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

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You are cordially invited to the public defense of my PhD thesis entitled:

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

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On Wednesday 14th June 2017 at 12:00 in the Agnietenkapel Oudezijds Voorburgwal 231, Amsterdam

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

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**Cover design:** The pictures on the cover page show the crystal structure of a protorhodopsin. Image on the front and back page shows the structure of its hexametric oligomer at the intracellular side and the extracellular side, respectively. Protons (H+) are being pumped from the intracellular side (front page) to extracellular side (back page), thereby passing through the whole thesis. The cover has been designed by Jos Arents and Que Chen.

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Chapter 1

General introduction:
Engineering a proton pumping rhodopsin as a complementary photosystem in *Synechocystis* sp. PCC6803

Que Chen, Klaas J. Hellingwerf
CHAPTER 1

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I Importance of oxygenic photosynthesis

“The Sun energizes the plants with six tastes, which constitute food for all living beings on the Earth. Thus, the food that supports the lives of creatures is filled with solar energy and the Sun is the father of all living beings”. (Mahabharata, 2.600 BC)

Solar energy is considered as the most abundant and most suitable form of sustainable energy on the earth’s surface. It powers our planet by a process known as photosynthesis, which captures and stores solar energy on a massive scale. Photosynthesis is believed to have developed very early in the history of life (1-3). It operates in a wide range of organisms, from bacteria to plants, and comes in two very different forms: tetrapyrrole- and retinal-based photosynthesis (4). The relevant tetrapyrroles are chlorophyll and bacteriochlorophyll.

During the last few decades, owing to an increasing size of the world population and an expanding economy, human society has been increasing its appetite for fuel, mostly fossil, petroleum-based fuel. However, as a consequence, worldwide problems, i.e. that of a limited fuel supply as well as an alarming level of CO$_2$ emission, have emerged. Thus, developing methods for the sustainable production of fuel and commodities has become one of the most imperative challenges of this century. A widely acclaimed proposal to achieve this is to convert solar energy with photosynthetic microorganisms, especially oxygenic photosynthetic microorganisms, like cyanobacteria and green algae, which are considered as the most promising cell factories (for a review see: (5)) for liquid fuels. Such phototrophic microorganisms have been engineered already for the production of biofuels and commodity products, like ethanol, 1-butanol, ethylene, fatty acids, bio-diesel, hydrogen, isoprene, etc. (6-8). Cyanobacteria, which have the advantage of having the highest oxygenic photosynthetic efficiency (9, 10), high growth rates, and a well-studied metabolism, attract most attention for producing a range of valuable carbon-based products, amongst others because this is possible via ‘direct conversion’, i.e. without the formation of biomass as an obligatory intermediate (11-13).

II Maximizing photosynthetic efficiency

To achieve success in setting up sustainability applications, organisms are required that convert solar energy with the highest possible efficiency. However,
the highest efficiency of natural oxygenic photosynthesis is about 12% (9, 10), as far as realized in photosynthetic micro-organisms, and less than 6% in land-plants (14); however, the measured values are often less than 5% and 1% for micro-organisms and land-plants, respectively (15, 16). The reasons for such a low photosynthetic efficiencies are multiple and complex. From a biological perspective, photosynthesis is an expensive process in terms of resources required, and organisms have evolved only the photosynthetic capacity that they need to serve their requirements within the limits of their environmental and developmental constraints. For thermodynamic reasons, this process is inefficient because visible photons represent a temperature of about 5,800 K and photosynthesis takes place at around 300 K. Beyond that, evolution has selected pigments for oxygenic photosynthesis that limit this process to the use of photons with a wavelength ≤ 700 nm (17-19), which covers only about half of the number of available photons from the sun that reach the surface of the earth (20). The light from this range is often referred to as the Photosynthetically Active Radiation (PAR). Among the organisms carrying out anoxygenic photosynthesis, there are many that are able to process the energy of photons of much longer wavelength, i.e. up to 1100 nm (21). Furthermore, in oxygenic photosynthesis, all PAR photons are utilized at the free energy level of a 680 nm photon.

![Figure 1](image.png)

**Figure 1**: The photon flux spectrum of solar radiation reaching Earth’s surface (plotted in black) and the transmission spectra of a natural photosynthetic organism, the cyanobacterium *Synechocystis* PCC6803 (green dot-dashed line). The transmission spectra show that cyanobacteria mainly absorb photons in the window 350nm-700nm. Reproduced and adapted from (28) with permission.
Multiple approaches have already been explored to increase the efficiency of (oxygenic) photosynthesis beyond its current biological-, thermodynamic- and kinetic limit. Examples are *i.e.* expression of an improved RuBisCO (22), antenna truncation (23), modulation of the ratio of formation of ATP and NADPH (24, 25), bypassing the carbon sink limitation (26), *etc.* To break its thermodynamic limit, a widely proposed method is the expansion of its effective absorption spectrum into the infrared region of the spectrum of electromagnetic radiation (*i.e.* beyond 700 nm). An expansion up to 750 nm would increase the number of available photons for oxygenic photosynthesis around 19% (27).

**III Natural infrared absorbing photosynthetic systems**

As alluded to above, however, solar energy conversion systems naturally exist that do function with the light of wavelengths > 700 nm. The most relevant examples for oxygenic photosynthesis (29-31), *i.e.* those based on chlorophyll d (*Chl d*) (32, 33) and chlorophyll f (*Chl f*) (18, 34, 35), capture photons in the range of 700 – 720 nm and 700 – 740 nm, respectively (Fig. 2 (29)). For the *Chl d*-utilizing cyanobacterium *Acaryochloris marina* it has been shown that it can indeed display a higher photosynthetic efficiency than a comparable chlorophyll a (*Chl a*)-utilizing oxygenic species (36). However, for bacteriochlorophyll (BChls) (37-39), which is the key pigment in the process of anoxygenic photosynthesis, variants are known which utilize light with a wavelength up to 1100 nm. This latter type of photosynthesis operates only a single photosystem (in these organisms called reaction center) that utilizes relatively reduced electron donors (*i.e.* H$_2$, H$_2$S or Fe$^{2+}$, succinate or malate) rather than H$_2$O, and therefore does not generate oxygen (40). Their photosystem is classified into either a type II reaction center or a type I reaction center, on the basis of its surrounding flow of electrons and the specific terminal electron acceptor used. A type II reaction center, *i.e.* P$_{870}$, normally drives cyclic electron flow, primarily for the generation of ATP (see review (41)), while a type I reaction center, *i.e.* P$_{840}^*$, drives linear electron transfer, *i.e.* from H$_2$ or H$_2$S and passes the electrons eventually to NAD(P)$^+$ so as to yield both ATP and NAD(P)H. It is notable that formation of NAD(P)H by a type II reaction center is possible, but only via involvement of the so-called ‘reversed electron transfer’, driven also by the energy from the proton gradient (41, 42).
Chapter 1

IV Engineering an infrared absorbing *Synechocystis* strain

Considering the above, to engineer an infrared absorbing *Synechocystis* strain, via the introduction of a heterologous infra-red absorbing photosystem, like a cyclic electron transfer system of an anoxyphototroph (28, 43) or via the heterologous expression of chlorophyll $d$ or $-f$, may seem like a straightforward approach. Indeed, a recent publication has reported the successful heterologous synthesis of Chl $f$ in the cyanobacterium *Synechococcus* 7002. However, the low level of production that was achieved presumably prevented phenotypic effects of the application of this approach. It should be noted that the infrared-absorbing bacteriochlorophyll-based photosystem can be envisioned to have either of two roles (Fig. 3): (i) it can function as an infrared-absorbing PSI, as initially envisioned (28), which, however, may lead to an unwanted decrease in the redox span driving electron transfer through the cytochrome b6/f complex (28), and (ii) it can function as a reaction center in a cyclic electron transfer chain as it occurs in anoxyphototrophs (43). This latter option will break-up electron transfer through the Z-scheme and NADPH will then have to be formed, with electrons derived from water oxidation, through the combined action of PSII and NDH-1. An infrared-absorbing cyclic electron transfer system can then provide the ATP, and jointly the two pumps can exploit the entire range of radiation radiating from the sun and impinging on our earth’s surface. We estimate that this second approach has best prospects.

Figure 2: Absorption spectra of photosynthetic pigments. Chlorophylls and bacteriochlorophylls are the main pigments used in natural light harvesting for photosynthesis. They all derive from tetrapyrrole but differ from each other in the extent of the double-bond conjugation and the number and nature of the substitutions on the pyrrole rings. These characteristics also determine their absorption spectra. Note that binding to selected proteins, as well as pigment-pigment interaction, can shift their absorbance maxima considerably to the red. Reproduced and adapted from (29) with permission.
Figure 3: Schematics of the functioning of PSII, together with PSI or one of three reengineered infrared-absorbing photosystems in (engineered) oxygenic photosynthesis. (A) The conventional Z scheme of oxygenic photosynthesis; (B) Reengineered photosynthesis by replacement of PSI with an infrared-absorbing PSI reaction center: electron transfer still follows the Z scheme but the reengineered photosystem I is able to use photons from the infrared region (>700 nm); (C, D) Photo-electrochemical energy capture diagram for (A) and (B), respectively. The lengths of the upward arrow mark the initial photoinduced ground-to-excited-state electrochemical energy change of the primary electron donor. The position of the arrows along the wavelength axis is fixed by their length and is approximately at the red-most absorption edge of the (bacterio)-chlorophylls. (E) A tandem-photosystem reengineered by replacing PSI with an infrared-absorbing reaction center from anoxygenic photosynthesis. (F) A tandem-photosystem reengineered by replacing PSI with an infrared-absorbing Proteorhodopsin. For the tandem-photosystems the linear electron flow, still driven by visible light (350-700 nm), is catalyzed by PSII plus NDH-1, for water-driven reduction of NADPH. The region of the solar spectrum driving each reaction center (RC) is indicated above it. Black arrows indicate reactions, blue arrows represent electron flow, and yellow arrows represent proton flow. Reproduced and adapted from (28, 43) with permission.
Heterologous expression of all the components of a cyclic electron transfer chain nevertheless is challenging, but it may be greatly facilitated through the use of so-called ‘pink plasmids’ (44). Furthermore, this approach can be simplified by initial experiments in which this complex proton pump is substituted by a red-shifted retinal based proton pump (45, 46). For this latter approach a well-characterized and -accessible cyanobacterium, *i.e.* *Synechocystis* sp. PCC6803 has been selected as the host organism, such that this engineering would grant *Synechocystis* with the ability to utilize infrared light and increase the energy output from natural photosynthesis, and its derivative processes such as ‘direct conversion’. Furthermore, the infra-red absorbing proton pump makes PSI obsolete, so that it is possible to use a PSI deletion strain. This will eliminate competition for the same photons between the two/three photosystems.

**V Retinal-based light-driven proton pumps**

The Rhodopsins are a family of light sensitive seven-transmembrane α-helix containing proteins that have a retinal molecule attached, via a Schiff-base linkage, to a lysine side chain in the interior of the protein.

This family contains type-I and type-II rhodopsins, which refers to microbial and animal rhodopsins, respectively, as they share practically no sequence similarity (47). With respect to function, the family I have members with either a sensory- (48), or a chemi-osmotic function in free-energy transduction (49). One representative example of the latter is light-driven proton pumping. Recently, however, new rhodopsins have been discovered that pump ions other than protons, like sodium- (50-53), lithium- (52), or chloride ions (54-56). Site-directed engineering can broaden this range of ion-selectivity even further to include Cs⁺ and K⁺ ions (57-59).

The best-studied light-driven retinal-based proton pump so far is bacteriorhodopsin from *Halobacterium salinarum*, which is also the first-discovered microbial rhodopsin (in 1971; for review see (60)). It pumps protons, driven by light absorption, and hence is able to generate a proton motive force (PMF) that is necessary for growth and can directly be used for such processes as ATP-synthesis, solute transport, flagella-based motility, *etc.* (61). Characterization of bacteriorhodopsin revealed its presence in the cytoplasmic membrane in the quaternary structure of a hexagonal array of trimers (62) and a fast dynamics of photo-isomerization and of proton pumping (~100 protons translocated per BR molecule per second) (63). Beyond that, its protein structure and
even the dynamical alterations in its structure during functioning after photo-activation have been well resolved (64). However, its poor expression level in many heterologous hosts limits its usefulness, be it that a recent publication showed improved overexpression of bacteriorhodopsin in *E. coli*, based on constructing chimeric proteins of bacteriorhodopsin and sensory rhodopsin II, and/or optimization of the absence of tertiary structure in the 5’ region of its corresponding mRNA (65). But these latter studies aimed at producing increasing amounts of bacteriorhodopsin for structural studies; no physiological characteristics of strains carrying such chimera were reported.

In 2000, the groundbreaking discovery of the proteorhodopsins was published, which significantly expanded the research field of light-driven proton pumps and offers more biological candidates for application in energy conver-
sion and “optogenetics” (66). The gene encoding the first proteorhodopsin was detected in the genome sequence of an uncultured γ-proteobacterium (from the SAR 86 group) from oceanic waters (67). Since then it has turned out that PRs are highly abundant in the oceans (68-72). Organisms containing them, including cyanobacteria (56, 73) abound in many other habitats as well (70, 74-76). Proteorhodopsins (PRs) form a subgroup of the family I of the rhodopsins that occur in the Domain of the Bacteria (77).

PRs can be classified, based on their absorption spectrum, into green-absorbing PRs (GPR, λmax ~525nm) and blue-absorbing PRs (BPR, λmax ~490 nm), which is assumed to be physiologically relevant with respect to the ecology of the host microorganisms (i.e. for organisms living near the surface and in the deep sea, respectively). This spectral tuning is controlled by a single amino acid at position 105 (equivalent to Leu93 in BR, Leu in GPR and Gln in BPR, respectively) (78). Significantly, GPRs generally have a 10 times higher turnover rate of proton pumping (as derived from the rate constants of the partial reactions of their photocycle; see Fig. 4) than BPR (79).

**Figure 5: characteristics of some proton pumps of interest.** (A) The absorption spectrum of bacteriorhodopsin (BR), adapted from the thesis of (94), proteorhodopsin (PR), and the dual chromophore containing *Gloeobacter* rhodopsin (GR). (B) GR homology model: Location of salinixanthin (green) and retinal (magenta) in *Gloeobacter* rhodopsin-based on the model of xanthorhodopsin (95) is indicated (constructed by Srividya Ganapathy). (C) Sequence alignment of important residues of some microbial rhodopsin of interest by using the information from (96). The top row of numbers refers to the bacteriorhodopsin sequence.
Although GPR contains the key conserved amino acid residues that also occur in bacteriorhodopsin and also undergoes a BR-like photocycle (80), a remarkable difference between their structures was discovered, i.e. the presence of a histidine in trans-membrane α-helix B (H75) (81), and the absence of the typical proton release group (PRG) of bacteriorhodopsin, which includes two glutamic acids on the extracellular side of the protein (82), which are thought be the explanation of its slower photo-cycle (10 protons translocated per PR molecule per second) (83, 84). Moreover, proteorhodopsin turned out to function as a hexameric or pentameric assembly (85), i.e. different from the hexagonal lattice of trimers characteristic for BR (62).

Experiments with organisms that express a proteorhodopsin have shown that its pumping activity can lead to: (i) an increase in growth rate under nutrient-limited conditions (68, 69, 86), (ii) to light-dependent production of ATP (87), (iii) to increased fixation of CO$_2$ (68, 88), and/or (iv) to increased cellular survival under starvation- or stress conditions (87, 89-92). Such enhancements, however, generally require nutrient-limitation or stress conditions that presumably translate into a limitation in the size of the proton motive force of the organism, before they exceed the value necessary for passing over the limit of detection of altered growth rate (93).

Yet another subgroup of eubacterial proton pumps, represented by xanthorhodopsin (XR), has attracted attention. This group has the distinctive feature that its members are able to bind two chromophores, retinal and a carotenoid (i.e. salinixanthin (95)). The carotenoid serves as a light-harvesting antenna that transfers the absorbed light energy to the retinal, so as to increase the absorption cross section of the pump for visible light about 4-fold (97). This subgroup includes a cyanobacterial rhodopsin identified from *Gloeobacter violaceus* PCC7421 (98), the so-called *Gloeobacter* rhodopsin (GR) (99). In vitro study has further shown that this protein is able to bind carotenoids with a 4-keto group, i.e. salinixanthin and echinenone (100-102). Furthermore, this protein is present in a primitive cyanobacterium that is able to carry out both Chl-a based phototrophy and, presumably, retinal-based phototrophy. Its existence brings up an interesting question on the co-evolution of the above two types of phototrophy, and also indicates that it is relevant to test the heterologous introduction of retinal-based phototrophy into a cyanobacterium with the aim of increasing the efficiency of (oxygenic) photosynthesis (see above)(103).
VI Scientific plan

This project comprises two steps, which are (i) to accomplish bio-incorporation of a suitable proton pump (which will serve as the third photosystem) into *Synechocystis* and (ii) to shift its absorbance maximum to longer wavelengths, beyond 700 nm. Experiments will first be performed with a native rhodopsin (when necessary, retinal would be supplemented to achieve holo-opsin formation), which is very stable and well-characterized, allowing us to optimize expression conditions and estimate the effect of rhodopsin expression on the phototrophic performance of *Synechocystis*. In the next stage, *Synechocystis*, transformed with a native or mutated form of rhodopsin, will be supplemented with selected modified retinal to establish whether this will lead to the production of a red-shifted rhodopsin. The most red-shifted rhodopsin can then be incorporated into wild-type *Synechocystis* and in PSI deletion-derivatives of this organism. This will reveal whether the red-shifted rhodopsin can be stably expressed, and most importantly, whether such a system can be energized cellular processes with light from beyond the PAR region.

Figure 6: Schematic representation of the project, exhibiting principle of and the connection between the three sub-lines. Subline 1 will be operated by University of Amsterdam while subline 2 and subline 3 will be executed by Leiden University.

A complementary project is carried out by our colleagues from Leiden University, who work towards constructing a strongly red-shifted rhodopsin. To achieve this (see Fig. 5 for a summary), one approach is to test a variety of retinal analogs that have been described to cause a red-shift of the absorbance band of retinal proteins (104-108), as well as to synthesize new retinal analogs by additional ring modifications (like 3-methoxy, 6-s-trans locked, aro-
matization, etc.). The other approach is to modify the amino acid sequence of the apo-protein via random mutagenesis, with the aim of further red-shifting the absorbance maximum of the holo-protein, while retaining high proton pump activity. Eventually, they will test combinations of a mutated rhodopsin and a retinal analog, with respect to the position of the absorbance band, pH of the Schiff base, proton pump activity, and thermal stability, so as to select the best for expression in *Synechocystis*.

**VII Scope of this thesis**

This thesis focuses on improving the photosynthetic efficiency of *Synechocystis* by introducing a far-red absorbing proton pump as a commentary photosystem. **Chapter 2** provides an overview of applications of artificial photosynthesis in cyanobacteria, especially the concept of 'direct conversion' through natural photosynthesis, and its advantages, limitations and potential further improvement. **Chapter 3** presents the expression and characteristics of proteorhodopsin in *Synechocystis* sp. PCC6803. Functional expression of proteorhodopsin, at a level of up to $10^5$ molecules per cell, contributes measurably to light-energy conversion in *Synechocystis*. Additionally, the formation of holo-protein of proteorhodopsin demonstrates that *Synechocystis* sp. PCC6803 has the capacity to synthesize all-trans retinal. **Chapter 4** investigates the expression level and oligomeric state of a faster retinal-based proton pump (*Gloeobacter* rhodopsin) in *Synechocystis*. It turns out that its lower expression level decreases its effectiveness in energy conversion as compared to proteorhodopsin, although it pumps protons twice faster and has a unique ability of binding an antenna chromophore. **Chapter 5** focuses on retinal metabolism in *Synechocystis*. A gene (*sll1541*) has been identified that is essential for retinal synthesis. Deletion of *sll1541* leads to a mutant deficient in retinal synthesis. Supplementing a red-shifted retinal analog to such a mutant (with an expression of apo-PR) generates a red-shifted proteorhodopsin in vivo. **Chapter 6** reveals that expressing PR, but not GR, brings a significant growth advantage to a ∆PSI strain of *Synechocystis*. To explore the mechanism underlying this stimulation, the photosynthetic efficiency and glucose consumption rate of this strain have been investigated and compared with the ∆PSI strain that does not express PR. **Chapter 7** summarizes the findings reported in this thesis and puts these in the perspective of current developments in the field of photosynthesis research.
Chapter 2

‘Direct conversion’: Artificial photosynthesis with cyanobacteria

Que Chen, Davide Montesarchio and Klaas J. Hellingwerf

This chapter has been published as:

Abstract:

Cyanobacteria, the only bacteria that can carry out oxygenic, *i.e.* plant-type, photosynthesis, can be engineered with the methods of synthetic biology so that they acquire the ability to convert CO$_2$ directly into biofuel and/or commodity chemicals, *i.e.* thereby bypassing the formation of the entire complex set of (macro)molecules that jointly form biomass. This approach has become known as ‘direct conversion’ and has been shown to be feasible for several products already, even upon significant scale-up. Here we explain and this concept of ‘direct conversion’ through natural photosynthesis and discuss its limitations and potential further improvement.

Key words:

natural photosynthesis, oxygenic photosynthesis, synthetic biology, CO$_2$ fixation, biofuel, commodity
I Introduction

In order not to obstruct a development towards a sustainable future, our society will have to make a fundamental transition from an economy that derives the main part of its energy from the net flow, and oxidation, of carbon from fossil sources into atmospheric CO$_2$, into an economy in which the global carbon cycle will have been transformed into a closed, *i.e.* circular form, driven principally by solar energy. Many adaptations will be necessary for such a transition to become feasible, including an overall reduction in energy consumption/dissipation, increased energy efficiency of many existing activities/technologies, and the large-scale use of renewable electricity. The latter form of energy may take a primary role in future society and can and must be generated with techniques directly or indirectly tapping from solar energy, like *i.e.* with photovoltaic panels, hydroelectric- and tidal power stations, and wind turbines. In addition to electricity, the solar energy can also be directly converted into chemical (free) energy, with the use of so-called ‘artificial leaves’ (109) in compounds like H$_2$, CO$_2$ and CH$_3$OH. This approach, however, has not yet resulted in large-scale demo plants, nor has it allowed the direct synthesis of molecules containing one or more carbon-carbon bonds.

Next to these man-made processes of harvesting solar energy, there is also an additional, natural, mechanism to achieve the same tapping of solar energy for human use: photosynthesis. This process is carried out by living organisms, ranging from the smallest microorganisms to plants, and is principally aimed at producing free energy to drive the synthesis/formation of new cells. For this synthesis, next to the basic building blocks and minerals required for biomass formation, also free energy is required in two forms: redox intermediates like NAD(P)H and phosphorylated intermediates like ATP (110), with the chemiosmotic free energy of a proton gradient as an exchangeable intermediate (111). Generally, and particularly during autotrophic growth (*i.e.* with CO$_2$ as the carbon source), the building blocks for the synthesis of new cells will be more oxidized than the average redox level of biomass, although there are well-known exceptions to this rule when *i.e.* reduced sugars like mannitol are fermented or when photoheterotrophic bacteria grow with *i.e.* butyrate as their carbon source (see further below). But we will restrict the discussion here to photosynthesis, and more particularly to photoautotrophy.

Surprisingly, and so far little appreciated, four different types of photosynthesis have developed during evolution (Fig. 1): Generally accepted terminology
has not yet crystallized for these different types, but mostly they are referred to as: retinal-based photosynthesis (A), anoxygenic photosynthesis (B, C) and oxygenic photosynthesis (D). For the sum of (B) to (D) also the terms: chlorophotosynthesis or chloro-phototrophy are being used (i.e. (112). In the field of microbiology the existence and discovery of retinal-based photosynthesis has blurred the distinction between chemotrophs and phototrophs because sequence analysis only of a newly discovered strain is not enough to decide between these two categories because of the existence of retinal proteins (i.e. rhodopsins) with either a sensing function or a function in proton pumping (i.e. photosynthetic energy transduction).

In photosynthesis of types A and B, light energy is converted into the free energy of hydrolysis of ATP only, via formation of a proton gradient as the high intermediate. The capacity to form NAD(P)H in these organisms is limited as this has to proceed via so-called ‘reversed electron transfer’, driven also by the energy from the proton gradient (111). In contrast, both in anoxygenic photosynthesis of type C and in oxygenic photosynthesis, light energy is directly converted not only into ATP, but also into redox carriers like NADPH. This is because the electrons activated/energized by photon energy are transported downhill through a linear pathway of electron-transfer components that even-

Figure 1: Four different types of photosynthesis: A: retinal photosynthesis, B, C: Anoxygenic photosynthesis and D: Oxygenic photosynthesis. The main photosynthetic complexes that operate in the four different types of photosynthesis are indicated as embedded in a bilayer membrane, connected with mobile electron/proton carriers. Also reference is made to the build-up of a proton motive force, as a result of the light-induced reactions (E/Z isomerization in A and electron transfer in B-D).
Artificial photosynthesis with cyanobacteria

II Oxygenic photosynthesis

The moderate redox midpoint potential of the electron donors that are suitable to facilitate anoxygenic photosynthesis (Fig. 1C) also assures that a single light-reaction suffices to bring an electron to a redox level so that it can be transferred spontaneously to NAD(P)H. In this latter aspect lies the principle difference with oxygenic photosynthesis. In this type of photosynthesis, water (abundantly available in many ecosystems), with its very high redox midpoint potential, can be used as the electron donor for NAD(P)H formation, which, however, because of the large redox-span, necessitates the involvement of two successively operating photosystems, i.e. the PSII and PSI, that together with the connecting redox components plastoquinone, the cytochrome b_{6}/f complex and plastocyanine, form the Z-scheme of oxygenic- or plant-type photosynthesis (Fig. 1D).

Illumination of the components of the Z-scheme embedded in the thylakoid membranes of plants and microorganisms accordingly gives rise to the formation of NADPH and ATP, such that, ideally, with the input of 8 photons, 2 molecules of NADPH and 3 molecules of ATP will be formed. Stress conditions, however, may alter this ratio (see further below under D). This 2::3 stoichiometry exactly matches the requirements of the metabolic pathway that functions...
to channel the main carbon-source building block of photosynthesis, \textit{i.e.} \( \text{CO}_2 \), into new (metabolic intermediates for) cell material/biomass. This metabolic pathway is known as the Calvin-Benson cycle \((115)\) and involves as the key enzyme ‘the most abundant protein on earth’, RubisCO, that catalyzes the incorporation of \( \text{CO}_2 \) into ribulose-1, 5-bis-phosphate, to form two molecules of 3-phosphoglycerate. Accordingly, for the synthesis of one molecule of phosphoglycerate 24 photons will be necessary and its formation is accompanied by the uptake of three molecules of \( \text{CO}_2 \) and an equimolar release of oxygen. The 3-phosphoglycerate can then be converted into any of the complex set of biomolecules necessary to make a new cell, primarily via the lower part of glycolysis in combination with the tricarboxylic acid cycle, to form fatty- and amino acids, and the gluconeogenic pathway to form sugars.

Oxygenic photosynthesis has evolved to become the dominant type of photosynthesis, both in the terrestrial environment (via plants) and in the aquatic environment (via macro- and micro-algae). In cyanobacteria, this process is characterized by a relative overabundance of PSI (as compared to PSII), the occurrence of multiple forms of chlorophyll \( \textit{i.e.} \ a, d, f \) and phycobilisome antennae that fill the gap between the two main absorption bands of chlorophyll. Their photosynthesis is very efficient, amongst others because of the presence of carboxysomes, which largely prevents photorespiration by RubisCO, but their energy metabolism is complex because of the strongly interactive nature of respiratory and photosynthetic electron flow in their interconnected thylakoid and cytoplasmic membranes \((117)\).

Only in selected ecological niches is it that retinal-based photosynthesis and anoxygenic photosynthesis have an important role. These niches can be differentiated with respect to the specific electron donor that is available, other than water. If very little to none is available, \textit{i.e.} in oligotrophic environments like in the open oceans or the surface of glaciers, organisms carrying out retinal photosynthesis may thrive \((90)\). In eutrophic waters, in which a significant amount of fixed carbon is present, presumably mostly as organic acids, anoxygenic photosynthesis of type that makes use of quinone electron acceptors (displayed in Fig. 1B) will abound, whereas when reduced inorganic material is abundant, photosynthesis of the type using iron-sulfur cluster acceptors (displayed in Fig. 1C) will dominate \((117)\).

For basic scientific research all these three forms of ‘non-oxygenic’ photosynthesis \( \textit{i.e.} \) those forms that do not use water as an electron donor and hence do not evolve oxygen) are important, particularly because they provide
well accessible model systems that allow detailed studies of the mechanisms operating in the more complex process of oxygenic photosynthesis. In this respect, the parallels between quinone-based and iron-sulfur cluster-based reaction centers (operating in purple-sulfur- and purple-non-sulfur bacteria, respectively; see Fig. 1B, C) and PSII and PSI, respectively, is very significant (two types). Regarding their biology, important unanswered basic questions still remain, particularly regarding the mutual competitiveness and coexistence of retinal-based- and (bacterio)chlorophyll-based photosynthesis (103).

III ‘Direct conversion’

A closing of the global carbon cycle as referred to in part A of this chapter inevitably implies an intricate involvement of, and major contribution by, natural (oxygenic) photosynthesis. This process by itself is responsible for the uptake of more than 100 gigatonnes of CO$_2$ from the atmosphere annually (118), be it that a significant part of it is directly emitted back into the atmosphere via various mechanisms of photorespiration. Nevertheless, a large fraction of the carbon (and oxygen) of this CO$_2$ is converted into the multitude of molecules that jointly form biomass. Although only part of this biomass can be made available for human use, this is very a significant part. This is first and foremost because it provides us both directly and indirectly (i.e. as feed for husbandry animals), with the food that we humans consume.

Nevertheless, a significant second use of this fixed carbon is that it provides us with new, renewable, (i.e. construction) materials and with renewable energy. In the development towards a sustainable society, this second type of use has become so important that it has generated the ‘food versus fuel’ controversy (119) on the topic of how to best use the products of natural photosynthesis. The reason behind this is that some of the very nutritious components of plants for human consumption, like starch and tri-glycerides, are also excellent starting substrates for conversion into liquid biofuel, the form of fossil energy that is most difficult to substitute with a renewable alternative. Yet society will continue to need this form of energy for i.e. aviation and heavy transport. But in addition to food and fuel, there is also a need to destine a significant part of the available biomass to the production of renewable (construction) materials.

Through the use of an approach that has been labeled by some as ‘bioraffinage’ (120), it may be possible to bring some relief in the competition for biomass between the production processes of food, materials and fuel. This is because many forms of biomass contain parts that are less nutritional, but that are nev-
Nevertheless chemically reasonably well-defined and processable, such as the cellulose-, the lingo-cellulose- and the lignin fraction. A biorefinery approach can, therefore, be applied both to the primary product of photosynthesis, as well as to the agricultural waste that remains after food- and feed production. This approach also may be an important aspect of the strategy to make new ways of production of renewable materials and liquid fuels economically competitive (121). Nevertheless, in spite of all possibilities to modify and adjust the approach of traditional crop-based photosynthesis to the future needs for production of food, materials and liquid energy carriers, the inherent maximal efficiency of plant photosynthesis – which is 4 and 6.5% for photosynthesis in C(3) and C(4) plants, respectively (14), but in practice is often below 1% (122), or even lower – will not be enough to produce sufficient supplies.

It is therefore generally assumed that further improvement in the efficiency of the application of the process of natural photosynthesis will be necessary to make a successful transition to a closed global carbon cycle. Towards this end one can follow *i.e.* the series of ideas discussed in the landmark paper of Blankenship et al (123) in which various proposals are made to modify (genetically) the basic process of oxygenic photosynthesis to allow it to use a larger part of the spectrum of electromagnetic radiation from the sun (*i.e.* use of radiation from outside the PAR window), and also more efficiently. The latter may be achieved not only through the use of far-red absorbing chlorophylls (123), but possibly also via the use of red-shifted retinal-based proton pumps (124). However, to be able to exploit these possibilities to their fullest extent will require the efforts of many, over a period of many years. Also, some of the proposed adaptations, like the adjustment of the energy gap to be bridged in PSI, may fire back on the efficiency of proton transport by the components of the central part of the Z-scheme, because these proton-pumping reactions are generally assumed to require a significant amount of excess free energy to achieve complete coupling between electron and proton transport (125). And impairment of proton pumping will directly affect the overall efficiency of photosynthesis.

For these and other reasons, we have initiated a new approach in the application of oxygenic photosynthesis for the production of materials, including liquid fuel, which has subsequently been baptized ‘direct conversion’ (11). This approach implies the use of cyanobacteria, the only prokaryotic representatives that are able to carry out oxygenic photosynthesis, in engineered form to directly synthesize the preferred product. This has a number of key advantages, as summarized in Table 1. First, regarding the organisms se-
Artificial photosynthesis with cyanobacteria have the highest efficiency of oxygenic photosynthesis known so far (up to 10%; see ref (9); presumably in part because of their low maintenance energy requirement), and allow the use of simple and direct methods of genetic engineering, and relatively simple physiological engineering (i.e. because of the absence of subcellular compartments). The simple and straightforward genetic engineering is crucial because ‘direct conversion’ implies the application of genetically engineered cyanobacteria, such that the majority of the fixed carbon is directly channeled into a preferred product like ethanol (126), sucrose (127), butyraldehyde (128) or lactic acid (129). It can be achieved by introduction of a limited number of exogenous genes that jointly form a fermentative pathway to the desired product; usually not more than two to three genes. Its main advantage is that in this approach one bypasses the inefficiencies inherent in the anabolic reactions of biomass formation and the maintenance energy required for that (130), as well as the energy needed to process the resulting biomass to (a) desired product(s). Also, no energy needs to be invested into the synthesis of multicellular structures that do not contribute to the primary photosynthetic process (roots, trunks, etc.).

Table 1: Summary of the advantages of ‘direct conversion’ with cyanobacteria over competing applications of oxygenic photosynthesis.

- Higher absolute efficiency of photosynthesis (up to 10%)
- Smaller land use than crop-based photosynthesis because of complete growth season, completely surface-covering ‘canopy’, higher maximal efficiency, etc.
- Much lower water use than crop-based photosynthesis
- Re-use of minerals is straight-forward through processing of biomass via methane fermentation or hydro-thermal treatment of biomass
- No involvement of inefficient plant parts like roots and stems
- Straight-forward genetic and metabolic engineering because of prokaryotic nature of the organisms
- Direct conversion of CO$_2$ into product without the need for bioraffinage

During the past five to six years this synthetic-biology/genetic-engineering approach for direct conversion of CO$_2$ in cyanobacteria has been widely embraced in academia. For the synthesis of a wide range of compounds biosynthetic pathways have been added to the endogenous intermediary metabolism of particularly *Synechocystis* and *Synechococcus*, often in combination with adjustment of the endogenous metabolism to optimize the overall efficiency of the process (128). Meanwhile, for all the four products mentioned above, it has been demonstrated that the engineering was so successful that indeed
more than 50% of the fixed CO$_2$ molecules is directly converted into product. This makes such engineered cells qualify as a ‘cell factory’ for the respective product (Fig. 2), a cell factory which carries out a ‘one-pot green synthesis’ (131). Indeed, for some products, like ethanol and butanediol (132, 133) the partitioning of carbon over product and biomass has reached values of > 80%. Such high carbon partitioning values very significantly minimize the amount of waste biomass that is formed in parallel to the desired product and in the optimal case is processed either via anaerobic digestion or hydrothermal treatment for minerals recycling. The latter approach has the advantage that the minerals become available in their oxidized form (i.e. nitrate rather than ammonia), which is the most suitable form for the growth of cyanobacteria.

Figure 2: A schematic representation of a cyanobacterial cell factory. Exogenous genes can be added to and deleted from, the genome of a cyanobacterium in order to achieve and/or improve the production of specific chemicals. This has led to the introduction of the term “cell factory” where the bacterial metabolism can be transfigured to a factory assembly line. New assembly lines can also be added, already existing ones deleted or modified, in order to arrive at a more efficient production (system).

Concentrating on recent literature one can conclude (134-136) that the range of products that can be produced via ‘direct conversion’ with cyanobacteria is not limited to energy- or materials-related products. Rather, the range of products that one can make is as high as with any of the traditional organisms frequently used in biotechnology. This development has been stimulated by the recent developments in fossil fuel prices (see further below) and has amongst others led to proof that also compounds with high added value like
polyl sweeteners and flavor compounds (terpenes, alkaloids, etc.) are well-suited products for this approach, be it that carbon partitioning to these latter products has so far been modest at most (137-139). The versatility of cyanobacteria with respect to the provision of reducing equivalents (derived directly from the electron transfer systems in the thylakoid membrane in the form of NADPH) makes these latter organisms particularly suited for the synthesis of relatively reduced compound like the polyols and terpenes, because these phototrophic organisms are not subject to a closed redox balance like fermentative bacteria are, nor is the NAD(P)H subject to oxidation in a respiratory chain as in aerobic bacteria and yeasts.

The concept of ‘direct conversion’ can be extended to those applications in which a cyanobacterium is used for the light-driven conversion of CO₂ into some simple commodity chemical, like sucrose, glycerol, lactic acid, or glycolic acid, which then can be used by a second organism like *E. coli*, to synthesize a high-value-added product, for which a lot of dedicated biotechnological engineering is required that so far may not have been possible in a cyanobacterium. The two organisms can operate in separate compartments, *i.e.* to offer an anaerobic environment to delicate, *i.e.* oxygen-sensitive, biosynthetic enzymes (140). A mixed approach forms the methane directly from algal biomass (141).

**IV Optimization of direct conversion’ through natural photosynthesis**

Aspects that can be addressed with respect to optimization of ‘direct conversion’ through natural photosynthesis are many-fold, varying from very specific strategies for one particular product, to very generic aspects of the efficiency of metabolism and growth of the cyanobacterial host. In this section, we will address some aspects of both. First, after a preferred product has been selected the optimal tapping point of cyanobacterial intermediary metabolism has to be selected. Very often this will be pyruvate or fructose-6-phosphate (135). The next step is optimizing the enzymology and molecular biology of the heterologous pathway that must be introduced into the cyanobacterium to allow product formation (and if necessary: product export (127)). This step includes a sufficient (but not too high, to prevent protein-burden effects) gene-expression level and enzymology of the introduced enzymes (*i.e.* substrate affinities and molecular turnover numbers. The latter may *i.e.* be a problem with the more high-value-added products like terpenes (139). Also, it is important to have sufficient excess free energy dissipation in the product-forming
pathway to drive product formation to near completion (142), for which it often helps to engineer an ATP-consuming or CO$_2$-liberating step. These are the first-order priorities. These steps in most cases require detailed biochemical and metabolic engineering (129, 131, 143). Beyond that, one can – if necessary – embark on further optimization via approaches like speeding-up metabolism via substrate channeling (i.e. via the use of fusion- or cascade proteins) or via the creation of micro-compartments (i.e. like those involved in ethanolamine catabolism (144) if reactions need to be catalyzed that are not directly compatible with the intracellular milieu of cyanobacteria.

Beyond the introduced, heterologous metabolic pathway, also the organisms’ intrinsic metabolism can be optimized and optimally adjusted to the product forming pathway, to maximize the efficiency of the overall process. Cyanobacteria, in spite of their very strong specialization towards photoautotrophic metabolism, still have many redundant parallel metabolic pathways at their disposal, i.e. to catalyze multiple pathways for cyclic electron transfer (145) or sugar catabolism (i.e. glycolysis and the pentose-phosphate pathway). As not all these pathways have equal energetic efficiency, elimination of the least efficient ones should allow an efficiency increase. Similar arguments hold for the light-harvesting antennae of these organisms (146). Furthermore, through these adjustments, one may optimally tune the ratio of synthesis of NADPH and ATP (see the previous section), to the requirements of the specific product formed. Accordingly, it has been argued by Knoop and Steuer (147) that elimination of the pathways for cyclic electron transfer will increase the efficiency of formation of products like ethanol and ethylene, which only require input of NADPH as the high-energy intermediate in their synthesis and no ATP, because this elimination will decrease the ration of ATP over NADPH generated by the cells and therefore ‘force’ the cells to channel more reducing equivalents to product formation (instead of making more cells).

Also selected, i.e. strongly rate limiting/controlling steps, can be addressed, to remove bottlenecks in metabolism. This approach is best based on a sensitivity analysis of the main enzymes involved in product formation and can be achieved via overexpression of the relevant enzyme(s) (for published examples see i.e. RubisCO and pyruvate kinase (128, 132, 143). Ultimately one would like to arrive at a situation in which the control over product formation is evenly distributed over all reactions involved, but this would almost require a complete drawing-board design of such a cell factory (see further below).
V The use of large, closed, outdoor photobioreactors

These engineered cyanobacterial cell factories will then have to be grown and produce a product in large-scale photobioreactors under ambient environmental conditions of light intensity, temperature and circadian regime. Also, they will have to be incorporated into an integrated system for CO$_2$ supply, nutrient addition/recycling, gas (i.e. O$_2$ and CO$_2$) exchange, mixing and downstream processing for product recovery, to fully exploit the remainder of the advantages listed in Table 1. Nevertheless, with respect to the actual form of the photobioreactor, a large variety of choices can (and will have to) be made. First of all, because of the use of engineered, product-forming organisms, it will be unavoidable to use closed photobioreactors, rather than open ponds. The closed photobioreactors can be built in a multitude of forms, of which the basic types are: the column reactors, the flat-panel reactors and the tubular reactors (Fig. 3; for review see (148, 149). Furthermore, various modes of mixing and the introduction of CO$_2$, and stripping of excess oxygen, can be used (i.e. through gas supply in so-called “up-flow reactors” or the inclusion of a separate compartment in the reactor for gas exchange (150, 151)). Nevertheless, designs will always have to embody the restriction that the path-length of sunlight through the reactor should not be much more than several centimeters, because larger path-lengths will lead to complete darkness in a significant fraction of the photobioreactor. And in this non-illuminated volume, only energy dissipation can take place because of the maintenance energy requirement of the phototrophic organism.

Figure 3: Schematic outline of three basic types of closed photobioreactor, suitable for upscaling. The design of three representative types of closed photo-bioreactors commonly employed for cultivation of cyanobacteria or algae. (A) Column reactor, (B) front- and side-view of a flat-panel reactor and (C) tubular reactor.
Besides the sun (light), it is also imaginable that artificial light sources, like LEDs will be used for product formation via ‘direct conversion’, fueled by renewable electricity (152). A speculative and simplifying calculation, which assumes that best laboratory performance now will be achievable at large-scale within a few years, leads to a very interesting conclusion: One can cover a field of non-arable land with PV panels (power efficiency 45%), and convert the electricity generated by LEDs (power efficiency 70%) to 680 nm light, which then can be used by an engineered cyanobacterium for ‘direct conversion’ (with 25% efficiency) in any type of photobioreactor. This then will allow photosynthesis with efficiency higher than achievable with any current crop (i.e. 8% versus 6.5%; (14)). The use of LEDs may also significantly simplify photobioreactor design (152), which may considerably increase the robustness of the overall production system (i.e. regarding the possibility to create axenic conditions; see also next paragraph).

Regarding the use of the closed, large-scale, outdoor, photobioreactors further optimization aspects are contained in the procedures to select a very robust (but accessible for molecular-genetic engineering) host strain. Also, it will be of extreme importance to develop protocols for the axenic growth of these host strains in very large photobioreactors. This robustness primarily will have to be against environmental stresses like temperature (changes), light intensity, salinity and pH, which then, in turn, may greatly facilitate procedures for their large-scale axenic growth. However, these selection criteria may also have to include increased robustness against spontaneous mutation, because the genetic instability of product-forming strains has occasionally been reported (129, 153), particularly when plasmid-based genetic engineering is used (154). For this, the natural recombination systems of the host organism may be targeted and/or its CRISPR/Cas systems (155).

In ‘direct conversion’ one makes use of the characteristic of cyanobacteria that many low molecular weight products, like ethanol, butanol, short-chain alkanes, monoterpenes, etc. (135) are rapidly secreted into the extracellular medium, and if not that this can be facilitated by engineering of a selective transporter (i.e. (127)). The product therefore can be recovered from the spent medium of the photobioreactor rather than from the biomass. This product can either be soluble-, non-soluble- or volatile in the photobioreactor. In the latter two cases, this will allow exploitation of the phase separation of the product, which will have a dramatic effect on the ease of its downstream processing (from either the aqueous or the gas process stream) and the associated costs thereof.
VI Outlook

The key to the further development and optimization of ‘direct conversion’ via natural photosynthesis is a reliable computational simulation of the process. The developments in systems biology are bringing many innovative developments in the computational simulation of intermediary metabolism and growth of life at all forms life at the cellular level. Prominent among these is flux-balance analysis, a constraint-based form of modeling of the stoichiometric metabolic network of a particular organism, any of several optimization criteria (147). Kinetic aspects of such simulations, however, so far have been very much dependent on these optimization assumptions. On the other hand, the most advanced forms of kinetic modeling of oxygenic photosynthesis in microorganisms use the assumption of functional PSII/PSI units (156), which clearly is a crude approximation of the non-integer, often very high PSI/PSII ratio observed in many cyanobacteria. We, therefore, think that the way forward in these computational simulations will be to try and derive experimental values for the light-generated fluxes of NADPH and ATP and use these as input constraints in stoichiometric metabolic network models. To derive these fluxes from modeling electron transfer in the thylakoid membranes will be extremely complicated in wild-type cyanobacteria because of the many forms of energy dissipation and cyclic electron transfer (145).

The recent surge in the generation of harvesting of renewable electricity has created yet another priority in society: The transient storage of electrical energy in more stable form, because of the limited capacity of batteries to do so. Also for this problem ‘direct conversion’ may offer a solution because of the ability of cyanobacteria to efficiently photosynthesize with LED-generated 690 nm light (which may be as high as 25 to 30% power conversion). This would call for a new type of volumetric (or: 3D) photobioreactor in which electricity generates LED light so that everywhere in the reactor the light intensity will be in between the compensation point (157) and the intensity at which significant photoinhibition starts. With proper design, this would allow for the most efficient use of space and convenient operation of large-scale axenic cultures of cyanobacteria, which would be able to convert peak-shaved electricity into a liquid biofuel. In such applications living organisms like engineered cyanobacteria may turn out to be much better suited than i.e. chemical electrolysers, because they function as almost perfect rectifiers (129), in contrast to the chemical devices which suffer significantly from corrosion under such conditions of fluctuating loads. One could even go one step further and predict that with current laboratory-type photovoltaic cells available in the field, the most
efficient form of agriculture would be to plant these photovoltaic cells on non-arable land and grow cyanobacteria or algae in such a 3D reactor. The actual efficiency of conversion of harvested sunlight into a biological product might for this type of photosynthesis turn out to be appreciably higher than for any conventional crop.

All these different aspects of ‘direct conversion’ make it more and more relevant to put serious effort into the design of an organism optimally equipped to the task of converting CO$_2$ and solar energy into a specific product. This design can be approached from multiple angles, *i.e.* using genome reduction (*i.e.* to eliminate redundancy) or a bottom-up design with synthetic biology. Also, natural selection for further optimization may have a role in this process. Currently, it cannot be decided yet which of these approaches will bring the best chances for success. Nevertheless, it is worth pointing out that this approach may have a very important secondary benefit: The more an organism is designed to specifically carry out one particular task under one specific environmental condition, the smaller the chances are that that organism will survive in the natural environment. So, it may well be that serious work to design an organism for optimally efficient ‘direct conversion’ also contributes to increased environmental safety of this approach.

The concept of ‘direct conversion’ has meanwhile been adapted by several academic and commercial researchers, leading to a situation in which the first results of commercial production of ethanol via this approach are already appearing (http://www.algenol.com/ and http://www.jouleunlimited.com/). Nevertheless, to use ‘direct conversion’ to compete successfully on an economic basis in the production of liquid fuel derived from fossil sources is still a major challenge. Actually many hold that this will not be possible without a significant tax on the release of fossil CO$_2$, because energy products are among the very cheapest products traded in our society. For that reason, a large part of the research in the field of direct conversion has shifted to the production of materials with (considerably) higher added value. This is facilitated by the developments in ongoing research in cyanobacteria, which have shown that these organisms can rival with the classical workhorses of biotechnology, *i.e.* *Escherichia coli* and *Saccharomyces cerevisiae*, in the versatility of their ability to make a suite of products (134, 135, 145). The main difference being that the two classical workhorses make their products from sugar, whereas cyanobacteria can do that from CO$_2$. The limitation of *E. coli* and *S. cerevisiae* that they need a closed redox balance under fermentative conditions does not apply to the cyanobacteria and hence the latter organisms are particularly
Artificial photosynthesis with cyanobacteria

suitet to make the more chemically reduced products, like fuels, sweeteners, and terpene-based scents.

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Expression of holo-proteorhodopsin in Synechocystis sp. PCC6803

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Abstract:

Retinal-based photosynthesis may contribute to the free energy conversion needed for growth of an organism carrying out oxygenic photosynthesis, like a cyanobacterium. After optimization, this may even enhance the overall efficiency of phototrophic growth of such organisms in sustainability applications. As a first step towards this, we here report on functional expression of the archetype proteorhodopsin in *Synechocystis* sp. PCC6803. Upon use of the moderate-strength *psbA2* promoter, *holo*-proteorhodopsin is expressed in this cyanobacterium, at a level of up to $10^5$ molecules per cell, presumably in a hexameric quaternary structure, and with approximately equal distribution (on a protein-content basis) over the thylakoid and the cytoplasmic membrane fraction. These results also demonstrate that *Synechocystis* sp. PCC6803 has the capacity to synthesize all-trans retinal. Expressing a substantial amount of a heterologous opsin membrane protein causes substantial growth retardation in *Synechocystis*, as is clear from a strain expressing PROPS, a non-pumping mutant derivative of proteorhodopsin. Relative to this latter strain, proteorhodopsin expression, however, measurably stimulates its growth.

**Key words:**

proton pump, all-trans retinal, *apo*- and *holo*-protein, proton motive force, thylakoid membrane, cytoplasmic membrane
Introduction

Concerns about global warming and the depletion of fossil fuels have led to an increasing need for the development of alternative, more sustainable, methods to produce biofuel and commodity chemicals. Cyanobacteria, like all phototrophs, can utilize the energy from sunlight to fix CO\textsubscript{2} and produce a range of valuable carbon-based products via ‘direct conversion’ (158-160). The relatively high growth rate of these organisms, the use of water as the source of electrons and their genetic accessibility, has made them the preferred organisms for such applications.

Rhodopsins are light-sensitive seven-helix transmembrane proteins that bind a retinal molecule as their chromophore. This family has members with either a sensory or a chemiosmotic function in energy transduction. Bacteriorhodopsin from \textit{Halobacterium salinarum} is the archetype of the chemiosmotic-energy-transducing rhodopsins (60). It pumps protons, driven by light, and hence is able to generate a proton motive force (PMF). Proteorhodopsins (PRs) form a subgroup of the rhodopsins from the Domain of the Bacteria, which utilize light energy to translocate protons over a membrane against an electrochemical proton gradient. The gene encoding the first discovered member of this group was detected in the genome sequence of an uncultured γ-proteobacterium from oceanic waters (67). Since then, PRs have turned out to be highly abundant in the oceans (68, 70, 71, 161), and organisms containing them, including a cyanobacterium (73) can be found in many other habitats as well (70, 74, 75, 162). In \textit{vivo} experiments have shown that pumping of protons by PR can lead to increases in growth rate under nutrient-limited conditions (68, 69, 86), production of ATP (87), increased fixation of CO\textsubscript{2} (68, 88), and/or survival under starvation or stress conditions (87, 89-91). However, these enhancements generally require nutrient-limitation or stress conditions before they exceed experimental error.

PRs have also been heterologously expressed in non-photosynthetic hosts (61, 67, 163-166). For example, the introduction of a PR in Shewanella oneidensis increased the PMF, which resulted in increases in electrical current generation, lactate uptake, and survival under starvation conditions (164, 165). In \textit{Escherichia coli}, the PMF generated by a PR resulted in ATP synthesis (163), was able to drive the flagellar motor (61), and could be used to significantly increase the production of hydrogen by a co-introduced hydrogenase (166, 167). Very recently, functional expression of a proteorhodopsin in \textit{E. coli} was
shown to cause a minute increase in the growth rate of this organism when growing fermentatively (168). This stimulation, however, may be strictly limited to anaerobic conditions: Photo-activation of *Gloeobacter* rhodopsin under aerobic conditions led to a decrease in growth rate of the organism because of increased oxidative stress (169).

Cyanobacteria and green algae have a significantly lower absorption in the green part of the visible spectrum (*i.e.* 450-550 nm), a range that is well covered by the absorption spectrum of many PRs (170, 171). Introduction of PRs into such a system could lead to increased CO$_2$ fixation, which could ultimately increase production rates of interesting compounds by cyanobacterial cell factories (172). Hence, it has previously been suggested that PRs could supply additional free energy to oxy-phototrophic organisms (170, 171), even more so when it turns out to be possible to shift the window of absorption of this pigmented protein outside that of the PAR region (173). Several proposals have been made to increase the efficiency of oxygenic photosynthesis beyond its natural limits (*i.e* (28)). In the most recent proposal (43) PSI is substituted by an (infrared-light dependent) proton pump; NADPH would then have to be derived via the combined action of PSII and NAD(P)H-dehydrogenase (43). Functional expression of PR may aid in the engineering towards obtaining such strains.

As a first step towards this we have expressed the gene sequence of the proteorhodopsin from Monterey Bay ((67); also known as GPR, but hereafter referred to as PR) in the cyanobacterium *Synechocystis* sp. PCC6803 (hereafter *Synechocystis*). We first quantitated the amount of expressed apo-PR and its retinal chromophore, and characterized the sub-cellular distribution of the protein. Using the assay of growth (rate), we were then able to demonstrate an improvement in growth of the transgenic *Synechocystis* strain expressing PR, when compared with the control strain expressing a non-pumping proteorhodopsin, PROPS (174). To our knowledge, this is the first demonstration of a beneficial effect of PR on the growth of a cyanobacterium. By implication, we demonstrate for the first time that *Synechocystis* can synthesize all-trans retinal in *vivo*.
Materials & Methods

Strains and growth conditions

Strains of *Escherichia coli* were routinely grown at 37°C, either in liquid LB medium while shaking at 200 rpm, or on solid LB plates containing 1.5% (w/v) agar. Where appropriate, antibiotics were added to a final concentration of 100 μg • m⁻¹ for ampicillin, and 25 to 50 μg • ml⁻¹ for kanamycin.

All *Synechocystis* sp. PCC6803 strains used in this study were derived from a single wild-type strain (a glucose tolerant strain, obtained from D. Bhaya, Stanford University, Stanford, CA). Unless specified otherwise, *Synechocystis* was routinely grown at 30°C with continuous illumination by white light from fluorescent light sources at moderate intensities of approximately 30 to 50 μmol • m⁻² • s⁻¹ (= μmol photons • m⁻² • s⁻¹). Liquid cultures were grown in BG-11 medium (Sigma-Aldrich), supplemented with 50 mM sodium bicarbonate, and incubated with shaking at 120 rpm. For growth on solid medium, BG-11 plates were made with 1.5% (w/v) agar, 10 mM TES-KOH (pH 8.0), 5 mM glucose, and 0.3% (w/v) sodium thiosulfate. Where appropriate, kanamycin was added at a final concentration of 20 μg • ml⁻¹ in plates, or 40 μg • ml⁻¹ in liquid medium.

Growth of *Synechocystis* sp. PCC6803 was routinely monitored via the optical density at 730 nm (OD₇₃₀) on a Biochrom WPA Lightwave II spectrophotometer. All light intensities mentioned were measured with a LI-COR LI-250 light meter and a LI-COR Quantum Sensor.

Plasmid construction

PCR reactions for cloning were performed with the proofreading Pwo DNA Polymerase (Roche Diagnostics). Primers are listed in Table I. All digestions made use of restriction enzymes from the FastDigest range (Thermo Scientific; formerly Fermentas). T4 DNA Ligase (Thermo Scientific) was used for ligation. *Escherichia coli* XL1-Blue (Agilent Technologies; formerly Stratagene) or MC1061 (175) were used as cloning hosts. Constructed plasmids were verified using specific PCRs with Taq DNA Polymerase (Thermo Scientific), followed by additional verification via sequencing.

The cloning plasmid pJBS1250 was constructed by introducing a terminator and several cloning sites into the broad-host-range conjugation vector
pVZ321 (176). A fragment containing the bi-directional BBa_B0014 terminator was amplified from a template plasmid (172) using primers JBS300 and JBS301. The fragment was digested with AvrII and PstI, and DpnI was added to digest the template DNA. The plasmid backbone of pVZ321 was restricted with XbaI and PstI, and treated with Fast Alkaline Phosphatase (FastAP; Thermo Scientific). This removed an approximately 1.7 kb region, including the chloramphenicol acetyl transferase (cat) gene. The digested plasmid and the PCR product were ligated to generate pJBS1250. Note that XbaI and AvrII form compatible overhangs of which the ligation product is not recognized by either of the two enzymes, which allows the same enzymes to be used again in subsequent cloning steps.

The cloning plasmid was further extended with several promoter fragments, which all included a ribosome binding site (RBS), followed by an AvrII site (promoter-RBS-AvrII). A fragment containing the trc promoter was amplified from template plasmid pSEQ-Trc (172) with primers JBS302 and JBS303. Similarly, fragments containing the rnpB and the psbA2 promoters were amplified from template plasmids (177) with primers JBS339 and JBS340, and JBS341 and JBS342, respectively. All promoter-RBS-AvrII fragments were digested with EcoRI and XbaI. Plasmid pJBS1250 was digested with EcoRI and Spel, and treated with FastAp. The digested plasmid and fragments were subsequently ligated. Note that XbaI and Spel form compatible overhangs that do not result in the restoration of either restriction site after ligation. This procedure generated plasmids pJBS1253, with the relevant structure P_{trc}-RBS-AvrII-BBa_B0014; pJBS1310, with P_{rnpB}-RBS-AvrII-BBa_B0014; and pJBS1312, with P_{psbA2}-RBS-AvrII-BBa_B0014.

The gene coding for proteorhodopsin (GenBank accession AF279106.2 (67)) with a C-terminal poly-histidine tag (PR-His) was amplified with primers JBS306 and JBS311 from plasmid pKJ900 (178), which was a kind gift of Dr. K. H. Jung from the University of Seoul, Korea, while the gene encoding proteorhodopsin optical proton sensor (PROPS, PR D97N (174)) was generated by introducing a G-A transversion in the PR gene using standard fusion PCR approach with primer pair JBS306/JBS319 and JBS320/JBS307. The resulting gene product was subsequently amplified with primers JBS306 and JBS307. The amplified fragment was digested with XbaI, and ligated into AvrII-digested pJBS1253, pJBS1310, or pJBS1312. This created plasmids pJBS1255, with the relevant structure P_{trc}-RBS-PR-His-BBa_B0014, pQC005, with P_{rnpB}-RBS-PR-His-BBa_B0014, and pQC006, with P_{psbA2}-RBS-AvrII-BBa_B0014.
**Table 1 Primers used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Fragment</th>
<th>Sequence a</th>
<th>Reference</th>
</tr>
</thead>
</table>
| JBS300     | BBa_B0014        | taccctaggaattcgcggccggtctagtactagttactagca-
                          | cacttgccctacccctcgc          | This study                 |
| JBS301     | BBa_B0014        | tacctgcagccggccgctactagtagaggaataata-
                          | aaaaagcgcgttaata            | This study                 |
| JBS302     | P_{bc} -RBS-Avrl | tacgaattcgcggccgctactagactagtagttgacatta-
                          | atcactccggc                | This study                 |
| JBS303     | P_{bc} -RBS-Avrl | tacctgcagccggccgctactagactagtagttgacatt-
                          | gccgtctcttgtagaatattgctcgc | This study                 |
| JBS306     | PR-His/PROPS     | tactcttagaattataattactgtagacg              |                            | This study                 |
| JBS307     | PROPS            | tactcttagaattactgtagatggtgtagttactgtagac-
                          | cattccagagacttttaac         | This study                 |
| JBS311     | PR-His           | tactcttagaattactgtagatggtgtagttactgtagac-
                          | cattccagagacttttaac         | This study                 |
| JBS319     | PROPS_1          | ctgttagatcaaccataatgtatctaaat             |                            | This study                 |
| JBS320     | PROPS_2          | gtatttagatcataatgtgttagttactaag           |                            | This study                 |
| JBS339     | P_{mpB} -RBS-Avrl| tacgaattcgcggccgctactagtagttcacttagct-
                          | gcctcataaccccgtcctctcctcctctcctctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctctcctct
of 0.2 μm (Pall Corporation, Mexico) and placed on a BG-11 plate that had been supplemented with 5% (v/v) Luria-Bertani medium. The plate was incubated overnight at 30°C under low-light conditions. After the incubation the membrane was removed from the plate, and cells were washed off with 300 μl fresh BG-11 medium, which was then plated onto BG-11 plates with 20 μg • m⁻¹ kanamycin. Single colonies were picked and plated again, after which independent single colonies were tested for the presence of the construct with a vector-specific and a construct-specific PCR.

**Western blotting**

*Synechocystis* sp. PCC6803 strains containing one of the plasmids described above were cultured under standard conditions with illumination at the intensity of approximately 50 μmol • m⁻² • s⁻¹. Cells were harvested by centrifugation and re-suspended into a 20 mM sodium phosphate buffer at pH 8 with 500 mM NaCl, 20 mM imidazole, and 0.1% (w/v) n-dodecyl-β-D-maltopyranoside (DDM; GAMPRO Scientific). To this, 60% (w/v) 0.1-mm glass beads (Sigma-Aldrich) were added. The cells were subsequently disrupted in a Precellys 24 bead-beater (Bertin Technologies) by 20 s of beating at 6000 rpm, followed by 120 s on ice, which was repeated a total of ten times. An additional 15% (w/v) DDM was added to a final concentration of 1.5% (w/v), and the cell lysates were incubated overnight at 4°C under mild agitation. Insoluble material and glass beads were removed by centrifugation at 10,000 g and 4°C for 15 min. The protein concentration in the supernatant was determined with the Pierce BCA Protein Assay (Thermo Scientific) according to the manufacturer’s protocol.

Proteins in the cell lysates were separated via SDS-PAGE in ‘Any kD Mini-PROTEAN TGX Precast Gels’ (Bio-Rad) using standard procedures. When bands from samples needed to be compared, equal amounts of protein (5 μg unless specified otherwise) were loaded in each lane. The separated proteins were subsequently transferred to NitroPure nitrocellulose membranes (Osmonics). A pre-stained protein ladder (Thermo Scientific) was used to assess protein size and transfer efficiency. After transfer, the membranes were probed with monoclonal Anti-His6 antibodies (Roche) as primary antibody, and goat anti-mouse peroxidase (Thermo Scientific; formerly Pierce Biotechnology) as the secondary antibody. The bands were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Thermo Scientific) according to the manufacturer’s protocol, and an Odyssey Fc imaging system (LI-COR).
For quantification, band intensities were determined 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. To determine the content of PR-His in a particular sample, its band intensity was compared with a standard curve formed by the band intensities of a series of known amounts of PR-His on the same gel. The standards were prepared from PR-His isolated from *E. coli* (182), which was quantified spectroscopically at its visible absorption maximum (525 nm; see (183)). Relative PR content (% w/w) in a sample was calculated from the PR-His protein content of the corresponding band over the total protein content loaded in the same lane. In order to present the PR-His expression level as the number of PR molecules per cell, the number of PR-His molecules in a particular band was calculated based on the PR-His protein content, the molar mass of PR-His (28,359 g/mol (calculated with ApE v2.0.45)) and Avogadro’s number. Assuming that efficiency of cell disruption was 100%, the total protein content loaded on a gel was converted into the number of cells. The former was determined with the Pierce BCA Protein Assay while the latter was estimated based on the OD$_{730}$ of a sample, using the conversion factor that 1 ml culture of wild type *Synechocystis* with an OD$_{730}$ = 1 contains 108 cells, as determined with a Casy 1 TTC cell counter (Schärfe System GmbH, Reutlingen, Germany) (184).

Images were minimally processed to increase printability using Adobe Photoshop CS5: they were cropped to show only the relevant areas, and brightness and contrast were adjusted equally over the entire image in some cases.

**Membrane fractionation**

Thylakoid and cytoplasmic membranes were isolated based on the procedure described in (46) from 20 l batch cultures of *Synechocystis* (final OD$_{730}$ = 0.91), with the modification that a two-phase system consisting of 6.7% dextran T-500 and 6.7% polyethylene glycol 3350 (both from Sigma-Aldrich) was used, instead of 5.8% and 5.8%, respectively. The final membrane fractions were incubated overnight at 4°C under mild agitation in the presence of 1.5% (w/v) DDM. His-tagged PR protein was subsequently detected as described under “Western blots” above.

In this procedure the cytoplasmic membrane separates into two fractions: CM1, which may represent the attachment regions between the cytoplasmic and thylakoid membranes, and the main fraction CM2 (also referred to as
PM1 and PM2, respectively) (185). Here, we only analyzed the main fraction of the cytoplasmic membrane (CM2).

The purity of the two separated membrane fractions was estimated using antibodies against two different markers: anti-PixJ1 ((186, 187)); a kind gift from Birgitta Norling (School of Biological Sciences, Nanyang Technological University, Singapore, Singapore), and anti-PsbC (CP43) (Agrisera, Vännäs, Sweden). PixJ1 has been reported to be a specific marker protein for the cytoplasmic membrane, while CP43 is a specific marker for the thylakoid membrane. The Western-blotting procedure used for these two marker proteins was similar to the detection procedure for His-tagged PR protein described above, except that the proteins were separated in SDS-PAGE gels with 14% polyacrylamide plus 6 M urea, transferred to PVDF membranes (Westran Clear Signal 0.45 μm, Whatman), and treated with goat anti-rabbit peroxidase (Thermo Scientific) as secondary antibody.

**Retinal identification and quantification**

Retinal was isolated, identified and quantified using a procedure adapted from (188). Cell pellets were re-suspended in 1 M hydroxylamine at pH 8.0 in 50% (v/v) methanol, and disrupted via bead-beating as described above for the Western blot samples, prior to incubation at 30°C for 10 min. During these steps (opsin-bound) retinal was converted with hydroxylamine into the more stable retinal oxime (189). The resulting reaction mixtures were subsequently extracted at least three times with petroleum ether (40 to 60°C). After pooling of the organic phases, the petroleum ether was evaporated under N2. The extracted material was then dissolved in n-heptane (HPLC grade), and separated on an HPLC system with an EC 150/4.6 NUCLEOSIL 100-5 C18 column (MACHEREY-NAGEL), and n-heptane at 1 ml • min⁻¹ as mobile phase. Spectra of the eluting fractions were recorded using a UVD 340U UV/VIS diode array (Thermo Scientific; formerly Dionex).

To aid peak identification, all-trans retinal, all-trans retinol, and retinoic acid (all from Sigma-Aldrich) were used as standard compounds. Retinal oxime was synthesized from all-trans retinal by treatment with hydroxylamine, similar to the procedure used for the cell samples. For quantification, a dilution series of all-trans retinal was treated exactly the same as the samples, including the bead-beating, incubation, and extraction steps, to include reaction and extraction efficiencies in the standard curve. Elution of retinal oxime was monitored at 357.7 nm—the wavelength closest to its absorption maximum in the sys-
tem used. The obtained peaks were integrated using (Chromeleon 6.80 SP1 Build 2238), and the peak areas were used to generate a standard curve. Standards were always processed together with the samples to account for any variation. The stock solution of all-trans retinal was stored in n-heptane (HPLC grade) in darkness and was quantified spectroscopically before use at the visible absorption maximum of retinal (370 nm in n-heptane (190)). To determine the retinal content in a sample, the peak area of retinal oxime in the sample was compared with a standard curve formed by the peak area of a series of known amounts of retinal (oxime). To present retinal production as the number of retinal molecules per cell, the number of retinal molecules was calculated based on retinal content, the mass of retinal (284.4 g/mol) and Avogadro’s number. The number of cells was estimated as described for the Western blotting procedure above.

Isolation of proteorhodopsin from Synechocystis

His-tagged PR was isolated from the same batch of cells as used for the membrane fractionation experiments (see above). Cells were harvested by centrifugation and disrupted by bead-beating as described for the Western blot samples above. After the removal of insoluble material by centrifugation, the lysate was diluted in buffer A (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 0.1% DDM, pH 8.0), and filtered using a syringe filter (0.22 μm pore size). His-tagged PR was subsequently purified using a HisTrap FF Crude column with 5 ml column volume, and an ÄKTA FPLC system (all from GE Healthcare, Uppsala, Sweden). The protein was eluted with a gradient from buffer A to buffer B (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, 0.1% DDM, pH 8.0). Fractions containing the protein were collected, spectroscopically characterized, pooled, and immediately dialyzed against buffer A to remove excess imidazole. After dialysis, the protein was concentrated using a concentrator with a molecular-weight cut-off of 10 kDa (Spin-X UF 6 Concentrator, Corning). The concentrator was also used to exchange the buffer for buffer C (20 mM Tris-HCl, 100 mM NaCl, 0.1% DDM, pH 8.0) by repeated cycles of washing and concentrating the protein.

The concentrated protein was further purified on a Superdex 200 HR 10/30 (GE Healthcare, Buckinghamshire, UK) gel filtration column with buffer C as mobile phase. Spectra of fractions were measured on an Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies, Germany).
Mass spectrometry

Retinal was converted to retinal oxime and extracted using the same procedure as described above for the retinal identification and quantification. Fractions from the PR isolation (described above) and standards were treated in the same way. The resulting extract in n-heptane was dried under an argon flow, and re-dissolved in a mixture of 55% (v/v) methanol, 30% (v/v) acetonitrile and 0.1% (v/v) formic acid in water.

Mass spectrometric analysis was done with an AmaZon speed IonTrap (Bruker, Bremen, Germany) equipped with a nano-spray ESI source. Positive ion mode spectra between 50 and 600 m/z were measured of samples and standards by direct sample infusion with a syringe pump system. The typical flow rate was 3 μl per min. The m/z values of compounds of interest were selected manually or by the automated +MS2 selection of the IonTrap in order to generate their MS/MS spectra. Collision-induced dissociation (CID) was used as the fragmentation method. Compass Data Analysis software (version 4.1; Bruker) was used to process and analyze the raw data. The pump system was extensively washed with 55% (v/v) methanol, 30% (v/v) acetonitrile and 0.1% (v/v) formic acid in water after each run. A new analysis was not started until the background signal was restored to its initial value.

Functional assays

To determine the effect of PR expression on the growth of *Synechocystis* sp. PCC6803, cells were grown in BG-11 medium with 50 mM sodium bicarbonate grown at 30°C with shaking at 120 rpm, with the exception that cells were illuminated with light from a custom-made RGB (red, green, blue) light panel. This light panel was constructed using 40 LEDs of each color for 120 LEDs in total, and allowed tuning of the output light intensity of each color individually. The red, green, and blue LEDs emitted maximally at 635 nm, 527 nm, and 459 nm, respectively. The total light intensity used for these experiments was approximately 47 μmol • m⁻² • s⁻¹, made up out of 21 μmol • m⁻² • s⁻¹ red, 23 μmol • m⁻² • s⁻¹ green, and 3 μmol • m⁻² • s⁻¹ blue light. To apply stress to the cells in the form of partial dissipation of the proton motive force sub-maximal concentrations of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU), [3-chlorophenyl-hydrazono] malononitrile (CCCP) and sodium chloride were added to the BG-11 medium.
Three strains were compared in each experiment: cells containing the ‘empty’ plasmid pJBS1312, cells with the PR-His-encoding plasmid pQC006, and cells with the PROPS-encoding plasmid pQC011. To start an experiment, three 10-ml cultures were inoculated from a pre-culture for each strain in 50 ml Erlenmeyer flasks in a shaking incubator at 30 °C. Growth was monitored via cell density by measuring the OD_{750} of a small volume (150 μl) sample from each culture in a Multiskan FC Microplate Photometer (Thermo Scientific, Finland). For selected experiments, instead of OD_{750}, the number of cells per ml and average cell size was analyzed in triplicate with a Casy 1 Model TTC cell counter (Schärfe System GmbH, Reutlingen, Germany) with a 60 μm capillary (191).

Results

Expression of PR in Synechocystis

To explore the possibility of functional expression of PR in *Synechocystis*, we constructed a series of expression plasmids based on the broad host-range conjugation vector pVZ321 (176). Each plasmid carried a promoter, a ribosome binding site, the structural gene of PR, with an extension encoding a C-terminal poly-histidine tag (PR-His), and a bi-directional terminator (see Materials & Methods for further detail). We preferred a plasmid-based expression system over a genomic integration because this results in significantly (about 3-fold) higher expression levels for otherwise equal constructs (192).

To find the optimum expression level of PR, we compared three plasmids, each with a different promoter driving PR expression: the very strong trc promoter, the moderately strong *psbA2* promoter, and the weak *rnpB* promoter (172, 192, 193). The protein-expression level of PR-His in strains carrying these three plasmids was determined using Western blots with an anti-His-tag antibody, and quantified by comparing the intensities of the PR-His bands with a standard series of known quantities of PR-His isolated from *E. coli*. The wild type strain was also included in this experiment, as a control to confirm that the labelling is specific for the His-tagged proteorhodopsin (data not shown).

Contrary to expectations, the highest level of PR-His expression was found in the strain in which its expression was controlled by the *psbA2* promoter (data not shown). No expression could be demonstrated in the strain with *Ptrc*-driven expression, which was likely due to genetic instability of this construct, as after cultivation we were only able to re-isolate mutated plasmids from this
strain. Although the cause of this genetic instability was not further investigated, it is plausible that very high PR expression levels poses a serious burden to the cells, which is not sufficiently compensated by energy conversion via PR and would provide a significant competitive advantage to spontaneous mutants impaired in PR expression. The construct with the weak *mpB* promoter did express PR-His, but levels were only detectable on Western blots after an additional concentration step of the cell-free extracts using a HisTrap spin column (data not shown). Therefore, we chose to utilize the *psbA2* promoter for the remainder of this study.

![Graph](image1.png)

**Figure 1:** Expression of His-tagged PR in a batch culture of *Synechocystis* in various growth phases. Cells were grown in BG-11 medium at moderate light intensities (for further detail: see the text). A) The filled squares show the cell density as monitored via the OD$_{730}$. At several time points, sample was removed for PR-His quantification via Western blots and the results obtained are expressed as the grey bars (as the weight percentage of PR-His protein over the total protein content of the cell extracts (i.e. μg of PR / μg total protein × 100). B) The Western blot corresponding to this experiment. Lanes 1-5 contain the samples that represent the grey bars in panel A. Lanes 6-10 contain a decreasing amount of His-tagged PR purified from *E. coli*. The data shown are from a representative experiment.

To investigate the dependence of the PR-His expression level on the growth phase of *Synechocystis* growing in batch culture at a moderate light intensity (~ 50 μmol • m$^{-2}$ • s$^{-1}$, see Materials & Methods), we compared samples taken from different growth phases of such cultures. A representative result is shown in Fig. 1. We reproducibly found that the expression levels of PR-His rose slightly during the early growth phases, but declined again very late in the stationary phase.
Sub-cellular localization of PR in *Synechocystis*

Cyanobacteria such as *Synechocystis* have two distinct inner membrane fractions: the cytoplasmic membrane (CM) and the thylakoid membrane (TM), be it that these two components may be partially connected (187, 194-196). Since our expression system did not include a specific membrane-targeting signal, PR might localize in either one or in both of these membrane fractions. To investigate this, we fractionated the membranes of a PR-His expressing *Synechocystis* strain, using sucrose-density gradient centrifugation and a two-phase separation system (185). Of the two membrane fractions obtained equal amounts of protein (in μg) were applied on SDS-PAGE, and analyzed using Western blots with three different antibodies: An anti-His-tag antibody to detect PR-His; an antibody against PsbC (CP43), a marker for the TM fraction (197); and an antibody against PixJ1, a marker specific for the CM fraction (186, 187).

![Figure 2: Localization of His-tagged PR in the cytoplasmic membrane fraction (CM) and the thylakoid membrane fraction (TM) of *Synechocystis*. Membranes were separated by two-phase separation and analyzed on Western blots as described in Materials & Methods. A) Localization of PR in the membrane fractions. B) Localization of PsbC (CP43), a marker for the TM fraction. C) Localization of PixJ1, a marker for the CM fraction. Note that the image shown for each panel was derived from separate gels and blots, which were run under optimized conditions for the particular protein/antibody combination. Because of this, and because of the different antibodies used, the band intensities are not comparable between panels. However, an equal total protein amount of the TM and CM fractions was always loaded on each individual gel (10 μg, 10 μg and 60 μg for panels A, B and C, respectively). Thus, the band intensities of both fractions in the same panel can be directly compared. The experiment shown here was repeated with an independently grown culture and independently isolated membrane fractions, and had the same outcome.](image)

The results obtained, summarized in Fig. 2, show that the level of PR-His relative to the total amount of protein in the respective fraction is comparable for both fractions (panel A). The marker proteins PsbC (panel B) and PixJ1 (panel
C) are almost exclusively isolated in the TM and the CM fraction, respectively, which shows that the fractions were separated successfully. Taken together, these results suggest that PR-His does not appear to localize to any particular membrane fraction selectively, but rather integrates into the CM and TM fraction randomly.

It should be noted that the membrane fractionation procedure we employed results in membrane fractions of high purity, but not in their quantitative isolation. Therefore, we have not made attempts to quantify the amount of PR-His in the two membrane fractions in vivo, beyond the conclusion that their level on a total protein basis is approximately the same in the two types of (purified) membrane (Fig. 2A).

**Retinal synthesis in *Synechocystis***

The experiments described above established that the apo-PR protein can be expressed at significant levels in both major membrane fractions of *Synechocystis*. However, PR requires a retinal chromophore before it can become functional as a light-dependent proton pump. The gene product of sl1541 from *Synechocystis* has been shown to have apo-carotenoid-15,15'-oxygense (SynACO) activity in vitro (198). The SynACO enzyme is able to convert β-apo-carotenals and related molecules of various chain-lengths into retinal in vitro (198). However, the most common precursor of retinal, β-carotene appears not to be accepted by SynACO as its substrate in vitro (198-200), which is probably due to the selectivity of the enzyme’s substrate binding site (200, 201). It is not known whether or not the enzyme actually can produce retinal in vivo.

In order to test if *Synechocystis* has an endogenous pathway for synthesis of retinal, we adapted a retinal detection assay from (188) for analysis of samples from a batch culture. Briefly, cell extracts of *Synechocystis* were incubated with hydroxylamine, to allow formation of the stable retinal-derivative retinal oxime. This derivative was subsequently extracted using petroleum ether, and analyzed on a reverse-phase HPLC system, equipped with a C18 column and a diode-array detector (see Materials & Methods for further details).

Despite repeated attempts, we were not able to detect any traces of retinal in wild type *Synechocystis* nor in those strains that carried an ‘empty’ control plasmid (i.e. without PR expression; pJBS1312; top trace in Fig. 3A). Intriguingly, and in contrast to the results obtained with pJBS1312, we did detect a
peak at the retention time of retinal oxime in extracts from cells that expressed PR-His (i.e. pQC006; middle trace in Fig. 3A). In addition, the compound co-eluting with retinal oxime has a UV/Vis spectrum identical to retinal oxime (Fig. 3B) and in contrast to visual pigments and bacteriorhodopsin (189) appears to be extracted predominantly in the syn form. These results strongly suggest that Synechocystis has the capacity to endogenously synthesize retinal, but that this compound is in most conditions rapidly metabolized. When apo-PR is present, however, the retinal can bind to this protein to form holo-PR. This presumably stabilizes the retinal against further metabolism. Thus, through the expression of apo-PR we have been able to demonstrate for the first time that Synechocystis can synthesize retinal in vivo.

Because the pathway of retinal synthesis in vivo has not been characterized yet, it is not clear whether retinal is present in similar amounts throughout all phases of growth. Therefore, we analyzed the content of retinal in the samples that had been used for quantification of the amount of apo-PR-His expressed (Fig. 1). Samples were extracted and analyzed as described in the legend to Fig. 3. A known quantity of pure retinal was used to prepare a standard curve for each HPLC analysis experiment, which allowed an accurate quantification of the amount of retinal (oxime) that was isolated. The optical density at 730 nm (OD$_{730}$) and the volume of the sample were used to estimate the number of cells from which this quantity of retinal was extracted, allowing the calculation of the approximate number of retinal molecules per cell (see Materials & Methods for further details). It should be noted that this procedure uses several estimated conversion factors, and should be viewed as an approximation.

Similarly, the amount of PR-His protein in Western blot samples of the same cells (Fig. 1) was converted to an approximate number of PR molecules per cell. Like the estimation of the number of retinal molecules, also this procedure uses several estimated conversion factors (see Materials & Methods for details), and should be viewed as an approximation. The number of apo-PR molecules and the number of retinal molecules in each of the five samples indicated by grey bars in Fig. 1 were estimated accordingly. We then calculated their ratio to vary between 0.9 and 2.1 (with an approximate error between biological replicates of 0.2) over the first three samples indicated in Fig. 1. This ratio indicates that the amount of retinal available is in the right order of magnitude to allow the formation of detectable quantities of holo-PR. This conclusion is further supported by the isolation of pigmented PR from Synechocystis (see below). Surprisingly, at the end of the growth the amount of retinal in the cells increases steeply.
Binding of retinal to apo-proteorhodopsin

The appearance of a peak corresponding to retinal (oxime) in the HPLC traces of extracts from cells expressing PR (Fig. 3) strongly suggests that retinal is associated with PR in vivo. To further confirm this, we made use of the His-tag on PR to isolate this protein using a HisTrap column. The resulting protein fraction had a bright pinkish color (inset in Fig. 4A), as expected for holo-PR (67).

Figure 3: Extraction of retinal from *Synechocystis* that do (pQC006) and do not (pJBS1312) express PR. Retinal standards or cell extracts were incubated with hydroxylamine to allow the formation of retinal oxime, which was subsequently extracted with petroleum ether and detected with HPLC (see Materials & Methods for further detail). A) Time traces of the absorption at 357.7 nm—the wavelength of maximum absorption of retinal oxime in the system used. The arrow A at ~4.1 min indicates the maximum absorption of syn-retinal oxime, while the arrow B at ~4.5 min indicates the peak of anti-retinal oxime (198). B) The absorption spectrum of the maximum of the peak containing retinal oxime from the standard sample (dotted line) and the corresponding peak from the strain carrying pQC006 (solid line). The peak was absent in the strain carrying the empty plasmid pJBS1312 (dashed line). For display purposes, an arbitrary offset was added to the traces of panel A, and in panel B the absorption at 357.7 of the retinal oxime standard was normalized to the same absorption at 357.7 nm as the pQC006 sample.

The protein was further purified by molecular-sieve chromatography, and the absorption at 525 nm of all fractions was measured to determine the amount of holo-PR present in each sample. The resulting elution pattern showed that two main fractions were separated. In these two fractions PR is presumably present in either of the two relevant oligomeric states, *i.e.* the hexameric and
monomeric state, respectively, as is suggested by the absorption spectrum of the protein eluting in these two fractions (Fig. 4B). The UV/Vis absorption spectrum of the material eluting in fraction A is the same as for holo-PR; the material eluting in fraction B had a clearly red-shifted absorption maximum, consistent with the interpretation that this fraction contains monomeric PR (85) (see further Discussion).

To further confirm that the chromophore carried by the isolated and purified holo-protein indeed was retinal, we allowed the fractions isolated with the molecular-sieve chromatography (see Fig. 4) to react with hydroxylamine, and extracted the chromophore in the same way as done for the whole-cell extraction described above (see Fig. 3). The extracted chromophore was then analyzed with electrospray mass spectrometry, and compared with a standard. The MS/MS results obtained after selecting the mass of retinal oxime (m/z 300.3) (Fig. 5) clearly show that several of its dominant fragmentation products are identical. It should be noted that the selection window for MS/MS will also include other compounds with a similar mass, resulting in co-fragmentation of other compounds that may differ between the fraction and standard.

Figure 4: Isolation and fractionation of holo-PR from Synechocystis. His-tagged holo-PR was isolated using a HisTrap column, and further fractionated and purified using gel filtration chromatography. A) Absorption at 525 nm of fractions from the gel filtration column. The inset shows the protein as loaded on the gel filtration column. B) Absorption spectra of the two fractions indicated with arrows in panel A (solid line for fraction A; dashed line for fraction B). Spectra were normalized for visual comparison.
Thus, the results are not necessarily expected to be identical. However, in the corresponding fractions of strains that did not express PR-His, the m/z 300.3 signal with the fragmentation pattern as shown in Fig. 5 was not detectable.

**Figure 5: Identification of the chromophore via mass spectrometry.** The protein was first isolated with its associated chromophore using a HisTrap column, further purified using gel filtration (Figure 4), and finally the chromophore was extracted as retinal oxime with hydroxylamine. Several fractions were then analyzed using mass spectrometry. Fraction A is shown here, but results for fraction B were comparable. The bottom panel shows the mirrored spectrum of a retinal oxime standard.

### Assay of functional activity of PR in vivo

To test whether the expressed *holo*-PR would be active in vivo, and hence contribute to the organism’s energy conversion, we first tried to demonstrate such physiological activity of PR by measuring light-driven proton translocation. However, whereas this approach worked successfully in starved *E. coli* cells expressing PR, it did not in *Synechocystis*. This was mainly because *Synechocystis* exhibits a number of competing light-dependent proton translocation and metabolic reactions which have an effect on the extracellular pH, such as reactions involved in light-dependent electron transport and utilization of CO$_2$/HCO$_3^-$ (202, 203). It also turned out to be technically too challenging to demonstrate light-dependent proton translocation in isolated subcellular (thylakoid) membrane fractions of PR-expressing cells.

For that reason, we next attempted to demonstrate an effect of PR on the growth rate of *Synechocystis*. In order to reveal such a stimulatory effect,
and because the presence of an expression plasmid may cause growth retardation, we compared the strains expressing PROPS (pQC011) and PR (pQC006) with the strain containing the 'empty' plasmid (pJBS1312). Furthermore, we used not only regular BG-11 medium, but also conditions that tend to abolish the PMF. For the latter, we chose several stresses that decrease the PMF: growth with only green light, in the presence of an inhibitor (3-(3,4-dichlorophenyl)-1,1-dimethyleurea, DCMU) of photosynthesis; growth in the presence of a protonophorous uncoupler ([3-chlorophenyl) hydrazono] malononitrile, CCCP), with and without glucose, and growth in a high salinity medium (204). These experiments were carried out with 47 μmol • m⁻² • s⁻¹ RGB (red, green, blue) illumination, since green light is an indispensable element for PR-mediated proton translocation. Under standard conditions the control strain, *i.e.* *Synechocystis* containing the ‘empty’ plasmid pJBS1312, always had a higher growth rate than *Synechocystis* expressing PR (pQC006) or expressing the non-proton-pumping PROPS (*i.e.* the D97N mutant form of PR (174) from pQC011; see Fig. 6A for an example). This is probably because expressing PR/PROPS caused an energy burden for *Synechocystis*, and/or PR/PROPS occupied a large fraction of the available space in the thylakoid membrane, which may have led to an impairment of photosynthetic electron flow. Consistent with this it was observed that the PR/PROPS expressing strains contain less chlorophyll and phycobilisomes (between 10 and 20%) and these cells are slightly larger (~10% increase of mean diameter) than cells expressing pJBS1312, during exponential growth in BG-11 medium. Significantly, these data also show that pQC006 allows *Synechocystis* to grow slightly faster than pQC011 (Fig. 6A). To rule out that this faster growth would be due to alteration in light-scattering characteristics of the cells rather than to a true change in growth rate, the experiment was also analyzed via cell counting. Fig. 6B shows that this analysis leads to the same conclusion.

In a significant number of experiments in which cells were grown with PMF-stress (see above), it was observed that the growth rate of the PR(pQC006)-containing strain was even slightly higher than the control strain (data not shown).

Our data demonstrates that PR can slightly enhance the rate of photoautotrophic growth of *Synechocystis*, relative to a strain that expresses similar amounts of non-functional proteorhodopsin. Under these conditions, PR presumably generates additional PMF, which may aid the cells in generating extra ATP for maintenance and/or anabolism.
In this manuscript we report the heterologous (holo-protein) expression of a PR-based proton pump in the membranes of *Synechocystis* sp. PCC6803. The heterologous protein incorporates approximately evenly (on a protein-content basis) in both types of membrane of the cells (*i.e.* CM and TM). It could also make a contribution to the cell’s energy conversion in both membrane types. Furthermore, we demonstrated that this protein can make a measurable contribution to the conversion of solar energy into a useful form of metabolic energy for this organism (Fig. 6). It is relevant to note that PR presumably also functions well at high-salt conditions (92).

Expression of this proteorhodopsin was so far only directly detectable when using the *psbA2* promoter, which is of intermediate strength. With weaker promoters, detection of the protein with Western blots required specific pre-concentration steps, whereas the stronger *trc* promoter elicited genetic instability of the heterologous gene. The expression level of the *apo*-protein peaks at the late stages of (linear) growth in the batch cultures studied here, and then declines during stationary phase. A similar growth-phase dependence of PR expression was observed in the naturally PR-containing Dokdonia sp. MED134 (68). The drop in PR content during the stationary phase may be in part due to the promoter we selected, because it is known that *psbA2* expres-
Expression and transcript stability are light-intensity dependent (205-210), and so is heterologous expression driven by this promoter (211, 212). It is worth noting that several of the recently discovered proteorhodopsins in ‘chemotrophic’, often psychrophilic, bacteria only contribute detectably to growth rate or cell yield when the cells are stressed and/or in stationary phase (68, 69, 86, 87, 89-91).

Beyond the apo-protein, *Synechocystis* sp. PCC6803 also turned out to be able to synthesize its natural chromophore, retinal, which spontaneously forms a Schiff-base linkage with the apo-PR to form the holo-protein (Fig. 3-5). This fraction of protein-bound retinal then becomes protected against catabolism, which is the regular fate of both endogenously and exogenously-added retinal in this organism: micromolar concentrations of retinal added to a batch culture of *Synechocystis* are catabolized with a half time of between 1 and 2 h (data not shown). Synthesis and degradation of retinal have been studied before in *Synechocystis*, but only via in *vitro* enzyme assays (198-200, 213, 214).

Upon isolation of the heterologously expressed PR from *Synechocystis*, two distinct fractions of the protein were obtained with different (spectral) characteristics (Fig. 4). We interpret these differences as being a consequence of the oligomeric state of the protein, consistent with the results presented by Hussain et al. (85). These authors showed convincingly that the dominant form of organization of (heterologously expressed) PR molecules after solubilization in DDM is the quaternary structure of a hexameric ring, whereas a small fraction is isolated as monomeric PR molecules. They also reported a higher pKa for the monomeric state, which resulted in a red-shifted absorption spectrum as compared to the hexameric state (λmax 518 nm, alkaline, vs. 535 nm, acidic), which we also observed here. We therefore tentatively conclude that PR is predominantly present in a hexameric organization, both in the thylakoid and in the cytoplasmic membranes of *Synechocystis*. The small amount of monomeric PR may actually be caused by detergent solubilization (85, 215).

To show that PR is able to make a contribution to light-energy conversion in *Synechocystis*, we also made use of starvation/stressful conditions, just like it was initially done in *E. coli* (61) and in some of the organisms in which it is endogenously present (68, 69, 86, 87, 89-91). However, no fully consistent data have been obtained under these conditions so far. This is because the technical reproducibility of these experiments was less than with growth in BG-11 medium. The small contribution of PR to the energy metabolism in *Synechocystis* observed here will in part be due to its relatively slow turnover.
(i.e. ‘pumping’) rate, which may be close to only 10 protons per second (82, 83, 216). Taking the latter value as a starting point one can calculate that in *Synechocystis*, growing with a doubling time of about 8 h and carrying out predominantly linear electron flow, the retinal-based pump, when expressed at levels of up to 105 molecules per cell, contributes to proton motive force generation (and hence ATP production) less than 1% (this is assuming that one CO\textsubscript{2} fixed requires the pumping of 10 protons). Furthermore, we consistently observed that expression of the heterologous membrane protein PR-His decreased the growth rate of *Synechocystis* sp. PCC6803. Therefore, use of the proper control strain (i.e. the strain carrying PROPS (pQC011)) is crucial in these experiments. The calculation of the 1% stimulation is based on many simplifying assumptions, like neglect of the multiple (at least 6; see (217)) forms of cyclic electron flow. This positive effect, however, may be offset by increased formation of reactive oxygen intermediates, as recently shown in *E. coli* (169), because cyanobacteria are very sensitive to H\textsubscript{2}O\textsubscript{2} (218).

To further increase the potential of retinal phototrophy in *Synechocystis* various possibilities can be proposed. The first is to use a proteorhodopsin variant that pumps faster. Since the start of this project several routes towards improvement along this line have revealed themselves, like the use of proteorhodopsin variants that bind a carotenoid like echinenone (219), not only because of their pumping rate (173), but also because their antenna carotenoid increases their effective absorption cross section 3.5 fold (220). A second possibility may be to use the trimeric bacteriorhodopsin, which presumably will not form an extended hexagonal lattice, but will show a higher pumping rate. Bacteriorhodopsin, the archetype of the retinal-based proton pumps and retinal-based photosynthesis (60), pumps with a rate of about 100 protons per second (63). The recent description of a sodium-ion translocating bacterial rhodopsin (52) offers even more exciting opportunities to explore in relation to salt stress and phototrophy. It should be kept in mind that there is one example of an endogenous proteorhodopsin in a cyanobacterium: *Gloeobacter violaceus* contains a retinal-based proton pump next to its regular machinery for oxygenic photosynthesis (73). However, the physiological role of this rhodopsin has not been resolved (yet; see (221)).

The contribution of retinal-based proton pumping to the energy budget of *Synechocystis* would become much more significant once it would be possible to exploit the photons that are not utilized by wild-type cyanobacteria. For this, the absorbance maximum of the proteorhodopsin would have to be shifted to beyond 700 nm. Although progress is made in this direction (173) there is a
significant way still to go. In a seminal paper, written at the peak of the recent revival of interest in sustainability research (28), a comparison of ‘artificial’ and natural photosynthesis was made, together with a review of options to increase the efficiency of light energy conversion through rational engineering in both approaches. Although the possibility to exploit retinal photosynthesis is not mentioned, the option to use the near-infra-red part of the solar spectrum is recommended, in particular through exploiting cyanobacteria that synthesize chlorophyll d or f and/or further engineering pigment synthesis. In that paper the possibility to alter the free-energy gap to excite the two photosystems is also proposed as a way to exploit a larger part of the solar spectrum. However, those approaches have the disadvantage that they strongly reduce the amount of free energy available to drive electron transfer between the two photosystems. Such a near-infra-red light driven retinal-based proton pump will have sufficient free energy available to drive proton translocation at physiological levels of the proton motive force. Bacteriorhodopsin pumps only a single proton per photon (4, 222), whereas in purple bacteria like Rhodopseudomonas viridis photons with a wavelength close to 1,000 nm provide the organism with enough free energy to translocate two protons over their cytoplasmic membrane (see also (171)).

In a recent update of the discussion paper on the efficiency of oxygenic photosynthesis (43) it is proposed to cut electron transfer through the Z-scheme into two halves, which then should lead to: (i) linear electron flow via PSII plus NDH-1 for water-driven reduction of NADPH, and (ii) replacement of PSI by an infra-red absorbing system for proton pumping, like i.e. a cyclic electron transfer chain as it functions in purple-non-sulfur bacteria. This would then allow exploitation of all solar radiation for oxygenic photosynthesis. Functional expression of a proteorhodopsin in a PSI deletion strain may be a very significant step in such an engineering approach.

Strong emphasis on maximizing efficiency is necessary if natural photosynthesis is to be used for large-scale fuel production, i.e. via (cyanobacterial) cell factories. In this approach, however, there are many more aspects that can be further optimized, like i.e. expression of an improved RuBisCO (22), antenna truncation (223, 224), and a retinal-based proton pump to modulate the relative rate of ATP and NADPH synthesis and stimulate product formation (225).
Authors’ Contributions

JBvdS, QC and KJH designed experiments; QC, JBvdS and HLD performed experiments; JBvdS, QC and KJH wrote the paper; and SG and WJdG contributed to the writing of the paper and the overall experimental design.

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.

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Chapter 4

Functional expression of *Gloeobacter* rhodopsin in *Synechocystis* sp. PCC6803

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Abstract:

Proteorhodopsins are retinal-based, light-driven proton pumps that occur widespread in Nature. They predominantly appear to function in environments with high incident irradiance for phototrophy (and photosynthesis) of their host, by boosting the magnitude of the proton motive force across the organism’s cytoplasmic membrane. Their maximal absorbance is usually in the blue to the orange range but can be extended into the (far)red. Since they are also easily heterologously expressed, these pumps can play an important role in further attempts to increase the efficiency of oxygenic photosynthesis. Here we report on an extension of our initial studies towards this goal, by comparison of the effect of expressing two different bacterial rhodopsins (i.e. Proteorhodopsin and Gloeobacter rhodopsin) in the model cyanobacterium Synechocystis sp. PCC6803, with special emphasis on the pigments bound by the respective apo-opsins, and the oligomeric state of the corresponding holo-rhodopsins, in Escherichia coli and in the cyanobacterial membranes.

From these studies, we tentatively conclude that the two proton-pumping rhodopsins are predominantly present as hexamers and trimers, respectively. Furthermore, Gloeobacter rhodopsin is able to bind (an antenna) carotenoid, in addition to retinal, and also has a higher turnover rate of proton pumping at a given light intensity than Proteorhodopsin. The smaller amount of GR expressed, however, will decrease its effectiveness. It still remains to be established which of these two bacterial rhodopsins can give the highest stimulatory effect on the growth rate of their cyanobacterial host.

Key words:

bacterial rhodopsins, growth-phase dependence, oligomeric state, carotenoids, echinenone, canthaxanthin, 3-hydroxy-echinenone, pumping rate
Introduction

Rhodopsins are light-sensitive seven-helix transmembrane proteins that bind a retinal molecule as their chromophore. This family has members with either a sensory- (48), or a chemiosmotic function in free-energy transduction. Most members of this group utilize light energy to translocate protons over a membrane against an electrochemical proton gradient (77). More recently, however, bacterial rhodopsins have been discovered that pump ions other than protons, like sodium\(^+\) (50-53), lithium\(^+\) (52), or chloride ions (54-56). Site-directed engineering can broaden this range even further to even include Cs\(^+\) and K\(^+\) (57-59). The gene encoding the first discovered member of the Bacterial group of the proteorhodopsins was detected in the genome sequence of an uncultured \(\gamma\)-proteobacterium from oceanic waters (67). Since then, PRs have turned out to be highly abundant in the oceans (68-72) and organisms containing them, including cyanobacteria (56, 73) can be found in many other habitats as well (70, 74-76). Surprisingly, Proteorhodopsin turned out to function as a hexameric assembly (85), rather than the hexagonal array of trimers characteristic for bacteriorhodopsin from \textit{Halobacterium salinarum} (62).

In \textit{vivo} experiments with organisms that express a Proteorhodopsin have shown that its pumping activity can lead to an increase in growth rate under nutrient-limited conditions (68, 69, 86), production of ATP (87), increased fixation of CO\(_2\) (68, 88), and/or survival under starvation or stress conditions (87, 89-92). Such enhancements, however, generally require nutrient-limitation or stress conditions before they exceed the limit of detection. Nevertheless, the occurrence of Proteorhodopsin-mediated light-driven ATP synthesis implies that next to chlorophyll-based phototrophy and photosynthesis, there is a second type of phototrophy: retinal-based phototrophy (226, 227) and presumably even retinal-based photosynthesis (227). This type of photosynthesis is characterized by a very limited use of antenna pigments and is based on light-driven E/Z isomerization, rather than on light-driven charge-separation and electron transfer, as in anoxygenic- and oxygenic phototrophy and photosynthesis.

Which of the two types of photosynthesis (\textit{i.e.} the retinal-based or the chlorophyll-based), is most efficient, is very difficult to decide (4, 228), and may even depend on the abiotic environment of the cell in which this is tested (see also above). This issue has gained special importance within the context of the bio-based economy and the worries about global warming. These concerns have
brought the need for society to develop alternative, non-fossil based, sustainable, methods for fuel- and commodities production. Cyanobacteria play a crucial role in this development because they combine the ability to use water-derived electrons to reduce CO$_2$ and combine this with the highest oxygenic photosynthetic efficiency (9) and high growth rates. Genetic engineering of such organisms then allows one to produce a range of valuable carbon-based products via 'direct conversion' (11-13).

Because of the importance of oxygenic photosynthesis for a variety of sustainability applications, multiple proposals have been made to increase the efficiency of oxygenic photosynthesis beyond its current biological and thermodynamic limits (27, 43, 123). The most recent of these (43) proposes to transfer electrons from water to NADPH by PSII only, and combine this activity with a far-red light-driven proton pump that then would provide the complementary energy-rich intermediate for the 'dark reactions of photosynthesis' (i.e. ATP). Although in the latter publication it is proposed to use an (infra)red-light-absorbing and bacterio-chlorophyll-based cyclic electron transfer chain as this proton pump, a far-red absorbing proteorhodopsin (229) would equally qualify, particularly because heterologous expression of the latter is much more straightforward. For this reason, we have expressed holo-proteorhodopsin (the green proteorhodopsin from Monterey Bay (67) in Synechocystis and observed that this retinal-based proton pump can slightly but measurably increase the rate of photoautotrophic growth of the cyanobacterium Synechocystis sp. PCC6803 (46).

Here we report on the comparison of the functional expression of (green) Proteorhodopsin with Gloeobacter rhodopsin from Gloeobacter violacea in Synechocystis sp. PCC6803. The latter is of interest because of: (i) the higher turnover rate of its photocycle (i.e. 230 ms vs 140 ms of the largest decay time constant for the alkaline forms of Proteorhodopsin and Gloeobacter rhodopsin, respectively (73, 79, 82) (ii) its ability to bind carotenoids like salinixanthin and echinenone (100-102), which function as an antenna for the retinal-based proton pumping and in this way increase the absorption cross section of the pump for visible light about 4-fold (97).

As this beneficial effect of PR expression in Synechocystis was only very small it is important to find out whether more efficient or faster pumping, proton pumps can increase it. One of the possible candidates for this is Gloeobacter rhodopsin, i.e. because of its faster pumping rate. Here we characterize this Gloeobacter rhodopsin with respect to the holo-protein expression level in
Synechocystis, carotenoid binding and oligomeric state. The latter aspect was included because of the difficulty to functionally express bacteriorhodopsin in *i.e.* *Escherichia coli*, partly because it forms hexagonal arrays of protein trimers (62).

**Materials and Methods**

**Strains and growth conditions**

Strains of *Escherichia coli* were routinely grown in LB-Lennox (LB) liquid medium at 37°C with shaking at 200 rpm, or on solid LB plates containing 1.5% (w/v) agar. Where appropriate, antibiotics were added to a final concentration as follows: ampicillin (100 μg/ml) or kanamycin (25 to 50 μg/ml), either separately or in combination.

*Synechocystis* sp. PCC6803 (a glucose tolerant strain, obtained from D. Bhaya, Stanford University, USA) was routinely grown at 30°C with continuous illumination with white light at the moderate intensity of approximately 45 μmol • m⁻² • s⁻¹ (= μmol photons • m⁻² • s⁻¹). Liquid cultures were grown in BG-11 medium (Sigma-Aldrich), supplemented with 50 mM sodium bicarbonate, 25 mM TES-KOH (pH = 8) and appropriate antibiotics, and with shaking at 120 rpm (Innova 43, New Brunswick Scientific). The BG-11 agar plates were supplemented with 10 mM TES-KOH (pH= 8), 5 mM glucose, 0.3% (w/v) sodium thiosulfate, and 1.5% (w/v) agar. Where appropriate, antibiotics were added to a final concentration of: ampicillin (100 μg/ml) or kanamycin (25 to 50 μg/ml), either separately or in combination.

To exert salt stress, cells were grown in the presence of increasing concentrations of NaCl (up to 1 M). Cells were grown in BG-11 with 50 mM NaHCO3, 25 mM TES, pH = 8, at 30°C with shaking at 120 rpm, and illuminated with RGB light (red, green, blue) at a total light intensity of 47 μmol • m⁻² • s⁻¹ (containing 21 μmol • m⁻² • s⁻¹ red, 23 μmol • m⁻² • s⁻¹ green, and 3 μmol • m⁻² • s⁻¹ blue light). The red, green, and blue LEDs emitted maximally at 635 nm, 527 nm, and 459 nm, respectively. Growth was monitored via cell density by measuring the OD₇₅₀ of a small volume (≤ 150 μl) sample from each culture in a Multiskan FC Microplate Photometer (Thermo Scientific, Finland).
Strain construction

The gene coding for *Gloeobacter* rhodopsin (Genebank accession number NP_923144, (102)) with a C-terminal 6×histidine tag (GR-His) was amplified with the proof-Reading Pwo DNA Polymerase (Roche Diagnostics) with primers JBS308: 5, tac gaattccggccgccctctag ATGTTGATGACCGTATTTTCTTC 3, and JBS312: 5, tac ctgcagcggccgctactagta CTA GTGATGGTGATGGTGATGGGAGATAAGACTGCCTCCC 3, from plasmid pKJ900 (73, 178), which was a kind gift of Dr. K.H. Jung from the University of Seoul, South Korea. The obtained fragment was digested with XbaI (Thermo Scientific) and then ligated by T4 DNA Ligase (Thermo Scientific) into AvrII-digested pJBS1312, which is a derivative of the broad-host-range conjugation vector pVZ321, with the relevant structure: PpsbA2-RBS-AvrII-BBa_B0014 (46). This newly constructed plasmid was named pQC012, with the relevant structure: PpsbA2-RBS-GR-His-BBa_B0014. *E. coli* XL1-Blue (Agilent Technologies) was used as cloning host. Constructed plasmids were verified with primers JBS308 and JBS315: 5, GATGTATGCTCTTCTGCTC 3, using colony PCRs by 2 × MyTaq Polymerase (Bioline), followed by additional verification via sequencing.

This plasmid was conjugated into wild-type *Synechocystis* sp. PCC6803 via tri-parental mating, following the description in (46). *E. coli* J53/RP4 (180, 181) was used as a helper strain and *E. coli* XL1-Blue carrying pQC012 was used as the donor strain.

The construction of plasmid pQC006 (with the structure: PpsbA2-RBS-PR-His-BBa_B0014), pQC011 (with a structure: PpsbA2-RBS-PROPS -BBa_B0014), as well as the generation of the *E. coli* and *Synechocystis* strains containing these plasmids, has been described in (46).

Isolation of His-tagged *Gloeobacter* rhodopsin from *E. coli* and *Synechocystis* cells

The GR-His expressing *E. coli* strain (*i.e.* *E. coli* with pQC012) was grown in the presence of all-trans retinal at a final concentration of 10 µM. Cells were harvested by centrifugation (11,000 g, 10 min, 4 °C) and re-suspended into binding buffer A (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 1.5% (w/v) n-dodecyl-β-D-maltopyranoside (DDM; GAMPRO Scientific), pH = 8), and then disrupted by sonication. The obtained lysate was stirred at 4 °C overnight, followed by centrifugation (10,000g, 30 min at 4 °C) to get rid of cell debris. The obtained cell-free lysate was loaded on a His-Trap column (His-
Trap FF Crude 5ml), connected to an ÄKTA FPLC system (GE Healthcare). Histidine-tagged protein was eluted with elution buffer B (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, 0.1% (w/v) DDM, pH 8). The UV/Vis spectrum of the main eluted fractions has been recorded on an Agilent 8453 UV–vis spectrophotometer (Agilent Technologies, Germany). The fractions containing protein were collected and dialyzed overnight against buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% (w/v) DDM, pH = 8.0) at 4°C. The purity of the protein was verified by the ratio of absorption at 280 nm over 541 nm. For fractions with a high purity, the protein concentration was determined via the absorption at 541 nm with an extinction coefficient of 50,000 L mol⁻¹ cm⁻¹ (102). The isolation and purification of GR-His from *Synechocystis* cells (*Synechocystis* with pQC012) followed the same protocol except that all-trans retinal was not added to the culture.

**Reconstitution of GR-His with carotenoids in vitro**

*Holo-Gloeobacter* rhodopsin isolated from *E. coli* (see above) was dialyzed and dissolved in a buffer (25 mM MOPS, 100 mM NaCl, 0.02% DDM, pH = 7.2) (101) and then aliquoted for storage at – 20 oC. Echinenone, 3-hydroxy-echinenone (both were a kind gift from Dr. J. Lugtenburg, Leiden University, The Netherlands), and canthaxanthin (Sigma-Aldrich, >95% pure) were used as the carotenoids in the carotenoid-binding test. All of the above carotenoids were dissolved in acetone (AR grade) and added into a GR-His sample at a 1:1 molar ratio, at the final concentration of 4 µM. Absorption spectra were measured at various points in time with an Agilent 8453UV–vis spectrophotometer (Agilent Technologies, Germany).

For calculations, an extinction coefficient of 50,000 L mol⁻¹ cm⁻¹ at 541 nm (102) was used for *Gloeobacter* rhodopsin in the DDM-containing buffer (see above), while 119,000 L mol⁻¹ cm⁻¹ was used for all above-mentioned carotenoids in acetone (101).

**Measurement of the Proton-Pumping Activity of GR-His**

Light-dependent rates of proton extrusion of *Escherichia coli* cells was measured in a darkened temperature-controlled vessel (230) of 1.9 ml at 25°C with a pH electrode (Mettler Toledo type no. 51343164) and a white light source (Schott KL 1500 LCD) connected via an optical fiber. Stirring was performed by an overhead constant-stirring device, which also allows solute addition.
**E. coli** XL1 cells expressing PR-His or GR-His (*i.e.* containing plasmid pQC006 (46) or pQC012) were grown in LB-Lennox medium with kanamycin (25 µg/ml) overnight at 37°C, and 200 rpm. The culture was then diluted 100-fold and grown again to an OD600 of 0.35. Retinal was then added at a final concentration of 10 µM and growth was continued overnight in darkness. To stop protein synthesis, chloramphenicol was added to the cells at a final concentration of 50 µg/ml and incubated for 2 hours. Next, carotenoids (echinenone, 3-hydroxy echinenone, or canthaxanthin) dissolved in acetone, were added to the culture at a final concentration of 10 µM. After overnight incubation with carotenoids, cells were harvested by centrifugation and washed three times. Finally the cells were re-suspended in starvation buffer (10 mM NaCl, 10 mM MgSO4, 0.1 mM CaCl2, 10 mM Tris-HCl, pH = 7.0). The cells were starved in darkness at room temperature for 4 - 5 days at 60 rpm. 1 ml of this cell suspension was centrifuged in the dark (*i.e.* in a black tube), washed 3 times with measurement solution (10 mM NaCl, 10 mM MgSO4, 0.1 mM CaCl2, and 250 mM KCl) and diluted to an OD600 of 1.5 with measurement solution. The initial rate of proton extrusion elicited by illumination was measured at different light intensities (from 250 to 6,000 μmol • m⁻² • s⁻¹) by measuring the extracellular pH for about 30 sec in the light, and during re-equilibration during about 20 minutes in the dark. The light intensity was calibrated with a LI-COR Radiation Sensor. The pH response was calibrated with the addition of 5 µl 0.01 N oxalic acid. The proton extrusion activity of *E. coli* cells without addition of all-trans retinal was used as a control.

To determine the expression level of PR or GR in *E. coli* production strains, a small amount of cells (approximately 100 OD600 cells, equal to 100 ml culture with OD600 of 1.0) were disrupted with 100-µm glass beads (Sigma) using a Precellys®24 bead beater (Bertin Technologies) in 1 ml buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 1.5% (w/v) DDM, pH = 8) and then stirred overnight at 4°C. The obtained cell extract was centrifuged to discard cell debris at 10,000 g and 4°C for 15 minutes. The resulting supernatant was considered as the GR or PR sample, and its protein concentration was determined by spectrometry at 525 nm for PR and 541 nm for GR, on the basis of the molar extinction coefficient of 44,000 M⁻¹ cm⁻¹ for PR (231) and 50,000 M⁻¹ cm⁻¹ for GR.

**Dependency of GR-His expression and retinal production on growth phase**

To investigate the dependency of the retinal production and the level of GR-
His expression on the growth phase of a culture, a batch culture of *Synechocystis* containing pQC012 was harvested at different growth phases, for quantification of the GR-His expression level (by quantitative Western blot) and retinal production (by HPLC analysis).

**Quantitative Western blotting**

Cells were harvested and disrupted with 100-μm glass beads (Sigma) using a Precellys®24 bead beater (Bertin Technologies) in a buffer containing 20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 1.5% (w/v) DDM, pH = 8, and then stirred overnight at 4°C. The obtained cell extract was centrifuged to discard cell debris at 10,000 g and 4°C for 15 minutes. The supernatant was used as the GR sample; its protein concentration was determined with the Pierce BCA Protein Assay (Thermo Scientific) according to the manufacturer’s protocol.

Proteins in the cell-free lysates were separated via SDS-PAGE in ‘Any kD Mini-PRETEAN TGX Precast Gels’ (Bio-Rad) or ExpressPlus Page gels 8-16% (GenScript) using the same procedures as described before (46). Purified GR-His from *E. coli* was quantified by spectrophotometry, based on its absorption maximum at 541 nm and a molar extinction coefficient of 50,000 L mol⁻¹ cm⁻¹ (102); then loaded in different volumes on the same gel as the standard. For quantification, band intensities of GR-His samples were determined using ImageJ v1.49a (W. S. Rasband, U.S. National Institutes of Health, http://imagej.nih.gov/ij/) and compared with a series of standards. To estimate the number of GR molecules per cell, we assumed that the efficiency of cell disruption was 100%. The total protein content (as measured with the Pierce BCA Protein Assay) loaded on a gel was converted into the corresponding number of cells, on the basis of the OD₇₃₀ of a sample, using the conversion factor that 1 ml culture of wild-type *Synechocystis* with an OD₇₃₀ = 1 contains 10⁸ cells, as determined with a Casy 1 TTC cell counter (Schärfe System GmbH, Reutlingen, Germany)(232).

**Retinal identification and quantification**

Retinal was identified and quantified in the same batches of samples as those used for quantification of GR-His expression. Cell pellets were re-suspended in 1 M hydroxylamine at pH 8.0 in 50% (v/v) methanol and disrupted via vortexing for 1 min, prior to incubation at 30°C for 10 min. The resulting reaction mixtures were subsequently extracted at least three times with petroleum ether (boiling point 40 to 60°C) and finally dissolved in n-heptane (HPLC
The extracted pigments were separated on an HPLC system with an EC 150/4.6 NUCLEOSIL 100-5 C18 column (MACHEIY-NAGEL), and n-heptane (HPLC grade) at 1 ml • min⁻¹ as the mobile phase. Spectra of the eluting fractions were recorded using a SPD-M30A diode array detector (Shimadzu Nexera X2, Japan).

All-trans retinal purchased from Sigma-Aldrich was used as a standard compound for pigment identification and quantification. After reacting with hydroxylamine, all-trans retinal was converted into the more stable compound retinal oxime (233). Elution of retinal oxime was monitored at 354 nm in our system. The obtained peaks were integrated using the software Labsolution (Shimadzu, Japan). To determine the retinal content in a sample, the peak area of retinal oxime in the sample was compared with that of a series of known amounts of retinal (oxime). To present retinal production as the number of retinal molecules per cell, the number of cells was estimated as described for the Western blotting procedure above.

Size-exclusion chromatography (SEC)

A Superdex 200 Increase column 10/300GL (GE Healthcare) was used with an AKTA FPLC system to perform size-exclusion chromatography. GR-His isolated from *E. coli* and *Synechocystis* was first concentrated with a 10 kD spin filter (Corning) and then 0.1 ml of each sample was (separately) loaded on the column. The protein was eluted with filtered elution buffer (25 mM MOPS, 100 mM NaCl, 0.02% DDM, pH = 7.2) with a flowrate of 0.4 ml/min and collected in fractions. The GR-His protein isolated from *E. coli* was reconstituted with echinenone, as described above, to investigate the effect of carotenoid binding on the oligomeric state of GR.

Results and Discussion

Stimulation of growth of *Synechocystis* under salt stress by Proteorhodopsin

We previously have demonstrated that, in regular BG-11 medium, PR provides a small but measurable enhancement of photoautotrophic growth of *Synechocystis*, as compared to the corresponding strain expressing PROPS (the D97N mutant form of PR (46, 202). With the aim of further exploring this effect, we also compared the growth rates of these three strains under some stress conditions that dissipate (most of) the proton motive force (PMF)
generated by the chlorophyll-based photosynthesis machinery of the cells, like: growth with only green light, with and without low concentrations of glucose; growth in the presence of an inhibitor of photosynthetic electron transfer (3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU); growth in the presence of a protonophorous uncoupler of the PMF ([3-chlorophenyl] hydrazono] malononitrile, CCCP); and growth in a high-salinity medium.

Under most conditions described above, we observed that *Synechocystis* containing an empty plasmid (pJBS1312) always had a higher growth rate (as deduced from the OD$_{730}$ of the cultures) and cell count than *Synechocystis* expressing PR (pQC006) or expressing its non-proton-pumping derivative PROPS; the same trend as shown under standard condition in BG-11 medium (46). The best situation encountered was that pQC006 could enhance the growth rate to the level of wild-type cells (with pJB1312) but never overtake the wild type without the self-replicative empty plasmid. This is probably because expressing PR/PROPS in *Synechocystis* causes an energy burden or space limitation in the thylakoid membrane, thereby decreasing photosynthetic efficiency in the relevant mutants.

![Figure 1: PR-stimulated growth in high-salt conditions.](image)

*Figure 1: PR-stimulated growth in high-salt conditions.* *Synechocystis* with the empty plasmid pJBS1312 (triangles), plasmid pQC006 that leads to expression of PR-His (squares), or plasmid pQC011, expressing the non-proton-pumping PROPS (202) homologue of PR (circles). Cells were grown in standard medium with 1 M additional NaCl added. Data shown are from a representative experiment from a series of five independent experiments. Error bars represent the standard deviation of biological replicates within the representative 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.
When the three strains were exposed to different levels of salinity (0.8 M (data not shown) and 1 M NaCl), the *Synechocystis* strain expressing PR (pQC006) consistently displayed a higher growth rate and cell density than the *Syn-echocystis* strain expressing PROPS (pQC011), or expressing no opsin at all (empty plasmid pJBS1312). A representative result of these experiments is shown in Fig. 1. In this experiment, we observe a slightly larger stimulatory effect of PR on growth in *Synechocystis* under high salinity stress. It is known from literature that salt stress strongly induces cyclic electron transfer around PSI (234), which is the endogenous alternative light-driven proton pump for generating additional amounts of ATP (without simultaneous generation of NADPH, since that would bring redox stress to the cells), presumably to secrete sodium ions that leak into the cells under these stress conditions (234). The additional PMF (or ATP) generated by PR could further accelerate the process of sodium expulsion, thereby better-restoring growth.

However, the stimulatory effect of PR on growth so far has been rather small (a few percent at most), but could be further increased via various approaches. The most straightforward one is to use a proteorhodopsin variant that pumps faster than PR, like *Gloeobacter* rhodopsin (GR), which has both a higher photo-cycle rate (73) than PR (82) and additionally binds one molecule of carotenoid as an antenna pigment, thereby increasing GR’s effective absorption cross section 3.5 fold (97).

**Isolation of His-tagged *Gloeobacter* rhodopsin from *E. coli***

The poly-histidine (6× His) tag engineered at the C-terminus of GR (GR-H) allows the isolation and purification of GR from *E. coli* by using a His-Trap column. The results shown in Fig. 2, *i.e.* the color (magenta-red) and the spectra (with maximum absorption at 541 nm) of the main fractions (9 - 11) suggest an efficient isolation procedure of GR-His with the His-trap chromatography. Beyond that, we obtained one fraction (Frac 5) containing cytochrome. Furthermore, to determine the purity of GR-His in the main fractions, we estimated the ratio of absorption at 541 nm over 280 nm (A541/A280) of each fraction (see Table 1). Assuming that the fraction with the highest A541/A280 ratio only contains GR-His protein, the ratio of A541/A280 of this particular fraction should be equal to the ratio of the molar extinction coefficient (ε) of GR at 541 nm and 280 nm. The estimation of ε280 is based on the convention that the molar extinction coefficient at 280 nm of a particular protein is related to its tryptophan (W), tyrosine (Y) and cysteine (C) amino acid content, and can be calculated from the formula: ε280 = (nW × 5500) + (nY × 1490) + (nC × 125).
In this formula, \( n \) refers to the number of the corresponding amino acid. On the above basis, \( \varepsilon_{280} \) of GR-His is equal to 55,350 M\(^{-1}\) cm\(^{-1}\), while \( \varepsilon_{541} \) is 50,000 M\(^{-1}\)cm\(^{-1}\)(102). Therefore, a ratio of 0.9 of \( A_{541}/A_{280} \) would indicate an absolutely pure fraction of GR-His. The three main fractions show a constant value of \( \sim 0.7 \) of this ratio, which indicates a high purity. Although this numerical value is not identical to the theoretical number, one should realize that the theoretical number is an approximation.

![Figure 2: The spectra of the main fractions of a purification run of his-tagged Gloeobacter rhodopsin from E. coli with a His-Trap column.](image)

### Table 1: relative purity of the PR-containing fractions from the Ni-affinity column

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>F9</th>
<th>F10</th>
<th>F11</th>
</tr>
</thead>
<tbody>
<tr>
<td>280</td>
<td>0.466786</td>
<td>0.650316</td>
<td>0.522788</td>
</tr>
<tr>
<td>541</td>
<td>0.326077</td>
<td>0.465747</td>
<td>0.370724</td>
</tr>
<tr>
<td>( A_{541}/A_{280} )</td>
<td>0.698558</td>
<td>0.716186</td>
<td>0.709129</td>
</tr>
</tbody>
</table>

**Reconstitution of Gloeobacter rhodopsin with carotenoids in vitro**

Protein homology analysis (GR has 50% identical amino acid residues with xanthorhodopsin (100)) and successful reconstitution of *Gloeobacter* rhodopsin with salinixanthin (Imasheva et al., 2009) and echinenone (Balashov et al., 2010) have proven that *Gloeobacter* rhodopsin is a light-driven proton pump with a dual chromophore system (*i.e.* a carotenoid and all-trans retinal). The presence of a carotenoid in a rhodopsin is advantageous, as the carotenoid appears to act as an antenna system to increase the effective absorp-
tion cross section of the protein. The selectivity of *Gloeobacter* rhodopsin with respect to carotenoid binding indicates that the 4-keto group of the carotenoid plays a key role in the successful binding and (resonance) energy transfer between the carotenoid and the retinal chromophore (102).

Intriguingly, *Synechocystis* naturally produces a considerable variety and amount of carotenoids, including the 4-keto derivatives: echinenone; 3',-hydroxy echinenone and canthaxanthin (235, 236). Therefore, prior to exploring the issue in *vivo*, *i.e.* whether or not, and if so which, carotenoids will bind to GR in *Synechocystis*, we have characterized the binding of carotenoids to GR in *vitro*.

![Figure 3](Image)

**Figure 3:** Time course of the reconstitution of *Gloeobacter* rhodopsin (4 µM) with A): echinenone (4 µM); B): 3-hydroxy-echinenone (4 µM) and C): canthaxanthin (4 µM). The spectra were recorded in buffer containing 20 mM Tris-HCl, 80 mM NaCl, 0.08% (w/v) DDM, pH = 8.0.

Echinenone is the most promising candidate for such binding as it is considered as one of the main carotenoids (18%) produced by *Synechocystis* (235), and the association between echinenone and GR has been reported in the literature (101). Another two interesting candidates would be 3'-hydroxyechinenone, and canthaxanthin, as their presence has been confirmed in *Synechocystis* by HPLC (236), although they are not the main carotenoid products. However, due to the fact that 3'-hydroxyechinenone is not commercially
available, it has not been included in this experiment; instead, 3-hydroxyechinenone has been tested.

Fig. 3 presents the spectra of the reconstitution of GR-His with the three carotenoids as a function of time. The addition of echinenone to *Gloeobacter* rhodopsin at a molar ratio of 1:1 produced a shifted and broad absorption peak in 10 minutes, of which the maximum was blue-shifted from 540 nm to 530 nm. Moreover, extended incubation (21 h at room temperature) resulted in a further shift in the absorption maximum to 491 nm. This latter shift was accompanied by the appearance of fine structure in the spectrum with peaks at 428, 458 nm and 541 nm, typical for a carotenoid molecule embedded in a hydrophobic environment. Comparatively, reconstitution of *Gloeobacter* rhodopsin with 3-OH echinenone proceeded faster (in 3 hours) and showed a sharper absorbance maximum at 458 nm, but with a less-explicit shoulder at 541 nm. In contrast, reconstitution with canthaxanthin generated less clear carotenoid fine-structure, but with 8 nm blue shift of the maximum of the peak (from 541 nm to 533 nm).

In accordance with the previous observation that salinixanthin, with the additional 2'-hydroxy group and glycoside moiety, bound to *Gloeobacter* rhodopsin much faster than echinenone (101), our results also clearly show that an additional hydroxy group accelerated the binding of 3-hydroxy echinenone to *Gloeobacter* rhodopsin, while an extra 4'-keto group (*i.e.* in canthaxanthin) prevented this and perhaps even impairs functional binding to GR. This illustrates that the hydroxy group definitely plays a role in the binding process, although the further detail of the mechanism is still unknown. However, considering that an extra 4'-keto group (canthaxanthin) impaired the binding, presumably the polarity of the group at the 4' position profoundly influences the binding.

The binding between carotenoids and GR-His was not very stable in aqueous solutions of DDM (*i.e.* a high off-rate), because the carotenoids disassociated from GR-His during incubation in buffer containing 0.1% DDM.

**Effect of carotenoid-binding on the molecular turnover rate of proton-pumping of *Gloeobacter* rhodopsin**

Comparing the photocycle turnover rates of proteorhodopsin and *Gloeobacter* rhodopsin shows that the former has a slower photocycle (~230 ms of the largest decay time constant for the alkaline form) (73, 79, 82), which implies a
lower rate of proton pumping. To directly compare the proton pumping activity of proteorhodopsin and *Gloeobacter* rhodopsin, we measured the proton pumping rate of GR and PR in intact *E. coli* cells under various intensities of white light (from 250 μmol • m$^{-2}$ • s$^{-1}$ to 6000 μmol • m$^{-2}$ • s$^{-1}$, see Fig. 4). The proton pumping rate initially increased linearly with the light intensity (≤ 1500 μmol • m$^{-2}$ • s$^{-1}$), and then gradually saturated with higher light intensities. Fig. 4 shows that under the conditions selected GR displays a two-fold higher molecular proton-pumping rate than PR, with a maximum rate of 4.8 protons per GR molecule per second.

![Figure 4: Comparison of proton pumping rate of PR and GR expressed in E. coli.](image)

Measurements were carried out in a single experiment.

An important question is whether or not association with carotenoids can improve the proton pumping activity of GR-His. In order to investigate this, we measured the proton pumping rate of holo-GR-His (all-trans retinal bound GR-His) in *vivo* under conditions with and without the addition of echinenone; 3-OH echinenone or canthaxanthin. However, our attempts to reconstitute holo-GR-His with carotenoids in *vivo* in *E. coli* were not successful. Spectra of intact *E. coli* cells expressing holo-GR showed no differences with and without the addition of carotenoids. Presumably, the outer membrane of *E. coli* prohibits the uptake of carotenoids from the medium into the cells, or the carotenoids may only bind GR in *E. coli* very weakly.

**Dependency of GR-His expression and retinal production on growth phase**

To explore the possibility of functional expression of GR holoprotein in *Synecocystis* and its effect on the photo-autotrophic growth rate of this organ-
ism, the Synechocystis strain containing pQC012 (for GR-H expression) was cultivated in batch culture at a moderate light intensity (~ 45 μmol • m⁻² • s⁻¹). Cells were collected at different growth phases for quantification of the GR-expression level and the level of retinal production, respectively.

Figure 5: Retinal production- and expression level of His-tagged apo-GR in a batch culture of Synechocystis in various growth phases. Cells were grown in the BG-11 medium at a moderate light intensity. Solid line: Growth curve monitored via the OD₇₃₀. Bars: Amount of apo-PR-His per cell, quantified via Western blots and amount of retinal per cell, quantified via HPLC. The number of cells per sample was determined using the conversion factor that 1 ml culture of wild-type Synechocystis with an OD₇₃₀ 1 contains 10⁸ cells, as determined with a Casy 1 TTC cell counter (Schärfe System GmbH, Reutlingen, Germany) (184). The data shown are from three biological replicates. Error bars represent the standard error of the mean, and are only visible when they exceed the size of the symbols.

The results summarized in Fig. 5 show that GR-His had the highest expression level (~9.5×10⁴ molecules per cell) in the linear growth phase, which is shifted to an earlier phase as compared to PR-His in Synechocystis, the expression of which reached a peak at the transition between linear growth and the stationary phase (46). Moreover, retinal production increased during the subsequent growth phases and reached a peak (at ~8.4 ×10⁴ molecules per cell) at the early stationary phase, and was then followed by a decrease. In contrast, the PR-expressing strain (Synechocystis with pQC006) showed an overall increasing trend in retinal production and displayed a sharp increase in the late stationary phase (data not shown). In addition, the changes in retinal production level did not correlate with those of GR-His expression. Significantly, the number of functional GR-His molecules clearly depends on the growth phase of the cells. Therefore, in i.e. the linear growth phase of Synechocystis, in which a large fraction of GR-His is present as apo-GR-His, exogenous ad-
dition of all-\textit{trans} retinal could well increase the amount of functional GR so as to further increase the extent of the beneficial effect of this retinal-based proton pump on light-energy conversion in the cyanobacterium.

\textbf{Isolation of His-tagged \textit{Gloeobacter} rhodopsin from \textit{Synechocystis}}

To explore the possibility that carotenoids would bind to \textit{holo}-GR-His in \textit{vivo} in \textit{Synechocystis}, GR-His was isolated from the \textit{Synechocystis} strain containing pQC012 (for GR-H expression) by using a His-Trap column (see Materials and Methods). The relevant elution fractions showed a yellow-brown color with a spectrum containing a broad absorption peak in the range of 350 - 550 nm. In addition, a peak with a maximum absorption at 678 nm in the same fraction strongly suggests the presence of contaminating (\textit{i.e.} Chl a-binding) proteins. The co-elution with a small Chl a-binding protein has been consistently observed in fractionations of His-tagged proteins from \textit{Synechocystis}. A corresponding fraction from a retinal-deficient \textit{Synechocystis} strain (JBS14003; unpublished results) containing pQC006 (for \textit{apo}-PR-His expression) was used as a reference to correct for the presence of this contaminant. The spectrum corrected with this reference spectrum should represent the spectrum of His-tagged GR from \textit{Synechocystis}. Fig. 6 clearly shows that the corrected spectrum contained an absorption peak in the range of 400 to 600 nm, with a maximum at 486 nm rather than 540 nm. A closer look at the spectra shows that two shoulders are present at 458 nm and 540 nm. These latter characteristics strongly suggest the binding of carotenoids to GR-His. HPLC analysis of pigment extracts of this purified GR-His protein fraction further confirmed this finding as we detected a peak with a close retention time (0.3 minutes later) as echinenone, but with a different UV/Vis spectrum, \textit{i.e.} a blue-shift of its maximum absorption of a few nm as compared to echinenone (data not shown). However, not enough information so far is available to propose a tentative structure of this/these compound(s). We consider it likely, however, that it is a derivative of echinenone, as \textit{Synechocystis} displays a very complex carotenoid metabolism, and conversion of echinenone is certainly possible. Moreover, we also detected a peak of retinal oxime from these extracts of the GR-His fraction, which confirms that all-\textit{trans} retinal had been bound by GR-His. Therefore, based upon the analysis of the spectrum and the results of chromophore extraction, we conclude that GR-His expressed in \textit{vivo} in \textit{Synechocystis} has bound all-\textit{trans} retinal well as (a) carotenoid(s). Although the structure of the carotenoid(s) is still unknown, we were able to isolate (a) carotenoid(s) bound to \textit{holo}-GR from \textit{Synechocystis} in a buffer with 0.1% DDM, which implies that the binding of the carotenoid(s) to GR-His is
rather strong. Therefore, these carotenoids would fit better into the carotenoid-binding pocket of GR than echinenone. Revealing their structure will help to delineate the carotenoid-binding pocket of GR.

![Graph showing spectra eluted from a Ni²⁺-affinity column for purification of His-tagged GR from Synechocystis.](image)

**Figure 6:** Spectra eluted from a Ni²⁺-affinity column for purification of His-tagged GR from *Synechocystis*. A strain deficient in retinal synthesis (i.e. JBS14003, obtained via deletion of sl1541 and slr1648), and conjugated with plasmid pQC006 (for the expression of PR-His), was cultivated in BG-11 medium and served as the control. Wild-type *Synechocystis* conjugated with plasmid pQC012 (for the expression of GR-His) was investigated for the formation of holo-PR.

The oligomeric state of *Gloeobacter* rhodopsin isolated from *E. coli* and from *Synechocystis*

Transmembrane proteins often form functional oligomers, as oligomer formation can provide increased structural- and proteolytic stability may facilitate membrane insertion, and allows functional cooperativity (237). Proteorhodopsin and Bacteriorhodopsin both function in the form of oligomers (i.e. hexamers (85) and a two-dimensional array of trimers (62), respectively). The quaternary structure of *Gloeobacter* rhodopsin, in particular when isolated from *Synechocystis* has so far remained unexplored.

The oligomeric state of GR-His isolated from *E. coli* and from *Synechocystis*, both purified by His-Trap affinity chromatography was analyzed by size-exclusion chromatography on a Superdex 200 column.

As shown in Fig. 7, three peaks (labeled a, b, and c) stood out in the elution pattern, which indicates the co-existence of three oligomeric structures for GR-His isolated from *E. coli*, which is comparable to results published elsewhere (238). Therefore, we assigned these three peaks (a, b, c) according to size as representing oligomers, trimers and monomers of GR-His, respective-
ly. Apparently, of these, the trimer is the dominant species. CD-spectroscopy of equivalent samples has confirmed this assignment (229). In addition, Tsukamoto et al. showed that the quaternary structure of GR-His is pH dependent: The monomer state dominates at acidic pH (pH <4), while the ratio of trimers over monomers increased with increasing pH, and the oligomers have a considerable population only when pH >7.5 (238).

Binding a carotenoid could stimulate the formation of oligomers of a rhodopsin, as such a hydrophobic pigment molecule may bind at the interface between monomers so as to connect and assemble them into oligomers (239). As GR-His also can be present in either of three oligomeric states at different ratios in a pH-dependent manner, it is interesting to know whether binding of a carotenoid has a significant influence on oligomer formation of GR-His. To explore this, GR-His was reconstituted with echinenone in vitro as described in Materials and Methods and analyzed by size-exclusion chromatography. The change in the ratio of the three structural isomers was estimated in the eluted samples by comparing the results obtained with and without the addition of echinenone. No significant changes in the relative composition of three structural isomers were observed, which strongly indicates that echinenone does not play a crucial role in the formation of the quaternary structure of GR-His (data not shown). The SEC chromatography of GR-His isolated from *Synechocystis* (Fig. 7) showed a different monomer/oligomer distribution than GR-His isolated from *E. coli*, although they both presumably do form the same type of oligomers. Besides this, for GR, in addition, a large fraction showed up in front of the eluting oligomers. Overall these results indicate that the

![Figure 7: SEC analysis of GR-His dissolved in buffer with 20mM Tris-HCl, 0.1 M NaCl, 0.1%DDM, pH 8.](image-url) Three peaks, named a, b, and c, were identified as an oligomer, trimer and monomer, respectively.

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Chapter 4

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same oligomeric states are present in GR-His isolated from *E. coli* and from *Synechocystis*. Further spectral analysis showed that the material eluting at 7 – 9 ml mainly contained contaminating proteins (*i.e.* Chl a-binding protein), while between 10 and 13 ml, GR-His eluted from the column with two chromophores bound (*i.e.* retinal and a carotenoid; data not shown).

**Conclusions and Perspectives**

Our previous study demonstrated that functional expression of Proteorhodopsin in *Synechocystis* stimulates the growth rate of this organism, only with a few percent (46). In an attempt to further increase the energy contribution from retinal based phototrophy, *Gloeobacter* rhodopsin was heterologously expressed in *Synechocystis*. Although this latter rhodopsin shows a higher pumping rate (two-fold higher than PR) and has a unique ability to bind (an antenna) carotenoid (which can further increase its pumping rate), its lower expression level, however, will decrease its effectiveness and may offset its advantage in pumping rate, so that GR contributes approximately to the same extent to light-energy conversion of *Synechocystis* as Proteorhodopsin.

To be able to better quantitate the bio-energetic contribution of the two rhodopsins to the energy metabolism of *Synechocystis*, and compare which of the two is more effective, expression of these two rhodopsins in (a) mutants of *Synechocystis* may be a promising approach. The results of physiological studies strongly suggest that PR activity in its endogenous host is most beneficial when cells are grown under harsh (stress) conditions. However, the growth data collected from those conditions on *Synechocystis* are of lesser technical reproducibility than those obtained with growth in standard conditions. The use of a mutant, which is subject to severe energy limitation because of a genetic lesion, could solve this problem.

Among such mutants, we consider a PSI deficient *Synechocystis* strain as the most promising one. Cyclic electron flow around PSI essentially also functions as a light-driven proton pump, and therefore works to generate extra proton motive force and/or ATP synthesis, without the coupled formation of NADPH (145). Considering this, one may expect that functional expression of proteorhodopsin in a PSI deletion strain could partially restore the impaired production of ATP and therefore growth of such a mutant to the corresponding wild-type levels. This idea even makes more sense if linear electron flow in a PSI deletion strain could be adjusted to occur via PSII plus NDH-1 only, for water-driven reduction of NADPH, as proposed in (43).
As compared to Gloeobacter rhodopsin, bacteriorhodopsin even pumps much faster (~ 100 protons/BR/s; (63). However, the poor expression level of bacteriorhodopsin in many heterologous hosts limits its usefulness. A recent report showed improved overexpression of bacteriorhodopsin in E. coli, based on constructing chimeric proteins of bacteriorhodopsin and a sensory rhodopsin, and/or optimization of the absence of tertiary structure in the 5’ region of its mRNA (65). This opens up the possibility to try expression of bacteriorhodopsin derivatives also in Synechocystis, although there is no guarantee that functional holo protein will be formed from such chimera’s. More exciting is the possibility to functionally express a far-red-shifted proteorhodopsin which can absorb and function with the light of a wavelength beyond 700 nm (i.e. a proteorhodopsin with maximum absorption at 750 nm; (229)). This will allow us to construct a Synechocystis strain which can (also) utilize infrared light. Replacement of PSI by such an infrared-absorbing proton pump is a promising strategy to increase the maximum areal energetic efficiency of oxygenic photosynthesis.

Acknowledgements

This project was carried out within the research program of BioSolar Cells (BSC core project grant C2.9 to WJdG and KJH), co-financed by the Dutch Ministry of Economic Affairs. The authors would like to acknowledge Prof. Dr. Johan Lugtenburg for the supply of the carotenoids.

Authors’ contributions

QC and KJH designed experiments; QC and JCA performed experiments; QC, JCA and KJH wrote the paper; and SG and WJdG contributed to the writing of the paper and the overall experimental design.

Conflict of interest

The authors declare that they have no conflict of interest. KJH is a 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.
Retinal metabolism in *Synechocystis* sp. PCC6803 and the formation of *holo*-proteorhodopsin

Que Chen, Jeroen B. van der Steen, Aloysius F. Hartog, Srividya Ganapathy, Willem J. de Grip, and Klaas J. Hellingwerf
Abstract:

In many pro- and eukaryotic genera retinal-based proton pumps (i.e. rhodopsins) are expressed to equip the cells with the ability of light-driven ATP synthesis, which the cells can use during growth and/or starvation. Such proton pumps also occur in several cyanobacterial genera such as Gloeobacter violaceus and Cyanothece.

As these retinal-based proton pumps may play an important role in artificially increasing the efficiency of oxygenic photosynthesis (45), it is of interest to characterize retinal metabolism in cyanobacteria. Here we have studied this process in the model cyanobacterium Synechocystis sp. PCC6803. It has been proposed, based on the outcome of in vitro enzyme activity assays, that two enzymes play a role in retinal synthesis: SynACO and SynDiox2, whereas CYP120A1 has been identified as an enzyme that can hydroxylate the C16, C17 methyl groups of retinal as an initial step in retinal catabolism. Furthermore, the aldehyde retinal may be converted into its corresponding alcohol or carboxylic acid by various more-or-less specific dehydrogenases.

We have studied the in vivo role of these five enzyme(s) (activities) presumably involved in retinal metabolism. We confirm the role of SynACO as the decisive enzyme for retinal synthesis in Synechocystis, via asymmetric cleavage of β-apo-carotenal, while SynDiox2 competes for the same substrates with SynACO, but only measurably contributes to retinal synthesis in the stationary phase via an as yet unknown mechanism. Knocking out the gene encoding SynACO fully abolishes the ability of Synechocystis to synthesize retinal. Such mutants can be used for the reconstitution of holo-proteorhodopsins with exogenously added retinal or retinal analogues, as we have demonstrated in this study with all-trans 3, 4-dehydroretinal and the 3-methylamino-16-nor-1, 2, 3, 4-didehydroretinal analogue.

In vivo degradation of retinal can occur through chemical oxidation as well as via physiological pathways. Apparently, in wild-type Synechocystis, retinal degradation is faster than retinal biosynthesis, since retinal is only detected in cells expressing apo-PR, where it is protected against degradation in holo-PR. In the linear growth phase excess apo-PR is available, as is evident from quantitation of the cellular levels of retinal and apo-PR, an increase of the retinal content is observed in this phase in the knock-out mutant QCSY002 (deletion of gene slr0574) also in the late stationary phase. Our results thus suggest that slr0574 plays a role in the retinal degradation pathway. Prelim-
inary results obtained with \[^{13}\text{C}]-\text{NMR} \text{ analysis, however, suggest that also conversion to retinol plays a role.}

**Key words:**

retinal biosynthesis; retinal degradation; retinal analogue; infra-red absorption; retinal supplementation
Introduction

The vastly increasing societal demand for sustainable energy makes it necessary to convert solar energy as efficiently as possible. An important goal in the life sciences, therefore, is to achieve an increase in the energy-conversion efficiency of oxygenic photosynthesis. A widely proposed approach to the latter is by expanding the absorption spectrum of oxygenic photosynthesis into the (far-)red region of the spectrum of electromagnetic radiation (27, 240, 241), as this type of photosynthesis so far is limited to the use of photons in the 350 – 700/750 nm range (17-19). Such an expansion can be achieved by introduction of a heterologous photosystem, like a cyclic electron transfer system of an anoxyphototroph (240, 241) or a retinal-based proton pump (45, 46), provided that these proton-pumping photosystems can exploit far-red photons. The latter approach, i.e. the use of a retinal-based proton pump, may be simplest of the two, in terms of requirements in the fields of synthetic biology and physiological adjustment.

Retinal-based photosynthesis is mediated by proton-pumping prokaryotic rhodopsins. These are hepta-helical transmembrane proteins with a covalently bound all-trans retinal chromophore (for review see i.e. (242)). Our previous study has demonstrated that in Synechocystis sp. PCC6803 (hereafter: Synechocystis), a model organism for studies of oxygenic photosynthesis, a retinal-based proton pump can contribute measurably to energy conversion for the growth of the organism (46). Remarkably, this study revealed that Synechocystis has the capacity to synthesize all-trans retinal (46). This brings up the question which biochemical pathway is used for retinal synthesis and -degradation in Synechocystis. This topic becomes even more important if one wants to generate transgenic Synechocystis strains with a retinal-based proton pump which can utilize far-red light (>700 nm), because this will presumably require – next to the use of retinal analogues (243) – deletion of the endogenous all-trans retinal biosynthetic pathway.

Retinal metabolism has been extensively studied, amongst others, in animals, (green) algae, fungi, archaeabacteria, and eubacteria. So far, three different pathways have been identified as being involved in retinal biosynthesis, but all via a poly-isoprenoid derived intermediate. Firstly, in animals, a β-carotene-15, 15’-oxygenase (usually abbreviated as 15,15’ BCO or BCO) is commonly employed to generate all-trans retinal through symmetrical oxidative cleavage of β-carotene at the C15-C15’ double bond (244, 245). Secondly, halobacteria
use two non-carotenoid oxygenases (the putative membrane protein \textit{Brp} and the \textit{Brp}-like protein \textit{Blh}) to synthesize all-	extit{trans} retinal from β-carotene (246). Thirdly, selected microorganisms utilize apo-carotenoids (but not carotenoids) as the precursor of all-	extit{trans} retinal. Examples are the cyanobacteria \textit{Nostoc} sp. PCC7120 and \textit{Fusarium fujikuroi} (247, 248).

Gene sequence comparison shows that in \textit{Synechocystis} two genes have similarity with a carotenoid cleavage dioxygenase (CCD) (249), which are referred to as \textit{slr1541} ((Syn)Diox1, or SynACO), and \textit{slr1648} (SynDiox2), respectively. It has been shown that the enzyme SynACO, in \textit{vivo}, can degrade β-apo-carotenals, but not β-carotene, with a wide tolerance with respect to (i) the chain length (\textit{i.e.} between C25 and C35) and (ii) functional end-groups (\textit{i.e.} aldehydes and alcohols; (250). Incubation of the purified SynACO enzyme with carotenoid extracts from \textit{Synechocystis} did not provide convincing evidence on the nature of the physiological substrate nor product of this enzyme in \textit{vivo}. The authors proposed that a C3-hydroxylated apo-carotenal with a C27 or C30 chain-length could function as its substrate (250). The crystal structure of SynACO shows that the structure of the substrate-binding pocket of this enzyme is consistent with this substrate specificity (251). The function of SynDiox2 has not been characterized yet, except that it has been claimed that activity of SynDiox2 leads to accumulation of β-13-carotenone (252), which would imply that SynDiox2 functions to cleave β-apo-carotenals. Then, with respect to substrate specificity, it would compete with SynACO.

Current knowledge of retinal degradation suggests that retinal in \textit{vivo} is either oxidized into retinoic acid or reduced to retinol. The former reaction is catalyzed by members of the aldehyde dehydrogenase 1 superfamily (ALDH) (253); while the latter reaction can be catalyzed by alcohol dehydrogenase (ADH), retinol dehydrogenase (RDH) and aldo-keto reductase (AKR) (254). Very little information, however, is available with respect to the question which ALDHs and/or ADHs from \textit{Synechocystis} can react with retinoids as their substrate.

Based on gene analysis and substrate specificity identified in in \textit{vivo} assays (255-257), for retinal degradation we decided to specifically investigate in this study the aldehyde dehydrogenase SynAlh1 (encoded by \textit{slr0091}), although in \textit{vivo} assays show that it only oxidizes apo-carotenals (chain length ≥ C25) and alkanals, but not retinal, into the corresponding acids (258), and the enzyme AdhA, a medium-chain alcohol dehydrogenase, encoded by \textit{slr1192}, as this enzyme has been shown to be active towards aromatic primary alco-
hols, and preferentially reduces aldehydes rather than oxidiz alcohols (257). Beyond these two enzymes, also the cytochrome P450 enzyme CYP120A1, encoded by slr0574, has been included in these studies, as its in vitro characterization has led to the suggestion that it accepts not only retinoic acid, but also retinal as a substrate and is able to introduce a single hydroxyl group at the C16 or C17 position of this latter substrate (252).

In conclusion, in the present study, we have characterized the role of sll1541 and slr1648 in retinal synthesis, and slr0091, slr0574, and slr1192 in retinal degradation in *Synechocystis*. We show that SynACO is an indispensable enzyme for retinal synthesis, while SynDiox2 seems to convert the same substrate(s) (*i.e.* apo-carotenoids) as SynACO, but presumably into a wider range of products than only retinal. SynDiox2, however, may be important for retinal biosynthesis in the late- or stationary phase of growth. As for retinal degradation, we show that slr0574 plays a crucial role in the retinal catabolic pathway.

Moreover, we also show reconstitution of apo-PR, expressed in a retinal-free *Synechocystis* strain, upon supplementation with retinal analogues into holo-proteorhodopsin. This paves the way to generate PR-expressing strains that can harvest infra-red light (>700 nm,) by supplementing the cyanobacterium with a strongly red-shifting retinal analogue.

**Materials & Methods**

**Strains and growth conditions**

Strains of *Escherichia coli* were routinely grown in LB-Lennox (LB) liquid medium at 37°C with shaking at 200 rpm, or on solid LB plates containing 1.5% (w/v) agar.

*Synechocystis* sp. PCC6803 (a glucose tolerant strain, obtained from D. Bhaya, Stanford University, USA) was routinely grown at 30°C with continuous illumination by white light at moderate intensities of approximately 45 μmol • m⁻² • s⁻¹ (≡ μmol photons • m⁻² • s⁻¹). Liquid cultures were grown in BG-11 medium (Sigma-Aldrich), supplemented with 50 mM sodium bicarbonate, 25 mM TES-KOH (pH 8) and appropriate antibiotics, and with shaking at 120 rpm (Innova 43, New Brunswick Scientific). The BG-11 agar plates were supplemented with 10 mM TES-KOH (pH= 8), 5 mM glucose, 20 mM sodium thiosulfate, and 1.5% (w/v) agar.
Where appropriate, antibiotics were added to the following final concentration: ampicillin (100 μg/ml), kanamycin (25 to 50 μg/ml), chloramphenicol (35 μg/ml), streptomycin (10 μg/ml), and spectinomycin (25 μg/ml), either separately or in combination.

**Strain construction**

Genomic sequences of slr1541, slr1648, slr0091, slr0574 and slr1192, which encode SynACO, SynDiox2, SynAlh1, CYP120A1, and AdhA, respectively, were derived from CyanoBase (259). Unless noted otherwise, PCRs were performed with the proofreading Pwo DNA Polymerase (Roche Diagnostics) or the Herculase II fusion enzyme (Agilent Technologies). Plates were incubated under low-intensity continuous illumination in a humidified incubator.

Null mutants of slr1541 (strain JBS14001) were constructed by double-homologous recombination with a fusion PCR product consisting of three fragments: a fragment of approximately 1400 bps adjacent to slr1541 (hom1), a fragment containing an omega antibiotic-resistance cassette, and a fragment of approximately 1400 bps adjacent to the complementary side of slr1541 (hom2). The hom1 and hom2 fragments were amplified from genomic DNA with primers JBS391 & JBS392, and JBS395 & JBS396, respectively, which introduced overlaps with the omega fragment. The omega fragment was amplified from pAVO-cTM1254 (260) with primers JBS393 & JBS394, which introduced overlaps with both the hom1 and the hom2 fragment. The three fragments were fused together in a PCR of 15 cycles without additional primers, after which primers and extra dNTPs were added and the PCR was continued for an additional 25 cycles.

Null mutants of slr1648 (strain JBS14002) were constructed using the same approach, except that a chloramphenicol resistance cassette was used as the marker. Primers JBS397 and JBS398, and JBS401 and JBS402 were used to amplify the corresponding hom1 and hom2 fragment from genomic DNA. Primers JBS399 & 400 were used to amplify the chloramphenicol resistance cassette from plasmid phaAHCmH (261).

The resulting fragments were gel-purified using the QIAGEN QIAquick Gel Extraction Kit (QIAGEN) or the Bioline ISOLATE II PCR and Gel Kit (Bioline) according to the instructions provided by the manufacturers.
Null mutants of \textit{slr0091} (strain QCSY001) were constructed by double-homologous recombination with a plasmid pQC016, that derived from plasmid pWD013 containing the omega antibiotic-resistance cassette. For pWD013 plasmid construction, upstream (hom1) and downstream (hom2) homologous regions (approximately 1000 bps each) of \textit{slr0091} were amplified from \textit{Syn
echocystis} genomic DNA with primers Adh-up-Fwd / Adh-up-Rev and Adh-
down-Fwd / Adh-down-Rev, respectively. Those primers also introduce overlaps between hom1 and hom2, and thus the generated fragments were fused together with Pfu DNA Polymerase (Thermo Scientific). After gel extraction and purification (Zymo Research), an extra adenosine (“A”) was added as the 3’ overhang of the fusion fragment, using Taq DNA Polymerase (Thermo Scientific). Then, this fragment was ligated to the BioBrick “T” vector pFL-SN (262). The omega antibiotic-resistance cassette, amplified with primers QC37/
QC38, was inserted between hom1 and hom2 of plasmid pWD013, using the XbaI restriction enzyme.

A null mutant of \textit{slr0574} (strain QCSY002) was constructed by double-homologous recombination with plasmid pQC015, carrying three fragments: A fragment of approximately 1000 bps adjacent to \textit{slr0574} (hom1), a chloramphenicol resistance cassette, and a fragment of approximately 1000 bps adjacent to the complementary side of \textit{slr0574} (hom2). Hom1 and hom2 were amplified from genomic DNA by primers QC43/QC44, and QC47/QC48, respectively, and then introduced into plasmid PFL-XN/Cm (+) (262), which contains a chloramphenicol resistance cassette, by plasmid restriction with the enzymes Nhel /PstI and XbaI, respectively.

Genome segregation

For transformations with mutagenic plasmids and linear DNA fragments, \textit{Syn
echocystis} sp. PCC6803 was grown until an OD$_{730}$ (optical density at 730 nm) of 0.2 to 0.3. Cells were then concentrated by centrifugation to an OD$_{730}$ of 2.5 in a volume of 100 μl of fresh BG-11 plus 20 mM TES-KOH (pH 8.0) in a sterile 1.5 ml Eppendorf cup. To this, a maximum of 10 μl of purified fusion PCR product or 1 μg of plasmid DNA was added. The mixture was incubated at 30°C in the light in a shaking incubator (regular growth conditions) for 5 to 8 h. Cells were then incubated on BG-11 plates containing 10 mM TES-KOH (pH 8.0), 5 mM glucose and 20 mM sodium thiosulfate, and supplemented with the corresponding antibiotic(s) at a low concentration. Single colonies were next plated on plates containing increasingly higher concentrations of antibiotic to promote genome segregation. The final concentrations of the antibiot-
ics used were: a mix of 25 μg/ml spectinomycin and 10 μg/ml streptomycin for the sll1541 mutant and the slr0091 mutant, 65 μg/ml chloramphenicol for the slr1648 mutant and the slr0574 mutant, and 20 μg/ml Zeocin for the slr1192 (deletion) mutants. Full segregation for all these strains was confirmed with PCR tests using MyTAQ polymerase (Bioline) with flanking primers JBS391 and JBS396 for Δsll1541, JBS397 and JBS402 for Δslr1648, Adh-up-Fwd and Adh-down-Rev for Δslr0091, QC43 and QC48 for Δslr0574.

The double null mutants of sll1541 and slr1648 (strain JBS14003) and of slr0091 and slr0574 (strain QCSY003) were created by transforming the segregated single mutants with the appropriate fusion PCR product or plasmid, using an identical protocol as described above. After full segregation, the continued presence of the first null mutation was confirmed by PCR as well.

Conjugation

The relevant strains were conjugated with plasmid pQC006 (46) (encoding His-PR) or plasmid pJBS1312 (46) (empty-plasmid control) as described in (46). The presence of the plasmids and the continued presence of the null mutations of sll1541, slr1648, slr0091, slr0574 and slr1192 were confirmed with appropriate PCR tests after the conjugation procedure.

Retinal identification and quantification

To investigate the retinal content of selected mutants, and its dependence on the cellular growth phase, batch cultures were grown under the same conditions (see growth conditions, above) and cells were removed at different growth phases for retinal quantification. Retinal was isolated, identified and quantified essentially as described before (46). In short, cell pellets were first processed to react with hydroxylamine so as to convert retinal to the more stable compound retinal oxime (233). The obtained extract was separated on an HPLC system with an EC 150/4.6 NUCLEOSIL 100-5 C18 column (MACHEREY-NAGEL), and n-heptane (HPLC grade) at 1 ml/min as the mobile phase. The retinal content was determined by the peak area of the oxime form of retinal, and compared with that of a series of known amounts of retinal (oxime).

To precisely quantify retinal A1 (all-trans retinal) and retinal A2 (all-trans 3,4-dehydroretinal) in a sample, the peak area was integrated at 354.2 nm and 367.8 nm, respectively, where the oxime forms of retinal A1 and A2 maxi-
mally absorb. Quantitative analysis of the molar ratio of retinal A1 and A2 in a sample, or in a mixture, was calculated based on the peak areas and the extinction coefficients of A1 and A2, taken as 49,000 and 44,000 M$^{-1}$·cm$^{-1}$, respectively (265, 266).

To present retinal production in units of the number of retinal molecules per cell, the number of cells was estimated on basis of the conversion factor that 1 ml culture of wild-type *Synechocystis* with an OD$_{730}$ = 1 contains $10^8$ cells, as determined with a Casy 1 TTC cell counter (Schärfe System GmbH, Reutlingen, Germany) (232).

**Isolation of His-tagged proteo-opsin from *Synechocystis***

His-tagged protein from *Synechocystis* cells was isolated and purified by using a HisTrap FF Crude column with 5 ml column volume, and an ÄKTA FPLC system (all from GE Healthcare, Uppsala, Sweden). Cell pellets were disrupted by use of a bead beater and the purification procedure essentially followed the protocol described in (46).

When necessary, all-*trans* retinal or the retinal analog (all-*trans* 3,4-dehydroretinal (retinal A2) or 3-methyl-amino-16-nor-1,2,3,4-didehydroretinal (MMAR)) was added in a solution of ethanol, separately or in combination, to the culture at the final concentration of 20 µM, when cell density of culture (OD$_{730}$) had reached approximately 2. Then, retinal or the retinal analog was added every 24 hours for two consecutive days.
Table 1: Strains or plasmids constructed for this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Source or reference</th>
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<td>Strains</td>
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<td><em>Synechocystis</em> sp. PCC6803</td>
<td>WT: A glucose tolerant <em>Synechocystis</em> sp. PCC6803</td>
<td>from D. Bhaya, Stanford University, Stanford, CA</td>
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<td>JBS14001: Spc&lt;sup&gt;R&lt;/sup&gt;, Str&lt;sup&gt;R&lt;/sup&gt;; Δsll1541:: Ω&lt;sup&gt;R&lt;/sup&gt;; chromosomal deletion of gene <em>sll1541</em></td>
<td>This study</td>
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<td>JBS14002: Cam&lt;sup&gt;R&lt;/sup&gt;; Δsll1648:: Cm&lt;sup&gt;R&lt;/sup&gt;; chromosomal deletion of gene <em>sll1648</em></td>
<td>This study</td>
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<td>JBS14003: Spc&lt;sup&gt;R&lt;/sup&gt;, Str&lt;sup&gt;R&lt;/sup&gt;, Cam&lt;sup&gt;R&lt;/sup&gt;; Δsll1541:: Ω&lt;sup&gt;R&lt;/sup&gt;, Δsll1648:: Cm&lt;sup&gt;R&lt;/sup&gt;; chromosomal deletion of gene <em>sll1541</em> and <em>sll1648</em></td>
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<td>A kind gift from Prof. T. Pembroke, Limerick, Ireland</td>
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<td><em>Escherichia coli</em></td>
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<td>Agilent Technologies</td>
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<td>J53/RP4: Helper strain</td>
<td>(263, 264);</td>
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<td>pQC006: kan&lt;sup&gt;R&lt;/sup&gt;; pJBS1312-based expression of pR; C-terminal 6×histidine tagged</td>
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<td>pFL-SN: Amp&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;; BioBrick “T” vector with XcmI on each side</td>
<td>(262)</td>
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<td>pFL-XN: Amp&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;; BioBrick “T” vector with Spel and Nhel and XbaI on one side of chloramphenicol cassette</td>
<td>(262)</td>
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<td>pWD013: pFL-SN derivate, Amp&lt;sup&gt;R&lt;/sup&gt;, containing upstream and downstream homologous regions of the <em>sll0091</em> gene</td>
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<td>pQC016: pFL-SN derivate, Amp&lt;sup&gt;R&lt;/sup&gt;, Spc&lt;sup&gt;R&lt;/sup&gt;, Str&lt;sup&gt;R&lt;/sup&gt;; containing <em>sll0091</em> gene upstream and downstream homologous regions and a Ω resistance cassette in between</td>
<td>This study</td>
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<td>pQC015: pFL-SN derivate, Amp&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;; containing <em>sll0574</em> gene upstream and downstream homologous regions and a chloramphenicol resistance cassette in between</td>
<td>This study</td>
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<sup>a</sup> Ω is short for the omega resistance cassette; Cm<sup>R</sup> represents the chloramphenicol resistance; while Amp<sup>R</sup> means the ampicillin resistance cassette; Kan<sup>R</sup> for kanamycin resistance; Zoe<sup>R</sup> for the zeocin resistance; Spc<sup>R</sup> for spectinomycin resistance and Str<sup>R</sup> for streptomycin resistance.
Results

Retinal synthesis in *Synechocystis*

Two genes, *i.e.* slt1541 (encoding SynACO) and slr1648 (SynDiox2), have so far been identified as a carotenoid cleavage dioxygenase (CCD) in *Synechocystis* (249). In *vitro* assays have shown that the product of both genes can convert β- apo-carotenals, but with different products as a result (see also Fig. 1): all- *trans* retinal for SynACO (250) and β-13-carotenone for SynDiox2 (252). However, due its rapid catabolism and limited in *vivo* stability, significant amounts of retinal were not detected in *Synechocystis* until recently. This situation changed after heterologous expression of a microbial rhodopsin in this cyanobacterium (*i.e.* a proteorhodopsin; hereafter: PR). It was observed that this allows the apo-form of PR to bind and protect all- *trans* retinal so that it can accumulate as the bound chromophore of holo-PR in the cells (45, 46).

**Figure 1: Tentative scheme of retinal synthesis and degradation in *Synechocystis*.** Summarizing available information from in *vitro* obtained data from the literature (250, 252, 257, 267). Solid arrows represent reactions that have been demonstrated in *vitro*, while dotted lines represent hypothetical pathways in *Synechocystis*. Moreover, the pathways in red and blue represent the presumed pathways for synthesis and degradation of retinal in *vivo*.

Based on the availability of the apo-PR expression system, as well as the knowledge from in the in *vitro* analyses, we have designed experiments to elucidate the pathway of retinal biosynthesis and degradation in *Synechocystis* cells in *vivo*. Accordingly, we first concentrated on the role of slt1541 and slr1648 in retinal synthesis. Our strategy was to quantify retinal content in various mutants, all containing plasmid pQC006 (which drives high-level expression of histidine-tagged apo-PR; (46)). These mutants additionally would carry
a deletion in one or both of the above genes (i.e. sll1541 and/or slr1648) introduced through natural–transformation based deletion mutagenesis. The resulting strains are referred to as: JBS14001, JBS14002, and JBS14003. Wild-type Synechocystis carrying plasmid pQC006 is the positive control in these experiments. To explore the effect of deletion of these genes on the growth of Synechocystis, batch cultures were grown at a moderate light intensity (~45 μmol • m² • s⁻¹) in BG-11 medium, supplemented with the antibiotic for plasmid maintenance (see Materials and Methods). Fig. 2A shows that, under our conditions, no significant difference in growth rate was observed between the wild-type control and the three mutants, be it that mutants JBS14001 and JBS14003 show a slightly higher final OD than the wild type.

![Graph A](image1.png)

**Figure 2:** All-trans retinal production in four Synechocystis strains grown in batch culture as a function of growth phase. Cells were grown in the BG-11 medium at the moderate light intensity. A) Growth curves monitored via the OD₇₅₀ for comparison of the wild type strain with deletion strains in sll1541 (JBS14001), slr1648 (JBS14002) and sll1541 plus slr1648 (JBS14003). Gene sll1541 and slr1648 encodes enzyme SynACO and SynDiox2, respectively. All strains were conjugated with plasmid pQC006 (for PR-His expression). Error bars represent the standard deviation of 3 biological replicates (n=3) and are only visible when they exceed the size of the symbols. B) all-trans retinal production, expressed in a bar graph as the number of all-trans retinal molecules per cell. Samples were taken at 26 h, 58 h, 146 h, and 218 h for all-trans retinal quantification by HPLC analysis. Error bars represent the standard deviation of 5 replicates.

From such experiments, cells were harvested at various time points to reveal the dependency of the retinal content on the growth phase of Synechocystis. After harvesting the cells, their retinal content was quantified by means of HPLC analysis (46). These results revealed that no retinal could be detected in the mutants JBS14001 and JBS14003, both of which carry a deletion of sll1541 (encoding SynACO). In contrast, strain JBS14002, carrying a deletion of slr1648 (encoding SynDiox2), had a slightly higher retinal content than the wild type, in both the exponential phase and in the linear growth phase.
Chapter 5

(represented by the samples taken after 26 h and 58 h, respectively), but still one-third less than wild type in the late stationary phase (i.e. after 218 h). These results strongly suggest that slt1541 plays the decisive role in retinal synthesis in Synechocystis; while SynDiox2 presumably consumes the same precursor(s) as SynACO, to convert these substrates into products other than all-trans retinal.

The typical growth-phase dependency of the retinal content observable in Fig. 2B in both the wild type and in JBS1402, i.e. that retinal levels dropped slightly during the linear phase of growth (at the 58 h time point), and then increased as cells gradually enter stationary phase, has been observed repeatedly. Significantly, in the wild-type strain, a sharp increase in retinal content was seen in the late stationary phase. Particularly slr1648 (SynDiox2) is probably important for this phase of retinal production (Fig. 2B).

Purification of histidine-tagged Proteorhodopsin from a mutant strain deficient in retinal synthesis

As the data presented in Fig. 2B clearly show that mutants with a deletion of slt1541 lose the ability to produce all-trans retinal, this allows for experiments with the aim of altering the chromophore of holo-PR in vivo. The success of this approach can easily be traced because of the typical property of the native holo-PR, i.e. the main absorption band with a maximum at 516 nm under slightly alkaline conditions. The corresponding experiment was carried out with strain JBS14003 (carrying a deletion in slt1541 and slr1648) containing plasmid pQC006 (for apo-PR-His expression). Cultures were grown under a mixture of red and blue light at a moderate combined light intensity (~ 35 μmol·m⁻²·s⁻¹, with or without exogenous addition of 10 μM all-trans retinal. Wild-type Synechocystis conjugated with plasmid pQC006, as a control strain, was grown under the same conditions, but without the addition of all-trans retinal. His-tagged proteorhodopsin was purified from harvested cells as described before (see Materials and Methods) and the UV/visible absorption spectrum of the eluted fractions was recorded by spectrophotometry.

As expected, the relevant fractions from the control strain (Synechocystis wild type (WT) + pQC006) showed a pink appearance (data not shown), and their spectra contained an absorption peak in the range of 400 to 600 nm, with a maximum at 516 nm (Fig. 3, dotted curve). These characteristics clearly suggest the presence of significant amounts of holo-PR. Significantly, none of these characteristics was observed for the corresponding fractions from the
strain JBS14003, containing plasmid pQC006 (Fig. 3, solid curve). Furthermore, when this mutant JBS14003 (plus pQC006) is supplied with exogenous all-trans retinal, as shown in Fig. 3 (dashed curve), the relevant eluted fractions do show the typical absorption spectrum of holo-proteorhodopsin. This leads us to conclude that slr1541 is indispensable for retinal synthesis in Synechocystis sp. PCC6803: Deletion of slr1541 completely halted the synthesis of all-trans retinal and subsequent formation of holo-PR. The peaks in these spectra in the range of 350 to 450 nm and 650 to 700 nm that consistently showed up in PR-containing fractions obtained from Synechocystis, show that small amounts of (presumably protein-bound) chlorophyll a (and possibly carotenoids) are present in these samples.

Reconstitution of apo-PR with a red-shifted retinal analogue in vivo

The previous experiment has shown that it is possible to regenerate holo-proteorhodopsin in Synechocystis cells by incubating a retinal chromophore together with apo-PR in a strain deficient in retinal synthesis (i.e. JBS14003 + pQC006). Because of our interest in a red-shifted, and retinal-based proton pump in Synechocystis (45), we selected the retinal analogue MMAR (Fig. 4), which was shown to red-shift the absorbance band of PR by about 50 nm relative to native retinal, with additional tailing out to about 850 nm (229). Thus we supplemented a Synechocystis batch culture with MMAR for reconstitution of apo-PR in intact cells of JBS14003 + pQC006.
The UV/Vis absorption spectrum of His-tagged proteorhodopsin, reconstituted with MMAR purified from *Synechocystis*, shows a main broad absorption peak with a maximum at 570 nm, and a low-energy shoulder from 700 to ~850 nm. This is the first time that a photo-active protein has been isolated from *Synechocystis* which can absorb light of wavelengths beyond 750 nm. This spectrum also reveals the presence of traces of contaminating chlorophyll (see also above). The absorption of these contaminants overlaps with our protein of interest in the range of 650-700 nm. In order to record a precise spectrum of PR from *Synechocystis* reconstituted with MMAR, we subtracted the spectrum of the apo-PR-His fraction from *Synechocystis* (from strain JBS14003 + pQC006). The spectrum of MMAR-reconstituted PR, isolated from *E. coli*, served as the positive control. Based on this, we conclude that the main absorbance band of these two proteorhodopsin analogues (i.e. isolated from *Synechocystis* and *E. coli*) is factually identical (Fig. 4B). The difference in the strength of the 280 nm band may be due to the correction procedure or to differences in the level of contamination with apo-protein and/or host proteins. These results also demonstrate that this MMAR retinal analogue is not metabolically modified prior to incorporation into PR.

![Figure 4: Incorporation of a retinal analogue in apo-proteorhodopsin in vivo. A strain deficient in retinal synthesis, JBS14003, conjugated with plasmid pQC006 (for expression of PR-His), was inoculated in BG-11 medium, with or without exogenous addition of the retinal analogue MMAR (chemical structure on top). Spectra were normalized on the 570 nm absorbance. A) MMAR-containing PR-His purified from *Synechocystis*; MMAR-containing PR-His purified from *E. coli*; and apo-PR-His purified from *Synechocystis* (for the explanation of the symbols: see inset in the figure). B) The corrected spectrum of MMAR-containing PR purified from *Synechocystis* and from *E. coli*.](image-url)
Binding affinity of apo-PR for retinal A1 and retinal A2 in *Synechocystis*

We exploited the fact that PR has binding affinity for both retinal A1 and all-trans 3,4-dehydroretinal (retinal A2) (Ganapathy et al., 2015) to investigate the relative affinity of apo-PR in *Synechocystis* for these two chromophores. Hereto, we utilized two mixtures with different ratios of A1 and A2 as the substrate for in vivo reconstitution. HPLC analysis of both mixtures, after their conversion to the oxime form (with hydroxylamine), showed that both samples contained two fractions, eluting at 2.85 min and 3.17 min, respectively (Fig. 5A). On the basis of their spectra, these two components have been identified as the oxime-form of A1 and A2, respectively (Foster et al., 1993). Quantitative analysis, on the basis of peak area and the respective extinction coefficient, suggests that their molar ratio in these mixtures is: $A1: A2 = (25.4 \pm 3.8): (74.6 \pm 3.8)$ and $(15.2 \pm 0.31): (84.8 \pm 0.31)$, respectively.

**Figure 5: Analysis of the binding specificity of apo-PR for retinal A1 and A2 in *Synechocystis*.** A) Elution pattern of the two chromophores from the HPLC system as measured via the absorption at 354.2 nm (solid line) and 367.8 nm (dashed line) — the absorbance maxima of the oxime form of retinal A1 and A2, respectively. B) The absorption spectrum of the two peaks separated by HPLC, which confirms that the compound eluting at 2.852 min is the oxime form of retinal A1 (solid line), while the compound eluting at 3.176 min is the oxime form of retinal A2 (dotted line). C) Incorporation of a mixture of retinal A1 and A2 into apo-proteorhodopsin in *Synechocystis* in vivo. A retinal-synthesis deficient strain, JBS14003, conjugated with plasmid pQC006 (for expression of PR-His), was inoculated with a mixture at a different molar ratio of all-trans retinal A1 and all-trans retinal A2. Holo-PR was then isolated from *Synechocystis*.
We supplemented the above two mixtures of retinal A1 and A2 with apo-PR in two separate cultures of Synechocystis (JBS14003+ pQC006). Upon isolation of the reconstituted and purified holo-PR-His, both samples showed an absorption peak with a maximum at 560 nm, but with a shoulder at 518 nm. This confirmed the binding of both retinal A2 and A1 to apo-PR-His. The ratio of the absorption maxima at 518 nm and 560 nm varied depending on the composition of the retinal mixture provided to the cells. HPLC analysis of chromophores re-isolated from the purified holo-PR-His samples from these cells showed that the chromophore composition (i.e. the ratio of retinal A1 over A2) of (21.5 ± 1.4) : (78.5 ± 1.5) and (16.1 ± 0.19) : (83.9 ± 0.19) of these protein samples is very similar to the chromophore ratio’s used for the in vivo reconstitution (see above paragraph). We therefore conclude that apo-PR has about the same affinity for these two chromophores and that the specificity of their binding does not change when PR is expressed in Synechocystis, while in the latter organism a significant part (i.e. about 50%; (46)) of the protein is embedded in the thylakoid membrane.

Retinal degradation in Synechocystis

Our previous study proved that Synechocystis has the ability to synthesize all-trans retinal, but apparently also rapidly degrades it because retinal cannot be detected in Synechocystis cells unless these cells heterologously express a proteorhodopsin. This indicates that Synechocystis may have developed (a) system(s) to efficiently degrade retinal. Current literature suggests that retinal degradation in vivo is mainly initiated either by enzymes from the aldehyde dehydrogenase 1 superfamily (ALDH) (253) into retinoic acid, or by an alcohol dehydrogenase (ADH), a retinol dehydrogenase (RDH) and/or an aldo-keto reductase (AKR) (254) into retinol. Synechocystis has several members in both the ALDH family and the ADH family, but no information was available so far in the literature with respect to how effectively ALDHs or ADHs in Synechocystis would convert retinoids like retinal.

To explore retinal catabolism in Synechocystis in vivo, we decided to investigate the role of ALDHs, ADHs, and CYP450s in retinal degradation by knocking out the relevant genes. Based on gene analysis and substrate specificity identified in vitro assays (255-257), the most promising candidates for this part of our study are aldehyde dehydrogenase SynAlh1, encoded by slr0091, a medium-chain alcohol dehydrogenase (AdhA), encoded by slr1192 and the cytochrome P450 isoform CYP120A1, encoded by slr0574.
With an attempt to see a significant increase in retinal content after deleting relevant genes, we first quantified the retinal content in mutants with a deletion of one or more of the above genes (i.e. slr0091, slr0574, slr0091 and slr0574, and slr1192, in the following referred to as strain QCSY001; QCSY002; QCSY003 and UL025; see also Table 1), while wild-type Synechocystis was taken as the control. However, although we took samples at four different growth phases from the culture of each strain, no retinal was detected in any of the mutants, or in WT, at any growth phase. This result is consistent with our previous observation that, in Synechocystis, heterologous expression of proteorhodopsin is strictly required for the protection of retinal against degradation.

Figure 6: Cellular retinal content in batch cultures of a PR-expressing Synechocystis strain as a function of the growth phase of the culture. Cells were grown in the BG-11 medium at the moderate light intensity. A) Growth curve of the wild type, conjugated with plasmid pQC006 (for PR-His expression), and monitored via the OD_{730}. B) all-trans retinal content and apo-PR expression level as a function of growth phase in wild-type + pQC006. Both are expressed as bars in units of the number of molecules per cell. Samples were taken after 74 h, 96 h, 138 h, 192 h, and 265 h for all-trans retinal quantification by HPLC analysis and for (apo-)PR-His quantification via Western blots. The data shown are from a representative experiment.

For that purpose, we conjugated our PR-expression plasmid pQC006 into all the above mentioned retinal degradation deletion mutants. As retinal is chemically rather unstable, particularly in the light, retinal accumulation due to disruption of degradation can be visible only when a significant fraction of apo-PR is existing in cells, so that apo-PR can stabilize retinal and make it detectable. Our previous study showed that both the apo-PR expression level and the retinal content in WT, conjugated with pQC006, changes during the subsequent growth phases (46), hence a time window has to be identified in which the apo-PR level is relatively high.
Fig. 6B shows a clear difference in the retinal content and level of apo-PR expression as growth progresses in WT + pQC006. The level of apo-PR expression continuously increased and reached a peak in the early stationary phase, followed by a continuous decrease. In parallel, the retinal content showed an overall increasing trend, but with a significant decrease at the start of the linear growth phase. By calculating the molar ratio of apo-PR to retinal, we found, among those five sampling points, that the highest ratio of apo-PR to retinal was present in the linear growth phase. Two independent biological experiments yielded a molar ratio apo-PR/retinal in that growth phase of 1.9 and 2.3. Therefore, we concluded that a time point in the linear growth phase provides a suitable time window to monitor a potential increase in retinal content in mutants impaired in their ability to degrade retinal. Beyond that, the late stationary phase also may be informative in this respect, as in this growth phase a high retinal content is consistently detected in WT+pQC006. Hence, this could also be a time window, where any effect of the null mutations could become apparent.

The retinal-degradation experiment was performed with five Synechocystis strains, carrying a deletion of gene slr0091 (encoding SynAlh1; strain QCSY001); slr0574 (encoding CYP120A; QCSY002); slr0091 and slr0574 (encoding SynAlh1 and CYP120A, respectively; QCSY003); or slr1192 (en-
coding AdhA; UL025), and the wild-type served as the control. All these strains were conjugated with plasmid pQC006 (for PR-His expression). From each culture, a batch of cells was harvested in the linear growth phase (OD$_{730}$ ~ 0.95) and in the stationary phase (OD$_{730}$ between 3 and 4) for all-trans retinal quantification. HPLC analysis of those samples shows that the retinal content of QCSY002 + pQC006 was higher than that of the WT + pQC006 in both linear growth phase and stationary phase (Fig. 7), which implies that the product of gene slr0574 is involved in retinal degradation.

**Discussion**

Retinoids (in particular retinal, retinol, and retinoic acid) are critical molecules for most forms of life with respect to vision, normal embryonic development, and for control of cellular growth, differentiation, energetics and death (242, 268, 269). Sequence alignment shows that genes with significant similarity to BCO I/BCO II (β-carotene-cleaving enzymes) are widely spread among the cyanobacteria (250, 270). Consistent with that, studies on cyanobacterial blooms in eutrophic lakes have revealed that retinal was widely detected in many of the 39 species of freshwater cyanobacteria and algae identified (271). Beyond that, earlier findings on the occurrence of retinylidene receptors in Calothrix (272); Anabena (273) Leptolyngbya (274); Nostoc sp. PCC7120 (275) and Gloeobacter violaceus PCC 7421 (276) confirmed the widespread occurrence of retinoids in cyanobacteria.

However, relatively little information on retinoid metabolism (and biological function; but see *i.e.* (273)) was documented for members of the cyanobacteria, although the characteristics of relevant enzymes from Nostoc sp. PCC7120 and Synechocystis sp. PCC6803 have been extensively investigated in *vitro*. Therefore, we have initiated a study to elucidate the metabolism of retinal in *vivo* in the model cyanobacterium *Synechocystis* sp. PCC6803, to start filling this gap and pave the way for further studies of retinoid metabolism and function in (engineered) cyanobacteria.

Our investigation on retinal synthesis shows that deletion of *sll1541* (encoding SynACO) completely impaired the ability of the cells to synthesize all-trans retinal in *Synechocystis*, which suggests that SynACO is decisively involved in retinal synthesis. This result confirmed its enzymatic activity identified in *vitro*. Beyond that, we also observed that deletion of *slr1648* (encoding SynDiox2) resulted in a considerable stimulation of retinal production during early growth stages. Enzymatic characterization of the activity of SynDiox2 has led to the
claim that its activity leads to the accumulation of β-13-carotenone. A subsequent study on NSC3 (also named NosDiox2, a homologue of SynDiox2) proved that NSC3 also consumes β-apo-carotenal, but cleaves it at the C-13 C-14 or C-13’ C-14’ double bond, so that it synthesizes β-apo-carotenone (277). Together with our data, this implies that SynDiox2 in Synechocystis actually competes with SynACO for the same substrates, so that deletion of slr1648 can drive more flux through SynACO, to produce more retinal. Strikingly, the study on NSC3 revealed a new cleavage position at the C15-C15’ double bond with certain substrates (277). However, no retinal was found in our strain JBS14001 + pQC006, the mutant in which SynACO had been deleted but SynDiox2 is still present. This enzyme, therefore, did not measurably cleave carotenoids at the C15 C15’ double bond, which would have directly generated retinal in Synechocystis, probably due to a lack of this substrate near the active site of the enzyme.

Moreover, Fig. 2 presents a clear growth-phase dependency of the retinal content in Synechocystis. A higher retinal content was observed in the stationary phase for both WT and JBS14002 (each provided with pQC006). This could be a consequence of the fact that both slr1648 and sll1541 have a higher transcription level in the stationary phase than in the exponential phase (278), which would allow the cells to synthesize more retinal. Another possibility is that retinal is protected by PR from degradation during growth and accumulated to a high content in the stationary phase while simultaneously PR is slowly enzymatically degraded. Presumably, the C-terminal his-tag will be one of the first elements to be removed but the retinal binding pocket may withstand extensive proteolysis and keep its function (279), until eventually, the binding pocket will fall apart. This obliterates binding of the anti-his-tag antibody in Western blotting but could still allow stabilization of retinal against degradation, so that a large excess of retinal over intact proteorhodopsin could be present.

Furthermore, a higher retinal content in the stationary phase was observed in WT + pQC006 than in JBS14002 + pQC006, whereas the transcription level of slr1648 reached a maximum under nitrogen deprivation conditions (278). We, therefore, consider it likely that slr1648 significantly contributes to retinal synthesis in the late stages of growth because of the higher transcription level, induced by nitrogen deprivation. However, how SynDiox2 positively affects retinal production is still unclear. Possible explanations include supply or delivery of higher affinity or higher Vmax substrates to SynACO.
In addition, a pattern observed in both WT + pQC006 and JBS14002 + pQC006 shows that net retinal production of *Synechocystis* decreased slightly in the linear growth phase (*i.e.* at 58 h). Possibly, in this light-limited growth phase, carotenoids are directed towards the assembly of more photosynthetic machinery, thereby less flux of carotenoids is available for retinal synthesis.

Investigation of retinal degradation in *Synechocystis* is a delicate task, as retinal itself is chemically rather unstable. Our experiments on the stability of retinal in *Synechocystis* cultures have shown that its half-life is less than 2 hours. In addition, we found $10^4 \sim 10^5$ molecules retinal per cell in WT + pQC006, but no retinal in WT cells, which implies that *Synechocystis* has the capability to efficiently synthesize and degrade (free) retinal. Moreover, deletion of related genes encoding presumed degradation enzymes did not lead to strong accumulation of retinal, which indicates the existence and a high capacity of a different mechanism, *i.e.* chemical- and multiple biochemical routes of degradation. Nevertheless, via the use of the expression of *apo-PR*, we observed that *slr0574* is involved in retinal degradation.

To determine the role of relevant genes in the retinal degradation pathway, a more straightforward approach would be to investigate the content and composition of retinoids (*i.e.* retinal, retinol and retinoic acid) separately among the various mutants and the WT. However, due to the instability of retinoids, complexity and overlap between different degradation mechanisms, quantitative estimation on retinoids is challenging. Instead, we propose to trace the fate of retinal in *vivo* in *Synechocystis* via supplementing $[^{13}C]$ retinal into the culture. Preliminary results have been obtained by the degradation of 20 µM 3-$[^{13}C]$ all-trans retinal degradation in a concentrated suspension ($OD_{730} = 5$) *Synechocystis* cells, harvested at the end of the linear phase of growth. Under those conditions, in a 24 h period, significant conversion of the retinal into retinol was observed (Q. Chen et al., unpublished observation). Neither the converse conversion, *i.e.* from retinol into retinal, nor from retinoic acid to retinal could be observed in wild type cells of *Synechocystis*, nor could any *holo-PR* be isolated from such incubations. The latter was also true for cultures of the SynACO deletion mutant JBS14001.

Another highlight of our study is that we show that a photo-active protein could be isolated from *Synechocystis* which can absorb light with wavelengths beyond 750 nm. While this protein has a low absorbance cross-section in the near infra-red, its pumping activity has been confirmed at 730 nm ((229)). Thereby, our work paves new ways to generate *Synechocystis* strains which
can exploit photons beyond 750 nm for (oxygenic) photosynthesis. This latter conclusion is reinforced by our recent observation that PR can also accelerate phototrophic growth in a PSI-deletion strain of *Synechocystis* (chapter 6).
Supplementary material:

Table S1: the primers used in this study

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</table>

a Ω is short for the omega resistance cassette; Cm for the chloramphenicol resistance cassette. Forward primers are indicated with (F); reverse primers with (R). b Sequences that overlap with the sequence of the target fragment are underlined. Sequences that overlap with the adjacent fragment for the fusion PCR are in italics.
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 CO₂ 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 cyanobacteria 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 (ΔPSI strain). In such a ΔPSI 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, i.e. to provide 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 (i.e. proteorhodopsin (PR) and Gloeobacter rhodopsin (GR)) on both photoautotrophic and photomixotrophic growth of a ΔPSI strain of 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 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. Gloeobacter rhodopsin did not measurably enhance the growth rate of the 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.

Materials & Methods

Strains and growth conditions

Strains of 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 ΔPSI-derivative of 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 μmol • m⁻² • s⁻¹ (containing 3 μmol • m⁻² • s⁻¹ red, 25 μmol • m⁻² • s⁻¹ green, and approximately 0.3 μmol • m⁻² • 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'-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 ΔPSI *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 ΔPSI 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 × 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 $^{32}\text{O}_2$ and $^{36}\text{O}_2$, 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 36O$_2$ 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>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<td>(23)</td>
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<td>(24, 25);</td>
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<td>(22)</td>
</tr>
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<td>pQC006</td>
<td>kan R; pJBS1312-based expression of PR, C-terminal 6×histine tagged</td>
<td>(22)</td>
</tr>
<tr>
<td>pQC012</td>
<td>kan R; pJBS1312-based expression of GR, C-terminal 6×histine tagged</td>
<td>(22)</td>
</tr>
</tbody>
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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 × 10^8 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 (∆PSI + pJBS1312) consistently maintained a larger cell size than the rhodopsin expressing strains (PR-His or GR-His, refers to strain ∆PSI + pQC006 and ∆PSI + 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)

Figure 2: PR-stimulated growth in ΔPSI Synechocystis strains in the absence of glucose. Growth comparison among two strains: ΔPSI Synechocystis with the empty plasmid pJBS1312 (triangles), or plasmid pQC006 that leads to expression of PR-His (circles). Both strains were grown in BG-11 medium with 10 mM glucose added. Cells were harvested and then washed three times with fresh medium before being re-suspended in fresh BG-1 medium (i.e. without 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 (OD₇₃₀; A); the number of cells per ml (B); and mean cellular diameter (C); the last two characteristics were measured with a Casy Counter.
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.

![Graph A: The abundance of gene encoding PR vs. Time (h)](image)

![Graph B: The abundance of gene encoding GR vs. Time (h)](image)

**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.

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 $32\text{O}_2$ and $36\text{O}_2$ as a function of time. If a reaction is initiated with the only H$_2^{16}$O present, any increase in the concentration of $32\text{O}_2$ 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 $36\text{O}_2$ 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 ($\leq 25$ 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 $\geq 75$ 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 O$_2$ per 10$^9$ 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 ∆PSI 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 ∆PSI 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 ∆PSI 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 ∆PSI strain only occurred upon illumination with green light, and not with orange-red light. Under photomixotrophic conditions, the PR-expressing strain (∆PSI + 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. ∆PSI + 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 (535nm) 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. 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 ∆PSI strain of *Synechocystis*, that suffers from severe energy limitation, because of this genetic lesion in light-dependent linear- and cyclic electron flow. In the ∆PSI 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 ∆PSI strain showed no significant difference in growth rate as compared to the ∆PSI 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 ∆PSI strain (∆PSI+ pQC012) relative to an ‘empty’ plasmid-carrying strain (∆PSI+ 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 (∆PSI+ pQC006) and the ‘empty’ plasmid-carrying strain (∆PSI+ 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 ∆PSI 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 (∆PSI+ 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 ∆PSI Synechocystis**

To explore the mechanism by which PR may stimulate the growth of the ∆PSI 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 ($\lambda_{\text{max}} = 535 \text{ nm}; \leq 200 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), 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 ∆PSI strains expressing PR. Oxygen uptake, however, in this ∆PSI 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 + pQC006) 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 + pQC006) 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 OD₇₃₀. 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 OD₇₃₀), 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.** The main energy transducing complexes from the thylakoid membrane of this strain are shown, including their proton-translocation stoichiometry. The full and broken arrows in the NDH-1 complex represent the more and less favoured direction of catalysis, respectively.

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 μmol • m⁻² • s⁻¹ • OD₇₃₀⁻¹. 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’.

Acknowledgments

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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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Chapter 7

General discussion: Potential applications of PR-based phototrophy and the challenges in exploring its physiological effect in vivo

Que Chen, Filipe Branco dos Santos, Klaas J. Hellingwerf
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I Ion-pumping rhodopsins in cyanobacteria

Light-driven ion-pumping rhodopsins are widely distributed in microorganisms (71, 319, 320) and encompass three members: outward H\(^+\) pumps (60, 66, 95, 99), outward Na\(^+\) pumps (50-53) and inward Cl\(^-\) pumps (54-56). One representative example of each membrane is H\(^+\) pump (BR) from *Halobacterium salinarum* (60); Na\(^+\) pump rhodopsin (KR2) from flavobacterium *Krokinobacter eikastus* (321) and inward Cl\(^-\) pump (HR) from *Halobacterium salinarum* (322); see Figure 1). Studies on ion-pumping rhodopsins have emerged since the 1970s, Light-driven Na\(^+\) and Cl\(^-\) pumps were identified very soon afterward (60, 322, 323). But only recently, was it recognized that rhodopsins have the capacity of also pumping other ions such as Li\(^+\) (52) or SO\(_4^{2-}\) (324). Site-directed engineering can broaden this range of ion-selectivity even further to include Cs\(^+\) and K\(^+\) ions (57-59), via increasing the size of selectivity filter pore. Moreover, the functional conversion from Na\(^+\) or Cl\(^-\) to H\(^+\) pumps or Na\(^+\) to Cl\(^-\) pumps has been achieved (325, 326).

Figure 1. Functional classification of ion-pumping rhodopsins. Structures of bacteriorhodopsin (BR; PDB ID: 1C3W); halorhodopsin (HR; PDB ID: 1E12); Krokinobacter rhodopsin 2 (KR2; PDB ID: 3X3C), are shown as cylinder models. The colored cylinders denote helices forming the ion-conducting pathway in BR, HR, KR2. Adapted from (329) with permission.

Cyanobacteria have been shown to contain sensory rhodopsins, as light sensors, for modulating biological activities like phototaxis or the circadian clock (319, 320, 327). In contrast, only a few ion-pumping rhodopsins have been reported from this phylum, like xanthorhodopsin-like proton pumps from *Gloeobacter violaceus* PCC 7421 (289) and *Oscillatoriales cyanobacterium* JSC-12
(328), and the inward-directed Cl- pump (MrHR) from cyanobacterium *Mastigocladopsis repens* (325). Although so far only a few examples are available from oxyphototrophs (note that many more examples are available from anoxyphototrophs), the co-existence of chlorophyll $a$ (Chl $a$) based phototrophy and retinal-based phototrophy in a single organism, brings up an interesting question on the evolution of the above two types of phototrophy (see below). Moreover, this co-existence also indicates a promising possibility of the heterologous introduction of retinal-based phototrophy into a cyanobacterium with the aim of enhancing the photosynthetic efficiency (103).

### II Stability of gene expression mediated by different expression system in *synechocystis*

Both expression vectors and chromosomal integration are widely applied to introduce foreign genes into cyanobacteria. Each approach has its unique (dis)advantages, and the choice between them depends on the overall goal and practical conditions (*i.e.* the level of overexpression required, the number of genes of interest, the genetic background of the strain, precise control of gene copy number, the utilization of resistance genes, *etc.*). Generally speaking, introducing a foreign gene via a plasmid vector is less time-consuming, in comparison with the integration of genes into the genome, especially when working with a strain containing multiple copies of its chromosome. However, plasmids cannot offer an equally precise control of gene copy number as chromosomal integration, even in selective medium. As the optimal plasmid copy number in bacterial cells is often a trade-off between a desired gene dosage effect and the corresponding metabolic burden, and also affected by many additional factors, *i.e.* the gene inserted, growth conditions and growth stage (298, 299). These characteristics limit the utility of expression vector when introducing genes involved in key metabolic pathways. Moreover, the widespread use of antibiotic cassettes as a selective pressure agent, especially in pilot scale photobioreactors, is a concern because of the risk of spreading antibiotic resistance.

Utilization of an endogenous plasmid as expression vector attracts great interest, as this approach utilizes the high stability of gene expression over multiple sub-cultures, even after the removal of selective pressure (330), as well as, a faster gene segregation process. Moreover, via a high-copy-number plasmid, expression levels could be achieved approximately 10 times higher than achievable via chromosomal insertion (330). Very importantly, endogenous plasmids are recognized as ideal expression vectors to produce products in
the stationary phase, as both the plasmid copy number and expression level of most genes encoded by these plasmids increases during the entry into a stationary phase of *Synechocystis* sp. PCC6803 (331).

In this project, we aim to engineer PR as a complementary photosystem in *Synechocystis*. This requires the host to express PR at the highest possible level and through all growth phases. With this in mind, we decided to express PR via a high-copy-number plasmid rather than via chromosol integration, as about 12 copies of the genome is generally assumed to be present in each *Synechocystis* cell (332). Moreover, we chose a broad-host-range RSF1010 replicon (pVZ321) (333) as an expression vector, which allows one to express PR and measure the function of PR in a range of bacterial hosts. This characteristic brings us a lot of conveniences when it comes to measuring the proton pumping activity of PR. Light-driven proton pumping activity by PR can be detected in *E. coli* (see chapter 3 and chapter 4) but not in *Synechocystis*, as green light also triggers the light reactions, and coupled proton translocation, of the native photosystems.

However, in chapter 6, we reported the phenomenon that loss of the PR gene from plasmid pQC006 occurred when cells entered the stationary phase. Initially, this was investigated in the stationary phase of a mono-culture of strain ∆PSI+ pQC006. Next, however, we also observed this phenomenon in a mixed culture of strain ∆PSI+ pQC006 plus ∆PSI+ pJBS1312 in the stationary phase. The results above indicate that the exogenous plasmid shows considerable instability when its host cells enter the stationary phase. Presumably, this is caused by the metabolic burden of expressing PR and/or a structural instability of the plasmid vector (300).

**III Transcriptional regulation of gene expression in *Synechocystis***

Genetic instability is recognized as an increasing concern when engineering metabolism of *Synechocystis* for biotechnological applications. Many studies have shown that high production levels are often accompanied by a severe genetic instability (see review (334)), partially due to a high level of heterologous protein(enzyme) expression. To construct a cell-based production system with a high and stable production level over multiple generations, a well-characterized and tightly-regulated expression system is required. The easiest approach to build such an expression system is to develop well-characterized inducible promoters.
*Synechocystis* has the capacity to adjust the cellular transcriptional level in response to the environmental conditions, via its native-inducible promoters (*i.e.* light responsive (*psa*, *psb*, and *secA*), dark-inducible (*lrtA*), nitrate/nitrite inducible (*nirA*), and heavy metal-ions inducible promoters) (335). Among these inducible native promoters, some achievements on fine-tuning gene expression via heavy metal-ions inducible promoters have been reported recently (336-338). However, an obvious drawback of using native inducible promoter is that they could cause cross-talks with the host’s genetic background. To solve this problem, developing non-native inducible promoter holds significant importance, especially after it had been demonstrated that some inducible promoters characterized in *E. coli*, like the LacI-regulated P*trc* and P*tac* promoters, do not function in *Synechocystis*. Studies via systematic genetic engineering generated new functional and inducible promoters, like the TetR-regulated promoter (335), and the P*pcpG2* system (339). Moreover, another two inducible promoters: the nirA promoter of *Synechococcus* (340) and PA1lacO-1a (341) can be used as a tightly inducible system for controlling transgene expression in *Synechocystis*, and very importantly, the latter even shows a strength comparable to the P*trc* promoter.

In chapter 3, we compared the PR expression level from three plasmids, each with a different promoter driving PR expression: the LacI-regulated P*trc* promoter, the native P*psbA2* promoter and the RNase P subunit B promoter P*rnpB*. The P*trc* promoter is one of the strongest, and most commonly used, promoters to express and over-express genes in *Synechocystis*, while the P*psbA2* promoter is one of the strongest native promoters in *Synechocystis*, expressing the D1 subunit of Photosystem II. The P*rnpB* promoter is considered to be constitutive in *Synechocystis* under standard cultivation conditions. In terms of promoter strength, P*trc* is the strongest one while P*rnpB* is the weakest one among above three promoters.

Contrary to expectations, when introducing PR in *Synechocystis*, the highest level of PR-His expression, up to $10^5$ molecules per cell, was found in the strain in which the *psbA2* promoter controlled its expression. No expression could be demonstrated in the strain with P*trc*-driven expression, which was likely due to genetic instability of this construct, presumably due to a heavy metabolic burden or restrictions in the available membrane space. Nevertheless, compared to other organisms (see review (171)), we achieved 2 to 10 times higher PR expression levels in *Synechocystis*, likely because *Synechocystis* has two distinct inner membrane fractions: the cytoplasmic membrane (CM) and the thylakoid membrane (TM). Presumably, the multiple thylakoid-
membrane layers in *Synechocystis* significantly increase the available membrane surface for heterologous PR expression. In line with this assumption, in chapter 3, we show that PR-His integrates into the CM and TM fraction equally on a protein-content basis.

**IV The potential biotechnological application of PR-based phototrophy**

PRs have the capacity to convert solar energy to chemical energy in the form of a PMF, which as a secondary effect, can stimulate cellular enzymatic activities that depend on the PMF, like the ATP synthase, the flagellar rotary motor, active transport systems, voltage-gated and ion channels, *etc.* (see review (342, 343)). As a consequence, PRs expression could bring the biological host a benefit in many different respects, like growth rate, cell yield, biomass yield, cell survival, the rate of substrate uptake, motility, product extrusion, *etc.* (68, 69, 86, 87, 89-92). Such enhancements, however, generally require nutrient-limitation or stress conditions before they become detectable (93).

**IV.a Optimization of the ATP/NADPH ratio**

Cyanobacteria, as the most promising cell factories for a high-value commodity- and biofuel production (5-8, 11-13), are required to convert solar energy into biomass and/or products with the highest possible efficiency. The ATP/NADPH ratio, as the output of oxygenic photosynthesis and input of cellular anabolism, plays a crucial role in regulating the photosynthetic efficiency and the metabolic fluxes (344-346). Too extreme values of the ATP/NADPH ratio would both cause a stress reaction and hinder cellular fitness. To keep the ratio of the rates of formation of ATP and NADPH optimal, a system that can quantitatively optimize the ATP/NADPH ratio and is well-characterized and tightly-regulated is urgently needed.

It is widely accepted that, in *Synechocystis*, the ATP/NADPH-output ratio of linear electron flow (LEF) is 1.28, lower than the desirable ratio of 1.5 for the Calvin cycle (344, 345). To increase this ratio to 1.5, *Synechocystis* naturally developed several different forms of cyclic electron transfer (CEF), in which electrons follow a cyclic path and eventually lead to the production of ATP only, while consuming part of the light energy (145, 345). This mechanism allows the cells to optimize the actual ATP/NADPH ratio, by decreasing the rate of production of NADPH relative to the amount of ATP formed. With the
assumption that all the different forms of CEF have a tentative $2\text{H}^+/\text{e}^-$ stoichiometry, one can calculate that, to generate an ATP/NADPH ratio of 1.5 from photosynthesis, approximately 20% of the electrons from water splitting have to be handled through cyclic electron flow, which would lead to a corresponding decrease in the NADPH production rate of 20%, compared to conditions with LEF reactions only. However, introducing an isolated system for only ATP synthesis could also solve this problem, for example, PR-based phototrophy, which converts light energy into a proton motive force and next into ATP, via transient energy storage in the retinal chromophore (45), thereby stimulating ATP synthesis without interfering with LEF. Furthermore, our results in chapter 4 show that the pumping activity of PR is triggered by green light. More importantly, the pumping rate increases hyperbolically with light intensity up to very high intensities. These characteristics point out that engineering PR-based phototrophy in *Synechocystis* forms a promising opportunity to adjust the ATP/NADPH ratio in a quantitative way in *vivo*.

Because *Synechocystis* has a significantly lower absorption in the green than in the red and blue part of the visible spectrum (*i.e.* 450-550 nm) (27), a range that is, however well covered by the absorption spectrum of many PRs (67, 173), introduction of PRs into such organisms could supply additional free energy to this oxyphototrophic organism (170, 171, 344), when green photon are in excess. This system can even be more valuable to the cell when the window of absorption of PR is shifted beyond 700 nm (see further below).

**IV.b Artificial photosynthesis**

Cyanobacteria are less suitable biological hosts for production schemes that involve oxygen-sensitive enzymes, like hydrogenases and nitrogenases (347, 348), as these organisms evolve oxygen naturally during photoautotrophic growth. The conversion of heterotrophic bacteria to photoheterotrophs, by introducing PR-based phototrophy, would keep the advantage of the metabolic versatility of heterotrophic bacteria, and meanwhile, obtain a solar energy conversion system. Merging of these two assets could be leveraged to produce a bigger variety of useful commodities. This concept becomes even more attractive if one can engineer into heterotrophic bacteria the ability to fix CO$_2$. This latter capacity has already been transferred to *E. coli* (349, 350). However, such a system does require an electron donor, such as hydrogen or a reduced form of sulphur, nitrogen, iron or manganese.
IV.c Industrial application of bacterial rhodopsins

At the factory scale of biofuel production, biological contamination/invasion is a significant problem especially when the cultivation process is excessively expensive and time-consuming. A common solution to this problem is to grow cultures under extreme environmental conditions, like a very high (or very low) salt concentrations or pH (351, 352). Our results shown in chapter 4 demonstrate that PR improved the growth of *Synechocystis* under high salt conditions (0.8 M), presumably because the extra PMF (or ATP) generated by PR helps the cells to extrude Na$^+$ ions. Expression of PR, therefore, could increase the salt resistance of its host and thereby further optimize its competitiveness against invading organisms. Expressing PR can also stimulate the proton-substrate symporters, which either ease the uptake of substrates like sugars, Vitamin B1 (353-355) or the export of products, which would improve the productivity (356, 357), especially for toxic or harmful compounds. Beyond that, extrusion of products would also ease the following process of harvesting the biofuel.

IV.d Construction of an infrared-absorbing microbial rhodopsin

Another important application of PR is to construct an infrared-absorbing microbial rhodopsin. This not only holds significant importance in this project, *i.e.* in the attempt to complement a PSI-deletion strain, while simultaneously maximizing the photosynthetic efficiency, but is also desirable in biotechnological applications of optogenetics (66, 358), as infrared-absorbing radiation penetrates deep into tissues and is promising to be applied for the non-invasive remote manipulation of neural functions.

Moreover, microbial rhodopsins are more desirable than animal rhodopsins in optogenetic applications, because all-*trans* retinal is more abundant than 11-*cis* retinal in brain cells, and “photobleaching” does much less affect microbial rhodopsins (359). PR has been recognized as the most promising variant of the microbial rhodopsins, as its high abundance, genetic diversity, broad accessibility and the successful heterologous expression has been reported. Constructing an infrared-absorbing microbial rhodopsin on basis of PR would generate a broad-applicable and useful system. Currently, the studies on red-shifting the absorption spectrum of rhodopsins have been carried out via protein modification and/or chromophore substitution (229, 360, 361). Success in the sense of one infrared-absorbing PR, with significant pumping rate, has already been achieved (229).
V The challenges in estimation of the physiological effect of PR expression in vivo

Contrary to the widespread occurrence of PRs among microorganisms (70, 88, 161, 162, 227, 362, 363), PR-mediated enhancement of growth and starvation survival have currently been identified only in a few natural hosts (68, 69, 86, 87, 89-92). Given the great diversity of phylogenetic, genomic, and physiological backgrounds of natural PR-containing hosts, it would be no surprise if PR-based phototrophy might benefit its host via different mechanisms. Among those, promotion of starvation survival and stimulation of growth would be the two extremes, while some more subtle physiological or ecological benefits are still too delicate to explore. Furthermore, the interactions between a rhodopsin and other energy-transducing systems could make this task even more challenging.

Another important approach to explore the physiological effect of PR activity is by heterologously expressing PR in a model organism like *E. coli*. This approach has already led to valuable results (61, 163-166, 168). However, a considerable amount of negative, largely unpublished, results testify to the difficulties in estimating the physiological effect of PR phototrophy. Apart from the expression level of PR, and some environmental factors, below we describe some additionally important issues that matter in such an approach.

V.a A condition of a limited proton motive force is strictly required

Light-driven proton pumping results in the establishment of a proton motive force (PMF), comprised of both an electric field- (Δψ) and a proton concentration gradient- (ΔpH) component. The size of the PMF is, next to the rate of PMF-consuming processes, mainly dependent on the buffering capacity of the lumen or extracellular space and the counter-ion movements, such as efflux of cations (mainly K⁺, Mg²⁺) or influx of anions (Cl⁻), through ion channels. It has been recognized that the PMF has a dual function: beyond that, it contributes to the synthesis of ATP, it also drives down-regulation of the rate of electron transfer at the level of the cytochrome b6/f complex. Furthermore, a high ΔpH has been demonstrated to result in energy dissipation via non-photochemical quenching (NPQ) (see review: (346, 364)). This implies that a moderate increase in PMF could improve energy conversion and generate additional ATP, but an excessively large PMF, especially ΔpH, would then hinder electron transfer or reduce quantum efficiency, thereby slowing down the
photosynthetic machinery. These two opposite extremes of the function of the PMF may explain why the PR-mediated growth or survival stimulation was only observed under nutrient-limiting- or stress-conditions in which the PMF in limited. Another complication may be a strongly light-dependent expression level, just as for some cyclic electron transfer systems from anoxygenic photosynthesis (365).

Our MIMS data (in chapter 6) shows that, under the illumination with green light, but not with orange-red light, the PR-expressing strain mediated a lower light-driven oxygen evolution (30-50% less) than the control strain (a strain carrying an empty plasmid). This observation holds true for both WT Synechocystis and ΔPSI Synechocystis. This green light-mediated effect in the PR expressing strain can presumably be attributed to an increase in PMF. This reinforces the conclusion that PR is functional in both WT and ΔPSI Synechocystis. Nevertheless, when investigating the stimulatory effect of expressing holo-PR in Synechocystis, PR-mediated growth stimulation, compared to a control strain without PR, has only been clearly observed in ΔPSI Synechocystis (see chapter 6) and much less in WT Synechocystis (see chapter 3) under standard conditions. Beyond that, in chapter 4, it has been demonstrated that PR increased the growth rate of WT Synechocystis under high salt conditions (0.8 M). Overall, in line with the current reports, our data shows that the benefit of being able to use PR-based phototrophy has only been observed when the PMF in the PR-expressing cells is limited.

V.b Light intensity and light quantity matters the most

Obviously, light matters the most in studies exploring the effect of PR. Firstly, in chapter 4, our data shows that the pumping activity of PR/GR is tightly regulated by light and the pumping rate increases hyperbolically with light intensity. However, it does not mean that applying an extremely high light intensity to a biological host is desirable, as high light intensities can be a threat to trigger a toxic effect on the bacteria and/or induce a photo-inhibition reaction in phototrophs. Particularly, when working with a phototroph, one may have to consider the competition between the natural photosystem(s) and PR-mediated phototrophy, as well as the turnover rate of each system. For instance, the Z-scheme in Synechocystis has a turnover frequency in the order of hundreds per second (366), while approximately 10 per second is the maximal turn-over rate for PR (79, 82). In addition, the former system has a higher stoichiometry of 1.5 H⁺ transferred per photon than 1H⁺ per photon for PR. These characteristics indicate that PR would only boost the energy conversion when extra
photons \((i.e.\text{ in the 450-550 nm range})\) are available. In a better scenario, introducing a new photosystem which complements the absorption spectrum of the native photosystem(s) would bring a larger stimulatory effect, like introducing into *Synechocystis* an infrared-absorbed proteorhodopsin.

**V.c Availability of retinal is essential.**

A functional PR, as a light-driven proton pump, requires not only the apo-protein but also stoichiometric amounts of its chromophore, all-trans retinal. Retinal, however, is chemically unstable: micromolar concentrations of retinal added to a batch culture of *Synechocystis* are catabolized with a half-life between 1 and 2 h. In an attempt to observe the highest possible benefits of PR expression, one has to guarantee the availability of all-trans retinal to the newly synthesized apo-PR during an entire growth experiment. Regarding those biological hosts, which have an endogenous pathway to synthesize all-trans retinal, one should investigate the retinal production in a time course and ensure its sufficient production in accordance with apo-PR expression level.

In chapter 4, *sll1541* (encoding SynACO) has been identified as the determining gene for retinal synthesis in *Synechocystis*. Moreover, a clear growth-phase dependency of the cellular retinal content has been recognized in both chapter 3 and chapter 4. Retinal content and apo-PR or apo-GR expression level increase more or less in parallel before cells enter the stationary phase, while, a roughly 10 fold higher retinal content is observed in the stationary phase. The latter phenomenon could be a consequence of a higher transcription level of *sll1541* in the stationary phase and/or a protection of retinal against degradation in partially degraded PR molecules.

**VI On the evolution of photosynthesis**

At the time of discovery of the existence and function of bacteriorhodopsin, this retinal-based photosynthetic system was generally considered to be a peculiarity of ultra-saline, and often alkaline, environments like the Dead Sea in which organisms like *Halobacterium halobium* and *H. marismortui* are thriving (367). In the same period, the general consensus was that chlorophyll-based photosynthesis had its evolutionary roots in anoxygenic photosynthesis, which was known for decades to thrive in fresh-water lakes (368). Therefore, at the time, the idea could be entertained that retinal-based- and chlorophyll-based photosynthesis are adaptations to specific extracellular physico-chemical conditions. In line with this, the two types of photosynthesis were thought to
have separate origins in the Archaea and the Bacteria, respectively (369), as they initially appeared to cluster together in these two different clades of canonical phylogeny.

This changed with the discovery of more and more anoxyphototrophic bacteria in extreme environments, like some Ectothiorhodospiraceae, that also have a preference for growth in alkaline soda lakes (370). This led Hellingwerf et al. (371) to bring up the question of the evolutionary competitiveness of these two types of photosynthesis. A superficial comparison would lead to the expectation that the chlorophyll-based type would outcompete the retinal-based photosynthesis because of its higher efficiency supported by the 2 (versus 1) pumped protons per photon and a much broader absorption cross section. These considerations were later confirmed by much more detailed calculations of Kirchman and Hanson (372), who added the suggestion that the bacterial rhodopsins might provide selective advantages at aerobic conditions with very high light intensities. It is relevant to note that the impact of these calculations meanwhile also had significantly increased after the discovery that the proton-pumping bacterial rhodopsins have a wide phylogenetic distribution (see review: (67, 320, 342) in organisms that are abundant, particularly in the marine environment (70, 88, 161, 162, 227, 362, 363).

Meanwhile, the ‘mystery’ of the co-existence of the two types photosynthesis has been simplified and intensified by the observation that the genetic code required to express both systems occurs in several organisms. This holds for a number of cyanobacteria (289, 325, 328) that combines retinal-based photosynthesis with oxygenic photosynthesis, but also for several obligate aerobic anoxygenic photosynthetic bacteria (i.e. that carry out retinal-based and bacteriochlorophyll-based photosynthesis) of the Roseobacter clade (97, 373, 374). In these latter organisms both types of photosynthesis function as rather isolated metabolic units that carry out light-driven proton extrusion to contribute to the generation of the proton motive force (46).

As alluded to above, in phylogenetic terms, the origin of photosynthesis has for a long time been assumed to be tetrapyrrole-based (i.e. chlorophyll- or bacteriochlorophyll-based), and was possibly even at the very basis of the ‘origin of life’ (1). The development of the concept of the three Domains of Life (369) then implied that photosynthesis had originated twice: (i) the tetrapyrrole-based form in Bacteria; and (ii) the retinal/poly-isoprene based form in Archaea. The concept of the ‘tree of life’, however, suffers from some simplifications, like the neglect of events of horizontal gene transfer. Because of the
incongruence of the early results of the systematic- and phylogenetic classification, the possibility that lateral gene transfer might have obscured the phylogenetic classification was intensely discussed (i.e. (375)). However, yet another major event/m Mechanism has contributed to the emergence of life as we know it: The endo-symbiotic merging of organisms (376). The best-known example of the latter is the presumed engulfment of an α-proteobacterium by a wall-less archaeon (377).

Figure 2: The rings of life are summarized in this figure. The eukaryotes, shown in purple at the top of the rings, are the result of the convergence of multiple gene flows. The Proteobacteria are present in the upper left green ring representing the flow from the double membrane prokaryotes into the eukaryotes that introduced mitochondria and chloroplasts into the eukaryotes (shown in purple). The second flow of genes into the eukaryotes is shown in cyan at the top right. It corresponds to the gene flow that transported informational genes into the eukaryotic nucleus from the eocytes. This gene flow includes many proteins and RNAs that are involved in fundamental cell/ molecular processes that are unique to eukaryotes and eocytes. Examples include the eocyte/eukaryotic ribosomal apparatus for protein synthesis, the mechanisms for RNA transcription, and the unique chromatin structures that are used for the bundling of chromosomes into nucleosomes. The root of the rings of life is shown at the lower left of the figure. This set of rings leads to the Actinobacteria, to the Firmicutes, to the Halobacteria, and to the double-membrane prokaryotes, including the Proteobacteria. Copied (with permission) from (379).

With considerations about lateral gene transfer and endo-symbiotic events in mind Lake (378, 379) has formulated the hypothesis of the ‘rings of life’ (Fig. 2). This hypothesis suggests indeed a very early role of photosynthesis in the evolution of life, with presumable roles for both chlorophyll and carotenoids, which Lake (380) refers to as ‘photocytes’ (see a yellow branch in
Fig. 2). In this latter group of organisms, both types of photosynthesis may have originated as specific adaptations to environmental challenges. For the chlorophyll-based photosynthesis, this may have been under conditions in which organisms have to compete at limiting light intensities, and hence would benefit from large antenna systems. Life at the surface may have elicited the generation of poly-isoprenic carotenoids, which in the end may have led to the formation of retinal and retinal-based proton pumping. The latter process then may have originated to assist survival under conditions with high, stressful, light intensities.

Whether or not the hypothesis of a 'ring of life' will survive the scrutiny of further detailed (mathematical) analyses remains to be seen (see i.e. (381)). One of the possibilities is that this concept will be upgraded to the concept of the 'web of life' (382). Irrespective of the outcome of this, the co-existence of two metabolic units for light-driven proton pumping, with a completely different, almost antagonistic, molecular design concept, remains an interesting issue also to fuel heated evolutionary discussions.
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Summary
To achieve success in setting up sustainability applications, organisms are required that convert solar energy with the high efficiency. A widely proposed method to maximize the photosynthetic efficiency is the expansion of its effective absorption spectrum into the infrared region of the spectrum of electromagnetic radiation \(\text{i.e. beyond 700 nm}\). As the first step to this, this thesis worked on expressing of retinal-based phototrophy, like proteorhodopsin (PR) and \textit{Gloeobacter} rhodopsin (GR) into \textit{Synechocystis} PCC 6803 and ΔPSI derivate of \textit{Synechocystis}, thereafter exploring their stimulatory effect on the growth of the host. Beyond that, to functionally express PR and GR in \textit{Synechocystis}, this thesis also investigated the all-trans retinal metabolism in \textit{Synechocystis}.

In \textbf{chapter 1}, the importance of oxygenic photosynthesis in sustainability applications and the current approaches to improving the efficiency of oxygenic photosynthesis are briefly reviewed. Moreover, the (dis)advantages of three proposed methods on expanding the absorption spectrum of \textit{Synechocystis} to the infrared region have been intensively discussed. Beyond that, the chapter summarizes the studies on three popular light-driven proton pumps (BR, PR and GR).

In \textbf{chapter 2}, the different types of photosynthesis (oxygenic- and anoxygenic or chlorophyll based- and retinal based- photosynthesis) and their difference are summarized. Moreover, the conception of ‘direct conversion’ is explained through natural photosynthesis and its application in biofuel-production in Cyanobacteria, its limitations, and potential further improvement are intensively discussed.

In \textbf{chapter 3}, functional expression of the archetype PR in \textit{Synechocystis} sp. PCC 6803 is reported. Upon use of the moderate-strength psbA2 promoter, \textit{holo}-PR is expressed in this cyanobacterium, at a level of up to \(10^5\) molecules per cell, presumably in a hexameric quaternary structure, and with approximately equal distribution (on a protein-content basis) over the thylakoid and the cytoplasmic membrane fraction. These results also demonstrate that \textit{Synechocystis} sp. PCC 6803 has the capacity to synthesize all-trans-retinal. Expressing a substantial amount of a heterologous opsin membrane protein causes a substantial growth retardation \textit{Synechocystis}, as is clear from a strain expressing PROPS, a non-pumping mutant derivative of PR. Relative to this latter strain, PR expression, however, measurably stimulates its growth.
In **chapter 4**, as an extension of our initial studies, comparison of the effect of expressing two different bacterial rhodopsins (*i.e.* PR and GR) in the model cyanobacterium *Synechocystis* sp. PCC6803 is investigated, with special emphasis on the pigments bound by the respective apo-opsins, and the oligomeric state of the corresponding holo-rhodopsins, in *Escherichia coli* and in the cyanobacterial membranes. These studies suggest that the two proton-pumping rhodopsins were predominantly present as hexamers and trimers, respectively. Furthermore, GR is able to bind (an antenna) carotenoid, in addition to retinal, and also has a higher turnover rate of proton pumping at a given light intensity than PR. The smaller amount of GR expressed, however, would decrease its effectiveness. It still remains to be established which of these two bacterial rhodopsins can give the highest stimulatory effect on the growth rate of their cyanobacterial host.

In **chapter 5**, we have studied the in vivo role of these five enzyme(s) (activities) presumably involved in retinal metabolism in the model cyanobacterium *Synechocystis*. The results confirmed the role of SynACO as the decisive enzyme for retinal synthesis in *Synechocystis*, via asymmetric cleavage of β-apo-carotenal. Knocking out the gene encoding SynACO fully abolishes the ability of *Synechocystis* to synthesize retinal. Such mutants could be used for the reconstitution of holo-PR with exogenously added retinal or retinal analogues, as we demonstrated in this study with all-trans 3,4-dehydroretinal and the 3-methylamino-16-nor-1,2,3,4-didehydroretinal analogue. Furthermore, it suggests that *slr0574* played a role in the retinal degradation pathway. Preliminary results obtained with [13C]-NMR analysis, however, suggested that also conversion to retinol plays a role.

In **chapter 6**, to further increase the energy contribution from retinal-based phototrophy, two retinal-based proton pumps, PR and GR were expressed in a ΔPSI 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 PR can enhance the rate of photoheterotrophic growth of this *Synechocystis* derivative strain. In contrast, the GR 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 characteristics obtained are consistent with the concept that the proton-pumping PR provides the cells with the additional capacity to generate proton motive force. For GR 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 PR-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.

In **chapter 7**, we summarized the results in this thesis and emphasized the potential application of PR-based phototrophy in optimizing the ATP/NADPH ratio, artificial photosynthesis, in industrial biofuel-production and constructing an infrared-absorbed bacterial rhodopsin. Beyond that, we also pointed out the challenges in exploring the physiological effect of PR in vivo.
Samenvatting
Om de verduurzaming van onze samenleving te realiseren is een organisme nodig dat zonne-energie om kan zetten met hoge efficiëntie. Een breed gedragen voorstel voor het maximaliseren van de efficiëntie van fotosynthese is het vergroten van het effectieve spectrum dat gebruikt kan worden, met name in de richting van het infrarode deel van elektromagnetische straling (700 nm en langer). Een eerste stap hiervoor wordt uiteengezet in dit proefschrift met de beschrijving van retinal-gebaseerde fototropie, met behulp van proteorodopsine (PR) en Gloeobacter rodopsine (GR), welke tot expressie gebracht werden in Synechocystis sp. PCC 6803 en een daarvan afgeleide mutant waarin fotosysteem I ontbreekt (ΔPSI). Dit proefschrift verkent het stimulerende effect van deze rodopsines op de groei en de volledig-trans retinal-stofwisseling van de gastheer Synechocystis.

In hoofdstuk 1 worden het belang van oxygene fotosynthese in duurzame toepassingen en de huidige stand van de techniek voor het verbeteren van de efficiëntie ervan kort samengevat. Verder worden de voor- en nadelen van de voorgestelde methoden voor het uitbreiden van het bruikbare spectrum naar het infrarood uitvoerig besproken. Daarnaast vat het hoofdstuk het onderzoek naar drie populaire licht-gedreven protonpompen (BR, PR en GR) samen.

Hoofdstuk 2 vat de verschillende typen van fotosynthese, waarbij wel (chlorofyl) of niet (retinal) zuurstof ontwikkeld wordt, samen en beschrijft de verschillen daartussen. Voorts wordt het concept van ‘directe omzetting’ door natuurlijke fotosynthese en haar toepassing voor het produceren van biobrandstof in Cyanobacteriën uitgelegd. De beperkingen en potentiële verbeteringen worden uitvoerig besproken.

Hoofdstuk 3 rapporteert over de functionele expressie van het PR-archetype in Synechocystis. Bij gebruik van een promoter van gemiddelde sterkte, PpsbA2, wordt functioneel PR tot een aantal van $10^5$ moleculen per cel tot expressie gebracht, vermoedelijk in een hexamere quaternaire structuur met een evenredige verdeling (op basis van eiwitinhoud) over het thylakoid- en cytoplasmamembraan. Deze resultaten demonstrieren tevens dat Synechocystis de capaciteit heeft om volledig-trans retinal te synthetiseren. Expressie van een substantiële hoeveelheid opsin, een membraaneiwit, veroorzaakt een sterke vermindering van de groei in Synechocystis, zoals blijkt in een stam die PROPS, een niet-pompende afgeleide van PR, tot expressie brengt. In verhouding tot deze stam zorgt PR echter voor een meetbare stimulans op de groei.
In hoofdstuk 4 wordt, als extensie van ons initiële onderzoek, gekeken naar het verschil tussen twee verschillende bacteriële rodopsine (PR en GR) in de modelcyanobacterie *Synechocystis*. Met name de pigmenten die gebonden worden door de respectievelijk eiwitten om functioneel te worden, en hun quaternaire structuur in functionele vorm, zowel in *Escherichia coli* als in de cyanobacteriële membranen, zijn onderzocht. Deze studie suggereert dat de twee proton-pompende rodopsines voornamelijk bestaan uit respectievelijk hexameren en trimeren. Daarnaast blijkt dat GR in staat is een extra (antenne) carotenoïde te binden naast retinal en daarmee een hogere pompssnelheid weet te bereiken dan PR bij gelijke lichtintensiteit. De kleinere hoeveelheid GR die tot expressie gebracht wordt zou de effectiviteit verminderen ten opzichte van PR, maar het blijft een open vraag welke van deze bacteriële rodopsines de grootste stimulans op de groeisnelheid van hun gastheer oplevert.

In hoofdstuk 5 hebben we de in *vivo*-rol onderzocht van vijf enzymen die vermoedelijk betrokken zijn bij retinalmetabolisme in *Synechocystis*. De resultaten bevestigden de rol van SynACO als het kritieke enzym in de retinalsynthese door middel van het doorknippen van β-apo-carotenal. Het verwijderen van het gen dat codeert voor SynACO heft het vermogen om retinal te synthetiseren volledig op. Deze mutanten zouden gebruikt kunnen worden om functioneel PR te maken met extern toegevoegde retinal of analogen daarvan, zoals gedemonstreerd in deze studie waarin volledig-trans 3,4-dehydroretinal en 3-methylamino-16-nor-1,2,3,4-didehydroretinal deze rol op zich nemen. Verder suggereert dit dat het gen slr0574 betrokken is bij de afbraak van retinal. Voorlopige resultaten met [13C]-NMR-analyse suggereren echter dat omzetting in retinol ook een rol speelt.

In hoofdstuk 6 wordt beschreven hoe de expressie van PR en GR bij kan dragen aan de energievoorziening in ΔPSI. Metingen van de groeisnelheid, competitie en fysiologische karakterisering van rodopsine-bevattende stammen, in vergelijking met ΔPSI als controle, maken het mogelijk onomstotelijk vast te stellen dat de retinal-gebaseerde protonpomp PR de fotorheterotrofe groei kan verbeteren van deze afgeleide van *Synechocystis*. In tegenstelling tot PR liet GR dit stimulerende effect niet zien, ondanks het expressieniveau dat bereikt is, hoewel het een sterk effect had op de carotenoïdesynthese. De fysiologische karakterisering is consistent met het concept dat de protonpomp PR de cellen voorziet van extra capaciteit om een protonengradiënt te genereren. Voor GR is dit positieve effect mogelijk tenietgedaan door de negatieve consequenties van de expressie van dit heterologe eiwit. Het onvermogen om fotoautotroof te groeien van ΔPSI met PR is waarschijnlijk eerder een ki-
netische dan een thermodynamische limitatie van de NADPH-dehydrogenase NDH-1 in zijn NADP+-reducerende activiteit.

In hoofdstuk 7 vatten we de resultaten van dit proefschrift samen en benadrukken we de potentiele toepassing van PR-gebaseerde fototrofie voor het optimaliseren van de ATP/NADPH-ratio, kunstmatige fotosynthese, industriële biobrandstofproductie en het maken van een infrarood-absorberende bacteriële rodopsine. Daarnaast benoemen we de uitdagingen van het verkennen van de fysiologische effecten van PR in vivo.
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