Characterization of *Halobacterium halobium* Mutants Defective in Taxis

STEVEN A. SUNDBERG,^{1,2} MAQSUDUL ALAM,^{3,4†} MICHAEL LEBERT,⁴ JOHN L. SPUDICH,²
DIETER OESTERHELT,⁴ AND GERALD L. HAZELBAUER^{3*}

Cardiovascular Research Institute, University of California, San Francisco, California 94143¹; Department of Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461²; Biochemistry/Biophysics Program, Washington State University, Pullman, Washington 99164-4660³; and Max-Planck-Institut für Biochemie, D-8033 Martinsried, Federal Republic of Germany⁴

Received 1 November 1989/Accepted 29 January 1990

Mutant derivatives of *Halobacterium halobium* previously isolated by using a procedure that selected for defective phototactic response to white light were examined for an array of phenotypic characteristics related to phototaxis and chemotaxis. The properties tested were unstimulated swimming behavior, behavioral responses to temporal gradients of light and spatial gradients of chemoattractants, content of photoreceptor pigments, methylation of methyl-accepting taxis proteins, and transient increases in rate of release of volatile methyl groups induced by tactic stimulation. Several distinct phenotypes were identified, corresponding to a mutant missing photoreceptors, a mutant defective in the methyltransferase, a mutant altered in control of the methylesterase, and mutants apparently defective in intracellular signaling. All except the photoreceptor mutant were defective in both chemotaxis and phototaxis.

Halobacterium halobium is a motile archaeobacterium that exhibits phototaxis and chemotaxis (8, 27, 35). Attractants include light in the green to red regions (530 to 620 nm), glucose, histidine, asparagine, and other amino acids. Near-UV and blue light (350 to 500 nm) and phenol are repellents. The cells make net progress in spatial gradients of these attractants and repellents as the result of a biased random walk created by modulating the frequency of abrupt changes in direction that are caused by reversals of the rotary flagellar motor. The probability of reversals, which reorient the cell in a new direction of swimming, is reduced when the cell experiences over time an increase in attractant concentration (or intensity in the case of attractant light) or a decrease in repellent concentration (intensity of repellent light). Changes of the opposite signs result in increased probability of reversals. The sensory systems adapt; that is, responses to continued stimulation are transient.

In comparison to the enteric bacteria, little is known about the underlying molecular mechanisms of sensory behavior in any archaeobacterium, including *H. halobium*. Although specific chemoreceptors are likely to exist, none has yet been defined. Two photosensory receptors have been identified. Both are retinal-containing proteins distinct from the retinal-based, light-driven ion pumps bacteriorhodopsin and halorhodopsin (3, 31, 39). Sensory rhodopsin I (sR-I) detects attractant light in its sR-I₅₈₇ form and repellent light in its S₃₇₃ form (34). S₃₇₃ is a long-lived (0.8-s half-life) intermediate in the photocycle of excited sR-I₅₈₇. Thus, mediation of blue-light sensitivity by sR-I requires illumination at longer wavelengths to create a steady-state content of the S₃₇₃ form. In contrast, sR-II (also called phoborhodopsin or P₄₈₀) serves as a receptor for repellent blue and near-UV light independent of other illumination (14, 32, 39, 42). The two sensory pigments also differ in their time of appearance during growth in culture: sR-I starts to appear in the mid-log

phase, whereas sR-II is present at an essentially constant concentration in cells at all phases (18, 40). The sR-I and sR-II chromoproteins are similar in size (24, 32) and photochemical properties (4, 40) to the related light-driven ion pumps bacteriorhodopsin and halorhodopsin. These pumps catalyze electrogenic ion transport without involvement of other components. In contrast, sR-I and sR-II photoreactions result in nonelectrogenic transmission of signals to the flagellar motor through components of a sensory signaling system. The sR-I protein has been purified and found to have the specific photochemical properties expected from experiments with unpurified material (24). The deduced amino acid sequence of sR-I reveals substantial homology with the retinal-containing ion pumps (D. Oesterhelt, S. Schegk, and A. Blanck, Photochem. Photobiol. 49S:37S, 1989). Like the enteric bacteria, *H. halobium* possesses methyl-accepting taxis proteins (1, 2, 25, 26, 30). Methylation and demethylation of these proteins appear to be directly involved in both chemotaxis and phototaxis by *H. halobium*, probably at the level of adaptation (1, 30), and provide the only biochemical assay in that species for an activity of the sensory systems.

The mechanisms and components that link sensory receptors to flagellar motors in *H. halobium* have not yet been identified. Photoactivation of sensory rhodopsins does not induce changes in membrane potential (5), nor are such changes involved in transduction from these receptors; instead, intracellular signals appear to be carried by diffusion (17). In analogy to enteric bacteria (6, 7), the signal pathway might involve phosphorylation of cytoplasmic proteins, but calcium ion and cyclic GMP are also possible candidates (29). In any case, signals from chemoreceptors and photoreceptors are integrated (9, 35), indicating that at least one component must be common for the two signalling pathways. Kinetic models have been suggested for the regulation of switching by flagellar motors (14, 15, 28), but the molecular identities of the mathematical parameters are unknown.

The genetic approach of isolation and characterization of mutants defective in chemotaxis has proved invaluable for elucidation of sensory mechanisms in *Escherichia coli* and

* Corresponding author.

† Present address: Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow-117437, USSR.

Salmonella typhimurium (19). In those species, specifically nonchemotactic mutants were found to be defective in particular receptor proteins, and generally nonchemotactic mutants were usually defective in components involved in intracellular signal transduction or in sensory adaptation. In this report we present a detailed characterization of mutants in *H. halobium*; one mutant lacked photoreceptors, and the others were generally defective in chemotaxis and phototaxis.

MATERIALS AND METHODS

Strains and growth conditions. *H. halobium* Flx15 is a derivative of S9 that lacks bacteriorhodopsin and halorhodopsin but contains the sensory rhodopsins sR-I and sR-II (31, 37). Pho mutants were isolated from a population of Flx15 mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and selected for loss of photosensitivity (38). For methyl-³H labeling experiments these strains were grown in peptone medium by shaking at 37°C in the dark or under white-light illumination to the early log phase (approximately 2 days after inoculation) and for behavioral experiments to the late log phase (approximately 3.5 days) (38). There were no differences in behavioral responses of cells grown with or without illumination. For spectroscopic measurements, cultures were grown to the stationary phase and harvested, and membrane vesicles were prepared by using a modification of the sonication procedure of MacDonald and Lanyi (13), in which DNase was omitted and the buffer was 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.0) in 4 M NaCl.

Behavioral experiments. Cultures were diluted 100-fold with growth medium and incubated on a shaker for at least 2 h before use in behavioral studies. Cells were placed on microscope slides maintained at 37°C by a water-jacketed slide holder, and responses to light stimuli were observed by using infrared ($\lambda > 700$ nm) background illumination and the optical system of Sundberg et al. (37). Step stimuli were 4 s, repeated every 30 s. Light intensities were estimated as described previously (37). Images of swimming cells were recorded on videotape and processed by using a computerized cell-tracking system (Motion Analysis Corp., Santa Rosa, Calif.) and an analysis algorithm described previously (37).

Flash photolysis. Membrane vesicles were collected from the pH 7 buffer by centrifugation at $150,000 \times g$ for 1 h, and the pellet was placed in a split quartz cuvette with a 1.0-mm path length (Hellma Cells Inc., Jamaica, N.Y.) mounted at a 45° angle with respect to both the measuring beam and the laser pulse axis. Measuring light was provided by a 12-V, 55-W Philips tungsten-halogen lamp passed through an ISA (Metuchen, N.J.) model H20 1200 UV monochromator (dispersion, 4 nm/mm; 2-mm slit) before it reached the sample. The photomultiplier (model R928; Hamamatsu Corp., Bridgewater, N.J.) was protected from stray actinic light by appropriate interference filters (Ditric Optics, Hudson, Mass.). Actinic light was provided by a Phase-R (Durham, N.H.) model DL-1200V dye laser (approximately 300-ns pulse, approximately 0.15 J per pulse) with 50 μ M Orange2 in methanol for 492-nm light and 100 μ M Green2 in methanol-1% Ammonyx LO for 584-nm light. Monitoring light and laser pulses were nominally unpolarized. Transient signals from the photomultiplier were captured by using a Nicolet (Madison, Wis.) model 206 digital oscilloscope at a digitizing rate of 2 ms per point. Signals were stored with a Nicolet model 1180 data acquisition computer and averaged for up to

256 successive sweeps. The repetition rate for flashing was once every 10 s. All measurements were carried out at room temperature (20 to 22°C).

Analysis of methylation and demethylation of taxis proteins. Labeling of taxis proteins with [methyl-³H]methionine and analysis of labeled polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis or of volatile products of demethylation with a flow apparatus were as described by Alam et al. (1), except that puromycin was not present in cell suspensions labeled for flow assays or in the solutions used for that analysis.

RESULTS

Identification of mutants defective in taxis. The strains considered here were identified as phototaxis defective by screening mutagenized cells of *H. halobium* that had undergone a selection designed to enrich for phototaxis mutants (38). Eighty-two phototaxis-defective (Pho⁻) derivatives of strain Flx15 were identified among cells submitted to a procedure in which a flashing light that induced frequent reversals of swimming direction served as a trap for phototactic cells but not for mutants insensitive to photostimulation. The 82 isolates were unlikely to correspond to 82 independent mutations, since all were derived from a single batch of mutagenized cells grown to the stationary phase after mutagenesis. That batch was the source of five samples of cells that were submitted separately to five or six cycles of selection and growth. We surveyed 59 isolates, derived from four of the samples, for patterns of methyl-³H-labeled proteins and chose 11 for extensive characterization. Of those 11 at least 6 were likely to represent independent mutational events because they exhibited distinct phenotypes. Those phenotypes are summarized in Table 1. The various features examined are considered in detail below.

Behavioral phenotypes. The swimming behavior of mutant strains was determined by viewing motile cells under the microscope. The tactically wild-type parental strain Flx15 reversed every 20 to 30 s, thus exhibiting a pattern of random motility in which periods of smooth swimming were punctuated by reversals. Pho81 exhibited a similar pattern of random motility, whereas the other mutants swam smoothly without exhibiting any reversals during the periods of observation (Table 1). The chemotactic ability of each strain was assessed by using semisolid agar plates containing peptone (35). In such plates, cellular growth reduces the concentration of metabolized amino acids at the site of inoculation, thus creating a local gradient to which chemotactic cells respond by migrating to high concentrations, forming distinct rings of chemotactic cells. Pho81 formed chemotactic rings similar to those formed by Flx15, its chemotactically wild-type parent. The other mutants did not form rings and thus were apparently unable to perform effective chemotaxis toward any of the attractant amino acids to which wild-type cells responded in the peptone-containing plate.

Responses to changes in light intensity were determined at three wavelengths, 600 nm, 400 nm with orange background illumination, and 450 nm, respectively, corresponding at the intensities used to attractant light detected by sR-I, repellent light detected by sR-I in its S₃₇₃ form, and repellent light detected by sR-II (Fig. 1). The behavior of populations of swimming cells was recorded on video tape and analyzed by a computer program designed to detect reversals (37). Because of the long intervals between spontaneous reversals, induction of reversals by negative stimuli is more easily measured than suppression of reversal frequencies by posi-

TABLE 1. Properties of taxis mutants

Strain ^a	Swimming pattern	Behavioral response ^b			Receptor pigments		Methylation of methyl-accepting taxis proteins	Response by transient increase in demethylation ^b						
		600 nm, decrease	400 nm, ^c increase	450 nm, ^d increase	Amino acids ^e			570 or 600 nm		400 nm ^f		Amino acids ^g		
					sR-I	sR-II		Increase	Decrease	Increase	Decrease	Increase	Decrease	
														Increase
Flx15	Random ^h	+	+	+	+	+	+	+	+	+	+	+	+	+
Pho5	Smooth	-	-	-	+	+	± ⁱ	-	-	-	-	-	-	-
Pho18	Smooth	-	-	-	+	+	+	+	+	+	+	+	+	+
Pho37	Smooth	-	-	-	+	+	± ^j	+	+	+	+	+	+	+
Pho60	Smooth	-	-	-	+	+	-	-	-	-	-	-	-	-
Pho72	Smooth	-	-	-	+	+	-	-	-	-	-	-	-	-
Pho81	Random ^h	-	-	-	-	-	± ^k	-	-	-	-	+	+	+

^a Of 11 mutant derivatives characterized in detail, Pho1 had a phenotype like that of Pho5, Pho8 and Pho17 were like Pho18, Pho64 and Pho71 were like Pho72.

^b Response to step decreases and step increases as indicated. See legends to Fig. 1 and 4 for intensities and concentrations used. +, Response; -, no response.

^c In the presence of orange-red background illumination. +, Response greater than that observed in the absence of background illumination and thus attributable to sR-I.

^d Stimulation at 450 nm was in the absence of background illumination, and thus a response can be attributed to sR-II.

^e Formation of tactic rings on semisolid agar plates containing 1% peptone.

^f Stimulation at 400 nm was in the absence of background illumination and thus can be attributed to sR-II.

^g Stimulation was with 1% peptone.

^h Mean reversal frequencies of 0.034 and 0.037 per s for Flx15 and Pho81, respectively, were determined by information from >100 cell tracks.

ⁱ The extent of labeling was substantially reduced compared with that in the wild type; see Fig. 3.

^j A specific methyl-labeled band appeared to be missing in some electrophoretic analyses.

^k A specific doublet of methyl-labeled bands was missing; see Fig. 3.

FIG. 1. Behavioral responses to photostimuli. Response to a step decrease in light at 600 ± 20 nm ($I_{\max}$, approximately 78 W/m^2) and step increases in light at 450 ± 20 nm ($I_{\max}$, 350 W/m^2) or 400 ± 5 nm ($I_{\max}$, 40 W/m^2) in the presence of orange-red background illumination (600 ± 20 nm; $I_{\max}$, 49 W/m^2) were determined by computerized tracking of individual cells. Stimuli were delivered 1 s after the beginning of data acquisition, and the light remained on or off for the duration of the 5-s interval. Values for percent reversal were determined for 67-ms intervals.

tive stimuli. For this reason we tested strains for reversals induced by a decrease in attractant light or increase in repellent light at the three different wavelengths. The Pho mutants did not respond to light stimuli at any of the three wavelengths, even though intensities were sufficient to elicit a maximal response from the parent strain.

Presence of photoreceptors. The presence of photoactive sR-I and sR-II was assayed by flash photolysis measurements in which transient absorbance changes were monitored after excitation of isolated membrane by a laser pulse of approximately 300 ns (Fig. 2). A 584-nm pulse excited sR-I but not sR-II. The transient absorbance changes characteristic of sR-I after excitation decreased most near 590 nm and increased most near 380 nm; in both cases the relaxation half times were approximately 800 ms (3). We monitored A_{590} and observed a transient decrease and a relaxation half time characteristic of sR-I for each of the mutants except Pho81, which exhibited no change in absorbance (Fig. 2). We concluded that Pho81 lacked active sR-I, whereas the other mutants contained this photoreceptor. Because of variability in a number of parameters, including the intensity of the flash, the relative amount of sR-I in the different strains cannot be deduced from the relative magnitude of the absorbance changes. A 492-nm pulse excites sR-II, causing a characteristic decrease in A_{480} but not in A_{590} (39). By this criterion, Pho81 membranes did not contain photoactive sR-II. In membranes containing sR-I as well as sR-II, 492-nm light will activate both photoreceptors, and each will contribute to a transient decrease in A_{480} . For

FIG. 2. Content of sensory rhodopsins determined by flash photolysis. Absorbance changes in membrane vesicles prepared from the strains indicated were monitored at two wavelengths after an actinic flash (300 ns) delivered at the time indicated by the arrows. The changes observed in absorption of the Flx15 sample after flashes at 584 and 492 nm are characteristic of sR-I and sR-II, respectively. Traces are averages of 128 sweeps and have been digitally smoothed.

sR-I alone, the change of A_{480} will be a constant fraction of the change in A_{590} ; this fraction (approximately 0.16), which should be independent of the wavelength of excitatory light, can be determined by measurements after a 584-nm flash, which does not induce changes in sR-II. If sR-II is present, then the observed change in A_{480} (but not in A_{590}) should include a contribution of that photoreceptor, and the A_{480}/A_{590} ratio should exceed 0.16. All mutants that contained sR-I also contained sR-II by this criterion (Fig. 2 and Table 2). Analysis of the time course of relaxation at 480 nm provided additional evidence for the presence of sR-II in each strain except Pho81. The relaxation half time of sR-II has been reported to be between 140 and 300 ms (32, 40, 42),

TABLE 2. Ratios of absorbance changes after flash photolysis

Source of membrane	$\Delta A_{480}/\Delta A_{590}$ ratio ^a with excitation at:	
	584 nm	492 nm
Flx15	0.145	0.43
Pho5	0.165	0.34
Pho18	0.167	0.39
Pho37	0.151	0.44
Pho60	0.164	0.50
Pho72	0.150	0.57
Pho81	No change	No change

^a Values are averages of ratios from two independent experiments.

FIG. 3. Patterns of methyl-³H-labeled proteins in wild-type and mutant strains. The figure shows a fluorogram of a sodium dodecyl sulfate-polyacrylamide gel to which was applied samples of whole cells of Flx15 (lane WT), Pho5 (lane 5), Pho18 (lane 18), Pho37 (lane 37), Pho60 (lane 60), Pho64 (lane 64), Pho71 (lane 71), Pho72 (lane 72), or Pho81 (lane 81) radiolabeled with L-[methyl-³H]methionine (15 Ci/mmol) in the presence of puromycin to inhibit protein synthesis. The gel contained 11% acrylamide (0.073% bisacrylamide), and the buffer was at pH 8.2 (A) or 8.4 (B). Note that in these conditions of pH and bisacrylamide content, the M_r s of methyl-³H-labeled bands are shifted relative to those observed in gels with the usual values of these parameters (1). Only the relevant portion of the fluorogram is shown. M_r markers: β -galactosidase, 116,000; phosphorylase b, 97,400.

which is substantially shorter than the approximately 800-ms half time of sR-I. The relaxation half time of the decrease in A_{480} induced by a 492-nm flash was considerably shorter (approximately 300 ms for each of the strains tested) than the relaxation half time observed after a 584-nm flash (approximately 800 ms), and treatment of the relaxation time course as a sum of two exponential decays, one with a half time of 800 ms, identified a half time of the second component ranging from 120 to 215 ms for the various strains tested, in good agreement with the reported values for sR-II. Thus we concluded that all strains examined except Pho81 contained sR-I and sR-II.

Methyl-accepting taxis proteins. Tactically wild-type strains of *H. halobium* contain methyl-accepting taxis proteins that appear as a series of methyl-³H-labeled bands with apparent molecular weights between 90,000 and 135,000 in sodium dodecyl sulfate-polyacrylamide gels (1, 30). Some mutants defective in taxis (for example, Pho18) exhibited electrophoretic patterns of methyl-³H-labeled polypeptides that were indistinguishable from the parental patterns, whereas others exhibited striking differences (Fig. 3). In some cases (for example, Pho60 and Pho72; Fig. 3A), most radiolabeled bands were missing; the only remaining bands were the lightly labeled pair that appear to be unrelated to taxis (1, 30). This same pattern was observed for 14 of 58 Pho⁻ isolates representing one or more different mutational events. Additional examples are strains Pho64 and Pho71, which are shown in Fig. 3B. For samples of Pho5, several

methyl-³H-labeled bands were visible, but the intensity of the label was substantially less than that for the wild type. The intensity relative to a wild-type pattern varied from almost invisible at exposures like those in Fig. 3 to a maximal intensity exhibited by the sample in Fig. 3A. Four other Pho⁻ isolates exhibited a similar, less intense labeling. Pho81 lacked a specific *methyl*-³H-labeled electrophoretic species (30) that appeared on high-resolution gels (Fig. 3) as a doublet of one intense and one faint band. The most rapidly migrating band in the *methyl*-³H-labeled pattern from wild-type cells is faintly labeled in unstimulated cells but increases in intensity in cells stimulated with amino acids (1). That band is too faint to be detectable in Fig. 3A but is visible in Fig. 3B for strains Flx15, Pho18, and Pho81 but not for Pho37. Longer exposures of this and other gels indicated that Pho37 appeared to lack this particular labeled species. In some physiological conditions, *H. halobium* exhibits a series of *methyl*-³H-labeled bands with electrophoretic positions corresponding to molecular weights between 12,000 and 29,000. These species do not appear to be related to sensory phenomena but rather have characteristics of biosynthetic intermediates (1) and thus have not been considered here.

Sensory modulation of release of volatile methyl groups. Cells of *H. halobium* continually release methyl groups in volatile chemical form, and the rate of release is altered transiently by chemostimuli and photostimuli (1). The photoeffects are mediated by activity of sR-I in both its attractant and repellent forms as well as by activity of sR-II (33). Simultaneous photoactivation of sR-I attractant and sR-II repellent receptors resulted in no net change in methyl release, demonstrating that the phenomenon is regulated by an integrated signal. This phenomenon has many features in common with the well-documented effects in *E. coli* of sensory stimuli on methyl esterase activity (11); these effects contribute to the changes in transducer methylation that mediate adaptation. An unusual and as yet not understood feature in *H. halobium* is that both positive and negative stimuli result in an increased rate of methyl release. A pattern representative of tactically wild-type strains is shown for Flx15 (Fig. 4), illustrating the distinct transient increases in rate of release of volatile methyl groups after the addition or removal of attractant or repellent chemostimuli or photostimuli. Photostimulation directed toward sR-I was performed by using filters that provided maximum intensity at 570 nm and excluded wavelengths below 555 nm (0.01% cutoff) or with an interference filter that provided light at 600 ± 20 nm. Figure 4 includes examples of data from both types of stimulation. Among the mutants examined, Pho18 and Pho37 exhibited essentially normal responses to each of the stimuli tested (Fig. 4 and Table 1). Mutants lacking *methyl*-³H-labeled taxis proteins (Pho72 as well as Pho64 and Pho71, 2 additional representatives of the 14 isolates with the same phenotype) released methyl groups at a low rate when unstimulated, and that low rate did not change upon stimulation. These observations are consistent with the notion that the methyl-accepting taxis proteins are the source of volatile methyl groups released after sensory stimulation as well as of much of the volatile material released in the steady state (1). Pho60 exhibited an unstimulated rate of release that was often comparable to those of strains that contained active methyl-accepting taxis proteins, even though no *methyl*-³H-labeled taxis proteins were visible on fluorograms (Fig. 3), but stimulation had essentially no effect on the rate of release. For Pho5, which contained taxis proteins that could be radiolabeled with methyl groups (albeit weakly),

the addition of phenol caused the sharp, transient increase in release of volatile methyl groups that is characteristic of a wild-type response, but other stimuli elicited no detectable changes. Pho1, a mutant that resembled Pho5 in its low level of methyl-labeled taxis proteins, had an identical pattern of methyl release. Pho81 exhibited no changes in response to photostimuli and normal changes in response to chemostimuli; the data documenting this phenotype were presented by Alam et al. (1).

DISCUSSION

The isolation and characterization of behavioral mutants have provided a powerful tool for identifying molecular components and elucidating mechanistic links in the chemotactic system of enteric bacteria. Analysis of mutants has provided substantial insight into photoreception in the archaeobacterial species *H. halobium* (31, 34, 38). The work described here represents an application of the strategy of mutational analysis to characterization of other components of the sensory system of *H. halobium*. Even though the power of the genetic approach has been limited in this species by lack of a procedure for complementation analysis, important information can be gained from characterization of behavioral mutants. Some mutants characterized in this study can be viewed as analogs in *H. halobium* of tactic mutants previously identified in enteric bacteria (19). Such analogies would be reasonable, since there are clearly common features between the enteric and halobacterial sensory systems. However, it is certainly possible that in *H. halobium* there are components or features involved in sensitivity to light and integration of photostimuli and chemostimuli that do not exist in *E. coli*. In addition, the substantial phylogenetic distance between the enteric bacteria and halobacteria is reflected in numerous striking differences in cellular biochemistry. It is likely that this pattern will include the respective sensory systems. An example already documented is the difference noted above between *E. coli* and *H. halobium* in the patterns of release of volatile methyl groups after sensory stimulation.

Types of mutants defective in taxis. The mutants considered in this study were identified initially because of a defect in phototaxis. All but one are also defective in chemotactic responses. These pleiotropic phenotypes might reflect two independent mutations that alter separate components of the chemotactic and phototactic machinery, but we feel that it is unlikely that such double events would have predominated to the extent that no single but several different double mutants were isolated. Reversion analysis of Pho72 (30) indicates that a single genetic change causes both the chemotactic and phototactic defect in that strain. This may also be true for many, if not all, of the other mutants analyzed in this study. Thus, the Che⁻ Pho⁻ phenotypes imply that certain components or processes are crucial to both phototaxis and chemotaxis and that transductional pathways from the two classes of receptors share common components. However, since signals from both classes of receptors impinge on a common target, the flagellar motors, it is possible that a Che⁻ Pho⁻ phenotype could result from an alteration in one pathway that made the common target insensitive to effects of signals from the other pathway. For instance, a mutation in a photoreceptor that locked the cell in a smooth swimming behavior could block chemotaxis simply by eliminating the reversal behavior that chemostimuli would otherwise modulate. Such mutants might be expected to exhibit other stimulus-induced responses (for instance, changes in meth-

FIG. 4. Changes in rate of release of volatile ^3H -labeled methyl groups induced by photostimuli and chemostimuli. Cells were labeled with L-[methyl- ^3H]methionine (90 Ci/mmol), washed free of unincorporated radiolabeled methionine, placed on a filter in a flow apparatus, and subjected at room temperature to a continuous flow (1.5 ml/min) of *H. halobium* basal salts containing 0.1% arginine and 0.1 mM nonradioactive methionine. Fractions collected each 18 s were analyzed for volatile radioactivity. Cells were photostimulated by turning on (+) or off (-) a 570-nm green light (KG3 and OG570 filters, 380 W/m 2) or a 400-nm blue light (KG3 and BG12 filters, 8.5 W/m 2) with no background light to create the S_{773} form of sR-I. For Flx15 and Pho18 the first pair of stimuli were turning on and turning off a light at 600 $\pm$ 20 nm (63 W/m 2). These data were collected separately from the rest of the respective traces in experiments in which samples were collected for 24 s. Values for counts per minute have been adjusted to correspond to counts per minute per 18-s interval but are plotted at 24-s intervals. The two sets of data are separated by a break in the trace for those two strains. Chemoeffectors were added (+) by switching the inlet tube from buffer to buffer plus 1% Oxoid peptone or buffer plus 5 mM phenol and removed (-) by transferring the inlet tube back to buffer alone. a.a.'s, Amino acids.

ylation), even though swimming behavior was not influenced. Among the mutants characterized here, Pho18 and Pho37 exhibit a phenotypic pattern consistent with such a secondary effect.

As documented here and elsewhere, Pho81 is a photo-receptor mutant (30, 32, 38), and the alteration it carries appears to affect more than one gene. The strain lacks sR-I and sR-II, distinct proteins (24, 32) that are presumably the products of different genes (see above). It is also missing a particular electrophoretic doublet of methyl-accepting taxis protein (Fig. 3) that appears to be capable of reacting with retinal (32). Thus, Pho81 contains more than one genetic change or a single alteration that prohibits the expression of more than one gene.

Pho72 is defective in transfer of methyl groups and thus may be a methyltransferase mutant. No *methyl*-³H-labeled taxis proteins were observed in this strain. The steady-state rate of release of volatile, radiolabeled methyl groups was substantially depressed, and that rate did not change after sensory stimulation. In principle, this phenotype could result from lack of any one of three classes of molecules: the methyltransferase, the methyl-accepting taxis proteins (if methyl groups were never removed, then the cellular complement of methyl-accepting taxis protein would not acquire radiolabeled methyls), or the methyl-accepting taxis proteins. In *E. coli*, a mutant lacking methyltransferase or the entire complement of methyl-accepting proteins never tumbles, and a methyl-esterase mutant tumbles incessantly (19). Since Pho72 swims smoothly without reversals, the analogy with the enteric sensory system suggests that the defect in Pho72 is not in the methyl-esterase. If the multiplicity of methyl-labeled electrophoretic species in a wild-type strain reflects several different gene products, as implied by the absence of specific bands in the pattern for Pho81, then elimination of all labeled bands would require inactivation of several genes by multiple mutations or by elimination of a common requirement for expression. Selection for correction of the Che⁻ defect in Pho72 resulted in restoration of phototaxis and as well as of the entire wild-type pattern of *methyl*-³H-labeled taxis proteins (30), implying that the mutant phenotype is the result of a single genetic change. However, *E. coli* mutants defective solely in the methyltransferase exhibit distinct behavioral responses to negative temporal stimuli, although adaptation is extremely abnormal (22). The lack of behavioral response in Pho72 and the phenotypically identical derivatives Pho64 and Pho71 indicates that these mutants are not precise analogs of a specific methyltransferase mutant in the enteric bacteria. This could be because the mutation in Pho72 eliminates expression of several genes of the tactic system, or because lack of the methyltransferase results in a more extreme bias toward smooth swimming in *H. halobium* than in *E. coli*. Pho60 exhibited the same phenotype as Pho72, except that the unstimulated rate of release of volatile methyl groups was often as high as that in strains that contain methylated methyl-accepting taxis proteins. Perhaps Pho60 carries a different mutation in the same locus as Pho72. For all other mutants characterized, the pattern of *methyl*-³H-labeled proteins and the results of flow assays indicate that both the methyltransferase and the demethylase are present and, at least to some degree, functional.

Pho18 and Pho37 appear to be defective in intracellular signaling in a manner analogous to that of *E. coli* strains lacking CheY, the protein that enables tumble mode rotation of the flagellar motor (12, 23, 41), presumably by direct, physical interaction with flagellar proteins (21). Modulation of demethylase activity by all classes of sensory stimuli

indicates that Pho18 and Pho37 contain functional receptors and demethylase as well as an active link between the two. In *E. coli*, *cheY* mutations cause exclusively smooth swimming and eliminate any effect of sensory stimulation on swimming behavior, while leaving unperturbed receptors and the CheA-mediated modulation of demethylase activity (20, 36). The absence of one specific methyl-labeled band, observed for Pho37, would not be predicted from the analogy with *cheY* mutants and may not be directly related to a defect in intracellular signalling. The lack of this band does not necessarily mean that a specific methyl-accepting taxis protein is missing. It is well-documented in *E. coli* that distinct electrophoretic forms of methyl-accepting chemotaxis proteins are created by covalent modification, either deamidation or methylation (10, 16). Thus an altered balance among signaling components could conceivably shift the steady-state level of covalent modification to cause the disappearance of a particular methyl-labeled band. Alternatively, the missing band may reflect a second genetic change or a relationship not encountered in the enteric sensory system. The minor differences between the phenotypes Pho18 and Pho37 may reflect quantitative effects of particular mutant alleles.

Pho5 is defective in the circuitry that mediates sensory control of demethylase activity and in the extent to which methyl-accepting proteins acquire radiolabeled methyl groups. With the exception of phenol addition, the strongest stimulus tested, neither chemostimuli nor photostimuli affected the rate of release of volatile methyl groups. This pattern could reflect a defect in activation of the demethylase, which would result in lower steady-state activity of the enzyme (and thus fewer sites available for modification by radiolabeled methyl groups) and a lack of responsiveness to all but the strongest stimuli. The alteration could be in the enzyme itself or in a signaling component crucial for activation of the demethylase. The lack of sensory responsiveness to temporal stimuli argues for a defect in a signaling component.

We anticipate that the distinct taxis mutants characterized in this study will prove useful for the elucidation of the molecular mechanisms of the tactic system in *H. halobium*.

ACKNOWLEDGMENTS

The first two authors contributed equally to this work. This work was supported by Public Health Service grants GM29963 (to G.L.H.) and GM27750 (to J.L.S.) from the National Institutes of Health, by fellowship DRG-830 from the Damon Runyon-Walter Winchell Cancer Fund (to S.A.S. while in the laboratory of J.L.S.), and by a Federal Republic of Germany-USSR exchange program fellowship from the Deutsche Forschungsgemeinschaft (to M.A.). S.A.S. thanks Roberto Bogomolni and Walther Stoeckenius for support (Public Health Service grants GM34219 and DMB-8717193 to R.B. and training grant HL 07192 from the National Institutes of Health) and use of laboratory facilities. G.L.H. is the recipient of an American Cancer Society Faculty Research Award.

LITERATURE CITED

1. Alam, M., M. Lebert, D. Oesterhelt, and G. L. Hazelbauer. 1989. Methyl-accepting taxis proteins in *Halobacterium halobium*. *EMBO J.* 8:631-639.
2. Bibikov, S. I., V. A. Baryshev, and A. N. Glagolev. 1982. The role of methylation in the taxis of *Halobacterium halobium* to light and chemo-effectors. *FEBS Lett.* 146:255-258.
3. Bogomolni, R. A., and J. L. Spudich. 1982. Identification of a third rhodopsin-like pigment in phototactic *Halobacterium halobium*. *Proc. Natl. Acad. Sci. USA* 79:6250-6254.
4. Bogomolni, R. A., and J. L. Spudich. 1987. The photochemical

- reactions of bacterial sensory rhodopsin I. *Biophys. J.* **52**: 1071-1075.
5. Ehrlich, B., C. Schen, and J. L. Spudich. 1984. Bacterial rhodopsins monitored with fluorescent dyes in vesicles and *in vivo*. *J. Membr. Biol.* **82**:89-94.
 6. Hess, J. F., K. Oosawa, N. Kaplan, and M. I. Simon. 1988. Phosphorylation of three proteins in the signaling pathway of bacterial chemotaxis. *Cell* **53**:79-87.
 7. Hess, J. F., K. Oosawa, P. Matsumura, and M. I. Simon. 1987. Protein phosphorylation is involved in bacterial chemotaxis. *Proc. Natl. Acad. Sci. USA* **84**:7609-7613.
 8. Hildebrand, E., and N. Dencher. 1975. Two photosystems controlling behavioral response of *Halobacterium halobium*. *Nature (London)* **257**:46-48.
 9. Hildebrand, E., and A. Schimz. 1986. Integration of photosensory signals in *Halobacterium halobium*. *J. Bacteriol.* **167**: 305-311.
 10. Kehry, M. R., and F. W. Dahlquist. 1982. Adaptation in bacterial chemotaxis: CheB-dependent modification permits additional methylations of sensory transducer proteins. *Cell* **29**: 761-772.
 11. Kehry, M. R., T. G. Doak, and F. W. Dahlquist. 1984. Stimulus-induced changes in methyltransferase activity during chemotaxis in *Escherichia coli*. *J. Biol. Chem.* **259**:11828-11835.
 12. Kuo, S. C., and D. E. Koshland, Jr. 1987. Roles of *cheY* and *cheZ* gene products in controlling flagellar rotation in bacterial chemotaxis of *Escherichia coli*. *J. Bacteriol.* **169**:1307-1314.
 13. MacDonald, R. E., and J. K. Lanyi. 1975. Light-induced leucine transport in *Halobacterium halobium* envelope vesicles: a chemiosmotic system. *Biochemistry* **14**:2882-2889.
 14. Marwan, W., and D. Oesterhelt. 1987. Signal formation in the halobacterial photophobic response mediated by a fourth retinal protein (P₄₈₀). *J. Mol. Biol.* **195**:333-342.
 15. McCain, D., L. Amici, and J. L. Spudich. 1987. Kinetically resolved states of the *Halobacterium halobium* flagellar motor switch and modulation of the switch by sensory rhodopsin I. *J. Bacteriol.* **169**:4750-4758.
 16. Nowlin, D. M., J. Bollinger, and G. L. Hazelbauer. 1988. Site-directed mutations altering methyl-accepting residues of a sensory transducer protein. *Proteins* **3**:102-112.
 17. Oesterhelt, D., and W. Marwan. 1987. Change of membrane potential is not a component of the photophobic transduction chain in *Halobacterium halobium*. *J. Bacteriol.* **169**:3515-3535.
 18. Otomo, J., W. Marwan, D. Oesterhelt, H. Desel, and R. Uhl. 1989. Biosynthesis of the two halobacterial light sensors, P₄₈₀ and sensory rhodopsin, and variation in gain of their signal transduction chains. *J. Bacteriol.* **171**:2155-2159.
 19. Parkinson, J. S., and G. L. Hazelbauer. 1983. Bacterial chemotaxis: molecular genetics of sensory transduction and chemotactic gene expression, p. 293-318. *In* J. Beckwith, J. Davies, and J. A. Gallant (ed.), *Gene function in prokaryotes*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 20. Parkinson, J. S., and S. E. Houts. 1982. Isolation and behavior of *Escherichia coli* deletion mutants lacking chemotaxis functions. *J. Bacteriol.* **151**:106-113.
 21. Parkinson, J. S., S. R. Parker, P. B. Talbert, and S. E. Houts. 1983. Interactions between chemotaxis genes and flagellar genes in *Escherichia coli*. *J. Bacteriol.* **155**:265-274.
 22. Parkinson, J. S., and P. T. Revello. 1978. Sensory adaptation mutants of *E. coli*. *Cell* **15**:1221-1230.
 23. Ravid, S., P. Matsumura, and M. Eisenbach. 1986. Restoration of flagellar clockwise rotation in bacterial envelopes by insertion of the chemotaxis protein CheY. *Proc. Natl. Acad. Sci. USA* **83**:7157-7161.
 24. Schegk, E. S., and D. Oesterhelt. 1988. Isolation of a procaryotic photoreceptor: sensory rhodopsin from halobacteria. *EMBO J.* **7**:2925-2933.
 25. Schimz, A. 1981. Methylation of membrane proteins is involved in chemosensory and photosensory behavior of *Halobacterium halobium*. *FEBS Lett.* **125**:205-207.
 26. Schimz, A. 1982. Localization of the methylation system involved in sensory behavior of *Halobacterium halobium* and its dependence on calcium. *FEBS Lett.* **139**:283-286.
 27. Schimz, A., and E. Hildebrand. 1979. Chemosensory responses of *Halobacterium halobium*. *J. Bacteriol.* **140**:749-753.
 28. Schimz, A., and E. Hildebrand. 1985. Response regulation and sensory control in *Halobacterium halobium* based on an oscillator. *Nature (London)* **317**:641-643.
 29. Schimz, A., and E. Hildebrand. 1987. Effects of cGMP, calcium, and reversible methylation on sensory signal processing in halobacteria. *Biochim. Biophys. Acta* **923**:222-232.
 30. Spudich, E. N., C. A. Hasselbacher, and J. L. Spudich. 1988. Methyl-accepting protein associated with bacterial sensory rhodopsin I. *J. Bacteriol.* **170**:4280-4285.
 31. Spudich, E. N., and J. L. Spudich. 1982. Control of transmembrane ion fluxes to select halorhodopsin deficient and other energy-transduction mutants of *Halobacterium halobium*. *Proc. Natl. Acad. Sci. USA* **79**:4308-4312.
 32. Spudich, E. N., S. Sundberg, D. Manor, and J. L. Spudich. 1986. Properties of a second sensory receptor protein in *Halobacterium halobium* phototaxis. *Proteins* **1**:239-246.
 33. Spudich, E. N., T. Takahashi, and J. L. Spudich. 1989. Sensory rhodopsins I and II modulate a methylation/demethylation system in *Halobacterium halobium* phototaxis. *Proc. Natl. Acad. Sci. USA* **86**:7746-7750.
 34. Spudich, J. L., and R. A. Bogomolni. 1984. Mechanism of color discrimination by a bacterial sensory rhodopsin. *Nature (London)* **312**:509-513.
 35. Spudich, J. L., and W. Stoekenius. 1979. Photosensory and chemosensory behavior of *Halobacterium halobium*. *Photobiophys.* **1**:43-53.
 36. Stewart, R. C., C. B. Russell, A. F. Roth, and F. W. Dahlquist. 1988. Interaction of CheB with chemotaxis signal transduction components in *Escherichia coli*: modulation of the methyltransferase activity and effects on cell swimming behavior. *Cold Spring Harbor Symp. Quant. Biol.* **53**:127-140.
 37. Sundberg, S. A., M. Alam, and J. L. Spudich. 1986. Excitation signal processing times in *Halobacterium halobium* phototaxis. *Biophys. J.* **50**:895-900.
 38. Sundberg, S. A., R. A. Bogomolni, and J. L. Spudich. 1985. Selection and properties of phototaxis-deficient mutants of *Halobacterium halobium*. *J. Bacteriol.* **164**:282-287.
 39. Takahashi, T., H. Tomioka, N. Kamo, and Y. Kobatake. 1985. A photosystem other than PS370 also mediates the negative phototaxis of *Halobacterium halobium*. *FEMS Microbiol. Lett.* **28**:161-164.
 40. Tomioka, H., T. Takahashi, N. Kamo, and Y. Kobatake. 1986. Flash spectrophotometric identification of a fourth rhodopsin-like pigment in *Halobacterium halobium*. *Biochem. Biophys. Res. Commun.* **139**:389-395.
 41. Wolfe, A. J., M. P. Conley, T. J. Kramer, and H. C. Berg. 1987. Reconstitution of signaling in bacterial chemotaxis. *J. Bacteriol.* **109**:1878-1885.
 42. Wolff, E., R. A. Bogomolni, P. Scherrer, B. Hess, and W. Stoekenius. 1986. Color discrimination in halobacteria: spectroscopic characterization of a second sensory receptor covering the blue-green region of the spectrum. *Proc. Natl. Acad. Sci. USA* **83**:7272-7276.