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Photo-catalytic conversion of carbon dioxide to organic acids by a recombinant cyanobacterium incapable of glycogen storage.

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Materials and Methods:

Creation and verification of mutant lines

A specific ORF replacement of the *glgC* gene (slr1176) with a gentamicin-resistance gene was performed using homologous recombination. A gene deletion construct was created using fusion PCR that contained flanking regions of the *glgC* gene and the pUC119-gen plasmid (PCR primers described in table S2), using KOD Hot Start DNA Polymerase (Novogen) under standard conditions[18, 19] (data not shown). Transformation of wild type was performed using the created gene-deletion construct. Selection was performed on agar plates with BG-11 (10 mM $NaHCO_3$, gentamicin between 20 and 50 μg/mL). Segregation of the mutation was verified by PCR-product analysis (Fig. S1A) using primers listed in Table S2. Glycogen determination (described below), and western blot protein analysis have consistently and repeatedly demonstrated that the *glgC* mutant is a fully segregated mutant. A western blot is included as Figure S1B. Protein was isolated from logarithmically growing cultures, extracted, and quantified [19]. Proteins were separated using Mini Protean TGX Gels (Biorad), and blotted using Fast Semi Dry Blotter (Pierce) onto PVDF (Biorad) membranes. A custom peptide primary antibody for GlgC (YenZym Antibodies, LLC) was used in conjunction with Goat Anti-Rabbit secondary antibody (Pierce) and a CN/DAB Substrate Kit (Thermo Scientific).

Synechocystis sp. PCC 6803 strain Δ*glgC* psbA2::*glgC* was constructed from the Δ*glgC* line above with the pPSBA2KS vector from Wim Vermaas[20] altered by removal of the SalI site via partial digest and blunting to allow for retention of the kanamycin-resistance gene by Carrie Eckert (NREL). The *glgC* gene was amplified from genomic DNA isolated from WT by PCR and inserted into the vector between the NdeI and SalI restriction sites. Transformation was conducted by incubation of ~1 μg of the integration vector for 6 h with 200 μL cells (adjusted to optical density of 2.5 from logarithmic-phase cultures), followed by addition of 2 mL BG11, 24-h outgrowth in culture tubes under standard growth conditions, and plating of 200 μL on BG11 plates with 200 μg/mL kanamycin. The mutation was verified by PCR-product analysis (Fig. S1A) using primers listed in Table S2.

Strain Culturing and nitrogen starvation

Cultures were grown at 30°C in BG11 medium (ATCC medium 616) supplemented with 3μM $NiCl_2$, 20mM TES, and 100 mM $NaHCO_3$ under continuous illumination (~50 μE m^{-1} s^{-1}) in an atmosphere of 5% CO_2 in air. The antibiotics, gentamicin and kanamycin, were used at 5 and 50 μg/mL in liquid cultures for inoculation from plates. Thereafter, the antibiotics were removed for growth of cultures in BG11. For nitrogen starvation, the nitrogen source ($NaNO_3$) in BG11 was replaced with NaCl (mol:mol) with all other components the same (BG11-N medium). Cells from log-phase cultures in replete medium (OD_{730}, 0.6-0.8; DW, 0.3-0.5 g/L) were harvested by centrifugation, washed, resuspended in BG11-N, and placed under the same light and atmosphere conditions as above. Optical densities were measured at 730 nm (WPA Biowave II, Biochrom, USA). To monitor bleaching, absorbance of whole-cell suspensions of cultures were measured in a wavelength range of 300-800 nm each day with a spectrophotometer (DU800, Beckman Coulter, USA).

For precise growth comparisons between WT and Δ*glgC* strains (Fig. S2), optical density measurements at 735 nm were automated (measure every 5 minutes) in photobioreactors model FMT150 (Photon System Instruments, Czech Republic) supplying continuous light flux from white LEDs at 65μE m^{-1} s^{-1} and maintaining cultures at 30° C with continuous gas sparging under 2% CO_2 in air.

O_2-evolution rates

Following suspension in BG11-N and each day thereafter, aliquots of cultures (6 mL) were taken and resuspended in 2 mL of photosynthesis buffer (20mM TES, 100 mM $NaHCO_3$, and 230 μM K_2HPO_4). Whole cell O_2 evolution was measured with a Clark-type electrode system (Algi, USA) that included a white LED light source (1000 μE m^{-1} s^{-1}) in a water jacket for maintaining sample temperature at 25° C. Evolution rates presented were not adjusted for respiration rates, which were typically 15-25% of the initial evolution rates.

Dry weight determination

Clear glass bottles (Wheaton, USA) with an approximate capacity of 25 mL were cleaned, acid/base washed, and placed at 55°C for drying for several weeks. Exactly 50 mL of cells were harvested each day, transferred to 50 mL conical tubes, and centrifuged for 10 minutes at 4,000g in a swinging bucket rotor. Supernatants were carefully decanted, and approximately 5 mL of deionized water was used to resuspend each of the remaining cell pellets and rinse residual material from conical tubes. Cell suspensions and rinses were transferred to a pre-weighed glass bottle, which was returned to a 55°C oven for several weeks until all moisture had evaporated (indicated by constant cool dry mass). Weights were measured once for each culture per day. Error bars represent 3 separate cultures. Weights of extracellular metabolites were calculated based on the formula weight of the acids and concentrations determined in molarity by NMR as described below.

Glycogen determination

Glycogen was determined following a method adapted from Ernst *et al.*[21], scaled up for cell culture harvests in order to achieve detection limits below 0.2% cell dry weight. Briefly, 10-mL of cells were collected by centrifugation and base-boiled in 1 mL of 30% (w/v) aqueous KOH. Glycogen was then precipitated on ice by the addition of 5 mL cold absolute ethanol and collected by centrifugation, discarding supernatant. Glycogen isolates were digested by α-amylglucosidase (Sigma-Aldrich, USA) at 55°C in sodium acetate buffer (pH 4.75). Soluble glucose from digests was determined using a hexose-kinase assay kit (Sigma-Aldrich, USA).

Extracellular metabolite determination

Cell suspensions were collected and centrifuged, and 500 μL of the resulting cell-free supernatant was collected and combined with 100 μL of D_2O (Sigma-Aldrich, USA), containing 30 μg/mL TSP (3-(trimethylsilyl)propionic-2,2,3,3-D_4 acid sodium salt) in a Pyrex NMR tube. Proton NMR spectra were collected with a 500 MHz Bruker Avance-II spectrometer at 25°C; 256 scans were used with a relaxation delay of 1s with water-suppression.[8] All spectra were processed using MestReNova software v 6.0.4 (Mestrelab Research S.L., Santiago de Compostela, Spain). HPLC was also used to verify identities of the metabolites in cell-free medium by comparison of retention times of the peaks relative to standards prepared in BG11-N.

Carbon-13 isotope labeling

Cells cultured in (nutrient replete) BG11 were resuspended in BG11-N medium with some sodium bicarbonate replaced with ^{13}C sodium bicarbonate (Sigma-Aldrich, USA) as indicated by percentages (final concentration of ^{12}C + ^{13}C sodium bicarbonate, 100 mM). Cell-free medium following 1-day growth in BG11-N was collected, and proton NMR spectra were determined as above. Carbon spectra were collected on a 400-MHz Varian spectrometer at 25°C (18,000 scans with a relaxation delay of 1s). All spectra were processed using MestReNova software v 6.0.4 (Mestrelab Research S.L., Santiago de Compostela, Spain).

Figures S1-S3:

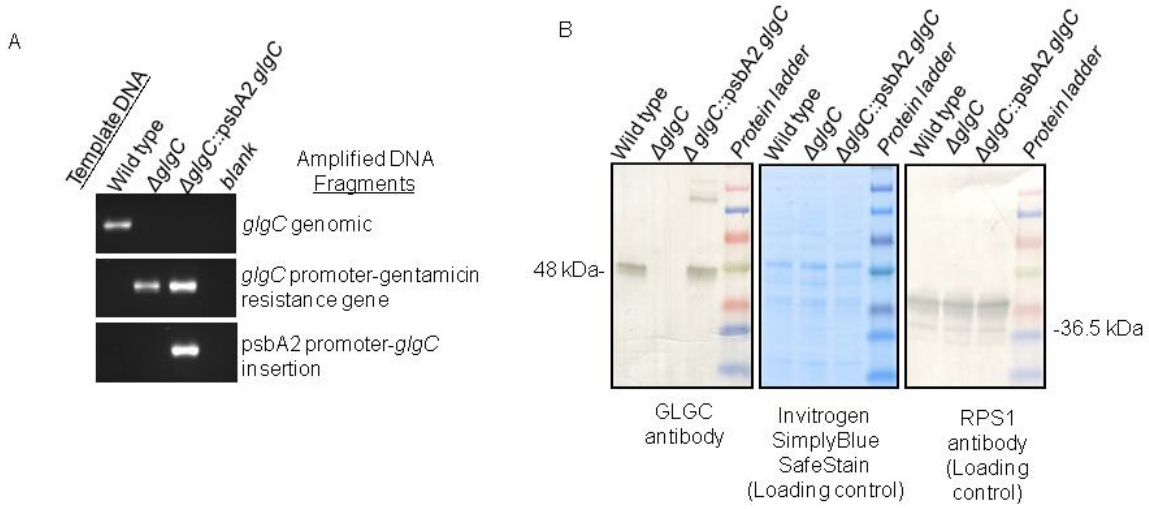

Fig. S1. Demonstration of the Δ*glgC* mutant and ectopically expressed rescue. The *glgC* genomic fragment is only detectable in the wild type genome **(A)**. The presence of transgenes is revealed by amplification of respective insertion junctions with primers that are 5' to the insertion site in the genome. All PCR fragments were of the expected lengths. A western blot using a GlgC-specific peptide antibody confirmed that the Δ*glgC* mutant was GlgC-protein null **(B)**. Predicted protein molecular weights are included (kDa) and correspond to observed masses. A nonspecific protein gel stain SimplyBlue SafeStain (Invitrogen) shows similar total protein loading, and RPS1 primary antibody (Agrisera) detection was included as a specific protein loading control for ~10 µg of total protein loaded per lane.

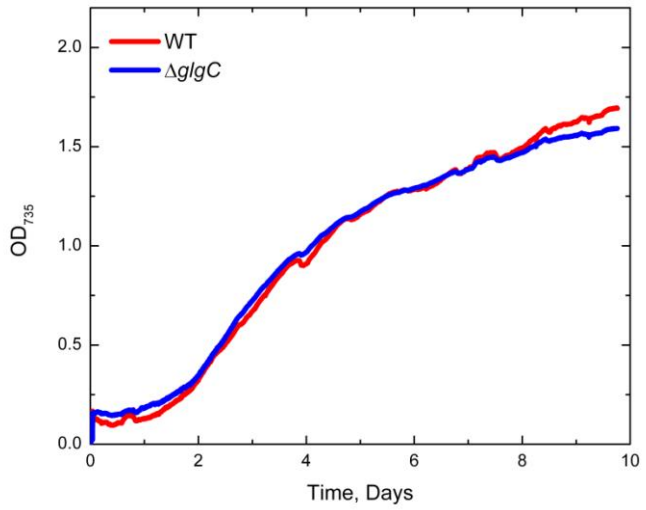

Fig. S2. Growth curves of WT and Δ*glgC* cultures in regular BG11 medium in photobioreactors under continuous illumination as monitored by absorbance at 735 nm every 5 minutes. Light intensity was 65 μE m^{-2} s^{-1}.

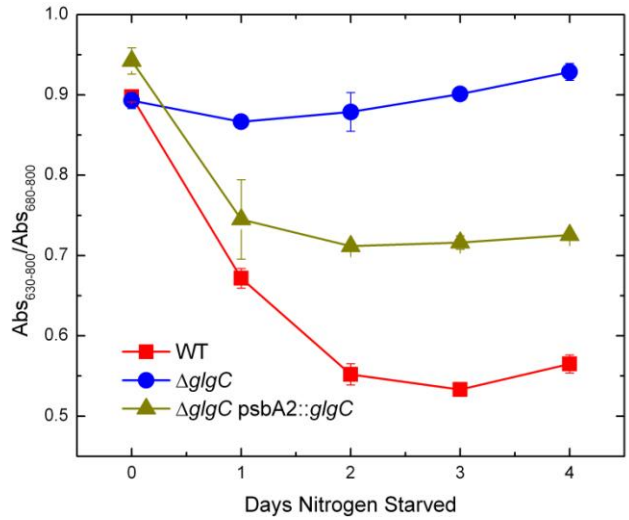

Fig. S3. Absorbance ratios of whole-cell suspensions at 630 nm (phycocyanin maximum) and 680 nm (chlorophyll a maximum) for *Synechocystis* sp. PCC 6803 WT, Δ*glgC*, and Δ*glgC* psbA2::*glgC* during a nitrogen-starvation growth regime as an indication of cell bleaching[6]. Phycocyanin degradation was not observed in Δ*glgC* cultures, but was apparent in WT and Δ*glgC* psbA2::*glgC* cultures. A slight decline in chlorophyll absorbance (~30% after four days) was observed for all cell lines causing an increase in the phycocyanin/chlorophyll absorbance ratios over time.

Tables S1-S2:

Table S1. Labeling ratios of α-ketoglutarate (AKG) and pyruvate from cyanobacterial cells in BG11-N prepared with listed ratios of ^{13}C-bicarbonate as determined by proton NMR.

^{13}C/C-bicarbonate in medium	^{13}C/C Pyruvate	^{13}C/C AKG
0.134	0.135 ± 0.008	n.d.[a.]
0.258	0.267 ± 0.006	0.30 ± .05

a. Not determined. Limited signal-to-noise ratios of AKG satellite signals preclude precise determinations at this concentration of labeling.

Table S2. PCR primers for fusion PCR and genomic segregation screening of mutant lines.

Fusion PCR Primers	
5'agpF	GTCATGCCAATGCCGTTATC
agp/gn atgR	CATCGTTGCTGCTGCGTAACATTTCGAAGTCAAGTTTAGAACAGAGG
agp/gn atgF	CCTCGGTTCTAAACTTGACTTCGAAATGTTACGCAGCAGCAACGATG
agp/gn taaR	GTGCGAGGAAAGAAACTGGCCTAAGGTGGCGGTACTTGGGTCG
agp/gn taaF	CGACCCAAGTACCGCCACCTAAGGCCAGTTTCTTTCCTCGCAC
3'agpR	GGTGAACGACAAAGCCAGTTA
Genomic Segregation Screening	
5'outagpF	CAGATGGCCCGCTGTTTATT
agpR	AACAACCAGAGGTATTGCCG
GentintR	AAGAAGCGGTTGTTGGCGC
PsbA2outF	CCCATTGCCCCAAAATACATC

Supplemental References:

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21. A. Ernst, H. Kirschenlohr, J. Diez and P. Boger, *Archives of Microbiology*, 1984, **140**, 120-125.