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Modification of carbon metabolism in *Synechococcus elongatus* PCC 7942 by cyanophage-derived sigma factors for bioproduction improvement

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Many cyanophages, which infect cyanobacteria, most of possess putative sigma factors that have high amino acid sequence homology with the σ70-type sigma factor present in cyanobacteria, allowing them to obtain energy and metabolites for their own propagation. In this study, we aimed to modify the carbon metabolism of *Synechococcus elongatus* PCC 7942 by expressing putative sigma factors from *Synechococcus* phages to improve bioproduction. Four cyanophage-derived putative sigma factors—putative RpsD4 from *Synechococcus* phage S-CBS1, putative RpoD and putative RpoS from S-CBS2, and putative RpsD4 from S-CBS3—were selected for this purpose. These were introduced into *S. elongatus* PCC 7942, and their expression was controlled with a theophylline-dependent riboswitch. The expression of the putative RpoD from S-CBS2 and putative RpsD4 from S-CBS3 resulted in a significant decrease in the growth rate of *S. elongatus* PCC 7942. In addition, metabolome analysis showed a 3.2-fold increase in acetyl-CoA concentration with the expression of the putative RpoD from S-CBS2 and a 1.9-fold increase with the putative RpsD4 from S-CBS3. The results of RT-qPCR showed that several sugar metabolism genes were repressed by the putative RpoD and activated by the putative RpsD4. In particular, the engineered strain overexpressing the putative RpsD4 and expressing phosphate acetyltransferase succeeded in improving the productivity of the model target product acetate to 217% of its previous value. To the best of our knowledge, this study is the first to modify the metabolism of *S. elongatus* PCC 7942 by expressing their putative sigma factors from cyanophages.

[**Key words:** Cyanobacteria; *Synechococcus elongatus* PCC 7942; Sigma factor; Cyanophage; Metabolic engineering]

In recent years, the production of useful chemicals using cyanobacteria has attracted a great deal of attention. Cyanobacteria can fix CO_2 in organic compounds through photosynthesis. Thus, by introducing synthetic metabolic pathways, they can be engineered to produce various chemicals, including isobutyraldehyde, isobutanol (1,2), ethanol (3), 2,3-butanediol (4,5), 2-methyl-1-butanol (6), 1-butanol (7,8), acetone (9,10), 3-hydroxybutyrate (11), ethylene (12,13), isoprene (14,15), 1,2-propanediol (16) and glycerol (17,18). In addition, we have successfully developed engineered cyanobacteria capable of producing 1,3-propanediol (19,20), isopropanol (21–23), and lactate (24) through the introduction of synthetic metabolic pathways. For cyanobacterial bioproduction, *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 are often used as host strains.

Despite the success of cyanobacterial bioproduction, the productivity of cyanobacterial systems remains far below industrial feasibility. In many cases, this is due to the low metabolic flux of glycolysis in producing pyruvate or acetyl-CoA, which are the starting metabolites for many synthetic metabolic pathways (25). One method for solving this problem in the case of cyanobacterial bioproduction is to change the carbon metabolism to a state suitable for target chemical production.

Sigma factor is a bacterial transcription initiator that binds to a specific sequence and regulates the transcription of multiple genes. The expression of a specific sigma factor greatly affects the phenotype of the cell, including its metabolism. The number of sigma factors varies among bacterial species, with *Escherichia coli* having seven sigma factors including σ70 (RpoD), the primary sigma factor responsible for exponential growth, and σ38 (RpoS), a stationary phase sigma factor, and σ28 (RpsD), a flagellar sigma factor (26,27). These seven sigma factors can be broadly divided into two groups: the σ70 family, members of which have a structure similar to that σ70, and the σ54 group, members of which exhibit distinct structures.

Cyanobacteria possess several sigma factors in the σ70 family. These σ70 family sigma factors can be broadly divided into three groups based on their amino acid sequences. Group 1 factors control the expression of genes essential for cell growth (28), while group 2 sigma factors are not essential for survival and are mainly classified into the four subclasses B, C, D, and E (in some cases M). Group 3 is a category for all other σ70 sigma factors. SigE is a sigma factor belonging to cluster E in *Synechocystis* sp. PCC 6803 that controls several sugar catabolism genes. By overexpressing SigE, it is possible to increase the transcription of the glycogen

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metabolism-related genes *glgP* and *glgX* and of the sugar catabolism-associated genes *opcA*, *zwf*, *gnd*, and *talC* 1.5−2.2-fold. As a result of changing the expression of these genes, the acetyl-CoA concentration increased 1.2-fold, and production of the important biopolymer polyhydroxybutyrate starting from this metabolite increased 2.5-fold (29−31). Modifying the carbon metabolism in *Synechocystis* sp. PCC 6803 by overexpressing SigE is a powerful strategy for bioproduction from acetyl-CoA. Unfortunately, unlike in the case of *Synechocystis* sp. PCC 6803, SigE has not yet been discovered in *S. elongatus* PCC 7942, another well-utilized host for cyanobacterial production (32).

When infecting cyanobacteria, cyanophages express their own genes and modify the host metabolism to a state suitable for their own propagation. Some cyanophages possess genes with high amino acid sequence homology to *zwf*, *gnd*, and *talC*, which are associated with the pentose phosphate pathway in cyanobacteria. It is thought that the host metabolism is modified by expressing these genes in order to generate the energy for phage propagation (33). In addition, several cyanophages encode photosynthesis-related genes, such as *psbA* and *hli* which are hypothesized to maintain the host's energy production during infection (34). There are also some cyanophages that infect *Synechococcus* and *Prochlorococcus* that, in order to modify the host metabolism, carry genes encoding putative sigma factors with high amino acid homology to the cyanobacterial sigma factors σ70 and σ38; in addition, these cyanophages possess genes homologous to the sugar catabolism genes *zwf* and *gnd* (35,36). In contrast, the *Synechococcus* phages S-CBS1, S-CBS2, and S-CBS3 do not have genes showing high homology to sugar catabolism genes such as *zwf* or *gnd*, but they do have putative sigma factors (35). It is speculated that the putative sigma factors from cyanophages that do not have genes homologous to cyanobacterial sugar catabolism genes may have a greater impact on the host metabolism than the putative sigma factors from cyanophages with such genes. Thus, the overexpression of the putative sigma factors from the *Synechococcus* phages S-CBS1, S-CSB2, and S-CBS3 in *S. elongatus* PCC 7942 might modify the host carbon metabolism in a way that is useful for bioproduction. However, to our knowledge, there have been no reports regarding the effects of the overexpression of cyanophage-derived putative sigma factors on bioproduction in engineered cyanobacteria.

In this study, we aimed to investigate the changes in metabolism, including in acetyl-CoA levels, induced by the introduction of putative sigma factors from cyanophages into *S. elongatus* PCC 7942. We selected four cyanophage-derived putative sigma factors from marine *Synechococcus* phages S-CBS1, S-CSB2, and S-CBS3, which are not known to infect the freshwater model strain *S. elongatus* PCC 7942 and do not possess other sugar metabolic enzyme-encoding genes, such as *zwf*, *gnd*, and *talC*. The expression levels of these putative sigma factors were tightly controlled using a theophylline-dependent riboswitch. First, we investigated the change in the expression of sugar catabolism genes and determined the intracellular metabolic profile induced by the overexpression of each putative sigma factor in *S. elongatus* PCC 7942. Next, we overexpressed these putative sigma factors with phosphate acetyltransferase to know the effects of these sigma factors to improve the bioproduction of acetate as a model bioproduct.

MATERIALS AND METHODS

Chemicals and reagents All chemicals were purchased from Sigma−Aldrich (St. Louis, MO, USA) or 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. ReverTra Ace qPCR RT kit and THUNDERBIRD SYBR qPCR Master Mix (Toyobo Co., Ltd.) were used for the analysis of mRNA expression by quantitative real-time PCR (RT-qPCR). Oligonucleotides were synthesized by Life Technologies Japan, Ltd. (Tokyo, Japan).

Culture media Modified BG11 medium supplemented with HEPES buffer for pH stabilization as previously reported (19), and hereafter referred to as BG11 medium, was used for cultivation of *S. elongatus* PCC 7942 (wild-type strain TA1297) (Life Technologies Corporation, Carlsbad, CA, USA). 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 medium as appropriate.

Growth 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, Osaka, Japan). Photon flux density was measured with IKS-27 (Koito, 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 medium in a 50-mL flask and incubated under fluorescent light on a rotor shaking at 150 rpm (NR-30 shaker, Taitec, Saitama, Japan). For determining the influence of sigma factor on cell growth and acetate production, pre-cultured cells at an OD_{730} of 1.0−2.0 were inoculated into 20 mL BG11 medium at an initial OD_{730} of 0.025 with 2 mM or 1 mM theophylline and 1 mM IPTG. Cultures were grown under the same conditions as those used for pre-culture. For measurements of intracellular metabolites and glycogen concentrations and RT-qPCR, pre-cultured cells at an OD_{730} of 1.0−2.0 were inoculated into 50 mL BG11 medium at an initial OD_{730} of 0.025 with 1 mM theophylline and 1 mM IPTG. Main cultures for metabolome analysis and RT-qPCR were grown under continuous aeration with air containing 1% carbon dioxide at 1.0 vvm (volume per volume per minute). To prevent evaporation during aeration, ambient carbon dioxide-mixed air was passed through MilliQ-water.

Selection of cyanophage-derived putative sigma factors Based on the NCBI's genetic information on cyanophages that do not have genes similar to host catabolism genes but only have putative sigma factors (NCBI, http://www.ncbi.nlm.nih.gov/genbank/), four genes were selected for use in this study: putative *rpsD4* (accession no. YP_004934627) from *Synechococcus* phage S-CBS1, putative *rpoD* (accession no. YP_004421515) and putative *rpoS* (accession no. YP_004421531) from S-CBS2, and putative *rpsD4* (accession no. YP_004421751) from S-CBS3. Genes codon-optimized for *E. coli* were synthesized for the four selected cyanophage-derived putative sigma factors (GenScript USA Inc., Piscataway, NJ, USA) (35) (Table S1).

***Ptrc*::*Riboswitch E**::*sigma factor* plasmid** The plasmids and strains constructed in this study are listed in Table S2. In cyanobacteria, leaky gene expression without induction has been reported in many cases under the control of only a promoter with an operator sequence and a repression protein (37). Because it was predicted that the intracellular metabolism would be dramatically modified by the expression of the cyanophage-derived putative sigma factors, it was desirable to control not only gene expression but also the translation of each expressed gene. In order to control the expression and translation of these putative sigma factors, the *Ptrc* promoter was used to control the level of transcription, and a theophylline-dependent riboswitch was used to control the level of translation. The *Ptrc* promoter is comprised of a promoter sequence and a LacI binding site for controlling gene expression via the addition of IPTG. The riboswitch (*Riboswitch E**) is composed of a stem-loop mRNA stabilizing sequence, a theophylline aptamer sequence, and an Shine−Dalgarno (SD) sequence. The *Ptrc* promoter and *Riboswitch E** without SD sequence were common to all of the constructed plasmids (38) (Fig. 1A). In addition, a silent mutation was introduced upstream of the structural genes for stable control by the inserted stem-loop riboswitch (Fig. 1B).

The common region from *Ptrc* to the stem-loop mRNA stabilization sequence was synthesized and amplified by PCR using two overlapping primers, T2140 and T2141 (Fig. 1A, Table S3). The fragment was digested and ligated into the *Xho*I−*Acc65*I site of pTA43, creating a common *Ptrc* promoter plasmid (pR0). The aptamer and SD sequence, including the upstream region of each structural gene, were synthesized as an oligonucleotide (Table S3). Each oligonucleotide and structural gene was linked by splicing by overlap extension (SOE)-PCR. The linked fragment was digested and ligated into the *Acc65*I−*Bam*HI site of pR0 to create each plasmid series as described below. Neutral site (NS), NS I- and NS II-targeting plasmids (pTA371 and pTA424) were derived from a previous study (19).

pTA1314 (*Ptrc*::*Riboswitch E**::*S-CBS1-rpsD4*, NS II-targeting plasmid), the aptamer region and *S-CBS1-rpsD4* structural gene were linked by SOE-PCR. The linked fragment was amplified using primers T2142-2148 and a mixture of T2144 (oligonucleotide) and pTA1215 (structural gene) as a template. The amplified sequence was digested and ligated into the *Acc*65I−*Bam*HI site of pR0 to create pR1 (*Ptrc*::*Riboswitch E**::*S-CBS1-rpsD4*). To introduce the silent mutation, *rpsD4* was amplified from pR1 using primers T2142-T2145 and T2146-T2148. The two PCR products were then purified and mixed. Modified *rpsD4* was amplified from the mixed products using primer T2142-T2148 by SOE-PCR. The amplified SOE product was digested and ligated into the *Acc*65I−*Bam*HI site of pR0 to create pTA1310. For creating the NS II-targeted plasmid, modified *rpsD4* with the *Ptrc*::*Riboswitch E** region was amplified by T2254-T2148 using pTA1310 as a template. The amplified sequence was digested and ligated into the *Aat*II−*Bam*HI site of pTA424 to create pTA1314.

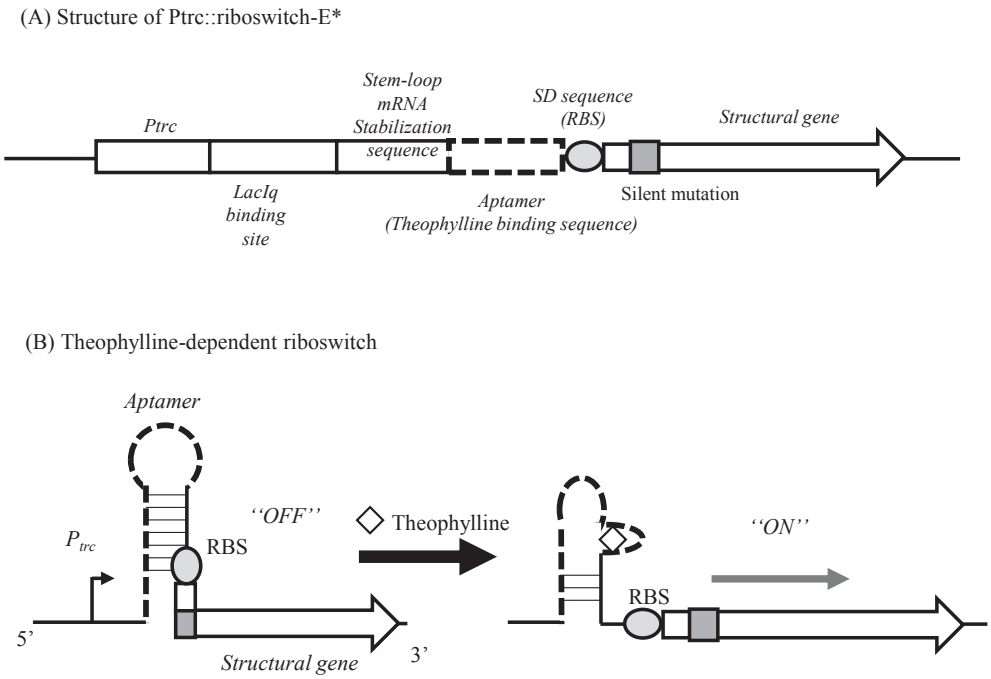

FIG. 1. Construction and control mechanism of the *Ptrc*::*Riboswitch E**::*sigma factor*. (A) Structure of the theophylline-dependent riboswitch. The theophylline-dependent riboswitch was constituted from an mRNA stabilizing sequence (stem-loop stabilization sequence), a theophylline aptamer binding sequence, and a Shine–Dalgarno (SD) sequence (ribosome binding site; RBS) for translational regulation. The promoter region was constituted from a *Ptrc* promoter with a LacI binding site for transcriptional regulation. (B) Mechanism of control by the theophylline-dependent riboswitch. Translational "on" or "off" switching is dependent on the presence of theophylline. The SD sequence is hidden and unable to bind to the site due to the secondary structure of the riboswitch. In the presence of theophylline, the binding of theophylline to the aptamer region leads to a conformational change in the secondary structure. The ribosome can then translate the downstream gene by binding to the exposed RBS.

pTA1315 (*Ptrc*::*Riboswitch E**::*S-CBS2-rpoD*, NS II-targeting plasmid) was constructed using the method described above. In brief, the linked fragment was amplified using primers T2142-2155 with a mix of T2151 and pTA1216 as template. The amplified sequence was digested and ligated into pR0 to create pR2, as stated above. To introduce the silent mutation, modified *rpoD* was amplified from the mixed PCR products (T2142-T2152 and T2153-T2155, amplified from pR2) using primer T2142-T2155. The amplified SOE product was digested and ligated into pR0 to create pTA1311. For creating the NS-II-targeted plasmid, pTA1315 was created from pTA1311 using primer T2254-T2155 and was then digested and ligated into pTA424.

pTA1316 (*Ptrc*::*Riboswitch E**::*S-CBS2-rpoS*, NS II-targeting plasmid) was constructed using the method described above. In brief, the linked fragment was amplified using primers T2142-T2164 with a mixture of T2158 and pTA1217 as template. The amplified sequence was digested and ligated into pR0 to create pR3, as stated above. To introduce two silent mutations, modified *rpoS* was obtained by two rounds of SOE-PCR. The first point mutation in *rpoS* was amplified from the mixed products (T2142-T2160 and T2159-T2164, amplified from pR3) using primer T2142-T2164. The product was digested and ligated into pR0 to create pR3'. Finally, modified *rpoS* was amplified from the mixed PCR products (T2142-T2162 and T2161-T2164, amplified from pR3') using primer T2142-T2164. The amplified SOE product was digested and ligated into pR0 to create pTA1312. For creating the NS-II-targeted plasmid, pTA1316 was created from pTA1312 using primer T2254-T2164 and was then digested and ligated into pTA424.

pTA1317 (*Ptrc*::*Riboswitch E**::*S-CBS2-rpsD4*, NS II-targeting plasmid) was constructed using the method described above. In brief, the linked fragment was amplified using primers T2142-T2171 with a mixture of T2167 and pTA1218 as template. The amplified sequence was digested and ligated into pR0 to create pR4, as stated above. To introduce the silent mutation, modified *rpsD4* was amplified from the mixed PCR products (T2142-T2169 and T2168-T2171, amplified from pR4) using primer T2142-T2171. The amplified SOE product was digested and ligated into pR0 to create pTA1313. For creating the NS-II-targeted plasmid, pTA1317 was created from pTA1313 using primer T2254-T2171 and was then digested and ligated into pTA424.

pTA1377 ($P_L$lacO1:ptaOptC, placIq: lacI, NS I-targeting plasmid), the *pta* gene derived from *E. coli* BW25113 was codon-optimized and synthesized (GenScript, Piscataway, NJ, USA) for efficient heterologous expression in *S. elongatus* PCC 7942. This gene was amplified from the synthetic gene using primer T2266-T954. The PCR products were digested and ligated into the *Avr*II–*Bam*HI site of pTA371 to create pTA1377.

Strain construction Genes were integrated into the *S. elongatus* PCC 7942 genome (TA1297) by homologous recombination. For gene integration into the NS I (39) and NS II (40) regions, both previously constructed and newly generated plasmids were used. All of the integrated genes added to the engineered strains in this study were sequenced for confirmation.

Measurement of intracellular metabolites and glycogen The intracellular metabolites of TA1297 (wild-type strain), TA2767 (overexpressing *S-CBS2-rpoD*) and TA2769 (overexpressing *S-CBS3-rpsD4*) cultured for 3 days were measured using a liquid chromatography with triple-quadrupole mass spectrometry (LC-QqQ-MS) high-performance liquid chromatography system (Agilent 1290 Infinity, MS: Agilent 6460 with Jet Stream Technology, Agilent Technologies, Waldbronn, Germany) controlled by MassHunter Workstation Data Acquisition software (Agilent Technologies). Sample preparation and LC-QqQ-MS analysis were performed as reported previously (41). Metabolites that were significantly increased or decreased in TA2767 and TA2769 were defined as those with a fold change $\geq$1.5 (i.e., $\leq$0.66 or $\geq$1.5 relative value) compared with the level in TA1297.

The glycogen concentrations of TA2767 and TA2769 cultured for 5 days were measured according to a previous method (42). Equal cell amounts (5 mL cell culture with OD_{730} = 1.0) were collected by centrifugation of the culture at 20,000× *g* for 10 min at 4°C. The glucose concentration in each sample was measured using a Biosensor BF-7 (Oji Scientific Instruments, Amagasaki, Hyogo, Japan).

Product concentration analysis The culture supernatant of TA2954 (wild-type strain TA1297 overexpressing *pta*), TA3198 (TA2767 overexpressing *S-CBS2-rpoD* and *pta*) and TA3200 (TA2769 overexpressing *S-CBS3-rpsD4* and *pta*) were centrifuged at 20,000× *g* for 10 min and filtered using a Minisart RC4 (Sartorius, Goettingen, Germany). The acetate concentration of the filtered supernatant was analyzed using a high-performance liquid chromatography system, as described previously (21).

RNA isolation and RT-qPCR Total RNA was isolated from TA1297 (wild-type strain), TA2767 (overexpressing *S-CBS2-rpoD*) and TA2769 (overexpressing *S-CBS3-rpsD4*) using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Cells were collected from each strain at 6 h after the addition of inducers (1 mM theophylline and 1 mM IPTG) by centrifugation of the culture at 20,000× *g* for 10 min at 4°C. The RNA concentration of each sample was confirmed using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

cDNA was synthesized from 2 μg total RNA using the ReverTra Ace qPCR RT kit. RT-qPCR was performed using Thunderbird SYBR qPCR Master Mix according to the manufacturer's instructions using the primers listed in Table S4. The quantification cycle (Cq) values for all transcripts in each sample were determined by real-time PCR on a LightCycler 96 (Roche). The expression level of *rnpB* (encoding RNaseP subunit B) was used as an internal standard, as in a previous report (43). Genes that were up- or downregulated in TA2767 and TA2769 were defined as those with a fold change $\geq$1.5 (i.e., $\leq$0.66 or $\geq$1.5 relative value) compared with levels in TA1297.

RESULTS

Cell growth of strains overexpressing cyanophage-derived putative sigma factors The growth of strains TA2766 (overexpressing *S-CBS1-rpsD4*), TA2767 (overexpressing *S-CBS2-rpoD*), TA2768 (overexpressing *S-CBS2-rpoS*), TA2769 (overexpressing *S-CBS3-rpsD4*), and TA1297 (wild-type strain) was compared with or without the addition of the inducers 2 mM theophylline and 1 mM IPTG (Fig. 2). Data in this and all following figures are represented as means ± S.D. of three individual experiments. When grown without the inducers, all strains with an integrated cyanophage-derived putative sigma factor showed almost the same growth as TA1297 (Fig. 2B–E). Moreover, TA2766 and TA2768 also showed almost the same growth as TA1297 even in the presence of the inducers (Fig. 2A, B, D). In contrast, considerable growth inhibition was observed in TA2767 and TA2769 in the presence of the inducers (Fig. 2C, E).

Intracellular metabolite concentration The intracellular metabolite concentrations of TA2766 (overexpressing *S-CBS1-*

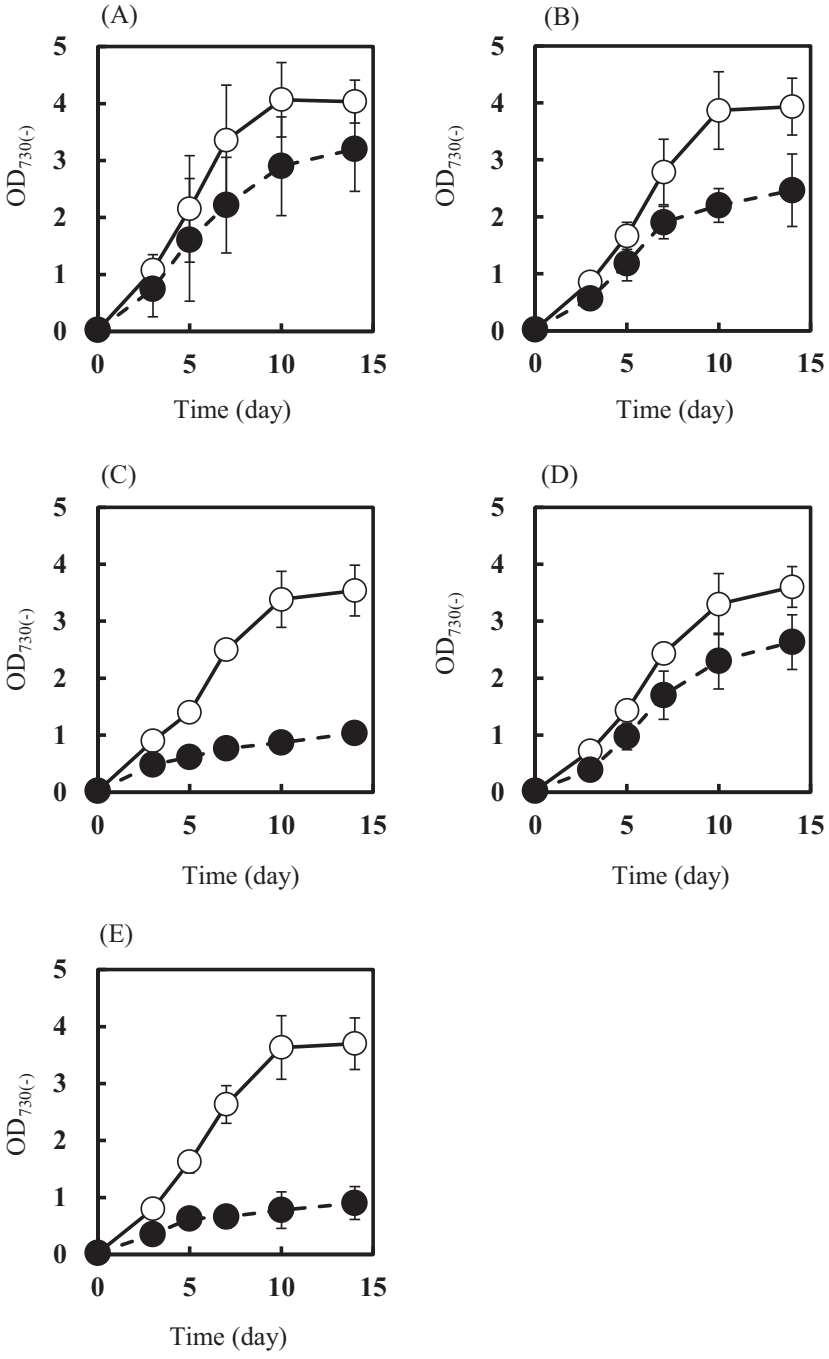

FIG. 2. Growth profiles of wild-type strain (TA1297) and engineered strains (TA2766, TA2767, TA2768, and TA2769). Open circles and solid lines represent data observed in BG11 culture without inducers. Closed circles and dotted lines represent data observed in BG11 culture with inducers (2 mM theophylline and 1 mM IPTG). (A) TA1297 (wild-type strain); (B) TA2766 (*S-CBS1-rpsD4*); (C) TA2767 (*S-CBS2-rpoD*); (D) TA2768 (*S-CBS2-rpoS*); (E) TA2769 (*S-CBS3-rpsD4*). Cell growth was determined at 730 nm (OD_{730}). Data are represented as means ± S.D. of three individual experiments.

rpsD4), TA2767 (overexpressing *S-CBS2-rpoD*), TA2768 (overexpressing *S-CBS2-rpoS*), TA2769 (overexpressing *S-CBS3-rpsD4*), and wild-type strain TA1297 were measured with and without inducers after 3 days (72 h) of culture at which point sufficient time had passed to allow changes in the metabolites following induction. For each metabolite, the ratio of the concentration in each strain in the presence of inducers to that in the absence of inducers was calculated. For measuring the intracellular metabolite concentration, the concentration of theophylline was modified to half that used in the cell growth comparison above. This is because the growth of TA1297 under continuous aeration was markedly inhibited by the addition of 2 mM theophylline, but there was no inhibition observed with the addition of 1 mM (Fig. S3). Therefore, the following experiments were investigated by induction using 1 mM theophylline and 1 mM IPTG. As the results of cultivation for TA2766, and T2768, there was no marked difference in the cell growth with and without inducers (data not shown). In contrast, the concentrations of metabolites derived from sugar catabolism and the Calvin cycle were increased in strains TA2767 and TA2769 with the addition of the inducers. This suggests that the cyanophage-derived putative sigma factors expressed in these two strains modify the host metabolism and suppress cell growth (Figs. 2C, E, and Fig. 3). Marked increases of 1.6-fold and 1.5-fold in glucose-1-phosphate (G1P), 3.3-fold and 1.3-fold in glucose 6-phosphate (G6P), 2-fold and 2-fold in fructose 6-phosphate (F6P), 2.4-fold and 1.6-fold in erythrose 4-phosphate (E4P), 3.9-fold and 6.5-fold in xylulose-5-phosphate (X5P), were observed in strains TA2767 and TA2769, respectively, with induction (Figs. 3 and S1). In particular, the concentration of acetyl-CoA produced via sugar catabolism was increased 3.2-fold and 1.9-fold in TA2767 and TA2769, respectively, with induction. In addition, other metabolites of the tricarboxylic acid (TCA) cycle, including citrate, fumarate, and malate, were increased 1.4-fold, 1.2-fold, and 1.7-fold, respectively, in TA2769 with induction. In contrast, metabolites of the TCA cycle showed almost no change in TA2767 after the addition of the inducers, same as those in TA1297 (Fig. S1). It was clear that in TA2767, the concentrations of metabolites pyruvate and R5P were decreased and increased by induction, respectively, whereas in TA2769, metabolites derived from the TCA cycle including sugar catabolism were increased by induction. These results suggest that these two putative sigma factors have different control mechanisms. The subsequent analyses were carried out only in TA2767 and TA2769, because TA2766 and TA2768 did not show marked differences in cell growth or metabolite concentrations after induction.

Glycogen measurements Accumulated glycogen levels at 5 days were measured in TA1297 (wild-type strain), TA2767 (overexpressing *S-CBS2-rpoD*) and TA2769 (overexpressing *S-CBS3-rpsD4*) cultures with or without inducers. As shown in Fig. 4, the glycogen level per cell of TA2767 was increased 1.5-fold with induction, while that of TA2769 was decreased to 0.5-fold with induction. In the wild-type strain TA1297, there was no significant difference in glycogen content per cell before and after induction.

RT-qPCR analysis RT-qPCR was carried out in order to investigate the expression levels of genes associated with glycogen metabolism and sugar catabolism in TA2767 (overexpressing *S-CBS2-rpoD*) and TA2769 (overexpressing *S-CBS3-rpsD4*). In RT-qPCR analysis, some gene expression levels in both strains showed large changes after 6 h of induction (Figs. 3 and S2). For each sample, we calculated the expression ratio of each gene with versus without inducers (Fig. S2).

In TA2767, the only increase in transcription observed was a 1.5-fold increase in *gnd* (expression ratio, 1.4) at 6 h after inducer addition compared with the level in the wild-type strain, TA1297 (0.9). Decreases in transcription were more common in TA2767, with the transcription of the glycogen metabolism genes *glgX* and *glgP* decreasing to about 0.4 and 0.2, respectively, at 6 h after induction and the transcription of *zwf*, *talC*, and *gpmB*, associated with sugar catabolism, decreasing to about 0.1, 0.3, and 0.3, respectively (reference values in TA1297: *glgX*, 0.6; *glgP*, 0.6; *zwf*, 0.4; *talC*, 0.5; *gpmB*, 0.7). Thus, in TA2767, induction resulted in the downregulation of sugar catabolism and glycogen metabolism genes, consistent with the increase in the glycogen concentration in this strain after induction. In contrast, in TA2769, marked increases in transcription at 6 h after induction were observed in *glgX* (2.7), *glgP* (2.6), *zwf* (1.7), *talC* (1.4), and *gpmB* (2.0) (Fig. S2). These results support the decrease in the glycogen concentration observed in TA2769. Consequently, these results strongly suggest that transfection with cyanophage S-CBS2 or S-CBS3 considerably modifies the host metabolism. These findings also indicate that the sigma factor RpoD from S-CBS2, introduced into TA2767, negatively controls host sugar catabolism genes, whereas RpsD4 from S-CBS3, introduced into TA2769, positively controls these genes.

Acetic acid production by strains transfected with cyanophage-derived putative sigma factors and a gene for acetic acid production The concentration of acetyl-CoA was increased in TA2767 (overexpressing *S-CBS2-rpoD*) and TA2769 (overexpressing *S-CBS3-rpsD4*) by induction, suggesting that these strains could be used with synthetic metabolic pathways to increase the production of chemicals from intracellular acetyl-CoA compared to that in the wild-type strain. In order to confirm this hypothesis, acetate was selected as a model target to be produced using intracellular acetyl-CoA. For this purpose, strains TA2954 (TA1297 wild type with *pta*), TA3198 (TA2767 with *pta*), and TA3200 (TA2769 with *pta*) were created by inserting the *E. coli pta* gene into the NS I site of TA1297, TA2767, and TA2769, respectively.

There was no remarkable difference in cell growth of TA2954 with and without inducers. In TA3198 and TA3200, these cell growths also showed a similar tendency with and without inducers, respectively. TA2954, TA3198, and TA3200 produced 0.52, 0.0, and 1.13 mM acetate (0.14, 0.0, 0.33 mM/OD_{730}), respectively, at 16 days after the addition of inducers Fig. 5. It should be noted that without introduction of the *pta* gene, wild-type strain TA1297, TA2767, and T2769 did not produce acetate with the addition of the inducers (data not shown). While both TA2767 and TA2769 exhibited higher concentrations of acetyl-CoA than wild-type strain TA1297, TA3200 produced about two times more acetate, whereas TA3198 produced no acetate. One reason for this result is that the RpoD from S-CBS2, introduced into TA2767, negatively controls host sugar catabolism genes, whereas the RpsD4 from S-CBS3, introduced into TA2769, positively controls these genes, as mentioned above.

DISCUSSION

Several proteins have been identified as factors in the proper regulation of sugar metabolism in cyanobacteria, including the sigma factor SigE, the two-component control system histidine kinases Hik8 and Rre37, PII signal transduction factors, and the transcription factor NtcA (32,44,45). However, no study has yet

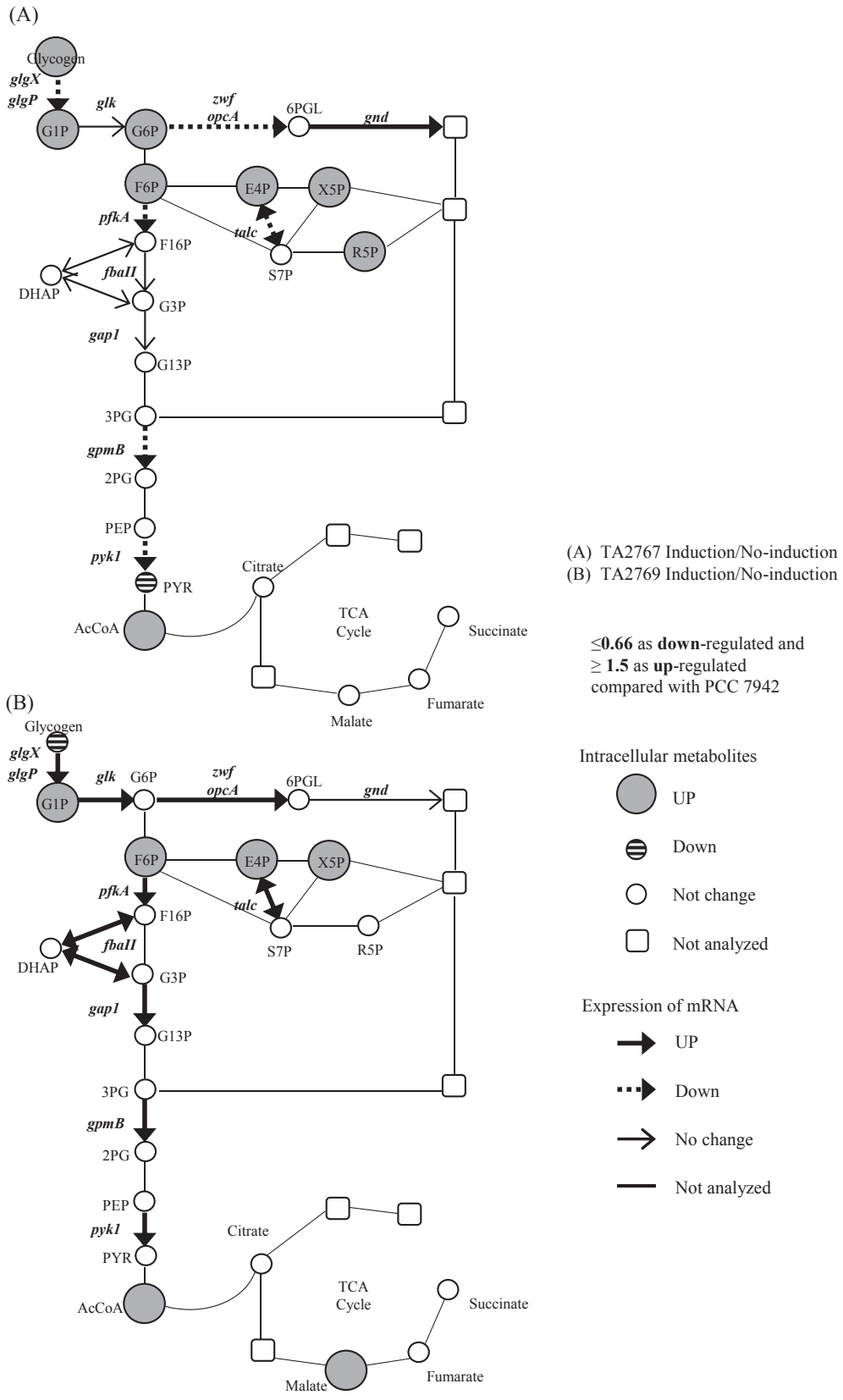

FIG. 3. Schematic showing metabolic changes in engineered strains after induction. Hatched circles and dotted lines indicate downregulation of metabolite concentration and mRNA expression, respectively (≤0.66). Shaded circles and solid lines indicate upregulation of metabolite concentration and mRNA expression, respectively (≥1.5). Open circles and thin lines indicate no change or not examined. This figure is a summary of Figs. S1 and S2. (A) TA2767 (*S-CBS2-rpoD*); (B) TA2769 (*S-CBS3-rpsD4*). Three individual experiments were performed with or without inducer (1 mM theophylline and 1 mM IPTG). G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; 6PGL, 6-phosphogluconolactonase; F6P, fructose-6-phosphate; 6PG, 6-phosphogluconate; E4P, erythrose 4-phosphat; X5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose-7-phosphate; F16P, fructose-1,6-bisphosphate; S7P, sedoheptulose-7-phosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde-3-phosphate; G13P, glycerate 1-3-phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; AcCoA, acetyl-CoA.

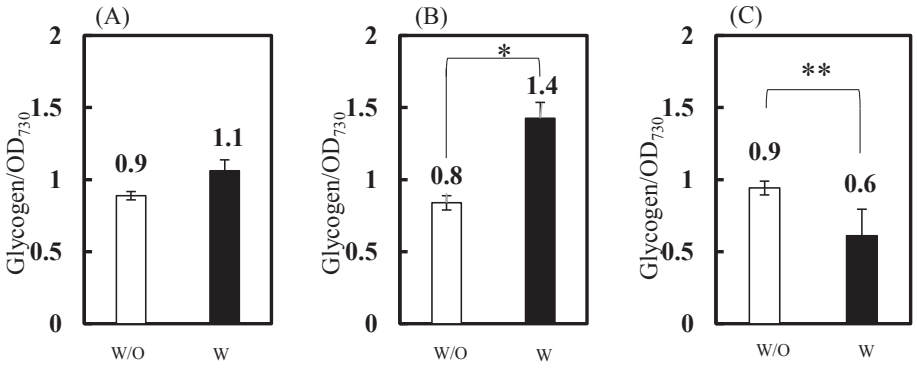

FIG. 4. Quantitative analysis of glycogen concentrations in engineered strains. Open and closed bars indicate values in 5-day culture without (W/O) or with (W) inducers (1 mM theophylline and 1 mM IPTG), respectively. (A) TA1297 (wild-type strain); (B) TA2767 (*S-CBS2-rpoD*); (C) TA2769 (*S-CBS3-rpsD4*). Data are represented as means ± S.D. of three individual experiments. One and two asterisks would mean that the *p*-value was under 0.05 ($P < 0.05$) and 0.1 ($P < 0.1$), respectively (two-tailed test).

explored the modification of cyanobacterial metabolism with cyanophage-derived putative sigma factors. After infection, cyanophages modify the host metabolism and employ it for their own propagation. In particular, it is predicted that cyanophages that do not possess genes highly homologous to metabolic genes and instead only possess putative sigma factor genes will dramatically modify the host metabolism in order to use it for their own propagation. In this study, four putative sigma factors from cyanophages were selected, and the metabolism of *S. elongatus* PCC 7942 was modified by the introduction of two of these putative sigma factors under the control of a theophylline-dependent riboswitch.

In TA2767, the overexpression of a cyanophage-derived putative sigma factor RpoD from cyanophage S-CBS2 suppressed the transcription of almost all metabolic genes measured in this study, including a marked decrease in the transcription of *glgP* (0.2) and *zwf* (0.1) at 6 h after induction (Fig. S2). The consumption of the energy storage substance glycogen was also reduced. In addition, in TA3198 (TA2767 with *pta*), acetate was not produced with induction (Fig. 5B). This strongly suggests that the putative RpoD from S-CBS2 is a sigma factor that negatively controls the transcription of sugar metabolism and glycogen metabolism genes. One of hypotheses explaining why the acetyl-CoA concentration in TA2767 increased despite suppression of glycogen and sugar metabolic catabolic genes might be a decrease in metabolic flux from acetyl-CoA to citrate. In addition, expression of the putative RpoD from S-CBS2 resulted in a marked increase in the concentration of R5P (2.2-fold), and it is possible that this is used in the synthesis of phage nucleic acids.

As in TA2767, marked differences in cell growth and the concentrations of intracellular metabolites were also observed after induction in TA2769, in which a putative RpsD4 from S-CBS3 was overexpressed. However, while the expression level of *gnd* was not significantly altered by induction in this strain, marked increases in transcription were observed in *zwf*, *talC*, *opcA*, and *gpmB*, which are associated with the oxidative pentose phosphate pathway. We speculate that these increases in transcription resulted in a decrease in the glycogen content per bacterial cell and increases in the concentrations of intracellular metabolites. By overexpression of a putative sigma-factor RpsD4 from cyanophage S-CBS3, especially, malate in TCA cycle, and acetyl-CoA was accumulated. This accumulation could be beneficial for protein synthesis of SCBS3. In addition, although it is unclear why there was no significant difference in acetate productions on TA2954 (wild-type strain TA1297 with *pta*) and TA3200 (TA2769 with *pta*) until 12 days, TA3200 produced two times more acetate than TA2954 at 16 days after the addition of inducers (Fig. 5A, C). It has already been reported that a strain of *Synechocystis* sp. PCC 6803 overexpressing SigE exhibited the positive regulation of genes associated with sugar metabolism, such as *zwf* and *opcA* (45). In the presence of an adequate source of nitrogen, after 24 h, the strain overexpressing SigE showed increases of 1.2-fold each in the transcription of the glycogen metabolism genes *glgP* (sll1356) and *glgX* (slr0237) compared with levels in the wild-type strain; similarly, increases were observed in the transcription of genes in the pentose phosphate pathway, including increases of 1.4-fold for *zwf*, 2.2-fold for *gnd*, and 1.6-fold for *talC* and *opcA* (45). Our findings suggest that the putative RpsD4 from S-CBS3 is a sigma factor that positively controls sugar-metabolizing genes in *S. elongatus* PCC 7942, controlling gene transcription in a similar way to that observed in strains of *Synechocystis* sp. PCC 6803 that overexpress SigE. As a future study, it is interesting to compare the acetate productivity of strains with overexpression of cyanophage-derived sigma factors and SigE.

In this study, we focused on the strains TA2767 (overexpressing *S-CBS2-rpoD*) and 2769 (overexpressing *S-CBS3-rpsD4*) with a drastic change in growth and intracellular metabolite concentration by over-expression of putative sigma factor. However, there is a possibility that the expression levels of sigma-factors in TA2766 (overexpressing *S-CBS1-rpsD4*) and TA2768 (overexpressing *S-CBS2-rpoS*) were lower than that of TA2767 and TA2769. As a future study, it is interesting to measure expression levels of these genes by RT-qPCR analysis.

To date, no sigma factor that controls several sugar-metabolizing genes, such as SigE belonging to cluster E in *Synechocystis* sp. PCC 6803, has been discovered in *S. elongatus* PCC 7942. However, in this study, we introduced exogenous genes highly homologous to endogenous sigma factors to modify the expression levels of genes related to metabolism and intracellular metabolite concentrations. In particular, we found that a strain overexpressing a putative RpsD4 from S-CBS3 increases intracellular metabolite concentrations, especially of acetyl-CoA, and produces about 2-fold acetate with *pta*. This study is the first example of the modification of the metabolism by the overexpression of a cyanophage-derived putative sigma factor. Our results suggest that the combination of a cyanophage-derived putative sigma factor and a synthetic metabolic pathway from acetyl-CoA may be a powerful strategy for

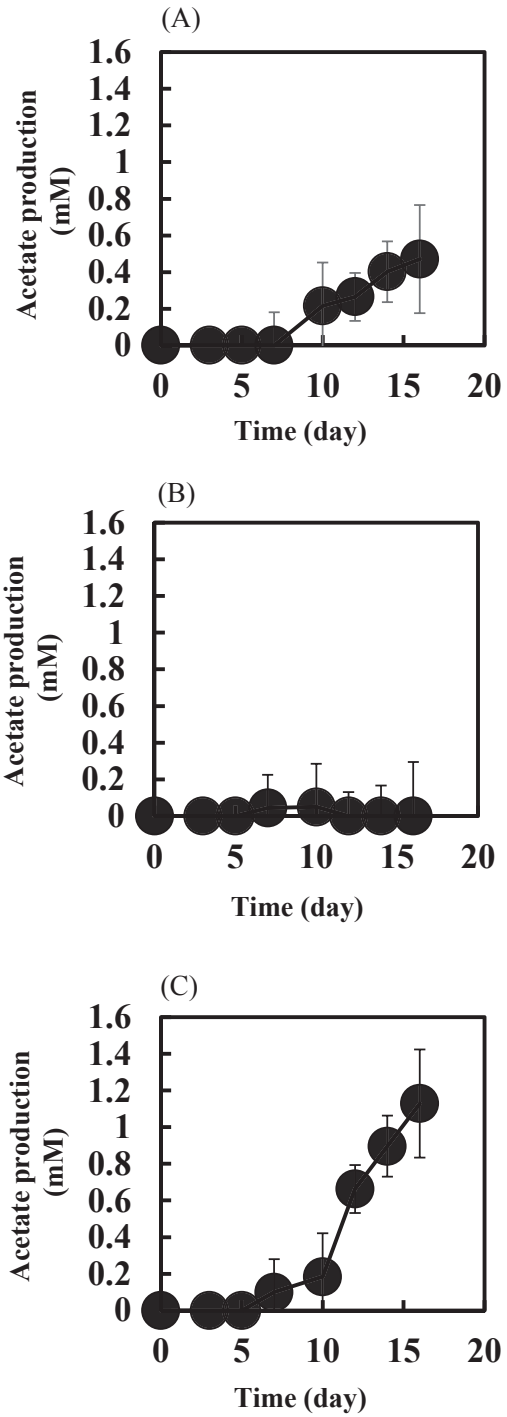

FIG. 5. Acetate production of engineered strains. Acetate titers during 16 days of incubation with inducers (1 mM theophylline and 1 mM IPTG). (A) TA2954 (wild-type strain with *pta*); (B) TA3198 (TA2767 with *pta*); (C) TA3200 (TA2769 with *pta*). Data are represented as means ± S.D. of three individual experiments.

increasing the production and titer of target chemicals using cyanobacteria.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jbiosc.2018.07.019.

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