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Temperature enhanced succinate production concurrent with increased central metabolism turnover in the cyanobacterium Synechocystis sp. PCC 6803

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1	Temperature enhanced succinate production concurrent with
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3	Synechocystis sp. PCC 6803
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24 **ABSTRACT**

25Succinate is a versatile petrochemical compound that can be produced by microorganisms, often from carbohydrate based carbon sources. Phototrophic 2627cyanobacteria including Synechocystis sp. PCC 6803 can more efficiently produce organic acids such as succinate without sugar supplementation, via photosynthetic 28production of glycogen followed by glycogen utilization, typically under dark 29conditions. In this study, Synechocystis 6803 bioproduction of organic acids under 30 dark anoxic conditions was found to increase with elevation of temperature from 3130°C to 37°C. The further enhancement of succinate bioproduction by 32overexpression of the rate limiting enzyme phosphoenolpyruvate carboxylase 33 resulted in improved glycogen utilization. To gain more insight into the 34mechanisms underlying the increased organic acid output, a novel temperature 35dependent metabolomics analysis was performed. Adenylate energy charge was 36 found to decrease along with elevating temperature, while central metabolites 37glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, glycerol 3-38phosphate, malate, fumarate and succinate increased. Temperature dependent ¹³C-39 labeling metabolomics analysis further revealed a glycolysis to TCA bottleneck, 40 41 which could be overcome by addition of CO₂, leading to even higher organic acid 42production. Optimization of initial cell concentration to 25 g-dry cell weight/L, in 43combination with 100 mM NaHCO₃ supplementation, afforded a succinate titer of more than 1.8 g/L, the highest reported autotrophic succinate titer. Succinate titers 44 remained high after additional knockout of *ackA*, resulting in the highest reported 45autotrophic D-lactate titer as well. The optimization of Synechocystis 6803 organic 46

47 acid production therefore holds significant promise for CO₂ capture and utilization.

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- 50 Key words:
- 51 Autofermentation; Cyanobacteria; Lactate; Metabolomics; Succinate; *Synechocystis*;
- 52 Temperature

54 **1. Introduction**

55The rapid development of the petrochemical industry in the 20th century has created a mass consumption and disposal social structure, leading to the over-56generation of greenhouse gasses including carbon dioxide (CO₂) and global climate 5758change. In order to address these problems and help build a sustainable lowcarbon future society, it is necessary to develop technologies to biologically 59produce fuels and commodity chemicals from renewable biomass. Recently, non-60 61 food-based lignocellulosic biomass that exists abundantly on land has attracted 62attention as a renewable feedstock for microbial fermentation to produce a wide 63 variety of chemicals. However, recalcitrant structures of lignocellulosic biomass make it difficult to generate fermentable sugars from component cellulose and 64 65hemicellulose with high yield.

Photosynthetic algae directly convert CO₂ to useful substances including lipid, starch, glycogen, pigments and organic acids (Aikawa et al., 2013; Dismukes et al., 2008; Hasunuma et al., 2016, Ho et al., 2017; Wijffels et al., 2013), which can be utilized to avoid the use of more expensive sugars as well as complicated plantbiomass decomposition processes. Furthermore, algae cultivation does not require agricultural resources such as farmland or fresh water (lijima et al., 2015).

The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) is the first algae to be genome decoded (Kaneko et al., 1996). Along with the development of genetic engineering tools, *Synechocystis* 6803 metabolic pathways have been successfully modified to enhance photosynthetic activity and to produce alcohols, diols, hydrocarbons, organic acids and other

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valuable chemicals (Angermayr et al., 2015; Formighieri et al., 2015; Hasunuma et al., 2014; Lai and Lan 2015; Lan and Wei, 2016; Wang et al., 2016).

Synechocystis 6803 inherently produces organic acids including D-lactate 79 and succinate by catabolism of the primary storage polysaccharide glycogen, which 80 accumulates during photosynthesis (Hasunuma et al., 2016). As carbon sources 81 such as sugars and glycerol are not necessary, this type of catabolism is referred to 82as autofermentation (McNeely et al., 2010). Lactate typically serves as a building 83 84 block for the synthesis of a bio-based plastic, polylactic acid (PLA). Notably, stereocomplex-type PLA composed of L- and D-lactate is promising due to a high 85melting point (ca. 230°C) (Ikeda et al., 1987). The current industrial biological 86 production of D-lactate (Wee et al., 2006) remains to be established and is 87 88 therefore considered to be more important than that of L-lactate.

Succinate is also an important raw material of poly(butylene succinate),
polyurethanes and other green sustainable plastics (Choi et al., 2015; Delhomme et
al., 2009). Various chemicals produced in the current petrochemical industry
including adipic acid, 1,4-butanediol, γ-butyrolactone, *N*-methylpyrrolidone and
tetrahydrofuran can be produced from succinate as well (Akhtar et al., 2014).

The metabolic pathways of autofermenting cyanobacteria are not well characterized as of yet. Recently, dynamic metabolomics using ¹³C labeling has clarified that succinate is biosynthesized from glycogen via glycolysis, the anaplerotic pathway and reductive TCA cycle in *Synechocystis* 6803 under dark anoxic conditions (Hasunuma et al., 2016). However, optimal culture environments to maximize production of succinate have not yet been determined.

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100 Temperature is an important control element in the fermentation process 101 to efficiently produce target molecules (Gourdon and Lindley, 1999; Shin et al., 102 2007). Yet, the influence of temperature regulation on the total metabolic system of 103 a microorganism has not been reported to our knowledge (Yurkovich et al., 2017).

The optimal growth temperature of *Synechocystis* 6803 is 30-32°C (Tasaka et al., 1996). In the present study of *Synechocystis* 6803, elevated temperatures were found to increase production of organic acids including D-lactate and succinate. The highest production of D-lactate and succinate was observed at 37°C to 40°C.

109So far, the optimal temperature range of various enzymes involved in 110 central metabolism has been investigated in vitro. However, in vivo metabolic turnover, which is dependent on activated-enzyme level and intracellular 111 112environment such as molecular crowding, differs from the *in vitro* data. Thus, in the 113present study, influence of elevated temperature on central metabolism was systematically analyzed using a dynamic metabolome analysis developed by 114115combining *in vivo* ¹³C labeling, metabolomics and mass distribution determination. This comprehensive approach afforded direct observation of *in vivo* kinetics and 116 carbon distribution in Synechocystis 6803. Elevating temperature to 37°C was 117found to enhance organic acid bioproduction in both wild type and 118 phosphoenolpyruvate carboxylase (PEPC) overexpressing Synechocystis 6803. 119 Furthermore, a glycolysis to TCA bottleneck was discovered and alleviated via 120addition of CO_2 , which significantly increased succinate titers to 1,802 mg/L. 121

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123 **2. Materials and Methods**

124 **2.1.** Strains and culture conditions

A recombinant Synechocystis 6803 strain, referred to as Ppc-ox, overexpresses the 125endogenous PEPC gene (ppc, sll0920) under the control of the trc promoter. The 126Ppc-ox strain was constructed from a wild type GT strain (Williams, 1988) as 127described previously (Hasunuma et al., 2016). Recombinant and GT strains were 128cultivated in BG11 medium (Rippka et al., 1979) with and without 50 mg/L 129kanamycin, respectively, under photoautotrophic conditions. Cell density was 130 observed by optical density at 750 nm (OD₇₅₀). Sampling volumes were determined 131132based on g-dry cell weight (DCW). DCW was measured after harvesting cells by 133filtration, washing with 20 mM ammonium bicarbonate, and lyophilization.

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135 **2.2** Construction of a recombinant strain

The Ppc-ox strain was transformed with pTCP1299 (Osanai et al., 2015) using a previously described method (Hasunuma et al., 2016) to yield strain Ppc-ox/ Δ *ackA*. Colonies resistant to 50 mg/L kanamycin and 34 mg/L chloramphenicol were selected. Knockout of *ackA* (*sll1299*) was confirmed by PCR using the specific primers 5'-TCAGCATTGATACCACTATGGGCTTCAC-3' and 5'-GACAGCCCAGAGACTCCGAGCAAACCGGA-3'.

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143 **2.3 Batch fermentation under anaerobic conditions**

After pre-cultivation in BG11 medium, cells were inoculated into modified-BG11 medium containing 50 mM Hepes-KOH (pH 7.8) and 5 mM NH₄Cl as a nitrogen

source instead of NaNO₃ at a biomass concentration of 0.1 g-DCW/L. 146 Photoautotrophic cultivation proceeded for 3 days under 1% (v/v) CO₂ and 147continuous light irradiation of 105-115 µmol photons/m²/s at 30°C as described 148previously (Hasunuma et al., 2016). After photoautotrophic cultivation, cells were 149transferred into 100 mM Hepes-KOH (pH 7.8) at biomass concentrations of 5-50 g-150DCW/L and cultivated at 30-40°C under anaerobic conditions as described 151(Hasunuma et al., 2016). Organic acids secreted into fermentation medium were 152analyzed with high-performance liquid chromatography (HPLC), while minor 153components were quantified with capillary electrophoresis-mass spectrometry 154(CE-MS) (CE, Agilent G7100; MS, Agilent G6224AA LC/MSD TOF; Agilent 155156Technologies, Palo Alto, CA, USA) again as described (Hasunuma et al., 2016).

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158 **2.4** Analysis of intracellular metabolites

Cells (5 mg DCW) were collected by filtration with 1-µm pore size PTFE disks 159(Omnipore; Millipore, Billerica, MA,). After washing with 20 mM ammonium 160 carbonate at 4°C, intracellular metabolites were extracted according to previous 161 methods (Hasunuma et al., 2016). To remove solubilized protein, the extract was 162163filtered through a 3 kDa cut-off membrane (Millipore). The metabolites were 164analyzed using a CE-MS system as described above. Intracellular glycogen was 165determined with HPLC after extraction from cells and enzymatic hydrolysis (Hasunuma et al., 2016). 166

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168 2.5 ¹³C-labeling metabolomics

Metabolite turnover in *Synechocystis* 6803 was determined by the combination of *in vivo* ¹³C-labeling and CE-MS analysis (Hasunuma et al., 2016). Cells were labeled in 100 mM Hepes-KOH (pH 7.8) containing 2 g/L [U-¹³C] glucose for 0.5-3 h. After cell collection and metabolite extraction, mass spectra of the intracellular metabolites were analyzed using CE-MS to observe mass shifts from ¹²C to ¹³C in metabolites. ¹³C fractions, ratios of ¹³C to total carbon, were calculated by relative isotopomer abundance (*m_i*) of metabolites incorporating *i*¹³C atoms as follows:

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$$m_i(\%) = \frac{M_i}{\sum_{j=0}^n M_j} \times 100$$

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C fraction (%) = $\sum_{i=1}^{n} \frac{i \times m_i}{n}$

where M_i represents the isotopomer abundance of metabolite incorporating i^{13} C atoms, and *n* is the number carbon atoms in the metabolite.

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181 **2.6 Enzyme assay**

182Enzyme assays were performed using cells cultivated for 24 h under dark anoxic 183condition as described in Section 2.3. Activities of PEPC were measured as described previously (Hasunuma et al., 2016). NAD-dependent malate 184 dehydrogenase (MDH) activity was determined as follows. About 50 mg-DCW of 185cells were collected by centrifugation at 6,000 x g for 10 min at 4°C. After 186 187discarding the supernatant and washing with extraction buffer (18 mM KH₂PO₄, 27 mM Na₂HPO₄, 15 mM MgCl₂, and 100 µM EDTA, pH 8.0), cells were disrupted in 3 188 189 mL of the extraction buffer by sonication. Cell debris was removed by

centrifugation at 20,000 x g for 20 min at 4°C to obtain protein extract as
supernatant. MDH assay was performed in 1.5 mL aliquots containing 50 mM TrisHCl (pH7.5), 200 µM NADH, 0.5 mM oxaloacetate and 250 µL of protein extract.
Replicate aliquots without oxaloacetate were used as controls. MDH activity was
determined by measuring NADH oxidation as a change in absorbance at 340 nm.
Protein content was determined using a BCA assay kit as described previously
(Hasunuma et al., 2013).

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198 2.7 qPCR analysis

Synechocystis 6803 cells, cultivated at 30°C and 37°C under anaerobic conditions 199 200for 24 h, were harvested by centrifugation at $10,000 \times g$ for 1 min and immediately 201frozen in liquid nitrogen. Total RNA was extracted from the frozen cells using Fruit-202mate for RNA Purification (Takara Bio Inc., Shiga, Japan) and a NucleoSpin RNA kit 203(Takara Bio Inc.) according to the manufacturer instructions. Complementary DNA 204 was synthesized from 30 ng of total RNA by using a ReverTra Ace qPCR RT Master 205Mix with gDNA Remover (TOYOBO, Osaka, Japan), and qPCR was performed by 206using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and Mx qPCR Systems (Agilent Technologies) as previously described (Ho et al., 2017). The qPCR primers used in 207this study were as follows: for rnpB, 5'-GTGGAACCGCTTGAGGAATTTG-3' and 5'-208TTTTGACAGCATGCCACTGG-3', for ppc, 5'-ACGATGCCAGTGATGTGTTG-3' and 5'-209 TTCAAACAGGGGCACAATGC-3', and for mdh, 5'-AGATTTGATGCTGCCCTTGC-3' and 2105'-AGTAAGGCGGCAATTTCAGC-3'. Relative transcript levels were evaluated using 211the level of the *rnpB* encoding RNA subunit of RNase P as a reference, and then 212

213 normalized by the levels of each gene at 30°C.

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216 3. Results
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3.1. Increased temperature enhances secretory production of organic acids

in *Synechocystis* cells

219Synechocystis 6803 cells were cultivated under photoautotrophic conditions and transferred to dark and anoxic conditions with an initial cell concentration of 5 g-220DCW/L to initiate secretion of organic acids by autofermentation. The 221222autofermentation of Synechocystis has been so far performed at only 30°C (Hasunuma et al., 2016; Ueda et al., 2016). In Fig. 1, effects of temperature on 223secretory production of organic acids were investigated after 72 h fermentation. 224Acetate and D-lactate increased with increase in the cultivation temperature. The 225production of succinate reached a maximum value at 37°C, which was 162.3 mg/L. 226227Other organic acids, which are secreted as minor products, such as fumarate, 2-228ketoglutarate, malate, nicotinate, pyruvate and shikimate increased by elevating temperature from 30 to 37°C (Supplementary Table 1). 229





represent the average (± SD) of three independent experiments.

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In autofermentation, D-lactate and succinate are biosynthesized via sugar 234catabolism (Hasunuma et al., 2016). The succinate biosynthetic route includes 235glycolysis, the anaplerotic pathway and the reductive TCA cycle in which PEPC 236encoded by *ppc* is a rate-limiting enzyme. In fact, overexpression of *ppc* improved 237succinate production in Synechocystis 6803 (Hasunuma et al., 2016). Thus, time-238239course analysis of organic acid production was carried out using the ppcoverexpressing strain, Ppc-ox (Fig. 2). Production of acetate, D-lactate and 240241succinate was enhanced by elevating temperature from 30°C to 37°C. Acetate productivity, shown as the slope of Fig. 2, increased together with the increase in 242243temperature. D-lactate production almost reached a plateau after 9 h cultivation at 24430°C, while elevated temperature resulted in a continuous increase in D-lactate 245even after 9 h of cultivation. Secretion of succinate was initiated after 6 h. The 246initial slopes of succinate production at 35°C and 37°C were higher than that of 24730°C. Ppc-ox exhibited higher production of acetate, D-lactate and succinate at 37°C, relative to that of the wild-type strain. 248





Fig. 2 Time-course of organic acid secreted and intracellular glycogen in Ppc-ox cultivated at 30°C (blue triangles), 35°C (green squares) and 37°C (red circles). Values represent the average (± SD) of three independent experiments.

The time course of intracellular glycogen content in Ppc-ox was compared using three different temperatures (Fig. 2). At 30°C, glycogen content decreased from 43% to 33%. When the cells were cultivated at 35°C and 37°C, glycogen content decreased to 19% and 11%, respectively after 96 h fermentation. Decrease in glycogen at higher temperatures was accompanied by reduction in OD₇₅₀ and less higher density molecules observed with a transmission electron microscope (TEM), while cell number remained constant (Fig. S1).

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263 **3.2.** The temporal and temperature dependent metabolome

264 In order to determine the effect of fermentation temperature on dynamic

265metabolism in Ppc-ox, time course analysis of intracellular metabolites was 266performed during autofermentation at three different temperatures. Metabolites 267involved in glycolysis, pentose phosphate pathway (PPP), and TCA cycle, as well as metabolism of amino acids, nucleotides and cofactors, are shown in Fig. 3 and Fig. 268269S2. Accumulation of hexose and pentose phosphates such as glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), fructose 1,6-bisphosphate (FBP), 6-270phosphogluconate (6PG), ribulose 5-phosphate (Ru5P), ribose 5-phosphate (R5P) 271and sedoheptulose 7-phosphate (S7P) was dependent of temperature except for 272273glucose 1-phosphate (G1P). G6P, F6P and FBP continued to increase over time at 37°C, while they remained almost constant in the latter half of the fermentation at 27427530°C. The pool size of G1P did not increase after 48 h at 37°C. FBP is converted to 276glycelaldehyde 3-phophate (GAP) and dihydroxyacetonephosphate (DHAP), which 277accumulated depending on the temperature. Glycerol 3-phosphate increased along 278with elevating temperature. The pool size of 3-phosphoglycerate (3PGA), 2-279phosphoglycerate (2PGA), and phosphoenolpyruvate (PEP) decreased over time, 280and this pattern was almost same among the 30°C, 35°C and 37°C groups. Pyruvate, D-lactate and pyruvate-derived amino acids such as alanine, leucine, isoleucine and 281valine increased dependent on temperature. On the other hand, acetyl-CoA 282(AcCoA) and metabolites synthesized via the oxidative TCA cycle (including citrate, 283284cis-aconitate and isocitrate) decreased with time, and demonstrated lower pool size at higher temperature. Malate, fumarate and succinate synthesized via the 285286reductive TCA cycle demonstrated higher pool size at elevated temperatures until 28724 h. Arginine, histidine and phenylalanine showed temperature dependent increases in pool size, while proline decreased with increasing temperature.

The time course of adenosine phosphate and cofactor pool sizes is shown in Fig. 3. Adenylate energy charge, defined as [(ATP) + 1/2 (ADP)] / [(ATP) + (ADP) + (AMP)] (Chapman et al., 1971), decreased along with elevating temperature. Elevated temperature resulted in a slightly lower NAD⁺ level than that of 30°C. Reduced NADH and NADPH were not detected with CE-MS due to their low abundance.

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Fig. 3 Time course of intracellular metabolite pool size in Ppc-ox cultivated at 30°C 298(blue triangles), 35°C (green squares) and 37°C (red circles). Abbreviations: AcCoA, 299 ADP-glucose; 300 acetyl-CoA; ADP-Glc, BPGA, 1,3-bisphosphoglycerate; DHAP, 301dihydroxyacetonephosphate; FBP, fructose 1,6-bisphosphate; F6P, fructose 6phosphate; GAP, glyceraldehyde 3-phosphate; G1P, glucose 1-phosphate; G6P, 302303 glucose 6-phosphate; PEP, phosphoenolpyruvate; 6PG, 6-phosphogluconate; 2PGA, 3042-phosphoglycerate; 3PGA, 3-phosphoglycerate; R5P, ribose 5-phosphate; Ru5P, 305ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate. Values represent the average (± SD) of three independent experiments. 306

308 3.3. Metabolite turnover under anaerobic conditions reveals a glycolysis to 309 TCA bottleneck

Time course analysis of metabolite pool sizes is insufficient to account for carbon flow and distribution, because the pool size is dependent on the rate of synthesis and utilization of metabolite. Even though a particular reaction is activated under a certain condition, the amount of active metabolite may remain unchanged under steady state conditions.

In the present study, to more comprehensively elucidate the effects of elevating temperature on central metabolism, *in vivo* kinetics of carbon assimilation were investigated using [U-¹³C] glucose as a tracer compound. Glucose incorporated into the cells is phosphorylated to G6P by glucokinase (Lee et al., 2005), and then metabolized via glycolysis and PPP.

As shown in Fig. 4, metabolites involved in glycolysis and PPP were immediately labeled with ¹³C after the initiation of the autofermentation. The ¹³C fraction is defined as the ratio of ¹³C to total carbon in metabolites, which is obtained by mass spectrometry. Since ADP-glucose, a precursor of glycogen (Fig. 3), and G1P are labeled with ¹³C, glucose might be utilized to synthesize glycogen. The incorporation of ¹³C into glycogen was not observed during 3-h ¹³C labeling, which should be due to the large pool size of glycogen.



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Fig. 4 Time-course changes in ¹³C fractions of metabolites following ¹³C-glucose addition to cultures of Ppc-ox cultivated at 30°C (blue triangles) and 37°C (red circles). Values represent the average (\pm SD) of three independent experiments. Statistical significance was determined using the Student's *t*-test (**P* < 0.05, ***P* < 0.01).

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In cyanobacteria, glycogen is hydrolyzed to G1P by glycogen phosphorylase (Aikawa et al., 2015), and then converted to G6P by phosphoglucomutase (Lindahl and Florencio, 2003). Therefore, both G6P derived from glycogen and ¹³C-G6P derived from [U-¹³C] can be utilized as carbon sources for organic acids biosynthesis.

Sugar phosphates including G1P, G6P, and F6P and triose phosphates such

as 3PGA demonstrated slightly higher ¹³C fractions at 30°C relative to that of 37°C, over the course of 3 h fermentation. On the other hand, elevated temperature resulted in significantly higher ¹³C-fractions of malate, fumarate and succinate when compared to that of 30°C. ¹³C fractions of pyruvate, lactate, AcCoA, isocitrate and 2-ketoglutarate were similar for both 30°C and 37 °C groups. Increased flux of PEP through the anaplerotic pathway could be clearly observed in the ¹³C fractions (Fig. S3).

¹³C enrichment is dependent on the pool size of metabolites. For instance, 347metabolites whose pool size is large require large amount of ¹³C to reach high ¹³C 348fraction value. Thus, the number of ¹³C atoms incorporated into metabolites was 349350calculated after 3 h cultivation by multiplying the ¹³C fraction and pool size (Table 3511). Oxaloacetate could not be detected due to its low abundance. With exception to 352G1P, metabolites involved in glycolysis decreased at 37°C relative to that of 30°C. In contrast, ¹³C atom numbers of malate, fumarate and succinate increased when 353354elevating temperature from 30°C to 37°C. These results indicate enhanced carbon 355influx to the reductive TCA cycle. Therefore, the metabolic bottleneck that was previously identified (Hasunuma et al., 2016) had become relaxed. This was 356accompanied by increases in the specific activity of PEPC and MDH (Fig. 5). mRNA 357levels of *ppc* and *mdh* increased together with the activity of these enzymes which 358are involved in the carbon influx (Fig. 6). ¹³C atom number of D-lactate also 359increased with elevating temperature. 360

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	364	Table 1	Number of ¹³ C atoms in metabolite
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Metabolite	¹³ C atom number (µmol/g-DCW)			
	30°C	37°C		
G1P	501.2 ± 15.8	685.2 ± 45.7*		
G6P	274.4 ± 27.5	236.6 ± 16.9		
F6P	230.8 ± 7.5	194.6 ± 14.9*		
3PGA	667.3 ± 63.6	484.0 ± 35.8*		
2PGA	47.2 ± 3.1	38.3 ± 4.2*		
PEP	200.3 ± 11.7	149.2 ± 9.4**		
Pyruvate	7.1 ± 2.6	5.4 ± 1.3		
AcCoA	128.6 ± 18.1	84.7 ± 9.5*		
Citrate	444.5 ± 61.4	409.8 ± 10.8		
<i>cis</i> -Aconitate	14.7 ± 3.5	10.0 ± 0.5		
Isocitrate	392.5 ± 58.3	480.6 ± 86.2		
2-Ketoglutarate	7.1 ± 1.2	10.0 ± 0.5*		
Malate	170.9 ± 33.5	596.7 ± 121.1*		
Fumarate	33.2 ± 6.6	115.2 ± 24.4*		
Succinate	157.7 ± 26.9	$354.9 \pm 60.0^*$		
Lactate	31.0 ± 7.2	59.2 ± 11.7*		

365 The number of ¹³C atoms incorporated into metabolites was measured 3 h after addition of ¹³C-

366 glucose by multiplying the ¹³C fraction, pool size and carbon number. Values represent the

367 average (± SD) of three independent experiments. Statistical significance was determined using

368 the Student's *t*-test (*P < 0.05, **P < 0.01).

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Fig. 5 Phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) specific activities measured after 24 h fermentation. Values represent the average (\pm SD) of three independent experiments. Statistical significance was determined using the Student's *t*-test (***P* < 0.01).



Fig. 6 Relative expression levels of *ppc* and *mdh* at 30°C and 37°C. Relative transcript levels were evaluated using the level of *rnpB* as a reference, and then normalized by the levels of each gene at 30°C. Values represent the average (\pm SD) of three independent experiments. Statistical significance was determined using the Student's *t*test (***P* < 0.01).

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In Fig. 4, the initial slope of ¹³C-fraction indicates turnover of metabolites. Organic acids showed slower turnover than sugar phosphates and triose phosphates at both temperatures. Therefore, a bottleneck is still present at one of the reactions between glycolysis and the TCA cycle in Ppc-ox grown at 37°C.

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390 3.4. Improvement of succinate bioproduction

391 PEPC forms the TCA intermediate oxaloacetate via reaction of the glycolysis392 intermediate PEP with bicarbonate. In order to improve succinate production from

the TCA cycle, sodium bicarbonate was added to the fermentation. As shown in Fig. 7, an additive effect of high temperature and NaHCO₃ addition on the secretory production of D-lactate and succinate was observed. Succinate production was the highest (400.8 mg/L) in the presence of 100 mM NaHCO₃ at 37°C in Ppc-ox. The highest D-lactate (1,097.9 mg/L) titer was obtained in the presence of 200 mM NaHCO₃. The addition of up to 300 mM NaHCO₃ resulted in increases of pH from 7.6 to no higher than 7.8.

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Fig. 7 Effect of NaHCO₃ addition on D-lactate, succinate and acetate production after 72 hours fermentation. Bars on the left hand side in blue and bars on the right hand side in orange indicate concentrations at 30°C and 37°C, respectively. Values represent the average (\pm SD) of three independent experiments.

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Effect of initial cell concentration on D-lactate and succinate production was also investigated at 37°C in the presence of 100 mM NaHCO₃ (Fig. 8). Production of D-lactate and succinate was dependent on the initial cell concentration, with the highest concentration of D-lactate (1,520.3 mg/L) and succinate (1,802.3 mg/L) produced using 25 g-DCW/L (Fig. 8). Both succinate and lactate titers stopped improving after the initial cell concentration was increased to 413 over 25 g-DCW/L.

Since acetate was a major byproduct during succinate production, further 414 optimization of succinate and lactate production was investigated by knocking out 415the acetate kinase (AK) gene (ackA, sll1299) in the Ppc-ox strain. This resulted in 416 significantly lower production of acetate byproduct, while D-lactate production 417418 increased to 10.7 g/L, the highest reported titer in cyanobacteria (Fig. 8, lower panels). Although succinate production in Ppc-ox/ $\Delta ackA$ decreased when using cell 419 420densities of 25 and 37.5 g-DCW/L, succinate levels slightly increased when using 5, 12.5 and 50 g-DCW/L. Interestingly, without ackA, levels of PEP, pyruvate and 421AcCoA all increased which is consistent with higher lactate production (Fig. 9). 422423Despite the increases in lactate and succinate production, knockout of ackA did not affect culture pH. This may be due to the presence of 100 mM Hepes-KOH, pH 7.8, 424425or by the additional NaHCO₃ during increased lactate production.

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Fig. 8 D-lactate, succinate and acetate produced after 72 hours fermentation at 37°C using 100 mM NaHCO₃ and various initial cell concentrations. Organic acid production in the Ppc-ox strain is shown in the upper 3 panels, and production in the *ackA* knockout of Ppc-ox is shown in the lower 3 panels. Values represent the average (± SD) of three independent experiments.



436
437 Fig. 9 Time-course of organic acid secretion and intracellular metabolites in Ppc-ox
438 (closed circles, also shown in Fig. 2) and Ppc-ox/ΔackA (open triangles) cultivated

anaerobically at 37° C without NaHCO₃ supplementation. Values represent the average (± SD) of three independent experiments. Effects of *ackA* deletion on organic acid production are shown in the upper panel, and effects on related intracellular metabolites are shown in the lower two panels. The middle panel shows the pathway to succinate discussed in this study.

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446 **4. Discussion**

In this study, temperature enhanced secretory production of organic acids in *Synechocystis* 6803 was observed under dark anoxic conditions. A metabolomics approach unveiled dynamic variations in the pool size and turnover of metabolites dependent on the fermentation temperature. To the best of our knowledge, this is the first comprehensive temperature dependent metabolomics study of a microorganism (Yurkovich et al., 2017).

Analysis of ¹³C-fraction turnover indicates that a bottleneck between PEP 453and the TCA cycle was relaxed by increasing temperature from 30°C to 37°C (Fig. 4544). The pool size of citrate, *cis*-aconitate and isocitrate was lower at 37°C, while 455malate, fumarate and succinate were increased by elevating temperature during 24 456h fermentation (Fig. 3). ¹³C atom number of malate, fumarate and succinate greatly 457increased with elevating temperature (Table 1). The TCA cycle in Synechocystis 4586803 is branched into oxidative and reductive routes. These results suggest that 459increase in temperature boosts the reductive TCA cycle to produce succinate. 460

461 A few studies have reported effects of temperature on bioproduction. For 462 example, production of succinate in *E. coli* strain SBS550MG was not significantly

affected by temperatures of 37°C or 43°C, whereas glucose concentration, CO₂ 463 464 concentration, pH and cell density were all found to be important factors (Martínez et al., 2011). For production of ascorbic acid-2-phosphate in Brevundimonas 465diminuta, cell density, pH and temperature were key variables, with an optimal 466 temperature of 40°C (Shin et al., 2007). Increase in temperature from 33 to 39.5°C 467 was found to induce glutamate production in Corynebacterium glutamicum 468 (Gourdon and Lindley, 1999). However, the metabolic pathways underlying the 469 effects of temperature increase on bioproduction have not been comprehensively 470 investigated. 471

472In the current study, glycogen was produced during photosynthesis, 473followed by utilization of glycogen and carbon dioxide as carbon sources. 474Overexpression of ppc increased glycogen utilization to 89% and also improved 475succinate production at 37°C. Throughout fermentation cell density remained 476 constant while OD₇₅₀ and TEM observed high density particles both decreased, indicating the consumption of glycogen (Fig. S1). Although the optimal 477478 temperature of Synechocystis 6803 PEPC was reported to be 30°C, the enzyme retained 80% of its activity at 40°C and was tolerant to allosteric inhibition (Takeya 479et al., 2017). 480

Global transcriptional analysis of *E. coli* indicates that a decrease in temperature from 37°C to 28°C results in an increase in TCA cycle encoding enzymes (Gadgil et al., 2005). Conversely, a 30°C to 45°C temperature increase led to increased glycolytic flux, decreased flux from the TCA cycle into anabolic pathways, and a decrease in adenylate energy charge (Wittmann et al., 2007).

486 AMP and ADP allosterically activate the *E. coli* glycolytic enzymes pyruvate kinase II (Kotlarz et al., 1975) and phosphofructokinase I (Babul, 1978), 487respectively (Li et al., 2017). Additionally, oxidative phosphorylation generates ATP 488 from NADH, which inhibits *E. coli* citric acid enzymes pyruvate dehydrogenase 489(Kim et al., 2008) and citrate synthase (Talgoy and Duckworth, 1979; Weitzman, 490 1981). Thus, higher concentrations of ATP slow down central metabolism (Kihira 491et al., 2011), and a 10% decrease in ATP concentration was enough to exert a 492493beneficial effect on succinate production (Li et al., 2017). Reduction of anaerobic 494 ATP levels via PEP synthase mediated futile cycling was also critical to obtain the 495impressive heterotrophic succinate titer of 31.87 g/L (Li et al., 2017). Accordingly, 496 in the current study, increasing temperature to 37°C had a dramatic effect on the 497 increase of ADP and AMP concentrations, and a moderate but significant effect on lowering ATP level. Therefore, the current study further emphasizes that lowering 498 499 adenylate energy charge is an effective strategy for increasing autotrophic 500succinate yields.

501Reducing anaerobic NADH/NAD+ ratio by overexpression of the fumarate reductase gene was reported to be important for increasing succinate yield in E. 502coli (Li et al., 2017). A high NADH/NAD⁺ ratio has also been reported to have a 503negative effect on bioproducton of glutamate in C. glutamicum (Gourdon and 504Lindley, 1999). Conversely, in the cyanobacteria Synechococcus sp. PCC 7002, 505knockout of nitrate reductase led to an increase in NADH/NAD⁺ ratio as well as 506glycogen, H₂, lactate and glycogen production (Qian et al., 2016). Cyanobacteria 507nitrogen reduction normally leads to NADH consumption and catabolism of 508

glycogen via glycogen phosphorylase and phosphoglucomutase. Accordingly,
glycogen metabolism and NADH/NAD⁺ ratio should be carefully investigated and to
further optimize the autotrophic production of succinate.

Under anaerobic conditions cyanobacteria run their TCA cycle backwards 512from oxaloacetate to succinate (Fig. 9), which generates NAD⁺ from NADH. In this 513study, overexpression of *ppc* in the metabolically engineered strain Ppc-ox provides 514more oxaloacetate for this route. Additional ackA knockout in Ppc-ox results in 515increased levels of glycolytic intermediates PEP, pyruvate and AcCoA, while TCA 516517intermediates to succinate decreased (Fig. 9). This indicates that in the Ppc-ox ackA 518knockout PEPC can no longer overcome the glycolysis to TCA bottleneck mentioned 519above, and therefore additional overexpression of the pyruvate carboxylase (PC) 520gene may be useful to further increase succinate titers (Meng et al., 2016). As NADH levels also increase in the Ppc-ox ackA knockout, the overexpression of 521522malate dehydrogenase or NADH dependent fumarate reductase genes are additional options to improve succinate production while at the same time 523524decreasing NADH/NAD⁺ ratio (Li et al., 2017).

Previous knockout of *ackA* together with overexpression of *SigE* and 200 mM KCl supplementation in *Synechococcus* 6803 resulted in an increase of succinate titers up to 141 mg/L and an increase of lactate titers up to 217.6 mg/L (Table 2, Ueda et al., 2016). In the current report, knockout of *ackA* further improved succinate titers over 10 fold using *Synechocystis* 6803 Ppc-ox. Although impressive succinate titers have been achieved using heterotrophic *E. coli* grown with glucose as an energy source (Li et al., 2017), production in autotrophic cells

must be optimized in order to develop a renewable process. Cyanobacteria 532succinate production has previously been optimized using a variety of strategies 533(Table 2). Using only photosynthetic conditions, succinate levels could be increased 534further in *Synechococcus elongatus* PCC 7942 to 430 mg/L (Lan et al., 2016). In the 535current autotrophic study, the highest autotrophic succinate titer of 1.8 g/L was 536achieved using the metabolically engineered strain Ppc-ox with overexpression of 537ppc in combination with a straightforward approach of carbon dioxide utilization, 538and elevation of fermentation temperatures to 37°C. 539

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543	Table 2	Comparison of	f cyanobacteria	succinate	bioproduction	studies.
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Study	Organism	Conditions	Succinate Titer (Cultivation time)	Succinate Productivity (Cultivation time used for calculation)
Li et al., 2016	Synechococcus elongatus PCC 7942	Photosynthetic, nitrogen starvation, <i>∆glgc,</i> +gltA, +ppc	435.0 μg/L (48 h)	9 μg/L/h (0-48 h)
Huang et al., 2016	Synechococcus elongatus PCC 7942	Photosynthetic, nitrogen starvation, repression of <i>glgc</i> , <i>sdhA</i> , <i>sdhB</i>	580-630 μg/L (48 h)	13 μg/L/h (0-48 h)
Osanai et al., 2015	Synechococcus sp. PCC 6803	Dark anaerobic, +sigE, ΔackA	~115 mg/L (96 h)	1.38 mg/L/h (0-96 h)
Ueda et al., 2016	Synechococcus sp. PCC 6803	Dark anaerobic, + <i>sigΕ, ΔackΑ</i> , 200 mM KCI	141.0 mg/L (72 h)	2 mg/L/h (0-72 h)
Lan et al., 2016	Synechococcus elongates PCC 7942	Photosynthetic, overexpression of 4 genes*	430 mg/L (192 h)	3.33 mg/L/h (72-96 h)
This study	Synechocystis sp. PCC 6803	Dark anoxic, Ppc-ox, NaHCO₃, 37°C	1.8 g/L (72 h)	25 mg/L/h (0-72 h)

544The study by Lan et al. utilized overexpression of α -ketoglutarate decarboxylase, 545 succinate semialdehyde dehydrogenase, citrate synthase and PEPC genes.

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746 Supplementary Materials

747Supplementary Table 1Organic acid produced after 72 h cultivation at 30°C and748 37° C. Values represent the average (± SD) of three independent experiments.749Statistical significance was determined using the Student's *t*-test (**P* < 0.05, ***P* <</td>7500.01).

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Organic acid	Production (mg/L)		
_	30°C	37°C	
Fumarate	11.27 ± 0.74	57.32 ± 8.82*	
2-Ketoglutarate	6.75 ± 0.14	13.67 ± 1.65*	
Malate	13.38 ± 1.13	46.69 ± 5.11**	
Nicotinate	0.07 ± 0.02	0.46 ± 0.03**	
Pyruvate	0.64 ± 0.04	1.68 ± 0.16**	
Shikimate	0.07 ± 0.01	0.14 ± 0.04	

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Fig. S1 Transmission electron micrographs of Ppc-ox cultivated at 30°C and 37°C for 96 h (a). (b) Time course of cell number at 30°C (blue circles), 35°C (purple circles) and 37°C (red circles). Values represent the average (\pm SD) of three independent experiments. (c) Decrease in OD₇₅₀ of cells grown at 30°C (blue circles), 35°C (purple circles) and 37°C (red circles). Values represent the average (\pm SD) of three independent experiments.



763Fig. S2Time course of intracellular amino acid pool size in Ppc-ox cultivated at764 30° C (blue triangles), 35° C (green squares) and 37° C (red circles). Values represent765the average (± SD) of three independent experiments.



Fig. S3 Time-course for mass distribution of metabolites near the pyruvate branch at 30°C and 37°C. Values are the average (\pm SD) of three independent experiments. Mi represents the relative isotopomer abundance for each metabolite in which *i*¹³C atoms are incorporated.