

Reviewed Preprint

v1 • June 17, 2026

Not revised

Reviewed Preprint

v2 • August 27, 2026

Revised by authors

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[§] shared first authorship

Competing interests: No competing interests declared

Funding: See [page 25](#)

Reviewing editor: Caetano Antunes, University of Kansas, United States

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Story about honest mistakes: The cyanobacterium *Synechocystis* has a promiscuous Entner-Doudoroff (ED) aldolase but no functional ED pathway

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eLife Assessment

This study presents **fundamental** results on questions related to the presence/absence of the Entner-Doudoroff pathway in cyanobacteria. In contrast to an earlier study, **compelling** evidence is given that *Synechocystis* PCC 6803 lacks both an Entner-Doudoroff pathway and a related bypass but contains a promiscuous aldolase. This study successfully reconciles data from different studies and lessons learned from a previous misconception.

<https://doi.org/10.7554/eLife.111485.2.sa3>

Abstract

In 2016, the glycolytic Entner-Doudoroff (ED) pathway was reported in cyanobacteria and plants (1). The claim was based on the biochemical characterization of its key enzyme the 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase also named ED aldolase (EDA), on protein sequence alignments, physiological data from cyanobacterial mutants, and the *in vivo* detection of an ED pathway specific metabolite (1). However, two enzymes 6-phosphogluconate (6PG) dehydratase (EDD) and EDA are unique to this route. A recent study suggests that EDD (Slr0452) from *Synechocystis* sp. PCC 6803 most likely encodes an enzyme involved exclusively in amino acid synthesis, indicating that a complete ED pathway would be missing (2). To address the presence or absence of the ED pathway in *Synechocystis*, we conducted extended biochemical and physiological studies, revisited old data and resolved contradictions. These investigations reveal that *Synechocystis* lacks both an ED pathway and a glucose dehydrogenase/glucokinase (GDH/GK) bypass but contains a promiscuous aldolase EDA. EDA prefers KDPG as substrate but also decarboxylates oxaloacetate (OAA) and cleaves 2-keto-4-hydroxyglutarate (KHG). Synthesis of KDPG from pyruvate and glyceraldehyde 3-phosphate (GAP) is catalyzed with very low efficiency. These *in vitro* data suggest that EDA might be involved in the phosphoenolpyruvate (PEP)-pyruvate-OAA node and proline catabolism, which requires further clarification. The previous misconception was based on missing enzymatic characterizations, the oversight of a secondary mutation in a deletion strain, and an outdated view on carbohydrate fluxes. We conclude with a list of lessons and provide a solid foundation for future investigations into the role of EDA in cyanobacteria and other photoautotrophs.

Significance statement

This study provides a retrospective on why, for many years, it was mistakenly assumed that the glycolytic Entner-Doudoroff (ED) pathway exists in the cyanobacterium *Synechocystis* sp. PCC 6803. It shows that the first enzyme of this pathway, ED dehydratase EDD is absent, while the second enzyme, 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase EDA, is present but is promiscuous, cleaving KDPG in addition to 2-keto-4-hydroxyglutarate (KHG) and decarboxylating oxaloacetate (OAA) *in vitro*. Finally, valuable lessons are drawn from prior misconceptions and experimental limitations. This study provides a solid foundation for future studies on the role of the ED aldolase in absence of the ED pathway in cyanobacteria and other photoautotrophs.

Introduction

The carbon metabolism of cyanobacteria includes anabolic processes that build carbohydrates, like the Calvin-Benson-Bassham (CBB) cycle, as well as catabolic processes such as glycolytic pathways, in which these carbohydrates are consumed to provide intermediates, energy and reducing equivalents. In the day-night rhythm, carbohydrate fluxes alternate between anabolic and catabolic directions. In 2016, it was claimed that cyanobacteria and plants possess the Entner-Doudoroff (ED) pathway as a glycolytic route besides classical glycolysis (also known as Embden-Meyerhof-Parnas (EMP) pathway) and the oxidative pentose phosphate (OPP) pathway (Fig. 1A) (1). The ED pathway utilizes two enzymes that are unique to this route. The first enzyme is 6-phosphogluconate (6PG) dehydratase, abbreviated as EDD (ED dehydratase), which converts 6PG to 2-keto-3-deoxy-6-phosphogluconate (KDPG). The second enzyme is KDPG aldolase, abbreviated as EDA (ED aldolase), which splits KDPG into pyruvate and glyceraldehyde 3-phosphate (GAP). This claim was mainly based on the biochemical characterization of purified KDPG aldolases (EDAs) from the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) and the plant *Hordeum vulgare*, in combination with protein sequence alignments, growth experiments with selected cyanobacterial deletion mutants and the detection of gluconate and KDPG in *Synechocystis* and *H. vulgare*, from which KDPG is a metabolite that is indicative of an active ED pathway (1). In the following years, *Synechocystis* mutants in which EDA was deleted either alone or in combination with other glycolytic enzymes, such as 6PG dehydrogenase (GND), which belongs to the OPP pathway, were characterized in detail and yielded contradictory results (3–7). These observations raised the question whether EDA could be either a promiscuous aldolase or an enzyme with moonlighting functions (3). Slr0452 from *Synechocystis* and its homologues in plants were considered to be EDDs (1). This consideration was based on protein sequence alignments, and in addition on a report of a promiscuous dihydroxy acid dehydratase (DHAD) with gluconate dehydratase (GAD) activity in *Sulfolobus solfataricus* also discussing similarities between the two dehydratases DHAD and EDD (1, 8). However, a recent study reported that the assumed EDDs in cyanobacteria and plants are instead in most cases exclusively DHADs (2). DHADs are specifically involved in the synthesis of the branched amino acids valine, leucine and isoleucine. Accordingly, this raises further questions about the presence of the ED pathway in cyanobacteria and plants.

The anabolic CBB cycle and the catabolic glycolytic EMP and OPP pathways share several enzymes, requiring a sophisticated regulation of metabolic routes. Under photomixotrophic conditions, organic carbon (e.g., glucose) is consumed from the medium and metabolized in parallel with CO₂ fixation, accelerating growth compared to photoautotrophic conditions (9). It was previously observed that growth of the *Synechocystis* mutant $\Delta pfk\Delta zwf$, in which the EMP and the OPP pathway were interrupted by deleting both phosphofructokinases (*pfk-A1* and *pfk-A2*) and glucose-6-phosphate dehydrogenase (*zwf*), was still able to enhance its growth in the presence of glucose (see Fig. 1B) (1). This observation suggested the presence of an alternative route for carbohydrate degradation. When bioinformatic protein sequence analyses revealed the presence of both enzymes that are unique to the glycolytic ED pathway: EDD (Slr0452) and the KDPG aldolase EDA (Slr0107), this contradiction seemed to be resolved. EDA was purified and verified biochemically as KDPG aldolase (1). In addition, two putative glucose dehydrogenases (GDHs) (Slr1709 and Slr1608) were identified through bioinformatic studies. Since ZWF, which supplies the substrate 6PG for

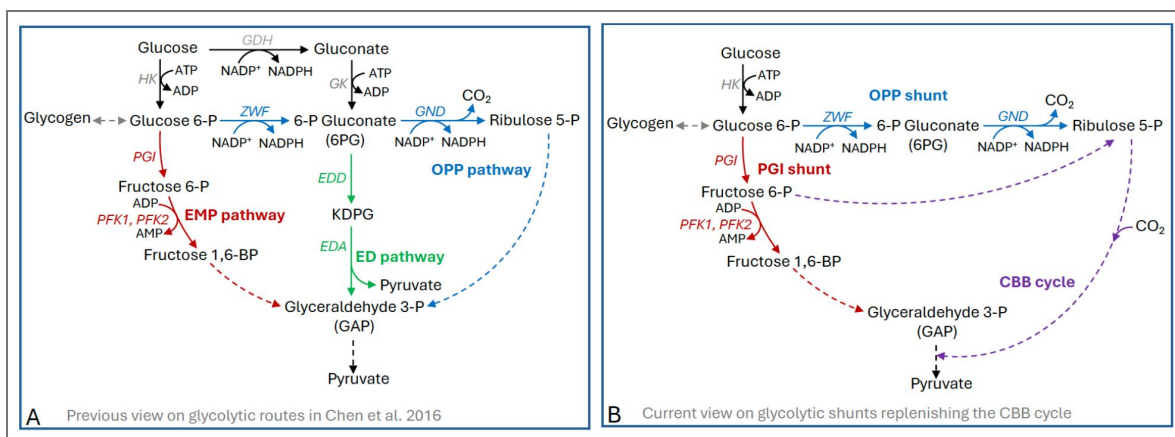


Figure 1. Previous and current view on glycolytic routes, glycolytic shunts and the CBB cycle in *Synechocystis*

(A) The central carbohydrate metabolism in *Synechocystis* as described by Chen et al (1). The shown GDH/GK bypass and ED pathway were assumed to be present based on the misinterpretation of experimental data (1). It was unclear at that time how glycolytic routes and the CBB cycle are organized under photomixotrophic conditions on glucose in continuous light. (B) Updated view where glycolytic routes and the CBB cycle are intertwined under photomixotrophic conditions so that glucose is fed via the glycolytic PGI and OPP shunt into the CBB cycle based on flux analyses (3). ED pathway and GDH/GK bypass are omitted based on results of this study. CBB, Calvin-Benson-Bassham; ED, Entner-Doudoroff; EDA, Entner-Doudoroff aldolase; EDD, Entner-Doudoroff dehydratase; EMP, Embden-Meyerhof-Parnas; GDH, glucose dehydrogenase; GK, gluconate kinase; GND, 6-phosphogluconate dehydrogenase; HK, hexokinase; KDPG, 2-keto-3-deoxy-6-phosphogluconate; OPP, oxidative pentose phosphate; PFK, phosphofructokinase; PGI, phosphoglucose isomerase; ZWF, glucose-6-phosphate dehydrogenase.

EDD, was deleted in $\Delta pfk\Delta zwf$, it was assumed that a putative GDH, together with a gluconate kinase (GK), would convert glucose (bypassing hexokinase (HK) and ZWF) to gluconate and further to 6PG (Fig. 1 [4](#)). Growth experiments with the respective deletion mutants seemed to support these assumptions ([1](#)). Furthermore, gluconate and KDPG were detected in cells of the *Synechocystis* wild type (WT) and $\Delta pfk\Delta zwf$ via IC-ESI-MS/MS ([1](#)). Hence, it was concluded that the accelerated growth of the $\Delta pfk\Delta zwf$ mutant was made possible by the existence of a previously overlooked glycolytic ED pathway in combination with a GDH/GK bypass ([1](#)). Notably, at that time, it was not known whether the CBB cycle and glycolytic routes are spatially separated in the cytoplasm or rather intertwined with sophisticated regulation to enable a switch between anabolic and catabolic reactions. Subsequently, flux analyses with labelled glucose under photomixotrophic conditions with WT and Δeda yielded interesting, but also contradictory results, as will be outlined in detail below ([3](#)). It was observed that glycolytic routes and the CBB cycle are highly intertwined in that sense that catabolic glycolytic routes are shortened to shunts that feed carbohydrates as anaplerotic reactions into the anabolic CBB cycle and thereby accelerate CO₂ fixation ([3](#), [4](#)). Two glycolytic shunts were identified: the OPP shunt, comprising the first two reactions of the OPP pathway catalyzed by ZWF and GND, and the PGI shunt, which includes only the first enzyme of the EMP pathway, phosphoglucose isomerase (PGI) (see Fig. 1 [4](#)) ([3](#), [4](#)). Whereas 27.6% of the labeled glucose was directed to the glycogen pool, the majority, 63.1%, was metabolized via the PGI shunt into the regenerative CBB cycle, and 9.3% were channeled via the OPP shunt to the CBB cycle ([3](#)). Contrary to our expectations, no flux via the ED pathway was detected, and no KDPG was measurable, despite the use of ultrasensitive LC-MS/MS.

The earlier assumption that the accelerated growth on glucose of the glycolytic $\Delta pfk\Delta zwf$ mutant requires the GDH/GK bypass and the ED pathway, had been proven wrong by the described flux analyses, as this growth behavior can easily be explained by the existence of the PGI shunt which is responsible for the main flux of glucose into the CBB cycle and is still intact in the $\Delta pfk\Delta zwf$ mutant (Fig. 1 [4](#)) ([3](#)). However, although there was no detectable flux via the ED pathway in the WT, carbohydrate fluxes were still altered in Δeda . The flux via the PGI shunt was not modified, but a higher portion of labeled glucose was directed to the glycogen pool, and the flux through the OPP shunt was reduced ([3](#), [4](#)). Further studies yielded similarly conflicting results concerning the existence of the ED pathway and particularly concerning the phenotypes of Δeda and the double mutant $\Delta eda\Delta gnd$. Both $\Delta eda\Delta gnd$ and Δeda were impaired in their ability to recover from nitrogen starvation, Δeda had a reduced ability to respond to CO₂ shifts, the reactivation of the dark arrested CBB cycle was delayed and growth of mutants in which either *eda* was deleted alone or in combination with other glycolytic enzymes was not understandable on the mere interruption of glycolytic fluxes ([4](#), [5](#), [7](#)). Therefore, these observations suggested that alterations in Δeda and $\Delta eda\Delta gnd$ might rather be based on regulatory aspects such as a moonlighting functions or substrate promiscuity of EDA instead of missing fluxes ([3](#)). Enzymes with substrate promiscuity accept a broad range of substrates because their active sites can accommodate molecules with different shapes or chemical groups. Therefore, they catalyze several reactions in addition to the primary, most efficient one ([10](#)). Moonlighting enzymes, on the other hand, perform additional functions that are independent of the catalytic activity and active site of an enzyme, such as protein-protein interactions, regulation of gene expression, or binding to RNA or DNA as transcription factors ([11](#)).

The most puzzling contradiction that remained was the observation that the intermediate 6-P gluconate (6PG) accumulated only in the double mutant $\Delta eda\Delta gnd$ (where both 6PG effluxes should be interrupted in the case of an existing ED pathway) but neither in the single mutant Δeda nor in Δgnd (see Fig. 1A [4](#)) ([4](#)).

In order to understand previous results in detail against the background of contradictory data and the new bioinformatic information that EDD is absent in most cyanobacteria ([2](#), [12](#)), we went back to old data, purified and tested the putative EDD (Slr0452), checked the existence of the previously presumed GDH/GK bypass, performed a series of control measurements with different mutants

and resolved the accumulation pattern of 6PG in deletion strains. A detailed biochemical characterization of the KDPG aldolase EDA (Slr0107) reveals its promiscuity and provides initial evidence for its possible role in the absence of EDD in the cyanobacterium *Synechocystis*.

Results and Discussion

In vitro characterization of the predicted EDD/DHAD in *Synechocystis*

Based on the biochemical characterization of the KDPG aldolase EDA in *Synechocystis*, we had previously assumed that Slr0452 encodes for an enzyme that is involved in both amino acid synthesis as dihydroxy acid dehydratase (DHAD) and the ED pathway as EDD, as it was shown that the bifunctional DHAD (DHAD, IlvD/EDD superfamily) in the (hyper)thermophilic archaeon *Sulfolobus solfataricus*, also accepts D-gluconate - and to a lesser extent, galactonate - as alternative substrates in addition to its canonical role in branched-chain amino acid biosynthesis (8). Similarly, DHAD from *Rhizobium leguminosarum* bv. *trifolii* exhibits activity toward several C5 and C6 sugar acids, including gluconate. In addition, and the DHAD from *Fontimonas thermophila* has been engineered to increase its gluconate-dehydration activity by an order of magnitude. Together, these observations underscore the intrinsic substrate promiscuity of IlvD enzymes in the IlvD/EDD superfamily (13, 14).

However, although at least some DHADs exhibit activity toward gluconate (sugar acids), they have not been shown to accept 6-phosphogluconate (6PG), the physiological substrate of EDD in the canonical ED pathway, from any organism. One possible explanation for this limited overlap in substrate specificity was proposed by Evans et al. (2), who suggested that gluconate, being smaller and unphosphorylated, may fit within the DHAD substrate-binding pocket normally occupied by dihydroxy-isovalerate. However, the lack of crystal structures of DHAD bound to dihydroxy-isovalerate or of EDD bound to 6PG precludes direct structural comparisons, leaving the precise geometric and electrostatic determinants of substrate discrimination between these enzymes unresolved. Of note, archaea like *S. solfataricus* utilizing modified, branched ED pathway versions involving the dehydration of gluconate to KDG, do not employ a DHAD but a dedicated gluconate dehydratase (GAD) (phylogenetically grouped within the enolase superfamily, specifically a subgroup related to the mandelate racemase/muconate lactonizing enzyme (MR/MLE)) to catalyze this conversion (15, 16). This indicates that DHAD side activity alone might be insufficient to sustain physiologically relevant flux through the modified ED pathway. To date, there is no evidence that DHAD can functionally replace a bona fide GAD from the enolase superfamily in these organisms *in vivo*.

A recent study supposes that Slr0452 from *Synechocystis* which was assumed to be an EDD is an exclusive DHAD based on the biochemical characterization of its homolog in soybean (*Glycine max*) (2). Notably, organisms harboring both DHAD and EDD activity typically encode an additional IlvD/EDD superfamily gene, likely originating from gene duplication. Thus, the presence of only a single DHAD candidate in *Synechocystis* argues against an EDD function. DHAD activity was indeed shown for Slr0452 in another study, however, its potential activity toward 6PG as a substrate was not evaluated, leaving unresolved whether Slr0452 encodes a strict DHAD or a bifunctional DHAD/EDD enzyme (17). To address this question, the *slr0452* gene from *Synechocystis* was heterologously expressed in *E. coli* and recombinant protein was purified (Fig. S1). For comparison, a DHAD from the cyanobacterium *Synechococcus elongatus* was purified and assayed under identical conditions as a positive control (12) (Fig. S2). Enzymatic assays confirmed that Slr0452 catalyzes the dehydration of 2R-dihydroxyisovalerate (2R-DHIV) to 2-ketoisovalerate (KIV), consistent with DHAD function (Fig. S3A). Slr0452 exhibited a specific activity of 2.0 U mg⁻¹, whereas DHAD from *S. elongatus* showed 0.8 U mg⁻¹ with 2R-DHIV as substrate (Fig. S3A).

To test for potential dual DHAD/EDD activity, Slr0452 was subsequently assayed using 6PG as substrate. A biochemically characterized EDD from *Caulobacter crescentus* (18) served as a positive control. Despite screening a range of divalent metal cofactors (Mg²⁺, Mn²⁺, Zn²⁺, Co²⁺, Cu²⁺, and

Fe²⁺), no measurable activity toward 6PG was detected for Slr0452 under any tested condition (Fig. S3B). In contrast, EDD from *C. crescentus* displayed robust 6PG dehydratase activity as expected (Fig. S2, Fig. S3B). To evaluate the possibility of a modified ED pathway in *Synechocystis*, Slr0452 and EDA (Slr0107) activities were also assayed using gluconate and KDG as substrates, respectively. *S. solfataricus* DHAD with GAD activity (SsoDHAD) and *Sulfolobus acidocaldarius* KD(P)G aldolase (SaciKD(P)GA), previously shown to catalyze reactions with gluconate and KDG, respectively (19, 20), were used as positive controls (Fig. S2, Fig. S4). Slr0452 and EDA from *Synechocystis* showed no activity on gluconate or KDG indicating that they are not involved in a modified ED pathway (Fig. S4).

Collectively, these results demonstrate that *slr0452* encodes a monofunctional, metal-dependent DHAD rather than an EDD or a bifunctional DHAD/EDD enzyme. This confirms that Slr0452 is exclusively involved in amino acid synthesis and does not catalyze the first reaction of the ED pathway as previously suggested (2, 17).

Above all, these results show that *Synechocystis* lacks a canonical 6-phosphogluconate (6PG) or gluconate dehydratase and therefore does not contain either the canonical or modified glycolytic ED pathway.

Testing for the existence of the previously presumed GDH/GK bypass

We furthermore tested for the existence of the previously presumed glucose dehydrogenase/gluconate kinase (GDH/GK) bypass as an alternative route to hexokinase and ZWF for gluconate and 6PG production. Two putative GDHs (Slr1709, Slr1608) were previously identified in the genome based on protein sequence alignments (1). Crude cell extracts were analyzed for GDH and GK activity; however, analysis of crude extracts from *Synechocystis* WT, Δzwf , as well as a putative GDH1 (Slr1709) overexpression mutant *slr1709:oe* revealed no detectable GDH activity under either photoautotrophic or photomixotrophic conditions (Fig. S5, Fig. S6, Fig. 2A-B).

Unfortunately, we were not able to overexpress putative GDH2 (Slr1608). Crude cell extracts of Δzwf were tested on the assumption that the GDH/GK route, if existent, might be upregulated in this mutant. The *slr1709:oe* overexpression mutant was tested to ensure sufficient enzyme amounts. Cell crude extracts of Δzwf and purified putative GDH1 (Slr1709) were furthermore tested for GDH activity in an *in vitro* enzyme assay (Fig. S6, Fig. 2B). In this analysis, the two quinones 2,3-dimethoxy-5-methyl-1,5-benzoquinone (DMB) and duroquinone (DQ) were also tested as electron acceptors in addition to NADP⁺ and NAD⁺ since some glucose dehydrogenases are known to reduce quinones instead of NAD(P)⁺. No significant amount of GDH activity was detected for any tested electron acceptor in the GDH eluate, as well as in a cell extract of photomixotrophically grown Δzwf (Fig. 2B). Taken together, these results indicate that GDH activity, which converts glucose to gluconate, is absent in *Synechocystis* *in vivo*. However, the inability to detect enzymatic activity is never a completely safe proof for its absence as the conditions might not have been chosen correctly, the enzyme activity might be below detection limits, or the purified enzyme might have lost its activity during purification. Furthermore, we were not able to purify and test the second putative GDH2 (Slr1608). Therefore, the second enzyme of the putative GDH/GK route was sought. The search for homologues of GK in the *Synechocystis* genome, using GKs from *E. coli* and *Pseudomonas aeruginosa* as baits, was not successful. In line with the *in silico* analyses, no gluconate kinase activity was detectable in either crude cell extracts from WT or Δzwf (Fig. 2C). These results furthermore strengthen the assumption that a GDH/GK bypass is absent in *Synechocystis*.

In order to further investigate if the GDH/GK bypass is absent from all cyanobacteria, we conducted a second blast (BlastP) analysis. Similarity thresholds were set at a query score > 30 %, an identity score > 30 % and an e-value < 1⁻¹⁰. At these threshold settings we found 261 cyanobacteria that possess putative homologs of GKs from *E. coli* and *P. aeruginosa* (Table S1). We searched for homologs of GDHs in these 261 cyanobacteria next to *Synechocystis*. As baits the

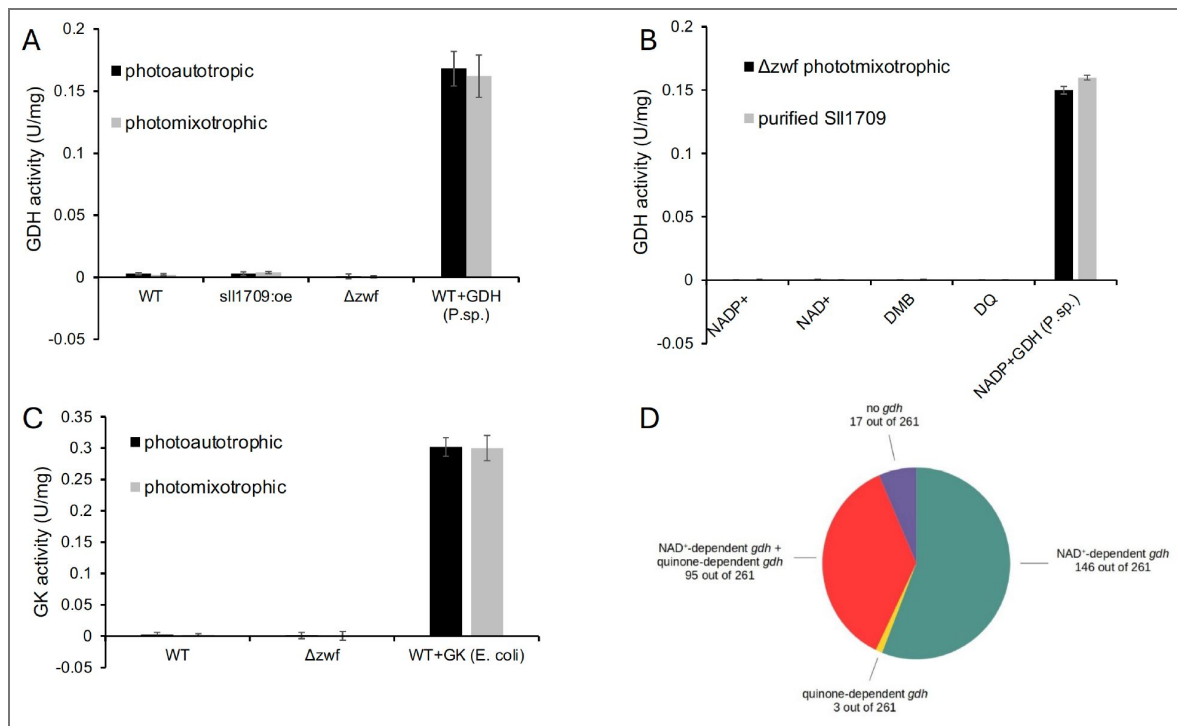


Figure 2. Evaluation of the proposed glucose dehydrogenase/gluconate kinase (GDH/GK) bypass in *Synechocystis* and representative cyanobacteria.

(A) GDH activity measurements in crude cell extracts of *Synechocystis* WT, Δzwf and a strain overexpressing putative GDH1 (*sl11709:oe*) under photoautotrophic or photomixotrophic conditions. As a positive control, 0.05 U glucose dehydrogenase from *Pseudomonas* sp. was added to WT cell extract. (B) GDH activity measurements in crude cell extracts of photomixotrophically grown Δzwf cultures and with purified putative GDH1 (Sll1709). $NADP^+$, NAD^+ , and the two quinones DMB (2,6-Dimethoxy-1,4-benzoquinone) and DQ (2,3-Dimethoxy-1,4-naphthoquinone) were tested as electron acceptors. As a positive control, 0.05 U glucose dehydrogenase from *Pseudomonas* sp. was added to Δzwf cultures and to purified putative GDH1 (Sll1709). (C) Gluconate kinase activity measurements in cell extracts of *Synechocystis* WT and Δzwf under photoautotrophic or photomixotrophic conditions. As a positive control, 0.05 U gluconate kinase from *E. coli* was added to WT cell extract. (D) Blast analyses of putative glucose dehydrogenase (GDH) in cyanobacteria. Protein databases of 261 cyanobacteria that also carry a putative gluconate kinase (GK) were analyzed for GDH homologues.

NAD⁺-dependent GDH 1GCO from *Bacillus megaterium* and the quinone-dependent GDH from *Acinetobacter calcoaceticus* were used (21, 22). While the results again showed a rather poor similarity for both putative GDHs (Slr1709 and Slr1608) from *Synechocystis* and thereby confirmed the lack of GDH activity in enzyme tests, good homologies for proteins were found for 244 (93 %) of the other 261 analyzed cyanobacteria (Fig. 2D, Table S1). Out of these 244 cyanobacteria that likely possess both a putative GDH and a putative GK homologue, we picked two strains, namely *Spirulina* sp. SIO3F2 and *Lyngbya aestuarii* and overexpressed and purified their putative GDHs (NEO87985.1 and WP_238987112) and the putative GK from *Lyngbya aestuarii* (WP_023068790.1) in *E. coli* (Fig. S7 to Fig. S9). Purification of the putative GK from *Spirulina* sp. SIO3F2 (NEO86388.1 MAG) was not successful. Enzyme activity assays showed a clear GDH activity for both the GDH from *Spirulina* and the GDH from *Lyngbya* (Table 1). Both NADP⁺ and NAD⁺ could function as cofactors, while higher activity was observed with NADP⁺ for both enzymes. The *Spirulina* and *Lyngbya* GDHs also used 2-Deoxy-D-glucose as substrate with 20-40% residual activity compared to glucose. Marginal activity with D-mannose was observed with the *Lyngbya* GDH (Table 1). All other tested substrates including fructose 6-phosphate, D-fucose, glucose 6-phosphate, lactose, D-glucosamine, D-lyxose, D-allose, D-arabinose, L-arabinose, D-xylose, L-xylose, and D-ribose were not used as substrates.

For both the GK from *Lyngbya* and *Spirulina*, expression in *E. coli* could be observed (Fig. S9), however, purification was only achieved for the GK from *Lyngbya*. For the *Lyngbya* GK a specific activity of 24.8 U/mg could be determined (Table 1). In combination with already shown GDH activity, this proves the presence of the GDH/GK bypass in this cyanobacterium.

Taken together, these results indicate that there is no GDH/GK bypass in *Synechocystis*, whereas it seems to be present in other cyanobacteria, as e.g. *Lyngbya*.

Based on these results, we currently have no explanation for the gluconate that was detected in previous IC-ESI-MS/MS measurements in *Synechocystis* (1). Supplementation of the growth medium with gluconate did not enhance growth of the WT, which furthermore supports the absence of a gluconate kinase or alternatively gluconate transport into the cells (Fig. 3A). An additional control was performed by testing photomixotrophic growth of a hexokinase mutant (Δhk , *sll0539*) assuming that a GDH/GK bypass if present might be upregulated in this mutant.

Two enzymes (Slr0539 and Slr0329) were previously identified as putative glucose phosphorylating enzymes, with Slr0539 as the main contributor, while the deletion of Slr0329 did not affect glucose uptake or photomixotrophic growth (23, 24). Therefore, only Δhk ($\Delta sll0539$) was studied (25). Enzyme assays confirmed that the Δhk mutant lost its ability to phosphorylate glucose (Fig. 3B). It was furthermore unable to metabolize glucose from the medium and to accelerate its growth (Fig. 3C-D). The described phenotypes could be complemented in $\Delta hk::hk$. These data further support the assumption that a GDH/GK bypass is absent in *Synechocystis*, because it should allow glucose utilization in the absence of hexokinase (Hk, Slr0539) otherwise.

Investigation into the causes why 6PG accumulates in $\Delta eda\Delta gnd$ in the absence of the ED pathway, but not in Δeda or Δgnd

As our data indicate that the GDH/GK bypass is not present in *Synechocystis*, the only known source for 6PG would be the reaction catalyzed by ZWF (see Fig. 1). Based on this assumption, the accumulation of 6PG in $\Delta eda\Delta gnd$ in contrast to Δeda and Δgnd is unexpected in the absence of an existing ED pathway (see Fig. 1) (4). This pattern of 6PG enrichment had led to believe for many years, despite conflicting results, that there must be at least a small flux through the ED pathway in *Synechocystis* (4). So, to resolve this apparent contradiction, we attempted to resolve the origin of the 6PG accumulation pattern in $\Delta eda\Delta gnd$, Δeda and Δgnd .

In order to test if ZWF is responsible for 6PG production in $\Delta eda\Delta gnd$, this enzyme was deleted in addition resulting in the mutant $\Delta eda\Delta gnd\Delta zwf$. 6PG accumulation was abolished in $\Delta eda\Delta gnd\Delta zwf$ as expected, indicating, that ZWF activity was responsible for 6PG production in $\Delta eda\Delta gnd$ (Fig. 4A).

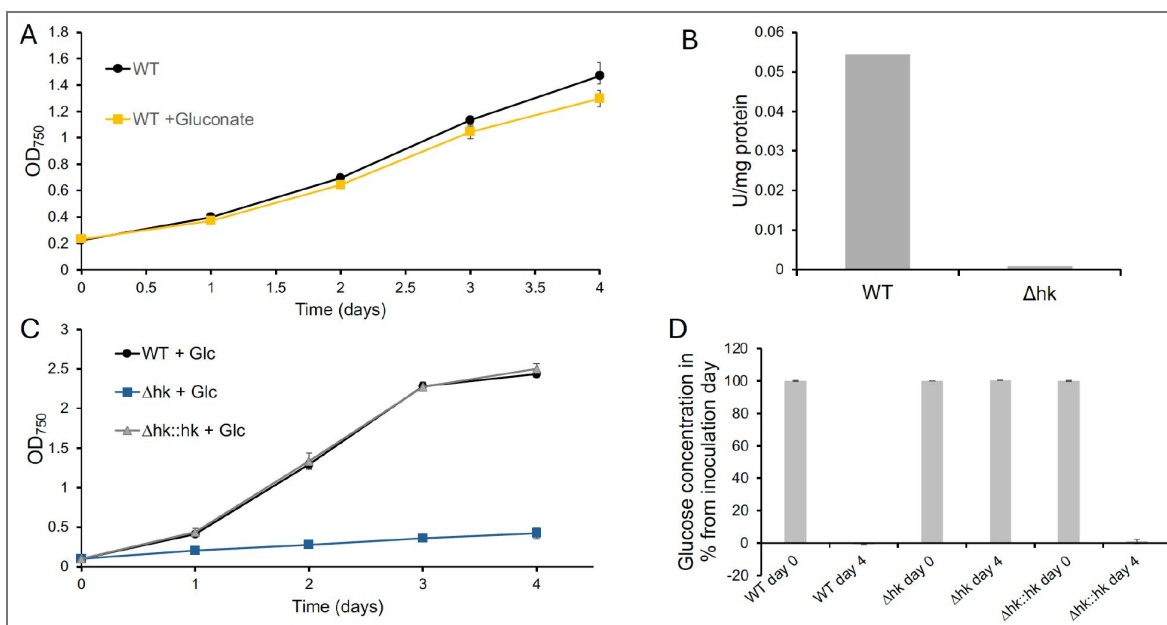
Table 1. Specific activities of the two cyanobacterial GDHs from *Spirulina* and *Lyngbya* and the gluconate kinase from *Lyngbya*.

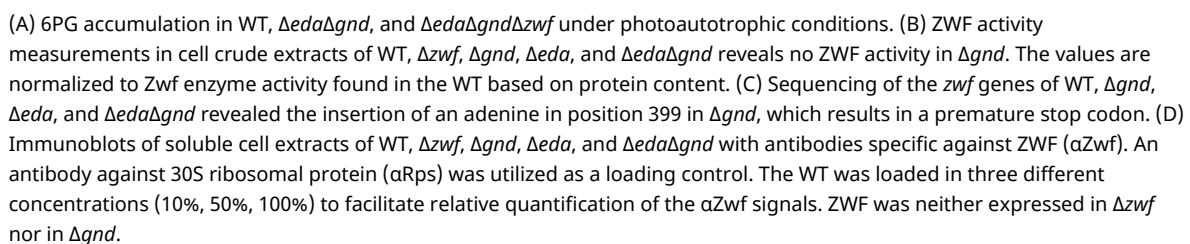
Enzyme assays were performed after recombinant expression in *E. coli* and following purification.

Glucose dehydrogenase (GDH)	Activity (U/mg) with NADP ⁺	Activity (U/mg) with NAD ⁺
GDH (NEO87985.1) from <i>Spirulina</i> sp. SIO3F2	5.63 (Glucose) 2.12 (2-Deoxy-D-glucose)	0.15 (Glucose)
GDH (WP_238987112) from <i>Lyngbya aestuarii</i>	20.2 (Glucose) 4.04 (2-Deoxy-D-glucose) 0.26 (D-Mannose)	2.95 (Glucose)
Gluconat kinase (GK)	Activity (U/mg) with ATP	
GK (WP_023068790.1) from <i>Lyngbya aestuarii</i>	24.79 (10 mM Gluconate)	

Figure 3. Utilization of gluconate and glucose as carbon source for growth in WT, Δhk ($\Delta sl10539$), and $\Delta hk::hk$.

(A) Growth of WT in the absence and presence of gluconate. (B) Hexokinase activity in WT and Δhk with 10 mM glucose. (C) Photomixotrophic growth of WT, Δhk , and $\Delta hk::hk$. (D) Glucose concentration (%) in the growth medium after inoculation (day 0; 10 mM glucose) and on day 4 in WT, Δhk , and $\Delta hk::hk$.





We subsequently tested ZWF activities in $\Delta eda\Delta gnd$, Δeda , Δgnd , and Δzwf as a control and found to our surprise that ZWF activity was completely lacking in Δgnd (Fig. 4B). Sequencing of the *zwf* gene in Δgnd , revealed an insertion of an adenine in position 399, which results in a premature stop codon, and obviously completely abolishes ZWF activity in the Δgnd mutant, whereas the mutants $\Delta eda\Delta gnd$ and Δeda lacked this insertion in their *zwf* genes (Fig. 4C). Immunoblots with specific antibodies against ZWF confirmed that ZWF is present in WT, Δeda and $\Delta eda\Delta gnd$ but is no longer expressed in Δgnd (Fig. 4D). Whereas $\Delta eda\Delta gnd$ showed ZWF activity that resembled WT levels, ZWF activity was reduced to approximately 60% in Δeda (Fig. 4B). This observation is in line with the reduced photomixotrophic flux through the OPP shunt in this mutant that we had observed earlier (3). The construction of corresponding complemented mutants ($\Delta eda::eda$) is underway to test whether these differences are indeed due to the absence of EDA.

In summary, these data demonstrate that the accumulation pattern of 6PG in $\Delta eda\Delta gnd$, Δeda and Δgnd is not caused by an active ED pathway, as previously assumed. Instead, the pattern can be explained by a secondary mutation in *zwf* in the Δgnd mutant which abolishes 6PG production in the Δgnd mutant.

In vitro characterization of EDA (Sll0107)

Taken together, all data so far support the conclusion that the ED pathway is absent in the cyanobacterium *Synechocystis*. One remaining puzzling observation is the reported detection of KDPG in *Synechocystis* in one study using IC-ESI-MS/MS, which can be indicative of an active ED pathway, whereas another study using LC-MS/MS failed to detect this metabolite (1, 3). Analytical artifacts can never be fully excluded. To clarify whether EDA (Sll0107) might catalyze the reversible aldol condensation of pyruvate and GAP to KDPG, which could explain the detection of KDPG in the absence of EDD and to test for potential promiscuity of EDA with several substrates, the *sll0107* gene (KEGG annotation) was expressed in *E. coli*. Although SDS-PAGE and immunoblot analysis confirmed the expression of a ~23 kDa protein, it could not be eluted from the Ni-TED resin, and crude extracts lacked detectable KDPG aldolase activity. These observations suggested expression of a non-functional or truncated protein. Genomic inspection of *sll0107* (*eda*) revealed an alternative start codon (genome position 2,972,167) 72 nucleotides upstream of the annotated start codon (genome position 2,972,095) (Fig. 5). Comparative sequence and AlphaFold 3 analyses (26) using *E. coli* and *C. crescentus* KDPG aldolases (27) as a reference, showed that only the upstream-initiated longer sequence retained the N-terminal α -helix characteristic of class I KDPG aldolases (Fig. 5A).

Therefore, the extended Sll0107 version was expressed in *E. coli*, purified and resulted in active protein (Fig. S10–S12). SDS-PAGE showed a subunit mass of ~25 kDa, consistent with the calculated 23 kDa, and size-exclusion chromatography indicated a native mass of ~64 kDa, corresponding to a homotrimeric assembly typical of bacterial class I KDPG aldolases (13, 29, 30).

Recombinant Sll0107 was assayed using the LDH-coupled continuous assay and exhibited robust activity toward its canonical substrate KDPG, catalyzing its cleavage into pyruvate and GAP with a catalytic efficiency of $24.4 \text{ s}^{-1} \text{ mM}^{-1}$ (Fig. 6, Table 2). Inspired by recent reports that metal-independent class I KDPG aldolases from the cyanobacterium *Synechococcus elongatus* also accept oxaloacetate (12), we tested this substrate and found that EDA (Sll0107) catalyzed its cleavage to pyruvate and CO_2 with a catalytic efficiency of $0.58 \text{ s}^{-1} \text{ mM}^{-1}$ (Fig. 6, Table 2). Since elevated proline levels were previously reported in Δeda , we next examined activity with 2-keto-4-hydroxyglutarate (KHG), an intermediate of the proline degradation pathway as substrate (5, 31). EDA efficiently cleaved KHG to pyruvate and glyoxylate with a catalytic efficiency of $0.18 \text{ s}^{-1} \text{ mM}^{-1}$ (Fig. 6, Table 2).

EDA exhibited a strong preference for KDPG as its primary substrate but also catalyzed reactions with OAA and KHG, albeit with substantially lower catalytic efficiency. The effect of NADP^+ on EDA activity was examined, as NADP^+ has been reported to act as an activator of *S. elongatus* EDA (12). Under the assay conditions used in this study, the addition of NADP^+ at concentrations up to 3 mM

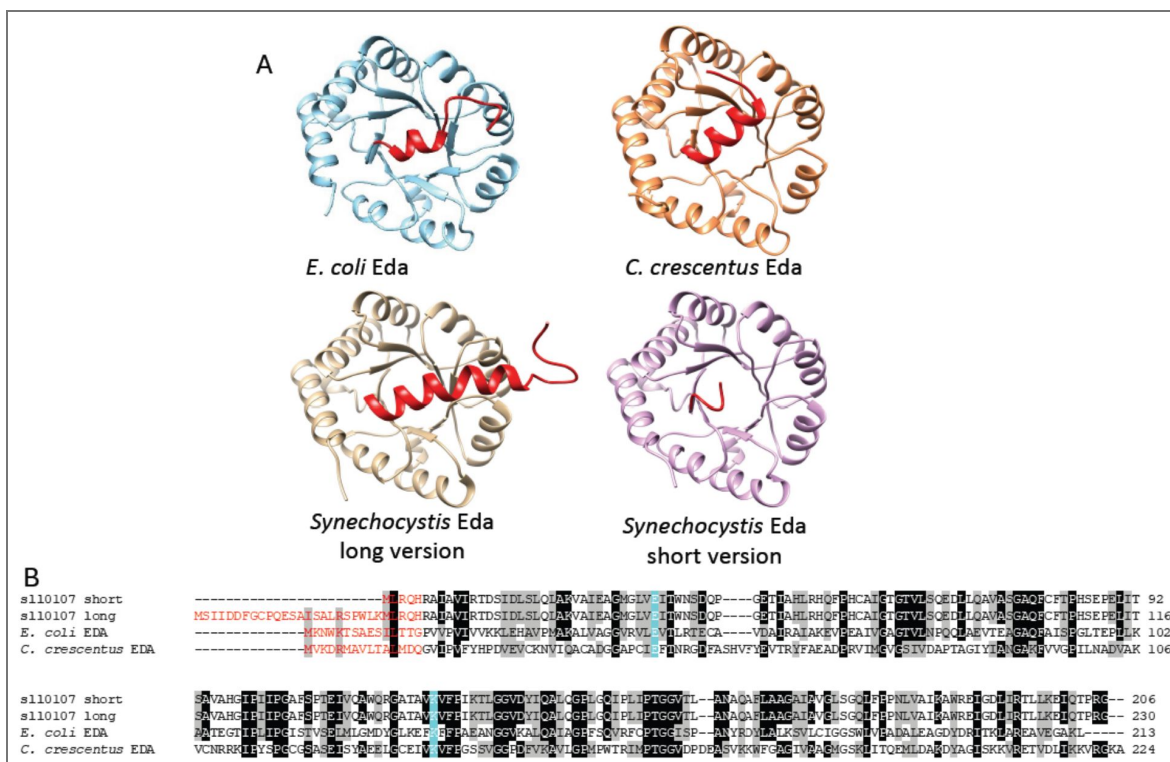


Figure 5. Structural comparison and sequence alignments of EDA enzymes including the genome-annotated version and an N-terminally extended version with an alternative start codon of the *Synechocystis* enzyme.

(A) Structures of *E. coli* and *C. crescentus* EDA enzymes are compared with AlphaFold 3 models of the N-terminally extended (long) and genome-annotated (short) *Synechocystis* EDA variants. The conserved N-terminal α -helix is highlighted in red. Amino acid sequences were retrieved from KEGG, AlphaFold 3 models were generated using AlphaFold Server v3.0 (26) and structures were visualized and edited using UCSF ChimeraX. (B) Sequence alignment highlighting the N-terminal residues of the conserved α -helix in red. The catalytic lysine responsible for Schiff-base formation and the glutamate acting as the acid/base catalyst are indicated in cyan. The alignment was performed using Clustal Omega (28) and manually visualized in BioEdit.

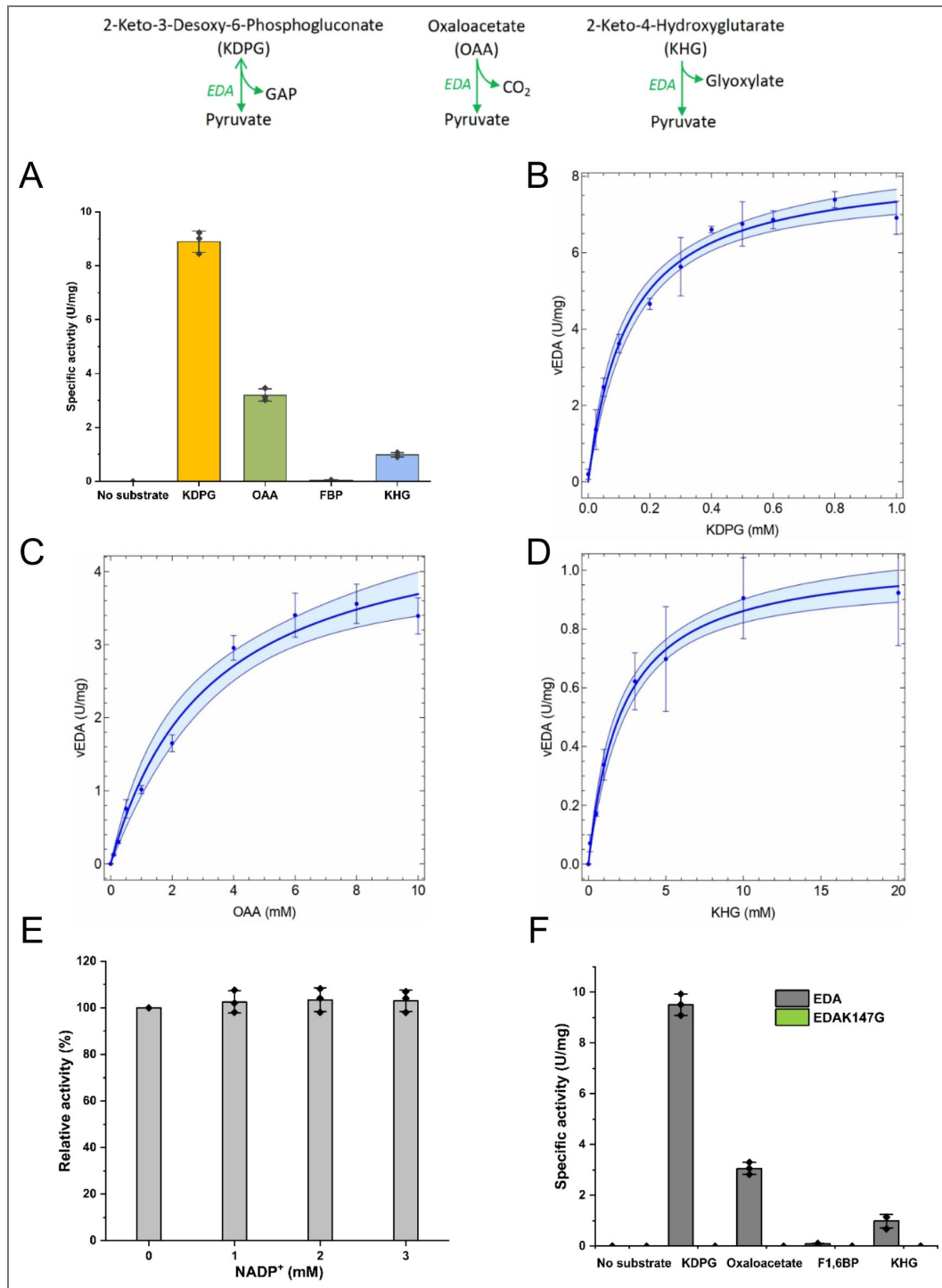


Figure 6. Substrate specificity and characterization of EDA (SlI0107) from *Synechocystis*.

(A) Substrate specificity was assessed with 5 mM of each KDPG, oxaloacetate, FBP, and KHG using the continuous coupled assay (see Methods). **(B-D) Enzymatic characterization of EDA using KDPG, OAA and KHG as substrates.** The activity of EDA was measured across a range of (B) KDPG (0–1 mM), (C) OAA (0–10 mM) and (D) KHG (0–20 mM) concentrations. Colored bands indicate 95% confidence, and data are shown as mean ± standard deviations from three technical replicates. The kinetic parameters are given in [table 2](#). **(E) Effect of NADP⁺ on EDA activity.** Different NADP⁺ concentrations (0, 1, 2 and 3 mM) were tested for their effect on EDA activity. Assays were performed with sub-saturating KDPG concentration (0.2 mM). The relative activity (%) in comparison to the control without effector (100%; specific activity of 4.3 U/mg) is shown. **(F) Effect of K147G mutation on EDA activity.** Enzymatic activity of wild-type EDA and the catalytic mutant EDAK147G was measured with 5 mM KDPG, oxaloacetate, FBP, and KHG using continuous coupled assays (see Methods). Data are presented as mean ± SD from three technical replicates (n = 3).

Substrates	V_{max}	K_m	k_{cat}	k_{cat}/K_m
KDPG	8.28 ± 0.25 U mg ⁻¹	0.13 ± 0.01 mM	3.2 s ⁻¹	24.4 s ⁻¹ mM ⁻¹
OAA	4.87 ± 0.37 U mg ⁻¹	3.19 ± 0.64 mM	1.86 s ⁻¹	0.58 s ⁻¹ mM ⁻¹
KHG	1.05 ± 0.03 U mg ⁻¹	2.18 ± 0.24 mM	0.4 s ⁻¹	0.18 s ⁻¹ mM ⁻¹
FBP	No activity			
Malate	No activity			
Pyruvate + D-GAP	0.18 ± 0.01 U mg ⁻¹ (2.2% of the KDPG cleavage activity)			
Pyruvate + CO ₂	No activity			
Pyruvate + glyoxylate	No activity			

Table 2. Kinetic parameters of EDA (SII0107) from *Synechocystis*

did not result in any measurable increase in EDA activity (Fig. 6E [↗](#)). These findings are consistent with curated enzyme data reported in BRENDA Enzyme Database ([32](#)), in which NADP⁺ is not listed as an activator for EDA.

To assess reaction reversibility, a hallmark of class I aldolases, reported for archaeal KD(P)G aldolases ([15](#)), we tested the enzyme in the condensation direction using (i) pyruvate + GAP for KDPG formation, (ii) pyruvate + CO₂ for oxaloacetate, and (iii) pyruvate + glyoxylate for KHG synthesis. Only a low level of reverse activity, 0.18 U/mg (~2% of the cleavage activity) was detected for KDPG formation using a discontinuous TBA assay, whereas no reverse activity was observed for oxaloacetate or KHG synthesis (Fig. S12 [↗](#)).

Given that a soybean KDPG aldolase was recently reported to accept fructose 1,6-bisphosphate (FBP) ([2](#)) we also tested FBP as a substrate. However, EDA (SlI0107) displayed no detectable activity with FBP (Fig. 6A, 6F [↗](#), Table 2 [↗](#)). Also, malate was not used as substrate (Table 2 [↗](#)).

Class I KDPG aldolases are metal-independent enzymes that utilize a catalytic lysine residue to form a Schiff base intermediate during catalysis. In EDA this lysine (Lys147, based on the extended *slI0107* sequence) was identified as essential for activity toward multiple sugar acid substrates ([33](#)). To generate a catalytically inactive variant, Lys147 was substituted with glycine (K147G), which lacks both side-chain and net charge. The mutant enzyme EDAK147G was expressed and purified at comparable levels to the WT protein but exhibited no detectable activity with any tested substrate (Fig. 6F [↗](#)).

Taken together, these data show that the KDPG aldolase EDA (SlI0107) of *Synechocystis* is an aldolase with substrate promiscuity, which *in vitro* cleaves KDPG to GAP and pyruvate, OAA to CO₂ and pyruvate, and KHG to glyoxylate and pyruvate (Fig. 6 [↗](#), Table 2 [↗](#)). In addition, it synthesizes KDPG from GAP and pyruvate with very low efficiency (~2% of the cleavage activity) (Fig. S12 [↗](#)), which might explain the detection of KDPG in our initial study ([1](#)).

Since 6PG dehydratase (EDD) seems to be absent in *Synechocystis*, the canonical substrate of EDA, 2-keto-3-deoxy-6-phosphogluconate (KDPG) is not produced, and no alternative enzymatic source of KDPG is known to the best of our knowledge. Our *in vitro* enzyme assays therefore suggest that EDA may instead participate in alternative metabolic reactions, potentially linked to the PEP-pyruvate-OAA node and/or proline catabolism, as illustrated in Figure 7 [↗](#). However, further studies are required to clarify the physiological role of EDA *in vivo*.

Conclusions

Our data demonstrate that *Synechocystis* lacks a functional ED pathway, due to the absence of both EDD and the proposed GDH/GK bypass, which is also supported by photomixotrophic flux analyses ([3](#)). Instead, it possesses a promiscuous ED aldolase, whose physiological role remains to be elucidated. The dehydratases EDD and DHAD have a common origin, a high degree of amino acid and structural similarity, and the same catalytic mechanism. However, they differ in two domains that contribute to substrate selection, which is why EDD and DHAD are enzymes with high substrate specificity that cannot replace one another ([2](#)). Apart from four identified cyanobacteria (*Nostoc* sp. 335mG, *Synechococcus moorigangali* CMS01, *Leptolyngbya valderiana* BDU 20041, and *Leptolyngbya* sp. 15MV) that possess potential EDDs (based on the described domains, PXA97457.1 for *Nostoc*; MBV5257376.1 for *S. moorigangali*; OAB60313.1 for *L. valderiana*; QYU66659.1 for *Leptolyngbya* sp), most cyanobacteria lack EDD despite the presence of EDA, which likewise applies to algae, moss and plants ([2](#), [31](#)). Our data demonstrate that Slr0452 in *Synechocystis*, which we previously assumed as EDD ([1](#)), lacks 6PG dehydratase activity and instead dehydrates DHIV exclusively, consistent with recent reports ([17](#)). The same experimental results were recently obtained for the cyanobacterium *S. elongatus* PCC 7942 that also lacks EDD but contains EDA ([12](#)). This raises the question of what role EDA could play in the absence of EDD and the ED pathway in cyanobacteria, moss, algae and plants. In soybean (*Glycine max*), GmEDA was found to catalyze three reactions *in vitro*: firstly, the cleavage of KDPG to GAP and pyruvate, secondly the cleavage of fructose 1,6-bisphosphate (FBP) to dihydroxyacetone phosphate (DHAP) and GAP and thirdly the condensation of erythrose 4-phosphate (E4P) and GAP to sedoheptulose 1,7-bisphosphate (SBP) ([2](#)).

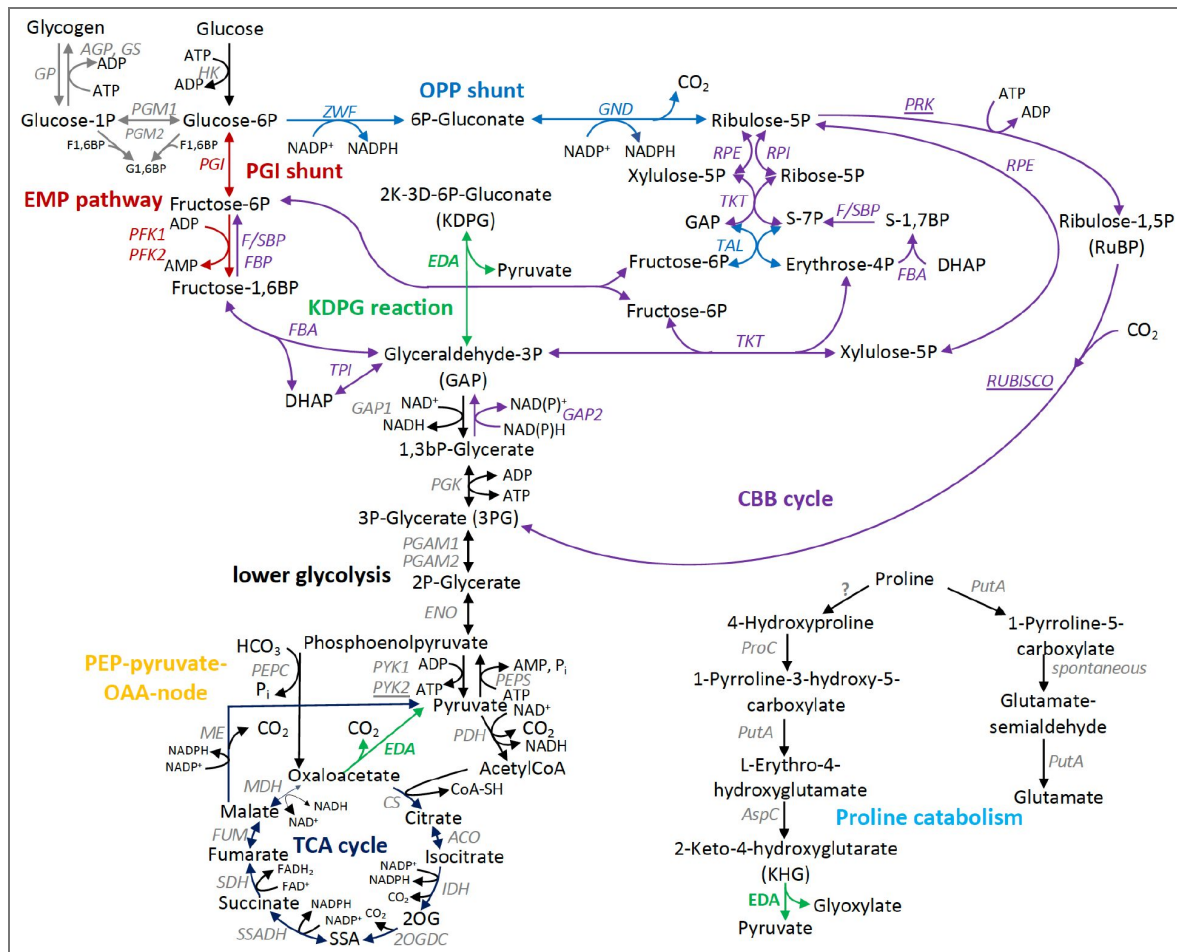


Figure 7. Proposed metabolic map of the central carbon metabolism in *Synechocystis* with all suggested *in vivo* roles of the promiscuous aldolase SII0107 (EDA) highlighted in green, based on substrate promiscuity and activities observed *in vitro*.

ACO, Acontinase; AGP, ADP-glucose pyrophosphorylase; CBB, Calvin-Benson-Bassham; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; EMP, Embden-Meyerhof-Parnas; EDA, Enter-Doudoroff aldolase; ENO, enolase; F6P, fructose 6-phosphate; FBA, fructose-bisphosphate-aldolase; FBP, fructose 1,6-bisphosphate; FBPase, fructose-1,6-bisphosphatase; F/SBPase, fructose-1,6-bisphosphatase/sedoheptulose-1,7-bisphosphatase; FUM, fumarase; GAP, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBP, glucose 1,6-bisphosphate; GND, 6-phosphogluconate dehydrogenase; GP, glycogen phosphorylase; GS, glycogen synthase; HK, hexokinase; IDH, isocitrate dehydrogenase; ME, malic enzyme, MDH, malate dehydrogenase; OPP, oxidative pentose phosphate; PDH, pyruvate dehydrogenase complex, PEPC, phosphoenolpyruvate carboxylase, PEPS, phosphoenolpyruvate synthetase, PFK, phosphofructokinase; PGAM, phosphoglycerate mutase; PGI, phosphoglucose isomerase; PGK, phosphoglycerate kinase; PGL, 6-phosphogluconolactonase; PGM, phosphoglucomutase; PRK, phosphoribulokinase; PUTA, proline dehydrogenase; PYK, pyruvate kinase; R-5P, ribose 5-phosphate; RPE, ribulose-5-phosphate epimerase; RPI, ribose-5-phosphate isomerase; SDH, succinate dehydrogenase; TalB, transaldolase; TKT, transketolase; TPI, triosephosphate isomerase; X-5P, xylulose 5-phosphate; ZWF, glucose-6-phosphate dehydrogenase.

Accordingly, the idea was put forward that GmEDA is a promiscuous aldolase that catalyzes aldolase reactions in the CBB cycle and glycolysis *in vivo*, which is well in line with its localization in chloroplasts (2). In diatoms the ED pathway was suggested to operate in mitochondria providing GAP and pyruvate to lower glycolysis and the TCA cycle, based on bioinformatic analyses, bacterial complementation experiments and measurements on the conversion of 6PG to pyruvate in soluble protein extracts (34–36). However, as neither the EDD nor EDA from diatoms were thoroughly characterized biochemically yet, further studies would be informative. The proteomes of three marine *Prochlorococcus* and three marine *Synechococcus* strains were investigated upon exposure to glucose (37). Protein levels of EDA were either downregulated or not effected while proteins involved in OPP shunt and CBB cycle were upregulated indicating their involvement under photomixotrophy in these cyanobacteria possibly similar to *Synechocystis* (3, 4, 37). However, flux analyses are still missing for the marine strains.

Based on our *in vitro* data *Synechocystis* EDA might be connected to the TCA cycle and the PEP-pyruvate-OAA node by decarboxylating oxaloacetate to pyruvate and/or in proline catabolism by splitting KHG to glyoxylate and pyruvate (Fig. 7C). The latter assumption is supported by earlier findings of elevated proline levels in Δ eda mutants of *Synechocystis* (5). For EDA from *S. elongatus* PCC 7942, it has recently been demonstrated that, in addition to cleaving KDPG, it also decarboxylates oxaloacetate (12). In contrast to *Synechocystis* EDA, EDA from *S. elongatus* PCC 7942 prefers OAA over KDPG. The catalytic efficiency of *S. elongatus* PCC 7942 EDA on oxaloacetate is rather low ($\text{OAA } 0.437 \text{ s}^{-1} \text{ mM}^{-1}$), however, its activity can be enhanced by NADP^{+} (12). The *Synechocystis* EDA showed a 42-fold higher catalytic efficiency with KDPG compared to oxaloacetate ($\text{KDPG } 24.4 \text{ s}^{-1} \text{ mM}^{-1}$, $\text{OAA } 0.58 \text{ s}^{-1} \text{ mM}^{-1}$) and no activation by NADP^{+} (1–3 mM) was observed. As the *S. elongatus* PCC 7942 Δ eda mutant was compromised in terms of photosynthesis and growth under diurnal day/night cycles, the authors hypothesized that *S. elongatus* PCC 7942 EDA might play a role in balancing NADPH/NADP^{+} ratios and photosynthesis by modulating TCA cycle activities (12). For *Synechocystis* EDA, the catalytic efficiency for KDPG compared to KHG was 136-fold higher ($\text{KDPG } 24.4 \text{ s}^{-1} \text{ mM}^{-1}$, $\text{KHG } 0.18 \text{ s}^{-1} \text{ mM}^{-1}$). However, EDA displayed no detectable activity with FBP (Fig. 6C), which is consistent with the fact that class I KDPG aldolases are members of a different enzyme class (EC 4.1.2.14). Likewise, class I and class II FBP aldolases (EC 4.1.2.13) are not known to exhibit activity toward KDPG (16, 30, 38).

Interestingly, EDA was able to condense GAP and pyruvate to KDPG *in vitro* with low efficiency. It remains to be investigated whether this reaction could play a role *in vivo*, with KDPG potentially acting as a regulatory metabolite at low concentrations. This could explain why KDPG was detected in WT and Δ pfk Δ zwf in one study (1), although this observation could not be replicated later (3). Based on the current data, it is not possible to determine whether the detected KDPG was due to a measurement artifact or not. The accumulation pattern of 6PG in WT, Δ eda, Δ gnd and Δ eda Δ gnd, which contradicted the flux analyses and had been interpreted as evidence of an active ED metabolic pathway in *Synechocystis* (3, 4), could be explained by a secondary mutation in the Δ gnd mutant (Fig. 4C) and now agrees with the flux analyses.

Our systematic re-examination of data on the proposed ED pathway in *Synechocystis* demonstrates how complementary approaches can contribute to elucidating cyanobacterial metabolism. By combining detailed biochemical enzyme characterization, genetic deletion and complementation, careful mutant validation, and taking into account metabolic flux analyses (3), we were able to clarify that neither EDD nor the GDH/GK bypass is present, and that the ED pathway is incomplete and not functional in *Synechocystis*. These integrated studies further revealed the presence of a promiscuous KDPG aldolase whose broader metabolic involvement now opens exciting new research directions.

This study also highlights the importance of best practices to strengthen metabolic pathway reconstruction and provides the following lessons: Although protein sequence alignments provide valuable information, they cannot replace detailed biochemical enzyme tests, which are required to verify the predicted enzyme functions and to further test for substrate promiscuity. Furthermore, to demonstrate the presence of a metabolic pathway, it is advisable to characterize all enzymes of this pathway biochemically, rather than limiting analysis to individual key

enzymes, and to also include flux analyses. Routine complementation of deletion mutants and sequencing of selected genes or the entire genome are effective means of identifying secondary mutations that can lead to misleading phenotypes (39). A final lesson is to exercise great caution when interpreting data and drawing conclusions, even if the data sets appear completely logical and conclusive at first glance (1). Over time, contradictory results have accumulated regarding the claim that there is an ED pathway in *Synechocystis*. In the present study, we have succeeded in clarifying a number of these contradictions. Taken together, these advances provide a solid foundation for future investigations into the *in vivo* role of the promiscuous aldolase EDA in cyanobacteria.

Materials and Methods

Growth experiments

Synechocystis sp PCC 6803 WT (glucose tolerant strain) and mutants (Table S2) were grown in 200 ml BG11 medium (pH 8) in custom-made glass tubes placed in a photobioreactor (manufactured by Willi Hilke, Uslar, Germany), at 28°C under constant light (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Standard BG11 medium contained: 17.6 mM NaNO_3 , 5 mM Tris, 0.304 mM MgSO_4 , 0.245 mM CaCl_2 , 0.189 mM NaCO_3 , 0.175 mM K_2HPO_4 , 46.3 μM H_3BO_3 , 31.2 μM citric acid, 22.8 μM FeNH_4 citrate, 9.1 μM MnCl_2 , 2.79 μM Na_2EDTA , 1.61 μM NaMoO_4 , 0.77 μM ZnSO_4 , 0.32 μM CuSO_4 , 0.17 μM $\text{Co}(\text{NO}_3)_2$ according to (40). FeNH_4 citrate was added after autoclaving. Cultures were gassed with filter-sterilized ambient air. Photomixotrophic cultures were supplemented with 10 mM D-glucose. Aliquots were taken under sterile conditions every 24 h to measure optical density photometrically at 750 nm.

Generation of mutants

Constructs for deletion mutants, complementation of Δhk and edaK147G were made by Gibson Assembly cloning (41), using the primers listed in Table S3. An antibiotic resistance cassette was placed in the gene to be deleted and was fused for homologous recombination to two DNA fragments approximately 200 bp directly up- and downstream of the gene and cloned into pBluescript. *Synechocystis* was transformed with these constructs to replace the gene with the antibiotic resistance cassette via homologous recombination. Mutants were checked via PCR for segregation.

Generation of a putative GDH1 (*sll1709*) His-tagged over-expression mutant (*sll1709:oe*) and overexpression of GDH homologues of *Spirulina* and *Lyngbya*

Two DNA fragments from the genome of *Synechocystis* were synthesized by Genescript: 1. a fragment containing 212 bp up- and 212 bp downstream of the *sll1709* start codon, with a *Bam*HI, *Xho*I and *Nde*I site in its middle and 20 bp of overlapping sequences with the pBluescript SK(+) vector at its ends (Fig. S5A). 2. a fragment containing a modified *petE* promoter, followed by His-tag, TEV cleavage recognition and linker encoding sequences, various restriction sites and 20 bp of overlapping sequences with the pBluescript SK(+) vector at its ends (Fig. S5B). Both fragments were separately cloned into the pBluescript SK(+) vector by Gibson Assembly cloning. A kanamycin antibiotic resistance cassette was inserted into the *Eco*RV site of the plasmid containing the modified *petE* promoter. Both plasmids were then digested with *Bam*HI and *Nde*I and the promoter cassette was ligated into the alkaline phosphatase treated GDH1 plasmid to yield the final mutagenesis vector. This plasmid was transformed into *Synechocystis* and mutant segregation was confirmed by PCR analysis. GDH homologues of *Spirulina* and *Lyngbya* were overexpressed in *E.coli* (Fig. S7-8).

P3-His-GDH1 (sll1709) over-expression and His-tag purification

For the purification of GDH1 from *Synechocystis*, a 6 L photoautotrophic culture of the P3-His-GDH1 overexpression strain was grown in a 10 L glass flask at 28°C, illuminated with constant light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$) and gassed with filter sterilized ambient air to an OD750 of about 1. Cells were harvested by centrifugation at a RCF (relative centrifugal force) of $3,992 \times g$ in a Beckman Coulter with a JLA-8.1000 Rotor for 20 minutes at 4°C. Initially, His-GDH1 over-expression in the 6 L culture was assessed by SDS PAGE analysis followed by immunoblotting with a His-tag specific antibody (Fig S6C) as described in (42). A specific band could be detected in the over-expression mutant, confirming expression and stable accumulation of the over-expressed and N-terminally His-tagged GDH1 protein. A small-scale purification (using a 50-mL aliquot of the large culture and treating this sample as described below for the remaining sample) demonstrated that the protein could be purified (Fig S6C). For the large-scale purification the remaining cells were resuspended in lysis buffer (50 mM NaPO₄ pH=7.0; 250 mM NaCl; 1 tablet complete protease inhibitor EDTA free (Roche) per 50 mL) and broken by passing them through a French Press cell at 1250 p.s.i. twice. Unbroken cells and membranes were removed by centrifugation in a Beckman ultracentrifuge using a 70 Ti rotor at 30.000 rpm for 45 min at 4°C. The decanted soluble extract was adjusted to a volume of 45 mL with lysis buffer and incubated with 5 mL TALON cobalt resin (Takara) for 1 h at 4°C. The resin was then washed extensively with 200 mL lysis buffer and subsequently with 100 mL lysis buffer containing 5 mM imidazole. Bound proteins were eluted with 10 mL elution buffer (50 mM NaPO₄ pH=7.0; 250 mM NaCl; 250 mM imidazole) and after dialysis overnight against the reaction buffer 50 mM Tris pH=7.5; 20 mM MgCl₂, the protein was concentrated in a Vivaspinn Turbo 4 Ultrafiltration Unit (5 kDa MWCO). Commercially available Glucose dehydrogenase from *Pseudomonas* sp. (19359, Sigma) was resuspended in the reaction buffer for control measurements. Finally, protein concentrations were determined by Bradford assay using the Roti-Quant reagent (Carl Roth) and adjusted to 0.25 mg/mL. Samples taken during the purification procedure were assessed by SDS PAGE analysis followed by immunoblotting with antibodies specific against the His-tag.

Preparation of crude cell extracts

Enzyme activity was measured in crude cells extracts. For the generation of crude cell extracts of *Synechocystis*, an OD volume of 30-50 OD*mL sample culture was pelleted by centrifugation (5 min, 9000 g, 4 °C) and the pellet resuspended in 0.5 mL 100 mM Tris buffer (pH 7.6). The suspension was then transferred to centrifugation tubes with screwed lids (max 500 µl/cup) and glass beads (0.17-0.18 mm diameter; Sartorius, Göttingen, Germany) were added so that 1-2 mm liquid remained above the dispersion. Samples were vortexed for 10 min at 4 °C. Afterwards, the glass beads were pelleted by centrifugation in two consecutive centrifugation steps (1 min, 1000 g, 4 °C and 10 min, 1600 g, 4 °C). The supernatant was transferred to new centrifugation cups in between centrifugations. In order to precipitate cell debris, the supernatant was centrifuged (15 min, 18000 g, 4 °C) one more time. The supernatant constituted the final cell extract and was measured for its protein concentration and kept on ice until further enzyme activity measurements.

Protein quantification

Protein concentrations were determined according to Bradford (43). 1 µL protein sample (cell extract) was mixed in triplicates with 799 µL water. Afterwards, 200 µL Bradford reagent, consisting of 100 mg/L Coomassie Serva Blue G-250 (Bio-Rad, Munich, Germany), 5 % (v/v) ethanol and 8.5 % (v/v) H₃PO₄ was added to all samples as well as to 800 µL bovine serum albumin (BSA) standards in concentrations of 2, 5, 10, 15 and 20 µg/mL. After an incubation at RT for 10 min, absorption at 595 nm was measured and sample protein concentration calculated by using the standard curve.

Glucose dehydrogenase activity measurements

For glucose dehydrogenase (GDH) activity measurements in *Synechocystis*, NADPH turnover was monitored photometrically at 340 nm. Measurements were carried out in 50 μ L crude cell extract or purified GDH in 100 mM Tris/HCl buffer (pH 7.4) in the presence of 20 mM glucose, 5 mM NAD(P)⁺ and 10 mM MgCl₂. Reactions were started by addition of glucose. The kinetics of the reaction were monitored with a double-beam spectrophotometer (Uvikon 810, Kontron, Augsburg, Germany) for 5-10 minutes. In case quinones were tested as electron acceptors, the GDH reaction was enzymatically coupled to the gluconate kinase (GK) and 6PG dehydrogenase (GND) reaction, the latter providing the required NADP⁺ turnover. This was achieved by adding 5 mM ATP, 0.02 U gluconate kinase from *E. coli* (Megazyme, Wicklow, Ireland) and 0.005 U 6-phosphogluconate dehydrogenase from *E. coli* (Megazyme, Wicklow, Ireland). The two tested quinones 2,3-dimethoxy-5-methyl-1,5-benzoquinone (DMB) and duroquinone (DQ) were supplied in a concentration of 5 mM. As a positive control, 0.05 U glucose dehydrogenase from *Pseudomonas sp.* (Sigma Aldrich, Steinheim, Germany) was added.

The GDH activity of recombinant enzymes from *Spirulina sp.* SIO3F2 and *Lyngbya aestuarii* was determined after GST (Glutathione S-Transferase) - His tag removal by TEV protease cleavage and purification. Activity was determined spectrophotometrically by monitoring glucose dependent reduction of NAD(P)⁺ to NAD(P)H at 340 nm and 30°C (extinction coefficient of NAD(P)H=6.22 mM⁻¹cm⁻¹). The standard assay mixture (final volume of 500 μ L) contained 0.1 mM Tris/HCl (pH 7.7 at 30°C), 10 mM MgCl₂, 5 mM NAD(P)⁺, 20 mM Glucose, and 2.5 – 5 μ g of recombinant protein. To determine the substrate specificity glucose was substituted by other sugars (for details see text).

Gluconate kinase activity measurements

For gluconate kinase (GK) activity measurements in *Synechocystis* crude cell extracts the GK reaction was enzymatically coupled to 6-phosphogluconate dehydrogenase (GND) reaction, the latter providing NADP⁺ reduction, which was monitored photometrically at 340 nm. Measurements were carried out in 50 μ L crude cell extract in 100 mM Tris buffer (pH 7.4) in the presence of 20 mM gluconate, 5 mM NADP⁺, 10 mM MgCl₂, 5 mM ATP and 0.005 U 6-phosphogluconate dehydrogenase. Reactions were started by addition of gluconate and kinetics were monitored with a double-beam spectrophotometer (Uvikon 810, Kontron, Augsburg, Germany) for 5-10 minutes. As a positive control, 0.05 U gluconate kinase from *E. coli* (Megazyme, Wicklow, Ireland) was added.

Recombinant GK activity in *E. coli* was determined spectrophotometrically in a continuous coupled pyruvate kinase/lactate dehydrogenase (PK/LDH) assay by monitoring NADH oxidation at 340 nm (30 °C) (extinction coefficient of NAD(P)H=6.22 mM⁻¹cm⁻¹). Gluconate phosphorylation was followed indirectly via ADP formation, which was converted by PK/LDH in a NADH-dependent reaction. The standard assay (500 μ L) contained 100 mM Tris/HCl (pH 7.7), 10 mM MgCl₂, 0.2 mM NADH, 1 mM PEP, 2 mM ATP, 1-10 mM gluconate, auxiliary enzymes PK and LDH (1U each), and 0.7-15 μ g purified recombinant enzyme (after GST-tag cleavage).

Glucokinase/hexokinase activity measurements

For glucokinase/hexokinase (GLK/HK) activity measurements, the GLK/HK reaction was enzymatically coupled to the glucose-6-phosphate dehydrogenase (ZWF) reaction, whose NADP⁺ turnover was monitored photometrically at 340 nm. Measurements were carried out in 50 μ L crude cell extract in 100 mM Tris buffer (pH 7.4) in the presence of 10 mM glucose or fructose, 5 mM NADP⁺, 10 mM MgCl₂, 5 mM ATP and 0.005 U 6-phosphogluconate dehydrogenase from *E. coli* (Megazyme, Wicklow, Ireland). Reactions were started by addition of glucose and kinetics were monitored with a double-beam spectrophotometer (Uvikon 810, Kontron, Augsburg, Germany) for 5-10 minutes.

Quantification of 6-phosphogluconate

Quantification of 6-phosphogluconate (6PG) was achieved photometrically by enzymatic conversion of sample 6PG to ribulose-5-phosphate and CO₂, which goes hand in hand with equimolar formation of NADPH. 5 to 50 OD*mL cell culture was pelleted by centrifugation (5 min, 9000 g, 4 °C) and cell lysis was achieved by solving the pellet in 1 mL 0.2 M HCl, followed by a 15 min incubation at 95 °C. The lysates were centrifuged (10 min, 18000 g, 4°C) and the supernatants transferred to new tubes where they were neutralized by addition of 1 mL 1 M Tris/HCl buffer (pH 8.0). Samples were then split into two (each sub-pool 900 µL), that were supplied with 90 µL of an 11.11 mM NADP⁺ solution. 10 µL of a 5 U/mL 6-PG dehydrogenase from (Megazyme, Wicklow, Ireland) solution was added to one of the samples, whereas 10 µL of water was added to the second sample as a control. All samples were then incubated at 37 °C for 30 min and the absorption at 340 nm was measured using a double-beam spectrophotometer (Uvikon 810, Kontron, Augsburg, Germany). 6PG standards were prepared (0 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM and 0.8 mM) and measured in the same way as samples. Δabsorption values at 340 nm of standards were used to create a standard curve, which was then used to convert sample Δabsorption values into 6PG concentrations. The resulting 6PG concentration (mM) was converted into specific cellular 6PG content (µg/OD*mL) by multiplying it with the molar mass and dividing it by the respective OD volume.

Quantification of glucose

For quantification of medium glucose concentrations, 1 mL *Synechocystis* culture was pelleted by centrifugation (5 min, 10000 g, RT) and 50 µL of supernatant were transferred to a new cup. If medium glucose concentration was expected to exceed 5 mM, samples were diluted. 50 µL glucose standards with the following concentrations were prepared: 0 mM, 0.625 mM, 1.25 mM, 2.5 mM and 5 mM. Furthermore, a 'NADP buffer', containing 150 mM NADP⁺, 150 mM ATP, 300 mM MgCl₂ in 100 mM Tris-HCl (pH 7.4,) was prepared. 940 µL 'NADP buffer' were added to all samples and standards, which were then transferred to acryl-cuvettes and absorption at 340 nm was measured in a double-beam spectrophotometer (baseline absorption) (Uvikon 810, Kontron, Augsburg, Germany). After that, 0.17 U Hexokinase as well as 0.085 U glucose-6-phosphate dehydrogenase were added to all samples and standards. Samples and standards were then incubated at 37 °C for 60 min to ensure complete enzymatic conversion. Finally, absorption at 340 nm was measured again (endpoint absorption). Baseline absorbance values were subtracted from endpoint readings to calculate ΔA₃₄₀. A glucose standard curve was generated from the standards and used to determine glucose concentrations in the culture supernatants.

Blast analysis

In order to search for the existence of a gluconate kinase in the genome of *Synechocystis* and cyanobacteria in general, the fasta protein sequences of the well characterized gluconate kinases from *Escherichia coli* (KIG95398.1) and from *Pseudomonas aeruginosa* (WP134279162.1) were used as baits for the protein/protein blast tool at NCBI. Similarly, the fasta sequences of the NAD⁺ dependent glucose dehydrogenase 1GCO from *Bacillus megaterium* and the quinone dependent glucose dehydrogenase 1C9U from *Acinetobacter calcoaceticus* were used as baits for the search of gluconate dehydrogenases in cyanobacteria. Next to *Synechocystis*, 261 cyanobacterial species were analyzed. Homology thresholds were set at a query score > 30 %, an identity score > 30 % and an e-value < 1⁻¹⁰.

SDS PAGE and immunoblot analyses

To assess the level of ZWF protein in various strains, soluble protein extracts were prepared and analysed by SDS-PAGE and immunoblotting. 5 µg of protein were loaded per lane on a 12.5% (w/v) polyacrylamide BisTris gel using MES running buffer (250 mM MES, 250 mM Tris, 5 mM EDTA, 0.5 % (w/v) SDS). Gels were Coomassie-stained or electroblotted onto nitrocellulose membrane. For immunoblotting, 5% (w/v) milk powder in 1x PBS-T was used as blocking solution and all washing steps were performed with 1x PBS-T. For detection a ZWF antibody (αZwf, Agrisera, Sweden, Phyto

AB, PHY5113A) in a 1:5k dilution in 1x PBS-T (phosphate-buffered saline with Tween-20) and a horseradish peroxidase-conjugated secondary antibody (anti-rabbit, 1:10k in 1x PBS-T Cytiva (phosphate-buffered saline with Tween 20, 0.1 µm sterile filterized), Thermo Fisher Scientific, Germany) were used. As a loading control an antibody against 30S ribosomal protein S1 (αRps, Agrisera, Sweden) was utilized.

Gene cloning and protein overexpression

Genes were codon-optimized for expression in *E. coli*. Codon-optimized genes from *Synechocystis* (*eda*, *sll0107*, longer *sll0107* variant, extended by 24 amino acids, *edd/dhad*, *slr0452*) and *Synechococcus elongatus* PCC 7942 (*dhad*, *syc0898_c*) and *Caulobacter crescentus* (*eda*, *ccna_0156* and *edd*, *ccna_02134*) for expression in *E. coli* were synthesized by BioCat GmbH (Germany). The *dhad* gene from *Sulfolobus solfataricus* (*sso3107*), encoding a DHAD with GAD activity was synthesized by Eurofins (Eurofins Genomics, Ebersberg, Germany). The *sll0107* and longer *sll0107* variant were inserted into pet15B vectors encoding an N-terminal 6xHis-tag, while *slr0452* and *syc0898_c* genes were inserted into pET24a vectors encoding a C-terminal 6xHis-tag. The *saci_0225* gene from *Sulfolobus acidocaldarius* (encoding KDPG aldolase) was cloned into the pET15b vector using *NdeI* and *BamHI* restriction sites. The primers used for cloning are listed in Table S3. Genes encoding *eda* (*ccna_01562*) and *edd* (*ccna_02134*) from *C. crescentus* were cloned into pET28b and pET15b using *NdeI/BamHI* and *NdeI/HindIII*, respectively. All constructs were verified by Sanger sequencing (LGC Genomics, Berlin, Germany). For protein expression, plasmids were transformed into *E. coli* Rosetta (DE3) (Agilent Technologies). Cultures were grown in Terrific Broth (TB; 22 g L⁻¹ yeast extract, 12 g L⁻¹ tryptone, 4 mL L⁻¹ glycerol) supplemented with the appropriate antibiotics (100 µg mL⁻¹ ampicillin or kanamycin, and 30 µg mL⁻¹ chloramphenicol) at 37 °C with shaking at 180 rpm. Protein expression was induced at an OD₆₀₀ of 0.6–0.8 by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), followed by incubation at 18 °C for 18–22 h. Cells were harvested by centrifugation (15 min, 8630 × g, 4 °C) and stored at -70 °C until further use.

EDA (KDPG aldolase) mutation at the active center

Lysine at position 147 in EDA (*Sll0107*) was substituted with glycine (K147G). Site-directed mutagenesis was performed as described (44). Q5® High-Fidelity DNA Polymerase and its recommended reaction buffer (NEB) were utilized for the amplification process. The primers used for mutagenesis are listed in Table S3, with the nucleotide substitutions introducing the K147G exchange underlined. As a template the codon-optimized *eda* gene from *Synechocystis* (*sll0107*) that was synthesized by BioCat GmbH (Germany), was utilized. Successful mutagenesis was confirmed by DNA sequencing (LGC genomics, Berlin, Germany). The resulting EDA variant was named EDAK147G.

Protein purification

Recombinant *Synechocystis* EDA (both versions), EDAK147G, and DHAD (*Slr0452*), recombinant *Caulobacter crescentus* CcEDA and CcEDD, *Sulfolobus acidocaldarius* *SaciKD(P)GA*, and *Sulfolobus solfataricus* *SsoDHAD* were purified by immobilized metal affinity chromatography (IMAC) followed by size-exclusion chromatography (SEC) for EDA and *Slr0452*. Frozen cell pellets (2.4 g wet weight) were resuspended in 50 mM HEPES–NaOH (pH 7.8, 30 °C), 300 mM NaCl at a ratio of 1 g wet cells per 3 mL buffer. For EDD/DHADs purification buffer was supplemented with 10 mM MgCl₂ and 1 mM DTT. Cells were disrupted by sonication (3 × 5 min; amplitude 50; cycle 0.5) using a UP200S homogenizer (Hielscher Ultrasonics, Brandenburg, Germany). Cell debris was removed by centrifugation (45 min, 21,130 × g, 4 °C). His-tagged proteins, except *Synechocystis* DHAD (*Slr0452*), were purified from the supernatant using Ni–TED columns (Macherey–Nagel, Düren, Germany) according to the manufacturer's protocol. *Synechocystis* DHAD (*Slr0452*) was purified on a self-packed Ni–NTA affinity column. Elution fractions containing the target protein were pooled and concentrated using Vivaspin 20 centrifugal concentrators (Sartorius Stedim Biotech; 30 kDa cutoff for EDD/DHAD and 3 kDa cutoff for EDA). Concentrated samples of *Synechocystis* EDA (6 mg

protein) were further purified by SEC on a HiLoad 16/600 Superdex 200 prep grade column (Cytiva, Marlborough, MA, USA) equilibrated with 50 mM HEPES–NaOH (pH 7.8, 30 °C), 300 mM NaCl and additionally contained 10 mM MgCl₂ and 1 mM DTT for DHADs purification. Protein-containing fractions (4.5 mg after SEC) were analyzed by SDS–PAGE and enzymatic activity assays. Purified proteins were stored at –70 °C in 25% (v/v) glycerol. Protein concentrations were determined by the Bradford assay (45) using bovine serum albumin (Merck, Darmstadt, Germany) as the standard.

Determination of the native molecular mass of EDA

Following IMAC purification, as stated above, the column was calibrated under identical buffer conditions using standard proteins from the LMW and HMW gel-filtration calibration kits (Cytiva, Marlborough, MA, USA): Aprotinin (6.5 kDa), Ribonuclease A (13.7 kDa), Ovalbumin (44 kDa), Aldolase (158 kDa), and Ferritin (440 kDa), with blue dextran to determine the column void volume. The native molecular mass of EDA was estimated from the resulting calibration curve.

In vitro EDA enzyme characterization

The catalytic activities of *EDA*, *EDAK147G*, *SaciKD(P)GA*, and *CcEDA* in the KDPG cleavage direction were determined using a continuous, coupled spectrophotometric assay. EDA catalyzes the cleavage of 2-keto-3-deoxy-6-phosphogluconate (KDPG) into pyruvate and glyceraldehyde 3-phosphate (GAP). Pyruvate formation was monitored indirectly by its reduction to lactate using lactate dehydrogenase (LDH; rabbit muscle, Merck, Darmstadt, Germany), coupled to the oxidation of NADH to NAD⁺. The decrease in absorbance at 340 nm was recorded in 96-well plates (BRANDplates®, BRAND, Wertheim, Germany) using a Tecan Infinite M200 plate reader (Tecan Group AG, Männedorf, Switzerland) at 30 °C. NADH concentration was quantified against a standard curve ranging from 0–0.7 mM.

For KDPG cleavage assays (200 µL final volume), reactions contained 0.1 M HEPES–NaOH (pH 7.8, 30 °C), 0.7 mM NADH, 2 mM KDPG, 2 U LDH, and the indicated enzyme (typically 1 µg EDA, *EDAK147G*, *SaciKD(P)GA*, or 0.06 µg *CcEDA*). Substrate specificity toward oxaloacetate (OAA) and 2-keto-4-hydroxyglutarate (KHG) was assayed under the same conditions as KDPG assay, as they all generate pyruvate as one of the two product, however, for the KHG assay, 6 µg of EDA was used.

FBP cleavage activity forming GAP and DHAP was assayed using a coupled system containing glycerol-3-phosphate dehydrogenase (G3PDH; rabbit muscle, Merck, Darmstadt, Germany), which converts the DHAP product to glycerol 3-phosphate coupled to the oxidation of NADH to NAD⁺. Reaction mixtures (200 µL) contained 0.1 M HEPES–NaOH (pH 7.8, 30 °C), 0.7 mM NADH, 5 mM FBP and 2 U G3PDH. FBP aldolase (from *Saccharomyces cerevisiae*; Merck, Darmstadt, Germany) served as the positive control. Reactions were initiated by addition of enzyme, and all measurements were performed in triplicate. Negative controls omitted either enzyme or substrate. To obtain the kinetic parameters (Table 2), a classical Michaelis-Menten rate equation was fit to the complete dataset using NonlinearModelFit function in Wolfram Mathematica v14.2. EDA activity in the reverse (condensation) direction—forming KDPG from pyruvate and D-GAP was quantified using the colorimetric thio-barbituric acid (TBA) assay as described (46). Reactions (1 mL) contained each 20 mM pyruvate and 10 mM D-GAP in 0.1 M HEPES–NaOH (pH 7.8, 30 °C) with 15 µg EDA or 3 µg *SaciKD(P)GA*. *SaciKD(P)GA* was assayed at 70°C. At two-minute intervals, 50 µL aliquots were quenched with 5 µL of 12% trichloroacetic acid. Absorbance of the TBA–KDPG adduct was measured at 549 nm ($\epsilon = 67.8 \text{ mM}^{-1} \text{ cm}^{-1}$), and *SaciKD(P)GA* served as a positive control. EDA activity in reverse direction – forming OAA was assayed using malate dehydrogenase (MDH, *S. cerevisiae*, Merck, Darmstadt, Germany) that forms malate from OAA by oxidation of NADPH to NADP⁺. EDA activity in reverse direction – forming KHG from pyruvate and glyoxylate was assayed by measuring the pyruvate consumption in a discontinuous assay. Pyruvate (5 mM) and glyoxylate (5 mM) were incubated with EDA (5 µg) in 1 mL of 0.1 M HEPES–NaOH buffer (pH 7.8) at 30 °C. At defined time points (0, 2, 4, 6, 8, 10, 20, and 30 min), 50 µL aliquots were withdrawn and quenched by the addition of 5 µL of 12% (v/v) TCA. Samples were immediately placed on ice and centrifuged at 8,130 × g for 10 min to remove precipitated protein. Supernatants (50 µL) were

diluted into 950 μL of 0.1 M HEPES–NaOH (pH 7.8). Pyruvate consumption was quantified by a lactate dehydrogenase (LDH)–coupled assay using 0.7 mM NADH and 1 U of LDH (rabbit muscle; Merck, Darmstadt, Germany). The decrease in NADH absorbance reflected the conversion of pyruvate to lactate by LDH and was used to compare pyruvate levels across time points. It was ensured that auxiliary enzymes were not rate limiting. One unit (1 U) of enzyme activity is defined as 1 μmol substrate consumed or product formed per minute.

To test whether EDA exhibits activity toward 2-keto-3-deoxygluconate (KDG), an additional continuous coupled assay was employed. Reaction mixtures (200 μL final volume) contained 0.1 M HEPES–NaOH (pH 7.8, at 30 $^{\circ}\text{C}$), 20 mM gluconate, 20 μg *Sulfolobus solfataricus* DHAD (SsoDHAD; SSO3107), 10 mM MgCl_2 , and 1 mM DTT. The mixtures were incubated with 20 μg of either SsoDHAD at 50 $^{\circ}\text{C}$ or Slr0452 at 30 $^{\circ}\text{C}$ for 2 h. Reactions were initiated by the addition of EDA or *Sulfolobus acidocaldarius* KD(P)GA (SaciKD(P)GA; Saci_0225) as positive control, which converts the reaction product 2-keto-3-deoxygluconate (KDG) into pyruvate and glyceraldehyde. Pyruvate formation was detected as described above for the KDPG cleavage assay. All assays were performed in triplicate. Negative control reactions were performed in the absence of either the enzyme or the substrate.

***In vitro* EDD enzyme assay**

EDD catalyzes the dehydration of 6PG to form KDPG in the classical Entner–Doudoroff pathway. Enzyme activity was monitored using a continuous, coupled spectrophotometric assay in which the KDPG product was subsequently cleaved by excess CcEDA to yield pyruvate and GAP. Pyruvate formation was detected as described above for the KDPG cleavage assay. NADH concentrations were quantified against a standard calibration curve (0–0.7 mM). Reaction mixtures (200 μL final volume) contained 0.1 M HEPES–NaOH (pH 7.8, 30 $^{\circ}\text{C}$), 0.7 mM NADH, 10 mM MgCl_2 , 5 mM 6PG, 5 μg CcEDA (recombinantly produced and characterized in this study, 150 U mg^{-1}), 2 U LDH, and the respective amounts of Slr0452 (20 μg) as the test sample and CcEDD (5 μg) as positive control (47). To test whether Slr0452 exhibits activity toward gluconate, as reported for the bifunctional EDD/DHAD from *Sulfolobus solfataricus* (19), an additional continuous coupled assay was employed. Reaction mixtures (200 μL final volume) contained 0.1 M HEPES–NaOH (pH 7.8, at 30 $^{\circ}\text{C}$), 0.7 mM NADH, 10 mM MgCl_2 , and 1 mM DTT. The mixtures were incubated with 20 μg of either *S. solfataricus* DHAD (SsoDHAD) at 50 $^{\circ}\text{C}$ or Slr0452 at 30 $^{\circ}\text{C}$. Reactions were initiated by the addition of *Sulfolobus acidocaldarius* KD(P)GA aldolase, which converts the reaction product 2-keto-3-deoxygluconate (KDG) into pyruvate and glyceraldehyde (20). Pyruvate formation was detected as described above for the KDPG cleavage assay. All assays were performed at least in triplicate. Negative controls omitted either the enzyme or substrate. It was ensured that auxiliary enzymes were not rate limiting.

***In vitro* DHAD enzyme assay**

Dihydroxyacid dehydratase (DHAD; *ilvD*) catalyzes a key step in the biosynthesis of branched-chain amino acids (BCAAs: leucine, isoleucine, and valine). The activities of Slr0452 and *S. elongatus* DHAD (SeDHAD) were assayed using the physiological substrate 2R-dihydroxyisovalerate (DHIV; Merck, Darmstadt, Germany), which is dehydrated to yield 2-ketoisovalerate (KIV). Product formation was quantified using a discontinuous colorimetric assay as described previously (17).

Reaction mixtures (100 μL) contained 50 mM Tris–HCl (pH 7.8), 1 mM dithiothreitol (DTT), 10 mM MgCl_2 , and 1 mM 2R-DHIV. Reactions were initiated by the addition of 0.25 μM enzyme, incubated aerobically at room temperature for 30 min, and terminated with 25 μL of 2 M HCl. The resulting KIV was derivatized by addition of 50 μL of saturated 2,4-dinitrophenylhydrazine (DNPH; Häberle, Germany) in 2 M HCl and incubated for 30 min at room temperature. Subsequently, 25 μL of 10 M NaOH were added, samples were mixed, and precipitates were removed by centrifugation (5,000 $\times g$, 10 min). Supernatants (100 μL) were transferred to clear 96-well microplates, and absorbance of

the KIV-DNPH adduct was measured at 540 nm. Quantification was based on a KIV-DNPH calibration curve (0–1 mM), which exhibited linear response across this range. All enzyme assays were performed in triplicate.

Bioinformatic analysis

Crystal structures were obtained from the Protein Data Bank (PDB), and structural models were retrieved from the AlphaFold Protein Structure Database (version 3.0) (26). Structural analyses and comparative assessments were performed using UCSF ChimeraX (48). Amino acid sequences for alignment were retrieved from UniProt, and multiple sequence alignments were generated using the EMBL-EBI Job Dispatcher framework (28), followed by manual refinement and editing in BioEdit.

Data availability

All study data are included in the article and SI Appendix. In addition, the kinetic data and model simulations are available as Excel files and Mathematica notebooks on the FAIRDOMHub (<https://fairdomhub.org/investigations/775>) and are publicly available.

Acknowledgements

This study was supported by grants from and the German Science Foundation (DFG Gu1522/2-2, Gu1522/5-1, SI 642/14-1, SI 642/14-2, FOR2816, GRK2749/1). We acknowledge financial assistance from the DSI/NRF in South Africa (grant NRF-SARCHI 82813 to J. L. S.). We thank Markus Brand, Frauke Caliebe, Friederike Hörsch, Carolin Kremer and Maria Patti for engaged discussions.

Additional information

Authors' contributions

RO, MT, RF, AM, MB, and CP, performed the experiments; MB, CB, JLS, BS, KG supervised, RO, MT, RF, AM, MB, CP, CB, JLS, MH, BS, and KG analyzed data; RO, AM, and KG wrote the original draft; all authors wrote, reviewed, and edited; BS, and KG conceptualized the study; BS and KG acquired funding.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the author(s) used ChatGPT (Open AI 2025), ChatGPT Team (5.1) and DeepL in order to optimize language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Funding

Funder	Grant reference number	Author
Deutsche Forschungsgemeinschaft (DFG)	SI 642/14-2	Ravi Shakar Ojha
Deutsche Forschungsgemeinschaft (DFG)	Gu1522/2-2	Marius Theune
Deutsche Forschungsgemeinschaft (DFG)	GRK2749/1	Ruben Fritsche
Deutsche Forschungsgemeinschaft (DFG)	Gu1522/5-1	Alexander Makowka
Deutsche Forschungsgemeinschaft (DFG)	SI 642/14-1	Carmen Peraglie
DSI/NRF in South Africa	NRF-SARCHI 82813	Jacky L Snoep
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Additional files

[Supplementary Information](#) 

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Peer reviews

Reviewer #1 (Public review):

Strengths:

Thorough reanalysis of the experimental results obtained in previous studies, which led to the publication of the PNAS paper in 2016.

New experimental evidence to confirm that enzymes previously considered as participating in the ED, actually are not catalyzing the ED biochemical reactions, but are involved in other metabolic pathways. Also, the authors completely discarded the occurrence of the GDH/GK shunt in *Synechocystis* PCC 6803. Generally speaking, the manuscript is very clearly written, with a precise description of the previous findings, the mistakes which took place in the 2016 paper, and the strategies they have used to address those issues, in order to reach a thoroughly revised vision of the glucose metabolic pathways in *Synechocystis* PCC 6803. In this regard, the drawings shown in Figures 1 and 7 are very helpful for the reader to follow the story and understand the possible metabolic transformations depending on the working hypothesis.

Also, I commend the authors for openly describing previous mistakes. In this paper, they reassess past observations under the light of more recent findings, and to integrate the information in this manuscript. The scientific conclusions are solid and very interesting, and besides they use the opportunity to offer valuable advice to researchers. This is especially focused on the importance of careful biochemical characterization of enzymes, which should always be carried out when studying proteins which have been identified as a specific enzyme on the basis of sequence homology. In a similar way, they found that an insertional mutant was the cause for the absence of specific metabolites, which had been attributed to particularities of a metabolic pathway in that mutant, when it was actually due to a nucleotide insertion; given that currently, genome sequencing is an affordable technique, this kind of mistakes can now be easily prevented by confirming the correct generation of the mutant by DNA sequencing, as proposed by the authors in a recently published preprint (Theune et al, bioRxiv 10.64898/2026.04.08.717167).

Weaknesses:

The authors propose that EDA might be involved in the PEP-pyruvate-OAA node, or in the proline metabolism, but this requires further experimental work for clarification; what their results indicate clearly is that this enzyme is not actually catalyzing the transformation of KDPG to GAP, which is the second specific enzyme of the ED pathway. But the real physiological function in this cyanobacterium is still unconfirmed.

Another aspect which could be improved is that the recombinant expression of some genes was carried out in *E. coli*; even if this is a useful and valid research strategy, in studies like this (where there is a strong focus on the physiological function of enzymes in the original organism, *Synechocystis* PCC 6803), I think it would have been more appropriate to express the 6803 genes in another cyanobacterium easily amenable for genetic transformation and gene expression, which would produce the protein in a physiological environment more similar to another cyanobacterium (compared to *E. coli*, which is an heterotrophic bacterium). I am not sure this would change any of the obtained results, but certainly would confer additional robustness to the enzymatic results.

Comments on revised version.

The authors have provided satisfactory replies to all my suggestions and corrections, and I have no further changes to suggest.

<https://doi.org/10.7554/eLife.111485.2.sa2>

Reviewer #2 (Public review):

Summary:

The study presents novel results on the presence of the Entner Doudoroff pathway in *Synechocystis* sp. PCC 6803. In contrast to an earlier study, compelling evidence is given that this strain lacks both an ED pathway and a glucose dehydrogenase/glucokinase bypass but contains a promiscuous aldolase, which also decarboxylates oxaloacetate and cleaves 2-keto-4-hydroxyglutarate (as it occurs in proline degradation). The study concludes with successfully reconciling data of different studies and with lessons learned from the previous misconception.

Strengths:

Solid biochemical data is presented to reconcile contradicting data of earlier studies and to serve as basis for disclosing possible functions of a promiscuous aldolase. Earlier misconceptions and lessons to be learned are well discussed.

Weaknesses:

The materials and methods section is rather lengthy, suffering from a lack of conciseness and repetitions, and nevertheless misses some specifications.

Comments on revised version.

The materials and methods section has been significantly improved. The revised manuscript is now recommended for publication as it is.

<https://doi.org/10.7554/eLife.111485.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

Summary:

*Some of the authors proposed in a PNAS paper in 2016 the occurrence of the EntnerDoudoroff (ED) pathway in cyanobacteria and plants, on the basis of several lines of biochemical and genetic evidence. However, more recent results indicated that one of the two specific enzymes of the ED pathway (EDD) is missing in *Synechocystis* PCC 6803. The authors carried out additional experiments, which demonstrated that EDD is missing, and one of the enzymes (ED aldolase) is a promiscuous enzyme which seems to be involved in proline metabolism and is not actually participating in the ED pathway as initially believed. The results described in this paper are strong evidence that this new interpretation is appropriate, and therefore, it corrects the previous proposal, providing an honest description of the reasons why the authors had reached the wrong conclusion about the existence of the ED pathway in cyanobacteria and plants.*

We thank Reviewer 1 for the summary and comments. Based on our finding that EDA is a promiscuous aldolase that, in addition to the cleavage of KDPG to GAP and pyruvate (a reaction of the ED pathway) catalyzes other reactions *in vitro*, we proposed potential *in vivo*

functions of EDA, including its involvement in proline metabolism. However, these assumptions require further experimental testing. We do not yet have definitive findings regarding the function of the promiscuous aldolase EDA in *Synechocystis* *in vivo*, but respective studies are currently underway.

Strengths:

Thorough reanalysis of the experimental results obtained in previous studies, which led to the publication of the PNAS paper in 2016.

*New experimental evidence to confirm that enzymes previously considered as participating in the ED actually are not catalyzing the ED biochemical reactions, but are involved in other metabolic pathways. Also, the authors completely discarded the occurrence of the GDH/GK shunt in *Synechocystis* PCC 6803. Generally speaking, the manuscript is very clearly written, with a precise description of the previous findings, the mistakes which took place in the 2016 paper, and the strategies they have used to address those issues, in order to reach a thoroughly revised vision of the glucose metabolic pathways in *Synechocystis* PCC 6803. In this regard, the drawings shown in Figures 1 and 7 are very helpful for the reader to follow the story and understand the possible metabolic transformations depending on the working hypothesis.*

Also, I commend the authors for openly describing previous mistakes. In this paper, they reassess past observations in light of more recent findings and to integrate the information in this manuscript. The scientific conclusions are solid and very interesting, and besides, they use the opportunity to offer valuable advice to researchers. This is especially focused on the importance of careful biochemical characterization of enzymes, which should always be carried out when studying proteins which have been identified as a specific enzyme on the basis of sequence homology. In a similar way, they found that an insertional mutant was the cause of the absence of specific metabolites, which had been attributed to particularities of a metabolic pathway in that mutant, when it was actually due to a nucleotide insertion; this could have been easily prevented by confirming the correct generation of the mutant by DNA sequencing.

We thank the reviewer for this kind comment. We agree that biochemical characterization of enzymes as well as DNA sequencing to check deletion mutants, are important and valuable tools. As outlined in the manuscript and additionally in more detail in a recently submitted article, which is available at bioRxiv (Theune et al. 2026, doi:

<https://doi.org/10.64898/2026.04.08.717167>) and is currently under review at PLOS One, we suggest that genome sequencing of deletion mutants in combination with complemented strains as controls are required to minimize the risk of misinterpretation based on secondary mutations (1). During the early stages of our research on the ED pathway, and later as well when we were already trying to resolve the conflicting results that had accumulated concerning the ED pathway, genome sequencing for *Synechocystis* mutants was not affordable as a routine procedure (2-4). Therefore, we could not have easily prevented this misconception based on this technique at that time. However, we strongly encourage genome sequencing of deletion mutants (in combination with complemented strains) as routine procedures these days (1).

Weaknesses:

The authors propose that EDA might be involved in the PEP-pyruvate-OAA node, or in the proline metabolism, but this requires further experimental work for clarification; what their results indicate clearly is that this enzyme is not actually catalyzing the transformation of KDPG to GAP, which is the second specific enzyme of the ED pathway. But the real physiological function in this cyanobacterium is still unconfirmed.

As stated above and in the manuscript, we agree that the *in vivo* role of EDA requires further experimental work which is in progress. However, our results demonstrate that EDA splits KDPG into GAP and pyruvate *in vitro*, but we assume that this reaction does not play a role *in vivo* due to the absence of its substrate.

Another aspect which could be improved is that the recombinant expression of some genes was carried out in E. coli; even if this is a useful and valid research strategy, in studies like this (where there is a strong focus on the physiological function of enzymes in the original organism, Synechocystis PCC 6803), I think it would have been more appropriate to express the 6803 genes in another cyanobacterium easily amenable for genetic transformation and gene expression, which would produce the protein in a physiological environment more similar to another cyanobacterium (compared to E. coli, which is an heterotrophic bacterium). I am not sure this would change any of the obtained results, but it certainly would confer additional robustness to the enzymatic results.

Synechocystis is easily amenable to genetic manipulation, and we agree that expression and purification of all enzymes from this host would have been ideal. However, the first characterization of *Synechocystis* EDA was performed with proteins that were purified from *Synechocystis* and showed activity on KDPG at comparable rates as proteins that were purified from *E. coli* in this study (2). Moreover, most biochemical characterizations of EDAs from archaea, bacteria and plants were performed after recombinant expression in *E. coli* and yielded highly active enzyme as in the case of *Synechocystis* in this study (5-7). Therefore, we currently have no reason to worry that the expression in *E. coli* might affect the enzymatic activity of EDA. The main reason for utilizing *E. coli* as an expression strain in this study was to gain higher yields of protein for in-depth analyses.

Bibliography:

I think the list of papers used in this manuscript is complete and up to date. However, I do miss recent papers which addressed one aspect that was proposed in the original 2016 PNAS paper: the authors wrote, "We therefore suggest that Prochlorococcus might oxidize glucose via the ED pathway under mixotrophic conditions, as shown for Synechocystis." Recent studies checked this hypothesis and have shown that the ED pathway seems to be also missing in Prochlorococcus and marine Synechococcus, and I think this manuscript is a good place to cite them, since these results are consistent with the findings of this paper.

We will include a references from Moreno-Cabezuelo et al. 2023 (DOI: 10.1128/spectrum.03275-22) in which the proteomes of three marine *Prochlorococcus* and three marine *Synechococcus* strains were investigated upon exposure to glucose (8). Protein levels of EDA were either downregulated or not affected while proteins involved in OPP pathway and CBB cycle were upregulated. The authors of this study conclude that this indicates that the latter processes rather than the ED pathway are involved in photomixotrophy in these strains. However, flux analyses are still missing.

Reviewer #2 (Public review):

Summary:

The study presents novel results on the presence of the Entner-Doudoroff pathway in Synechocystis sp. PCC 6803. In contrast to an earlier study, compelling evidence is given that this strain lacks both an ED pathway and a glucose dehydrogenase/glucokinase bypass but contains a promiscuous aldolase, which also decarboxylates oxaloacetate and cleaves 2-keto-4-hydroxyglutarate (as it occurs in proline degradation). The study

concludes with successfully reconciling data from different studies and with lessons learned from the previous misconception.

Strengths:

Solid biochemical data are presented to reconcile contradicting data of earlier studies and to serve as a basis for disclosing possible functions of a promiscuous aldolase. Earlier misconceptions and lessons to be learned are well discussed.

Weaknesses:

The materials and methods section is rather lengthy, suffering from a lack of conciseness and repetition, and nevertheless misses some specifications.

We thank Reviewer 2 for the kind summary and comments and will improve the materials and methods part accordingly in a revised version.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

Some additional aspects that could be improved:

(1) L182-184: Are there any known in vitro attempts to determine whether some DHADs can accept 6PG as substrate? If not, did the authors check this possibility in the lab?

To the best of our knowledge, as mentioned in the manuscript, some DHADs are tested for their activity towards gluconate and some other substrates but not 6PG. It was suggested that since gluconate is smaller it might fit in the catalytic site of DHADs normally occupied by DHIV (7). We discussed this point in the manuscript (Line 177-185).

In this study we tested the DHAD Slr0452 from *Synechocystis* and DHAD from *Synechococcus* with 6PG as substrate but the enzyme did not catalyze 6PG dehydration. The result for Slr0452 is shown in Figure S3.

*(2) L234-241: This paragraph shows how important it is to avoid relying only on sequence alignments to assign functions to proteins (and enzymes in particular). The authors stress this idea elsewhere in the paper, but I think it should receive even more attention in a manuscript like this. Physiological characterization of the protein function is paramount, especially in the case of enzymes. Also, the GDH1 overexpression mutant was done in *E. coli*; this adds an additional layer of uncertainty, since the protein processing in *E. coli* might not be entirely identical to that carried out in cyanobacteria, as I mentioned above. This, in turn, could be one of the reasons for not finding the expected enzymatic activity. This comment is also relevant for the results shown in Table 1 (page 12).*

As outlined following lines L234-241 we tested crude cell extracts from *Synechocystis* WT, a *Synechocystis* strain overexpressing putative GDH1 (Sl1709) and a Dzwf deletion mutant which can be assumed to upregulate a GDH/GK bypass if present in *Synechocystis* for GDH activity but did not find any. This strongly indicates that *sl1709* does not code for an active GDH in *Synechocystis*. We thereafter also overexpressed GDH1 in *E. coli* and again could not detect any GDH activity. In case of GDH1, enzyme activity was therefore tested both in *Synechocystis* and *E. coli* and yielded similar results.

*(3) L309-310: "we currently have no explanation for the gluconate that was detected in previous IC-ESI-MSMS measurements in *Synechocystis*". This is a serious issue, given how important this evidence was for the conclusions of the 2016 PNAS paper and the*

hypothesis of the ED pathway in cyanobacteria. I would suggest that the authors provide some possible explanations, even if it is based on studies from other teams.

It would be very speculative and therefore in our eyes not helpful to search for explanations in this case as indeed the measurements were done in another lab. One explanation might be that 6P gluconate got dephosphorylated and yielded gluconate as an artifact. However, as we have no experimental validation for this idea and furthermore cannot test it. We therefore prefer to not further comment on this aspect.

(4) L347: The presence of an insertion in the sequence of zwf in the Δgnd mutant is a welcome explanation for the observed results: abolition of 6PG production in this mutant. This is another important message to stress in this manuscript: construction of mutants should always be double checked by DNA sequencing of the relevant genomic regions, to ensure that these kinds of side problems do not appear, leading to confusing results.

We agree with the reviewer and would get even one step further and rather suggest combining whole-genome sequencing with complemented mutants as it is difficult to know all relevant genomic regions that should be sequenced. We discuss this issue in even more detail in another manuscript that is currently available as online preprint in bioRxiv and is under review (9). This work was now added as a citation in line 566 and the end of the following statement (line 564-566): Routine complementation of deletion mutants and sequencing of selected genes or the entire genome are effective means of identifying secondary mutations that can lead to misleading phenotypes (9).

(5) L418-419 and Table 2: The results observed for the authors (i.e., that EDA could also catalyze reactions with OAA and KHG, albeit with substantially lower catalytic efficiency than with KDPG), is a matter for concern: if their hypothesis is correct (meaning that this EDA is fundamentally involved in the PEP-pyruvate-OAA node and/or proline metabolism, and not with the ED pathway), then why should it keep in the evolution of these organisms such a strong preference for KDPG, when it is not being used physiologically for the ED pathway)? Furthermore, the Km for KDPG is lower than for OAA or KHG, leading to a very big difference in the Kcat/Km values.

To solve these questions further respective studies are underway. As EDD is absent from *Synechocystis* no KDPG should be available in the cells so that catalytic activity on KDPG should be irrelevant *in vivo*. The *in vivo* role of Eda requires further clarification.

Hereafter, I will mention some aspects, following the instructions of eLife, which are related to suggestions for improved experiments/data/analyses, improvements of writing and presentation, and minor corrections to text/figures.

(1) L81: I would modify the text to "Accordingly, this raises further questions..."

Thanks for this hint. We modified the text accordingly.

(2) L189: Add "pages" after "following".

Thanks for pointing this out. We replaced "following" by "below".

(3) Page 7: The whole beginning of the Results section is actually more discussion than description of results, and the first mention of figures appears in L207 of page 8. Given the content of the paper, I think the authors might reconsider using "Results and Discussion" rather than different, specific sections for Results and Discussion. This is one of the papers where I think the combined use of both makes sense and will allow an easier understanding of the message.

We thank the reviewer for this valuable suggestion and changed the heading to Results and Discussion.

(4) L181: Add reference regarding the IlvD/EDD superfamily.

We added the following references in lines 172-176 and 185-189:

- (1) Melse, O., Sutiono, S., Haslbeck, M., Schenk, G., Antes, I., & Sieber, V. (2022). Structure Guided Modulation of the Catalytic Properties of [2Fe- 2S]-Dependent Dehydratases. *ChemBioChem*, 23(10), e202200088.
- (2) Ren, Y., Vettenranta, E., Penttinen, L., Jänis, J., Rouvinen, J., & Hakulinen, N. (2025). The engineered dimer of L-arabinonate dehydratase from *Rhizobium leguminosarum* bv. *trifolii*: The role of intersubunit interactions in IlvD/EDD family. *Biochemical and Biophysical Research Communications*, 757, 151610.
- (3) Ahmed, H., Ettema, T. J., Tjaden, B., Geerling, A. C., Van Der Oost, J., & Siebers, B. (2005). The semi-phosphorylative Entner–Doudoroff pathway in hyperthermophilic archaea: a reevaluation. *Biochemical Journal*, 390(2), 529-540. ff
- (4) Bräsen, C., Esser, D., Rauch, B., & Siebers, B. (2014). Carbohydrate metabolism in Archaea: current insights into unusual enzymes and pathways and their regulation. *Microbiology and Molecular Biology Reviews*, 78(1), 89-175.

(5) Figure 2: The data shown in column plots in Fig 2A, B and C, and 4B, could be presented in tables, which would save space while providing the same information: basically, very little/no activity in some cases vs high levels of activity in others.

We would like to keep the column plots as we still think that they visualize our data well.

(6) L255 "Unfortunately, we were not able to overexpress putative GDH2". It would be interesting to give more details about the possible reasons for this fact.

We tested different growth conditions for recombinant GDH2 expression. The expression culture was incubated at 37 °C for 3 hours as well as overnight at 18 °C for overnight after induction. Both experiments did not resolve the expression problem.

(7) L409-410: I think this sentence should include a brief part explaining the kind of assay used to test this activity.

We added now that the LDH-coupled continuous assay was used (see line 403).

(8) Figure 6E: Please give the specific activity in U/mg, as in Fig 6F, instead of percents.

100% is given in U/mg units in the figure legend as “control without effector (100 %; specific activity of 4.3 U/mg)”. For easy comparison of effectors, the relative activity (%) is often used. We would therefore prefer to keep the current data presentation.

(9) L576: Provide the origin of the utilized PCC 6803 strain, given there is a certain level of variability in this strain (glucose tolerance, etc). Also, even if there are some methods which are very widely used, I think the Materials and Methods section should either properly describe them or else cite the source. For instance, BG11 medium is mentioned, but no further information is given.

We included the information that the glucose-tolerant *Synechocystis* strain was utilized and added the receipt of and a citation for BG11 medium (10).

(10) L582 *Generation of mutants: This section mentions the Gibson Assembly cloning method, but I miss further information to allow the reader to reproduce the methodology with as many details as possible, or at least cite papers which do so.*

We added a reference in which Gibson Assembly is described (11). Together with the primers listed in Table S3 the generation of mutants is now reproducible.

(11) L609 *Please give information in g, not rpm, for centrifugation. Also, mention the model and brand of the centrifuge and rotors used. Also, immunoblotting is very loosely described. This is also valid for other sections, for instance, L618, L636.*

We now added the following information: Cells were harvested by centrifugation at an RCF (relative centrifugal force) of 3,992 x g in a Beckman Coulter with a JLA-8.1000 Rotor for 20 minutes at 4°C. We now added a reference (12) in which immunoblotting is described in more detail.

(12) L613: *Describe the "small scale purification".*

We now added the information that the small-scale purification was performed using a 50-ml aliquot of the large culture which was treated as described below for the remaining sample.

(13) L619-620: *Describe the composition of the lysis buffer.*

The composition of the lysis buffer is already described as follows: lysis buffer (50 mM NaPO₄ pH=7.0; 250 mM NaCl; 1 tablet complete protease inhibitor EDTA-free

(Roche) per 50 mL)

(14) L691: *Specify which amounts of auxiliary enzymes in coupled enzymatic assays were used.*

Thanks for pointing this out. We have now integrated the information that 1U of each of the auxiliary enzymes was utilized in the coupled enzymatic assays.

(15) L716: *The authors mention several times using a "double beam spectrophotometer". Please provide the model and brand.*

Model and brand were now added for the double-beam spectrophotometer (Uvikon 810, Kontron, Augsburg, Germany).

(16) L717 and 718: *define "Δabsorption".*

In line 715, the information is given that absorption was measured at 340 nm, "Δabsorption" is accordingly the Δabsorption at 340 nm. This information was added.

(17) L723: *"Synechocystis" should be in italics.*

Synechocystis is now written italics.

(18) L749-759: *This section should be described in more detail: preparation of protein extracts, SDS, immunoblotting, etc, or cite references of the same team where these methods were properly described.*

In this section the listed methods are already described in detail.

(19) 798-799: *"frozen cell pellets". Please provide numbers to specify the amount of material used.*

Thanks for pointing this out. We now added the information that frozen cell pellets with a wet weight of 2.4 g wet weight were resuspended.

(20) L871-872: *"It was ensured that auxiliary enzymes were not rate-limiting. One unit (1 U) of enzyme activity is defined as 1 μ mol substrate consumed or product formed per minute" is repeated several times in the manuscript (L 907-909, L936-938). I would advise using it the first time, and on other occasions, refer to the same conditions as described above.*

We have accordingly circumvented the repetition of 1 U definition from the manuscript.

Reviewer #2 (Recommendations for the authors):

(1) *Interpunctuation, especially comma placement, should be improved.*

We improved interpunctuation, especially comma placement to the best of our knowledge.

(2) *Line 63: delete the first "which".*

"Which" was deleted.

(3) *Lines 81/82: revise sentence.*

We revised the sentence to: Accordingly, this raises further questions about the presence of the ED pathway in cyanobacteria and plants.

(4) *Line 164: "presumed" instead of "presumes".*

The word was changed accordingly.

(5) *Line 228: by others? especially in reference 1?*

We deleted by others as the reference is given.

(6) *Line 240: "or" instead of "no".*

"no" was replaced by "or"

(7) *Figure 2: The axes are not well visible, and the explanation for the positive control in panel B is missing in the legend.*

Axes from figures 2, 3 and 4 were enlarged. For Figure 2B the following information was added in the legend: As a positive control, 0.05 U glucose dehydrogenase from *Pseudomonas* sp. was added to Δzwf cultures and to purified putative GDH1 (Sll1709). Axes from figures 2, 3 and 4 were enlarged.

(8) *The investigation on the general absence/presence of the GDH/GK bypass in cyanobacteria may not be necessary for this study.*

We included this data in this manuscript as the mistaken assumption that the GDH/GK bypass exist in *Synechocystis* lead among other observations to the misinterpretation of an existing ED pathway in *Synechocystis*. We would therefore prefer to keep these data in the manuscript.

(9) *Line 316: delete "on".*

"on" was deleted.

(10) *Line 352: delete "or".*

“or” was deleted.

(11) Lines 352/353: ZWF expression level appears to be reduced accordingly. This should be stated.

We agree that Zwf expression might be lower, however, we are not entirely sure if this is truly valid and would rather test this assumption further as described in the following lines.

(12) Figure 4: The axes are not well visible.

Axes from figures 2, 3 and 4 were enlarged.

(13) Line 363: values are not only normalized to protein content, but also to activity found for the WT.

We now added: The values are normalized to Zwf enzyme activity found in the WT based on protein content.

(14) Line 408: no separate subsection required.

The title for a new subsection was deleted.

(15) Lines 437-439: These are results descriptions, which should not be placed in the legend, but in the main text, as is partially done.

We deleted these result descriptions in the legend.

(16) Lines 441/442: formatting: one or no bracket pair.

The brackets were corrected.

(17) Lines 443/444: refer to Table 2 instead of giving the values in the legend to avoid duplication.

We deleted the values and now refer to Table 2.

(18) Line 503: delete “identified”.

We deleted the second “identified” in the sentence and changed the wording to: Apart from four identified cyanobacteria that possess potential EDDs. In addition, we also added the names of the four cyanobacteria that were found including the sequence IDs of the putative EDDs.

(19) Lines 529/530: revise sentence and format.

We added one sentence and revised the following sentence: In contrast to *Synechocystis* EDA, EDA from *Synechococcus* prefers OAA over KDPG. The catalytic efficiency of *Synechococcus* EDA on oxaloacetate is rather low ($\text{OAA } 0.437 \text{ s}^{-1} \text{ mM}^{-1}$), however, its activity can be enhanced by NADP^+ (13).

(20) Line 542: revise sentence.

We revised the sentence to: It remains to be investigated whether this reaction could play a role *in vivo*, with KDPG potentially acting as a regulatory metabolite at low concentrations.

(21) Line 577: The glass tubes used for cultivation should be specified.

We now added the following information: Custom-made glass tubes were placed in a photobioreactor (manufactured by Willi Hilke, Uslar, Germany).

(22) Lines 584-585: unclear, was the resistance cassette not placed in the gene to be deleted?

Yes, the resistance cassette was placed in the gene to be deleted and was fused for homologous recombination to two DNA fragments approximately 200 bp directly upstream and downstream of the gene. This information was now added.

(23) Line 607: cultivation equipment to be specified.

The following information was now added: For the purification of GDH1 from *Synechocystis*, a 6 L photoautotrophic culture of the P3-His-GDH1 overexpression strain was grown in a 10 L glass flask at 28°C, illuminated with constant light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$) and gassed with filter sterilized ambient air to an OD_{750} of about 1.

(24) Line 670: GTS should be specified.

Thank you for this hint. This was a typo. GST was meant not GTS. This was now corrected and GST was specified as Glutathione S-Transferase.

(25) Line 679: delete "gluconate kinase (GK) and".

The second gluconate kinase (GK) was deleted and sentence was revised to:

For gluconate kinase (GK) activity measurements in *Synechocystis* crude cell extracts the GK reaction was enzymatically coupled to 6-phosphogluconate dehydrogenase (GND) reaction, the latter providing NADP^+ reduction, which was monitored photometrically at 340 nm.

(26) Line 686: again GK activity? Difference unclear. Was the previously described procedure for GND activity determination?

GK activity measurements in *Synechocystis* crude cell extracts and GK activity measurements with recombinant enzyme that was expressed in *E. coli* were done in two different labs with different protocols. Therefore, the first description refers to measurements with *Synechocystis* while the second measurement refers to measurements with *E. coli*. This is now specified more clearly.

(27) Type/supplier of spectrophotometers and centrifuges used should be given.

Model and brand were now added for the double-beam spectrophotometer (Uvikon 810, Kontron, Augsburg, Germany). As this study was performed in two different labs over a period of 8 years including one lab moving to a new location, it is now impossible to specify all centrifuges that were utilized. Even though we agree that it would be good to provide this information, we now would have difficulties to be specific.

(28) Consider the referencing of published methods to streamline the materials and methods section.

We now streamlined the materials and methods section by deleting repetitions as outlined below. However, as protein expression, protein purification and enzymatic tests were performed in different labs, in some cases several protocols are given.

(29) Line 757: give specifics of anti-rabbit antibody and define PBS-T and PBS-T Cytiva.

Specifics were added to the text.

(30) Lines 761ff: It is not given for all genes used how they were derived. All synthesized?

We now added detailed information for all genes.

| (31) Line 762: codon-optimized for? *E. coli*?

The information was added that genes that were expressed in *E. coli* were codon-optimized for *E. coli*.

| (32) Lines 782-786: *Rationals for experimental strategies do not belong to materials and methods sections, but to results sections.*

The part was deleted in the materials and methods section and transferred to the results section.

| (33) Lines 818/819: *repetitive.*

We removed the repetition and refer to the purification method as stated above in the materials and methods section.

| (34) Lines 847-851: *True for all EDA-type assays? Kinetic parameters are shown in Table 2 rather than Table 1.*

Yes, true for all EDA-type assays. We changed the Table number to 2.

| (35) Line 863: delete "in".

"in" was deleted

| (36) Lines 888-893: *sounds repetitive.*

The lines were modified accordingly.

| (37) Lines 908/909: *repetitive.*

The repetitive comment on the definition of 1U was deleted.

| (38) Lines 928-938: *repetition*

The repetition was deleted.

References

- (1) M. Theune et al., Easy-to-use whole-genome sequencing workflows and standardized practices to uncover hidden genetic variation in *Synechocystis* PCC 6803 wild-type and knock-out strains. *bioRxiv* 10.64898/2026.04.08.717167, 2026.2004.2008.717167 (2026).
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- (10) R. Y. Stanier, R. Kunisawa, M. Mandel, G. Cohen-Bazire, Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriol Rev* 35, 171-205 (1971).
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- (13) N. Xie, C. Sharma, K. Rusche, X. Wang, Phosphoketolase and KDPG aldolase metabolisms modulate photosynthetic carbon yield in cyanobacteria. *The Plant cell* 10.1093/plcell/koae291 (2024).

<https://doi.org/10.7554/eLife.111485.2.sa0>