Engineering retinal-based phototrophy via a complementary photosystem in Synechocystis sp. PCC6803

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Combining retinal-based and chlorophyll-based (oxygenic) photosynthesis: Proteorhodopsin expression increases growth rate and fitness of a ΔPSI-strain of *Synechocystis* sp. PCC6803

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Abstract:

Two retinal-based proton pumps, proteorhodopsin and *Gloeobacter* rhodopsin were expressed in a PSI-deletion strain of *Synechocystis* sp. PCC6803. Growth-rate measurements, competition experiments and physiological characterization of the rhodopsin-expressing strains, relative to the ΔPSI control strain, allow us to unambiguously conclude that the retinal-based proton pump proteorhodopsin can enhance the rate of photoheterotrophic growth of this *Synechocystis* derivative strain. In contrast, the *Gloeobacter* rhodopsin did not show this stimulatory effect despite the expression levels achieved. The latter rhodopsin did, however, strongly modulate levels of carotenoid synthesis in the transformed strain. The physiological characterization included measurements of the residual glucose concentration in the medium and analysis of oxygen uptake- and production rates, recorded with membrane-inlet mass spectrometry. The results obtained are consistent with the concept that the proton-pumping proteorhodopsin provides the cells with the additional capacity to generate proton motive force. For *Gloeobacter* rhodopsin this positive effect on the physiology of *Synechocystis* presumably is negated by negative consequences of the expression of this heterologously expressed protein. The inability of the proteorhodopsin-expressing ΔPSI strain of *Synechocystis* to grow photoautotrophically is most likely due to a kinetic, rather than a thermodynamic, limitation of its NADPH-dehydrogenase NDH-1 in its NADP+-reducing activity.

Key words:

Photosynthesis efficiency; microbial rhodopsin; glucose consumption; oxygen evolution; oxygen uptake; growth rate
Introduction

The urgent need to produce sustainable liquid energy carriers, and the concerns about the increasing CO$_2$ emission levels, have focused attention on the use of photosynthetic microorganisms, especially oxygenic photosynthetic microorganisms, as a remedy. Cyanobacteria and green algae can serve as efficient cell factories to produce renewable commodity chemicals, feedstock, and biofuels. To optimize such sustainability-oriented applications, organisms are required to convert solar energy with the highest possible efficiency. Multiple approaches have been explored to achieve this, such as: increasing the expression level of RuBisCO (22), truncation of light-harvesting antennae (23), modulation of the ratio of formation of ATP and NADPH (24, 25), and bypassing sink limitation (26). Yet, another widely proposed method is to expand the range of the effective spectrum of electromagnetic radiation that can be used for oxygenic photosynthesis. The most promising approach for this is to expand this spectrum into the far-red- or even infrared region, as oxygenic photosynthesis so far is largely limited to the use of photons with a wavelength ranging from 350 to 700 nm (17-19). The latter range covers only about half of the number of available photons from the sun that reach the surface of the earth (20).

It should be noted, though, that some solar energy conversion systems naturally exist that do function with the light of wavelengths > 700 nm. The best examples (29-31) are those based on chlorophyll $d$ (Chl $d$) (32, 33) and Chl $f$ (18, 34, 35), which capture photons in the range of 700 – 720 nm and 700 – 740 nm, respectively. However, for bacteriochlorophyll (37-39), variants are known, which even utilize light with a wavelength of up to 1100 nm (note that even photons of the latter wavelength have enough energy to drive photophosphorylation and phototrophic growth). For the Chl $d$-utilizing cyanobacterium Acaryochloris marina, it has been shown that it can indeed display a higher photosynthetic efficiency than a comparable Chl $a$-utilizing cyanobacterium (36). Furthermore, a recent publication reported the successful heterologous synthesis of Chl $f$ in the model cyanobacterium Synechococcus 7002 (35). The low level of production of the exogenous chlorophyll, however, presumably prevented the emergence of a corresponding phenotypic trait in energy metabolism.

Also in anoxygenic photosynthesis quantum yields can be very close to unity (280). Accordingly, to engineer an infrared-absorbing derivative of the cyano-
bacterium *Synechocystis*, introduction of an infrared-absorbing cyclic electron transfer system of an anoxyphototroph, as a substitute for PSI (28, 43), seems like the most promising approach. Of note is the fact that - for autotrophic growth - this will also require functioning of an alternative linear electron transfer chain to form NADPH with electrons liberated from water, composed of PSII and NDH-1 only (23). From the studies inspired by “thinking pink” (44), it is known that the many genes that are required to express an anoxygenic photosynthetic cyclic electron transfer system can be found in a superoperon (281) and can be trans-conjugated, be it presumably at low frequency (24).

Therefore, it is relevant to note that an alternative, much simpler, device is available, in the form of an infrared-absorbing derivative of proteorhodopsin (229), to substitute for the cyclic electron transfer system. Although natural infrared-absorbing retinal-based proton pumps have not been identified yet, the proteorhodopsin mutant PR-D212N/F234S (hereafter, PR-DNFS), reconstituted with the all-trans retinal analog 3-methylamino-16-nor-1,2,3,4-didehydroretinal (MMAR), has recently been reported to show a broad and complex absorbance band with a maximum around 740 nm. Surprisingly, it still retains appreciable proton pump activity under near-infrared illumination (*i.e*. with 730 nm LED) (229). In combination with the protocol that we have developed for functional expression of holo-proteorhodopsin in *Synechocystis* sp. PCC6803 (46) and the identification of a key gene that controls retinal synthesis in vivo in *Synechocystis* (chapter 5), it should become possible to introduce a functional near-infrared-absorbing proton pump into *Synechocystis*, by expressing the proteorhodopsin mutant (PR-DNFS) into a *Synechocystis* mutant that is deficient in retinal synthesis, and supplementing the cells with the retinal analog MMAR during cultivation, so as to achieve formation of functional far-red absorbing holo-protein.

Recently (28, 43), it has been proposed that the most convenient way to introduce such an infrared-absorbing proton pump into *Synechocystis* is to use a PSI-deletion strain (∆PSI strain). In such a ∆PSI strain visible light (350-700 nm) can be used by PSII to form plastoquinol and far-red light can be used to generate proton motive force. Reversal of NDH-1 activity, driven by the proton motive force in a ‘reversed electron transfer process’ may lead to the formation of NADPH under these conditions. This would minimize the competition for photons between the two light-dependent systems and ultimately allow biological exploitation of all available solar radiation in a single (engineered) organism.
Here we present the results of the first steps of this innovative alternative solution, \textit{i.e.} to provide \textit{Synechocystis} with an infrared-absorbing retinal-based proton pump. Such a retinal-based proton pumping system is easier to manipulate genetically and allows one to combine, as well as to modulate, each photosystem separately (45, 46). In this study, we investigated the physiological effect of expressing a bacterial rhodopsin that absorbs visible light (\textit{i.e.} proteorhodopsin (PR) and \textit{Gloeobacter} rhodopsin (GR)) on both photoautotrophic and photomixotrophic growth of a \textit{ΔPSI} strain of \textit{Synechocystis} sp. PCC6803. We report that it can be unequivocally concluded that the retinal-based proton pump proteorhodopsin is able to increase the growth rate of the \textit{ΔPSI} strain under selected conditions of photomixotrophic growth. In addition, growth rate, glucose consumption rate, rates of oxygen evolution and oxygen uptake, as well as absorption spectra were measured to explore the mechanism behind this growth-rate-enhancing effect. \textit{Gloeobacter} rhodopsin did not measurably enhance the growth rate of the \textit{ΔPSI} strain, but it did appreciably increase the absorption of this strain in the range from 400 to 500 nm, suggesting that its expression modulates carotenoid synthesis.

\textbf{Materials & Methods}

\textbf{Strains and growth conditions}

Strains of \textit{Escherichia coli} were routinely grown in lysogeny broth (LB) liquid medium at 37°C with shaking at 200 rpm, or on solid LB plates containing 1.5\% (w/v) agar.

The \textit{ΔPSI}-derivative of \textit{Synechocystis} sp. PCC6803 (a glucose tolerant strain (282, 283) was routinely grown at 30°C with continuous illumination under red, green and blue light (RGB-light) at a total light intensity of 28.3 \(\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}\) (containing 3 \(\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}\) red, 25 \(\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}\) green, and approximately 0.3 \(\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}\) blue photons). The red, green and blue LEDs emitted maximally at 635 nm, 527 nm, and 459 nm, respectively. Liquid cultures were grown in BG-11 medium (Sigma-Aldrich), supplemented with 10 mM glucose, 50 mM Piperazine-N, N\textsuperscript{-}bis(3-propanesulfonic Acid) (PIPPS), pH 8.0, and appropriate antibiotics, and with constant shaking at 120 rpm (Innova 43, New Brunswick Scientific). The BG-11 agar plates were supplemented with 25 mM PIPPS (pH 8), 10 mM glucose, 0.3\% (w/v) sodium thiosulfate, and 1.5\% (w/v) agar.
Where appropriate, the antibiotics were added to a final concentration of 25 - 50 μg/ml (kanamycin) or 35 μg/ml (chloramphenicol), either separately or in combination.

**Conjugation**

Plasmids were transferred to the ∆PSI Synechocystis strain via tri-parental mating as described before (46). These plasmids included pQC012 (for expression of His-GR; chapter 4); pQC006 (for expression of His-PR) (46) and plasmid pJBS1312 ('empty' plasmid control; (46)). The presence of the plasmids was confirmed by PCR after the conjugation procedure.

**Effect of expression of a bacterial rhodopsin on phototrophic growth of the ∆PSI strain of Synechocystis**

Pre-cultures of the tested strains were grown photomixotrophically as described above (see: Strains and growth conditions). To start an experiment, an identical number of cells of each strain, taken from cultures growing exponentially, were harvested and washed three times with fresh medium, and then inoculated into three 10-ml cultures for each strain in triplicate. To compare the growth under photomixotrophic growth, the medium was supplemented with 10 mM glucose. While carrying out an experiment under photoautotrophic growth, the medium had no additional glucose.

Growth was monitored via cell density by measuring the OD$_{730}$ of a small volume (150 μl) sample from each culture in a SPECTROstar Nano Microplate Photometer (BMG LABTECH GmbH, Germany), as well as via the number of cells per ml as determined with a Casy 1 Model TTC cell counter (Schärfe System GmbH, Reutlingen, Germany) with a 60 μm diameter capillary.

**Measurement of glucose content**

The samples for analysis of glucose content were collected from the three cultures that were used to compare the rate of photomixotrophic growth. Concurrently with the OD measurements, 100 μl of each culture was harvested for quantification of the residual glucose content of the medium. Cells were removed by filtration with Ultrafree-MC filters (MILLIPORE, U.S.A) via centrifugation for 2 min at 14,000 rpm at 4°C. Glucose content in the resulting supernatant was determined using the D-Fructose/D-Glucose Assay Kit (Megazyme, U.S.A), in 96-well plates with a microplate photometer. This
measurement was carried out according to the manufacturer’s instructions at 30°C in a SPECTROstar Nano Microplate Photometer (BMG LABTECH GmbH, Germany).

The rate of glucose consumption was calculated in terms of micromoles of glucose consumed per $10^9$ cells per hour. As the dynamics of glucose consumption and cell proliferation change along with the residual glucose content, we calculated the glucose consumption rate for each specific time window (i.e. between $t_{n-1}$ and $t_n$; $t$ corresponds to the time of measurement, $n$ refers to the number of measurements). In each time window, the amount of consumed glucose was calculated from the decrease in glucose content per ml culture, while the number of cells was taken as the average number of cells per ml measured at $t_{n-1}$ and $t_n$.

**Growth competition**

To start a competition experiment, cells of two strains: ΔPSI Synechocystis containing the ‘empty’ plasmid (pJBS1312) and ΔPSI Synechocystis expressing a microbial rhodopsin (PR-His or GR-His, expressed from plasmid pQC006 and plasmid pQC012, respectively), both in the exponential- or early linear growth phase, were harvested and washed three times with fresh BG-11 medium supplemented with 10 mM glucose. Then an identical number of cells from each strain was mixed together and inoculated into 10 ml of medium. The experiment was carried out in BG-11 medium supplemented with 10 mM glucose at an illumination of 28 μmol • m$^{-2}$ • s$^{-1}$ RGB light. The growth of mixed culture was monitored via cell density by measuring the OD$_{730}$ in WPA Lightwave II spectrophotometry (Biochrom, UK). An aliquot of the culture was diluted to an OD$_{730}$ of 0.1 in 10 ml of fresh medium every two days, in order to maintain exponential growth. This dilution process was executed 8 times. During the experiment, 1 ml of culture was removed every day for cell density measurement and the abundance of the rhodopsin gene (i.e. PR or GR) was measured with PCR. Accordingly, in a period of 16 days, the culture was diluted 1000 times. Then 10 µl of the diluted culture was plated on a BG-11 plate (supplemented with 10 mM glucose). The plates were incubated at 5 μmol • m$^{-2}$ • s$^{-1}$ white light at 30 °C. The colonies that formed were used to determine the -relative abundance of each strain by identification through colony PCR. Each experiment was carried out with three completely independent biological replicates.
Analysis of the growth-competition experiments by PCR

To analyze the outcome of a competition experiment between a strain expressing bacterial rhodopsin (i.e. PR or GR) and the strain carrying the 'empty' plasmid, the number of each type of cells in the mixed culture was assayed by quantifying the relative abundance of the gene encoding PR or GR by quantitative PCR, relative to a corresponding fragment from the 'empty' plasmid (see further below). To construct a calibration curve, standards were prepared by mixing the cells of two strains (i.e. ΔPSI expressing PR or GR, and ΔPSI carrying the 'empty' plasmid) in a series of known ratios, varying from 0 to 100% of PR- or GR-expressing cells, in steps of 10%. Equal numbers of cells of each sample were added to a PCR reaction mixture as the template, and primers JBS315/JBS316, which specifically bind to the plasmid backbone, rather than to the PR or GR open reading frame, were used for PCR amplification (Table S1).

Cells in samples harvested from a competition experiment were washed three times and were re-suspended in Mill-Q water. The number of cells in each sample was determined with a Casy counter. For proper comparison, the parameters of the PCR reactions, i.e. the amount of template ($1.82 \times 10^5$ cells), the number of amplification cycles (35 cycles), annealing temperature (55 °C), and the extension time (45 seconds) were optimized and kept constant throughout all analyses. During the entire analysis, including the PCR reactions, the agarose gel electrophoresis and the staining procedure, standards and samples were run in parallel.

Amplified product from the 'empty' plasmid (pJBS1312), from the PR-His encoding plasmid (pQC006) and from the GR-His encoding plasmid (pQC012), had a size of 578 bps, 1352 bps and 1493 bps, respectively. After staining the PCR products with ethidium bromide, the intensity of each band was measured using ImageJ v1.49a (W.S. Rasband, U.S. National Institutes of Health, http://imagej.nih.gov/ij/) by taking the total intensity of a band area corrected for the background intensity of an adjacent area of equal size. The band-intensity of a PCR fraction of cells expressing PR-His in a particular standard mixture was plotted against the abundance of the particular strain in that fraction that was used to generate a calibration curve.
Membrane-inlet mass spectrometry

Rates of net oxygen production and oxygen consumption were measured by membrane-inlet mass spectrometry (MIMS), essentially as described in (284), via the concentration of the oxygen isotopes $^{32}$O$_2$ and $^{36}$O$_2$, respectively. In short, MIMS measurements were performed in a 10-ml air-tight cuvette containing a Synechocystis culture, at a cell density ($OD_{730}$) of 0.4 for wild type Synechocystis and 1.0 for ΔPSI Synechocystis. Prior to the experiment, the culture was dark adapted for 30 minutes and then briefly (~ 10 sec) flushed with N$_2$ to lower the prevalent O$_2$ concentration. Subsequently, the cuvette was closed and $^{36}$O$_2$ was added into the reaction cuvette. The samples were illuminated with orange-red light (640 nm) or green light (535 nm) at intensities ranging from 0 to 200 μmol • m$^{-2}$ • s$^{-1}$. Cells were subjected to illumination with a low light intensity of 5 μmol • m$^{-2}$ • s$^{-1}$ for 10 minutes to assure light adaptation and then to all subsequent light intensities for 3 minutes. After testing the different light intensities, dark respiration was measured during a period of 3 minutes. The raw signal of oxygen concentrations derived from MIMS measurements has been corrected for oxygen consumption by the mass spectrometer itself, via normalization to the Argon signal.

Cells from the exponential growth phase were collected and re-suspended in fresh medium supplemented with 25 mM bicarbonate, with and without the addition of 2 mM glucose for the ΔPSI and WT strain, respectively. To measure the PSII-dependent oxygen evolution in the ΔPSI strain during photoautotrophic growth, cells grown in a pre-culture in BG-11 medium plus 10 mM glucose were washed and then re-suspended in fresh medium without additional glucose. This culture was grown photoautotrophically for 24 hours prior to measurements.

UV/Vis Absorption spectroscopy

Cells of the strain of interest were harvested, washed three times with, and then re-suspended in, fresh BG-11 medium supplemented with 10 mM glucose. Absorption spectra of intact cells were measured with a SPECORD® 210 PLUS spectrophotometer (Analytik Jena, Germany) to minimize the contribution of light scattering. The spectra were normalized based on their light scattering in the range of 730 – 850 nm.
### Table 1: Strains or plasmids constructed for this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristicsa</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>Synechocystis</em> sp. PCC6803</td>
<td></td>
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<tr>
<td>PSI-less</td>
<td>CamR; ΔpsaAB:: CmR; a PSI deletion strain derived from glucose tolerant <em>Synechocystis</em> sp. PCC6803</td>
<td>(23)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
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<tr>
<td>XL1-Blue</td>
<td>Cloning host</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>J53/RP4</td>
<td>Helper strain</td>
<td>(24, 25);</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pJBS1312</td>
<td>kanR; expression vector, pVZ321 origin, P<em>psbA2</em></td>
<td>(22)</td>
</tr>
<tr>
<td>pQC006</td>
<td>kanR; pJBS1312-based expression of PR, C-terminal 6×histine tagged</td>
<td>(22)</td>
</tr>
<tr>
<td>pQC012</td>
<td>kanR; pJBS1312-based expression of GR, C-terminal 6×histine tagged</td>
<td>(22)</td>
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a Ω is short for the omega resistance cassette; CmR represents the chloramphenicol resistance; while AmpR means the ampicillin resistance cassette; KanR for kanamycin resistance; SpcR for spectinomycin resistance and StrR for streptomycin resistance.
Results

Effect of bacterial rhodopsin expression on photomixotrophic growth of a ΔPSI Synechocystis

We have tested the separate contribution of PR and GR to energy metabolism and growth of the ΔPSI Synechocystis strain in an attempt to clearly demonstrate the potential effect of retinal-based photosynthesis in a (chlorophyll-containing) oxyphototroph (46). As a first step, we compared how much each of these two bacterial rhodopsins can contribute towards increasing the growth rate of the ΔPSI strain in batch culture in Erlenmeyer shake flasks. To exclude an effect of the expression plasmid, we compared the growth rate of three strains: a strain expressing GR (ΔPSI + pQC012), another expressing PR (ΔPSI + pQC006), and a control strain containing the ‘empty’ plasmid (ΔPSI + pJBS1312). We deliberately supplied lower incident light intensity of red and blue light (a combined intensity of ~3 μmol • m⁻² • s⁻¹, as the ΔPSI strain is rather sensitive to inhibition by light (283)), in combination with relatively intense green light (~25 μmol • m⁻² • s⁻¹), as light of this color (λmax = 527 nm with 34 nm full width at half maximum) can activate proton pumping in PR and GR, and is poorly absorbed by the photosynthetic apparatus of cyanobacteria (27). Growth was monitored via cell density (i.e. OD₇₃₀) and the number of cells per ml (Fig. 1).

The PR expressing strain (ΔPSI + pQC006) had an appreciably higher growth rate than the strain containing the ‘empty’ plasmid (ΔPSI + pJBS1312), both judged from the slope of Log (OD₇₃₀) versus time as well as in the plot of the log of the number of cells per ml versus time. (Fig.1, panels A and B, respectively). Calculated growth rates based on cell density from the relevant time window (from 19.6 to 44.5 hours) suggest that expressing PR allows the ΔPSI strain a growth advantage of approximately 30%. The same trend was also observed when it comes to direct cell count (a more direct measure of cell proliferation). The PR-expressing strain (ΔPSI + pQC006) started with an OD₇₃₀ that was 0.03 lower than the ‘empty’ plasmid containing strain (ΔPSI + pJBS1312), but because of its faster growth, it ended up with a slightly higher OD₇₃₀ than the strain containing the ‘empty’ plasmid (0.8 and 0.74, respectively) prior to entry into the stationary phase.

However, contrary to PR, the GR-expressing strain (ΔPSI + pQC012) initially grew appreciably slower than the strain containing only the ‘empty’ plasmid during the first 95.2 hours of cultivation. Just before entering stationary phase
this strain overtook the strain containing ‘empty’ plasmid (ΔPSI + pJBS1312), generating a slightly higher cell yield than the ‘empty’ plasmid strain (1.95×10^8 and 1.56×10^6 cells per ml culture, respectively). We did not observe, however, any stimulatory effect of GR expression on the maximal growth rate of the PSI-deletion strain.

Beyond that, we observed that cells of the ‘empty’ plasmid strain (ΔPSI + pJBS1312) consistently maintained a larger cell size than the rhodopsin expressing strains (PR-His or GR-His, refers to strain ΔPSI + pQC006 and ΔPSI + pQC012, respectively) during all stages of growth (Fig. 1C). The difference in cell size likely explains why, at the zero-time point, the OD_730 (reflecting cell scattering) of the strain containing the ‘empty’ plasmid (ΔPSI + pJBS1312) was higher than that of the PR- or GR-expressing strain, although all the cultures contained the same number of cells. Remarkably, this result is opposite to our finding in wild-type Synechocystis (WT), for which we previously reported that the PR expressing cells have a larger size than the ‘empty’ plasmid containing WT strain. This apparent discrepancy could be a result of glucose metabolism (see further Discussion).

Figure 1: PR-stimulated growth in ΔPSI Synechocystis strains. Growth comparison among three strains: ΔPSI Synechocystis with: the empty plasmid pJBS1312 (triangles), plasmid pQC006 that leads to expression of PR-His (circles), or plasmid pQC012 for expression of GR-His (squares). All strains were grown in BG-11 medium supplemented with 10 mM glucose. Error bars represent the standard deviation of biological replicates within the experiment (n = 3) and are only visible when they exceed the size of the symbols. Time point zero indicates the time of inoculation of the cultures from a pre-culture growing linearly in the same medium. Cell density at 730 nm (OD_{730}; A); the number of cells per ml (B); and mean diameter (C); the last two characteristics were measured with a Casy Counter.
Effect of proteorhodopsin expression on photoautotrophic growth of the ΔPSI Synechocystis

A recent paper (43) proposes that in the ΔPSI strain, electrons liberated by PSII could flow through the PQ pool and NDH-1, to lead to the formation of NAD(P)H, be it that this presumably would cost some proton motive force (based on the proton translocation stoichiometries of the electron transfer chain complexes involved (i.e. PSII and NDH-1); see further: Discussion). Because expression of PR provides the cells with an additional pathway for proton motive force generation, this could be expected to allow photoautotrophic growth of the ΔPSI strain.

To test this hypothesis, cells of the PR-expressing ΔPSI strain (ΔPSI + pQC006) and the 'empty' plasmid containing strain (ΔPSI + pJBS1312) were tested for their capacity to grow photoautotrophically with 28.3 μmol • m⁻² • s⁻¹ RGB light. The results obtained show a small but clear increase in both cell number and optical density for the PR-expressing strain (ΔPSI + pQC006)
during the initial phase of the experiment, but a smaller increase in cell number and a constant optical density for the strain containing the 'empty' plasmid (ΔPSI + pJBS1312) (Fig.2 panels A and B). Regarding cell size, both strains showed a continuously decreasing trend. But still, cells of the 'empty' plasmid strain (ΔPSI + pJBS1312) were appreciably larger than the PR-expressing strain (ΔPSI + pQC006) (Fig.2C). All in all, our data confirm that the ΔPSI strain of *Synechocystis* has lost the capacity for sustained photoautotrophic growth. Expression of PR seems initially to enhance its growth in terms of both optical density and number of cells, but this stimulatory effect was only observed in the initial stage (roughly 24 hours) of the experiment. This may be due to continuing cell division of the (large) cells pre-grown with glucose (see further Discussion).

**Competition based on growth rate between strains with and without expression of a bacterial rhodopsin**

To further characterize the growth advantage provided by expression of a bacterial rhodopsin in the ΔPSI derivative of *Synechocystis* sp. PCC6803, we designed a growth competition experiment (Fig. 3), for a more direct comparison. In these competition experiments, the abundance of the gene encoding PR increases from approximately 30%, to gradually and hyperbolically reaching more than 95% after 16 days in the growth conditions tested (Fig. 3A). If sufficient time would have been available, the PR-expressing strain (i.e. ΔPSI + pQC006) would have fully out-competed the control strain. From the progress of this displacement, and estimating that the growth rate of the control strain (i.e. (ΔPSI + pJBS1312)) is 0.035 h⁻¹ under the conditions selected, one can calculate that the PR-expressing strain grows about 20% faster (i.e. at 0.042 h⁻¹) under these conditions than the control strain. A colony PCR analysis showed that all the single colonies obtained from samples taken on the final (16th) day of the experiment were cells carrying the gene encoding PR (40 colonies per plate were tested, with three biological replicates), thus confirming the expectation that the PR-expressing strain will completely take over the culture.

In contrast, growth competition between the GR-expressing strain (ΔPSI + pQC012) and the same control strain (i.e. ‘empty’ plasmid containing strain (ΔPSI + pJBS1312)) shows that the abundance of gene encoding GR continued to fluctuate around 60% through the entire experiment (Fig.3B). PCR analysis of 120 single colonies, derived from samples taken after the final dilution, showed that 58% of the colonies (40 colonies per plate tested, with three
biological replicates) carried the GR-encoding gene. These results show that expression of GR did not bring a detectable stimulatory growth advantage to the ΔPSI strain under the conditions selected.

Figure 3: The competition for growth rate between rhodopsin expressing ΔPSI Synechocystis strains relative to ‘empty’ plasmid carrying strain. A): The relative fraction of the gene encoding PR during the competition experiment, quantitated using PCR. B): The relative fraction of the gene encoding GR during the competition experiment, quantitated using PCR. Growth was monitored via cell density at 730 nm (OD$_{730}$, gray). Error bars represent the standard deviation of biological replicates within the experiment (n = 3), and are only visible when they exceed the size of the symbols. Time point zero indicates the time of inoculation of the cultures from a pre-culture growing linearly in the same medium.

Assay of the residual glucose content in spent medium

During photomixotrophic growth of ΔPSI Synechocystis, the glucose most likely serves both as a carbon source and as an energy source. When growing in green light the PR-expressing strain (ΔPSI + pQC006), due to its unique capacity to generate extra proton motive force/ATP with green illumination, would be able to convert more glucose into biomass, while the ‘empty’ plasmid containing control strain (ΔPSI + pJBS1312) will probably oxidize more glucose through respiration to produce the necessary ATP. Accordingly, the PR-expressing strain (ΔPSI + pQC006) is expected to show a lower rate of glucose consumption during growth.

To test this hypothesis, we monitored the glucose consumption of each strain by measuring the residual extracellular glucose content during photomixotrophic growth in a batch culture. From the results obtained (Fig. 4) it is clear that the glucose content decreased measurably faster in cultures of the ‘empty’ plasmid containing strain (ΔPSI + pJBS1312) than in the PR-expressing strain (ΔPSI + pQC006), although after 92.5 hours the glucose was exhausted in both cultures. Surprisingly, the glucose content in the culture of the GR-ex-
pressing strain (ΔPSI + pQC012) was consumed even slower. If one assumes that during this photomixotrophic growth the three strains consume the same amount of CO$_2$, this implies that expression of the bacterial rhodopsin indeed increases the cell yield on glucose.

![Graph](image)

Figure 4: Kinetics of glucose consumption of the three strains studied in the analysis of growth rates (Fig. 1). The residual glucose concentration in the culture was measured using a glucose-content determination kit. Panel A shows the dynamics of the remaining concentration of glucose in the spent medium of the three strains. Panels B and C show the glucose consumption per $10^9$ cell per hour, and the glucose consumption per OD$_{730}$ per hour, respectively. Error bars represent the standard deviation of biological replicates within the experiment (n = 3) and are only visible when they exceed the size of the symbols. Time point zero indicates the inoculation of the cultures from a pre-culture growing linearly in the same medium.

Comparison of the cell-specific (Fig. 4B) and the biomass-specific (i.e. based on OD$_{730}$; Fig. 4C) glucose consumption rate among the three strains shows that the ‘empty’ plasmid containing strain (ΔPSI + pJBS1312) and the GR-expressing strain (ΔPSI + pQC012) have similar glucose consumption rates (~ 2 µmol per $10^9$ cells per hour), while the PR-expressing strain (ΔPSI + pQC006) consumed glucose much slower, at a rate varying from 0.7 to 1.8 µmol per $10^9$ cells per hour. Intriguingly, the glucose consumption rate of the PR-expressing strain showed a low rate initially (≤ 0.9 µmol per $10^9$ cells per hour) up until ~50 hours, which then gradually increased to 1.8 µmol per $10^9$ cells per hour as cells entered stationary phase at around 90 hours (compare Fig. 1A). This
finding is consistent with the PR expression pattern in WT *Synechocystis*, which showed that the PR expression level sharply decreased when cells enter the stationary phase (46). Furthermore, it suggests that PR particularly contributes towards energy conversion of cells during the exponential growth phase.

**Analysis of oxygen metabolism with membrane-inlet mass spectrometry (MIMS)**

The signals that can directly be derived from MIMS measurements are the change in the concentration of 32O2 and 36O2 as a function of time. If a reaction is initiated with the only H216O present, any increase in the concentration of 32O2 directly reflects the rate of oxygen evolution by PSII. If simultaneous oxygen consumption takes place, the observed rate of oxygen production has to be increased with the simultaneous rate of oxygen consumption. The latter can be derived from the rate of 36O2 oxygen consumption, *i.e.* when the two oxygen isotopes are present at equal concentrations, much higher than the Km of the oxygen consuming enzymes (like respiratory oxidases, the Flv1/3 proteins, Rubisco, etc.).

In an earlier study (284), we have used MIMS to characterize oxygen metabolism in WT *Synechocystis* and two of its mutants impaired in oxygen metabolism. In that study, we used red light (659 nm) to initiate oxygen evolution. As cyanobacteria contain a multitude of pigments and hence respond differently to different light regimes (*i.e.* (29) and V.M. Luimstra, P. van Alphen, et al., unpublished observations), we first compared the response of the WT strain, with and without expression of PR, with respect to its response to orange (640 nm) and green (535 nm) light (Fig. 5A and 5 B). The results of our MIMS measurements show that low-intensity orange-red light (≤ 25 μmol • m−2 • s−1) mediated a decrease in oxygen uptake rate in *Synechocystis*, as compared to the rate of dark respiration, which is comparable to the results of our previous study (284). Moreover, we observed the Mehler-like reaction (30) only under orange-red light at intensities ≥75 μmol • m−2 • s−1, but not with a green light up to 200 μmol • m−2 • s−1. This implies that green light, even at 200 μmol • m−2 • s−1, did not trigger the high-light induced Mehler-like reaction (285). The rate of light-driven oxygen evolution-saturated between 100 and 150 μmol • m−2 • s−1 of orange-red light and was almost 3 fold faster than with green light: 200 μmol • m−2 • s−1 of green light and 75 μmol • m−2 • s−1 of orange-red light mediate roughly the same oxygen evolution rate of 10 μmol O2 per 109 cells per hour.
Comparison of the WT strain with and without PR expression shows that with orange-red actinic light, as expected, there is no appreciably observable difference, because the light of this wavelength is poorly absorbed by this bacterial rhodopsin (229). Increasing intensities of green light increasingly reduce light-induced oxygen evolution (up to about 30%) in the PR-expressing strain (WT+ pQC006). This decrease presumably is a result of the increased proton motive force generated by PR, which will particularly inhibit electron transfer through the cytochrome b6/f complex (see further: Discussion). In contrast, the rate of oxygen uptake of the PR-expressing strain is not inhibited by green light, and very close to zero when subjected to any of the tested light intensities. Thus, Figs. 5A and 5B also allow the conclusion that PR expression by itself does not affect oxygen uptake in WT *Synechocystis* under phototrophic growth conditions.

The measurements on the ΔPSI strain show that not only during photomixotrophic growth (Figs 5C and 5D), but also under photoautotrophic conditions, *i.e.* upon glucose starvation (Figs. 5E and 5F), the ΔPSI strain of *Synechocystis* retains some capacity to evolve oxygen (about 30% of wild-type *Synechocystis*), and this ability is saturated at 75 μmol • m$^{-2}$ • s$^{-1}$ of green light and 25 μmol • m$^{-2}$ • s$^{-1}$ of orange-red light, under photomixotrophic growth. Higher intensities of green light (*i.e.* > 100 μmol • m$^{-2}$ • s$^{-1}$) and orange-red light (> 75 μmol • m$^{-2}$ • s$^{-1}$) were saturating under photoautotrophic conditions and saturation levels are about half of those in the presence of glucose. Moreover, we did not observe the Kok-effect (*i.e.* light-inhibited respiration (286)) in the ΔPSI strain under illumination with either green light or orange-red light, presumably because of the inability of this strain to channel electrons to PSI, prior to transfer to an oxidase.

In agreement with observations on the wild-type strain, an appreciable difference caused by PR expression in the ΔPSI strain only occurred upon illumination with green light, and not with orange-red light. Under photomixotrophic conditions, the PR-expressing strain (ΔPSI + pQC006) had an appreciably lower rate of oxygen uptake (from 30 to 50% less, dependent on light intensity), as well as a lower oxygen evolution rate (30% to 40% lower) than the corresponding strain containing the ‘empty’ plasmid (*i.e.* ΔPSI + pJBS1312; Fig. 5D). This is the clearest indication that PR generates proton motive force over the thylakoid membrane and accordingly inhibits electron transfer driven by: (i) respiratory electron transfer to cytochrome c oxidase (see: Discussion) and (ii) PSII dependent oxygen evolution.
Under photoautotrophic conditions, the small remaining rate of oxygen evolution, as well as uptake, is slightly inhibited in the PR-expressing ΔPSI strain, when illuminated by orange-red as well as green light, (Fig. 5E and F). This is not according to expectation, but can be caused by a slightly disturbing effect of PR expression on electron transfer in the thylakoid membranes of this strain.

Figure 5: Light-driven net oxygen production and consumption, as measured with a membrane-inlet mass spectrometer (MIMS), of wild type and ΔPSI *Synechocystis*, with and without PR expression. Strains carrying the empty plasmid pJBS1312 or plasmid pQC006 were compared for an analysis of the effect of PR-His expression. Oxygen exchange was measured with MIMS under the illumination with orange-red light (640 nm), or green light (535 nm) at intensities varying from 0 to 200 μmol photons m⁻² s⁻¹. Prior to measurements, the cells were washed once and re-suspended in fresh BG-11 medium supplemented with 25 mM NaHCO₃. Cells were dark adapted for 30 minutes prior to the experiment. WT/ HCO₃⁻: wild type *Synechocystis* grown with the addition of HCO₃⁻; ΔPSI/ glu: ΔPSI *Synechocystis* grown with the addition of glucose; ΔPSI/ HCO₃⁻: ΔPSI *Synechocystis* grown with the addition of HCO₃⁻; ○: Oxygen evolution of strain carrying empty plasmid pJBS1312; ▲: Oxygen evolution of strain carrying plasmid pQC006 (PR-His); ●: Oxygen uptake of a strain carrying empty plasmid pJBS1312; ■: Oxygen uptake of a strain carrying plasmid pQC006 (PR-His).
Absorption spectroscopy

To analyze the effect of expression of a bacterial rhodopsin on the spectral features of the ΔPSI strain, absorption spectra of intact cells were measured and plotted in the form of absolute and difference spectra (Fig. 6). The ΔPSI strain shows an appreciable decrease in absorption of its 680 and 440 nm bands because of the loss of a large fraction of its chlorophyll a, due to the deletion of the PSI core proteins. Hence the absolute spectra are dominated by the absorbance of the phycobilisomes (PBS), absorbing maximally at 625 nm. Expression of GR (and to a small extent of PR) caused a large decrease of absorption in this main absorbance band. Beyond that, the GR-expressing strain also showed increasing absorption in the spectral range from 400 to 550 nm (Fig. 6B). These results show that the GR-expressing strain (ΔPSI + pQC012) has a strongly increased carotenoid content. On basis of an expression level of $< 10^5$ rhodopsin molecules per cell in WT Synechocystis, this increase in carotenoid content is super-stoichiometric with the GR content of the cells. It is probably due to the unique ability of this particular rhodopsin to bind carotenoids (95, 101, 102), in particular, carotenoids containing a keto-group at the 4 position. The decreased absorption at 625 nm in both strains indicates a decrease in the level of the PBS pigments.

![Figure 6: Absorption spectra of ΔPSI Synechocystis equipped with a retinal based proton pump.](image)

Cells were grown under 3 μmol • m$^{-2}$ • s$^{-1}$ red/blue and 25 μmol • m$^{-2}$ • s$^{-1}$ green light in BG-11 media, supplemented with 10 mM glucose. Prior to measurement, cells were washed with, and then re-suspended in, fresh BG-11 medium. Absorption spectra were recorded by using a Specord spectrophotometer, and data was normalized at 730 nm. A) Absorption spectra; B) difference spectra of all strains against the ΔPSI +pJBS1312 strain.
Discussion

Growth advantage provided by PR expression

Our previous study (46) demonstrated that functional expression of PR in *Synechocystis* stimulates the growth rate of this organism, be it only with a few percent. To further increase the energy contribution from retinal based phototrophy, and to compare, which of the two available proton pumps (*i.e.* PR or GR) has higher efficacy in energy conversion, in this study we expressed PR and GR in a ΔPSI strain of *Synechocystis*, that suffers from severe energy limitation, because of this genetic lesion in light-dependent linear- and cyclic electron flow. In the ΔPSI strain, we observed that expressing PR brought a much higher stimulatory effect on growth rate (*i.e.* a 30% higher growth rate) than in wild-type *Synechocystis*. Unexpectedly, expressing GR in the ΔPSI strain showed no significant difference in growth rate as compared to the ΔPSI strain carrying the ‘empty’ plasmid. Presumably GR does contribute to energy conversion (Figs. 1 and 3), be it perhaps only with a few percent, *i.e.* appreciably less than PR, so that the benefit of its expression just compensates its energy cost plus possible deleterious effects that expression of this proton pump may have (see Figs. 1, 3, 4 and 6).

As GR was demonstrated to have a higher maximal pumping rate (two-fold higher than PR; (73, 79), chapter 4), and a unique ability to bind (an antenna) carotenoid (which increases its absorption cross section and thereby could further increase its pumping rate (95, 97, 101, 102)), and a high expression level (about $8 \times 10^4$ molecules per cell in WT *Synechocystis* (which corresponds to 80% of the PR expressing level in WT *Synechocystis*; chapter 4 (46)), one would expect a higher contribution to light-energy conversion from GR- than from PR-mediated proton pumping. Strikingly, however, the difference spectrum of a GR-expressing ΔPSI strain (ΔPSI+ pQC012) relative to an ‘empty’ plasmid-carrying strain (ΔPSI+ pJBS1312), reveals much higher absorption in the range of 400 to 550 nm, and a lower absorption peak at 625 nm (Fig. 6B), indicating a higher accumulation level of carotenoids and a decrease in PBS content, respectively. The latter effect was observed in both the GR- and the PR-expressing strain, presumably because expressing PR as well as GR occupies membrane space, which could lead to a decrease of the content of the endogenous photosystem(s). The content of PBS, as an antenna system of PSII, may decrease as a result of this. Furthermore, the GR-expressing strain shows an appreciably lower PBS content than the
PR-expressing strain, even though the former has a lower rhodopsin expression level. The oligomeric state of the expressed rhodopsin in vivo can play a crucial role in this as GR has an oligomeric state (i.e. trimers (238), chapter 4) different from PR (pentamers/hexamers (85)).

In view of the fact that GR has the capacity to bind a keto-carotenoid as antenna chromophore (both echinenone and hydroxy-echinenone can be bound; chapter 4), we propose here that GR may compete with, and take away, part of the keto-carotenoids from other keto-carotenoid binding proteins, which then may lead to overexpression of carotenoids.

Notably, additional characteristics of a proton pumping rhodopsin, such as its oligomeric state, pH-dependent pumping activity, as well as its voltage- and delta-pH dependent vectoriality (287-289), will also influence its function in vivo. As GR distinctly differs in such characteristics from PR, these differences could also contribute to the failure of GR in enhancing the growth rate of the ΔPSI strain of Synechocystis.

Cells of the PR-expressing ΔPSI strain have a smaller diameter than the ‘empty’ plasmid carrying ΔPSI strain (see Fig. 1C), which, intriguingly, is contrary to the finding that expression of PR in WT Synechocystis cells leads to an increase in cell size (46). Furthermore, cells of the ΔPSI strain (diameter ~ 3 µm) turn out to be larger than WT Synechocystis (~ 2 µm), when carrying the same plasmid. This suggests that glucose metabolism has a direct influence on cell size. Indeed, it has been reported that cells of WT Synechocystis show an increased cell size when grown photomixotrophically in the presence of glucose, an effect that was not observed in cells lacking the glucose uptake transporter GlcP (290). We have confirmed this cell-size observation with the wild type Synechocystis strain used in this study (data not shown). An increase in cell size can be caused i.e. by an increase in cytosolic osmolarity (290-292) and/or an increase in cytosolic alkalization (293, 294), due to glucose metabolism. The degree of swelling may increase with the glucose uptake and/or consumption rate. Cells of the ‘empty’ plasmid carrying ΔPSI strain, which have a higher glucose consumption rate (see Fig. 4), indeed did swell more than the PR expressing ΔPSI strain. This hypothesis also may explain why, with the depletion of glucose in the medium, the ΔPSI strain (both with and without expression of PR) showed a sharp decrease in cell size (see Fig.2C). It is relevant to note that the effect of PR expression on cell size in the WT strain is smaller than the effect of glucose, and presumably, is not related. Rather, it could be due to the general observation that application of stress
tends to increase the size of *Synechocystis* cells (295). In relation to this, it is worthwhile to note that deletion of PSI changes the structure of the thylakoid membrane from concentric sheets to a tubular assembly. Whether this could lead to an increase in cell size remains unknown (296). Additional factors like increasing growth rate would also lead to an increase in cell size (297), but presumably also via an unrelated mechanism, unless one would consider high growth rate also as a stress-inducing condition.

**The copy number of the plasmids is dependent on the specific heterologous gene expressed**

In the competition experiments, although we inoculated the same number of cells for each strain at the start of the experiment, the ratio of the number of plasmids in the test was not 50:50%; instead, it was 31:69% between pQC006 and pJBS1312 and 60:40% for pQC012 and pJBS1312. This indicates that the plasmid copy number of each plasmid is (slightly) different. The optimal plasmid copy number in bacterial cells for engineering purposes is a trade-off between a desired gene dosage effect and the corresponding metabolic burden. However, this copy number can be affected by many additional factors, *i.e.* the gene inserted, growth conditions and growth stage (298, 299). Although cells of all strains were harvested from the late-exponential/early linear growth phase in our experiment, it is reasonable to assume that the condition of the cells, as well as of the pre-culture, could be slightly different, especially in terms of glucose concentration and cellular metabolism. Thus, a slight deviation from the 50:50% ratio at the beginning of the experiment, of the number of the two types of plasmids over that of the corresponding cells is understandable. This deviation, however, does not interfere with the accuracy of our measurements, as we focus on the relative change in the ratio of the two plasmids during the entire course of the growth experiment. Furthermore, the colony test performed on the cells sampled at the end of the competition experiments, independently confirmed the outcome of the competition experiments.

We also carried out a competition experiment between the PR expressing strain (ΔPSI+ pQC006) and the ‘empty’ plasmid-carrying strain (ΔPSI+ pJBS1312) in a batch culture, which was grown continuously for 5 days without repeated dilution. The growth conditions such as medium composition, remaining glucose concentration, illumination conditions, *etc.*, were the same as in the competition experiment we report above. In this latter experiment, we observed that the fraction of the PR-expressing ΔPSI strain increased until the
culture entered the stationary phase (after the first 3 days), and then gradually decreased in the stationary phase (during the next two days). This phenomenon has been observed independently in two biological replicates. PCR analysis of cells of a monoculture of strain (ΔPSI+ pQC006) in the stationary phase showed two bands that correspond to PR-encoding plasmid pQC006 and ‘empty’ plasmid pJBS1312; sequencing of each band further verified this interpretation. These results indicate that a high frequency of loss of the inserted gene (i.e. a structural instability of the PR encoding gene) occurs in plasmid pQC006 when its host cells enter the stationary phase. Therefore, we cannot fully exclude the possibility that some cells (expressing PR or GR) harvested from a pre-culture already contained a small fraction of ‘empty’ plasmid. The mechanism behind this can be manifold (for a review see: (300)); presumably due to contribution from the metabolic burden of expressing PR, increased expression of recombination enzymes, or a structural instability of the plasmid. As the pumping activity of PR decreases with decreasing light intensity, the lower light intensity per OD$_{730}$ in the stationary phase may lower the positive selection for its maintenance. Therefore, the likelihood that this gene will be lost, increases. Moreover, because plasmid pQC006 contains the same restriction site flanking the PR gene on either side, this may aid PR gene excision by homologous recombination between these direct repeats.

**PR expression increases the growth rate of ΔPSI Synechocystis**

To explore the mechanism by which PR may stimulate the growth of the ΔPSI strain, we investigated the glucose consumption rate and the rate of oxygen evolution and consumption between strains that do and do not express PR. PR serves as a light-driven proton pump, thereby supposedly primarily generating additional PMF. Generally, a high PMF over the thylakoid membrane would slow down electron transport at the level of the cytochrome b6/f complex (301) via the ‘back-pressure’ effect (302). This explains why, upon illumination with green light (λmax = 535 nm; ≤ 200 μmol • m$^{-2}$ • s$^{-1}$), linear electron flow via the components of the Z-scheme of oxygenic photosynthesis in the WT strain, was strongly inhibited. Intriguingly, although electron transfer through the Z scheme is broken (43), oxygen evolution and the coupled electron transfer is still inhibited by green light in ΔPSI strains expressing PR. Oxygen uptake, however, in this ΔPSI strain, presumably via the cytochrome b6/f complex and the cytochrome c oxidase (303) is also strongly inhibited via expression/activation of PR (Fig. 5). This suggests that there is the little capacity of electron acceptors to draw from these electrons liberated by light in PSII in the PSI-deletion strain.
In agreement with the results of our earlier study (284), we have observed inhibition of respiration with (low intensities of) orange-red light in the wild-type cells (Fig. 5A). Moreover, in the wild-type cells, we observed the Mehler-like reaction only with illumination with orange-red light (from 75 μmol • m^-2 • s^-1 onwards), and not with green light. This is the main pathway for oxygen uptake when excess reducing power is generated. However, in the ΔPSI strain, the oxygen consumption is mediated primarily by the cytochrome c oxidase (303). Consistent with the above, we observed a green-light specific inhibition in this rate of oxygen consumption (Compare Figs. 5C and D), based on the proton-pumping nature of this pathway of electron transfer. Expression of PR provides the ΔPSI strain with an extra pathway for proton motive force generation and/or ATP synthesis, thereby allowing this strain to function in the light with a lower rate of respiration than the ‘empty’ plasmid-carrying strain (ΔPSI + pJBS1312). By consequence, the PR-expressing strain (ΔPSI + pQCO06) is able to convert more glucose into biomass rather than oxidize it for ATP production and because of that may have higher levels of ATP. This may explain why the PR expressing strain grows faster and has a higher biomass yield. Beyond that, and in agreement with the above interpretation, the MIMS data indicates that the PR-expressing strain (ΔPSI + pQCO06) show 30 - 50% lower respiratory activity than the ‘empty’ plasmid-carrying strain (ΔPSI + pJBS1312), when illuminated with green light from 75 μmol • m^-2 • s^-1 to 200 μmol • m^-2 • s^-1 per OD_{730}. This is in agreement with the observation that expressing PR enhances the growth rate by 30% in a batch culture grown with 25 μmol • m^-2 • s^-1 green light.

The effect of PR expression on photoautotrophic ‘growth’ of the ΔPSI strain of Synechocystis

The effect of PR expression on photoautotrophic growth of the ΔPSI strain of Synechocystis was ambiguous (Fig. 2). When pre-grown photomixotrophically, cells were washed and re-suspended in a medium without glucose, to test their capacity for photoautotrophic growth. They showed a slow rate of cell proliferation, but without a simultaneous increase in biomass (as reflected in the OD_{730}), which suggests that these cells were not, or at least, hardly able to fix CO₂ under these conditions. This is in line with findings in Chlamydomonas reinhardtii (304). Moreover, our MIMS data confirm that PSII-dependent electron flow does exist in the ΔPSI strain under photoheterotrophic and autotrophic conditions. This electron transfer would generate proton motive force, and hence lead to the synthesis of ATP. Hence, the proliferation observed is presumably limited to division of cells without an increase in total
biomass. Furthermore, the absence of CO₂ fixation indicates that, although ATP is synthesized, NAD(P)H is not formed in a ΔPSI strain in the absence of glucose. Appreciably, expression of PR, which can increase the size of the proton motive force in green light, only stimulated ‘growth’ during the first 24 hours (Fig. 2).

Presumably, during these first 24 hours, cells still contain some residual metabolic intermediates, in a condition, in which PR could drive considerable ATP synthesis. Those residual intermediates, like glucose-1-phosphate, succinate, keto-glutarate, etc., could then serve as the substrates for synthesis of a restricted amount of biomolecules during the first 24 h after glucose depletion, to facilitate limited further cell division.

**Light-dependent electron transfer from water in the PSI deletion strain**

As explained by Ort et al. (43), in a ΔPSI strain the combination of PSII and NDH could constitute an alternative linear electron transfer pathway (the ‘broken Z-scheme’) in which electrons can be transferred from water to an acceptor like NADPH (Fig. 7). Although exact bookkeeping of the translocated protons in this pathway is complex because of the involvement of vectorial protons (due to the involvement of NADP+), literature expresses consensus about the fact that this electron transfer requires PMF energy, rather than the PMF-yielding electron transfer through the regular Z-scheme. This also makes sense thermodynamically because two photons are involved in the latter process, rather than one in this ‘broken Z-scheme’. This is due to the fact that the NDH complex has a proton to electron stoichiometry of ~2 for proton pumping (305-307), whereas PSII has a stoichiometry of only 1. It is therefore likely that in a ΔPSI strain photoautotrophic NADPH formation will be a thermodynamically uphill process and therefore impossible. Consequently, such strains will be able to transfer electrons derived from water to acceptors only at the redox level of plastoquinol, and hence, will not be expected to be able to grow photoautotrophically.

The observations reported here, particularly those made with the MIMS technique are consistent with the interpretation that the heterologously expressed PR does pump protons across the energy transducing membranes (i.e. the thylakoid- and the cytoplasmic membrane), in which also PSII and the NDH-1 are housed. These latter enzymes will therefore also be affected by the additional, retinal-based, proton pumping. As the intrinsic capacity of retinal-based proton pumps to generate PMF is large (their Av; (287, 288, 308)), these lat-
ter enzymes will presumably be subject to an increased PMF. This increased PMF would be expected to inverse the thermodynamics of NADPH formation, and allow ‘reverse electron transfer’ just like it can occur in purple bacteria (309, 310). We perceive the fact that no continued, steady, autotrophic growth is observed as evidence for the absence of NADPH formation under these conditions. This then suggests that NDH from *Synechocystis* has kinetic limitations in the catalysis of NADPH synthesis (Fig. 7).

![Figure 7: Schematic representation of the modified electron transfer pathway in the ΔPSI strain of *Synechocystis* sp. PCC6803 expressing Proteorhodopsin.](image)

Purple bacteria, when growing photomixotrophically on a relatively oxidized carbon source like acetate or oxalate, or photoautotrophically *i.e.* with thiosulfate as their electron donor, do catalyze a major metabolic flux from quinols (like ubiquinol or menaquinol, depending on the specific species involved; (311, 312)) to NAD(P)H, either directly, or via the involvement of an (energy-linked) transhydrogenase. In view of the above, it therefore seems that the proposal by Ort et al (43), for the construction of an organism that can use the entire solar spectrum for oxygenic photosynthesis must be extended with the exchange of the cyanobacterial NDH-1 for a corresponding enzyme from a purple bacterium, assuming that the cyanobacterium will have endogenous transhydrogenase activity.
**Oxygen-release and uptake as measured with MIMS in the PSI deletion strain**

Our studies with MIMS have confirmed that deletion of PSI did not halt electron transfer driven by water splitting and resulting in oxygen evolution in PSII (283, 313). We confirm quantitatively that the PSI-deletion strain does mediate oxygen evolution. This light-stimulated oxygen evolution is accompanied by oxygen uptake of approximately the same magnitude. Moreover, our data indicate that, to measure a net oxygen evolution of the ΔPSI strain in green light, when grown photomixotrophically, one has to offer the strong actinic light of about \(100 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \cdot \text{OD}_{730}^{-1}\). Net oxygen evolution of the ΔPSI strain grown photoautotrophically is very much reduced.

Although PSII dependent oxygen evolution in ΔPSI *Synechocystis* has been frequently reported (283, 303, 313), be it at widely divergent rates, the underlying electron transfer pathway has only recently been resolved. It is widely accepted that this process strictly requires the plastoquinone (PQ) pool and involves an oxidase, based on its sensitivity to KCN (303, 313-315). However, the type of oxidase operating in this process has been a matter of debate. Recent studies on the respiratory oxidases of *Synechocystis* have shown that both cytochrome c oxidase (Cox) and quinol oxidase (Cyd) can be involved, while Cox contributes the most during dark respiration and under chemoheterotrophic growth, and Cyd acts an alternative pathway to complement Cox when the PQ pool is over-reduced (303, 316-318).

**Strategies for the construction of an infrared-absorbing *Synechocystis*: towards the ‘Rainbow strain’**

To engineer in *Synechocystis* the capacity to absorb infra-red light, it was initially proposed to replace PSI by an infrared-absorbing bacteriochlorophyll-based cyclic electron transfer system, which would have either of two roles: (i) it could function as an infrared-absorbing PSI (i.e a Type I reaction center), as initially envisioned (28). This, however, might lead to an unwanted decrease in the redox span driving electron transfer through the cytochrome b6/f complex; or (ii) as an alternative it could function as a cyclic electron transfer chain as it does in anoxyphototrophs (i.e Type II reaction center) (43), so as to break the Z-scheme into two halves: linear electron flow through PSII, the PQ pool and NDH-1, for formation of NADPH; and cyclic electron flow through the heterologous cyclic electron transfer system for ATP generation. It is our estimate that this latter option has the best prospects, be it that the heterolo-
gous expression of all the components of a cyclic electron transfer chain is challenging. Nevertheless, the existence of ‘pink plasmids’ (44) suggests that Nature may already have foreseen our wish to embark on such engineering. Furthermore, this approach can be simplified by replacing PSI initially by a red-shifted retinal based proton pump (45, 46, 229), which then may provide a well-characterized and accessible phototrophy in the first ‘rainbow strain’.

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Conflict of Interest

The authors declare that they have no conflict of interest. KJH is scientific advisor to the start-up company Photanol BV. This does not create a conflict of interest nor does it alter the authors’ adherence to accepted policies on sharing data and materials.
Supplementary material:

Table S1: the primers used in this study

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