

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Thermophilic cyanobacteria are capable of carbon fixation at temperatures up to 73°C, the highest measured temperature of any photooxytroph. When compared to the thermal tolerance of other life-forms, such as hyperthermophillic Archea, this is a relatively low maximal temperature. It seems that a crucial component of the oxygenic photosynthetic apparatus is unable to evolve greater thermal tolerance. Studying *Thermosynechococcus elongatus* as a model and utilizing membrane inlet mass spectrometry (MIMS) with stable isotopes, we have assessed photosynthetic fluxes of O₂ and CO₂ to probe the upper thermal limits of photooxytrophic organisms. Through comparisons of relative thermal damage to respiratory oxygen uptake vs photosynthetic oxygen evolution, we are defining the key site of thermal weakness. High precision physiological measurements made with the MIMS system have also allowed us to further characterize *Thermosynechococcus elongatus*. Initial findings indicate that high affinity inorganic carbon transporters involved in the carbon concentrating mechanism are inducible, and we report the $K_{0.5}$ and $V_{max}$ of these systems.
Deletion of PsbT in *Synechocystis* sp. PCC 6803 Results in Increased Turnover of D1 and Causes QA\(^{-}\) Oxidation To Be Blocked by Formate and Dimethyl-p-Benzoquinone

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PsbT (~3.5 kDa) is located at the monomer-monomer interface of photosystem II (PS II). Inactivation of *psbT* resulted in a strain where oxygen evolution supported by dimethyl-p-benzoquinone (DMBQ) in the presence of K\(_3\)Fe(CN)\(_6\) was highly susceptible to high-light-induced stress. These cells were able to recover from photodamage when transferred from high- to low-light conditions and recovery was dependent on protein synthesis. Furthermore, \(^{35}\)S-methionine-labeling studies indicated a more rapid turnover of the D1 protein in ∆PsbT cells than in wild type. However, when oxygen evolution was supported by the addition of HCO\(_3^-\), following high light treatment, no impairment in oxygen evolution was observed in the ∆PsbT mutant. Furthermore, the addition of formate (HCO\(_2^-\)) to ∆PsbT cells — that had not been exposed to high light — abolished oxygen evolution supported by DMBQ and K\(_3\)Fe(CN)\(_6\) and this effect was reversed by the addition of HCO\(_3^-\). Moreover, QA\(^{-}\) oxidation in ∆PsbT cells was slowed from an overall half-time of 0.2 ms to 4.0 ms; this was extended to 10 ms in the presence of formate and to >100 ms in the presence of DMBQ. HCO\(_3^-\) reversed the effect of formate and partially reversed the effect of DMBQ: none of these treatments impaired QA\(^{-}\) oxidation in the wild type. These results indicate that removal of PsbT from PS II leads to a remodeling of the acceptor side of PS II so as to: (i) slow electron transfer between QA and QB; (ii) introduce a bicarbonate-reversible formate inhibition of QA\(^{-}\) oxidation, and (iii) results in the PS II-specific electron acceptor DMBQ becoming a potent inhibitor of QA\(^{-}\) oxidation. We hypothesize that PsbT plays a structural role in optimizing the quinone-iron environment on the acceptor side of PS II.
Flavodiiron proteins are distributed spatially to protect both photosynthesis and nitrogen fixation in filamentous heterocystous cyanobacteria

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Flavodiiron proteins (FDP), involved in detoxification of O2 or NO in anaerobic Bacteria and Archaea, were found in all so far sequenced cyanobacterial species. In unicellular, non-N2-fixing cyanobacteria Synechocystis sp. PCC 6803 two out of four FDPs, Flv1 and Flv3, function in photoreduction of O2 directly to water (cyanobacteria-specific Mehler reaction) obtaining electrons on the reducing side of Photosystem I1,2,3.

Filamentous, N2-fixing, heterocystous cyanobacteria possess two extra copies of Flv1 and Flv3 proteins. In Anabaena sp. PCC 7120 each Flv1/Flv3 protein is encoded by the family of two genes, “A” and “B”. We have found previously that the flv1A and flv3A genes were up-regulated strictly in vegetative cells at low CO2 and high light conditions4.

Membrane Inlet Mass Spectrometry measurements using 18O2 isotope reveals that light-induced O2 uptake is completely inhibited in Δflv1A and Δflv3A mutants (similarly to Δflv1 and Δflv3 of Synechocystis). As a consequence, both mutants grown in high (3%) CO2 conditions demonstrate the over-reduction of PQ pool and decreased activity of both photosystems. We have also shown that Δflv1A and Δflv3A are not able to grow under fluctuating light condition which creates excess pressure on photosynthetic apparatus3.

The Flv3A protein appears to be specifically important for the wellbeing of cells under ambient CO2 conditions. Due to increased light-sensitivity, the Δflv3A mutant requires activation of protective mechanisms for the maintenance of the photosynthetic apparatus. The mutant contains more carotenoids, reduced antennae, and modifies the “state transition” system. Moreover it requires the activation of photorespiration which, to our knowledge, first time demonstrates the oxygenating function of RUBISCO in cyanobacteria under native growth conditions.

In contrast to the “A” genes, the flv1B and flv3B genes were shown to be expressed after N step-down and the corresponding proteins were located exclusively in heterocysts4, special cells where micro-oxic conditions are maintained for the fixation of atmospheric N2. The NifH, NifD, and NifK subunits of nitrogenase, the key enzyme of N2-fixation, are strongly down-regulated in the mutant strain lacking the Flv3B protein. Consequently, growth of the Δflv3B mutant was strongly impaired in N2-fixing conditions and the mutant cells contained less chlorophyll and reduced antennae.

Importantly, isolated heterocysts of Δflv3B have an increased rate of dark respiration, but completely lack the light-induced O2 uptake, whereas in heterocysts of WT the rate of O2 uptake under the light is about 40% higher than in darkness. Therefore, we propose that the “heterocyst-specific” copy of the Flv3 protein protects enzymes of N2-fixation by reducing O2 and maintaining the micro-oxic conditions.

It is highly conceivable that the appearance of additional copies of genes encoding flavodiiron proteins in the genomes of filamentous, N2-fixing, heterocystous cyanobacteria and the spatial specificity of protein expression allow both the protection of photosynthesis in vegetative cells and the maintenance of the efficient N2-fixation in heterocysts.

1 Helman et al. (Current Biology, 2003)
2 Allahverdiyeva, Ermakova et al. (Journal of Biological Chemistry, 2011)
3 Allahverdiyeva, Ermakova et al. (PNAS, 2013)
4 Ermakova et al. (FEBS Letters, 2013)

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In order to succeed in habitats exposed to high doses of UV radiation, such as soil and rock surfaces, cyanobacteria must use various UV radiation defense mechanisms. Sunscreens serve as passive preventative mechanisms that allow them to stop UV before it reaches the cellular machinery and DNA, or produces reactive oxygen species. The indole-alkaloid scytonemin, found exclusively among cyanobacteria, is one such sunscreen. It is a brownish-yellow, lipid-soluble pigment located in the extracellular matrix of the cells that absorbs within the UV-A range (315-400 nm). Although the production of scytonemin is induced by UV-A, so far nothing is known about the transcriptional factors involved in this process. The discovery of a gene cluster (scy) responsible for the production of scytonemin in *Nostoc punctiforme* ATCC 29133 [1], opened the door to molecular biology studies. One of our aims is to unveil the functional role of each scy gene product and the proteins involved in the scytonemin regulation.

The *in silico* analysis of the enzymes encoded by scyD, scyE, and scyF suggests that these are periplasmic proteins involved in the late steps of the pathway [2]. Since the initial monomer synthesis and condensation are cytoplasmic [3], to be true this would be a distinctive case of compartmentalization of a biosynthetic pathway among prokaryotes. Plasmids for the in-frame deletion of *N. punctiforme* scyD, scyE, and scyF were constructed and conjugations for the transformation of *N. punctiforme* are currently being performed. The generation of *N. punctiforme* in-frame deletion mutants for these genes will allow assessing the role of each encoded protein in the scytonemin biosynthetic pathway. The cellular localization of ScyD, ScyE, and ScyF will be evaluated by the construction of GFPuv fusions to the C-terminal of each protein and subsequent microscopy analysis. Constructions of scyX-gfp fusions expressed under the lacZ promoter in a cyanobacterial replicative plasmid were performed and these constructs will be transferred to *N. punctiforme* via electroporation.

5'RACE (Rapid Amplification of cDNA Ends) was used for the identification of the scy operon promoter region. A TSP (Transcription Start Point) was identified 227 bp upstream scyA start codon. An *in silico* analysis of the putative scy promoter region revealed the presence of a -10 box but was unsuccessful in identifying any conserved motifs for the binding of known transcriptional regulators. Furthermore, in an attempt to discover proteins involved in the UV sensing and response, we started by targeting the analysis of *N. punctiforme* genes predicted to encode phytochrome-related proteins that exhibit a ground-state absorption in the UV to violet spectral region.

Evolution and biogenesis of photosystem I in the siderophilic cyanobacterium *Leptolyngbya* sp. JSC1

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Key word: Cyanobacteria; Photosystem I; Iron limitation

*Leptolyngbya* sp. JSC1 (hereafter JSC1) was isolated from a floating cyanobacterial mat inhabiting an iron-depositing, circum-neutral hot spring in Yellowstone National Park. This filamentous strain is siderophilic: it requires high concentration of iron for growth and accumulates both intracellular and extracellular iron precipitates. Interestingly, based on genomic sequence, it contains two *psaAB* operons (*psaAIBI, psaAIIBII*) and one other *psaBIII* gene, suggesting its specificity in regulation and biogenesis of PSI reaction centers. JSC1 also has four *isiA* genes, which encode the iron-stress-induced protein IsiA. Besides the conserved *isiAI-isiB* operon that is upregulated by iron deficiency in most cyanobacteria, the other three *isiA* genes are arranged in a cluster of ‘*isiAII-isiAIII-isiaPsaL-cpcG-isiaIV*’, in which gene *isiA*PsaL encodes a fusion protein composed of IsiA and PsaL domains. Considering that iron is a critical factor in the regulation of PSI biogenesis, we studied the PSI composition and function in JSC1 grown under normal and iron-stress conditions by a variety of methods. Compared to the model organism *Synechococcus* sp. PCC 7002, iron-replete JSC1 cells have a higher PSI/PSII ratio with the maximum fluorescence emission from PSI at 727 nm, as measured by 77K fluorescence emission spectroscopy. PSI complexes isolated from iron-replete JSC1 occur as IsiA-free monomers and trimers. In response to iron depletion, JSC1 cells accumulated less PSI with the chlorophyll fluorescence emission maximum was blue-shifted to 722 nm. Further analysis revealed that expression and association of IsiA protein into PSI occurred in iron-depleted cells. A novel PSI complex was observed that contained both IsiAIII and the fusion protein IsiAPsaL. The chlorophyll content per P700 of the PSI-IsiAIII-IsiAPsaL complex is ~2 fold higher than that of the iron-replete PSI trimers; this result suggested that these complex had an enlarged chlorophyll light-harvesting antenna. The fusion protein IsiAPsaL is likely to stabilize the association of and probably also facilitate the energy transfer from IsiA antenna to PSI reaction center, which could compensate for the loss of phycobilisomes and PSI resulting from iron deficiency. Meanwhile, as PsaAII and PsaBII were the only PSI core subunits detected under both iron-replete and iron-depleted conditions. Studies concerning the regulation of the *psaAIBI* operon is undergoing and might ultimately provide insights into understanding the impact of environmental factors on PSI evolution and biogenesis.
Global-warming induces a replacement of keystone cyanobacteria in Western North American soils

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Shifts in plant and animal geographical ranges are one of the most commonly predicted outcomes of global climate change. On warming, species are faced with either migrating towards cooler areas or sustaining range losses. Such predictions have so far excluded microorganisms, not only because their geographic distributions remain largely undescribed, but also because it is unknown if environmental parameters sensitive to global change, such as temperature or water availability, help at all in shaping their large scale distributions. We analyzed DNA-based, continental-scale compositional surveys of North American biocrusts and found that a clear replacement in dominance exists between the cyanobacteria Microcoleus vaginatus and M. steenstrupii, key pioneers to biocrust formation. Statistical analyses of climatic, geographic, edaphic and microbial compositional data, indeed point to temperature as a major driver of the segregation of M. vaginatus dominance to colder climates. Moreover, the responses to temperature of enrichment cultures and of a collection of cultivated strain representative of these two taxa clearly support this contention, with M. vaginatus strains being more psychrotolerant and less thermotolerant than those of M. steenstrupii, regardless of their geographic origin. In view of predicted climate drift in the US Southwest, M. steenstrupii dominance is likely to replace that M. vaginatus in much of the studied area within the next few decades, with yet to be determined ecological consequences for soil fertility and erodibility.

Three cyanobacteriochromes synthesize and degrade c-di-GMP in *Thermosynechococcus*

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Cyanobacteriochromes are cyanobacterial photoreceptors that respond to light from near-UV to red region. Their photosensory GAF domain draws growing attention as an optogenetic tool with regard to its small size and diverse optical properties. However, the molecular mechanisms of cyanobacteriochrome signaling remain much less explored. Some cyanobacteriochromes regulate complementary chromatic acclimation via two-component phosphorelay system. Other cyanobacteriochromes regulate phototaxis probably via protein-protein interaction or cyclic dimeric GMP (c-di-GMP) signaling. c-di-GMP is a bacterial universal second messenger involved in sessile/planktonic lifestyles and motility, but the study of c-di-GMP signaling in cyanobacteria has just started. We previously reported that Tlr0924 is a blue- and green-light responsive cyanobacteriochrome in a thermophilic cyanobacterium *Thermosynechococcus*. Tlr0924 synthesizes c-di-GMP by its diguanylate cyclase activity when irradiated with blue light and induces cell aggregation under low temperature. Besides Tlr0924, there are two putative cyanobacteriochromes (Tlr1999 and Tlr0911), which may be involved in c-di-GMP signaling in *Thermosynechococcus*.

Here, we report the biochemical characterization of Tlr1999 and Tlr0911. Tlr1999 shows photoconversion between blue- and teal-light responsive states. Tlr1999 shows phosphodiesterase activity to degrade c-di-GMP. The activity is enhanced by teal light and GTP but reduced by blue light. These results suggest that Tlr1999 serves as a teal light sensor to turn off the c-di-GMP signaling. Tlr0911 shows photoconversion between blue- and green-light responsive states. Tlr0911 shows both diguanylate cyclase and phosphodiesterase activities. Blue light irradiation enhances the diguanylate cyclase activity and decreases the phosphodiesterase activity. Green light irradiation enhances the phosphodiesterase activity and decreases the diguanylate cyclase activity. This is first evidence that a photoreversible photoreceptor indeed regulates two different signal-output activities in response to two different light signals. This cyanobacterium may integrate the three photoreceptors under ambient light conditions to regulate c-di-GMP signaling for sessile lifestyle such as cell aggregation.
Identification of Two Genes Required for Long-Chain Hydrocarbon Production in Cyanobacterium *Anabaena* sp. PCC 7120

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Oil reserves worldwide are limited, and as prices have risen, renewable fuels have become increasingly important. Is there a biofactory that can convert carbon dioxide, water and sunlight into fuels? Yes, several species of cyanobacteria are known to produce alkanes and alkenes from carbon dioxide, water and sunlight. These liquid hydrocarbons are the major component of oil. This provides a more sustainable approach to producing biofuels than using grains or biomass that could compete for food. However, native cyanobacteria do not produce these hydrocarbons in sufficient yield for commercial deployment. This research is to understand how cyanobacteria can photosynthetically produces fuels using only atmospheric gases and water. Two putative alkane genes in *Anabaena* sp. PCC 7120 that produce alkanes through a fatty acid pathway were knocked out by inserting a *gfp-spec* cassette within the genes. Subsequently, the engineered genetic sequence was homologeously integrated into the chromosome of *Anabaena* 7120. Through a double crossover, the functional alkane genes were replaced by the inactivated gene segment. The GC/MS data comparing cell extractions of the mutant and wildtype *Anabaena* 7120 reveal that 6-methyl octadecane (C\textsubscript{19}H\textsubscript{40}) produced by the wildtype was not found in the knockout mutant, suggesting that one or both of the alkane genes that were knocked out are part of a biosynthetic pathway for the production of 6-methyl octadecane. A complementation experiment with reintroduction of the functional genes into the mutant is being performed to ascertain the role of these two genes.
Improved genetic tools for cyanobacteria

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Cyanobacteria provide an excellent platform for the production of renewable bioproducts including biofuels. New strains with potential for large-scale growth and improved genetic tools are needed to fully exploit cyanobacteria for biotechnology. A search for strains with superior growth traits led to the identification of *Leptolyngbya* sp. BL0902, which shows robust growth in a range of temperatures, salt and urea concentrations, alkalinity, and at high solar irradiance. Its growth-rate can rival that of *Arthrospira* strains, and importantly it showed culture stability in large outdoor ponds. *Leptolyngbya* accumulates higher lipid content and a higher proportion of monounsaturated fatty acids than *Arthrospira* strains. Unlike *Arthrospira* strains, for which only limited genetic methods are available, *Leptolyngbya* sp. BL0902 is genetically tractable and has been shown to be amenable to efficient conjugal transfer from *E. coli* of broad host range self-replicating plasmids, expression of antibiotic resistance markers and reporter genes, and transposon tagging (Taton et al 2012, PLoS ONE 7: e30901). We are currently developing genetic tools for *Leptolyngbya* sp. BL0902 and other cyanobacterial strains based on the idea that improved, modular, standardized, and well-characterized genetic tools will be required to exploit diverse production strains. Inspired by the recent development of synthetic biology technologies, we devised a strategy that allows the efficient construction of modular vectors from up to 4 donor vectors, each vector harboring an essential module. The resulting modular vectors are designed for the component parts to be easily replaced or additional parts to be easily inserted. Different types of vectors can be assembled, including autonomously replicating vectors and suicide vectors for gene knockout and gene expression from the chromosome. A library of donor vectors carrying standardized parts has been made and tested in several cyanobacterial strains. The library of vector parts includes origins of replication for *E. coli*, origins of transfer for conjugation, origins of replication and neutral sites for various cyanobacteria, antibiotic markers, expression cassettes with different promoters, and reporter cassettes. Assembled modular vectors allow a thorough characterization of different parts in a number of cyanobacterial strains.
The effects of light-dark cycles on the metabolism of *Cyanothece* sp. ATCC 51142 and *Cyanothece* sp. ATCC 7822

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*Cyanothece* is a genus of cyanobacteria that performs photosynthesis in the light and fixes N\textsubscript{2} during the dark when grown in the absence of combined nitrogen. During N\textsubscript{2} fixation, it produces H\textsubscript{2}, a promising candidate for bio-fuel usage. *Cyanothece* sp. ATCC 51142 (*Cyanothece* 51142) displays strong temporal regulation when grown under twelve hours of light and twelve hours of dark (12hL: 12hD), but it is unknown how this strain responds when grown under 16hL: 8hD, and 8hL: 16hD. The effects of light-dark cycles on another strain in this genus, *Cyanothece* sp. ATCC 7822 (*Cyanothece* 7822), have not been detailed, and this study aims to identify what metabolic changes occur when both strains are grown under varying light-dark conditions. Our hypothesis is that by altering the exposure to light-dark, the cultures are still able to maintain circadian patterns, and that 16hL: 8hD conditions will yield higher H\textsubscript{2} production than that from 8hL: 16hD and 8hL: 12hD. However, when grown under short days and long nights, less glycogen accumulates, which leads to less substrate for respiration. This in turn means less respiration for ATP and O\textsubscript{2} scavenging for nitrogenase, thus lowering nitrogenase activity and H\textsubscript{2} production.

Samples were collected for three days to evaluate the H\textsubscript{2} production (using a GC), nitrogenase activity (using a GC), photosynthetic O\textsubscript{2} evolution rates (using a Clark electrode), cellular respiration rates (using a Clark electrode), and cellular glycogen concentrations (through biochemical methods). We determined that, when grown in 16hL: 8hD and 12hL: 12hD, *Cyanothece* 7822 and 51142 are capable of producing H\textsubscript{2} and maintaining strong circadian patterns. However, the actual circadian patterns varied when grown in 8hL: 16hD. From this, we conclude that when *Cyanothece* 7822 and 51142 were grown in short light and long dark periods, regulation shifts from a circadian pattern. However, the net H\textsubscript{2} produced under all three L: D conditions remained approximately the same.
Biorefineries typically release one third of the carbohydrate carbon as CO₂ during fermentation, as well as significant amounts of low grade heat. Ideally, a photosynthetic organism could be engineered to convert these unused resources into high value chemicals. The current microalgae production model suffers from technical challenges including harvest cells, extract oils, and then convert them into a final product. We circumvented these challenges by engineering cyanobacteria as a cellular factory to directly produce and secrete fuel alcohols using sunlight and CO₂ as the feedstock. Our model products in this project are ethanol and linalool (C₁₀H₁₈O). Ethanol is widely used biofuel currently produced from corn or sugarcane. Linalool, a naturally-occurring C₁₀-terpene alcohol emitted as a volatile compound from many flowers, has a potential for drop-in fuels because of its higher energy density and other features for combustion.

Cyanobacteria have native metabolic pathways to photosynthetically convert CO₂ and H₂O to a variety of reduced carbon compounds including pyruvate (the precursor of ethanol) and geranyl diphosphate (GPP, the precursor of linalool). However, cyanobacteria lack the enzymes for converting these precursors to final products. In this research, both the linalool synthase gene from a linalool-emitting plant and the ethanol synthesis genes from a ethanol-producing bacterium were fused to *Anabaena* promoters and subcloned into an *Anabaena* shuttle vector for transformation of *Anabaena*. The transgenic *Anabaena* has been confirmed to produce and secrete either ethanol or linalool. In summary, our engineered cyanobacteria can convert CO₂ (released from corn-based ethanol plants) directly into fuel alcohols (ethanol or linalool), driven by solar energy and waste biorefinery heat.
Genetic Engineering of the Filamentous Cyanobacterium *Anabaena* 7120 for the Photosynthetic Production of Limonene

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Limonene (C\(_{10}\)H\(_{16}\)) is a combustible, cyclic monoterpene found in the rinds of citrus fruits, and is an important chemical in pharmaceuticals, cosmetics, and biofuels. Current methods for limonene extraction from plant material are economically inefficient, therefore limonene has been a target compound for production using microbial engineering. It is catalyzed by the enzyme limonene synthase (LimS) through the C\(_{10}\) pre-cursor geranyl diphosphate (GPP) in the non-mevalonate pathway (MEP) in bacteria. This metabolic pathway is also shared by *Anabaena* sp. PCC 7120, a filamentous nitrogen-fixing cyanobacterium capable of converting CO\(_2\) and H\(_2\)O to high-value compounds. Although *Anabaena* 7120 contains all the genes involved in the MEP pathway, it lacks a limonene synthase in the final catalytic reaction from GPP to limonene. Our novel approach is to metabolically engineer *Anabaena* 7120 as a cellular factory to continuously over-produce and excrete limonene into a photo-bioreactor media using CO\(_2\) as a sole carbon source. To accomplish this, a synthetic MEP operon containing the limonene synthase gene was inserted into an expression vector containing a strong *Anabaena* promoter for maximal protein expression of the operon in the cyanobacteria host. Expression of LimS in *Anabaena* 7120 was verified using Western blotting, and the presence of excreted limonene was detected in both the culture headspace and medium using gas chromatography-mass spectrometry (GCMS) analysis.
Cyanobacterial platform for production of plant secondary metabolites

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Cyanobacteria are being actively used for bioproduction in recent years. However, the application of them for enhancing production of plant secondary metabolites is essentially lacking. Under current technology level (both growth and processing), we believe, this is a particularly attractive field because of the existence of extensive multifunctional membranes and high content of reductants for biosynthesis of plant secondary metabolites in the cells. Our laboratory has been exploring the suitability of using Synechocystis PCC 6803 for production of coumaric acid, caffeic acid and other plant secondary metabolites that exhibit beneficial effects on human health as anticancer, antioxidant, anti-virus and anti-inflammatory agents. The sam8 gene, coding for a tyrosine ammonia-lyase was genetically engineered into Synechocystis sp. PCC 6803 and the strain was found to accumulate no or trace amounts of p-coumaric acid. Upon deletion of a putative degrading enzyme from the genome of the strain, the accumulation of p-coumaric acid was detected using LC/MS and the maximum titer reached ~ 82.6 mg/L. The enzyme p-coumarate 3-hydroxylase (C3H) converts p-coumaric acid into caffeic acid. A ref8 gene (coding for C3H) was targeted into the genome of Synechocystis sp. PCC 6803. The successful insertion of ref8 gene was verified by polymerase chain reactions. The expression of the transgene was confirmed by western blot analyses. Upon feeding of p-coumaric acid to the cyanobacterial culture, production of caffeic acid was measured by HPLC and LC/MS. Production improvement was achieved by optimization of the ref8 codons and by deletion of the degrading enzyme. The caffeic acid production yield was increased from 5.2 to 7.3 mg/L by codon optimization, and the yield was further increased to 72.4 mg/L upon deletion of the degrading enzyme. This is the first report so far that has demonstrated the suitability of using cyanobacteria for production of plant phenylpropanoids.
A biophotovoltaic approach to understanding extracellular electron transport by *Synechocystis* sp. PCC6803

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Biological photovoltaic systems utilise photosynthetic organisms to transfer electrons from water to an anode, generating electrical power or driving cathodic electrosynthesis [1-2]. However, there is a lack of knowledge of the intra- and extracellular route(s) photosynthetically generated electrons take to the electrode. Understanding the inherent electron transfer pathway(s), in particular the site(s) at which electrons exit the photosynthetic electron transport chain, will aid genetic engineering approaches to controllably increase flux to the electrode and introduce exogenous, redox matched electron transport components to enhance electron export. Previous studies using the model freshwater cyanobacteria *Synechocystis* sp. PCC6803 have been limited by its inability to form a biofilm and a requirement for membrane-permeable mediators to shuttle electrons from the organism to the anode [3-5]. We have developed a simple, mediatorless bioelectrochemical setup for rapid and reproducible measurement of photocurrent from *Synechocystis* cells immobilised on an inorganic electrode. Photocurrent scales with cell density and light intensity, and analysis of mutant strains and the use of inhibitors confirm water splitting is the major source of photogenerated electrons. Parallel determination of oxygen evolution and fluorescence kinetic measurements allows normalisation of photocurrent to the rate of water splitting and electron transfer by PSII. A number of candidate mutants are being tested with the aim of identifying components involved in anodic electron transfer.

Unravelling the mysteries of Mg-Protoporphyrin IX Monomethylester (oxidative) Cyclase, the third committed enzyme in chlorophyll biosynthesis

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Chlorophyll is essential for harvesting and trapping solar energy in photosynthetic organisms. The majority of the enzymes in the chlorophyll biosynthetic pathway have been characterised in detail, however one enzyme, the magnesium-protoporphyrin monomethylester cyclase (cyclase), has remained an enigma for over sixty years. The cyclase is responsible for the formation of the chlorophyll isocyclic ring (ring E), which differentiates chlorophylls from other tetrapyrrole molecules. Until recently, only one component of the cyclase was known, the subunit known as acsF. In this work we present the isolation, identification, characterisation and crystal structure of Ycf54, another subunit of the cyclase, from the cyanobacterium *Synechocystis PCC 6803*. Additionally a series of *in vivo* point mutations have been used to map the site on Ycf54 required for interaction with the *Synechocystis* AcsF subunit, Sll1214.
Effects of BP Oil Spill and Dispersant on Photosynthesis and Metabolism of Cyanobacteria

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The Macondo well released million barrels of gas and oil into the Gulf of Mexico due to the sinking of the Deepwater Horizon drilling platform in 2010. Approximately 1.6 million gallons of dispersant (eg. Corexit 9500) has been used to break up the oil into small droplets and hopefully to reduce the damage of oil spill on environment and wildlife. However, the roles of dispersants are controversial and may cause more toxic effects than the oil spill. The response to the oil spill requires the multi-disciplinary efforts, including oceanography, biology, chemistry, and engineering [1]. We have previously investigated the response of cyanobacteria to environmental factors including high light and elevated temperatures [2]. In this work we intend to explore the effects of oil spill (crude oil) and dispersant (Corexit 9500) on the photosynthetic performance and metabolism in cyanobacteria including *Synechocystis* sp. PCC 6803 and *Acaryiochloris marina* using bioanalytical techniques. Experimental procedures for probing the effects of oil spill and dispersant on cyanobacteria were established (Figure 1). Cyanobacterial cells were grown in the absence and presence of oil spill and dispersant. Growth and photosynthetic performance of cyanobacteria were examined by UV-vis-NIR spectrometry, SDS-Page, and oxygen evolution measurement. The volatile metabolic components of cyanobacteria were detected and identified by GCMS. In addition, FTIR probed the changes of molecular structures in pigments, proteins, and whole cells during the exposure to oil spill and dispersant. This project may provide molecular information on the responses of cyanobacteria to the Deepwater Horizon oil spill, which might provide useful information in probing and developing strategies to address the events.

Figure 1. Effects of oil spill and dispersant on cyanobacteria.
Left panel: *Synechocystis* sp. PCC 6803; Middle panel: *Acaryiochloris marina*; Right panel: “CTR” is control, “O” is control+oil, “OD” is control+oil+dispersant


**Water stress protein A (WspA) in cyanobacterial *Nostoc* species**

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*Nostoc* is a group of nitrogen-fixing cyanobacteria found in a variety of environments and often forms colonies composed of filaments of moniliform cells in jellylike sheath. The terrestrial cyanobacterium *Nostoc commune* has a marked capacity to tolerate simultaneous stresses of desiccation, UV irradiation, extreme temperatures and oxidation, and can survive long-term in a desiccated state. *N. commune* forms visible colonies in which cellular filaments are embedded within extracellular matrices (ECM) [1]. The major component of ECM is extracellular polysaccharides (EPS), accounting for 80% by weight of desiccated *N. commune* colonies, and it is thought that cyanobacterial EPS play a pivotal role in protecting cells from various stresses in severe environments. The major protein component in *N. commune* ECM is a water stress protein (WspA) [2]. More than 70% of the total soluble proteins in desiccated *N. commune* colonies are WspA and its relatives, and synthesis and secretion of WspA were induced by desiccation or UV irradiation [1, 2]. The aquatic *Nostoc verrucosum* forms macroscopic colonies, which consist of cellular filaments and ECM materials. In addition, WspA was present in *N. verrucosum* as a major soluble protein component, suggesting that the production of ECM and WspA are not directly linked to extreme desiccation tolerance in *N. commune* [3].

In this study, WspA in the third *Nostoc* species, a freshwater cyanobacterium *N. sphaericum* was investigated, since *N. sphaericum* forms round and solid thalli which are different from jellylike macroscopic colonies of *N. commune* and *N. verrucosum*. *N. verrucosum* and *N. sphaericum* cells were desiccation sensitive, in contrast to the extreme desiccation tolerance in *N commune* cells. The ECM proteins were extracted from *N. sphaericum* thalli and separated by SDS-PAGE. The proteins with sizes corresponding to those of the WspA in *N. commune* and *N. verrucosum* were not detected by staining with CBB. The wspA or wspA-like genes were not found in the genomes of any other cyanobacterial species including *N. punctiforme* which is closely related to *N. commune*. However, cloning and sequencing of the PCR product amplified from chromosomal DNA of *N. sphaericum* with wspA-specific primers indicated that the wspA-like gene(s) was present in the genome of this particular *Nostoc* species. These results suggest that the presence of WspA is not a unique feature to characterize *N. commune* and further studies are necessary to elucidate the origin and function of WspA in the genus *Nostoc*.

An assessment of the capability of Synechococcus sp PCC 7002 metabolic network for the production of Bio-chemicals.

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Cyanobacteria have diverse metabolic capabilities that enable them to produce a variety of industrially relevant bio-chemicals and biofuel precursors from water and CO\textsubscript{2} by harnessing solar energy. Since they are prokaryotes they are easy target for genetic manipulation. Metabolic engineering of cyanobacteria can help in the effective utilization of the system towards cheaper production of biochemical and biofuels. In this work we present an assessment of the capabilities of the metabolic network of a marine cyanobacterium, Synechococcus sp. PCC 7002. The strain has a short doubling time and is capable of withstanding high salt concentration and high light intensity. We use a metabolic model of Synechococcus sp. PCC 7002, isyp611 [1, 2] to assess the theoretical yield of 12 biosynthetic precursors and 20 amino acids [3] (both in the light and dark conditions). These biosynthetic precursors and amino acids are the precursors for many biofuels and industry relevant bio-chemicals. The theoretical yields are estimated using Flux Balance Analysis (FBA) approach to analyze the metabolic network. The theoretical yields estimated are the maximum achievable limit for metabolic engineering. Next we report the constraints for each of these metabolites by interpretation of the shadow prices or reduced cost (Mathematical dual of linear optimization used in FBA) associated with each metabolites [3,4]. The constraints are in terms of stoichiometry, redox potential (NADPH), and energy (ATP) [4]. Shadow price or reduced cost is a parameter associated with the linear programming used in FBA and can be used for the analysis of the above mentioned constraints associated with the production of a particular metabolite. A metabolite can have one or more of the above three constraints. Knowledge about these constraints assists in framing the strategy for metabolic engineering. We also assess the balance between optimal growth and the biochemical production. Finally we report the effect of the presence of a reduced carbon source in the form of glycerol.

References:

Extending Proteomics into Cyanobacteria Characterization

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Previous global proteomic analysis across both *Synechocystis* sp. PCC 6803 and *Cyanothece* sp. ATCC 51142 have demonstrated the capability to quantitatively track and accurately identify the dynamic nature of specific protein abundances due to diurnal rhythms and altered environmental perturbations [1-3]. Additionally, coupling both transcriptomic and proteomic observations has provided a more complete perspective of the mechanisms of energy conservation and utilization for these cyanobacteria. As current proteomic technologies and approaches continue to extend levels of detection and sensitivity, with increased robustness and throughput for quantitative measurements, we have continued to extend our studies to address additional questions in cyanobacterial biology. Data will be reported from a study identifying and quantifying specific proteins localized to either the plasma (PM) or thylakoid (TM) membrane in *Synechocystis* 6803. Using advanced methods, we were able to detect more than 600 different proteins that were significantly observed in either PM or TM. Interestingly, of this group, many more proteins were localized to PM compared to TM. These data begin to elucidate the protein structural organization of the cell.

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Characterization of 4(1H)-quinolone (4-1HQ) and 2-nonyl-1-hydroxy-4(1H)-quinolone (NQNO) as inhibitors of photosynthetic electron transfer and signaling

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The cytochrome bf complex of photosynthesis performs key energy conversion and regulatory reactions, which involve two binding sites for quinone molecules (Qₚ and Qₙ), on the positive and negative sides of the membrane, respectively, with corresponding high- and low-potential electron transfer chains. Inhibitors are important for investigating such reactions but relatively few are available for cytochrome bf complexes. 2-nonyl-1-hydroxy-4(1H)-quinolone (NQNO) binds the quinone-reductase (Qₙ) site and slows electron flow through the low-potential chain, but is not available commercially. We have now synthesized NQNO and a related precursor, 4(1H)-quinolone (4-1HQ), and used a unique, pump-probe, kinetics spectrophotometer (BioLogic JTS-10) to investigate the impacts of these inhibitors on electron transfer reactions in Synechococcus sp. PCC 7002 cyanobacteria. In contrast to NQNO, 4-1HQ resulted initially (within ~50 ms of illumination) in oxidation, rather than reduction, of the low-potential chain. Interestingly, NQNO as well as 4-1HQ slowed electron flow through the bf complex (measured as cytochrome f/c₆ re-reduction) after short (9 ms) illumination periods. However, after longer illumination (e.g. 10 sec) only 4-1HQ remained effective as an inhibitor of electron flow through the cytochrome bf complex. In contrast, NQNO became ineffective as an inhibitor of steady-state electron flow although the low-potential chain (b-hemes) remained reduced. Overall, these data indicate that 4-1HQ binds the cytochrome bf Qₚ-site and inhibits quinol oxidation at this site rather than quinone reduction at the Qₙ site. Further, the initial inhibition by NQNO of electron flow through the bf complex, and subsequent loss of inhibition, suggest that NQNO binding to the Qₙ-site, or reduction of the low-potential chain, results in a change in conformation or binding that renders NQNO ineffective. This has interesting implications for electron transfer and signaling reactions mediated by the cytochrome bf complex. These inhibitors will help dissect electron transfer and signaling reactions, including possible conformational changes and formation of cytochrome bf ‘supercomplexes’ with photosystem I or other proteins.
Production of 2’-Hydroxy 2-Myxol Fucoside in Response to pH, Blue Light Intensity, and Temperature in the Thermophilic Cyanobacterium, Thermosynechococcus, BP-1

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Abstract

The thermophilic cyanobacterium, Thermosynechococcus, BP-1, has a bifurcated carotenoid pathway in which a rare glycosylated compound, 2’-hydroxy 2-myxol fucoside (HMF), is produced in abundance under certain environmental parameters. In order to determine optimal conditions for the production of HMF and other carotenoid intermediates, a screening experiment with varying blue light intensity, temperature, and pH parameters was conducted. Surface plots were generated for the response of intermediate compounds in each pathway based on regression analysis of the parameters and their interactions. An inverse relationship was discovered between the β-carotene pathway and the HMF pathway with β-carotene decreasing due to a drop in temperature, increase in pH and increase in blue light intensity, and with HMF levels rising under the same conditions. Meanwhile, intermediate compounds of each pathway, zeaxanthin and myxol fucoside, respectively, accumulated as temperature increased, suggesting possible down-regulation of 2,2’-β-hydroxylase (crtG). Work is currently underway to study differential gene expression using RNA-seq and real-time qPCR of selected screening conditions for analysis of global and local biochemical regulation of carotenogenesis in BP-1.
Converting *E. coli* into a Nitrogen Bio-Fertilizer Using a Cyanobacterial *nif* Cluster: an iGEM project

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**Abstract:**

The production of a bio-fertilizer could benefit the agricultural industry by decreasing the need for energy intensive chemical nitrogen fixation processes. The *nif* cluster of the unicellular cyanobacterium *Cyanothece* 51142 consists of 29 genes that construct and regulate a nitrogenase protein complex, which catalyzes the fixation of atmospheric nitrogen. This year, our iGEM team aims to harness the power of *nif* to produce ammonia in *Escherichia coli*. After synthesizing a *nif*-containing plasmid (35 kbp) using the DNA assembler method (Shao et al 2009) and transforming that plasmid into *E. coli*, our team tested for nitrogenase activity using the acetylene reduction assay. The transformed *E. coli* were then compared to wild-type under limited nitrogen conditions to check for a competitive advantage. Future tests will evaluate the expression of various nitrogenase subunits, such as *nifD* and *nifK*. Our team also aims to further characterize the promoter sequences of the *Cyanothece* 51142 *nif* cluster. Between the *cysE* and *nifB* genes, there is a 958 bp uncharacterized, bidirectional promoter region of particular interest. We are currently using fluorescent reporters to identify key regions within this promoter sequence, and plan to test its function in various environmental conditions.

**Reference:**

Characterization of the role of CpeF in phycoerythrin biosynthesis in *Fremyella diplosiphon* strain UTEX 481

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Phycoerythrin (PE), present on the outer phycobilisome (PBS) rods in *Fremyella diplosiphon*, contains five covalently attached phycoerythrobilin (PEB) chromophores for efficient photosynthetic light capture. Chromophore ligation on phycobiliprotein α and β subunits (two PEB on α-PE and three PEB on β-PE) occurs through bilin lyase catalyzed reactions. It is believed that each bilin attachment site is chromophorylated by specific bilin lyases. The cpeF gene in *F. diplosiphon* is orthologous to a gene that was previously named mpeV from marine *Synechococcus* species [1]. This cpeF gene and the cpeYZ lyase genes (which encode the lyase that attaches PEB to α-82 of PE in *F. diplosiphon* [2]) belong to the CpcEF family of bilin lyases.

To better understand the function of cpeF in native cyanobacteria, we characterized PE purified from a cpeF deletion mutant (ΔcpeF) in *F. diplosiphon* and compared it with wild type (WT). The green phenotype of ΔcpeF is indicative that the mutant produced less PE than the brown-colored WT cells. PE purified from the ΔcpeF mutant cells was analyzed using absorbance and fluorescence emission spectroscopy, SDS-PAGE, Western blot and mass spectrometry. PE purified from ΔcpeF showed a slight shift in molecular weight, possibly indicating a loss of one or more PEB chromophores. Mass spectrometry analysis suggests PEB is missing from the Cys-48/Cys-59 and Cys-165 sites on β-PE peptides. The results obtained thus far from the mutant analysis suggest that CpeF plays a role in β-PE chromophorylation and biosynthesis. Preliminary results from recombinant heterologous co-expression in *E. coli* suggest that CpeF (MpeV) is the lyase responsible for the doubly-ligated chromophore at Cys-48/59 at rings A and D, respectively.

References


ETHYLENE AFFECTS THE PHOTOTACTIC RESPONSE IN CYANOBACTERIA

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Ethylene receptors in plants are thought to have been acquired following the endosymbiotic event that led to chloroplasts. In plants, ethylene functions by binding to ethylene receptors to elicit downstream responses. Analysis of cyanobacteria genomes shows that many have proteins with putative ethylene-binding domains with sequences similar to ethylene receptors in plants.[1] Additionally, many cyanobacteria species have high-affinity, saturable ethylene binding activity.[1] Synechocystis sp. PCC 6803, is one of these cyanobacteria with high-affinity ethylene binding activity.[2] Here, we seek to elucidate the biochemical nature of ethylene binding in Synechocystis and determine the physiological role of ethylene in this organism.

In Synechocystis, slr1212 encodes a multidomain protein, SynETR1, which contains a predicted ethylene binding domain.[2] By exogenously expressing a truncated version of the protein with only the ethylene binding domain, we have shown that SynETR1 is a bona fide ethylene binding protein. Additionally, we found that copper enhances ethylene binding as is seen in ethylene receptors from plants. Previous reports showed that SynETR1 is a light receptor mediating the phototactic response of Synechocystis.[3-5] From this, we hypothesized that ethylene regulates phototaxis through SynETR1. We confirmed this by showing that ethylene alters phototaxis towards white light. Disruption of this gene alters both phototaxis and responses to ethylene. Thus, SynETR1 appears to be an ethylene receptor. We are currently characterizing ethylene binding to SynETR1 and further characterizing the physiological role of this protein.

5. Song et al. (2011) PNAS 108, 10780-10785
Pili-mediated electron donation to iron oxides in *Synechocystis* sp. PCC 6803

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There is evidence that bacterial type 4 pili may facilitate electron donation to extracellular electron acceptors [1,2]. This electron pathway is thought to be crucial for maintaining respiration in the absence of oxygen [3], but may also make iron complexed in iron oxides available for bacteria. As iron is an essential element for growth of the cyanobacteria *Synechocystis* sp. PCC 6803, we investigate the role of pili in iron utilization by growth experiments, where iron oxides constitute the exclusive iron source. Our experiments with wild type and a mutant strain, in which the gene coding for the main pili (sll1694) has been inactivated, indicate that pili are required for accessing iron in iron oxides. This finding is consistent with a role of pili in converting insoluble ferric (Fe^{3+}) iron oxides into soluble ferrous (Fe^{2+}) iron.

The growth of *Halomicronema hongdechloris* - a filamentous cyanobacterium containing chlorophyll f - under different light conditions

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Light is the one important energy source for photosynthetic organism growth. Its quality and quantity strongly affect the photosynthesis efficiency. For *Halomicronema hongdechloris* (*H. hongdechloris*), chlorophyll a (Chl a) QY peak absorbs light mainly from 650-700 nm; while Chlorophyll f (Chl f), the most red-shifted chlorophyll up to date, extends its absorption range to 760 nm; and phycobiliproteins absorb the light region between 600-670 nm[1]. To investigate the effect of light quality and quantity on the cell development of *H. hongdechloris*, four different light qualities (white light, mono-wavelength 730, 660 and 625 nm LEDs) with various light intensities were applied for establishing different light cultural conditions. The highest growth rate was observed under 730 nm light with intensity of 20 µE or white light with intensity 40 µE (doubling time of 4.4±0.5 days). Chl f is a red-light induced chlorophyll, which is only detected in the cells grown under 730 nm. The pigment composition of *H. hongdechloris* showed similar profiles as most Chl a-containing cyanobacteria, using phycobilins as their major antenna. No Chl f is detected under white light and 625 nm LED light irradiation. According to the differences of pigment profile under various light conditions, the morphological features and photosynthetic membrane structures were studied using confocal fluorescence microscopy. Using a long-pass 692 nm filter, the distribution of Chl f-binding protein complexes was observed only from the cells grown under 730 nm light. This is strikingly different from cells grown under other wavelengths of light. Strong fluorescence between 600 and 680 nm was only observed at the septa and weaker fluorescence throughout the rest of the cell grown under 730 nm light. These images suggest that phycobiliproteins are mainly distributed in septa regions. Chlorophylls are on both side of the cell membranes under 730 nm light, while both phycobiliproteins and chlorophylls are equally distributed among the cell membrane under white light or red light of 625 and 660 nm. The further analysis of cell membrane arrangement was examined using transmission electron microscopy (TEM). It shows agreement with the differences of phycobilinprotein distribution in cells grown under different light conditions.

Design, engineering, and construction of photosynthetic microbial cell factories for renewable solar fuel production

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There is an urgent need to develop sustainable solutions to convert solar energy into energy carriers used in the society. In addition to solar cells generating electricity, there are several options to generate solar fuels. Native and engineered cyanobacteria have been as model systems to examine, demonstrate, and develop photobiological \( \text{H}_2 \) production. More recently, the production of carbon-containing solar fuels like ethanol, butanol, and isoprene have also been demonstrated [1]. We have initiated a development of a standardized genetic toolbox, using a synthetic biology approach, to custom-design, engineer and construct cyanobacteria to produce a desired product [2]. One bottleneck is a controlled transcription of introduced genetic constructs.

I will present and discuss recent progress in the design, construction and use of (i) cyanobacteria for renewable solar fuel production, and (ii) artificial genetic elements for the regulation of transcription in cyanobacteria. Specific focus will be on the native system present in cyanobacteria to generate hydrogen, metabolic modulations [3] and genetic engineering ([4, 5] for enhanced hydrogen production, as well as the introduction of custom-designed, non-native hydrogenases. In addition, I will report on the development of engineered TetR-regulated promoters with a wide dynamic range [6]. By altering only few bases of the promoter in the narrow region between the -10 element and transcription start site significant changes in the promoter strengths, and consequently in the range of regulations, were observed.


Grazing by protists is a major stressor of prey species in ecological systems. In aquatic environments, cyanobacteria are grazed upon by ciliates, flagellates and amoebae. Cyanobacteria are currently being developed to produce biofuels and other high-value commercial products, and decimation of production crops by grazers is a concern. To study interactions between cyanobacteria and protozoan grazers, we have isolated amoebae from natural aquatic environments and experimental production ponds. One predator-prey model system we study is natural amoebal isolate HGG1 grazing upon the filamentous cyanobacterium *Anabaena* sp. PCC 7120. When co-cultured in liquid media, HGG1 ingests *Anabaena* and replicates, clearing the cyanobacterial culture. Observation of feeding behavior by video microscopy has revealed that HGG1 can sever *Anabaena* filaments and ingest cells at the ends of filaments. Consistent with this feeding behavior, we see shortened and damaged filaments after co-culture. To examine the response of *Anabaena* to grazing, we performed proteomic analysis on PCC 7120 cultured with and without HGG1. Initial proteomic analysis revealed upregulation of All3983, a protein that contains a putative signal sequence for secretion and three tandem Surface Layer Homology (SLH) domains. All3983 has been shown to be present in the outer membrane fraction of *Anabaena* sp. PCC 7120 (1). We confirmed that *all3983* mRNA expression is upregulated during grazing. To expand on this approach, we have examined the proteomic response of *Anabaena* after 0, 2, 6, and 12 hours of co-culture with HGG1. This proteomic approach could reveal defense response strategies against predation mounted by *Anabaena* during grazing.

An Evolutionary Fitness Enhancement Conferred by the Circadian System in Cyanobacteria

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Circadian clocks are found in a wide variety of organisms from cyanobacteria to mammals. Many believe that the circadian clock system evolved as an adaption to the daily cycles in light and temperature driven by the rotation of the earth. Studies on the cyanobacterium, *Synechococcus elongatus* PCC 7942, have confirmed that the circadian clock in resonance with environmental cycles confers an adaptive advantage to cyanobacterial strains with different clock properties when grown in competition under light-dark cycles. The results thus far suggest that in a cyclic environment, the cyanobacterial strains whose free running periods are closest to the environmental period are the most fit and the strains lacking a functional circadian clock are at a competitive disadvantage relative to strains with a functional clock. In contrast, the circadian system provides little or no advantage to cyanobacteria grown in competition in constant light. To explain the potential mechanism of this clock-mediated enhancement in fitness in cyanobacteria, several models have been proposed; these include the limiting resource model, the diffusible inhibitor model and the cell-to-cell communication model. None of these models have been excluded by the currently available experimental data and the mechanistic basis of clock-mediated fitness enhancement remains elusive.
Metabolic engineering requires delicate tuning of gene expression for optimization of metabolic flux through biosynthetic pathways. *Synechococcus sp.* PCC 7002 (PCC 7002) shows a great deal of promise as a photosynthetic biorefinery due to its fast growth and broad media tolerance, including high nutrient wastewater, however few genetic tools are currently available for metabolic engineering. We are constructing PCC 7002 specific promoter and ribosome binding site libraries as well as inducible expression systems. A limited number of endogenous promoters have been characterized in PCC 7002 and expression levels of foreign promoters in cyanobacteria are poorly correlated to relative expression in the original host. To address this, we created and characterized the first promoter library for PCC 7002 gene expression. First we identified a minimal version of the strong promoter regulating the phycocyanin subunit gene \( cpcB \) from *Synechocystis sp.* PCC 6803. Its relative strength and transcriptional start site were characterized. We used this minimal promoter as the template for error prone PCR mutagenesis, generating a library that spans a 3-log range of protein expression. We have used this library to investigate the architecture of the \( cpcB \) promoter through sequence analysis of library members and directed mutagenesis. The promoter library was also moved into *Escherichia coli* in order to compare relative expression between these species. We are currently constructing a complementary ribosome binding site library and using these tools to optimize commodity chemical biosynthesis and bioremediation of high nitrogen and phosphorus wastewater.
Cyanobacteria are photosynthetic microbes that prosper under a wide range of environmental conditions. Their sturdiness is based on the successful combination of effective metabolic pathways that hold great promise for use in sustainable production of biofuels and other bioproducts [1]. Due to its rapid doubling time (~4 hours), its ease of genetic manipulation, and accumulation of different storage compounds (glycogen and cyanophycin), the unicellular cyanobacterium *Synechococcus* sp. PCC 7002 represents a robust research organism [2,3,4,5,6]. Thus, metabolic engineering of this organism can be used as a powerful tool to produce renewable energy resources and valuable metabolic products. The aim of this study is to increase the total carbohydrate content of *Synechococcus* cells and to use this biomass for biofuel fermentation. This will be done by various approaches including nutrient limitation and genetic modification of *Synechococcus* followed by enzymatic treatment and subsequent fermentation of the sugar-enriched cyanobacterial biomass to ethanol by an industrial production strain (Thermosacc) of the yeast *Saccharomyces cerevisiae*. *Synechococcus* cells were grown under standard conditions (38°C, 250 µE m⁻² s⁻¹, 1% v/v CO₂) in A+ medium containing different concentrations of sodium nitrate. A total sugar content of 58% w/w of the dry weight was obtained when *Synechococcus* cells were grown under nitrogen limitation (0.24 g L⁻¹ NaNO₃) for 48 hours to an optical density (OD₇₃₀) of 5.9 compared to standard conditions (1 g L⁻¹ NaNO₃) that led to a total sugar content of 29% w/w at an OD₇₃₀ of 11. Lysis of *Synechococcus* cells were carried out using a treatment with cell-wall-degrading lysozyme at 37°C followed by a treatment with polyglucose-degrading alpha-amylases at 60–85°C. Following the enzymatic hydrolysis the produced glucose was fermented to ethanol by *S. cerevisiae*. The conversion yield of total glucose in the cyanobacterial biomass to ethanol was 70–90%. These results show that cyanobacterial biomass can be efficiently used as feedstock for microbial bioethanol fermentation.

Interactions between extracellular polymeric substances (EPS) produced by *Cyanothece* sp. CCY 0110 and heavy metals

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Many cyanobacterial strains can synthesize and secrete extracellular polymeric substances (EPS) that can remain associated to the cell or be released into the environment (RPS-released polysaccharides). The particular features of cyanobacterial EPS, namely the presence of two different uronic acids, sulphate groups and high number of different monosaccharides (usually 6-10), makes them promising for biotechnological applications such as the removal of heavy metals from polluted waters. For the successful implementation of systems based on cyanobacterial EPS, it is necessary to unveil the pathways utilized for synthesis and export, identify the physiological/environmental factors that influence the synthesis and/or the characteristics of the polymers, and characterize the interactions between the cells/EPS with the metal ions. Previously, an *in silico* analysis of cyanobacterial genome sequences was performed and a putative mechanism for the last steps of EPS production/export was proposed [1]. In addition, the physiological/environmental conditions that promote RPS production by the unicellular marine N₂-fixing cyanobacterium *Cyanothece* sp. CCY 0110 were evaluated. The results revealed that this strain is among the most efficient EPS producers and that the amount of RPS is mainly related to the number of cells (growth), rather than to the amount produced by each cell. Light is a key parameter, with high light intensity enhancing significantly RPS production. The polymer produced by *Cyanothece* is highly complex, composed by 9 different monosaccharides (including 2 uronic acids), peptides and sulphate groups and shows remarkable thermo stability [2]. The effects of different concentrations of Cu²⁺, Cd²⁺, Pb²⁺, Li⁺ and Cr³⁺ in the growth/EPS production by *Cyanothece* were also assessed. As expected, cell growth was negatively affected by the presence of the metals, with the cells being more sensitive to copper (cell death at 0.2 ppm), followed by lead, cadmium and lithium (cell death at 70 ppm). The addition of chromium to the culture medium triggered the formation of cell aggregates, strongly affecting growth. In general, carbohydrate production followed the pattern of growth, with the RPS constituting 50 to 60% of the amount of total carbohydrates. The differential tolerance of *Cyanothece* cells to the metals is probably correlated to mechanisms of metal uptake and accumulation [3]. Therefore, the proteomes of the cells grown in the presence or absence of different metals were compared using Isobaric tag for relative and absolute quantitation (iTRAQ) technology.

Construction of Genome-Scale Metabolic Models for a Family of Cyanobacteria

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The \textit{Cyanothece} genus of cyanobacteria has been shown to be capable of both high levels of hydrogen production and nitrogen fixation. These organisms can fix nitrogen by temporally separating the process from photosynthesis. Previously we published the \textit{iCyt773} model for \textit{Cyanothece} ATCC 51142. Here we demonstrate the use of a workflow to quickly develop genome-scale models for \textit{Cyanothece} 7424, 7425, 7822, 8801, and 8802, using the existing \textit{iCyt773} model. Homologs between 51142 and the new species are identified using a bidirectional BLAST search, and these homologs are used to evaluate the gene-protein-reaction (GPR) associations of the reactions in \textit{iCyt773} to determine which exist in the metabolism of the new species. Annotations from Uniprot, NCBI Protein Clusters, and the RAST method are used to identify additional reactions from the SEED database that should be included in the models. All reactions in the developed models are both charge and elementally balanced, and GPR relationships were developed using both the \textit{iCyt773} GPRs and the retrieved annotations.

The five newly created models share a core set of 988 reactions, which accounts for between 76\% and 86\% of the reconstructions. Comparisons yield shared sets of reactions that mirror the species phylogeny, with the closest related sets, 7424/7822 and 8801/8802 having the highest similarities with .934 and .991 respectively. 7425, the species phylogenetically most distant from the others, has the highest number of unique reactions, with 145. Comparisons between the models also yield notable differences, such as the diversity in the nitrogen metabolism pathways. The glyoxylate shunt of the TCA cycle, an alkane biosynthetic pathway, polyhydroxyalkanoate metabolism, and a pathway for the nonfermentative synthesis of higher alcohols are all present in some of the 5 species. This model development workflow includes both reactions and details common to the organisms as well as reactions that are not present within the reference model. The use of a more curated model enables the newly developed reconstructions to produce biomass components not found in other models created using automated methods. These reconstructions allow for further comparisons among the species, and similar evaluations can be made for other groups of microorganisms using this workflow.
Flavodiiron proteins Flv1 and Flv3 are essential for cyanobacterial growth under fluctuating light

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Flavodiiron proteins (FDPs) function in O₂ and NO detoxification in anaerobic prokaryotes. FDP genes have also been identified in certain oxygenic photosynthetic bacteria and eukaryotes. The genome of the cyanobacterium *Synechocystis* sp. PCC 6803 contains four genes encoding FDPs: *sll1521* (Flv1), *sll0219* (Flv2), *sll0550* (Flv3) and *sll0217* (Flv4). Flv2 and Flv4 are present only in β-cyanobacteria while Flv1 and Flv3 types of FDPs are found in all cyanobacteria. Flv2/Flv4 heterodimer is involved in photoprotection of photosystem (PS) II [1]. Instead, Flv1/Flv3 heterodimer function in photoreduction of O₂ to H₂O without concomitant formation of reactive oxygen species, called Mehler-like reaction [2].

Our recent studies show that Flv1 and Flv3 are indispensable under fluctuating light [3]. Sudden short-term fluctuations in light intensity are typical in aquatic environments and faced by most photosynthetic microorganisms. Under fluctuating light—when the growth light is repeatedly interrupted with high-light pulses—Δflv1 and Δflv3 mutants arrest cell growth. Various biophysical and molecular biology approaches demonstrated malfunction of the PSI complex in these mutants. Strong acceptor-side limitation of PSI during rapid short-term increases in light intensity results in generating reactive oxygen species which leads to oxidative damage of PSI. Flv1 and Flv3 proteins function as an important electron sink during overreduction of electron transport chain thus safeguarding PSI under fluctuating light.

Effects of antenna truncation on productivity and Photosystem II function

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Phycobilisomes (PBSs) are large pigment-protein complexes that serve as light-harvesting antenna associated with the thylakoid membrane of cyanobacteria, increasing absorption and transfer of light energy to the photosystems. PBSs in Synechocystis sp. PCC 6803 are hemidiscoidal complexes made up of three cylindrical allophycocyanin cores and six phycocyanin rods radiating from the core. These are arranged in a manner to optimize the directional energy transfer towards the thylakoid membrane. It has been hypothesized that highly efficient light harvesting antenna systems cause inefficient use of available light energy on a culture-wide basis. Therefore, antenna truncation has been suggested as a strategy for increasing photosynthetic productivity in green algae (1).

To explore the role of antenna truncation in cyanobacteria, a set of light harvesting antenna mutants with partial and total reduction of PBS were compared to wild type (WT) to determine the effects on the photosynthetic productivity and Photosystem II (PSII) function. These mutants, CB, CK and PAL, have reduced pigment and protein content: CB and CK are linker polypeptide and complete phycocyanin rod knockouts, respectively, and the PAL mutant lacks assembled PBSs (2).

A growth comparison of these strains was performed in precision controlled photobioreactors and biomass accumulation was measured. The rates of photosynthesis and respiration were also determined for estimating the photosynthetic productivity in each strain. Interestingly, progressive truncation of antenna resulted in an overall decrease in the productivity (3). To study the effects of antenna truncation on the expression of the photosystems, the ratio of PSI to PSII was determined using low temperature spectroscopy and western blotting. The relative amounts of functional PSII were evaluated by measuring steady state oxygen evolution rates as well as flash induced oxygen evolution. These data collectively indicated that antenna mutants express increasing levels of PSII to compensate for the loss of PBSs. The increased PSII titer leads to increased oxygen evolution rates, although these mutants demonstrated impaired S-state transitions. Therefore, the reduction in photoautotrophic productivity observed in antenna truncation mutants is likely due to the physiological changes in the light harvesting and delivery to PSII.


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Development of carbon-neutral bioproducts and biofuels is an imperative for sustainable economic development, global ecology, and national security. Cyanobacteria can help meet this need because they efficiently capture enormous amounts of solar energy and convert atmospheric carbon dioxide (CO₂) into carbon polymers. We have introduced optimized isoprene synthase and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway genes into fast-growing, *Synechococcus* PCC 7002 cyanobacteria and produced isoprene (a C₅H₈ precursor for synthetic rubber and aviation fuels) at rates significantly greater than those previously published. Introduction of a MEP pathway isopentenyl isomerase gene increased isoprene production by ~15-fold, demonstrating that modifications of this pathway can substantially increase isoprenoid yields. Toward further improvement, we have 1) synthesized additional MEP pathway genes, 2) inactivated genes for glycogen synthesis, and are 3) down-regulating light-harvesting capacity for increased cell density, 2) pursuing regulated gene expression to mitigate inhibitory effects, and 3) engineering additional isoprenoid products. Inactivation of glycogen synthesis resulted in only a modest increase in isoprene, suggesting that carbon may be diverted to soluble sugars as recently demonstrated by Xu et al. (2013 *Metabolic Engineering* 16, 56). Thus further modifications may be needed to funnel additional carbon into the MEP pathway. The halo-tolerant *Synechococcus* PCC 7002 grow well under 100% CO₂ and full sunlight, indicating that waste waters and industrial gas effluents may be used as nutrient and CO₂ sources. A provisional patent application (T120019US) has been filed, and a company, Algoma Algal Biotechnology LLC, established to further pursue isoprenoid products.
Direct Coupling of Photosynthesis to Ethanol Production in Cyanobacteria

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The idea of using cyanobacteria for continuous intracellular ethanol production arose before 1999 when John Coleman first published ethanol production in an engineered strain of Synechococcus PCC 7942. Based on this research approach we achieved and investigated ethanol production in Synechocystis PCC 6803 and generated strains producing significant amounts of ethanol. Here, we present data about the correlation of ethanol production and photosynthesis as well as results regarding cultivation conditions optimal for high yield ethanol production. At the moment the company aims to transfer laboratory results into the field.
Characterization of the Putative Bilin Lyase CpeT from *Fremyella diplosiphon* (UTEX 481).

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*Fremyella diplosiphon* is a cyanobacterium that undergoes Type III Chromatic acclimation (CAIII). When green-light adapted cells are transferred to red light, phycoerythrin (PE) synthesis is halted and a specific set of Phycocyanin (PC-2) genes is induced. When cells that have been adapted to red light are then transferred to green light, PE genes (and their bilin lyase and linker genes) are expressed and the inducible PC-2 genes are not expressed [1]. PE is composed of two subunits with five phycoerythrobilin chromophores covalently attached at Cys residues by enzymes called bilin lyases. We are interested in understanding how PE is biosynthesized in *F. diplosiphon*, focusing on the characterization of the bilin lyases responsible for PEB ligation.

CpeT [2] is a putative bilin lyase which is similar to the known lyase CpcT [3]. Based on *CpcT*’s role of ligating phycocyanobilin to Cys-153 on β-PC, we hypothesized that CpeT is the PEB lyase for the Cys-165 position on β-PE [3]. Phycoerythrin (PE) and whole phycobilisomes (PBS) were isolated and purified from WT and ΔcpeT deletion mutant strains of *F. diplosiphon* in order to characterize the function of *CpeT*. Purified PE samples from WT and ΔcpeT were analyzed using absorbance and fluorescence spectroscopy and MALDI-TOF mass spectroscopy. The phenotype of the ΔcpeT mutant is very dramatic with very low levels of PE being made under Green Light. Purified PBS samples from WT and ΔcpeT were analyzed using SDS-PAGE, absorbance and fluorescence spectroscopy, and Western blots. These analyses suggested that the ΔcpeT mutant produced much less PE and more PC than the WT.

The results of the MALDI MS/MS analyses of PE purified from ΔcpeT cultures showed that four of the five PEB chromophores were present. The PEB at Cys-165 on β-PE was not detected. However, after growing cells for several months, the PE levels started to increase, and analyses of this PE sample showed the presence of all five PEB. A possible explanation for this is that the mutants in the second sample set may have had a subsequent mutation which allowed them to make more PE. An explanation for the low very low PE phenotype is that CpeT may have an unknown regulatory role in PE synthesis. Alternatively, if CpeT is a bilin lyase for Cys-165 on β-PE, perhaps the PEB attachment at the Cys165 position must occur first in an ordered process within cyanobacteria, as has been suggested [4]. We are also trying to confirm that CpeT is a bilin lyase by recombinant protein expression in *E. coli*.

References

Presence of a functional low-molecular weight thiol pool within the thylakoid lumen in the cyanobacterium Synechocystis sp. PCC 6803

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The thylakoid lumen provides the optimal physical and chemical environment for water oxidation during oxygenic photosynthesis. While many parameters, e.g. the inorganic ion concentration and pH, are known to contribute greatly to photosynthetic efficiency, little is known about the lumenal redox environment despite the plethora of redox reactions occurring within this space. To investigate how the lumen maintains redox poise, we used the ΔgshB mutant of the cyanobacterium Synechocystis sp. PCC 6803. ΔgshB lacks glutathione (1), a key component of the soluble redox buffering system. Biochemical analysis of membranes isolated from WT and ΔgshB strains revealed a fraction of the total thiol content was retained in the membrane fraction following extensive washing. Based on these results, we estimate the low-molecular weight thiol concentration in the lumen of cyanobacteria to be between 2 and 20 µM. Furthermore, by measuring photosynthetic electron transfer rates in whole cells and isolated membranes, we found that the rate of P700⁺ reduction is approximately 2-fold faster in the ΔgshB strain compared to the WT and the genetically complemented ΔgshB/T2086 strains. These results indicate that changes in the composition of the lumenal thiol pool can directly influence photosynthesis. We propose that these thiols function to maintain redox homeostasis in the thylakoid lumen of cyanobacteria.

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Engineering Photosystem I for Enhanced Electron Transport Rates In Vitro for Applied Photosynthesis

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Photosystem I has been shown to be a robust photoactive nanoparticle capable of generating both hydrogen and electricity in vitro. Both of these processes require the electrons to be transferred from soluble metalloproteins, such as plastocyanin and cytochrome c, to the oxidized special pair (P700+) of the reaction center. Much of what we know about this process has been via the studying of model single cell organisms such as cyanobacteria. Cyanobacteria are important for carbon fixation and oxygen production and also offer a powerful and facile genetic system. Moreover, as oxygenic organisms they utilize the Z-scheme employing both PSII and PSI resembling that found in plants. Currently, the thermophilic, rod-shaped, unicellular cyanobacterium, Thermosynechococcus elongates BP-1 is of great interest in this field due to its fully sequenced genome and the success in crystallizing many highly thermostable protein complexes, including both PSI and PSII. An additional benefit of this organism is its ability to be genetically transformed via electroporation. Recently we have developed a genetic system to allow homologous recombination in cyanobacteria T. elongatus. Using this genetic approach we can manipulate individual PSI subunits by site-specific mutagenesis, adding epitope/affinity tags, and disrupting the gene products by insertional disruption. Using these molecular techniques we will create a portfolio of tools for improve the molecular properties of PSI such that it is more active as a hydrogen-evolving nanoparticle. My current project is to engineer the PSI’s surface chemistry. For example, we are making complementary changes on both the PSI and cytochrome c6 surface so that they interact more quickly in solution. Specifically, we have made a psaF modification that may allow more rapid docking of the electron donor, cytochrome c6 to the lumenal surface of PSI. In utilizing this tighter, more rapid interaction with the electron donor, we are also in the process of creating poly-Cyt c6s that would result in a continuous electron transfer to P700+ due to the extended lumenal exposure of the linked cytochromes. The electron transfer rate can then be tested with a Pulsed LED Spectrometer (JTS-10) by measuring the re-reduction profile of P700-. The kinetics of this electron transport step is being investigated as a function of cytochrome content, temperature, pH, ionic strength, and post selective mutagenesis. These results will be discussed in light of our understanding of how Cyt c6 and PSI interact.
Conversion of CO₂ to Chemicals in Cyanobacteria

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Conversion of CO₂ for the synthesis of chemicals by photosynthetic organisms is an attractive target for establishing independence from fossil carbon reserves. However, synthetic pathway construction and characterization of metabolism in cyanobacteria, is still in its infancy compared with model fermentative organisms. We systematically developed the 2,3-butanediol (23BD) biosynthetic pathway in *Synechococcus elongatus* PCC 7942 as a model system to establish design methods for efficient exogenous chemical production in a photosynthetic host. We identified 23BD as a target chemical with low toxicity and designed an oxygen-insensitive, cofactor-matched biosynthetic pathway coupled with irreversible enzymatic steps to create a driving force toward the target, increasing titers to 2.38 g/L. On a large scale it is desirable to supplement photosynthetic strains with naturally derived energy molecules such as sugars, to boost chemical production during diurnal and natural low light cycles. Many cyanobacteria, including our model system, have been considered obligate phototrophs, strictly depending upon the generation of photosynthetically derived energy for biomass production. We determined that sugar transporter systems are the necessary genetic factors to install heterotrophy in *S. elongatus* PCC 7942. After modification, continuous growth was possible under diurnal (light/dark) conditions using saccharides such as glucose, xylose, and sucrose. This work demonstrates that developing strong design methods and characterizing the limitations of metabolism can continue to increase chemical production in cyanobacteria.
A gene regulation mechanism that allows synthesis of two ferredoxin:NADP oxidoreductase isoforms from a single gene in the cyanobacterium Synechocystis sp. PCC 6803

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Ferredoxin:NADP oxidoreductase (FNR), encoded by the petH gene, provides NADPH for CO2 fixation in photoautotrophic cells and oxidizes NADPH in heterotrophic cells. Whereas there is only one petH gene copy in the cyanobacterium Synechocystis sp. PCC6803, two FNR isoforms accumulate (FNR_L and FNRS). It was proposed that FNR_L fulfills functions in linear electron transport while FNRS is involved in cyclic electron transport and respiration. In addition, FNRS was shown to be the product of an internal translation initiation within the FNRL open-reading frame (1).

Deletions in the 5'noncoding region, of petH (FNR-encoding gene) suggested that each isoform is produced from a specific mRNA. 5'-end mapping of the petH transcripts confirmed this fact and showed that under standard conditions -when FNR_L accumulates- two mRNAs carrying similar leaders (32 and 53 bases) are transcribed; while under nitrogen starvation - when FNRS accumulates- an mRNA, carrying a longer leader (126 bases), is transcribed (2).

We also showed that the global nitrogen regulator NtcA binds to the upstream region of petH. Mutations 42 nucleotides upstream from the long transcript 5'end resulted in the abolition of FNRS accumulation and in identifying the NtcA binding site.

Toeprinting assays on both mRNA showed that translation-initiation complexes locations were mapped to the FNRL initiator codon in the short mRNA and to the FNRS initiator codon in the longer mRNA. These results suggested that the translation regulation does not require a specific factor; but it’s rather due to a spontaneously occurring secondary structure, adopted by the longer leader.

Secondary structural probing, along with deletions and point mutations revealed the presence of different structures in both mRNAs. The short mRNA adopts a structure that blocks FNRS initiation codon and allows FNRL translation while the long mRNA adopt a structure that blocks FNRL initiation codon and allows initiation at FNRS initiation codon. These results were also confirmed using Toeprinting assays.

Thus we have uncovered a novel gene-regulation mechanism by which two isoforms are produced from a single gene in the cyanobacterium Synechocystis sp. PCC 6803. Similar petH regulation mechanisms were found in other cyanobacteria such as Anabaena PCC7120 and Synechococcus PCC7002.

Temperature stress and RNA helicase expression: Multiple levels of (auto)regulation

Albert RR Rosana, Danuta Chamot, Denise S. Whitford and George W. Owttrim

Temperature fluctuation is a constant stress for free-living organisms. Synechocystis sp. PCC 6803 encodes a single DEAD-box RNA helicase, crhR, whose expression is regulated by environmental stresses that alter the redox status of the electron transport chain, including temperature downshift. RNA helicases are associated with regulation of gene expression by catalyzing the rearrangement of RNA secondary structure with CrhR representing a unique class of RNA helicases that are capable of both unwinding and annealing RNA duplexes. Here, temperature regulation of crhR expression is shown to involve a complex regulatory network functioning at a minimum of six control points at the post-transcriptional, translational and potentially post-translational levels. Regulation was controlled by three CrhR-dependent autoregulatory- and three CrhR-independent-mechanisms. Furthermore, autoregulation is extended to the rimO-crhR operon where a defect in RNA processing contributes to differential accumulation of the two cistrons. The observation that temperature induction of crhR only requires translation elongation, combined with CrhR association with a degradosome-polysome complex localized to the thylakoid membrane may also contribute to the regulatory network. Disruption of this network has profound morphological and physiological implications. Combined with our previous demonstration that CrhR regulates sRNA metabolism, the research provides insights into the mechanisms by which alteration of RNA secondary structure is associated with temperature regulation of gene expression, findings important for the manipulation of cyanobacteria in biotechnological applications.
Characterization of a squalene hopene cyclase deficient strain of *Synechocystis PCC6803*

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Squalene is a natural triterpene hydrocarbon having several beneficial properties in pharmacy, medicines and cosmetics. Squalene is synthesized in all types of cells because it is a crucial intermediate for hopanoid biosynthesis in bacteria and eukaryotic sterols. Hopanoids are pentacyclic triterpenoids that act to stabilize membranes and regulate their fluidity and permeability. The synthesis of hopanoids depends on the enzyme squalene hopene cyclase (Shc) that catalyzes the formation of hopene from squalene.

In the unicellular cyanobacterium *Synechocystis* PCC6803, the gene *slr2089* is predicted to encode Shc. Sequence analysis shows that the protein encoded by *slr2089* contains five copies of QW motifs and an aspartate-rich amino acid motif DXDD which are unique to this enzyme class. It has been shown that inactivation of *slr2089* results in a strain (∆shc) which, unlike the *Synechocystis* wild-type (WT), accumulates squalene in the cells\(^1\).

To understand the functional role of Shc and the hopanoids which has been never explored before in the cyanobacterium, the ∆shc strain was compared with the WT strain under different pH- and temperature stress conditions. ∆shc strain showed high sensitivity to acidic pH (pH 6) and to low temperature (15\(^°\)C) with lower growth rate and significantly lower chl\(\alpha\) accumulation in the cells after seven days of adaptation to the specific stress conditions. Squalene accumulation in the ∆shc strain was observed to be significantly lower irrespective of any stress conditions. Complementation of the ∆shc strain with *slr2089* expressed from a plasmid vector resulted in reduced amount of squalene accumulation in the cells, confirming that the accumulation of squalene observed in the ∆shc strain is due to inactivation of Shc.

We hypothesized lack of Shc would lead to membrane damage due to lack of hopanoids. Hopanoids have been shown to localize in the outer membrane and thylakoid membrane of cyanobacteria\(^2\). In our preliminary studies, cell images from transmission electron microscopy (TEM) showed loosening of outer cell membrane in ∆shc strain in comparison to WT cells, indicating a possible functional role for hopanoids in maintaining cell membrane integrity in *Synechocystis* PCC6803.

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A Cobalamin-Regulated Fluorescent Reporter System for the Verification of a Putative Cobalamin Uptake System in *Synechococcus* sp. PCC 7002

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An endogenous plasmid-based, vitamin B₁₂ (cobalamin)-regulated, fluorescent reporter system was developed in a *Synechococcus* sp. PCC 7002 (*Synechococcus* 7002) strain genetically modified for cobalamin-independent growth. This system allowed for the in vivo verification of the cobalamin uptake system in this cyanobacterium. Wild-type *Synechococcus* 7002 has a strict requirement for exogenous cobalamin because it lacks the ability to synthesize this vitamin de novo. Cobalamin is a required cofactor for the synthesis of L-methionine by cobalamin-dependent methionine synthase (MetH). Complementation of *Synechococcus* 7002 with a cobalamin-independent methionine synthase (*metE*) gene from a closely related cyanobacterium, *Synechococcus* sp. PCC 73109 (*Synechococcus* 73109), rendered *Synechococcus* 7002 capable of cobalamin-independent growth. Furthermore, facile deletion of the native *metH* gene in the cobalamin-independent *Synechococcus* 7002 strain indicated that cobalamin is only required for MetH activity. *Synechococcus* 73109 possesses genes for both MetE and MetH, and a cis-acting cobalamin riboswitch exists for transcriptional regulation of *metE*. Cobalamin riboswitches are 5' untranslated region elements that can control expression at either the translational or transcriptional level via allosteric changes induced by various forms of cobalamin. The promoter region of *metE* from *Synechococcus* 73109 was fused upstream of the enhanced yellow fluorescent protein gene (eYFP) and transformed into the cobalamin-independent *Synechococcus* 7002 strain grown in the absence and presence of exogenous cobalamin. Whole-cell fluorometric screening demonstrated repression of the eYFP reporter in the presence of cobalamin. Cobalamin is a rather large molecule and is unable to passively traverse the cell membrane, hence an active transport system is required for its translocation into the cell. Comparative genome analysis predicted a putative B₁₂ uptake (*btu*) system in *Synechococcus* 7002 composed of an inner membrane ATP-binding cassette (ABC) transporter and a TonB-dependent, outer membrane transporter. Knock-out mutations of the individual putative *btu* genes, which had been misannotated as siderophore uptake genes, were used to verify the in silico predictions using the in vivo reporter system described above. The TonB-dependent outer membrane component (*btuB*), the periplasmic (*btuF*), and the permease (*btuC*) components of the ABC transporter in the inner membrane were verified in vivo. A predicted ATPase subunit (*btuD*) of the ABC transporter could not be verified. These results were also confirmed by comparing global transcriptional profiles of the cobalamin-independent *Synechococcus* 7002 strain grown in the presence and absence of cobalamin, which demonstrated that three genes identified are also regulated by a cobalamin-riboswitch. This study demonstrates the importance of experimental validation of in silico predictions and provides a general scheme for in vivo verification of similar systems.
Quantitative metabolite analysis for a cyanobacterial strain *Synechococcus* sp. PCC 7002.

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*Synechococcus* sp. PCC 7002 is a fast growing (~3h doubling time with ammonia and 4h doubling time with nitrate), tolerant to high salt and high light intensities, naturally transformable strain with completely sequenced genome [1]. These characteristics make the strain an ideal system for metabolic engineering. Plants and photosynthetic organisms are photoautotrophs which use sunlight and CO₂ to produce complex molecules like metabolites and sugars. This cyanobacterium *Synechococcus* sp. PCC 7002 is capable of photoheteterotropic growth with glycerol. Quantitative $^{13}$C labeling pattern analysis of both terminal and internal metabolites at mixotropic and photoautotropic conditions can be used to estimate fluxes in the system. We obtain the labeling pattern for various metabolites under steady state conditions and perform steady state $^{13}$C-MFA (metabolic flux analysis). A newly developed technique of isotopically nonstationary MFA (INST-MFA) can be used to estimate flux analysis of photoautotropic metabolism [2]. The results provide new insights into the metabolic networks through the labeling pattern and carbon transition pattern. The response or change in the central carbon metabolism to different conditions is analyzed. The reconstruction of a metabolic model with fluxes has the potential to fill gaps in the understanding of the regulatory network. A genome scale metabolic model is published for the *Synechococcus* sp. PCC 7002 with computational predictions for knock out strains for enhanced product formation [3]. We compare the predictions using MFA analysis to computationally predicted mutants.

References:

Transcriptional Regulation of Nitrogenase Genes Is Influenced by RNA Secondary Structure

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Anabaena variabilis is a filamentous nitrogen-fixing cyanobacterium with three separate nitrogenases. It fixes nitrogen aerobically by spatially separating the oxygen-labile enzyme, nitrogenase, from oxygen-evolving photosynthesis in specialized cells called heterocysts. The primary Mo-nitrogenase, Nif1, and the V-nitrogenase, Vnf, are expressed in heterocysts under N-limiting conditions, and their differential expression is regulated by Mo availability. The second Mo-dependent nitrogenase, Nif2, fixes nitrogen in vegetative cells only under anoxic conditions.

The gene clusters for the Nif1 and Nif2 nitrogenases are conserved between these two systems and this gene organization is highly conserved among cyanobacteria. Previous work showed that the nifB1 promoter serves as the primary promoter for nifB1S1U1H1D1K1, with a second, promoter found in nifU1 contributing about 20% to transcription (Ungerer et al., 2010). There is no promoter directly upstream of nifH1, but there is a transcript processing site near the start of nifH1. An xisA mutant, which cannot remove the nifD1 excision element, thus separating both the nifB1 and nifU1 promoters from nifK1 and other downstream genes, produced no transcript for nifK1 or nifE1, but made some transcript for nifN1 and genes downstream of nifN1, indicating that there is a weak promoter in the coding region of nifE.

As has been shown for the nif1 genes, in the nif2 gene cluster the intergenic regions of nifUH2 and nifKE2 could not drive expression of downstream nif2 genes. Thus, it appears that the nifB2 promoter served as the primary promoter for the nif2 operon. No other strong promoters were identified in either the nif1 or the nif2 cluster.

Although the nif1 genes are all transcribed from the same primary promoter, transcript half-life differs among the genes, suggesting that transcript stability may be important for regulating the amount of transcript, which is highly variable among the genes. The region just downstream from the transcript processing site upstream of nifH1 contains two stem-loop structures (Ungerer et al., 2010). A set of mutations that abolished the first stem-loop structure resulted in a strain that showed only 10% of the wild-type level of nifH1 transcript and greatly reduced nitrogenase activity. A second set of mutations that restored a stem-loop structure, but with a different nucleotide sequence, resulted in a strain with wild-type levels of nifH1 transcript and normal nitrogenase activity. Thus, it appears that secondary structure of the transcript is important in controlling the amount of nifH1 transcript.

Proteomic Study on Akinetes and Heterocysts in *Anabaena cylindrica*

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The vegetative cells of *Anabaena cylindrica* can differentiate into both heterocysts and akinetes in medium AA/8 with and without fixed nitrogen. The heterocyst is accepted to be the site for nitrogen fixation in heterocystous cyanobacteria. The akinete is a spore-like cell capable of germination and of withstanding certain environmental extremes such as cold, desiccation, and phosphate limitation. The first akinete-specific protein AvaK from *Anabaena variabilis* was previously reported (Zhou & Wolk, 2002. *J. Bacteriol.* 184: 2529-32), but no proteomic study of akinetes has been presented.

*Anabaena cylindrica* was selected for proteomic analysis of akinetes because its akinetes are large and easily isolated. The akinetes and heterocysts were harvested by passing the *A. cylindrica* culture grown in AA/8 medium through a High Pressure Homogenizer at 4500 psi twice to completely break the vegetative cells, and then purified by CsCl density gradient centrifugation, respectively (Wolk & Simon, 1969. *Planta* 86:92-97). The total proteins extracted from the purified akinetes and heterocysts were subjected to SDS-PAGE, in-gel tryptic digestion and LC-MS/MS for protein identification. LC-MS/MS identified a total of 634 proteins from the purified akinetes and a total of 924 proteins from the purified heterocysts, including 107 proteins found exclusively in akinetes while 417 proteins including *nif* gene products are heterocyst specific. Since we, in collaboration with C. Peter Wolk (see abstract by Zhu, Wolk, and Zhou in this workshop), recently succeeded in genetic transformation of *A. cylindrica*, the identification of an akinete-specific proteome should enable us to pursue a genetic study of akinete formation.
The functional significance of the CyanoQ N-terminal lipid modification

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The CyanoQ protein has been shown to be a component of cyanobacterial Photosystem II (PSII), but this subunit is not resolved within the current PSII X-ray structure [1]. Thus, little is known as to how this component interacts with the other extrinsic proteins on the luminal side of PSII (PsbO, PsbV, and PsbU) or the intrinsic PSII components. Unlike the extrinsic proteins resolved in the current PSII structural models, CyanoQ is a lipoprotein. Upon cleavage of the transit peptide that signals translocation into the thylakoid luminal space, the N-terminal cysteiny1 residue of the mature CyanoQ protein is modified with a lipid group, tethering this otherwise soluble protein to the thylakoid membrane. To probe the functional significance of the lipid anchor, mutants of the CyanoQ protein have been generated to eliminate the N-terminal cysteiny1 residue and consequently the lipid modification. Substitution of this cysteine residue with serine results in a significant decrease in the amount of detectable CyanoQ protein and altered processing of the CyanoQ precursor.

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Synechococcus sp. PCC 7002: A Superior Host for Converting Carbon Dioxide into Free Fatty Acids

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Cyanobacteria are excellent hosts for renewable biofuel production, as they naturally fix carbon dioxide to form complex hydrocarbon molecules. The potential for cyanobacterial-based biofuel production has been demonstrated by the genetic engineering of cyanobacteria for the production of a variety of fuels and fuel precursors, including ethanol, butanol, isoprene, free fatty acids (FFAs), and alkanes. While these biofuels have been successfully produced by cyanobacterial hosts, the high titers required for economical production of these low-value commodities have yet to be achieved. Cyanobacterial host tolerance of the target fuel is often a limiting factor, as demonstrated in the production of FFAs by Synechococcus elongatus PCC 7942.

This study investigates the potential of Synechococcus sp. PCC 7002 as host for the production of FFAs. A total of 6 engineered strains of Synechococcus sp. PCC 7002 were constructed for FFA production. Metabolic targets include gene knockout of acyl-CoA synthetase (fadD), expression of thioesterases ('tesA and fat1), overexpression of RuBisCO (rbcLS), and overexpression of acetyl-CoA carboxylase (accBCDA). The engineered strains of Synechococcus sp. PCC 7002 were compared to similarly engineered strains of S. elongatus PCC 7942. Analysis of excreted FFA concentration and host physiological parameters indicated that Synechococcus sp. PCC 7002 is a superior host for FFA production compared to S. elongatus PCC 7942. The improved physiological response of Synechococcus sp. PCC 7002 to FFA production was also found to be temperature dependent, with lower temperatures (30°C) leading to healthy cell cultures and higher temperatures (38°C) resulting in compromised cell physiology. While this study demonstrates the capability of Synechococcus sp. PCC 7002 as host for FFA production, further investigation is necessary to determine the underlying mechanisms contributing to the high FFA tolerance of this cyanobacterium.
Glycosylated mycosporine-like amino acids and the genotypes in the terrestrial cyanobacterium *Nostoc commune*

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Cyanobacteria have two types of sunscreen pigments, scytonemin and mycosporine-like amino acids (MAAs). These secondary metabolites are thought to play multiple roles against environmental stresses and their antioxidative properties may be necessary for the protection of biological molecules against the oxidative damages induced by UV radiation. In the terrestrial cyanobacterium *Nostoc commune*, the antioxidant activity and vitrification property of these pigments are thought to be requisite for the desiccation and rehydration processes in anhydrobiosis [1].

We have identified three typical glycosylated MAAs in *N. commune* [2,3]. An MAA with an absorption maximum at 335 nm was identified as a pentose-bound porphyra-334 derivative with a molecular mass of 478 Da. Another identified MAA had double absorption maxima at 312 and 340 nm and a molecular mass of 1050 Da. Its unique structure consisted of two distinct chromophores of 3-aminocyclohexen-1-one and 1,3-diaminocyclohexen and two pentose and hexose sugars. These chromophores have distinct absorption maxima, so that this hybrid MAA molecule can absorb a wider range of UV-A/B radiation. The third glycosylated MAA with an absorption maximum at 322 nm was identified as a two hexose-bond palythine-threonine derivative with a molecular mass of 612 Da. These glycosylated MAAs had strong radical scavenging activity *in vitro* [2,4]; the 1050-Da hybrid MAA contributed approximately 27% of the total radical scavenging activities in a water extract of *N. commune* [2].

*N. commune* is known to be genetically diverse, and four major genotypes of *N. commune* have been reported in Japan [4]; however, the morphological features of the macroscopic colonies and microscopic trichomes are almost identical, and the genotypes are indistinguishable without a determination of molecular taxonomical markers [4]. During an investigation of MAA contents in field-isolated *N. commune* colonies, we observed three different UV-absorption spectra in the water extracts from colonies at different sampling locations. According to their UV-absorbing spectra and HPLC chromatograms, we thought that the glycosylated MAA patterns could be a feasible chemotaxonomic marker with which to characterize *N. commune*. Thus the genotypes and specificities of the types of the glycosylated MAAs were investigated. In conclusion, *N. commune* are classified into three groups, namely, the 478-Da MAA producer (the genotype A), the 1050-Da MAA producer (the genotype B/C) and the 612-Da MAA producer (the genotype D), as these groups are genetically different chemical races of *N. commune*.

Genetic Improvements and the Gas Compositions for Sustained Nitrogenase-based Photobiological H₂ Production by Heterocystous Cyanobacteria

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In our system, the enzyme of H₂ production is nitrogenase (N₂ase), and the presence of hydrogenase (H₂ase) activity should be eliminated because it reabsorbs the produced H₂. We have created several H₂ase mutants (ΔHup) from the heterocystous Nostoc sp. PCC 7120 and sp. PCC 7422. The Nostoc sp. PCC 7422 ΔHup mutant accumulated H₂ to 20-30% (v/v) in 3 to 8 days, and the efficiency of light energy conversion into H₂ was 3.7% vs visible light (calculated to be about 1.7% vs. total solar radiation). When Nostoc sp. PCC 7422 ΔHup mutant cells were transferred to a combined N-free medium under 80% N₂, H₂ production activity declined in a few days by the sufficiency of the combined nitrogens due to active N₂ fixation by N₂ase. By decreasing N₂ concentration to 0.5-1% in Ar + 5% CO₂, the H₂ production activity was maintained at high levels for more than several weeks.

We have created 49 site-directed N₂ase mutants from Nostoc sp. 7120 ΔHup by replacing the amino acid residues assumed to be located near the FeMoco[1]. H₂ production activities of some of the mutants were not decreased by 80% N₂[2], indicating the possibility of the cost reduction of gas (Ar) in practical application in the future.

W.A. Amos concluded that if the product cost of the bioreactor exceeds US $ 100 per m², photobiological H₂ production by the green alga Chlamydomonas will not be economically viable. As inexpensive bioreactors, we have proposed the use of the ones composed of several layers of plastic film[3]. We found that some of commercially available films had low H₂ permeability, which can be the candidate materials for inexpensive bioreactors.[4]

References
Revisiting the cell-type specific proteomes of N₂-fixing filaments of the heterocyst forming cyanobacterium *Nostoc punctiforme* ATCC29133 using ICAT labeling

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We here demonstrate how the use of multiple peptide labeling methods could potentially broaden our understanding of the physiology of *Nostoc punctiforme*. Using cell-type fractionation of N₂-fixing filaments and "isotope-coded affinity tags" (ICAT) labeling of the total Cys proteome we have so far managed to identify hundreds of proteins and quantify the cell-type specific expression of over 400 proteins. The proteins were extracted 24 hours after ammonium step down and the identified proteins differ significantly from previously reported iTRAQ data of steady state cultures (Ow et al., 2009), with a substantial fraction of unique hits. We are currently working to significantly increase our detection and expand our understanding of the cell-type specific proteomes of both vegetative cells and heterocysts in this multicellular bacterium. We also aim to move beyond protein quantification and start to map redox regulation of thiol-groups associated with functional proteins which play important roles in the day-to-day control of biological processes.

Ref:

Blue-green chromatic acclimation in marine Synechococcus is controlled by two putative AraC-class transcription factors

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Cyanobacteria of the genus Synechococcus are ubiquitous in oceans and are responsible for a significant portion of the primary productivity and oxygen production on Earth. These organisms contain phycobilisomes, which contain phycobiliproteins to which light harvesting molecules, known as chromophores, are attached. Some marine Synechococcus undergo blue-green chromatic acclimation, adjusting the composition of their phycobilisomes to optimize photon capture to drive photosynthesis in different light conditions.

During this type of chromatic acclimation, different chromophores are reversibly attached to three different positions on phycobiliproteins. Blue light absorbing chromophores and green light absorbing chromophores are attached in their respective light conditions. Part of this process is controlled by the lyase-isomerase MpeZ, which converts a green light absorbing chromophore into a blue light absorbing chromophore and attaches it to one specific phycobiliprotein site. The transcript levels of \textit{mpeZ} are higher in blue light than green light.

Using a reverse genetic approach, we have identified two genes encoding putative AraC-family transcription factors whose presence in a number of Synechococcus strains is strongly correlated with the ability to undergo blue-green acclimation. These genes are contiguous in all of the genomes in which they occur and appear to form an operon. The interruption of each of these genes leads to a complete loss of this response, as judged by changes in phycobilisome composition. However, the phenotypes of two mutant classes are completely opposite from each other. An interruption in the first of these two genes results in mutant cells that behave as if they are always in green light, regardless of the ambient light color. Interruption of the second gene results in mutant cells that act as if they are always in blue light. Our results provide important genetic and biochemical information about the signal transduction pathway controlling the globally important process of blue-green chromatic acclimation in marine Synechococcus.
A Cyanobacterial Phylogenetic Tree Using PS I and PS II Protein Sequences

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A molecular phylogenetic tree that is quantitative and reproducible can be constructed using comparison of sequences of 12 proteins from Photosystem I (PS I) and 20 proteins from PS II reaction centers. Distances between different taxa are expressed as $\Delta$ values, which reflect the number of sites $N$ where mismatches in amino acids occur between aligned protein sequences between pairs of taxa. For 50 taxa there are $50 \times 49 = 2450$ such pairs for each protein. The $\Delta$ values are then normalized by dividing by $L$, the number of amino acids expressed for each protein, and multiplied by 1000. $\Delta = 1000/L$. The numbers thus reflect normalized mismatches in parts per thousand (per mil) for each protein or group of proteins and for each pair of taxa. This tree is more useful and informative than previous trees using data from a wider variety of cyanobacterial genes, including those of 16S ribosomal RNA. [1,2] The tree based on genes for reaction center proteins is very different from one using 16S rRNA sequences or other cyanobacterial proteins.

Whereas previous trees have been limited to presentation in two-dimensions on a printed page, the new approach provides a basis for multidimensional trees that grow dimensionally together with the number of taxa considered. A 5-dimensional tree for 50 taxa is described, but it has already been straightforwardly expanded to include an additional 50 taxa recently added to the database. A preview of a game for introducing multi-dimensional phylogenetic trees to beginning biology students will be presented.

The tree is organized into three major clades: Clade A includes most Synechococcus taxa; Clade B includes Nostoc, Cyanothece and related taxa; Clade C consists entirely of Prochlorococcus taxa. A few taxa, such as those of thermophiles, one from Japan and two from the Octopus Hot Spring in Yellowstone National Park, are not integral to any of the tree major clades, but are distant from each. The thermophiles from the Octopus Hot Spring are close to one another, but distant from the one from the Japanese hot spring.

Within each Major clade, secondary clades can be constructed that further define the structure of the tree. Taxa, such as Acaryochloris and Prochlorothrix that have been considered to be “outliers” are found to fit comfortably in Clade B. Evidence in the new tree can be interpreted in terms of aspects of the pattern evolution of the cyanobacteria. Features are present for some clades that may have arisen from lateral transfer via PS RC viruses. [3] The relation between the RC sequences of Thermosynechococcus elongatus BP-1, from which the reaction centers have been crystallized and subjected to X-ray crystallography, and those from the non-thermophile nearest on the tree, Cyanothece PCC7425, provides a basis for understanding the structural basis of thermophilic stability. [4]


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Fructose is an antibiotic or a growth substrate for Synechocystis sp. PCC6803 depending on the pathway it is taken up into the cells

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Many cyanobacteria are obligate photo-litho-autotrophs. For those that are able to grow organo-heterotrophically, only a limited number of organic substances, mostly sugars, can support this growth mode. Many cyanobacteria use glucose or fructose as heterotrophic substrates. Until recently, a peculiar situation was known in Synechocystis sp. PCC6803: The sugar carrier Gtr (also known as GlcP) can transport 4 sugars into the cells that have very different effects on the cells: 1) glucose was the only known heterotrophic substrate, its uptake allows both photo-organo-heterotrophic and chemo-organo-heterotrophic growth, 2) 3-O-methyl-glucose and 3) 6-deoxy-glucose are transported at essentially the same rates as glucose, but are not metabolized and have no detectable effect on cell physiology after entry into the cells, 4) fructose is transported by the Gtr protein with a lower affinity than glucose and acts as an antibiotic that kills the cells. The mode of the toxic action of fructose is not yet understood, but it has been known for some time that glucose - despite being able to support heterotrophic growth - also has a toxic effect on Synechocystis sp- PCC6803. In this context we recently discovered that the uptake rate of glucose is dramatically decreased when Synechocystis sp. PCC6803 is pre-incubated with glucose, both under mixotrophic and under photo-organo-heterotrophic conditions. Compared to many other bacteria, it is a very unusual behaviour that an organo-heterotrophic substrate is decreasing - instead of increasing - its own uptake rate. It is easy to produce a mutant strain (called Synechocystis GS1) that homozygously has lost the wild-type gtr gene. This strain is incapable of organo-heterotrophic growth on glucose and also resistant to fructose. In another cyanobacterium, Anabaena sp. PCC7120, which for decades was thought to be an obligate photo-litho-autotroph, we recently discovered that photo-organo-heterotrophic and even chemo-organo-heterotrophic growth is possible, when unusually high amounts of fructose (up to 200mM) are supplied [1]. The uptake protein has not (yet) been identified, but the annotation of the total genomic sequence contains a number of putative sugar transporters. Some of these have sequence homologues in Synechocystis sp. PCC6803. We therefore incubated Synechocystis GS1 with very high concentrations (50 - 200mM) of fructose and discovered photo-organo-heterotrophic (but not chemo-organo-heterotrophic) growth of this strain. We are currently working to identify the carrier responsible for fructose uptake in strain GS1. When similarly high concentrations of fructose are applied to the Synechocystis sp. PCC6803 wild type strain, cell death is observed.

[1]
Biogenesis of the thylakoid membrane in *Synechocystis* sp. PCC6803

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The model organism *Synechocystis* sp. PCC6803 (*Synechocystis*) has been extensively used in many different studies regarding photosynthesis and respiration. However, it is a matter of intense debate whether the thylakoid membranes (TM) are completely separate entities from the plasma membrane (PM) or if, on the other hand, there is a (permanent or temporary) connection between them.

Using a combination of sucrose density gradients and liquid two-phase partitioning, we separated “light” (PM1) and “heavy” (PM2) plasma membrane fractions from the TM and studied their respective protein and pigment composition. We show that PM1 is a specific type of plasma membrane, due to its much higher carotenoid content in comparison to PM2. We have used proteomics techniques to localize several of the carotenoid synthesizing enzymes, which seem to be preferentially associated with the plasma membrane. We analyzed in detail the regulation of the enzyme responsible for the synthesis of the main precursor lipid, monoglucosyldiacylglycerol synthase (synMGS). This enzyme is unique in its class in that its activity responds to the lipid environment mainly through sulfoquinovosyldiacylglycerol, rather than phosphatidylglycerol, as is the case for similar enzymes.

We utilized pulse-chase labeling to study photodamage-induced repair of D1 in the different membrane classes. pD1 seems to be inserted and processed exclusively either in the TM or in a membrane subfraction co-purifying with it. We are currently using different techniques in order to localize CtpA within the membrane subfractions, both in normal conditions as well as in response to high light stress.

Overall, our results paint a complex picture of the process of thylakoid membrane biogenesis and highlight the intricate regulation needed for the co-ordination of the different synthetic pathways in order to assemble functional thylakoid membranes.
Improving *Anabaena* sp. Strain PCC 7120 for biodiesel production by down-regulation of *pepc* gene expression

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Abstract: Biodiesel from microalgae holds promise as one of renewable energy instead of fossil resource, and the key step is preparation of the microalgal strains. This paper summarized ten essential characteristics of ideal microalgae for biodiesel production, and presented the “Metabolic Pivot Hypothesis” including three rational considerations for constructing microalgae feedstock of biodiesel. Although tremendous species of microalgae exist in nature, up to now there is no report about the ideal species from nature, and we have to reconstruct new organisms with genetic manipulation. The eukaryotic algae possess outstanding advantages, however, since 1978 the prepared biodiesel from them has been not industrialized. Maybe prokaryotic cyanobacteria are able to complement part of the shortage of eukaryotic algae. Following the hypothesis N$_2$-fixing filamentous cyanobacteria, *Anabaena* sp. strain PCC 7120 was selected as gene manipulation platform, and *pepc* gene was chosen as the target, which encodes phosphoenolpyruvate carboxylase (PEPC). After the transformation with the “Reverse Vector Method” (RVM), comparing with wild type of *Anabaena* 7120, the expression of *pepc* gene in the reverse mutant was down-regulated proved by RT-qPCR, and 67% of PEPC activity was abated, also soluble protein content was decreased by 29%, but lipid content was increased by 93%. The effects on the forward mutant were just opposite to the reverse mutant. These data indicated PEPC of cyanobacteria had the function of regulating lipid and protein metabolic pathways. Moreover, the reverse mutant contained fatty acids with shortened carbon chain and more unsaturated, and alkanes with more kinds and amounts. The responses of net photosynthesis to light intensities, temperatures and pHs proved that the reverse mutant increased the tolerances to some stress conditions. These data implied that down-regulation of *pepc* expression affected more physiology and biochemical processes, and PEPC may locate a metabolic pivot site. All of above data and growth of the mutants showed the RVM may be an effective, gentle and simple gene manipulation way.
Novel O-antigen Synthesis Gene Mutants That Confer Resistance to Grazing in a Model Cyanobacterium-Amoeba System

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Predatory grazing activities on photosynthetic organisms are one of the primary threats to the sustainability of high-density biomass cultures in large, open ponds for the production of cyanobacterial and microalgal biofuels. These contaminants destroy valuable biomass and prevent stable, continuous production of biofuel crops. We previously reported the establishment of a model predator-prey system using the amoeba HGG1 and the cyanobacterium *Synechococcus elongatus* PCC 7942, and its application to the discovery of “rough” mutants that are impaired in O-antigen synthesis and transport, are resistant to grazing, and autoflocculate (1). Screening of a library of transposon insertional mutants covering ~88% of the *S. elongatus* genome for a rough appearance identified four previously uncharacterized candidate genes. Knockouts of these genes produce resistance to HGG1 grazing, while complementation with a wild-type copy of the gene restores grazer susceptibility and the smooth phenotype. Bioinformatics analysis and gel electrophoresis of outer membrane preparations of one of the mutants, SynPCC7942_2292, indicate that this gene encodes the ligase responsible for the periplasmic transfer of the O-antigen from its lipid anchor to lipid A. Bioinformatics suggests that two of the genes, SynPCC7942_1903 and SynPCC7942_1904, produce glycosyltransferases that likely participate in the polymerization of the O-antigen along with the previously reported synthesis terminator, SynPCC7942_1905. The product of the fourth gene, SynPCC7942_2293, is predicted to have a single transmembrane helix and a domain distantly related to pyruvate kinase. Further biochemical analysis of the functions of these genes is ongoing.


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Due to the growing demand for power and detrimental effects of the utilization of fossil fuels (e.g. pollution, climate change, and political instability), photosynthetic organisms are being explored for alternative energy production. Strategies to utilize the high efficiency photochemical charge separation of Photosystem I (PSI) while eliminating lower efficiency processes involved in the conversion of NADPH to biomass, which then would still have to be processed into fuel, are being developed. This project focuses on engineering and incorporation of PSI into a bio-inorganic photovoltaic device (BIPV) that will produce electricity. Previous studies showed that ZnO-binding peptide (ZOBiP) fused PSI subunits (PsaD and PsaE) from *Mastigocladus laminosus* could replace Wild type (WT) PSI subunits of PSI isolated from *Thermosynechococcus elongatus* (*T.e.*), and that this chimeric complex could donate electrons to methyl viologen. The goal of this project is to introduce metal-oxide binding peptides onto the N-termini of ferredoxin (Fd) of *T.e.* as well as onto PsaD and PsaE of *T.e.* and *Synechocystis* sp. PCC 6803 (*Syn 6803*). It is thought that PSI containing native ZOBiP-PsaD and PsaE will assemble better, leading to a more productive complex. These ZOBiP-PSI subunits have been produced in vitro. WT and ZOBiP PsaD and PsaE subunits have been characterized with Western blotting, circular dichroism, and MALDI-TOF. WT PsaD and PsaE subunits have been replaced in vitro with ZOBiP-PsaD and ZOBiP-PsaE with around 50% efficiency. While WT PsaD and PsaE bind quite well to ZnO nanoparticles, WT PSI does not. ZOBiP-PSI will bind ZnO, and the results of binding will be compared to WT PSI ZnO binding. Plasmids have been constructed that will allow for the transformation of *T.e.* and *Syn 6803*, and production of ZOBiP-PsaD or ZOBiP-PsaE by the organisms. The ZOBiP will allow for self-orientation of PSI onto ZnO, while ZnO nanowires will increase the surface area of the biological heterojunction, and will increase the amount of electricity produced and harnessed from the BIPV. In the second approach to this goal a TiO$_2$-binding peptide (TOBiP) has been introduced on the N-terminus of Fd from *T.e*. This will allow for the crosslinking of TOBiP-Fd to PSI and for the self-organization of that complex onto TiO$_2$ nanowires, which offers the same advantages of the ZnO ones. (Support from: Director’s Strategic Initiative, Army Research Laboratory; NSF IGERT DGI-0801470: NSF NIRT DBI-0403781; NSF EPSCoR EPS-1004083)

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Light quality dependent alteration of reactive oxygen species (ROS) levels plays a role in the photoregulatory mechanism controlling cellular morphogenesis during complementary chromatic adaption (CCA) in *Fremyella diplosiphon*

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*Fremyella diplosiphon* has an ability to alter its cellular morphology and change the protein composition of its major light-harvesting complex, i.e. phycobilisomes (PBSs), according to ambient light quantity and quality to maximize the ecologically important process of photosynthesis [1]. This phenomenon is termed as complementary chromatic adaptation (CCA). Cells of *F. diplosiphon* are spherical, and inducible phycocyanin constitutes the outer rod of PBSs under red light (RL), whereas rectangular cell shape and phycoerythrin in outer rods of PBSs are characteristic of growth in green light (GL)-enriched environment [2]. WT-pigmented strain SF33 of *F. diplosiphon* was found to accumulate reactive oxygen species (ROS) under both GL and RL growth conditions; however, the level of oxidative stress was found to be higher under RL compared to GL [3]. The higher level of ROS under RL was associated with the RL-specific spherical cellular morphology of *F. diplosiphon*. *F. diplosiphon* adopts a spherical cellular morphology under RL by decreasing the cell length, however, the width of cells remain unaffected [3]. The spherical morphology of cells under RL was reversed to a GL-specific rectangular morphology, i.e. increase in cell length, in the presence of the antioxidant ascorbic acid, which was associated with a decrease in ROS levels [3]. Further experiments conducted to study the temporal dynamics of changes in ROS levels and cellular morphology also indicated a strong correlation between ROS levels and cell morphology at early stages of acclimation of white-light (WL)-grown cells to growth under RL or RL-grown cells to growth under GL [4]. The transition towards spherical morphology under RL was associated with an increase in ROS levels; by contrast, the GL-associated transition to rectangular morphology of RL-adapted cultures was coupled with a decrease in ROS levels [4]. Together, these observations suggest the involvement of RL-dependent increase in oxidative stress in the regulation of cellular morphogenesis during CCA in *F. diplosiphon*.


Fine tuning n-butanol production pathway in *Synechococcus spp* using Promoter Engineering

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Cyanobacteria are oxygen evolving photosynthetic blue green algae that fix CO\(_2\) and play an important role in harvesting solar energy. Recent research focuses on developing Cyanobacteria as a cell factory to convert fixed CO\(_2\) into biofuels and other valuable compounds. Butanol is considered as a sustainable fuel alternative over ethanol due to its higher energy density. Butanol is also less volatile and less corrosive compared to ethanol. Butanol pathway has already been expressed in *Synechococcus elongates pcc 7492*.

Promoter strength plays an important role in determining levels of gene expression. Most of the promoters available for study consist of constitutive or strong promoters. These may not be ideal for expression of heterologous genes. Promoter Engineering is based on randomization of the promoter sequences and has been successfully employed to construct promoter libraries to optimize levels of gene expression in various organisms. Promoter engineering yields promoters of all possible strengths. This allows best promoter-gene combination to be explored.

In this study, we randomize a 17 bp spacer sequence between -35 and -10 box of *Synechococcus* promoter. This provides promoters of various strengths, identified using a reporter gene. These promoters will be coupled to each gene of n-butanol pathway to obtain best promoter-gene combination. In this study the rate limiting step in the pathway can also be identified and fine-tuned for better expression of n-butanol pathway. An optimally expressed pathway will improve productivity of n-butanol in *Synechococcus spp*.

References:


Cellular and Functional Specificity among Ferritin-like proteins in *Nostoc punctiforme*

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The ability to control the level of reactive oxygen species (ROS) is crucial for cell fitness and determines the robustness of an organism, which is the motivation of our research. The robustness, as defined as the capability to adapt to environmental perturbations, has a huge importance for life but is also decisive for an efficient production of biomass and biofuels. Ferritin-like proteins including ferritins, bacterioferritins and Dps proteins are part of the bacterial defense against oxidative stress as well as of iron homeostasis. The genome of the heterocyst-forming cyanobacterium *Nostoc punctiforme* encodes five ferritin-like proteins. We report a multidimensional characterization of these proteins, demonstrating differences in physiological function and in cell localization. The phylogenetic and bioinformatics analyses suggest both structural and physiological differences among the ferritin-like proteins, which were clustered as four Dps proteins and one bacterioferritin. The expression of the five genes responded differently to hydrogen peroxide treatment. Analysis of gene-deletion and over-expressing strains implied that one specific Dps has a major role in the cells tolerance against hydrogen peroxide and that others were involved in tolerance against additional stressful conditions. Examination of Promoter-GFP reporter fusions of the ferritin-like genes showed cell specific and growth condition specific expression. Our study provides a comprehensive analysis of cyanobacterial ferritin-like proteins, combining functional differentiation and cellular specificity within this important group of proteins, which gives new understanding of division of labor in a multicellular cyanobacterium.
Chemical Imaging of Cyanobacteria: A Picture is Worth a Thousand Words

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Since its emergence in the 1990’s, chemical imaging has been broadly defined as the capability to create a map of the chemical state of a sample from spectral, spatial, and temporal information[1]. With advances in detection sensitivity and multivariate analysis algorithms, chemical imaging has matured and has been successful at providing quantitative biochemical information at the single cell and subcellular levels for a variety of living systems, including cyanobacteria [2,3,4]. In this work we illustrate the application of hyperspectral confocal fluorescence and hyperspectral line-scan Raman microscopy of living cyanobacteria to assess photosynthetic pigment dynamics in response to changing environmental parameters. Hyperspectral fluorescence and Raman microscopies are capable of interrogating specific molecular information that when coupled with advance multivariate analysis methods can differentiate light harvesting pigments, including phycobilins, chlorophylls, and carotenoids based on their spectral properties. These enabling technologies result in spatially-resolved information about the function and connectivity of photosystem components that is unavailable with other techniques. Using a diverse set of cyanobacteria, we will illustrate the widespread applicability and potential of quantitative chemical imaging for more fully illuminating several important biological processes such as carotenoid biogenesis, fatty acid metabolism, and environmental adaptation. Advantages of combining single cell-based methods which are inherently capable of assessing heterogeneity of cellular response with the traditional benchtop biochemical assays that report bulk measurements will be discussed.


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Conservation and molecular characterization of freshwater cyanobacterial Germplasm of IBSD, Imphal, Manipur falling under Indo-Burma biodiversity hotspot

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Abstract

The recent realization of immense biotechnological potential has made cyanobacteria the organisms of choice for research and development in the fields of biofertilizers, food and feed, energy production, fine chemicals, pharmaceuticals and bioremediation etc. This necessitates a study of cyanobacterial diversity for finding strains with different potentials and characteristics to suit their varied applications. However, with the exception of a few reports, cyanobacterial diversity studies in the north-eastern region of India are scarce although the region is well known as one of the biodiversity hot-spots in the world for its exceptionally large floral and faunal diversity. Lack of enough taxonomic criteria has led to incorrect assignment of cyanobacteria in different groups. Morophologically similar strains differ greatly at molecular level and vice versa. Such changes become evident in field isolates maintained under artificial culture conditions. This include morphological features and physiological characteristics e.g. pigment composition, variation in vacuole formation, akinete production etc. Hence use of phenotypic characters in combination with molecular markers as part of a robust approach seems to be a better method to understand molecular affiliation and systematics of cyanobacteria and the composition of natural cyanobacterial communities. Molecular techniques based on PCR amplification targeting conserved regions inside the 16S rRNA gene have allowed determination of phylogenetic affiliations among cyanobacteria and development of modern cyanobacterial taxonomy. A total of 1133 cyanobacterial strains were encountered from different ecological habitats of Northeast region of India. The isolates belong to thirty-four (34) genera viz. Aphanothece (02), Aphanocapsa (03), Gloeocapsa (01), Myxosarcina (01), Oscillatoria (32), Trichodesmium (01), Lyngbya (130), Microcoleus (01), Spirulina (03), Phormidium (213), Hydrocoleum (01), Limnothrix (25), Porphyrosiphon (01), Anabaenopsis (04), Anabaena (268), Pseudanabaena (07), Nostoc (188), Cylindropermum (19), Wollia (02), Aulosira (17), Scytonema (08), Plectonema (69), Tolypothrix (04), Microchaete (30), Calothrix (56), Dichothrix (06), Rivularia (01), Westiellopsis (23), Hapalosiphon (11), Nostochopsis (02), Mastigocladosus (01), Stigonema (01), Seguenzea (01) and Mastigocladus (01) have been deposited to the Freshwater Cyanobacterial and Microalgal Repository at IBSD, Imphal, Manipur (a facility of national importance created by the Department of Biotechnology, Govt. of India with ref. no.: BT/PR 11323/PBD/26/171/2008 Dated 31-03-2009). These isolates were characterized at the morphological as well as at the biochemical level. All these cyanobacterial isolates are being maintained in agar slants and liquid medium. In addition to these, few selected isolates are also being cryopreserved using 3 different cryoprotectants viz., 10% glycerol, 8% DMSO and 5% methanol. Revival rate of the cryopreserved isolates were monitored for every 6 months using standard revival protocols and found to be satisfactory. The potent isolates were selected and further characterized by PCR based molecular methods using markers such as RAPD and 16S rRNA for identification. This study proved to be highly efficient in distinguishing closely related strains revealing greater cyanobacterial diversity in all locations than initially appeared under morphological investigations.
Temperature dependence of photoinhibition of PSII

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Photoinhibition, light induced decay of photosystem II (PSII) activity, occurs under all light intensities. It has been suggested that photoinhibition depends on charge recombination reactions between the reduced electron acceptors and the S-states of the oxygen evolving complex ($S_{2/3}Q_{A/B}^-$ recombination reactions). Recombination reactions produce the triplet state of the primary donor and thus promote the formation of singlet oxygen ($^1O_2$). According to the recombination hypothesis, photoinhibition depends on $^1O_2$ formed in this way.

To test the recombination hypothesis, we compared the temperature dependence of recombination reactions and the temperature dependence of photoinhibition in isolated thylakoid membranes. $S_{2/3}Q_{A/B}^-$ recombination is a thermally activated reaction occurring via three competing pathways. The temperature dependence of each pathway was measured with thermoluminescence, and the temperature dependence of photoinhibition was measured by assaying the rate of oxygen evolution after illuminating isolated thylakoids at different temperatures. The results show that the rate constant of photoinhibition increases with temperature from 5 to 35 °C but the increase is much weaker than the temperature dependence of the recombination pathway associated with the production of $^1O_2$.

Both chlorophylls and the manganese ions of the oxygen evolving complex act as photoreceptors of photoinhibition. As artificial manganese complexes tend to have low absorption at long wavelengths where chlorophylls absorb well, it might be possible that different wavelengths cause photoinhibition with different mechanisms. To test this, we measured the temperature dependence of photoinhibition in blue (<450 nm, more absorption by manganese) and red (>600 nm, more absorption by chlorophyll) lights. However, the temperature dependence of photoinhibition did not depend on wavelength. The similarity of photoinhibition at different wavelengths indicates that the manganese and chlorophyll dependent mechanisms of photoinhibition function in concert rather than in parallel.

Photoinhibition can also be induced by illuminating thylakoid membranes with single turnover flashes. The photoinhibitory efficiency of nanosecond laser pulses is high if the pulses are separated by long time intervals whereas pulse trains with short intervals between pulses cause less photoinhibition. This behavior has been interpreted to indicate participation of $S_{2/3}Q_{A/B}^-$ recombination reactions in laser-pulse-induced photoinhibition. We measured the temperature dependence of photoinhibition induced with 532-nm, 12 mJ, 4-ns laser pulses, and found that photoinhibition induced with such pulses showed no apparent temperature dependence. Thus, recombination reactions are not involved in laser-pulse-induced photoinhibition.
The omega subunit of the RNA polymerase directs transcription efficiency in cyanobacteria

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The catalytic core of the RNA polymerase (RNAP) in most eubacteria is composed of two α subunits, a β, β’, γ, and ω subunit, and for transcription initiation a regulatory σ subunit is recruited. In cyanobacterial lineage (and also in plastids) β’ is split to two parts, the N-terminal part is called as a γ subunit and C-terminal part is a β’ subunit. We have studied the role of the tiny, 7 kDa, ω subunit. Studies with E. coli and some pathogenic eubacteria have suggested that this non-essential subunit might have a chaperone-like function assisting β’ to join the core complex as a last assembly step. Similar function in cyanobacteria would require simultaneous contact of the ω subunit with both β’ and γ subunits.

The ω subunit is encoded by the rpoZ gene in cyanobacteria. The cyanobacterial ω subunit is shorter than that of E. coli although it contains an N-terminal extension. We inactivated the rpoZ gene in Synechocystis sp. PCC 6803. The ΔrpoZ inactivation strain grew as fast as the glucose tolerant control strain in our standard conditions (constant light 40 µmol photons m⁻² s⁻¹; 32 °C; ambient air) but mutant cells were more yellowish than cells of the control strain. Chlorophyll and phycobilin contents of the ΔrpoZ strain were normal but it had a high carotenoid content, and cells appeared yellowish. DNA microarray analyses in standard growth conditions revealed 187 up-regulated and 212 down-regulated genes in ΔrpoZ compared to the control strain. Down-regulated genes included ones that are highly expressed in the control strain, like genes encoding subunits for Rubisco, ATP synthase, NDH-1 complexes and other carbon concentrating mechanisms. Up-regulated genes, in turn, mainly belong to categories hypothetical or unknown function, and showed only low expression in standard conditions. These results suggest that the presence of the ω subunit in RNA polymerase core favors the expression of highly expressed household genes which are mainly recognized by the primary SigA σ factor. The ΔrpoZ strain did not survive in conditions (high CO₂ or slightly elevated temperate) that support rapid growth of the control strain.
Metabolic engineering of *Synechocystis* sp. PCC 6803 for production of chemicals and fuels

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Global warming and decreasing fossil fuel reserves have prompted great interest in the synthesis of chemicals from renewable resources. In an effort to address these concerns, we performed metabolic engineering of the cyanobacterium *Synechocystis* sp. strain PCC 6803 to develop a strain that can synthesize isobutanol under both autotrophic and mixotrophic conditions. With the expression of two heterologous genes from the Ehrlich pathway, the engineered strain can accumulate 90 mg/liter of isobutanol from 50 mM bicarbonate in a gas-tight shaking flask. The strain does not require any inducer or antibiotics to maintain its isobutanol production. In the presence of glucose, isobutanol synthesis is moderately promoted (titer = 114 mg/liter). Based on isotopomer analysis, we determined that, compared to the wild-type strain, the mutant strain significantly reduced its glucose utilization and mainly employed autotrophic metabolism for biomass growth and isobutanol production. Since isobutanol is toxic to the cells and may also be degraded photochemically during the cultivation process, we employed *in situ* removal of isobutanol. This resulted in a final net concentration of 298 mg/liter of isobutanol under mixotrophic culture conditions. Finally, we will present our recent results for D-lactate production via a novel D-lactate dehydrogenase by *Synechocystis* 6803, in which >1g/L D-lactate can be produced under phototrophic conditions.


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The \textit{sll1951} Gene Encodes the Surface Layer Protein of \textit{Synechocystis} sp. strain PCC 6803

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\textit{Sll1951} is the surface layer (S-layer) proteins in the cyanobacterium \textit{Synechocystis} sp. strain PCC 6803. This large, hemolysin-like protein was found in the supernatant of a strain that was deficient in S-layer attachment. A \textit{sll1951} deletion mutation was introduced into \textit{Synechocystis} and was easily segregated to homozygosity under laboratory conditions. In thin-section and negative-stain transmission electron microscopy a \(\sim 30\) nm wide S-layer lattice covering the cell surface was readily visible in wild-type cells but was absent in the \(\Delta sll1951\) strain. Instead, the \(\Delta sll1951\) strain displayed a smooth lipopolysaccharide surface as its most peripheral layer. In the presence of chaotropic agents the wild type released a large (>150 kDa) protein into the medium that was identified as \textit{Sll1951} by mass spectrometry of trypsin fragments; this protein was missing in the \(\Delta sll1951\) strain. In addition, \textit{Sll1951} was prominent in crude extracts of the wild type, indicating that it is an abundant protein. The carotenoid composition of the cell-wall fraction of the \(\Delta sll1951\) strain was similar to that of the wild type, suggesting that the S-layer does not contribute to carotenoid binding. Although the photoautotrophic growth rate of the \(\Delta sll1951\) strain was similar to that of the wild-type strain, the viability of \(\Delta sll1951\) was reduced upon exposure to lysozyme treatment and hypo-osmotic stress, indicating a contribution of the S-layer to the integrity of the \textit{Synechocystis} cell wall. This work identifies the S-layer protein in \textit{Synechocystis}, and shows that, at least under laboratory conditions, this very abundant, large protein has a supportive but not a critical role in the function of the cyanobacterium.
Gene regulatory interactions and ultradian rhythms in global transcription of *Cyanothece* sp 51142 under continuous light.

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Cyanobacteria, a group of photosynthetic prokaryotes, oscillate between day and night time metabolism with concomitant oscillations in gene expression in response to light/dark cycles. The oscillations in gene expression have been shown to sustain in constant light (LL) with a free running period of 24 h in a model cyanobacterium *Synechococcus elongatus* PCC 7942 (1,2). However, equivalent oscillations in metabolism have not been reported under LL in this non-nitrogen fixing cyanobacterium (3). *Cyanothece* sp. ATCC 51142, a unicellular diazothrophic cyanobacterium, temporally separates oxygenic photosynthesis from oxygen sensitive nitrogen fixation when grown in 12 h alternate light/dark condition (LD) (4, 5). Approximately 30% of the genes of this organism have been shown to oscillate under diurnal cycles (5). We recently demonstrated that *Cyanothece* 51142 grows better under highly turbulent regime, possibly due a simulated flashing light effect (6). We show that global gene expression and metabolism oscillate in *Cyanothece* 51142 under LL with a free running period of ~ 11 h under these growth conditions (6). Over 40% of its genes show oscillations with this periodicity. The metabolism alternates between photosynthesis and respiration with concomitant glycogen accumulation and consumption, respectively. The genes that peak at dawn and dusk in LD peak at the beginning of photosynthesis and respiratory phases, respectively, in LL. The circadian rhythm of this organism appears to be more robust with peaking of genes in anticipation of the ensuing daytime or nighttime activities. Upon building a relevance network model of periodic genes, we show the temporal separation of the key cellular processes such as nitrogen fixation, carbon fixation and energy metabolism. Using our approach named GlobalMIT (7), which admits a polynomial time complexity and uses information theoretic scoring metric named mutual information test (MIT), we studied the gene expression dataset for potential regulatory interactions (8). We have mapped the circadian clock genes and their underlying genetic interactions from the well enumerated cyanobacterium *Synechococcus* 7942 to study the core circadian clock in *Cyanothece* 51142 (5) employing comparative genomics techniques (7). Further, we construct a predictive model using Inferelator (10), a differential equation based approach. The model, trained on previously published *Cyanothece* 51142 microarray data (5) and tested on our collected microarray data, is capable of predicting accurately the global change in periodicity from ~24h to ~11h in *Cyanothece* under prolonged continuous light condition.

References  
Light induced movement of cyanobacterial cells

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Synechocystis sp. PCC 6803 exhibits flagellar-independent “twitching motility” that allows bacteria to move over moist surfaces using type IV pili. Mutants that lost type IV pili are non-motile. In order to use optimal light qualities and quantities for photosynthesis, they are able of directed movement along a light gradient. Regulation of phototactic motility is complex and involves many different gene products, including amongst others different photoreceptors, the RNA chaperone Hfq and adenylate cyclases. \( \Delta hfq \) mutants are non-motile and most of their type IV pili were missing. Consequently, they have lost also their natural competence for transformation by exogenous DNA. Microarray experiments revealed several mRNAs and small regulatory RNAs belonging to the cAMP receptor protein regulon that are differentially expressed in \( \Delta hfq \) mutants. Employing recombinant FLAG- and eYFP-tagged fusion proteins we could identify the motor ATPase PilB that energizes pilus extension as a possible Hfq interaction partner and show that Hfq is localized at the cell periphery in co-immunoprecipitation and fluorescence microscopy studies, respectively. Expression analyses and interaction studies will be discussed in connection with RNA-based regulatory processes in Synechocystis sp. PCC 6803.

In addition, we demonstrate a biological function of the Cph2 photoreceptor in motility. Cph2, a bilin-binding photoreceptor protein from the cyanobacterium Synechocystis sp. PCC 6803 with multiple GAF domains, inhibits phototaxis towards blue light. We show that Cph2 perceives blue light by its third GAF domain. This GAF3 domain belongs to the cyanobacteriochrome family and undergoes photoconversion between green and blue-light absorbing states. The two N-terminal GAF domains support photoconversion between red and far-red absorbing states. Cph2 differs from most other phytochromes by harboring EAL and GGDEF domains, both associated with turnover of the second messenger c-di-GMP. Upon blue light irradiation the GAF3 domain together with the downstream GGDEF domain shows elevated c-di-GMP synthesis in vitro compared to green light irradiation. A photochemically inactive mutant protein exhibits light independent c-di-GMP production. Overexpression of the GAF-GGDEF module in Synechocystis sp. PCC 6803 leads to blue-light dependent inhibition of motility, expression of the N-terminal Cph2 fragment harboring the EAL domain as well as an E. coli EAL domain protein complement this phenotype. C-di-GMP has been shown to regulate flagellar and pili-based motility in several bacteria. Here we provide the first evidence that this second messenger is also involved in light dependent regulation of cyanobacterial phototaxis.

Understanding Carboxysomes through Synthetic Biology

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The carboxysome is a bacterial microcompartment found in cyanobacteria that consists of a proteinaceous shell encapsulating ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and carbonic anhydrase (CcaA). Cyanobacteria depend on the carboxysome to concentrate carbon dioxide near the active site of RuBisCO to minimize the unproductive side reaction with oxygen. The genes that encode the shell and two internal components of the carboxysome are typically found in a single operon (ccm operon) necessary for carboxysome formation and survival of the cell in atmospheric CO₂ concentrations. We are using DNA fabrication to construct a synthetic library of ccm operons consisting of nearly 40,000 possible combinations of ccm genes from phylogenetically diverse cyanobacterial species. The synthetic library will be transformed into a strain of Synechococcus sp. PCC 7942 deficient in the native ccm operon to determine which combinations of genes produce novel carboxysomes with enhanced phenotypes. This work also takes advantage of gene synthesis to make E. coli codon-optimized genes to produce protein for use in crystallography studies. Here we present recently obtained structures of Ccm proteins from various species of cyanobacteria. Ultimately, through application of synthetic biology tools this research will enhance the knowledge of carboxysome structure and create carboxysomes with novel properties.
Repression of phycoerythrin expression by a novel blue-green photoreceptor in the red-green chromatically acclimating cyanobacterium *Fremyella diplosiphon*

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*Fremyella diplosiphon* changes the composition of the phycobiliproteins its photosynthetic light harvesting antennae, or phycobilisomes, in response to shifts in the ratio of red to green ambient light through a process called type III chromatic acclimation. In green light, *F. diplosiphon* expresses high levels of a red-pigmented phycobiliprotein that absorbs green light, phycoerythrin (PE), and low levels a green-pigmented phycobiliprotein that absorbs red light, phycocyanin (PC), allowing efficient absorption of green light by the phycobilisomes. In red light, PE expression is repressed and PC expression is activated. The Rca system is the only pathway known to regulate PC activation in red light. However, two pathways repress expression of the PE-encoding genes: the Rca system and the Cgi system. The well-studied Rca pathway controls the cell's responsiveness in both red and green light at the level of transcription, repressing the operon that encodes PE during growth in red light. The Cgi system also represses PE expression in red light, but post-transcriptionally, and the components of the pathway are currently unknown.

We have conducted a transposon mutant screen to identify factors that contribute to PE regulation, including those that are part of the Cgi system. This screen was carried out using cells that lack the photoreceptor of the Rca system, RcaE. Cells lacking RcaE display a blackish phenotype due to the presence of intermediate levels of both PE and PC in the cells. Transposon mutants disrupted in PE repression during growth in red light display a brown or red phenotype due to the increased levels of PE in these cells.

Five independent red-brown mutants had transposon insertions in a previously undescribed operon containing genes encoding for one response regulator and two histidine kinases. We have named this the *dpx* operon for its role in the decrease in phycoerythrin expression. Homologues of the *dpx* operon are present in at least 15 other cyanobacterial species, including several that do not produce PE, suggesting that this group of regulatory genes controls multiple processes in cyanobacteria.

The first gene of the *dpx* operon, *dpxA*, is predicted to encode a histidine kinase with an N terminal GAF domain. GAF domains often bind chromophores and act as light-sensing proteins. Sequence analysis suggested that DpxA is a blue-green photoreceptor, because it contains both of the cysteine residues required for chromophore attachment, as well as a DXCF motif, which has been universally correlated with the capacity for blue-green sensing in cyanobacteriochromes, a group of photoreceptors within the phytochrome superfamily. We have demonstrated that the GAF domain of DpxA binds a chromophore and switches between a green-absorbing form (P$_G$) with a maximum absorbance of 567 nm and a blue-absorbing form (P$_B$) with a maximum absorbance of 494 nm. An in-frame, clean deletion of *dpxA* in an *rcaE* background results in a mutant with increased levels of PE during growth in red light. Current work is analyzing whether or not DpxA's blue-green light sensing capability has a role in its ability to repress PE synthesis.
Live cell characterization of temporal redox dynamics by chemical profiling in the photoautotroph *Synechococcus* sp. PCC 7002


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The dynamic reversible reduction and oxidation of cysteine thiols is thought to be a particularly important means of regulating protein function. We have developed reduced thiol and sulfenic acid-targeting chemical probes that are taken up by living cells. Hence, they can be applied to interrogate “redox dynamics” *in vivo*. The probes have three important features: (1) an electrophilic iodoacetamide or maleimide group to covalently label reduced cysteine thiols in proteins, or a dimedone for covalent capture of sulfenic acids; (2) spacer/binding regions that impart cell permeability, and (3) an alkyne handle for ‘click chemistry’ conjugation of probe-labeled proteins to an azido-functionalized biotin for streptavidin enrichment of probe-labeled proteins, or an azido-fluorophore for cell imaging.

We applied these probes directly to living cells of *Synechococcus* sp. PCC 7002. The following experimental conditions were applied: a C-limited steady-state continuous culture was deprived of CO$_2$ for 45 minutes to impose C-starvation. At this time, CO$_2$ was reintroduced into the sparging gas and samples were taken for probe labeling and PAM fluorometry at times ranging from 0.5 to 60 minutes. We have also performed a second experiment in which the redox response to a temporal change in light intensity was evaluated.

Under the C-replenishment conditions, changes in redox status of proteins were detected in 30 seconds and there were multiple temporal cycles of oxidation and reduction over the first 20 minutes. For about 45% of the labeled proteins, there was less than a 10-fold change in detection over the time course, but the remainder showed quite large changes over time. Approximately 175 proteins were detected by *in vivo* labeling; only 75 of these had been previously identified as redox-regulated in either *Synechocystis* 6803 or *Arabidopsis* chloroplasts. All major metabolic subsystems contained proteins whose redox status changed over time. For example, the labeled proteins included 21 involved in photosynthesis, 9 of 11 Calvin cycle enzymes, 11 involved in transcription or its regulation, 30 in translation and 9 redox regulators.

The analysis of PAM fluorometry data indicated that during the first 12 minutes after CO$_2$ was resupplied, photosynthetic processes were restored, but that downstream metabolic processes were not fully capable of consuming the products of the photosystems. During this interval, there were 2 cycles of reduction/oxidation. Subsequently, there was a better match between metabolic subsystems, and CTDE-sensitive proteins became reduced.

In summary, our results reveal a series of newly identified proteins that undergo dynamic redox changes, and potentially govern a wide range of biological processes including signal transduction, ROS remediation, photosynthesis, metabolism, and protein synthesis. Many of these proteins undergoing dynamic redox transformations participate in the major components for the production or consumption of reductant and/or energy in photosynthetic organisms. Thus, our *in vivo* approach reveals new redox-susceptible proteins, in addition to validating those previously identified *in vitro*. 
Construction of a recipient strain of *Synechococcus* sp. PCC 7002

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Some strains of Cyanobacteria have long been used as research models for understanding the processes related to photosynthesis and carbon metabolism, and now they also have important applications in synthetic biology and new energy development. While *Synechocystis* sp. PCC 6803 has been more researched and engineered genetically, we are evaluating if it is advantageous to use less explored *Synechococcus* sp. PCC 7002 as a research tool for it has been found to be able to grow faster and appears to have higher transformation efficiency, and may be easier to isolate PSII particles. Its higher PSII/PSI ratio and higher optimal growth temperature may also lead to improvements in structure-function studies of PSII. Moreover, the differences in growth rate and photosystem homeostasis between *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 may lend understanding to environmental adaptation and evolutionary diversity.

Similar strategy as what have been done in *Synechocystis* sp. PCC 6803 is used to delete three *psbA* genes and generate His-tagged *psbB* gene in *Synechococcus* sp. PCC 7002, but instead of using one antibiotic for each gene manipulation, we employ counterselection to recycle antibiotics after confirming the completion of segregation. To do this, two plasmids, pRL277 and pRL278, which have Sp\(^R\) and Km\(^R\) cassette adjacent to *sacB* gene, respectively, are used. We also use Gibson Assembly to generate similar constructs for other antibiotics, and these fragments and upstream and downstream homologous fragments are assembled using Gibson Assembly or Fusion PCR. The full-length synthetic DNA fragments are introduced into *Synechococcus* sp. PCC 7002 by natural transformation. The resulting strain with three *psbA* deletions and His-tagged *psbB* is a good material for more advanced research.


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Introduction of *Acaryochloris marina* genomic libraries into *Synechocystis* sp. PCC 6803 and a FACS-based screening strategy for chlorophyll d-producing *Synechocystis* cells

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Objective: Develop genomic libraries that can be introduced into *Synechocystis* sp. PCC 6803 (S6803), and an assay to select for altered phenotype detectable by FACS

S6803 is a genetically tractable cyanobacteria that is increasingly used for biotechnology applications in recent years. To genetically modify S6803, a high-throughput library in conjunction with a high-throughput screening system is desirable. We have established a method to select for S6803 cells exhibiting altered emission spectra by flow cytometry. This method enables recovery of rare transformants of interest out of a large background of other transformants. This method was originally developed to identify a gene from *A. marina*, a chlorophyll d-producing cyanobacteria, by introducing genomic libraries from *A. marina* into S6803. The development of both *E. coli*-dependent and independent genomic libraries are currently underway.

This strategy can be readily adapted to generate genomic and metagenomic libraries from various fastidious organisms with valuable traits, and metagenomes from environments where useful genes are enriched. With appropriate screening strategies, S6803 can be used as a chassis to discover and utilize novel genes for future applications.
Isolation and description of a globally distributed cryosphere cyanobacterium

*Oscillatoria* sp. KNUA009 from Antarctica

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Cyanobacteria are distributed in a wide variety of environmental niches playing indispensable role in cycling of carbon and nitrogen by using sunlight and air. Due to their remarkable tolerance to a great range of environmental conditions, cyanobacteria are abundant in almost every conceivable environment, including the polar and alpine habitats. In this study, a previously uncultured cyanobacterium, strain KNUA009, was axenically isolated from a meltwater stream on Barton Peninsula, King George Island, in the South Shetland Islands, Antarctica. Molecular evidences showed that the isolate belongs to groups of globally distributed cryosphere cyanobacterial clones and this new isolate represents the first laboratory culture to be assigned to these groups. Strain KNUA009 was able to thrive at low temperatures ranging between 5 and 20°C, but did not survive at temperatures of 25°C and above. As the isolate morphologically resembled *Oscillatoria* species, it is suggested that this cyanobacterium may represent a new species clade with cold resistance within the genus *Oscillatoria*. It was also observed that strain KNUA009 autotrophically produced saturated palmitic acid (C₁₆:₀, 34.6 ± 0.4%) as one of its cellular fatty acids. Since cyanobacteria have gained increasing attention in recent years due to their higher photosynthetic efficiency and oil yield compared to terrestrial sources, strain KNUA009 may serve as an attractive candidate for biofuel production in the winter seasons. In addition, it should be noted that the isolate was also capable of synthesizing ω-3 fatty acid (C₁₈:₃ ω3, 17.8 ± 0.8%) which is a nutritionally important ω3 fatty acid. Therefore, strain KNUA009 may also be promoted as an alternative source to fish oil for ω3 fatty acid production.
Cyanobacteria are the only known prokaryotic organisms that perform plant-like oxygenic photosynthesis, but the structure of their central carbon metabolism has remained elusive. This study directly profiles the photomixotrophic metabolism of *Synechocystis sp.* PCC 6803 using both isotopic pulse-chase experiments and $^{13}$C-Metabolic Flux Analysis (MFA). Our fluxomics results indicate that the *in vivo* enzyme reaction ($\alpha$-ketoglurate $\rightarrow$ succinate) is present, however, the absolute carbon flux through this reaction is below the detection limit by $^{13}$C-MFA (relative flux is less than 1% of total carbon uptake). Moreover, $^{13}$C-based flux analysis indicates that the glyoxylate shunt is inactive (glyoxylate is synthesized from photorespiration) and measurable flux exists through the oxidative pentose phosphate pathway under photomixotrophic conditions.
Analysis of metabolic fluxes under N-starvation in Synechocystis sp. PCC6803

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Cyanobacteria are poised to be at the forefront of microbial chemical factories due to their high carbon fixation rates and ability to be genetically engineered. Especially attractive is the fact that with light as the energy source, the chemical production is predominantly renewable. While most cyanobacteria generally secrete few compounds during photoautotrophic growth, they are capable of secretion during mixotrophic or heterotrophic growth. Recently, several groups have metabolically engineered pathways into cyanobacteria for the production of native and foreign compounds. However, the overall titer and yields are still suboptimal compared to commercial bioprocesses with industrial microbes. Metabolic flux analysis is a critical tool used to identify rate-limitations, metabolic regulation and flux control at branchpoints in microbes. Thus, the analysis of metabolic fluxes will be of immense assistance in the rational design of engineered pathways in cyanobacteria. We are investigating the metabolic response to N-deplete conditions in Synechocystis sp. PCC6803 wild type and a glucose-1-phosphate adenylyltransferase glgC mutant, which lacks the ability to store glycogen [1].

We are taking both computational and experimental approaches to study the response to N-starvation in photoautotrophic conditions. Flux balance analysis is being implemented with different objective functions in place of the commonly used maximization of growth rate. We will present computationally derived flux maps for both strains based upon objection functions of maximizing ATP, maximizing carbon uptake rate, or minimizing total flux. Experimentally, we determined changes in the overall macromolecular biomass composition after N-starvation. The glgC mutant differed from wild type by significantly degrading intracellular protein and polysaccharide contents within 48 hours. On the other hand, lipid amounts were relatively unchanged for both strains. One of the key aspects we are attempting to quantify is the amount of overflow metabolism that is redirected from catabolism from intracellular carbon vs. newly fixed CO₂. To truly assess this, we are employing our recently developed ¹³C isotopically instationary flux analysis [2].


Synechococcus sp. UTEX 2973, a new cyanobacterial chassis for synthetic biology and metabolic engineering applications

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Photosynthetic microbes are of considerable interest in biotechnological applications due to their ability to grow photoautotrophically using sunlight and CO₂ as energy sources. Important and desirable traits for such model systems are fast growth and amenability to genetic manipulation. Here we describe a new cyanobacterial strain, Synechococcus sp. UTEX 2973, that displays rapid growth rates, with a doubling time of less than two hours under optimal conditions. Synechococcus UTEX 2973 can be readily transformed by conjugation and fully segregated genetic mutants can be acquired more quickly than other model cyanobacteria such as Synechocystis sp. PCC 6803. The whole genome of this strain was sequenced and compared to the genomes of its close relatives Synechococcus elongatus PCC 6301 and PCC 7942. Significant differences were found, including a large deleted genomic region that may be related to the unique attributes of this strain.

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Engineering a synthetic CO$_2$-fixing photorespiratory Bypass in Cyanobacteria

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Autotrophic CO$_2$ fixation in Cyanobacteria occurs primarily by the Calvin-Benson (CB) cycle and by its key enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). This enzyme, however, has an imperfection, because it is not able to discriminate very well between carbon dioxide and oxygen. The incorporation of oxygen instead of carbon dioxide results in photorespiration and leads to the formation of 2-phosphoglycolate (2-PG). This toxic compound has to be removed or, ideally, recycled. The first step of detoxification is the dephosphorylation of 2-PG to glycolate. To salvage glycolate Cyanobacteria can make use of several strategies. They can simply excrete glycolate, oxidize it completely to CO$_2$ via glyoxylate and oxalate, or recycle it like plants in a complex reaction sequence converting it into 3-phosphoglycerate, which is then used to replenish the CB cycle. In any case photorespiration always results in a significant loss of previously fixed carbon. Underscoring the huge impact of photorespiration, disruptions of these pathways in cyanobacterial mutants usually result in growth retardation and/or high CO$_2$ requiring phenotypes. We are attempting to engineer a glycolate/glyoxylate salvaging pathway that actually involves an additional CO$_2$ fixing step. This may be accomplished by making use of enzymes that are involved in the 3-hydroxipropionate bi-cycle, an alternative autotrophic CO$_2$ fixation pathway found in *Chloroflexus aurantiacus*. One turn of this designed cyclic photorespiratory bypass consumes glyoxylate, fixes bicarbonate, and finally provides pyruvate which can be used directly for biosynthesis or to replenish the CB cycle.
Synthetic Biology Tools for Differential Gene Expression in Cyanobacteria

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The pressure to decrease dependence on petroleum has spurred researchers to investigate the application of synthetic biology to develop bio-based methods of chemical and fuel production. Cyanobacteria have emerged as a promising platform for these efforts, as they are genetically pliable and do not require a sugar source or arable land for growth. But, in application, the utility of using cyanobacteria for chemical production is reduced by a lack of functional metabolic engineering tools. Particularly, there is a shortage of tools for predictable control of gene expression. Our goal is to develop methods for differential expression of native and heterologous genes in the cyanobacterium \textit{Synechococcus} sp. PCC 7002. Synthetic biology was used to modify a native cyanobacterial promoter for tetracycline-based induction, an asRNA system was adapted for use in PCC 7002, and we aim to investigate the role of Hfq in asRNA function. We have constructed a tetracycline-dependent induction system based on the \textit{cpcB} promoter that allows for variable control of gene expression in cyanobacteria. Initially, this system showed strong repression, but limited induction. We looked at the effects of operator placement and spacing on expression, decreased expression of the repressor to maximize induction, and investigated the time dependence and light sensitivity of induction. Further modifications are necessary to optimize this system, but it could be of use in a variety of metabolic engineering projects in cyanobacteria. Specifically, we plan on using this tetracycline dependent induction system to control expression of trans acting antisense RNA (asRNA). asRNA functions by binding to the ribosome binding site of the mRNA transcript and inhibiting translation—this post-transcriptional control would provide a method to fine-tune gene expression, turn down native pathways, and increase the complexity of genetic circuits. We adapted the IS10 asRNA module from \textit{E. coli} into PCC 7002 and demonstrated its functionality, but we are working on improving the repression of translation by increasing the ratio of asRNA to the target mRNA. We aim to develop additional asRNA systems in cyanobacteria as an alternative to the IS10 module, using native cyanobacteria and \textit{E. coli} asRNA scaffolds. Moreover, we are investigating the role of Hfq in the function of asRNA in PCC 7002. Hfq is a small RNA chaperone that is involved in asRNA binding to the target mRNA, as well as mRNA degradation. It is known that PCC 7002 has an Hfq homolog, but it's uncertain what its targets are. We plan to knock out the native Hfq in PCC 7002 and determine the effects on our current IS10 asRNA system, as part of a more in-depth exploration of the role of Hfq in RNA stability and asRNA function. These tools and basic science knowledge will increase the utility of cyanobacteria in developing sustainable methods for chemical and fuel production.
Deletion of *Synechocystis* sp. PCC6803 Leader Peptidase LepB1 Affects Photosynthetic Complexes and Respiration.

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*Synechocystis* sp. PCC 6803 possesses two leader peptidases in the genome, LepB1 (Sll0716) and LepB2 (Slr1377), responsible for the processing of signal peptide-containing proteins. Both proteins contain the conserved motifs (or “boxes”) similar as in the other organisms, including the invariant amino acid residues demonstrated to be essential for catalytic activity. Deletion of the gene for LepB1 results in an inability to grow photoautotrophically, while the deletion of LepB2 is lethal.

Here we show, using a combination of Blue Native/SDS-PAGE, Western blotting and iTRAQ analysis, that lack of LepB1 strongly affects the cell’s ability to accumulate WT levels of both photosystem I (PSI) and cytochrome (Cyt) b6f complexes, though photosystem II (PSII) is unchanged. In particular, PsaF, one of the PSI subunits with signal peptide, was found incorporated into PSI in its unprocessed form, which could influence the assembly and/or stability of PSI. Thus, imbalance in the ratios of PSI and Cyt b6f to PSII leads to an imbalanced photosynthetic electron flow up- and down-stream of the plastoquinone pool, resulting in the observed light sensitivity of the mutant. Since both strains were cultured under unusually dim light with glucose and showed a comparable growth rate, we assume the energy source was mainly through the respiration chain. CydAB was previously shown to be one of the terminal oxidases in respiratory chain in *Synechocystis*. The observed two-fold increase in expression of CydA in the mutant may compensate for the functional loss of Cyt b6f complex. Changes encountered by the cells given with different energy supplies (light or/and glucose) were also investigated. iTRAQ analysis revealed a number of other changes accompanying the mutation, primarily a strong induction of the one uncharacterized Rieske protein PetC3, as well as a significant decrease in phycobiliproteins and chlorophyll/heme biosynthesis enzymes. Some proteins involved in stress response were also found to be significantly induced in LepB1 mutant, such as two of the poly(3-hydroxyalkanoate) synthases, Slr1829 and Slr1830, an effect confirmed by the increase in PHA content in the mutant. Two of the potential substrates for LepB1 were confirmed by the accumulation of their unprocessed forms in the mutant and providing insights of the specificity of these two leader peptidases in *Synechocystis*. 

The functional assembly of phycocyanin is involved in multiple electron transfer pathways regulating photosynthesis in Synechocystis sp. PCC 6803

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Phycocyanin is an important component of phycobilisomes, which are responsible for light-harvesting in cyanobacteria. The covalent attachment of phycocyanobilin to phycocyanin is catalyzed by phycocyanin lyase. The photosynthetic properties and protein complexes involved in photosynthetic electron transfer were characterized in wild type and mutants defective in holophycocyanin accumulation either through inactivation of phycocyanin α-subunit lyase (Δsll1051) or disruption of the phycocyanin operon (CK). Due to the lack of functional phycocyanin, both mutants have lower variable fluorescence yield, indicating less electron input from PS II to the plastoquinone (PQ) pool. The P700\(^{+}\) re-reduction rate was slower in Δsll1051 mutant but faster in CK than that of WT, indicating that fewer electrons cycled back to the PQ pool in Δsll1051 and vice versa in CK. This result corresponded to the amount of NDH-1 complexes that accumulated. The NDH-1 complex has been shown to be important to cyclic electron transfer in cyanobacteria. Further analysis of the different forms of NDH-1 complexes by blue-native PAGE demonstrated that, when compared to wild type, Δsll1051 has the same amount of NDH-1L but much lower amounts of NDH-1M and NDH-1S, an inducible component of the CO\(_2\)-concentrating mechanism (CCM). When compared to wild type, CK has more NDH-1L but similar amounts of NDH-1M and NDH-1S. The phycobilisome linker polypeptide CpcD is responsible for attachment of ferredoxin-NADP\(^{+}\) oxidoreductase large form (FNRL\(_{L}\)) to the thylakoid membrane. FNRL amounts were detected by immunoblots. When compared to wild type, CK has less total FNRL and substantial proteolysis of FNRL\(_{L}\), whereas Δsll1051 has normal amounts of FNRL. Impaired FNRL in CK indicates a deficiency of reducing equivalent production, which subsequently affects cellular respiration, carbon fixation and other metabolic pathways. These data suggest that proper assembly of phycocyanin has multiple impacts on regulation of photosynthesis via light absorption, redox state of the PQ pool, cyclic electron flow and the CCM.

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Vipp1 is required for the biogenesis of Photosystem I rather than thylakoid membranes in *Synechococcus* sp. PCC 7002

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The mechanism of biogenesis of thylakoid membranes in cyanobacteria is presently not understood, but the product of the *vipp1* gene (vesicle-inducing protein in plastids 1) has been suggested to play an important role in this process. Previous studies in *Synechocystis* sp. PCC 6803 reported that the *vipp1* (sll0617) gene could not be fully deleted and thus was essential (1, 2). We show here that the *vipp1* (SynPCC7002_A0294) product is not essential in the cyanobacterium *Synechococcus* sp. PCC 7002 by constructing a fully segregated *vipp1* null mutant. Spectroscopic studies show that Photosystem I (PS I) was undetectable in the *vipp1* mutant, but Photosystem II (PS II) complexes were still assembled and were active. Thylakoid membranes were still observed by electron microscopy in the *vipp1* mutant, and very similar, vestigial thylakoid membranes were observed in a *psaAB* mutant that lacks PS I. When the *vipp1* mutant strain was complemented with an orthologous *vipp1* gene from *Synechocystis* sp. PCC 6803 that was expressed from the strong *PcpcBA* promoter, PS I content and activities were restored to normal levels, and cells produced thylakoids that were indistinguishable from those of wild type. Transcription profiling showed that *psaAB* transcripts were lower in abundance in a *vipp1* mutant. The results from this study show that vestigial thylakoids can be produced in the absence of Vipp1, but normal thylakoid biogenesis in *Synechococcus* sp. PCC 7002 requires the expression and biogenesis of PS I, which in turn requires Vipp1.

References:

Characterization of an uptake hydrogenase deletion mutant strain of
Cyanothece sp. PCC 7822

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Cyanothece sp. PCC7822 (Cyanothece 7822) is a unicellular diazotrophic cyanobacterium. Under N2-fixing conditions, nitrogenase converts nitrogen to NH4+, along with the important by product H2, which is normally reabsorbed by the uptake hydrogenase (HupLS). Hydrogen production and nitrogenase activity demonstrated circadian behavior during growth under 12L/12D cycle and N2-fixing conditions. Seven of the Cyanothece strains have been sequenced and we know that these organisms have a very versatile metabolism, including the ability to make a variety of different biofuels. However, we were unable to generate a useable genetic system in Cyanothece 51142, the most heavily studied system. However, we were successful in generating knock-out mutants in Cyanothece 7822 using a single-stranded DNA technique [1]. We have also been able to complement such a knock-out strain using the same method.

The mutant ΔhupL (the large subunit of the uptake hydrogenase) was constructed by inserting a neomycin/kanamycin (Nm/Km) antibiotic cassette in the gene. We studied the impact of the ΔhupL mutant under conditions in which cells were transferred from nitrogen-replete to nitrogen-deprived conditions. The uptake hydrogenase activity was absent, as expected. However, we determined that the nitrogenase was also inactive and we detected almost no hydrogen production. These results were quite different from the uptake hydrogenase mutants studied in the filamentous cyanobacteria strains [2]. The mutant cells are more circular in morphology and about 1.7-fold larger in volume relative to the wild type cells, which are oval and longer in BG11 medium. In order to study the expression of hydrogenase and nitrogenase, semi-quantitative RT-PCR was performed on the WT, mutant, and the complemented strains under the nitrate step-down condition. The hupL complementation strain demonstrated both the recovery of nitrogenase activity and H2 production equivalent to those of the WT, suggesting that the initial mutant was in a single gene (hupL). It was suggested that HupLS activity may have four beneficial functions for the organism: (a) it minimizes the loss of ATP in H2-formation by nitrogenase, since ATP is regained in respiration with H2 as electron donor; (b) it removes O2 by the respiratory “Knallgas” reaction and protects nitrogenase against; (c) it prevents a deleterious build-up of a high concentration of H2 in cells such as heterocysts (H2 in high concentrations affects N2-fixation); (d) it provides additional reductant to photosynthetic and respiratory processes. [3]. We suggest that, in Cyanothece 7822, the uptake hydrogenase plays a particularly important role in the removal of O2 from the nitrogenase, especially under our current cultivation conditions, which have resulted in the production of less exopolysaccharide to exclude oxygen from the cytoplasm. Thus, the removal of HupLS has an immediate effect on nitrogenase activity for aerobically grown cells. Research supported by a grant from US DOE Genomics:GTL program.

Genetic Transformation of Anabaena cylindrica ATCC 29414

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Unlike Anabaena sp. PCC 7120, whose vegetative cells can differentiate only into N₂-fixing heterocysts, vegetative cells of A. cylindrica can differentiate also into another distinct type of cell, the spore-like akinete. Akinetes develop within the same filament, and normally adjacent to heterocysts. We report the first successful transformation of A. cylindrica. Further development of transformation of A. cylindrica should permit genetic analysis of akinetes and of their juxtaposition to heterocysts.

The gene acaK is a homolog of the previously identified akinete marker gene, avaK (Zhou & Wolk, 2002. J. Bacteriol. 184: 2529-32). acaK and its upstream region were jointly PCR-amplified from A. cylindrica genomic DNA. The 1.5-kb PCR product was fused to a promoter-less gfp gene in pAM1956 (Wu et al., 2004. J Bacteriol. 186: 6422-9), a derivative of Nostoc plasmid pDU1. The resulting construct, pZR963, was transferred into A. cylindrica ATCC 29414 by conjugation. Transformants were selected on AAN medium supplemented with 25 µg kanamycin ml⁻¹. Five colonies observed in a Petri dish were confirmed as exconjugants by colony PCR. In these transformants, akinetes formed earlier, and in greater abundance, than in the wild-type strain in medium AA/8 with and without fixed nitrogen. GFP-based fluorescence from PacaK–gfp originated primarily in mature akinetes or developing akinetes. To further study the role of AcaK in akinete formation, we are trying to inactivate acaK in A. cylindrica and have made a Pnir–acaK construction (Pnir, a nitrate-inducible promoter) in a pDU1-based plasmid and are transferring it to A. cylindrica to determine whether nitrate can induce akinete formation.
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