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Color discrimination in halobacteria: Spectroscopic characterization of a second sensory receptor covering the blue-green region of the spectrum

(halobacteria/rhodopsin/phototaxis/retinal/archaeobacteria)

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ABSTRACT **_Halobacterium halobium_ is attracted by green and red light and repelled by blue-green and shorter wavelength light. A photochromic, rhodopsin-like protein in the cell membrane, sensory rhodopsin sR_{587}, has been identified as the receptor for the long-wavelength and near-UV stimuli. Discrepancies between the action spectrum for the repellent effect of blue light and the absorption spectrum of sR_{587} and its photocycle intermediate S_{373} strongly suggest the existence of an additional photoreceptor for the blue region of the spectrum. Transient light-induced absorbance changes in intact cells and cell membranes show, in addition to sR_{587}, the presence of a second photoactive pigment with maximal absorption near 480 nm. It undergoes a cyclic photoreaction with a half-time of 150 msec. One intermediate state with maximal absorption near 360 nm has been resolved. The spectral properties of the new pigment are consistent with a function as the postulated photoreceptor for the repellent effect of blue light. The phototactic reactions and both pigments are absent when retinal synthesis is blocked; both can be restored by the addition of retinal. These results confirm and extend similar observations by Takahashi *et al.* [Takahashi, T., Tomioka, H., Kamo, N. & Kobatake, Y. (1985) *FEMS Microbiol. Lett.* 28, 161–164]. The archaeobacterium *H. halobium* thus uses two different mechanisms for color discrimination; it uses two rhodopsin-like receptors with different spectral sensitivities and also the photochromicity of at least one of these receptors to distinguish between three regions covering the visible and near-UV spectrum.**

Halobacterium halobium uses light energy to drive ion fluxes across its membrane. This biological light-energy transduction is mediated by two small retinylidene proteins, bacteriorhodopsin (bR) and halorhodopsin (hR), which have broad absorption bands near 570 nm (for recent reviews, see refs. 1 and 2). In addition, the cells show sensory light reactions. They are attracted by long-wavelength light, green and red, and repelled by short-wavelength light and, thus, migrate to regions of illumination that are optimal for their light-energy transduction systems (3, 4). These phototactic responses are also mediated by retinal pigments (5). Spudich and Bogomolni have recently identified a third retinylidene protein, sensory rhodopsin (sR). It is similar in its physical and chemical properties to bR and hR and mediates both the attractant effect of green and red and the repellent effect of near-UV light (6, 7). sR has a strong absorption band centered at 587 nm and undergoes a cyclic photoreaction with a long-lived intermediate, which absorbs maximally at 373 nm. The intermediate, S_{373}, is rapidly photoconverted back to sR_{587}, and this has a repellent effect, whereas absence of near-UV light causes an attractant effect.

Discrepancies have been noted between action spectra predicted from the absorption spectrum of sR_{587} and its photocycle intermediate S_{373} and those actually observed in several strains of *H. halobium*. Several small maxima or shoulders between 540 and 640 nm in the broad action-spectrum peak for the attractant response are not present in the absorption spectrum of sR (8, 9, §); more importantly, a repellent effect of blue light may be observed under conditions where no significant amount of S_{373} is expected to be present or to absorb light, and the crossover from the attractant to the repellent effect varies but usually occurs near 500 nm (4, 5), whereas the isosbestic point in the sR_{587}/S_{373} difference spectrum occurs at 420 nm (6). These observations prompted the search for a repellent receptor, which absorbs at longer wavelength than does S_{373} and does not require long-wavelength background illumination.

MATERIALS AND METHODS

Unless noted, all experiments were carried out with *H. halobium* Flx3 and strains derived from it. Flx3 has been selected because it lacks detectable amounts of hR and bR but shows essentially the same phototactic responses as its parent and most other *H. halobium* strains (10). Cells were grown in complex medium, alternating growth in suspension and on swarm plates for selection of highly motile cells (4). For experiments, cells from a 100-ml suspension culture at the end of the logarithmic growth phase were subjected to a short low-speed centrifugation to remove large and relatively immobile cells. The cells in the supernatant were centrifuged onto a cushion of Fluorolube (Hooker Industrial & Specialty Chemicals, Niagara Falls, NY) and then were diluted to the desired concentration of 2×10^9 to 2×10^{10} cells·ml^{-1} with either complex medium supplemented with 0.1% arginine, or with the same salt solution lacking the peptone but containing 1% arginine. Aliquots were sealed in 0.1- to 0.2-mm-deep chambers on a light microscope slide.

The phototactic reactions were observed with a Zeiss or Leitz light microscope equipped with a substage darkfield condensor and with attachments for epifluorescence work. Light of wavelength >750 nm was used for darkfield illumination, and the epicondensor was used to project light from a high-pressure xenon lamp through a monochromator into a 200-μm-diameter spot in the center of the darkfield. Varying the wavelength and/or intensity of this light will cause cells to move preferentially into or out of the spot and, therefore,

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Abbreviations: bR, bacteriorhodopsin; hR, halorhodopsin; sR, sensory rhodopsin.

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increase or decrease the intensity of >750-nm light scattered from it. Measuring the intensity changes of light scattered from the central spot with a photomultiplier protected by a 750-nm long-pass filter as a function of wavelength and intensity yielded action spectra for the phototactic responses of the cells. Care was taken to keep the actinic light intensity in the linear range of the response; we measured only the initial rate of change before local changes in cell concentration began to affect the results. A detailed description of the technique used will be published elsewhere.

Flash-induced absorbance changes in membrane preparations were measured by using a spectrometer of our own construction (11, 12) with colinear measuring and exciting beams polarized at the magic angle. Data were acquired, stored, and processed in a Nicolet 1180 computer. Membrane suspensions were prepared in 4 M NaCl (13); membrane pellets were obtained and mounted in split quartz cuvettes as described (6).

RESULTS

Fig. 1 compares action spectra of *H. halobium* Flx3 phototactic responses to a difference spectrum of a membrane preparation from cells of the same strain. The spectrum 7 ms after illumination with >580-nm light was subtracted from the spectrum before illumination (Fig. 1*A*). The positive and negative bands in the difference spectrum closely correspond to the absorption bands of S_{373} and sR_{587} because these were the dominant species present at that time in the illuminated sample, and there is little overlap of their absorption bands. As predicted by the Spudich–Bogomolni model (7), a red background light was required to elicit the blue-light repellent response (Fig. 1*B*). The maxima in the action and absorption spectra coincide reasonably well and thus support the Spudich–Bogomolni model; however, the crossover from an attractant to a repellent response occurs at 450 nm, whereas the absorption spectrum shows the crossover near 420 nm.

While the discrepancy in the crossover might be explained by assuming a higher amplification of the repellent signal, this explanation is insufficient to account for a number of other observations. First, a strong blue-light repellent effect can often be elicited without simultaneous application of long-wavelength background light and the maximum of this blue-light repellent effect without long-wavelength background light is found between 450 and 480 nm. Second, the wavelength of the crossover point and the relative extent of the long-wavelength attractant and short-wavelength repellent effects vary widely—and so far not entirely reproducibly—from one preparation to another. The cells from *H. halobium* Flx3 KM-1, a spontaneous mutant of Flx3, which appears white because it lacks the main carotenoids present in wild-type strains and Flx3, consistently showed a larger repellent response to blue-green light than did their parent strain. Their long-wavelength attractant response was weaker, and the crossover to the repellent effect, which does not require the presence of background light, occurred at 535–550 nm (Fig. 2). Such a response cannot be attributed to S_{373}, which has no significant absorbance beyond 440 nm and can be present only in low concentrations under the illumination conditions used here. An additional photoreceptor that is different from the system sR_{587}/S_{373} must be present in these cells at relatively high effective concentrations.

Cells showing the opposite type of extreme in their spectral

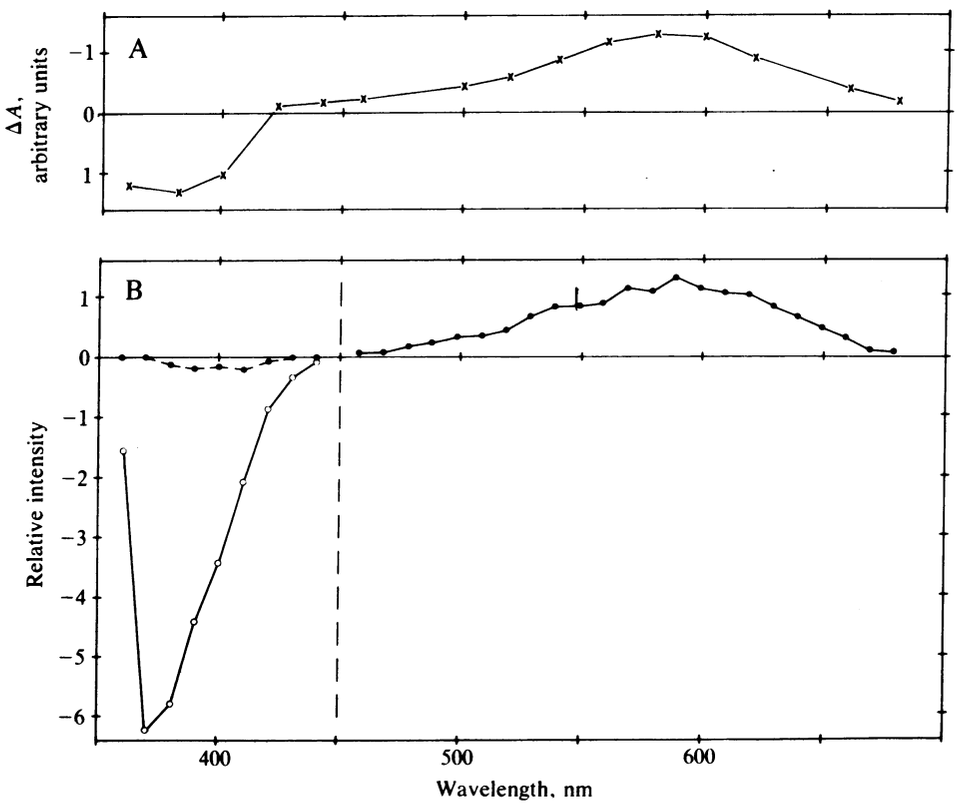

FIG. 1. (*A*) For comparison, the flash-induced difference spectrum for sR_{587} from a cell-membrane preparation is shown. A more detailed spectrum obtained under the same conditions is shown in Fig. 4*A*. (*B*) Action spectrum for phototaxis of *H. halobium* Flx3 cells. Here and in Figs. 2 and 3, the initial change of scattered light intensity is plotted in arbitrary units vs. the wavelength. Shown is the sum of six spectra obtained with cells from six different cultures. The large repellent response was obtained only when a long-wavelength background light was applied [Schott 550-nm long-pass filter (○)]. Without background light, only a small response is seen in the near UV region (●). Here we have selected cell populations that show a minimal repellent response in the blue-green region of the spectrum.

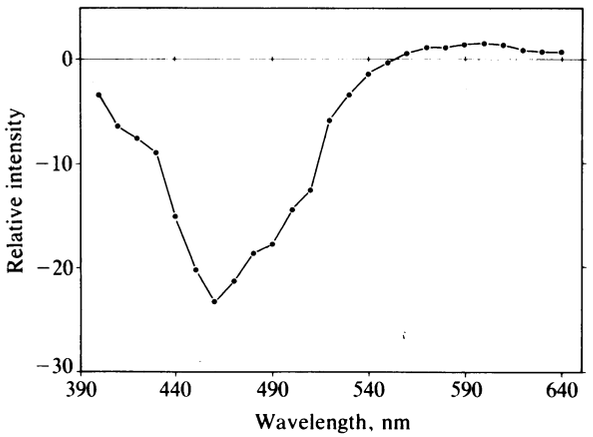

FIG. 2. Action spectrum for Flx3 KM-1 cells without background illumination.

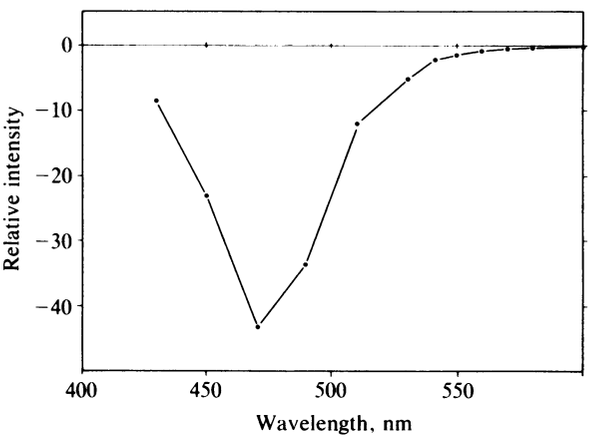

FIG. 3. Action spectrum for Flx3 KM-1 cells with saturating background illumination at 590 nm (Schott narrow-band interference filter).

sensitivity are shown in Fig. 1. Here a strong attractant effect was elicited by red and green light, but without background illumination it disappeared between 460 and 440 nm, and no significant repellent effect was observed in the blue region of the spectrum.‡ Typically, the cells showed a behavior intermediate between these extremes.

It was possible to record the action spectrum for the postulated new receptor in the blue-green region independently of the reaction to sR_{587} excitation by saturating sR_{587} with 590-nm background light. The repellent effect then extended to ≈600 nm (Fig. 3). Apparently, the postulated blue-light photoreceptor, which generates a maximal repellent effect near 480 nm, and sR_{587} were both present in different proportions in all our preparations. The blue-light receptor probably also contains a retinal chromophore, because it repeatedly has been shown in the past (4, 5), and we confirmed in this investigation (data not shown), that the repellent effect of the blue-green light requires the presence of retinal; its function can be restored in retinal synthesis-blocked cells by the addition of retinal. If this additional "blue" receptor resembles the other retinylidene proteins bR, hR, and sR, we should be able to detect it directly through its light-induced absorbance changes.

We examined the transient absorbance changes in Flx3 KM-1 membrane suspensions and pellets after excitation at two actinic wavelengths, 475 nm and 580 nm. Absorbance changes induced by saturating 580-nm flashes showed the kinetics and wavelength dependence characteristic of sR (Fig. 4). The difference spectrum at 2 msec after excitation had a broad depletion with a minimum at 590 nm, a structured absorption increase peaking at 375 nm, and an isosbestic point near 420 nm. Excitation with saturating 475-nm flashes caused absorbance changes in the 570- to 720-nm region indistinguishable in amplitude and kinetics from those elicited by the 580-nm laser flash. At shorter wavelengths, however, an additional absorption decrease with maximum amplitude around 480 nm and markedly faster kinetics appeared. The difference spectrum obtained with 580-nm light was subtracted from the 475-nm light-induced spectrum to extract this new component. The resulting difference spectrum has a maximum depletion at 480 nm, an isosbestic point at 390 nm, and a maximum absorption increase at 360 nm (Fig. 4). The difference in the transient absorbance changes induced by 475- and 580-nm excitation shows the decay kinetics of the 380-nm transient ($t_{1/2} \approx 150$ msec) closely following the regeneration of 480-nm absorbance (Fig. 5). Clearly, Flx3 KM-1 membranes contain a second photoactive pigment in addition to sR. It cycles with $t_{1/2} \approx 150$ ms and absorbs maximally near 480 nm, and its cyclic photoreaction includes at least one relatively long-lived intermediate state with an absorption peak near 360 nm. We also detected this pigment in Flx3 but, because of the relatively much larger absorbance changes of sR_{587} in these membranes, could not resolve it as well.

‡The weak repellent effect centered at 400 nm, which is near the limit of detection and has not been observed consistently, will require further investigation. Because of the use of glass optics, the relatively low intensity of the xenon lamp, and reduced sensitivity of our light measurements, the error in the action spectra rapidly increases below 420 nm. A more detailed exploration of this region will require improvements in our instrument.

DISCUSSION

The absorption characteristics of the second photoactive pigment found in Flx3 and Flx3 KM-1 membranes are the properties expected of the photoreceptor for blue-green light. The general shape of the spectrum is very similar to that of the other retinal pigments bR, hR, and sR. Its absorption peaks near 480 nm and extends beyond 560 nm on the long-wavelength side. This explains the repellent effect of

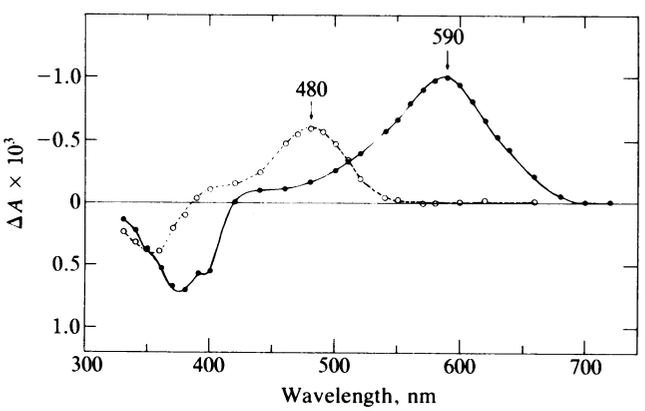

FIG. 4. Flash-induced difference spectra of Flx3 KM-1 membranes under the conditions described in Fig. 5. ●, Amplitude of absorbance changes 2 msec after excitation with a saturating 580-nm laser pulse. This spectrum is essentially identical to that previously assigned to sR_{587} (6, 21). ○, Differences between the amplitudes of absorbance changes induced by saturating 480-nm and 580-nm laser pulses, measured 2 msec after excitation. They are caused by the photoreaction of the new pigment $sR\text{-}II_{480}$. For further details, see the text. Note that absorbance decreases are shown as upward deflections.

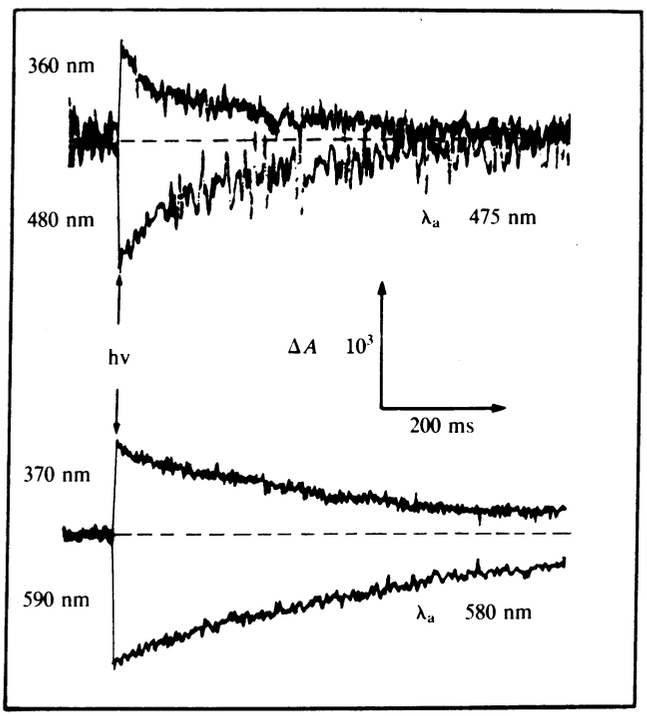

FIG. 5. Flash-induced absorbance changes in a Flx3 KM-1 membrane pellet mounted in a 0.5-mm pathlength split quartz cuvette in 4 M NaCl (pH 6.8) at 22°C (laser flash was 10 nsec at 1 millijoule per pulse). Traces are the average of 64 flashes at a repetition rate of 0.15 Hz. The arrows indicate the time of laser excitation. Lower traces: absorbance changes at 370 and 590 nm, elicited by a saturating 580-nm pulse. Upper traces: absorbance changes of the new photoactive species, at 360 and 480 nm, obtained by subtracting the absorbance changes induced by a saturating 580-nm pulse from those induced by a saturating 475-nm pulse as described in the text.

green light obtained with Flx3 KM-1 cells. The maximum repellent effect without long-wavelength background light is usually found near 460 nm. This presumably is due to simultaneous excitation of sR_{587}, which has significant absorbance between 500 and 470 nm and should decrease the effect of blue-green light in this region. We conclude that *H. halobium* Flx3 contains a second sensory retinal pigment, which mediates a repellent effect in the blue-green region of the spectrum. In addition to resolving the major discrepancies between absorption and action spectra outlined in the Introduction, the discovery of the receptor for blue-green light also offers a ready explanation for the observation that a mutant *H. halobium* strain has been isolated that responds to short-wavelength light but not to long-wavelength light and for similar observations, which have been used as arguments against the Spudich–Bogomolni model (14, 15). Since these and other phototaxis results (16–18), which now must clearly be ascribed to the presence of an independent pigment absorbing in the blue-green region, were obtained with the parent strain of Flx3 and other *H. halobium* strains, the new pigment is obviously not limited to the Flx3 and Flx3 KM-1 mutants. However, in cells with the wild-type concentrations of carotenoids and/or bR and hR, it is difficult, if not impossible, to detect by spectroscopy. We also have found a spectroscopically similar, if not identical, retinal pigment in haloalkaliphilic bacteria (19).

While this work was in progress, Takahashi *et al.* (20) described a mutant strain of Flx3 that showed no attractant effect in long-wavelength light but gave light-induced absorbance changes attributable to sR_{587}. It had an action spectrum for repellent light with a maximum at 470–480 nm. Membrane suspensions of these cells showed a large and faster absorbance decrease at 486 nm when excited at 450 nm as compared to 590 nm. The authors also noted that Flx3 with dim red background light had a higher repellent sensitivity at 470 nm than 370 nm. Since early exponential-phase cells of the mutant strain required retinal for this response, they concluded that it was due to a second sensory retinal pigment, absorbing maximally at 470–480 nm. This, of course, generally agrees with our observations and conclusions except that, so far, we have not seen cells that respond to stimuli in the blue region, have spectroscopically detectable sR_{587}, but do not respond to long-wavelength stimulation. Our mutant Flx3 KM-1 apparently contains larger amounts of the new pigment and/or smaller amounts of sR_{587} than its parent Flx3, but sR_{587} is usually detectable both spectroscopically and functionally. Its proportions tend to increase at the end of the exponential growth phase and in early stationary phase. Selecting cells on swarm plates for strong responses to blue light yields populations that, after the first transfer to liquid medium, show hardly any evidence of sR_{587}; but, after a second or third transfer, sR_{587} reappears in the usual amounts. While different amplification may contribute to the spectral sensitivity, in general we have found good agreement between the amount of pigments seen spectroscopically and phototactic responses.

Functionally, the "blue" pigment covers a spectral region where sR_{587} is not very effective and where a strong repellent action should be advantageous for the cells. The data presented here do not show that it is actually different from sR_{587}; the difference in absorption bands and kinetics could conceivably arise from differences in the environment of the pigment. However, bleaching and reconstitution experiments indicate a chemically similar molecule with a slightly but distinctly different apparent molecular weight (unpublished data). Since it too is a sensory pigment, we propose to name the new pigment $sR\text{-}II_{480}$ and rename the first-found pigment $sR\text{-}I_{587}$ and to use the subscripts to indicate intermediates in their photocycles.

Thus, we have shown that the color discrimination system in *H. halobium* is even more complex than formerly recognized. The discrimination between green or red and near-UV light is based on the photochromic properties of $sR\text{-}I_{587}$ (7). Such a mechanism has the obvious limitation that long-wavelength light must be present to obtain sensitivity in the near-UV, and the rigid coupling of sensitivities to the two wavelength ranges will clearly make optimal control of the cells' responses difficult, if not impossible, in some cases. An independent second pigment that overlaps in spectral sensitivity at both ends of its spectrum with the other two not only fills an important gap in the sensitivity spectrum but also should allow the cell more freedom in its reaction repertoire. It is interesting to note that the archaeobacterium *H. halobium* has two different mechanisms for color discrimination: one based on photochromicity closely resembles the phytochrome system found in plants, and the other uses two independent pigments, which is the same mechanism used in the eyes of animals for color discrimination. In addition, $sR\text{-}II_{480}$ may of course also use a photochromicity mechanism, and this possibility will have to be explored.

Similar results have been obtained independently in John Spudich's laboratory and the same conclusions have been reached. We thank John Spudich for informing us of these results and for discussion. Kathleen McGinnis has isolated the Flx3 KM-1 mutant; she and Gisela Latzke prepared the cells and membranes. The work was begun when W.S. was on sabbatical leave at the Max-Planck-Institut für Ernährungsphysiologie in Dortmund and was supported by the award of an Alexander v. Humboldt Prize. The results have been presented in part at the 13th Aharon Katzir–Katchalsky Conference on "Sensing and Response in Microorganisms," March 17–22, 1985, and at the 30th Meeting of the American Biophysical

Society, February 9–13, 1986. The spectroscopic work in San Francisco was supported by National Institutes of Health Grants GM-27057 and GM-34219, and National Science Foundation Grant DMB-84-44103.

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