Title:

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

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Abbreviations:

DDM, n-dodecyl-β-D-maltopyranoside; pmf, proton motive force; PR(s), Proteorhodopsin(s); PR-His, PR with a C-terminal poly-histidine tag; PROPS, proteorhodopsin optical proton sensor; GR(s), Gloeobacter rhodopsin(s); GR-His, GR with a C-terminal poly-histidine tag; BR(s), Bacterial rhodopsin(s); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP,[(3-chlorophenyl) hydrazono] malononitrile; E, echinenone; 3-OH echinenone, 3-hydroxy echinenone; Can, canthaxanthin; *E. coli*, *Escherichia coli*; LB, LB-Lennox Broth; SEC, size exclusion chromatography

Abstract:

Proteorhodopsins are light-driven proton pumps that occur widespread in Nature, where they function predominantly in environments with high incident irradiance for phototrophy. Their maximal absorbance is usually in the blue but can be extended into the (far)red range. Since they can heterologously be expressed, they can play an important role in further attempts to increase the efficiency of photosynthesis. Here we report further studies towards this goal, by comparing the 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.

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.

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- (Spudich, Zacks, & Bogomolni, 1995), or a chemiosmotic function in free-energy transduction. Bacteriorhodopsin from *Halobacterium salinarum* is the archetype of the chemiosmotic-energy-transducing rhodopsins (Lanyi, 1978). It pumps protons, driven by light absorption, and hence is able to generate a proton motive force (pmf). Proteorhodopsins (PRs) form a subgroup of the rhodopsins that occur in the Domain of the Bacteria (Fuhrman, Schwalbach, & Stingl, 2008). Most members of this group utilize light energy to translocate protons over a membrane against an electrochemical proton gradient (Fuhrman et al., 2008). More recently, however, bacterial rhodopsins have been discovered that pump ions other than protons, like sodium- (Balashov et al., 2014; Inoue et al., 2013; Jung, 2012; Kwon et al., 2013), lithium- (Inoue et al., 2013), or chloride ions (Hasemi, Kikukawa, Kamo, & Demura, 2016; Schobert & Lanyi, 1982; Yoshizawa et al., 2014). Site-directed engineering can broaden this range even further to even include Cs^+ and K^+ (Gushchin et al., 2015; Konno et al., 2015; Sasaki, Brown, Chon, & Kandori, 1995). The gene encoding the first discovered member of the Bacterial group of the proteorhodopsins was detected in the genome sequence of an uncultured γ-proteobacterium from oceanic waters (Beja et al., 2000). Since then, PRs have turned out to be highly abundant in the oceans (Campbell, Waidner, Cottrell, & Kirchman, 2008; Finkel, Béjà, & Belkin, 2013; Gómez-Consarnau et al., 2007; Palovaara et al., 2014; Rusch et al., 2007) and organisms containing them, including cyanobacteria (Hasemi et al., 2016; Miranda et al., 2009) can be found in many other habitats as well (Atamna-Ismaeel et al., 2012; Atamna-Ismaeel et al., 2008; Koh et al., 2010; Rusch et al., 2007). Surprisingly, Proteorhodopsin turned out to function as a hexameric assembly (Hussain, Kinnebrew, Schonenbach, Aye, & Han, 2015), rather than the hexagonal array of trimers characteristic for bacteriorhodopsin from *Halobacterium salinarum* (Blaurock & Stoeckenius, 1971).

In 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 (Gómez-Consarnau et al., 2007; Kimura, Young, Martinez, & DeLong, 2011; Palovaara et al., 2014), production of ATP (Steindler, Schwalbach, Smith, Chan, & Giovannoni, 2011), increased fixation of CO_2 (Gonzalez et al., 2008; Palovaara et al., 2014), and/or survival under starvation or stress conditions (Akram et al., 2013; Feng, Powell, Wilson, & Bowman, 2013; Gómez-Consarnau et al., 2010; Steindler et al., 2011; Z. Wang et al., 2012). 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 (Bryant & Frigaard, 2006; Sabehi et al., 2005) and presumably even retinal-based photosynthesis (Sabehi et al., 2005). 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 (i. e. the retinal-based or the chlorophyll-based), is most efficient, is very difficult to decide (Hellingwerf, Crielaard, & Westerhoff, 1993; Kirchman & Hanson, 2013), 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 (Janssen, Tramper, Mur, & Wijffels, 2003) and high growth rates. Genetic engineering of such organisms then allows one to produce a range of valuable carbon-based products via 'direct conversion' (Angermayr, Hellingwerf, Lindblad, & de Mattos, 2009; Ducat, Way, & Silver, 2011; Machado & Atsumi, 2012).

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 (Blankenship et al., 2011; M. Chen & Blankenship, 2011; Ort et al., 2015). The most recent of these (Ort et al., 2015) 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 (Srividya et al., 2015) 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 (Beja et al., 2000) 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 (Q. Chen et al., 2016)

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 (Dioumaev et al., 2002; Miranda et al., 2009; W. W. Wang, Sineshchekov, Spudich, & Spudich, 2003) (ii) its ability to bind carotenoids like salinixanthin and echinenone (Balashov et al., 2010; Imasheva, Balashov, Choi, Jung, & Lanyi, 2009; Luecke et al., 2008), 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 (Balashov et al., 2005).

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, e.g. because of its faster pumping rate. Here we characterize this Gloeobacter rhodopsin with respect to 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 e.g. *Escherichia coli*, partly because it forms hexagonal arrays of protein trimers (Blaurock & Stoeckenius, 1971).

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 μE · 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 NaHCO$_3$, 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 μE · m^{-2} · s^{-1} (containing 21 μE · m^{-2} · s^{-1} red, 23 μE · m^{-2} · s^{-1} green, and 3 μE · 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 OD$_{750}$ 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, (Imasheva et al., 2009)) 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 gaattcgcggccgcttctag ATGTTGATGACCGTATTTTCTTC 3′ and JBS312: 5′ tac ctgcagcggccgctactagta CTA GTGATGGTGATGGTGATG GGAGATAAGACTGCCTCCC 3′ from plasmid pKJ900 (Kim, Waschuk, Brown, & Jung, 2008; Miranda et al., 2009), 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 (Q. Chen et al., 2016). 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 (Q. Chen et al., 2016). *E. coli* J53/RP4 (Bachmann, 1972; Jacob & Grinter, 1975) 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 (Q. Chen et al., 2016).

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 541nm with an extinction coefficient of 50,000 L mol^{-1} cm^{-1} (Imasheva et al., 2009). 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) (Balashov et al., 2010) and then aliquoted for storage at – 20 °C. 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 (Imasheva et al., 2009) 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 (Balashov et al., 2010).

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 (Kraayenhof et al., 1982) 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 (Q. Chen et al., 2016) 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 OD_{600} 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 $MgSO_4$, 0.1 mM $CaCl_2$, 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 $MgSO_4$, 0.1 mM $CaCl_2$, and 250 mM KCl) and diluted to an OD_{600} 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 µE · 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 OD_{600} cells, equal to 100 ml culture with OD_{600} 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 (Friedrich et al., 2002) 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-PROTEAN TGX Precast Gels' (Bio-Rad) or ExpressPlus Page gels 8-16% (GenScript) using the same procedures as described before (Q. Chen et al., 2016). 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} (Imasheva et al., 2009); 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 OD_{730} of a sample, using 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)(Schuurmans et al., 2014).

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 grade). The extracted pigments were 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^{-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 standard compound for pigment identification and quantification. After reacting with hydroxylamine, all-*trans* retinal was converted into the more stable compound retinal oxime(Groenendijk, De Grip, & Daemen, 1979). 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 (Katoh, Sonoda, Katoh, & Ogawa, 1996)(Q. Chen et al., 2016). 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 (Q. Chen et al., 2016). 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.

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 *Synechocystis* 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 (van Thor et al., 2000), 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 (van Thor et al., 2000). 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 (Miranda et al., 2009) than PR (Dioumaev et al., 2002)and additionally binds one molecule of carotenoid as an antenna pigment, thereby increasing GR's effective absorption cross section 3.5 fold (Balashov et al., 2005).

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 (fraction 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 ε_{280} 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: ε_{280} = (nW × 5500) + (nY × 1490) + (nC × 125). In this formula n refers to the number of the corresponding amino acid. On the above basis, ε_{280} of GR-His is equal to 55,350 M^{-1} cm^{-1}, while ε_{541} is 50,000 $M^{-1}cm^{-1}$(Imasheva et al., 2009). Therefore, a ratio of 0.9 of A541/A280 would indicate an absolutely pure fraction of GR-His. The three main fractions show a constant value of ~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.

Reconstitution of Gloeobacter rhodopsin with carotenoids *in vitro*

Protein homology analysis (GR has 50% identical amino acid residues with xanthorhodopsin (Luecke et al., 2008)) 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 absorption 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 (Imasheva et al., 2009).

Intriguingly, *Synechocystis* naturally produces a considerable variety and amount of carotenoids, including the 4-keto derivatives: echinenone; 3''-hydroxy echinenone and canthaxanthin (Makino et al., 2008; Zhang, Selão, Selstam, & Norling, 2015). 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*.

Echinenone is the most promising candidate for such binding as it is considered as one of the main carotenoids (18%) produced by *Synechocystis* (Zhang et al., 2015), and the association between echinenone and GR has been reported in the literature (Balashov et al., 2010). Another two interesting candidates would be 3'-hydroxyechinenone, and canthaxanthin, as their presence has been confirmed in *Synechocystis* by HPLC (Makino et al., 2008), 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. 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 541nm, 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 541nm 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 (Balashov et al., 2010), 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 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 (showing e.g. a high off-rate), because the carotenoids disassociated from GR-His during incubation in buffer containing 0.1% DDM.

The 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) (Dioumaev et al., 2002; Miranda et al., 2009; W. W. Wang et al., 2003), 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 μE m^{-2} s^{-1} to 6000 μE m^{-2} s^{-1}, see Fig. 4). The proton pumping rate initially increased linearly with the light intensity ($\leq$ 1500 μE 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.

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 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 *Synechocystis* and its effect on the photo-autotrophic growth rate of this organism, the *Synechocystis* strain containing pQC012 (for GR-H expression) was cultivated in batch culture at a moderate light intensity (~ 45 $\mu E \cdot m^{-2} \cdot s^{-1}$). Cells were collected at different growth phases for quantification of the GR-expression level and the level of retinal production, respectively.

The results summarized in Fig. 5 show that GR-His had the highest expression level (~9.5×10^4 molecules per cell) in the linear growth phase, which is shifted to an earlier phase ascompared to PR-His in *Synechocystis*, the expression of which reached a peak at the transition between linear growth and the stationary phase (Q. Chen et al., 2016). Moreover, retinal production increased during the

subsequent growth phases and reached a peak (at ~8.4 ×10^4 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 e.g. the linear growth phase of *Synechocystis*, in which a large fraction of GR-His is present as apo-GR-His*s*, exogenous addition of all-*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.

Isolation of His-tagged Gloeobacter rhodopsin from *Synechocystis*

To explore the possibility that carotenoids would bind to holo-GR-His *in vivo* in *Synechocystis*, GR-His was isolated from the *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 (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 *Synechocystis.* A corresponding fraction from a retinal-deficient *Synechocystis* strain (JBS14003; unpublished results) containing pQC006 (for apo-PR-H 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 *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, 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 *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-*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 *vivo* in *Synechocystis* has bound all-*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 holo-GR from *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.

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 (Cymer & Schneider, 2012). Proteorhodopsin and Bacteriorhodopsin both function in the form of oligomers (i.e. hexamers (Hussain et al., 2015) and a two-dimensional array of trimers (Blaurock & Stoeckenius, 1971), 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 over 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 (Tsukamoto et al., 2013). Therefore, we assigned these three peaks (A, B, C) according to size as representing oligomers, trimers and monomers of GR-His, respectively. Apparently, of these, the trimer is the dominant species. CD-spectroscopy of equivalent samples has confirmed this assignment (S. Ganapathy et al., unpublished experiments). 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 (Tsukamoto et al., 2013).

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 (Yoshimura & Kouyama, 2008). 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 under Materials and Methods and analyzed by size-exclusion chromatography. The change in ratio of the three structural isomers was estimated in the eluted samples by comparing the results obtained with and without

addition of echinenone. No significant changes in the relative composition of three structural isomers was observed, which strongly indicates that echinenone does not play a crucial role in the formation of quaternary structure of GR-His (data not shown). The SEC chromatography of GR-His isolated from *Synechocystis* (Fig. 7B) 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 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 (Q. Chen et al., 2016). 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 PS-I deficient *Synechocystis* strain as the most promising one. Cyclic electron flow around PS-I 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 (Branco Dos Santos, Du, & Hellingwerf, 2014). Considering this, one may expect that functional expression of proteorhodopsin in a PS-I 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 PS-I deletion strain could be adjusted to occur via PS-II plus NDH-1 only, for water-driven reduction of NADPH, as proposed in (Ort et al., 2015).

As compared to Gloeobacter rhodopsin, bacteriorhodopsin even pumps much faster (~ 100 protons/BR/s; (Singh & Caplan, 1980). 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 (Bratanov et al., 2015). This opens up the possibility to try expression of bacteriorhodopsin derivatives also in *Synechocystis*, although there is no guarantee that functional holoprotein 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 light of a wavelength beyond 700 nm (i.e. a proteorhodopsin with maximum absorption at 750 nm; S. Ganapathy et al, unpublished results). This will allow us to construct a *Synechocystis* strain which can (also) utilize infrared light. Replacement of PS-I by such an infrared-absorbing proton pump is a promising strategy to increase the maximum areal energetic efficiency of oxygenic photosynthesis.

Author 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 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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