Background on S. elongatus PCC 7942

## Synechococcus elongatus Circadian Cycles & Metabolism

Cyanobacteria are photoautotrophs, meaning that they are dependent on light energy for carbon fixation and growth. Because of this fact, cyanobacteria have evolved a complex circadian clock which oscillations modify the in response to the challenges of low light periods. S. elgonatu PCC 7942 is a model strain for the study of circadian rhythms and with research elucidating the complex regulatory patterns that PCC 7942 has developed to cope with periods of light starvation and the resulting physiological stress. This complex cascade of regulatory interactions begins with the KaiBC circadian proteins, which transmit cell state information to the master regulator RpaA [1, 2] which in turn interactions with a secondary master circadian regulator RpaB [3] binding a large number of downstream global regulators dominated by sigma factors, triggering a complex cascade of regulatory action [4]. This regulatory response to darkness downregulates DNA replication [5, 6] photosystems, and modulates core metabolism from performing CO2 fixation and glycogen synthesis to utilizing stored glucose via the oxidative pentose phosphate pathway [7].

Diagram

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Figure 1. Overview of the photosynthesis mechanisms of *S. elongatus* PCC 7942. The primary electron transport flow is annotated in green, cyclical electron flow is annotated in red – this alternative pathway is used as a mechanism for handling reducing overload.

### Photosynthesis, aerobic respiration, & ROS response

Cyanobacteria acquire energy via photosynthesis. The cytoplasmic membrane contains a respiratory chain but separates photosynthetic complexes in the thylakoid. The first step of photosynthesis is accomplished by photosystem II (PSII), a multimeric protein composed of 19 subunits. Of these subunits the D1/D2 subunits compose the reaction center which bind several cofactors [8, 9] enabling the oxidization of water and reduction of the terminal electron acceptor plastoquinone (PQ). The reaction center proteins are encoded by genes psbA1 (three copies) and PsbD1 (two copies). Where it has been shown that under normal light conditions protein D1:1 is constitutively expressed, but in response to photoinhibition, alternative form D1:2 with 25% higher quantum yield [10] replaces D1:1 allowing the bacteria to overcome photoinhibitory damage to PSII [11]. Closely associated with the D1/D2 reaction center are two chlorophyll-containing proteins CP42 (psbC) and CP47 (PsbB). Further the subunit cytochrome b599 encoded by PsbE and PsbF has been implicated in secondary electron transfer pathways as a mechanism of protecting PSII against photoinhibition [12]. While other proteins subunits are involved their role is less characterized (psbH MNO TU IJKLVWX). After reduction of PQ by PII, electrons are transported from the PQ pool to the cytochrome b6f complex, a protein composed of four core subunits; cytochrome f (PetA), cytochrome b6 (PetB), an iron-sulfur protein (PetC) and subunit IV (PetD). With small subunits consisting of PetG, PetM, and PetN. Here the role of the cytochrome is to transport electrons to the luminal side of the thylakoid membrane to a soluble electron carrier in the form of plastocyanin [13]. Photosystem I (PS I) then catalyzes a light driven electron transfer from plastocyanin to ferredoxin or flavodoxin in iron limited conditions [14] on the stromal side of the thylakoid membrane. PSI can exist in a trimeric or monomer form where the trimeric form has been shown to be prominent under conditions of low light intensity [15]. The core protein subunits of PS I are two homologues PsaA and PsaB which contain the majority of antenna chorophylls [14]. The final step in the photosynthesis electron transport chain is accomplished by ferredoxin-NADP oxidoreductase (petH) which catalyzes the electron transport from a reduced ferredoxin/flavodoxin to NADP+ producing NADPH at the terminal stage of the electron transport chain. The proton gradient driven by the catalysis of water by PII is consequently used to generate ATP via the F0F1 ATP synthase where ATP synthesis is catalyzed by the F1 complex (AtpH, AtpG, AtpC, AtpD, AtpA) linked to the transmembrane movement of protons catalyzed by the F0 complex (AtpB, AtpF2, AtpF, AtpE). A second pathway, cyclic electron flow, bypasses PII and utilizes electrons from PS I to reduce ferrodoxin as in regular photosynthesis, but instead transfers electrons to NDH-1 which is responsible for transferring electrons through ferrodixin back into the plastoquinone pool.

Reactive oxygen species (ROS), including singlet oxygen (1O2), superoxide anions (O2-), hydrogen peroxide (H2O2), and hydroxyl radicals (OH-), are byproducts of photosynthesis and aerobic respiration [16]. Effective scavenging of these products is essential to prevent oxidative damage to lipds proteins and DNA [17] yet at basal levels ROS are essential and function as signaling molecules [18]. To manage ROS, bacteria have evolved complex mechanisms. In S. elongatus PCC 7942 several enzymes serve this role including the bifunctional catalase and peroxiredoxins, acting on H2O2 and a broad range of peroxides (ROOH) respectively [16]. In heterotrophic bacteria, the activation of ROS scavenging enzymes is activated by OxyR in E. Coli [19] and PerR in B. subtillus [20]. While no homologous of OxyR have been identified, PerR homologous have been found to regulate some genes responsible for ROS response in Synechostitis 6803 [21, 22], however PerR is not believed to be the master regulator in the process, not activing all genes responsible for ROS scavenging [16]. Hikk33 was another regulator shown to respond to H2O2 stress, but again did not activate all responsible genes. Three other Hiks were shown to respond to H2O2 including Hik16, 34, and 41 [23].

### Carbon Metabolism

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Figure 2. During the daytime CO2 is assimilated via the Calvin-Benson cycle. To avoid less efficient photorespiration, cyano have evolved a CO2 concentrating mechanisms in the form of the carboxysome which transport bicarbonate across the membrane and transformed into CO2 internally to form a high CO2 concentration environment which then react with Rubsico fixing CO2 into G3P. ATP and NADPH are next used to reduce GAP which can further be assimilated into glycogen stores. In the nighttime glycogen stores are then degraded to form intermediates G6P which can be used to generate reducing power in the oxidative phosphate pathway important for managing ROS stress produced via aerobic respiration. Sucrose can also be formed from the degradation products glycogen. Sucrose is an important osmolyte expressed in the presence of salt stress to balance the osmatic pressure in the cell.

Cyanobacteria acquire inorganic carbon in the form of CO2 and bicarbonate (HCO3-). Cyanobacteria have evolved mechanisms for concentrating CO2 through a concentrating mechanism (CCM) to provide an elevated CO2 concentration around the primary CO2 fixing enzyme Rubisco which is encapsulated in micro-compartments within the cell known as the carboxysome [24]. At least four mechanisms are responsible for the transport of CO2 and bicarbonate, two of which are constitutive and the other two being inducible under carbon limited growth. The final result regardless is the accumulation of bicarbonate in the cell. Bicarbonate can then be transported into the carboxysome where carbonic anhydrase catalyzes to conversion of bicarbonate to CO2 to match the rate of CO2 fixation. Under normal conditions (2-5% CO2) constitutive transport of CO2 is accomplished via the NDH-1 complex encoded by ndhF4-chpX and ndhD4 while bicarbonate is acquired via an Na+ dependent BicA [25, 26]. Under low CO2 conditions (20-50 ppm CO2) a high affinity NDH-1 complex encoded by the ndhF3-ndhD3-chpY operon expressed for the uptake of CO2 while the BCT1 ABC transporter encoded by cmpABCD and Sbt which encodes an Na+ transporter is responsible for the uptake of bicarbonate under limiting conditions [25]. Carbon limited conditions is accompanied by increased Rubisco activity [27] and increase in carboxysome content [28, 29]. The carboxysome protein complex is formed by the proteins cccmO, CcmL, CcmP, ccmK2, ccmK3 and ccmK while McdAB system is responsible to positioning carboxysomes [30]. The LysR family TF cmpR is responsible for activating the genes responsible for high affinity bicarbonate transport [25, 31] while another LysR family TF ndhR is responsible for repressing high affinity uptake targeting, Sbt and the high affinity NDH-1 in Synechocystis PCC 6803 [25].

After CO2 fixation, the Calvin Benson cycle continues to the reduction and regeneration stage in which 1 mol of glyceraldehyde 3-phosphate (G3P) is produced for every three mol CO2. The Calvin Benson. Cycle (CBBC) is tightly regulated on multiple levels. At the transcriptional level the LysR family TF rbcR is responsible for the activation of CBB genes in Synechocystis PCC 6803 [25]. At the protein level CBBC is regulated by the master regulator CP12, a redox-sensitive protein which is bound to Gap2 and Prk in night cycles, inhibiting the CBBC until it is reduced under the resumption of light catalyzed reactions reactivating the CBBC cycle [7, 32, 33]. G3P is also an intermediate of the glycolysis/gluconeogenesis which can subsequently feed to glycogenesis, the process of storing glucose in the form of the glucose polymer glycogen. During daytime, glycogen is accumulated in preparation for night [7, 34] and as to assimilate excess reducing power under high light intensity conditions [16, 35] where mutation in glycogen biosynthesis genes glgA, glgC, or glgP would be detrimental to light dependent growth. During the night metabolism is shifts to degrade glycogen, is metabolized glucose via the oxidative pentose phosphate pathway where the priority is generating reducing power in the form of NADPH necessary for detoxification of ROS species that accumulated before transition to night and loss of ability to detoxify ROS when photosynthesis is not active [7, 36].

### Nitrogen metabolism

Diagram

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Figure 3. Nitrogen metabolism of S. elongatus PCC 7942. Ammonium is the primary form of metabolite which can be metabolized however cyanate as well as nitrate and nitrite can be transported into the cell and metabolized into amminum. the GS-GOGAT pathway is responsible for assimilating nitrogen into the metabolism via glutamate which can then be metabolized into a variety of amino acids products including glutathione which is reduced via NADPH as a mechanism of ROS reduction. Oxoglutarate is synthesized via the TCA cycle and acts as an important mechanism for regulation nitrogen metabolism by PII & the NtcA master nitrogen regulator which modulates expression of transporters and nitrogen assimilatory pathways.

*S. elongatus PCC 7942*  is capable of importing ammonium, nitrate, and CNO via the amt1/amtB, nrtABCD, and cynABD transporters respectively [37]. Ammonium serves as the preferential nitrogen source, with transcription of alternative nitrogen source transporters being downregulated by its presence [38, 39]. Alternative nitrogen sources are catalyzed to ammonium which can subsequently be utilized by the cell. Cyanate requires bicarbonate as a second substrate to catalyze a reaction which produces CO2 and ammonia catalyzed by cynate lyase (cynS) [40]. Nitrate reduction is accomplished in a two-step process, the reduction of nitrate to nitrite by the ferredoxin-nitrate reductase (narB) and the reduction of nitrate to ammonium by ferredoxin-nitrite reductase. In cyanobacteria ferredoxin is reduced by photosystem I and acts as the preferred electron donor with reduced flavodoxin acting as an alternate in iron limited conditions [41]. *S. elongatus PCC 7942* encodes two ammonium/methylammonium transporters, amt1 and amtB both controlled by NtcA with amtB being expressed under low nitrogen conditions [42, 43]. Ammonium can then be assimilated via the GS/GOGAT pathway in a two-step process catalyzed by glutamine synthetase and glutamate synthease with 2-oxoglutarate as a co-substrate. In *S. elongatus* 7942 2-oxoglutarate is formed as a byproduct of the TCA cycle and acts as a key signaling molecule in the cell for nitrogen metabolism where the TCA cycle is largely nonessential with the exception of the first three steps which form 2-oxoglutarate [44]. Under nitrogen limiting conditions 2-oxoglutarate increases which in turn modulates the functionality of several proteins involved in nitrogen metabolism [45, 46]. NtcA, the global transcriptional regulator of nitrogen metabolism is tightly bound to the activity of both PII-interacting protein X (PipX) and the P-II signal transduction protein (glnB) where NtcA must bind both 2-oxoglutarate and PipX to become activated. Under low 2-oxoglutarate concentration PII signal transduction proteins can further form complex with N-acetyl glutamate kinase [47, 48] and actively compete for the binding of PipX excluding access of NtcA [49]. When bound the PII-PiPX complex structure suggests the proteins play a further role in regulatory interactions. Utilizing a three-hybrid yeast assay Labella et al.,2016 co-precipitated the TF PlmA [50]. PlmA is a cyanobacterial specific subfamily of the GntR class of transcription factors. Associated with plasmid maintenance in *Anabaena* sp. Strain PCC 7120 and photosystem stoichiometry [51] Attempts to inactivate plmA confirm that the gene plays an essential role in *S. elongatus*. Further, western plot assays demonstrate abundance of the regulator independent of nitrogen regime suggesting the existence of a large PlmA regulon that is uncharacterized.

## Sucrose secretion & co-culture candidates

*S. elongatus PCC 7942*, is a freshwater cyanobacteria adapted to low ionic strength environments, under osmotic stress conditions – high salinity and drought [52] – PCC 7942 catalyzes two reactions to produce sucrose from CBB cycle intermediates via sucrose phosphate synthase (sps) and sucrose phosphate phosphatase (spp). Sucrose acts as a compatible solute in PCC 7942, balancing the osmolyte pressure inside and outside of the cell, sucrose is the sole compatible solute produced and at a concentration of 200 mM NaCl sucrose concentrations are raised 30-40 fold in the cell [53]. A sucrose secreting engineered strain of PCC 7942 was engineered by heterologous expression of a symporter of protons and sucrose (cscB) which exhibited increase biomass production rate in coordination with enhanced P II activity, carbon fixation, and chlorophyll content [54]. When explored for how this sucrose secreting strain interacts with heterotrophic partners via flux balance models it was shown that *S. elongatus* PCC 7942 was capable of supporting the growth of heterotrophic partners via the exchange of sucrose ad other metabolic intermediates including alcohols, organic acids, and amino acids [55].

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