

# The photorespiratory glycolate metabolism is essential for cyanobacteria and might have been conveyed endosymbiotically to plants

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Photorespiratory 2-phosphoglycolate (2PG) metabolism is essential for photosynthesis in higher plants but thought to be superfluous in cyanobacteria because of their ability to concentrate CO<sub>2</sub> internally and thereby inhibit photorespiration. Here, we show that 3 routes for 2PG metabolism are present in the model cyanobacterium *Synechocystis* sp. strain PCC 6803. In addition to the photorespiratory C<sub>2</sub> cycle characterized in plants, this cyanobacterium also possesses the bacterial glycerate pathway and is able to completely decarboxylate glyoxylate via oxalate. A triple mutant with defects in all 3 routes of 2PG metabolism exhibited a high-CO<sub>2</sub>-requiring (HCR) phenotype. All these catabolic routes start with glyoxylate, which can be synthesized by 2 different forms of glycolate dehydrogenase (GlcD). Mutants defective in one or both GlcD proteins accumulated glycolate under high CO<sub>2</sub> level and the double mutant  $\Delta\text{glcD1}/\Delta\text{glcD2}$  was unable to grow under low CO<sub>2</sub>. The HCR phenotype of both the double and the triple mutant could not be attributed to a significantly reduced affinity to CO<sub>2</sub>, such as in other cyanobacterial HCR mutants defective in the CO<sub>2</sub>-concentrating mechanism (CCM). These unexpected findings of an HCR phenotype in the presence of an active CCM indicate that 2PG metabolism is essential for the viability of all organisms that perform oxygenic photosynthesis, including cyanobacteria and C<sub>3</sub> plants, at ambient CO<sub>2</sub> conditions. These data and phylogenetic analyses suggest cyanobacteria as the evolutionary origin not only of oxygenic photosynthesis but also of an ancient photorespiratory 2PG metabolism.

It is well established that the photorespiratory C<sub>2</sub> pathway, whereby 2-phosphoglycolate (2PG) is metabolized (1), is essential for photosynthesis in the majority of plants (2). In contrast, the functioning of the C<sub>2</sub> pathway and its importance are still under discussion for cyanobacteria. These organisms were the first to have evolved oxygenic photosynthesis, and endosymbiotic engulfment of an ancient cyanobacterium led to the evolution of plant chloroplasts (3). In cyanobacteria, as in C<sub>3</sub> plants, the primary carbon fixation is catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Ribulose 1,5-bisphosphate reacts with either CO<sub>2</sub>, leading to the formation of 2 molecules of 3-phosphoglycerate (3PGA), or O<sub>2</sub>, generating 3PGA and 2PG. The latter compound is toxic to plant metabolism because it inhibits distinct steps in the carbon-fixing Calvin-Benson cycle (4, 5). Therefore, plants employ the so-called photorespiratory glycolate pathway (or C<sub>2</sub> cycle), which degrades 2PG and converts 2 molecules of 2PG into 1 molecule each of 3PGA, CO<sub>2</sub>, and NH<sub>4</sub><sup>+</sup> (1, 6, 7). In a typical C<sub>3</sub> plant, the ammonium is refixed at the expense of ATP, and 25% of the carbon entering the path is released as CO<sub>2</sub>. Generally, the photorespiratory cycle is indispensable for C<sub>3</sub> plants, because mutations in single steps of the C<sub>2</sub> cycle resulted in high-CO<sub>2</sub>-requiring (HCR) phenotypes (2, 8–10).

In contrast to plants, early studies on cyanobacterial 2PG metabolism indicated its absence or the occurrence of only the initial steps engaged in glycolate formation (11). This was difficult to understand because the affinity of the cyanobacterial

Rubisco for CO<sub>2</sub> is considerably lower than that of C<sub>3</sub> plants (12). Today, it is widely accepted that the low CO<sub>2</sub> affinity of Rubisco is compensated by an efficient inorganic carbon (C<sub>i</sub>)-concentrating mechanism (CCM) that raises the concentration of CO<sub>2</sub> in close proximity to Rubisco (13–15). Mutants impaired in functional components of the CCM, such as the carboxysomes (16–19) or transport and internal accumulation of C<sub>i</sub> (20–22), show very low apparent photosynthetic affinity for external C<sub>i</sub> and, thus, exhibit a HCR phenotype. These findings clearly revealed the essential function of the CCM for cyanobacterial survival under the present atmosphere and prompted the widely accepted notion that oxygenase activity of Rubisco is almost totally repressed in cyanobacteria. Therefore, metabolism of 2PG, the immediate product of this oxygenase function, seemed to be unnecessary in these organisms.

Recently, we provided evidence for combined action of a plant-like C<sub>2</sub> cycle and a bacterial-like glycerate pathway (23) to metabolize 2PG in the cyanobacterial model strain *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). Mutants defective in specific steps involved in these routes displayed growth retardation and accumulated intermediates of the photorespiratory metabolism already under high CO<sub>2</sub> conditions [air enriched with 5% CO<sub>2</sub> (HC)]. However, contrary to C<sub>3</sub> plants, even the double mutants in the 2 known 2PG degrading routes operating in *Synechocystis*, the C<sub>2</sub> cycle and glycerate pathway, were able to grow under ambient CO<sub>2</sub> conditions [ambient air with 0.035% CO<sub>2</sub> (LC)]. This ability was attributed to the activity of the CCM, which depresses the formation and hence metabolism of 2PG. Alternatively, this ability could also suggest the existence of additional routes for 2PG breakdown. Gene expression profiling, where the mRNA levels in LC- and HC-grown cells were compared, revealed the existence of hundreds of C<sub>i</sub>-regulated genes (24, 25). Interestingly, some of the genes up-regulated under low CO<sub>2</sub> encode for enzymes that form a third route of 2PG metabolism via a series of decarboxylations: glyoxylate is converted into oxalate, then to formate, and finally to CO<sub>2</sub>.

In the present study, we show unequivocally that an active 2PG metabolism not only exists but is essential for cyanobacterial growth in the present O<sub>2</sub>-containing atmosphere, despite the existence of the CCM. Also, the essential nature of the 2PG metabolism and its occurrence in all present-day cyanobacteria implies that this metabolism already existed in ancient cyanobacteria and might have been the starting point for the evolution of

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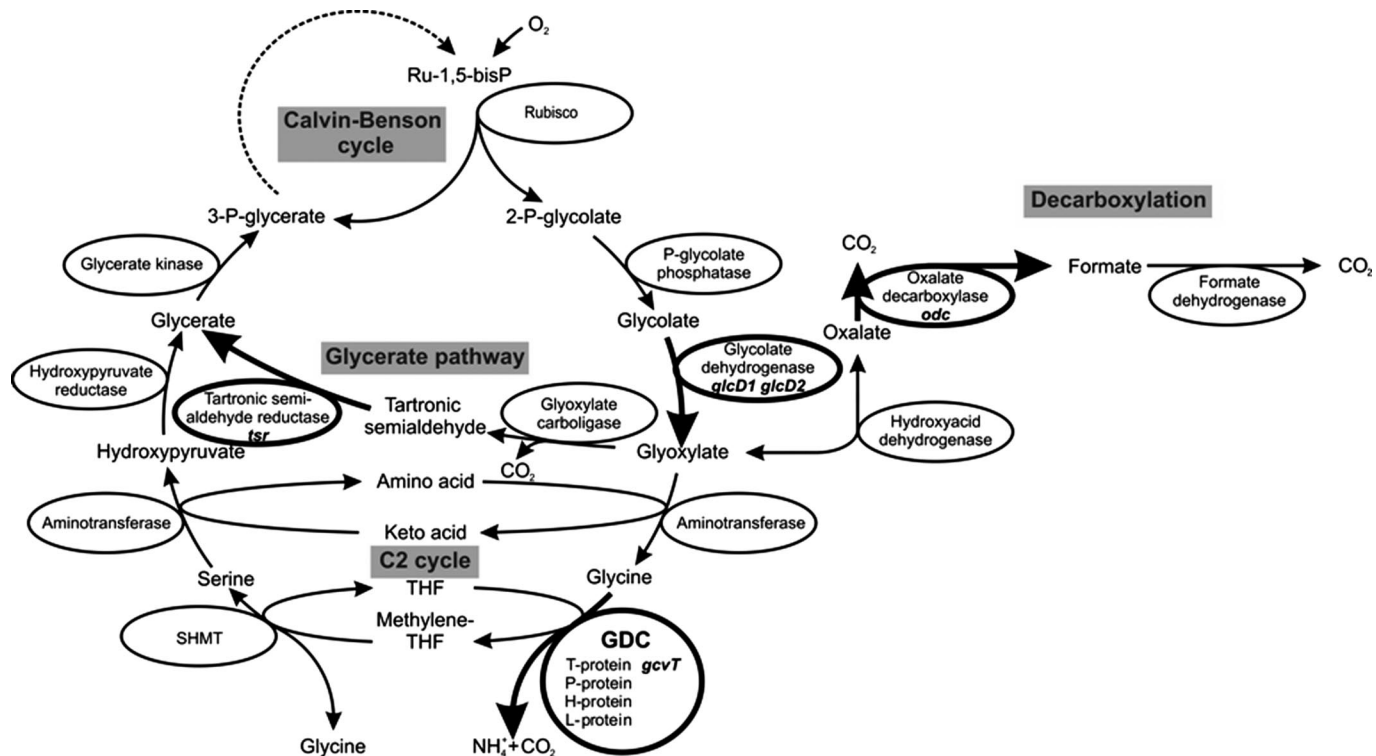


Fig. 1. A scheme displaying the 2PG metabolism in *Synechocystis* sp. strain PCC 6803, which employs 3 different routes: C2 cycle, glycerate pathway, and the decarboxylating branch. Enzymatic steps mutated in strains used for this study are indicated in bold.

the plant photorespiratory 2PG metabolism after the engulfment of the primary cyanobacterial endosymbiont.

## Results

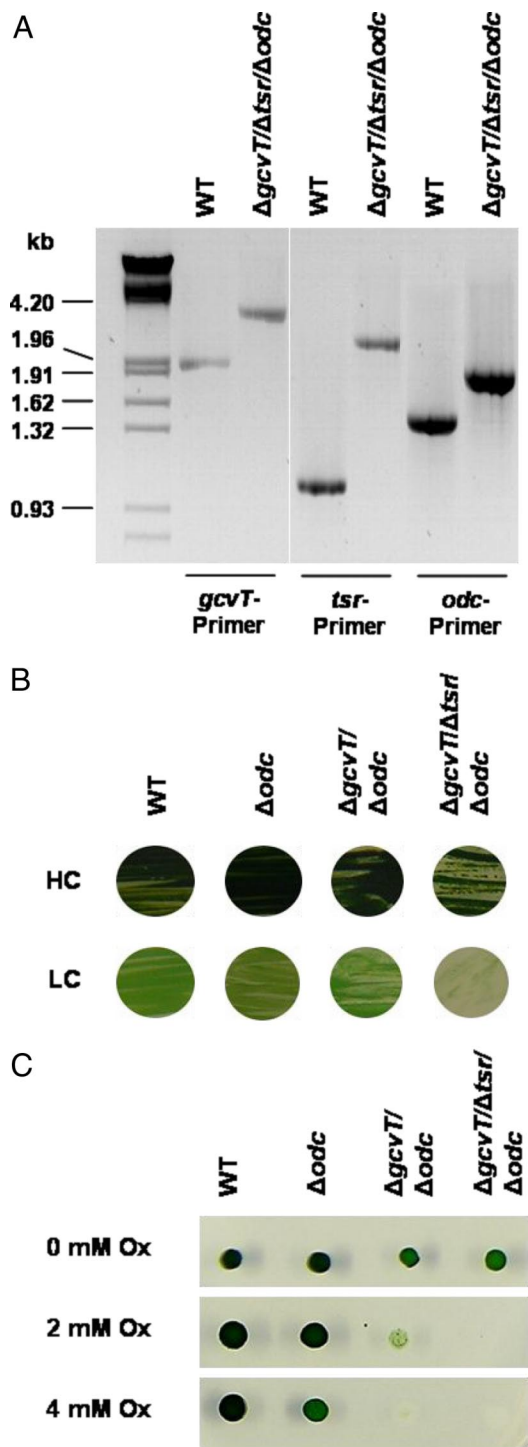
**A Triple Mutant in 3 Branches of 2PG Metabolism Exhibits an HCR Phenotype.** To examine the functioning and cooperation of all of the proposed photorespiratory pathways (Fig. 1), we raised a mutant in which these routes of 2PG metabolism were specifically inactivated downstream from glyoxylate. This mutant generation included the inactivation of *odc* encoding the oxalate decarboxylase involved in the decarboxylation branch, of *gcvT* encoding the T-protein of glycine decarboxylase in the C2 cycle, and *tsr* encoding the tartronic semialdehyde reductase in the glycerate pathway. The triple mutant  $\Delta gcvT/\Delta tsr/\Delta odc$ , isolated under HC, is completely segregated. PCR analysis proved that all of the WT copies of these 3 genes were inactivated, bearing the inserted cartridges encoding different antibiotics in all of the chromosome copies (Fig. 2A). Surprisingly, the triple mutant exhibited a HCR phenotype: it was unable to grow at air level of  $CO_2$  (Fig. 2B). In contrast, the single or double mutants defective in the decarboxylation branch and/or in the plant-like C2 cycle could acclimate and grow under LC.

Such a HCR phenotype is typical for plant photorespiratory mutants, for example the C3 plant *Arabidopsis thaliana* (2, 8–10), which do not possess a CCM. However, it was not expected in cyanobacteria, where the up-regulation of the CCM activity by LC is supposed to severely suppress photorespiration (13, 14). It is noteworthy that the HCR characteristic of the triple mutant was lost after a few cycles of cultivation at LC. However, with freshly generated triple-mutant clones, we were able to reproduce several times the initial HCR phenotype and its subsequent loss. These data clearly suggested that photorespiratory metabolism is essential for the ability of *Synechocystis* to grow under LC. A further apparent indication of the cooperation of these 3 metabolic routes was found in experiments where we tested the resistance toward external oxalate. An increasing sensitivity to oxalate ( $\Delta odc < \Delta gcvT/\Delta odc <$

$\Delta gcvT/\Delta tsr/\Delta odc$ ; Fig. 2C) was observed, suggesting that oxalate can also be converted back to glyoxylate and subsequently degraded by the C2 cycle and/or glycerate pathway.

**Identification of an Alternative Glycolate Dehydrogenase.** In an earlier study (23), we showed that a mutant  $\Delta gldD$  defective in a subunit of GlcD could grow under LC, albeit some 50% slower than the WT. In view of the HCR phenotype of the triple mutant, the growth characteristics of mutant  $\Delta gldD$  raised the possibility of a potential bypass for glycolate oxidation in cyanobacteria. A search in the *Synechocystis* genome identified a gene, *slr0806*, potentially coding an alternative GlcD. The amino acid sequence of Slr0806 shares 46% similarity with GlcD1 (Slr0404) from *Synechocystis* and from *Escherichia coli*. Also, Slr0806 harbors a conserved consensus sequence GXGXXG [supporting information (SI) Fig. S1] thought to serve as a flavin-binding domain (26). In addition to GlcD1, the GlcD complex is comprised of a second dehydrogenase subunit GlcE (26), which is  $\approx 42\%$  similar to Slr0806. Also, phylogenetic analysis showed that Slr0806-like proteins from several microorganisms clearly cluster with the GlcD/E-group (Fig. S1) and is present in all of the available cyanobacterial genome sequences. Therefore, we assumed that Slr0806 may form a second GlcD, designated GlcD2, which acts together with GlcD1 in glycolate oxidation. This enzyme activity could explain the viability of  $\Delta gldD1$  mutant at LC in contrast to the HCR phenotype of the triple mutant.

To verify this assumption, we inactivated *gldD2* in *Synechocystis* generated a double mutant in which both potential GlcDs were defective. The double mutant  $\Delta gldD1/\Delta gldD2$  was isolated under HC, and PCR analyses proved that all the WT-copies of *gldD1* and *gldD2* were inactivated (data not shown). To examine possible involvement of GlcD2 in glycolate conversion, we quantified the internal glycolate levels in the WT and the mutants by using HPLC; 3 h after transfer from HC to LC, the glycolate content in the WT cells was very low ( $\approx 0.02 \mu\text{mol}$  of



**Fig. 2.** Genotypic and phenotypic characterization of the triple mutant  $\Delta gcvT/\Delta tsr/\Delta odc$ . (A) Complete segregation of the mutant  $\Delta gcvT/\Delta tsr/\Delta odc$  was verified by PCR with gene-specific primers (see [SI Table 1](#)). (B) Growth of WT, single mutant  $\Delta odc$  blocked in the decarboxylation branch, double mutant  $\Delta odc/\Delta gcvT$  blocked in the decarboxylation branch and C2 cycle, and triple mutant  $\Delta gcvT/\Delta tsr/\Delta odc$  blocked in all 3 branches of 2PG metabolism (see Fig. 1), respectively, under HC or LC. Strains were plated on BG11, pH 7, solidified by 0.9% Kobe agar, and incubated under continuous illumination of  $30 \mu\text{mol}$  of photons per  $\text{s}$  per  $\text{m}^2$  at  $30^\circ\text{C}$  for 7 d. (C) Resistance of WT, single mutant  $\Delta odc$ , double mutant  $\Delta odc/\Delta gcvT$ , and triple mutant  $\Delta gcvT/\Delta tsr/\Delta odc$  toward oxalate (Ox). Strains were plated on BG11 agar plates, pH 8, supplemented by different amounts of oxalate. Cells were incubated under continuous illumination of  $30 \mu\text{mol}$  of photons per  $\text{s}$  per  $\text{m}^2$  at  $30^\circ\text{C}$  and HC for 7 d. [C reproduced with permission from ref. 41].

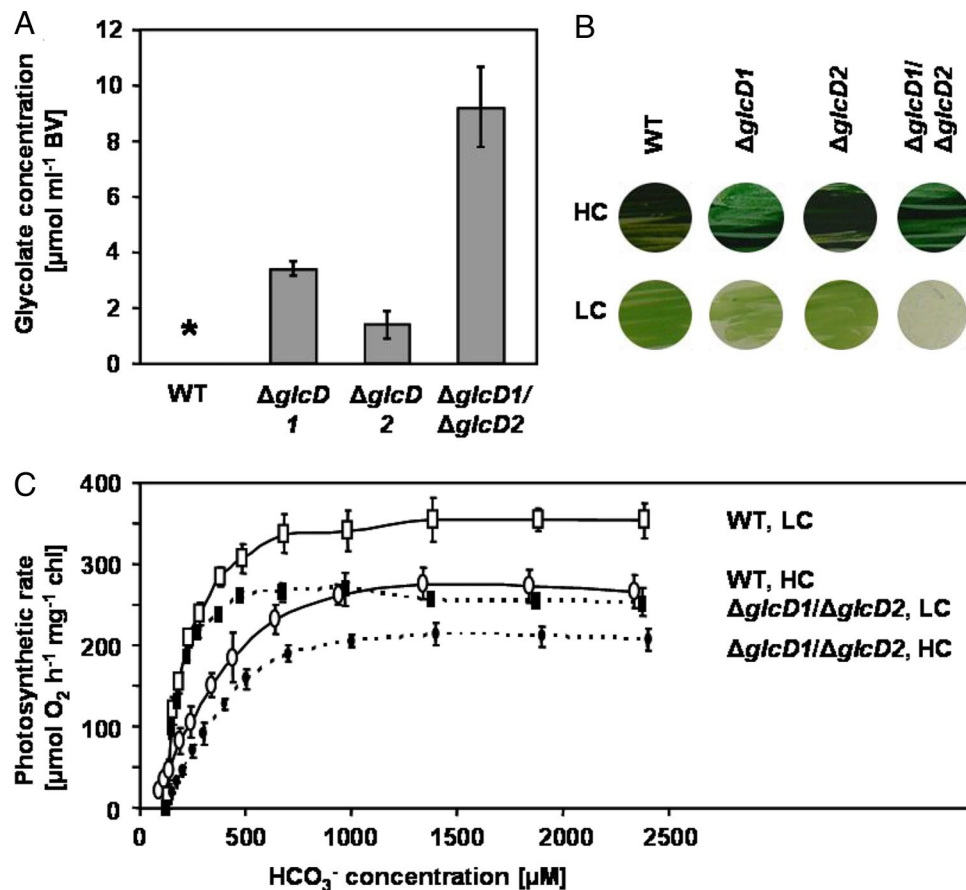
glycolate per mL of cell volume). It became  $\approx 200$ -fold higher in mutant  $\Delta glcD2$  and even higher in mutant  $\Delta glcD1$  (Fig. 3A). Importantly, compared with the single mutants, the double mutant  $\Delta glcD1/\Delta glcD2$  accumulated far more glycolate (Fig. 3A). It should be noted that glycolate was detected in cells of mutants  $\Delta glcD1$  and  $\Delta glcD1/\Delta glcD2$  even when grown under HC, whereas only traces were observed in HC-cells of mutant  $\Delta glcD2$  and none in extracts from WT-cells grown under HC.

**The Double Mutant Impaired in both GlcD1 and GlcD2 Exhibits a HCR Phenotype.** Because the level of glycolate accumulated was higher in mutant  $\Delta glcD1$  than in  $\Delta glcD2$  (Fig. 3A), we conclude that the former is the major GlcD in *Synechocystis*. This assumption is supported by the fact that the single  $\Delta glcD1$  mutant, but not  $\Delta glcD2$ , grew slower than the WT under both HC and LC. Interestingly, the double mutant  $\Delta glcD1/\Delta glcD2$  could grow under HC, albeit somewhat slower than the WT, but could not grow under LC (Fig. 3B). In contrast to the triple mutant, the HCR phenotype of the double mutant was stable for many generations. After a few days under LC, the cell suspensions of mutant  $\Delta glcD1/\Delta glcD2$  were completely bleached and could not recover after transfer back to HC. The HCR phenotype of this double mutant corresponds to the same phenotype described above for the triple mutant  $\Delta gcvT/\Delta tsr/\Delta odc$  impaired in the 2PG metabolism downstream of glyoxylate. The fact that the newly-constructed double and triple mutants showed a HCR phenotype clearly indicates and supports our view that a photorespiratory 2PG metabolism is essential for cyanobacteria at LC despite the action of the CCM.

**Characterization of Photosynthesis in the GlcD1/D2 Mutants.** In earlier studies, HCR phenotypes were observed in CCM mutants of cyanobacteria defective in the ability to accumulate  $\text{C}_i$  internally (22, 27) or in the structural organization of the carboxysomes (16, 28, 29). In both cases, the phenotype emerged from the very low apparent photosynthetic affinity for external  $\text{C}_i$ . Analysis of the photosynthetic parameters showed that this was not the case in the  $\Delta glcD$  mutants (Fig. 3C). When grown under HC, the apparent photosynthetic affinity for extracellular  $\text{C}_i$  and the  $V_{\text{max}}$  were somewhat lower in the  $\Delta glcD1/\Delta glcD2$  mutant as compared with WT. The  $K_{1/2}$  for  $\text{C}_i$  of the double mutant was  $\approx 0.35 \text{ mM HCO}_3^-$ , whereas this value increased  $\approx 100$  times to  $40 \text{ mM HCO}_3^-$  in the  $\Delta ccnM$  mutant (19) or to  $>20 \text{ mM HCO}_3^-$  in the  $\Delta 5$  mutant (30), where the carboxysomes were absent and all the genes encoding known  $\text{C}_i$  uptake systems were inactivated, respectively. Therefore, the HCR phenotype of the double mutant cannot be explained by a reduced affinity of the cell for  $\text{C}_i$ . In this respect, the double mutant  $\Delta glcD1/\Delta glcD2$  resembled the  $\Delta purK$  mutant of *Synechococcus* sp. strain PCC 7942, where the HCR phenotype emerged from an inability to produce purines under LC (31). Last, after exposure of mutant  $\Delta glcD1/\Delta glcD2$  to LC for 6 h, the photosynthetic  $K_{1/2}$  ( $\text{C}_i$ ) decreased considerably as in the WT (Fig. 3C), indicating that the induction of a more efficient CCM was not sufficient to enable growth under LC. Interestingly, the photosynthetic  $V_{\text{max}}$  of the mutant was lower than that of the WT, particularly under LC, possibly reflecting inhibition of Calvin-Benson cycle enzymes by the accumulating 2PG (4, 5).

## Discussion

Results presented here point to a common concept for C3 plants and cyanobacteria, namely that 2PG metabolism is an essential partner for oxygenic photosynthesis in  $\text{O}_2$ -containing environments. We believe this to be a novel view arising from the generation and characterization of 2 different sets of cyanobacterial mutants impaired in 2PG metabolism. To gain a comprehensive molecular description of the 2PG metabolism in cyanobacteria, we have identified the complete decarboxylation of glyoxylate as the third route for 2PG degradation (Fig. 1) and constructed the triple mutant  $\Delta gcvT/\Delta tsr/\Delta odc$  that showed the



**Fig. 3.** Phenotypic characterization of single mutants and a double mutant of *Synechocystis* defective in GlcDs. (A) Quantification of intracellular glycolate in cells of single- ( $\Delta\text{gld1}$ ,  $\Delta\text{gld2}$ ) and double- ( $\Delta\text{gld1}/\Delta\text{gld2}$ ) mutants in the glycolate converting step. Samples were taken 3 h after shift from HC to LC and glycolate was quantified by HPLC. \*, WT cells contained only traces of glycolate under these conditions. (B) Growth of WT, single mutants  $\Delta\text{gld1}$  or  $\Delta\text{gld2}$ , and double mutant  $\Delta\text{gld1}/\Delta\text{gld2}$  under HC or LC. Strains were plated on BG11, pH 7, solidified by 0.9% Kobe agar, and incubated under continuous illumination of 30  $\mu\text{mol}$  of photons per s per  $\text{m}^2$  at 30 °C for 7 d. (C) Photosynthesis rates of cells of the WT and the double mutant  $\Delta\text{gld1}/\Delta\text{gld2}$  at different concentrations of  $\text{HCO}_3^-$  as a source for C<sub>i</sub>. The cells were grown in liquid BG11 medium at HC or transferred to aeration by ambient air (LC) for 6 h.

HCR phenotype. This phenotype, which contradicted the ability of mutant  $\Delta\text{gldD}$  to grow under LC, urged us to identify an alternative GlcD, GlcD2, involved in the conversion of glycolate to glyoxylate. The generation of the double mutant  $\Delta\text{gld1}/\Delta\text{gld2}$  resulted again in the HCR phenotype and confirmed that 2PG conversion is indeed essential for growth.

Although the newly identified GlcD2 is similar to the earlier recognized GlcD1, it could not fully replace GlcD1 in the  $\Delta\text{gld1}$  mutant, because the latter mutant accumulated glycolate and vice versa. In addition, the significant increase in glycolate in the double mutant defective in both GlcDs also supports the notion that both GlcD proteins are active in glycolate oxidation. Correspondingly, the HCR phenotype was only obtained in the double mutant where the conversion of glycolate to glyoxylate is completely blocked. The high accumulation of glycolate may have been toxic for the cells, as was shown for higher plants (10). Accumulation of glycolate in these mutants indicated that the oxygenase activity of Rubisco was not fully inhibited even under the HC applied here. Also, because the photosynthetic parameters were close to those observed in the WT (Fig. 3C) and differed from those observed in “classical HCR mutants” defective in CCM, we conclude that the functioning and even activation of the CCM was insufficient to allow growth of GlcD mutants at air level of  $\text{CO}_2$ . Recently, it was shown that 2PG, the product of oxygenase reaction by Rubisco, serves as a signal to trigger acclimation of cyanobacteria to LC (32). In agreement

with this observation, we found indications for LC acclimation already under HC in cells of the GlcD1 mutant, which accumulates glycolate and possibly also increased amounts of 2PG under HC (25).

Apparently, the glyoxylate produced by GlcD1 and GlcD2 is metabolized by the cooperation of 3 different routes operating in *Synechocystis* and presumably other cyanobacteria. The presence of the third route, the complete decarboxylation of 2PG, was verified here by the generation and characterization of the triple mutant  $\Delta\text{gcvT}/\Delta\text{tsr}/\Delta\text{odc}$ , which is unable to grow under LC. The observed unstable HCR phenotype of the triple mutant is not exceptional. Pseudoreversions of mutants originally showing HCR phenotype were reported in several cases including a mutant lacking carboxysomes (33) and a double mutant in the 2 2PG phosphatases of *Synechocystis* (23). It is important to note that these mutants neither excreted 2PG nor glycolate (23), an observation supported by the marked glycolate accumulation inside the GlcD mutants.

In view of the HCR phenotype of the double and triple mutants we conclude the following: (i) the 2PG metabolism in *Synechocystis* comprises 3 cooperating routes, the C2 cycle, the glycerate pathway, and complete decarboxylation; (ii) the 2PG metabolism is active and essential in cells grown under atmospheric level of  $\text{CO}_2$ , indicating that the CCM does not block photorespiration as efficiently as was postulated; and (iii) the main function of 2PG metabolism seems to be related to the reduction of the amount of toxic intermediates to below critical

**Table 1. Proteins involved in 2-phosphoglycolate metabolism in the cyanobacteria *Synechocystis* sp. strain PCC 6803 (ORF in 6803) and *Nostoc* sp. strain PCC 7120 (ORF in 7120), and the presence of close homologs (e-values higher than  $e^{-25}$ ) in *Prochlorococcus* sp. strain SS120 (SS120) and *Arabidopsis thaliana* (A. thal.)**

Protein	ORF in 6803	ORF in 7120	Present in SS120	Present in A. thal.	C2 cycle in A. thal.	Cyano origin?
2-phosphoglycolate phosphatase	slr0458 sll1349	– alr4944	+ +	– –	At5 g36790*	
Glycolate DH						(At5g06580*)
GlcD1	sll0404	alr5269	–	+		
GlcD2	slr0806	all4443	+	–		
Glycolate oxidase	–	all0170	–	+	At4g18360 <sup>†</sup>	<b>At4 g18360</b>
Serine/glyoxylate aminotransferase	sll1559	alr1004	+	+	At2g13360 <sup>†</sup>	
Glycine decarboxylase						
P-protein	slr0293	all4607	+	+	At4g33010 <sup>‡</sup>	<b>At4g33010</b>
T-protein	sll0171	all4609	+	+	At1g11860 <sup>‡</sup>	(At3g16950*)
H-protein	slr0879	all4608	+	+	At2g35370 <sup>‡</sup>	
L-protein	slr1096	alr4745	+	+	At3g17240 <sup>‡</sup>	
Serine hydroxymethyl-transferase	sll1931	alr4806	+	+	At4g37930 <sup>‡</sup>	(At4g32520*)
Glutamate/glyoxylate aminotransferase	slr0006	alr2765	+	–	At1g23310 <sup>†</sup>	
Hydroxypyruvate reductase	sll1908	alr1890	+	+	At1g68010 <sup>†</sup>	(At1g17745*)
Glycerate kinase						
Bacterial type	slr1840	–	–	–		
Plant type	–	alr2873	+	+	At1g80380*	<b>At1g80380</b>
Glyoxylate carboligase	sll1981	all3555	+	–		
Tartronic semialdehyde reductase	slr0229	alr3358	+	+		
Hydroxyacid reductase	sll1556	alr0058	–	+		
	sll0891	alr4322	–	+		
Oxalate decarboxylase	sll1358	–	–	–		
Formate dehydrogenase	sll1359	–	–	–		

The column "C2 cycle in A. thal." lists proteins that are experimentally proven to participate in the plant-like photorespiratory C2 cycle. The column "Cyano origin?" indicates genes for enzymes acting in the plant C2 cycle that possibly originate from the cyanobacterial endosymbiont because these plant proteins cluster closest to homolog proteins from cyanobacteria. Amino acid similarity searches and cluster analyses were done by using the BLAST algorithm (40) at the National Center for Biotechnology Information ([www.ncbi.nlm.nih.gov/blast/Blast.cgi](http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)) or CyanoBase (<http://bacteria.kazusa.or.jp/cyanobase/>). ORFs written in bold are probably of cyanobacterial origin and involved in the plant C2 cycle, whereas ORFs in brackets probably originated also from cyanobacteria but are not directly involved in the plant C2 cycle because these proteins are located in other cellular compartments. +, present; –, absent.

\*The mature protein is predicted to be localized in the *Arabidopsis* chloroplast.

<sup>†</sup>The mature protein is predicted to be localized in the *Arabidopsis* peroxisome.

<sup>‡</sup>The mature protein is predicted to be localized in the *Arabidopsis* mitochondrion.

threshold levels, although we cannot rule out the importance of 3PGA regeneration.

The third conclusion is supported by the close correlation observed between growth retardation and accumulation of glycolate (shown here) or glycine (34). Similar indications exist for C3 plants, where the extent of phenotypical changes correlated with the amount of internally accumulated intermediates of 2PG metabolism (10). Our suggestion that metabolism of 2PG is not only beneficial but essential for cyanobacteria is supported by the fact that all the enzymes necessary for the plant-like C2 cycle, as well as for the glycerate pathways, are present in all the presently-known complete genome sequences. This includes the smallest genomes of marine *Prochlorococcus* (Table 1) and *Synechococcus* strains, which are thought to possess only genes essential for their survival in a constant environment as photoautotrophic organisms (35).

Last, in view of our findings, we propose that the 2PG metabolism is an essential partner of oxygenic photosynthesis early on from its evolution in cyanobacteria. This hypothesis does not necessarily contradict the suggestion that the oxygenic photosynthesis evolved 2–3 billion years ago in an oxygen-free atmosphere. It is quite likely that inside the cyanobacterial cell oxygen concentration could rise significantly (15), leading to an elevated oxygenase activity of Rubisco already in ancient cyanobacteria. This may have been the case particularly within mats or stromatolith-like structures, where the ancient cyanobacterial cells were shielded by extracellular polysaccharides and inorganic matter. Also, it was suggested that the CCM evolved only ≈400 million years ago (14, 15), certainly long after the primary

engulfment of an ancient cyanobacterium by the endosymbiosis ≈1.2 billion years ago (3, 36). If this is the case, it was necessary to develop the means to overcome the formation and accumulation of toxic amounts of intermediates such as 2PG, glycolate, or glycine soon after the evolution of oxygenic photosynthesis.

Taking this hypothetical scenario into consideration, it is possible that an active photorespiratory 2PG metabolism existed already in ancient cyanobacteria and was transferred into present-day higher plants by the engulfment of the primary cyanobacterial endosymbiont. To examine this assumption, we performed phylogenetic analyses of genes for the cyanobacterial 2PG metabolism searching for homologous proteins in the *Arabidopsis* genome (Table 1). Phylogenetic trees, where the cyanobacterial and corresponding plant proteins cluster closest together, are usually taken as evidence that those genes might have been transferred by means of endosymbiosis into the plant genome (3, 36). Such genomic searches suggested that glycolate oxidase and the plant-type glycerate kinase (Fig. S2), which are missing in *Synechocystis* but present in other cyanobacteria such as *Nostoc* sp. strain PCC 7120 and the P-protein subunit of glycine decarboxylase complex, originated from cyanobacteria. Also, close homologs were found for GlcD1, the L-protein subunit of glycine decarboxylase complex, serine hydroxymethyltransferase, and hydroxypyruvate reductase. However, these plant homologs are localized in other cells compartments (e.g., the cyanobacterial-like serine hydroxymethyltransferase is directed to the chloroplast; see Table 1) and are probably not directly involved in the present day plant-type C2 cycle. These analyses also proposed the presence of proteins probably homolo-

gous to enzymes of the bacterial-type glycerate pathway in Arabidopsis, but their function has yet to be characterized (Table 1).

## Materials and Methods

**Strains and Culture Conditions.** The cyanobacterial strains used in this work are listed in Table S1. The glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was obtained from N. Murata (National Institute for Basic Biology, Okazaki, Japan) and served as the WT. Cultivation of mutants was performed at 50  $\mu\text{g}\cdot\text{mL}^{-1}$  kanamycin (Km), 20  $\mu\text{g}\cdot\text{mL}^{-1}$  spectinomycin (Sp) or at 25  $\mu\text{g}\cdot\text{mL}^{-1}$  chloramphenicol (Cm) as required. Axenic cultures of *Synechocystis* ( $\approx 10^8$  cells per mL) were grown photoautotrophically in batch cultures (3-cm glass vessels with 5-mm glass tubes for aeration) at 29 °C under continuous illumination of 130  $\mu\text{mol}$  of photons per s per  $\text{m}^2$  (warm light; Osram L58 W32/3) bubbling (flow rate  $\approx 5$  mL $\cdot\text{min}^{-1}$ ) with air enriched with  $\text{CO}_2$  (HC) in the BG11 medium at pH 7.0.  $\text{C}_i$  limitation was set by transferring exponentially growing cultures ( $\text{OD}_{750}$  0.9, volume 130 mL) from bubbling with  $\text{CO}_2$ -enriched air to bubbling with ambient air ( $\approx 0.035\%$ , LC). Growth was monitored by measurements of the optical density at 750 nm ( $\text{OD}_{750}$ ). Agar plates (BG 11, pH 7, solidified by 0.9% Kobe agar) were incubated under continuous illumination of 30  $\mu\text{mol}$  of photons per s per  $\text{m}^2$  at 30 °C for 7 d in air or HC. Contamination by heterotrophic bacteria was checked by spreading of 0.2 mL of culture on LB plates. The *E. coli* strain TG1 (37), cultured in LB medium at 37 °C, was used for routine DNA manipulations.

**Generation of Mutants.** To generate mutation in the ORF *slr0806* (designated *glcD2*), the Sp resistance cartridge derived from pUC4S was integrated into the coding sequence at the unique *Bam*HI restriction site. The products were

checked by restriction analysis. Plasmid DNA of these constructs was isolated from *E. coli* by using the illustra plasmidPrep Mini Spin Kit (GE Healthcare);  $\approx 1$   $\mu\text{g}$  of DNA was used for transformation of *Synechocystis* and Sp-resistant clones were selected (38). To show alterations in the genotype, PCR with gene-specific oligonucleotides (see Table S1) was carried out by using the Taq-PCR Master Mix (Qiagen).

**Quantification of Internal Glycolate Concentrations.** Glycolate was extracted from frozen cyanobacterial cell pellets of 50 mL of culture with 80% ethanol at 65 °C for 3 h. After centrifugation, the supernatants were dried by lyophilization and redissolved in 350  $\mu\text{L}$  of water. The content of glycolate was determined by HPLC in ion-exclusion mode as described in ref. 23.

**Characterization of Photosynthesis.** The rate of  $\text{CO}_2$ -dependent  $\text{O}_2$  evolution as a function of  $\text{C}_i$  concentration was determined by using a Clark type  $\text{O}_2$  electrode (PS2108, Passport dissolved  $\text{O}_2$  sensor) essentially as described in ref. 39. The cells were harvested by centrifugation and resuspended in a  $\text{CO}_2$ -free medium containing 10 mM NaCl and 20 mM Hepes, pH 7.5. They were then placed in the  $\text{O}_2$  electrode chamber at 30 °C, 300  $\mu\text{mol}$  of photons per s per  $\text{m}^2$  and allowed to use the  $\text{C}_i$  in their medium until they reached the  $\text{CO}_2$  compensation point. Aliquots of  $\text{NaHCO}_3$  of known concentrations were injected to raise the  $\text{C}_i$  concentration by known increments while measuring the resulting rise in the rate of  $\text{O}_2$  concentration in the chamber.

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