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ORIGINAL PAPER

Photoheterotrophic growth of unicellular cyanobacterium *Synechocystis* sp. PCC 6803 *gtr*⁻ dependent on fructose

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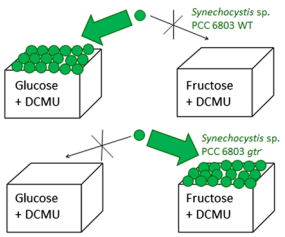
Abstract

Although cyanobacteria have specialized for a photolithoautotrophic mode of life during evolution many cyanobacterial strains have been identified as being capable of photoheterotrophy or even chemoheterotrophy. The mutant strain of *Synechocystis* sp. PCC 6803, which lacks the *gtr* gene coding for the strain's glucose/fructose permease, has been believed to be a strict photolithoautotroph in the past as it has lost the wild type's facility to use external glucose for both photoheterotrophy and light-induced chemoheterotrophy. However, recent experiments revealed the strain's capacity to use fructose for mixotrophic and photoheterotrophic growth, a sugar which is toxic for the wild type. Both the growth rate and the amount of fructose incorporated into the cells increased along with the fructose concentrations in the surrounding medium. Furthermore an increase of the total carbon mass of the cells within a liquid culture over a period of photoheterotrophic growth could be demonstrated. Contrary to the wild type, glucose could not be used for photoheterotrophic growth, and chemoheterotrophic growth failed with fructose as well as with glucose.

Graphic abstract



Keywords Carbohydrates · Membranes · UV/Vis spectroscopy · Transport

Introduction

The major interest in cyanobacteria rests on their capacity for oxygenic photosynthesis. This process has been acquired later in evolution by some eukaryotes caused by endosymbiosis with cyanobacteria-like prokaryotes [1]. Characteristic for cyanobacteria is the photolithoautotrophic growth mode using light as their energy source, water as their electron source, and carbon dioxide as their carbon source.

Although many cyanobacteria are strict photolithoautotrophs unable to use other carbon compounds than CO_2 as their sole carbon source, some have the capacity for photoheterotrophy and some can even grow chemoheterotrophically [2–4]. In some cases the inability to grow heterotrophically may be due to the incapacity for taking up organic substrates (for a review see [5]). For instance *Synechocystis* sp. PCC 6803 loses its capacity for both photoheterotrophic [2, 3] and light-activated chemoheterotrophic growth [6] on glucose when the *gtr* gene encoding the glucose transporter

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[7] is knocked out [8, 9]. When this gene was introduced into *Synechococcus* sp. PCC 7942 on a self-replicating plasmid, the new transgenic strain acquired the capacity for photoheterotrophic growth on glucose; however, this plasmid could not be maintained stably [10]. Integration of the *gtr* gene into the chromosome of PCC 7942 led to a strain for which glucose was highly toxic [10]. Similarly glucose was toxic for *Anabaena* sp. PCC 7120 if the *Synechocystis gtr* gene was introduced on a stably replicating plasmid [11].

Wolk and Shaffer [12] demonstrated that *Anabaena* sp. ATCC 29413 can grow in permanent darkness if fructose is added to the growth medium. Ungerer et al. [13] introduced the gene locus *frtRABC* encoding the transport system for fructose from *Anabaena* sp. ATCC 29413 into the closely related *Anabaena* sp. strain PCC 7120, which had been characterized earlier as strictly photolithoautotrophic [3]. The resulting derivative of *Anabaena* sp. PCC 7120 acquired the capacity for chemoheterotrophic growth on fructose [13]. Later the strain was discovered to grow without any genetic modification both photoheterotrophically and chemoheterotrophically on fructose, if very high concentrations (50–200 mM) were added to the growth medium [11]. These results led us to study the behaviour of a mutant strain of *Synechocystis* sp. strain PCC 6803, whose *gtr* gene encoding the glucose/fructose permease had been deleted [7]. Strain PCC 6803 *gtr*$^-$ exhibited both mixotrophic and photoheterotrophic growth dependent on high concentrations of fructose.

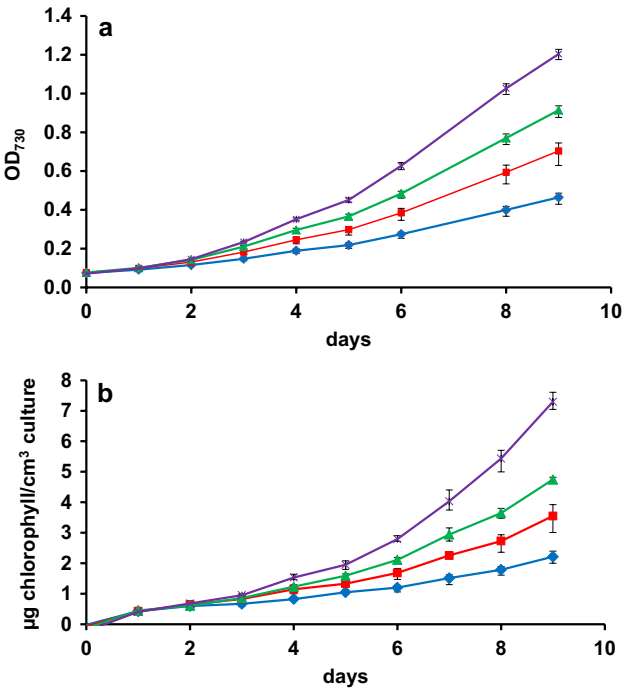

Fig. 1 Mixotrophic growth of *Synechocystis* sp. PCC 6803 *gtr*$^-$ (**a**) OD_{730} values, (**b**) chlorophyll concentration: blue diamond: 0 mM, red square: 50 mM, green triangle: 100 mM, violet cross mark: 200 mM fructose, the bars indicate the deviation within the triplicate of measurements (color figure online)

Results and discussion

Synechocystis sp. PCC 6803 *gtr*$^-$ can grow mixotrophically and photoheterotrophically on high concentrations of fructose

We tested *Synechocystis* sp. strain PCC 6803 *gtr*$^-$ [7], which had been considered to be an obligate photolithoautotroph prior to the results described in this paper. Strain PCC 6803 *gtr*$^-$ was cultivated at various concentrations of fructose (50 mM, 100 mM, and 200 mM) in the presence (photoheterotrophic conditions) as well as in the absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (mixotrophic conditions). DCMU blocks the electron transfer from photosystem II to the quinone pool thereby preventing oxygenic photosynthesis [14–16]. In addition to OD_{730} values (Figs. 1a, 2a) we also measured the chlorophyll concentration/cm^3 culture (Figs. 1b, 2b) as a further control to exclude the possibility that the long durations of the experiments as well as the high sugar concentrations may enhance the danger that the cultures have turned xenic.

Both mixotrophic (Fig. 1) and photoheterotrophic growth (Fig. 2, Tables 1S and 2S) were found with high concentrations of fructose, which indicates the strain's capacity to grow in the absence of oxygenic photosynthesis. DCMU-resistant mutants in diverse cyanobacterial strains have been known for a long time [15, 17]; however, in the photoheterotrophic experiments, no growth occurred in the negative control, where DCMU but not fructose had been added (Fig. 2, Tables 1S and 2S).

In contrast to fructose 200 mM glucose did not support mixotrophic or photoheterotrophic growth. Since *Anabaena* sp. PCC 7120 can grow in permanent darkness dependent on high concentrations of fructose [11] we tested whether *Synechocystis* sp. PCC 6803 *gtr*$^-$ is able to grow in the dark on 200 mM glucose or fructose. No growth at all was observed for both sugars either in complete darkness or at a daily illumination of 5 min. The latter mode has been discovered to be essential for chemoheterotrophic growth of *Synechocystis* sp. strain PCC 6803 wild type dependent on glucose [6]. Nieves-Morion and Flores [18] have identified five genes encoding ABC sugar transporter components in PCC 7120 and single mutations in each of them resulted in a reduction of mixotrophic growth dependent on fructose. No homologous genes are present in *Synechocystis* sp. PCC 6803.

Since low concentrations (10 mM) of fructose are toxic for *Synechocystis* sp. PCC 6803 wild type [8], the concentrations that enabled photoheterotrophic growth of strain PCC 6803 *gtr*$^-$ (Fig. 2) were also tested on *Synechocystis* sp. PCC

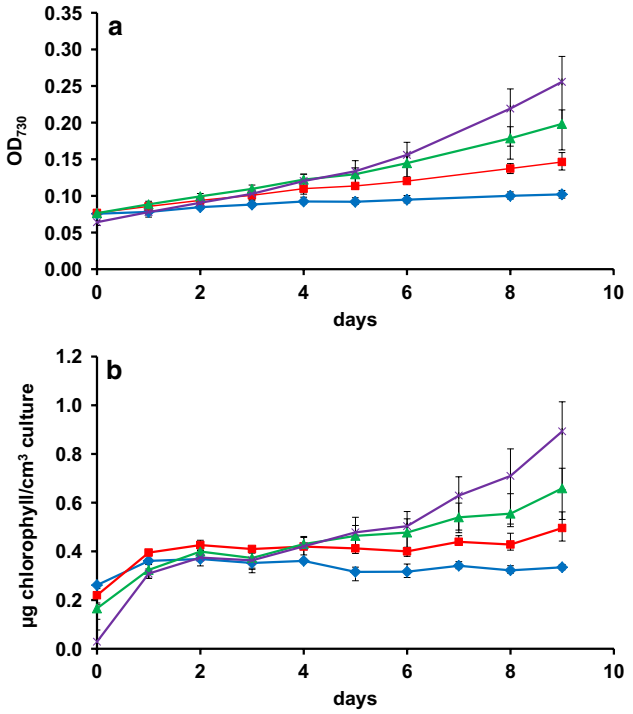

Fig. 2 Photoheterotrophic growth of *Synechocystis* sp. PCC 6803 *gtr*⁻ (**a**) OD_{730} values, (**b**) chlorophyll concentration: blue diamond: 0 mM, red square: 50 mM, green triangle: 100 mM, violet cross mark: 200 mM fructose, the bars indicate the deviation within the triplicate of measurements (color figure online)

6803 wild type both in the presence and absence of DCMU. Under none of the tested conditions the cells could survive.

Fructose is taken up by *Synechocystis* sp. PCC 6803 *gtr*⁻

The uptake of radioactively labelled fructose by *Synechocystis* sp. PCC 6803 *gtr*⁻ was tested at different external fructose concentrations (1 mM, 10 mM, and 100 mM). The strain took up fructose from the surrounding medium and incorporated it inside the cells (Fig. 3a). A higher fructose concentration in the medium also increased the amount of fructose taken up by strain PCC 6803 *gtr*⁻, although the increase of uptake was not proportional to the surrounding concentrations (Fig. 3a). Although glucose did not stimulate photoheterotrophic growth in PCC 6803 *gtr*⁻, it was taken up roughly 8 times faster compared to fructose (Fig. 3b, c). Therefore, the growth supporting effect of fructose but not of glucose is rather caused by metabolic instead of uptake mechanisms.

According to these data an unidentified carrier protein must allow the import through the cytoplasmic membrane, since large polar molecules like fructose cannot pass the cytoplasmic membrane without such a transporter. The

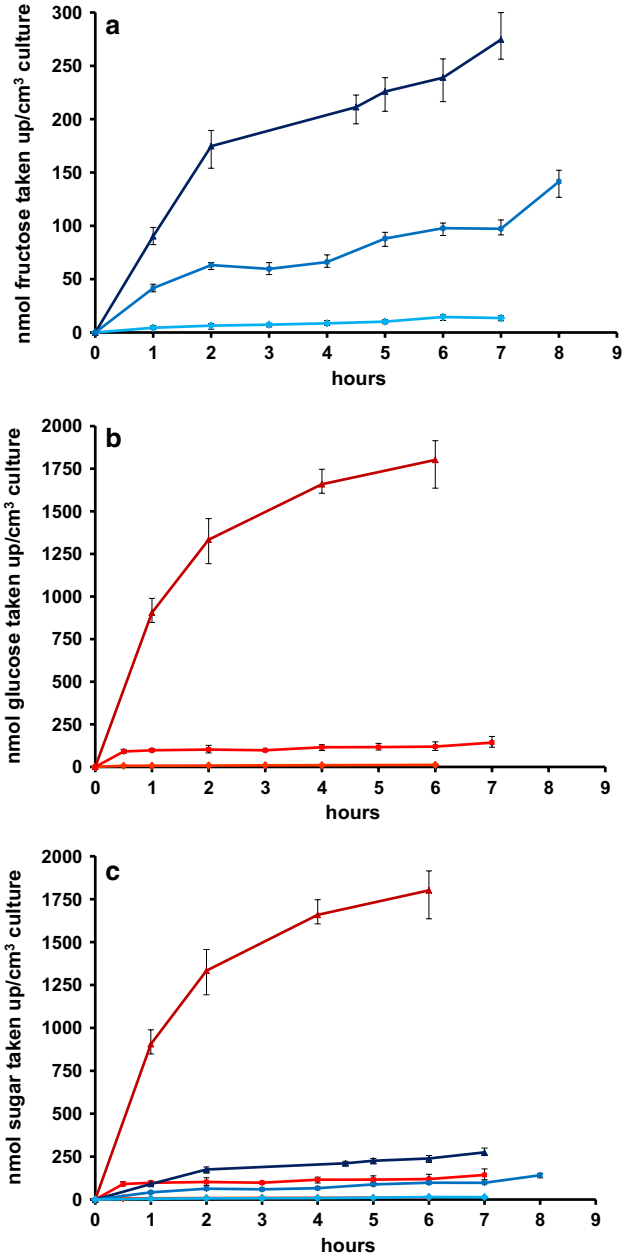

Fig. 3 Uptake of **a** fructose, **b** glucose and **c** fructose compared with glucose under different external sugar concentrations by *Synechocystis* sp. PCC 6803 *gtr*⁻; light blue diamond: 1 mM fructose, blue square: 10 mM fructose, dark blue triangle: 100 mM fructose, light red diamond: 1 mM glucose, red square: 10 mM glucose, dark red triangle: 100 mM glucose, the bars indicate the deviation within the triplicate of measurements (color figure online)

possibility that simple diffusion is the way of entry into the cells can also be excluded, because for simple diffusion the uptake rate of fructose should increase proportionally to the concentration in the surrounding medium, which is not the case (Fig. 3a). This fact rather indicates an import of this sugar by a specific transporter protein. The total sequence

of PCC 6803 does not contain a sugar transporter beyond the *gtr* gene so that the transporter(s) responsible for the entry of fructose and/or glucose into *Synechocystis* sp. PCC 6803 *gtr*$^-$ may have another primary function. Niederholtmeyer et al. [19] demonstrated that the glucose importer Glf of *Zymomonas mobilis* [20] acted as an exporter within *Synechococcus* sp. PCC 7942 if the intracellular glucose concentration was artificially increased. Therefore fructose may be imported as well by an actual exporter for the carbohydrate portion of LPS (lipopolysaccharides of the outer membrane), if there is an excess of a similar substrate like fructose in the surrounding medium.

It is currently unknown, why the entering fructose molecule cannot be used for dark growth of *Synechocystis* sp. PCC 6803 *gtr*$^-$ as it is the case for *Anabaena* sp. PCC 7120. A number of instances is known from the literature [10, 19, 21], where the entry of a sugar into a cyanobacterium using different transporters leads to drastically different effects (growth or death). For example *Anabaena* sp. PCC 7120 wild type can utilize 200 mM fructose and to a much lesser extent 200 mM glucose for mixotrophic growth, while 200 mM fructose and even 5 mM glucose are toxic for PCC 7120 mutant strain containing the *gtr* gene from *Synechocystis* sp. PCC 6803 on a stably self-replicating plasmid [11].

The glucose carrier Gtr from *Synechocystis* sp. PCC 6803 has been identified as interacting with glucose and to a lesser extent with fructose [8]. Inactivation of the *gtr* gene in PCC 6803 abolished both the wild type's capacity for photoheterotrophic growth and light-activated chemoheterotrophic growth on glucose and its sensitivity towards fructose [8, 9, 22]. In contrast to the PCC 6803 wild type, which was even a little sensitive against its only known heterotrophic substrate glucose under mixotrophic conditions [8], no such characteristics could be observed for fructose on strain PCC 6803 *gtr*$^-$. Concentrations of fructose, which supported photoheterotrophy (Fig. 2), also allowed mixotrophy in PCC 6803 *gtr*$^-$ (Fig. 1).

Photoheterotrophic growth is due to fructose as carbon source

To rule out the possibility that any contaminant of commercially available fructose is the source for the growth of *Synechocystis* sp. PCC 6803 *gtr*$^-$ the fructose used was analysed by HPLC/ESI–MS. The data revealed that the fructose used contained a contamination of 0.16% glucose. Beside glucose no further organic contaminants in significant amounts were found.

To exclude the possibility that photoheterotrophic growth is supported by the detected glucose contamination the increase of the total carbon mass in the cells during photoheterotrophic growth was measured applying elemental analysis. Several cultures of *Synechocystis* sp. PCC 6803 *gtr*$^-$ were inoculated with the same density in pure BG11T medium. After they had reached an OD_{730} between 0.8 and 0.9 200 mM fructose and DCMU were added. For half of these cultures the absolute carbon amount present in the cells was determined immediately after fructose/DCMU had been added, while the other cultures grew further under photoheterotrophic conditions. The carbon amount present in their cells was measured 10 days later when they had reached an OD_{730} between 4.1 and 4.2 (for details see "Experimental"). The dry masses of each culture, the % and the absolute mass of carbon are listed in Table 1.

The amount of carbon incorporated into the cells during 10 days of photoheterotrophic growth can be calculated as the difference between the carbon present in the cells immediately after the addition of fructose and DCMU (4.19 mg) and the carbon present in the cells after 10 days (15.77 mg), which means that 11.58 mg carbon must have been incorporated from the surrounding medium. The 200 mM fructose present in 50 cm^3 medium contains 60 mmoles or 720 mg carbon, which are much more than the incorporated 11.58 mg. The glucose added as contaminant in each culture contains 0.096 mmoles or 1.152 mg carbon, while the BG11 medium [3] also contains a total of 0.0538 mM citric acid + citrate, which corresponds to 0.016 mmoles or 0.194 mg carbon in the photoheterotrophic cultures. Together, the glucose contamination, the citric acid and the citrate contain 1.346 mg carbon in the surrounding medium and therefore cannot supply the 11.58 mg carbon incorporated into the cells. As a consequence the photoheterotrophic growth must be due to fructose.

Table 1 Analysis of carbon mass of *Synechocystis* sp. PCC 6803 *gtr*$^-$ cultures in response to DCMU and fructose: each experiment was performed three independent times. The average values and standard deviations were calculated

	Mass of pellets after drying/mg	% of carbon	Absolute mass of carbon/mg
Synechocystis sp. PCC 6803 *gtr*$^-$ cultures harvested and washed at $OD_{730}=0.8$ immediately after addition of fructose and DCMU	8.45 ± 0.86	49.53 ± 0.65	4.19 ± 0.46
Synechocystis sp. PCC 6803 *gtr*$^-$ cultures harvested and washed 10 days after the addition of fructose and DCMU	33.41 ± 3.24	47.14 ± 0.41	15.77 ± 1.42

Conclusion

Despite having been classified as a strict photolithoautotroph for decades, *Synechocystis* sp. PCC 6803 *gtr*⁻ can use fructose for mixotrophic as well as for photoheterotrophic growth and fructose is able to enter the cells of this mutant strain. Combining these results with previous research about *Anabaena* sp. PCC 7120 we believe that more cyanobacteria than hitherto believed are facultative (photo)heterotrophs and among these strains fructose may be a widespread substrate.

Experimental

Cyanobacterial strains used

- *Synechocystis* sp. PCC 6803 *gtr*⁻ [7]
- *Synechocystis* sp. PCC 6803 wild type

Cultivation of the strains

PCC 6803 *gtr*⁻ was cultivated either in 50 cm^3 liquid BG11 medium [3] supplemented with 10 mM 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (TES) in 100 cm^3 Erlenmeyer flasks or on solid plates of 20–30 cm^3 BG11 supplemented with 10 mM TES, 3 g/dm^3 $Na_2S_2O_3 \cdot 5\ H_2O$ and 1.5% Difco bacto-agar in petri dishes. The liquid cultures were shaken at 150 rpm, 32 °C and an average illumination of 50 µE m^{-2} s^{-1} in a New Brunswick shaker 2300. The solid cultures were incubated in a Heraeus Vötsch climat chamber at 32 °C. When necessary, 20 $µg/cm^3$ of kanamycin was added to liquid or solid cultures. Photoheterotrophic growth was tested in the presence of 10 µM DCMU and varying concentrations of glucose or fructose. The capacity for mixotrophic growth was tested with glucose or fructose in the absence of DCMU. For experiments concerning chemoheterotrophic growth the Erlenmeyer flasks containing the cultures were wrapped with aluminum foils to prevent exposure to light.

Measurements of growth

Growth was determined by measuring the OD_{730} as well as the chlorophyll concentration/cm^3 culture. For the latter 1 cm^3 culture was centrifuged for 10 min at 14,000 rpm. The supernatant was removed and the pellet was resuspended in 1 cm^3 methanol. After centrifugation for 10 min at 14,000 rpm the OD_{665} of the supernatant was determined. To obtain µg chlorophyll/cm^3 culture the OD_{665} values were divided by 0.0745 [23]. Every day samples were taken. All experiments were performed in triplicate and the average values and the standard deviations were calculated.

Measurement of sugar uptake into the cells of *Synechocystis* sp. PCC 6803 *gtr*⁻

Cells were cultivated under photolithoautotrophic conditions until they had reached an $OD_{730} \sim 1.0$. They were centrifuged for 10 min at 4000 rpm. The supernatant was removed and the pellet was washed in BG11T medium and centrifuged again for 10 min at 4000 rpm. Then the cells were resuspended in BG11T medium to an $OD_{730} = 1.10$ cm^3 of this suspension were transferred into a cell chamber, where they were rotated by a magnetic stirrer and permanently illuminated. After an incubation phase of 10 min the required amount of stable glucose or fructose and 50 nCi of uniformly labeled ^{14}C-D-glucose or uniformly labeled ^{14}C-D-fructose from Hartmann Analytic (Braunschweig, Germany) were added. In regular intervals 1 cm^3 samples were taken and transferred onto a Whatman nitrocellulose filter (25 mm diameter, pore size 0.45 µm) connected to an oil vacuum pump apparatus. The supernatant was sucked off and the filter with the attached cells was washed immediately afterwards with 5 cm^3 BG11T medium. Then the filter was transferred into a scintillation tube and dissolved in 10 cm^3 scintillation cocktail (60 g naphthalene and 4 g 2,5-diphenyl-1,3,4-oxadiazol dissolved in 1 dm^3 dioxan). As a negative control 10 cm^3 scintillation cocktail without a sample were measured. For determination of the total radioactivity 1 cm^3 unfiltrated suspension was mixed with 10 cm^3 scintillation cocktail in a tube. The samples were evaluated in a Perkin-Elmer Tri-Carb 2800 TR liquid scintillation counter. All experiments were performed in triplicate and the average values and the standard deviations were calculated.

HPLC instrumentation and chromatographic conditions for HPLC/ESI–MS analysis of the fructose reagent

HPLC/ESI–MS analysis was performed on a Dionex HPCL Ultimate 3000 system equipped with LPG3400A low-pressure gradient pump, WPS-3000SL autosampler, TCC3000 thermostatted column compartment and VWD-3400 variable wavelength detector. Separation was performed on a Phenomenex (Torrance, California, United States) column, Rezex RCM-Monosaccharide Ca^{2+}, 300 × 7.80 mm, particle size 8 µm.

The mobile phase was 100% water (VWR Chemicals, manufactured by: VWR international S.A.S. Fontenay-sous-Bois Cedex, France) at a flow rate of 0.6 cm^3/min with a split-flow volume of 0.12 cm^3/min into the mass spectrometer. Run time was 30 min under isocratic conditions at 80 °C.

Fructose was dissolved in double-distilled water in a concentration of 100 µg/cm^3 and 20 mm^3 were injected. The separated sugars were detected by mass spectrometer instrument (maXis classic QqTOF, Bruker Daltonik, Bremen, Germany). The scan range was 50–350 Da at a scan rate of 1 Hz. Capillary voltage was 4500 V, nebular gas flow was 1.2 bar, dry gas flow was 6.0 dm^3/min N_2, and the transfer capillary temperature was at 180 °C.

Measurement of total carbon mass of a culture

Six 50 cm^3 cultures of PCC 6803 *gtr*$^-$ were inoculated at an OD_{730} of ~ 0.1 and grown to an OD_{730} between 0.8 and 0.9. Then 10 µM DCMU and 200 mM fructose were added. Three of the cultures were immediately harvested, while the other cultures were further grown. The latter were harvested after they had reached an OD_{730} ~ 4.2. When harvested the cultures were centrifuged for 10 min at room temperature. The pellets were resuspended in 50 cm^3 pure H_2O and centrifuged once more for 10 min at room temperature. The pellets were resuspended in 1 cm^3 pure H_2O and centrifuged once more for 10 min at room temperature. All supernatant was removed and the pellets were dried in the vacuum desiccator for 2 h. The amount of carbon in the dried pellet was determined by elemental analysis (see "Determination of the amount of carbon present in the cell"). The total carbon amount of each culture was calculated by mass of the pellet x % of carbon/100. Since all experiments have been performed in triplicate both the average and standard deviation were calculated.

Determination of the amount of carbon present in the cells

The amount of carbon was determined at the laboratory for microanalysis services at the faculty of chemistry of the University of Vienna. In short: the amount of carbon in the sample was determined using C, H, N, S analysis in a Eurovector EA 3000 CHNS-O Elemental Analyser (built 2009) with sulphanilamide as a standard substance. The samples were exposed to O_2 at 1000 °C. The resulting gases were transported through a gas chromatograph with high-purity helium as the carrier gas. The amount of CO_2 was determined using a thermal conducting detector (TCD).

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