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

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Chapter 1

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

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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 CO₂ 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: 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.* H₂, H₂S or Fe²⁺, succinate or malate) rather than H₂O, 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.* P₇₇₀, normally drives cyclic electron flow, primarily for the generation of ATP (see review (41)), while a type I reaction center, *i.e.* P₈₄₀, drives linear electron transfer, *i.e.* from H₂ or H₂S 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).
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 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 (350-700nm), 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, $\lambda_{\text{max}} \sim 525 \text{nm}$) and blue-absorbing PRs (BPR, $\lambda_{\text{max}} \sim 490 \text{ nm}$), 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, pK$_a$ 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.