Malic Enzyme, not Malate Dehydrogenase, Mainly Oxidizes Malate That Originates from the Tricarboxylic Acid Cycle in Cyanobacteria

Noriaki Katayama,a Kaori Iwazumi,a Hiromi Suzuki,a Takashi Osanai,a Shoki Itoa

School of Agriculture, Meiji University, Kawasaki, Kanagawa, Japan

ABSTRACT Oxygenic photoautotrophic bacteria, cyanobacteria, have the tricarboxylic acid (TCA) cycle, and metabolite production using the cyanobacterial TCA cycle has been spotlighted recently. The unicellular cyanobacterium Synechocystis sp. strain PCC 6803 (Synechocystis 6803) has been used in various studies on the cyanobacterial TCA cycle. Malate oxidation in the TCA cycle is generally catalyzed by malate dehydrogenase (MDH). However, Synechocystis 6803 MDH (SyMDH) is less active than MDHs from other organisms. Additionally, SyMDH uses only NAD⁺ as a coenzyme, unlike other TCA cycle enzymes from Synechocystis 6803 that use NADP⁺. These results suggest that MDH rarely catalyzes malate oxidation in the cyanobacterial TCA cycle. Another enzyme catalyzing malate oxidation is malic enzyme (ME). We clarified which enzyme oxidizes malate that originates from the cyanobacterial TCA cycle using analyses focusing on ME and MDH. In contrast to SyMDH, Synechocystis 6803 ME (SyME) showed high activity when NADP⁺ was used as a coenzyme. Unlike the Synechocystis 6803 mutant lacking SyMDH, the mutant lacking SyME accumulated malate in the cells. ME was more highly preserved in the cyanobacterial genomes than MDH. These results indicate that ME mainly oxidizes malate that originates from the cyanobacterial TCA cycle (named the ME-dependent TCA cycle). The ME-dependent TCA cycle generates NADPH, not NADH. This is consistent with previous reports that NADPH is an electron carrier in the cyanobacterial respiratory chain. Our finding suggests the diversity of enzymes involved in the TCA cycle in the organisms, and analyses such as those performed in this study are necessary to determine the enzymes.

IMPORTANCE Oxygenic photoautotrophic bacteria, namely, cyanobacteria, have the tricarboxylic acid (TCA) cycle. Recently, metabolite production using the cyanobacterial TCA cycle has been well studied. To enhance the production volume of metabolites, understanding the biochemical properties of the cyanobacterial TCA cycle is required. Generally, malate dehydrogenase oxidizes malate in the TCA cycle. However, cyanobacterial malate dehydrogenase shows low activity and does not use NADP⁺ as a coenzyme, unlike other cyanobacterial TCA cycle enzymes. Our analyses revealed that another malate oxidation enzyme, the malic enzyme, mainly oxidizes malate that originates from the cyanobacterial TCA cycle. These findings provide better insights into metabolite production using the cyanobacterial TCA cycle. Furthermore, our findings suggest that the enzymes related to the TCA cycle vary from organism to organism and emphasize the importance of analyses to identify the enzymes such as those performed in this study.

KEYWORDS aerobic respiration, cyanobacteria, malate dehydrogenase, malic enzyme, tricarboxylic acid cycle
metabolite production from carbon dioxide (1, 2). The non-nitrogen-fixing unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (*Synechocystis* 6803) has many characteristics that make it suitable as a model organism, such as natural transformation capability (3), whole-genomic information (4), and tolerance for cryopreservation. Therefore, *Synechocystis* 6803 has been widely used in both basic and applied studies.

The tricarboxylic acid (TCA) cycle is one of the most important metabolic pathways for the generation of energy and amino acids. Cyanobacteria do not possess a 2-oxoglutarate dehydrogenase complex that catalyzes the conversion of 2-oxoglutarate to succinyl coenzyme A (succinyl-CoA) in the TCA cycle (5, 6). Therefore, the cyanobacterial TCA cycle was thought for over 4 decades to be incomplete. However, in 2011, it was discovered that 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase catalyze the conversion of 2-oxoglutarate to succinate in the cyanobacterial TCA cycle (7). Since then, the cyanobacterial TCA cycle has been considered complete, and various studies regarding the cyanobacterial TCA cycle have been conducted. In *Synechocystis* 6803, the γ-aminobutyric acid shunt also contributes to the conversion of 2-oxoglutarate to succinate in the TCA cycle (8). The glyoxylate cycle, which is a variant of the TCA cycle, is important for acetate assimilation in some nitrogen-fixing cyanobacteria (9). In recent years, studies of metabolite production using the TCA cycle in *Synechocystis* 6803 have been well performed (10–14). However, in *Synechocystis* 6803, metabolic flux through the TCA cycle is lower than that through other carbon metabolic pathways, such as glycolysis (15). Understanding the biochemical properties of the cyanobacterial TCA cycle is necessary to improve metabolic flux through the TCA cycle. Until 2016, only isocitrate dehydrogenase was biochemically analyzed among the TCA cycle enzymes in *Synechocystis* 6803 (see Table S1 in the supplemental material) (16). Recently, biochemical analyses of TCA cycle enzymes in *Synechocystis* 6803 have been performed (Table S1) (17–22).

Malate dehydrogenase (MDH; EC 1.1.1.37) catalyzes the following reversible redox reactions in the TCA cycle: malate $+$ NAD$^+$ $\rightarrow$ oxaloacetate $+$ NADH. *Synechocystis* 6803 MDH (*Sy*MDH) has lower malate oxidation activity than MDHs from other organisms and specifically catalyzes the reductive reaction (18). In addition, *Sy*MDH uses only NAD$^+$ as a coenzyme and has no enzymatic activity for NADP$^+$ (18), a coenzyme of the other two enzymes that catalyze the NAD(P)H-generating reaction in the TCA cycle in *Synechocystis* 6803 (isocitrate dehydrogenase and succinic semialdehyde dehydrogenase) (Table S1) (16, 21). These results suggest that MDH seldom catalyzes malate oxidation in the cyanobacterial TCA cycle, and the enzyme(s) that oxidizes malate that originates from the cyanobacterial TCA cycle remains unclear.

The other enzyme conserved in most organisms that can catalyze malate oxidation is malic enzyme (ME; EC 1.1.1.38, 1.1.1.39, 1.1.1.40). ME catalyzes the following reversible redox reaction: malate $+$ NAD(P)$^+$ $\rightarrow$ pyruvate $+$ NAD(P)H $+$ CO$_2$. A *Synechocystis* 6803 mutant which carries a transposon insertional mutation in the me gene exhibits poor growth under photoautotrophic conditions (23), and me gene-disrupted mutants cannot grow under dark heterotrophic conditions (15). These results suggest that ME is necessary for normal growth in cyanobacteria. However, biochemical characteristics of cyanobacterial ME, such as the malate oxidation activity, coenzyme specificity, and reaction specificity, have not yet been revealed. Thus, it is unclear whether ME oxidizes malate that originates from the TCA cycle in cyanobacteria.

In this study, we revealed that ME, not MDH, mainly oxidizes malate that originates from the TCA cycle in cyanobacteria using analyses focusing on ME and MDH.

**RESULTS**

*Sy*ME showed higher activity than *Sy*MDH for malate oxidation when using NADP$^+$ as a coenzyme. First, we performed biochemical analysis of *Sy*ME. *Sy*ME was purified as a His-tagged protein (see Fig. S1 in the supplemental material). *Sy*ME showed the highest activity at 50°C and pH 8.3 when NADP$^+$ was utilized as a coenzyme (Fig. 1A). Similar to other bacterial MEs (24–26), *Sy*ME activity strongly depended on monovalent and divalent cations (particularly NH$_4^+$ and Mn$^{2+}$) (Fig. S2). The $S_{0.5}$
(half-saturation concentration) values of SyME for NH₄Cl and MnCl₂ were 18.1 mM and 0.0072 mM, respectively (Fig. 1B). Therefore, we defined the optimum conditions of SyME as follows: 50°C and pH 8.3 in the presence of 100 mM NH₄Cl and 0.5 mM MnCl₂.

We compared the kinetic parameters under optimum conditions (SyME, 50°C and pH 8.3; SyMDH, 50°C and pH 8.0) (18) between SyME and SyMDH (Table 1). Similar to SyME, SyMDH was purified as a His-tagged protein (Fig. S1). Both SyME and SyMDH activities increased depending on the concentrations of malate and NAD(P)⁺ (Fig. 2A and B). The $S_{0.5}$ and $k_{cat}$ (turnover number) of SyME for malate were lower and higher than those of SyMDH, respectively (Table 1). The $k_{cat}/S_{0.5}$ (catalytic efficiency) of SyME for malate was 264-fold higher than that of SyMDH (Table 1). In contrast to SyMDH, SyME specifically showed enzymatic activity for NADP⁺ rather than NAD⁺; the $k_{cat}/S_{0.5}$ for NADP⁺ was 437-fold higher than that for NAD⁺ (Table 1 and Table S2). The $k_{cat}/S_{0.5}$ of SyME for NADP⁺ was 2,673-fold higher than that of SyMDH for NAD⁺ (Table 1). SyME also showed no enzymatic activity in the reductive reaction of the conversion of pyruvate to malate. However, SyMDH showed high specificity for the reductive reaction of the conversion of oxaloacetate into malate (Fig. 2B and C); the $k_{cat}/S_{0.5}$ values for oxaloacetate and NADH were 57-fold and 264-fold higher than those for malate and NAD⁺, respectively (Table 1).

Similarly, the differences in the kinetic parameters between SyME and SyMDH were confirmed at the optimum growth temperature of Synechocystis 6803 (30°C) (27) (Table 2). SyME showed higher catalytic efficiency in malate oxidation than SyMDH when using NADP⁺ as a coenzyme (Table 2). In contrast to SyME, SyMDH showed high specificity for the reductive reaction (Table 2).
Mechanism of Malate Oxidation in Cyanobacteria

TABLE 1 Kinetic parameters of cyanobacterial malic enzymes and malate dehydrogenases under optimum conditions

| Reaction and enzyme | Substrate or coenzyme | $S_{0.5}$ (mM) | $k_{cat}$ (s⁻¹) | $k_{cat}/S_{0.5}$ (s⁻¹ mM⁻¹) | $n_s$ |
|---------------------|-----------------------|----------------|-----------------|-----------------------------|-------|
| Malate oxidation    | SyME                  | Malate         | 0.46 ± 0.06*    | 77.2 ± 5.9**              | 169 ± 10** | 1.76 ± 0.25* |
|                     |                       | NADP⁺          | 0.015 ± 0.001*  | 55.7 ± 3.3**              | 3689 ± 23** | 2.14 ± 0.29* |
|                     | SyMDH                 | Malate         | 1.61 ± 0.37     | 1.0 ± 0.1                 | 0.64 ± 0.09 | 0.93 ± 0.06 |
|                     |                       | NAD⁺           | 1.34 ± 0.30     | 1.8 ± 0.1                 | 1.4 ± 0.2   | 0.72 ± 0.03 |
|                     | ArME                  | Malate         | 0.30 ± 0.03*    | 42.8 ± 0.9**              | 144 ± 14**  | 1.15 ± 0.07* |
|                     |                       | NADP⁺          | 0.034 ± 0.003** | 49.5 ± 2.0**              | 1458 ± 69** | 1.03 ± 0.11* |
|                     | ArMDH                 | Malate         | 0.034 ± 0.002   | 0.25 ± 0.004              | 7.3 ± 0.3   | 1.49 ± 0.08 |
|                     |                       | NAD⁺           | 0.30 ± 0.02     | 0.50 ± 0.01               | 1.7 ± 0.1   | 0.66 ± 0.01 |
|                     | NoME                  | Malate         | 0.41 ± 0.02*    | 30.3 ± 1.2**              | 75 ± 0.4**  | 1.57 ± 0.06** |
|                     |                       | NADP⁺          | 0.014 ± 0.004*  | 33.4 ± 3.2**              | 2533 ± 436* | 1.07 ± 0.28 |
|                     | NoMDH                 | Malate         | 1.88 ± 0.56     | 3.5 ± 0.4                | 2.0 ± 0.4   | 0.86 ± 0.10 |
|                     |                       | NAD⁺           | 8.45 ± 1.95     | 6.3 ± 0.5                | 0.77 ± 0.10 | 0.75 ± 0.02 |

Reructive reaction

| Reaction and enzyme | Substrate | $S_{0.5}$ (mM) | $k_{cat}$ (s⁻¹) | $k_{cat}/S_{0.5}$ (s⁻¹ mM⁻¹) | $n_s$ |
|---------------------|-----------|----------------|-----------------|-----------------------------|-------|
| SyMDH               | Oxaloacetate | 0.11 ± 0.002* | 4.0 ± 0.04**    | 36 ± 1**                   | 1.71 ± 0.07** |
|                     | NADH      | 0.018 ± 0.002* | 6.6 ± 0.5**    | 364 ± 19**                 | 1.75 ± 0.27** |
| ArMDH               | Oxaloacetate | 0.041 ± 0.001* | 1.4 ± 0.03**   | 34 ± 0.4**                 | 1.84 ± 0.08** |
|                     | NADH      | 0.0085 ± 0.0025** | 1.7 ± 0.1**   | 214 ± 50**                 | 1.14 ± 0.52 |
| NoME                | Pyruvate  | 6.55 ± 0.38** | 0.79 ± 0.03**   | 0.12 ± 0.003**             | 1.52 ± 0.01 |
|                     | NADPH     | 0.0074 ± 0.001 | 0.85 ± 0.03**   | 116 ± 10**                 | 0.94 ± 0.09 |
| NoMDH               | Oxaloacetate | 0.026 ± 0.002* | 1.1 ± 0.03*    | 42 ± 2**                   | 1.65 ± 0.05** |
|                     | NADH      | 0.0093 ± 0.0001* | 1.3 ± 0.1**    | 142 ± 8**                  | 1.10 ± 0.55** |

*The activities of SyME and ArME for the reductive reaction were not detected. These kinetic parameters represent the mean ± standard deviation calculated from three independent saturation curves. Asterisks above the kinetic parameters in malate oxidation represent statistically significant differences between the kinetic parameters of ME and MDH in malate oxidation obtained using Welch’s t test (*, $P < 0.05$; **, $P < 0.005$). Asterisks above the kinetic parameters in the reductive reaction represent statistically significant differences between the kinetic parameters in malate oxidation and the reductive reaction obtained from Welch’s t test (*, $P < 0.05$; **, $P < 0.005$).

The explanation of each kinetic parameter is as follows: $S_{0.5}$, half-saturation concentration (concentration at 50% $V_{max}$); $k_{cat}$, turnover number; $k_{cat}/S_{0.5}$, catalytic efficiency; $n_s$, Hill coefficient.

SyME, not SyMDH, continuously catalyzed the sequential enzymatic reaction with fumarase. In the TCA cycle of higher plants, the interaction between adjacent enzymes is often important for sequential enzymatic reactions (28). Malate is generated from fumarate via a reaction catalyzed by fumarase (Fum) in the TCA cycle (Fig. 3A). We performed a coupled activity assay using Fum from Synechocystis 6803 (SyFum) at room temperature (23°C) (Fig. 3B). SyFum was purified as a His-tagged protein (Fig. 5I). After adding fumarate to the reaction mixture as a starting substrate, we monitored the amount of NAD(P)H produced by measuring the absorbance at 340 nm (Fig. 3B). When SyME was used as a malate oxidation enzyme, the absorbance at 340 nm increased over time; that is, the sequential enzymatic reaction with SyFum proceeded continuously (Fig. 3B). However, when SyMDH was used, the absorbance at 340 nm hardly changed; that is, the sequential enzymatic reaction with SyFum did not proceed (Fig. 3B).

Synechocystis 6803 strain lacking the me gene (ΔME strain) accumulated malate in the cells. To compare the catalytic activities of SyME and SyMDH in vivo, we constructed a Synechocystis 6803 strain lacking the me gene (ΔME strain) and citH gene encoding SyMDH (ΔMDH strain) (Fig. 4A and B) and measured the intracellular malate levels in these mutants. Since the deletion of the me gene in Synechocystis 6803 makes it impossible to grow under dark heterotrophic conditions (15), these mutants were cultivated under photoautotrophic conditions (Fig. 4C). The intracellular malate level in the ΔME strain was approximately 3-fold higher than that in the wild-type glucose-tolerant (GT) strain of Synechocystis 6803 (Fig. 4D). In contrast, the intracellular malate level in the ΔMDH strain was approximately the same as that in the GT strain (Fig. 4D). To confirm the malate accumulation in the ΔME strain, we...
introduced the \textit{me} gene into the ΔME strain, i.e., the ME-complement (ME-Comp) strain (Fig. 4A). The intracellular malate level in the ME-Comp strain was the same as that in the GT strain (Fig. 4E).

\textbf{ME was highly conserved in cyanobacterial genomes compared with MDH.} Our biochemical analysis indicated that ME had higher malate oxidation activity than MDH in \textit{Synechocystis} 6803. We performed bioinformatic analyses (BLAST analysis and phylogenetic analysis) using cyanobacterial ME and MDH sequences to examine the conservation of these enzymes among cyanobacteria (Table S3; Fig. 5 and Fig. S3). BLAST analyses of ME and MDH for all sequenced cyanobacteria (130 species) revealed that 78\% (102 species) and 51\% (66 species) of the sequenced cyanobacteria possessed ME and MDH, respectively (Table S3). In addition, 51\% (66 species) possessed both ME and MDH; that is, none of the cyanobacteria possessed only MDH (Table S3). Cyanobacteria are morphologically divided into unicellular and filamentous types. In some filamentous cyanobacteria, vegetative cells can differentiate into specialized cells to survive environmental stress (29). Phylogenetic analysis of cyanobacterial MEs revealed that the amino acid sequences of cyanobacterial MEs were highly preserved per each group.

\textbf{FIG 2} Saturation curves of \textit{Synechocystis} 6803 malic enzyme (ME) (\textit{SyME}) and \textit{Synechocystis} 6803 malate dehydrogenase (MDH) (\textit{SyMDH}) for substrates and coenzymes. (A) Saturation curves of \textit{SyME} for malate (left) and NADP$^+$ (right). These measurements were performed at 50°C and pH 8.3. In the measurement of the saturation curve for malate, the NADP$^+$ concentration was fixed at 0.5 mM. In the measurement of the saturation curve for NADP$^+$, the malate concentration was fixed at 3 mM. The concentrations of NH$_4$Cl and MnCl$_2$ were 100 mM and 0.5 mM, respectively. Error analyses of the curve fittings revealed that the coefficients of determination ($R^2$) of the saturation curves for malate and NADP$^+$ were 0.99219 and 0.96929, respectively. (B) Saturation curves of \textit{SyMDH} for malate (left) and NAD$^+$ (right). These measurements were performed under the optimum conditions of \textit{SyMDH} (50°C and pH 8.0) (18). In the measurement of the saturation curve for malate, the NAD$^+$ concentration was fixed at 8 mM. In the measurement of the saturation curve for NAD$^+$, the malate concentration was fixed at 4 mM. Error analyses of the curve fittings revealed that the $R^2$ of the saturation curves for malate and NAD$^+$ were 0.99445 and 0.99743, respectively. (C) Saturation curves of \textit{SyMDH} for oxaloacetate (left) and NADH (right). These measurements were performed at 50°C and pH 8.0. In the measurement of the saturation curve for oxaloacetate, the NADH concentration was fixed at 0.1 mM. In the measurement of the saturation curve for NADH, the oxaloacetate concentration was fixed at 0.5 mM. Error analyses of the curve fittings revealed that the $R^2$ of the saturation curves for oxaloacetate and NADH were 0.99525 and 0.98133, respectively. All data plots are the mean ± standard deviation calculated from three independent experiments.
The enzymes (Table 1) were calculated based on their saturation curves (Fig. S6 and S7). They were purified from Arthrospira platensis based on the cyanobacterial morphological characteristics (Fig. 5 and Fig. S3). Arthrospira platensis was conserved in other cyanobacteria.

The kinetic parameters of SyME and SyMDH were performed at the intracellular pH of Synechocystis 6803 (pH 7.8) (S2). In the measurements of the saturation curves of SyME, the concentrations of NH4Cl and MnCl2 were fixed at 100 mM and 0.5 mM, respectively. In the measurement of the saturation curve of SyME for malate, the NADP+ concentration was fixed at 0.5 mM. In the measurement of the saturation curve of SyME for NAD(P)H, the malate concentration was fixed at 3 mM. The activity of SyME for the reductive reaction was not detected. In the measurement of the saturation curve of SyMDH for malate, the NAD+ concentration was fixed at 8 mM. In the measurement of the saturation curve of SyMDH for malate, the NAD+ concentration was fixed at 8 mM. In the measurement of the saturation curve of SyMDH for NAD+, the malate concentration was fixed at 4 mM. In the measurement of the saturation curve of SyMDH for oxaloacetate, the NADH concentration was fixed at 0.1 mM. In the measurement of the saturation curve of SyMDH for NADH, the oxaloacetate concentration was fixed at 2 mM. These kinetic parameters represent the mean ± standard deviation calculated from three independent saturation curves. Asterisks above the kinetic parameters in malate oxidation represent statistically significant differences between the kinetic parameters of SyME and SyMDH in malate oxidation obtained using Welch’s t test (*, P < 0.05; **, P < 0.005). Asterisks above the kinetic parameters in the reductive reaction represent statistically significant differences between the kinetic parameters in malate oxidation and the reductive reaction obtained from Welch’s t test (*, P < 0.05; **, P < 0.005).

![Image](https://example.com/image)

**TABLE 2** Kinetic parameters of *Synechocystis* 6803 malic enzyme and malate dehydrogenase at 30°C

| Reaction and enzyme | Substrate or coenzyme | $S_{0.5}$ (mM) | $k_{cat}$ (s⁻¹) | $k_{cat}/S_{0.5}$ (s⁻¹ M⁻¹) | $n_m$ |
|---------------------|-----------------------|----------------|-----------------|---------------------------|-------|
| Malate oxidation    | SyME                  |                |                 |                           |       |
|                     | Malate                | 0.92 ± 0.06** | 39.1 ± 1.7**   | 43 ± 2**                  | 2.74 ± 0.39* |
|                     | NADP⁺                 | 0.014 ± 0.002*| 42.1 ± 3.5**   | 3.017 ± 209**            | 1.17 ± 0.24 |
|                     | NAD⁺                  | 1.79 ± 0.27   | 2.46 ± 0.20    | 1.4 ± 0.1                | 1.69 ± 0.35 |
|                     | Malate                | 0.23 ± 0.03   | 0.72 ± 0.02    | 3.1 ± 0.3                | 0.98 ± 0.04 |
|                     | NAD⁺                  | 0.35 ± 0.04   | 0.64 ± 0.02    | 1.8 ± 0.2                | 0.92 ± 0.06 |
| SyMDH               | Oxaloacetate          | 0.63 ± 0.07*  | 12.9 ± 0.5**   | 20 ± 1**                 | 1.03 ± 0.02 |
|                     | NADH                  | 0.0071 ± 0.0019*| 10.4 ± 1.1** | 1.512 ± 274*            | 0.72 ± 0.17 |

*The measurements of saturation curves of SyME and SyMDH were performed at the intracellular pH of Synechocystis 6803 (pH 7.8) (S2). In the measurements of the saturation curves of SyME, the concentrations of NH4Cl and MnCl2 were fixed at 100 mM and 0.5 mM, respectively. In the measurement of the saturation curve of SyME for malate, the NADP⁺ concentration was fixed at 0.5 mM. In the measurement of the saturation curve of SyME for NAD(P)H, the malate concentration was fixed at 3 mM. The activity of SyME for the reductive reaction was not detected. In the measurement of the saturation curve of SyMDH for malate, the NAD⁺ concentration was fixed at 8 mM. In the measurement of the saturation curve of SyMDH for malate, the NAD⁺ concentration was fixed at 8 mM. In the measurement of the saturation curve of SyMDH for NAD⁺, the malate concentration was fixed at 4 mM. In the measurement of the saturation curve of SyMDH for oxaloacetate, the NADH concentration was fixed at 0.1 mM. In the measurement of the saturation curve of SyMDH for NADH, the oxaloacetate concentration was fixed at 2 mM. These kinetic parameters represent the mean ± standard deviation calculated from three independent saturation curves. Asterisks above the kinetic parameters in malate oxidation represent statistically significant differences between the kinetic parameters of SyME and SyMDH in malate oxidation obtained using Welch’s t test (*, P < 0.05; **, P < 0.005). Asterisks above the kinetic parameters in the reductive reaction represent statistically significant differences between the kinetic parameters in malate oxidation and the reductive reaction obtained from Welch’s t test (*, P < 0.05; **, P < 0.005).

Based on the cyanobacterial morphological characteristics (Fig. 5). The amino acid sequences of cyanobacterial MDHs were also highly preserved per each group (Fig. S3).

**Differences in biochemical characteristics between ME and MDH observed in *Synechocystis* 6803 were conserved in other cyanobacteria.** Finally, we performed biochemical analyses of MEs and MDHs from two cyanobacterial species, namely, *Arthospira platensis* NIES-39 and Nostoc sp. strain PCC 7120, which belong to the other groups based on the cyanobacterial morphological characteristics (Fig. 5 and Fig. S3). They were purified as glutathione S-transferase (GST)-tagged proteins (Fig. S1). Biochemical analyses of these enzymes were generally conducted under each optimum condition; however, the measurement temperature of MDHs was standardized to the optimum temperature of SyMDH (50°C) to avoid the decomposition of oxaloacetate under high-temperature conditions (Fig. S4 and S5).

The kinetic parameters of *Arthospira platensis* NIES-39 and Nostoc sp. PCC 7120 enzymes (Table 1) were calculated based on their saturation curves (Fig. S6 and S7). The $k_{cat}/S_{0.5}$ of *Arthospira platensis* NIES-39 ME (ArME) for malate was 20-fold higher than that of *Arthospira platensis* NIES-39 MDH (ArMDH) (Table 1). ArME and ArMDH specifically used NADP⁺ and NAD⁺, respectively, as coenzymes (Table 1 and Table S2). The $k_{cat}/S_{0.5}$ of ArME for NADP⁺ was 858-fold higher than that of ArMDH for NAD⁺ (Table 1). ArME showed no enzymatic activity for the reductive reaction (Table 1). In contrast, ArMDH showed high specificity for the reductive reaction (Table 1). The $k_{cat}/S_{0.5}$ of Nostoc sp. PCC 7120 ME (NoME) for malate was 38-fold higher than that of Nostoc sp. PCC 7120 MDH (NoMDH) (Table 1). NoME and NoMDH specifically used NADP⁺ and NAD⁺, respectively, as coenzymes (Table 1 and Table S2). The $k_{cat}/S_{0.5}$ of NoME for NADP⁺ was 3,290-fold higher than that of NoMDH for NAD⁺ (Table 1). NoME and NoMDH showed a high specificity for oxidative and reductive reactions, respectively (Table 1).
DISCUSSION

In this study, we examined which enzyme oxidizes malate that originates from the TCA cycle in cyanobacteria.

SyME showed markedly higher catalytic efficiency in malate oxidation than SyMDH at both 50°C and 30°C (Tables 1 and 2). The absolute intracellular concentration (molar concentration) of malate has not been reported in cyanobacteria but has been reported in *Escherichia coli* (1.7 mM) (30). Although SyME showed a lower affinity for malate than SyMDH at 30°C, the *S*0.5 of both enzymes (SyME, 0.92 mM; SyMDH, 0.23 mM) was lower than the intracellular concentration of malate in *E. coli* (Table 2). These results mean that the difference in the affinity for malate between SyME and SyMDH hardly affects the difference in the malate oxidation activity between SyME and SyMDH in *vivo*. Therefore, we consider that SyME shows higher malate oxidation activity than SyMDH in *vivo*. Similar to isocitrate dehydrogenase and succinic semialdehyde dehydrogenase from *Synechocystis* 6803 (16, 21), SyME showed high specificity for NADP⁺ rather than NAD⁺ (Tables 1 and 2 and see Table S2 in the supplemental material). In *Synechocystis* 6803, the intracellular concentration of NADP⁺ (0.614 μmol/g dry cell weight) is approximately the same as that of NAD⁺ (0.514 μmol/g dry cell weight) (31). These results indicate that SyME uses NADP⁺, not NAD⁺, as a coenzyme in *vivo*. Unlike MEs from higher plants (32–34), SyME showed no enzymatic activity for the reductive reaction (Tables 1 and 2). In higher plants, the TCA cycle enzymes form protein complexes (metabolons), and channeling of metabolites is performed between adjacent enzymes (28). The results of the coupled activity assay with SyFum suggested that channeling of malate was not performed between SyMDH and SyFum (Fig. 3B). Thus, SyME has more suitable properties for oxidizing malate that originates from the TCA cycle than SyMDH.

Unlike the deletion of the *citH* gene, deletion of the *me* gene increased the intracellular malate level under photoautotrophic conditions (Fig. 4D). Previous metabolic flux

---

**FIG 3** Coupled activity assays with fumarase (Fum) from *Synechocystis* 6803 (SyFum). (A) Model drawing of malate metabolism in the tricarboxylic acid (TCA) cycle. (B) Results of coupled activity assays with SyFum. The red circles and blue triangles indicate the results using *Synechocystis* 6803 malic enzyme (ME) (SyME) and *Synechocystis* 6803 malate dehydrogenase (MDH) (SyMDH) as enzymes catalyzing malate oxidation, respectively. All data plots are the mean ± standard deviation calculated from three independent experiments.
analysis in _Synechocystis_ 6803 revealed that under photoautotrophic conditions, there are fluxes from malate both to oxaloacetate and to pyruvate (35). _Synechocystis_ 6803 accumulates malate in the cells under photoautotrophic conditions rather than dark conditions (36). These results suggest that under photoautotrophic conditions, SyME constantly catalyzes malate oxidation, while SyMDH can catalyze malate oxidation against its reaction specificity when malate is accumulated in the cells. In addition, previous metabolic flux analyses in _Synechocystis_ 6803 revealed that under mixotrophic conditions (37) and nitrogen-limited conditions (35), the reaction between malate and oxaloacetate and that between malate and pyruvate proceed in reductive and oxidative directions, respectively. These results reflect the differences in biochemical characteristics between SyME and SyMDH revealed in this study. From the above, we concluded that SyME, but not SyMDH, mainly oxidizes malate that originates from the TCA cycle (named the ME-dependent TCA cycle) (Fig. 6). In the ME-dependent TCA cycle, oxaloacetate is generated from phosphoenolpyruvate via the reaction catalyzed by phosphoenolpyruvate carboxylase (PEPC), which all photoautotrophs possess (Fig. 6).

The results of the BLAST analyses of ME and MDH in cyanobacteria suggested that ME is a more important enzyme for cyanobacterial carbon metabolism than MDH (Table S3). Cyanobacteria that possess only ME, not MDH, are candidates for exclusively possessing the ME-dependent TCA cycle, not the classical TCA cycle (named the MDH-dependent TCA cycle). However, to prove that only the ME-dependent TCA cycle acts in these cyanobacteria, further in vivo analyses such as metabolic flux analysis are also required. In the cyanobacteria that possess both ME and MDH, the differences in the
biochemical characteristics of ME and MDH, such as the malate oxidation activity, coenzyme specificity, and reaction specificity, were highly preserved (Table 1). These results suggest that the ME-dependent TCA cycle acts as an alternative to the MDH-dependent TCA cycle in these cyanobacteria.

The ME-dependent TCA cycle generates three molecules of NADPH rather than NADH (Fig. 6). In cyanobacteria, both the respiratory chain and the photosynthetic electron transport chain are present on the thylakoid membrane (38, 39). Cyanobacterial NAD(P)H dehydrogenase (complex I), which is involved not only in oxidative phosphorylation but also in cyclic electron flow during photosynthesis, receives electrons from the reduced ferredoxin (40). Cyanobacterial ferredoxin-NADP\(^+\) reductase uses NADPH, not NADH, as a coenzyme (41, 42). Thus, NADPH is utilized as an electron carrier in complex I through ferredoxin reduction in the cyanobacterial respiratory chain. This is consistent with our opinion that the cyanobacterial TCA cycle is an NADPH-generating pathway. In cyanobacteria, the oxidative pentose phosphate (OPP) pathway also acts as an NADPH-generating pathway under dark conditions (15, 43). In Synechocystis 6803, the OPP pathway metabolites accumulate immediately after a shift from light to dark conditions (44). The OPP pathway enzymes catalyzing NADPH-generating reactions in Synechocystis 6803 are strongly inhibited by citrate in the TCA cycle (21, 45). Therefore, we consider that when the flux through the OPP pathway decreases owing to the accumulation of citrate, the flux through the ME-dependent TCA cycle increases to generate NADPH.

In the ME-dependent TCA cycle, malate is converted to pyruvate (Fig. 6). In
cyanobacteria, pyruvate kinase (PK), which catalyzes the reaction that produces pyruvate from phosphoenolpyruvate, is inhibited by ATP, which is abundantly generated via the light reaction of photosynthesis (46, 47). Under photoautotrophic conditions, the Synechocystis 6803 mutant, which carries a transposon insertional mutation in the me gene, exhibits a lower growth rate than the GT strain; however, the growth rate in the mutant is restored by the addition of pyruvate (23). These results indicate that the ME-catalyzed reaction is important for pyruvate generation under photoautotrophic conditions in cyanobacteria. Under photoautotrophic conditions, not only the TCA cycle but also purine and urea metabolism generates fumarate in Synechocystis 6803 (12). However, previous metabolic flux analysis in Synechocystis 6803 revealed that under photoautotrophic conditions, the flux from succinate to fumarate in the TCA cycle is 4-fold higher than those from the other metabolites to fumarate (35). Therefore, we presume that under photoautotrophic conditions, the ME-dependent TCA cycle acts independently without being significantly affected by purine and urea metabolism.

In this study, we revealed that ME, but not MDH, mainly oxidizes malate that originates from the cyanobacterial TCA cycle. This finding suggests that the enzymes involved in the TCA cycle vary from organism to organism, and analyses focusing on enzymes, such as those performed in this study, are necessary to identify the reactions related to the TCA cycle.

MATERIALS AND METHODS

Construction of expression vectors that were transformed into E. coli cells. The genomic regions containing genes encoding SyME (slr0721), SyMDH (slr0891), SyFum (slr0018), ArME (NIES39_A01760), ArMDH (NIES39_M01090), NoME (alr4596), and NoMDH (alr3432) were commercially synthesized by Eurofin Genomics Japan (Tokyo, Japan). The regions containing the genes encoding SyME, SyMDH, and SyFUM were cloned into the BamHI-XhoI site of the pQE80L vector (Qiagen, Venlo, Netherlands). The regions containing the genes encoding ArME, ArMDH, NoME, and NoMDH were cloned into the BamHI-XhoI site of the pGEX6P-1 vector (GE Healthcare, Little Chalfont, UK).

Affinity purification of His-tagged proteins. The expression vectors containing the His-tagged Synechocystis 6803 enzyme (SyME, SyMDH, or SyFUM) were transformed into E. coli BL21(DE3) competent cells (BioDynamics Laboratory Inc., Tokyo, Japan). Thereafter, a shaking culture of 3.2 L of E. coli cells was conducted in LB medium overnight (25°C and 150 rpm) while inducing the expression of the His-tagged protein with 1 μM isopropyl-β-D-1-thiogalactopyranoside (Wako Chemicals, Osaka, Japan). After shaking culture, the E. coli cells were transferred to 20 mL of equilibrating buffer (20 mM Tris-HCl and 500 mM NaCl; pH 8.0) and disrupted by sonication (model VC-750; Eyela, Tokyo, Japan) for 2.5 min at 20% intensity. The suspension was then centrifuged (9,100 × g for 20 min at 4°C), and 1.5 mL of Talon metal affinity resin (TaKaRa Bio, Shiga, Japan) was added to the soluble fraction containing the His-tagged protein.

FIG 6 Schematic model of the malic enzyme (ME)-dependent tricarboxylic acid (TCA) cycle in cyanobacteria. PEPC, phosphoenolpyruvate carboxylase; PK, pyruvate kinase; PDH, pyruvate dehydrogenase; PS, pyruvate synthase; CS, citrate synthase; ACN, aconitase; ICD, isocitrate dehydrogenase; 2OGDC, 2-oxoglutarate decarboxylase; SSADH, succinic semialdehyde dehydrogenase; SDH, succinate dehydrogenase; FUM, fumarase.
The mixture was shaken gently for 30 min on ice to absorb the His-tagged protein in the resin. To remove the supernatant, the mixture was centrifuged (2,300 × g for 5 min at 4°C), and the residual resin bound to the GST-tagged protein was washed five times using 1 mL of washing buffer (20 mM Tris-HCl, 500 mM NaCl, and 5 mM imidazole; pH 8.0). Subsequently, the resin was washed five times using 1 mL of equilibrating buffer. After the His-tagged protein was eluted five times in 700 μL of His tag elution buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl and 150 mM imidazole), it was concentrated using a Vivaspin 500 30-kDa-molecular-weight-cutoff (MWCO) concentrator (GE Healthcare, Chicago, IL, USA).

Affinity purification of GST-tagged proteins. The expression vectors containing each GST-tagged cyanobacterial enzyme (ArME, ArMDH, NoME, or NoMDH) were transformed into E. coli BL21(DE3) competent cells. In the purification of ArME, ArMDH, and NoME, a shaking culture of 3.2 L of E. coli cells was conducted in LB medium overnight (25°C and 150 rpm) while inducing the expression of the GST-tagged protein with 1 μM isopropyl-β-D-1-thiogalactopyranoside. In the purification of NoMDH, a shaking culture of 3.2 L of E. coli cells was performed in LB medium for 5 days (10°C and 150 rpm) while inducing the expression with 1 μM isopropyl-β-D-1-thiogalactopyranoside. The shaken E. coli cells were transferred to 20 mL of PBS-T containing 100 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4·12H2O, 1.47 mM KH2PO4, 0.005% Tween 20. E. coli cells in PBS-T were disrupted by sonication (model VC-750) for 3.5 min at 20% intensity. The suspension was then centrifuged (5,800 × g for 10 min at 4°C), and 1 mL of glutathione-Sepharose 4B resin (GE Healthcare Japan, Tokyo, Japan) was added to the soluble fraction, including the GST-tagged protein. The mixture was shaken gently for 30 min on ice to absorb the GST-tagged protein in the resin. Subsequently, 1 mM ATP/MgSO4·7H2O was added to the mixture, which was shaken gently for 30 min at 37°C. To remove the supernatant, the mixture was centrifuged (5,800 × g for 2 min at 4°C). The residual resin binding to the GST-tagged protein was washed 15 times using 1 mL of PBS-T containing 1 mM ATP/MgSO4·7H2O. The GST-tagged protein was eluted five times in 1 mL of GST elution buffer (50 mM Tris-HCl [pH 8.0] and 10 mM reduced glutathione). Thereafter, the proteins were concentrated using a Vivaspin 500 concentrator with an MWCO of 50 kDa (Sartorius, Göttingen, Germany) or 30 kDa.

Enzyme assays. In the assays of ME and MDH activities for the oxidative reaction, NAD(P)H production was monitored as an increase in absorbance at 340 nm using a Shimadzu UV-1850 spectrometer (Shimadzu, Kyoto, Japan). The oxidative reaction catalyzed by ME was conducted in a 1-mL assay solution [100 mM Tris-HCl (pH 7 to 9), various concentrations of L-malate, various concentrations of NAD(P)H, various concentrations of NAD(P)H, various concentrations of MnCl2, and 100 pmol enzymes]. The oxidative reaction catalyzed by MDH was conducted in a 1-mL assay solution [100 mM Tris-HCl (pH 7.0 to 9.0) or N-cyclohexyl-3-aminopropanesulfonic acid (CAPS)–NaOH (pH 9.5 to 11.0), various concentrations of L-malate, various concentrations of NAD(P)H, and 30 to 250 pmol enzymes]. After incubating the assay solution without L-malate at various temperatures for 5 min, L-malate was added to the solution to start the reaction. In the assays of ME and MDH activities for the reductive reaction, NAD(P)H consumption was monitored as a decrease in absorbance at 340 nm. The reductive reaction catalyzed by ME was conducted in a 1-mL assay solution (100 mM Tris-HCl, various concentrations of sodium pyruvate, various concentrations of NADPH, 50 mM K2HCO3, 100 mM NH4Cl, various concentrations of MnCl2, and 25 to 200 pmol enzymes). The reductive reaction catalyzed by MDH was conducted in a 1-mL assay solution (100 mM Tris-HCl, various concentrations of oxaloacetate, various concentrations of NADH, and 10 to 100 pmol enzymes). After incubation of the assay solution without pyruvate or oxaloacetate at the optimum temperature for each enzyme for 5 min, pyruvate or oxaloacetate was added to the solution to start the reaction. One unit of enzymatic activity was defined as the amount of enzyme that could convert 1 μmol of substrate per minute.

Calculation of kinetic parameters. Except for $k_{cat}$, the kinetic parameters were calculated by curve fitting based on the Hill equation (below) (48) using Kaleida Graph ver. 4.5.

$$v = \frac{V_{max} [S]^{nH}}{[S]^{nH} + K_{S} + S^{nH}}$$

The $k_{cat}$ values were calculated by dividing the maximum reaction velocity ($V_{max}$) by the molar number of enzymes.

Coupled activity assays with SyFum. The composition of a 1-mL assay solution when using SyME as a malate oxidation enzyme was as follows: 100 mM Tris-HCl (optimum pH of SyFum, pH 7.5) (20), 0.163 mM fumarate, 0.614 mM NADP+, 100 mM NH4Cl, 0.5 mM MnCl2, 100 pmol SyFum, and 100 pmol SyME. The composition of a 1-mL assay solution when using SyMDH as a malate oxidation enzyme was as follows: 100 mM Tris-HCl (pH 7.5), 0.163 mM fumarate, 0.514 mM NAD+, 100 pmol SyFum, and 100 pmol SyMDH. The concentrations of these substrates and coenzymes were the ratio of intracellular concentrations in Synechocystis 6803 (29). After starting the reaction at room temperature (23°C) by adding fumarate, NAD(P)H production was monitored as an increase in absorbance at 340 nm for 10 min using a Shimadzu UV-1850 spectrometer.

Construction of vectors that were transformed into Synechocystis 6803 cells. The genomic region containing the me gene (sll0721) with N-terminal EcoRI and C-terminal XhoI sites was synthesized and cloned into the pEX-A2J1 vector containing the Amp promoter by Eurofin Genomics Japan. The genomic region containing the citH gene (sll0891) with N-terminal BamHI and C-terminal XhoI sites was synthesized and cloned into the pEX-A2J1 vector by Eurofin Genomics Japan. Regarding the vector used for the construction of the ME-Comp strain, 500 bp upstream and 100 bp downstream of the me gene were introduced into N-terminal NdeI and C-terminal HpaI sites, respectively, and the region was cloned into the pTTP2031V vector by Eurofin Genomics Japan.
Cyanobacterial strains and culture conditions. A GT strain of Synechocystis 6803 isolated in 1988 (49) was used as the wild-type Synechocystis 6803 strain. The GT strain was cultivated in 70 mL of modified BG-11 medium consisting of BG-11 liquid medium (50) buffered with 20 mM HEPES-KOH (pH 7.8). Both the ΔME and ΔMDH strains were cultivated in 70 mL of the modified BG-11 medium containing 0.3 μg/mL of chloramphenicol. The ME-Comp strain was cultivated in 70 mL of the modified BG-11 medium containing 0.8 μg/mL of kanamycin. The cultivation was conducted at 30°C under continuous white light (50 μmol photons/(m² · s⁻¹)) with the cultures bubbled with 1% (vol/vol) CO₂ in the air. The cell densities were calculated as optical density at 730 nm (OD730) using a Shimadzu UV-2700 spectrophotometer (Shimadzu, Kyoto, Japan), and the OD730 at the start of cultivation was fixed at 0.4.

Immunoblotting. Antibodies against SyME and SyMDH were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Immunoblotting was conducted using 12% SDS-PAGE gels as described previously (51).

Extraction of malate from Synechocystis 6803 cells. After 3 days of cultivation, Synechocystis 6803 cells (OD730 × culture volume [mL] = 150) were collected by centrifugation (5,800 × g for 2 min at 25°C). The cells were suspended in 600 μL of 60% (vol/vol) methanol, and the suspension was mixed using a Twin Mixer TM-282 (Asone, Osaka, Japan) for 15 min. The suspension was centrifuged (20,400 × g for 5 min at 4°C), and 500 μL of the supernatant was collected. The supernatant was added to an Amicon Ultra 3-kDa-cutoff filter (Merck, Billerica, MA, USA) and centrifuged (20,400 × g for 60 min at 4°C). After centrifugation, 350 μL of the filtrate was dried using a CVE-2000 centrifugal evaporator (Eyela, Tokyo, Japan). The residue was dissolved in 30 μL of 100 mM Tris-HCl buffer (pH 8.0). The concentration of malate in the sample was measured using an E-kit for liquid L-malate (J. K. International, Tokyo, Japan).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.2 MB.
FIG S2, TIF file, 0.1 MB.
FIG S3, TIF file, 0.2 MB.
FIG S4, TIF file, 0.1 MB.
FIG S5, TIF file, 0.1 MB.
FIG S6, TIF file, 0.1 MB.
FIG S7, TIF file, 0.1 MB.
TABLE S1, DOCX file, 0.02 MB.
TABLE S2, DOCX file, 0.02 MB.
TABLE S3, XLSX file, 0.02 MB.

ACKNOWLEDGMENTS

This study was supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan, a grant to S.I. from JSPS KAKENHI Grant-in-Aid for JSPS Fellows (grant number 20J20651), a grant to T.O. from ALCA from the Japan Science and Technology Agency (grant number JPMJAL1306), JSPS KAKENHI Grant-in-Aid for Scientific Research on Innovative Areas (grant number 16H06559), Grant-in-Aid for Scientific Research (B) (grant number 20H02905), and Grant-in-Aid for Challenging Research (Pioneering) (grant number 20K21294).

N.K. performed experiments and analyzed data; H.S. analyzed data; K.J. performed experiments; T.O. performed experiments and wrote the manuscript; S.I. designed the research, performed experiments, analyzed data, and wrote the manuscript.

REFERENCES

1. Katayama N, Iijima H, Osanai T. 2018. Production of bioplastic compounds by genetically manipulated and metabolic engineered cyanobacteria. Adv Exp Med Biol 1080:155–169. https://doi.org/10.1007/978-981-13-0854-8_7.

2. Oliver NJ, Rabinovitch-Deere CA, Carroll AL, Nozzi NE, Case AE, Atsumi S. 2016. Cyanobacterial metabolic engineering for biofuel and chemical production. Curr Opin Chem Biol 35:43–50. https://doi.org/10.1016/j.copcb.2016.08.023.

3. Yu Y, You L, Liu D, Hollinshead W, Tang YJ, Zhang F. 2013. Development of Synechocystis sp. PCC 6803 as a phototrophic cell factory. Mar Drugs 11:2894–2916. https://doi.org/10.3390/md1110802894.

4. Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hiroswa M, Sugiiura M, Somanato S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naro K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, Tabata S. 1996. Sequence analysis of the genome of the unicellular cyanobacterium Synechocystis sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res 3:109–136. https://doi.org/10.1093/dnares/3.3.109.

5. Pearce J, Carr NG. 1967. The metabolism of acetate by the blue-green algae, Anabaena variabilis and Anacystis nidulans. J Gen Microbiol 49: 301–313. https://doi.org/10.1099/00221287-49-2-301.
25. Kawai S, Suzuki H, Yamamoto K, Inui M, Yukawa H, Kumagai H. 1996. Purification and properties of the malic enzyme from Synechocystis sp. PCC 6803. Protein Expr Purif 139:21

26. Driscoll BT, Finan TM. 1997. Properties of NADP+ - and NADP+-dependent malic enzymes of Rhizobium (Sinorhizobium) melliferi and differential expression of their genes in nitrogen-fixing bacteroids. Microbiology (Reading) 143:489–498. https://doi.org/10.1099/00221287-143-2-489

27. Takeya M, Gombos Z, Nishiyama Y, Mohanty P, Obha T, Ohki K, Murata N. 1996. Targeted mutagenesis of acyl-lipid desaturases in Synechocystis: evidence for the important roles of polysaturated membrane lipids in growth, respiration and photosynthesis. EMBO J 15:6416–6425. https://doi.org/10.1093/emboj/15.24.6416

28. Zhang Y, Beard KFM, Swart C, Bergmann S, Krömer JO. 2020. Trans-4-hydroxy-L-proline production by the cyanobacterium Synechocystis sp. PCC 6803 for application in the food industry. Biochem J 477:1903–1912. https://doi.org/10.1042/BCJ20200038

29. Nishii M, Tsunoda T, Katayama N. 2010. Cyanobacterial coenzyme F420 dehydrogenase from the unicellular cyanobacterium Synechocystis sp. PCC 6803. J Biochem 147:403–409. https://doi.org/10.1262/jb.1000097

30. Bennett BD, Kimball EH, Gao B, Minter S, Awiapo DC, Brandhorst J, Vang Y, Nowaczyk S, Hiltz R, Behrendt J, Engel BD, Sétif P, Ikegami T, Krüger I, Nowaczyk S, Klockow D, Nowaczyk S, Krüger I, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Klockow D, Nowaczyk S, Kloc...
45. Ito S, Osanai T. 2018. Single amino acid change in 6-phosphogluconate dehydrogenase from Synechocystis conveys higher affinity for NADP+ and altered mode of inhibition by NADPH. Plant Cell Physiol 59:2452–2461. https://doi.org/10.1093/pcp/pcy165.

46. Knowles VL, Smith CS, Smith CR, Plaxton WC. 2001. Structural and regulatory properties of pyruvate kinase from the cyanobacterium Synechococcus PCC 6301. J Biol Chem 276:20966–20972. https://doi.org/10.1074/jbc.M008878200.

47. Haghighi O. 2021. In silico study of the structure and ligand preference of pyruvate kinases from cyanobacterium Synechocystis sp. PCC 6803. Appl Biochem Biotechnol 193:3651–3671. https://doi.org/10.1007/s12010-021-03630-9.

48. Dixon M, Webb EC. 1979. Enzymes, p 400–402. Longman, London, United Kingdom.

49. Williams JGK. 1988. Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in Synechocystis 6803. Methods Enzymol 167:766–778. https://doi.org/10.1016/0076-6879(88)67088-1.

50. Rippka R. 1988. Isolation and purification of cyanobacteria. Methods Enzymol 167:3–27. https://doi.org/10.1016/0076-6879(88)67004-2.

51. Osanai T, Oikawa A, Numata K, Kuwahara A, Iijima H, Doi Y, Saito K, Hirai MY. 2014. Pathway-level acceleration of glycogen catabolism by a response regulator in the cyanobacterium Synechocystis species PCC 6803. Plant Physiol 164:1831–1841. https://doi.org/10.1104/pp.113.232025.

52. Nakamura S, Fu N, Kondo K, Wakabayashi KI, Hisabori T, Sugiura K. 2021. A luminescent Nanoluc-GFP fusion protein enables readout of cellular pH in photosynthetic organisms. J Biol Chem 296:100134. https://doi.org/10.1074/jbc.RA120.016847.