A Rapid Population Method for Action Spectra Applied to Halobacterium halobium

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We have developed a simple and rapid technique for measuring the action spectra for phototaxis of populations of microorganisms and applied it to halobacteria. A microscope with a dark-field condenser was used to illuminate the cell suspension in a sealed chamber with light of wavelength >750 nm; in this region of the spectrum, the halobacteria show no phototactic response. A 150-μm spot of light from a xenon arc lamp, whose wavelength and intensity can be varied, was projected through the objective lens into the center of the dark field. The objective lens imaged this measuring spot through a 780-nm cut-off filter on an aperture in front of a photomultiplier. The intensity of the scattered 750-nm light, and therefore the photomultiplier current, is proportional to the number of cells in the measuring spot. A third lamp provided background light of variable wavelength and intensity through the dark-field condenser. To minimize secondary effects due to large changes in cell density, we recorded the initial changes in the photomultiplier current over 1 min after the actinic light had been switched on. By plotting the rate of change against wavelength, we obtained action spectra after the proper corrections for changes in light intensity with wavelength were applied and saturation effects were avoided.

Many motile microorganisms can sense changes in light intensity and will move toward or away from a light source. These phototactically activated responses in bacteria are usually called phototaxis, in analogy to chemotaxis, a similar and much more extensively investigated mechanism. We shall follow this convention, even though phototaxis is used somewhat differently by photobiologists mainly concerned with eucaryotic organisms (for a comprehensive review, see reference 7).

Action spectra provide the most important criteria for the initial characterization of photoreceptors (for a recent review, see reference 9). Spectra for the light-controlled movements of microorganisms may be obtained by observing either single cells or cell populations. Observation of single cells is indispensable for an analysis of the photoreactions; however, this observation needs a large number of measurements, is likely to introduce bias through selection of cells, and, if fully automated, requires substantial programming and computer time (8, 20, 21). Complete action spectra are much more easily obtained by monitoring changes in cell density as a function of spatial differences in light intensity and wavelength. We developed the microscopic method described here to obtain action spectra for the phototactic responses of halobacteria, which, until now, have been obtained mainly by observations of single cells (2, 3, 22, 23) or by a very slow macroscopic technique (14). The basic technique was already used by Engelmann, the discoverer of bacterial phototaxis (6), who observed the accumulation of cells in a field of attractant light. An improved recording phototaxigraph was introduced by Diehn (4). This instrument measured the changes in optical density caused by changes in cell density in the illuminated volume of a cell suspension. Its measuring beam had a cross section of several square millimeters. We used a microscope and monitored the changes in cell density when a spot of actinic light was projected onto a cell suspension by recording the intensity changes of a nonactinic measuring light scattered from this spot.

The same equipment can also be used for following the reactions of single cells; it is commercially available from several manufacturers and requires only minor modifications. Results obtained with this method, which led to the discovery of a second photoreceptor in Halobacterium halobium, have been published (24). Here we describe the instrument and its application in detail. The method should also prove valuable for the study of phototactic reactions in other microorganisms.

MATERIALS AND METHODS

Instrument. We used an Orthoplan microscope (E. Leitz GmbH, Wetzlar, Federal Republic of Germany) equipped for incident light fluorescence microscopy and photometry (Fig. 1). The specimen was illuminated alternatively or simultaneously by two Leitz 100-W quartz iodine lamps through a substage dark-field condenser with an aperture from 0.8 to 0.95, which produced an illuminated field of 2-mm diameter in the object plane. One of the lamps provided the measuring light of wavelength >750 nm through a Schott RG 780 filter; the others served for visual observation or, with the appropriate interference filters, as a source of constant background illumination.

The actinic light source was the 150-W XBO lamp of a Universal Monochromator Illuminator (Oriel Corp., Stratford, Conn.), combined with a 7340 monochromator and a 7271 grating (Oriel Corp.). Light from the monochromator exit slit was transferred via a rectangular (0.7- by 10-mm) to
circular (4-mm), fused silica fiber optic cable to the incident light illuminator of the microscope so that the circular cable end replaced the filament of the lamp in the standard housing. The spectral resolution was 3.0 nm, and the light intensity was varied by the insertion of neutral density filters. The field aperture of the incident-light condenser was adjusted so that a 170-μm diameter area in the center of the field of view was uniformly illuminated through the 32/65 P (E. Leitz GmbH) oil immersion objective lens. This measuring spot could therefore be illuminated simultaneously by the actinic light and through the substage dark-field condenser with >750-nm measuring light and with a beam splitter, simultaneously with actinic background light, if required.

The field aperture of the microscope photometer was adjusted so that all the light from the measuring spot but none from the surrounding area reached the photomultiplier, which was also protected from the scattered actinic and background light by a Schott RG 780 filter. This procedure assured that only the >750-nm measuring light scattered by the cells in the measuring spot was monitored. The intensity of this light, in a first approximation, is proportional to the number of cells in the spot. The photomultiplier current could, therefore, be used to record the changes in cell number in response to changes of the actinic light.

The Leitz Orthoplan microscope is delivered with filters in the incident-light illuminator, which must be removed. The standard half-silvered mirror, which reflects the light into the objective lens, can be used. A more efficient solution is to replace this mirror with a dichroic mirror (cold mirror) with transmission at >700 nm. Because the cold mirror has a transmission band in the near UV, a second dichroic mirror with a transmission limit at shorter wavelength (UV mirror) is required. Both mirrors have to be aligned carefully so that they illuminate the same area in the specimen, or the apertures defining the measuring spot have to be realigned each time the mirrors are switched. The dichroic mirrors are not available as standard equipment.

To obtain large-area dark-field illumination (Fig. 2), we used an automobile headlight reflector inverted over a photographic ring flashlight. The chamber with the cell suspension (see below) was placed on the opening for the light bulb, and photographs were taken at a magnification of ×6.4 with a Polaroid camera equipped with a Zeiss Tessovar lens.

**Data acquisition and evaluation.** The actinic light was controlled by a Compur electronic shutter and a home-built timer which also triggered data acquisition via a strip chart recorder and/or a computer (model 205-A; Nicolet Instrument Co., Madison, Wis.). The data were recorded on disk and later transferred to a computer (model 3032; The Perkin-Elmer Corp., Norwalk, Conn.) for further analysis. Only the initial slope of the scattering change was used (see Results) and analyzed via least-square methods.

The calibration of the light flux through the actinic spot for different wavelengths was obtained by mounting a silicon photodiode in the position of the specimen chamber (see below). The total light flux was thus measured, including all optically active elements which might influence the experiment and the normalization of the data. The relative responsiveness of the diode over the range from 350 to 700 nm was obtained by comparing the output current to the light flux measured via a precision light flux probe (KLC model dc...
1010 lightmeter; Karl Lambrecht Co., Chicago, Ill.). To measure the absolute light flux in the microscope, the same light beam was focused onto the diode and onto the light flux probe of the KLC lightmeter through a 590.4-nm interference filter with half width of 2.6 nm (Schott Glasswerke, Mainz, Federal Republic of Germany).

The responses of the cells at all wavelengths were normalized to a reference wavelength (see Results) and corrected for fluctuations in actinic light intensity.

The calibration factors for the different wavelengths were then used to correct the action spectrum in the program for data analysis of the Perkin-Elmer 3032 computer, and the corrected action spectrum was plotted via a standard routine developed at the Max Planck Institute, Dortmund (available from B. Hess on request).

The light flux in the actinic spot used in the experiments under nonsaturating conditions was in the range of $8 \times 10^{11}$ to $1.6 \times 10^{12}$ photons $\cdot$ mm$^{-2} \cdot$ s$^{-1}$ at 590 nm, i.e., the maximum of the action spectrum. This light intensity is comparable with or lower than the intensities used by other workers (5, 11, 12, 22, 23).

**Cell growth conditions.** For the experiments reported here, we used the *H. halobium* mutant Flx3 or strains derived from it to avoid possible interference from other photoactive pigments. These strains lack bacteriorhodopsin and halorhodopsin but contain sensory rhodopsin(s) (sR); Flx3R also lacks retinal synthesis (15, 16).

Cells were grown in complex medium at 37 to 40°C under illumination with white light. To maintain high motility, growth in suspension and on swarmplates was alternated (18). For the phototactic experiments, these cells were grown in 100-ml shake cultures and harvested at the end of their logarithmic growth phase (usually after 65 to 90 h). Strain Flx3R showed no phototactic responses, but the responses were present after 100 µl of $10^{-3}$ to $10^{-5}$ M retinal solution in ethanol had been added to a 100-ml suspension and the cells had been incubated overnight or for at least 1 h before the experiment started.

**Preparation of cell suspensions.** Usually, cells showed the largest phototactic responses near the end of their logarithmic growth phase, but cultures then also contained a variable but significant number of large, poorly mobile cells. To remove these cells, 30 ml of the culture was centrifuged at 2,000 $\times$ g for 9 min in the swinging bucket rotor of a Heraeus Christ Minifuge 2. The cells from the supernatant were centrifuged at 2,000 $\times$ g for 30 min onto a cushion of Fluorolube (Hooker Industrial, Niagara Falls, N.Y.), a water-immiscible liquid of high density, on which they formed a distinct band. Most of the band was carefully removed with a pipette and suspended to a concentration of $2 \times 10^7$ cells $\cdot$ ml$^{-1}$ in basal salt, i.e., the growth medium without peptone. At this cell concentration, the suspension rapidly became anaerobic. It has been reported that blocking the respiratory chain does not affect or even increases the sensitivity of the phototactic response (1). However, 0.2% arginine must be added to the basal salt solution to ensure a sufficient energy supply for the cells (5). Aliquots of the suspension were transferred to 0.1-mm-deep chambers (10 by 20 mm) on a microscope slide, and the chambers were closed with cover slips and sealed with molten paraffin wax. All preparative steps up to this point were carried out at room temperature. Temperature control of the experiments was provided by placing this observation chamber in a home-built thermostated holder, which fitted onto the microscope stage.

While filling the chambers, care must be taken to avoid trapping air; *H. halobium* is also aerotactic and accumulates near air bubbles (Fig. 2). Air bubbles can give rise to long-lasting, strong base-line drifts or large changes in base line when the specimen is moved. Qualitatively, we observed the following behavior. After 1 h, a faint ring of accumulated cells was seen around the air bubble (Fig. 2a). This ring slowly became narrower as the cell density increased (Fig. 2b and c). After about 4 h, the accumulation of cells disappeared, presumably because the oxygen in the air bubble had been used up. This observation agreed with the $\approx 20\%$ reduction of the air bubble volume. We have, on other occasions, observed that the cells first aggregated in a ring of higher density some distance from the air bubble and that this ring slowly moved toward the bubble until the ring touched the bubble. The determination of the causes for these variations in behavior will require further work under better-defined conditions, especially temperature control.

**RESULTS**

Halobacteria are attracted by long-wavelength and visible light and repelled by short-wavelength, visible, and near-UV light (3, 18). The resulting accumulation or depletion of cells in the measuring spot can be observed directly (Fig. 3A and B) or recorded as the change in photomultiplier current (Fig. 3C). At constant light intensity, the change in photomultiplier current is linear for approximately 1 min and then slowly decreases. The decreasing rate of change is at first mainly due to the changes in cell concentration near the edge of the measuring spot, but later, chemotactic effects may contribute (18). However, the initial rate of change in current is linearly dependent on light intensity until light saturation is approached and may therefore be used to measure action spectra (Fig. 4).

Complete spectra were routinely obtained by first recording the response of the cells near an expected maximum in the spectrum at relatively high actinic light intensity. The light intensity was then decreased by the insertion of neutral density filters until the response was in the linear range, which typically required at least a 1.0-optical-density filter. Then the wavelength was changed, in 10-nm steps usually, and in 5-nm steps in critical regions. For spectral regions in which less actinic light intensity was available, neutral density filters were removed and the responses were recorded for at least one wavelength with and without the filter to ensure that linearity was maintained. For each measurement, the measuring spot was moved to a new area of the chamber with the specimen stage. The experiments showed that neither the position in the chamber nor the depth at which the objective lens was focused was critical.

The noise in the measurements was mainly determined by two factors: the random movements of cells into and out of the measuring spot and the active movements of cells swimming within the measuring spot. This latter part of the noise was also detected in shallow chambers, when the cells with their flagella stuck to the surfaces of the chamber but were still rotating. As expected, as cell number increased, the noise increased to a maximum value and then decreased. With dead cells, which collected mainly at the bottom of the chamber, no significant noise was present at the signal amplification used.

A complication arose because the response of the cells in the chamber decreased slowly over several hours, for still-unknown reasons. To correct for this effect, the measurement at the first recorded wavelength was used as a standard
and repeated after every five measurements. The decrease in response was usually found to be linear and amounted to as much as a factor of two in 3 to 4 h. The responses at all wavelengths were normalized to the nearest standard wavelength. At much-longer times, the response began to decrease suddenly and dropped to near zero within 10 to 15 min. Typical recordings show unexpected undershoots and overshoots of the traces during the first 20 s after the actinic light had been turned on or off (Fig. 5). Possible explanations are considered in Discussion. We measured as the initial rates the slope of the curves after the under- or overshoots when the traces crossed the dark level.

Data for a complete action spectrum from 350 to 680 nm (Fig. 6A) was obtained in approximately 2.5 h from the time the cell suspension was sealed in the specimen chamber. The strain used, Flx3, contains only minor amounts of sR-II. As expected from previous observations on single cells (17), a pronounced repellent effect of short-wavelength light was obtained only with a long-wavelength background illumination. The action spectrum corresponded well to the absorpt-
The action spectra of the phototaxis receptor pigment sR-I$_{587}$ and its short-wavelength photoproduct S-I$_{373}$ (24). The spectrum in Fig. 7 was obtained with a strain that has lost the response to sR-I$_{587}$ but retained the response to sR-II$_{480}$. For most strains, contributions from both receptors are seen. For noise reduction, several action spectra were averaged. The variations observed for individual spectra are shown in Fig. 6B for the attractant response.

**DISCUSSION**

In general the action spectra obtained with the method described here are very similar to the spectra obtained by others from single-cell observations; discrepancies in the interpretations of the spectra have recently been discussed (19; W. Stoeckenius, E. Hildebrand, and A. Schimz, Letter, Trends Biochem. Sci. 11:402-403, 1986).

In principle, it should be possible to derive the light response of the population quantitatively from the responses.
of single cells, which are due to modulations of their spontaneous reversal frequencies by changes in the light intensity (18). The derivation is not possible, so far, because the conditions near the edge of the measuring spot, e.g., the light gradient, are not well defined enough and the conditions under which single-cell reactions have been observed are quite different. Qualitatively, the observed response was roughly what one would expect from single-cell observations, except for the over- and undershoots of the responses when the actinic light was turned on and off. Of these responses, the paradoxical off reactions were much more pronounced and may be explained, at least in part, by the recently discovered fact that cells, after a light-induced reversal, are refractory to a reversal-inducing stimulus and recover their reactivity only slowly after several seconds (10–13). Cells swimming inside the actinic spot near its edge in an outward direction will reverse when an attractant light is turned off. Cells swimming inward have just experienced a reversal at the edge and will not react to the decrease in attractant light intensity. Thus, when the light is turned off, all cells will swim inward, and since the effective light spot due to light scattering is somewhat larger than the image of the condenser aperture, a transient increase in light scattering intensity should be observed. Indeed, a small increase of the photometer aperture so that it included a narrow ring outside the condenser aperture image largely abolished the overshoot. An analogous argument can be used to explain the overshoot in the depletion effect when a repellent light is turned off. The much less pronounced paradoxical responses seen after the actinic light had been switched on may simply be due to a change in the interval between reversals, which would be expected to cause a transient small decrease in cell number in the measuring spot for attractant light and an increase for repellent light.

The response pattern may be further complicated by the summation of simultaneous stimuli and the inverse responses seen in single cells under certain conditions (10, 11). Since we used the initial rate of the scattering changes, our signal was determined only by cell concentration changes in the periphery of our measuring spot. The central part only raises the light level and contributes noise. An obvious way to increase the signal-to-noise ratio would be to use one or more concentric rings instead of a solid circle of light. Preliminary experiments confirmed this view. However, implementations of this approach require the manufacture of special apertures and modifications of the condenser and photometer; the optimal arrangement still remains to be determined.

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LITERATURE CITED