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Chapter 2

Prokaryotic Phototaxis

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and Klaas J. Hellingwerf**

Summary

Microorganisms have various mechanisms at their disposal to react to (changes in) their ambient light climate (i.e., intensity, color, direction, and degree of polarization). Of these, one of the best studied mechanisms is the process of phototaxis. This process can be described as a behavioral migration-response of an organism toward a change in illumination regime. In this chapter we discuss three of these migration responses, based on swimming, swarming, and twitching motility, respectively. Swimming motility has been studied using a wide range of techniques, usually microscopy based. We present a detailed description of the assays used to study phototaxis in liquid cultures of the phototrophic organisms *Halobacterium salinarum*, *Halorhodospira halophila*, and *Rhodobacter sphaeroides* and briefly describe the molecular basis of these responses. Swarming and twitching motility are processes taking place at the interface between a solid phase and a liquid or gas phase. Although assays to study these processes are relatively straightforward, they are accompanied by technical complications, which we describe. Furthermore, we discuss the molecular processes underlying these forms of motility in *Rhodocista centenaria* and *Synechocystis* PCC6803. Recently, it has become clear that also chemotrophic organisms contain photoreceptor proteins that allow them to respond to their ambient light climate. Surprisingly, light-modulated motility responses can also be observed in the chemotrophic organisms *Escherichia coli* and *Acinetobacter calcoaceticus*. In the light-modulated surface migration not only “che-like” signal transduction reactions may play a role, but in addition processes as modulation of gene expression and even intermediary metabolism.

Key words: *Halorhodospira halophila*, *Ectothiorhodospira*, *Halobacterium salinarum*, *Rhodobacter sphaeroides*, *Synechocystis*, *Rhodocista centenaria*, *Rhodospirillum centenum*, Swimming motility, Swarming motility, Twitching motility, Photoactive yellow protein, Sensory rhodopsin, Phytochrome, BLUF, Redox sensing

1. Introduction

Prokaryotic microorganisms have various modes at their disposal for their own dispersal, as well as to be able to be attracted and/or repelled by a wide range of abiotic and biotic signals. The best known of these is the swimming behavior, relevant in aqueous environments, in which cells are propelled by one or more rotating flagella. Other types of migration in this environment are based on floatation, i.e., on the difference in density between the cells and their surroundings, which can be affected by, e.g., glycogen synthesis and the formation or collapse of gas vesicles.

Besides in the aqueous phase, bacteria can migrate also on the interface between a solid phase on the one hand and a liquid phase or a gas phase on the other, in a multitude of ways. These have been referred to in literature with terms as: swarming, gliding, twitching, sliding, etc. (1). Only for swarming and twitching motility has the molecular basis for the particular mode of migration been satisfactorily resolved. Swarming cells are propelled also by rotating flagella, but these latter organelles during swarming are often much more numerous, expressed on a per-cell basis, and in addition they are peritrichously inserted into the cytoplasmic membrane. In twitching motility the displacement of the cells is effected by expanding and contracting surface appendages, called pili (2), usually referred to as Type IV pili).

Besides random migration for dispersal, many motile bacteria can also migrate in a specific direction, steered by environmental signals by which they are attracted or repelled. The prototype system in this respect is the chemotaxis (Che) system from enterobacteria like *Escherichia coli*, which allows organisms to show both excitability and adaptation. This system uses: (1) multiple extracellular and intracellular (ligand) sensing domains (mostly organized in a large array in one of the poles of the cell) as part of the so-called methyl-accepting chemotaxis proteins (MCPs), (2) two-component system-based phosphoryl transfer reactions to activate at least one small response regulator that modulates flagellar rotation and in addition a methyl-esterase, and (3) methanol release as a reporter of the adaptation process (see ref. 3 for a review). All three types of motility discussed here (i.e., swimming (3), swarming (4), and twitching (5) motility) can become directional by signal input from a Che-like signal transduction system (see also Fig. 1 and (6)).

The ability to migrate in space is important not only for the microorganism, but also for plant and animal hosts that may suffer a pathogen's infection. Ample evidence has been provided that the ability to migrate correlates with the virulence of many pathogenic bacteria (see e.g. (2, 7, 8) for some recent references).

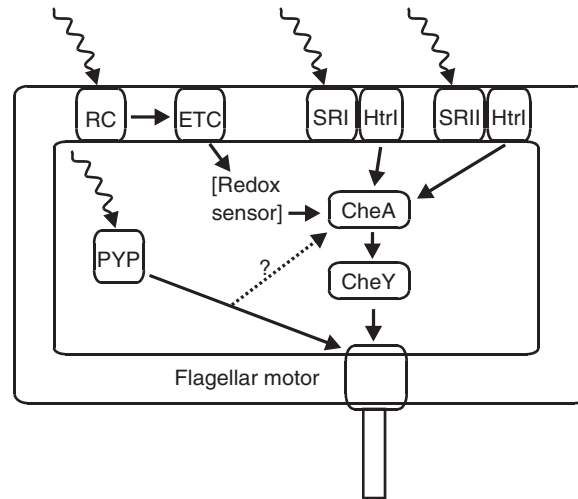


Fig. 1. Summary of available knowledge on the phototaxis signaling pathways in *H. salinarum*, *R. sphaeroides*, and *H. halophila* in a Che-like reaction scheme. *H. salinarum* contains the photoreceptors SRI and SRII, which are complexed in the membrane to their signal transducers HtrI and HtrII. These transducers modulate the autokinase activity of CheA and thus modulate the phosphorylation status of CheY. Phototaxis of *R. sphaeroides* proceeds via its photosynthetic reaction center (RC) and electron transfer chain (ETC) via a putative redox sensor. Positive phototaxis in *H. halophila* occurs via a similar pathway, while its negative phototaxis is triggered by photoactive yellow protein (PYP). The signal transduction pathway for PYP is unknown; one candidate is the Che system. Possible adaptation mechanisms have been omitted from this figure.

In this contribution we will discuss the behavioral responses of bacteria in response to changes in their illumination regime, in a range of processes loosely referred to as “phototaxis.” Whereas originally this term was used to refer to a behavioral reaction of an individual organism *toward* a light source (9), we will here use the term for any cellular or multicellular migration response toward a change in the illumination regime. In this discussion, however, we will restrict our discussion to examples based on the three well-resolved modes of migration: swimming, swarming, or twitching motility. Use of the term “phototaxis” in this way implies that it is a process also affected by intermediary metabolism and modulation of gene expression.

1.1. Phototaxis in Liquid Cultures

Phototaxis has been examined in some detail in a number of photosynthetic prokaryotes (9), particularly halophilic Archaea, purple photosynthetic Proteobacteria, and Cyanobacteria (for purple bacteria and Archaea, see Fig. 1). Two very different mechanisms of photosensing are at the basis of these phototaxis responses: some responses are triggered via light absorption by the photosynthetic machinery, while others involve dedicated photosensory proteins.

Interactions and spectral overlap between these two modes of sensing provide another layer of complexity in these photosensory pathways. The final output in terms of motility is also varied: for free-swimming bacteria it ranges from light-induced changes in swimming direction to transient stops by attractant removal, and in bacteria moving on a solid surface from gliding of entire bacterial colonies to changes in twitching motility. Only for phototaxis in halophilic Archaea is detailed information available on the entire photosensory signaling pathway.

The motility of *Halobacterium salinarum* (previously *Halobacterium halobium*) is based on its five to eight flagella, placed in tufts at the cell poles (10–12). It swims equally well in both directions (clockwise and counterclockwise rotation of the flagellum) along its long cell axis, at a relatively slow speed of approximately 2.5 $\mu\text{m/s}$, and reverses its swimming direction about every 10–15 s, or sometimes stops. The duration of a typical straight run of a cell roughly corresponds to the time scale on its swimming direction will be randomized. The ability of *H. salinarum* to swim equally well in both directions, and to reverse swimming direction is different from the situation found in *E. coli*, where counterclockwise rotation of the peritrichous flagella causes the assembly of a flagellar bundle, resulting in swimming. Counterclockwise rotation results in the disassembly of the flagellar bundle, causing “tumbling” of *E. coli*. The frequency of these reversals is regulated by chemical and light stimuli. In between reversals *H. salinarum* usually swims following nearly straight paths. Highly motile strains of *H. salinarum* have been obtained by selection (13).

The functioning of the photoreceptors and signal transduction pathways that trigger phototaxis in *H. salinarum* has been analyzed to a high degree of sophistication (14, 15). Phototaxis is initiated by the archaeal rhodopsins sensory rhodopsin I (SRI) and sensory rhodopsin II (SRII; see Fig. 1). These retinal-containing proteins membrane proteins are complexed to signal transducer proteins, strongly resembling the methyl-accepting chemotaxis proteins (MCPs) that serve as the receptors for chemotaxis in *E. coli*. These halobacterial transducer proteins (HtrI and HtrII) interact with cytoplasmic signal transduction components that again strongly resemble those of *E. coli*, and that relay signals to the flagellar motor complex by changes in the phosphorylation state of CheY. The molecular mechanism of the photoactivation of SRI and SRII, and the relay of the signal from SRII to its transducer HtrII have been studied at near-atomic resolution (16). SRI functions as the receptor for positive phototaxis to orange light. In addition, its blue-shifted photocycle intermediate is the signaling state for a negative phototaxis in response to near-UV light. Sensory rhodopsin II triggers negative phototaxis toward blue light.

Rhodobacter sphaeroides (previously *Rhodospseudomonas sphaeroides*) swims by means of a single flagellum located in the center (not at the pole) of the cell (17). This single flagellum only rotates in the clockwise direction and always pushes the cell. Cells switch (typically every ~10 s) between periods of swimming (clockwise rotation of the flagellum) and periods during which the flagellum does not rotate, and the cells consequently stop swimming for approximately 1 s. Thus, the intermittent stops of *Rb. sphaeroides* are equivalent to tumbling in *E. coli*. *Rb. sphaeroides* can swim at speeds up to 80 $\mu\text{m/s}$. The duration of the periods of swimming and stalled flagellar motion is altered under the influence of external stimuli such as light. Its flagellar resetting bias in non-stimulated cells is 0.85, as opposed to the 0.5 bias in *E. coli*. By consequence *Rb. sphaeroides* responds significantly more strongly to negative stimuli than to positive stimuli (18).

Phototaxis in *Rb. sphaeroides* is triggered by the effects of the photosynthetic machinery on the rate of electron transfer (9). Thus, in contrast to the situation in *H. salinarum*, phototaxis in *Rb. sphaeroides* does not involve a dedicated photosensor. The rate of electron transfer is presumably sensed by an as yet unidentified receptor, and relayed into the complex set of Che proteins in *Rb. sphaeroides* (Fig. 1). Thus, phototaxis responses in *Rb. sphaeroides* can be regarded as a form of redox taxis and are modulated by factors affecting electron transport, such as the presence or absence of oxygen (19). The *Rb. sphaeroides* genome encodes nine transmembrane chemoreceptors (MCPs) and four putative cytoplasmic MCPs, four CheA proteins, and six CheY proteins (20). A number of proteins from this Che system have been shown to be required for phototaxis in *Rb. sphaeroides*, showing that the signal transduction chains for phototaxis and chemotaxis converge at this level (21). A similar situation holds for phototaxis and chemotaxis in *R. centenum* (see later).

Halorhodospira halophila (previously *Ectothiorhodospira halophila*) swims by means of two polar flagella. Like *H. salinarum*, it swims equally well in both directions (22). Relatively straight (or slightly circular) runs are separated by a reversal in swimming direction; occasionally cells can undergo brief stops. The rate of swimming is variable and can reach up to 100 $\mu\text{m/s}$. Attractant photostimuli increase the frequency of reversals in swimming direction, while repellent photostimuli suppress reversals for periods longer than 5 s.

H. halophila exhibits both positive and negative phototaxis (22). The positive phototaxis response is triggered through the photosynthetic machinery, as is the case for *Rb. sphaeroides*. The wavelength dependence of the negative phototaxis response has indicated that photoactive yellow protein (PYP) functions as the dedicated photoreceptor for this response. While the mechanism for the light activation of purified PYP has been unraveled in

great depth (23), the signal transduction chain linking PYP to the flagellar motor has not yet been resolved (Fig. 1).

1.2. Phototaxis on (Semi)Solid Surfaces

1.2.1. Overview

It has become clear that many organisms are capable of (photo) tactic behavior not only in liquid cultures, but also on (semi)-solid surfaces. Later, we will review current knowledge on well-studied cases of phototactic motility of bacteria on solid surfaces, mostly the surface of an agar plate but a glass surface can also be used. The general method to study this is to spot a small volume (typically 1–5 μ l) of a liquid bacterial culture in a well-defined growth phase on a low-concentration agar plate. The exact agar concentration needed for highest motility varies depending on the organism studied, but is generally 0.5–0.8%. Usually, the lowest agar concentration yields the largest colony diameter (24). Besides the concentration of agar, various additional factors can influence the rate of movement of a bacterial colony over a semisolid surface. For example, the presence of surfactants can increase motility significantly (25). Niu et al. showed that addition of 0.02% Tween80 could lead to an up to fivefold increase of colony diameter of swarming *E. coli* cells. Surfactants, either excreted by the cells themselves, or added to the soft agar, also enhance (swarming) motility (26). Also, the brand of agar used has been shown to influence motility. For example, Toguchi et al. compared motility on Eiken agar and Difco agar (26). Colony diameter was significantly larger on Eiken agar, and surfactant defects in bacterial strains could be overcome by using Eiken agar instead of Difco, due to some unknown parameter of the Eiken agar, such as superior wettability.

Furthermore, it cannot be stressed enough that these responses on solid surfaces are influenced by many external factors, apart from those mentioned earlier. Extreme care should be taken to precisely monitor and regulate environmental factors such as humidity and temperature. Also the growth phase of the organism of interest has been shown to influence motility. Furthermore, light can influence taxis directly through phototaxis, but also indirectly, by changing parameters such as temperature and humidity. Control experiments for the effect of illumination are therefore best performed on plates wrapped in black cloth. Recently, photoreceptor proteins, and corresponding light responses, have been shown to be present in a wide range of chemotrophic organisms (27). Because in this case the light only serves as an environmental signal and not as a source of energy, as can be the case in phototrophic organisms, this may simplify detailed (mathematical) description of phototaxis responses (28).

As compared to experiments of population responses on an agar surfaces, which is the most extensively applied technique, studies of migration in a glass microscope chamber (29) have the advantage that responses can be recorded in a few minutes, so

that dominant effects of gene expression, in the response toward alteration of the illumination regime, can be excluded.

1.2.2. Swarming Motility in *Rhodocista centenaria*

The purple photosynthetic bacterium *Rhodocista centenaria* (formerly *Rhodospirillum centenum*) can undergo a differentiation from swimming cells to swarming cells. Whereas swimming cells in liquid medium contain only a single polar flagellum, cells grown on solid surfaces develop numerous lateral (peritrichous) flagella in addition to the polar flagellum (30, 31). In liquid culture *R. centenaria* cells use their single flagellum for a light response that is independent of the direction of incoming light, i.e., it is not a phototactic response in the strict meaning of this term (32), but rather very similar to the phototaxis responses in other purple bacteria. However, on solid surfaces, groups of cells of this organism have been shown to exhibit a genuinely phototactic response: cells were shown to exhibit positive phototaxis toward light of long wavelengths (>800 nm), and a negative phototactic response toward light of shorter (<600 nm) wavelengths (30). The molecular signal transduction pathway underlying the swarming response has been elucidated in great detail. *R. centenaria* has three *che* operons, of which two have been shown to be involved in both swimming and swarming cell motility. Where the *che1* operon directly regulates taxis in a manner similar to the *Escherichia coli che* paradigm, the *che2* operon is involved in flagellar biosynthesis (33). Presumably, this response is not based on a dedicated photosensory protein, but rather on sensing of (the rate of) electron flow through the photosynthetic electron transfer chain (34). However, because both wavelengths for positive and for negative responses include wavelengths of bacteriochlorophyll absorption, there must be a more complex regulatory mechanism at the basis of these processes (9). Internal colony light gradients may also play an important role in this light sensing.

1.2.3. Twitching Motility in *Synechocystis*

Also in cyanobacteria, a phototaxis response of cells involved in surface migration has been identified and studied in great detail. *Synechocystis* sp. PCC6803, the first phototrophic organism with a fully sequenced genome (35), is able to migrate through twitching motility. Various types of photoresponses are known to occur in this organism, including both negative- and positive phototaxis. Most published phototaxis experiments in *Synechocystis* are performed using soft agar plates, although also glass slide-based methods have been described (29). On solid surfaces, *Synechocystis* cells can move and form expanding, flat, and irregularly shaped colonies. This type of movement has been shown to be dependent on type IV pili. *Synechocystis* has two, morphologically different types of pili, thick and thin, but only the thick pili, for which the structural gene is *pilA1*, are required for motility (36). In several other bacterial species thin pili have an opposite function, i.e., to attach the cells to a solid surface (37).

To capture a light signal, ultimately resulting in a phototactic response, *Synechocystis* has various possible photoreceptor proteins at its disposal: The presence of the phytochromes Cph1 and Cph2, binding a linear tetrapyrrole, has been relatively long known. In vitro studies have shown that Cph1 absorbs predominantly red light, as most phytochromes, but the absorption of Cph2 is mainly in the blue part of the spectrum (38); the flavin-binding BLUF protein PixD (Slr1694) (39) is a typical blue-light receptor. The third phytochrome-like protein in this organism is PixJ1. Although in vitro experiments have suggested that this is a blue/green photoreversible light receptor (40), in vivo experiments suggest that it has authentic red/far-red photoreversibility (29). Interestingly, PixJ1 in its carboxy-terminal region shows homology to MCPs, whereas the other orfs in this operon (*slr0038*–*slr0043*) all show homology to Che proteins, involved in flagellar switching in chemotaxis and in type IV pili biogenesis. The best-studied light response in *Synechocystis* is positive phototaxis (41). Disruption of *pixD* abolishes the attraction of the organism by red light; it even results in a repellent effect of light of this color, i.e., from positive to negative phototaxis (39)(Fig. 2), as does deletion of *pixJ1* (41, 42).

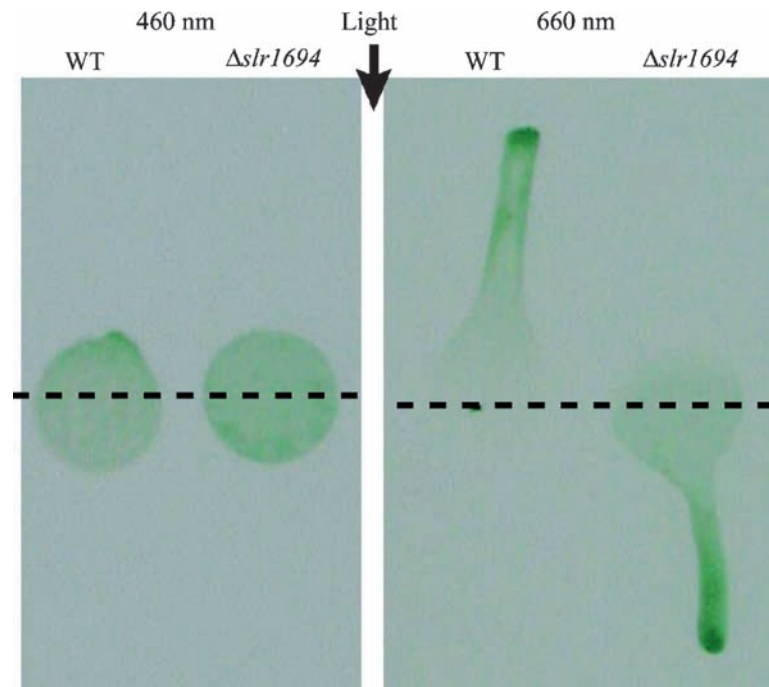


Fig. 2. Phototactic movements of colonies of the *slr1694* gene-disrupted mutant ($\Delta slr1694$) and its parent strain, *Synechocystis* PCC-P. 1 μ l of each cell suspension was spotted and grown for 44 h under lateral illumination (arrow) at 460 nm (left) and 660 nm (right). The dotted line shows the initial position of inoculation before the illumination. Reprinted with permission from ref. 39.

Both the various photoreceptor proteins that provide input signals for the phototaxis signal transduction cascade, and the resulting responses of pili synthesis and retraction that form the output of this phototaxis process have been characterized in some detail. The intermediate signal transduction steps that link the photosensory input signals to changes in pili structure are still less well understood. A tentative working model to unify the majority of the observations available from literature on this system is shown in **Fig. 3**. The signaling cascade starts with photoactivation of PixD, of which the molecular basis has been resolved in high detail: Both yeast two-hybrid screens (39) and biochemical experiments (43) have shown that PixD interacts with the PatA-like response regulator PixE. Light absorption alters the multimeric state of the PixD-PixE complex, resulting in the release of PixE molecules from the complex, presumably enabling these proteins to propagate the signal (43). In a large-scale analysis of protein-protein interaction in *Synechocystis*, glycogen phosphorylase and glucokinase were identified as additional interaction partners of PixD (CyanoBase, <http://beta.kazusa.or.jp:3018/genes/show/slr1694>; see also ref.44), directly linking light activation with glucose catabolism and intermediary metabolism. PixD may modulate the activity of glucokinase and glycogen phosphorylase, with the result that cAMP levels might increase in the light. An effect of light on cAMP levels (which are mostly regulated by the adenylate cyclases Cya1 and Cya2) has been demonstrated (45). Increases in cAMP levels by blue-light stimuli are mediated by Cya1 (46), but Cya1 activity itself is insensitive to light (47). cAMP levels can directly regulate PilA expression via the cyclic AMP-binding protein SYCRP1 (48). Light signaling through cAMP levels and SYCRP1 specifically regulates phase 3 of the response to a light-stimulus, in which cells move rapidly in fingerlike projections (49). Pili expression is primarily governed by the stress sigma factor, σ^F (50). Expression of this group 3 sigma factor is subject to autoregulation, and to activation by cAMP-activated SYCRP1 (48). σ^F recognizes the promoter sequences of *pilA1* (51), and, interestingly, also the promoter of the phytochrome-encoding gene *slr0041* (51). This latter photoreceptor is responsible for the red/far-red photoreversibility of the attractant response of *Synechocystis* toward low and moderate light intensities ((29), see also **Fig. 3**). In view of the similarity between σ^B from *Bacillus subtilis* and σ^F , it is of significant interest to find out which other stress signals, besides salt (52), activate this latter sigma factor.

1.3. Light-Regulated Motility in Nonphototrophic Bacteria

Whereas light responses were long thought to be present only in phototrophic bacteria, recently, more and more light-regulated responses have been observed in chemotrophic bacteria as well (27). Especially because of the wealth of genomic data becoming

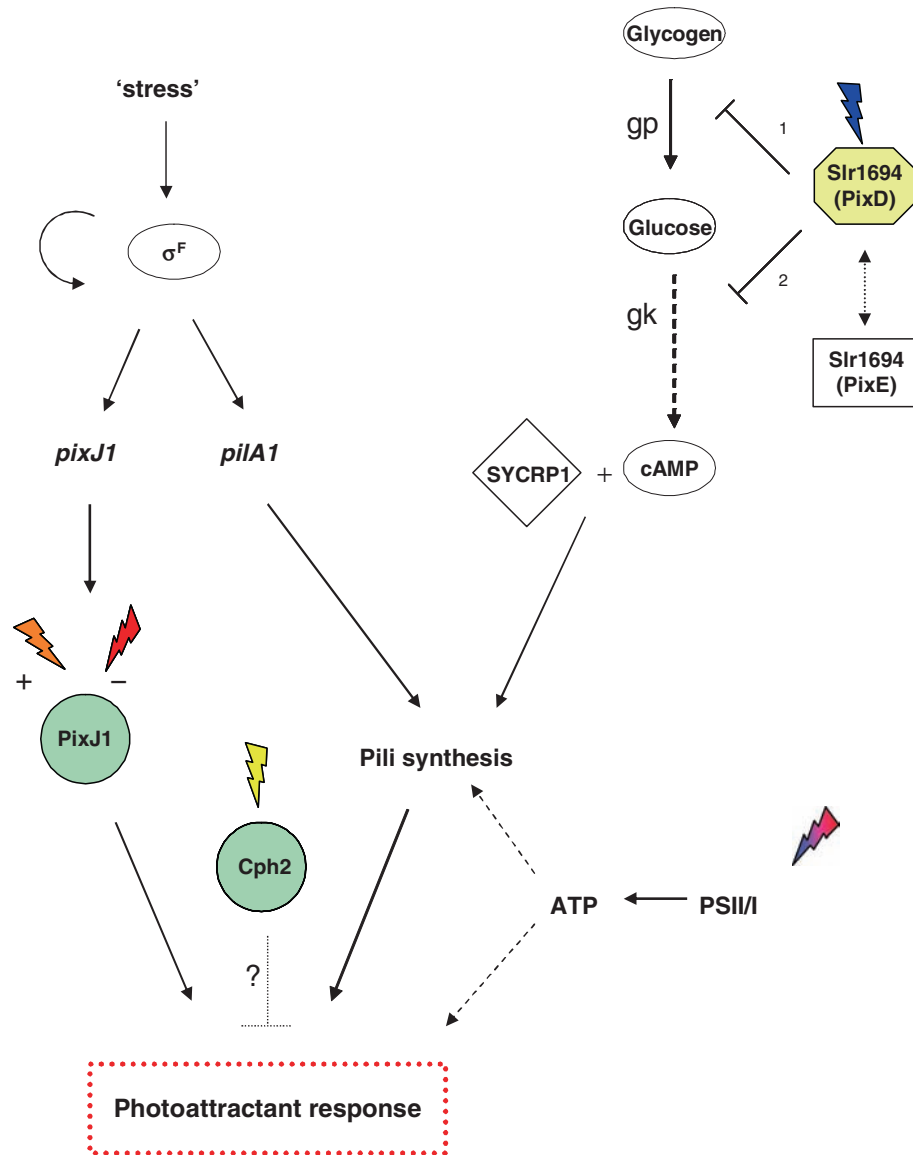


Fig. 3. Working model of regulation of phototaxis in *Synechocystis* sp. PCC6803. See text for detailed description. “1” and “2” indicate interference with the reactions catalyzed by glycogen phosphorylase (gp) and glucokinase (gk), as found by yeast two-hybrid screening methods (44), which is relieved upon blue-light activation of PixD. The circular arrow upstream of σ^F refers to the process of autoactivation to which this sigma factor is subjected. PixJ1 (=TaxD1) is a photoreversible phytochrome (29).

available, and the identification of genes encoding homologs of known photoreceptor proteins in genomes, it is now becoming clear that many chemotrophic bacteria may exhibit previously unsuspected light responses.

The first example is the well-studied bacterium *Escherichia coli*, which encodes in its genome the BLUF-domain containing protein

YcgF (as its only photoreceptor protein). This recently discovered photoreceptor protein binds flavin to yield a blue-light photoreceptor (53) of the BLUF type. *E. coli* serves as the model system for chemotaxis, but until now, no physiological light-regulated motility response has been described in this organism (in contrast to nonphysiological effects of visible light; see e.g., ref. 54). Recently, however, it was shown that light regulates both adhesion on solid surfaces and motility in this organism (Key et al., unpublished results; see also ref. 55). Presumably, the YcgF protein modulates, through signaling-state formation in its BLUF domain, the activity of its output domain, which shows homology to EAL domains. This in turn may affect the level of the signaling molecule bis-(3'-5')-cyclic-diguanosine monophosphate (c-di-GMP) in the cell (56). The observations are most straightforwardly explained by assuming that light-stimulated exopolysaccharide synthesis impairs flagella-based swimming motility.

Acinetobacter sp. ADP1, a common soil bacterium that shows an interesting system for natural transformation (57), does not possess flagella but instead uses polar thick fimbriae for surface migration through twitching motility (58). The organism has thin pili too, but these seem to function in adhesion rather than in migration (M. Bitrian, unpublished observation; compare (37)). Recently, we observed that migration through twitching motility in *Acinetobacter* sp. ADP1 is modulated by illumination with visible light (Bitrian et al., unpublished results); however, the molecular basis of this effect has not yet been resolved. Interestingly, the genome of this organism encodes four photoreceptor proteins of the BLUF-type (27).

A third example was recently published by Oberpichler et al. (7). Light turns out to inhibit motility of *Agrobacterium tumefaciens* (and several other *Rizobiaceae* species) on LB soft agar "swimming plates" via inhibition of the synthesis of the major flagellin subunits FlaA,B. Surprisingly, none of the three tested photosensory proteins encoded in the genome of the organism (i.e., two bacteriophytochromes and a cryptochrome) seems to be involved in this response.

In the following sections, the methods for phototaxis measurements in liquid cultures of *Halobacterium salinarum*, *Rhodobacter sphaeroides*, and *Halorhodospira halophila* will be described in detail.

2. Materials

2.1. Growth of *Halobacterium salinarum*

1. Peptone medium (59): 250 g NaCl, 20 g MgSO₄·7H₂O, 2 g KCl, 0.2 g CaCl₂, and 10 g bacteriological peptone are required per liter.

2. Trace metal solution (1 ml/1 peptone medium): 3 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 23 g FeCl_2 , 4.4 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ dissolved in 1 l of 0.01 N HCl.
3. A filter apparatus for sterilizing the trace metal solution.
4. Bacto-Agar (Difco).
5. Erlenmeyer flasks.
6. A rotary shaker thermostatted at 38°C (for example a New Brunswick Model G-2 gyrorotary shaker set at 240 rpm).

2.2. *Halobacterium salinarum* Phototaxis Measurements

1. Microscope slides and cover slips.
2. Interference filters in the range from 390 to 650 nm.
3. Infrared-sensitive camera (Newvicon, 1 in., RCA Product Div., Lancaster, PA).
4. Optical shutter (Vincent Associates, Rochester, NY).
5. Infrared transmitting beam splitter (Edmund Scientific, Barrington, NJ).
6. Computerized cell-tracking system (Motion Analysis Systems, Inc., Santa Rosa, CA).

2.3. Growth of *Rhodobacter sphaeroides*

1. 10× concentrated growth medium for *Rb. sphaeroides*(60): 34.8 g K_2HPO_4 , 5.0 g $(\text{NH}_4)_2\text{SO}_4$, 40.0 g succinic acid, 1.0 g L-glutamic acid, 0.4 g L-aspartic acid, 5.0 g NaCl, 2.0 g nitrilotriacetic acid, 3.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.334 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.020 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 ml $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ (1% solution), 1 ml trace elements solution, and 1 ml vitamin solution are required per liter.
2. Trace element solution (per 100 ml): 1.765 g EDTA, 10.95 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.54 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.392 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.248 g $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.114 g H_3BO_3 .
3. Vitamin solution (per 100 ml): 1.0 g nicotinic Acid, 0.5 g thiamine HCl, 0.010 g biotin.
4. Casamino acids (Difco Laboratories) and a 1 M KCl solution.
5. A flat glass growth chamber (approx. 2 mm thick) to ensure homogeneous illumination of the bacterial culture.
6. An illuminated incubation area thermostatted at 30°C.

2.4. Surface Tethering of *Rhodobacter sphaeroides*

1. 10 mM HEPES buffer of pH 7, containing 100 µg/ml chloramphenicol to arrest protein synthesis. This solution is used to wash the cells before tethering to a glass surface.
2. Optically flat capillaries (Camlabs, Cambridge, UK).
3. Sigmacote (Sigma-Aldridge, Poole, UK), a hydrophobic agent used to coat the capillaries.
4. Antifilament antibodies to tether the cells to the glass surface of the optically flat capillaries (0.1 × 1 mm, Camlabs, Cambridge, U.K.).

2.5. *Rhodobacter sphaeroides* Phototaxis Measurements

1. A long-bandpass filter (>950 nm) placed in the optical path of the microscope before the bacterial sample. This light is used to observe the bacteria without driving photosynthesis.
2. A quadrant photodiode (PIN-SPOT 4DMI, UDT Sensors, Inc., Hawthorne, CA) to detect rotation of the tethered cells.
3. A 100-W tungsten-halogen light source equipped with a 500–820-nm bandpass filter (~ 270 Wm $^{-2}$ light intensity, measured using a quantum radiometer, e.g., from Li-Cor, Lincoln, Nebraska) to provide photostimuli to the cells that initiate photosynthesis.
4. An optical shutter to allow precise timing of the photostimulation of the cells.
5. A beam splitter to allow the coaxial illumination of the cells with >950 nm observation light and 500–820-nm photostimulation light (Fig. 4).

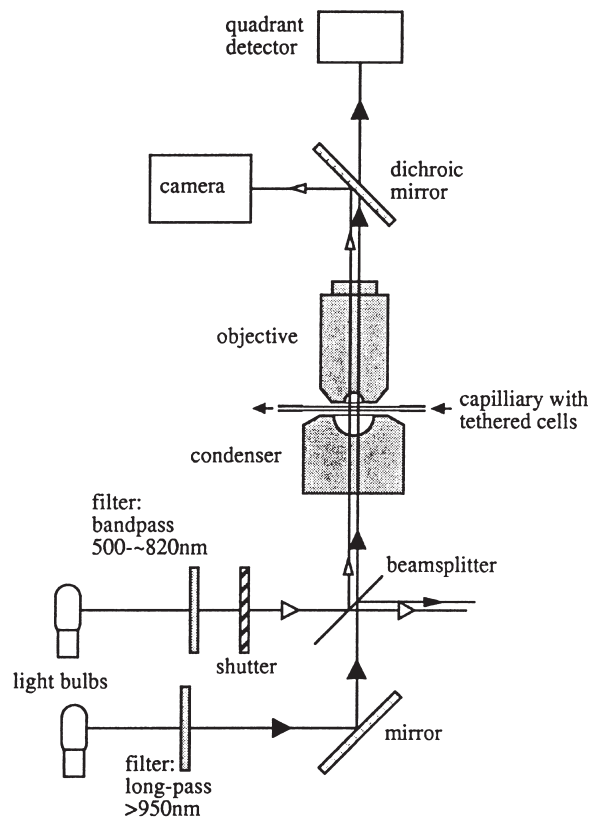


Fig. 4. Experimental setup used for phototaxis measurements on tethered cells of *Rhodospirillum rubrum*. Light for microscopic detection of the cells (>950 nm) and for providing photostimuli to the cells (500–820 nm) are controlled independently. The rotation of individual cells is quantified using a quadrant photodiode. Reprinted with permission from ref. 18.

2.6. Growth of *Halorhodospira halophila*

1. For growth of *H. halophila* (61): 0.8 g KH_2PO_4 , 0.8 g NH_4Cl , 0.05 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2 g NaAcetate, 200 ml 1 M Na_2CO_3 pH 9.0, 1 g $\text{Na}_2\text{S}_2\text{O}_3$, 0.1 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 130 g NaCl, and 1 ml trace solution (see next) are required per liter.
2. Trace solution (per 1 l): 1.8 g $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.25 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 70 mg $\text{MgCl}_2 \cdot 4\text{H}_2\text{O}$, 0.1 g ZnCl_2 , 0.5 g H_2BO_3 , 30 mg $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 10 mg $\text{NaSeO}_3 \cdot 5\text{H}_2\text{O}$.
3. Glass screw-cap tubes or bottles with a rubber ring to ensure anaerobic conditions during growth.
4. Bacto-agar (DIFCO).
5. AnaeroGen Compact transparent pouches (An0010C) from Oxoid (Cambridge, U.K.) with atmospheric generation system for anaerobic growth on agar plates.
6. A thermostatted water bath illuminated by 60-W Tungsten light bulbs (approximately $50\text{--}75 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) for growth of liquid cultures.
7. A thermostatted incubator containing light bulbs for illumination of bacterial agar plates.

2.7. *Halorhodospira halophila* Population Phototaxis Assay

1. Optically flat glass capillaries (see earlier).
2. Broadband optical filters and narrow bandwidth optical interference filters for providing phototaxis stimuli and for microscopic detection of the cells.

2.8. *Halorhodospira halophila* Phototaxis Measurements: Step-Up and -Down Responses and Wavelength Dependence

1. Optically flat glass capillaries.
2. A broadband filter transmitting light above 550 nm, but not in the range 400–500 nm.
3. Narrow bandwidth (9 nm) interference filters in the spectral range 400–500 nm.
4. A Schott KL 1500 halogen lamp (150 W) with an optical fiber.

3. Methods

H. salinarum and *Rb. sphaeroides* can be grown chemotrophically in the dark or photosynthetically in the light. The observed photoresponses will depend critically on the conditions under which these cells are grown. For example, responses triggered by SRI dominate in *H. salinarum* grown under anaerobic photosynthetic conditions, while SRII is the main photoreceptor after aerobic chemotrophic growth. Thus, great care should be taken

in choosing growth conditions for cell cultures to be examined for phototaxis responses.

Phototaxis responses can be observed using a number of distinct assays: (1) the pattern of accumulation into or away from a light spot with a specified spectral composition projected into a bacterial culture (22, 32); (2) the effects of sudden changes in light intensity on the motility response of single cells tethered to a glass surface via their flagella (18, 19); (3) the taxis response of individual free-swimming bacteria in a population of cells after a sudden change in light intensity (22, 62, 63). The wavelength dependence of these responses can yield critical information on the photoreceptor that triggers the observed response.

The microscopic observation of bacteria in phototaxis experiments introduces a possible complication: the light used to observe the cells can generate phototactic signals. Thus, it is critical that the light used to observe the cells microscopically is chosen to be in a spectral range that does not trigger the phototaxis responses under study. An additional complication in the case of positive phototaxis in purple bacteria is that the energy status of the cells needs to be sufficient to allow swimming to occur, and the “photoreceptor” for photosynthesis and phototaxis is the same: the photosynthetic machinery. In the case of *H. salinarum*, the blue-shifted S_{373} intermediate, which is formed by attractant orange light, is the photoreceptor for a negative phototaxis response to near-UV light. Thus, the photoresponse toward near-UV light is only observed when the cells are simultaneously illuminated with light that initiates the photocycle of SRI (64). These “spectral complications” need to be carefully taken into account when designing experiments.

3.1. Phototaxis of *Halobacterium salinarum*

3.1.1. Growth of *Halobacterium salinarum*

1. To prepare the peptone medium, the components are dissolved in distilled water in the order listed, and the pH is adjusted to pH 7.0 with 4 N NaOH, using a pH electrode with a low sodium error.
2. The medium is sterilized by autoclaving and cooled down to room temperature.
3. The trace metal solution is prepared in 0.01 N HCl, filter sterilized, and added to the autoclaved peptone at 1 ml/l peptone medium.
4. The cells are grown aerobically in the dark at 38°C in Erlenmeyer flasks (typically 700 ml in a 2-l flask) using a rotary shaker.
5. For phototaxis assays, the cells are grown to the late exponential phase, diluted ~100-fold in fresh medium, and grown for ~2 h for use in motility measurements. The phototaxis response that will be observed depends critically on the strain of *Halobacterium salinarum* that is used (see **Note 1**).

3.1.2. *Halobacterium salinarum* Phototaxis Measurements

6. To maintain the cells, they are grown on slants containing the peptone medium plus 1.5% Bacto-Agar.

1. The cells are placed on a microscope slide, covered with a cover slip, and observed with dark field optics using >700 nm (730–850 nm) light using a 100-W tungsten-halogen lamp (62, 65). Images are recorded using an infrared-sensitive camera mounted on the microscope. During the phototaxis measurement, the cells are maintained at 37°C using a water-jacketed and thermostatted slide holder on the microscope stage.
2. The cells are exposed to light stimuli from a 150-W Xe arc lamp, 200-W Hg arc lamp, or 100-W Hg–Xe lamp. The light from these lamps was passed through interference filters in the region 390–650 nm and an electronically controlled optical shutter. The filtered light from these lamps is combined with the infrared monitoring beam using a visible reflecting, infrared transmitting beam splitter. Cells can be exposed to either step-up or step-down responses lasting 3–90 s or light pulses lasting 10–100 ms.
3. The recorded images of swimming cells are analyzed using the EV1000 computerized (66) cell-tracking system to extract the time-dependent reversal frequency of the cells (Fig. 5; see for example ref. 67).

3.2. Phototaxis of *Rhodobacter sphaeroides*

3.2.1. Growth of *Rhodobacter sphaeroides*

1. Add the components of the 10× medium to 850 ml of distilled water while stirring. Bring the volume to 1 l with distilled water. The pH will be in the range 4.5–4.9. The solution can be frozen for later use.
2. Add the components of the trace element solution to 85 ml of distilled water while stirring. Bring the volume to 100 ml with distilled water. Store the solution at 4°C.
3. Add the components of the vitamin solution to 85 ml of distilled water while stirring. Bring the volume to 100 ml with distilled water. Store the solution at 4°C.

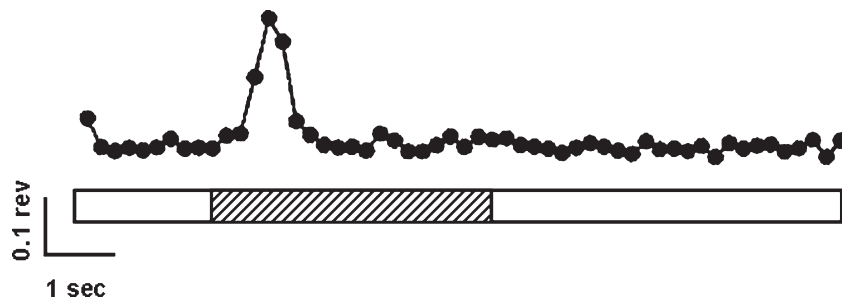


Fig. 5. Detection of a repellent response in *Halobacterium salinarum* to a step-down in orange light intensity. The reversal frequency in a population of cells is determined as a function of time using computerized motion analysis software. The step-down stimulus is indicated by the *hatched bar*. Reprinted with permission from ref. 67.

4. Add 100 ml of the 10× medium to 800 ml distilled water.
5. Adjust to pH 7.0 with KOH.
6. Add 2 g casamino acids per liter 1× medium (optional).
7. Bring volume to 1 l with distilled water.
8. Sterilize by autoclaving.
9. *Rb. sphaeroides* cells (*see Note 2*) are grown anaerobically at 30°C in clear and flat glass bottles with airtight caps under bright light illumination (approximately 100 W/m² in the range 400–800 nm).
10. The cells are harvested in early exponential phase at low-to-intermediate cell densities.

3.2.2. Surface Tethering of *Rhodobacter sphaeroides*

1. The optically flat capillaries are coated with Sigmacote.
2. Anaerobic tethering buffer (10 mM Na-HEPES, pH 7.2 containing 100 µg/ml chloramphenicol) is prepared by sparging with nitrogen gas (*see Note 3*).
3. One milliliter of cell culture is harvested by centrifugation and resuspended in anaerobic tethering buffer. The resuspended cells can be illuminated for up to 1 h to ensure anaerobic conditions.
4. A 5-µl volume of cells is incubated for 30 min at 30°C with 2 µl of polyclonal antibody (135 µg of protein per ml) directed against *Rb. sphaeroides* flagellin (*18, 19*). This polyclonal antibody was obtained by shearing the cells to break off flagellar filaments. Purified flagellar filaments are used to generate a polyclonal antibody in New Zealand white rabbits. This polyclonal antibody is adsorbed using a nonflagellated strain of *Rb. sphaeroides* to remove antibodies directed against the cell body. The antibody is stored at –15°C.
5. The cell-antibody mixture is then introduced into the coated optically flat capillaries, which are then sealed with Vaseline and incubated for 20 min to allow attachment of the flagella to the glass surface. Since the flagellum is tethered to the glass surface, flagellar rotation will result in the rotation of the cell body with respect to the tethering point on the glass surface. This rotary motion of the cell is measured.

3.2.3. *Rhodobacter* *sphaeroides* Phototaxis Measurements

1. The cells tethered to the surface of the optically flat capillary are observed using a Nikon Optiphot (or similar) microscope with a 100× oil immersion phase contrast objective using light >950 nm to image the cells (*18*).
2. This light is projected onto the quadrant photodiode such that the image of the tethered cell is aligned to have the center of cell rotation.
3. The currents from the four quadrants of the photodiode (labeled *a–d*) are sampled at 128 Hz, and used to calculate

the rotation of the bacterium as expressed in terms of X and Y signals calculated as follows:

$$X = ((a + d) - (a + c)) / (a + b + c + d)$$

$$Y = ((a + b) - (c + d)) / (a + b + c + d)$$

4. The signals for a specific time range are selected. The extremes of the signals of X and Y in the data are fitted to envelopes, and these envelopes are subtracted from the data to correct for drift in the signals. The data are scaled to a scale from -1 to 1 .
5. The resulting corrected X and Y signals are used to calculate the speed of rotation of the cells using the following procedure. The angle θ is calculated as $\arctan(Y/X)$, and converted into $\cos \theta$ and $\sin \theta$. The speed of bacterial rotation is then calculated as $1/2\pi (d\theta/dt)$.
6. Just before starting the experiments, the cells are illuminated with light that is absorbed by the photosynthetic machinery.
7. The cells are exposed repeatedly by light from the second light source (in the range 500–820 nm) using an optical shutter.
8. The effects of the illumination of the cell on the rotation speed of individual bacteria as a function of time after opening or closing the optical shutter are determined.

3.3. Phototaxis of *Halorhodospira halophila*

3.3.1. Growth of *Halorhodospira halophila*

1. *H. halophila* cells are grown in solution in the growth medium described by (61). The $MgCl_2$, $CaCl_2$, Na_2S , NH_4Cl , and trace solution are added from autoclaved stock solutions to an autoclaved stock solution of the other components to avoid precipitation of salts. After mixing, the pH is adjusted to 8.5 with a filter-sterilized solution of 3 M HCl. Typically, cells are inoculated in prewarmed medium with 5% volume from a liquid culture in the late-exponential or stationary phase with an OD of ~ 1.5 at 540 nm. The cells are incubated at 41–43°C in a thermostatted water bath, with the well-sealed tubes or bottles submerged $\sim 80\%$ into the water bath, and illuminated with Tungsten light bulbs (see Note 4). Depending on the light intensity, the doubling time can be ~ 10 h.
2. For growth of *H. halophila* on agar plates, at least 0.6% (wt/vol) agar is added to the Imhoff medium prepared as described earlier. After inoculation of the plates with *H. halophila* cells, the agar plates are sealed in AnaeroGen Compact transparent pouches (An0010C) from Oxoid (Cambridge, U.K.). The atmospheric generation system for these pouches is used to generate anaerobic growth conditions. The sealed pouches are incubated at 40–43°C under illumination with a 60-W Tungsten lamp inside the incubator until deep red colonies of *H. halophila* appear.

3.3.2. *Halorhodospira*
halophila Population
Phototaxis Assay

1. *H. halophila* cells from a liquid culture in the late exponential phase with a high percentage of motile cells (*see Note 5*) are incubated in a glass capillary. The open ends of the capillary are sealed with Vaseline to create an anaerobic environment in the capillary (22).
2. The cells are exposed to green light for ~30 min that excited photosynthesis to ensure the energy status of the cells.
3. The light of the microscopy (for example a 100-W halogen lamp in a Nikon Optiphot microscope) is passed through an optical filter to provide a light climate of choice, and through a diaphragm to illuminate only a portion of the cell suspension that is viewed using an $\times 125$ phase contrast objective.
4. The cell suspension is exposed to this light climate for ~10 min. The light intensity reaching the bacteria can be measured using a photometer, for example a Unit SKP200 photometer with an SKP215 sensor head (Skye Instruments, Portree, Isle of Skye, Scotland).
5. The diaphragm is then manually rapidly adjusted to homogeneously illuminate the entire area in view, and then bacterial culture is photographed. This procedure can be repeated with various optical filters to document the wavelengths involved in the phototaxis responses. Note that the same spectral composition is used to photostimulate and detect the cells (*see Note 6*).
6. Phototaxis responses will result in a nonhomogeneous distribution of bacteria in the field of view (**Fig. 6**). Positive phototaxis results in the accumulation of bacteria in the circle that was illuminated during the 10-min incubation period. This is the case for *H. halophila* when green light is projected into the culture (22). Negative phototaxis results in a reduction in cell density from the illuminated spot. A combination of negative and positive phototaxis, as is the case for *H. halophila* when blue light is used (**Fig. 6**), results in the accumulation of bacteria at the edge of the illuminated light spot (22).

3.3.3. *Halorhodospira*
halophila Cellular Photo-
taxis Measurements:
Step-Up and -Down
Responses and
Wavelength Dependence

1. *H. halophila* cells from a liquid culture in the late exponential phase are incubated in a glass capillary with its ends sealed with Vaseline to create an anaerobic environment in the capillary. The cells are then exposed to green light for ~30 min (22).
2. A broadband filter with an absorbance maximum near 450 nm is inserted into the optical path of the microscopy (before the cell sample), and the cells are adapted for ~10 min to these light conditions.
3. To measure the cellular response to a step-up in blue-light intensity, the broadband filter is rapidly removed (manually)

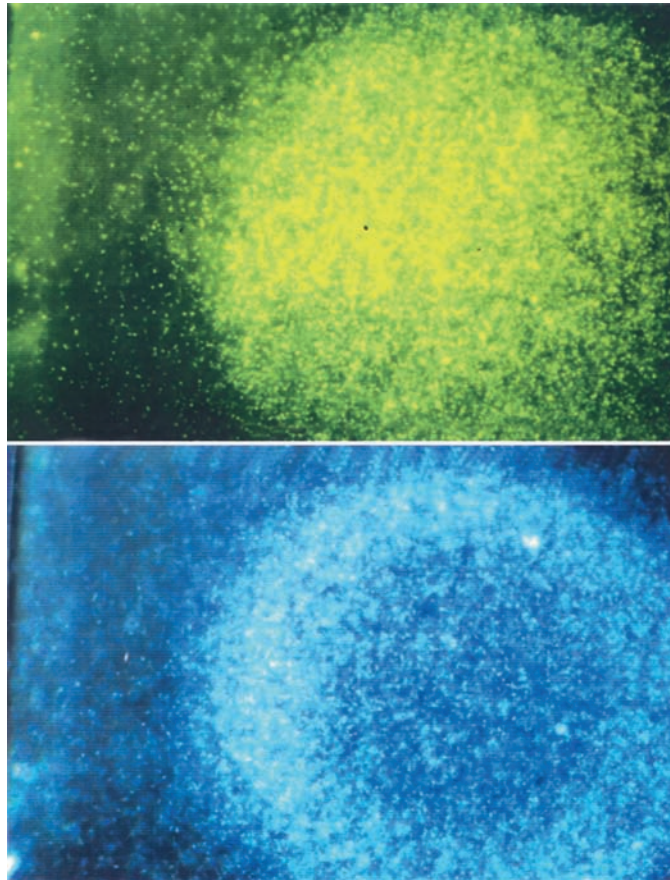


Fig. 6. Population phototaxis assay for *Halorhodospira halophila*. A green-light spot (*top*) causes cells to accumulate in the light due to positive phototaxis. A blue-light spot (*bottom*) triggers both positive phototaxis toward the light and negative phototaxis away from intense blue light, resulting in a *ring-shaped* accumulation pattern. Reprinted with permission from **ref. 22**.

from the optical path, and the resulting response of the cells is monitored and recorded on a video recorder for subsequent analysis (either manually or preferably using cell-tracking software).

4. To measure the response to a step-down in light intensity, the same broadband filter can be rapidly inserted into the light beam.
5. To measure the wavelength dependence of the step-up response, the cells are detected using light above 550 nm that is saturating for photosynthesis and are exposed to this light for ~10 min before starting the measurement.
6. A Schott KL 1500 halogen lamp (150 W) with an optical fiber is used to illuminate the capillary. Narrow bandwidth interference

filters are used to select photoexcitation wavelengths in the range 400–500 nm. The light intensity transmitted by each filter is determined using a photometer and adjusted to be in the same using neutral density filters. The effect of 4-s exposures of the cells (achieved using an optical shutter and shutter control box, Vincent Associates, Rochester, NY) is determined as described under **step 3**.

3.4. Concluding Remarks

Whereas during the last two decades of the previous century behavioral responses in bacteria were routinely interpreted within the framework of the *E. coli* chemotaxis response, this approach now is no longer tenable. Not only do we know examples of Che-like proteins that are not involved in motility responses, like the Wsp-system of *Pseudomonas aeruginosa* (68) which plays a role in biofilm formation, but in addition, light-modulated effects on bacterial motility have now been documented in which no Che-like proteins seem to play a role (see earlier). For this reason, resolving the molecular basis of these responses has become more challenging. From now on also effects of intermediary metabolism and modulation of gene expression have to be considered as possible underlying mechanisms.

An additional challenge is to find out which of the candidate photosensory proteins plays a role in each of the various responses recently discovered in chemotrophic bacteria. Nevertheless, these latter systems also bring the attraction of using light stimuli to optimally control the biological system under study.

4. Notes

1. A large number of strains of *H. salinarum* are available, containing different combinations of mutations in genes relevant for phototaxis responses (69, 70). Complications due to overlapping photosynthetic responses triggered by bacteriorhodopsin (BR) and halorhodopsin (HR) and phototactic responses triggered by SRI and SRII can be avoided by using strains lacking BR and HR, respectively. In addition, strains are available containing only SRI or SRII, allowing photoreponses triggered by each individual photoreceptor to be studied. Strains that are deficient in retinal biosynthesis can be used for studies on the effects of retinal analogs (added to the medium) on phototaxis responses. Finally, methods for selecting highly motile cells are available and have been used to select highly motile strains of *H. salinarum*.
2. Important differences exist between strains of *Rb. sphaeroides*. Strains RK1 and WS8-N respond differently to blue-light stimuli,

and only *Rb. sphaeroides* RK1 contains a *pyp* gene, but this gene is not involved in the observed blue-light response (63).

3. Since the absence or presence of oxygen will affect the rate of electron transport, the concentration of this gaseous compound can strongly influence the observed phototaxis responses. Thus it is critical to control the oxygen concentration that the cells are exposed to during the phototaxis measurements.
4. The emission spectrum of the lamp being used for photosynthetic growth should be considered. *H. halophila* appears to grow better when using tungsten lamps than fluorescent lamps.
5. Loss of motility during growth of *H. halophila* in the laboratory can present an important challenge to studies of its phototaxis responses. Sprenger et al. (22) solved this problem by selecting a motile strain using the following approach. A cell suspension was concentrated by centrifugation and resuspension in small amount of spent medium. This suspension was mixed with an equal volume of 1.5% low-melting agarose cooled to ~45°C. This mix was placed in a hole (removed from the plate using a flame-sterilized metal spoon) in the center of a 0.6% (wt/vol) agar plate with Imhoff's medium and 2 mM Na₂S·9H₂O. The plates were incubated upside down under anaerobic conditions at 40°C for 3 weeks. Deeply purple-colored swarming cells extending from center plug are then cut from the plate using a flame-sterilized knife and diluted into fresh medium.
6. This can represent a problem if obligately phototrophic cells such as *H. halophila* are studied, and light is used that is not absorbed by the photosynthetic machinery. A dedicated photosensory protein may trigger a phototaxis response to this light, but the low-energy status of the cells in this light may preclude the detection of this response. In this case, side illumination of the capillary using an additional light source equipped with a light guide can be used to provide photosynthetically active light. This approach can also be used to separate positive and negative phototaxis responses to distinct colors of light.

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