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Construction of a novel D-lactate producing pathway from dihydroxyacetone phosphate of the Calvin cycle in cyanobacterium, *Synechococcus elongatus* PCC 7942

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Using engineered cyanobacteria to produce various chemicals from carbon dioxide is a promising technology for a sustainable future. Lactate is a valuable commodity that can be used for the biodegradable plastic, polylactic acid. Typically, lactate production using engineered cyanobacteria was via the conversion of pyruvate in glycolysis by lactate dehydrogenase. In cyanobacteria, the metabolic flux in the Calvin cycle is higher than that in glycolysis under photoautotrophic conditions. The construction of a novel lactate producing pathway that uses metabolites from the Calvin cycle could potentially increase lactate productivity in cyanobacteria. In order to develop such a novel lactate production pathway, we engineered a cyanobacterium *Synechococcus elongatus* PCC 7942 strain that produced lactate directly from carbon dioxide using dihydroxyacetone phosphate (DHAP) via methylglyoxal. We confirmed that wild-type strain of *S. elongatus* PCC 7942 could produce lactate using exogenous methylglyoxal. A methylglyoxal synthase gene, *mgsA*, from *Escherichia coli* was introduced into *Synechococcus elongates* PCC 7942 for conversion of DHAP to methylglyoxal. This engineered strain produced lactate directly from carbon dioxide. Genes encoding intrinsic putative glyoxalase I, II (Synpcc7942_0638, 1403) and the lactate/H^+ symporter from *E. coli* (*lldP*) were additionally introduced to enhance the production. For higher lactate production, it was important to maintain elevated extracellular pH due to the characteristics of lactate exporting system. In this study, the highest lactate titer of 13.7 mM (1.23 g/l) was achieved during a 24-day incubation with the engineered *S. elongatus* PCC 7942 strain possessing the novel lactate producing pathway.

[**Key words:** Carbon dioxide; Cyanobacteria; D-Lactate; Photosynthesis; Glyoxalase; Dihydroxyacetone phosphate]

The world's energy supply is highly dependent on fossil resources despite the excessive consumption of such fuel, leading to numerous environmental issues. It is crucial to reduce our dependency on fossil resources for both energy generation and chemical production for a sustainable future. One option to solve such problems is the use of biomass for the biological production of fuels and chemicals (1,2). This approach has become feasible by advances in genetic engineering that allow modification of microorganisms such as *Escherichia coli* and yeast to either enhance production of natural chemicals or to even generate entirely foreign substances (3,4). One of the most important limiting factors affecting the use of these organisms is the need for an abundant carbon source to feed the manufacturing process. So far edible biomass (for example, corn and sugarcane) (5) and inedible lignocellulosic biomass (for example, wood wastes and energy crops) (6) were used for bioproduction. However, commonly used heterotrophic microorganisms are unable to directly assimilate these biomass, so it must first be enzymatically or chemically saccharified to simple sugars (7,8). An additional issue is that growing the required biomass can take a considerable amount of time, again limiting potential.

In contrast to heterotrophic microorganisms, cyanobacteria are photoautotrophic and feed by fixing carbon dioxide to organic compounds using solar energy. Similar to other organisms, genetic engineering approaches have also been developed in cyanobacteria for the production of chemicals, although in this case, products are manufactured directly from carbon dioxide. Some of these valuable products include isobutyraldehyde, isobutanol (9,10), ethanol (11), 2,3-butanediol (12,13), 2-methyl-1-butanol (14), 1-butanol (15,16), acetone (17,18), 3-hydroxybutyrate (19), ethylene (20,21), isoprene (22,23), 1,2-propanediol (1,2-PDO) (24), and glycerol (25,26). Our prior work has also demonstrated biological production of isopropanol (27) and 1,3-PDO (28) using *S. elongatus* PCC 7942. However, the currently achievable chemical titers by engineered cyanobacteria are significantly lower than those from heterotrophic microorganisms (29,30). For example, even the most efficient ethanol production with engineered cyanobacteria has not been able to exceed 10 g/l (11). Therefore, while direct production of chemicals from carbon dioxide is an attractive and promising technology for a sustainable future, increased production titers are necessary if it is to become practical and cost effective.

Lactate is one of the major chemicals produced by engineered microorganisms. It is widely used and can be found in a range of food additives, cosmetics, and various pharmaceuticals (31,32). Demand as a substrate for biodegradable plastics (in the form of poly-lactic acid; PLA) is expected to increase as the world moves

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towards a sustainable future (33,34). Structurally, the asymmetric carbon in the lactate molecule yields two optical isomers, L-lactate and D-lactate. Importantly, the physical properties of PLA are affected by optical purity so it is vital to produce optically pure lactate for PLA synthesis (35). Lactate can be produced chemically by hydrolysis of lactonitrile, a byproduct of acrylonitrile production. However, lactate produced by this method is a mixture of the two optical isomers, affecting the quality of any resultant PLA (36). When produced biologically, lactate is generated from conversion of pyruvate by lactate dehydrogenase (LDH) (37). The specific optical character of the lactate isomer produced strictly depends on enzymatic characteristics, allowing optically pure lactate to be manufactured. The lactic acid bacteria, *Lactobacillus* sp. and *Lactococcus* sp. can naturally produce lactate (38), but these strains have complex auxotrophic requirements and the purity of produced lactate depends on incubation conditions. To overcome these drawbacks, LDH has been introduced into more user friendly microorganisms such as *E. coli* and yeast. This has led to more effective biological production of optically pure lactate (39).

Lactate can be produced directly from carbon dioxide using genetically engineered cyanobacteria. As with *E. coli* and yeast, the introduction of LDH enables cyanobacteria to produce lactate from cellular pyruvate. The highest lactate titer of 20.5 mM (1.84 g/l) has been achieved using an engineered *Synechocystis* sp. PCC 6803 strain possessing LDH (40). The highest production rate (2.2 mM/day) was obtained using another cyanobacterium strain, *Synechococcus* sp. PCC 7002 (41). This production rate was achieved by combining LDH introduction with additional genetic engineering using CRISPRi to increase central carbon flux. Another approach to increase lactate production may be to utilize a metabolic pathway that naturally possesses a higher carbon flux. One example is the Calvin cycle of photoautotrophically grown cyanobacteria, which typically shows a greater flux than glycolysis (42,43). The reason of greater flux in Calvin cycle is that many parts of fixed carbon by photosynthesis were utilized to reproduce ribulose-1,5-bisphosphate for next carbon fixation. Chemical production using metabolites in the Calvin cycle of cyanobacteria would therefore be expected to lead to higher titers of chemical production. Previously, production of 1,2-PDO, sucrose, glycerol, and 1,3-PDO have all been achieved using metabolites (DHAP or fructose-6-phosphate) in the Calvin cycle (19,24,25,28,44). The titers of these chemicals were indeed higher than the other chemicals produced using metabolites from other pathways.

In this study, we constructed a novel lactate producing pathway that uses dihydroxyacetone phosphate (DHAP), a metabolite from the Calvin cycle, rather than pyruvate (Fig. 1). The pathway composed of methylglyoxal synthase, lactate/H^+ symporter, and glyoxalase I, II was constructed in *S. elongatus* PCC 7942. In the constructed pathway, any reducing power like as NADH is not necessary for lactate production and DHAP in Calvin cycle showing greater flux (42,43) is used as substrate. Previously, we constructed 1,3-PDO and glycerol producing pathway from DHAP in this strain (28). We fully investigated the effectiveness of the novel pathway in an engineered cyanobacterium *S. elongatus* PCC 7942 strain. To the best of our knowledge, it is the first time to construct a lactate producing pathway using DHAP for lactate production in cyanobacteria and even any bacteria.

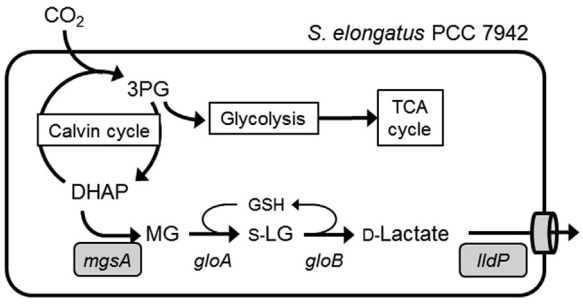

FIG. 1. Overview of the novel lactate producing pathway constructed in this study. Introduced genes are highlighted in italics. Shaded background indicates exogenous gene (*mgsA* and *lldP*) from *E. coli*. White background indicates intrinsic gene (*gloA* and *gloB*). 3PG, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; MG, methylglyoxal; s-LG, s-lactoylglutathione; GSH, glutathione.

MATERIALS AND METHODS

Chemicals and reagents All chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise specified. Restriction enzymes, phosphatase (New England Biolabs; Ipswich, MA, USA), ligase (Rapid DNA Ligation Kit, Roche, Mannheim, Germany), and DNA polymerase (KOD Plus Neo DNA polymerase, Toyobo Co., Ltd., Osaka, Japan) were used for cloning. Oligonucleotides were synthesized by Life Technologies Japan, Ltd. (Tokyo, Japan).

Culture media Modified BG11 medium supplemented with 20 mM HEPES-NaOH (pH 7.5) for pH stabilization was used for cultivation of *S. elongatus* PCC 7942 (Life Technologies Corporation, Carlsbad, CA, USA). Hereafter, this medium was referred to as BG11-HEPES medium. The composition of BG11-HEPES medium used in this study was the same as previously reported (27). In some experiments, HEPES-NaOH (pH 7.5) buffer was eliminated and substituted for 20 mM CHES-NaOH (pH 9.0) or CAPS-NaOH (pH 10.0). Hereafter, these buffer replaced mediums were referred to as BG11-CHES and BG11-CAPS medium. To prepare the plate medium, 1.5% (w/v) of Bacto Agar (Difco Laboratories, Franklin Lakes, NJ, USA) was added. Antibiotics (10 μg/ml kanamycin and 20 μg/ml spectinomycin) were added to BG11 mediums as appropriate.

Growth and production conditions All cyanobacterial cultures were grown under fluorescent light (100 μmol photon m^{-2} s^{-1}) at 30°C in a growth chamber (MLR-325H-PJ; Panasonic Corporation, Osaka, Japan). Photon flux density was measured with an IKS-27 (Koito Manufacturing, Tokyo, Japan). Cell density (OD_{730}) was measured using an Infinite 200 PRO (Tecan, Männedorf, Switzerland). For pre-culture, cells were inoculated into 20 mL BG11-HEPES medium in a 50-mL flask, and incubated under fluorescent light on a rotor shaking at 150 rpm (NR-30 shaker; Taitec Corporation, Saitama, Japan). Pre-cultured cells at OD_{730} of 1.0–2.0 were inoculated into 20 mL BG11 mediums containing 1 mM of IPTG at an initial OD_{730} of 0.025. Cultures were grown under same conditions as pre-culture.

Strain construction The bacterial strains used in this study are listed in Table S1.

Genes were integrated into the *S. elongatus* PCC 7942 genome by homologous recombination. For gene integration into the neutral site (NS) I (45) and NS II (46), the previously constructed and newly generated plasmids were used. The integrated genes in the engineered strains were confirmed by sequence analysis.

Plasmids construction Plasmids and primers used in this study are listed in Tables S1 and S2.

The gene encoding methylglyoxal synthase (*mgsA*) was amplified from *E. coli* BW25113 genome using primers T1514-T1590. The amplified sequence was digested by Acc65I-BglII. The digested PCR products were ligated into Acc65I-BamHI site of pTA424 to create pTA1501 ($P_L$lacO1::*mgsA*, NS II-targeting plasmid).

mgsA amplified from *E. coli* BW25113 genome using primers T1514-T1515 was digested by KpnI-NheI. The gene encoding lactate/H^+ symporter (*lldP*) amplified from *E. coli* BW25113 genome with primers T2576-T2577 was digested by NheI-BamHI. Digested PCR products were ligated into the KpnI-BamHI site of pTA424 to create pTA1567 ($P_L$lacO1::*mgsA-lldP*, NS II-targeting plasmid).

A NS I targeting plasmid (pTA1562) was newly constructed based on commercial pZ vectors purchased from EXPESSYS. The antibiotic resistant cassette of pZE22-MCS was switched from kanamycin resistance to spectinomycin resistance to create pTA1471. The upper and lower regions of NS I were amplified from *S. elongatus* PCC 7942 genome using primers T2489-T2490, and T2491-T2492, respectively. The amplified fragments were digested by AvrII-SpeI. The digested NS I upper and NS I lower fragments were ligated into the SpeI and AvrII sites of pTA1471 respectively to create pTA1476. *lacI* regulated by a *P*laclq promoter was amplified using primers T2662-T2664 and digested by AvrII-SpeI. The digested fragment was ligated into the AvrII site of pTA1476 to create pTA1562.

The genes encoding putative glyoxalase [*gloA* (Synpcc7942_0638) and *gloB* (Synpcc7942_1403)] were amplified from the *S. elongatus* PCC 7942 genome using primers T2783-T2784 and T2785-T2786, respectively. The amplified *gloA* and *gloB* sequences were digested by KpnI-NheI and NheI-BamHI, respectively. The digested sequences were ligated into KpnI-BamHI site of pTA1562 to create pTA1697 ($P_L$lacO1::*gloAB*, *P*laclq::*lacI*, NS I-targeting plasmid).

Product analysis The supernatant was obtained by centrifugation (20,000 ×g, 10 min, 4°C) and filtered using Minisart RC4 (Sartorius, Goetingen, Germany). The filtered sample was subjected to quantitative analysis. Lactate was analyzed using a high-performance liquid chromatograph (LC-20AD, Shimadzu, Kyoto, Japan) equipped with an autosampler and electric conductivity detector

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