The Plant-Like C2 Glycolate Cycle and the Bacterial-Like Glycerate Pathway Cooperate in Phosphoglycolate Metabolism in Cyanobacteria[1]

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The occurrence of a photorespiratory 2-phosphoglycolate metabolism in cyanobacteria is not clear. In the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803, we have identified open reading frames encoding enzymes homologous to those forming the plant-like C2 cycle and the bacterial-type glycerate pathway. To study the route and importance of 2-phosphoglycolate metabolism, the identified genes were systematically inactivated by mutagenesis. With a few exceptions, most of these genes could be inactivated without leading to a high-CO_2-requiring phenotype. Biochemical characterization of recombinant proteins verified that *Synechocystis* harbors an active serine hydroxymethyltransferase, and, contrary to higher plants, expresses a glycolate dehydrogenase instead of an oxidase to convert glycolate to glyoxylate. The mutation of this enzymatic step, located prior to the branching of phosphoglycolate metabolism into the plant-like C2 cycle and the bacterial-like glycerate pathway, resulted in glycolate accumulation and a growth depression already at high CO_2. Similar growth inhibitions were found for a single mutant in the plant-type C2 cycle and more pronounced for a double mutant affected in both the C2 cycle and the glycerate pathway after cultivation at low CO_2. These results suggested that cyanobacteria metabolize phosphoglycolate by the cooperative action of the C2 cycle and the glycerate pathway. When exposed to low CO_2, glycine decarboxylase knockout mutants accumulated far more glycine and lysine than wild-type cells or mutants with inactivated glycerate pathway. This finding and the growth data imply a dominant, although not exclusive, role of the C2 route in cyanobacterial phosphoglycolate metabolism.

In all phototrophic organisms, photosynthetic carbon fixation is catalyzed by the bifunctional enzyme Rubisco. Since both CO_2 and O_2 serve as substrates, 2-phosphoglycolate (2-PG) is produced in addition to 3-phosphoglycerate, which is metabolized in the Calvin cycle. 2-PG inhibits the Calvin-cycle enzyme triosephosphate isomerase (Husic et al., 1987; Norman and Colman, 1991) and needs to be metabolized rapidly. In higher plants, this is accomplished by the action of at least 10 different enzymes comprising the photorespiratory glycolate pathway, also known as the C2 cycle (Tolbert, 1997). Though quite well studied in higher plants, the existence and function of this pathway in cyanobacteria is not clear. It is widely accepted that the activity of the cyanobacterial CO_2-concentrating mechanism (CCM) raises the level of CO_2 in close proximity to Rubisco and thereby efficiently inhibits oxygenase activity (Kaplan and Reinhold, 1999; Giordano et al., 2005; Badger et al., 2006). Nevertheless, glycolate excretion could be detected in some cyanobacterial strains, e.g. *Anabaena cylindrica*, *Anabaena variabilis*, *Anabaena flos-aquae*, *Coccochloris peniocystis*, *Nostoc* sp. 73102, *Phormidium molle*, or *Plectonema boryanum*, particularly after transfer from high CO_2 to air or exposure to elevated oxygen concentrations (Norman and Colman, 1988; Renström and Bergman, 1989). Furthermore, the activities of several C2-cycle enzymes, namely, 2-PG phosphatase (PGP), Glu-glyoxylate aminotransferase, and hydroxypyruvate reductase (HPR), were demonstrated in extracts of cyanobacterial cells (Norman and Colman, 1988, 1991; Renström and Bergman, 1989). Also, a thylakoid-associated glycolate dehydrogenase (Glc) activity was found (Sallal and Codd, 1975; Grodzinski and Colman, 1976). In contrast, significant activity of the Ser hydroxymethyltransferase (SHMT), which catalyzes the Gly-to-Ser interconversion, was not observed (Codd and Stewart, 1973). The supposed absence of SHMT was supported by inhibitor experiments, in which the SHMT inhibitor isonicotinyl hydrazide did

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not block cyanobacterial glycolate metabolism (Miller et al., 1971; Cheng et al., 1972; Codd and Stewart, 1973; Ingle and Colman, 1976; Norman and Colman, 1988). Therefore, the significance and fate of intermediates of the glycolate pathway in cyanobacteria is not well known. It has been suggested that the glycolate pathway is incomplete and that an alternate pathway must exist to metabolize the glycolate formed (Norman and Colman, 1988).

Such an alternative might be the bacterial glycerate pathway. It was shown that extracts of *A. cylindrica* catalyze the decarboxylation of glyoxylate to tartronic semialdehyde by glyoxylate carboligase (GCL) with subsequent reduction of tartronic semialdehyde to glycerate by tartronic semialdehyde reductase (TSR; Codd and Stewart, 1973). Nevertheless, these earlier studies also did not reveal a clear biochemical pathway for the salvage of 2-PG carbon to Calvin-cycle metabolites.

With the completion of many cyanobacterial genome sequences, it became possible to search, in silico, for the existence of genes encoding enzymes homologous to those comprising the plant C2 cycle. Surprisingly, for most of the enzymes known to be involved in the C2 cycle, genes encoding homologous proteins could be found in the genome of *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis*; Kaneko et al., 1996; see Fig. 1), *Anabaena* sp. PCC 7120, or *Prochlorococcus* spp. (http://www.kazusa.or.jp/cyanobase) and others. The presence of these genes, even in the smallest genome of *Prochlorococcus* spp., might indicate that the glycolate metabolism is essential for the photosynthetic mode of life in cyanobacteria, as it is in plants.

In the first attempt to investigate, at the molecular level, the importance of the C2 cycle in cyanobacteria, *Synechocystis* mutants impaired in the Gly decarboxylase complex (GDC) were generated (Hagemann et al., 2005). In contrast to plants and other organisms, the GDC seemed to be dispensable in *Synechocystis* even under conditions stimulating glycolate formation (Hagemann et al., 2005). This result could be interpreted in three ways: (1) due to the presence of the CCM, the proportion of total carbon flux through the C2 cycle is rather low; (2) glycolate could be metabolized

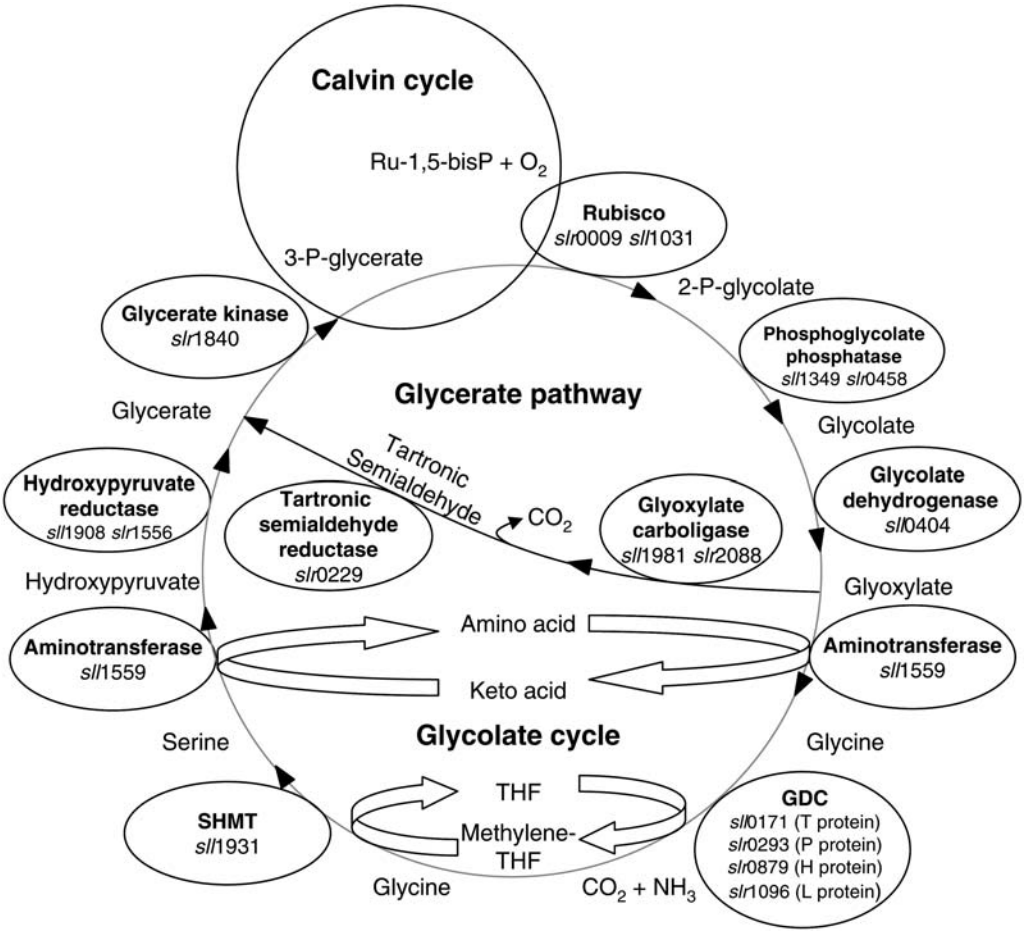

Figure 1. Pathways of 2-PG metabolism by the plant-like photorespiratory C2 cycle (outer circle, glycolate cycle) or the bacterial-like glycerate pathway (inner branch). The metabolites involved, enzymatic steps, and putative genes encoding the relevant enzymes in *Synechocystis* (http://www.kazusa.or.jp/cyanobase/Synechocystis/index.html) are shown. Ru-1,5-bisP, Ribulose-1,5-bisphosphate.

by an alternate route, e.g. by the glycerate pathway; and (3) the hypothetical glycolate pathway might be essential under the changing and complex conditions in nature but dispensable under laboratory conditions.

In this study, the route and importance of glycolate metabolism in cyanobacteria was systematically analyzed using *Synechocystis* as a model organism. All the open reading frames (ORFs) showing considerable similarities to proteins known to participate in the glycolate metabolism in plants and bacteria were target of a mutational approach (see Table I). Growth and the level of specific metabolites under both high and low CO_2 were analyzed in several of these mutants, which were selected according to the position of the affected enzyme in the two candidate pathways for glycolate metabolism. As a result of this analysis, clear indications were found for the parallel action of the plant-like C2 cycle and the bacterial-like glycerate pathway.

RESULTS AND DISCUSSION

Genes Encoding Enzymes Involved in 2-PG Metabolism in *Synechocystis*

Genes of *Synechocystis* homologous to those encoding enzymes involved in the C2 cycle of plants or the glycerate pathway of bacteria are shown in Table II. For PGP, HPR, and GCL, the *Synechocystis* genome appears to encode two proteins of identical enzymatic activity, which might catalyze the same reaction during 2-PG metabolism. It is also remarkable that the enzymes listed in Table II represent a mixture of plant- and bacterial-type enzymes. For example, *Synechocystis* harbors a bacterial-type glycerate kinase, which is distinct from the glycerate kinase present in plants, fungi, and some other cyanobacteria, such as *Anabaena* sp. strain PCC 7120 (Boldt et al., 2005). The same is true for PGP and Glc, which show no similarity to the corresponding enzymes from Arabidopsis. In contrast, Ala:glyoxylate aminotransferase (AGT), the four GDC subunits, SHMT, and HPR are clearly related to plant proteins (Table II).

From this in silico analysis, we conclude that genes encoding all the enzymes known to participate in glycolate metabolism in other organisms exist in *Synechocystis* and other cyanobacteria, too (results of corresponding searches in other cyanobacterial genomes are not shown). These findings allowed the construction of a hypothetical cyanobacterial glycolate cycle, comprising a complete C2 cycle, which overlaps with and is short-circuited by two enzymatic steps of the glycerate pathway (Fig. 1).

Synechocystis Exhibits Glc and SHMT Activities

To gain direct experimental support for this hypothesis, we first examined the biochemical functionality of two enzymes of this putative 2-PG-metabolizing pathway, encoded by *glcD* (*sll*0404) and *shm* (*sll*1931)

Table I. *Strategies applied to inactivate selected ORFs in Synechocystis*

The oligonucleotides were used to amplify the sequences encoding the candidate genes together with flanking regions. The insertion sites for the kanamycin (Km^R)-, chloramphenicol (Cm^R)-, or spectinomycin-resistance (Sp^R) cartridges are given. ORFs *sll*1349 and *slr*0458 were obtained as two PCR fragments. The resistance genes were inserted in between added restriction sites leading to deletions (D) of 267 bp in *sll*1349 and 59 bp in *slr*0458, respectively. ORF *sll*0171 was cut with *Msc*I, leading to deletion of 1.0 kb. In the case of ORF *slr*2088, restriction with *Sna*BI led to a deletion of 0.24 kb. All other insertion sites were unique. Additionally introduced restriction sites are underlined in the oligonucleotide sequences.

ORF	Oligonucleotides	Insertion Site	Inserted Cartridge
*sll*1349	Fw1 5′-TTG GGC GGA ACG GGC CG-3′		Km^R
	Rev1 5′-GGG GGA TCC AGA ATG CGG TAA TCC CG-3′	*Bam*HI	
	Fw2 5′-TAA CTC TGC TCA ACC AA-3′	D	
	Rev2 5′-GGG CTG CAG ACT AGT AAC CAG GGC ATG-3′	*Pst*I	
*slr*0458	Fw1 5′-TGA CCT CGA TGG AGT AT-3′		Cm^R
	Rev1 5′-GGG GGA TCC AGA ATG CGG TAA TCC CG-3′	*Bam*HI	
	Fw2 5′-GAG TGG GTT TAT GGT CG-3′	D	
	Rev2 5′-GGG GCA TGC CTC CTC ACT AAA GCT CC-3′	*Sph*I	
*sll*0404	Fw 5′-CTC GAG ATG GCC ATT TTC TCC-3′	*Eco*RV	Km^R
	Rev 5′-GAA TTC TCA ATA AAT TTC CTC-3′		
*sll*1559	Fw 5′-CTC GAG ATG GAT AAT AAG CAA-3′	*Stu*I	Sp^R
	Rev 5′-GAA TTC TTA ACC TTT AGC CAA-3′		
*sll*0171	Fw 5′-AGA CCT GAA GGA AGC TGT AG-3′	*Msc*I, D	Sp^R
	Rev 5′-GAG GAA GTG GTG CAC AGG TT-3′		
*sll*1931	Fw 5′-GCT ATT ACG GCG GCT GTG AA-3′	*Sma*I	Km^R
	Rev 5′-CCA TGA CGG CCA CAA CTG AA-3′		
*sll*1981	Fw 5′-CCG GAT TCG TTA GGC TAG-3′	*Afe*I	Km^R
	Rev 5′-AGT TAG CGT CGA TTT GGT-3′		
*slr*2088	Fw 5′-CGA TTG AGT TAA AAT TAG-3′	*Sna*BI, D	Km^R
	Rev 5′-GGT CAA GAA AAA CCG AGG-3′		
*slr*0229	Fw 5′-ATA AGT CAG AGA AGT GAA-3′	*Hpa*I	Km^R
	Rev 5′-CCA TGT TTA CTC CAG TAA-3′		

Table II. *Sequence comparison of proteins participating in phosphoglycolate turnover with candidate proteins from Synechocystis using PSI- and PHI-BLAST (Altschul et al., 1997)*

The similar proteins from bacteria or Arabidopsis are in most cases biochemical characterized.

Protein (Abbreviation)	Organism	Accession No.	Literature	Similarity e Value	ORF in *Synechocystis*
Phosphoglycolate phosphatase (PGP)	*A. eutrophus*	P40852	Schäferjohann et al. (1993)	$3e^{-08}$	*slr*0458
				$9e^{-06}$	*sll*1349
Glycolate dehydrogenase subunit D (GlcD)	*E. coli*	AAC76015	Pellicer et al. (1996)	$3e^{-126}$	*sll*0404
Ala:glyoxylate aminotransferase (AGT)	Arabidopsis	At2g13360	Liepman and Olsen (2001)	$3e^{-59}$	*sll*1559
GDC, P protein (GcvP)	Arabidopsis	At2g26080	H. Bauwe (unpublished data)	0	*slr*0293
GDC, T protein (GcvT)	Arabidopsis	At1g11860	H. Bauwe (unpublished data)	$7e^{-52}$	*sll*0171
GDC, H protein (GcvH)	Arabidopsis	At2g35120	H. Bauwe (unpublished data)	$2e^{-28}$	*slr*0879
GDC, L protein (GcvL)	Arabidopsis	At3g16950	H. Bauwe (unpublished data)	$1e^{-164}$	*slr*1096
Ser hydroxymethyltransferase (SHMT)	Arabidopsis	At4g37930	Voll et al. (2006)	$6e^{-94}$	*sll*1931
Hydroxypyruvate reductase (HPR)	Arabidopsis	At1g68010	H. Bauwe (unpublished data)	$2e^{-28}$	*sll*1908
				$2e^{-26}$	*slr*1556
Glycerate kinase (GLYK)	*E. coli*	AAB93855	Cusa et al. (1999)	$3e^{-64}$	*slr*1840
Glyoxylate carboligase (GCL)	*E. coli*	AAA23864	Chang et al. (1993)	$1e^{-94}$	*slr*2088
				$3e^{-58}$	*sll*1981
Tartronic semialdehyde reductase (TSR)	*E. coli*	P77161	Cusa et al. (1999)	$3e^{-35}$	*slr*0229

identified in the *Synechocystis* genome. The putative GlcD was selected, since in plants the C2 cycle employs a glycolate oxidase and, despite high similarities to bacterial glycolate dehydrogenases, the ORF *sll*0404 is annotated as a subunit of glycolate oxidase in CyanoBase (http://www.kazusa.or.jp/cyanobase/Synechocystis/cgi-bin/orfinfo.cgi?title=Chr&name=sll0404&iden=1). The occurrence of SHMT in cyanobacteria is not clear at all (Norman and Colman, 1988). The respective genes, *glcD* (*sll*0404) and *shm* (s*ll*1931), were heterologously expressed in *Escherichia coli*. Western-blot analyses, using a specific Strep-Tactin antibody, verified the expression and purification of the recombinant proteins, designated Syn-GlcD and Syn-SHMT, of 57 and 50 kD, respectively (Fig. 2). Specific activities were measured in crude extracts from *E. coli* cultures overexpressing these genes and with purified enzymes.

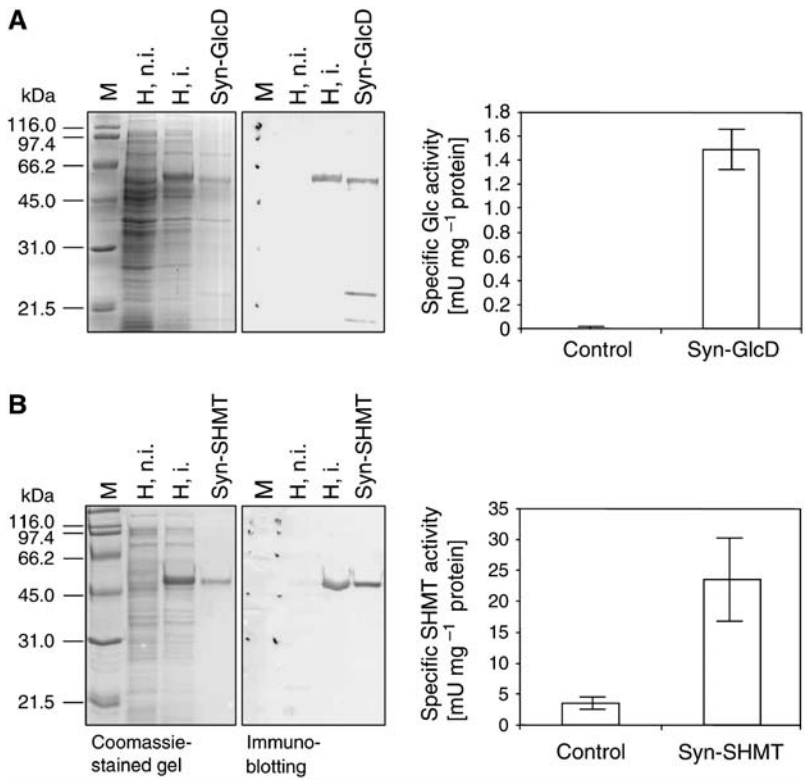

Figure 2. Overexpression of *glcD* and *shm* from *Synechocystis* in *E. coli* to verify their biochemical activities. A, GlcD from *Synechocystis* with a molecular mass of 57 kD. B, SHMT from *Synechocystis* with a molecular mass of 50 kD. The relevant genes were cloned in IBA6, overexpressed, purified by the fused Strep-tag, and the activity tested (see ''Materials and Methods''). M, Molecular mass standard (broad range; Bio-Rad); H, n.i., homogenate not induced; H, i., homogenate induced; Control, raw extract from not induced *E. coli* culture; Syn-GlcD, purified Sll0404; Syn-SHMT, purified Sll1931.

In agreement with the protein's primary structure, which indicates a flavin-binding site and shows highest similarities with glycolate dehydrogenase from *E. coli* (Table II), the GlcD protein showed NAD^+-dependent glycolate dehydrogenase but no glycolate oxidase activity. The specific activity of affinity-purified Syn-GlcD was distinctly higher in comparison with controls prepared from *E. coli* cultures grown under identical conditions but without the cyanobacterial *glcD* (Fig. 2). These data correspond to earlier reports on the occurrence of Glc in cyanobacteria (Sallal and Codd, 1975; Grodzinski and Colman, 1976) and show very clearly that, in *Synechocystis*, conversion of glycolate to glyoxylate is catalyzed by a glycolate dehydrogenase. This differs from the situation in plants where photorespiratory glycolate is converted to glycolate by glycolate oxidase. However, it should be mentioned that the genome of another cyanobacterium, *Anabaena* sp. strain PCC 7120, in addition to GlcD, encodes a protein (All0170), which is very similar to the plant-type peroxisomal glycolate oxidase.

SHMT activity was not recognized in earlier studies using crude cyanobacterial extracts (Codd and Stewart, 1973) or the SHMT inhibitor isonicotinyl hydrazide (Cheng et al., 1972; Ingle and Colman, 1976; Norman and Colman, 1988). However, it must be noted that the estimation of SHMT activity is difficult since the methylene acceptor molecule tetrahydrofolate (THF) is not very stable in aqueous O_2-containing solution. In our experiments, using recombinant cyanobacterial SHMT, the specific activity of overexpressed Syn-SHMT was 7 times higher in comparison with control cell extracts from *E. coli* cells without the cyanobacterial *shm* (Fig. 2B). This finding provides clear evidence for the presence of a functional SHMT in *Synechocystis*, which is encoded by *sll*1931 as annotated in CyanoBase.

Mutational Approach to Study the Involvement of Specific Proteins in 2-PG Metabolism

To clarify the participation and importance of proteins possibly involved in 2-PG metabolism, we next generated mutants in almost all enzymatic steps of the hypothetical 2-PG-metabolizing pathway depicted in Figure 1. In the first reaction, the 2-PG produced by the oxygenase activity of Rubisco is converted to glycolate by the activity of PGP. Schäferjohann et al. (1993) expressed the *cbbZ* gene from the nonphotosynthesizing bacterium *Alcaligenes eutrophus* and showed that the gene product exhibits PGP activity. *Synechocystis* possesses two ORFs, *sll*1349 and *slr*0458, which encode proteins with homology to *cbbZ* (http://www.kazusa.or.jp/cyanobase/Synechocystis/index.html). Inactivation of each of these genes resulted in completely segregated mutants, whose growth characteristics under various conditions, including high or low CO_2 levels, did not differ from that of the wild type (data not shown). A double mutant where both genes were inactivated grew very slowly on agar plates maintained at 3% CO_2 but not under air level of CO_2. The colonies obtained were yellowish and we could not grow the cells in liquid cultures to examine PGP activity. One colony that possibly emerged following a suppression mutation grew well on an agar plate and in liquid culture. It showed normal appearance and normal PGP activity (data not shown). While the data described here do not provide conclusive evidence, they lend support to the notion that PGP activity is essential even under high CO_2 conditions since it should prevent 2-PG inhibition of triosephosphate isomerase (Norman and Colman, 1991).

We then generated mutants impaired in GlcD (*sll*0404, *glcD*), the aminotransferase (*sll*1559), the T protein of GDC (*sll*0171, *gcvT*), SHMT (*slr*1931; *shm*), the two putative GCLs (*sll*1981 or *slr*2088, *gcl*), and TSR (*slr*0229, *tsr*; compare Table I). After selection of antibiotic-resistant *Synechocystis* colonies and progressive segregation by increasing drug concentrations, the genotypes were examined using PCR. Analysis of the DNA from single and double mutants, where *glcD*, *gcvT*, *gcl*, and *tsr* or *gcvT*/*gcl* and *gcvT*/*tsr* were inactivated, respectively, showed only the fragments corresponding to the inactivated gene but not fragments derived from the wild-type genes. Representative results for mutants Δ*glcD* and Δ*gcvT* are shown in Figure 3A. These data indicated complete segregation of the chromosomal copies and suggested that under the growth conditions used here, the Glc, GDC, GCL, and TSR were dispensable for cyanobacterial metabolism. These results also indicate

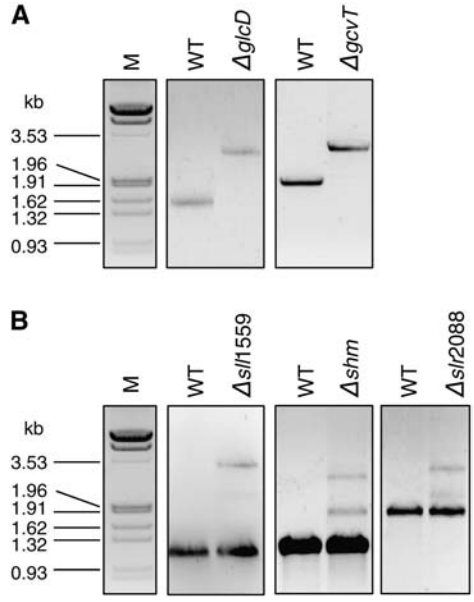

Figure 3. Segregation of the *Synechocystis* mutants Δ*glcD* and Δ*gcvT* (A) and Δ*sll*1559, Δ*shm*, and Δ*slr*2088 (B) was checked by PCR using total DNA of the mentioned strains and the gene-specific primers given in Table I. Abbreviations in A: M, λ-DNA *Eco*RI/*Hin*dIII; WT, wild-type 1.5 kb or 2.0 kb for *glcD* and *gcvT*, respectively. The modified (due to the insertions) *glcD* is 2.7 kb and *gcvT* is 3.0 kb, as expected. Abbreviations in B: M, λ-DNA *Eco*RI/*Hin*dIII; WT, wild-type 1.2 kb, 1.3 kb, or 2.0 kb; Δ*sll*1559, 3.2 kb; Δ*shm*, 2.5 kb; Δ*slr*2088, 3.0 kb.

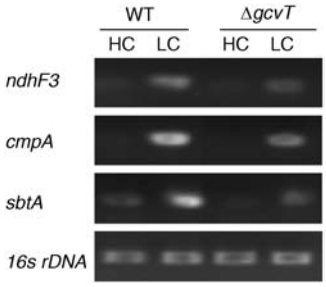

Figure 4. Influence of carbon limitation on the expression of certain genes in the wild type (WT) and a Δ*gcvT* mutant of *Synechocystis*. Total RNA was obtained from cells grown at 5% CO_2 (HC) or air level of CO_2 (LC). The transcript abundance was assessed by RT-PCR using the gene-specific primers given in the "Materials and Methods" section.

that the hypothetical C2 cycle as well as the glycerate pathway are not essential for viability under ambient CO_2 conditions at laboratory conditions, which, at first sight, may support the view that oxygenase activity of Rubisco is nearly zero in cyanobacteria.

In contrast, only partial segregation could be achieved for the genomes of those mutants where we attempted to inactivate *sll*1559, *shm*, or *slr*2088. In all these latter cases, a dominant fragment of the expected wild-type size was observed in addition to a faint band corresponding to the mutated gene copies (Fig. 3B). Raising the CO_2 concentration during cultivation hardly affected the degree of segregation, suggesting essential functions for the putative aminotransferase Sll1559, SHMT, and the hypothetical GCL Slr2088. A similar result was reported for mutations in the L-protein subunit of GDC, which also could not be completely segregated (Engels and Pistorius, 1997). Apparently, the respective gene products fulfill essential tasks in the cyanobacterial cell. For the L protein, such a task is probably its additional function as a subunit of the pyruvate dehydrogenase complex. SHMT, as in other organisms, might be essential for one-carbon metabolism. The Sll1559 and Slr2088 proteins are most probably involved in basic amino acid metabolism and therefore not dispensable (Kouhen and Joset, 2002).

CCM Activity and Expression of Low-CO_2-Dependent Genes in a GDC Mutant

It was possible that the lack of a clear phenotype in some of the *Synechocystis* mutants impaired in either the C2 cycle or the glycerate pathway is due to enhanced CCM activity or faster acclimation of the mutants to low levels of CO_2 in comparison with the wild type. To test this possibility, we used a membrane-inlet mass spectrometer to measure inorganic carbon uptake (Tchernov et al., 1997, 2003) and reverse transcription (RT)-PCR to assess the level of genes whose expression is strongly affected by the concentration of CO_2 (Shibata et al., 2002; McGinn et al., 2004; Wang et al., 2004). Detailed analyses did not show marked differences between the rate of CO_2 uptake in high-CO_2-grown wild type and mutants impaired in GlcD or GDC. Displacements of CO_2 concentration from equilibrium in the light, consequent of CO_2 uptake and inorganic carbon cycling (Tchernov et al., 2003), were essentially similar in the wild type and the mutants. Furthermore, acclimation of the cells to air level of CO_2 enhanced the rate of CO_2 uptake in both the wild type and the mutant to the same extent (data not shown).

Analysis of the abundance of transcripts originating from *ndhF3*, *cmpA*, and *sbtA*, which are known to be up-regulated in *Synechocystis* cells exposed to a low level of CO_2 (Shibata et al., 2002), were analyzed in wild type and selected mutant cells. In Figure 4, we show only representative data from the wild type and a mutant impaired in *gcvT* grown under high CO_2 and transferred to air level of CO_2 for 1 h. In principal, the mutant affected in the T-protein subunit of GDC showed similar CO_2-dependent regulation as wild-type cells. Under high CO_2, we did not find transcripts; however, a shift to air level of CO_2 resulted in the induction of these genes, even though a weaker transcription of these genes was detected in the mutant (Fig. 4). Taken together, the data from the membrane-inlet mass spectrometer and the analysis of the abundance of transcripts did not support the possibility that ability to withstand these mutations (albeit with some decline in the growth rates in mutants Δ*glcD* and Δ*gcvT*; see below) rested on enhanced CCM activity and thereby reduced metabolic flux in the glycolate pathway.

Growth Experiments

To find out whether there exist more subtle effects on the metabolic performance, we examined the growth rates under 5% CO_2 or air level of CO_2 for those mutants where the genes encoding GlcD (*glcD*), T protein of GDC (*gcvT*), or TSR (*tsr*) were individually inactivated. To distinguish between the effects on 2-PG turnover brought about by either the C2 cycle or the glycerate pathway or both (see Fig. 1), we also included the double mutant Δ*gcvT*/Δ*tsr* in these analyses.

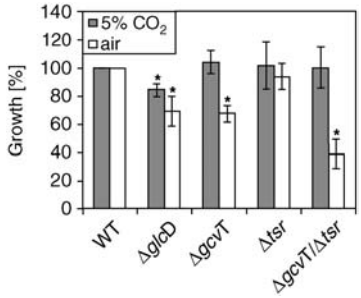

Figure 5. Influence of carbon limitation on growth of wild type (WT) and cells bearing mutations in the indicated genes of *Synechocystis*. Growth was measured as increase in OD_{750} over time. Means and SDs from three independent experiments are shown. Growth rates of the wild-type cells grown under 5% CO_2 or air were set to 100% (0.072 ± 0.007 h^{-1} and 0.021 ± 0.005 h^{-1}, respectively; *, statistically significant growth difference between wild-type and mutant cells, $P \leq 0.05$).

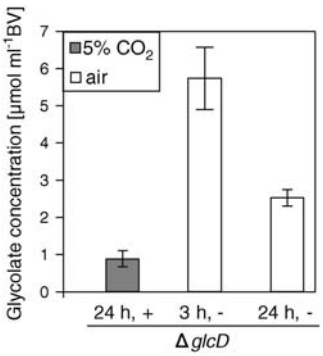

Figure 6. Glycolate content in the cells of mutant Δ*glcD* of *Synechocystis*. Samples were taken 3 or 24 h after transfer of high-CO_2-grown cells into air or after 24 h growth at 5% CO_2, respectively. Low-molecular-mass compounds were isolated from cell pellets, and glycolate was detected and quantified by HPLC.

When aerated with 5% CO_2 in air, the wild type and most of the mutants showed similar growth rates. The only exception was mutant Δ*glcD* (Fig. 5), where both the C2 cycle and the glycerate pathway (Fig. 1) were inactivated. This mutant strain grew significantly poorer and reached only about 85% of the wild-type growth rate. This can be interpreted as the first hint that 2-PG metabolism occurs in *Synechocystis* and is important even under high CO_2 conditions. When the wild-type and mutant cultures were transferred to air level of CO_2, their growth was significantly reduced. The least affected strains were wild type (growth rate of wild-type cells at 5% CO_2: 0.072 ± 0.007 h^{-1} growth rate of wild-type cells at air level of CO_2: 0.021 ± 0.005 h^{-1}) and the single mutant Δ*tsr* whose growth rate was reduced to one-third as compared to growth of high CO_2 cells. The growth rate of the other strains was only 40% to 70% that of the wild type under low CO_2 level. Here, mutants Δ*glcD* and Δ*gcvT* showed only about 70% of the growth rate of wild-type cells. Growth of the double mutant Δ*gcvT*/Δ*tsr*, where both branches of the hypothetical 2-PG metabolism are impaired, was even more reduced under air conditions (40% compared to wild type), unlike the corresponding single mutants Δ*gcvT* and particularly Δ*tsr* (Fig. 5).

Apparently, low CO_2 conditions trigger the differences between the wild type and several mutants impaired in glycolate metabolization. This shows that, under these conditions, the presence of a fully functional 2-PG metabolism becomes more important and provides strong support to the suggestion that the two branches of 2-PG metabolism, as shown in Figure 1, are indeed metabolically active in *Synechocystis*.

Levels of Glycolate, Gly, Ser, and Lys

We next analyzed whether the observed growth retardation of several strains is accompanied by a corresponding accumulation of intermediates of 2-PG metabolism. To this end, we examined the levels of glycolate and selected amino acids in wild-type and mutant cells grown under 5% CO_2 as well as in cells transferred for a few hours from high to low CO_2 conditions (Figs. 6 and 7). As reported for other cyanobacterial strains (Colman, 1989), extracts of *Synechocystis* cells grown at standard conditions with 5% CO_2 were essentially free of glycolate. In contrast to wild type and the other mutant strains, intracellular glycolate accumulation was observed only in the case of mutant Δ*glcD* (where the conversion of glycolate to glyoxylate should be inhibited). Glycolate accumulation in this mutant occurred even at 5% CO_2 (Fig. 6). Glycolate was at undetectable levels not only in samples from wild type and mutants Δ*gcvT* and Δ*tsr*, where either the C2 cycle or the glycerate pathway is inhibited, but also in the double mutant Δ*gcvT*/Δ*tsr*, where both routes are inhibited (data not shown). The significant glycolate accumulation in mutant Δ*glcD* even after 24 h in air, i.e. long after the CCM induction should have been completed (Woodger et al., 2005), suggests that the CO_2 concentration in close proximity to Rubisco within the carboxysomes may not be high enough to completely abolish the enzyme's oxygenase activity.

In some filamentous cyanobacterial strains, glycolate excretion was observed under selected conditions (Norman and Colman, 1988; Renström and Bergman, 1989), which, in principle, could also explain the undetectable low intracellular amounts of glycolate in *Synechocystis* and most of the analyzed mutants. To consider this possibility, too, we analyzed the glycolate contents of medium from low-CO_2-grown Δ*glcD* and wild-type cells and did not find any sign of glycolate accumulation (data not shown). We conclude that *Synechocystis* does not excrete significant amounts of

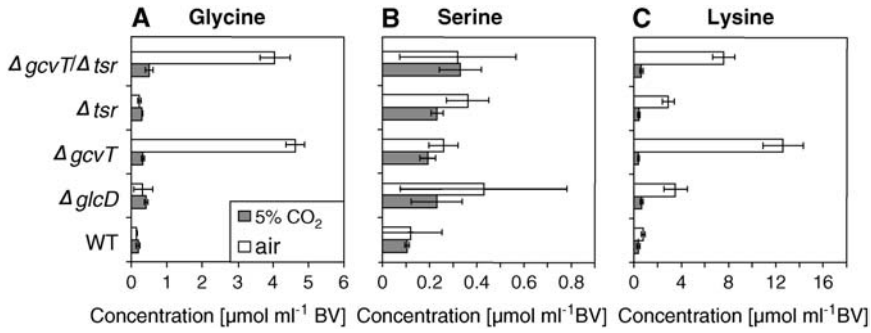

Figure 7. The intracellular content of Gly (A), Ser (B), and Lys (C) in cells of the wild type and mutants of *Synechocystis* grown under high (5%, gray columns) or ambient CO_2 (white columns) concentration. Low-molecular-mass compounds were isolated from cell pellets, and amino acids were detected and quantified by HPLC.

glycolate corresponding to the observed intracellular glycolate accumulation in mutant Δ*glcD*. Accordingly, the complete lack of a significant glycolate accumulation in wild-type cells under low and high CO_2 levels can therefore only be explained by the presence of an active glycolate metabolism in *Synechocystis*. The lack of glycolate accumulation in other mutants, particularly in the double mutant Δ*gcvT*/Δ*tsr*, may be attributed to accumulation of other intermediates like glyoxylate or tartronic semialdehyde, which we could not analyze. It may also indicate the existence of additional routes for the metabolization of glycolate and/or glyoxylate. For example, complete decarboxylation of glycolate via formate was proposed in previous studies (Norman and Colman, 1992).

The plant-type C2 cycle, by aminotransferase reactions and by the Gly-to-Ser interconversion via GDC and SHMT, is closely related to amino acid metabolism. The low level of glycolate in the Δ*gcvT*/Δ*tsr* mutant therefore prompted us to examine the possibility of changes in the level of selected amino acids (Fig. 7). When grown under 5% CO_2 in air, the intracellular levels of Gly, Ser, and Lys were not very strongly affected by the various mutations examined here. In contrast, when exposed to air level of CO_2, several of the mutants accumulated large quantities of Gly and Lys, but not Ser. The relatively small effect of CO_2 concentration on the accumulation of Ser is consistent with the data obtained with higher plants where the ratio of Gly to Ser is strongly affected by photorespiratory conditions, mainly due to altered levels of Gly (Snyder and Tolbert, 1974; Heineke et al., 2001; Novitskaya et al., 2002). Under air conditions, the intracellular contents of Gly and Lys were strikingly changed in the single Δ*gcvT* and in the double Δ*gcvT*/Δ*tsr* mutant, where the T-protein subunit of GDC was inactivated. These mutants accumulated much more Gly and Lys than did wild-type cells exposed to low CO_2. This may explain the slower growth of these mutants under low CO_2 (see Fig. 5) since elevated Gly concentrations appear to be toxic for *Synechocystis* (Hagemann et al., 2005). On the other hand, the normal growth phenotype of air-grown mutant Δ*tsr* is consistent with the lower levels of Gly and Lys observed in this mutant, relative to Δ*gcvT* (Fig. 7). The close correlation between the levels of Gly and Lys in the mutants points to the presence of a metabolic link between these two amino acids. Theoretically, Gly can be converted to Lys via the homoserine or, alternatively, via the malate-oxalacetate-Asp route. Most of the necessary enzymatic steps are obviously encoded in the *Synechocystis* genome (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.ad.jp/dbget-bin/get_pathway?org_name=syn&mapno=00300).

CONCLUSION

The data provided here give evidence that *Synechocystis* and probably many cyanobacteria not only produce significant amounts of 2-PG but also possess a complete 2-PG metabolism. Obviously, the cyanobacterial CCM is not efficient enough to completely abolish 2-PG production by Rubisco. We also show that the genes, which were assumed to encode the necessary enzymatic steps by comparisons to similar proteins from plants and bacteria, seem to be correctly assigned. The 2-PG metabolism is branched at the glyoxylate metabolizing reaction into the plant-type photorespiratory C2 cycle and the glycerate pathway found in bacteria. The directed mutation of several of the involved individual genes did not reduce viability. This is in contrast to plants, where most photorespiratory mutants do not grow at all at ambient CO_2 conditions (e.g. Bauwe and Kolukisaoglu, 2003). However, the deactivation of enzymes acting prior to the branchpoint of glyoxylate metabolization to either the C2 cycle or the glycerate pathway resulted in growth retardation even under high CO_2 conditions. Therefore, both pathways appear to work in parallel and cooperate by sharing common intermediates and several enzymatic steps. This joint operation avoids accumulation of potentially toxic substances, such as Gly or 2-PG, to levels that may lead to death as it was found in corresponding plant mutants (Heineke et al., 2001; Boldt et al., 2005). The metabolic regulation between these two routes of 2-PG processing is not known. However, the fact that mutants impaired in GDC accumulated much higher Gly than those defective in the glycerate pathway may indicate that the metabolic flux via the GDC is more significant than that via the glycerate pathway, but also raises the possible existence of additional routes to dissipate the formed glyoxylate.

MATERIALS AND METHODS

Strains and Culture Conditions

The Glc-tolerant *Synechocystis* sp. strain PCC 6803 was obtained from Prof. N. Murata (National Institute for Basic Biology, Okazaki, Japan) and served as the wild type. Axenic cultures were grown on agar plates at 30°C under constant illumination using agar-solidified BG11 medium (Rippka et al., 1979) buffered with 20 mM TES-KOH or 20 mM HEPES-NaOH to pH 8.0. Transformants were initially selected on media containing 10 μg mL^{-1} kanamycin (Km), 4 μg mL^{-1} spectinomycin (Sp), or 5 μg mL^{-1} chloramphenicol (Cm; Sigma), respectively, whereas the segregation of clones and cultivation of mutants were performed at 50 μg mL^{-1} Km, 20 μg mL^{-1} Sp, or 15 μg mL^{-1} Cm. For the physiological characterization, axenic cultures (about 10^8 cells mL^{-1}) were grown photoautotrophically in batch cultures at 29°C under continuous illumination of 130 μmol photons m^{-2} s^{-1} (warm light; Osram L58 W32/3) bubbling with air enriched with CO_2 (5% CO_2 in air) in the BG11 medium at pH 8.0. Cultivation at ambient CO_2 concentration (0.035% CO_2 in air) was performed by bubbling with air in BG11 medium at pH 7.0. Growth was monitored by measurements of the optical density at 750 nm (OD_{750}). Contamination by heterotrophic bacteria was checked by spreading of 0.2 mL of culture on LB plates.

The *Escherichia coli* strain TG1 (Sambrook et al., 1989), cultured in LB medium at 37°C, was used for routine DNA manipulations. *E. coli* strain BL21 Gold was used for heterologous expression of selected genes.

DNA Manipulations

Total DNA from *Synechocystis* was isolated according to Hagemann et al. (1997). All other DNA techniques, such as plasmid isolation, transformation of

E. coli, ligations, and restriction analysis (restriction enzymes were obtained from Life Technologies and New England Biolabs), were standard methods (Sambrook et al., 1989). PCR with gene-specific oligonucleotides (see Table I) was carried out using the Taq-PCR Master Mix (Qiagen). PCR products were cloned into pGEM-T (Promega). Sequence comparisons were made using the PSI- and PHI-BLAST software packages (Altschul et al., 1997).

Isolation of RNA and RT-PCR Analyses

Cultures were grown to a cell density of OD_{750} = 0.5 under 5% CO_2 and transferred to air level of CO_2 for various durations. Analyses of transcript abundances of *ndhF3*, *cmpA*, *sbtA*, and 16S rRNA genes were performed using RT-PCR on RNA isolated from the wild type and various mutants. The cells were lysed and total RNA was prepared as done by Katoh et al. (2001). One hundred nanograms of total RNA was used in the reverse transcriptase reaction using Superscript II (Invitrogen) and random hexamer primers to synthesize cDNA, followed by PCR amplification using gene-specific primers [*cmpA* (*slr*0040), Fw GAG CGG ATT GGG TAG ATA A, Rev GGT CAA TAT CGG TGT CAG GA; *sbtA* (*slr*1512), Fw CCT GGC TAA ACT GCC TAA C, Rev GGC TTG GTA AAT ATG CCC AG; *ndh*F3 (*sll*1732), Fw TTC CCG CCA CTA TTC TAA GG, Rev GGT AGG GCA ATA AGG GAT GC; *rnpB*, served as internal standard, Fw AGC GTC CGT AGG TGG TTA T, Rev GTG TCA GTT TCA GCC CAG T] for 12 to 25 cycles depending on the abundance of the transcript.

Generation of Mutants

To generate mutations in the selected ORFs, by insertion or deletion, the Km, Sp, or Cm resistance cartridges were integrated into the coding sequences at selected restriction sites (see Table I). The products were checked by restriction analysis. Plasmid DNA of these constructs was isolated from *E. coli* using the GFX micro plasmid prep kit (Amersham). About 1 μg of DNA was used for transformation of *Synechocystis*, and Km-, Sp-, and Cm-resistant clones, respectively, were selected (Hagemann et al., 1997).

Purification of Recombinant Syn-GlcD and Syn-SHMT

The genes encoding Syn-GlcD and Syn-SHMT were amplified by PCR using chromosomal *Synechocystis* wild-type DNA, gene-specific primers (Syn-GlcD: Fw 5′-CTC GAG ATG GCC ATT TTC TCC-3′ and Rev 5′-GAA TTC TCA ATA AAT TTC CTC-3′; Syn-SHMT: Fw 5′-CTC GAG GTG AAT CAA ACC AAC-3′ and Rev 5′-CAG CTG TCA GGC GAT CAC CGC-3′), and the proofreading Pfu polymerase. Each forward primer contained an extension with an additional *Xho*I site (underlined). PCR fragments were cloned into pGEM-T. The generated plasmids pG-GlcD and pG-SHMT were cleaved with *Xho*I/*Nco*I and *Xho*I/*Pst*I, respectively. After gel elution the fragments were ligated into the plasmid pASK-IBA6 (IBA) between the *Xho*I and *Nco*I or *Pst*I sites, respectively. The in-frame insertions led, after induction by anhydrotetracycline, to the expression of fusion proteins with an N-terminal Strep-tag, which facilitated protein purification using Strep-Tactin sepharose (IBA). *E. coli* BL 21 Gold strains containing IBA6-Syn-GlcD and IBA6-Syn-SHMT constructs, respectively, were grown in LB medium to an OD_{600} of 0.6. Expression of the fusion proteins was induced by addition of anhydrotetracycline (200 ng mL^{-1}). The cultures were allowed to grow for 16 h at 20°C. The cells were harvested by centrifugation and pellets were resuspended in buffer W (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, IBA). Under ice-cooling, the suspensions were sonicated (6 × 10 s, 90 W) and the homogenates were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was used for affinity chromatography following the protocol for Strep-tag protein purification under native conditions (IBA). The fractions of expression and purification were checked by western blotting using Strep-Tactin alkaline phosphatase conjugate (IBA) for detection.

Estimation of Enzyme Activities

Glycolate dehydrogenase (GlcD) activity was assayed by measuring the reduction of NAD^+ as an increase of absorption at λ = 340 nm. Cell extracts or elution fractions of 100 μL (1 μg protein μL^{-1}) were added to a mixture comprising 50 μL of 3 mg mL^{-1} NAD^+ and 840 μL of 40 mM potassium phosphate buffer, pH 7.0. The reactions were initiated by adding 10 μL of 0.8 M glycolate adjusted to pH 6.0.

SHMT activity was assayed according to Taylor and Weissbach (1965). The rate of methylene-THF production from ^{14}C-labeled Ser by SHMT was monitored. The produced methylene-THF was extracted after conversion to a dimedon complex with toluene and used for liquid scintillation counting, while the remaining substrate Ser and the second product Gly remained in the water phase. The complete assay mixture contained the following in a total volume of 1 mL: 10 μL 3-[^{14}C]-L-Ser (2.11 GBq $mmol^{-1}$, Amersham), 40 μL of 100 mM L-Ser, 50 μL of 0.5 mM pyridoxal phosphate, 50 μL of 1% (w/v) THF, 50 μL of 50 mM KCl, and 200 μL of protein (2.5 $\mu g/\mu L$) in 0.4 M potassium phosphate buffer of pH 7.4. All components except radioactive Ser and THF were filled in a degassed tube and incubated for 5 min at room temperature. After addition of ^{14}C-L-Ser and removal of 0.1-mL reference sample, the reaction was initiated by adding the substrate THF. After 5, 10, and 15 min, samples of 0.1 mL were taken and mixed with 0.1 mL of 0.1 M formaldehyde, 0.15 mL of 1 M sodium acetate, pH 4.5, and 0.15 mL of 0.4 M dimedon (in 50% ethanol). The mixtures were shaken and heated for 10 min at 100°C and stored on ice for 5 min. The dimedon complex was then extracted by vigorous shaking with 3 mL of toluene. Phase separation was reached by centrifugation for 5 min at 5,000 rpm. Two milliliters of the supernatant were prepared with 2 mL of scintillation mix in scintillation vials for counting.

Quantification of Internal Amino Acids and Glycolate Concentrations

Free amino acids were extracted from frozen cyanobacterial cell pellets of 2 mL of culture with 80% ethanol at 65°C for 3 h. After centrifugation, the supernatants were dried by lyophilization and redissolved in 8 mM Na_2HPO_4, pH 6.8, containing 2.5% tetrahydrofurane. Individual amino acids were assayed after derivatization with *o*-phthaldialdehyde (Gollan et al., 1992) as described by Geigenberger et al. (1996) using a Hypersil 120 ODS column (4.6 × 150 mm; Knauer) connected to a Class-*Vp* HPLC system (Shimadzu) with a fluorescence detector (RF-10AXL; Shimadzu).

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. The operating parameters used were as follows: HPLC column, Aminex HPX-87H 300 × 7.8 mm (Bio-Rad); mobile phase, sulfuric acid 0.006 M; temperature, 65°C; and detection by refractive index. The measurements were carried out using an HPLC equipment of Knauer GmbH. Possible glycolate excretion was checked by the detection of glycolate in the medium. Fifty milliliters of suspension was taken from the cultures and cells were harvested by centrifugation (10 min, 4,000 rpm, 4°C). The supernatant was dried by lyophilized and redissolved in 800 μL of water before HPLC analysis.

All experiments were repeated at least three times using independent cell cultivations. Pair-wise *t* test was applied for the statistical comparison of mean values.

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