Photosynth Res (2013) 118:25–36
DOI 10.1007/s11120-013-9941-z

REGULAR PAPER

Analysis of carbohydrate storage granules in the diazotrophic cyanobacterium *Cyanothece* sp. PCC 7822

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Received: 23 May 2013 / Accepted: 2 October 2013 / Published online: 19 October 2013
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Abstract The unicellular diazotrophic cyanobacteria of the genus *Cyanothece* demonstrate oscillations in nitrogenase activity and H_2 production when grown under 12 h light–12 h dark cycles. We established that *Cyanothece* sp. PCC 7822 allows for the construction of knock-out mutants and our objective was to improve the growth characteristics of this strain and to identify the nature of the intracellular storage granules. We report the physiological and morphological effects of reduction in nitrate and phosphate concentrations in BG-11 media on this strain. We developed a series of BG-11-derived growth media and monitored batch culture growth, nitrogenase activity and nitrogenase-mediated hydrogen production, culture synchronicity, and intracellular storage content. Reduction in $NaNO_3$ and K_2HPO_4 concentrations from 17.6 and 0.23 to 4.41 and 0.06 mM, respectively, improved growth characteristics such as cell size and uniformity, and enhanced the rate of cell division. Cells grown in this low NP BG-11 were less complex, a parameter that related to the composition of the intracellular storage granules. Cells grown in low NP BG-11 had less polyphosphate, fewer polyhydroxybutyrate granules and many smaller granules became evident. Biochemical analysis and transmission electron microscopy using the histocytochemical PATO technique demonstrated that these small granules contained glycogen. The glycogen levels and the number of granules per cell correlated nicely with a 2.3 to 3.3-fold change from the minimum at L0 to the maximum at D0. The differences in granule morphology and enzymes between *Cyanothece* ATCC 51142 and *Cyanothece* PCC 7822 provide insights into the formation of large starch-like granules in some cyanobacteria.

Keywords *Cyanothece* · Cyanobacteria · Glycogen granules · Biohydrogen production · Carbon:nitrogen ratio · Nitrogen:phosphate ratio · N_2 fixation

Abbreviations

PHB	Polyhydroxybutyrate
TEM	Transmission electron microscopy
PATO	Periodic acid–thiocarbohydrazide–osmium

Introduction

Cyanobacteria are important model organisms for key biological processes such as carbon sequestration, nitrogen fixation, and hydrogen production. Much of the current interest in cyanobacteria and their potential to produce biofuels is based upon the relationship of fossil fuels and human activity—the large increase of energy-related CO_2 emissions in the atmosphere and the potential scarcity or high cost of fossil fuels. Photosynthesis results in the production of energy and reducing equivalents that can be used for the fixation of CO_2 into reduced carbon compounds and for processes such as N_2 fixation in diazotrophic cyanobacteria. All cyanobacteria contain hydrogenases and diazotrophic strains also contain nitrogenases that can generate H_2 and this

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capability has recently been exploited in both heterocystous and unicellular strains (Ananyev et al. 2008; Bandyopadhyay et al. 2010; Min and Sherman 2010b). There are obvious reasons for making cyanobacteria a production organism. Their ability to directly convert solar energy into liquid or gaseous fuels, minimal nutrient requirements (Ruffing 2011), and the use of atmospheric CO_2 as a carbon source provide economic and environmental incentives. Advances in areas such as metabolic modeling (Knoop et al. 2010; Saha et al. 2012) and cultivation (Burrows et al. 2008) have prompted advances in engineering cyanobacteria for biofuel production. Recent research on cyanobacteria has yielded successful production systems for various bioenergy compounds including fatty acids (Liu et al. 2011), isoprene (Lindberg et al. 2010), 1-butanol (Lan and Liao 2012), and hydrogen (Bandyopadhyay et al. 2011; Min and Sherman 2010b).

Cyanothece spp. are unicellular diazotrophic cyanobacteria that temporally separate photosynthesis and nitrogen fixation (Bandyopadhyay et al. 2013). During the day, light energy is utilized via photosynthesis to generate energy and reductant. During this process, CO_2 is fixed via the Calvin cycle and stored in glycogen granules that form between the thylakoid membranes. During the night, photosynthesis shuts down and respiration utilizes the stored glycogen as a substrate to produce additional cellular energy and to remove molecular oxygen from the cell. The lack of intracellular O_2 permits the oxygen sensitive nitrogenase enzymes, which are expressed at the onset of the dark, to enzymatically fix atmospheric nitrogen (Sherman et al. 1998). This process also produces hydrogen as a by-product and is responsible for the majority of the H_2 evolved (Bandyopadhyay et al. 2011; Min and Sherman 2010b). *Cyanothece* sp. PCC 51142 (henceforth *Cyanothece* 51142) has been studied extensively due to its well-defined diurnal cycles between phototrophy and diazotrophy and because it has particularly impressive hydrogen production rates. Although *Cyanothece* sp. PCC 51142 has provided considerable insight into how major processes of the cell relate to one another, it has proven recalcitrant for the development of a genetic system due to its tendency for illegitimate recombination and the resulting difficulty in generating mutations (Min and Sherman 2010a).

The genomic sequences of seven members of the *Cyanothece* genus have been finished and all can produce reasonably high levels of H_2 under suitable conditions. However, a stable genetic system only has been developed for *Cyanothece* sp. PCC 7822 (henceforth *Cyanothece* 7822 (Min and Sherman 2010a). This cyanobacterium also has a propensity to form intracellular granules of polyhydroxyalkonoate (PHA) (excess carbon storage and electron sink), polyphosphate (phosphate storage), and cyanophycin (nitrogen storage as a co-polymer of the amino acids asp and arg). Recent proteomic analysis has yielded insights into how *Cyanothece* 7822 differs from *Cyanothece* 51142 (Aryal et al. 2013) under both nitrogen-replete and nitrogen-deficient conditions and highlighted pathways that may provide suitable targets for genetic manipulation. Due to the availability of genomic sequence information, capacity for genetic modifications, and aforementioned metabolic characteristics, *Cyanothece* 7822 is a prime candidate to use a chassis for biofuel production.

While efforts to improve the facility of genetics and mutant segregation in *Cyanothece* 7822 are ongoing, the aim of this study is to improve the physiological and growth characteristics and to gain a better understanding of global cellular processes. The growth medium used for cultivation of many cyanobacteria, BG-11 (Allen 1968), is a relatively rich media for phototrophic organisms and provides nitrate and phosphate in excess (Ananyev et al. 2008; Burrows et al. 2008; Chen et al. 2008). The molar nitrate and phosphate ratio is much higher than the canonical Redfield ratio (16:1) (Ernst et al. 2005), as well as the proposed range of nitrate:phosphate (N:P) appropriate for nitrogen fixing species suggested by Klausmeier et al. (2004). Therefore, adjustments to BG-11 started with reductions in nitrate and phosphate both in the amount and in the relative molar abundance, using the Redfield ratio as a starting point. We demonstrate that the revised media led to improved growth and to a reduction in intracellular storage granules. In turn, the less complex morphology enabled us to determine that glycogen was stored in granules that are distinct in size and shape from those found in *Cyanothece* 51142.

Materials and methods

Cultivation

Cyanothece 7822 was cultured in BG-11 medium (Allen 1968) in 100 ml cultures in 250 ml volume Erlenmeyer flasks at 30 °C. Shaking was maintained at 125 rpm with 30–40 μmol photons m^{-2} s^{-1} of light from cool white fluorescent bulbs. For experimental procedures, cultures were inoculated in modified BG-11 media with nitrate and phosphate adjustments as listed in Table 1. Stock cultures were grown in continuous light conditions for 7 days and sub-cultured to $\sim 1.5 \times 10^6$ cells ml^{-1} (OD730 = 0.12,

Table 1 BG-11 media modified by lowering N and P concentrations

Media	$NaNO_3$ (mM)	K_2HPO_4 (mM)	N:P
BG-11	17.65	0.23	77
Low NP BG-11 (1)	4.41	0.06	74
Low NP BG-11 (2)	2.2	0.06	37

Chl = ~0.5 μg ml^{-1}). When indicated, cultures were grown under 12 h light–12 h dark light regimen to induce diurnal cycling in nitrogen-deficient media.

Measurement of growth and cellular size and shape

Growth was measured by optical density (A_{730}) with a Perkin–Elmer Lambda 40 spectrophotometer and through direct cell counting using a Petroff–Hauser counter. The chlorophyll (Chl) concentration of the cells was determined by measuring light absorbance at 750, 678, and 620 nm and using the equation: $[Chl] = 14.97(A_{678} - A_{750}) - 0.615(A_{620} - A_{750})$ (Min and Sherman 2010b).

In addition, the population structure of cultures grown in various media and conditions was investigated using a BD influx cell sorter (BD Biosciences, San Jose, CA, USA) measuring individual cell size (forward scatter, FSC) and internal complexity (side scatter, SSC). Samples were analyzed at the Environmental Molecular Sciences Laboratory (EMSL) at Pacific Northwest National Laboratory (Richland, WA, USA). At times, cells were transported at 4 °C and briefly thawed at room temperature prior to analysis.

Analysis of intracellular storage granules

Intracellular glycogen content was measured quantitatively using a colorimetric biochemical assay and histocytochemically on the transmission electron microscope (TEM). For quantitative measurements, 1 ml of culture was harvested, resuspended in 0.2 ml of deionized water, and digested with 0.4 ml of 40 % (wt/vol) KOH at 90 °C for 1 h. The digestion was allowed to cool and two volumes of cold 100 % ethanol was added and the mixture was stored overnight at −20 °C allowing for carbohydrate precipitation. The precipitate was centrifuged for 30 min at 12,000 rpm in a microfuge and 0.1 ml of concentrated H_2SO_4 was added and the solution was further incubated at room temperature for 10 min. Samples were diluted with 0.9 ml of deionized water and carbohydrates were measured according to the anthrone technique described by Schneegurt et al. (1994). Aliquots of 0.5 ml of the dissolved precipitates were added to test tubes containing 1 ml of freshly prepared anthrone reagent, consisting of concentrated H_2SO_4 with 2.0 g/L anthrone, and immediately vortexed. The reaction was refluxed for 10 min at 90 °C, cooled, and the resulting solution was used to acquire the A_{625} absorbance value and compared to that of a standard curve.

For TEM analysis, cells from cultures used for the physiology measurements were concentrated via centrifugation and thin sections were prepared for intracellular glycogen detection through application of a specific post staining procedure based on the PATO method (Hanker et al. 1964; Sherman and Sherman 1983). Cells were sampled at four time points throughout the 12 h light–12 h dark period after 72 h growth in nitrogen-deficient BG-11 and low NP BG-11 media. Cells were pre-stained with 0.15 % ruthenium red in 0.1 M Na-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 a wash 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, enrobed in 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 + carb on film. Grids were post stained 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, USA) 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 a 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, Spot 3, 200 μm condenser aperture, and with a 50 μm objective aperture. Images were captured on Kodak SO-163 film.

Measurement of nitrogenase activity and hydrogen evolution

Nitrogenase activity was assayed via a modified acetylene reduction technique, whereby 12–30 ml of culture was sealed in 66-ml clear glass bottles in air or sparged with argon for 1 min to generate an anoxic environment and immediately sealed with a rubber stopper. To each sample bottle, 3 ml of acetylene gas was injected. Bottles were incubated in conditions consistent with their respective time points; D0 representing the end of the 12 h light period, D3 representing 3 h into the 12 h dark period, and three other time points in the light (L0, L3, and L11) representing 0 h, 3 h, and 11 h into the light period, respectively. After incubation for 24 h, a 0.2 ml headspace gas was used to analyze the amount of acetylene reduced to ethylene using a HP 5890 Series II gas chromatograph (Hewlett Packard, Ltd.) equipped with a 6-ft Poropak N column and a flame ionization detector using nitrogen as a

carrier gas. The temperature of the injector, detector, and oven were 100, 150, and 100 °C, respectively. Duplicate samples were analyzed for each set of conditions. To calculate the amount of acetylene reduced, the peak area values were divided by the calculated conversion factor of 13,736.77, duration of incubation, and amount of chlorophyll to result in units of μmol C_2H_2 reduced per mg chlorophyll per hour (Colon-Lopez et al. 1997; Min and Sherman 2010b; Schneegurt et al. 1994).

For hydrogen evolution, 12–30 ml of culture was transferred to 66-ml glass bottles similar to the preparation for the nitrogenase assays and measured in air or sparged with argon for 1 min to provide an anoxic environment. Bottles were immediately sealed and incubated under continuous light at 30 °C, shaking at 125 rpm. After 24 h, 0.2 ml of headspace gas was injected into a HP 5890 Series II gas chromatograph (Hewlett Packard, Ltd.) outfitted with a thermal conductivity detector and a molecular sieve column (HP Molesieve, catalog number 19095P-MS9). Nitrogen was used as a carrier gas. The molar quantity of H_2 produced was calculated by the equation determined via standard curve: [moles H_2 = (peak area + 10,774)/(889,044)]. The rate of H_2 production was calculated based on moles of H_2 produced per mg chlorophyll per hour of incubation period (μmol H_2/mg Chl h) (Min and Sherman 2010b).

Phylogenetic analysis of glycogen branching enzymes in *Cyanothece* spp.

Sequence alignment was performed with MacVector™ (Oxford Molecular Ltd., Cary, NC, USA) software (v 12.6.0) with the ClustalW algorithm using the default settings. A neighbor-joining tree was generated and bootstrap analysis with 1,000 replicates was performed. Scores >50 % are indicated at the branch points. The GenBank accession numbers for the sequences are as follows: *Cyanothece* 51142 cee_2248 (YP_001803664), cce_4595 (YP_001806009), cce_1806 (YP_001803222); *Cyanothece* sp. CCY 0110 CY0110_25931 (ZP_01730776), CY0110_29634 (ZP_01728659), CY0110_28144 (ZP_01728224); *Cyanothece* 7822 cyan7822_2889 (YP_003888122), cyan7822_1547 (YP_003886814); *Cyanothece* sp. PCC 8801 PCC8801_0452 (YP_002370704), PCC8801_2353 (YP_002372522); *Cyanothece* sp. PCC 8802 Cyan8802_0465 (YP_003136257), Cyan8802_2403 (YP_003138111); *Cyanothece* sp. PCC 7424 PCC7474_1226 (YP_002376542), PCC7424_4362 (YP_002379596); *Cyanothece* PCC 7425 Cyan7425_4120 (YP_002484795); *Synechocystis* sp. PCC 6803 sll0158 (NP_442003), slr0237 (NP_440018); *Synechococcus elongates* PCC 7942 Synpcc742_1085 (YP_400102); At time of publication *Cyanothece* BH63E ATCC 51472 was still in DRAFT without GenBank accession identities.

Results

Effects of media modifications on growth

Cyanothece 7822 was grown in a series of BG-11 derived media with various changes in macronutrients (Table 1). Decreasing the available nitrogen source from 17.65 to 4.41 mM and reducing the phosphate from 0.23 to 0.06 mM (low NP BG-11) resulted in improved growth, as measured by cell counting, optical density, and chlorophyll content at the end of 168 h incubation under continuous light (Table 2). Adjustments to the nitrate:phosphate ratio (N:P) had an insignificant effect on growth once N and P levels were decreased to 25 % of normal BG-11. When the N:P was cut in half (from 77:1 to 37:1) by further reducing the nitrate content an additional twofold, no significant change in growth was observed when compared to low NP BG-11 (data not shown). Furthermore, cultures grown in reduced nitrogen-deficient BG-11 displayed no significant difference in nitrogen fixation and hydrogen evolution capabilities compared to cells grown in regular nitrogen-deficient BG-11 (Table 3). The results for nitrogenase activity and H_2 production are virtually identical for cells grown in the different

Table 2 Effects of BG-11 and low NP BG-11 on growth of *Cyanothece* 7822

Media	Cell no.	OD730	[Chl]
BG-11	$6.5 \pm 2.8 \times 10^6$	0.58 ± 0.1	4.62 ± 1.5
Low NP BG-11	$1.3 \pm 1.6 \times 10^7$	0.70 ± 0.05	6.01 ± 0.7

Growth measurements were made after incubation in continuous light for 168 h

Table 3 Specific activities for hydrogen evolution and nitrogenase activity

	NF BG11		Reduced NF BG11	
	H_2 (μmol/mg Chl/h)	N_2 (μmol reduced/mg Chl/h)	H_2 (μmol/mg Chl/h)	N_2 (μmol reduced/mg Chl/h)
L11	46.3 ± 25.8^A	28.2 ± 7.5^a	48.3 ± 28.8^A	36.7 ± 12.8^a
D0	34.5 ± 18.1^A	35.0 ± 11.8^a	37.8 ± 11.1^A	42.4 ± 8.9^a
D3	9.4 ± 9.1^A	0.9 ± 0.6^a	14.8 ± 13.9^A	0.8 ± 0.5^a
L0	0.0 ± 0.0^A	0.0 ± 0.0^a	0.0 ± 0.0^A	0.0 ± 0.0^a
L3	2.5 ± 4.9^A	5.2 ± 6.3^a	6.6 ± 7.2^A	4.0 ± 4.6^a

Cells were grown in nitrogen-deficient regular NF and modified NF BG-11 for 3 days under 12 h light–12 h dark conditions. Experimental error was based on four technical replicates. One-way ANOVA and comparison of means using Tukey method of 95 % confidence intervals indicate that there is no significant difference in activities between cells grown in the different nutrient conditions

[A] Nitrogenase activity measurements

[a] Hydrogen evolution measurements

media. In addition, cultures grown in both media had identical variations in the peak of activity of about 2 h from experiment to experiment. The peak of activity varied from L11 to D3, which is typical for such circadian oscillations and which is the reason for the high standard deviation. These results prompted us to standardize on this low NP BG-11 (containing fourfold reduction in N and P) for further investigations on intracellular storage content.

Effects of $NaNO_3$ and KH_2PO_4 reduction on cellular state homogeneity in the population

Flow cytometry analysis of *Cyanothece* 51142 cell populations in batch culture displayed a homogeneous composition with a single, tight population. On the contrary, *Cyanothece* 7822 grown in regular BG-11 was composed of two distinct populations that differed in terms of both individual cell size and internal complexity (Fig. 1a). This characteristic of *Cyanothece* 7822 was lost when grown in the low NP BG-11. The fourfold reduction in N and P shifted the cells in batch culture to become a single tight population, similar to that seen with *Cyanothece* 51142 (Fig. 1b). This observation was confirmed by light microscopic observations; cells of *Cyanothece* 7822 grown in low NP BG-11 were smaller, rounder, and more uniform in size (Fig. 1d), whereas cells grown in BG-11 were larger, more egg-shaped, and more variable in size (Fig. 1c).

Culture population analysis was also performed on *Cyanothece* 7822 cultures grown in nitrogen-deficient low NP BG-11 at various time points during a 12 h light–12 h dark diurnal cycle to induce synchronized photosynthesis and nitrogen fixation. Samples displayed consistent similarity in cell size across the four time points tested, L0, L3, D0, and D3 (Fig. 2a), but slight variations in internal complexity were observed (Fig. 2b). These slight variations in complexity, along with the stability in cell size, demonstrate the dynamic nature of the ultrastructure across the diurnal cycle when the organism shifted metabolically from photosynthesis to respiration within a culture population that is well synchronized.

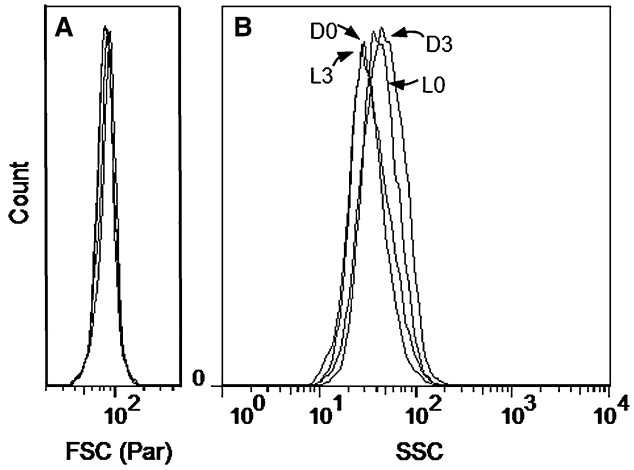

Fig. 2 **a** Individual cell size of *Cyanothece* 7822 as measured by forward scattering; **b** internal complexity in *Cyanothece* 7822 cells grown in nitrogen-deficient low NP BG-11 media during the 12 h light–12 h dark cycle as measured by side scattering. Culture samples were taken at four time periods: D0 (beginning of dark period), D3, L0 (end of dark period) and L3

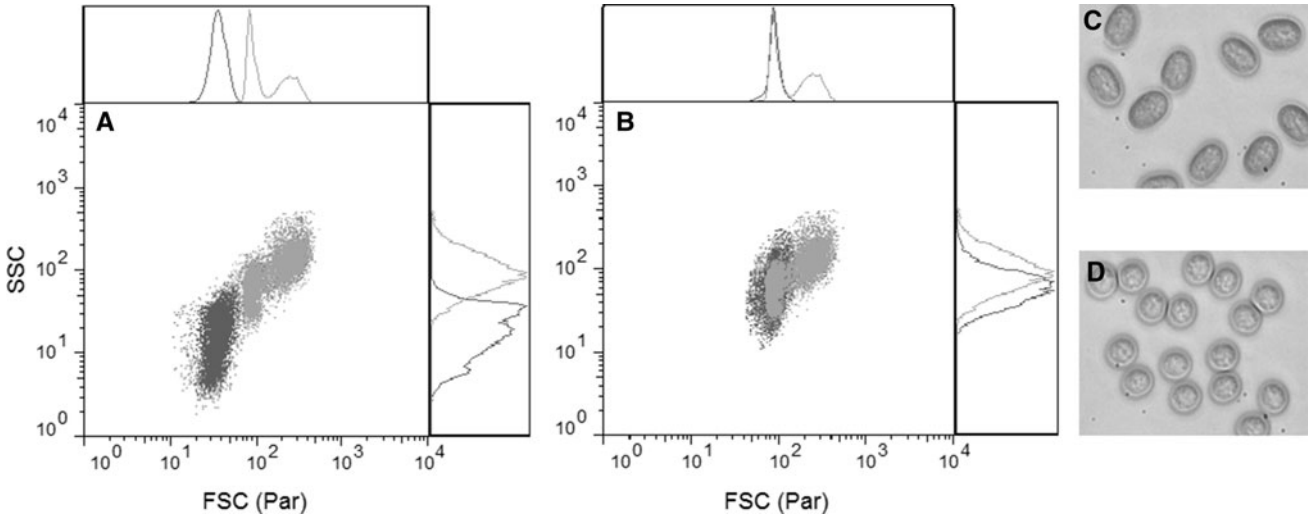

Fig. 1 **a** Distribution of cells in batch culture by individual size (*x*-axis) and internal complexity (*y*-axis) of individual *Cyanothece* 51142 (*dark gray*) and *Cyanothece* 7822 cultures grown in BG-11 (*light gray*); **b** *Cyanothece* 7822 grown in low NP BG-11 (*dark gray*) exhibiting a population architecture shift to a single population compared to *Cyanothece* 7822 grown in regular BG-11 (*light gray*); **c** high magnification light micrographs of *Cyanothece* 7822 grown in regular BG-11 and **d** low NP BG-11 media. *Error bars* represent standard deviation from three replicate samples

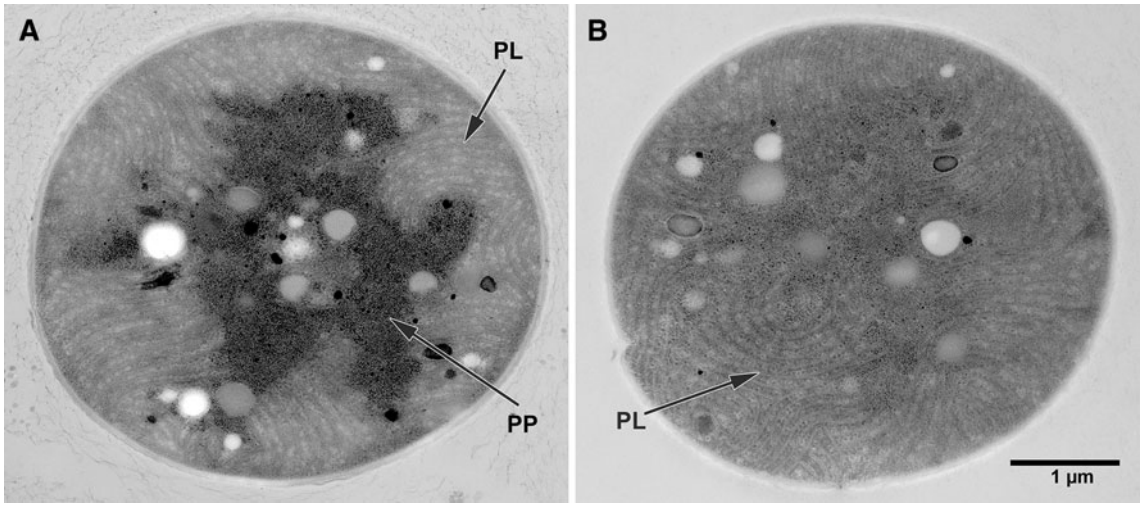

Fig. 3 Electron micrographs of *Cyanothece* 7822 cells grown in BG-11 medium (**a**) and low NP BG-11 medium (**b**). Cultures for both types were fixed by high pressure freezing for best preservation of cellular ultrastructure. *PP* polyphosphate, *PL* photosynthetic lamellae

Effects of $NaNO_3$ and KH_2PO_4 reduction on ultrastructure and storage granules

Results from flow cytometry analysis were verified and extended using TEM. Electron micrographs of *Cyanothece* 7822 grown in BG-11 had shown the presence of numerous storage granules in the cell, including those composed of polyphosphate, polyhydroxybutyrate, and cyanophycin (Bandyopadhyay et al. 2013). *Cyanothece* 7822 cultures were studied by both high pressure freezing (HPF) and chemical fixation to generate a better understanding of intracellular ultrastructure. HPF preserves cellular structure the best, because the flash freezing prevents loss of intracellular material. However, we also needed to utilize histochemical techniques that depend upon chemical fixation, so we compared cells prepared by the two techniques. HPF was performed on cells grown in regular BG-11 (Fig. 3a) and those grown in low NP BG-11 (Fig. 3b). In many of the BG-11 cells, there was dark material in the nucleoplasmic area and some cells contained many dark granules, both of which we interpret as polyphosphate. The cells grown in low NP BG-11 showed this dark material far less frequently and generally had fewer granules present. The population of cells seen in the electron microscope were much more similar to each other in the low NP BG-11 than in regular BG-11, consistent with the flow cytometry results.

The comparison of HPF versus the microwave chemical fixation procedure for *Cyanothece* 7822 is shown in Fig. 4. The cells were from a culture that had been grown in regular BG-11 and the cells were taken from late-log phase. The major differences routinely observed between the two techniques can be seen in the nucleoplasmic region. In the microwave procedure, there are lighter areas that we interpret were caused by the loss of intracellular material (compare Fig. 4b vs. a). This leads to slight shrinkage in the chemically-fixed cell and the relative sizes were typical of what was seen in such comparisons. This led to a slightly higher density of material in the photosynthetic membrane regions. In addition, there were typically more "holes" in place of granules; the results suggested that many of these holes were caused by the loss of polyphosphate from granules during microtomy. There were typically more presumptive polyhydroxybutyrate (PHB) granules in the chemically-fixed cells (Fig. 4b), presumably because the PHB was kept in a more native form when fixed by HPF (Fig. 4a).

In order to detect the glycogen granules in situ within *Cyanothece* 7822, we utilized a well-understood histochemical procedure, the PATO technique that depends upon periodic acid, thiosemicarbazide, and osmium tetroxide to generate a black deposit after reaction with glycogen (Hanker et al. 1964; Sherman and Sherman 1983). We had demonstrated some years ago that this procedure could quantitatively identify glycogen granules in *Synechococcus* sp. PCC 7942 (Sherman and Sherman 1983) and it was also successful in *Cyanothece* 7822 (Figs. 5, 6). Figure 5 compares the PATO-stained granules in *Cyanothece* 7822 cells grown under N_2-fixing conditions in a 12 h light–12 h dark diurnal cycle at L0 and D0 time points (Fig. 5). All of the dark-staining granules are composed of glycogen and it is obvious that these granules are more numerous and densely packed in cells at D0, which is at the end of the photosynthetic period, compared to L0, which is at the end of the 12 h dark period. Figure 5c represents the D0 control without the addition of periodic

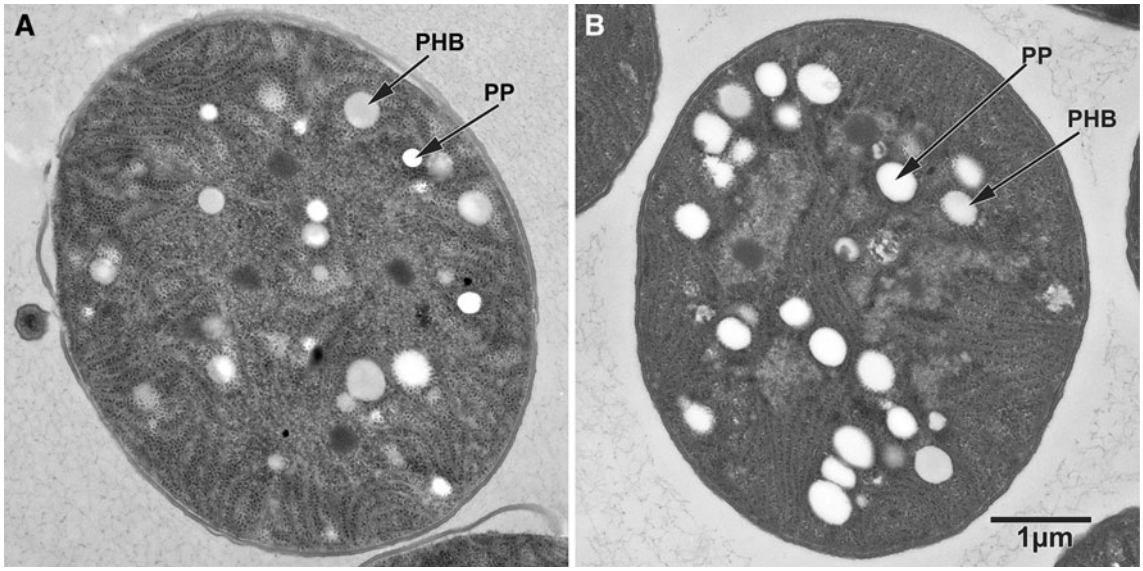

Fig. 4 Electron micrographs of *Cyanothece* 7822 grown in regular BG-11 and prepared by high pressure freezing (**a**) or by the microwave chemical fixation procedure (**b**)

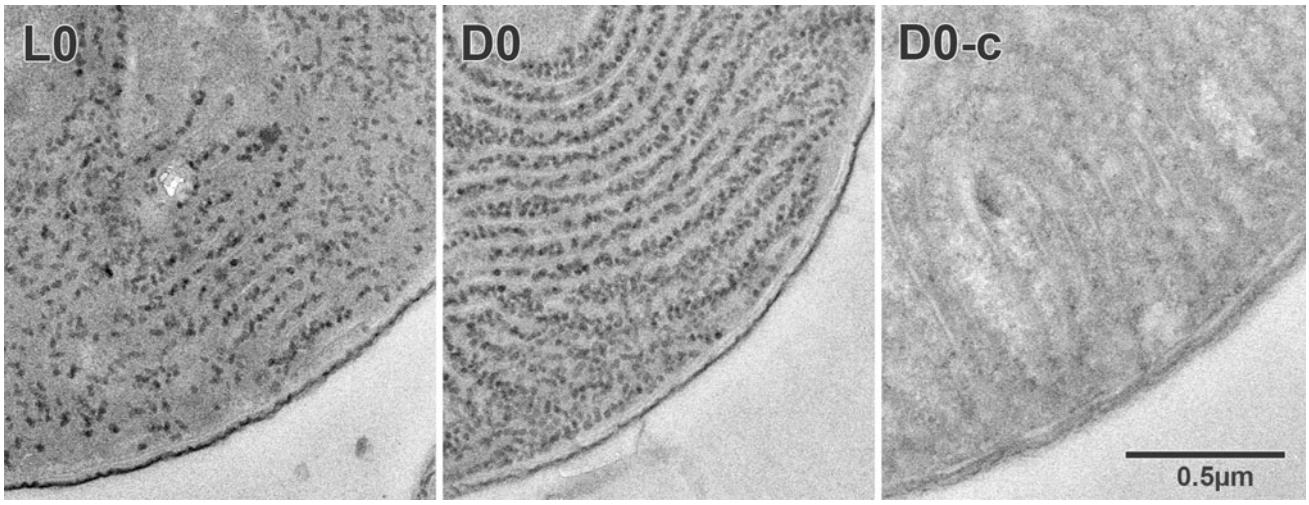

Fig. 5 Electron micrographs of *Cyanothece* 7822 stained using the PATO technique with the microwave chemical fixation procedure. Cells were grown under N_2-fixing conditions with a 12 h light–12 h dark cycle. (*Left*) *L0* the end of the dark period, (*center*) *D0* the end of the light period, and (*right*) untreated control from D0

acid and thiosemicarbazide; we consider the white patches to represent areas that contain glycogen granules.

This procedure was then used to quantitate glycogen granule composition when cells were grown under N_2-fixing conditions so that we could compare granules to the amount of glycogen detected via biochemical procedures. Figure 6 shows cells from L0, L3, D0, and D3 after PATO treatment. It is once again clear that cells at L0 have the lowest number of glycogen granules, whereas cells at the other times contain more granules. Micrographs from this experiment were used for quantitative analysis of glycogen content. The results indicated that the glycogen content peaked at the end of the light period with ~5,000 individual granules (per TEM section) and successively decreased to a minimum of ~1,500 granules at the end of the dark period. Biochemical analysis correlated these observations with a maximum glycogen content of 11 μg/mL at the end of the light period and a minimum of 4.8 μg/mL at the end of the dark period. In both cases, a similar fold change of approximately 3.3- to 2.3-fold was observed for both granule number and glycogen composition, respectively (Fig. 7). We conclude from this correlation and the strong staining using the PATO technique that these small granules contain glycogen.

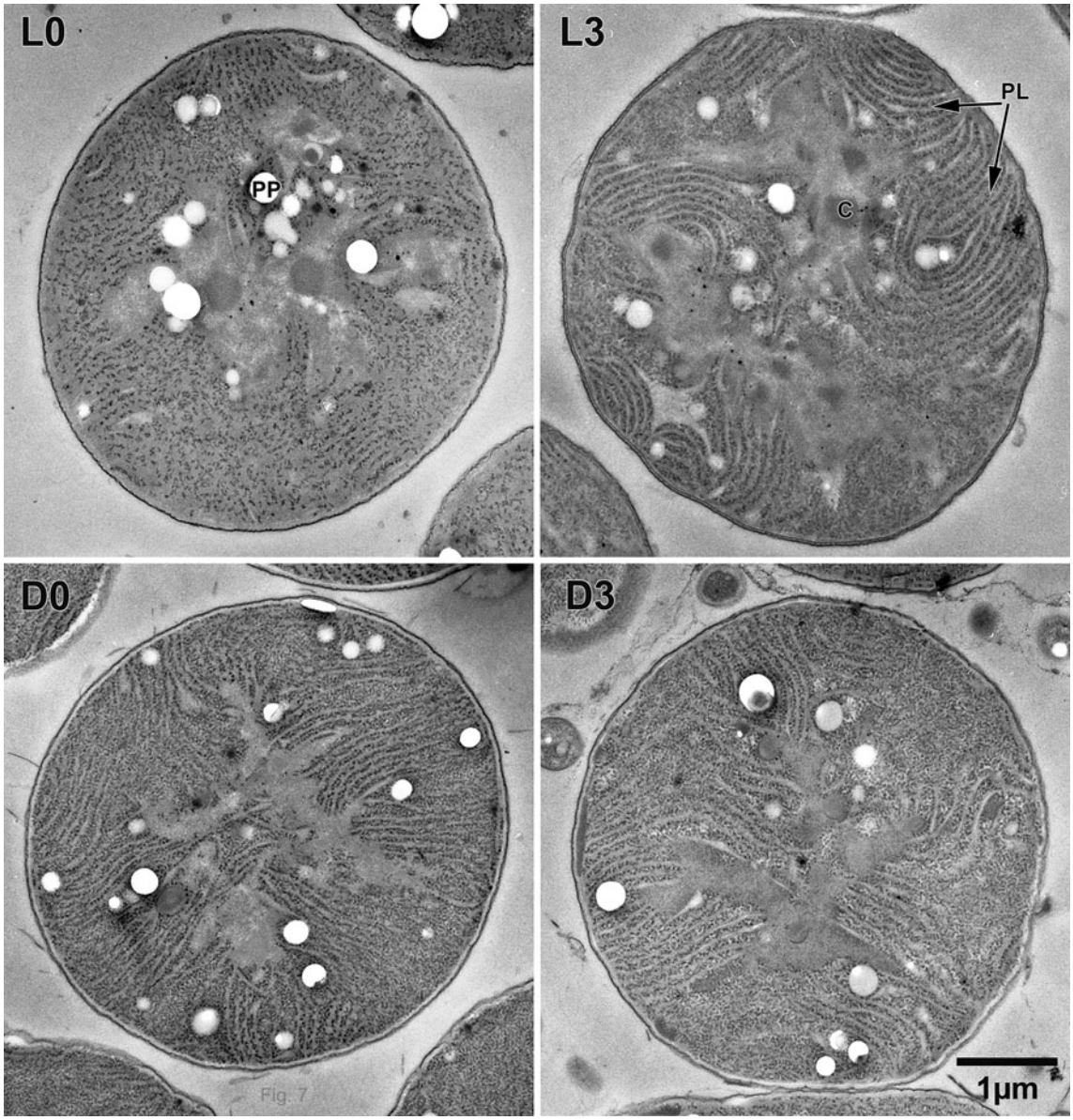

Fig. 6 Comparison of PATO-treated *Cyanothece* 7822 grown under N_2-fixing conditions with a 12 h light–12 h dark diurnal pattern. L0 to D3 represent the time period during N_2-fixing conditions at which samples were taken. *PL* photosynthetic lamellae, *PP* polyphosphate

Discussion

The goal of this study was to obtain culture conditions for *Cyanothece* 7822 that would improve the growth properties and minimize the amount of macronutrients (C, N and P) that were stored in large intracellular inclusion bodies. This was done successfully with the bonus of identifying glycogen granules quite different from those seen in *Cyanothece* 51142. The composition of the growth medium is an often-overlooked parameter, but it can have major implications on the cultivation of cyanobacteria. Whether it is for biomass production, influencing natural processes like nitrogen fixation, or stimulating the production of valuable pathway intermediates, the composition of the growth medium can have as much an impact as genetic modifications and the induction of metabolic pathways. *Cyanothece* 7822 is a phycoerythrin (PE)-rich cyanobacterium isolated from rice-field soil in India (Min and Sherman 2010a). PE-rich cyanobacteria dominate in low nutrient environments, where nutrient levels are quite different that that found in BG-11, and have developed highly efficient uptake and retention mechanisms for nitrogen (Herrero et al. 2001), carbon (Badger and Price 2003), and phosphorous (Ritchie et al. 2001). In fact, the use of BG-11 has been reported to be inhibitory to growth for PE-rich species like *Synechococcus* spp. (Ernst et al. 2005). Specifically,

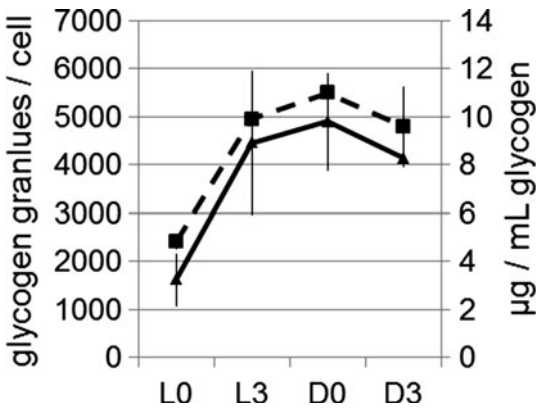

Fig. 7 Comparison of glycogen content and glycogen granules in *Cyanothece* 7822 grown under N_2-fixing conditions in 12 h light–12 h dark. *Error bars* represent standard deviations from replicate samples. ■ --- ■ Glycogen content; ▲ ___ ▲ Glycogen granules measured from PATO-stained cells. *Error bars* represent standard deviation from three replicate samples

BG-11 is rich in both nitrate and phosphate and has a N:P far higher than the Redfield ratio (16:1). The Redfield concept is a species-specific biogeochemical phenomenon (Rhee and Gotham 1980) that links the cellular mole ratio of carbon, nitrogen, and phosphorous to that found in the environment and is typically used to estimate ocean productivity and nutrient status of aquatic systems (Bertilsson et al. 2003). This ratio can also be considered the biochemical optimum for phytoplankton and ranges from ~10:1 to the ~40:1 for cyanobacteria (Klausmeier et al. 2004).

Based on these findings, various reductions of both nitrate and phosphate were made. Although altering the N:P ratio had little effect on the growth, a overall fourfold reduction in nitrate and phosphate did have a positive effect on growth and lowered flask-to-flask variability (the standard deviation of OD measurements among replicate cultures grown in the reduced media was cut in half). In addition, cells continued to display high nitrogenase activity and good capacity for hydrogen production. Since the low NP BG-11 media included reduction in nitrate from ~17.65 to 4.41 mM, the transition from nitrogen-replete to nitrogen-deficient media, and attaining cultures growing diazotrophically, required less time and in our experience no prior growth in nitrogen-free media was necessary before measuring nitrogenase activity. Cultures of *Cyanothece* 7822 achieved high biomass more rapidly by growing in low NP BG-11, followed by transition to nitrogen-deficient BG-11.

The reduction in N and P concentrations directly affected the internal storage content of the cells. The excess N and P in BG-11 resulted in nitrogen storage in cyanophycin granules, carbon into PHB granules, and polyphosphate into excess material seen in the nucleoplasmic region or in polyphosphate granules. Indeed, one of the important findings from this study was the different appearance of polyphosphate in electron micrographs of cells prepared by HPF (more dark staining material in the nucleoplasm) or by chemical fixation (more and larger granules, some containing dark material, but most observed as holes). Growth in low NP BG-11 had much less polyphosphate and the nucleoplasm was generally free of the dark-staining material. This explained a long-standing puzzle as to why some cells contained this material and others did not. Cells from cultures grown in low NP BG-11 also had fewer large granules, such as cyanophycin and PHB, thus indicating that cells did not need to store as much nitrogen or carbon under these conditions.

The relative lack of these large granules revealed a large number of smaller granules between the photosynthetic membranes. These had been seen previously, but it was assumed that one type of the larger granules was composed of glycogen, as in *Cyanothece* 51142 (Schneegurt et al. 1994; Schneegurt et al. 1997). These granules resembled the β-particles of glycogen that we had identified in *Synechococcus* sp. PCC 7942 many years earlier (Sherman and Sherman 1983) and we immediately demonstrated that they could be stained by the PATO technique. In addition, glycogen granules fluctuated under N_2-fixing conditions during the light–dark periodicity and correlated quite nicely with the total glycogen content that was determined biochemically.

It was surprising that a species within the *Cyanothece* genus contained different morphological types of glycogen granules. The assembly and degradation of the glycogen granules in *Cyanothece* 51142 are important features for the storage of potential energy and carbon from the light to the dark—the granules are formed via photosynthesis and are mobilized in the dark as a substrate for respiration to make ATP and to reduce intracellular oxygen to protect nitrogenase (Schneegurt et al. 1994; Schneegurt et al. 1997). The highly synchronized pattern of formation and mobilization of these granules is critical for the highly synchronized pattern of photosynthesis and N_2 fixation. The smaller glycogen granules in *Cyanothece* 7822 also demonstrated a synchronized formation and degradation as cells went from light to dark under N_2-fixing conditions, such that the underlying protective mechanism for nitrogenase was present.

Nonetheless, the structure of the two types of granules is of interest and has been studied in some detail by Ball and collaborators (Ball et al. 2011). Most cyanobacteria make soluble glycogen that is stored in β-granules. Some strains, such as *Cyanothece* 51142, were shown to store the material as semi-amylopectin. Amylopectin is composed of highly ordered, repeated clusters of glucose moieties due to densely localized α-1,6 branch points along glucan chains with intervals of 9–10 nm. This type of structure is responsible for the water-insoluble characteristics of these

starch-like granules (Suzuki et al. 2013). However, in glycogen, the α-1,6 branch points are uniformly distributed and the density of the branches becomes very high at the periphery of the molecule, thus limiting their size to about 42 nm in diameter, compared to over 100 nm in diameter for the starch-like granules (Ball et al. 2011; Schneegurt et al. 1994).

The genes for many of the biochemical processes have been identified (Suzuki et al. 2013), and the differences between the two *Cyanothece* strains is notable. The storage of α-glucan occurs through the sequential actions of ADP-glucose pyrophosphorylase (AGPase), glycogen/starch synthase (GS/SS), and a branching enzyme (BE) (Preiss 1984; Suzuki et al. 2010). Investigations of the genomic sequences of both species revealed that both contain two genes for GS/SS (cyan7822_4734 and cyan7822_2570 in *Cyanothece* 7822, and cce_0980 and cce_3396 in *Cyanothece* 51142), but vary slightly in the number of AGPase and branching enzymes genes. *Cyanothece* 51142 contains two AGPase genes (cce_0987 and cce_2658) and three BE genes (cce_1806, cce_2248, and cce_4595), whereas *Cyanothece* 7822 has one AGPase gene (cyan7822_2049) and two BE genes (cyan7822_2889 and cyan7822_1547).

Genomic sequence analysis has determined that the number of genes coding for enzymes in the glycogen biosynthesis pathway is variable among cyanobacterial species. *Synechococcus elongatus* PCC 7942 has the simplest composition with just one of each gene. Others, like *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120, are similar to *Cyanothece* 7822, having one gene for AGPase and two genes for GS/SS (Suzuki et al. 2010). We suggest that the different forms of polysaccharide storage observed in *Cyanothece* 7822 and *Cyanothece* 51142 are due to the actions of the branching enzymes. It is likely that the concerted actions of the three BE isoforms in *Cyanothece* 51142 give rise to the starch-like granules observed, as postulated by (Suzuki et al. 2013). Moreover, the sequence relationship among branching enzymes found in members of the *Cyanothece* genus, *Synechocystis*, and *Synechococcus* shows a correlation between species that harbor three BE isoforms with the formation of semi-amylopectin and species that have either only BE1 or both BE1 and BE2 (Fig. 8). At the moment, only the unicellular diazotrophic cyanobacteria found in benthic or oceanic regions seem to produce the semi-amylopectin granules and all have the three BE enzymes, as shown in Fig. 8.

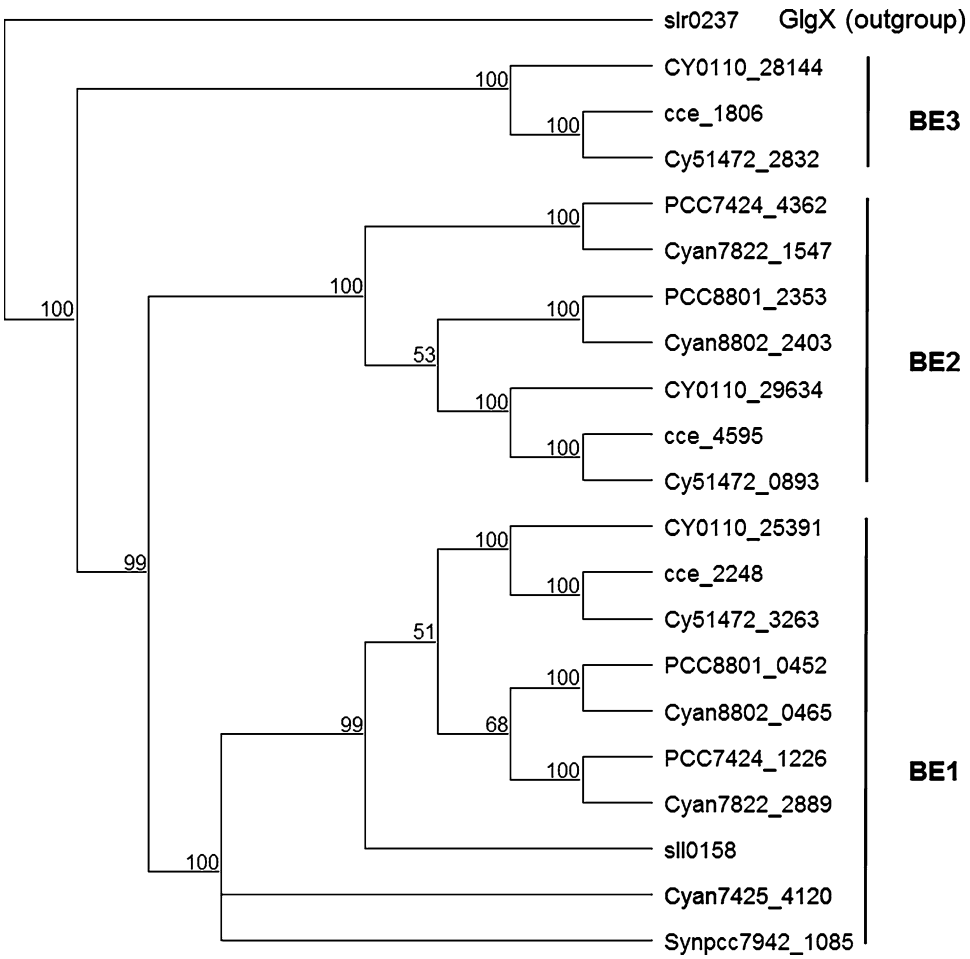

Fig. 8 Neighbor-joining tree showing amino acid sequence relationships among branching enzymes in the *Cyanothece* spp. with sequenced genomes

From analysis of the amino acid sequences, the most likely scenario is that, in *Cyanothece* 51142, BE1 (773 aa) duplicated to form BE2 (651 aa) and that either BE1 or BE2 then duplicated again to form BE3 (647 aa). The major differences among the three proteins are a large N-terminal deletion of 97 amino acids for BE2 and 111 amino acids for BE3. There are also various length deletions at the C terminus. BE1 and BE2 demonstrated 58 % identity and 74 % similarity, whereas BE1 versus BE3 or BE2 versus BE3 were only 29 and 44 % identical and conserved, respectively. Obviously, BE3 has changed the most and is likely critical for the formation of the large starch-like granule formation. Further biochemical and molecular analysis will be required to determine the enzymatic basis for the different forms of glycogen storage among these strains. Although it is likely easier to synchronize the degradation of a smaller number of large granules (as in *Cyanothece* 51142), compared to a larger number of small granules (as in *Cyanothece* 7822), both approaches seem to work well. Nevertheless, *Cyanothece* 51142 shows greater synchrony and higher peaks for both nitrogenase activity and H_2 production, indicating a value for the large starch-like granules.

Acknowledgments This work was supported in part by a grant from the DOE Genomics: GTL Program (DE 09-19 PO 2905402N; Himadri Pakrasi, principal investigator) and in part by a grant from the Membrane Biology EMSL Scientific Grand Challenge Project at the W.R. Wiley Environmental Molecular Science Laboratory, a national scientific user facility sponsored by the U.S. DOE Office of Biological and Environmental Research Program and located at the Pacific Northwest National Laboratory. Battelle operates PNNL for the DOE under Contract DE-ACO5-76RLO 1830.

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