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Original Research Article

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 CO_2 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, 12 h:12 h 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 d. Also achieving chemical production under dark conditions, this work enhances the feasibility of using cyanobacteria as industrial chemical-producing microbes.

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1. Introduction

The ever increasing consumption of petroleum products has led to an increase in CO_2 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 CO_2 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 CO_2 (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 exclusively used continuous lighting in laboratory conditions when measuring productivity. Natural sunlight is freely available for any photo-dependent chemical platform and would aid the commercial viability of production. However, natural lighting includes periods of darkness during which cyanobacteria naturally stop growing, fixing carbon, and producing biochemicals. This decreases the production time for many otherwise highly productive phototrophic organisms to 8–14 h per day, depending on geographic location. While many heterotrophs sense light (Elias-Arnanz et al., 2011), the growth and maintenance of photoautotrophs depends solely on light, and thus these organisms have developed several sophisticated sense/response and metabolic regulatory mechanisms (Diamond et al., 2015; Gan et al., 2014) including: global transcription regulation based on circadian rhythms (Vijayan et al., 2009), light sensitive cyanobacterialchrome activation (Hirose et al., 2013), and quorum sensing pathways (Sharif et al., 2008). Of these mechanisms, circadian-rhythm based regulation has been extensively studied in *S. elongatus*. All cyanobacteria exhibit this fundamental three-protein clock system wherein the auto-phosphorylation state of the complex induces signaling cascades (Cohen and Golden, 2015). This system has profound effects on all facets of gene regulation (Ito et al., 2009) by interacting with global regulators (Markson et

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al., 2013), sigma factors (Nair et al., 2002), and topological genome features (Vijayan et al., 2009). These complex systems make large-scale modification of the photoautotrophic cellular environment difficult to predict and engineer. The diversity of the metabolic capabilities of photoautotrophic cyanobacteria is not completely understood, as sophisticated engineering tools for cyanobacteria are still being 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, 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 photoheterotophs may prove difficult. Such limitations may be avoided by rewiring the metabolism of an obligate photoautotroph, an organism strictly reliant on light and CO_2 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). ^{13}C 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).

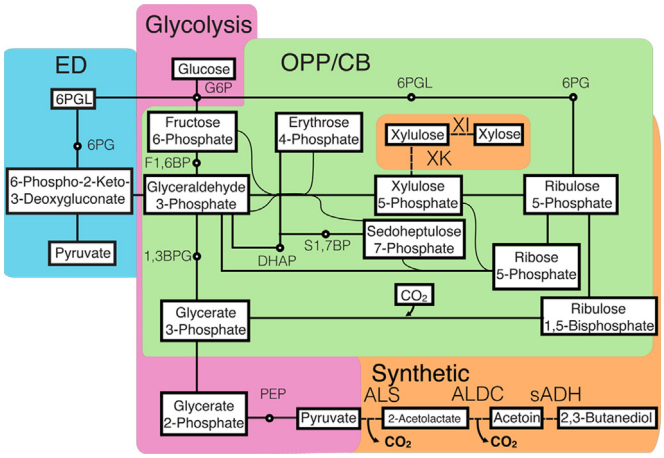

Fig. 1. Overview of central metabolic pathway engineering. An abbreviated schematic of central metabolites of glycolysis, the Entner-Doudoroff (ED) pathway, the oxidative pentose phosphate (OPP) pathway and the Calvin-Bensen (CB) cycle are shown. Solid lines indicate native genes and dashed lines indicate 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), CO_2 (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).

Table 1
Strains and Plasmids used in this study.

		Genotype	References
Strains	**Strain #**		
AL757	**1**	NSIII: *lacI*^{q}; *P*LlacO1::*alsS-alsD-adh*; *gent*^{R}	(Oliver et al., 2013)
AL2243	**2**	**1**+NSI: *lacI*^{q}; *P*trc::*galP*; *spec*^{R}	This study
AL2244	**3**	**1**+NSI: *lacI*^{q}; *P*trc::*xylE-xylA-xylB*; *spec*^{R}	This study
Plasmids			
pAL40		NSI targeting vector, *lacI*^{q}; *P*trc::*galP*; *spec*^{R}	(McEwen et al., 2013)
pAL70		NSI targeting vector, *lacI*^{q}; *P*trc::*xylE-xylA-xylB*; *spec*^{R}	(McEwen et al., 2013)
pAL300		NSIII targeting vector, *lacI*^{q}; *P*LlacO1::*alsS-alsD-adh*; *gent*^{R}	(Oliver et al., 2013)

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 μmol photons m^{-2} 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

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