Impaired glycogen synthesis causes metabolic overflow reactions and affects stress responses in the cyanobacterium *Synechocystis* sp. PCC 6803

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The biosynthesis of glycogen or starch is one of the main strategies developed by living organisms for the intracellular storage of carbon and energy. In phototrophic organisms, such polyglucans accumulate due to carbon fixation during photosynthesis and are used to provide maintenance energy for cell integrity, function and viability in dark periods. Moreover, it is assumed that glycogen enables cyanobacteria to cope with transient starvation conditions, as observed in most micro-organisms. Here, glycogen accumulates when an appropriate carbon source is available in sufficient amounts but growth is inhibited by lack of other nutrients. In this study, the role of glycogen in energy and carbon metabolism of phototrophic cyanobacteria was first analysed via a comparative physiological and metabolic characterization of knockout mutants defective in glycogen synthesis. We first proved the role of glycogen as a respiratory substrate in periods of darkness, the role of glycogen as a reserve to survive starvation periods such as nitrogen depletion and the role of glycogen synthesis as an ameliorator of carbon excess conditions in the model organism *Synechocystis* sp. PCC 6803. We provide striking new insights into the complex carbon and nitrogen metabolism of non-diazotrophic cyanobacteria: a phenotype of sensitivity to photomixotrophic conditions and of reduced glucose uptake, a non-bleaching phenotype based on an impaired acclimation response to nitrogen depletion and furthermore a phenotype of energy spilling. This study shows that the analysis of deficiencies in glycogen metabolism is a valuable tool for the identification of metabolic regulatory principles and signals.

**INTRODUCTION**

Glycogen is a highly branched polymer of glucose which serves as a carbon and energy reserve in most organisms (Preiss & Romeo, 1994). The synthesis of glycogen in cyanobacteria and of starch in plants and algae starts with the use of ADP-glucose as the glycosyl donor (Ball & Morell, 2003). The synthesis of ADP-glucose from glucose-1-phosphate and ATP, catalysed by ADP-glucose pyrophosphorylase (AGPase), also referred to as glucose-1-phosphate adenylyltransferase, defines both the first committed step of glycogen synthesis and the major rate-controlling step of glycogen synthesis. AGPase is finely tuned through allosteric activation by 3-phosphoglycerate, the product of the carboxylating reaction of ribulose-1,5-bisphosphate carboxylase/oxygenase, and is inhibited by orthophosphate. This regulation of AGPase in cyanobacteria most likely evolved to couple storage polysaccharide synthesis to photosynthetic CO₂ fixation, and was conserved during chloroplast evolution for starch biosynthesis in plants (Ball & Morell, 2003). The glucose moiety of ADP-glucose is transferred to the non-reducing end of a linear α-1,4 glucane primer, a reaction catalysed by glycogen synthases. The α-1,6-glycosidic branches are introduced into the growing polymer by the branching enzyme (Preiss & Romeo, 1994).

In many micro-organisms, glycogen plays a major role in the survival under adverse conditions. Accumulation of glycogen is enhanced when a macronutrient, e.g. a nitrogen or sulfur source, is limiting but a carbon source is present in sufficient amounts. Various bacterial species accumulate glycogen in the stationary phase, when growth stops because of either depletion of a nutrient, such as nitrogen, sulfur or phosphate, or an unfavourable pH (Preiss, 1984). Glycogen enables the cells to cope with transient starvation conditions that may result in a significant loss of viability in glycogen-deficient strains (Preiss, 1984). Several studies have associated bacterial glycogen metabolism with environmental survival, symbiotic performance, colonization and virulence, but the exact roles of the polymer may differ from strain to strain (Wilson *et al.*, 2010).
In cyanobacteria, the ability for glycogen synthesis appears to be conserved since the respective genes are present in all cyanobacterial genomes annotated so far (Beck et al., 2012). However, the number of isoenzymes involved in glycogen biosynthesis and degradation varies between cyanobacterial species. It is thought that cyanobacteria store a portion of the photosynthetically generated carbohydrate as glycogen, which is assumed to be the substrate of endogenous metabolism during periods of darkness (Stanier & Cohen-Bazire, 1977). It has been hypothesized that glycogen acts as a dynamic reserve in cyanobacteria with the dual function as a reserve and a buffer substance (Carr, 1988).

One way to understand the function of glycogen in phototrophic cyanobacteria is to generate a biological system that lacks genes that are involved in glycogen synthesis. First knockout mutagenesis of AGPase and glycogen synthase in Synechococcus elongatus PCC 7942 and of AGPase in Synechocystis sp. PCC 6803 revealed a role for AGPase and glycogen in acclimation and tolerance to salt and oxidative stress (Miao et al., 2003a; Suzuki et al., 2010). However, important aspects of the role of glycogen metabolism in central carbon metabolism and in acclimation processes to macronutrient changes have not yet been investigated. In this study, we address (i) the role of glycogen as a respiratory substrate in periods of darkness, (ii) the role of glycogen as a reserve to survive starvation periods such as nitrogen depletion and (iii) the role of glycogen synthesis as an ameliorator of carbon excess via a comparative physiological and metabolic analysis of knockout mutants defective in AGPase or in both glycogen synthases in the model organism Synechocystis sp. PCC 6803 (hereafter referred to as Synechocystis).

**METHODS**

**Bacterial strains and growth conditions.** The strains of Synechocystis used in this study were propagated on BG11 agar plates (Rippka et al., 1979). Liquid cultures of Synechocystis wild-type and mutant strains were grown at 28 °C under continuous illumination with white light in a range of 60–100 μmol photons m⁻² s⁻¹ (as indicated in the respective experiments) and continuous aeration (0.03 %, v/v, CO₂) in BG11 medium containing 20 mM HEPES buffer (pH 8.0) and 17.6 mM sodium nitrate as nitrogen source (+ N), or in BG11o which lacks sodium nitrate (−N). Nitrogen deprivation was achieved by a repeated washing-centrifugation procedure and resuspension in BG11o medium. Cultures were split and nitrate was added again to one of them (control). Photomixotrophic growth was achieved by the addition of glucose in a final concentration of 5 mM to the cell pellets with and without nitrogen. Nitrogen deprivation was achieved by the addition of glucose in a final concentration of 5 mM to the cell pellets with and without nitrogen. Nitrogen deprivation was achieved by the addition of glucose in a final concentration of 5 mM to the cell pellets with and without nitrogen.

**Determination of glycogen content.** For isolation of glycogen, cell pellets of 1–3 ml culture were washed three times with sterile water, suspended in 30 % (w/v) KOH and incubated at 95 °C for 2 h. Precipitation of glycogen was achieved by the addition of ice-cold ethanol to a final concentration of 70–75 % (v/v) and subsequent incubation on ice for at least 2 h. After a centrifugation step (10 min, 4 °C, 10,000 g) the glycogen pellet was washed twice with 70 and 98 % (v/v) ethanol, and dried in a speed-vac (10 min, 60 °C). The isolated glycogen was dissolved in water and quantified using the phenol-sulfuric acid method with glycogen standards.

**Analysis of growth, pigmentation, photosynthetic capacity and cell size.** Growth characteristics of the cultures during exponential growth (OD750 0.2–1.5) were analysed by daily measurement of OD750 and of the chlorophyll content (Tandeau de Marsac & Hounard, 1988). Whole cell absorbance spectra were recorded in the wavelength range from 550 to 750 nm on a Shimadzu UV-2401PC spectrophotometer equipped with an integrating sphere and were corrected for residual cell scattering at 750 nm and normalized to the chlorophyll a peak at 683 nm. Cultures used for the estimation of growth rates did not contain antibiotics to reduce side effects. The growth rate was calculated in the exponential growth phase as the linear regression of the natural logarithm of chlorophyll content (μg chlorophyll (ml cell suspension)⁻¹) versus time. Photosynthetic capacity of cell suspensions containing 15 μg chlorophyll ml⁻¹ was determined; the suspensions were washed and resuspended in BG11 with 25 mM NaHCO₃. Oxygen evolution rates were measured with a Clark-type (DW1) oxygen electrode (Hansatech) at 25 °C illuminated with white light (intensity of 300 μmol photons m⁻² s⁻¹). Particle size distribution was measured with the laser diffraction particle analyser ALV ASYSETTE 22 (Fritsch). Cell concentration was determined by counting in a Neubauer counting chamber.

**Viability test.** For spot assay experiments, cells were grown to OD750 0.5–0.7 and adjusted to a chlorophyll content of 5 μg ml⁻¹ at the beginning of the experiment. Aliquots (7 μl) of three different dilutions (1:1, 1:10, 1:100) were dropped on BG11 agar plates; the plates were incubated at 28 °C under continuous or alternating (12 h light/12 h dark) illumination (60 μmol photons m⁻² s⁻¹) for 7 days.

**Plasmids and mutagenesis.** For insertion mutagenesis, the glgC ORF (locus sll1176), the glgA₁ ORF (locus sll0945) and the glgA₂ ORF (locus sll1393) were amplified with #glgC-fw (5’-GTTGTTGTGTGGCAATTCG) and #glgC-rv (5’-GCCAGCTCTAGGGTAAA), with #glgA₁-fw (5’-CGACGGTTAGACTTCTTCTT) and #glgA₁-rv (5’-CCCGGGAACGGTGCAAC) and with #glgA₂-fw (5’-GGCGAGGGGAATTCTTCCG) and #glgA₂-rv (5’-GGGGATAATGAGCAGAAGTTT), ligated into the pJET1.2 vector (Fermentas) or pUC19 (New England Biolabs) and disrupted by deletion-insertion mutagenesis (for glgC) or insertion mutagenesis (for glgA₁ and glgA₂) of a chloramphenicol resistance cassette from pACYC184 (New England Biolabs) or a kanamycin resistance cassette from pUC4K (GE Healthcare) in sense as well as in antisense orientation to the respective gene in order to check for polar effects. The constructs were used to transform Synechocystis as described by Ermakova et al. (1993). Transformants were restreaked six times and analysed by Southern blot hybridization to detect the level of segregation of genome copies.

**Southern blot analysis.** Genomic DNA of Synechocystis was extracted as described by Franche & Damerval (1988). After digestion of DNA with the restriction endonucleases NcoI, XbaI, Smal and EcoRI (Fermentas), electrophoresis and gel blotting to nylon membranes (Amersham Hybond-N, GE Healthcare) were performed. DNA probe fragments, obtained by PCR for glgC with #glgC150-fw (5’-TCCGTCGATATTCTGCTCA) and #glgC330-rv (5’-AGTGCCCTGTTAAAACATCG) and from restriction with NcoI and XbaI of pUC19/glgA₁ and pUC19/glgA₂, respectively, were labelled with [α-³²P]dCTP with the DecaLabel DNA labelling kit (Fermentas) as recommended by the manufacturer. DNA:DNA hybridizations were performed according to standard protocols (Sambrook et al., 1989).
glucogen was resuspended in 100 mM sodium acetate (pH 4.5) and enzymically hydrolysed to glucose by treatment with 2 mg amyloglucosidase ml\(^{-1}\) (Roche Diagnostics) at 60 °C for 2 h. Glucogen content was determined with the glucose reagent (Hexokinase method) from Thermo Fisher Scientific according to the manufacturer’s instructions.

**Quantification of extracellular glucose.** The extracellular glucose content was determined in 10 μl medium with the glucose reagent (Hexokinase method) from Thermo Fisher Scientific.

**Determination of extracellular metabolites.** For analysis of extracellular metabolites the filtered (costar Spin-X 0.22 μm Nylon PP Tube; Corning) supernatants of cell suspensions were directly analysed by ion exclusion chromatography using a Shim-pack SCR-102H column and Guard Column SCR-102H (Shimadzu) with 4 mM p-toluensulfonic acid aqueous solution as mobile phase, a flow rate of 0.8 ml min\(^{-1}\) and a column temperature of 46 °C. Electroconductivity of organic acids was analysed by the post-column pH-buffered addition of 16 mM Bistris, 100 μM EDTA in 4 mM p-toluensulfonic acid aqueous solution. Identification of the respective peaks and quantification via calibration curves was achieved using commercial standards (Sigma Aldrich). Additionally, optical tests were performed for quantification of extracellular pyruvate (Lamprecht & Heinz, 1984) and 2-oxoglutarate (Senior, 1975). Both methods gave essentially identical results. Glutamate was quantified in a colorimetric assay using glutamate dehydrogenase, NAD, iodonitrotetrazolium and phenazine methosulfate (500 nm).

**RESULTS**

**Knockout of glucogen synthesis**

Genes responsible for glucogen synthesis in cyanobacteria have been identified by their similarity to the respective homologues for AGPase and glucogen synthase from *E. coli*, plants and green algae. The genome of *Synechocystis* encodes one AGPase homologue (*glgC: slr1176*) and two glucogen synthase homologues (*glgA: sll0945, sll1393*) (Nakao et al., 2010). ORF *sll0945* encodes a bacterial-type glucogen synthase, homologues of which are present in all cyanobacteria, subsequently referred to as *glgA* (Cid et al., 2002). The additional (starch-type) glucogen synthase (*sll1393*) is referred as *glgA\(_2\)* according to the annotated homologue in *Synechococcus* sp. PCC 7002.

Complete inhibition of glucogen synthesis was achieved by mutagenesis of either AGPase (*AglcG*) or simultaneous inactivation of both glucogen synthases (*ΔglgA\(_1/ΔglgA\(_2\)*), respectively. The knockouts were verified by Southern blot analyses (not shown). The inability of the mutants to synthesize glucogen was checked after nitrogen depletion, conditions that trigger massive glucogen accumulation in wild-type cells as a short-term response of the general stress acclimation process (see Fig. 4a below). By contrast, single knockout mutants of glucogen synthases, *ΔglgA\(_1\)* and *ΔglgA\(_2\)*, respectively, were still able to accumulate similar amounts of glucogen as the wild-type (not shown). Consequently, either the double knockout mutant *ΔglgA\(_1/ΔglgA\(_2\)*) or the single knockout mutant *ΔglgC* was used to analyse the impact of incapacities in glucogen synthesis on the cellular stress responses and on the carbon and nitrogen metabolism.

**Reduced photosynthetic capacity and high-light-sensitive phenotype**

Mutants unable to synthesize glucogen were not impaired in their photoautotrophic growth. On agar plates (Fig. 1a) as well as in liquid cultures (Fig. 2a) a similar growth behaviour was observed for the wild-type and all knockout mutants. Growth rates of 0.32 μg chlorophyll (ml cell suspension\(^{-1}\)) per day for wild-type and 0.30 μg chlorophyll (ml cell suspension\(^{-1}\)) per day for the mutants were measured under constant illumination with 40 μmol photons m\(^{-2}\) s\(^{-1}\) and constant aeration with 0.03 % (v/v) CO\(_2\) (Fig. 2a). Even though no significant growth differences were detectable, obvious phenotypes of glucogen-deficient mutants (*ΔglgA\(_1/ΔglgA\(_2\)\* ΔglgC*) were observed under strict photoautotrophic conditions: (i) elevated sensitivity to high light intensities (not shown) and to low inocula (Fig. 1a) and (ii) significant reduction in photosynthetic capacity. Oxygen evolution rates of 212 ± 8 (S.D.) μmol O\(_2\) h\(^{-1}\) (mg

![Fig. 1. Growth and viability under light–dark cycle conditions.](image-url)
Impairment of viability under stress conditions

Drastic phenotypes occurred when cells were stressed by varying conditions such as alternating light–dark periods (A), photomixotrophic growth in the presence of CO₂ and glucose (B), and nitrogen starvation (C).

(A) Restricted viability under alternating light–dark periods. Synchronized cells of Synechocystis (wild-type) accumulated glycogen in minor levels of 2.0–2.3 μg glucose equivalents (μg chlorophyll)⁻¹ during the 16 h light period (140 μmol photons m⁻² s⁻¹) and dissipated barely half of it to levels of 1.2–1.3 μg glucose equivalents (μg chlorophyll)⁻¹ during the 8 h dark period. The growth rate (wild-type) was slightly reduced to 0.22 μg chlorophyll (ml cell suspension)⁻¹ per day compared with strict photoautotrophic conditions. In contrast, cells incapable of glycogen synthesis (ΔglgA₁/ΔglgA₂; ΔglgC) were restricted in their viability under this light–dark regime (Fig. 1b). The loss in viability became most evident in spot assays where a minimal inoculum of cells has been tested. Based on identical inocula, the colour intensity of the colony spots differed significantly between wild-type and mutants after only a few days of growth. The wild-type cells formed deep blue–green colonies even from highly diluted inocula (Fig. 1b, 1:100). Much larger inocula of mutant cells were required to obtain colonies of similar cell density (Fig. 1b, 1:1).

(B) Photomixotrophy-sensitive phenotype. Synechocystis is capable of growing photomixotrophically and uses both CO₂ and glucose as carbon source in parallel under these conditions. In this case, it is assumed that the synthesized glycogen is a product not only of photosynthesis but also of glucose conversion (Smith, 1982). Under the conditions tested, glycogen levels of 1.0–2.0 μg glucose equivalents (μg chlorophyll)⁻¹ were determined in the presence of 5 mM glucose and 0.03 % (v/v) CO₂, similar to the value under strictly photoautotrophic conditions (see Fig. 4d). Simultaneous assimilation of CO₂ and glucose led to an enhanced exponential growth rate of 0.38 μg chlorophyll (ml cell suspension)⁻¹ per day in wild-type (Fig. 2b), whereas the growth rates of both mutants (ΔglgA₁/ΔglgA₂; ΔglgC) were reduced to 0.20 μg chlorophyll (ml cell suspension)⁻¹ per day under identical conditions (Fig. 2b). Moreover, the glucose uptake rates differed between wild-type and glycogen-deficient mutants. Based on the addition of 5 mM glucose to each culture, glucose levels were diminished to half within 50 h in wild-type cultures, whereas in cultures of mutants impaired in glycogen synthesis this level was not reached before 100 h (Fig. 2b). On agar plates containing glucose, cells with impaired glycogen synthesis were severely restricted in their viability and were unable to resume growth even after several days (not shown).

(C) Loss of viability under nitrogen starvation. Nitrogen depletion strongly promoted glycogen accumulation in the wild-type and of 174 ± 7 (sd) [μmol O₂ h⁻¹ (mg chlorophyll)⁻¹] in ΔglgA₁/ΔglgA₂ and ΔglgC mutants were determined under saturating illumination with white light.
wild-type cells. Here, glycogen levels of $67.5 \pm 3.7 \mu g$ glucose equivalents (µg chlorophyll)$^{-1}$ were reached under light intensities of 100 µmol photons m$^{-2}$ s$^{-1}$ after 48 h of nitrogen deprivation (Fig. 4a below). Long-term nitrogen depletion led inevitably to a loss of viability for mutants that lacked the capacity for glycogen synthesis ($\Delta$lggA$\_1$/ΔlggA$\_2$, ΔlggC). In spot assays, cells of cultures starved for nitrogen for up to 7 days were transferred to nitrogen-containing agar plates (Fig. 3). The wild-type cells formed blue–green colonies even at high dilutions (Fig. 3b: 1 : 100) and even after a prolonged period of nitrogen starvation (Fig. 3b). In contrast, viability of mutant cells ($\Delta$lgg$\_1$/ΔlggA$\_2$, ΔlggC) was so strongly affected within a few days of nitrogen depletion that regrowing was impeded (Fig. 3b).

‘Overflow metabolism’: release of pyruvate and 2-oxoglutarate

To prove the role of glycogen synthesis as an ameliorator of carbon excess, metabolite profiles of culture supernatants of the wild-type and of the knockout mutants incapable of glycogen synthesis ($\Delta$lgg$\_1$/ΔlggA$\_2$, ΔlggC) were determined. Under conditions of nitrogen deprivation or photomixotrophic growth, the mutant cells massively lost energetically relevant metabolites such as pyruvate and 2-oxoglutarate. After 48 h of nitrogen starvation, pyruvate levels of 0.4–0.6 mM and 2-oxoglutarate levels of 0.14–0.17 mM were measured in the culture supernatants (Fig. 4b, c, white bars), similar to recently published results (Carrieri et al., 2012). Glucose addition also led to an accumulation of pyruvate to levels of 1.0–1.4 mM and of 2-oxoglutarate to levels of 0.11–0.13 mM in the medium of these mutant cultures within 48 h (Fig. 4e, f, white bars). These energetically relevant metabolites were not detected under either strictly photoautotrophic conditions in the supernatants of glycogen-deficient mutants (Fig. 4, grey bars) or the tested stress conditions in the supernatants of wild-type cells (Fig. 4) or of the single knockout mutants $\Delta$lgg$\_1$ and $\Delta$lgg$\_2$ (not shown). Both lysis and general leakage effects could be excluded: first, no phycobiliproteins were detectable in the supernatants by spectrophotometric analysis. Second, the highly abundant intracellular amino acid glutamate was not detected in any of the supernatants (not shown).

Upon nitrogen starvation, wild-type cells did not accumulate glycogen when kept in darkness, and not even traces of energetically relevant metabolites were detectable in the supernatants of glycogen-deficient mutants under this condition. However, glycogen accumulation in wild-type cells as well as metabolite release from cells incapable of glycogen synthesis was detectable about 5 h after switching on the light (not shown). The release of pyruvate and 2-oxoglutarate by the glycogen-deficient mutants is thus a light-dependent process, like glycogen accumulation in wild-type cells.

**Fig. 3.** Viability test under nitrogen-depleted conditions. (a) The chlorotic process in cells of wild-type (WT) and of mutants defective in glycogen synthesis ($\Delta$lgg$\_1$/ΔlggA$\_2$, ΔlggC) in response to nitrogen starvation. (b) Viability test of nitrogen-starved cells of wild-type (WT) and mutants defective in glycogen synthesis ($\Delta$lgg$\_1$/ΔlggA$\_2$, ΔlggC) under nitrogen-replenished conditions. Differentially prolonged starved cells (0, 1, 2, 4, 7 days) were plated at different dilutions [1 : 1 (undiluted), 1 : 10, 1 : 100] on BG11 agar, pH 8.0 and incubated under continuous illumination of 60 µmol photons m$^{-2}$ s$^{-1}$ at 28 °C for 7 days. The effect was verified by four independent spot assays.
Impaired acclimation response upon nitrogen depletion: the non-bleaching phenotype

Unicellular, non-diazotrophic cyanobacteria such as Synechocystis undergo substantial changes in response to nutrient starvation. Two characteristics are the completion of cell division in the absence of further cell growth and the degradation of the light-harvesting antennae, the phycobilisomes, by a proteolytic process termed chlorosis (Schwarz & Forchhammer, 2005). Chlorosis becomes evident by a bleaching process of the cells that results in a colour change from blue-green to yellow-green (Fig. 3a, wild-type). Knockout mutants that lack the ability for glycogen synthesis were not able to degrade phycobilisomes. These cells maintained their greenish-blue colouring despite nitrogen depletion (Fig. 3a, ΔglgA1/ΔglgA2; ΔglgC). In whole cell absorbance spectra, the phycocyanin peak (628 nm) was unchanged in the ΔglgA1/ΔglgA2 and ΔglgC mutants (Fig. 5), whereas in the wild-type (Fig. 5) and in the single knockout mutants ΔglgA1 and ΔglgA2 (not shown) a distinct decline in the phycocyanin peak was detectable within a few hours of nitrogen depletion. Moreover, mutant cells incapable of glycogen synthesis (ΔglgA1/ΔglgA2, ΔglgC) were not able to divide after nitrogen deprivation. Total cell concentration as well as the chlorophyll content per cell remained nearly constant under these conditions. In contrast, wild-type cells duplicated once whilst their chlorophyll content dropped from about 8.0 ± 0.6 × 10⁻⁹ to 4.4 ± 0.7 × 10⁻⁹ µg per cell.

DISCUSSION

Knockout of glycogen synthesis in cyanobacteria

The ability to synthesize glycogen seems to be conserved in cyanobacteria. The genes glgC (encoding the AGPase), glgA (encoding the glycogen synthases) and glgB (encoding the branching enzyme), which are involved in the synthesis of the polymer, have been found in each of the so-far annotated cyanobacterial genomes (Beck et al., 2012; Nakao et al., 2010). In unicellular, non-diazotrophic cyanobacteria such as Synechocystis sp. PCC 6803 (this study) and S. elongatus PCC 7942 (Suzuki et al., 2010), glycogen synthesis was abrogated by the independent knockout of either AGPase or glycogen synthase(s). Neither glycogen (this study, Fig. 4) nor the respective enzymic activities (Suzuki et al., 2010) were detectable in cellular extracts of the mutants. Thus, both enzyme activities are essential for glycogen synthesis, and AGPase is the only source of ADP-glucose for glycogen and probable glycosylglycerol biosynthesis in Synechocystis.
enzymes involved in starch metabolism (Patron & Keeling, 2005). Both types of synthases are conserved in free-living cyanobacteria, was lost in the very early loss of glycogen synthesis during endosymbiosis (Nowack et al., 2008). All recent Archaeplastida synthesize starch, a glucose polymer that is structurally and physically different from glycogen (Ball et al., 2011). Due to an increasing detection of starch-like polymers in present-day cyanobacteria and due to the phylogenetic origin of the granule-bound starch synthase from bacteria, it is assumed that ancient cyanobacteria were capable of starch and/or glycogen synthesis. In line with this, a few enzymes of the cyanobacterial polyglucan (glycogen) metabolism clearly have a common origin with enzymes involved in starch metabolism (Patron & Keeling, 2005). Synechocystis sp. PCC 6803 possesses two isoenzymes of polyglucan synthase: ORF sl0945 (glgA1) clusters together with eubacterial glycosyltransferases and ORF sl1393 (glgA2) clusters together with plant and algal soluble (not granule bound) starch synthases (Cid et al., 2002; Patron & Keeling, 2005). Both types of synthases are involved in the polyglucan synthesis in Synechocystis, as demonstrated by the fact that both genes had to be inactivated for complete inhibition (see Results). Moreover, single knockout mutagenesis revealed that both synthases can replace the activity of the other, and synthesize similar amounts of polyglucans under the tested physiological conditions (see Results). Potentially distinctive roles of these isoenzymes in polyglucan (glycogen or starch-like) biosynthesis were not detectable with the applied methods and thus remain undefined.

**Essential role of glycogen synthesis in dark–light cycles**

Probably the most important changes which cyanobacterial metabolism has to accommodate to are the transitions between light and dark periods. Cells incapable of glycogen synthesis are obviously more sensitive to regular dark–light transitions than wild-type cells (Fig. 1). The impaired viability (reduced survival rate) of such cells supports the idea of glycogen’s dual role as reserve and buffer substance (Carr, 1988).

Dark–light transitions involve changes in cytoplasmic pH and redox state, as well as changes in the intracellular concentration of specific metabolites and metal ions (Smith, 1982). These factors mainly regulate the switch between assimilatory (photosynthetic) and catabolic pathways in the cyanobacterial cell (Smith, 1982). Transitions from dark to light stress the cells, as reflected in an increased transcript accumulation of stress genes (Gill et al., 2002). We assume that both glycogen and glycogen synthesis have crucial roles in this acclimation process, both acting as buffers and cellular tools for compensation of such stressful energetic transitions, mainly to ameliorate and to avoid futile cycles during the process of changing photosynthetic activity and of metabolic switching, similar to what is assumed for starch synthesis in plants (Sun et al., 1999).

In addition to this regulatory function, the glycogen pool might be the main endogenous reserve in Synechocystis that provides maintenance energy for cell integrity, function and viability in dark periods. Based on this assumption, the inactivation of glycogen synthesis should result in a non-viable genotype, as predicted by flux-balance analysis of the Synechocystis metabolic network (Knoop et al., 2010). Here, glycogen metabolism appears to be vital to produce all precursors required for biomass formation and to regenerate all metabolites and cofactors, especially in dark periods. However, the complete segregation genotype of both AGP and glycogen synthase mutants of Synechocystis suggests the presence of other available (minor) endogenous respiratory substrates. For instance, starch-less mutants of Nicotiana sylvestris accumulate sufficient soluble neutral sugars during the day which obviously provide respiratory substrates in the dark (Huber & Hanson, 1992). Thus, other available substrates such as sugars, lipids or chlorophyll may partly compensate for the lack of glycogen. However, the restricted viability of the Synechocystis mutants (Fig. 1) and

![Fig. 5. Progression of phycobilisome degradation under conditions of nitrogen depletion. Progression of phycobilisome degradation (phycoerythrin peak at 628 nm) was monitored by the analysis of whole cell absorbance spectra of wild-type (WT), ΔglgA1/ΔglgA2 and ΔglgC mutants after 0 h (solid line) and 24 h (dashed line) of nitrogen depletion. After nitrogen step-down strains were continuously illuminated with 100 μmol photons m⁻² s⁻¹. Spectra were normalized to the chlorophyll peak (683 nm). The effect was verified in 10 independent experiments.](image-url)
the reduced respiratory oxygen consumption by *S. elongatus* PCC 7942 (Suzuki et al., 2010) indicate that this polymer is the major respiratory substrate in cyanobacteria. We nevertheless assume that glycogen and glycogen synthesis is primarily needed as a buffer substance and an ameliorator in acclimation processes whenever metabolic switches in amphibolic pathways occur. The polymer likely has a similar role during transitions of cyanobacteria to periods of nitrogen starvation or to photomixotrophic growth. Both conditions conceivably mimic the physiological states of cells under light–dark transitions (Lee et al., 2007) and will be discussed below in more detail.

**Essential role of glycogen synthesis for the energy and carbon metabolism**

Phenotypes that result from the inability to synthesize glycogen are very prominent: (i) reduced oxygen evolution rates (see Results), (ii) glucose-sensitivity (Fig. 2b), (iii) a non-bleaching phenotype (Fig. 5) and (iv) overflow of pyruvate and 2-oxoglutarate (Fig. 4). All detected characteristics occur explicitly in the light when glycogen synthesis is needed and significant carbon fluxes are sensed by the cell. In cyanobacteria, photosynthesis and carbon/nitrogen assimilation are linked via the ferredoxin/thioredoxin system. Prominent targets of the thioredoxin system are enzymes of carbon and nitrogen assimilation, such as ribulose-1,5-bisphosphate carboxylase/oxygenase and glutamine oxoglutarate aminotransferase, as well as all enzymes involved in glycogen synthesis, such as phoshoglcomutase, AGPase, glycogen synthase and branching enzyme (Lindahl & Florencio, 2003) (see Fig. 6 for an overview). This regulatory principle explains the fact that glycogen synthesis and thus the phenotypes of glycogen deficiency occur only in the light and stop immediately in the dark.

The significant reduction of photosynthetic capacity (see Results) in the glycogen-deficient mutants is a common feature of all currently published polyglucan-free mutants in photosynthetic organisms (Eichelmann & Laik, 1994; Miao et al., 2003b; Sun et al., 1999; Suzuki et al., 2010). The real cause has not yet been identified. We assume a role of glycogen in photosynthetic reactions as has been suggested for starch (Eichelmann & Laik, 1994; Sun et al., 1999). Glycogen synthesis has been proposed to act as an electron acceptor and an ameliorator during periods of changing photosynthetic activities. It has also been suggested that glycogen metabolism is involved in anaplerotic reactions of the Calvin–Benson–Bassham cycle during acclimation to changing CO₂ concentrations (Eisenhut et al., 2008). Therefore, glycogen metabolism might be involved both in maintaining the redox homeostasis and in the acclimation to changes in photosynthetic activity.

**Glycogen’s essential role in stress responses**

(1) **Metabolic switch into the ‘dormant state’: accumulation of reserves.** Various micro-organisms including species of the cyanobacterial orders *Nostocales* and *Stigonematales* are capable of forming specialized resting cells in response to macronutrient starvation. In contrast, *Oscillatoriales* and *Chroococcales* such as *Synechocystis* lack the capacity to form such spore-like cells. Nevertheless, their cells also switch stringently from a growth-related mode (active state) to a maintenance/survival mode without growth and multiplication (dormant state) (Kaprelyants et al., 1993). During the transition of *Synechocystis* from an active to a dormant state, several metabolic processes are substantially changed (Krasikov et al., 2012; Aguirre von Wobeser et al., 2011). Here, a massive accumulation of carbon reserve polymers like glycogen (Fig. 4a) and polyhydroxybutyrate (PHB), degradation of phycobiliproteins (Fig. 5) and an activation of catabolic pathways are observed (Schwarz & Forchhammer, 2005). The simultaneous synthesis of both carbon reserve polymers by *Synechocystis* and some other cyanobacteria is an exception to the principle that only one kind of carbon reserve material is formed by a given species. In this context, it has to be emphasized that a mutant of *Synechocystis* defective in PHB synthesis (ΔphaAB) employs in response to nitrogen starvation a similar metabolic change, including phycobilisome degradation like the wild-type, and was only marginally affected in its viability (Taroncher-Oldenburg & Stephanopoulos, 2000) (see overview in Fig. 6). By contrast, mutants defective in glycogen synthesis seem to be unable to switch: they are incapable of cell division and of the chlorotic response (Fig. 5). Moreover, these cells lost their viability after a few days. These data indicate that in *Synechocystis* glycogen synthesis plays a more decisive role in the metabolic changes during the acclimation response and in viability maintenance during starvation periods than the other carbon reserve PHB. The long survival time of *Synechocystis* correlates with the small average chain length of cyanobacterial glycogen and sustains glycogen’s global role as a durable energy reserve that is essential for bacterial survival and persistence to stress conditions (Preiss, 1984; Wang & Wise, 2011).

(2) **Chlorotic response.** The observed non-bleaching phenotype (lack of degradation of phycobilisomes) of mutants incapable of glycogen synthesis (Fig. 5) is usually assigned to the absence, modification or inhibition of proteins that are involved in the nitrogen signalling cascade (Schwarz & Forchhammer, 2005). Just a few metabolic knockouts are known to cause a non-bleaching phenotype (Lahmi et al., 2006; Morrison et al., 2005). Here, manipulations of pyruvate metabolism via knockout of alanine dehydrogenase or of phosphotransacetylase in a Δhik33 (Δdspa) background result in a deceleration but not in a total inability of phycobilisome degradation, as is observed in cells impaired in glycogen synthesis (see overview Fig. 6). Even 7 days of nitrogen starvation did not change the greenish colour of glycogen-deficient mutant cells (Fig. 3a). Changes in metabolite levels by manipulation of the pyruvate metabolism, such as knockouts of alanine dehydrogenase or glycogen synthesis, obviously influence the progression of phycobilisome degradation (Fig. 4a, overview in Fig. 6). We
assume that additional metabolic signals within the pyruvate metabolism contribute to the acclimation response in cyanobacteria. To what extent molecular processes such as transcription, RNA processing, translation, post-translational modifications and/or protein–protein interactions of the nitrogen signalling cascade are affected by these manipulations is as yet unknown.

(3) Energy spilling reactions. Cells incapable of glycogen synthesis get into an acute dilemma since energy and ADP

\[ \text{ATP} \rightarrow \text{ADP} + \text{Pi} \]

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carbon excess cannot flow into glycogen synthesis. To release the excess, cells lose significant amounts of pyruvate and 2-oxoglutarate (Fig. 4). The amount of carbon released into the medium by the glycogen-deficient mutants as pyruvate and 2-oxoglutarate was calculated roughly to correspond to 30–60 % of the carbon that would have been used for glycogen synthesis by the wild-type under similar conditions (Fig. 4). Massive release of carbon compounds under conditions of unbalanced growth is well known as ‘overflow’ metabolism in heterotrophic bacteria. The ‘overflow’ metabolism is a form of ‘energy spilling’ (Russell, 2007). Characteristic of the overflow metabolism is the excretion of partially oxidized metabolites such as 2-oxoglutarate, pyruvate, succinate and gluconate, capsular material and protein into culture medium (Russell & Cook, 1995). The mechanism of membrane passage of these compounds is unclear for *Synechocystis*. Certainly, it does not reflect a general leakage from the cells since a concomitant release of glutamate, which is known to be a highly abundant free amino acid in *Synechocystis* (Eisenhut et al., 2008), was not observed in the culture medium (see Results). Our data indicate that glycogen synthesis is an essential cellular tool for integration of energy and carbon homeostasis to avoid energy spilling reactions in *Synechocystis*.

(4) The mystery of photomixotrophic sensitivity: role of glycogen synthesis for stressful amphibolic reactions in response to energetic and carbon transitions. Simultaneous assimilation of CO₂ and glucose (mixotrophy) results in a unique situation inside the cell. Directly opposed pathways are used in parallel: glycolysis and gluconeogenesis as well as reductive (Calvin cycle) and oxidative pentose phosphate pathways by sharing both enzymes and reaction intermediates (Kaplan et al., 2008) (see overview in Fig. 6). Such conditions increase the growth rate of *Synechocystis* (Fig. 2b) and other cyanobacteria. In contrast, cells incapable of glycogen synthesis have a significantly reduced growth rate under these conditions (Fig. 2b). On agar plates containing glucose, the cells lost their viability and were unable to resume growth within a few days. This phenotype is referred to as ‘photomixotrophic sensitivity’. The respective wild-type, however, retained the property of growth in the presence of glucose under the otherwise identical conditions. The photomixotrophic sensitivity phenotype is also known from other metabolic knockouts such as glycolytic unidirectional glycerol-3-phosphate dehydrogenase 1 (*gap1*) (Koksharova et al., 1998; Osanai et al., 2007) (see overview in Fig. 6). It is assumed that glucose uptake inflicts lethality and leads to a disturbed carbon flux and balance in such mutants (Kaplan et al., 2008). The exact mechanism is as yet unclear. Our data on overflow metabolism (Fig. 4) indicate that blocks in glycogen synthesis lead to a severely disturbed carbon flux and balance. We assume that glycogen synthesis enables the cell to cope with intrinsic bottlenecks or deficits in the form of anabolic and anaplerotic reaction sequences and thus allows essential metabolic switches in response to energetic and carbon transitions. To what extent metabolic routes other than photosynthetic reactions are impaired by knockout of glycogen synthesis is as yet unclear. It is known that the darkness-dependent glucose sensitivity is based primarily on inhibition of the oxidative pentose phosphate pathway by knockout of glucose-6-phosphate dehydrogenase (G6PDH) (Jansén et al., 2010). The oxidative pentose phosphate pathway is involved in not only glycogen mobilization through night periods but also the metabolism of organic substrates into glycogen in the light (Smith, 1982). Therefore, we assume a close interaction between oxidative pentose phosphate pathway and glycogen metabolism. The G6PDH is controlled by fluctuating ATP and NADPH concentrations as well as light-induced pH changes in the extra-thylakoidal environment (Grossman & McGowan, 1975). Glucose addition usually changes the cytosolic pH and gene expression (Ryu et al., 2004). We assume that a block of oxidative pentose phosphate pathway and/or a pH shift cause an inhibition of G6PDH and thus of glucose metabolism in cells defective in glycogen synthesis. The putative impact of an acidification of growth medium on viability, caused by the overflow of pyruvate and 2-oxoglutarate, was excluded by optimal HEPES buffering at pH 8.0 and controlling. Dilution effects in batch cultures (Fig. 2b) might attenuate putative acidification in contrast to more concentrating effects (slow diffusion) on solidified media.

The glucose uptake is also repressed in glycogen-deficient mutants (Fig. 2b). This crucial effect does not correlate with the level of growth restriction (Fig. 2b). We assume that a currently unknown negative feedback loop from central carbon metabolism acts on the glucose uptake
system similar to that observed for trehalose metabolism in *Saccharomyces cerevisiae* (Teusink et al., 1998).

**Conclusion**

In conclusion, cells incapable of glycogen synthesis are an ideal tool for identification of metabolic regulatory principles and signals. These mutants will most likely contribute enormously to the understanding of complex carbon and nitrogen metabolism and of acclimation responses in cyanobacteria.

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