# The Entner–Doudoroff pathway is an overlooked glycolytic route in cyanobacteria and plants

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**Glucose degradation pathways are central for energy and carbon metabolism throughout all domains of life. They provide ATP, NAD(P)H, and biosynthetic precursors for amino acids, nucleotides, and fatty acids. It is general knowledge that cyanobacteria and plants oxidize carbohydrates via glycolysis [the Embden–Meyerhof–Parnas (EMP) pathway] and the oxidative pentose phosphate (OPP) pathway. However, we found that both possess a third, previously overlooked pathway of glucose breakdown: the Entner–Doudoroff (ED) pathway. Its key enzyme, 2-keto-3-deoxygluconate-6-phosphate (KDPG) aldolase, is widespread in cyanobacteria, moss, fern, algae, and plants and is even more common among cyanobacteria than phosphofructokinase (PFK), the key enzyme of the EMP pathway. Active KDPG aldolases from the cyanobacterium *Synechocystis* and the plant barley (*Hordeum vulgare*) were biochemically characterized in vitro. KDPG, a metabolite unique to the ED pathway, was detected in both in vivo, indicating an active ED pathway. Phylogenetic analyses revealed that photosynthetic eukaryotes acquired KDPG aldolase from the cyanobacterial ancestors of plastids via endosymbiotic gene transfer. Several *Synechocystis* mutants in which key enzymes of all three glucose degradation pathways were knocked out indicate that the ED pathway is physiologically significant, especially under mixotrophic conditions (light and glucose) and under autotrophic conditions in a day/night cycle, which is probably the most common condition encountered in nature. The ED pathway has lower protein costs and ATP yields than the EMP pathway, in line with the observation that oxygenic photosynthesizers are nutrient-limited, rather than ATP-limited. Furthermore, the ED pathway does not generate futile cycles in organisms that fix $CO_2$ via the Calvin–Benson cycle.**

glucose degradation | Entner–Doudoroff-pathway | Embden–Meyerhof–Parnas pathway | oxidative pentose phosphate pathway | endosymbiotic gene transfer

The breakdown of glucose is central for energy and biosynthetic metabolism throughout all domains of life. The Embden–Meyerhof–Parnas (EMP) pathway (glycolysis) and the oxidative pentose phosphate (OPP) pathway are the backbones of eukaryotic carbon and energy metabolism (1, 2). They generate ATP, NAD(P)H, and biosynthetic precursors for amino acids, nucleotides, and fatty acids. Prokaryotes, in contrast, exhibit a broad diversity in sugar oxidation pathways (3–5). These routes differ in ATP yield, in the enzymes and cofactors involved, and in the chemical intermediates of the pathways. The most common glycolytic routes in prokaryotes are the EMP, ED, and OPP pathways (Fig. 1). The key enzyme unique to the ED pathway is 2-keto-3-deoxygluconate-6-phosphate (KDPG) aldolase (Eda), whereas phosphofructokinase (PFK) is unique to the EMP pathway in the catabolic direction (3, 6). KDPG as a metabolite is exclusively found in the ED pathway (Fig. 1). The first two steps of the OPP pathway are catalyzed by glucose 6-phosphate-dehydrogenase (Zwf) and 6-phosphogluconate dehydrogenase (Gnd). As the pentose phosphate pathway can either run in its oxidative mode (OPP pathway) to oxidize carbohydrates or in its reductive mode (Calvin–Benson cycle) to fix $CO_2$, it is tightly regulated. Zwf plays a central role in the fine-tuning of the OPP pathway and the Calvin–Benson cycle to prevent futile cycles (7–9). However, Zwf is not unique to the OPP pathway, as its product 6-P gluconate can also be further metabolized in the ED pathway. Cyanobacteria and plants are known to oxidize carbohydrates via either the EMP pathway or the OPP pathway (Fig. 1) (10–12).

## Growth Behavior of Mutants Missing Selected Enzymes of Known Glycolytic Routes in *Synechocystis*

The cyanobacterium *Synechocystis* sp. PCC 6803 is able to assimilate glucose from its environment and to switch from autotrophy to mixotrophy, thereby significantly enhancing its growth (Fig. S1). To evaluate the physiological significance of known glycolytic routes in cyanobacteria, we deleted the genes for both isoforms of *pfk* - *pfkB1* and *pfkB2* - and *zwf* from the genome of *Synechocystis* (Fig. S2*A*). For clarity, the mutant *ΔpfkB1ΔpfkB2* will be named *Δpfk* throughout this report. The *Δpfk* and *Δzwf* mutants and the *ΔpfkΔzwf* mutant exhibited enhanced growth under mixotrophic (light and glucose) compared with autotrophic (light) conditions (Fig. 2*A*), despite lacking essential enzymes for both the EMP pathway and the OPP pathway, indicating that this cyanobacterium has an additional route of glucose degradation. *Synechocystis* is thought to phosphorylate glucose via glucokinase to glucose-6P, which is then further oxidized either via the EMP pathway or the OPP pathway (Fig. 1) (13). We wondered whether the *ΔpfkΔzwf* mutant might bypass glucokinase via a concerted action of a glucose dehydrogenase (Gdh) and a gluconate kinase to form 6P-gluconate, which could then be further metabolized via Gnd in the OPP pathway (Figs. 1 and 2*B*). We found a *gdh* candidate

### Significance

**Life on Earth is substantially driven by a circuit of photosynthesis and glucose oxidation. Photosynthesizers capture sunlight and store its energy in the bonds of carbohydrates. Oxidation of carbohydrates provides organisms with a source of ATP and organic carbon for the synthesis of cellular building blocks. Our data provide strong evidence that the Entner–Doudoroff pathway of glucose degradation, which has been previously long overlooked, operates in cyanobacteria and plants. Phylogenetic analyses reveal that the cyanobacterial ancestor of plastids transferred this glycolytic route, via endosymbiotic gene transfer, to the plant lineage.**

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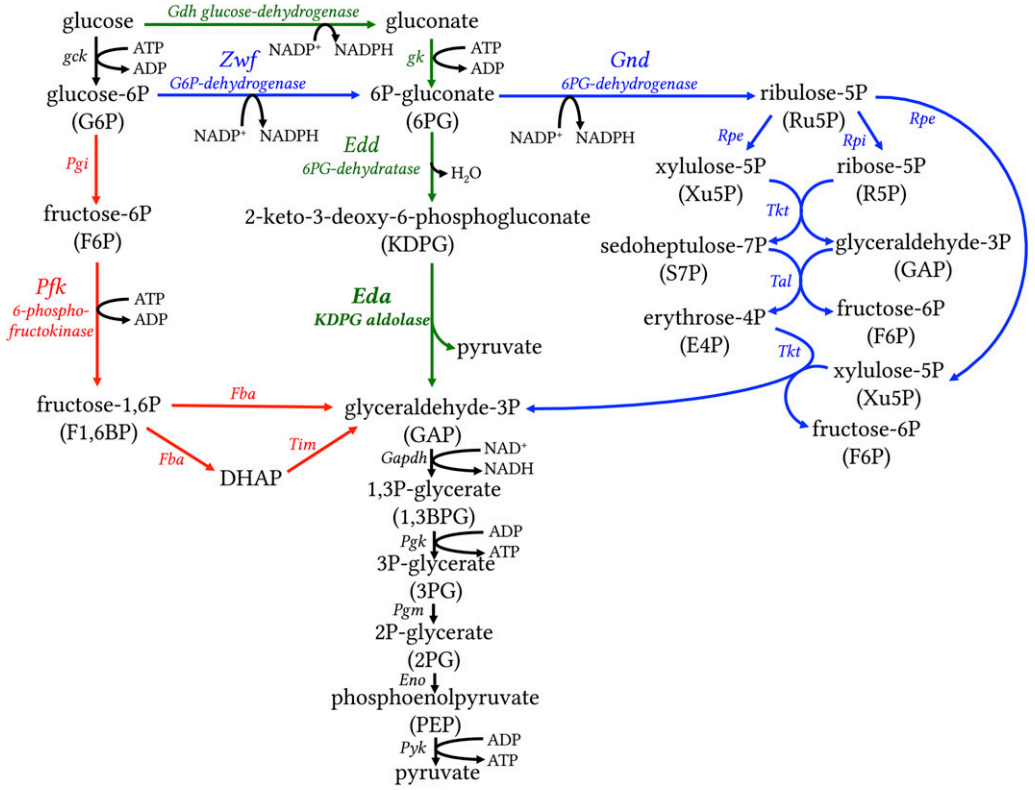


**Fig. 1.** The most common glycolytic routes in prokaryotes. EMP, often simply referred to as glycolysis, is shown in red with key enzyme PFK; the OPP pathway is shown in blue with enzyme Zwf; and the ED pathway is shown in green with key enzyme KDPG aldolase (Eda). Reactions that are shared by all pathways are shown in black. The EMP and OPP pathways are known to operate in cyanobacteria and plants. We show that in addition, the ED pathway operates in cyanobacteria and plants. Eda, KDPG aldolase; Edd, phosphogluconate dehydratase; Eno, enolase; Fba, fructose bisphosphate aldolase; Gdh, glucose dehydrogenase; Gk, gluconate kinase; Gnd, 6-phosphogluconate dehydrogenase; Pfk, 6-phosphofructokinase; Pgi, phosphoglucose isomerase; Pgk, phosphoglycerate kinase; Rpe, ribulose-5-phosphate epimerase; Rpi, ribulose-5-phosphate isomerase; Tal, transaldolase; Tim, triosephosphate isomerase; Tkt, transketolase; Zwf, glucose-6-phosphate dehydrogenase.

(*sll1709*) in the *Synechocystis* genome (Fig. S3), which we deleted, resulting in the mutants *Δgdh* and *gdh/ΔgdhΔpfkΔzwf*. Whereas the putative *gdh* could be completely deleted from the WT, this was not possible in *ΔpfkΔzwf*, indicating that the gene is essential in this mutant (Fig. S2 *B* and *C*). *Δgdh* had an aberrant growth phenotype under mixotrophic conditions, and *gdh/ΔgdhΔpfkΔzwf* could no longer enhance its growth under mixotrophic conditions (Fig. 2*B*). To test whether 6P-gluconate is metabolized in the OPP pathway via Gnd, *gnd* was deleted from *ΔpfkΔzwf* to interrupt the assumed bypass at a later step in the reaction sequence (Fig. S2*D*). However, *ΔgndΔpfkΔzwf* grew under autotrophic conditions and still showed enhanced growth under mixotrophic conditions (Fig. 2*C*), strongly suggesting that *Synechocystis* possesses an additional, thus far unrecognized, pathway of glucose degradation.

## Evidence for an Operating ED Pathway in the Cyanobacterium *Synechocystis*

A search of the *Synechocystis* genome for genes of the ED pathway uncovered candidates for 6PG-dehydratase (*edd, slr0452*) and the key enzyme KDPG aldolase (*eda, sll0107*) (Table S1).

The candidate KDPG aldolase (Sll0107) of *Synechocystis* was tested for enzyme activity in cell-free homogenates. *sll0107* was cloned for overexpression in *Synechocystis* into p2M2-Pcpc560ter (14). KDPG aldolase activity was not detected in cell-free homogenates of the *Δeda* mutant but was measured in cell-free homogenates of *Δeda/cpc::eda*$^{Synechocystis}$, in which Sll0107 was overexpressed. In addition, Sll0107 was purified with a his-tag via a Ni-NTA column. Both enzyme assays showed the same biochemical characteristics. A $K_M$ value of 0.095 mM for the KDPG aldolase of *Synechocystis* was obtained (Fig. 3*A* and Fig. S4*A*). No activity was measurable when 2-keto-3-deoxygluconate was tested as a substrate in the same concentration range. This establishes that Sll0107 does not operate in a nonphosphorylating ED pathway (15) but is a functional KDPG aldolase of the phosphorylating ED pathway.

Deletion mutants for *sll0107* were constructed. To determine whether the *ΔgndΔpfkΔzwf* mutant bypasses the EMP and OPP pathway via the ED pathway, the KDPG aldolase (*eda*) was deleted both in this mutant and in the WT, resulting in *ΔedaΔgndΔpfkΔzwf* and *Δeda* (Fig. S2*E*). *Δeda* was impaired in its growth under mixotrophic conditions, but more important, *ΔedaΔgndΔpfkΔzwf* showed no enhanced growth under mixotrophic conditions (Fig. 2*D*). In contrast to *eda* (*sll0107*), the gene *edd* (*slr0452*) could not be completely deleted from all genome copies of *Synechocystis* (Fig. S2*F*). We hypothesize that the encoded enzyme might be involved in the synthesis of the branched chain amino acids valine and isoleucine, as well as the conversion of gluconate to KDPG, as it was shown for the dihydroxyacid dehydratase of the archaeon *Sulfolobus solfataricus* (16). However, the merodiploid mutant *edd/Δedd* had a similar growth phenotype as *Δeda* (Fig. 2*E*). Furthermore, gluconate, the metabolite of the assumed bypass via Gdh and gluconate kinase, was present in WT and *ΔpfkΔzwf*. Most important, KDPG, the metabolite unique to the ED pathway, could be detected in the WT and *ΔpfkΔzwf* (Fig. 3*B*) We checked the *ΔpfkΔzwf* mutant for any glucose-6P dehydrogenase (G6PDH) activity. As expected, no G6PDH activity was detectable, in contrast to the WT (Fig. S4 *B* and *C*). The KDPG in *ΔpfkΔzwf* thus has to originate from a pathway not involving Zwf. Collectively, this indicates that the assumed bypass is present, and it clearly establishes that the ED pathway operates in *Synechocystis* in vivo.

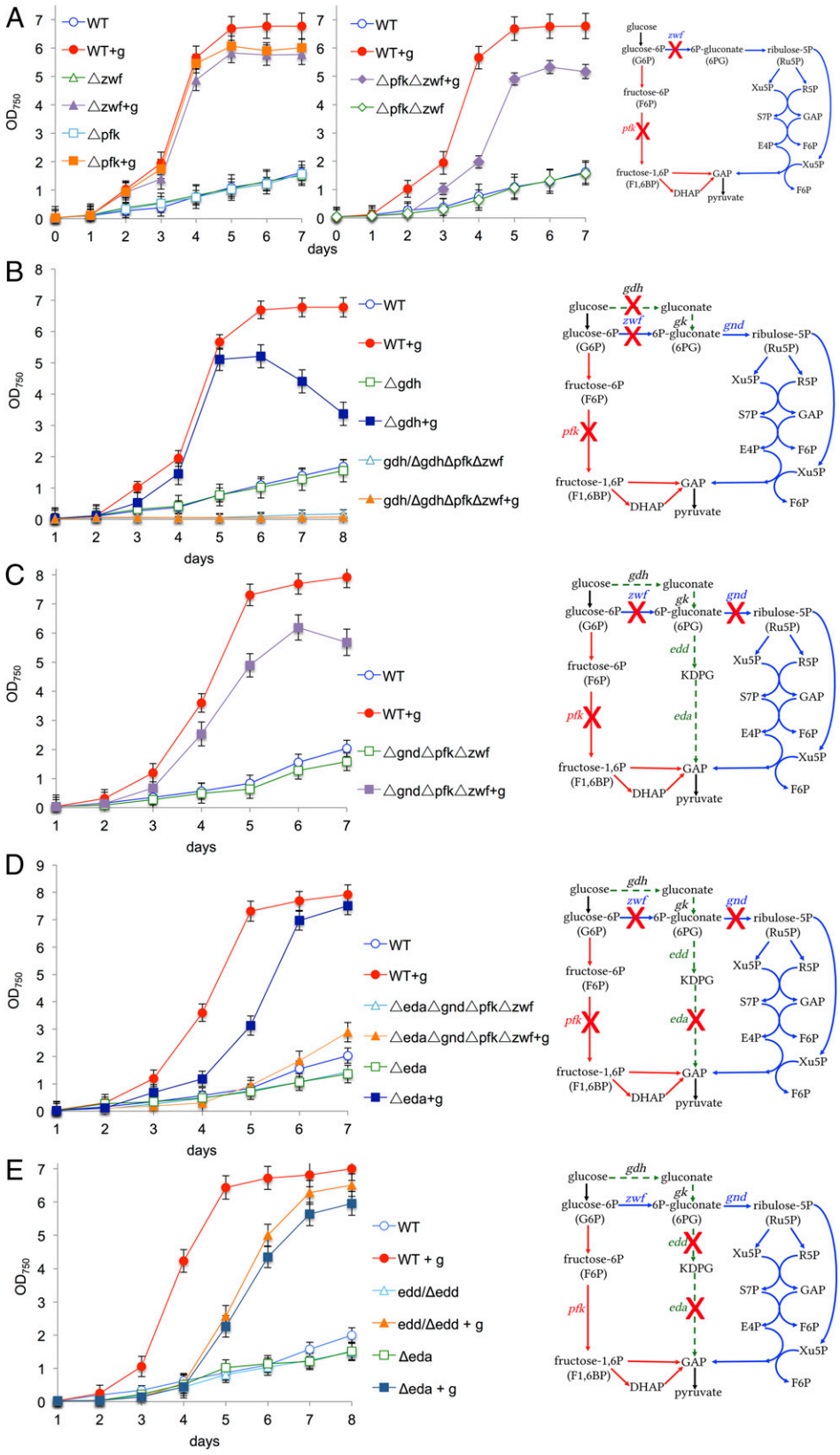


**Fig. 2.** Growth of *Synechocystis* WT and different mutants (as indicated) under autotrophic and mixotrophic (+g) conditions in continuous light. (*A–E*) Curves are accompanied by simplified schemes of glycolytic routes: EMP pathway in red, OPP pathway in blue, and ED pathway in green. Crosses indicate deleted genes. Error bars represent the SD from three independent cultures, each measured in triplicate. Each growth experiment was repeated independently at least three times to ensure reproducibility. Thus, in total, nine cultures were measured. In the graph, the data of one growth experiment are shown.

## Physiological Significance of the ED Pathway in *Synechocystis*

To evaluate the physiological importance of the ED pathway compared with the EMP and OPP pathways in *Synechocystis*, *Δeda*, *Δpfk*, *Δzwf*, and *Δgnd* were grown under mixotrophic conditions in continuous light, and additionally under autotrophic conditions in a dark/night cycle, probably the most common condition encountered in nature. Under both these conditions, *Synechocystis*

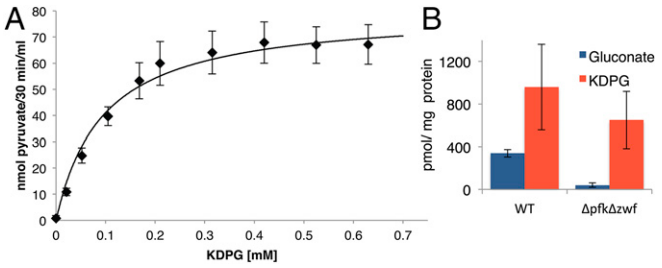


**Fig. 3.** The cyanobacterium *Synechocystis* possesses an active KDPG aldolase and ED pathway. (*A*) Activity assay for KDPG aldolase (Eda) from *Synechocystis*. Concentration dependence of the conversion of KDPG to pyruvate. The $K_M$ was determined to 0.095 mM. (*B*) KDPG, the unique metabolite of the ED pathway, and gluconate were detected via IC-ESI-MSMS in mixotrophically grown *Synechocystis* WT and Δ*pfk*Δ*zwf* cells. Error bars represent the SD from three replicates. Each experiment was repeated at least three times independently.

suffered most in growth when the ED pathway was interrupted compared with other glycolytic routes (Fig. 4 *A* and *B*).

## Occurrence of the ED Pathway in Cyanobacteria

The ED pathway has not previously been described in cyanobacteria, but BLAST searches revealed that KDPG aldolase is widespread among cyanobacteria (Table S1).

In fact, 92% of all sequenced cyanobacteria possess a KDPG aldolase, but only 57% possess PFK. 49% harbor both a KDPG aldolase and PFK, 8% possess only PFK, and 43% possess the KDPG aldolase but no PFK (Table S1). This distribution pattern indicates that the ED pathway is an important pathway of glucose degradation in cyanobacteria and is even more widespread than the EMP pathway in this group.

## Occurrence of the ED Pathway in Moss, Fern, Algae, and Plants

To check whether the ED pathway might also occur in other oxygenic photosynthesizers, Blast analyses were performed. Both Edd and KDPG aldolase were found to be present in moss and fern and widespread in algae and plants (Table S2 and Figs. S5 and S6). Phylogenetic analyses reveal that both plant enzymes branch with their homologs from cyanobacteria, indicating that they were acquired from the ancestors of plastids via endosymbiotic gene transfer (Figs. S5 and S6).

## Evidence for an Operating ED Pathway in the Plant Barley (*Hordeum vulgare*)

To test whether the putative plant KDPG aldolase is functional, the candidate gene BAJ87430 from barley (*H. vulgare*) was amplified from seedling cDNA. The protein was overexpressed in *Escherichia coli*, purified to apparent homogeneity via Ni-NTA, and subjected to a coupled enzyme test using lactate dehydrogenase. It had a $K_M$ value of 0.35 mM for the conversion of KDPG to GAP and pyruvate (Fig. 5*A*), close to the $K_M$ of the KDPG aldolase from other organisms (17, 18). In addition, 2-keto-3-deoxygluconate was tested as a substrate in the same concentration range to test whether the enzyme might participate in a nonphosphorylating ED pathway (15). No enzyme activity was detectable with 2-keto-3-deoxygluconate, indicating that higher plants, similar to cyanobacteria, possess a KDPG aldolase from the phosphorylating ED pathway. Glycolytic routes are especially important in periods of growth for the supply of ATP, NAD(P)H, and biosynthetic precursors. Therefore, mRNA was isolated from germinating barley seedlings during a period of 8 d and subjected to RT-PCR. The KDPG aldolase gene was expressed throughout this period during germination and early seedling development (Fig. 5*B*). Furthermore, KDPG, the metabolite unique to the ED pathway, was detected in roots of barley seedlings (Fig. 5*C*), indicating in vivo that this pathway operates in plants.

Collectively, our data thus indicate that the ED pathway operates in cyanobacteria and plants.

## Discussion

Most enzymes involved in the EMP pathway are bidirectional, either serving the degradation of glucose (glycolysis) or working in the opposite direction in gluconeogenesis. However, PFK is unique to the oxidative direction of the EMP pathway (6), so its absence from the genome most likely results in the lack of this pathway, even if all other enzymes belonging to the EMP pathway are present. All open ocean species that thrive in nutrient-depleted habitats, such as *Prochlorococcus*, *Crocosphaera*, *Synechococcus*, and *Trichodesmuim*, possess a KDPG aldolase but are devoid of a PFK (Table S1). Those few cyanobacteria (8%) that lack a KDPG aldolase thrive in freshwater, which is generally richer in nutrients. The cyanobacterium *Prochlorococcus* was recently found to take up glucose from its environment (19). As its genome lacks PFK, the key enzyme of the EMP pathway, the authors concluded that *Prochlorococcus* metabolizes glucose in the light exclusively via the OPP pathway (19, 20). However, the OPP pathway is known to be down-regulated in the light and fine-tuned under mixotrophic conditions, so as to prevent a futile cycle with the Calvin–Benson cycle (21, 22). We therefore suggest that *Prochlorococcus* might oxidize glucose via the ED pathway under mixotrophic conditions, as shown for *Synechocystis*. If one molecule of glucose is metabolized via the EMP pathway, the cell gains two ATP and two NADH; if it is metabolized via the ED pathway, it gains one ATP, one NADH, and one NADPH. At first sight, the EMP pathway looks beneficial, as its ATP yield is higher. However, cyanobacteria in the open ocean should not be ATP-limited, as they perform photosynthesis, and light is unlikely to be a limiting resource in this habitat. The ED

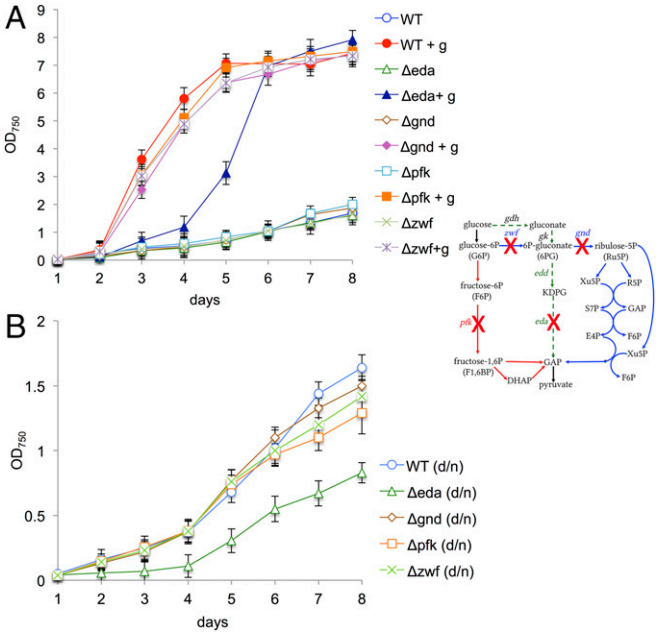


**Fig. 4.** Exclusively, deletion of KDPG aldolase delays growth under mixotrophic conditions in continuous light and autotrophic conditions in a day/night cycle. Growth of *Synechocystis* WT and single mutants, in which different key enzymes of glycolytic routes were deleted, as indicated in the simplified schemes: EMP pathway in red, OPP pathway in blue, and ED pathway in green. Crosses indicate deleted genes. (*A*) Cultures grown under autotrophic and mixotrophic (+g) conditions in continuous light. (*B*) Cultures grown under autotrophic conditions in day/night cycle (d/n). Error bars represent the SD from three independent cultures, each measured in triplicate. Each growth experiment was repeated independently at least three times to ensure reproducibility. In the graph, the data of one growth experiment is shown.

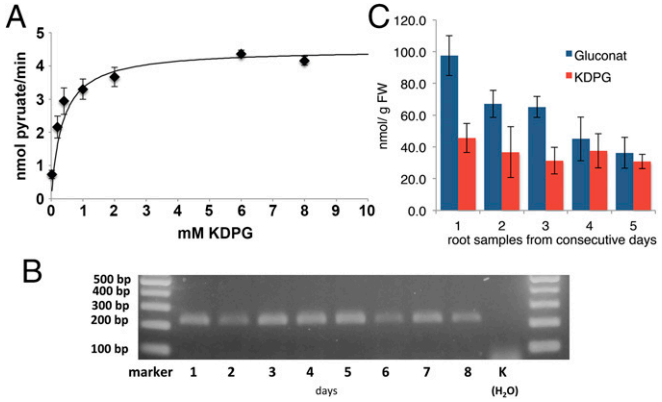


**Fig. 5.** The plant barley (*H. vulgare*) possesses an active KDPG aldolase, which is expressed throughout early seedling development and an active ED pathway. (*A*) Activity assay for KDPG aldolase (Eda) from *H. vulgare*. Concentration dependence of the conversion of KDPG to pyruvate. The $K_M$ was determined to 0.35 mM. (*B*) RT-PCR was carried out with RNA from germinating *H. vulgare* seedlings collected over a period of 8 d with primers specific for the KDPG aldolase transcript (expected size with intron, 466 bp; without intron, 222 bp). (*C*) KDPG, the unique metabolite of the ED pathway and gluconate were detected via IC-ESI-MSMS in roots of *H. vulgare* seedlings. Error bars represent the SD from three replicates. Each experiment was repeated at least three times independently.

pathway has a higher thermodynamic driving force than the EMP pathway because of its lower ATP yield, and it furthermore requires 3.5 times less enzymatic protein to achieve the same glycolytic flux as the EMP pathways (3). Flamholz et al. thus argue that the choice between the EMP pathway and the ED pathway is a tradeoff between ATP yield and protein costs (3). Cyanobacteria, algae, and plants in general are nutrient-limited, rather than ATP-limited. Algae, plants, and cyanobacteria even possess non-energy-conserving bypasses in their respiratory chains (12, 23) that should enable the oxidation of carbohydrates for biosynthetic purposes when ATP is oversupplied. It therefore makes perfect sense that cyanobacteria rely on the ED pathway rather than on the EMP pathway, and that cyanobacteria from the open ocean, such as *Prochlorococcus*, that streamline both their genome and proteome to adapt to nutrient-depleted conditions (24, 25), even seem to omit the upper part of the EMP pathway. Further research is needed to substantiate these hypotheses.

Cyanobacteria have long been thought to degrade glucose mainly via the OPP pathway, rather than the EMP pathway (10, 13, 21, 26). Furthermore, cyanobacterial glycolytic routes were perceived of being foremost important under heterotrophic conditions in the dark (10, 13). However, evidence is accumulating that mixotrophy (uptake of organic carbon in the light) among phototrophic cyanobacteria and algae has been dramatically underestimated, especially in aquatic habitats (27, 28). Under these conditions, however, glycolytic routes have to run in parallel to the Calvin–Benson cycle. The diatom *Phaeodactylum tricornutum* was recently shown to harbor functional forms of Edd and Eda belonging to the ED pathway (29). In contrast to the ED pathway, both the EMP and OPP pathways share several intermediates with the Calvin–Benson cycle (Fig. S7). A simultaneous operation of Calvin–Benson cycle and either the OPP pathway or the EMP pathway might thus result in futile cycles that slow down $CO_2$ fixation rates, thereby enhancing oxidative stress (26). This is not true for the ED pathway (Fig. S7) and might explain why *Δeda* compared with *Δpfk*, *Δzwf*, and *Δgnd* grew slower under mixotrophic conditions (Fig. 4*A*). In addition, the ED pathway provides cyanobacterial cells, obviously with a selective advantage under autotrophic conditions in a dark/night cycle (Fig. 4*B*), which might explain its high prevalence in photosynthesizing cells. The fact that the ED pathway is widespread in both cyanobacteria and plants indicates that it serves an important function. Otherwise, it would have been lost in evolution long ago. However, flux analyses are needed to quantify the relative contributions of EMP, OPP, and ED pathways to the degradation of carbohydrates in cyanobacteria and plants.

The present results show that cyanobacteria and plants possess an active ED pathway that was transferred from the cyanobacterial ancestors of plastids to the plant lineage. Oxygenic phototrophs thus possess three, instead of two, carbohydrate degradation pathways (Fig. 1), whereof the ED pathway is a previously overlooked glycolytic route of high physiological significance in cyanobacteria.

## Materials and Methods

Full protocols are available in the *SI Materials and Methods*.

**Growth Conditions *Synechocystis*.** For growth experiments, *Synechocystis* sp. PCC 6803 WT and mutants of this strain were grown in 250 mL BG11 medium in glass tubes at 28 °C under constant light (50 μE/m$^2$/s). The cultures were aerated with filter-sterilized air. Mixotrophic cultures were supplemented with 10 mM (final concentration) glucose. For light/dark cycles, cultures were subjected to a cycle of 12 h darkness and 12 h light (50 μE/m$^2$/s) under constant aeration.

**Growth Conditions of *H. vulgare* for RT-PCR Experiments and Ion Chromatography Electron Spray Ionization Tandem Mass Spectrometry Analysis.** After stratification, *H. vulgare* (Golden Promise) seeds were grown on moist nutrient-free vermiculite at 21 °C under continuous light (250 μE/m$^2$/s). Whole seedlings were collected every 24 h from day 1 to day 8 for RT-PCR experiments, and roots from seedlings were collected from day 2 to day 6 for Ion chromatography electron spray ionization tandem mass spectrometry (IC-ESI-MSMS) analysis.

**Generation of *Synechocystis* Deletion Mutants.** Constructs for the deletion mutants were made by PCR fusion, as described (30). The primers used are shown in Table S3. In principle, an antibiotic resistance cassette was fused to approximately 200 bp directly up- and downstream of the gene to be deleted. The constructs were ligated in pCRII-TOPO (Invitrogen), sequenced, and transformed into *Synechocystis* to replace the respective gene by the antibiotic resistance cassette. Mutants were checked by Southern hybridization (Fig. S2) (30).

**Extraction and Identification of Metabolites via IC-ESI-MSMS Measurements.** Metabolites were extracted from *Synechocystis* and barley (*H. vulgare*) roots via methanol:chloroform:water. The separation and detection of metabolites was carried out using an ion chromatography system (Dionex Thermo Fisher) connected to a triple quadrupole mass spectrometer QQQ6490 (Agilent Technologies). Quantification was performed with authentic standards at different concentrations.

**Overexpression and Purification of KDPG Aldolase of *Synechocystis* sp. PCC 6803.** For homologous overexpression of the KDPG aldolase (*sll0107*), ORF was cloned into p2M2-Pcpc560ter (14) with a C-terminal $His_6$-tag (for primers, see Table S3) and transformed into *Δeda*, resulting in the mutant *Δeda/cpc::eda*$^{Synechocystis}$. To purify the enzyme cells of the overexpression strain, *Δeda/cpc::eda*$^{Synechocystis}$ were harvested, resuspended in 100 mM Tris at pH 8.0 and 150 mM NaCl, and broken with glass beads (diameter, 0.17–0.18 mm) by vortexing 10 min at 4 °C. After centrifugation at 800 × *g* for 1 min at 4 °C, the liquid phase was removed and centrifuged at 1,300 × *g* for 10 min at 4 °C. The resulting homogenate was checked for unbroken cells under the microscope and, if necessary, centrifuged a second time at 1,300 × *g* for 10 min at 4 °C to remove unbroken cells. The homogenate was applied on a Ni-NTA column. After it completely entered the column, it was washed four times with two column volumes (cvs) wash buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole at pH 8). Subsequently, the protein was eluted from the column by applying six times 0.5 cvs elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM imidazole at pH 8) and collecting the eluate in 0.5-cv fractions.

**Overexpression and Purification of KDPG Aldolase of *H. vulgare* (Golden Promise).** cDNA from a barley seedling was subjected to a PCR with primers specific to the KDPG aldolase of *H. vulgare* (BAJ87430; AK356212) (Table S3).

The ORF was fused to a C-terminal $His_6$-tag and ligated into the pMAL-pIII (NEB), resulting in pMALeda$^{H.vulgare}$His. This vector was transformed into *E. coli* [BL21(pLys3)] and induced with 0.4 mM IPTG for 24 h. After harvesting, the cells were resuspended in lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole at pH 8) and incubated with 1 mg/mL lysozyme for 30 min on ice. Cells were lysed by six 10-s ultrasound bursts on ice with 10-s intermittent cooling periods. To remove cell debris, the homogenate was

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centrifuged at 20,000 × *g* for 15 min, and the supernatant was loaded on a Ni-NTA column. The column was run as described earlier for the purification of the KDPG aldolase from *Synechocystis*.

**KDPG Aldolase (Eda) in Vitro Measurements.** KDPG aldolase activity was measured by the production of pyruvate from KDPG. The isolated KDPG aldolases from *Synechocystis* and barley were used in 1-mL assays that contained 10 mM NADH, 5 U lactate dehydrogenase, and KDPG in a range from 0.02 to 8 mM in 100 mM Tris, 150 mM NaCl at pH 8 at 32 °C. The measurements were started by the addition of KDPG, and the consumption of NADH was followed at 366 nm.

In addition, KDPG aldolase from *Synechocystis* was also measured directly in cell homogenates of the overexpression strain *Δeda/cpc::eda*$^{Synechocystis}$. In that case, homogenates were prepared by breaking the cells with glass beads, as described for the purification of the KDPG aldolase from *Synechocystis* (see above).

The total extract was used directly for KDPG aldolase measurements. Aliquots of the extract were incubated with different KDPG concentrations (0.02–0.7 mM) for 30 min. After 30 min, the samples were centrifuged for 5 min at 20,000 × *g* to remove the membranes. Subsequently, the amount of pyruvate generated was determined by measuring the absorption change at 366 nm after adding 10 mM NADH and 5 U lactate dehydrogenase. The extract of the mutant *Δeda* was used as a control and showed in all cases no production of pyruvate.

**Glucose-6P Dehydrogenase (zwf) in Vitro Measurements.** Glucose-6P dehydrogenase activity was measured in *Synechocystis* WT and *ΔpfkΔzwf* by the conversion of NADP to NADPH on addition of glucose-6P. A kinetic and an endpoint determination were carried out.

**Phylogenetic Analyses.** Homologous amino acid sequences were selected by an all-against-all bidirectional best BLAST hit approach. Pairwise hits were clustered into families using MCL (31). Multiple sequence alignments were performed with MAFFT (32), and phylogenetic trees were generated with PhyML (33).

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1. Müller M, et al. (2012) Biochemistry and evolution of anaerobic energy metabolism in eukaryotes. *Microbiol Mol Biol Rev* 76(2):444–495.
2. Stincone A, et al. (2014) The return of metabolism: Biochemistry and physiology of the pentose phosphate pathway. *Biol Rev Camb Philos Soc* 90(3):927–963.
3. Flamholz A, Noor E, Bar-Even A, Liebermeister W, Milo R (2013) Glycolytic strategy as a tradeoff between energy yield and protein cost. *Proc Natl Acad Sci USA* 110(24): 10039–10044.
4. Bräsen C, Esser D, Rauch B, Siebers B (2014) Carbohydrate metabolism in Archaea: Current insights into unusual enzymes and pathways and their regulation. *Microbiol Mol Biol Rev* 78(1):89–175.
5. Siebers B, Schönheit P (2005) Unusual pathways and enzymes of central carbohydrate metabolism in Archaea. *Curr Opin Microbiol* 8(6):695–705.
6. Bennett BD, et al. (2009) Absolute metabolite concentrations and implied enzyme active site occupancy in Escherichia coli. *Nat Chem Biol* 5(8):593–599.
7. Pelroy RA, Rippka R, Stanier RY (1972) Metabolism of glucose by unicellular blue-green algae. *Arch Mikrobiol* 87(4):303–322.
8. Née G, Zaffagnini M, Trost P, Issakidis-Bourguet E (2009) Redox regulation of chloroplastic glucose-6-phosphate dehydrogenase: A new role for f-type thioredoxin. *FEBS Lett* 583(17):2827–2832.
9. Cossar JD, Rowell P, Stewart WDP (1984) Thioredoxin as a modulator of glucose-6-phosphate dehydrogenase in a $N_2$-fixing cyanobacterium. *Microbiology* 130:991–998.
10. Knowles VL, Plaxton WC (2003) From genome to enzyme: Analysis of key glycolytic and oxidative pentose-phosphate pathway enzymes in the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Cell Physiol* 44(7):758–763.
11. Johnson X, Alric J (2013) Central carbon metabolism and electron transport in *Chlamydomonas reinhardtii*: Metabolic constraints for carbon partitioning between oil and starch. *Eukaryot Cell* 12(6):776–793.
12. Plaxton WC, Podesta FE (2006) The functional organization and control of plant respiration. *Crit Rev Plant Sci* 25(2):159–198.
13. Jansen T, et al. (2010) Characterization of trophic changes and a functional oxidative pentose phosphate pathway in Synechocystis sp PCC 6803. *Acta Physiol Plant* 32(3): 511–518.
14. Zhou J, et al. (2014) Discovery of a super-strong promoter enables efficient production of heterologous proteins in cyanobacteria. *Sci Rep* 4:4500.
15. Reher M, Fuhrer T, Bott M, Schönheit P (2010) The nonphosphorylative Entner-Doudoroff pathway in the thermoacidophilic euryarchaeon *Picrophilus torridus* involves a novel 2-keto-3-deoxygluconate- specific aldolase. *J Bacteriol* 192(4):964–974.
16. Kim S, Lee SB (2006) Catalytic promiscuity in dihydroxy-acid dehydratase from the thermoacidophilic archaeon *Sulfolobus solfataricus*. *J Biochem* 139(3):591–596.
17. Fong S, Machajewski TD, Mak CC, Wong C (2000) Directed evolution of D-2-keto-3-deoxy-6-phosphogluconate aldolase to new variants for the efficient synthesis of D- and L-sugars. *Chem Biol* 7(11):873–883.
18. Scopes RK (1984) Use of differential dye-ligand chromatography with affinity elution for enzyme purification: 2-keto-3-deoxy-6-phosphogluconate aldolase from *Zymomonas mobilis*. *Anal Biochem* 136(2):525–529.
19. Muñoz-Marín MdelC, et al. (2013) *Prochlorococcus* can use the Pro1404 transporter to take up glucose at nanomolar concentrations in the Atlantic Ocean. *Proc Natl Acad Sci USA* 110(21):8597–8602.
20. Gómez-Baena G, et al. (2008) Glucose uptake and its effect on gene expression in *prochlorococcus*. *PLoS One* 3(10):e3416.
21. Pelroy RA, Bassham JA (1972) Photosynthetic and dark carbon metabolism of glucose by unicellular blue-green algae. *Arch Mikrobiol* 86(1):25–38.
22. Takahashi H, Uchimiya H, Hihara Y (2008) Difference in metabolite levels between photoautotrophic and photomixotrophic cultures of *Synechocystis* sp. PCC 6803 examined by capillary electrophoresis electrospray ionization mass spectrometry. *J Exp Bot* 59(11):3009–3018.
23. Mullineaux CW (2014) Co-existence of photosynthetic and respiratory activities in cyanobacterial thylakoid membranes. *Biochim Biophys Acta* 1837(4):503–511.
24. Steglich C, et al. (2008) The challenge of regulation in a minimal photoautotroph: Non-coding RNAs in *Prochlorococcus*. *PLoS Genet* 4(8):e1000173.
25. Dufresne A, et al. (2003) Genome sequence of the cyanobacterium *Prochlorococcus marinus* SS120, a nearly minimal oxyphototrophic genome. *Proc Natl Acad Sci USA* 100(17):10020–10025.
26. Narainsamy K, Cassier-Chauvat C, Junot C, Chauvat F (2013) High performance analysis of the cyanobacterial metabolism via liquid chromatography coupled to a LTQ-Orbitrap mass spectrometer: Evidence that glucose reprograms the whole carbon metabolism and triggers oxidative stress. *Metabolomics* 9(1):21–32.
27. Moore LR (2013) More mixotrophy in the marine microbial mix. *Proc Natl Acad Sci USA* 110(21):8323–8324.
28. Zubkov MV, Tarran GA (2008) High bacterivory by the smallest phytoplankton in the North Atlantic Ocean. *Nature* 455(7210):224–226.
29. Fabris M, et al. (2012) The metabolic blueprint of *Phaeodactylum tricornutum* reveals a eukaryotic Entner-Doudoroff glycolytic pathway. *Plant J* 70(6):1004–1014.
30. Hoffmann D, Gutekunst K, Klissenbauer M, Schulz-Friedrich R, Appel J (2006) Mutagenesis of hydrogenase accessory genes of *Synechocystis* sp. PCC 6803. Additional homologues of hypA and hypB are not active in hydrogenase maturation. *FEBS J* 273(19):4516–4527.
31. Enright AJ, Van Dongen S, Ouzounis CA (2002) An efficient algorithm for large-scale detection of protein families. *Nucleic Acids Res* 30(7):1575–1584.
32. Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* 30(14):3059–3066.
33. Guindon S, et al. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Syst Biol* 59(3):307–321.
34. Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Microbiology* 111:1–61.
35. Lancashire PD, et al. (1991) A uniform decimal code for growth stages of crops and weeds. *Ann Appl Biol* 119(3):561–601.
36. Tatusov RL, Koonin EV, Lipman DJ (1997) A genomic perspective on protein families. *Science* 278(5338):631–637.
37. Rice P, Longden I, Bleasby A (2000) (EMBOSS: The european molecular biology open software suite. *Trends Genet* 16(6):276–277.
38. Larkin MA, et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23(21): 2947–2948.
39. Abascal F, Zardoya R, Posada D (2005) ProtTest: Selection of best-fit models of protein evolution. *Bioinformatics* 21(9):2104–2105.
40. Felsenstein J (1996) Inferring phylogenies from protein sequences by parsimony, distance, and likelihood methods. *Methods Enzymol* 266:418–427.
41. Altschul SF, et al. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25(17):3389–3402.
42. Allard J, Grochulski P, Sygusch J (2001) Covalent intermediate trapped in 2-keto-3-deoxy-6- phosphogluconate (KDPG) aldolase structure at 1.95-A resolution. *Proc Natl Acad Sci USA* 98(7):3679–3684.