**2,3 Butanediol Production in an Obligate Photoautotrophic Cyanobacterium in
Dark Conditions via Diverse Sugar Consumption**

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Abstract

Cyanobacteria are under investigation as a means to utilize light energy to directly recycle CO₂ into chemical compounds currently derived from petroleum. Any large-scale photosynthetic production scheme must rely on natural sunlight for energy, thereby limiting production time to only lighted hours during the day. Here, an obligate photoautotrophic cyanobacterium was engineered for enhanced production of 2,3-butanediol (23BD) in continuous light, 12h:12h light-dark diurnal, and continuous dark conditions via supplementation with glucose or xylose. This study achieved 23BD production under diurnal conditions comparable to production under continuous light conditions. The maximum 23BD titer was 3.0 g·L⁻¹ in 10 days. Also achieving chemical production under dark conditions, this work enhances the feasibility of using cyanobacteria as industrial chemical-producing microbes.

Keywords: Cyanobacteria, Metabolic Engineering, Obligate Photoautotrophy, 2,3-Butanediol

1. Introduction

The ever increasing consumption of petroleum products has led to an increase in CO₂ emissions and growing concern regarding ecological and human health (Pinkerton and Rom, 2014; Schmidt and Archer, 2009). The utilization of photosynthetic microbes as a means to directly convert CO₂ to fuels and chemicals is gaining popularity as a renewable alternative to petroleum based chemicals. Unlike renewable production strategies derived from plants such as corn, microbial chemical production does not require arable land and so does not compete with food crops (Berla et al., 2013; Hays and Ducat, 2015; Oliver and Atsumi, 2014). Cyanobacteria, ubiquitous photosynthetic microorganisms, are primary producers of biomass in many ecosystems (Díez and Ininbergs, 2014) and have been engineered for the production of valuable fuels and chemicals directly from CO₂ (Machado and Atsumi, 2012). Owing to their genetic tractability, a variety of heterologous pathways for the production of useful chemicals have been integrated into cyanobacteria (Desai and Atsumi, 2013) including: alcohols (Atsumi et al., 2009; Deng and Coleman, 1999), acids (Liu et al., 2011), alkanes (Schirmer et al., 2010), alkenes (Guerrero et al., 2012; Takahama et al., 2003; Ungerer et al., 2012), diols (Oliver et al., 2013), isoprenoids (Lindberg et al., 2010), and esters (Kaiser et al., 2013).

A variety of cyanobacterial strains have been utilized as microbial cell factories, but the obligate photoautotrophic organism *Synechococcus elongatus* PCC 7942 (*S. elongatus*) has been engineered to produce various chemicals at higher titers and productivities relative to other cyanobacteria (Atsumi et al., 2009; Ducat et al., 2012; Oliver et al., 2013). However, nearly all cyanobacterial production studies have

49 exclusively used continuous lighting in laboratory conditions when measuring
50 productivity. Natural sunlight is freely available for any photo-dependent chemical
51 platform and **would** aid the commercial viability of production. **However**, natural lighting
52 includes periods of darkness **during which** cyanobacteria naturally stop growing, fixing
53 carbon, and producing biochemicals. This decreases the production time for many
54 otherwise highly productive phototrophic organisms to 8-14 hours per day, depending
55 on geographic location. While many heterotrophs sense light (Elias-Arnanz et al., 2011),
56 the growth and maintenance of photoautotrophs depends solely on light, and thus these
57 organisms have developed several sophisticated sense/response and metabolic
58 regulatory mechanisms (Diamond et al., 2015; Gan et al., 2014) including: global
59 transcription regulation based on circadian rhythms (Vijayan et al., 2009), light sensitive
60 cyanobacterialchrome activation (Hirose et al., 2013), and quorum sensing pathways
61 (Sharif et al., 2008). Of these mechanisms, circadian-rhythm based regulation has been
62 extensively studied in *S. elongatus*. All cyanobacteria exhibit this fundamental three-
63 protein clock system wherein the auto-phosphorylation state of the complex induces
64 signaling cascades (Cohen and Golden, 2015). This system has profound **e**ffects on all
65 facets of gene regulation (Ito et al., 2009) by interacting with global regulators (Markson
66 et al., 2013), sigma factors (Nair et al., 2002), and topological genome features (Vijayan
67 et al., 2009). These complex systems make **large-scale** modification of the
68 photoautotrophic cellular environment difficult to predict and engineer. The diversity of
69 the metabolic capabilities of photoautotrophic cyanobacteria is not completely
70 understood, as sophisticated engineering tools for cyanobacteria are still being
71 established (Berla et al., 2013; Heidorn et al., 2011; Nozzi et al., 2013).

One possible solution for improving chemical productivity in cyanobacteria is to use a photoheterotrophic organism supplemented with a fixed carbon feedstock. This approach may contribute to additional complexity in a phototrophic production facility and reintroduce common issues with traditional fermentative production schemes, such as increased probability for microbial contamination. However, efficient photobioreactor engineering and design is nascent, and substantial increases in productivity may compensate for the additional limitations. Recently, the facultative photoheterotrophic cyanobacterium, *Synechocystis* sp. PCC 6803 (*Synechocystis*), has been engineered for sugar-supplemented production of chemicals including isobutanol and lactic acid (Varman et al., 2013a; Varman et al., 2013b). The addition of glucose to *Synechocystis* production increased isobutanol titer from 90 mg·L⁻¹ to 114 mg·L⁻¹. However, in the isobutanol production strain, glucose consumption was reduced 65-80%, disabling effective conversion to product. In lactic acid production, glucose supplementation resulted in no improvement in productivity or titer. More recently, *Synechocystis* has been engineered to consume xylose in addition to the native substrate, glucose, for augmentation of heterologous ethylene production (Lee et al., 2015). This strain showed increased growth in heterotrophic (dark) conditions as well as up to 1.6 fold increase in mixotrophic (lighted) ethylene production compared to strictly photoautotrophic growth and production. However, titers presented in that study range between 3 mg·L⁻¹ and 15 mg·L⁻¹, falling behind previous engineering efforts in *S. elongatus* (Atsumi et al., 2009; Ducat et al., 2012; Oliver et al., 2013).

Optimizing sugar consumption by overcoming native regulation in natural photoheterotrophs may prove difficult. Such limitations may be avoided by rewiring the

metabolism of an obligate photoautotroph, an organism strictly reliant on light and CO₂ for growth (Fig. 1). We have previously shown that one of the causes of obligate photoautotrophy in *S. elongatus* is the poor uptake of extracellular fixed carbon sources through native membrane transporters (McEwen et al., 2013). *S. elongatus* can be rendered photoheterotrophic by heterologous expression of the glucose transporter gene *galP* or the xylose degradation genes *xylEAB* from *Escherichia coli*. However, the sugar consumption of these strains lagged behind natural photoheterotrophic microorganisms (McEwen et al., 2013). The two-fold goal of this study was the improvement of sugar consumption of the engineered *S. elongatus* strains, and the utilization of that consumption for the production of the valuable chemical 2,3-butanediol (23BD).

2. Materials and Methods

2.1. Reagents

Glucose, xylose, 23BD, and isobutanol were obtained from Sigma-Aldrich (St. Louis, MO). ¹³C labeled glucose was obtained from Cambridge isotope laboratories (Andover, MA). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Fischer Scientific (Hanover Park, IL). Phusion polymerase was purchased from New England Biolabs (Ipswich, MA). Gentamicin was purchased from Teknova (Hollister, CA); Spectinomycin was purchased from MP Biomedicals (Santa Ana, CA).

2.2. Strain construction

Strains used in this study are listed in Table 1. Transformation of *S. elongatus* was carried out as described (Golden et al., 1987). Transformants were selected on BG11

agar plates supplemented with the required antibiotics. Complete chromosomal segregation for the introduced fragments was achieved through propagation of multiple generations on a selective agar plate and verification by colony PCR. Correct recombinants were confirmed by PCR and sequencing of the purified genomic DNA in order to verify integration of targeting genes into the chromosome.

2.3. Culture conditions

Unless otherwise specified, all *S. elongatus* strains were cultured in BG11 medium at 30°C with rotary shaking (100 rpm) and light ($65 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in the PAR range) provided by four 86 cm 20 W fluorescent tubes 5 cm above the cell cultures whenever lights were on. Light intensity was measured using a PAR quantum flux meter (Model MQ-200, Apogee Instruments). For 23BD production experiments, cells were diluted at time = 0 to an OD_{730} of 0.2 except for high cell density experiments ($\text{OD}_{730} = \sim 5$) and grown in 10 mL of production media (BG11 containing 20 mM HEPES (pH 7.0), 0.1 mM IPTG, 10 mM NaHCO_3 , $10 \text{ mg}\cdot\text{L}^{-1}$ thiamine, $10 \text{ mg}\cdot\text{L}^{-1}$ gentamicin, and $20 \text{ mg}\cdot\text{L}^{-1}$ spectinomycin when necessary). During the production experiments, $5 \text{ g}\cdot\text{L}^{-1}$ of the appropriate sugar and 10 mM NaHCO_3 were added as required. Cell growth was indirectly monitored by measuring OD_{730} in a Microtek Synergy H1 plate reader (BioTek), under the assumption that OD_{730} correlates with cell biomass in the culture. Samples were diluted to between OD_{730} 0.2 and 0.9 for accurate cell density measurements.

2.4. 23BD production in continuous or diurnal lighting conditions

Cells were grown in 10 mL of production media in 20 mL glass tubes with air exchange caps. Every 12 h, 10% of the culture (1 mL) was removed, the pH was adjusted to 7.5 +/- 0.5 with 10N HCl, and 1 mL of production media containing 100 mM NaHCO_3 was

added. In the long term production experiments, when glucose was completely consumed (Day 4, 7, and 10), replacement production media containing 100 mM NaHCO₃ and 50 g·L⁻¹ glucose was used.

2.5. 23BD production in continuous dark conditions

Cells were inoculated into 25 mL of production media in 250 mL baffled glass flasks with air exchange caps, and grown in diurnal lighting conditions for 60 h. Subsequently, cultures were incubated in the dark by wrapping the flasks with aluminum foil. Every 24 h the cultures were placed in lighted conditions for 15 minutes. Then the 10% culture removal, pH adjustment, and addition of fresh medium were performed as described above.

2.6. Quantification of glucose and xylose

Glucose and xylose in culture supernatant were quantified using a high performance liquid chromatograph (LC-20AB, Shimadzu, Columbia, MD) equipped with an autosampler (SIL-20AC), Fast Acid Analysis Column (Biorad, Hercules, CA, USA), and a differential refractive detector (RID-10A). Five mM H₂SO₄ served as the mobile phase at a flow rate of 0.6 mL min⁻¹ at 65°C for 15 minutes.

2.7. 23BD quantification

Culture supernatant samples were analyzed by a gas chromatograph (GC) (Shimadzu) equipped with a flame ionization detector and Cyclodex-b column (30 m, 0.32 mm internal diameter, 0.25 mm film thickness; Agilent Technologies). 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. The temperature of the injector and detector was 250°C. Isobutanol was used as an internal standard.

2.8. GC/MS analysis of ^{13}C labeled 23BD

Samples were analyzed by gas chromatography (GC)-mass spectrometry (MS) (Shimadzu) equipped with SHR5XLB column (30 m, 0.25 mm internal diameter, 0.25 μm film thickness; Restek) and a quadruple mass selective detector operated at 70 eV. For each analysis, the GC oven temperature was held at 40°C for 3 min, increased with a gradient of 45°C min⁻¹ until 300 °C. The temperature of the injector was set at 225°C. The ion source (electron ionization [EI]) temperature was set at 200°C. 23BD detection was performed using selective ion mode ($m/z = 45, 46$ and 47). Although the molecular mass of 23BD is 90 g·mol⁻¹, the signal of the molecular ion ($m/z = 90$) of 23BD is extremely low with our MS settings, and we were unable to improve the signal. Thus, we compared the peak at $m/z = 45$ with the peaks at $m/z = 46$ and 47 to determine the ^{13}C -labeling percentage of 23BD. These peaks correspond to fragmented ions consisting of two carbons. The peak at $m/z = 45$ is for ions with two ^{12}C carbons, while those at $m/z = 46$ and 47 are for ions with one and two- ^{13}C labeled carbons, respectively. The relative intensity in the total of the three peaks were determined as X , X_{+1} , and X_{+2} . Finally, ^{13}C -labeling percentage of 23BD was defined as “%_{glucose} = $(0.5 \times X_{+1} + X_{+2}) / (X + X_{+1} + X_{+2}) \times 100$. Assuming that all carbons of 23BD originate from either glucose or CO₂, %CO₂ was yielded by “%CO₂ = 100-%_{glucose}.”

3. Results

3.1. Effect of sugar supplementation under continuous light conditions

Strain 1, harboring 23BD production genes (*PLlacO1::alsS-alsD-adh*), and

Strain 2, harboring both 23BD production and glucose transporter genes

(*PLlacO1::alsS-alsD-adh*, *P_{trc}::galP*) were cultivated in the presence and absence of glucose in 10 mL culture tubes (**Table 1 & Fig. 2**). **Strain 2** did not exhibit any growth or production on glucose alone (without sodium bicarbonate), thus 10 mM sodium bicarbonate was added to all subsequent experiments. Concentrations of NaHCO_3 greater than 10 mM did not yield any improvement to growth or production. For **Strain 2**, final biomass accumulation as measured by OD_{730} and final 23BD titer ($95 \text{ mg}\cdot\text{L}^{-1}$) increased by 92% and 66% (from $57 \text{ mg}\cdot\text{L}^{-1}$), respectively, upon the addition of glucose (**Fig. 3a**). In contrast, the control strain, **Strain 1**, showed no significant difference in 23BD titer ($72 \text{ mg}\cdot\text{L}^{-1}$) with the addition of glucose. Using the same conditions we evaluated the effects of xylose on **Strain 1** and **Strain 3**, harboring the genes for 23BD production and xylose transport/degradation (*PLlacO1::alsS-alsD-adh*, *P_{trc}::xylE-xylA-xylB*) (**Table 1 & Fig. 2**). A similar trend was exhibited wherein an increase in 23BD titer with xylose addition was only apparent in **Strain 3**, increasing from $39 \text{ mg}\cdot\text{L}^{-1}$ to $75 \text{ mg}\cdot\text{L}^{-1}$ compared to no significant difference for **Strain 1** at $41 \text{ mg}\cdot\text{L}^{-1}$ (**Fig. 3b**).

3.2. 23BD production in high cell density conditions

In a large scale production scheme, maximizing cell density may be advantageous for more efficient chemical production. However, chemical production from very dense cultures of obligate photoautotrophic organisms at large scale may be difficult due to decreased availability of light per cell stemming from limited light penetration into the culture and mutual cell shading. Sugar supplementation in dense cultures may overcome the associated difficulties of high cell density phototrophic cultures. Therefore, the starting cell density of **Strains 1** and **2** was increased to $\text{OD}_{730} \sim 5$ ($\sim 1.1 \text{ gDCW}\cdot\text{L}^{-1}$), and tested in continuous lighting conditions for growth and 23BD

production. Upon the addition of glucose, the cell growth of **Strain 2** increased 93% and 23BD titer by 1,050% to a final titer of 1,003 mg·L⁻¹ (**Fig. 3c**). Glucose was completely consumed within 96 h, and the maximum consumption rate of glucose for **Strain 2** was 67 mg·L⁻¹·h⁻¹ while the maximum 23BD production rate was 25 mg·L⁻¹h⁻¹ (**Fig. 3c**). **Strain 1** showed negligible glucose consumption and no significant increase in final 23BD titer (192 mg·L⁻¹) or biomass accumulation (**Fig. 3c**). Analogous conditions were applied to **Strain 3** (**Fig. 3d**). The maximum increase of 23BD titer and biomass upon addition of xylose to **Strain 3** was 813% (to 1138 mg·L⁻¹) and 179%, respectively, while **Strain 1** showed no increase in titer (158 mg·L⁻¹) (**Fig. 3d**). The xylose consumption rate for **Strain 3** was 67 mg·L⁻¹·h⁻¹ and the maximum productivity was 27 mg·L⁻¹·h⁻¹ (**Fig. 3d**).

3.3. Effect of sugar supplementation under diurnal light conditions

To mimic natural sunlight conditions, diurnal lighting conditions were examined. Cells were cultivated in a 12h:12h light/dark (LD) cycle in the presence or absence of sugar (**Fig. 4**). Continuous 23BD production was observed only when glucose was added to **Strain 2**, while no difference could be measured with **Strain 1** (**Fig. 4a**). 23BD production in **Strain 3** also continued through the dark phase when xylose was added (**Fig. 4b**). Growth of **Strains 2** and **3** in the presence of glucose or xylose, respectively, tended to be slower in the dark than in the light. However, productivity was consistent for these strains regardless of lighting conditions (**Fig. 4**). For **Strain 1**, the dark phases impeded both growth and productivity (**Fig. 4**). The 23BD titer and biomass accumulation achieved by **Strain 2** increased with the addition of glucose by 202% and 149%, respectively (**Fig. 4a**). In the presence of xylose, **Strain 3** showed a 453%

improvement in titer and 184% improvement in cell growth (**Fig. 4b**). The maximum glucose consumption rate in a single 12 h period for **Strain 2** was $19 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ while the average consumption rate was $8 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$. The maximum xylose consumption rate in a single 12 h period for **Strain 3** was $28 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ while the average consumption rate was $11 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$. The 23BD productivities from **Strains 2** and **3** during the dark period ($2 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$) were comparable to those in the light (glucose; $3 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$, xylose; $2 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$). **Strain 1** showed no significant growth, sugar consumption, or 23BD production in the dark.

3.4. Effect of sugar supplementation under dark conditions

To evaluate the capability of **Strains 2** and **3** to grow and produce 23BD heterotrophically, continuous dark conditions were tested (**Fig. 5**). 23BD can theoretically be produced in continuous dark since both the carbon precursors and necessary reducing power (in the form of NADPH) may be generated by sugar catabolism. Light activated heterotrophic growth (LAHG) is a well characterized growth mode in *Synechocystis* wherein the strain is maintained in continuous dark conditions except for a 15 minutes light pulse every 24 hours (Anderson and McIntosh, 1991). This method of light pulsing was used to test the growth and 23BD production of **Strains 2** and **3** in the presence or absence of glucose or xylose. Cells were grown in a 12:12 LD cycle for the first 60 h, and then incubated in continuous dark for another 84 h, excepting the 15-minute light pulse every 24 h. When the appropriate sugar was added to **Strains 2** or **3**, significant increases of both biomass accumulation and 23BD concentration were observed and continued for the duration of the 84 h dark incubation (**Fig. 5**). **Strain 2** produced $155 \text{ mg}\cdot\text{L}^{-1}$ of 23BD in the dark with glucose (**Fig. 5a**), and

Strain 3 produced $183 \text{ mg}\cdot\text{L}^{-1}$ of 23BD in the dark with xylose (**Fig. 5b**). In continuous dark, neither **Strains 2** nor **3** grew or produced 23BD without added sugar, nor did **Strain 1** grow or produce 23BD regardless of the addition of sugar.

3.5. ^{13}C -labeled metabolites analysis

To determine the ratio of carbon from glucose and CO_2 in produced 23BD, ^{13}C -labeled metabolite analysis was applied to high density cultures incubated in continuous lighting conditions (**Fig. 6**). The isotopic composition of 23BD was determined by gas chromatography-mass spectrometry (GC-MS) after 120 h of production under continuous light with ^{13}C -labeled glucose. In **Strain 2**, 67% of carbons in 23BD contained ^{13}C , indicating that 67% of the carbons were from glucose and 33% were from CO_2 (**Fig. 6b**). In contrast, **Strain 1** metabolite analysis showed that 8% of the carbons in 23BD were from glucose and 92% were from CO_2 (**Fig. 6b**).

3.7. Long-term 23BD production experiments

The maximum possible chemical titer and the degree of sugar utilization are of great relevance to the industrial feasibility of cyanobacterial production platforms. Thus, a long-term, fed-batch scheme with or without glucose was applied to **Strain 2** in continuous light (**Fig. 7**). The experiment continued for as long as **Strain 2** exhibited an increase in total 23BD titer either with or without glucose. Production of 23BD was observed from **Strain 2** for 10 d. Glucose was replenished to $5 \text{ g}\cdot\text{L}^{-1}$ every 3-4 d, depending on when glucose had been depleted (**Fig. 7b**). The strain exhibited continuous growth throughout the 10 day experiment, and the maximum titer of 23BD was $3.0 \text{ g}\cdot\text{L}^{-1}$, with a maximum productivity of $22 \text{ mg}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ (**Fig. 7a&c**).

4. Discussion

In this study, we show that modification of a photoautotrophic cyanobacterium for sugar consumption leads to superior photoheterotrophic chemical productivity when compared to the naturally photoheterotrophic strain, *Synechocystis*. Recent metabolomic analysis of *Synechocystis* grown in the presence of glucose showed a decrease in Calvin Benson (CB) cycle metabolites and a reduction in carbon fixation (Narainsamy et al., 2013). This suggests that central carbon metabolism is strongly regulated by the presence of fixed carbon sources. This is supported by the decrease in glucose consumption and low reported productivities of isobutanol and lactic acid in engineered *Synechocystis* strains when in the presence of glucose (Varman et al., 2013a; Varman et al., 2013b). This is most likely owing to a variety of specialized natural regulatory elements including sigma factors and specific regulatory proteins influencing carbon metabolism that are absent in obligate photoautotrophic strains (Osanai et al., 2011). By engineering the naturally obligate photoautotroph *S. elongatus* for heterotrophic production, we show that glucose consumption may continue uninhibited for at least 10 d at a fairly constant rate, and that a significant portion of the carbons in the target chemical produced (67%) are from glucose.

The high cell density production studies capitalize on the key advantages of supplementing phototrophic organisms with sugar (**Fig. 3c&d**). The continued growth of **Strain 1** (without any sugar consuming modifications) at high cell density is limited by light penetration of the cell culture, while growth on sugar is only limited by diffusion into the cell interior. The facilitated import and metabolism of sugars by **Strains 2** (glucose) and **3** (xylose) provide a strong advantage in growth and production at high cell

densities by allowing a higher rate of carbon entry into metabolism, while simultaneously maintaining the phototrophic advantage of photosynthetically producing ATP and NADPH decoupled from carbon input.

Freely available, natural lighting (sunlight) conditions are diurnal in nature and of particular interest to industrial pursuits. In our study, the 23BD production from **Strains 2 and 3** grown with sugar increased in both light and dark periods compared to strains grown without sugar, leading to a 2 to 4 fold improvement in final titer (**Fig. 4**). Though the availability of reducing equivalences generated from photosynthesis decreases under diurnal conditions, the sugar-augmented strains were able to overcome that limitation and continue both growth and production in the dark phases. Continuous production in diurnal lighting with a strict photoautotroph demonstrates the broad versatility of genetic manipulation in this strain. However, it is unclear whether photo-dependent regulation is circumvented in the dark and carbon storage generated in light conditions is utilized for growth and production in these engineered photoheterotrophs, or if all carbon and reducing equivalences come from the consumption of sugar in dark conditions.

Carbon metabolism of externally supplied sugar in *S. elongatus* is unknown and of particular interest. For the naturally photoheterotrophic strain *Synechocystis*, sugar metabolism has been widely investigated under different trophic conditions (Nakajima et al., 2014; Takahashi et al., 2008; Yang et al., 2002). In the light, it was reported that glycolysis in *Synechocystis* was activated in the presence of glucose, while the oxidative pentose phosphate (OPP) pathway was induced in low light in response to the increased requirement for NADPH. Additionally, increased OPP pathway flux was

consistently observed to compensate in *Synechocystis* for the decreased NADPH supply in the dark. Diamond et al have suggested that for *S. elongatus*, the circadian clock regulator, KaiC, might be responsible for inhibiting the OPP pathway activity in the light, and for switching between an active CB cycle and an active OPP pathway during the light and dark phases, respectively, of diurnal conditions (Diamond et al., 2015). An understanding of carbon metabolism in our engineered photoheterotrophic *S. elongatus* strains would likely aid in strain and target chemical production optimization. However, additional studies are necessary to ascertain the mode of sugar degradation in these engineered strains since little is known about the relative metabolic flux between the CB, OPP, and Entner-Doudoroff (ED, closely linked to OPP) pathways in *S. elongatus* (Fig. 1).

Consumption of sugar in diurnal conditions for **Strains 2** and **3** averaged 44 $\mu\text{M}\cdot\text{h}^{-1}$ for glucose and 73 $\mu\text{M}\cdot\text{h}^{-1}$ for xylose, respectively, and allowed for significant increase in the total 23BD productivity, particularly in dark conditions. While each sugar equally contributed to an increase in 23BD production, the overall consumption of xylose was much greater than that of glucose in the light (**Fig. 3**). However, no significant difference of sugar consumption between glucose and xylose was observed during the dark phases. Further analyses are required to elucidate the detailed mechanisms that cause these differences in carbon consumption between glucose and xylose in a variety of light conditions.

The normalized 23BD titer for **Strain 2** was greater than for **Strain 1**, but no increase in carbons utilized from CO_2 was observed between the strains indicating that the increase in titer was due to the conversion of glucose to 23BD. To understand the

changes in metabolic flux between these strains, a detailed flux analysis is required. A variety of metabolomics approaches have been previously used to investigate carbon flux and carbon fixation in cyanobacteria (Narainsamy et al., 2013; Schwarz et al., 2011; Young et al., 2011), but none of these have included cyanobacteria engineered for photoheterotrophic chemical production. These systems level approaches may be well suited to examine the increased production abilities of the mutant strains engineered in this study (**Fig. 6**).

5. Conclusions

In this work we utilized an obligate photoautotrophic cyanobacterium rewired to act as a facultative photoheterotroph (McEwen et al., 2013), and examined how these additional carbon sources affect cell growth and 23BD production (Oliver et al., 2013) in a variety of lighting conditions. In previous work, continuous chemical production under diurnal conditions has not been successful with the well-characterized photoheterotrophic cyanobacterium *Synechocystis*. However, in this study we were able to achieve relatively efficient production both in dark and light conditions. We observed continuous production in diurnal cycles, demonstrating the utility of this engineering in natural lighting conditions. Utilizing sugar in mixed carbon source conditions, a maximum titer of $3.0 \text{ g}^{-1} \text{ L}^{-1}$ 23BD was achieved, which is comparable to that achieved under continuous light conditions. High sugar consumption and conversion to the target chemical product demonstrates the viability of engineering obligate photoautotrophic cyanobacteria to overcome some of the native limitations of facultative photoheterotrophic cyanobacteria to achieve efficient chemical production.

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References

- Anderson, S. L., McIntosh, L., 1991. Light-activated heterotrophic growth of the cyanobacterium *Synechocystis* sp. strain PCC 6803: a blue-light-requiring process. *J Bacteriol.* 173, 2761-2767.
- Atsumi, S., et al., 2009. Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat Biotechnol.* 27, 1177-80.
- Berla, B. M., et al., 2013. Synthetic biology of cyanobacteria: unique challenges and opportunities. *Front Microbiol.* 4, 246.
- Cohen, S. E., Golden, S. S., 2015. Circadian Rhythms in Cyanobacteria. *Microbiol Mol Biol Rev.* 79, 373-385.
- Deng, M. D., Coleman, J. R., 1999. Ethanol synthesis by genetic engineering in cyanobacteria. *Appl Environ Microbiol.* 65, 523-528.
- Desai, S. H., Atsumi, S., 2013. Photosynthetic approaches to chemical biotechnology. *Curr Opin Biotechnol.* 24, 1031-6.
- Diamond, S., et al., 2015. The circadian oscillator in *Synechococcus elongatus* controls metabolite partitioning during diurnal growth. *Proc Natl Acad Sci U S A.* 112, 1916-1925.
- Díez, B., Ininbergs, K., 2014. Ecological importance of cyanobacteria. In: Sharma, N. K., et al., Eds.), *Cyanobacteria*. John Wiley & Sons, Ltd, pp. 41-63.
- Ducat, D. C., et al., 2012. Rerouting carbon flux to enhance photosynthetic productivity. *Appl Environ Microbiol.* 78, 2660-2668.
- Elias-Arnanz, M., et al., 2011. Light-dependent gene regulation in nonphototrophic bacteria. *Curr Opin Microbiol.* 14, 128-135.

400 Gan, F., et al., 2014. Extensive remodeling of a cyanobacterial photosynthetic
 401 apparatus in far-red light. *Science*. 345, 1312-1317.
 402 Golden, S. S., et al., 1987. Genetic engineering of the cyanobacterial chromosome.
 403 *Methods Enzymol.* 153, 215-231.
 404 Guerrero, F., et al., 2012. Ethylene synthesis and regulated expression of recombinant
 405 protein in *Synechocystis* sp. PCC 6803. *PLoS One*. 7, e50470.
 406 Hays, S. G., Ducat, D. C., 2015. Engineering cyanobacteria as photosynthetic feedstock
 407 factories. *Photosynth Res.* 123, 285-95.
 408 Heidorn, T., et al., 2011. Synthetic biology in cyanobacteria engineering and analyzing
 409 novel functions. *Methods Enzymol.* 497, 539-579.
 410 Hirose, Y., et al., 2013. Green/red cyanobacteriochromes regulate complementary
 411 chromatic acclimation via a protochromic photocycle. *Proc Natl Acad Sci U S A*.
 412 110, 4974-4979.
 413 Ito, H., et al., 2009. Cyanobacterial daily life with Kai-based circadian and diurnal
 414 genome-wide transcriptional control in *Synechococcus elongatus*. *Proc Natl Acad*
 415 *Sci U S A*. 106, 14168-14173.
 416 Kaiser, B. K., et al., 2013. Fatty aldehydes in cyanobacteria are a metabolically flexible
 417 precursor for a diversity of biofuel products. *PLoS One*. 8, e58307.
 418 Lee, T. C., et al., 2015. Engineered xylose utilization enhances bio-products productivity
 419 in the cyanobacterium *Synechocystis* sp. PCC 6803. *Metab Eng.* 30, 179-189.
 420 Lindberg, P., et al., 2010. Engineering a platform for photosynthetic isoprene production
 421 in cyanobacteria, using *Synechocystis* as the model organism. *Metab Eng.* 12,
 422 70-79.

423 Liu, X., et al., 2011. Fatty acid production in genetically modified cyanobacteria. Proc
 424 Natl Acad Sci U S A. 108, 6899-6904.
 425 Machado, I. M., Atsumi, S., 2012. Cyanobacterial biofuel production. J Biotechnol. 162,
 426 50-56.
 427 Markson, J. S., et al., 2013. Circadian control of global gene expression by the
 428 cyanobacterial master regulator RpaA. Cell. 155, 1396-1408.
 429 McEwen, J. T., et al., 2013. Engineering *Synechococcus elongatus* PCC 7942 for
 430 continuous growth under diurnal conditions. Appl Environ Microbiol. 79, 1668-
 431 1675.
 432 Nair, U., et al., 2002. Roles for sigma factors in global circadian regulation of the
 433 cyanobacterial genome. J Bacteriol. 184, 3530-3538.
 434 Nakajima, T., et al., 2014. Integrated metabolic flux and omics analysis of
 435 *Synechocystis* sp. PCC 6803 under mixotrophic and photoheterotrophic
 436 conditions. Plant Cell Physiol. 55, 1605-1612.
 437 Narainsamy, K., et al., 2013. High performance analysis of the cyanobacterial
 438 metabolism via liquid chromatography coupled to a LTQ-Orbitrap mass
 439 spectrometer: evidence that glucose reprograms the whole carbon metabolism
 440 and triggers oxidative stress. Metabolomics. 9, 21-32.
 441 Nozzi, N. E., et al., 2013. Cyanobacteria as a platform for biofuel production. Front.
 442 Bioeng. Biotechnol. 1, 7.
 443 Oliver, J. W., Atsumi, S., 2014. Metabolic design for cyanobacterial chemical synthesis.
 444 Photosynth Res. 120, 249-261.

445 Oliver, J. W., et al., 2013. Cyanobacterial conversion of carbon dioxide to 2,3-butanediol.
 446 Proc Natl Acad Sci U S A. 110, 1249-1254.
 447 Osanai, T., et al., 2011. Genetic engineering of group 2 sigma factor SigE widely
 448 activates expressions of sugar catabolic genes in *Synechocystis* species PCC
 449 6803. J Biol Chem. 286, 30962-30971.
 450 Pinkerton, K. E., Rom, W. N., 2014. Global Climate Change and Public Health. Humana
 451 Press.
 452 Schirmer, A., et al., 2010. Microbial biosynthesis of alkanes. Science. 329, 559-562.
 453 Schmidt, G., Archer, D., 2009. Climate change: Too much of a bad thing. Nature. 458,
 454 1117-1118.
 455 Schwarz, D., et al., 2011. Metabolic and transcriptomic phenotyping of inorganic carbon
 456 acclimation in the Cyanobacterium *Synechococcus elongatus* PCC 7942. Plant
 457 Physiol. 155, 1640-1655.
 458 Sharif, D. I., et al., 2008. Quorum sensing in Cyanobacteria: N-octanoyl-homoserine
 459 lactone release and response, by the epilithic colonial cyanobacterium
 460 *Gloeotheca* PCC6909. ISME J. 2, 1171-1182.
 461 Somerville, C., 2006. Cellulose synthesis in higher plants. Annu Rev Cell Dev Biol. 22,
 462 53-78.
 463 Takahama, K., et al., 2003. Construction and analysis of a recombinant cyanobacterium
 464 expressing a chromosomally inserted gene for an ethylene-forming enzyme at
 465 the *psbAI* locus. J Biosci Bioeng. 95, 302-305.
 466 Takahashi, H., et al., 2008. Difference in metabolite levels between photoautotrophic
 467 and photomixotrophic cultures of *Synechocystis* sp. PCC 6803 examined by

468 capillary electrophoresis electrospray ionization mass spectrometry. J Exp Bot.
469 59, 3009-3018.

470 Ungerer, J., et al., 2012. Sustained photosynthetic conversion of CO₂ to ethylene in
471 recombinant cyanobacterium *Synechocystis* 6803. Energ Environ Sci. 5, 8998-
472 9006.

473 Varman, A. M., et al., 2013a. Metabolic engineering of *Synechocystis* sp. strain PCC
474 6803 for isobutanol production. Appl Environ Microbiol. 79, 908-914.

475 Varman, A. M., et al., 2013b. Photoautotrophic production of D-lactic acid in an
476 engineered cyanobacterium. Microb Cell Fact. 12, 117.

477 Vijayan, V., et al., 2009. Oscillations in supercoiling drive circadian gene expression in
478 cyanobacteria. Proc Natl Acad Sci U S A. 106, 22564-22568.

479 Yang, C., et al., 2002. Metabolic flux analysis in *Synechocystis* using isotope distribution
480 from ¹³C-labeled glucose. Metab Eng. 4, 202-216.

481 Young, J. D., et al., 2011. Mapping photoautotrophic metabolism with isotopically
482 nonstationary ¹³C flux analysis. Metab Eng. 13, 656-665.

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485 **Table 1 Strains and Plasmids used in this study**

		Genotype	Reference
Strains	Strain #		
AL757	1	NSIII: <i>lacI^q</i> ; <i>PLlacO1::alsS-alsD-adh</i> ; <i>gent^R</i>	(Oliver et al., 2013)
AL2243	2	1 + NSI: <i>lacI^q</i> ; <i>P_{trc}::galP</i> ; <i>spec^R</i>	This study
AL2244	3	1 + NSI: <i>lacI^q</i> ; <i>P_{trc}::xylE-xylA-xylB</i> ; <i>spec^R</i>	This study
Plasmids			
pAL40		NSI targeting vector, <i>lacI^q</i> ; <i>P_{trc}::galP</i> ; <i>spec^R</i>	(McEwen et al., 2013)
pAL70		NSI targeting vector, <i>lacI^q</i> ; <i>P_{trc}::xylE-xylA-xylB</i> ; <i>spec^R</i>	(McEwen et al., 2013)
pAL300		NSIII targeting vector, <i>lacI^q</i> ; <i>PLlacO1::alsS-alsD-adh</i> ; <i>gent^R</i>	(Oliver et al., 2013)

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Fig. 1. Overview of central metabolic pathway engineering. An abbreviated schematic of central metabolites of glycolysis, the Entner-Doudoroff (ED) pathway, the oxidative pentose phosphate (OPP) pathway and the Calvin-Bensen (CB) cycle are shown. Solid lines indicate native genes and dashed lines indicate heterologous genes necessary for the indicated pathway. Metabolite abbreviations are as follows: G6P (glucose-6-phosphate), F1,6BP (fructose-1,6-bisphosphate), 1,3BPG (1,3-bisphosphoglycerate), PEP (phosphoenolpyruvate), CO₂ (carbon dioxide), 6PGL (6-phosphogluconolactone), 6PG (6-phosphogluconate). Enzyme name and products are as follows: XI (xylose isomerase), XK (xylulose kinase), RuBisCo (ribulose-bisphosphate carboxylase), ALS (acetolactate synthase), ALDC (2-acetolactate decarboxylase), sADH (secondary alcohol decarboxylase).

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Fig. 2 Schematic of genome engineering in *S. elongatus*
(a) Schematic representation of the 23BD pathway genes integration into NSIII. (b) Schematic representation of the glucose or xylose transporter genes integration into NSI.

Fig. 3. Sugar utilization under continuous light conditions. Filled shapes indicate the presence of sugar, empty shapes indicate the absence of sugar. Error bars indicate the standard deviation of biological triplicates. (a) Time course for cell growth and production of **Strain 1** (*PLlacO1::alsS-alsD-adh*) and **Strain 2** (*PLlacO1::alsS-alsD-adh*, *Ptrc::galP*) with or without glucose grown from a starting cell density of $OD_{730} = 0.2$. (b) Time course for cell growth and production of **Strain 1** and **Strain 3** (*PLlacO1::alsS-alsD-adh*, *Ptrc::xylE-xylA-xylB*) with or without xylose from a starting cell density of $OD_{730} = 0.2$. (c) Time course for the cell growth, culture sugar concentration, and 23BD production of **Strain 1** and **Strain 2** with or without glucose from a starting cell density of $OD_{730} = 5.0$. (d) Time course for the cell growth, culture sugar concentration, and 23BD production of **Strain 1** and **Strain 3** with or without xylose from a starting cell density of $OD_{730} = 5.0$.

Fig. 4. Sugar utilization under diurnal (L/D) conditions. Cells were cultivated in 12 h light/12 h dark cycles. Grey shaded regions indicate periods of darkness. Filled shapes indicate the presence of sugar, and empty shapes indicate the absence of sugar. Error bars indicate the standard deviation of biological triplicates. (a) Cell growth, culture glucose concentration, and 23BD production over time for **Strain 1** (*PLlacO1::alsS-alsD-adh*) and **Strain 2** (*PLlacO1:: alsS-alsD-adh, Ptrc::galP*) from a starting cell density of OD₇₃₀ = 0.2. (b) Cell growth, culture xylose concentration, and 23BD production over time for **Strain 1** and **Strain 3** (*PLlacO1::alsS-alsD-adh, Ptrc::xylE-xylA-xylB*) from a starting cell density of OD₇₃₀ = 0.2.

Fig. 5. Sugar utilization under dark (D/D) conditions. Cells were cultivated in 12 h light/12 h dark cycles for the first 60 h, then transferred to complete darkness with the addition of a 15-minute light pulse once every 24 h. Grey shaded regions indicate periods of darkness. Filled shapes indicate the presence of sugar, and empty shapes indicate the absence of sugar. Error bars indicate the standard deviation of biological triplicates. (a) Cell growth and 23BD production over time for **Strain 1** (*PLlacO1::alsS-alsD-adh*) and **Strain 2** (*PLlacO1::alsS-alsD-adh, Ptrc::galP*) from a starting cell density of OD₇₃₀ = 0.2. (b) Cell growth and 23BD production over time for **Strain 1** and **Strain 3** (*PLlacO1::alsS-alsD-adh, Ptrc::xylE-xylA-xylB*) from a starting cell density of OD₇₃₀ = 0.2.

Fig. 6. ¹³C-labeled metabolite analysis. Completely ¹³C-labeled glucose was added to **Strain 1** (*PLlacO1::alsS-alsD-adh*), **Strain 2** (*PLlacO1::alsS-alsD-adh, P_{trc}::galP*). Cells were cultivated in continuous light for 120 h. Error bars indicate the standard deviation of biological triplicates. **(a)** Cell growth, culture glucose concentration, and 23BD production over time in continuous lighting conditions. **(b)** The percentage of 23BD produced from either glucose or CO₂ by each strain after 120 h where 100 equals the total 23BD production. **(c)** The total titer of 23BD after 120 h normalized by cell density as the sum of production from glucose and CO₂.

Fig. 7. Long term Production under continuous light conditions.

Strain 2 (*PLlacO1::alsS-alsD-adh*, *Ptrc::galP*) was subjected to long term production in continuous lighting conditions. Error bars indicate the standard deviation of biological triplicates. **Strain 2** (a) cell growth, (b) glucose concentration in culture supernatant, and (c) 23BD production over the course of 10 days. Glucose was replenished to 5 g·L⁻¹ on days 4, 7 and 10. Filled shapes indicate the presence of glucose and empty shapes indicate the absence of glucose.