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Metabolic engineering for isopropanol production by an engineered cyanobacterium, *Synechococcus elongatus* PCC 7942, under photosynthetic conditions

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Cyanobacteria engineered for production of biofuels and biochemicals from carbon dioxide represent a promising area of research in relation to a sustainable economy. Previously, we have succeeded in producing isopropanol from cellular acetyl-CoA by means of *Synechococcus elongatus* PCC 7942 into which a synthetic metabolic pathway was introduced. The isopropanol production by this synthetic metabolic pathway requires acetate; therefore, the cells grown under photosynthetic conditions have to be transferred to a dark and anaerobic conditions to produce acetate. In this study, we achieved acetate production under photosynthetic conditions by *S. elongatus* PCC 7942 into which we introduced the *pta* gene encoding phosphate acetyltransferase from *Escherichia coli*. The metabolic modification (via *pta* introduction) of the isopropanol-producing strain enabled production of isopropanol under photosynthetic conditions. During 14 days of production, the titer of isopropanol reached 0.55 mM (33.1 mg/l) with an intermediate product, acetone, at 0.21 mM (12.2 mg/l).

[**Key words:** Cyanobacteria; Synthetic metabolic pathway; Isopropanol; Photosynthetic conditions; Acetate]

Cyanobacteria fixing carbon dioxide into organic compounds by photosynthesis using solar energy may be a promising host for bioproduction. Introduction of synthetic metabolic pathways composed of multiple genes derived from other organisms has been effective at enabling hosts to produce various chemicals that they cannot synthesize naturally (1,2). Genetically engineered cyanobacteria can produce various chemicals directly from carbon dioxide, for example, isobutyraldehyde, isobutanol (3), 1-butanol (4–7), 2-methyl-1-butanol (8), acetone (9,10), ethylene (11,12), ethanol (13), isoprene (14), fatty acids (15), 3-hydroxybutyrate (16), 1,2-propanediol (17), 1,3-propanediol (18), and 2,3-butanediol (19,20). Furthermore, we have accomplished isopropanol production from cellular acetyl-CoA by *Synechococcus elongatus* PCC 7942 into which we introduced a synthetic metabolic pathway (21,22).

It has been reported that the metabolic flux of glycolysis is estimated to be smaller than that of the Calvin cycle in cyanobacteria under photoautotrophic conditions according to metabolic flux analysis (23). Some chemicals (acetone, 1-butanol, and 3-hydroxybutyrate) derived from cellular acetyl-CoA have been already produced by cyanobacteria carrying a synthetic metabolic pathway. Initially, 1-butanol production by engineered *S. elongatus* PCC 7942 (4) and acetone production by engineered *Synechocystis* sp. PCC 6803 (9) were reported. These types of biosynthesis were achieved only under dark and anaerobic conditions. It is known that many cyanobacteria have various fermentation pathways to obtain energy under dark and anaerobic conditions (24). In such conditions, the endogenous stores of energy (mainly glycogen) are degraded into CO_2, acetate, lactate, ethanol, and other compounds by activation of glycolysis. Later, it was suggested that the activation of glycolysis by a drastic shift in conditions from photosynthetic to fermentative is important for the types of production based on acetyl-CoA in cyanobacteria. We also achieved isopropanol production by engineered *S. elongatus* PCC 7942 under similar conditions (21,22). Nonetheless, for 1-butanol production, pathway optimizations (involving an oxygen-tolerant enzyme and the driving force generated by an ATP-consuming reaction) were integrated into the synthesis pathway, resulting in the highest titer of 404 mg/l (5,6). Furthermore, it is known that *Synechocystis* sp. PCC 6803 naturally produces poly-3-hydroxybutyrate (PHB), one of the major biodegradable plastics, for storage of energy derived from cellular acetyl-CoA (25). The gene deletion involved in the last step of PHB synthesis and introduction of a gene encoding the enzyme cleaving coenzyme A from 3-hydroxybutyryl-CoA enabled the production of 3-hydroxybutyrate (3-HB) with the titer of 533.4 mg/l, which is a precursor for the synthesis of the biodegradable plastics poly-hydroxyalkanoates and many fine chemicals under photosynthetic conditions (16). These reports indicated that the drastic shift in conditions to dark and anaerobic is not necessary for production of some chemicals derived from acetyl-CoA in engineered cyanobacteria.

The drastic condition shift from photosynthetic (for cell growth) to dark and anaerobic (for production) that was employed in our

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previous studies (21,22) was complicated and unsuitable for commercialization. Therefore, the next challenge for isopropanol production directly from carbon dioxide in cyanobacteria is production with simultaneous cell growth under photosynthetic conditions. The pathway introduced into *S. elongatus* PCC 7942 for isopropanol production can function under aerobic conditions in *Escherichia coli* (26). To accomplish the isopropanol production under photosynthetic conditions, we hypothesized that the acetate biosynthesis from carbon dioxide in the photosynthetic conditions is important for two reasons. First, the second reaction in the isopropanol-producing pathway (converting acetoacetyl-CoA into acetoacetate) requires acetate as a CoA receptor (Fig. 1). Second, we previously reported that the engineered *S. elongatus* PCC 7942 can synthesize isopropanol under photosynthetic conditions only when acetate is extrinsically supplied (21) or fermentatively produced under dark and anaerobic conditions before isopropanol production (22). It was reported that acetate is assimilated in a light-dependent manner and is utilized to produce cell components via acetyl-CoA by *Synechococcus* (27). Recently, the photosynthetic acetone production by engineered *S. elongatus* PCC 7942 was reported (10). Acetone is biosynthesized from cellular acetyl-CoA via the same three enzymatic reactions included in the isopropanol-producing pathway. For photosynthetic acetone production, the metabolic flux into acetyl-CoA from carbon dioxide was enhanced by introduction of a heterologous pathway composed of phosphoketolase and phosphate acetyltransferase converting xylulose-5-phosphate (Xu5P) into acetyl-CoA via acetyl-phosphate (acetyl-P). The combination of this pathway with the driving force of ATP consumption used in 1-butanol production led to photosynthetic acetone production with the highest titer of 22.5 mg/l in a photobioreactor with a gas-stripping-based recovery system. Although

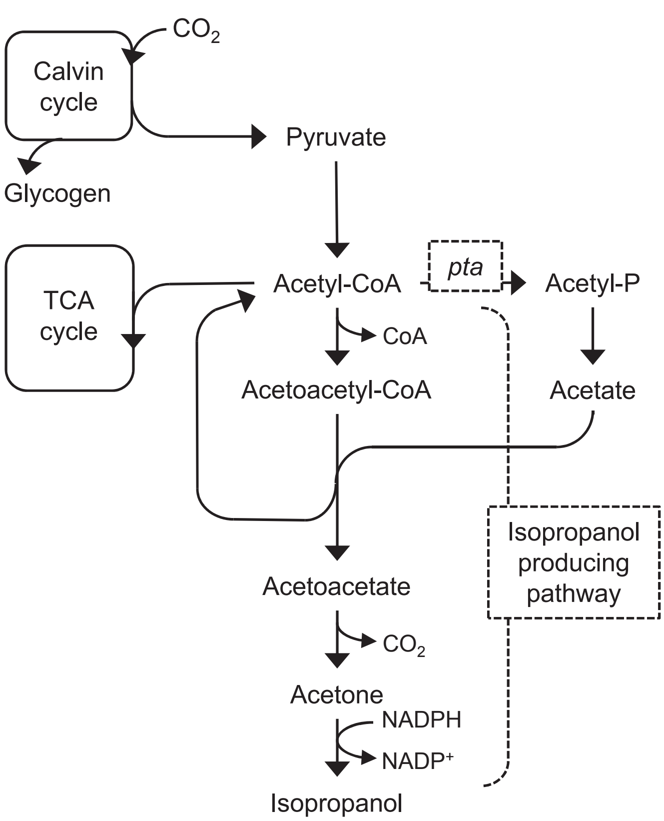

FIG. 1. The metabolic pathway for isopropanol production in a photosynthetic condition. The squares outlined by the dashed line represent the gene and pathway that were heterologously introduced for photosynthetic isopropanol production.

acetate production was not discussed in that report, we hypothesized that a part of acetyl-P from Xu5P might be converted into acetate. It was assumed back then that the acetate biosynthesis is essential for photosynthetic isopropanol production. In the present study, the *pta* gene for acetate metabolism in *E. coli* was introduced into *S. elongatus* PCC 7942 for acetate production. The additional modification introduced into the strain carrying a synthetic metabolic pathway for isopropanol biosynthesis enabled isopropanol production under photosynthetic conditions.

MATERIALS AND METHODS

Reagents All reagents were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan) unless specified otherwise. Restriction enzymes, phosphatase (New England Biolabs, Ipswich, MA), and DNA polymerase (KOD Plus Neo and KOD FX Neo DNA polymerase, Toyobo Co., Ltd., Osaka, Japan) were used for cloning. Oligonucleotides were synthesized by Life Technologies Japan, Ltd. (Tokyo, Japan).

Culture media and growth conditions The BG11 medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH (pH 7.5) was used for both growth and production unless stated otherwise. The composition of the medium was identical to that described elsewhere (21). For cultivation of a transformant, spectinomycin and kanamycin were added at 20 and 10 μg/ml (final concentration), respectively.

All cyanobacterial cultures were carried out under continuous light (100 μmol photon m^{-2} s^{-1}) at 30°C in a growth chamber (MLR-325H-PJ, Panasonic, Osaka, Japan). Cells inoculated into 20 ml of BG11 in a 50-ml flask were incubated with rotary shaking (150 rpm; NR-30, TAITEC, Saitama, Japan) under white light-emitting diode (LED) light (LC-LED450W, TAITEC) as preculture. When optical density at 730 nm (OD_{730}) of the preculture reached 1.0–2.0, the cells were inoculated into 50 ml of BG11 in a test tube to attain the initial OD_{730} of 0.025. The cells were incubated under fluorescent light with continuous aeration with air containing 1% of carbon dioxide.

The concentrations of inorganic phosphate and nitrate were measured by means of the QuantiChrom Phosphate Assay Kit (DIPI-500, BioAssays Systems, CA, USA) and Nitrite/Nitrate Colorimetric Method (Cat. No. 11 746 081 001, Roche, Basel, Switzerland), respectively.

Production conditions A culture grown under photosynthetic conditions (20 ml) was transferred into a 50-mL flask, which was then tightly capped. All cyanobacterial cultures for production were incubated with rotary shaking (150 rpm; NR-30, TAITEC) under white LED light (100 μmol photon m^{-2} s^{-1}; LC-LED450W, TAITEC) at 30°C in a growth chamber (MLR-325H-PJ, Panasonic). In some cases, sodium acetate (pH 7.5) or acetone was added to the culture medium at a final concentration of 10 mM or 1 g/l, respectively. Every day, 1 ml of the culture was removed from the flask for sampling, and pH of the culture was adjusted to approximately pH 7.0 with HCl. One-tenth volume of 100 mM $NaHCO_3$ dissolved in BG11 supplemented with appropriate concentrations of IPTG, spectinomycin, and kanamycin was added as a supply of carbon dioxide to the culture medium every 2 days.

Plasmid and strain construction for isopropanol production All plasmids and strains constructed and used in this study are listed in supplemental files (Tables S1 and S2, respectively). All amplified genes for plasmid construction and all genes integrated into the genome were sequenced for verification.

pTA821 digested by BamHI and dephosphorylated served as a vector. The PCR product amplified from pTA634 using primers T570 (5′-GCCAT CGGAT CCGAA GGAGA TATAC ATATG AAAGG GTTTG CC-3′) and T571 (5′-GCCAT CGGAT CCGTT ACAGG ATAAC AACCG CCTTA ATCAG ATC-3′) and digested with BamHI was used as an insert. The ligated product where *sadh* was inserted in the same direction as that of other genes (*thl*, *atoAD′*, and *adc*) was named pTA869 (PlacIq::*lacI*, $P_L$lacO1::*thl*, *atoAD′*, *adc*, *sadh*, $Spec^r$; the NS I-targeting plasmid).

The codon usage of *pta*-coding phosphate acetyltransferase derived from *E. coli* was optimized for *S. elongatus* PCC 7942. The codon-optimized *pta* was synthetized by DNA2.0 and the plasmid carrying codon-optimized *pta* was designated as pTA564. pTA424 (18) digested with Acc65I and BamHI and dephosphorylated served as a vector. The PCR product amplified from pTA564 using primers T953 (5′-GCCAT CGGTA CCATG TCACG CATTA TTATG TTGAT CCCC-3′) and T954 (5′-GCCAT CGGAT CCCTA TTGCT GCTGC GCGCT TTG-3′) and digested with Acc65I and BamHI was used as an insert. The ligated product was designated as pTA567 ($P_L$lacO1::*pta*, Km^r; the NS II-targeting plasmid).

The reconstructed isopropanol-producing pathway was integrated with *lacI* and a spectinomycin resistance gene into the NS I (28) genomic region of a wild-type strain of *S. elongatus* PCC 7942 (TA1297) by means of pTA869, resulting in strain TA1795 (PlacIq::lacI, $P_L$lacO1::*thl*, *atoAD′*, *adc*, *sadh*, $Spec^r$, NS I).

The *pta* gene was integrated with a kanamycin resistance gene into the NS II (29) genomic region of TA1795 by means of pTA567, resulting in strain TA2503 (PlacIq::*lacI*, $P_L$lacO1::*thl*, *atoAD′*, *adc*, *sadh*, $Spec^r$, NS I, $P_L$lacO1::*pta*, Km^r, NS II).