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

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Chapter 7

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

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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 C\(^-\) 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 *Gloeo-bacter 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 ΔPSI+ pQC006. Next, however, we also observed this phenomenon in a mixed culture of strain ΔPSI+ pQC006 plus ΔPSI+ 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 (lrlA), 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 Ptrc and Ptac 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 PcpcG2 system (339). Moreover, another two inducible promoters: the nirA promoter of Synechoccus (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 Ptrc promoter.

In chapter 3, we compared the PR expression level from three plasmids, each with a different promoter driving PR expression: the LacI-regulated Ptrc promoter, the native PpsbA2 promoter and the RNase P subunit B promoter PrmpB. The Pptrc promoter is one of the strongest, and most commonly used, promoters to express and over-express genes in Synechocystis, while the PpsbA2 promoter is one of the strongest native promoters in Synechocystis, expressing the D1 subunit of Photosystem II. The PrmpB promoter is considered to be constitutive in Synechocystis under standard cultivation conditions. In terms of promoter strength, Pptrc is the strongest one while PrmpB 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 Pptrc-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 $2H^+/e^-$ 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 photons 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-\textit{trans} retinal is more abundant than 11-\textit{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\(^+\), Mg\(^{2+}\)) 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. 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/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 archeon (377).

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

**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 chromatins 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).
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.