The photorespiratory glycolate metabolism is essential for cyanobacteria and might have been conveyed endosymbiontically 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_2 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 C2 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_2-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_2 level and the double mutant Δ*glcD1*/Δ*glcD2* was unable to grow under low CO_2. The HCR phenotype of both the double and the triple mutant could not be attributed to a significantly reduced affinity to CO_2, such as in other cyanobacterial HCR mutants defective in the CO_2-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 C3 plants, at ambient CO_2 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 C2 pathway, whereby 2-phosphoglycolate (2PG) is metabolized (1), is essential for photosynthesis in the majority of plants (2). In contrast, the functioning of the C2 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 C3 plants, the primary carbon fixation is catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Ribulose 1,5-bisphosphate reacts with either CO_2, leading to the formation of 2 molecules of 3-phosphoglycerate (3PGA), or O_2, 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 C2 cycle), which degrades 2PG and converts 2 molecules of 2PG into 1 molecule each of 3PGA, CO_2, and NH_4^+ (1, 6, 7). In a typical C3 plant, the ammonium is refixed at the expense of ATP, and 25% of the carbon entering the path is released as CO_2. Generally, the photorespiratory cycle is indispensable for C3 plants, because mutations in single steps of the C2 cycle resulted in high-CO_2-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_2 is considerably lower than that of C3 plants (12). Today, it is widely accepted that the low CO_2 affinity of Rubisco is compensated by an efficient inorganic carbon (C_i)-concentrating mechanism (CCM) that raises the concentration of CO_2 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_i (20–22), show very low apparent photosynthetic affinity for external C_i 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 C2 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_2 conditions [air enriched with 5% CO_2 (HC)]. However, contrary to C3 plants, even the double mutants in the 2 known 2PG degrading routes operating in *Synechocystis*, the C2 cycle and glycerate pathway, were able to grow under ambient CO_2 conditions [ambient air with 0.035% CO_2 (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_i-regulated genes (24, 25). Interestingly, some of the genes up-regulated under low CO_2 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_2.

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_2-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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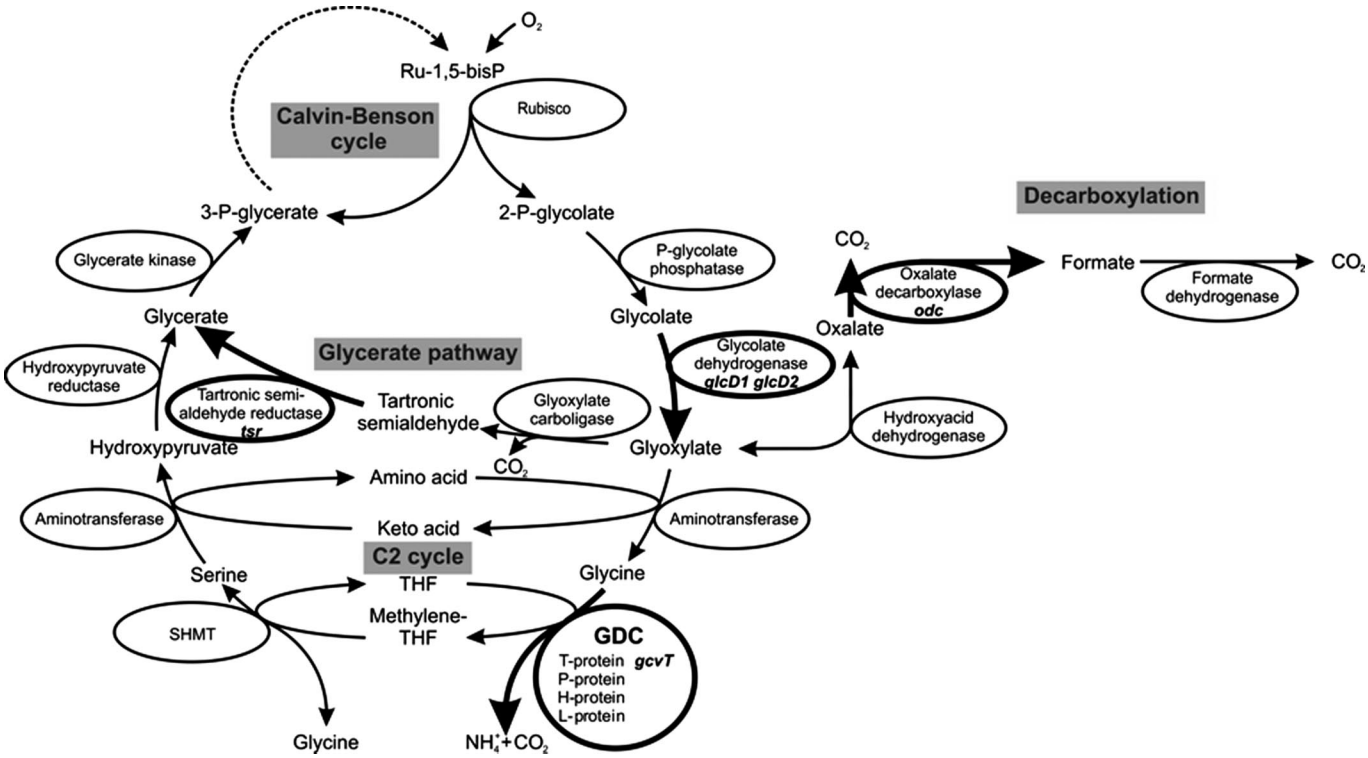

Fig. 1. A scheme displaying the 2PG metabolism in *Synechocystis* sp. strain PCC 6803, which employs 3 different routes: C2 cycle, glycerate pathway, and the decarboxylating branch. Enzymatic steps mutated in strains used for this study are indicated in bold.

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 decarboxlyation 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 Δ*gcvT*/Δ*tsr*/Δ*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. 2*A*). Surprisingly, the triple mutant exhibited a HCR phenotype: it was unable to grow at air level of CO_2 (Fig. 2*B*). 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 (Δ*odc* < Δ*gcvT*/Δ*odc* < Δ*gcvT*/Δ*tsr*/Δ*odc*; Fig. 2*C*) 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 Δ*glcD* 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 Δ*glcD* raised the possibility of a potential bypass for glycolate oxidation in cyanobacteria. A search in the *Synechocystis* genome identified a gene, *slr*0806, potentially coding an alternative GlcD. The amino acid sequence of Slr0806 shares 46% similarity with GlcD1 (Sll0404) 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 ≈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 Δ*glcD1* mutant at LC in contrast to the HCR phenotype of the triple mutant.

To verify this assumption, we inactivated *glcD2* in *Synechocystis* generated a double mutant in which both potential GlcDs were defective. The double mutant Δ*glcD1*/Δ*glcD2* was isolated under HC, and PCR analyses proved that all the WT-copies of *glcD1* and *glcD2* 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 (≈0.02 μmol of

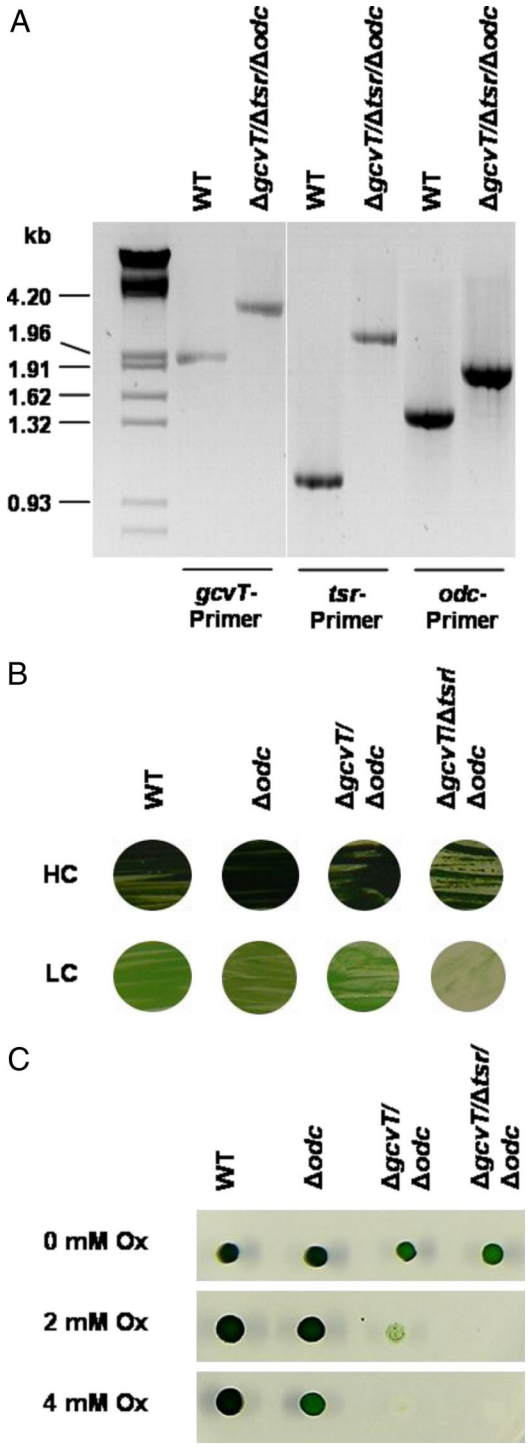

Fig. 2. Genotypic and phenotypic characterization of the triple mutant Δ*gcvT*/Δ*tsr*/Δ*odc*. (*A*) Complete segregation of the mutant Δ*gcvT*/Δ*tsr*/Δ*odc* was verified by PCR with gene-specific primers (see SI Table 1). (*B*) Growth of WT, single mutant Δ*odc* blocked in the decarboxylation branch, double mutant Δ*odc*/Δ*gcvT* blocked in the decarboxylation branch and C2 cycle, and triple mutant Δ*gcvT*/Δ*tsr*/Δ*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 μmol of photons per s per m^2 at 30 °C for 7 d. (*C*) Resistance of WT, single mutant Δ*odc*, double mutant Δ*odc*/Δ*gcvT*, and triple mutant Δ*gcvT*/Δ*tsr*/Δ*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 μmol of photons per s per m^2 at 30 °C and HC for 7 d. [*C* reproduced with permission from ref. 41].

glycolate per mL of cell volume). It became ≈200-fold higher in mutant Δ*glcD2* and even higher in mutant Δ*glcD1* (Fig. 3*A*). Importantly, compared with the single mutants, the double mutant Δ*glcD1*/Δ*glcD2* accumulated far more glycolate (Fig. 3*A*). It should be noted that glycolate was detected in cells of mutants Δ*glcD1* and Δ*glcD1*/Δ*glcD2* even when grown under HC, whereas only traces were observed in HC-cells of mutant Δ*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 Δ*glcD1* than in Δ*glcD2* (Fig. 3*A*), we conclude that the former is the major GlcD in *Synechocystis*. This assumption is supported by the fact that the single Δ*glcD1* mutant, but not Δ*glcD2*, grew slower than the WT under both HC and LC. Interestingly, the double mutant Δ*glcD1*/Δ*glcD2* could grow under HC, albeit somewhat slower than the WT, but could not grow under LC (Fig. 3*B*). 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 Δ*glcD1*/Δ*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 Δ*gcvT*/Δ*tsr*/Δ*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 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 C_i. Analysis of the photosynthetic parameters showed that this was not the case in the Δ*glcD* mutants (Fig. 3*C*). When grown under HC, the apparent photosynthetic affinity for extracellular C_i and the V_{max} were somewhat lower in the Δ*glcD1*/Δ*glcD2* mutant as compared with WT. The $K_{1/2}$ for C_i of the double mutant was ≈0.35 mM HCO_3^-, whereas this value increased ≈100 times to 40 mM HCO_3^- in the Δ*ccmM* mutant (19) or to >20 mM HCO_3^- in the Δ5 mutant (30), where the carboxysomes were absent and all the genes encoding known 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 C_i. In this respect, the double mutant Δ*glcD1*/Δ*glcD2* resembled the Δ*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 Δ*glcD1*/Δ*glcD2* to LC for 6 h, the photosynthetic $K_{1/2}$ (C_i) decreased considerably as in the WT (Fig. 3*C*), indicating that the induction of a more efficient CCM was not sufficient to enable growth under LC. Interestingly, the photosynthetic V_{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 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 Δ*gcvT*/Δ*tsr*/Δ*odc* that showed the

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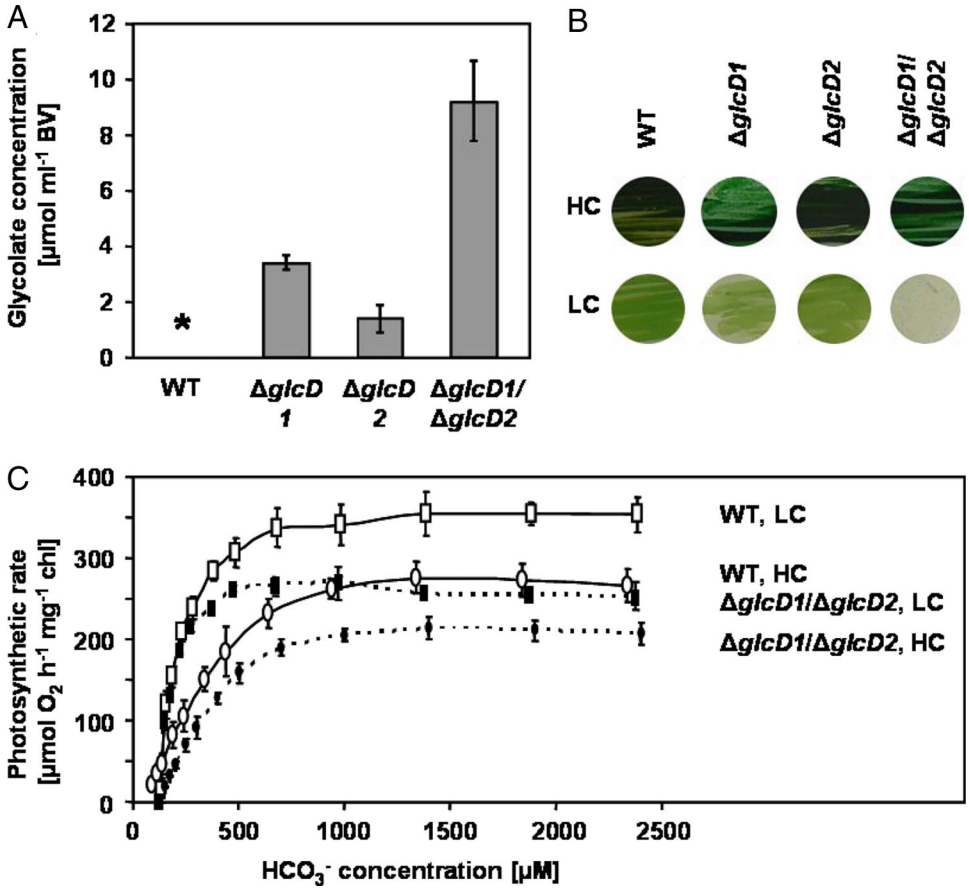

Fig. 3. Phenotypic characterization of single mutants and a double mutant of *Synechocystis* defective in GlcDs. (*A*) Quantification of intracellular glycolate in cells of single- (Δ*glcD1*, Δ*glcD2*) and double- (Δ*glcD1*/Δ*glcD2*) mutants in the glycolate converting step. Samples were taken 3 h after 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 Δ*glcD1* or Δ*glcD2*, and double mutant Δ*glcD1*/Δ*glcD2* under HC or LC. Strains were plated on BG11, pH 7, solidified by 0.9% Kobe agar, and incubated under continuous illumination of 30 μmol of photons per s per m^2 at 30 °C for 7 d. (*C*) Photosynthesis rates of cells of the WT and the double mutant Δ*glcD1*/Δ*glcD2* at different concentrations of HCO_3^- as a source for C_i. 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 Δ*glcD* 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 Δ*glcD1*/Δ*glcD2* 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 Δ*glcD1* 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. 3*C*) 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 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 Δ*gcvT*/Δ*tsr*/Δ*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 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	–	+	–	At5 g36790*	
	sll1349	alr4944	+	–		
Glycolate DH						(At5g06580‡)
GlcD1	sll0404	alr5269	–	+		
GlcD2	slr0806	all4443	+	–		
Glycolate oxidase	–	all0170	–	+	At4g18360†	**At4 g18360**
Serine/glyoxylate aminotransferase	sll1559	alr1004	+	+	At2g13360†	
Glycine decarboxlyase						
P-protein	slr0293	all4607	+	+	At4g33010‡	**At4g33010**
T-protein	sll0171	all4609	+	+	At1g11860‡	(At3g16950*)
H-protein	slr0879	all4608	+	+	At2g35370‡	
L-protein	slr1096	alr4745	+	+	At3g17240‡	
Serine hydroxymethyl-transferase	sll1931	alr4806	+	+	At4g37930‡	(At4g32520*)
Glutamate/glyoxylate aminotransferase	slr0006	alr2765	+	–	At1g23310†	
Hydroxypyruvate reductase	sll1908	alr1890	+	+	At1g68010†	(At1g17745*)
Glycerate kinase						
Bacterial type	slr1840	–	–	–		
Plant type	–	alr2873	+	+	At1g80380*	**At1g80380**
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) 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.

†The mature protein is predicted to be localized in the *Arabidopsis* peroxisome.

‡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 μg·mL^{-1} kanamycin (Km), 20 μg·mL^{-1} spectinomycin (Sp) or at 25 μg·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 μmol of photons per s per m^2 (warm light; Osram L58 W32/3) bubbling (flow rate $\approx$5 mL·min^{-1}) with air enriched with CO_2 (HC) in the BG11 medium at pH 7.0. C_i limitation was set by transferring exponentially growing cultures (OD_{750} 0.9, volume 130 mL) from bubbling with CO_2-enriched air to bubbling with ambient air ($\approx$0.035%, LC). Growth was monitored by measurements of the optical density at 750 nm (OD_{750}). Agar plates (BG 11, pH 7, solidified by 0.9% Kobe agar) were incubated under continuous illumination of 30 μmol of photons per s per 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 *slr*0806 (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 μg 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 μL 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 CO_2-dependent O_2 evolution as a function of C_i concentration was determined by using a Clark type O_2 electrode (PS2108, Passport dissolved O_2 sensor) essentially as described in ref. 39. The cells were harvested by centrifugation and resuspended in a CO_2-free medium containing 10 mM NaCl and 20 mM Hepes, pH 7.5. They were then placed in the O_2 electrode chamber at 30 °C, 300 μmol of photons per s per m^2 and allowed to use the C_i in their medium until they reached the CO_2 compensation point. Aliquots of $NaHCO_3$ of known concentrations were injected to raise the C_i concentration by known increments while measuring the resulting rise in the rate of O_2 concentration in the chamber.

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1. Tolbert NE (1997) The C-2 oxidative photosynthetic carbon cycle. *Ann Rev Plant Physiol Plant Mol Biol* 48:1–25.
2. Somerville CR (2001) An early Arabidopsis demonstration. Resolving a few issues concerning photorespiration. *Plant Physiol* 125:20–24.
3. Deusch O, *et al.* (2008) Genes of cyanobacterial origin in plant nuclear genomes point to a heterocyst-forming plastid ancestor. *Mol Biol Evol* 25:748–761.
4. Husic DW, Husic HD, Tolbert NE (1987) The oxidative photosynthetic carbon cycle or C2 cycle. *CRC Crit Rev Plant Sci* 5:45–100.
5. Norman EG, Colman B (1991) Purification and characterization of phosphoglycolate phosphatase from the cyanobacterium *Coccochloris peniocystis*. *Plant Physiol* 95:693–698.
6. Bowes G, Ogren WL, Hageman RH (1971) Phosphoglycolate production catalyzed by ribulose diphosphate carboxylase. *Biochem Biophys Res Commun* 45:716–722.
7. Ogren WL (1984) Photorespiration—Pathways, regulation, and modification. *Ann Rev Plant Physiol* 35:415–442.
8. Boldt R, *et al.* (2005) D-GLYCERATE 3-KINASE, the last unknown enzyme in the photorespiratory cycle in Arabidopsis, belongs to a novel kinase family. *Plant Cell* 17:2413–2420.
9. Engel N, *et al.* (2007) Deletion of glycine decarboxylase in Arabidopsis is lethal under nonphotorespiratory conditions. *Plant Physiol* 144:1328–1335.
10. Schwarte S, Bauwe H (2007) Identification of the photorespiratory 2-phosphoglycolate phosphatase, PGLP1, in Arabidopsis. *Plant Physiol* 144:1580–1586.
11. Colman B (1989) Photosynthetic carbon assimilation and the suppression of photorespiration in the cyanobacteria. *Aquatic Botany* 34:211–231.
12. Badger MR (1980) Kinetic properties of ribulose 1,5-bisphosphate carboxylase/oxygenase from *Anabaena variabilis*. *Arch Biochem Biophys* 201:247–255.
13. Kaplan A, Reinhold L (1999) CO_2 concentrating mechanisms in photosynthetic microorganisms. *Annu Rev Plant Physiol Plant Mol Biol* 50:539–570.
14. Badger MR, Price GD, Long BM, Woodger FJ (2006) The environmental plasticity and ecological genomics of the cyanobacterial CO_2 concentrating mechanism. *J Exp Bot* 57:249–265.
15. Raven JA, Cockell CS, De La Rocha CL (2008) The evolution of inorganic carbon concentrating mechanisms in photosynthesis. *Philos Trans R Soc London Ser B* 363:2641–2650.
16. Marcus Y, Schwarz R, Friedberg D, Kaplan A (1986) High CO_2 requiring mutant of *Anacystis nidulans* R2. *Plant Physiol* 82:610–612.
17. Omata T, Ogawa T, Marcus Y, Friedberg D, Kaplan A (1987) Adaptation to low CO_2 level in a mutant of *Anacystis nidulans* R2 which require high CO_2 for growth. *Plant Physiol* 83:892–894.
18. Marco E, Martinez I, Ronen-Tarazi M, Orus MI, Kaplan A (1994) Inactivation of *ccmO* in *Synechococcus* sp. strain PCC 7942 results in a mutant requiring high levels of CO_2. *Appl Environ Microbiol* 60:1018–1020.
19. Berry S, Fischer JH, Kruip J, Hauser M, Wildner GF (2005) Monitoring cytosolic pH of carboxysome-deficient cells of *Synechocystis* sp. PCC 6803 using fluorescence analysis. *Plant Biol* 7:342–347.
20. Omata T, *et al.* (1999) Identification of an ATP-binding cassette transporter involved in bicarbonate uptake in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *Proc Natl Acad Sci USA* 96:13571–13576.
21. Bonfil DJ, *et al.* (1998) A putative HCO_3^- transporter in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *FEBS Lett* 430:236–240.
22. Price GD, Woodger FJ, Badger MR, Howitt SM, Tucker L (2004) Identification of a SulP-type bicarbonate transporter in marine cyanobacteria. *Proc Natl Acad Sci USA* 101:18228–18233.
23. Eisenhut M, *et al.* (2006) The plant-like C2 glycolate cycle and the bacterial-like glycerate pathway cooperate in phosphoglycolate metabolism in cyanobacteria. *Plant Physiol* 142:333–342.
24. Wang HL, Postier BL, Burnap RL (2004) Alterations in global patterns of gene expression in *Synechocystis* sp. PCC 6803 in response to inorganic carbon limitation and the inactivation of *ndhR*, a LysR family regulator. *J Biol Chem* 279:5739–5751.
25. Eisenhut M, *et al.* (2007) Long-term response toward inorganic carbon limitation in wild type and glycolate turnover mutants of the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Plant Physiol* 144:1946–1959.
26. Pellicer MT, Badía J, Aguilar J, Baldomà L (1996) *glc* locus of *Escherichia coli*: Characterization of genes encoding the subunits of glycolate oxidase and the glc regulator protein. *J Bacteriol* 178:2051–2059.
27. Ogawa T, Kaplan A (2003) Inorganic carbon acquisition systems in cyanobacteria. *Photosyn Res* 77:105–115.
28. Ogawa T, Marco E, Orus MI (1994) A gene (*ccmA*) required for carboxysome formation in the cyanobacterium *Synechocystis* sp. strain PCC6803. *J Bacteriol* 176:2374–2378.
29. Schwarz R, Reinhold L, Kaplan A (1995) Low activation state of ribulose-1,5-bisphosphate carboxylase/oxygenase in carboxysome-defective *Synechococcus* mutants. *Plant Physiol* 108:183–190.
30. Xu M, *et al.* (2008) Properties of mutants of *Synechocystis* sp. strain PCC 6803 lacking carbon sequestering systems. *Plant Cell Physiol*, in press.
31. Schwarz R, Lieman-Hurwitz J, Hassidim M, Kaplan A (1992) Phenotypic complementation of high CO_2-requiring mutants of the cyanobacterium *Synechococcus* sp. strain PCC 7942 by inosine 5′-monophosphate. *Plant Physiol* 100:1987–1993.
32. Nishimura T, *et al.* (2008) Mechanism of low CO_2-induced activation of the *cmp* bicarbonate transporter operon by a LysR family protein in the cyanobacterium *Synechococcus elongatus* strain PCC 7942. *Mol Microbiol* 68:98–109.
33. Emlyn-Jones D, Woodger FJ, Andrews TJ, Price GD, Whitney SM (2006) A *Synechococcus* PCC7942 Delta ccmM (Cyanophyceae) mutant pseudoreverts to air growth without regaining carboxysomes. *J Phycol* 42:769–777.
34. Eisenhut M, Bauwe H, Hagemann M (2007) Glycine accumulation is toxic for the cyanobacterium *Synechocystis* sp. strain PCC 6803, but can be compensated by supplementation with magnesium ions. *FEMS Microbiol Lett* 277:232–237.
35. Dufresne A, *et al.* (2003) Genome sequence of the cyanobacterium *Prochlorococcus marinus* SS120, a nearly minimal oxyphototrophic genome. *Proc Natl Acad Sci USA* 100:10020–10025.
36. Martin W, *et al.* (2002) Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc Natl Acad Sci USA* 99:12246–12251.
37. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
38. Hagemann M, Schoor A, Jeanjean R, Zuther E, Joset F (1997) The *stpA* gene form *Synechocystis* sp. strain PCC 6803 encodes the glucosylglycerol-phosphate phosphatase involved in cyanobacterial osmotic response to salt shock. *J Bacteriol* 179:1727–1733.
39. Kaplan A, Marcus Y, Reinhold L (1988) Inorganic carbon uptake by cyanobacteria. *Methods Enzymol* 167:534–539.
40. Altschul SF, *et al.* (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402.
41. Eisenhut M, *et al.* (2008) Oxalate decarboxylase is involved in turnover of 2-phosphoglycolate in *Synechocystis* sp. strain PCC 6803. *Photosynthesis: Energy from the sun*, eds Allen JF, Gantt E, Golbeck JH, Osmond B (Springer, Netherlands).