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Redirecting carbon to bioproduction via a growth arrest switch in a sucrose-secreting cyanobacterium

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ABSTRACT

Cyanobacteria are microbes with high photosynthetic efficiencies, making them a promising target for the production of sustainable bioproducts directly from solar energy and carbon dioxide. The most common efforts to increase cyanobacterial bioproduction involve diverting cellular resources away from cellular biomass and towards a heterologous pathway, for example through nutrient starvation or knockout of genes in competing metabolic pathways. Here we show that an inducible cell growth arrest switch can be used to increase the partitioning of carbon to an engineered sucrose sink. Specifically, we show that overexpression of Regulator of Phycobilisome-Associated B (RpaB), an essential response regulator in *Synechococccous elongatus* PCC *7942*, allows for inducible arrest of cell growth and is associated with a > 2-fold higher specific productivity of a heterologous sucrose secretion pathway. Finally, we show that sucrose export can partially relieve photosynthetic feedback inhibition imposed by the RpaB dependent growth arrest, allowing sucrose-secreting strains to maintain higher photosynthetic efficiencies. This work provides a novel conceptual framework for improving photosynthetic productivity and cyanobacterial bioproduction.

1. Introduction

Cyanobacteria are photosynthetic organisms with promising potential for sustainable production of high-value and commodity bioproducts. Cyanobacteria capture solar energy and utilize it to fix carbon dioxide which may be incorporated into target metabolites. In comparison to plants and algae, cyanobacteria have higher photosynthetic efficiencies and are genetically tractable, making them excellent candidates for engineering strains that generate high-value products directly from solar energy and CO_2 [1]. Among cyanobacteria, the obligate phototrophic, freshwater species, *Synechococcus elongatus* PCC *7942* (hereafter *S. elongatus*) has a relatively well-developed molecular toolkit, which has facilitated the engineering of model cyanobacteria for the production of a large range of valuable compounds [2].

Two general strategies are routinely used to reroute cellular resources towards a target metabolic pathway in photosynthetic microbes: 1) increasing the withdrawal of core carbon intermediates by improving pathway stoichiometry and balancing the rate limiting enzymes [3–7], or; 2) restricting the flux of carbon to native metabolic pathways that compete for cellular resources [8–10]. The first strategy requires tailored engineering of enzyme activities and expression that is specific to a given pathway. By contrast, the latter approach can be applied to improve production of many end products in production strains. For example, the main carbon storage product, glycogen, often accounts for 10–20% of total fixed carbon in growing cyanobacteria [11], therefore, suppression of flux to this product can lead to redirection of these cellular resources to alternative natural or engineered metabolic sinks. Indeed, a genetic knockout of glucose-1 phosphate adenylyl transferase (*glgC*) creates glycogen deficient strains which have been shown to have improved production of numerous products (e.g. sucrose- [10], isobutanol- [8], limonene [9] and lactate [12]).

Aside from genetic mutation, another common strategy for restricting flux to endogenous carbon sinks is through the use of a culture medium that is limited for key nutrients required for cell growth

Abbreviations: RpaB, regulation of phycobilisome associated B; CscB, sucrose permease; G3P, glyceraldehyde 3-phosphate; PBS, phycobilisome; PC, plastocyanin; PQ, plastoquinone; SPS, sucrose phosphate synthase; ΦII, quantum yield of PSII; qP, photochemical quenching; IPTG, isopropyl β-D-1-thiogalactopyranoside; PET, photosynthetic electron transport; Theo, theophylline

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[13–16]. In both cyanobacteria and algae, perhaps the best-studied example of this is cultivation under nitrogen-limited conditions, which restricts cellular capacity to form key metabolites needed for protein synthesis and cell growth. This leads to global changes in the transcriptome and proteome, changes carbon partitioning, slows cellular growth rates, and encourages overall accumulation of products with a higher C:N ratio [17]. Such an approach has been routinely used to increase the accumulation of many products, including lipids, polyhydroxyalkanoates, alcohols, and hydrogen gas [18–22].

Yet, inhibiting metabolic sinks through pathway deletion or nutrient deprivation can create new problems that may limit their application at scale. For example, ΔglgC cells exhibit sensitivity to stress, including high light sensitivity, which can lead to unviable cultures in the long-term, presumed to be partially due to their inability to store excess light energy (source) in glycogen reserves (sink) [23,24]. Similarly, exposure to nutrient limitation can lead to a gradual decline in photosynthetic activity, efficiency, and cell viability [25,26]. Thus, this approach requires nutrient cycling to maintain the productivity of cultures for long-term culturing. Since liquid handling costs for typical continuous culture of cyanobacteria and algae are a significant barrier to commercialization [27,28], additional media cycling requirements might further exacerbate economic concerns of scaling cyanobacterial cultures.

Recently, overexpression of the sensor histidine protein kinase, regulator of phycobilisome-associated B (RpaB), was shown to lead to growth arrest [29]. RpaB is an essential two component response regulator that is phosphorylated through interactions with the environmentally-responsive sensor kinase, NblS. RpaB then transduces the signal in part by binding the promoter regions of downstream genes [30]. While many aspects of RpaB's signaling network remain to be elucidated, it is thought that NblS-RpaB activate an adaptive response in cyanobacteria under a variety of stress conditions, including high-light, salt-stress, and cold shock [31–33]. In *S. elongatus*, overexpression of RpaB arrests cell growth, decreases the cell length to width ratio [29] and suppresses the circadian rhythm [34]. Consistent with canonical response regulators, RpaB has a highly conserved residue (D56) in its receiver domain that can accept a phosphate group to regulate its function. Mutants mimicking a constitutively phosphorylated (D56E) or non-phosphorylatable (D56A) form of RpaB have been previously characterized [34,35]. Mutations to this residue in the endogenous copy of RpaB are lethal, while over-expression of RpaB-D56A or RpaB-D56E in a background containing a wildtype copy have also been shown to result in growth arrest [34–36].

We explored the utility of RpaB as a genetic switch that could suppress cell growth and thereby promote partitioning of carbon to heterologous production pathways. We characterized the effects of RpaB-dependent cell growth arrest in a production strain of *S. elongatus*, specifically the effect on photosynthetic activity and sucrose export. We show that modulation of RpaB activity not only allows for growth arrest in *S. elongatus*, but also increases carbon partitioning to an engineered metabolic sink (i.e. secreted sucrose). Finally, we observed that RpaB-overexpressing cells remained viable for many days, despite reduced growth rates. Although RpaB-expressing cultures exhibited decreased quantum yield of photosystem II (PSII), this diminished photosynthetic efficiency is partially rescued by relief of feedback inhibition when sucrose is exported. Altogether, we demonstrate a novel growth suppression switch that may be used to enhance specific productivity in cyanobacterial cultures.

2. Materials and methods

2.1. Strain construction

The constructs for expressing *sps* or *cscB* (*S. elongatus*$_{sps}$) were constructed as described previously [4]; see Fig. 1b for a cartoon schematic of this construct design. Briefly, *cscB* was cloned into the Neutral Site 3 (NS3) vector, and *sps* was cloned into Neutral Site 2 (NS2) [37,38]; both of these constructs are designed for homologous recombination into the genome and IPTG-inducible gene expression in *S. elongatus*. In order to construct an integrating vector that would allow for independent control of the translation of RpaB, we modified the Neutral Site 1 vector [37], integrating a promoter (Ptrc) and the theophylline riboswitch upstream of *rpaB*. The promoter elements were synthesized from IDT DNA (gBlocks), based on prior published designs [39], although it was designed to mutate the *lacO* site (Fig. 1), thereby abolishing the ability of LacI to bind within the *rpaB* promoter element. The *rpaB* was cloned from *S. elongatus* genomic DNA. Complete plasmid maps and sequences are available from Addgene with the indicated part number: RpaB-Ox (Addgene #106932), RpaB-D56E (Addgene #106934) or RpaB-D56A (Addgene #106933), NS2-SPS (Addgene #80458).

2.2. Strain growth conditions

Synechococcus elongatus PCC 7942 was grown in a Multitron Infors HT Incubator with ~100 μmol photons $m^{-2} s^{-1}$ of light at 32 °C and supplemented with 2% CO_2. Illumination was provided from Sylvania 15 W Gro-Lux fluorescent bulbs. Cultures of 50 mL in 125 mL baffled flasks were agitated at 150 rpm in BG11 + 1 g/L HEPES at pH 8.3 adjusted with NaOH. During routine maintenance of cyanobacterial cultures, flasks were back-diluted daily to 0.3 OD_{750} for ≥2 days prior to initiation of an experiment – or to another target OD, as indicated in the text. Where appropriate, 1 mM IPTG or 500 uM theophylline was added to induce *cscB* and *sps* expression or RpaB translation, respectively. Antibiotics were used to select for transformants but not during experiments: chloramphenicol - 25 mg L^{-1}, kanamycin - 50 mg L^{-1}, spectinomycin - 50 mg L^{-1}. Antibiotics were removed prior to conducting any of the reported experiments to minimize any unintended effects.

2.3. Sucrose quantification

Sucrose production and export was triggered by addition of 1 mM IPTG, which induced *sps* and/or *cscB* expression. Cells were collected and pelleted (17,000 g) and the supernatant was recovered and analyzed using the Sucrose/D-Glucose Assay Kit (Megazyme: K-SUCGL). Cell numbers were quantified by flow cytometry, BD-Accuri (BD Biosciences), while cell density was evaluated by OD_{750nm} on a Genesys 20 photospectrometer (Thermo).

2.4. Protein quantification

Samples were collected at times described in the text by centrifugation at 13,000 rpm on a benchtop centrifuge to collect the cell pellet. Total protein was extracted in Laemmli buffer by boiling for 20 min. Protein quantification was performed with Coomassie Plus Protein Assay Reagent (Thermo Fisher Scientific No. 23236) according to the manual and equal protein concentrations were loaded for SPS-PAGE. Gels were stained with Coomassie stain and imaged. Quantification was performed with the Gel Analyzer plugin from ImageJ.

2.5. Fluorescence measurements

ΦII and qP measurements and calculations were performed using an Aquapen AP-C 100 (Photon Systems Instruments, Drasov, Czech Republic) using blue LEDs and the NPQ1 protocol as previously described [4]. 2 mL of dark adapted samples were normalized for chlorophyll content and were used for the measurement. All measurements are the average of at least 3 biological replicates ($n \geq 3$) unless noted.