The Eubacterium *Ectothiorhodospira halophila* Is Negatively Phototactic, with a Wavelength Dependence That Fits the Absorption Spectrum of the Photoactive Yellow Protein

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The motile, alkalophilic, and extremely halophilic purple sulfur bacterium *Ectothiorhodospira halophila* is positively photophobic. This response results in the accumulation of bacteria in light spots (E. Hustede, M. Liebergesell, and H. G. Schlegel, *Photochem. Photobiol.* 50:809–815, 1989; D. E. McRee, J. A. Tainer, T. E. Meyer, J. Van Beeumen, M. A. Cusanovich, and E. D. Getzoff, *Proc. Natl. Acad. Sci. USA* 86:6533–6537, 1989; also, this work). In this study, we demonstrated that *E. halophila* is also negatively phototactic. Video analysis of free-swimming bacteria and the formation of cell distribution patterns as a result of light-color boundaries in an anaerobic suspension of cells revealed the existence of a repellent response toward intense (but nondamaging) blue light. In the presence of saturating background photosynthetic light, an increase in the intensity of blue light induced directional switches, whereas a decrease in intense blue light gave rise to suppression of these reversals. To our knowledge, this is the first report of a true repellent response to light in a free-swimming eubacterium, since the blue light response in *Escherichia coli* and *Salmonella typhimurium* (B. L. Taylor and D. E. Koshland, Jr., *J. Bacteriol.* 123:557–569, 1975), which requires an extremely high light intensity, is unlikely to be a sensory process. The wavelength dependence of this negative photoresponse was determined with narrow band pass interference filters. It showed similarity to the absorption spectrum of the photoactive yellow protein from *E. halophila*.

In free-swimming prokaryotes, behavior at the molecular level involves the measurement of the value of certain chemical or physical parameters in the course of time, as the cell swims in spatial gradients of stimulants and/or repellents (for reviews, see references 2 and 7). Protein molecules with a sensory function, either chemo- or photoreceptors or specific indicators of cellular metabolism, such as the chemiosmotic proton gradient or the pool of certain metabolic intermediates, inform the cell about its present physiological situation. This information is compared with a previously sensed situation, and the change is evaluated as either favorable or unfavorable. Whereas the direction of the swimming of the cell with respect to the spatial gradient is random and remains so, uninfluenced by tactic processes, the time during which the cell continues swimming in a given direction is dependent on whether the integrated sensory signals are positive or negative. In the former case, the cell's tendency to switch its direction is suppressed, whereas in the latter case (i.e., when the sum is negative), it is enhanced.

The process of chemotaxis in *Escherichia coli* has been extensively studied (2, 7). Chemotaxis toward a large number of attractant and repellent chemicals is mediated by four transmembrane chemoreceptors present in the cytoplasmic membrane. Attractant and repellent signals are passed on from these receptors, via a protein phosphorylation cascade, to the tumble generator that controls the direction of flagellar rotation. In parallel, individual receptor molecules adapt to

the changes in the concentration of excitatory compounds via a reversible methylation of the chemoreceptors. This adaptation process enables bacteria to perform efficient chemotaxis over a wide range of attractant or repellent concentrations.

Enteric bacteria, notably *E. coli* and *Salmonella typhimurium*, also change their swimming behavior upon exposure to blue light of very high intensity (32). When *S. typhimurium* cells were exposed to 2,000 to 5,000 W of light m⁻² between 390 and 530 nm, they tumbled continuously for several seconds. Upon longer exposure, the cells swam smoothly for half a minute, after which they became nonmotile. This loss of motility was irreversible. The addition of riboflavin as a photosensitizer caused the bacteria to respond at much lower light intensities, indicating that photooxidation of endogenous flavonoid compounds at nonphysiological light intensities is responsible for this blue light response.

Recent experimental data suggest that protein methylation is also involved in the tactic responses of the archaeobacterium *Halobacterium halobium* (1, 27). In this species, besides chemotaxis, both positive and negative phototactic reactions can be observed. In these responses, two specific membrane-associated photosensory proteins function as photoreceptors (29). These proteins, which both carry retinal as a chromophoric group, are known as sensory rhodopsin I (SRI) and SRII. Both photosensors enter a photocycle upon absorption of a photon. SRI absorbs maximally at 587 nm in its ground state and can be photoconverted into a long-lived intermediate, with an absorption maximum at 373 nm. This intermediate can either be photoconverted into the ground state or decay thermally in the dark via a relatively

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slow reaction (half-life = 80 or 750 ms, respectively). The protein transduces both an attractant response to light at 587 nm and a repellent response to light at 373 nm (28). SRII absorbs maximally at 487 nm and mediates a repellent response to blue light: an increase in blue light induces directional switches, whereas a decrease suppresses them (29). Its photocycle includes a long-lived intermediate absorbing at 350 nm and a third intermediate, between the UV-absorbing photointermediate and the ground state, absorbing maximally at 530 nm (30, 33). A similar protein is probably present in *Natronobacterium pharaonis* (4).

The process of phototaxis in phototrophic purple bacteria has also been studied. Here, we use the word phototaxis according to the definition used in reference 29. These bacteria show a strong attractant response to all photosynthetically active wavelengths: the action spectrum precisely matches the absorption spectrum of the bacteriochlorophyll and carotenoid pigments of the photosynthetic apparatus (5). It is a positive photophobic response: the probability of directional switching increases strongly when a swimming cell enters an area of lower light intensity. With several species of purple bacteria, it has been demonstrated that a change in the rate of photosynthetic electron transport is necessary for this positive photophobic response, since mutants lacking the photosynthetic reaction center showed no phototactic responses (3). Experiments with proton and potassium ionophores indicated that it is not the change in the rate of electron transport but rather the resulting change in the size of the electrochemical proton gradient that is needed for a phototactic response (3, 6). The fact that these bacteria can accumulate in an area with only a slightly higher (approximately 1%) light intensity than the surroundings is due to the fact that there is a very strong tendency to reverse in response to a decrease in photosynthetically useful light (2).

In 1985, a study of the soluble redox carrier proteins in *Ectothiorhodospira halophila* led to the isolation of a 14-kDa, water-soluble yellow protein of unknown function (18). The protein has a broad and slightly asymmetric absorption peak in the blue region of the spectrum ($\epsilon = 45.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 446 nm). Its absorption spectrum is redox independent, but the visible absorption peak is bleached under protein-denaturing conditions, e.g., low pH, giving rise to a new absorption peak at 345 nm (18). Subsequently, it was demonstrated that upon absorption of a photon, the protein enters a photocycle in which a long-lived photointermediate absorbing in the near UV decays thermally to the ground state on the seconds time scale (half-life = 0.7 s [22]). Thus, this photoactive yellow protein (PYP) is the first eubacterial protein showing photophysical similarities to the rhodopsins known from both archaeobacteria and eukaryotes. The influence of solvent hydrophobicity and viscosity on the kinetics of the bleaching and recovery reactions in the PYP photocycle suggests that the protein changes its conformation during the photocycle, temporarily exposing a hydrophobic domain on its surface (21). The process of photobleaching has a sufficiently high quantum yield (0.64) to be biologically significant (21); however, so far no direct evidence has been obtained with respect to the biological function of this unusual protein. The three-dimensional structure of PYP has been resolved by X-ray crystallography (17). Its structure consists of two parallel planes of β -sheet secondary structure, known as a β -clam. The chromophore is located in between these sheets, but its chemical structure has not yet been resolved.

The photochemical similarities between PYP and the

sensory rhodopsins, together with the fact that both *E. halophila* and *H. halobium* thrive in extremely saline brines and thus face the same risks of photodestruction and/or dehydration, have led to the working hypothesis that PYP may function as a photoreceptor in negative phototaxis in *E. halophila*. It was known that *E. halophila*, like all motile purple phototrophic bacteria studied so far, shows a normal positive photophobic response to photosynthetically active light (2, 12, 17). In this report, we present evidence indicating that *E. halophila*, in addition, shows a repellent response at physiological light intensities. No such response has been described before for any eubacterium. The wavelength dependence of this response is in accordance with the hypothesis that PYP functions as its photoreceptor.

MATERIALS AND METHODS

Growth of bacteria. *E. halophila* BN9626, from which T. E. Meyer first isolated PYP (18), was kindly provided by J. F. Imhoff and was used for all experiments described in this report; a number of experiments have been repeated with type strain SL1, which was kindly provided by T. E. Meyer. These latter experiments yielded identical results. Organisms were grown in batch culture in the medium described by Imhoff (13). A motile strain was selected by mixing a very concentrated cell suspension with an equal volume of cooled molten agarose (1.5% [wt/vol] in deionized water). This mixture was introduced into a hole in the middle of a 0.6% (wt/vol) agar plate containing Imhoff's medium with 0.05% (wt/vol) $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (2.25 mM) and incubated in the light under a CO_2 - N_2 atmosphere for 3 weeks at 39°C. After that period, a small feather-shaped extension from the (deeply purple-colored) inoculated area was observed, which contained motile bacteria. Motility varied considerably from batch to batch. However, in all samples investigated, motility was between 75 and 90%. Cells from the late exponential growth phase grown in batch culture (Imhoff's medium supplied with 2 mM sodium sulfide as the electron donor, 40°C, in white light supplied by 60-W tungsten bulbs) showed optimal motility after several hours of incubation in green or orange light in an anaerobic suspension.

Photography of cell distribution patterns in capillaries in and around colored light spots. A cell suspension taken from a batch culture was incubated in a glass capillary (0.1 by 1 mm; optically flat microslides; Camlab, Cambridge, United Kingdom). The open ends of the capillary were sealed with petrolatum. The cells were exposed to green light in the capillary for several hours to optimize motility. Light from the microscope light source (a 100-W, 12-V halogen lamp; Naiva) was passed through an optical filter and a diaphragm, which resulted in the projection of a colored light spot into the anaerobic bacterial suspension. The illuminated area was extended by adjusting the diaphragm manually before photographing the field. In this way, the cell distribution over the previously illuminated spot and its dark surroundings could be visualized under homogeneous illumination. The bacteria were observed with a Nikon Optiphot microscope by using phase-contrast optics at a magnification of $\times 125$ and photographed with a Nikon FX-35A camera. The distribution patterns were stable for at least 30 min.

Determination of the mean reversal frequency of a cell population via analysis of video recordings of free-swimming bacteria. A cell suspension, containing a high proportion of highly motile bacteria, was observed in an anaerobic capillary with a Nikon Optiphot microscope equipped with a

Panasonic TV camera by using phase-contrast optics. Fifty frames were recorded per second on a U-Matic videotape, with a 2,000-fold magnification on the screen. Cells were observed in green light. The light intensity in the visible region of the 100-W, 12-V halogen lamp (Naiva) was measured with a Unit SKP200 photometer with an SKP215 sensor head for a quantum type response (Skye Instruments, Portree, Isle of Skye, Scotland). The total measured visible emission of the lamp was $4,650 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. The total intensity of visible light (500 to 600 nm) transmitted by the green interference filter used (for the transmission spectrum; see Fig. 1B) was $305 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. (Note that 50 to 75 μmol of white light $\text{m}^{-2} \cdot \text{s}^{-1}$ from a 60-W tungsten light bulb was saturating for photosynthetic growth.) A step-up in blue light was brought about by removing the green interference filter from the light beam. After 2 min, a step-down in blue light intensity was given by reintroducing the filter into the light beam. The significant fraction of nonmotile bacteria and the high swimming speed and spiral shape of motile bacteria did not allow computer analysis of swimming behavior. Therefore, the video recordings were analyzed manually by dividing the field into squares to facilitate random selection of motile bacteria. For practical reasons, the step-up and step-down responses were analyzed differently.

Step-up response. The change in the probability of directional switching per 0.2-s interval upon removal of the green filter was determined with a U-Matic video recorder at 50 frames per second by randomly selecting 50 motile cells and scoring them as "switching" or "not switching" during the interval. This was done by dividing the screen into 50 rectangles. Starting in the top left corner of the screen, these rectangles were analyzed one by one until 50 suitable cells had been selected. A cell was counted only if (i) it showed normal motility and (ii) its path could be clearly observed during the relevant time interval. The number of switching cells divided by 50 was called the reversal frequency.

Step-down response. Recordings of the step-down stimulus, which was given to the same bacterial suspension by reintroducing the green interference filter after 2 min, were analyzed on a Panasonic NV-8500 video recorder at 24 frames per second, in analogy to the step-up response. However, because the bacteria apparently had adapted to the increase in light intensity, the observed response consisted of a strong decrease of the probability of directional switches compared with the unstimulated situation. Therefore, in order to include a sufficient number of directional switches, a more extended period of time (10 s) had to be analyzed. It was not feasible to assess the percentage of nonreversing bacteria during this long period and thus to determine the probability of switching. The relative change in this parameter was determined by counting the total number of reversals in a selected part of the field in 0.25-s intervals, thereby assuming that the amount of cells in this area was essentially constant during the 10-s period considered. This assumption is justified because no light boundary or spatial light gradient was present. The number of reversals was determined for every 0.25-s interval from 5 s before to 5 s after the step-down stimulus.

Wavelength dependence. For the determination of the wavelength dependence of the step-up photophobic response, free-swimming bacteria from a batch culture, harvested in the late exponential growth phase, were incubated for at least 30 min anaerobically in yellow-green light (negligible irradiation below 500 nm) in order for the cells to become adapted to this light regimen. Swimming bacteria were recorded on VHS videotape at 24 frames per second

with an Olympus IMT2 microscope connected to a Video-Scope model TM 860-N video camera. A Schott KL 1500 halogen lamp (15 V, 150 W) equipped with an optical fiber was used for side illumination of the capillary. Narrow-bandwidth interference filters (G572 series: 400, 420, 440, 460, 500, and 520 nm; bandwidth, 9 nm; Oriel, Stamford, Conn.) were used to select different wavelengths between 400 and 520 nm. A 4-s period centered around the moment of the step-up stimulus was analyzed on a Panasonic NV-8500 video recorder. The number of reversals per 0.25-s interval was determined. The relative increase in the number of reversals (R) was calculated by subtracting the total number of reversals in the 2-s period before the stimulus (B) from the total number in the 2-s period after the stimulus (A) and dividing this difference by the number of reversals in the 2 s before the stimulus: $R = (A - B)/B$. The absolute light intensity of the stimulus in the cell suspension could not be determined in this experiment; it was not possible to measure the precise light intensity reaching the bacteria within the capillary. As an alternative, the light transmission through each of the interference filters was measured at the end of the optical fiber with a Macam model 3000 photometer and with a YSI-Kettering model 65A radiation meter; it varied between 53.5 and 82 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. These differences were corrected for by calculating the value of R at a light intensity of 50 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. This approach is based on the assumption that there is a linear correlation between light intensity and the number of reversals induced within this range of intensities.

RESULTS

Cell distribution patterns. *E. halophila* cells swim by means of two single polar flagella; the spiral-shaped cell was seen to rotate around its long axis. To change the direction of swimming, the direction of flagellar rotation is reversed. Between two reversals, bacteria swam in straight paths or in smooth circles. Short stops (typically 0.1 s in duration) were also observed. The swimming velocity was quite variable, but it could exceed $100 \mu\text{m} \cdot \text{s}^{-1}$. Distribution patterns of bacteria caused by light spots of attractant (green plus infrared) and repellent (blue) spectral compositions are presented in Fig. 1. Figure 1A shows a pattern that was the result of positive photophobic taxis only, upon projection of a green light spot (for the transmission spectrum of the filter, see Fig. 1B) into a suspension of motile bacteria. A similar pattern (data not shown) was obtained with both a red spot (>50% transmission above 650 nm) and a dark blue spot (with minor transmission between 430 and 460 nm and >50% transmission above 760 nm). Figure 1C shows the pattern that was obtained when a spot which had been filtered by a broad-band blue filter was projected (for the transmission spectrum, see Fig. 1B). This pattern is different from the former. The highest density of bacteria was observed in a broad zone around the boundary of the spot. The same pattern was obtained with an unfiltered white spot of high intensity ($4,650 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$; data not shown). Apparently, reversals were induced not only when cells left the illuminated area but also when they entered it, suggesting that the light passing through this filter was effective both as a repellent and as an attractant photostimulus. In order to investigate this possibility, the following experiments were performed.

Direct observation of individual bacteria crossing an orange-white boundary projected into the cell suspension. Figure 2 shows that when bacteria swam from an area of orange

FIG. 1. Accumulation patterns of *E. halophila* caused by light spots of different spectral compositions. Light spots of different colors were projected into an anaerobic bacterial suspension, and the effect on the distribution of cells was studied microscopically. A positive accumulation pattern of bacteria was obtained when a spot of green plus infrared light (for transmission spectra of the filters used, see panel B) was used (A). The observed pattern was caused by positive phototaxis alone. A different pattern was obtained when a blue (see panel B) spot was used (C). In this case, the bacteria showed both an attractant and a repellent response. Magnification, $\times 125$.

light (>560 nm) into an area in which, in addition, light of shorter wavelengths was present, the fraction of cells that reversed within 1 s significantly increased compared with cells that remained in the orange area. On the other hand, the fraction of cells reversing within 1 s was much lower for cells that had just entered the orange area than for those that remained in the white part. These observations indicate that the observed movement of bacteria from areas with a higher intensity of blue light to areas with a lower intensity of blue light was caused by an induction of directional switches after a step-up of blue light and suppression after a step-down. Indeed, it could be seen, when observing individual cells microscopically, that when they entered the white area the

induced reversal usually caused the bacteria to reenter the orange area. The difference in measured light intensity in the orange and white areas was rather small: 80 versus 107 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Approximately 9.5 μmol of this difference $\text{m}^{-2} \cdot \text{s}^{-1}$ consisted of light transmitted by a yellow filter which could not induce reversals, as could be concluded from direct microscopic observation. Thus, a strong repellent photoresponse was observed in this batch of bacteria in response to a step increase of less than 20 μmol of blue light intensity $\text{m}^{-2} \cdot \text{s}^{-1}$.

Time-based video analysis of a population of bacteria upon a temporal green-white or white-green transition. The effect of the removal of a green filter from the light beam of the microscope on the reversal frequency of a population of free-swimming bacteria is shown in Fig. 3. The fraction of the cells that switched per 0.2-s period increased sharply within 0.4 s after the step-up in light intensity and fell back to approximately the prestimulus value of 0.06 within 1 s. Whether this rapid return of the reversal frequency to the prestimulus level reflects an adaptational process or whether it is the result of a refractory period which all bacteria enter simultaneously immediately after a flash-induced directional switch (as has been observed with *H. halobium* [16]) cannot be determined from these data. It should be noted, however, that almost 100% of the motile bacteria responded to the stimulus by reversing within 0.4 s. A single time point at 5 s after the stimulus (value, 0.22) confirms the impression obtained when observing the cells directly under the microscope in this type of experiment, i.e., that a step-up in blue light increases the probability of switching for at least several seconds.

Figure 4 shows the effect of reintroducing the green filter after 2 min of exposure to white light. The cumulative number of reversals observed per 0.25-s period is plotted against time. There was a significant decrease in the probability of reversals after a temporal transition from white to green light. This confirms our direct observation of a change from a relatively "tumbly" population to one with cells swimming in smooth circles. The observation of individual bacteria swimming in yellow light, after a blue flash, revealed that these cells generally reversed for the first time at least 10 s after the flash.

Wavelength dependence of the step-up response. Figure 5A shows the results of a video analysis experiment carried out to investigate the wavelength dependence of the induction of reversals by an increase in blue light. The bacteria were recorded in saturating photosynthetic light (>540 nm). They were given a step-up light stimulus by side illumination of the capillary, using narrow-bandwidth interference filters to select wavelengths between 400 and 520 nm. The total number of reversals during the 2-s period before and after the step-up was determined for each wavelength. When the poststimulus values are compared with the mean and standard error of the mean of the prestimulus values (19.8 ± 3.1), it appears that only the poststimulus values obtained at 500 and 520 nm are within the 95% confidence level (t test); all other values are outside the 99% confidence level. Evidently, light above 500 nm has no detectable effect on the probability of directional switching, whereas a maximal effect is observed with light around 440 nm.

These data have been replotted in Fig. 5B, after correction for differences in photon flux among the various filters. In this figure, the relative increase in the number of reversals, resulting from a step-up at a given wavelength, is shown. The absorption spectrum of PYP, the hypothetical primary

FIG. 2. Effect of swimming across an orange-white color boundary on probability of directional switching. A motile suspension of bacteria, into which an orange-white color boundary was projected, was studied microscopically. Randomly selected individual bacteria were scored as "reversing" (solid bars) or "not reversing" (shaded bars) within 1 s. Abbreviations: O-W, swimming from the orange into the white area; W-O, swimming from the white into the orange area; O-O, swimming within the orange area; W-W, swimming within the white area.

photoreceptor in the negative photoresponse (see Discussion), is plotted in the same figure for comparison.

DISCUSSION

A new type of photobehavior has been demonstrated for a eubacterial species of purple phototrophic bacteria, *E. halophila*. This organism exhibits a negative photophobic response to blue light between 400 and 500 nm. In contrast to the positive photophobic response that has been demonstrated for all motile purple bacteria studied so far (12), this negative response consists of an induction of reversals after an increase in blue light and suppression after a decrease in

blue light. The latter effect, suppression of directional switches, has not yet been assessed in positive phototaxis for purple bacteria, although there is some evidence that this may occur (2). Reversal suppression has been demonstrated, however, in chemotaxis in *E. coli* (8) and, significantly, in SRII-mediated negative phototaxis toward blue light in *H. halobium* (29). The latter system is very sensitive, since a single photon can induce a reversal (15).

All light above 500 nm was ineffective in triggering the repellent response in *E. halophila*, which excludes bacteriochlorophyll and the carotenoid pigments as the primary photoreceptors. On the other hand, the positive photophobic

FIG. 3. Increase in the reversal frequency caused by a step-up in blue light intensity. A green interference filter (see Fig. 1B for the transmission spectrum) was removed at time zero, and the reversal frequency (the fraction of 50 randomly selected bacteria that reversed during a 0.2-s period) was determined as a function of time. The light stimulus clearly resulted in a burst of reversals in the population.

FIG. 4. Decrease in the total number of observed reversals in response to a step-down in blue light intensity. Two minutes after the removal of the green interference filter (Fig. 3), the filter was reintroduced at time zero. The cumulative number of reversals is plotted as a function of time.

response in this species, which was demonstrated by the accumulation of free-swimming bacteria in light spots projected into the suspension (this work) and by reversible migration into the light in a glass capillary partly covered with foil (17), correlates its wavelength dependence with the absorption spectrum of the photosynthetic pigments, as in *Rhodospirillum rubrum* (5). In addition, positive phototaxis in *E. halophila* was effective at a much lower light intensity than the negative phototactic response, which is in agreement with the observation that most purple sulfur bacteria, including members of the genus *Ectothiorhodospira*, show maximal positive phototactic sensitivity at 40 mW of white light m^{-2} (12). In order to determine the wavelength dependence of the negative phototactic response in *E. halophila* for comparison, light intensities between 14 and 23 $\text{W} \cdot \text{m}^{-2}$ were used.

In enteric bacteria, blue light of high intensity can induce constant tumbling (32), as can repellent chemostimuli (8). This response was not observed in some chemotaxis-deficient mutants. However, a considerable light intensity is needed to elicit this response: 2,000 to 5,000 W of blue light m^{-2} was the minimal amount required for the response, compared with less than 14.5 W of 440-nm light m^{-2} (total irradiation was measured; an unknown fraction of this amount reached the bacteria), which yielded a significant repellent response in *E. halophila* (Fig. 5). Furthermore, the blue light response in enteric bacteria led to an irreversible loss of motility (and possibly cell death) after approximately 30 s of light exposure, whereas incubation of *E. halophila* in white light for 2 min led to normal swimming behavior and high phototactic sensitivity, since reinsertion of a green filter caused immediate suppression of directional switches. Under similar experimental conditions, *E. coli* RP 437 (25) showed no response (26). For the reasons mentioned above, we propose that a separate blue light photosensory system showing only superficial resemblance to the blue light response in enteric bacteria is present in *E. halophila*. The

latter is probably an artifact of unnaturally high light intensity, although it may provide information about the mechanism of true (chemo)sensory processes in these species.

It is possible that the repellent response to blue light that *E. halophila* exhibits is not mediated by any specific photosensory molecule. Possibly, an artifact comparable to the response in enteric bacteria is evident at a much lower light intensity as a result of the high sensitivity of flagellar rotation to, for instance, a slight decrease in photosynthetic electron transport. However, microscopic observation of photosynthetically grown *R. rubrum* and *Rhodobacter sphaeroides* 2.4.1 did not reveal any reversal or stopping response upon excitation with blue light (26). Extreme sensitivity of photosynthetic electron transfer to this intensity of blue light is therefore not a general property of phototrophic purple bacteria. This argues in favor of the involvement of PYP in negative phototaxis as the primary photoreceptor.

If *E. halophila* shows negative phototaxis to blue light at light intensities likely to be encountered in nature, how might this photoresponse be of selective advantage for this bacterium? The strain of *E. halophila* that was most extensively used in this study was isolated from the Wadi Natrun in Egypt (14). This environment is characterized by a very high pH (10.5) and a saturating concentration of salt (35.7% [wt/vol]). The lake is strongly irradiated by sunlight (2,500 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) and is mostly covered by a layer of crystalline salts, while the water underneath is reddish because of the phototrophic bacteria. It is from this extreme environment that this first eubacterial species demonstrated to be negatively phototactic originates. Although it is still completely uncertain whether other eubacteria which demonstrate a similar photobehavior exist, this hypersaline environment may be a clue to why *E. halophila* is so evidently negatively phototactic. It may be that in an almost completely anoxic water column, at high solar irradiation, a strongly positively phototactic bacterium could follow a light gradient into conditions under which the light intensity

FIG. 5. Wavelength dependence of the step-up photophobic response. The wavelength dependence of the increase in the probability of reversing in response to a step-up in light intensity was determined as follows. Motile bacteria were observed in green light (>540 nm), saturating for photosynthesis. Photostimuli of different wavelengths were given by side illumination. Photon fluxes varied between 53.5 and $82 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at different wavelengths. The experiments were carried out with a single cell suspension within a period of 10 min. In panel A, the total number of reversals during 2 s before (solid bars) and after (shaded bars) the step-up is plotted for each wavelength. The dashed lines indicate the 95% confidence level (t test) calculated from the six prestimulus values. The same data were used to calculate the relative increase in the number of reversals caused by the step-up stimuli after normalization to a photon flux of $50 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (B [solid triangles]). The absorption of PYP is plotted in the same panel for comparison.

becomes photodestructive. The additional repellent photoresponse which we have demonstrated with *E. halophila* could prevent this.

Interestingly, the only other free-swimming bacteria which display a negatively phototactic response, archaeobacteria of the genus *Halobacterium*, also thrive in hypersaline

environments. Under anaerobic conditions in the light, these facultatively phototrophic bacteria also display both a positive and a negative phototactic response. Apparently, negative phototaxis is of selective advantage to all bacteria in highly irradiated and thus hypersaline environments. However, for those species of bacteria which are attracted to the

water surface by positive tactic (e.g., aerotactic or phototactic) responses (like *E. halophila* and *H. halobium*), a photorepellent response could be essential.

Quite a number of resemblances between the photoreceptor chromoproteins involved in phototaxis in these two species can be noted. In both organisms, the pigments involved in positive phototaxis are closely related to those involved in photosynthesis. In purple bacteria, photosynthesis generates a proton motive force which is the basis of the positive photophototactic response (see the introduction). In halobacteria, the photosynthetic pigments (bR and hR) show sequence similarity to the receptor for positive phototaxis (SRI) and, even more significantly, have similar (about 570 nm) absorption maxima. In both organisms, negative phototaxis to blue light is mediated by a different photoreceptor which is not involved in photosynthesis. In halobacteria, this photoreceptor is SRII. We propose that PYP is the photoreceptor in negative phototaxis in *E. halophila*, for two main reasons. First, *E. halophila* combines two unusual photobiological features: it contains a unique photoactive protein of unknown function and displays a new type of eubacterial phototaxis. Second, the absorption spectrum of PYP matches the wavelength dependence of negative phototaxis. Definite proof of this hypothesis, however, can be obtained only via a genetic approach.

The only other eubacterium which has been reported to contain a PYP is *Rhodospirillum salexigens*, a halophilic purple phototroph like *E. halophila* (19). We expect this protein to be involved in negative phototaxis, like the *E. halophila* PYP. The predicted negative photoresponse in *R. salexigens* is currently under investigation.

It is interesting that there exist a number of additional similarities between the halobacterial rhodopsins and PYP. The general features of the photophysics (photocycle [see the introduction and references 22 and 23], fluorescence properties [9, 20], and low temperature photoreactions [9]) are very similar for all these proteins (31). However, the structures of PYP and of bR, hR, and SRI are completely different: PYP has a β -clam structure and is water soluble, whereas the three bacterial rhodopsins are predominantly composed of transmembrane α -helices. While these rhodopsins show significant sequence similarity, there is no similarity at all with PYP (data not shown). No such structural data are available for SRII. However, washing of membrane preparations containing both SRI and SRII with the nonionic detergent Tween 20 resulted in the selective loss of SRII photocycling activity from these membranes, suggesting that SRII has characteristics different from SRI (24). Unlike all four bacterial rhodopsins, the PYP chromophoric group is probably not identical to retinal (11, 22).

Since we propose that PYP, like SRII, is a repellent photoreceptor, it is relevant to compare these two proteins in more detail. Both pigments absorb maximally in the blue region of the spectrum (SRII, 487 nm; PYP, 446 nm) and have long-lived photointermediates with an altered polypeptide conformation. Recently, it was shown that the sum of the time-integrated concentrations of the two conformationally activated intermediates of the SRII photocycle correlates with the amount of reversal induction, indicating that these are signalling states in negative phototaxis (34). PYP also has a long-lived photointermediate with an altered polypeptide conformation, which has been postulated to trigger a negative phototactic response (21). Finally, both SRII and PYP are synthesized constitutively (10, 24). Whether these proteins resemble one another because they are the result of convergent evolution or because they are

derived from a common ancestral protein is unknown. To answer this question, more data on the polypeptide structure of SRII and the nature of the chromophore of PYP are needed.

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