Metabolome Phenotyping of Inorganic Carbon Limitation in Cells of the Wild Type and Photorespiratory Mutants of the Cyanobacterium *Synechocystis* sp. Strain PCC 6803

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The amount of inorganic carbon represents one of the main environmental factors determining productivity of photoautotrophic organisms. Using the model cyanobacterium *Synechocystis* sp. PCC 6803, we performed a first metabolome study with cyanobacterial cells shifted from high CO₂ (5% in air) into conditions of low CO₂ (LC; ambient air with 0.035% CO₂). Using gas chromatography-mass spectrometry, 74 metabolites were reproducibly identified under different growth conditions. Shifting wild-type cells into LC conditions resulted in a global metabolic reprogramming and involved increases of, for example, 2-oxoglutarate (2OG) and phosphoenolpyruvate, and reductions of, for example, sucrose and fructose-1,6-bisphosphate. A decrease in Calvin-Benson cycle activity and increased usage of associated carbon cycling routes, including photorespiratory metabolism, was indicated by synergistic accumulation of the fumarate, malate, and 2-phosphoglycolate pools and a transient decrease in Calvin-Benson cycle activity and increased usage of associated carbon cycling routes, including photorespiratory metabolism. The unexpected accumulation of 2OG with a concomitant decrease of glutamine pointed toward a complete dephosphorylation of the PII regulatory protein at LC characteristic for nitrogen-replete conditions. Moreover, mutants with defined blocks in the photorespiratory metabolism leading to the accumulation of glycolate and glycine, respectively, exhibited features of LC-treated wild-type cells such as the changed 2OG to glutamine ratio and PII phosphorylation state already under high CO₂ conditions. Thus, metabolome profiling demonstrated that acclimation to LC involves coordinated changes of carbon and interacting nitrogen metabolism. We hypothesize that *Synechocystis* has a temporal lag of acclimating carbon versus nitrogen metabolism with carbon leading.

Cyanobacteria belong to the oldest life-forms on earth and are regarded as the inventors of oxygenic photosynthesis. During endosymbiosis, an ancient cyanobacterium became the chloroplast and initiated the evolution of the plant kingdom (see Deusch et al., 2008). Cyanobacteria are still globally important, because they are responsible for a substantial part of the annual primary production in the oceans (Partensky et al., 1999). In all photosynthetic organisms, Rubisco catalyzes the initial reaction in primary carbon (C) fixation by condensing atmospheric CO₂ and ribulose-1,5-bisP, yielding two molecules of 3-phosphoglycerate (3PGA). Through the action of the Calvin-Benson cycle, 3PGA is converted into triosephosphates, which serve as the initial C skeleton for the formation of many cellular intermediates and glycogen, the C storage form in cyanobacteria. In darkness or cases of C starvation, it is mobilized via Glc by glycolysis, the oxidative pentosephosphate pathway (OPP), and the incomplete tricarboxylic acid (TCA) cycle (Cooley et al., 2000; Mendzhul et al., 2000). But importantly, the primary function of the TCA cycle is the synthesis of Glu in cyanobacteria (Zhang et al., 2006). One of the main environmental factors determining the productivity of photoautotrophic organisms is the amount of available inorganic C (Cᵢ), which is fluctuating in recent times and throughout earth history. Because the solubility of the different forms of Cᵢ in water is restricted, cyanobacteria are permanently challenged by a shortage in this essential nutrient. Importantly, cyanobacterial Rubisco displays a rather low affinity toward CO₂ compared to plant Rubisco (Badger, 1980). To compensate for the shortage of Cᵢ, cyanobacteria developed an efficient CO₂ concentrating mechanism (for review, see Kaplan and Reinhold, 1999; Badger et al., 2006). In contrast to the common opinion, it was recently demonstrated that the efficiency of the CO₂ concentrating mechanism is not sufficient to completely suppress Rubisco’s side activ-

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ity as oxygenase (Eisenhut et al., 2006). As a result of the oxygenase reaction, 2-phosphoglycolate (2PG) is formed besides 3PGA, identical to the initial step of the photosynthetic response of plants (Tolbert, 1997). 2PG inhibits the Calvin-Benson cycle enzymes, phosphofructokinase (Kelly and Latzko, 1977) and triosephosphate isomerase (Husic et al., 1987; Norman and Colman, 1991) and must hence be instantly degraded. While higher plants perform for these purposes the photosynthetic C2 cycle (Tolbert, 1997), cyanobacteria show variability in their 2PG metabolism. The cyanobacterial model strain Synechocystis sp. PCC 6803 (hereafter Synechocystis) metabolizes 2PG additionally to the C2 cycle via the glycerate (Eisenhut et al., 2006) and probably the decarboxylation pathway (Eisenhut et al., 2007a).

In contrast to our detailed knowledge on components and the mechanism of C\textsubscript{i} accumulation, relatively little is known about the sensing of available C\textsubscript{i}. It was shown that the acclimation to C\textsubscript{i} limitation includes coordinated responses at transcriptome (Wang et al., 2004; Eisenhut et al., 2007a) and proteome system levels (Zhang et al., 2007). Various hypotheses have been put forward concerning the question of what is the signal that induces these responses (see Kaplan and Reinhold, 1999; Wooger et al., 2005; Badger et al., 2006). The possibility that photosynthetic intermediates like 2PG could be used as an internal signal to trigger acclimation to low C\textsubscript{i} values was supported very recently (Nishimura et al., 2008). This finding sparked the interest to obtain a more comprehensive knowledge on the metabolic remodeling within cells confronted by changing ambient C\textsubscript{i} concentrations using modern metabolomic tools. In contrast to plant physiology, which has embraced modern metabolomic approaches aimed at functional genomics (e.g. Trethewey et al., 1999; Fiehn et al., 2000; Roessner et al., 2001; Fernie et al., 2004) and new insights into plant environmental stress acclimation (e.g. Guy et al., 2008), only a few metabolomic approaches have been published using microbial (e.g. Koek et al., 2006) or cyanobacterial models (Yang et al., 2002; Shastri and Morgan, 2007).

The aim of this study is to understand the metabolic acclimation of cyanobacterial cells in response to reduced availability of C\textsubscript{i} by metabolomic phenotyping of non-steady-state metabolite pools (Roessner et al., 2001). We focus on the sequestering of C to different metabolic routes and apply routine gas chromatography-time of flight-mass spectrometry (GC-TOF-MS)-based metabolite profiling of a fraction comprising predominantly primary metabolites of the central metabolism (Fiehn et al., 2000). Additional to wild-type cells, this technology was applied to mutants, which have defined defects in the 2PG metabolism, resulting in the accumulation of glycolate and Gly, respectively. Moreover, these mutants exhibited few changes in their gene expression pattern under elevated C\textsubscript{i} that were typical for the wild type at C\textsubscript{i} starvation (Eisenhut et al., 2007a). We herewith attempt to functionally characterize these changes on the metabolomic level.

**RESULTS**

**Cultivation and Terms of Sampling**

First, we examined the general effect of low CO\textsubscript{2} (LC; ambient air, about 0.035% CO\textsubscript{2}) conditions on the growth of wild-type and mutant cells. For that purpose, the cultures were grown under high CO\textsubscript{2} (HC; air with 5% CO\textsubscript{2}) conditions and then transferred to LC for different times. During the first 3 h, cells stopped growth transiently, but it resumed after 24 h (data not shown). Finally, the shift from HC to LC conditions resulted in significantly lower growth rates compared to HC conditions for all strains. The two mutants Δglc\textsubscript{D} and Δgev\textsubscript{T} showed similar growth responses, but mutant Δglc\textsubscript{D} exhibited slow growth already under HC conditions (see also Eisenhut et al., 2006). We decided to compare samples from HC-grown cells with samples from cells shifted for 3 h (short-term acclimation) or for 24 h (long-term acclimation) to LC conditions.

**Nontargeted Classification of Wild-Type and Mutant Metabolic Phenotypes**

The changes of non-steady-state metabolite pool sizes were estimated by a nontargeted GC-MS-based metabolite profiling protocol initially established for the analysis of higher plant organs. Preliminary experiments showed that this GC-MS method was also suitable for the analysis of cellular extracts of the cyanobacterium Synechocystis when coupled to fast sampling by filtration and subsequent shock-freezing in liquid nitrogen. Initially, for the nontargeted metabolic phenotyping, the whole data set of more than 10,000 detected metabolite fragments was taken into consideration. These analyses demonstrated that in the above-mentioned mutants compared to wild type and shifted from HC to LC conditions, respectively, a major reprogramming of Synechocystis metabolism occurred in response to reduced availability of C\textsubscript{i} (Fig. 1). Independent component analysis (ICA), which is a nonsupervised method used for multivariate sample classification (e.g. Daub et al., 2003), revealed that the mutant metabolite phenotypes were more distinct under LC conditions, as the mutant samples were clearly separated from wild type and each other under LC conditions compared to HC (Fig. 1A). As ICA selects the optimum bimodal sample distributions based on the major variances of a given data set, short-term (3 h) and long-term (24 h) acclimation to LC resulted in clear differences (Fig. 1B). This clear distinction among our chosen time points may indicate that short-term responses become superseeded by a slower acclimation process. In both short- and long-term responses, the mutants appeared to react more extremely than the wild type; indeed, mutant samples populated almost exclusively the two bottom quadrants of the IC3/IC4 plot (Fig. 1B). Most strikingly, the mutant phenotype under HC appeared to be similar to wild type under LC, as was...
indicated by the proximity of mutant LC samples and wild-type HC samples (compare with Fig. 1B, arrow, white triangles and diamonds close to black circles).

Based on the initial ICA analyses, those mass spectral features were selected that showed the highest significant changes in the wild-type profiles when shifted from HC to LC conditions (Fig. 1C). In addition, we tested for similar metabolic observations comparing mutants under HC conditions with wild type shifted to LC. These analyses showed a specific set of metabolic features that were significantly changed in wild type only under LC but present in both mutants already under HC conditions (Fig. 1C).

Thus, the so-called nontargeted fingerprinting analysis revealed that both mutants of 2PG metabolism exhibit already at HC conditions partial features of the HC to LC shift found in wild-type cells. This behavior we define as a mutant phenocopy of wild-type cell HC to LC shift.

In these fingerprinting analyses, multiple co-eluting fragments represent a single metabolite, which may be identified by comparison of retention behavior and respective mass spectral properties to an authenticated reference library. Only a subset of 74 observed components from GC-TOF-MS profiles can currently be identified among a majority of yet-unidentified meta-
bolic components (for the complete data set of identified metabolites, see Supplemental Table S1).

Composition of Metabolite Classes after Shifting from HC to LC

The identified metabolites belonged to diverse chemical classes of primary metabolism, such as amino acids, other nitrogen (N)-containing compounds, organic acids, polyhydroxy acids, fatty acids, phosphates, and sugars (Supplemental Table S1). The composition of observable metabolite classes, as determined by the sum of all respective normalized metabolite responses from GC-TOF-MS profiling, was influenced by the shift of growth conditions and choice of mutant. In HC-grown wild-type cells, amino acids dominated with 55% of the total normalized response, followed by organic acids (25%) and N compounds (7%). After 24-h treatment with LC, the abundance of amino acids increased, while the organic acid fraction decreased (Fig. 2). Strikingly, the detected sugars were concomitantly reduced by approximately 50% under LC conditions. Thus, an apparent activation of N metabolism under LC conditions at the expense of C metabolism was found as expected.

Similar trends of the organic acids and sugars were observed for the ΔgldC and ΔgcvT mutants. The comparison of the mutants to the wild type exhibited a slightly lower abundance of sugars in both mutants under HC conditions, while, in contrast to the wild type, the abundance of phosphorylated intermediates appeared to almost double in both mutants under LC conditions. A specific feature of the ΔgldC mutant appeared to be the high level of amino acids, already 62% under HC conditions, and no further increase when shifted to LC (Fig. 2). Following this evidence of global metabolic rebalancing, we extended our investigations toward metabolite-targeted detailed analyses and investigation of the observed mutant phenocopy.

Changes in Central Metabolic Routes after Shifting from HC to LC

The survey of our metabolic inventory (Supplemental Table S1) indicated good coverage of central C and N metabolism, which is dominated by the Calvin-Benson cycle and the associated 2PG metabolism as well as glycolysis, OPP, and an incomplete TCA cycle (Fig. 3). The N metabolism is connected to the C metabolism via the incomplete TCA cycle, which is thought to produce C skeletons for amino acid biosynthesis (Zhang et al., 2006). The second most important interface of N and C metabolism is the 2PG detoxification pathway, where ammonium is generated by a 2 to 1 conversion of Gly to Ser. This ammonium will typically be scavenged by reassimilation into the Gln pool. Differences in key metabolite pools varied between 0.1- and 10-fold, which further supported our finding of a global metabolic reprogramming of intermediates (Supplemental Table S1 and following figs.).

Generally, when shifting a cyanobacterial cell from HC to LC, the nutrient balance should be shifted from a high to a significantly lower C to N ratio. Correspondingly, we expected and confirmed at a global level (Fig. 2) a long-term change toward higher levels of N intermediates at the expense of C-containing metabolites. However, it has been shown that HC-grown cells store a high amount of glycogen, which completely disappears during the first 24 h after transfer to LC (e.g. Eisenhut et al., 2007a). Therefore, the short-term (3 h) response of HC to LC transition is characterized by a transient carbohydrate anaplerosis through glycogen breakdown, while the long-term HC to LC transition after 24 h ultimately requires acclimation to carbohydrate shortage.

As expected, after shifting the cells from HC into LC, the amount of 2PG was permanently increased (Fig. 4A), because LC conditions result in higher O₂ to CO₂ ratios and stimulate oxygenase activity of Rubisco. However, the capacity of the branched 2PG metabo-

Figure 2. Changes of composition of metabolite classes in cells of the Synechocystis wild type and ΔgldC and ΔgcvT mutants grown 24 h under HC (A) or LC (B) conditions. Relative abundance of metabolite classes was estimated by the sum of all observed normalized responses per metabolite class (compare with Supplemental Table S1).
lism in wild-type cells seems to be sufficient for quick detoxification. The C2 cycle intermediate glycolate did not rise above detection limit, and other metabolites of the pathway, such as Gly, were not changed. Only Ser had a transient increase that may be interpreted as a short-term restriction of the NH2-group transfer to acceptors brought about by the requirement for rapid ammonium sequestration from the preceding C2 cycle step.

Additionally, the shift to LC conditions seems to transiently block Calvin-Benson cycle activity, because the amount of 3PGA increased 8-fold but reverted to normal after 24 h acclimation to LC (Fig. 4B). The accumulated Calvin-Benson cycle intermediates appear to be permanently redirected into glycolysis as indicated by the 7- to 10-fold increase of dihydroxyacetone-phosphate (DHAP), 2PGA, and phosphoenolpyruvate (PEP) pools already after 3 h shift to LC (Fig. 4B). While the amount of PEP and 2PGA remained high even after 24 h of LC, the top part of glycolysis, namely Glc-6-P/Fru-6-P to DHAP, returned nearly to HC levels. The notable exception is Fru-1,6-bisP, which shows a late reduction of pool size at 24 h under LC. Moreover, 6-P-gluconate, an OPP intermediate, was transiently increased, indicating the role of glycolyin breakdown in carbohydrate anaplerosis of the Calvin-Benson cycle during the early stages of LC acclimation. The required carbohydrate resources to replenish the Calvin-Benson cycle appeared to be not much changed under different Cj conditions, 2-oxoglutarate (2OG) was drastically, approximately 10- to 20-fold, increased and Gln reduced below 0.2-fold, while Glu appeared to be under homeostasis. Furthermore, after long-term acclimation, the balance of the TCA cycle acids was changed to low citrate and succinate and accumulated fumarate and malate, respectively (Fig. 4C).

The pools of most amino acids remained nearly constant or were reduced. The only exceptions were increases of Ser, β-Ala, and Trp. The strongest decreases besides Gln were observed in the Ala and Asp pools branching off from pyruvate and oxaloacetate, respectively. In addition the pools of the N-acetylated amino acids, N-acetyl Ser and N-acetyl Glu appear to become strongly reduced (Supplemental Table S1). In conclusion, the observed apparent global shift toward amino acids (Fig. 2) must be caused by a predominant depletion of C metabolite pools.

Mutation in 2PG Metabolism Results in a Partial Phenocopy of the Wild-Type HC to LC Shift

The mutants ΔgldD and ΔgcvT were chosen for a targeted lesion of the 2PG pathway and demonstration of the importance of this pathway for optimal growth under LC conditions. These mutants are not only characterized by defined changes of the gene expression pattern (Eisenhut et al., 2007a) but also exhibit the respective substrate precursor accumulation at metabolic level (Fig. 5). Indeed, high Gly was observed in mutant ΔgcvT in accordance with its genetic defect in the T-protein subunit of Gly decarboxylase (Hagemann et al., 2005). As indicated by above ICA, the difference
of Gly levels compared to wild-type cells was moderate under HC conditions but became a dominant feature under LC conditions (Fig. 5A). Similarly, glycolate accumulation (Fig. 5B) was detectable only in cells of the other mutant defective in a glycolate dehydrogenase (GlcD), which converts glycolate into glyoxylate. As reported before (Eisenhut et al., 2006), we could detect glycolate already in HC-grown cells, and the level increased further under LC conditions. In conclusion, both mutants interfered with 2PG degradation initiated by the Rubisco oxygenase reaction but at two subsequent steps.

In both cases, the respective precursor pools were increased, indicative of a substantial rate of 2PG production already under HC conditions. Furthermore, 2PG increased even more under LC conditions, an observation that is in agreement with the assumption that the flux through 2PG should be increased under LC conditions. Most strikingly, we confirmed at pool size level that both mutants exhibited a partial phe- nocy of the LC phenotype already under HC conditions and responded more extremely when shifted to LC (Fig. 1C). Especially the 2OG pool, but also the 2-oxoisocaproate pool, an intermediate of ile metabolism utilized by branched-chain amino acid amino- transferase, exhibited a high accumulation under HC and a further increase under LC in both mutants. This phenomenon was extended, however at a minor magnitude, into other constituent pools of the incomplete TCA cycle, namely the aconitate, fumarate, and malate pools (Fig. 6). Other similar aspects were reduced Asp and N-acetyl-Ser pools, low Suc and dihydroxyacetone compared to high DHAP, and increased pools of both gluconate-6-P and 1-O-methyl-β-D-glucopyranoside (Supplemental Table S1). Interestingly, the amount of Fru-1,6-bisP was decreased by at least 0.5-fold in cells of both mutants, ΔgldC and ΔgcvT, in comparison to wild-type cells under all investigated growth conditions. This metabolite is central to both the Calvin-Benson cycle and glycolysis. Thus, Fru-1,6-bisP concentration may act as a possible metabolic signal under changing C conditions, which induce either higher 2PG production or reduced 2PG catabolism, such as engineered by our chosen enzyme deletions.

Changes of Metabolite Pools Common to the ΔgldC and ΔgcvT Mutants

The mutants ΔgcvT and ΔgldC also showed the accumulation of intermediates from the first steps of glycolysis or OPP pathways. Specifically, Glu-6-P and Fru-6-P, Glc, and gluconate-6-P accumulated (Fig. 7) to significantly higher amounts, while only Fru-1,6-bisP was reduced. More or less regardless of the growth conditions, the levels of these sugar-phosphates were 2 to 4 times higher than in wild-type cells (Fig. 7, A and B), with a slight indication that under short-term acclimation these pools may be transiently higher. Some amino acids exhibited similar changes in both mutants under HC conditions, specifically Gly, Ala, Met, Gln, Leu, and Phe were increased (Supplemental Table S1).

Changes of Metabolite Pools Specific for the ΔgldC Mutant

The ΔgldC mutant demonstrated specific features that may explain the marked growth defect already under HC conditions. An increased use of alternative routes for C metabolism was indicated through the observed accumulation of DHAP (Fig. 7B). However, because 2PG is a potent inhibitor of triosephosphate isomerase, the rise in DHAP might also be due to the impaired conversion into glyceraldehyde-3-phosphate. Unfortunately, glyceraldehyde-3-phosphate was be-
low detection limits, and we were not able to quantify those levels. Additionally to glycolate, the cells of mutant \( \Delta \text{gldC} \) contained higher amounts of lactate, which may indicate that the GlcD also accepts lactate as substrate. This was shown to be a typical feature of related GlcDs from bacteria, algae, and Arabidopsis (\textit{Arabidopsis thaliana}; Bari et al., 2004).

Changes in the Phosphorylation Status of the PII Protein

The PII protein is regarded as the central regulator to coordinate C and N metabolism (Forchhammer, 2008). In cyanobacteria such as \textit{Synechocystis}, the homotrimERIC protein can be phosphorylated at each subunit, leading to four possible phosphorylation states, which are detectable after separation of proteins under native conditions by immunoblotting experiments with a specific PII antibody (Forchhammer and Tandeau de Marsac, 1994; see Fig. 8). We compared the phosphorylation of PII under HC and LC conditions in combinations with different N sources. Wild-type cells behaved as was shown before (Forchhammer and Tandeau de Marsac, 1994, 1995; Kloft et al., 2005). They showed highest PII phosphorylation under N-deficient conditions, regardless if grown at HC or LC. Addition of nitrate resulted in a gradual decrease of phosphorylation under HC conditions, while LC generally induced PII dephosphorylation in nitrate-grown cells (Fig. 8). Similar changes were observed for the mutant \( \Delta gcvT \), while cells of the mutant \( \Delta \text{gldC} \) exhibited interesting differences. In cell extracts of the \( \Delta \text{gldC} \) mutant, the PII phosphorylation was always weaker compared to wild type. The dephosphorylation of PII characteristic of LC cells of wild type seems to have already started in cells of mutant \( \Delta \text{gldC} \) under HC conditions (Fig. 8). This behavior supports the conclusion drawn from our metabolome results, where mutant cells exhibited at HC a partial phenotype of the wild-type cells HC to LC shift.

DISCUSSION

The above data show that applying nontargeted GC-TOF-MS-based metabolite profiling allowed an overview of central C and N metabolism as well as discovery of differential metabolic pool sizes at different C\(_i\) levels. The expected high importance of C\(_i\) supply for the whole metabolism of cyanobacteria is fully supported by our data set. The statistical analyses of all detected compounds showed that most of them are significantly changed when comparing HC- and LC-grown wild-type cells of \textit{Synechocystis}. The effects on metabolite composition are much more pronounced...
moreover, we also compared wild-type cells and two defined mutants defective in reactions of the 2PG metabolism with regard to their response to changes in C\textsubscript{i} level. these mutants showed only minor transcriptional differences after shifts from HC to LC in comparison to wild-type cells (Eisenhut et al., 2007a). here, we found that many key metabolites of central metabolism are significantly changed in cells of ΔglcD and Δgcvt, respectively. these changes are not only related to the primary defect of the respective mutants in defined subsequent steps of the 2PG metabolism (Figs. 5–7) but also extend to crucial intermediates of primary metabolism. in conclusion, as found previously for higher plant systems, the newly developed techniques for high throughput metabolome analysis provide a powerful tool to support functional genetics. Moreover, metabolomic analyses contribute unique information on experimental systems, which are predominantly under metabolic control, as became evident in this assessment of the \text{C}_i acclimation of \textit{Synechocystis}.

as shown above, the comparison of HC- and LC-grown wild-type cells revealed marked changes in many identified key metabolites (compare with Figs. 4–7). we found indications for a transient reduction of Calvin-Benson cycle activity, while alternative routes of primary C metabolism seem to become activated,
including a more prominent role of the alternative \( C_\text{i} \) fixation by PEP carboxylase, as suggested before (Yang et al., 2002). Despite the general difficulty in drawing unambiguous conclusions on the activity of different metabolic routes from data on metabolite pool sizes, where an increase may result from either or both enhanced synthesis and decreased consumption, we believe that the aforementioned interpretations provide valid hypotheses, because they are often supported by our previous data on altered gene expression patterns. These transcriptome data also indicated a reduction in the expression of Calvin-Benson cycle enzymes, including Rubisco in LC-grown wild-type cells and an up-regulation of enzymes involved in alternative routes of primary C metabolism such as those for the synthesis of citrate, 6-phosphogluconate, or the degradation of glycogen (Eisenhut et al., 2007a).

We diagnosed further a block of N assimilation, which is indicated by the decreased Gln pool compared to the substantial increase in 2OG. This result, also substantiated by \( \Delta glcD \) and \( \Delta gccT \) mutant analyses (Fig. 6), was not expected, because the shift to LC should increase the N to C ratio compared to HC conditions. Traditionally, the lack of N is thought to cause increased 2OG levels (e.g. Muro-Pastor et al., 2005), and the lack of \( C_\text{i} \) should induce reduced availability of this key metabolite. Our observations are further supported by previous transcriptome analyses of the same system, where correspondingly decreased mRNA levels were detected for many genes involved in N assimilation, such as \( narB, niaA, glnA \), and the \( nrt- \) as well as \( mao\)-operons under LC conditions (Eisenhut et al., 2007a). The down-regulation of de novo N assimilation may simply result from the decreased growth rate of \( Synechocystis \) at LC conditions. However, the decrease in N assimilation could be more directly linked to increased ammonia release by activation of 2PG metabolism via the C2 cycle route. This pathway produces ammonia proportional to 2PG, but detoxification can only be effective if ammonia is rapidly reassimilated. The possible increase of cellular ammonia levels may signal to the cell a temporal N excess leading to the observed shutdown of N assimilatory genes. Moreover, N assimilation needs excess energy, which may be limiting for a cell in the process of acclimation to LC and the inherent reduced \( CO_2 \) availability. Last but not least, the shift to LC is accompanied by the mobilization of glycogen (Eisenhut et al., 2007a), the major organic C store in the cyanobacterial cell. The short-term glycogen breakdown employs OPP and glycolysis as anaplerotic pathways, leading to the accumulation of 2PGA and 2OG. We argue that in contrast to 2PGA, the latter cannot be further metabolized due to the block of N assimilation and the incomplete TCA cycle.

The 2OG pool is generally believed to play a crucial role as an internal indicator for the N status in cyanobacterial cells, including \( Synechocystis \), where a high 2OG pool is a signal for high C and/or low N resulting in the activation of N-regulated genes via the transcriptional factor NtcA (e.g. Muro-Pastor et al., 2005). Another key protein to regulate the C/N balance in cyanobacteria is the so-called PII protein (Forchhammer, 2008). It was shown that the PII protein binds 2OG besides ATP, leading to different activity states. Moreover, the PII phosphorylation is also directly regulated by 2OG levels, which activate the kinase activity and inhibit the phosphatase acting on PII (Forchhammer, 2008). This regulatory mechanism results in low phosphorylation under N excess and C-limiting conditions, respectively, and in highest PII phosphorylation states under N starvation. The expected changes of PII protein phosphorylation were fully reproduced in our study investigating HC- and LC-treated cell material in the presence or absence of nitrate. However, complete dephosphorylation of PII protein was observed in LC-grown cells, which showed simultaneously a clear accumulation of 2OG compared to HC-grown cells. Obviously, the phosphorylation state of PII protein and possibly other related N starvation signals are not exclusively sensed via 2OG in vivo. During the first 24 h after the shift from HC into LC conditions, an additional modulating or superseding control factor of PII phosphorylation needs to be proposed.

Another novel result appears to be the fact that mutants under HC conditions displayed specific metabolic features of LC-shifted wild-type cells. We define this observation as a partial LC wild-type phenocopy of the mutant cells already at HC. This partial phenocopy may be carefully interpreted as a further indication of additional, so-far-hidden signaling processes. The mechanisms in sensing a \( C_\text{i} \) limitation are poorly understood. It has been shown that signaling is correlated with the size of the internal \( C_\text{i} \) pool and involves oxygen (Woodger et al., 2005). But the true signal(s) is still a matter of investigation. The accumulation of the photosynthetic metabolites glycolate and Gly does not influence the transcription of typical LC-induced genes like \( cmpA \), \( sbtA \), or \( cupA \) (Woodger et al., 2005; Eisenhut et al., 2007a), encoding high-affinity \( C_\text{i} \) uptake systems. In contrast, 2PG, the immediate product of Rubisco’s oxygenase activity, was shown to act in vitro as a metabolic co-inducer of the \( cmpR\)-operon, encoding the high-affinity ATP-binding cassette transporter BCT1 (Nishimura et al., 2008). However, at present, it is not clear whether or not internal glycolate and Gly concentrations, respectively, might be used to sense \( C_\text{i} \) availability leading to the observed changes in the metabolome. These alterations could also result from potential inhibitory effects of those metabolites. Glycolate is well known as a metabolic inhibitor of enzymes of the Calvin-Benson cycle (Kelly and Latzko, 1977; Husic et al., 1987; Norman and Colman, 1991). Furthermore, high Gly amounts are toxic by reducing the availability of Mg\(^{2+} \) ions, which are essential for many enzyme activities (Eisenhut et al., 2007b). Therefore, changes in the activity of a specific or multiple Calvin-Benson cycle enzymes may act as an alternative signal used to monitor \( C_\text{i} \) status in a cyanobacterial...
cell. Clearly, we cannot rule out that one of the downstream metabolites found to be accumulated in the two mutants defective in 2PG metabolism may serve as a potential signal.

In conclusion, our work has led to novel insights into the metabolic processes of LC acclimation and C_i sensing. The comprehensive overview of central C and N metabolism indicates that C_i availability has a global influence on cyanobacterial cellular activities. Our comparison of wild-type and mutant cells defective in 2PG metabolism at HC and LC conditions, respectively, showed that the latter exhibited indications for a deacclimation toward low C_i already at HC conditions in its metabolic profile. This could be taken as an indication for a direct or indirect role of certain metabolites in C_i sensing.

MATERIALS AND METHODS

Strains and Culture Conditions

The Glc-tolerant strain of *Synechocystis* served as the wild type and was obtained from Prof. Murata (National Institute for Basic Biology, Okazaki, Japan). The generation and characterization of the mutants ΔglcD, bearing a defect in the GlcD coding gene sll0404, and ΔglnT, bearing a defect in gene sll0171 coding for the T-protein subunit of the Gly decarboxylase complex, have been described elsewhere (Hagemann et al., 2005; Eisenhut et al., 2006). For all experiments, axenic cultures of the cyanobacteria were grown photoautotrophically in batch cultures using 3-cm glass vessels with 5-mm glass tubes for aeration (bubbling flow rate was 5 mL min^{-1}) and 29°C growth temperature under continuous illumination of 130 μmol photons s^{-1} m^{-2} (warm light, Osram L58 W32/3). For shift experiments, cells were precultured with air enriched with 5% CO_{2} (HC) in BG11 medium (Rippka et al., 1979), pH 7.0. Cells were harvested by centrifugation and resuspended in fresh BG11 at an optical density at 750 nm (OD_{750}) of 0.8. After 1 h cultivation under HC conditions, C_i limitation was set by transferring those exponentially growing cultures to bubbling with ambient air containing 0.035% CO_{2} (LC). To prove PI protein phosphorylation, cells were shifted to HC and LC conditions in BG11 medium without combined N (replacing NaNO_{3} by equimolar amounts of NaCl) and nitrate-containing standard BG11, respectively. Growth was monitored by measurements of the OD_{750}. Cultivation of mutants was performed at 50 μg mL^{-1} kanamycin or 20 μg mL^{-1} spectinomycin. Contamination by heterotrophic bacteria was checked by spreading 0.2 mL of culture on Luria-Bertani plates. All experiments were repeated using at least three independent cell cultures.

Sampling Procedure for Metabolite Profiling

Samples of 5 to 10 mL cells, equivalent to about 10^8 cells mL^{-1}, were taken from the cultivation vessels and separated from the medium by filtration (0.45-μm nitrocellulose filters, Schleicher and Schuell) in the light without any subsequent washing. The cell pellets on the filters were put into 2-mL Eppendorf tubes and immediately frozen in liquid nitrogen and stored at −80°C. Time until metabolic inactivation by freezing was about 20 s. Thus, cells were separated from the bulk medium, but minor negligible contaminations of secreted metabolites may still have been present.

Metabolites were extracted from the deep frozen cells by a premixture of 300 μL methanol (final concentration approximately 80%), 30 μL nonanedioic acid methylster (2 mg mL^{-1} chloroform), 30 μL of ribitol (0.2 mg mL^{-1}), and Δ_{2,3,3,3-Ala} (1 mg mL^{-1}, standards in methanol). Each sample was mixed thoroughly at least 1 min for complete suspension, aged 15 minutes at 70°C, brought to room temperature mixed with 200 μL chloroform, and agitated again 5 min at 37°C. Finally, phase separation was induced by 400 μL distilled water and the upper polar fraction retrieved by 5 min centrifugation at 14,000 rpm using an Eppendorf 5417 microcentrifuge. A total volume of 320 μL was concentrated and dried in 1.5-mL microtubes by 12 h of vacuum centrifugation.

Nontargeted GC-EI-TOF-MS Profiling Analysis

The chemical derivatization, namely methoximation and subsequent trimethylsilylation, was performed manually (Fiehn et al., 2000). Samples were processed using a Factor Four VF-5 ms capillary column of dimensions, 30-m length, 0.25-mm i.d., and 0.25-μm film thickness with a 10-m EZ-guard pre-column (Varian BV) mounted to a 6890N gas chromatograph (Agilent), which was operated in splitless injection mode. Mass spectrometric data were acquired through a Pegasus III TOF mass spectrometer (LECO Instruments). Detailed GC-electrospray ionization-TOF-MS settings were as reported previously (Erban et al., 2007).

GC-EI-TOF-MS Compound Identification and Data Processing

GC-TOF-MS chromatograms were processed by TagFinder-Software (Luedemann et al., 2008) after export from the ChromaTOF software (version 1.00, Pegasus driver 1.61, LECO) to NetCDF file format. A complete matrix of peak heights representing arbitrary mass spectral ion currents of all available mass features was used for initial numerical analysis (e.g., Fig. 1). Peak heights were corrected by the amount of cells using OD_{750} and to the recovered amount of the internal standard, ribitol, to yield normalized responses. These normalized responses were used to calculate response ratios, subsequently also called relative content, compared to the respective choice of control condition. Identification of metabolite-specific mass fragments from this data matrix was manually supervised. Compounds were identified using the TargetFinder plug-in of TagFinder-Software and a reference library of mass spectra and retention indices from the collection of the Golm Metabolome Database (http://csbdb.mpimp-golm.mpg.de/csdb/hmdb/gmd_sm.html). Koprivova et al., 2005). Thresholds for automated mass spectral pre-matching were set at factor >650 and retention indices deviation <1.0%. Identifications were confirmed manually by co-chromatography of defined reference mixtures (Strehmel et al., 2008). The full resulting data set of identified metabolites and respective normalized responses may be found within Supplemental Table S1.

Metabolite-Targeted Measurement of Amino Acid Concentrations by HPLC

Gln determination using the above GC-TOF-MS is impaired due to chemical conversion of Gln into pyroglutamate. Residual Gln can still be used for relative quantification but may be close to detection limits, while the use of pyroglutamate gives nonselective information on the sum of Gln and pyroglutamate and may contain trace contributions from Glu. For this reason, free amino acids were extracted from frozen cyanobacterial cell pellets with 80% ethanol at 65°C for 3 h. After centrifugation, the supernatants were dried by lyophilization and resolved in 8 μL Na_{2}HPO_{4}, pH 6.8, containing 2.5% tetrahydrofuran. Individual amino acids were assayed after derivatization with o-phthalaldehyde and analyzed by HPLC as described by Hagemann et al. (2005).

Calculations and Statistical Data Mining

Data management, data transformation, and basic calculations were performed using Microsoft Office Excel 2003 options. The TMA multiple-experiment viewer (Saeed et al., 2003) and the MetaGeneAlyse Web service (Daub et al., 2003) were employed for visual and statistical data assessment, e.g. ICA, one- and two-way ANOVA, Kruskal-Wallis, or Mack-Skilling tests.

Monitoring of PI Protein Phosphorylation Status

For these experiments, 50 mL of cells were harvested from the culture vessel by centrifugation. The cell pellets were immediately frozen and stored at −20°C. Total proteins were extracted by sonication in 10 mL HEPES buffer, pH 7.5, containing phenylmethylsulfonyl fluoride. Equal amounts of soluble proteins (5–10 μg) obtained after centrifugation at 22,000 g at 4°C were separated in 15% acrylamide gels under native conditions and subsequently blotted onto nylon membranes. The PI protein bands representing the different phosphorylation states were detected by a PI-specific antibody (received from Prof. K. Forchhammer, University of Tübingen), which was
visualized using an alkaline-phosphatase-linked secondary antibody (Forchhammer and Tandeau de Marsac, 1994). The protein extraction, PAGE, and immunoblotting are described in more detail by Fulda et al. (2002).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Table S1. This table presents the metabolite inventory of identified compounds from *Synechocystis* sp. PCC 6803 wild type and ΔgcvD and ΔgcvT mutants under HC and LC conditions, respectively.

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LITERATURE CITED


