Biochemical characterisation of fumarase C from a unicellular cyanobacterium demonstrating its substrate affinity, altered by an amino acid substitution

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The tricarboxylic acid cycle produces NADH for oxidative phosphorylation and fumarase [EC 4.2.1.2] is a critical enzyme in this cycle, catalysing the reversible conversion of fumarate and L-malate. Fumarase is applied to industrial L-malate production as a biocatalyst. L-malate is used in a wide range of industries such as food and beverage, pharmacy chemistry. Although the biochemical properties of fumarases have been studied in many organisms, they have not been investigated in cyanobacteria. In this study, the optimum pH and temperature of Synechocystis 6803 fumarase C (SyFumC) were 7.5 and 30 °C, respectively. The $K_m$ of SyFumC for L-malate was higher than for fumarate. Furthermore, SyFumC activity was strongly inhibited by citrate and succinate, consistent with fumarases in other organisms. Substitution of alanine by glutamate at position 314 of SyFumC changed the $k_{cat}$ for fumarate and L-malate. In addition, the inhibitory effects of citrate and succinate on SyFumC activity were alleviated.

Phylogenetic analysis revealed cyanobacterial fumarase clades divided in non-nitrogen-fixing cyanobacteria and nitrogen-fixing cyanobacteria. SyFumC was thus biochemically characterised, including identification of an amino acid residue important for substrate affinity and enzymatic activity.

Fumarase, or fumarate hydratase [EC 4.2.1.2], is an enzyme in the tricarboxylic acid (TCA) cycle and is conserved in all organisms. Fumarase catalyses the reversible conversion of fumarate and L-malate. Two classes of fumarases are found in prokaryotes, namely Class I fumarases that are iron-dependent proteins, and Class II fumarases which resemble eukaryotic enzymes belonging to the aspartase/fumarase superfamily. *Escherichia coli* possesses three genes encoding fumarases, fumA, fumB, and fumC, which are differently regulated by oxygen and growth conditions. In *E. coli*, FumA and FumB are Class I enzymes and FumC is a Class II enzyme and no homology exists between the two classes of fumarases. The FumC protein of *E. coli* resembles fumarases found in other bacteria such as *Bacillus subtilis* as well as in mammals; it is an iron-independent enzyme and relatively heat-stable compared to FumA, and its biochemical properties are similar to those of mammalian fumarases. The crystal structures of *E. coli* and *Saccharomyces cerevisiae* FumC have been resolved and their activity and regulatory domains determined. Fumarase is thus a well-studied enzyme; however, recent studies have demonstrated that human fumarases are localized not only in mitochondria but also in the cytosol and function in response to DNA damage. A Class II fumarase in *B. subtilis* also functions in response to DNA damage by producing L-malate, which regulates translation of RecN. For the application, malate is used mainly in the food and beverage industry as an acidulant, flavor enhancer, food additive and a precursor for pharmaceutical chemicals. Improvement of the thermostability of *Corynebacterium glutamicum* fumarase enhances L-malate production and an enzyme membrane reactor with immobilized fumarase is used to produce L-malate. For the metabolic engineering, the production of L-malate is increased by expressing yeast fumarase in *Aspergillus oryzae*, suggesting that fumarase is a rate-limiting enzyme of malate production. In this way, the importance of fumarases is conserved across the kingdoms and recognized in both basic and applied sciences.

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Cyanobacteria are photosynthetic prokaryotes and are known as a model for photosynthetic organisms. Cyanobacteria fix carbon dioxide and this activity influences the global carbon cycle, with cyanobacteria contributing nearly 30% of the global net primary production\(^{19}\). Moreover, engineered cyanobacteria can produce industrially relevant chemicals from fixed carbon dioxide and are suitable for biofuel and bulk chemical production\(^{17,18}\). Among cyanobacteria, *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis* 6803) is a unicellular, non-nitrogen-fixing cyanobacterium, the genome of which was first sequenced in 1996\(^{19}\). Substrains of this species are used for studies of photosynthesis and for metabolic engineering\(^{20-22}\). Transcriptome analyses using microarrays can elucidate the mechanisms of environmental stresses and identify regulatory factors that transduce environmental signals\(^{23-25}\) and transcriptional regulators of *Synechocystis* 6803 carbon metabolism have been identified using these techniques\(^{26-28}\). Subsequently, metabolomic analyses of *Synechocystis* 6803 was performed and the carbon distribution and flux in response to environmental changes and genetic manipulation were demonstrated\(^{29-32}\). Fluxome analyses showed that TCA cycle and of acetyl-CoA metabolite pool sizes are smaller in *Synechocystis* 6803 than in *E. coli*\(^{30,32}\). Furthermore, fluxes in the TCA cycle remain low under all conditions tested\(^{30,33}\), indicating that the TCA cycle of unicellular cyanobacteria is unique among bacteria.

The cyanobacterial TCA cycle was thought to be incomplete because it lacked 2-oxoglutarate dehydrogenase, which generates succinyl-CoA from 2-oxoglutarate. Biochemical analysis based on *Synechococcus* sp. PCC 7002, however, has demonstrated that marine cyanobacteria possess the enzymes 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase, which generate succinate from 2-oxoglutarate by two enzymatic reactions\(^{35}\). In *Synechocystis* 6803, additional enzymatic reactions involving the γ-aminobutyric acid (GABA) shunt generate succinate from 2-oxoglutarate\(^{35}\). In this shunt, 2-oxoglutarate is converted to glutamate, followed by conversion to GABA\(^{36}\) which is then converted to succinyl-semialdehyde, and finally to succinate\(^{37}\). In addition to these bypasses, succinate is also produced in the reductive TCA cycle of *Synechocystis* 6803 under dark, anaerobic conditions\(^{38}\). Phosphoenolpyruvate carboxylase (PEPC), which generates oxaloacetate from phosphoenolpyruvate, is a rate-limiting enzyme for succinate production under these conditions\(^{39}\). Generally, PEPCs in bacteria and plants are inhibited by aspartate and malate; however, PEPC in *Synechocystis* 6803 is uniquely insensitive to these metabolites\(^{37}\). Substitution of the glutamate residue with lysine at position 954 restores these inhibitory effects, similar to that seen for PEPC in a nitrogen-fixing cyanobacterium\(^{39}\). These recent studies demonstrate that TCA cycle enzymes in cyanobacteria possess unique properties, making biochemical characterisation of these enzymes not only intriguing, but also indispensable for understanding their metabolism.

In the present study, biochemical analysis of fumarase C from *Synechocystis* 6803 (SyFumC) revealed that the reversible reactions are regulated by TCA cycle metabolites and further showed the importance of the alanine residue at position 314 for substrate affinity.

**Results**

**Affinity purification and biochemical characterisation of SyFumC.** Genome sequence analysis indicated that *Synechocystis* 6803 does not possess a Class I fumarase but does express a Class II fumarase, SyFumC. To evaluate the biochemical properties of SyFumC, glutathione-S-transferase-tagged SyFumC (GST-SyFumC) was expressed in *E. coli* cells and purified from the soluble fraction by affinity chromatography (Figs 1a and S1). The enzymatic activity of SyFumC using fumarate as a substrate (hereafter referred to as “the enzymatic activity towards fumarate”) was highest at pH 7.5 (Fig. 1b); with l-malate as a substrate (hereafter referred to as “the enzymatic activity towards l-malate”), the enzymatic activity of SyFumC was also highest at pH 7.5, but was less affected by variation in pH. Enzymatic activities towards both fumarate and l-malate were highest at 30 °C and were inhibited at 60 °C (Fig. 1c). SyFumC activity towards l-malate was less sensitive to temperature variation than that toward fumarate (Fig. 1c). Therefore, for the ensuing experiments, SyFumC enzymatic assays were performed at pH 7.5 and 30 °C.

Substrate saturation curves were obtained using different concentrations of fumarate (Fig. 2a) and l-malate (Fig. 2b) as substrates. The *Km* of SyFumC for fumarate and l-malate was 0.244 ± 0.026 and 0.478 ± 0.112 mM, respectively; the *kcat* of SyFumC for fumarate and l-malate was 101.2 ± 6.7 and 42.3 ± 4.3 s\(^{-1}\), respectively; and the *kcat/Km* of SyFumC for fumarate and l-malate was 415.1 ± 17.0 and 90.3 ± 11.3 s\(^{-1}\)M\(^{-1}\), respectively (Table 1).

**Identification of effectors altering SyFumC activity.** Effectors of SyFumC activities were also evaluated. Citrate and succinate are competitive inhibitors of fumarases from pig heart, *E. coli*, and *Pisum sativum*\(^{36-40}\); therefore, the effect of citrate and succinate on SyFumC was examined at various concentrations (2–8 mM) under conditions of substrate saturation (Fig. 3a,b). SyFumC activity towards both fumarate and l-malate reactions decreased in a concentration-dependent manner in the presence of both citrate and succinate (Fig. 3a,b). The enzymatic activity of SyFumC towards fumarate and l-malate decreased to 45–58% of control in the presence of 4 mM succinate or 4 mM citrate (Fig. 3a,b). The *Km* and *kcat* for fumarate and l-malate were 101.2 ± 6.7 and 42.3 ± 4.3 s\(^{-1}\), respectively (Table 1). The *Km* and *kcat* for fumarate and l-malate in the presence of 4 mM citrate were 0.616 ± 0.037 mM and 34.9 ± 1.8 s\(^{-1}\), and 0.921 ± 0.042 mM and 28.0 ± 0.4 s\(^{-1}\), respectively (Table 1). The *kcat/Km* for fumarate and l-malate in the presence of 4 mM succinate was 311.5 ± 12.4 and 57.0 ± 4.0, respectively; and in the presence of 4 mM citrate, it was 160.2 ± 8.8 and 30.4 ± 1.8 s\(^{-1}\)M\(^{-1}\), respectively (Table 1).

Additional effectors of SyFumC enzymatic activity were examined under conditions of substrate saturation. We selected 11 effectors which regulate the activities of enzymes in the TCA cycle and pyruvate metabolism in this cyanobacterium\(^{37,41}\). Among the 11 effectors tested, Co\(^{2+}\) and Zn\(^{2+}\) decreased SyFumC activity towards fumarate and l-malate (Fig. 4a,b). At 1 mM, Co\(^{2+}\) decreased SyFumC enzymatic activity towards fumarate and l-malate to 66.5% and 57.4% of control, respectively (Fig. 4a,b). The presence of 1 mM and 10 mM Zn\(^{2+}\) decreased SyFumC enzymatic activity towards fumarate to 0.99% and 1.99% of control, respectively (Fig. 4a,b); similarly, 1 mM and 10 mM Zn\(^{2+}\) decreased SyFumC enzymatic activity towards l-malate to 0.94% and 1.89% of control (Fig. 4a,b).
Figure 1. Determination of optimum conditions for *Synechocystis* sp. PCC 6803 fumarase C (SyFumC). (a) GST-tagged SyFumC after purification. Purified proteins were electrophoresed on 8% SDS-PAGE gel and stained using InstantBlue reagent. (b) Effect of pH on SyFumC activity. Data represent the means ± SD obtained from three independent experiments. For the enzyme assay, 15 pmol of SyFumC was used. One unit of SyFumC activity was defined as the consumption of 1 µmol fumarate or l-malate per min. Light blue circles and orange triangles represent activity specific for fumarate and l-malate, respectively. (c) Effect of temperature on SyFumC activity. Data represent the means ± SD obtained from three independent experiments. For the enzyme assay, 15 pmol of SyFumC was used. One unit of SyFumC activity was defined as the consumption of 1 µmol fumarate or l-malate per min. Light blue circles and orange triangles represent activity specific for fumarate and l-malate, respectively.

Figure 2. *In vitro* enzyme assays for SyFumC using fumarate and l-malate as substrates. (a) Enzyme activity was measured by varying the fumarate concentration. The data represent the means ± SD obtained from three independent experiments. Light blue squares represent activity specific for fumarate. Yellow and green squares represent activity specific for fumarate with 4 mM citrate and 4 mM succinate, respectively. (b) Enzyme activity was measured by varying the l-malate concentration. The data represent the means ± SD obtained from three independent experiments. Orange triangles represent activity specific for l-malate. Navy and purple triangles represent activity specific for l-malate with 4 mM citrate and 4 mM succinate, respectively.
At 10 mM, pyruvate and Mn\(^{2+}\) both decreased SyFumC enzymatic activity towards l-malate (Fig. 4a,b). SyFumC activity could not be measured in the presence of 10 mM Co\(^{2+}\), 2-oxoglutarate, and phosphoenolpyruvate due to interference of absorption by solvents (Fig. 4a,b).

Amino acid substitution and substrate specificity of SyFumC. Amino acid sequences were compared by multiple sequence alignment analysis, using amino acid sequences of fumarases from other cyanobacteria, *E. coli*, *Mycobacterium tuberculosis*, and *Arabidopsis thaliana* (Fig. 5a). The amino acid residue at position 314 of SyFumC was found to be alanine; however, except for *Synechococcus elongatus* and *Leptolyngbya* sp., the equivalent residues of other fumarases are either glutamate or aspartate (Fig. 5a).

The impact of the alanine residue at position 314 of SyFumC was examined by substituting glutamate for alanine at position 314, and the recombinant protein was named SyFumC\(_{A314E}\). The SyFumC\(_{A314E}\) protein was expressed in *E. coli* and purified by affinity chromatography (Figs 5b and S2). The optimal pH for SyFumC\(_{A314E}\) enzymatic activity towards fumarate was pH 7.3 and the optimal temperature was 30 °C (Fig. 5c). SyFumC\(_{A314E}\) was inactivated at 60 °C (Fig. 5d). Glutamate substitution had less impact at optimal pH and temperature using l-malate as a substrate (Fig. 5c,d). The maximum enzymatic activity of SyFumC\(_{A314E}\) for fumarate at

| Table 1. Kinetic parameters of SyFumC using fumarate and l-malate as substrates at 30 °C and pH 7.5. Parameters were calculated using the equations described in the Materials and Methods. Cit and Suc represent citrate and succinate, respectively. The data represent the means ± SD from three independent experiments. |

| Enzyme | Effector | \(K_m\) (mM) | \(k_{cat}\) (s\(^{-1}\)) | \(k_{cat}/K_m\) (s\(^{-1}\) mM\(^{-1}\)) |
|--------|----------|-----------|----------------|------------------|
| Using fumarate as a substrate |
| SyFumC | None | 0.244 ± 0.026 | 101.2 ± 6.7 | 415.1 ± 17.0 |
| SyFumC | 4 mM Suc | 0.256 ± 0.017 | 79.5 ± 2.2 | 311.5 ± 12.4 |
| SyFumC | 4 mM Cit | 0.409 ± 0.034 | 65.4 ± 2.1 | 160.2 ± 8.8 |
| SyFumC\(_{A314E}\) | None | 0.179 ± 0.036 | 37.8 ± 3.0 | 214.8 ± 27.9 |
| SyFumC\(_{A314E}\) | 4 mM Suc | 0.214 ± 0.025 | 44.0 ± 2.7 | 206.3 ± 10.7 |
| SyFumC\(_{A314E}\) | 4 mM Cit | 0.391 ± 0.036 | 47.4 ± 2.1 | 121.7 ± 6.0 |
| Using l-malate as a substrate |
| SyFumC | None | 0.478 ± 0.112 | 42.3 ± 4.3 | 90.3 ± 11.3 |
| SyFumC | 4 mM Suc | 0.616 ± 0.073 | 34.9 ± 1.8 | 57.0 ± 4.0 |
| SyFumC | 4 mM Cit | 0.921 ± 0.042 | 28.0 ± 0.4 | 30.4 ± 1.8 |
| SyFumC\(_{A314E}\) | None | 0.442 ± 0.020 | 21.6 ± 0.1 | 49.0 ± 2.0 |
| SyFumC\(_{A314E}\) | 4 mM Suc | 0.329 ± 0.030 | 12.7 ± 0.7 | 38.7 ± 1.6 |
| SyFumC\(_{A314E}\) | 4 mM Cit | 0.360 ± 0.020 | 10.4 ± 0.04 | 29.0 ± 1.7 |

At 10 mM, pyruvate and Mn\(^{2+}\) both decreased SyFumC enzymatic activity towards l-malate (Fig. 4a,b). SyFumC activity could not be measured in the presence of 10 mM Co\(^{2+}\), 2-oxoglutarate, and phosphoenolpyruvate due to interference of absorption by solvents (Fig. 4a,b).
Figure 4. Effect of various metal ions and metabolites on SyFumC activity using fumarate and l-malate as substrates. Enzymatic activity was measured with various metabolites or metal ions under conditions of substrate (fumarate and l-malate) saturation. (a) The grey bars represent the control data. Red and blue bars represent concentrations of 1 mM and 10 mM metabolites or metal ions, respectively, using fumarate as a substrate. (b) The grey bar represents the control data. Green and dark blue bars represent, respectively, using l-malate as a substrate. NO, no enzymatic activity was detected; ND, enzymatic activity not determined in this study. The data represent the means ± SD from three independent experiments. Asterisk (*) indicates P < 0.05. Abbrevations; 2-OG, 2-oxoglutarate; PEP, phosphoenolpyruvate; Asp, aspartate; Pyr, pyruvate.

Figure 5. Multiple amino acid sequence alignment of Class II fumarases and determination of optimum conditions for Synechocystis sp. PCC 6803 fumarase C with glutamate substituted for alanine (SyFumC_A314E). (a) The multiple alignment of 13 amino acid sequences from Class II fumarases performed by CLC Sequence Viewer. The region containing the GSSxxPxKxN sequence, called an SS loop (indicated by a blue arrow), is shown. The green arrow indicates the alanine at position 314 in Synechocystis sp. PCC 6803. (b) GST-tagged SyFumC_A314E after purification. Purified proteins were electrophoresed on 8% SDS-PAGE gel and stained using InstantBlue reagent. (c) Effect of pH on SyFumC_A314E activity. Data represent the means ± SD obtained from three independent experiments. For the enzyme assay, 15 pmol of SyFumC_A314E was used. One unit of SyFumC activity was defined as the consumption of 1 µmol fumarate or l-malate per min. Light blue circles and orange triangles represent activity specific for fumarate and l-malate, respectively. (d) Effect of temperature on SyFumC_A314E. For the enzyme assay, 15 pmol of SyFumC_A314E was used. One unit of SyFumC activity was defined as the consumption of 1 µmol fumarate or l-malate per min. Light blue circles and orange triangles represent activity specific for fumarate and l-malate, respectively.
Figure 6. SyFumC_A314E enzyme assays using fumarate and l-malate as substrates. (a) Enzyme activity was measured by varying the fumarate concentration. The data represent the means ± SD obtained from three independent experiments. Light blue squares represent activity specific for fumarate. Yellow squares and green squares represent activity specific for fumarate with 4 mM citrate and with 4 mM succinate, respectively. (b) Enzyme activity was measured by varying the l-malate concentration. The data represent the means ± SD obtained from three independent experiments. Orange triangles represent activity specific for l-malate. Navy triangles and purple triangles represent activity specific for l-malate with 4 mM citrate and 4 mM succinate, respectively.

the optimal pH and temperature decreased to 28.1% and 48.6% of SyFumC activity, respectively (Fig. 5c,d); the maximum enzymatic activity of SyFumC_A314E for l-malate at the optimal pH and temperature decreased to 41.5% and 41.6% of SyFumC activity, respectively (Fig. 5c,d).

The enzymatic activity of SyFumC_A314E was measured with various substrate concentrations to obtain saturation curves and calculate the kinetic parameters (Fig. 6a,b). The $K_m$ values for SyFumC_A314E towards fumarate and l-malate were 0.179 ± 0.036 and 0.442 ± 0.020 mM, respectively (Table 1), showing reductions to 73.4% and 92.5% of SyFumC values for fumarate and l-malate, respectively (Table 1). The $k_{cat}$ values for SyFumC_A314E towards fumarate and l-malate were 37.8 ± 3.0 and 21.6 ± 0.1 s⁻¹, respectively (Table 1).

The $K_m$ and $k_{cat}$ for SyFumC_A314E were examined in the presence of 4 mM succinate or 4 mM citrate. The $K_m$ for SyFumC_A314E using fumarate and l-malate as substrates in the presence of 4 mM succinate was 0.214 ± 0.025 and 0.329 ± 0.030 mM, respectively (Table 1). The $K_m$ for SyFumC_A314E in the presence of 4 mM citrate, using fumarate and l-malate as substrates, was 0.391 ± 0.036 and 0.360 ± 0.020 mM, respectively (Table 1). The $k_{cat}$ values for SyFumC_A314E with fumarate and l-malate as substrates, in the presence of 4 mM succinate, were 44.0 ± 2.7 and 12.7 ± 0.7 s⁻¹, respectively (Table 1). The $k_{cat}$/$K_m$ values for SyFumC_A314E with fumarate and l-malate as substrates, in the presence of 4 mM citrate, were 28.0 ± 0.4 and 10.4 ± 0.04 s⁻¹, respectively (Table 1). The $k_{cat}$/$K_m$ ratios for SyFumC_A314E with fumarate and l-malate as substrates were 206.3 ± 10.7 and 38.7 ± 1.6, respectively, in the presence of 4 mM succinate, and 121.7 ± 6.0 and 29.0 ± 1.7 s⁻¹·mM⁻¹, respectively, in the presence of 4 mM citrate (Table 1).

Finally, 11 effectors (metabolites and metal ions) were tested for their impact on SyFumC_A314E activity. At 1 mM, Zn²⁺ eliminated SyFumC_A314E enzymatic activity towards fumarate and l-malate (Fig. 7a,b). Also, at 1 mM, Co²⁺ decreased SyFumC_A314E enzymatic activity towards fumarate and l-malate to 54.6% and 47.6% of control, respectively (Fig. 7a,b). SyFumC_A314E enzymatic activity towards fumarate and l-malate was abolished with 10 mM Mn²⁺ (Fig. 7a,b). SyFumC_A314E enzymatic activity towards fumarate was marginally upregulated in the presence of 1 mM phosphoenolpyruvate (Fig. 7a); and at 10 mM, pyruvate promoted SyFumC_A314E enzymatic activity towards fumarate while enzymatic activity towards l-malate was suppressed (Fig. 7a,b).

Discussion

In this study, we performed the first biochemical analysis of class II fumarase in cyanobacteria, revealing that the modification of a residue at position 314 relieved the inhibition by citrate and succinate and reduced $k_{cat}$ of SyFumC. Previous studies have shown that the optimal pHs for fumarases from the thermophilic archaeabacterium Sulfolobus solfataricus and from marine microorganisms are 8.0 and 8.5, respectively. SyFumC is more active at a lower pH than these fumarases (Fig. 1b). The optimal pHs for fumarases from the eukaryotes Saccharomyces cerevisiae and Rhizopus oryzae are pH 7.5 and 7.2⁵,⁶, respectively, and are thus similar to that for SyFumC (Fig. 1b). The growth pH ranges for S. solfataricus and S. cerevisiae are pH 1.0–5.8 and pH 4.0–4.5, respectively,⁷,⁸, values inconsistent with the optimal pH for fumarases. Synechocystis 6803 shows optimal biomass production at pH 7.5⁹, a pH for growth consistent with optimal enzymatic activity.

The optimal temperature for SyFumC activity was 30°C, which is similar to that of fumarases from R. oryzae (30°C) and the mesophilic Streptomyces coelicolor (30°C), but lower than for bacterial and archaeal fumarases in Streptomyces lividans (45°C), thermophilic Streptomyces thermovulgaris (50°C) and Thermus thermophilus.

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Fluxome analysis indicates that TCA cycle fluxes in Synechocystis does not naturally produce energy sources. Metabolic reaction catalysed by a fumarase is inclined to the flux of 13C-atoms in malate was higher than in fumarate by approximately 5-fold at 37°C in microorganisms. Fumarases from other organisms like E. coli, Corynebacterium glutamicum, S. solfataricus, and marine microorganisms are similar; the substrate specificity of Synechocystis fumarases using fumarate as a substrate except FumC_A314E activity towards fumarate and l-malate (85°C), S. solfataricus (85°C), and marine microorganisms (55°C). S. thermovulgaris, T. thermophilus, and S. solfataricus are thermophilic organisms that thrive in hot springs, and higher optimal temperatures are not unexpected. The optimal temperature for Synechocystis 6803 growth is 30–32°C, which is close to the optimal temperature for SyFumC activity. The optimal temperatures for two other Synechocystis enzymes, SyPEPC and d-lactate dehydrogenase, are 30°C and 30–40°C, respectively. The optimal temperature for SyFumC enzymatic activity is thus similar to that of other Synechocystis enzymes involved in primary carbon metabolism.

The $K_m$ for SyFumC enzymatic activity towards l-malate was higher than that for fumarate (Table 1). Fumarases from other organisms like E. coli, Corynebacterium glutamicum, S. solfataricus, and marine microorganisms are similar; the $K_m$ ranges for fumarate and l-malate are 0.12–0.48 and 0.30–3.15 mM, respectively. Intracellular metabolite analysis using 13C-labeled glucose Synechocystis 6803 demonstrated that the number of 13C-atoms in malate was higher than in fumarate by approximately 5-fold at 37°C in Synechocystis 6803. This 13C flux analysis indicates that the conversion from fumarate to l-malate is stronger than from l-malate to fumarate under aerobic conditions. Previous reports showed that the absolute concentration of l-malate (µmol/g dry cell weight) is higher than that of fumarate in Synechocystis 6803, indicating that SyFumC preferentially catalyses the reaction generating l-malate from fumarate. A different study shows that intracellular fumarate and malate pool sizes peak at 24 h under dark, anaerobic conditions, and then decrease during cultivation under the same conditions. Carbon flow in the TCA cycle changes from using fumarate to using l-malate during dark, anaerobic incubation. Nevertheless, intracellular l-malate concentrations (µmol/g dry cell weight) are still higher than fumarate concentrations. The substrate specificity of SyFumC for fumarate well explains these results (Table 1). The $K_m$ of a malate dehydrogenase from Synechocystis 6803 (SyMDH) that catalyses the reversible conversion of l-malate and oxaloacetate is approximately 210-fold higher for the oxidative than for the reductive reaction and the catalytic efficiency of SyMDH is higher for the reductive than for the oxidation reaction. Thus, both SyMDH and SyFumC preferentially catalyse reactions that produce l-malate. A previous study suggested that organic acids in the TCA cycle, including l-malate, play critical roles in the storage of carbon sources under nitrogen starvation. SyFumC and SyMDH may preferentially convert metabolites to l-malate to promote this process.

The $k_{cat}$ for SyFumC enzymatic activity towards l-malate was lower than those for fumarase C from E. coli and Class II fumarases from marine microorganisms and T. thermophilus, and Homo sapiens (Table 2). Fluxome analysis indicates that TCA cycle fluxes in Synechocystis 6803 remain constant under dark, photoheterotrophic, photomixotrophic, and photoautotrophic conditions, suggesting that the cyanobacterial TCA cycle does not naturally produce energy sources. Metabolic reaction catalysed by a fumarase is inclined to the flux from fumarate to malate in all condition. In addition, molar-based, widely targeted metabolic profiling analysis reveals that concentrations of TCA cycle metabolites are lower than those of glycolysis metabolites. We compared fumarases of organisms in different kingdoms (Table 2). The $k_{cat}$ of SyFumC is lower than $k_{cat}$ of other microorganisms fumarases. $K_m$ of SyFumC using fumarate as a substrate is lower than $K_m$ of other microorganisms fumarases using fumarate as a substrate except $K_m$ for fumarate of E. coli (Table 2). Since the specific activity of SyFumC is lower than those of other organisms, the turnover number limits SyFumC activity in vitro (Table 2). These results were consistent with a less active TCA cycle in cyanobacteria. On the other hand, $k_{cat}/K_m$
of SyFumC using l-malate was higher than its of C. glutamicum fumarase (Table 2). The result indicates the reductive TCA cycle is relatively efficient compared to other organisms, consistent with a previous study. Citrate is known as a competitive inhibitor of fumarases from pig heart and E. coli, and citrate also inhibited SyFumC activity (Fig. 3, Table 1). Mitochondrial fumarase of a higher plant, P. sativum, is inhibited by 53% in the presence of 20 mM citrate, using l-malate as a substrate. The activities of A. thaliana mitochondrial and cytosolic fumarases decrease to 15–37% in the presence of citrate. Succinate is also known as an inhibitor of fumarases of pig and P. sativum, and it also inhibited SyFumC (Fig. 3). Succinate at 100 mM inhibits P. sativum fumarase; the activity decreases to 55% using l-malate as a substrate. The sensitivity of SyFumC to citrate and succinate was nearly identical with either fumarate and l-malate as substrates (Fig. 3a,b). Absolute quantification of metabolites (µmol/g dry cell weight) in Synechocystis 6803 shows that intercellular concentrations of citrate, succinate, fumarate, and malate were 2.16, 0.323, 0.163, and 0.182, respectively. These concentrations suggest that citrate and succinate are inhibitory under typical physiological conditions and the reduced SyFumC activity may be one cause of the low fluxes through the TCA cycle observed in Synechocystis 6803.

Additionally, SyFumC activity was also inhibited by several metabolites and divalent cations (Fig. 4a,b). P. sativum mitochondrial fumarase activity towards l-malate was decreased to 14% at 10 mM pyruvate. A. thaliana mitochondrial fumarase is activated to 178% by pyruvate using l-malate as a substrate. At 10 mM, pyruvate marginally inhibited SyFumC activity towards l-malate (Fig. 4b), and the effect of pyruvate on fumarases is different among photosynthetic organisms. Zn$^{2+}$ strongly inhibited SyFumC enzymatic activity using fumarate and l-malate as substrates (Fig. 4a,b). This inhibition is greater than reported for a fumarase from a marine microorganism (FumF). FumF enzymatic activity reduces to 45% by 5 mM Zn$^{2+}$. In contrast, a fumarase from R. oryzae is not inhibited by Zn$^{2+}$. The addition of 10 mM Ca$^{2+}$ was less inhibitory by other divalent cations for SyFumC and SyFumC_A314E activity towards l-malate (Figs 4b and 7b), but the R. oryzae fumarase is slightly stimulated by 9.0 mM Ca$^{2+}$ using l-malate as a substrate. SyFumC was differently inhibited by metabolites and metal ions. These results indicated that the regulation by metabolites and metals was not conserved among fumarases. Three TCA cycle enzymes (S/MDH, isocitrate dehydrogenase, and citrate synthase) in Synechocystis 6803 are regulated by divergent cations, particularly Mg$^{2+}$, Zn$^{2+}$, and Ca$^{2+}$. SyFumC was severely inhibited by Zn$^{2+}$, not by Mg$^{2+}$, indicating the different regulatory manner of enzymatic activities in the TCA cycle in this cyanobacterium.

The alanine at position 314 is close to the sequence called the SS loop, a motif associated with substrate binding and catalytic activity. The optimal pH and temperature for SyFumC_A314E are similar to the optima for SyFumC (Fig. 5c,d), but substrate affinity was enhanced by the A314E substitution (Fig. 6). The $K_m$ values for SyFumC_A314E activity towards fumarate and l-malate were not significantly different to those for SyFumC activity towards the same substrates (Table 1). Compared to SyFumC, the $k_{cat}$ for SyFumC_A314E activity towards fumarate and l-malate decreased to 0.37-fold and 0.51-fold, respectively (Table 1). The $k_{cat}$ for an R. eoli recombinant FumC with substituting glutaminase for glutamate at position 315 near the SS loop was decreased by 10-fold when using fumarate and l-malate as substrates, but no effect was observed for $K_m$ values. This glutamate residue at position 315 in E. coli is correspond to a glutamate at position 316 in SyFumC (Fig. 5). These results indicate that amino acids near the SS-loop determine the maximum rate of reaction of bacterial fumarases. Other group demonstrate that the alanine at position 347 in S. coelicolor fumarase (correspond to the glutamine at position 354 in SyFumC) is important for thermostability, and the improvement of the thermostability in SyFumC can be future theme for cyanobacterial fumarases.

Phylogenetic analysis revealed that SyFumC belongs to non-nitrogen-fixing cyanobacterial clade (except Trichodesmium erythraeum) being different from nitrogen-fixing cyanobacteria and Gram-negative bacteria (Fig. 8). Combined with previous data, the present results demonstrate that important amino acid residues are conserved between enteric bacteria and non-nitrogen-fixing cyanobacteria. However, succinate inhibited the SyFumC activity (Fig. 2a), while fumarase in C. glutamicum is not inhibited by succinate, indicating the sensitivity of fumarases to the effectors are different among the clades. These differences were derived from several amino acid substitutions; the inhibitory effects of citrate and succinate on SyFumC activity were reduced by the alanine to glutamate amino acid substitution (Figs 2a and 6a). The effects of some metabolites were also changed after the amino acid substitution. Pyruvate at 10 mM and phosphoenolpyruvate at 1 mM activated enzymatic activity with fumarate as a substrate (Fig. 7a), and 10 mM pyruvate and 1 mM 2-oxoglutarate inhibited SyFumC_A314E activity. Additionally, 10 mM pyruvate elicited different effects depending on whether fumarate or l-malate were

| Substrate | Fumarate | l-malate |
|-----------|----------|----------|
| Organisms | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$) | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$) |
| Synechocystis sp. PCC 6803 (This study) | 0.24 | $1.0 \times 10^5$ | $4.1 \times 10^5$ | 0.48 | $4.2 \times 10^4$ | $9.0 \times 10^3$ |
| Corynebacterium glutamicum (pH 7.0/TES-NaOH) | 0.58 | $4.30 \times 10^5$ | $1.1 \times 10^5$ | 1.80 | $1.1 \times 10^4$ | $6.1 \times 10^3$ |
| Escherichia coli (Native) | 0.21 | $1.1 \times 10^5$ | $5.6 \times 10^4$ | 0.86 | $5.9 \times 10^4$ | $6.9 \times 10^3$ |
| Homo sapiens | NR | NR | NR | 1.90 | $1.5 \times 10^5$ | $8.0 \times 10^3$ |
| Marine sample (FumF) | 0.48 | $9.1 \times 10^2$ | $1.9 \times 10^4$ | 3.20 | $3.2 \times 10^2$ | $1.0 \times 10^3$ |
| Streptomyces lividans (pH 7.3) | NR | NR | NR | 15.0 | $2.7 \times 10^5$ | $1.8 \times 10^3$ |
| Thermus thermophilus | NR | NR | NR | 1.00 | $1.0 \times 10^5$ | $1.0 \times 10^3$ |
Figure 8. Phylogenetic analysis of bacterial class II fumarases. Amino acid sequences of 31 bacterial fumarase obtained from GenBank were aligned using CLC Sequence Viewer ver. 8.0. A maximum-likelihood tree based on 431 preserved amino acid residues was designed using PhyML online (http://www.atgc-montpellier.fr/phyml/). Bootstrap values were calculated by 500 replications.

used as substrate (Fig. 7a,b). Pyruvate and PEP are compounds upstream of fumarase in the TCA cycle, and it is considered to be activated to acquire reducing power for fumarate. The present results show that SyFumC has a higher affinity for fumarate than for l-malate. The enzymatic activity of SyFumC towards fumarate and l-malate was inhibited by citrate and succinate. Current study, a biochemistry of a fumarase in a new clade, contributes to understanding the diversity of the TCA cycle in bacteria, and potentially leads to the metabolic engineering using cyanobacteria.

Methods

Vector construction and expression of recombinant proteins. The genomic region of Synechocystis 6803 including the fumC (slr0018) open reading frame (ORF) was amplified by PCR using the KOD -plus- neo DNA polymerase (Toyobo, Osaka, Japan) and the following primer set: forward, 5′-GAAGGTCGTGGGATCATGGTAAATTCCCACCGC-3′; and reverse, 5′-GATGCGGCCGCTCGAGCTAGTCAGCAATCGGGG-3′; the BamHI and XhoI restriction enzymes were obtained from TakaraBio (Shiga, Japan). The resultant fragment was cloned into the BamHI-XhoI sites of pGEX5X -1 (GE Healthcare Japan, Tokyo, Japan). Amino acid substitution was performed commercially by TakaraBio (Shiga, Japan). For SyFumC_A314E, the region +940–942 from the start codon in the fumC ORF was changed from GCC to GAA.

These vectors were transformed into E. coli DH5α cells (TakaraBio) and 5 L of transformed E. coli were cultivated in LB medium at 30°C with shaking (150 rpm); protein expression was induced overnight in the presence of 0.01 mM isopropyl-β-D-1-thiogalactopyranoside (Wako Chemicals, Osaka, Japan).

Affinity purification of recombinant proteins. Affinity chromatography for protein purification was performed as previously described [3]. Harvested DH5α cells suspended in 40 mL PBST (1.37 M NaCl, 27 mM KCl, 81 mM Na2HPO4·12H2O, 14.7 mM KH2PO4, and 0.05% Tween 20) were disrupted by sonication (VC-750, Yamato Scientific, Tokyo, Japan) for 2 min at 4°C. The supernatant was removed and the resin was re-suspended in 700 μL of PBST. After washing five times, recombinant proteins were eluted five times with 700 μL of GST elution buffer [50 mM Tris-HCl (pH 8.0), 10 mM reduced glutathione]. Proteins were concentrated with a Vivaspin 50 MWCO 50000 device (Sartorius, Göttingen, Germany), and protein concentrations were analysed with a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). SDS-PAGE was performed to analyse protein purification using InstantBlue (Expedion Protein Solutions, San Diego, CA).

Enzyme assay for SyFumC. SyFumC activity was measured using 15 pmol SyFumC mixed in 1 mL of an assay solution (100 mM Tris-HCl [pH 7.5] and 10 mM fumarate or 10 mM l-malate). Absorbance at 250 nm was monitored using a Shimadzu UV-1850 (Shimadzu, Kyoto, Japan). One unit of SyFumC activity was defined as the consumption or the generation of 1 μmol fumarate per min. K_m and V_max were calculated by curve fitting using KaleidaGraph v.4.5 software, and K_cat was calculated from the V_max. Results were plotted as graphs of rate of reaction against substrate concentration.
Statistical analysis. The P-values were calculated using paired two-tailed Student’s t-tests with Microsoft Excel for Windows (Redmond, WA, USA). All results were obtained from three or four independent experiments.

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Author Contributions
N.K. designed the research, performed the experiments, analysed the data, and wrote the manuscript. M.T. analysed the data. T.O. analysed the data and wrote the manuscript.

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