



Enhanced Nitrogen Fixation in a *glgX*-Deficient Strain of *Cyanothece* sp. Strain ATCC 51142, a Unicellular Nitrogen-Fixing Cyanobacterium

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ABSTRACT Cyanobacteria are oxygenic photosynthetic prokaryotes with important roles in the global carbon and nitrogen cycles. Unicellular nitrogen-fixing cyanobacteria are known to be ubiquitous, contributing to the nitrogen budget in diverse ecosystems. In the unicellular cyanobacterium *Cyanothece* sp. strain ATCC 51142, carbon assimilation and carbohydrate storage are crucial processes that occur as part of a robust diurnal cycle of photosynthesis and nitrogen fixation. During the light period, cells accumulate fixed carbon in glycogen granules to use as stored energy to power nitrogen fixation in the dark. These processes have not been thoroughly investigated, due to the lack of a genetic modification system in this organism. In bacterial glycogen metabolism, the *glgX* gene encodes a debranching enzyme that functions in storage polysaccharide catabolism. To probe the consequences of modifying the cycle of glycogen accumulation and subsequent mobilization, we engineered a strain of *Cyanothece* 51142 in which the *glgX* gene was genetically disrupted. We found that the Δ *glgX* strain exhibited a higher growth rate than the wild-type strain and displayed a higher rate of nitrogen fixation. Glycogen accumulated to higher levels at the end of the light period in the Δ *glgX* strain, compared to the wild-type strain. These data suggest that the larger glycogen pool maintained by the Δ *glgX* mutant is able to fuel greater growth and nitrogen fixation ability.

IMPORTANCE Cyanobacteria are oxygenic photosynthetic bacteria that are found in a wide variety of ecological environments, where they are important contributors to global carbon and nitrogen cycles. Genetic manipulation systems have been developed in a number of cyanobacterial strains, allowing both the interruption of endogenous genes and the introduction of new genes and entire pathways. However, unicellular diazotrophic cyanobacteria have been generally recalcitrant to genetic transformation. These cyanobacteria are becoming important model systems to study diurnally regulated processes. Strains of the *Cyanothece* genus have been characterized as displaying robust growth and high rates of nitrogen fixation. The significance of our study is in the establishment of a genetic modification system in a unicellular diazotrophic cyanobacterium, the demonstration of the interruption of the *glgX* gene in *Cyanothece* sp. strain ATCC 51142, and the characterization of the increased nitrogen-fixing ability of this strain.

KEYWORDS conjugation, cyanobacteria, glycogen, nitrogen fixation, photosynthesis

Cyanobacteria are photosynthetic microbes that are widely studied for their contributions to global carbon sequestration and nitrogen fixation (1). The cyanobacterial strains that are able to fix atmospheric N₂ to bioavailable forms have evolved strategies to separate the two incompatible processes. In some filamentous cyanobacteria, nitrogen fixation occurs in specialized cells called heterocysts, whereas photosynthesis is performed in the vegetative cells (2). In contrast, nitrogen-fixing unicellular

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cyanobacteria separate the incompatible processes of photosynthesis and nitrogen fixation temporally within the same cell (3). In these cells, light is harvested during the day and used by photosynthesis to fix atmospheric CO₂ and in turn to generate energy and reducing equivalents. Fixed CO₂ is stored in glycogen granules, which are used by respiration during the night to fuel energy-demanding processes like nitrogen fixation. The products of nitrogen fixation are stored in cyanophycin bodies, which are nonribosomally synthesized polymers of arginine and aspartic acid (4). Single-cell nitrogen-fixing cyanobacteria have been recognized as crucial players in all ecosystems, contributing significantly to carbon and nitrogen levels.

Of the unicellular nitrogen-fixing cyanobacteria, *Cyanothece* constitutes an important genus, with members exhibiting robust growth and high rates of nitrogen fixation (5). *Cyanothece* strains are known to occur worldwide in diverse ecosystems and have been studied extensively (6–9). One *Cyanothece* strain, *Cyanothece* sp. strain ATCC 51142, has been comprehensively studied at the genomic, transcriptomic, proteomic, ultrastructural, and physiological levels (10–13). This strain was found to have several novel attributes, including high levels of nitrogen fixation and hydrogen production (14–16). Despite these insights, until now genetic manipulation and development of a reliable system of genetic engineering have remained elusive in *Cyanothece* 51142, handicapping the potential to develop this strain further as a model system (17).

Carbon metabolism is central to cyanobacterial energy management, and carbon synthesis and degradation pathways have been shown to have significant effects on cellular processes (18, 19). The ability to store metabolic products in inclusion bodies for later mobilization is particularly crucial for unicellular diazotrophic cyanobacteria that maintain an active metabolic profile throughout the diel cycle. For example, mobilization of energy stored in glycogen granules is essential to drive the energy-expensive nitrogenase reaction at night.

Storage polysaccharides are found in bacteria, algae, and green plants, where they are key to survival (20). Soluble glycogen is found in most strains of bacteria, including cyanobacteria, whereas starch is synthesized by algae and plants as insoluble granules. After endosymbiosis of an ancestor of present-day cyanobacteria by a eukaryotic cell, starch metabolism in the host resulted from the merging of the pathways of storage polysaccharide production in the two organisms (21). In fact, probing these pathways has provided insights into the polysaccharide metabolism in the endosymbiont before endosymbiosis, the acquisition of starch metabolism in cyanobacteria, and the important role of the debranching enzyme (20, 22). While starch and glycogen are both constructed from α -1,4 linkages and α -1,6 branches, these two polymers differ greatly in appearance and characteristics. Unlike glycogen, which is limited in size to a maximum of \sim 40 nm, starch size is unlimited (20).

Interestingly, within the *Cyanothece* genus, two different types of polysaccharide storage bodies have been described (23, 24). In *Cyanothece* 51142, storage bodies formed as large granules (\sim 50 nm by \sim 150 nm) between the thylakoid membranes (13) are commonly referred to as glycogen granules (13, 25). In contrast, *Cyanothece* sp. strain PCC 7822 forms much smaller glycogen granules (26). The structures of the different types of granules were found to be semiamylopectin in *Cyanothece* 51142 and β -granules of glycogen in *Cyanothece* 7822 (26). Thus, members of the *Cyanothece* genus offer opportunities to dissect the carbon storage and degradation pathways and to elucidate their implications in the diel cycle.

Here we report the development and application of a reproducible genetic modification system in *Cyanothece* 51142 and the disruption of the glycogen-debranching gene *glgX* in this organism. In order to determine the effects of interference in the polysaccharide accumulation and degradation system during the diurnal cycle in this strain, we inactivated the *glgX* gene by targeted mutagenesis. We found that the Δ *glgX* strain exhibited a higher growth rate, compared to the wild-type (WT) strain, when grown under nitrogen-replete conditions and displayed a higher rate of nitrogen fixation under nitrogen-depleted conditions. Glycogen accumulated to higher levels at the end of the light period in the Δ *glgX* strain, compared to the WT strain. These data

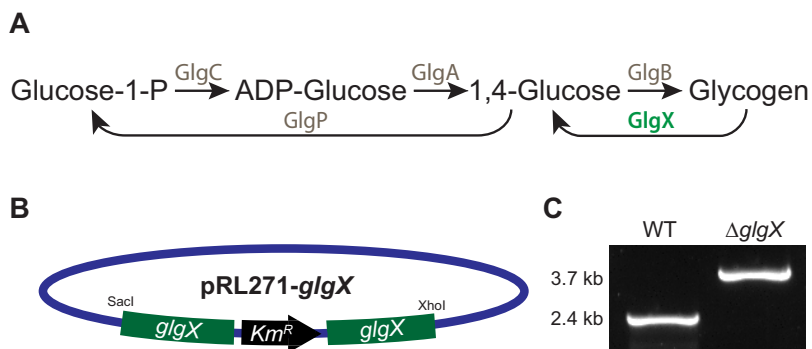


FIG 1 (A) Metabolic pathway of glycogen synthesis and degradation in *Cyanothece* 51142. The GlgX enzyme is shown in green. (B) Plasmid construct used for interruption of the *glgX* gene, showing restriction enzyme sites used for cloning. (C) PCR results showing the kanamycin resistance gene inserted into *glgX*, yielding a larger product than in the WT strain.

suggest that the larger glycogen pool maintained by the $\Delta glgX$ mutant is able to fuel greater growth and nitrogen fixation ability.

RESULTS

In bacteria, including cyanobacteria, glycogen synthesis is performed by glycogen synthase (GlgA), ADP-glucose pyrophosphorylase (GlgC), and the branching enzyme (GlgB) (Fig. 1A). The process begins with the addition of ADP to glucose to form ADP-glucose, which is then polymerized to the end of an α -1,4-linked glucan chain. The branching enzyme GlyB then links the chains together at the α -1,6 position. During degradation, the phosphorylases (GlgP) degrade the α -1,4 linkages but are unable to break the α -1,6 branch, so that degradation requires a specific debranching enzyme, GlgX (Fig. 1A). In *Cyanothece* 51142, there are two copies each of *glgA* (*cce_3396* and *cce_0890*), *glgB* (*cce_2248* and *cce_4595*), and *glgC* (*cce_0987* and *cce_2658*). The annotated degradation genes are *glgP* (*cce_1629*, *cce_5186*, and *cce_1603*) and *glgX* (*cce_3465*).

Earlier attempts to genetically transform *Cyanothece* 51142 cells were unsuccessful (17), despite the application of a variety of methods by different research groups. We employed a conjugation strategy to genetically transform *Cyanothece* 51142 cells that used modified media and plating methods. *Cyanothece* 51142 was isolated from the intertidal zones and was found to grow optimally in saltwater-based medium (ASP2 medium) (5). The strain was also found to be fastidious about growth on plates, forming colonies only on certain specific solidifying agents. The strain of *Cyanothece* 51142 that we used grew well in BG11 medium, although ASP2 medium continued to be the medium of choice for optimal growth. Comparison of conjugation results indicated that the use of BG11 medium favored the conjugation process and BG11 plates allowed cells to grow at low titer and to form colonies more readily, compared to growth on ASP2 plates.

For conjugation experiments, we engineered a version of the pRL271 plasmid by introducing a kanamycin resistance cassette into the *Agel* site within the *glgX* open reading frame (Fig. 1B) and then we used a modified version of the conjugation protocol developed for *Anabaena* sp. strain PCC 7120 to introduce this plasmid into *Cyanothece* 51142 (27). PCR was used to verify that the resistance cassette was inserted into the gene (Fig. 1C).

We grew the WT and $\Delta glgX$ strains under different conditions, with and without added nitrogen and in continuous light and alternating 12-h light/dark cycles. For these experiments, ASP2 medium with and without added sodium nitrate (NaNO_3) was used. In the absence of NaNO_3 , *Cyanothece* 51142 cells transition to nitrogen fixation. The mutant strain grown in ASP2 medium under nitrogen-fixing conditions with a 12-h light/dark regimen was consistently more yellow-green in color, which was reflected in

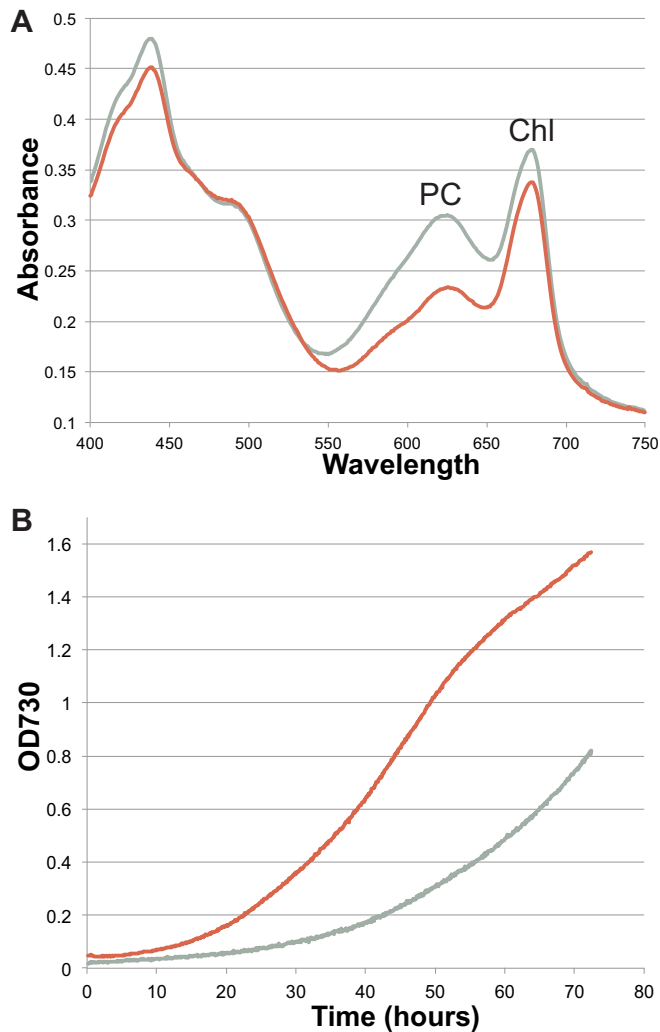


FIG 2 (A) Absorption spectra of the WT (gray line) and $\Delta glgX$ (red line) strains grown under nitrogen-fixing conditions. The absorbance for both chlorophyll (Chl) at 680 nm and phycocyanin (PC) at 625 nm was lower in the $\Delta glgX$ strain than in the WT strain, with the difference being much greater for phycocyanin. (B) Growth of the WT and $\Delta glgX$ strains with added nitrate at 38°C and 3% CO₂, showing the faster growth of the mutant strain.

changes in the absorption spectrum for phycocyanin at 625 nm (Fig. 2A). We calculated the chlorophyll and phycobilin contents in these two strains (28) and found that the $\Delta glgX$ mutant exhibited a 12% lower chlorophyll content, compared to the WT strain. A much greater difference was noted for the phycocyanin content, with the $\Delta glgX$ mutant having a 2-fold decrease in the phycocyanin content, compared to the WT strain.

Growth of the WT and $\Delta glgX$ strains with added nitrogen was compared under different conditions, including temperatures of 30°C and 38°C and with and without additional CO₂. Under all conditions assessed, the $\Delta glgX$ strain showed faster growth than the WT strain. In Fig. 2B, the fastest growth conditions are shown, i.e., 38°C and 3% CO₂; under these conditions, the $\Delta glgX$ mutant was able to grow considerably faster than the WT strain.

We compared, by transmission electron microscopy (TEM), the cellular ultrastructure of the WT and mutant strains grown under nitrogen-fixing conditions during both the light and dark periods (Fig. 3). In WT cells at the end of the light period (peak of glycogen accumulation), granules were observed as mostly uniformly shaped electron-opaque structures of 110 to 220 nm, measured along the long axis (mean, 167 nm;

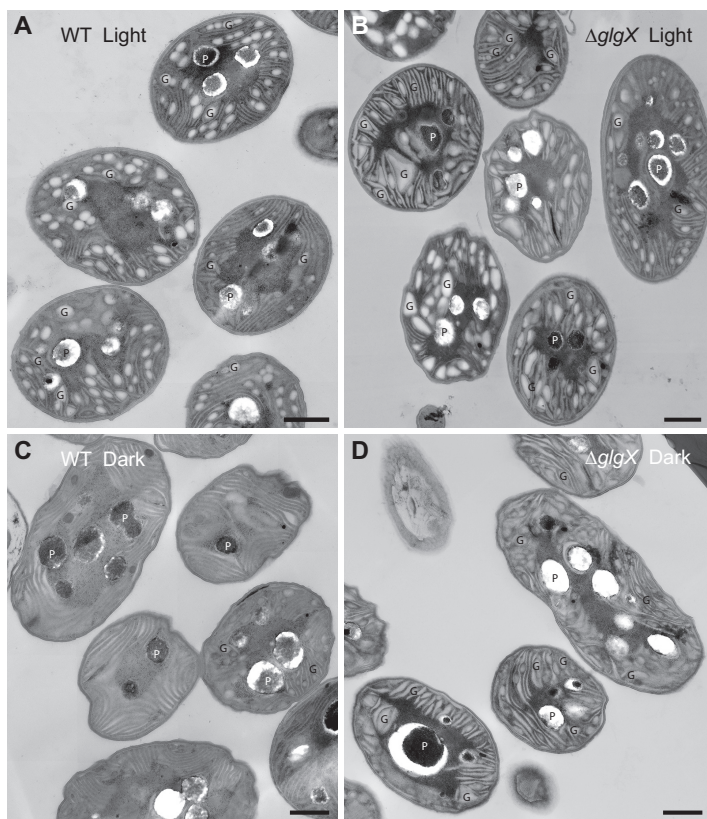


FIG 3 Electron microscopy images of *Cyanothece* 51142 WT (A and C) and $\Delta glgX$ (B and D) cells grown under nitrogen-fixing conditions and harvested at the end of the light (A and B) and dark (C and D) periods. Glycogen granules (G) and polyphosphate bodies (P) are labeled. Bar = 500 nm.

standard deviation [SD], 31 nm [$n = 23$]) (Fig. 3A). In comparison, in the $\Delta glgX$ strain at the same time point, glycogen granules were less uniform in size and shape and could be observed as larger, less regular structures ranging from 123 to 347 nm in length (mean, 227 nm; SD, 56 nm [$n = 34$]) (Fig. 3B). Late in the dark period (expected minimum of glycogen content), the WT cells showed almost no glycogen granules (Fig. 3C), whereas most of the $\Delta glgX$ cells showed remaining glycogen (Fig. 3D). As a further determination of glycogen size, we measured the average area of the granules in cells in electron micrographs at these time points. We found that the area of granules in the mutant was 57% larger than that in the WT strain at the end of the light period ($\Delta glgX$: mean, $0.025 \mu\text{m}^2$; SD, $0.012 \mu\text{m}^2$ [$n = 55$]; WT: mean, $0.014 \mu\text{m}^2$; SD, $0.004 \mu\text{m}^2$ [$n = 53$]). In the dark, few granules were present for measurement in the WT strain, but the remaining granules were similar to those seen in the light (mean, $0.010 \mu\text{m}^2$; SD, $0.003 \mu\text{m}^2$ [$n = 23$]); in the $\Delta glgX$ strain, the granules remained larger and more numerous than those in the WT strain (mean, $0.018 \mu\text{m}^2$; SD, $0.005 \mu\text{m}^2$ [$n = 58$]).

Measurements of glycogen contents (Fig. 4) showed that the $\Delta glgX$ strain maintained overall higher levels of glycogen throughout the diurnal cycle, compared to the WT strain. In both the WT and $\Delta glgX$ strains, glycogen levels decreased during the dark period and increased throughout the light period. Interestingly, the peak was earlier in the $\Delta glgX$ strain than in the WT strain.

Rates of nitrogen fixation, as assayed by reduction of acetylene to ethylene, were measured to determine whether the disruption of *glgX* would affect the nitrogen-fixing ability (Fig. 5). The $\Delta glgX$ strain displayed considerably higher rates of nitrogen fixation under both aerobic and anaerobic (argon-sparged) conditions. When glycerol was added to the medium as a carbon source, the enhanced nitrogen-fixing ability of the $\Delta glgX$ strain was no longer observed.

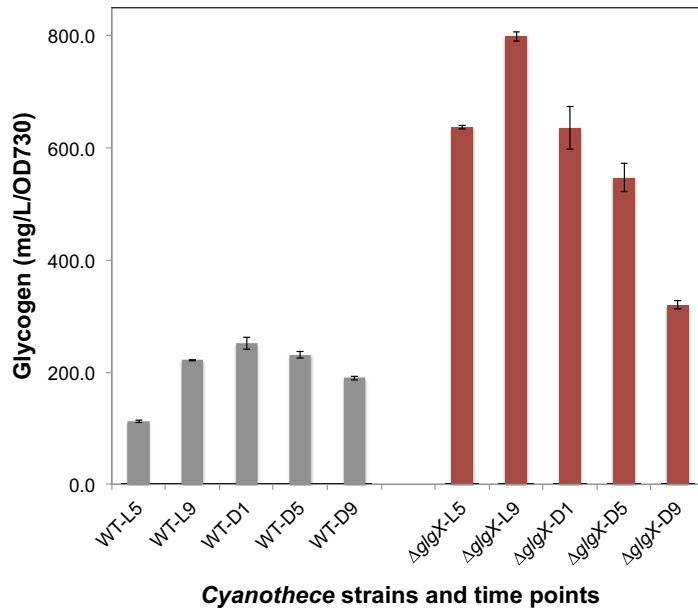


FIG 4 Glycogen contents measured in *Cyanotheca* 51142 WT (gray) and $\Delta glgX$ (red) cells grown under nitrogen-fixing conditions and harvested at different time points during the light (L)/dark (D) cycle. Error bars indicate the SDs from the averages of technical replicates. Representative data are shown, from a total of three biological replicates.

DISCUSSION

Despite intense interest and attempts over decades, a genetic transformation system in *Cyanotheca* 51142 has remained elusive (17). Due to the lack of genetic tools in this organism, important questions regarding the consequences of mutagenesis of genes involved in critical processes, including carbon storage and nitrogen fixation, have gone unanswered until now. In fact, among the sequenced *Cyanotheca* strains (3), successful genetic manipulation has been reported only for *Cyanotheca* sp. strain PCC 7822. In that strain, a $\Delta nifK$ mutant was generated by transformation with single-stranded DNA and was shown to be unable to grow without added nitrate (17). Also in *Cyanotheca* 7822, a $\Delta hupL$ strain was constructed by the same method, and the mutant strain demonstrated negligible nitrogenase activity and hydrogen production (29). Despite these successes in *Cyanotheca* 7822, genetic manipulation in this strain remains difficult and unpredictable. In this study, we have established a straightforward and reproducible system of genetic manipulation in *Cyanotheca* 51142.

In order to generate genetic mutants in *Cyanotheca* 51142, we used a modified conjugation protocol that allowed cells to be grown in liquid BG11 medium and plated on BG11 plates, in contrast to previous work in which cells were grown in ASP2 liquid

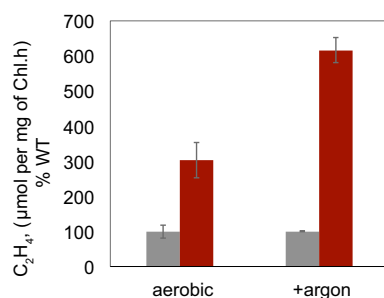


FIG 5 Nitrogenase activity measured in *Cyanotheca* 51142 WT (gray) and $\Delta glgX$ (red) cells grown under nitrogen-fixing conditions and incubated under aerobic or anaerobic conditions. Representative data are shown as the averages of three biological replicates, and error bars show the SDs from the averages.

and solid media. A main difference between the two types of solid media lies in the type of agar used; standard bacterial agar is used in BG11 plates, whereas ASP2 plates typically incorporate Phytigel as the solidifying agent. In our experience, *Cyanothece* 51142 cells did not grow reproducibly at low titer and did not form colonies well on ASP2 plates made with Phytigel, and they could not grow at all on ASP2 plates made with many other solidifying agents. The relative ease with which cells could be plated on BG11 plates and form colonies and the colonies could be picked and transferred to new plates was important for the overall success of our strategy. Therefore, the ability to grow *Cyanothece* 51142 on BG11 medium provided a considerable advantage in terms of cell growth after conjugation and subsequent colony formation. For growth experiments and other assays, ASP2 medium, the original growth medium for *Cyanothece* 51142, was used (5).

The presence of the pRL623 plasmid (30) containing the *Aval*, *Avall*, and *Avalll* methylase genes was found to be necessary for successful conjugation in *Cyanothece* 51142, and an examination of the restriction modification (RM) system in *Cyanothece* 51142 showed that these three components are present and likely play important roles in the RM system. In *Cyanothece* 51142, carbon is accumulated in large bodies found to be semiamylopectin, in contrast to the smaller, more conventional, glycogen granules found in some other *Cyanothece* strains, as well as in most other bacteria (24). The accumulation and subsequent utilization of these granules as part of the diurnal cycle have been well documented in *Cyanothece* 51142 (25, 31). We sought to understand the physiological characteristics of a strain in which the process of polysaccharide storage granule degradation was modified. We focused on the *glgX* gene because it existed in a single copy in *Cyanothece* 51142 and phylogenetic analysis showed that this gene is highly conserved in cyanobacteria (see Fig. S1 in the supplemental material).

The $\Delta glgX$ strain was generated by insertional inactivation of the *glgX* gene, and colony PCR showed that the resulting kanamycin-resistant strains were fully segregated after about 2 weeks (Fig. 1). The $\Delta glgX$ strain displayed several important phenotypic changes. The observed reduction in the phycocyanin peak in the $\Delta glgX$ strain (Fig. 2A) is in agreement with previous work (32) that demonstrated upregulation of genes involved in phycobilisome degradation (including *nbIA*) when cells had greater carbon contents. Another important change was faster growth under nitrogen-sufficient conditions with 3% CO₂, compared to the WT strain (Fig. 2B). Glycogen contents in the cells would be expected to be low under such growth conditions, and the faster growth suggests that the absence of GlgX in the modified strain enables CO₂ to be used more efficiently. Interestingly, in the only other $\Delta glgX$ strain created to date in cyanobacteria, insertional inactivation of *glgX* in *Synechococcus elongatus* PCC 7942, a nondiazotrophic strain, resulted in similar growth rates for the mutant and WT strains (18). It may be relevant that *Synechococcus* 7942 forms conventional cyanobacterial glycogen granules, not semiamylopectin as in *Cyanothece* 51142.

In *Cyanothece* 51142, carbon is accumulated and stored throughout the light period, reaching a peak at the end of the period, when it is mobilized for use throughout the dark period. This general pattern is observed in both the WT and $\Delta glgX$ strains, although the peak is shifted in the mutant (Fig. 4). However, the $\Delta glgX$ strain consistently maintains a greater amount of glycogen throughout the diurnal cycle, a result that is consistent with the findings for a $\Delta glgX$ mutant in *Escherichia coli* (33).

The most striking feature of the $\Delta glgX$ strain was the large increase in nitrogen fixation levels (Fig. 5). Under aerobic conditions, the $\Delta glgX$ strain showed up to 3-fold increases in nitrogen fixation, compared to the WT strain; with argon sparging, up to 6-fold increased rates were observed. When glycerol was added as a carbon source, a greater increase in nitrogen-fixing ability was seen in the WT strain than in the $\Delta glgX$ strain. This finding might indicate that a larger pool of carbon is available in the mutant cells, which allows higher rates of nitrogen fixation. Larger carbon pools in *Cyanothece* 51142 cells have been shown to lead to higher rates of nitrogenase activity (32). It is likely that the structure of the polysaccharide storage product is changed in the

absence of *glgX*, and this modified storage product may facilitate greater nitrogen fixation by an unidentified process. Interestingly, another gene (*cce_3194*) in *Cyanothece* 51142 is listed as a glycogen-debranching enzyme; it has no significant similarity to *glgX* (*cce_3465*). Although *cce_3194* is widely found in cyanobacteria, to our knowledge the role of this gene has not been characterized in any strain, and the gene is a candidate for future analysis.

MATERIALS AND METHODS

Strain cultivation and genetic modification. *Cyanothece* sp. strain ATCC 51142 was acquired from the American Type Culture Collection (ATCC) (Manassas, VA). The ATCC maintained and shipped *Cyanothece* 51142 on 616 medium, which has the same composition as BG11 medium (34). We found that the strain could grow on both BG11 medium and ASP2 medium (5). For conjugation experiments, *Cyanothece* 51142 cells were grown in BG11 medium with added nitrate, with shaking at ~150 rpm, under 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light in ambient air at 30°C. For physiological experiments, *Cyanothece* 51142 cells were grown in ASP2 liquid medium with or without added nitrate as required.

The ΔglgX strain was generated by insertion of a kanamycin resistance cassette into the *glgX* gene. The suicide plasmid pRL271-*glgX* was constructed by cloning ~2 kb of the *glgX* gene (*cce_3465*) into the *SacI* and *XhoI* sites in pRL271, and then a kanamycin resistance gene was cloned into the internal *AgeI* site in the *glgX* gene. Triparental conjugation was performed using pRL443 as the conjugal plasmid and pRL623 as the helper plasmid, with pRL271-*glgX* transformed into the HB101 strain already containing pRL623 (27). Overnight cultures of the *E. coli* strains were mixed with *Cyanothece* 51142 cultures grown in BG11 medium (200 μl at an optical density at 730 nm [OD_{730}] of 1.5, as measured with a μQuant plate reader [BioTek Instruments, Winooski, VT]). Mixed cells were incubated together for 2 h under low light at 30°C and then were plated onto HATF08250 filters (Millipore-Sigma, St. Louis, MO) on BG11 plates containing 5% (vol/vol) LB without antibiotic. After 3 days, filters were transferred to BG11 plates containing 50 $\mu\text{g/ml}$ kanamycin without LB; colonies appeared after ~9 days. Colonies were picked and grown on BG11 plates containing 10 $\mu\text{g/ml}$ kanamycin.

Measurement of cell growth. For growth measurements, *Cyanothece* cultures were maintained in ASP2 medium, with shaking at ~150 rpm, under 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light in ambient air at 30°C. Culture aliquots were then diluted to an OD_{730} of 0.05 in a Multi-Cultivator MC 1000-OD device (Photon Systems Instruments, Drasov, Czech Republic), and growth under continuous light-emitting diode (LED) light of 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was measured at 730 nm for 72 h or longer.

Transmission electron microscopy. Cells were grown in liquid ASP2 medium without nitrate, under 12-h light/dark conditions with 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, at 30°C for 5 days. Cells were harvested at the appropriate time points by centrifugation, resuspended in a small volume, and transferred to aluminum planchettes with 100- μm -deep wells. Cells were fixed by ultrarapid high pressure freezing using a Bal-Tec high pressure freezer (Bal-Tec, Manchester, NH), followed by freeze substitution in 2% osmium/acetone and embedding in Spurr's resin. Freeze substitution was performed for 3 days at -80°C, followed by slow thawing to room temperature. Thin sections (~80 nm) were cut and stained with uranyl acetate and lead citrate and were imaged using a LEO 912AB transmission electron microscope equipped with a ProScan digital camera. Length and area measurements were performed using iTEM software (Olympus Soft Imaging Solutions, Lakewood, CO).

Determination of glycogen contents. Cells were grown in liquid ASP2 medium without nitrate, under 12-h light/dark conditions with 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, at 30°C and collected for glycogen measurements at the appropriate time points, and the glycogen contents were measured using a hexokinase assay kit (Millipore-Sigma). Cells were pelleted and 30% (wt/vol) KOH was added to remove free glucose, followed by incubation at 95°C for 90 min. Glycogen was precipitated by the addition of absolute ethanol on ice for 2 h and was collected by centrifugation. Pellets were washed with absolute ethanol, dried at 60°C, and resuspended in 300 μl of 100 mM sodium acetate buffer (pH 4.75). A 25- μl volume of sample was retained for measurement of the glucose background level. The remaining glycogen sample was digested with amyloglucosidase for 25 min at 55°C. For the enzyme assay, 25 μl of sample was mixed with the assay reagent in a light-proof microtiter plate and incubated for 15 min at room temperature. NADPH levels were measured at 340 nm with a μQuant plate reader (BioTek Instruments).

Nitrogen fixation assay. Nitrogenase activity was measured using the acetylene reduction assay, as detailed in reference 14. Cultures were grown in liquid ASP2 medium without nitrate, under 12-h light/dark conditions with ~100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, at 30°C for 5 days. Ten-milliliter or 5-ml culture volumes were transferred to airtight 36-ml glass vials and incubated in a 5% acetylene atmosphere under light at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 30°C for 24 h. For anaerobic incubation, the glass vials were flushed with argon for 10 min. Gas samples were withdrawn from the vials, and ethylene production was measured using an Agilent 6890N gas chromatograph equipped with a Poropak N column and a flame ionization detector, with argon as the carrier gas.

Phylogenetic analysis. *GlgX* protein sequences were obtained from the JGI/IMG microbial database and aligned using ClustalW within MEGA 7. The phylogenetic tree was generated using MEGA 7 (maximum likelihood method). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02887-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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