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Metabolic and Microbial Community Engineering for Four-carbon Dicarboxylic acids Production from CO_2-derived Glycogen in the Cyanobacterium *Synechocystis* sp. PCC6803

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ABSTRACT

The four-carbon (C4) dicarboxylic acids, fumarate, malate, and succinate, are the most valuable targets that must be exploited for CO_2-based chemical production in the move to a sustainable low-carbon future. Cyanobacteria excrete high amounts of C4 dicarboxylic acids through glycogen fermentation in a dark anoxic environment. Enhancement of metabolic flux in the reductive TCA branch in the cyanobacterium *Synechocystis* sp. PCC6803 is a key issue in the C4 dicarboxylic acids production. To improve metabolic flux through anaplerotic pathway, we have created the recombinant strain PCCK, which expresses foreign ATP-forming phosphoenolpyruvate carboxykinase (PEPck) concurrent with intrinsic phosphoenolpyruvate carboxylase (Ppc) overexpression. Expression of PEPck concurrent with Ppc led to an increase in C4 dicarboxylic acids by autofermentation. Metabolome analysis revealed that PEPck contributed to an increase in carbon flux from hexose and pentose phosphates into the TCA reductive branch. To enhance the metabolic flux in the reductive TCA branch, we examined the effect of corn-steep liquor (CSL) as a nutritional supplement on C4 dicarboxylic acids production. Surprisingly, the addition of sterilized CSL enhanced the malate production in the PCCK strain. Thereafter, the malate and fumarate excreted by the PCCK strain are converted to succinate by the CSL-settling microorganisms. Finally, high-density cultivation of cells lacking the acetate kinase gene showed the highest production of malate and fumarate (3.2 g/L and 2.4 g/L with sterilized CSL) and succinate (5.7 g/L with non-sterile CSL) after 72 h cultivation. The present microbial community engineering is useful for succinate production by one-pot fermentation under dark anoxic conditions.

Keywords: Cyanobacteria, Anoxic Fermentation, C4-dicarboxylic acid, C4-dicarboxylic acid antiporter, microbial community

1. INTRODUCTION

The four carbon (C4) dicarboxylic acids succinate, fumarate, and malate are listed as the 12 most important building block chemicals in biorefineries by the U.S. Department of Energy[1]. Succinate is used as a raw material for the production of many important chemicals, including biodegradable polymers, such as polybutylene succinate and polyurethanes[2,3]. Fumarate and malate are valuable platform chemicals in the food industry used in the synthesis of biodegradable plastics and plasticizers[4,5]. The production of C4 dicarboxylic acids from biomass by microbial fermentation has been extensively studied in natural succinate overproducers such as *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, and *Mannheimia succiniciproducens*, or genetically modified model microorganisms *Escherichia coli*, *Corynebacterium glutamicum*, and *Saccharomyces cerevisiae,* achieving high production of a few dozen grams[2,6].

Compared to biomass-based production, CO_2-based chemical production significantly decreases total greenhouse gas emissions through direct carbon capture by CO_2-assimilating autotrophic organisms, such as photoautotrophic microorganisms[7]. Algae-based production lends itself to a sustainable low-carbon future because it grows well using only energy from light and CO_2[8,9]. Cyanobacteria are photosynthetic organisms capable of generating organic compounds from CO_2 through oxygenic photosynthesis. Cyanobacteria are unique in that they catabolize intracellular glycogen to excrete organic acids, including acetate, lactate, and succinate, into the medium during dark, anoxic fermentation[10,11] (Fig. 1). *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) is a unicellular and non-nitrogen-fixing cyanobacterium that is well-characterized in terms of physiology, biochemistry, and genetics[12]. Furthermore, advances in the genetic engineering of *Synechocystis* 6803 have enabled the successful improvement of photosynthetic activity and metabolic engineering for the production of a variety of chemicals[13–15]. Photoautotrophic production of succinate occurs via the oxidative branch[16–18]. Extensive biochemical and comprehensive metabolome analyses have shown that the metabolic flux of the oxidative TCA branch in *Synechocystis* 6803 is relatively low due to inefficient enzyme activity under dark anoxic conditions [10,19–21]. Meanwhile, malate dehydrogenase (MDH, encoded by *citH*) catalyzes the reductive reaction (from oxaloacetate to malate) more efficiently than the oxidative reaction[22,23]. Thus, the TCA branch flux is reversed upon shifting from light aerobic to dark anoxic conditions. Overexpression of the phosphoenolpyruvate carboxylase (Ppc) gene *pepc* or the malate dehydrogenase (MDH) gene *citH* enhances succinate production under dark anoxic

conditions[19,24] (Fig. 1). In addition, succinate production is also upregulated with increasing temperature (an increase from 30 °C to 37 °C)[25]. Up to 50% of the total glycogen accumulated can be converted to C4 dicarboxylic acids through metabolic engineering to promote autofermentation[24]. However, improving the conversion rate of glycogen toward C4 dicarboxylic acids remains challenging.

Phosphoenolpyruvate carboxykinase (PEPck) from the succinate producer *A. succinogenes* is known to catalyze the ADP-dependent conversion of phosphoenolpyruvate (PEP) to oxaloacetate[26–28], the overexpression of PEPck resulted in an increase in biomass and succinate flux of *Escherichia coli* in anaerobic growth[29], suggesting that PEPck expression is useful in redirecting the carbon distribution into the TCA reductive branch during fermentation. Furthermore, it is well known that the addition of nutritional supplements, including vitamins, minerals, and nitrogen sources, such as corn-steep liquor (CSL), enhances succinate production in *A. succinogenes* which utilizes the reductive TCA cycle to produce it[30]. Here, we report novel findings that increase C4 dicarboxylic acids through metabolic engineering of the anaplerotic pathway under anoxic dark fermentation. Specifically, expression of PEPck concurrent with Ppc expression resulted in an increase in the carbon flux of the TCA reductive branch. In addition, sterilized CSL effectively stimulated malate production. Furthermore, CSL-settling bacteria, especially the genus *Escherichia* in the CSL, promoted succinate production from malate and fumarate as carbon sources. It is suggested that microbial community engineering could successfully improve inefficient biomaterial productions by single species system[31]. The present study demonstrates the efficacy of a microbial consortium to increase succinate production from CO_2-derived glycogen in the cyanobacterium.

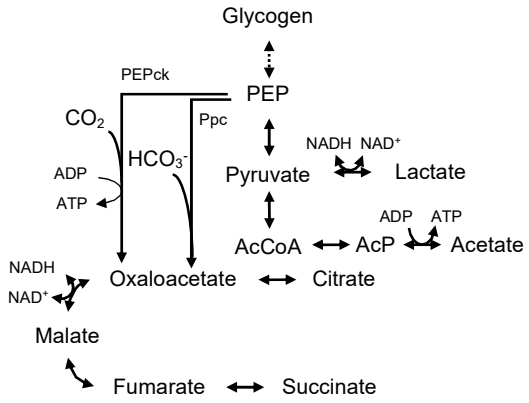


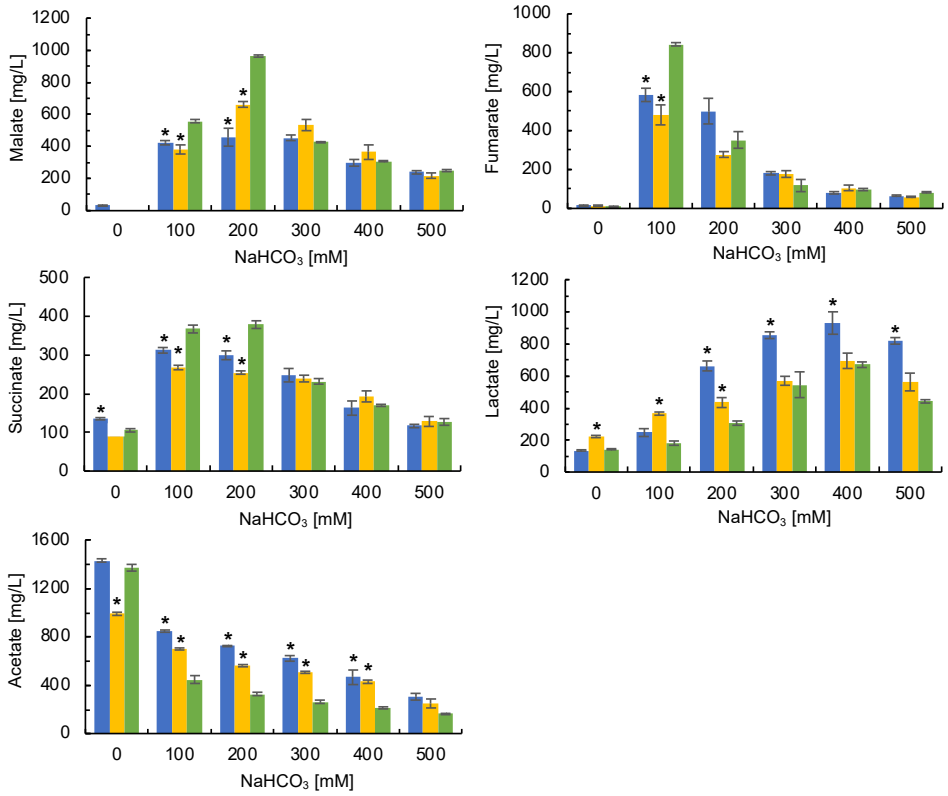
Fig. 1. Glycogen metabolic pathway to C4-dicarboxylic acids in *Synechocystis* 6803. The broken arrow represents pathways in which metabolic intermediates are formed. Abbreviations: PEP, phosphoenolpyruvate; AcP, Acetyl-phosphate; AcCoA, acetyl coenzyme A; PEPck, PEP carboxykinase; Ppc,

PEP carboxylase.

2. RESULTS

2.1. Effect of phosphoenolpyruvate carboxykinase overexpression on organic acid production

To overexpress the *pckA* gene, the gene was introduced by homologous recombination into the *slr0618* loci of the *Synechocystis* Ppc-ox strain. A control strain (CT) was constructed by integrating the chloramphenicol-resistance gene into the loci (Supplemental Fig. S1). PEPck activity was observed in the resultant PCCK strain but not in the Ppc-ox strain. The autofermentation of the resultant PCCK strain was carried out to initiate organic-acid secretion under dark anoxic conditions at 37 °C for 72 h in the presence of each concentration (100–500 mM) of $NaHCO_3$ as substrate for Ppc (Fig. 2). The highest succinate and fumarate production were observed in the PCCK strain treated with 100 mM $NaHCO_3$. Lactate levels increased in all strains in a dose-dependent manner. The lactate level of the Ppc-ox strain was >1.5-fold higher than that of the PCCK and Ppc/CT strains, suggesting that the addition of chloramphenicol contributed to the decrease in lactate levels. The concentrations of malate (557 mg/L), fumarate (843 mg/L), and succinate (368 mg/L) in the PCCK strain were quantitatively higher than those in the Ppc-ox strain, while the concentration of acetate (446 mg/L) was lower than that in the Ppc-ox strain. Expression of PEPck resulted in > 1.3- and 1.4-fold increases in malate and fumarate levels, respectively, at 72 h of fermentation with 100 mM $NaHCO_3$. Malate and succinate levels increased in a time-dependent manner during fermentation, whereas fumarate levels peaked at 72 h of fermentation (Fig. 3). The initial glycogen content before fermentation was comparable between the Ppc-ox and PCCK-ox strains (approximately 45%). Glycogen consumption rates were comparable to each other (18% remaining after 72 h). Overall, the expression of PEPck enhanced the TCA reductive branch by lowering acetate levels.



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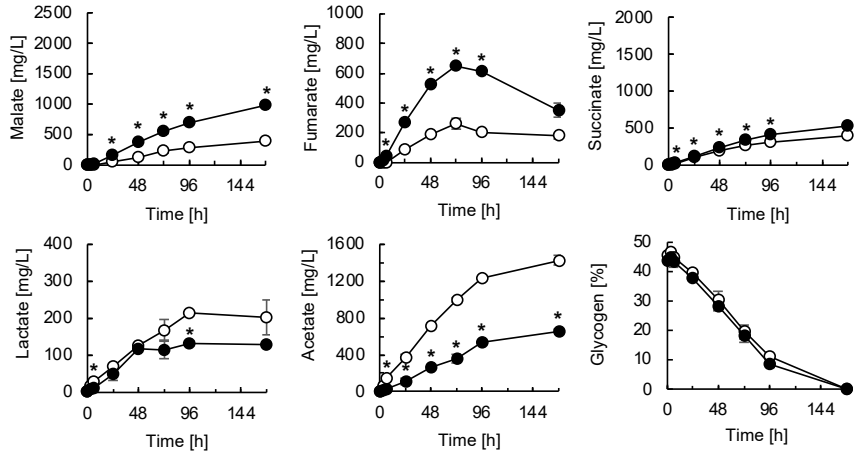
112 **Fig. 2.** C4-dicarboxylic acids, lactate, and acetate concentration in 4 g-DCW/L (equivalent to OD_{730} = 20)
113 of Ppc-ox (blue), Ppc/CT (yellow) and PCCK (green) cells after 72 h fermentation in the presence of each
114 concentration of $NaHCO_3$ under dark anoxic conditions. Values represent the average (± standard deviation)
115 of three biological replicates. Statistical significance over PCCK cells at each $NaHCO_3$ concentration was
116 determined using the Tukey−Kramer test (*$P < 0.01$).



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118 **Fig. 3.** Time-course changes of C4-dicarboxylic acids, lactate, acetate, and glycogen concentrations in 4 g-
119 DCW/L (equivalent to OD_{730} = 20) of Ppc-ox (white circle) and PCCK strains (black circle) under dark

anoxic fermentation with 100 mM $NaHCO_3$. Values represent the average (± standard deviation) of three biological replicates. Statistical significance between Ppc-ox and PCCK strains at each sampling point was determined using the student's *t*-test (*$P < 0.01$).

2.2. Metabolome analysis of the PCCK strain

To investigate the effect of PEPck on primary metabolism in recombinant *Synchocystis* 6803, intracellular metabolites of the cells were analyzed after 3, 6, 24, 48, 72, and 96 h of fermentation. The pool sizes of hexose, pentose, triose phosphates, organic acids, and acetyl-CoA were obtained by calculating their respective peak areas using CE-MS analysis. The pool sizes of hexose phosphates (G1P, G6P, F6P, and FBP), pentose phosphates (Ru5P and RuBP), and other Calvin-Benson-Bassham intermediates (6PG and S7P) in the PCCK cells were significantly lower than those in the Ppc-ox strain during fermentation (Fig. 4A). The triose phosphates PEP, 2PGA, and 3PGA in the Ppc-ox strain increased during 48-96 h of fermentation, peaking at 96 h. In addition, the levels of the metabolites of TCA oxidative branch, iso-citrate, citrate, and *cis*-aconitate, were comparably low during 0-24 h fermentation, while intracellular fumarate and malate accumulated in the PCCK strain during fermentation (Fig. 4B). Citrate transiently accumulated in the Ppc-ox strain during 0-6 h of fermentation. The pyruvate pool was not observed in the PCCK cells during fermentation; however, the pool size of acetyl-CoA was significantly higher than that of the Ppc-ox strain. Oxaloacetate was not determined because of its low abundance by mass spectrometry. We observed low adenosine phosphate levels in the PCCK strain. However, the cellular energy charge, calculated as the ratio of ATP over adenosine 5'-diphosphate (ADP) +ATP, was low in the PCCK strain (0.46 at 3 h and 0.45 at 6 h) compared to those in the Ppc-ox strain (0.62 at 3 h and 0.68 at 6 h) during the early phase of fermentation (0-6 h), associated with the low acetate level. However, it was comparable to each other in the mid- and late phases of fermentation (24–96 h) (Fig. 4C).

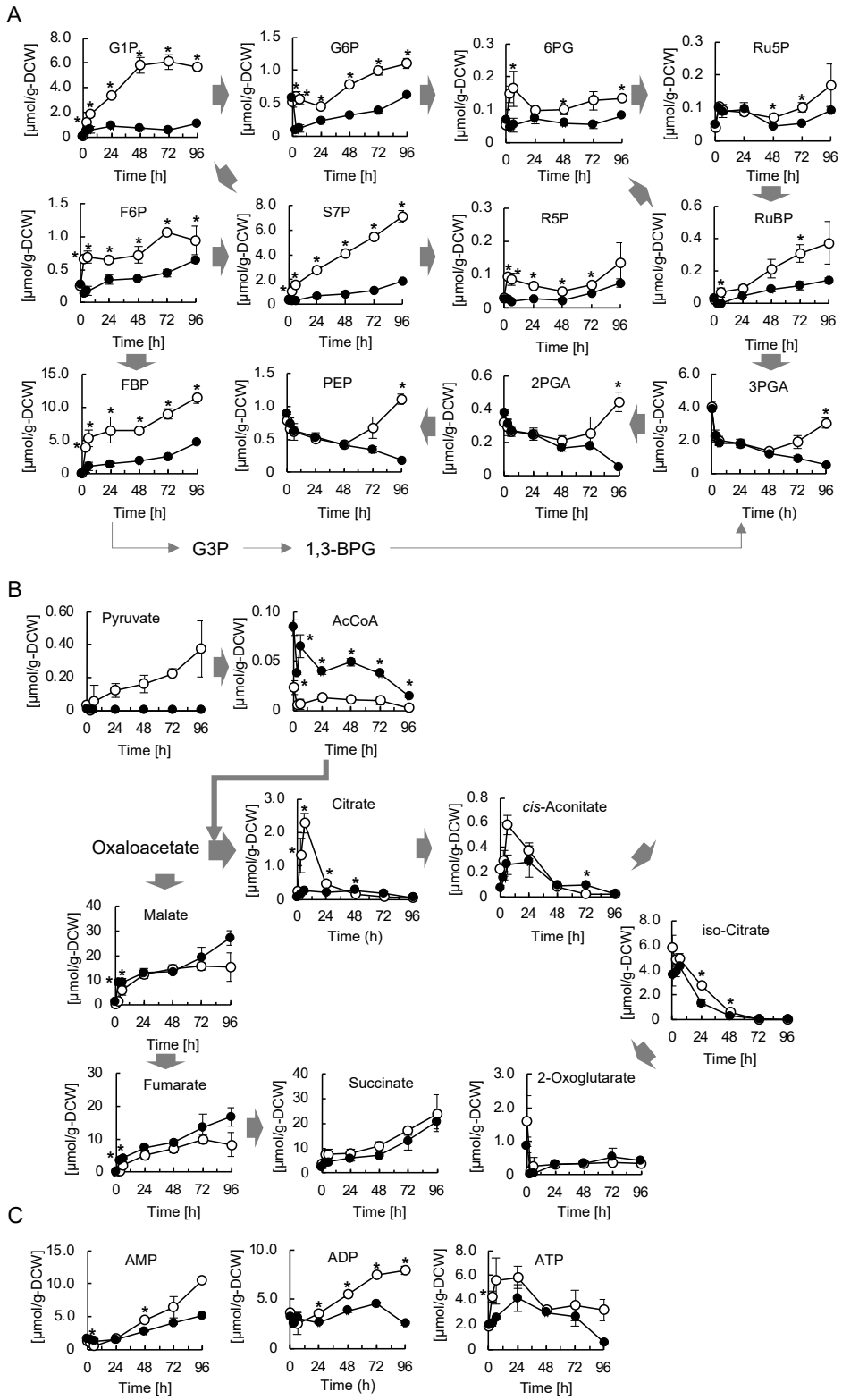


Fig. 4. Time-course changes of metabolites in hexose, pentose, and triose phosphates (A), TCA (B), and AMP, ADP, and ATP (C) in Ppc-ox (white circle) and PCCK strains (black circle) under dark anoxic fermentation with 100 mM $NaHCO_3$. Values represent the average (± standard deviation) of three biological replicates. Statistical significance between Ppc-ox and PCCK strains at each sampling point was determined

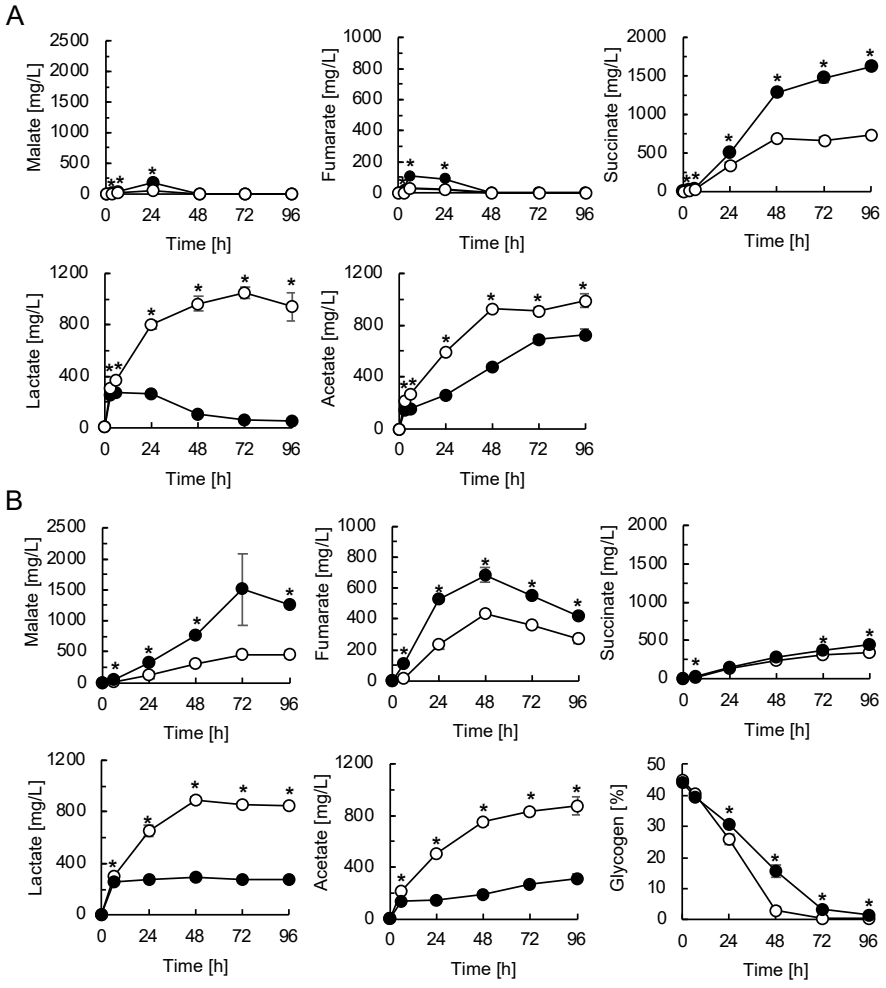
using the student's *t*-test (*P < 0.01).

2.3. Corn-steep liquor promotes production of C4 dicarboxylic acids

PEPck expression contributed to improvements in the production of C4 dicarboxylic acids by debottlenecking PEP metabolism to the reductive TCA branch. However, further improvement is needed to increase C4 dicarboxylic acid productivity with high glycogen consumption. The glycogen consumption rate could be increased by increasing the amount of $NaHCO_3$[19]. However, the carbon from glycogen is directed to lactate production due to insufficient influx into the cyanobacterial open TCA cycle. Nutrient supplementation is thought to enhance the carbon flux into the TCA cycle and encourage amino acid and protein synthesis[30], however the effect of the nutrient supplementation on cyanobacterial fermentation has not been studied. Of these nutrient supplements, CSL commonly known as "condensed fermented corn extractives", which contains mostly organic acids, amino acids, sugars, minerals, and vitamins, has been used as a fermentation nutrient supplement because of its high nutritional value and low cost. We first examined the effect of non-sterile CSL (raw CSL) (Solulys 095E) treated at 37 °C on the recombinant *Synechocystis* fermentation for 72 h at different concentrations of $NaHCO_3$. CE-MS analysis showed that 1 g/L Solulys CSL contained 138.3 mg/L lactate, 11.7 mg/L gluconate, 19.6 mg/L L-alanine, 16.0 mg/L L-leucine, and 6.8 mg/L succinate as major organic acids. As shown in Supplemental Fig. S2, succinate production was highest with 300 mM $NaHCO_3$, but lactate and acetate levels remained low from 100 to 400 mM $NaHCO_3$. Change in the C4 dicarboxylic acid produced over time in the recombinant *Synechocystis* strains fermented with 300 mM $NaHCO_3$ showed that succinate levels reached 1,621 mg/L at 96 h, whereas malate and fumarate were not observed after 48 h of fermentation (Fig. 5A). Next, we examined pretreatment conditions for the preparation of CSL suspensions by altering treatment temperatures (30, 40, 50, 60, and 80 °C) to extract active components as CSL components are not completely solubilized in water. Surprisingly, we observed a temperature-dependent increase in malate, but a decrease in succinate in PCCK autofermentation (Supplemental Fig. S3A). Malate accumulation was observed at higher temperatures. We found the succinate increase in the dark autofermentation with other commercially available CSLs, but no increasing effect on malate, fumarate, and succinate when 1 g/L yeast extract was tested (Supplemental Fig. S4), indicating that replenishing nitrogen sources is not the major reason for C4 dicarboxylic acids increases.

We sterilized by autoclaving and analyzed for C4 dicarboxylic acid production by autofermentation

177 of the PCCK strain with 300 mM $NaHCO_3$ in the presence of sterilized CSL. As expected, accumulation of
178 malate was observed in the PCCK strain when compared to that in the Ppc-ox strain, whereas succinate
179 levels between the PCCK and Ppc-ox strains were comparable (Fig. 5B), suggesting that the unknown active
180 component promoting succinate production was lost during sterilization. Acetate and lactate production was
181 significantly repressed in the PCCK strain compared to the Ppc-ox strain. In contrast to the changes in malate
182 levels, the fumarate levels in the PCCK strain were comparable to those with 100 mM $NaHCO_3$ in the
183 absence of sterilized CSL. Moreover, the glycogen consumption rate in the Ppc-ox strain (3.07 % after 48 h)
184 was higher than that in the PCCK strain (3.25 % after 72 h). This probably reflects the overproduction of
185 lactate and acetate as the final outputs of energy metabolism in the Ppc-ox strain. The activity of malate
186 dehydrogenase (MDH), which catalyzes the NADH-dependent reduction of oxaloacetate to malate, of the
187 PCCK strain in the presence of sterilized CSL was comparable to that in the absence of sterilized CSL
188 (Supplemental Fig. S5A).



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190 **Fig. 5.** Time-course changes of C4-dicarboxylic acids, lactate, acetate, and glycogen concentrations in 4 g-

DCW/L (equivalent to OD_{730} = 20) of Ppc-ox (white circle) and PCCK strains (black circle) under dark anoxic fermentation with raw CSL (A) and sterilized CSL (B) in the presence of 300 mM $NaHCO_3$. Values represent the average (± standard deviation) of three biological replicates. Statistical significance between Ppc-ox and PCCK strains at each sampling point was determined using the student's *t*-test (*$P < 0.01$).

2.4. Identification of causative microorganisms in CSL for succinate production

As mentioned above, some active components contributing to succinate production were susceptible to heat treatment, suggesting that succinate-producing microorganisms might be present in the CSL suspension. We streaked CSL suspensions treated at 37 °C on LB agar plates to isolate microorganisms, which would contribute to the succinate increase. The grown three colonies appeared in the LB agar plate at 37 °C were applied to 16S rDNA analysis to identify related bacteria (Supplemental Fig. S3B). The BLAST search results showed that each colony was derived from the respective strains closely related to the enteric bacteria *Enterobacter hormaechei*, *Escherichia hermannii*, and *Acinetobacter radioresistens* with greater than 99% similarity score. Next, we examined the effect of the addition of standard microorganisms distributed by the public bioresource institute (Biological Resource Center, National Institute of Technology and Evaluation, Japan) on *Synechocystis* fermentation with sterilized CSL (Fig. 6). The addition of *E. hermannii* was most effective for succinate production (1,410 mg/L) without producing malate and fumarate, in addition to the contribution of *E. hormaechei* to succinate production (814 mg/L), implying that several bacterial strains in raw CSL including *E. hormaechei*, contributed to succinate production (1,788 mg/L). The addition of *A. radioresistens* was ineffective not only for succinate production but also for malate and fumarate consumption. Malate and fumarate produced by cyanobacterial autofermentation was consumed by the addition of these microorganisms in the absence of sterilized CSL, resulting in the succinate production.

It has been reported that *E. coli* K-12 can catabolize malate or fumarate to succinate through the action of the fumarate/succinate antiporter system DcuABC under fermentation conditions[32]. In our study, the addition of *E. coli* K-12 BW25113 to *Synechocystis* fermentation led to succinate production, as in the case of *E. hermannii* (Fig. 6). On the other hand, the *E. coli* deletion of the *dcuB* gene, encoding the DcuB protein, which plays major roles as a component of the DcuABC complex in malate and fumarate uptake, resulted in the reduction of succinate level. However, malate and fumarate did not completely disappear and remained at about one-fifth or one-twentieth of those in sterilized CSL without adding any strain, indicating

that its malate and fumarate uptake ability was partially impaired.

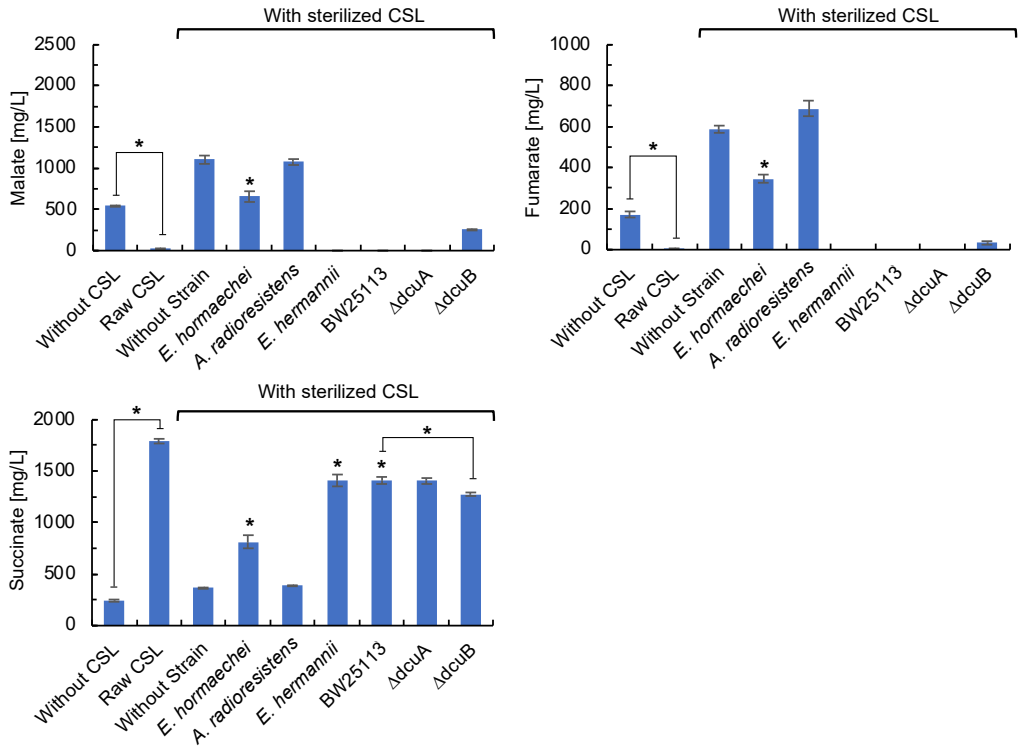

Fig. 6. Identification and characterization of succinate-producing microorganisms from raw CSL. C4-dicarboxylic acids production by the PCCK strain under dark anoxic fermentation in the presence or absence of raw CSL or in the presence of sterilized CSL with each strain as indicated. 300 mM $NaHCO_3$ was added to all tested vials. Values represent the average (± standard deviation) of three biological replicates. Statistical significance between without and with raw CSL was determined using the student *t*-test (*P < 0.01). In the bar graph of "with sterilized CSL", statistical significances of "without strain" among *E. hormaechei*, *A. radioresistens*, *E. hermannii*, and *E. coli* BW25113 and of *E. coli* BW25113 among ΔdcuA and ΔdcuB were determined using using the Tukey−Kramer test (*P < 0.01), respectively.

2.5. Evaluation of high-density fermentation for production of C4 dicarboxylic acids

Previous reports have clearly demonstrated that the initial concentration of *Synechocystis* cells is critical for increasing the succinate titer[24,25]. However, the acetate level usually increases as the initial concentration increases. Prior to high-density fermentation of the *Synechocystis* strains, we introduced the *pckA* gene into *slr0646* loci of the Ppc-ox/ΔackA strain that possesses a genetic background of deletion of endogenous *ackA* (*sll1299*) encoding acetate kinase in the Ppc-ox strain[19]. The constructed PEPck overexpression system is

regulated by P_{trc}, which shows higher transcriptional activity than P_{psbA2}[33]. The PEPck activity of PCCK-ox/ΔackA cells was approximately 3.5-fold higher than that of PCCK cells (Supplemental Fig. S5B). We then estimated the production of C4 dicarboxylic acids, lactate, and acetate in PCCK-ox/ΔackA and PCCK cells after 72 h of fermentation in the presence of sterilized or raw CSL at different initial cell concentrations (4, 10, and 20 g dry cell weight/L) (Fig. 7). As expected, the deletion of *ackA* resulted in a significant decrease in acetate concentration at any initial cell concentration; instead, fumarate (2,286 mg/L) or succinate (5,639 mg/L) peaked at 20 g DCW/L of PCCK-ox/ΔackA with sterilized CSL or raw CSL, respectively. In sterilized CSL, the malate concentration was very similar between the two strains (around 3,100 mg/L). Malate and fumarate were not observed following fermentation in the presence of raw CSL because of the fermentative action of CSL-settling bacteria.

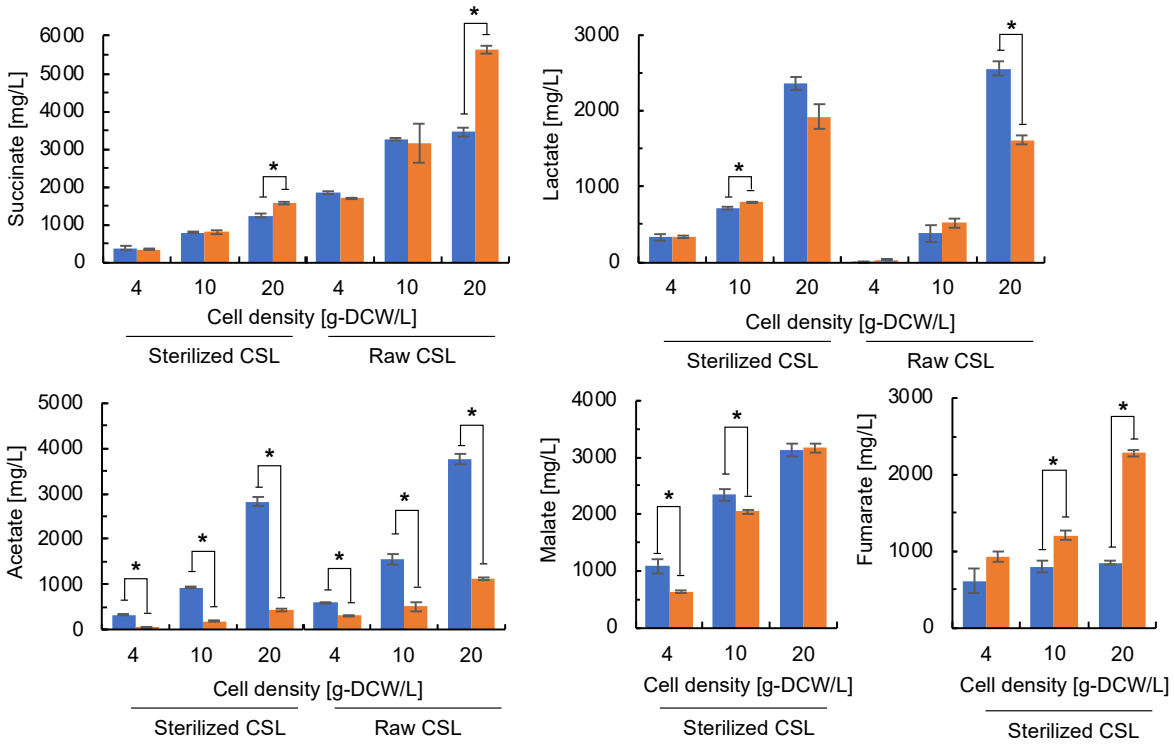

Fig. 7. Production of C4-dicarboxylic acids in PCCK (blue) or PCCK-ox/ΔackA (orange) strains after 72 h dark anoxic fermentation in the presence of 300 mM $NaHCO_3$ at different initial cell concentrations in the presence of sterilized or raw CSL. The cell densities of 4, 10, 20 g-DCW/L are equivalent to OD_{730} = 20, 50, and 100, respectively. Values represent the average (± standard deviation) of three biological replicates. Statistical significance between PCCK and PCCK-ox/ΔackA strains was determined using the student's *t*-test (*$P < 0.01$).

3. DISCUSSION

In the present study, we showed that PEPck expression concurrent with Ppc overexpression is effective for carbon influx from PEP into the reductive TCA branch through oxaloacetate production, thereby reducing lactate and acetate concentrations. *Synechocystis* 6803 possesses several routes for glucose catabolism, including the Embden–Meyerhof–Parnas, oxidative pentose phosphate (OPP), Entner–Doudoroff, and phosphoketolase pathways[3,34,35]. *Synechocystis* 6803 mainly drives the OPP pathway from glucose to triose phosphate, including PEP, which is a key metabolic branching point under dark anoxic conditions[35]. The most remarkable finding was that PEPck expression resulted in decreased pool sizes of sugar phosphates during fermentation, resulting in increased C4 dicarboxylic acids as final fermentation products. The low cellular energy charge of the PCCK strain during the early phase of fermentation, probably accompanied by the repression of high acetate production concomitant with ATP production, may reflect low pool sizes of sugar phosphates as ATP is considered a major allosteric inhibitor that represses the activities of glycolytic enzymes such as pyruvate kinase[36] and phosphoketolase[37].

The PCCK strain produces less lactate and more malate (Fig. 3), suggesting that the *Synechocystis* PCCK strain utilizes its reducing power more for the reductive TCA branch. Accumulation of acetyl-CoA and the low pool size of citrate in the PCCK strain clearly explains the interruption of citrate biosynthesis from oxaloacetate and acetyl-CoA, leading to a low flux of the oxidative TCA branch. D-lactate dehydrogenase plays a role in the utilization of the reducing power of NADH or NADPH by producing lactate from pyruvate during fermentation[38]. Meanwhile, the increased level of oxaloacetate promotes the NADH-dependent reductive activity of MDH, which leads to the catabolism of glycogen to maintain the intracellular redox balance. The pool sizes of intracellular malate, fumarate, and succinate are comparable significantly between the PCCK and Ppc-ox strains (Fig. 4). Because the intracellular pool size reflects an equilibrium among forward and reverse reactions and efflux rates, the higher C4-dicarboxylic acids levels in the PCCK strain would represent that the metabolic flux is enhanced by PEPck expression. While, the succinate production rate is slower than those of the fumarate and malate (Fig. 3), suggesting that the reduction of fumarate is the rate-limiting reaction in the reductive TCA branch.

We showed that the additive ingredient of sterilized CSL promotes malate production via the engineered anaplerotic pathway, with improved glycogen catabolism that supplies sugar phosphate or PEP in the presence of 300 mM $NaHCO_3$. In contrast to the PCCK strain, the Ppc-ox strain showed high glycogen

consumption, and carbon distribution was directed toward acetate and lactate production, resulting in the enhancement of energy metabolism. The question arises, why does CSL addition enhance the level of malate? The MDH activity was not enhanced by the addition of sterilized CSL. The addition of yeast extract into the cyanobacterial fermentation resulted in no increase on C4 dicarboxylic acids, the nitrogen or carbon sources would not be a major reason for the question. One possibility is that the unknown C4 dicarboxylic acid export mechanism is responsible. It has been reported that alkaline pH (pH 8.8) enhances the levels of succinate, malate, fumarate, and lactate by increasing the ratio of dissolved bicarbonate ions[39]. However, the extracellular pH during fermentation was approximately 8.4, irrespective of the presence or absence of CSL. It is also thought that divalent cations, such as calcium^{2+}, contained in CSL might be chelated to malate to change their permeation of the plasma membrane[40], because CSL contains 13.5%~17.5% minerals as a standard component.

Unexpectedly, the succinate increase in cyanobacterial autofermentation with raw CSL was due to succinate excretion by heterotrophic microorganisms with anaerobic malate and fumarate catabolic pathways. *E. coli* utilizes citrate, aspartate, tartrate, fumarate, and malate under anaerobic conditions[41]. The fumarate/succinate antiporter Dcu system (dicarboxylate uptake), which is highly conserved in enteric bacteria, including *A. succinogenes*[42], is responsible for the uptake of external C4-dicarboxylates, such as fumarate, malate, and aspartate. Fumarase and aspartase catalyze the conversion of L-malate and aspartate to fumarate, respectively, resulting in fumarate respiration, which generates a transmembrane proton potential to drive ATP synthesis. Succinate produced by fumarate respiration is then excreted by the fumarate/succinate antiporter, DcuB, as the final fermentation product. Our results clearly show that the fumarate/succinate antiporter is involved in malate and fumarate metabolism, similarly to the model microorganism *E. coli* K-12 BW25113 strain. Homologs of fumarate/succinate antiporters are found in *E. hormaechei* and *E. hermannii*, but not in *A. radioresistens*, reflecting the involvement of fumarate/succinate antiporters in succinate increased by each related strain isolated from raw CSL.

Previous report has shown that CitH overexpression results in increase in the reductive branch of the open cyanobacterial TCA cycle flux. The sums of excreted C4 dicarboxylic acids were 4.2 g/L succinate, 2.7 g/L fumarate, and 4.6 g/L malate from cells incubated at OD_{730} = 200 (equivalent to 40 g-DCW/L with 19.6 g/L glycogen) with product stripping by medium change[24]. In the present study, the total amount of C4 dicarboxylic acids with raw CSL and sterilized CSL are 5.6 g/L (succinate only) and 7.0 g/L (sum of 1.58

313 g/L succinate, 2.28 g/L fumarate, and 3.16 g/L malate from cells incubated at OD_{730} = 100 (equivalent to 20
314 g-DCW/L with 9.2 g/L glycogen), respectively. Also, the total amount of excreted major organic acids (sum
315 of C4-dicarboxylic acids, D-lactate, and acetate) were 8.3 g/L (D-lactate 1.61 g/L and acetate 1.12 g/L with
316 raw CSL) and 9.5 g/L (D-lactate 1.9 g/L and acetate 0.43 g/L with sterilized CSL). Considering that the
317 average molecular weights of succinate, fumarate, and malate are 123, the theoretical maximum of C4
318 dicarboxylic acid from six-carbon sugar and CO_2 is 1.78 mol/mol sugar or 1.17 g/g sugar. The conversion
319 ratio of the sum of C4 dicarboxylic acids (7.0 g/L) and the theoretical maximum of C4 dicarboxylic acid
320 (10.8 g/L), following calculation by 9.2 g/L glycogen accumulated in the PCCK-ox/ΔackA strain, was 65.5%,
321 1.3-fold higher than those in CitHox fermentation under optimized conditions[24]. In addition, the total
322 excreted organic acids should include organic acids accumulated during photosynthesis and/or derived from
323 the catabolism of other major carbon sources such as polyhydroxybutyrate and/or proteins[24]. When
324 compared to sterilized CSL, the total amount of C4 dicarboxylic acid produced with raw CSL was lower (the
325 conversion rate of succinate to total glycogen was 52%), indicating that malate and fumarate are utilized not
326 only as electron donors to produce energy under anaerobic conditions, but also as a carbon source for
327 assimilation under limited nutritional conditions in the CSL-settling bacteria in raw CSL.

328 Here, we demonstrated the increased production of C4 dicarboxylic acids at a cell density (20 g-
329 DCW/L) in the presence of CSL without product stripping. Microalgal biomass can also be utilized for
330 fermentation with succinate-producing microorganisms, such as *A. succinogenes*[43]. However, a pretreatment
331 process, acid-thermal hydrolysis of microalgal biomass for sugar extraction, is required. The present
332 microbial community concept would facilitate simplification of processes for the large-scale production of
333 C4 dicarboxylic acids from cyanobacterial biomass. The metabolic engineering of malate and fumarate
334 catabolic pathways in the succinate-producing microorganisms, such as *E. coli* K-12 strain, would contribute
335 to the improvement of succinate concentration in the microbial community. In order to bring cyanobacterial
336 bioproduction to socially useful levels, the photoautotrophic C4 dicarboxylic acid production system
337 accompanied by autofermentation as a final process, must be developed further to yield a sustainable
338 production system.

339

340 **4. CONCLUSIONS**

341 We have demonstrated that foreign PEPck expression enhanced the carbon flux from hexose and pentose

phosphates, which are metabolic intermediates from CO_2-derived glycogen, into the TCA reductive branch, as revealed by metabolome analysis. The combination of PEPck overexpression concurrent with Ppc overexpression in the presence of sterilized CSL facilitates the increase in excretion of malate and fumarate under the high-density fermentation. The intracellular glycogen concentration before fermentation was about the almost same compared to that of the previous study[24], but fumarate and malate production was about 1.5 times higher when the presence of sterilized CSL, and succinate production was less than 80% of the previous succinate titer, achieving the titer of 7.0 g/L from the theoretical maximum of 10.8 g/L at a cell density (20 g-DCW/L). Extracellular malate and fumarate could be converted to succinate by the microorganisms that are able to catabolize those organic acids through the fumarate/succinate antiporter Dcu system.

5. MATERIALS AND METHODS

5.1. Strains and culture conditions

Recombinant strains used in the present study were constructed based on the glucose-tolerant (GT) strain *Synechocystis* sp. PCC6803[44]. The recombinant *Synechocystis* sp. PCC 6803 strains were inoculated into modified-BG11 medium containing 5 mM ammonium chloride and 0.1 M HEPES-KOH (pH 7.8) in the presence or absence of 50 μg/mL kanamycin and/or 34 μg/mL chloramphenicol. The cultivation proceeded at 30 °C under continuous light irradiation of 105–115 μmol/m^2/s photons and 1% (*v/v*) CO_2. The cell density was determined by measuring the optical density at 750 nm (OD_{750}). Dry cell weight (DCW) was measured after harvesting the cells by filtration, followed by washing with 20 mM NH_4HCO_3, and lyophilization. All chemicals used were of analytical grade.

5.2. Construction of recombinant strains

Escherichia coli DH5α strain (TakaraBio Inc., Tokyo, Japan) was used as a host for gene cloning and plasmid amplification. The *Actinobacillus succinogenes pckA* gene (Uniprot ID: Q6W6X5) was obtained by gene synthesis (ThermoFisher Scientific, Waltham, MA), and the synthesized gene amplified by PCR using the primer pair PEPCK-Fw1 and PEPCK-Rv1. The amplified fragment was cloned into the *Nde*I sites of the plasmid pTCP2031[10], according to the manufacturer's instructions using the In-Fusion HD^R cloning kit (TakaraBio Inc.), yielding pTCP2031-PEPck. To construct an integration vector, the upstream and downstream regions (each 1,000 bp) of *slr0646* were amplified by PCR using the primer pairs uslr0646-Fw

and uslr0646-Rv for the upstream regions and dslr0646-Fw and dslr0646-Rv for the downstream regions. The amplified PCR fragments were cloned into *Mlu*I/*Hind*III site and *Eco*RI/*Xho*I site of the plasmid pSStrc-slr1556[19] using the In-Fusion HD^{R} cloning kit, resulting in plasmid pSStrc-0646. The *pckA* gene was amplified by PCR using the primer pair PEPCK-Fw2 and PEPCK-Rv2, and the resultant fragment was cloned into *Nde*I/*Sal*I site of pSStrc-0646, resulting in pSStrc-PEPCK.

The principles underlying specific gene integration or disruption in *Synechocystis* 6803 have been described previously[20]. The plasmid pTCP2031-Pepck was used for gene integration into the host strain, *Synechocystis* Ppc-ox[19], to yield PCCK. The plasmid pSStrc-PEPck was introduced into the host strain *Synechocystis* Ppc-ox/ΔackA[19], yielding the *Synechocystis* PCCK-ox/ΔackA strain. Integration of each gene cassette was confirmed by PCR. All primer sequences are listed in *SI Appendix* (Table S1).

5.3. Enzyme assay

Photoautotrophically grown cyanobacterial cells (4 g-DCW/L) were fermented at 37 °C under dark anoxic conditions, the crude extract of the fermentation cells was obtained using a method previously described[19]. Briefly, cells suspended in 50 mM potassium phosphate buffer were disrupted by sonication and centrifuged, and the resultant supernatant was collected as a crude extract. PEPck activity was measured by monitoring absorbance at 340 nm accompanying with the oxidation of NADH using V730-Bio spectrophotometer (JASCO Co., Tokyo, Japan) in a reaction mixture containing 0.1 M Mes (pH 6.6), 10 mM $MgCl_2$, 5.0 mM $MnCl_2$, 1.0 mM DTT, 10 mM Adenosine diphosphate (ADP), 75 mM $NaHCO_3$, 0.1 mM NADH, 20 U malate dehydrogenase, and the crude extract[28], the reaction was started by the addition of 10 mM phosphoenolpyruvate (PEP) then incubated for 10 min at 30 °C. The same mixture in the absence of ADP was used as a control to exclude ADP-independent carboxylation of PEP. MDH activity of crude extract was measured analyzed in a reaction mixture containing 100 mM potassium phosphate buffer (pH 8.0), 0.1 mM NADH, and 1 mM oxaloacetate at 30 °C. Protein concentrations were determined by Bradford dye-binding assay[45], using bovine serum albumin for standard curve generation.

5.4. Organic acid production by fermentation

Unless otherwise noted, autofermentation was performed as follows: recombinant *Synechocystis* sp. PCC 6803 strains were cultivated for 3 d at 30 °C under photoautotrophic conditions at 0.1 of optical density at

750 nm. The cultured cells were inoculated into 10 mL of 0.1 M HEPES-KOH (pH 7.8) containing 100 or 300 mM $NaHCO_3$ at initial cell concentrations of 4 g-DCW/L. Fermentation was conducted at 37 °C for four days under dark anoxic conditions (wrapped in foil and 100% N_2 bubbling). The accumulation of extracellular organic acids during fermentation was quantified using a high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and an RID-10A refractive index detector. The HPLC system was operated at 50 °C using 5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1}. The glycogen concentration was determined by measuring the glucose released from glycogen via enzymatic hydrolysis, as described previously[19]. The yield was calculated as the ratio of the corresponding C4 dicarboxylic acid amount to the amount of glucose consumed and the enzymatic hydrolysis of stored intracellular glycogen.

5.5. Analysis of intracellular metabolites

A 5 mg-DCW portion of cyanobacterial cells in the fermentation culture was collected at each sampling time (6, 24, 48, 72, and 96 h) by filtration using 1 μm pore size polytetrafluoroethylene (PTFE) disks (Millipore, Billerica, MA) and then immediate washing with 20 mM $(NH_4)_2CO_3$ pre-cooled to 4 °C. Intracellular metabolites were extracted and analyzed by capillary electrophoresis- mass spectrometry (CE-MS) (Agilent G7100; MS, Agilent G6224AA LC/MSD TOF; Agilent Technologies, Palo Alto, CA) according to a previously reported method[19].

5.6. Pretreatment of CSL solution for fermentation and isolation of CSL-settling bacteria

Powdered corn-steep liquor (CSL) (Solulys 095E, catalog number: C42166) was purchased from Oriental Yeast Co., Ltd., Osaka, Japan. The other CSLs were obtained from Spectrum Chemicals Australia (Catalog number: C3848; Astral Scientific Pty Ltd., NSW, Australia) and Sigma-Aldrich Co. LLC (Catalog number: C4648-500G; St. Louis, MO, USA). Unless otherwise stated, powdered CSL was dissolved in pure water and incubated at 37 °C for 24 h. The supernatant was collected from the CSL suspension by centrifugation (20,000-g for 10 min). Sterilized CSL was obtained by centrifugation after autoclaving the suspension. Five milliliters of the resulting supernatant were mixed with an equivalent volume of the *Synechocystis* solution with 0.1 M HEPES-KOH (pH 7.8) containing $NaHCO_3$ at initial cell concentrations of 8 g-DCW/L.

For isolation of CSL-settling bacteria, the supernatant of 1 g/L CSL suspension was streaked onto

Luria-Bertani agar medium and incubated for 24 h at 37 °C. Each colony grown in LB agar medium was arbitrarily picked and inoculated into LB liquid media based on colony morphology. The 16S rDNA of the isolated bacterium was amplified by PCR using the universal primer set 27F/1492R, and partial DNA sequences were analyzed by Sanger sequencing.

The related three strains, *Enterobacter hormaechei* (NBRC 105718), *Escherichia hermannii* (NBRC 105704), and *Acinetobacter radioresistens* (NBRC 102413) identified by BLAST-guided search and *E. coli* BW25113 and the knockout mutants *E. coli* ΔdcuA and *E. coli* ΔdcuA in Keio collection were purchased from Biological Resource Center, National Institute of Technology and Evaluation (NITE), Japan. Five milliliters of sterilized CSL supernatant suspended with each strain at 2.5 × 10^{-5} of an optical density at 600 nm, was mixed with an equivalent volume of the *Synechocystis* solution with 0.2 M HEPES-KOH (pH 7.8) containing 600 mM $NaHCO_3$ at initial cell concentrations of 8 g-DCW/L.

Conflicts of interest

There are no conflicts to declare

Author Contributions

Ryota Hidese: Investigation and Data Curation, Writing - Original Draft, Writing - Review & Editing; Mami Matsuda and Mamiko Kajikawa: Investigation and Data Curation; Akihiko Kondo: Supervision; Takashi Osanai and Tomohisa Hasunuma: Conceptualization, Writing - Review & Editing, Funding acquisition.

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Supporting Information

Supplementary Table S1 and Figures S1~S5

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