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Altering the Structure of Carbohydrate Storage Granules in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803 through Branching-Enzyme Truncations

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ABSTRACT

Carbohydrate storage is an important element of metabolism in cyanobacteria and in the chloroplasts of plants. Understanding how to manipulate the metabolism and storage of carbohydrate is also an important factor toward harnessing cyanobacteria for energy production. While most cyanobacteria produce glycogen, some have been found to accumulate polysaccharides in the form of water-insoluble α-glucan similar to amylopectin. Notably, this alternative form, termed "semi-amylopectin," forms in cyanobacterial species harboring three branching-enzyme (BE) homologs, designated BE1, BE2, and BE3. In this study, mutagenesis of the branching genes found in *Synechocystis* sp. strain PCC 6803 was performed in order to characterize their possible impact on polysaccharide storage granule morphology. N-terminal truncations were made to the native BE gene of *Synechocystis* sp. PCC 6803. In addition, one of the two native debranching enzyme genes was replaced with a heterologous debranching enzyme gene from a semi-amylopectin-forming strain. Growth and glycogen content of mutant strains did not significantly differ from those of the wild type, and ultrastructure analysis revealed only slight changes to granule morphology. However, analysis of chain length distribution by anion-exchange chromatography revealed modest changes to the branched-chain length profile. The resulting glycogen shared structure characteristics similar to that of granules isolated from semi-amylopectin-producing strains.

IMPORTANCE

This study is the first to investigate the impact of branching-enzyme truncations on the structure of storage carbohydrates in cyanobacteria. The results of this study are an important contribution toward understanding the relationship between the enzymatic repertoire of a cyanobacterial species and the morphology of its storage carbohydrates.

Photosynthetic carbon assimilation and subsequent storage of generated carbohydrates constitute a crucial process in cyanobacterial cells during diurnal growth. This process occurs in close proximity to the thylakoid membranes that are typically arranged around the central cytoplasmic space or project radially out from the cell poles (1). Most bacteria, including cyanobacteria, produce a form of soluble glycogen (2–4). In fact, the use of glycogen as the polysaccharide storage polymer is widespread in nature and can be found in bacterial, archaeal, and eukaryotic species (5). Glycogen biosynthesis occurs through the sequential actions of ADP-glucose pyrophosphorylase (GlgC) glycogen synthase (GlgA) and the branching enzyme (BE; GlgB) (Fig. 1). Briefly, glucose is activated via addition of ADP to become ADP-glucose, which then gets polymerized to the nonreducing end of an α-1,4-linked glucan chain. The growing glucose polymer chains are then linked together in the α-1,6 position by the branching enzyme (GlgB) via a hydrolytic cleavage reaction whereby an α-1,4 linkage is broken and inter- or intramolecular transfer reconnects the chain. These actions continue successively, eventually forming the glycogen granule. Breakdown of the glycogen granule occurs through the actions of two enzymes, a debranching enzyme (DBE; GlgX) and the glycogen phosphorylase (GPase and GlgP). (See reviews of glycogen [6] and starch [7] structures.)

The morphologies of glycogen and starch carbohydrates are quite different. The branching pattern found in glycogen allows for more spherical growth. This characteristic leads to densely packed glycosyl chains that get progressively more crowded toward the periphery. Due to this pattern, granule growth eventually becomes impossible as the space for interaction with the catalytic sites becomes limited. Mathematical modeling has predicted that because of these characteristics, the maximum value for glycogen is 42 nm in diameter (6). On the other hand, starch is comprised of amylopectin and amylose and can form much larger granule structures of osmotically inert and metabolically unavailable (until digested) glucose (Fig. 1B and E) (7–9).

While most cyanobacteria form bacterial glycogen, some species of cyanobacteria produce a different form of storage polysaccharide that is more insoluble and analogous to amylopectin found in starch. This variety of polysaccharide storage granule is

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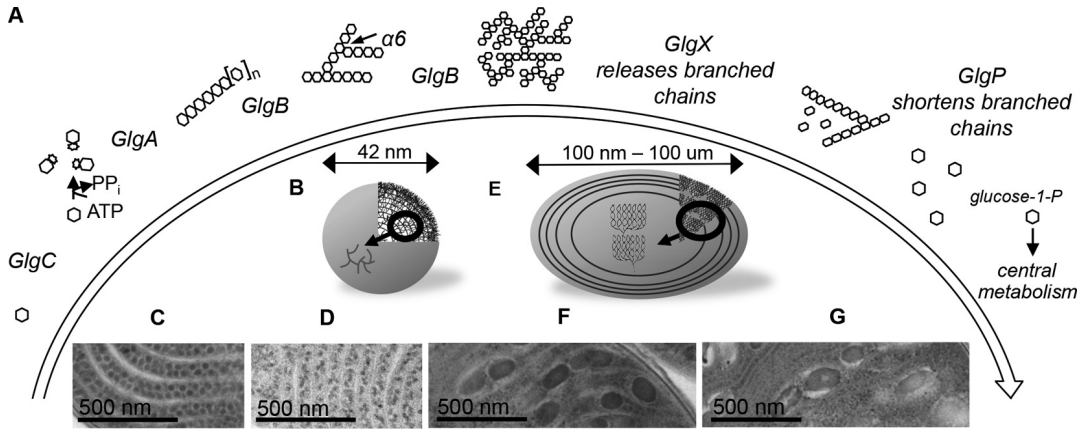

FIG 1 Glycogen biosynthesis and degradation pathways. (A) The process of glycogen biosynthesis occurs through the activities of three crucial enzymes: ADP-glucose pyrophosphorylase (GlgC), glycogen/starch synthase (GlgA), and a branching enzyme (GlgB). Degradation occurs via the debranching enzyme (GlgX) and glycogen phosphorylase (GlgP). (B) The short-chain branching in bacterial glycogen leads to an exponential increase in the density of chains further away from the core, leading to a maximum of 42 nm. This type of storage granule is seen in *Synechocystis* sp. PCC 6803 (C) and *Cyanothece* sp. PCC 7822 (D). For starch (E), the amylopectin cluster structure is generated through the asymmetric distribution of the branches, allowing for much larger granules to form. Semi-amylopectins from *Cyanothece* sp. ATCC 51142 and ATCC 51472 are shown in panels F and G, respectively. Panels B and E are adapted from reference 8 with permission.

referred to as "cyanobacterial starch" and is considered a semi-amylopectin due to its lower content of longer glucan chains compared to glucan chains found in true amylopectin. The accumulation of semi-amylopectin as storage polysaccharides has been found in a limited, yet growing, number of cyanobacterial species (10). One of the first descriptions of semi-amylopectin was made for the unicellular diazotrophic strain *Cyanothece* sp. strain ATCC 51142 (11). It was thought that all members of the *Cyanothece* genus stored carbohydrate material in this form, until we reported the presence of small glycogen granules in the strain *Cyanothece* sp. PCC 7822 similar to those stored by other bacterial and cyanobacterial species, such as *Synechocystis* sp. strain PCC 6803 (12). Examples of cyanobacterial glycogen from the genetic model species *Synechocystis* sp. PCC 6803 and from *Cyanothece* sp. PCC 7822, as well as semi-amylopectin from *Cyanothece* sp. ATCC 51142 and *Cyanothece* sp. ATCC 51472, can be seen from electron micrographs in Fig. 1.

A common method to characterize polysaccharide molecular architecture is to analyze the glucan chain length distributions using anion-exchange chromatography (13). This technique allows the degree of polymerization (DP), or the number of monosaccharide units (e.g., glucose) linked in a polysaccharide chain, to be measured. Semi-amylopectin, compared to glycogen, has a high relative amount of longer α-1,4-linked glucan chains, with a DP of ≥37 making up between 2 and 6%. In contrast, regular glycogen has <1% of α-1,4-linked glucan chains with a DP of ≥37, whereas true amylopectin from rice has ~6 to 7% of these long chains. When the DP profile of cyanobacterial glycogen is analyzed, the average relative percent is found to be between DP 6 and 8. The average DP of amylose ranges from 11 to 12. An average DP value of 11 to 12 has also been reported for cyanobacterial species that form semi-amylopectin. Analysis of storage polysaccharides from *Cyanothece* sp. ATCC 51142, *Cyanobacterium* strain Clg1, and *Cyanobacterium* strain NBRC 102756 revealed that their storage granules have a molecular mass virtually indistinguishable from that of amylopectin (9, 14). Moreover, the thermal properties, crystallinity, and branching structure are similar to those of amylopectin, and the semi-amylopectin material synthesized by these strains is organized in tandem cluster structures.

Why some cyanobacteria form different carbohydrate storage granules is unknown. Bioinformatic analysis of *Cyanothece*, *Cyanobacterium*, and *Synechocystis* species reveal varying copy numbers of all genes involved in the synthesis and degradation of storage polysaccharides (Table 1). Notably, all semi-amylopectin-forming strains contain three branching-enzyme gene copies, whereas bacterial glycogen-forming strains contain either one or two. Additionally, high variability in debranching-enzyme genes can be seen among the subgroups of strains. In this study, the differences between the amino acid sequences of the branching enzymes found in semi-amylopectin- and non-semi-amylopectin-forming cyanobacteria were investigated. The purpose of this study was to use genetic modifications to convert a strain of cyanobacteria that produced bacterial (β-granule) glycogen into a strain that produced starch-like semi-amylopectin.

MATERIALS AND METHODS

Growth and mutant construction. All cyanobacterial strains used in this study are listed in Table 2. *Synechocystis* sp. PCC 6803 strains were grown in 100-ml flasks containing BG-11 medium (15), whereas *Cyanothece* sp. ATCC 51142 (16) was grown in ASP2 medium (17, 18), both with shaking at ~125 rpm with illumination of ~30 to 40 μmol of photons $m^{-2}s^{-1}$ of light from cool white fluorescent bulbs. For experimental procedures, cultures were inoculated in identical media and, when stated, modified by subculturing in nitrate-free medium (BG-11 NF) or in medium with 0.01× nitrate. Growth was assayed using absorbance at 730 nm (Perkin-Elmer UV-Vis Lambda 40 spectrophotometer).

To generate mutations, genomic DNA was extracted from 100 ml of culture (~1×10^8 cells/ml). A Braun homogenizer and 0.5 ml of glass beads (0.1 to 0.2 mm) were used to lyse cells with four 30-s bursts at high speed, followed by centrifugation. The supernatant was then transferred to new tubes and sequentially washed by vortexing with 500 μl of phenol and chloroform. DNA was precipitated by adding 2 vol of ice-cold absolute ethanol. For the branching-enzyme constructs, pUC19 was used as a cloning backbone for sequential insertion of ~500-nucleotide (nt) fragments located immediately upstream of the native start codon and downstream of the coding sequence of the branching-enzyme gene *sll0158*.

TABLE 1 Copy number of glycogen metabolism genes in various members of *Cyanothece*, *Synechocystis*, and cyanobacteria known to produce starch-like granules

Genome	No. of glycogen metabolism gene:					Morphology type
	glgC	*glgA*	*glgB*	*glgX*	*glgP*	
Synechocystis sp. PCC 6803	1	2	1	2	1	Glycogen
Cyanothece sp. PCC 7822	1	2	2	4	2	Glycogen
Cyanothece sp. PCC 7424	1	2	2	4	2	Glycogen
Cyanothece sp. PCC 7425	1	2	2	1	2	Glycogen[a]
Cyanothece sp. PCC 8801	1	2	2	4	2	Semi-amylopectin[b]
Cyanothece sp. PCC 8802	1	2	2	4	2	Semi-amylopectin[c]
Cyanothece sp. ATCC 51472	2	2	3	3	3	Semi-amylopectin
Cyanothece sp. ATCC 51142	2	2	3	2	3	Semi-amylopectin
Cyanothece sp. CCY 0110	2	2	3		1	Semi-amylopectin
Cyanobacterium sp. Clg1		3	3	5	1	Semi-amylopectin + amylose
Cyanobacterium sp. NBRC 102756			3			Semi-amylopectin

[a] Based on TEM observations (40).
[b] Based on TEM observations (27).
[c] Based on TEM observations (41).

First, the upstream fragment was amplified using primers BEup/F and BEup/R and inserted in the PstI and XmaI sites. The downstream region, amplified using primers BEdown/F and BEdown/R, was inserted into the XmaI and EcoRI sites. The coding sequence of *sll0158* was PCR amplified with forward primers BE97/f and BE112/f and reverse primer BE/R1. These primers amplified the gene region corresponding to the 97th or 112th amino acid, introducing a start codon in either amino acid position 97 (yielding mutant gene *sll0158*Δ97) or 112 (yielding mutant gene *sll0158*Δ112), respectively. Mutant *sll0158* fragments were inserted between the upstream and downstream fragments using XmaI and BglII sites. A kanamycin resistance cassette from pRL448, amplified using primers BEKanF and BRKanR, was used for positive selection and inserted between the truncated branching-enzyme gene and downstream region.

A procedure similar to that described above was used to replace the native debranching enzyme (DBE) encoded by the gene *slr0237* with the DBE from *Cyanothece* sp. PCC 8801. Upstream and downstream fragments of the native DBE were amplified using primers U0237F/R and D0237F/R and inserted via the BamHI and SalI sites and SalI and PstI sites in pUC19, respectively. The coding sequence for the DBE from *Cyanothece* sp. PCC 8801 was amplified using the primer pair 8801-1045F/8801-1045R and inserted in between the upstream and downstream fragments via a SalI site. Next, a spectinomycin cassette (~2 kb), isolated from the plasmid pRL453, was inserted between the DBE gene, *PCC8801_1045*, and the downstream fragment of *slr0237* at the EcoRI site. *Synechocystis* sp. PCC 6803 was transformed using standard transformation protocols (19). Transformed colonies were selected on antibiotic plates and transferred over 2 to 4 months for segregation. Full segregation of the mutants was confirmed by PCR and at regular intervals thereafter. Sequences for primers used can be found in Table 3.

Determination of glycogen content. Intracellular glycogen content was measured using a colorimetric biochemical assay using anthrone described previously (11, 12, 16, 20, 21). Samples were measured at consistent daily time points using 2 ml of culture. The resulting 625-nm absorbance values were compared to that of a standard curve as described by Welkie et al. (12).

Preparation of polysaccharides. Cultures (500 ml) were grown for 3 to 4 days under continuous-light, low-nitrogen conditions (0.01× nitrate

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli NEB 5-alpha	DH5α derivative	New England Biolabs
Cyanobacteria		
Synechocystis sp. PCC 6803	Wild type	Lab collection
Δ97 mutant	Wild type transformed with pBE97; *sll0158*Δ97 Km^r	This study
Δ112 mutant	Wild type transformed with pBE112; *sll0158*Δ112	This study
Δ97x mutant	Wild type transformed with pBE97 and pGlgX2-8801; *sll0158*Δ97 Km^r *slr0237*::*PCC8801_1045* Km^r Sp^r	This study
Δ112x mutant	Wild type transformed with pBE12 and pGlgX2-8801; *sll0158*Δ112 *slr0237*::*PCC8801_1045* Km^r Sp^r	This study
Cyanothece sp. ATCC 51142	Wild type	Lab collection
Plasmids		
pRL448	Plasmid carrying Km^r cassette	42
pRL453	Plasmid carrying Sp/Sm Ω cassette	42
pBE97	97-amino-acid truncation construct for PCC 6803 BE containing a Km^r cassette	This study
pBE112	112-amino-acid truncation construct for PCC 6803 BE containing a Km^r cassette	This study
pGlgX2-8801	Construct with *glgX2* from PCC 8801 flanked by *slr0237* fragments and for Sp/Sm Ω cassette recombination	This study

TABLE 3 Primer sequences used in study

Primer name	Sequence (5′–3′)
BEup/F	GAGACTGCAGTTTCCACTCTCCCCAACTGC
BEup/R	GAGACCCGGGTCGGCTATGGTGATTTT
BEdown/F	GAGACCCGGGATATAAGATCTATAACTGAGTTAGC
BEdown/R	GAGAGAATTCGATCGCCAGAGGGCAAT
BE97/F	GAGACCCGGGAAGGATGATTTATGACCC
BE112/F	GAGACCCGGGATATGGACCTCCATGTGTTT
BE/R1	GAGAAGATCTCTAAGCTATGTTGCTAGC
BEKanF	GAGAGAAGATCTCGACTCTAGAGGATCTCAATGAA
BEKanR	GATATAAGATCTGGTCATTTCGAACCCCAGA
BEdown/F	GAGACCCGGGATATAAGATCTATAACTGAGTTAGC
BEdown/R	GAGAGAATTCGATCGCCAGAGGGCAAT
U0237F	GAGAGGATCCCGGTTTGTTTATTACGAC
U023R	GAGAGTCGACGGCAATTCCAAAAATTGT
D0237F	GAGAGTCGACGAGACTCGAGTCTAATTTACTGAGTTT
D0237R	GAGACTGCAGGCTAAGATTGCTAGAGATTT
8801-1045F	GAGAGTCGACAATGAACATAAAACTTTGGC
8801-1045R	GAGACTCGAGCCACCCTACGAGAATTACA
sp1045IN/F	AATGTAAGTTCGCCAGCCAGGACAGA
sp1045IN/R	GTAAATTAGACTCGAGCTTGAACGAATTGTTAGACAT TATTTGCC

BG-11 medium) to increase total glycogen storage. Cells were harvested by centrifugation at 10,000 × *g* and suspended with 20 ml of disruption buffer containing 50 mM imidazole-HCl (pH 7.4), 8 mM $MgCl_2$, and 12.5% (vol/vol) glycerol (9) and placed on ice. The suspension was passed through a French pressure cell three times at 16,000 lb/in^2 (16), with collection tubes submerged in ice to inhibit enzymatic activity as much as possible. After lysis, the solution was subjected to boiling for 10 min and then to centrifugation at 8,000 × *g* for 15 min. Glycogen was precipitated from the supernatant by the addition of 5 volumes of ice-cold absolute ethanol (22). Glycogen was recovered by centrifugation at 8,000 × *g* for 20 min, dried in a centrifugal vacuum evaporator (9), and stored at −20°C until processing.

Analysis of chain length distribution by HPAEC. To examine the branch pattern of glycogen produced by wild-type and mutant strains of *Synechocystis* sp. PCC 6803, isolated polysaccharide material from a cell lysate (100 mg) was dissolved in 10 ml of H_2O (10% solution [wt/vol]) and stirred overnight to disperse. Samples were then boiled for 1 h, followed by centrifugation at 12,000 × *g* for 10 min. The supernatant was diluted 10 times with 10 mM sodium acetate buffer (pH 4.5), and 1 μl each of isoamylase (0.1 U) and pullanase (0.72 U) (Megazyme, Wicklow, Ireland) was added to hydrolyze α-1,6 linkages. The reaction mixture was incubated at 40°C with shaking at 120 rpm for 48 h. The branch chain length distributions of glycogen were determined by a high-performance anion-exchange chromatography (HPAEC) system (GP40; Thermo Scientific, Sunnyvale, CA) equipped with an electrochemical detector (ED40; Thermo Scientific). A CarboPac PA100 anion-exchange analytical column (4 by 250 mm; Thermo Scientific) with a guard column (4 by 50 mm; Thermo Scientific) was used for separating 25-μl samples of debranched samples. Eluents A and B consisted of 150 mM NaOH and 150 mM NaOH containing 600 mM sodium acetate, respectively. The gradient of eluent B at 70 min (increased at 1%/min) was 0 to 70%.

Ultrastructure analysis. Cells were visualized in two ways in the transmission electron microscopy (TEM). For regular morphology, cells were fixed by high-pressure freezing (HPF) as described previously (12). To determine if granules contained glycogen, the histochemical PATO (periodic acid-thiocarbohydrazide-osmium) technique was used (23, 24). Cells were sampled at consistent time points after 72 h of growth in BG-11 NF medium. Cells were prestained with 0.15% ruthenium red in 0.1 M sodium cacodylate buffer, pH 7.0 (NaCaB) for 1 h at room temperature and subsequently washed. The primary staining consisted of 3.6% glutaraldehyde with 0.15% ruthenium red in NaCaB incubated for 1 h at room temperature. This was followed by washing with NaCaB and then a secondary staining procedure with 1% OsO_4 with 0.15% ruthenium red in NaCaB for 1 h at 4°C. Fixed samples were pelleted and coated with 2% agarose, and resulting pellets were cut into blocks for dehydration and embedding. The samples were dehydrated via a graded ethanol series and embedded in fresh Spurr resin. Thin sections were cut and retrieved with a 100-mesh nickel grid coated with a Formvar-carbon film. Grids were poststained by floating on droplets of 1% periodic acid for 1 h at room temperature, rinsed with water, floated on droplets of 1% thiosemicarbazide (Sigma-Aldrich, St. Louis, MO) for 1 h at room temperature, rinsed again with water, and allowed to air dry. Subsequently, the sections were exposed to OsO_4 vapors in small, closed petri plates for 7 h at room temperature, stained with 2% aqueous uranyl acetate for 5 min, rinsed with water, and air dried. A control for each time point was prepared by replicate preparation minus the periodic acid and thiosemicarbazide prior to final osmium vapor exposure. Samples were imaged with a Philips CM-100 TEM operated at 100 kV, with a Spot 3, 200-μm condenser aperture and with a 50-μm objective aperture. Images were captured on Kodak SO-163 film.

Phylogenetic analysis of glycogen biosynthesis enzymes in *Cyanothece*, *Synechocystis*, and *Cyanobacterium* spp. Amino acid sequences for branching and debranching enzymes were found in reference genomes by BLASTP analysis (http://www.ncbi.nlm.nih.gov/BLAST) to obtain all related sequences showing high similarity. Proteins that appeared incomplete were removed from analysis. Sequences were then aligned using the program Clustal Omega (ClustalO) using default settings at the time of analysis, with the number of iterations set to five (http://www.ebi.ac.uk/Tools/msa/clustalo/). The resulting alignment file was used to generate a neighbor-joining tree using the program MEGA-6 for mac (http://www.megasoftware.net/index.php). Bootstrap analysis with 1,000 replicates was performed to arrive at the tree generated. Additional alignment of the N-terminal and catalytic domain regions of the branching enzymes was performed similarly with ClustalO. The alignment file was then imported into ESPript 3 (http://espript.ibcp.fr/ESPript/ESPript/), and secondary-structure features were rendered from using PDB Chain ID 1M7X as a reference (http://www.rcsb.org/pdb), similar to analysis performed by Suzuki et al. (14).

RESULTS

Amino acid sequences and phylogenetic analysis of BE1, BE2, and BE3. The amino acid sequences for all branching enzymes found in the genomes of *Cyanothece* strains, including the known semi-amylopectin-forming strains *Cyanobacterium* sp. Clg1 and *Cyanobacterium* sp. NBRC 102756, were aligned for structure comparison. Three distinct groupings were identified, termed BE1, BE2, and BE3 (see Fig. S1 in the supplemental material). These groups were based on the fact that BE2 and BE3 lacked either 97 or 111 amino acids at the N terminus, consistent with observations made by others (10, 12), and that there were various insertions and deletions in the catalytic A-domain region (see Fig. S2) (14). Some regular glycogen-forming species, such as *Synechocystis* sp. PCC 6803, harbor only one BE gene, for BE1, whereas others, such as *Cyanothece* sp. PCC 7822, contain two (Table 1). All confirmed cyanobacterial strains known to form semi-amylopectin, with the exception of *Cyanothece* sp. PCC 8801, contained a third branching-enzyme isoform (BE3).

Figure 2 shows the protein sequence alignment at the amino termini of the branching enzymes. BE2 from *Cyanothece* sp. ATCC 51142 and *Cyanothece* sp. PCC 7822 both contained a 97-amino-acid truncation, whereas BE3 from *Cyanothece* sp. ATCC 51142 contained a 111-amino-acid truncation. The BE1 isoforms from *Cyanothece* sp. PCC 7822 and *Cyanothece* sp. ATCC 51142

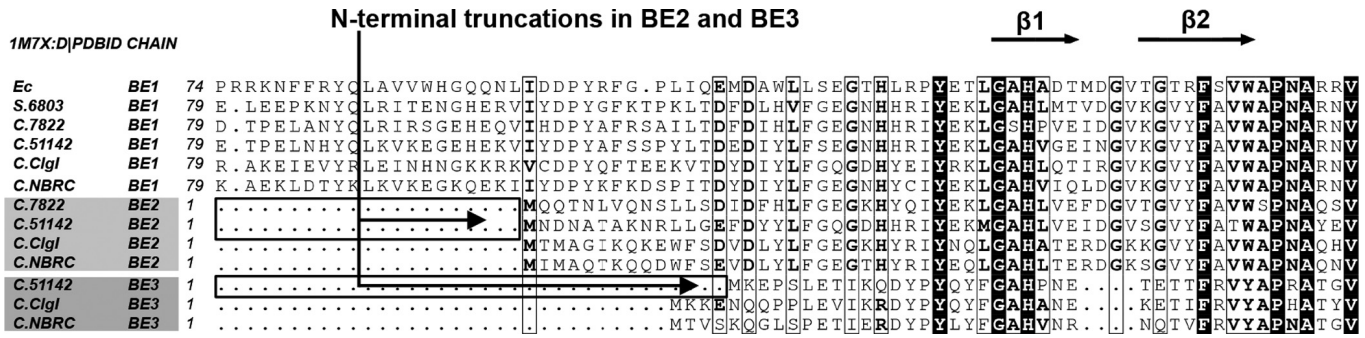

FIG 2 Multiple amino acid sequence alignment of the N-terminal domain from BEs from *Cyanothece* strains. Ec, *Escherichia coli*; *S.6803*, *Synechocystis* sp. PCC 6803; *C.7822*, *Cyanothece* sp. PCC 7822; *C.51142*, *Cyanothece* sp. ATCC 51142; *C.Clg1*, *Cyanobacterium* sp. Clg1; *C.NBRC*, *Cyanobacterium* sp. NBRC 102756. The sequence alignment was generated using ClustalO (43) and ESPript (http://espript.ibcp.fr) (44). Regions of catalytic domain based on the three-dimensional structure of BE from *E. coli* (PDB: 1M7X:D) are shown. Beta strands (β) are indicated.

were 77% identical to each other, whereas the BE2 isoforms from these strains were 65% identical. In addition, the BE1, BE2, and BE3 isoforms displayed a high sequence similarity with their respective counterparts across species (see Table S1 in the supplemental material).

Amino acid sequences and phylogenetic analysis of DBE and DBE-X2. Some studies have also shown a correlation between the polysaccharide granule morphology and the activity of the debranching enzyme. For example, Suzuki et al. (25) reported that deletion of the *glgX* gene in *Synechococcus elongatus* PCC 7942 resulted in an enrichment of very short chains with DP values within the range of 2 to 4 and increased rates of oxygen uptake in the dark. In addition, a variant copy of the debranching-enzyme gene found in the starch-forming *Cyanobacterium* strain Clg1 was demonstrated to be crucial for the accumulation of large, starch-like polysaccharides. This strain contains a gene that has been termed *glgX2*. Cells with a mutated *glgX2* gene accumulate small β-granule glycogen with morphology that resembles that of *Synechocystis* sp. PCC 6803 and *Cyanothece* sp. PCC 7822 (26). Interestingly, *glgX2* is not present in *Cyanothece* sp. ATCC 51142. This suggests that different combinations of BE and DBE genes may act in concert to produce various polysaccharide granule structures. The protein sequence alignment for all debranching enzymes from the genomes included in this study is depicted in Fig. S3 in the supplemental material.

The number of genes encoding the debranching enzyme was more variable than for branching-enzyme genes. For example, *Synechocystis* sp. PCC 6803 and *Cyanothece* sp. ATCC 51142 have two DBE genes, whereas *Cyanothece* sp. PCC 7822 has three. All five of these debranching-enzyme genes show low sequence similarity to *glgX2* from *Cyanobacterium* strain Clg1 (<50%). Yet, there is relatively close sequence identity between *glgX2* and debranching-enzyme genes from *Cyanothece* sp. PCC 8801, PCC 8802, PCC 7822, and PCC 7424, the highest being the genes from *Cyanothece* sp. PCC 8801 with >65%. Intriguingly, electron micrographs of *Cyanothece* sp. PCC 8801 show large storage granules present in the proximity of the thylakoids, similar to those found in *Cyanothece* sp. ATCC 51142 (27).

Mutagenesis of branching and debranching enzymes in *Synechocystis* sp. PCC 6803. In order to investigate the role of the N-terminal region of the branching enzyme, truncations similar to those found in the BE2 and BE3 of *Cyanothece* sp. ATCC 51142 were used to replace the single-copy BE (BE1) of *Synechocystis* sp. PCC 6803. This was accomplished with the use of primers that amplified the coding region of the branching enzyme starting at either 97 amino acids or 112 amino acids downstream of the usual start codon. The native amino acid at the 97th or 112th position was replaced (via a PCR primer) with a new start codon (AUG) (Fig. 3). Mutant segregation was confirmed through DNA gel

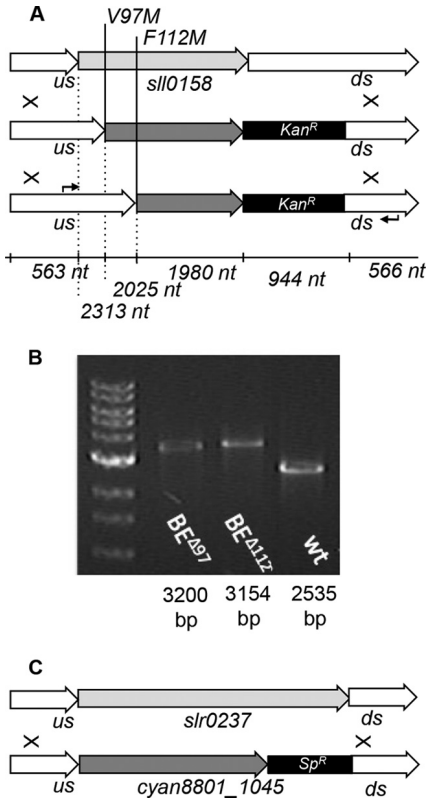

FIG 3 Branching-enzyme and debranching-enzyme mutagenesis. (A) Map showing the site of amino-terminal truncation mutagenesis. (B) Gel electrophoresis showing DNA fragments amplified by PCR indicate complete segregation of the modified genes in the Δ97 and Δ112 mutant strains. (C) Map showing the double homologous recombination replacement of the debranching enzyme *slr0237* with *PCC8801_1045*. *us*, upstream region; *ds*, downstream region; *nt*, nucleotide, Sp^R, spectinomycin resistance gene; ×, location of double homologous crossover. Arrowheads indicate the locations where the PCR primers used for verification of gene mutagenesis were annealed.

electrophoresis of PCR-amplified genomic regions containing inserted DNA.

Because the DBE gene *PCC8801_1045* from *Cyanothece* sp. PCC 8801 had the highest sequence similarity to the *glgX2* gene in *Cyanobacterium* sp. Clg1, it was also introduced into *Synechocystis* sp. PCC 6803. One of the two DBE genes of *Synechocystis* (*slr0237*) was replaced with *PCC8801_1045* though homologous recombination, maintaining transcriptional regulation from the native promoter. Mutant cells were segregated and verified as described for the BE truncation mutations. A genetic map for the construct used to introduce the *glgX2* gene from *Cyanothece* sp. PCC 8801 (*PCC8801_1045*) is shown in Fig. 3.

Growth and glycogen content in mutant strains. We first examined the effects of the truncated BE (Δ97 and Δ112) and the addition of the DBE from *Cyanothece* sp. PCC 8801 in the wild type and the Δ97 and Δ112 mutants on cell growth and physiology. No significant effect on growth or glycogen storage content in any of the mutant strains was observed under standard conditions of continuous light. At 72 h, growth of cultures went from an A_{730} of 0.15 to 0.38 ± 0.02. This was also the case when cells grown under standard conditions were washed and transferred to nitrate-free BG-11 medium under continuous illumination for 72 h to a final optical density of 0.33 ± 0.02. When stressed with depleted nitrogen, glycogen content increased in all strains, with no significant difference in glycogen accumulation between the wild type and mutant strains; glycogen contents were 7.4 ± 2.0 and 43.6 ± 5.5 μg/optical density at 730 nm (OD_{730}) at 72 h in regular and nitrate-free BG-11, respectively (data not shown).

Ultrastructure characteristics Δ97, Δ97x, and Δ112 mutants. To determine if the mutations to the BE and DBE of *Synechocystis* sp. PCC 6803 had an effect on the morphology of the glycogen granules, we examined cells grown under nitrogen-free conditions for 72 h using transmission electron microscopy. Nitrogen stress is known to induce glycogen accumulation in *Synechocystis* and allows for observation of cells with high levels of glycogen. Cells were fixed by high-pressure freezing (HPF), a procedure that was proven to provide the best preservation of cellular ultrastructure (12). In order to allow the staining of carbohydrates using the PATO technique (12, 23, 24), we also used chemical fixation via the microwave procedure. In Fig. 4, thin-section imaging of wild-type *Synechocystis* sp. PCC 6803 (Fig. 4A) and Δ97 (Fig. 4B), Δ97x (Fig. 4C), and Δ112 (Fig. 4D) mutant cells shows no discernible difference on granule morphology. PATO-stained wild-type cells (Fig. 4E) confirmed that the small granules seen within the thylakoid and cytoplasmic spaces filling the cell are carbohydrate material. The control for the PATO staining (Fig. 4F) depicts the wild-type cells prepared identically, but without the periodic acid and thiosemicarbazide treatment prior to final osmium vapor exposure.

Measurements made on the size of the granules formed within each strain were made using ImageJ software (28). The diameters of individual glycogen granules were measured manually ($n > 50$, from 3+ different TEM images each) and compared to those of the wild type. The average glycogen granule from wild-type *Synechocystis* sp. PCC 6803 was ~30 nm in diameter. The average glycogen granule from Δ97 mutant cells was comparable to that of the wild type, but Δ97x and Δ112 mutant cells had glycogen granules with a slight increase in size, with those of the Δ112 mutant being the largest, with an average diameter of ~35 nm (Table 4). The sizes of carbohydrate granules within the mutant strains described here are all within the diameter range characteristic of common glycogen granules and less than that of semi-amylopectin found in *Cyanothece* sp. ATCC 51142 and CCY 0110 and *Cyanobacterium* strains Clg1 and NBRC 102756 (9).

Chain length distribution of stored polysaccharides. There have been a number of studies describing the fate of carbohydrate storage and physiological effects on the host cells when glycogen metabolism genes are altered. Yoo et al. (29) showed that deletion of the branching enzyme in *Synechocystis* sp. PCC 6803 resulted in increases in α-glucan chains with DPs of 2 to 16 and decreases in larger chains with DPs of 17 to 44. Suzuki et al. (25) showed that disrupting the *glgX* gene in *Synechococcus elongatus* PCC 7942 resulted in an enrichment of short chains with DPs ranging from 2 to 4. This indicates that the debranching enzyme in cyanobacteria can contribute to the branching pattern of polysaccharide. Furthermore, work by Cenci et al. on *Cyanobacterium* cp. Clg1 provided additional evidence that debranching enzymes can influence polysaccharide storage structure (26). Moreover, expression of potato starch synthase III in a cyanobacterial host resulted in unexpectedly shorter and less longer α-1,4 chains than in the wild type (30).

In this study, the branch chain length distributions of the extracted α-glucans from wild-type *Synechocystis* sp. PCC 6803, *Cyanothece* sp. ATCC 51142, and mutant *Synechocystis* cells were analyzed via HPAEC (Fig. 5). Polysaccharides isolated from wild-type *Synechocystis* sp. PCC 6803 (Fig. 5A) showed the expected chain length distribution, with the most abundant glucan chain having a DP of 6, whereas the most abundant glucan chain lengths in polysaccharides isolated from *Cyanothece* sp. ATCC 51142 had a DP of 11. This is comparable to experimental results described previously (25, 31, 32). Comparison of these two strains shows how the semi-amylopectin synthesized by *Cyanothece* sp. ATCC 51142 had fewer short chains (DP, 2 to 10) and had more abundant longer chains (DP, 11 to 35) than the normal β-granule glycogen of *Synechocystis* sp. PCC 6803. Materials from the Δ97 (Fig. 5B), Δ112 (Fig. 5C), and Δ112x (Fig. 5D) mutant strains were similar to wild-type material in that the most abundant chain length corresponded to a DP 6, but Δ97 had reduced short-chain polyglucans and an increase in longer chains with a DP of >16. Both the Δ112 and Δ112x mutants showed results similar to those for the Δ97 strain, but with an increase in polyglucan chains with a DP of >22 and less of a decrease in short-chain glucans. Subtractive analysis of the relative percentage of each DP of the wild-type *Cyanothece* sp. PCC 51142 (Fig. 5E) and the Δ97 (Fig. 5F), Δ112 (Fig. 5G), and Δ112x (Fig. 5H) mutants revealed a decrease in short chains (DP, ~3 to 8) and an increase in long-chain content (DP, ~13 to 30) in the mutant strains, notably similar to that of the semi-amylopectin from *Cyanothece* sp. ATCC 51142. The number (DP_n) and weight average (DP_w) DP values for wild-type *Synechocystis* sp. PCC 6803 and the Δ97, Δ112, and Δ112x mutants and *Cyanothece* sp. ATCC 51142 reflect the distribution results (Table 5). The DP_n for the wild type was 8.9 ± 0.1, while for *Cyanothece* sp. ATCC 51142, the DP_n was 11.3. DP_n values for the Δ97, Δ112, and Δ112x mutants were intermediary, ranging between 9.6 ± 0.1, 9.3, and 9.4, respectively. We conclude that the enzymatic modifications described here led to an α-glucan storage structure in *Synechocystis* sp. PCC 6803 with similarities to semi-

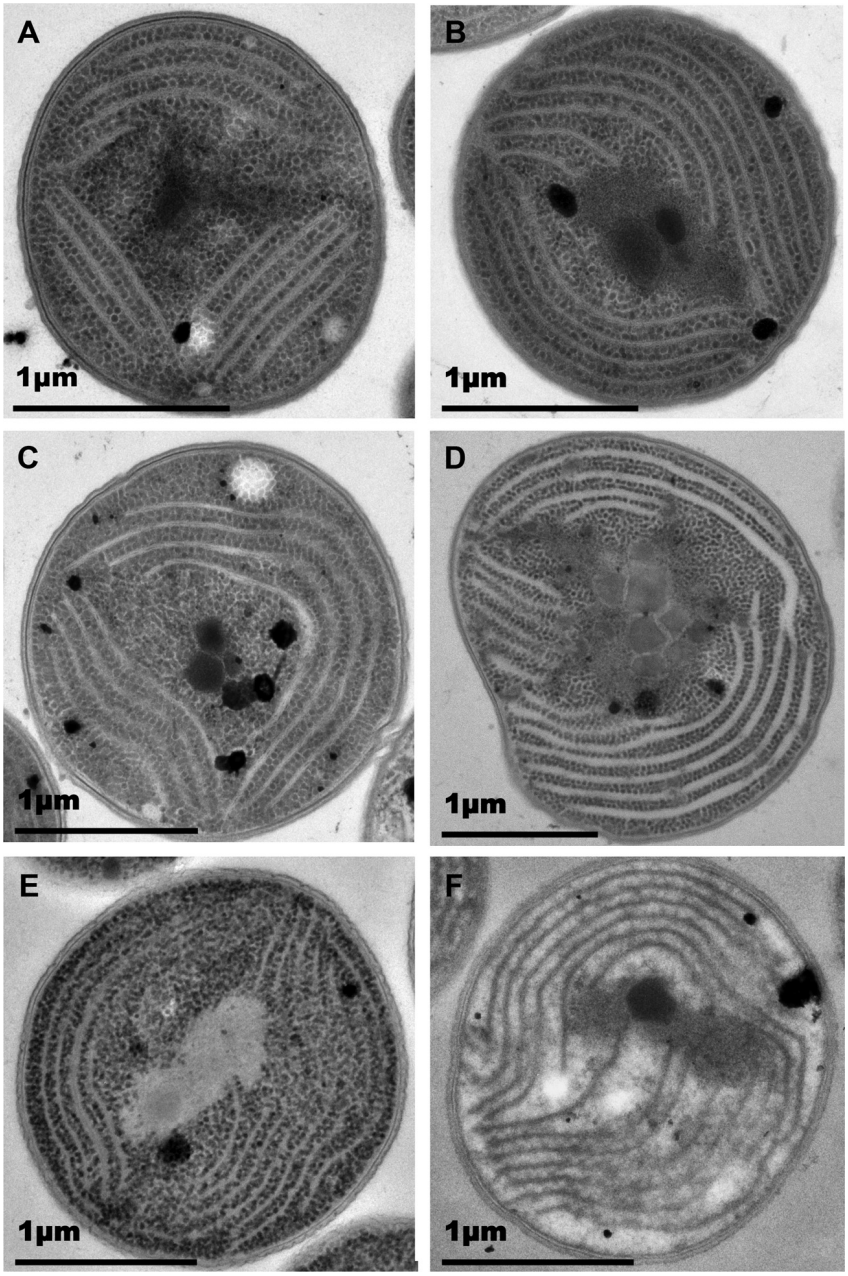

FIG 4 TEM of *Synechocystis* wild-type and mutant cells. Electron micrographs of *Synechocystis* sp. PCC 6803 mutant strains grown in nitrogen-free BG-11 medium and fixed by high-pressure freezing (HPF) are shown. Images show wild-type *Synechocystis* sp. PCC 6803 (A) and the Δ97 (B), Δ97x (C), Δ112 (D) mutants as well as the wild type stained using the PATO technique via microwave chemical fixation (E) and a wild-type PATO control (F). Magnification, ×25,000.

amylopectin, thus implicating branching-enzyme variation as a factor contributing to α-glucan storage morphology.

DISCUSSION

The nature of carbohydrate storage is a key element in the metabolism in cyanobacteria and in the chloroplasts of plants. This carbon storage process is at the intersection of photosynthesis, respiration, and central metabolism and has a profound effect on how cyanobacteria grow and maintain diurnal homeostasis. Glycogen metabolism is also crucial for establishing the proper intracellular environment for N_2 fixation in diazotrophic species, especially in the unicellular cyanobacteria. Its accumulation in all photosynthetic branches of the tree of life from green plants, algae, and cyanobacteria represents a carbon sink that can accumulate to high levels relative to the biomass and then be broken down and fed into biosynthetic pathways for chemical production. Since most starch- and semi-amylopectin-forming cyanobacterial species (e.g., *Cyanothece* sp.) have been refractory to site-specific genetic modifications (33), the elucidation of the functions of particular enzymes that contribute to the starch-like storage structure is a challenge. Although techniques to genetically modify *Cyanothece* sp. PCC 7822 have been developed (33), *Synechocystis* sp. PCC 6803 represents a strain with an excellent genetic system and simpler glycogen biosynthesis system, with only one branching

TABLE 4 Size comparison of glycogen granules

Strain	Avg glycogen granule diam (nm)[a]
Wild type	28.6 ± 2.9 A
Δ97 mutant	28.5 ± 3.1 A
Δ97x mutant	31.0 ± 3.7 B
Δ112 mutant	35.4 ± 3.3 C

[a] *n*, >50 to 60. Results are from one-way ANOVA Tukey pairwise comparisons, with a significance level of 0.05 indicated by letters. Means that do not share a letter are significantly different.

TABLE 5 Effects of glycogen branching- and debranching-enzyme modifications on glycogen storage and structure[a]

Strain	DP_n	DP_w	% increase of glycogen content after 72 h in LN[c] BG-11
Synechocystis sp. PCC 6803	8.9 ± 0.1	11.5 ± 0.3	NA
Δ97 mutant	9.6 ± 0.1	12.6 ± 0.1	~27
Δ112 mutant[b]	9.3	12.4	~22
Δ112x mutant[b]	9.4	12.8	~4
Cyanothece sp. ATCC 51142[b]	11.3	14.5	NA

[a] Values are means ± standard deviations obtained from three independent experiments. Average chain length was calculated based on peak area of each chain on HPAEC chromatograms. NA, not available (not measured).
[b] Only one experiment was performed.
[c] LN, low nitrate (0.01× nitrate).

enzyme and two DBEs. The mutations made to the BE and DBE in *Synechocystis* PCC 6803 described here clearly affected glycogen metabolism to a modest extent. The N-terminal truncations made to the native branching enzyme in *Synechocystis* sp. PCC 6803 led to a glycogen branching pattern with an increase in the abundance of longer glucan chains and a decrease in the abundance of shorter ones compared to the wild type. The observed increase and decrease of each respective polymer comprising the glycogen granules extracted from the mutant strains is in agreement with the chain length profiles of semi-amylopectin, although the percentage of the total distribution is not as great. Previous work by Yoo et al. introducing potato starch synthase III resulted in the opposite changes to the branching pattern, with decreases in longer chains and increases in shorted ones, at a scale similar to that seen in this study (30). Therefore, we suggest that the BE enzymes modified in this work are important in the starch-forming process, and we consider our results to be a step in the right direction for determining the enzymology of starch production. The addition of a heterologous DBE may also have had an additional impact on the

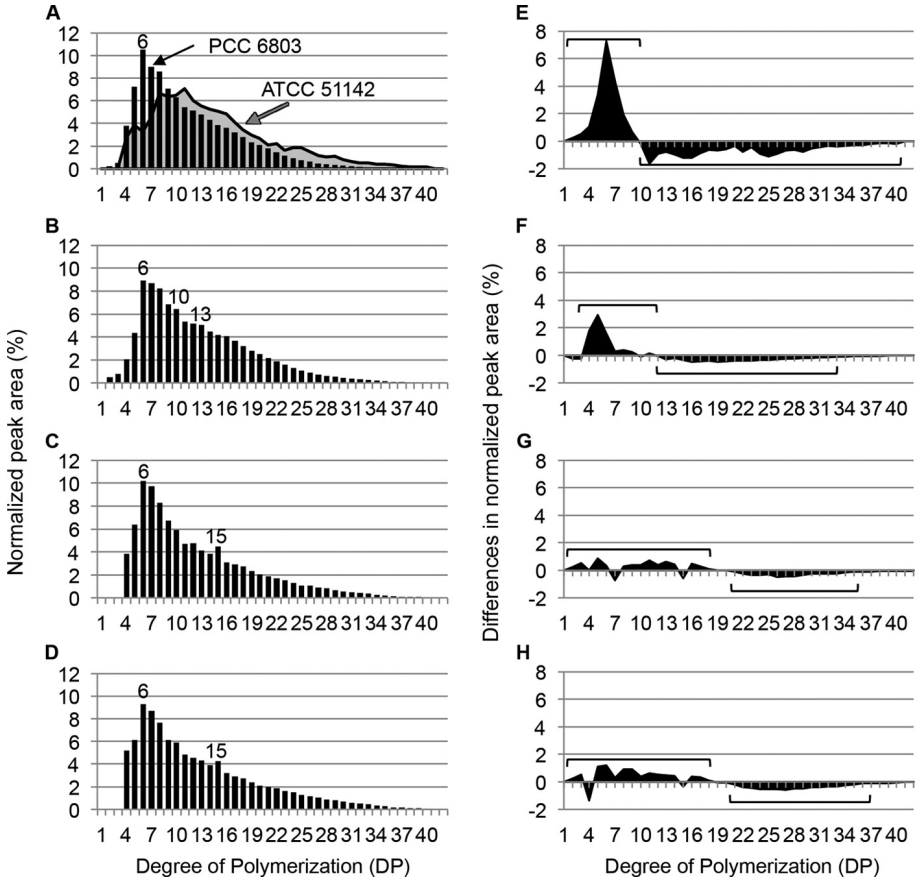

FIG 5 Chain length distributions of debranched α-glucans from wild-type and mutant *Synechocystis* sp. PCC 6803 strains. After isoamylase treatment to hydrolyze α-1,6 linkages, α-glucan chains were separated through HPAEC-PAD for wild-type PCC 6803 (bars) and *Cyanothece* sp. ATCC 51142 (shaded area) (A) and the Δ97 (B), Δ112 (C), and Δ112x (D) mutants. The peak area was calculated and normalized from the chain profile chromatogram. Subtractive analysis showing the difference in normalized peak area between the wild-type *Cyanothece* sp. PCC 51142 (E) and the Δ97 (F), Δ112 (G), and Δ112 (H) mutants revealed a decrease in short-chain content (DP, ~3 to 8) and an increase in long-chain content (DP, ~13 to 30) in the mutant strains, notably similar to that of the cyanobacterial α-glucan-containing *Cyanothece* sp. ATCC 51142. Changes in short and long branched chains (shaded regions) are shown by horizontal brackets.

chain length profile. Nonetheless, the scope of the changes indicate that other proteins, or potentially protein-protein interactions (30, 34), must be involved if all glycogen in *Synechocystis* sp. PCC 6803 is to be converted into semi-amylopectin as in *Cyanothece* sp. ATCC 51142.

One of the challenges to comparing the biochemistries of starch-, semi-amylopectin-, and glycogen-forming species of algae and bacteria is the complexity of the storage polysaccharide metabolic network across species. Compared to many eubacteria, cyanobacteria have an unusually high number of isoforms for each step in the glycogen metabolic pathway. Ball et al. (35) have recently examined the genomes of many sequenced cyanobacteria and eukaryotic algae that produce starch or semi-amylopectin and have developed a more complete and complex picture of the evolution of starch production. Indeed, they have rejected their earlier hypothesis that a single event resulted in starch acquisition by a host after an endosymbiotic event that led to plastid development in eukaryotic algae and subsequently in plants (26, 31, 36, 37). They have demonstrated by laborious genetic screens that an isoamylase-like DBE (termed GlgX2) in *Cyanobacterium* Clg1 was responsible for formation of a starch-like material. Their genomic analyses led them to conclude that starch acquisition had occurred frequently in cyanobacteria and eukaryotes and that the glycogen-to-starch transition has likely occurred numerous times (35). This *glgX2* gene product or any GH13-like DBE seems to be completely absent in *Cyanothece* sp. ATCC 51142, which likely means that the enzymes responsible for converting glycogen into starch (or starch-like material) are different in these two organisms. This conclusion largely agrees with our analysis of the copy number and type of enzyme isoforms for polysaccharide storage present in the *Cyanothece* genomes. This suggests that convergent evolution occurred numerous times to generate enzymes with similar properties.

One possible evolutionary pressure that led to semi-amylopectin and starch formation could have been the great oxygenation event (38). This global environmental transition triggered vast diversification among cyanobacteria. As environmental oxygen levels rose, the oxygen-sensitive nitrogenase enzymes present in some nitrogen-fixing bacteria required protection. Unicellular strains needed a way to efficiently decrease oxygen levels locally through strong bursts of respiration. One method to do this would be to evolve a more efficient storage polysaccharide structure that maximizes the stored carbohydrate pool. This idea is supported in a number of ways. First, an investigation of six *Cyanothece* strains revealed close associations between the respiratory rates, maintenance of intracellular oxygen levels, and N_2 fixation activities (39). For example, *Cyanothece* strains (such as *Cyanothece* sp. ATCC 51142) that contained semi-amylopectin had respiratory activities that began at the beginning of the dark period and peaked at the height of nitrogenase activity a few hours later. On the other hand, strains, such as *Cyanothece* sp. PCC 7822, that form glycogen were not nearly as well synchronized with regard to photosynthesis, respiration, or glycogen production and degradation. Thus, the altered carbohydrate storage material provided many evolutionary advantages in terms of more robust growth under adverse conditions. Second, mutants of green algae with defective isoamylase debranching-enzyme activities reverted to forming glycogen instead of starch, and as a consequence, their anoxic hydrogenase-mediated production of hydrogen was abolished. Once again, the carbohydrate storage product provided significant metabolic benefits.

Many open questions remain in this field. For instance, if *Cyanothece* sp. ATCC 51142 does not harbor an isoamylase-like DBE, then what is permitting semi-amylopectin formation? How active is the GlgX2 protein from *Cyanothece* sp. PCC 8801 compared to host GlgX1? Additionally, analysis of expression at the protein level and a more comprehensive mutant library would strengthen these results and possibly reveal additional possibilities for carbohydrate structure plasticity in these organisms. Moreover, a detailed analysis of the hypothetical genes and genes located on the extrachromosomal plasmids in *Cyanothece* strains is warranted and may reveal potential hydrolases and enzymes influential to polysaccharide storage structure. For example, there are 4 potential amylase enzymes in *Cyanothece* sp. ATCC 51142 (35). What role do they play, if any, in semi-amylopectin formation in this strain? Finally, random-mutagenesis studies of *Cyanothece* sp. ATCC 51142, similar to those described for *Cyanobacterium* strain Clg1 (26) that led to identifying the genes responsible for amylose production, could be performed to identify unknown genes involved in semi-amylopectin formation.

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