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Combinatorial optimization of cyanobacterial 2,3-butanediol production

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ABSTRACT

A vital goal of renewable technology is the capture and re-energizing of exhausted CO_2 into usable carbon products. Cyanobacteria fix CO_2 more efficiently than plants, and can be engineered to produce carbon feedstocks useful for making plastics, solvents, and medicines. However, fitness of this technology in the economy is threatened by low yields in engineered strains. Robust engineering of photosynthetic microorganisms is lagging behind model microorganisms that rely on energetic carbon, such as *Escherichia coli*, due in part to slower growth rates and increased metabolic complexity. In this work we show that protein expression from characterized parts is unpredictable in *Synechococcus elongatus* sp. strain PCC 7942, and may contribute to slow development. To overcome this, we apply a combinatorial approach and show that modulation of the 5′-untranslated region (UTR) can produce a range of protein expression sufficient to optimize chemical feedstock production from CO_2.

1. Introduction

Efforts are intensifying to bridge the gap between fossil carbon consumption and renewable supply. At the same time, rising consumption of organic (energetic) carbon is driving emissions of inorganic (exhausted) carbon into the atmosphere in the form of CO_2. Cyanobacteria, fast growing prokaryotes and simple photosynthetic organisms, can directly sequester CO_2 by biological conversion into organic carbon using sunlight. Cyanobacteria can be grown in brine and elevated temperatures removing competition with food crops for water and land supply. Despite high CO_2 fixation, cyanobacteria do not naturally accumulate useful feedstock chemicals (Machado and Atsumi, 2012). Recently, the application of synthetic biology – the assembly of disparate genetic elements for novel use – has led to an increasing number of strains in which chemical feedstock production is achieved (Bandyopadhyay et al., 2010; Ducat et al., 2011; Heidorn et al., 2011; Rabinovitch-Deere et al., 2013). However, these strains have failed to reach titers representative of their carbon fixation rates. Metabolic imbalance has been shown to contribute to toxicity and poor fitness during production in heterotrophic strains (Barbirato et al., 1996; Pfleger et al., 2006; Zelcbuch et al., 2013; Zhu et al., 2001), and may be particularly important in photosynthetic strains, which must maintain light harvesting complexes and the electron transport chain. However, rationally balancing protein expression requires precise predictive knowledge of the behavior of genetic elements, which even in highly studied model organisms remains a challenge (Ferreira et al., 2013; Kosuri et al., 2013; Mutalik et al., 2013a; Mutalik et al., 2013b). Efforts have been made to balance pathways in *E. coli* by randomly modulating enzyme concentrations through varied translational and transcriptional control elements (Blazeck et al., 2012; Blazeck et al., 2013; Pfleger et al., 2006; Zelcbuch et al., 2013). These investigations invariably depend on highly visible products such as reporter proteins or carotenoids, allowing for easy identification of a balanced phenotype from a pool of mutants. In contrast, designs for the synthesis of chemical feedstocks from CO_2 have focused on products such as ethanol, butanol, alkanes, and fatty acids, which do not result in an easily identified phenotype. Because feedstock chemical synthesis in the cell draws carbon away from metabolism, the phenotype of high production is lowered growth, which appears very similar to that for toxicity, preventing growth based assays (Oliver et al., 2013). Attempts to model and rationally optimize whole pathways *in silico* are advancing rapidly but are still limited by the complexity of metabolic response to heterogeneous pathways (Lewis et al., 2012). As has been seen in *E. coli*, and as we show in this work for cyanobacteria, characterization of parts with reporter proteins, and replacement with production enzymes changes expression levels unpredictably (Levin-Karp et al., 2013; Zelcbuch et al., 2013). We aimed to use a combinatorial approach with elements characterized *in situ* to produce a range of protein expression in

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S. elongatus to increase production of the 2,3-butanediol (23BD) pathway.

2. Materials and methods

2.1. Culture conditions

S. elongatus strains were cultured in BG-11 medium (Rippka et al., 1979) with the addition of 50 mM $NaHCO_3$ and 10 μg/ml gentamicin. Cells were grown at 30 °C with rotary shaking (100 rpm) and light (55 $\mu E\ s^{-1}m^{-2}$) provided by four 86 cm 20 W fluorescent tubes 5 cm above the cell cultures. Cell growth was monitored by measuring OD_{730}. For 23BD production, 10 ml of exponentially growing cultures were harvested by centrifugation at 1500 *g* for 10 min, resuspended to OD_{730} 0.1 in production media (BG-11 medium with the addition of 50 mM $NaHCO_3$, 10 μg/ml thiamine, and 10 μg/ml gentamicin), and allowed to recover for 24 h (~OD_{730} 0.3) before induction with 1 mM IPTG. The cells were grown for 72 h. A 2 ml sample was harvested by centrifugation at 15,000 *g* for 2 min and 23BD was quantified from the supernatant. For acetoin production, cultures were prepared as for 23BD with the exception that cultures were resuspended to OD_{730} 0.4 and induced with 1 mM IPTG immediately. The cells were grown for 24 h. A 360 μl sample was harvested by centrifugation at 1500 *g* for 10 min and acetoin was quantified from the supernatant. In cases where production rate was determined acetoin was measured every 12 h by the same method.

2.2. Plasmid construction

Plasmids used and constructed are listed in Supplementary Table S2. Oligonucleotides for constructions and verifications are listed in Supplementary Table S3. All plasmids were constructed by DNA assembly techniques using sequence and ligation independent cloning (SLIC) (Li and Elledge, 2007; Machado et al., 2012) in *E. coli* XL1-Blue (Agilent Technologies, Santa Clara, CA).

To construct plasmids with four different ribosome binding site combinations upstream of the *alsS* gene from *B. subtilis* and the *alsD* gene from *E. aerogenes*, we used the plasmid pAL302 (RBS U1 *alsS*-RBS U2 *alsD*) (Oliver et al., 2013) as template. Each RBS was replaced with RBS A–D (Supplementary Table S1).

The *adh* gene from *C. beijerinckii*, which was chemically synthesized by DNA2.0 Inc. (Menlo Park, CA) previously (Oliver et al., 2013), was flanked by the selected RBS sequences (RBS A–D) by PCR. The resulting PCR fragments were inserted to downstream of *alsD* gene on the neutral site (NS) targeting plasmids by SLIC (Supplementary Table S2).

To clone *alsS*, *alsD*, *adh*, *luc*, and *lacZ* genes individually under different RBS sequences (RBS U (U2 for *alsD* and U1 for others), A–D), each gene was flanked by the selected RBS by PCR. The PCR fragments were inserted into pAL312 (Oliver et al., 2013) by SLIC.

2.3. Transformation of S. elongatus

Transformation of *S. elongatus* was performed as previously described (Golden et al., 1987). A NS located between Synpcc7942_0893 (903,564–904,283 bp) and Synpcc7942_0894 (904,845–905,417 bp) in the *S. elongatus* chromosome was used for insertion of an expression cassette (Oliver et al., 2013) (Fig. 1). Strains were segregated several times by transferring colonies to fresh selective plates. Correct recombinants were confirmed for double crossover and gene fidelity by PCR and sequencing.

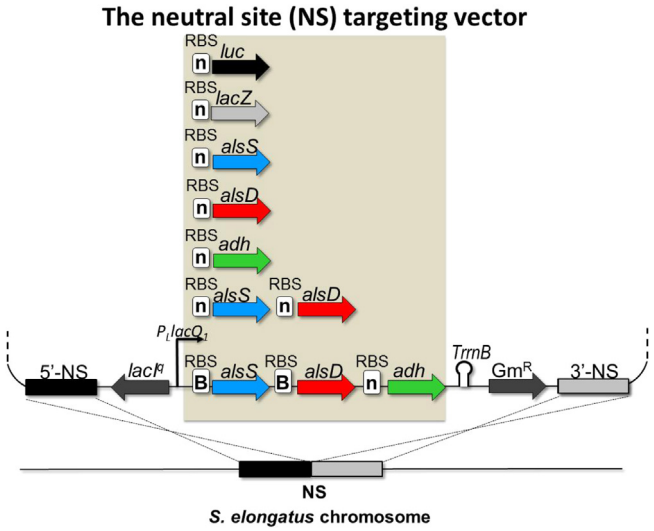

Fig. 1. Schematic representation of recombination to integrate the expression cassettes into the *S. elongatus* chromosome. Constructs with "n" represent a list of combinatorial strains each with varied RBS sequence of A, B, C, D, or U (Supplementary Table S1).

2.4. Acetoin and 23BD quantification

Acetoin was quantified by the method of Voges and Proskauer (1898), adapted to small volume on 96 well plates as described previously (Oliver et al., 2013). Triplicate measurements of no less than three standards, including at least one value each above, below, and within the desired range, were included in every assay.

23BD was analyzed with gas chromatography (GC) (Shimadzu) equipped with a flame ionization detector and an HP-chiral 20b column (30 m, 0.32-mm internal diameter, 0.25-mm film thickness; Agilent Technologies) as described previously (Oliver et al., 2013).

2.5. Enzyme assays

S. elongatus cells were collected at 24, or 72 h after induction by centrifugation (1,500 *g*, 10 min), washed in 50 mM potassium phosphate buffer (pH 7.5), and suspended in the same buffer. Crude extract were prepared with a bead beater (Mini Bead Beater eight (BioSpec Products, Inc., Bartlesville, OK)). The total protein determination was performed by Advanced Protein Assay Reagent (Cytoskeleton, Inc., Denver, CO).

Acetolactate synthase (ALS) activity was determined as described previously (Yang et al., 2000). The concentration of acetoin produced was quantified as described above, against a standard curve using pure acetoin. One specific unit of AlsS activity corresponds to the formation of 1 nmol of acetoin per mg of protein per minute.

Acetolactate decarboxylase (ALDC) activity was determined using the protocol for the ALS assay with the following modifications. The substrate was replaced with 2-acetolactate prepared from ethyl-2-acetoxy-2-methylacetoacetate as described (Bastian and Arnold, 2012). The acidification step was omitted, and reactions were quenched by the transfer of 20 μl of reaction by multichannel pipette to wells in a 96-well plate each containing 80 μl 2.5 M NaOH. Acetoin was immediately quantified by addition of reagent as described above. One specific unit of ALDC activity corresponds to the formation of 1 nmol of acetoin per mg of protein per minute. For the paired ALDC assay pyruvate was used as substrate, and the acidification step was omitted as described previously (Oliver et al., 2013).

Alcohol dehydrogenase (ADH) activity was determined by measuring the oxidation of NADPH. The reaction mixture contained