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A carbon sink pathway increases carbon productivity in cyanobacteria

John W.K. Oliver, Shota Atsumi *

Department of Chemistry, University of California, Davis, One Shields Ave, Davis, CA 95616, USA

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ABSTRACT

The burning of fossil reserves, and subsequent release of carbon into the atmosphere is depleting the supply of carbon-based molecules used for synthetic materials including plastics, oils, medicines, and glues. To provide for future society, innovations are needed for the conversion of waste carbon (CO_2) into organic carbon useful for materials. Chemical production directly from photosynthesis is a nascent technology, with great promise for capture of CO_2 using sunlight. To improve low yields, it has been proposed that photosynthetic capacity can be increased by a relaxation of bottlenecks inherent to growth. The limits of carbon partitioning away from growth within the cell and the effect of partitioning on carbon fixation are not well known. Here we show that expressing genes in a pathway between carbon fixation and pyruvate increases partitioning to 2,3-butanediol (23BD) and leads to a 1.8-fold increase in total carbon yield in the cyanobacterium *Synechococcus elongatus* PCC 7942. Specific 2,3-butanediol production increases 2.4-fold. As partitioning increases beyond 30%, it leads to a steep decline in total carbon yield. The data suggests a local maximum for carbon partitioning from the Calvin Benson cycle that is scalable with light intensity.

1. Introduction

Worldwide, CO_2 emissions are rising while natural carbon sinks are reaching capacity (Hoekstra and Wiedmann, 2014). In 2012 alone, 5.4 trillion metric tons of CO_2 was released into the atmosphere from the oxidation of fixed carbon (DOE, 2014). Currently 99% of chemicals synthesized in the US originate from non-renewable sources (McFarlane and Robinson, 2007). Technologies for converting CO_2 back to organic carbon are still in development, and large gains will need to be made to balance carbon fixation with carbon consumption. To meet this need, interest has been growing in the engineering of photosynthetic organisms for carbon fixation and production of chemicals to replace petroleum derived compounds (Hays and Ducat, 2014; Oliver and Atsumi, 2014). Cyanobacterial cultures offer a direct process for capturing light and concentrated CO_2 into biomass, and can be installed in locations that do not compete with food for water and land resources. This includes locations that can remediate waste gases from electricity generation and industry, which account for 53% of CO_2 emissions in the US (EPA, 2014). A growing number of engineered pathways converting CO_2 into useful products have been demonstrated in cyanobacterial hosts, and tools for design and genetic manipulation are becoming diverse (Camsund and Lindblad, 2014; Markley et al., 2014; Nozzi et al., 2013). However, carbon production rates from CO_2 capture remain low. Increasing productivity from engineered cyanobacteria is critical to capturing emissions on a large scale.

Photosynthesis is most often considered as being limited by either light, CO_2, or RuBisCO (Ducat and Silver, 2012). In an applied setting for exhaust carbon remediation, CO_2 and incident light can be provided in excess. During peak daylight hours, photosynthetically active radiation (PAR) can be many times higher than what is utilized by the cell (Kirst et al., 2014; Robertson et al., 2011). Light consumption in cyanobacterial cells (measured by oxygen evolution) is limited by turnover of NADPH, and can be increased up to 3 times its natural rate by decoupling electron transfer from NADPH turnover (Iwaki et al., 2006). This has led to predictions that increasing the rate of NADPH consumption in vivo by the introduction of an exogenous pathway could lead to increases in photosynthetic rates (Ducat et al., 2012; Oliver et al., 2013). NADPH is primarily consumed in the Calvin–Benson (CB) cycle. Under these conditions, overexpression of RuBisCO has been demonstrated to increase carbon yields (Atsumi et al., 2009; Iwaki et al., 2006). However, RuBisCO in natural cells is already highly expressed, meaning that gains from overexpression come at a high metabolic cost of nitrogen and to translation burden. Additionally, overexpressed RuBisCO is localized outside of the carboxysome, which lowers efficiency, and may cause cellular stress (Iwaki et al., 2006). Another approach to increasing carbon fixation is based on the idea that carbon consuming reactions associated with growth are slower than carbon fixation, thereby providing excess cellular carbon that prevents any greater fixation. This

* Corresponding author.
E-mail address: satsumi@ucdavis.edu (S. Atsumi).

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bottleneck could be relieved by introduction of a 'carbon sink', a high flux pathway that converts carbon into a molecule that is secreted from the cell and is not consumed by central metabolism. Thus decoupling growth from the maximum turnover of the CB cycle reactions has potential to increase both carbon fixation and light capture.

In this work we aimed to create a carbon sink through expression of a pathway encompassing each step from fixation to chemical product. In all engineered cyanobacterial pathways to date, except for that of 1,2-propanediol expressing *mgsA* and mannitol expressing *mtlD*, the exogenous pathway has been separated by at least two steps that remain under native regulation (Jacobsen and Frigaard, 2014; Li and Liao, 2013; Oliver and Atsumi, 2014). This allows the cell to respond to perturbations that may affect the pool of CB metabolites (CBMs). The combination of a sucrose exporter and salt stress have been shown to cause native metabolism to produce large amounts of sucrose, with an apparent increase in carbon fixation (Ducat et al., 2012). It is desirable to be able to control such an effect and direct carbon flux to products from exogenous pathways not related to sugar metabolism. To achieve this, we chose the previously established 2,3-butanediol (23BD) pathway in *Synechococcus elongatus* PCC 7942 (hereafter *S. elongatus*) as a carbon sink. This pathway has a high driving force, and an apparent limitation by intracellular levels of pyruvate (Oliver et al., 2013, 2014). 23BD is a model 4 carbon feedstock chemical, valuable for production of plastics, fuels, and antifreeze (Ji et al., 2011).

Previous metabolic engineering in cyanobacteria has supported the idea that carbon partitioned to secreted products will approach carbon fixation rates associated with biomass production by concurrently reducing growth (Ducat et al., 2012; Oliver et al., 2013). In well characterized fermentative hosts, such as *Escherichia coli*, conversion of carbon has approached 100% of theoretical yield. However, differences in the paradigm of carbon flux between photosynthetic and fermentative cells make the vision of redirecting carbon problematic. A fermentative cell can convert glucose into carbon, ATP and NADPH, at a rate corresponding to protein and glucose concentration. In contrast a photosynthetic cell depends also on the concentration of CBMs for the fixation of carbon, and a large number of proteins and metabolites involved in the light reactions for production of ATP and NADPH. Each carbon in pyruvate produced by glycolysis has passaged through 8 reactions since entering the cell, while in photosynthesis each carbon has undergone at least 27 enzymatic transformations before reaching pyruvate. Expression of pathway enzymes in fermentative cells may cause an asymptotic approach to maximum carbon partitioning (until a very high level of expression causes cell burden). However, in a photosynthetic cell, because CBMs must be maintained in concentrations required for the CB cycle, too much carbon flux toward pyruvate may deplete the CBM pool and prevent the fixation of more carbon, rather than just slowing growth. In this way there will be a local maximum of carbon fixation corresponding to an optimum partitioning of carbon from the CB cycle. In this work, we demonstrate that this is the case.

2. Materials and methods

2.1. Reagents

Chemicals used for standards; (*R*,*R*)-2,3-butanediol, and acetoin were obtained from Sigma-Aldrich. Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Fisher Scientific. Phusion polymerase was purchased from New England Biolabs. Gentamicin was purchased from Teknova; Spectinomycin was purchased from MP Biomedicals; Kanamycin was purchased from IBI scientific. Oligonucleotides were synthesized by Eurofins.

2.2. Culture conditions

Unless otherwise specified, *S. elongatus* strains were cultured in BG-11 (Rippka et al., 1979) medium with the addition of 50 mM $NaHCO_3$. Cells were grown at 30 °C with rotary shaking (100 rpm) and light (55 μmol/s/m^2 photons in the PAR range) provided by four 86 cm 20 W fluorescent tubes 5 cm above the cell cultures. Cell growth was monitored by measuring OD_{730}. Light was measured using a PAR quantum flux meter (Model MQ-200, Apogee Instruments).

For acetoin and 23BD production in *S. elongatus*, cells in exponential phase were diluted to an OD_{730} of 0.2 in 25 mL production media (BG-11 medium including 50 mM $NaHCO_3$, 10 mg/L thiamine, and 10 mg/L gentamicin) in 125 mL baffled shake flasks. 20 mg/L Kanamycin was added as required. Cultures were induced with 1 mM IPTG. Every 24 h, 10% of the culture volume was removed, the pH was adjusted to 7.5 ± 0.4 with 10 N HCl, and volume was replaced with 2.5 mL of BG-11 containing 0.5 M $NaHCO_3$, achieving a final concentration of 50 mM $NaHCO_3$ in the culture.

For 23BD production in high light experiments, 25 mL cultures were shaken in 125 mL baffled flasks on clear polycarbonate above four 86 cm 20 W fluorescent tubes, providing ~250 μmol/s/m^2 photons in the PAR range. Every 12 h 5% of media was removed, the pH was adjusted to 7.5 ± 0.4 with 10 N HCl, and volume was replaced with of BG-11 containing 0.5 M $NaHCO_3$.

2.3. Plasmid construction

Plasmids used in this study are listed in Table S1. Plasmids were constructed using sequence and ligase independent cloning (SLIC) (Li and Elledge, 2007). Fragments containing 3-gene operons were constructed using ligation and integrated into plasmids by PCR amplification of the ligation product and cloning by SLIC. Primers used to amplify fragments are listed in Table S2. Templates used for DNA amplification and fragment pairing for plasmid design are listed in Table S3. Partial pathways were constructed under control of the $P_{L}lacO_1$ promoter. Each pathway was flanked with homologous regions for recombination with neutral site II (Andersson et al., 2000) (Fig. S2). Three gene pathways (*Eco* and *End*) were constructed by sequential recombination, under control of the P_{trc} promoter (Fig. S3).

2.4. Transformation of S. elongatus

Transformation of *S. elongatus* was performed as described (Golden et al., 1987). Complete chromosomal segregation for the introduced fragments was achieved through propagation of multiple generations on a selective agar plate and verified by colony PCR. Correct recombinants were confirmed by PCR to verify integration of targeting genes into the chromosome.

2.5. 23BD quantification

Supernatant samples from cultures were analyzed with gas chromatography (GC) (Shimadzu) equipped with a flame ionization detector and a Cyclodex-b column (30 m, 0.32-mm internal diameter, 0.25-mm film thickness; Agilent Technologies). Samples were prepared by mixing 9 parts supernatant with 1 part internal standard (1-Pentanol). For each analysis the GC oven temperature was held at 105 °C for 1 min, increased with a gradient of 20 °C/min until 225 °C, and held for 3 min. Ultra high purity Helium was used as the carrier gas. The temperature of the injector and detector were set at 250 °C. Peaks were identified by matching retention time to standards.