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Methyl-accepting taxis proteins in *Halobacterium halobium*

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Methyl-accepting taxis proteins were identified and characterized in *Halobacterium halobium*, an archaebacterial species that is both chemotactic and phototactic. The data suggest direct involvement of methylation and demethylation in mechanisms of both chemotaxis and phototaxis and identify adaptation as the sensory process in which those reactions are likely to be involved. Analysis by electrophoresis and fluorography revealed methyl-accepting species, of apparent M_r between 90 000 and 135 000, that exhibited characteristics of sensory components. Those methyl-^{3}H-labeled species were absent in a mutant blocked in taxis. Methylation of specific bands increased after positive chemostimuli and decreased after negative stimuli. Other methyl-^{3}H-labeled bands, from 17 to 29 kd, exhibited features of biosynthetic intermediates, not of sensory components. Assay of rates of demethylation by measuring release of volatile forms of radiolabeled methyl groups revealed transient changes following chemo- or photostimuli that persisted for periods roughly equivalent to adaptation times. Negative chemostimuli induced increased rates of demethylation, as expected from fluorographic analysis, but positive chemostimuli also resulted in an increase. Photostimuli of either sign were followed by increases in rates of demethylation of shorter duration and lesser magnitude than chemostimuli-induced increases, a relationship that corresponded to differences in adaptation time.

Key words: chemotaxis/membrane proteins/phototaxis/receptor proteins/sensory adaptation

Introduction

Motile bacteria make net progress in favorable directions as the result of a biased random walk created by modulating the frequency of abrupt changes in direction. For *Escherichia coli*, the frequency of tumbles, short episodes of uncoordination that reorient the cell in a new direction of swimming, is reduced when the cell experiences an increase over time in attractant concentration or a decrease in repellent concentration. Response to changes of the opposite sign is more frequent tumbles. These responses are transient. This means that like most sensory cells, *E.coli* adapts to the continued presence of an active compound. A large body of data links protein methylation to the phenomenon of sensory adaptation in *E.coli* (Springer *et al.*, 1979). Changes in the extent to which a transmembrane receptor protein is occupied by ligand (attractant or repellant) result in initiation by the receptor of intracellular, excitatory signals that alter tumble frequency. An appropriate increase or decrease in the extent of methylation of specific glutamyl residues in the cytoplasmic domain of the receptor cancels the excitatory state of an attractant or repellant-occupied receptor, respectively, establishing an adapted state in which behavior is indistinguishable from the prestimulus pattern.

Halobacterium halobium is an archaebacterium that lives in high salt environments. The cells are polarly flagellated (Alam and Oesterhelt, 1984) and change direction as the result of reversals rather than tumbles as observed for the peritrichously flagellated *E.coli*. *H.halobium* exhibits both chemotaxis and phototaxis (Hildebrand and Dencher, 1975; Schimz and Hildebrand, 1979; Spudich and Stoeckenius, 1979). Chemostimuli and photostimuli each affect the reversal frequency of *H.halobium* in the same manner as chemostimuli affect the tumble frequency of *E.coli*. For both species, adaptation to maximal chemostimuli requires minutes (Springer *et al.*, 1979; Lebert, 1987). In contrast, adaptation to maximal photostimuli is more rapid, occurring over periods of tens of seconds (Spudich and Stoeckenius, 1979; Hildebrand and Schimz, 1985). Simultaneous application of combinations of stimuli results in responses that suggest integration of signals within and between the photosensory and chemosensory systems (Spudich and Stoeckenius, 1979; Hildebrand and Schimz, 1986). The receptors for the two classes of stimuli appear to be different. Photoreceptor activity is dependent upon vitamin A aldehyde (retinal), while chemoreceptor activity is not (Schimz and Hildebrand, 1979; Spudich and Stoeckenius, 1979), and a mutant strain lacking phototactic activity, even in the presence of added retinal, exhibits chemotaxis (Sundberg *et al.*, 1985). Two different retinal-containing proteins have been identified as photoreceptors. Sensory rhodopsin (SRI) in its SR_{587} form detects attractant light and in its SR_{373} form detects repellent light (Spudich and Bogomolni, 1984). Another pigment, P_{480} (SRII), also mediates response to repellent light (Takahashi *et al.*, 1985, Spudich *et al.*, 1986; Wolff *et al.*, 1986; Marwan and Oesterhelt, 1987). The photoreceptors are distinct from the two light-driven ion pumps of *H.halobium*, bacteriorhodopsin and halorhodopsin; cells missing both pumps still respond to both positive and negative photostimuli (Spudich and Spudich, 1982). Specific chemoreceptors have not been defined, but the array of tactically active compounds suggests that there should be several different receptors. Glucose, histidine and leucine are among the effective attractants while phenol is a repellent (Schimz and Hildebrand, 1979).

Methionine starvation eliminates reversals and thus tactic responses in *H.halobium* (Schimz and Hildebrand, 1979; Baryshev *et al.*, 1981), implying that methylation might play

a role in taxis by this species and that methyl-accepting taxis proteins, analogous to those found in enteric bacteria (Hazelbauer and Harayama, 1983; Simon *et al.*, 1985), might occur in *H.halobium*. In fact, treatment of cells with ethionine, which should lower intracellular pools of the methyl donor S-adenosylmethionine, extends adaptation times (Schimz and Hildebrand, 1987). Preliminary studies (Schimz, 1982; Bibikov *et al.*, 1982) provided indications that methyl-accepting proteins occurred in *H.halobium*, but only a few of the analytical procedures that have produced detailed information about methyl-accepting chemotaxis proteins of *E.coli* and *Salmonella typhimurium* were utilized. In this study we used a variety of sensitive techniques to analyze methyl-accepting proteins and the dynamics of methyl group turnover in *H.halobium*.

Results

Electrophoretic analysis of methyl-accepting species

Proteins that accept methyl groups from S-adenosylmethionine can be radiolabeled specifically *in vivo* by supplying [methyl-^{3}H]methionine to cells in which protein synthesis is blocked by an appropriate inhibitor. Fluorography of dried slabs of SDS–polyacrylamide gels provides high-resolution displays of methyl-^{3}H-labeled proteins. Examinations of proteins from *H.halobium* by this technique revealed only a few distinct electrophoretic species of methyl-^{3}H-labeled material (Figure 1). Besides a substantial quantity of radiolabel at the ion front (not visible in the figure), presumably reflecting small methylated molecules, there were two prominent groups of radiolabeled bands. A distinct group occurred in the region of apparent M_r between 90 000 and 135 000 (there was a single, faintly labeled band at ~70 000 that was not visible at the extent of exposure represented in Figure 1, but is discernible on later figures). A second, more dispersed group appeared as a ladder in the region between 17 000 and 29 000. In addition, several more diffuse bands appeared at lower apparent M_r. Both major sets of bands were found in the particulate fraction of lysed cells after high speed centrifugation (data not shown), a result consistent with a membrane location of the methyl-^{3}H-labeled material. The upper set of bands was observed in every experiment analyzing each tactically wild-type strain examined, with variation only in the spacing of bands, the result of different conditions of electrophoresis. Like the methyl-accepting chemotaxis proteins of *E.coli*, the set of high M_r bands exhibited altered migration relative to standard proteins in different conditions of electrophoresis (concentration of acrylamide, proportion of *bis* acrylamide or pH)—compare the positions of the bands relative to the M_r standards in the three types of gels shown in Figure 1. A number of mutant strains defective in both chemotaxis and phototaxis were found to be missing all but two faintly labeled bands among the array of methyl-^{3}H-labeled bands in the 90–135 kd region. The pattern from one such mutant is shown in Figure 1. A more extensive consideration of methylation patterns exhibited by taxis mutants will be presented elsewhere (M.Alam, S.A.Sundberg, J.L.Spudich,D., Oesterhelt and G.L.Hazelbauer, manuscript in preparation).

The pattern of methyl-^{3}H-labeled bands in the region of lower M_r was extremely variable. The region might contain no labeled bands, one or two diffuse bands or an extended ladder pattern reminiscent of oligosaccharides (Figure 2). In general, these bands were more intense for cells from actively growing cultures and less intense in cells from cultures in the stationary phase of growth. Determination

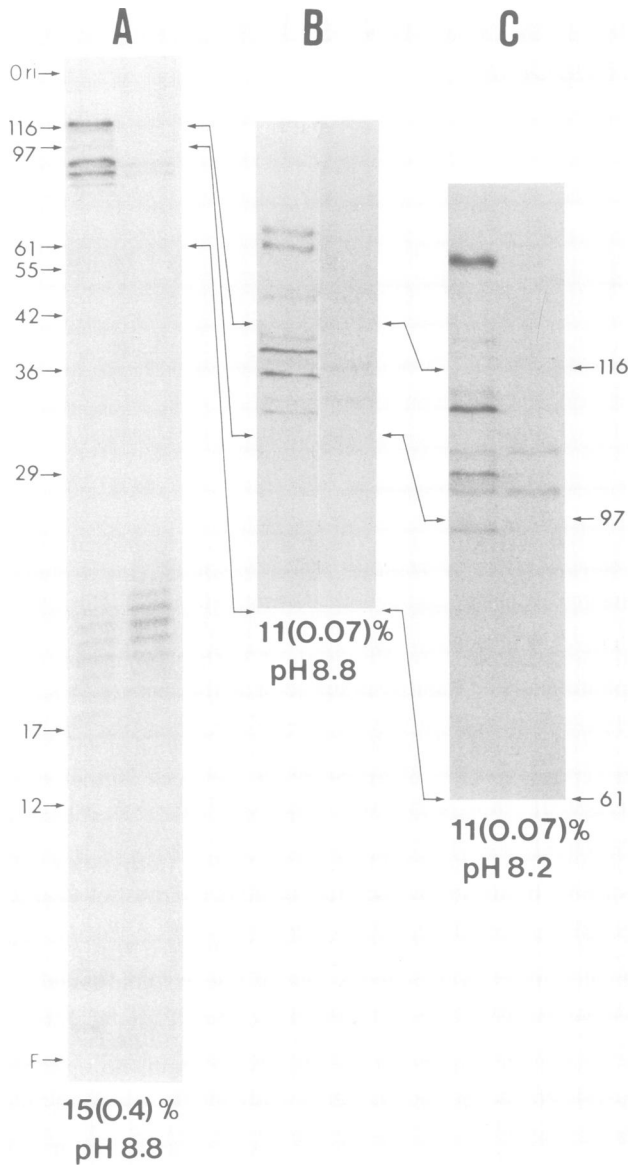

Fig.1. Patterns of methyl-^{3}H-labeled material from *H.halobium*. The figure shows fluorograms of lanes from SDS–polyacrylamide gels to which were applied samples of whole cells radiolabeled with [methyl-^{3}H]methionine in the presence of an inhibitor of protein synthesis. The gels for **panel A** contained 15% acrylamide (0.4% *bis*acrylamide) at pH 8.8 and displayed the entire complement of cellular protein while those in **panels B** and **C** contained 11% acrylamide (0.073% *bis*acrylamide) at pH 8.8 and 8.2, respectively, and thus expanded the spacing between bands in the cluster of higher M_r. Only the relevant sections of the latter gels are shown. Strain Flx15, wild-type for chemotaxis and phototaxis, but lacking bacteriorhodopsin and halorhodopsin (left-hand lane in each pair), and strain M402, a mutant defective in chemotaxis and phototaxis and also lacking bacteriorhodopsin (right-hand lane in each pair) were incubated for 60 min with [methyl-^{3}H]methionine and puromycin. Positions of markers of M_r are indicated in kd: β-galactosidase, 116.1; phosphorylase b, 97.4; α-amylase, 60.6; glutamic dehydrogenase, 55.5; actin, 41.7; carbonic anhydrase, 28.7; myoglobin, 17.2; cytochrome c, 12.4. The top of the separation gel and the ion front are indicated by 'Ori' and 'F', respectively.

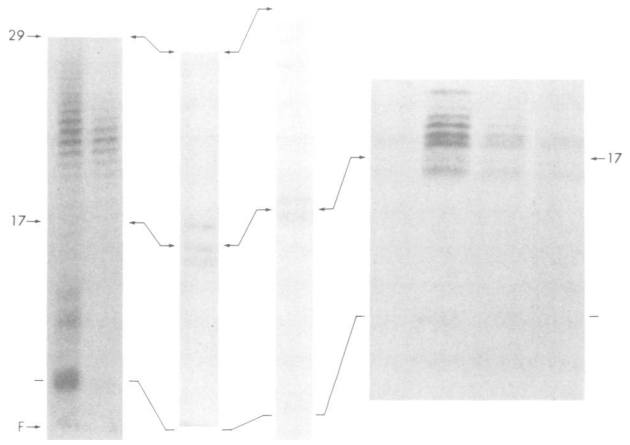

Fig. 2. Variation in patterns of methyl-^{3}H-labeled material in the 17−29 kd region. The figure is a composite of segments of fluorograms like the first pair of lanes in Figure 1. Only the area including the 17−29 kd region is shown. The unlabeled line marks the position of a prominent methylated species separated from the 'ladder' pattern. Markers for M_r are as in Figure 1.

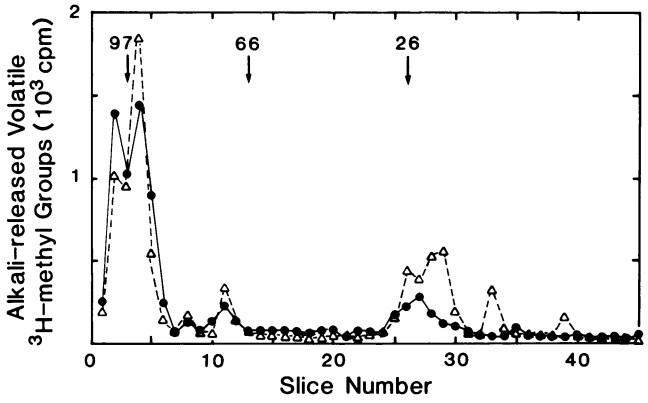

Fig. 3. Methyl-^{3}H-labeled material released in volatile form by alkali treatment. Flx15 cells were radiolabeled with [methyl-^{3}H]methionine in the presence (Δ) or absence (●) of puromycin and applied to an SDS−polyacrylamide (15%) gel. The 1 cm-wide lane was sliced in 2 mm segments. Each slice was treated with 0.5 M NaOH. Volatile radioactivity released by the treatment is plotted versus the slice number, beginning with the top of the gel. Positions on an adjacent lane are indicated for phosphorylase b (94), bovine serum albumin (66) and bacteriorhodopsin (26).

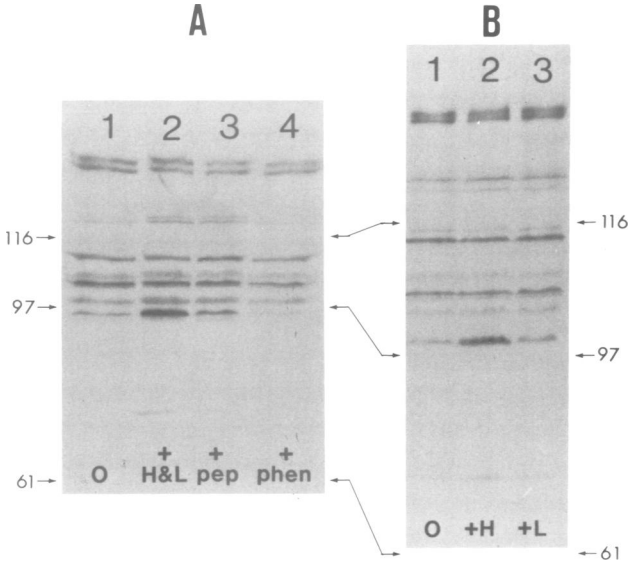

Fig. 4. Changes in methylation following chemostimulation. Strain Flx15 was labeled with [methyl-^{3}H]methionine for 45 min as described in the legend to Figure 1 and then stimulated with buffer (0), 10 mM histidine plus 5 mM leucine (+H & L), 10 mM histidine (+H), 5 mM leucine (+L), 1% Oxoid peptone (+pep), or 5 mM phenol (+phen). Reactions were terminated 2.5 min after stimulation by addition of 9 vols of ice-cold acetone. The figures are fluorograms of the segment of 11% acrylamide (0.073% *bis*acrylamide) gels at pH 8.8 (**A**) or pH 8.2 (**B**) including the radiolabeled bands in the 90−135 kd region. Markers for M_r are as in Figure 1.

of the amount of alkali-labile methyl-^{3}H-groups in the 17 000−29 000 M_r region revealed that for cells treated with an inhibitor of protein synthesis radiolabeled groups accumulated in this region (see below). These observations lead us to suspect that much, if not all of the methyl-^{3}H-labeled material in the 17 000−29 000 M_r region represented methylated forms of oligosaccharides, attached to lipids or other molecules, that accumulate in cells unable to transfer them to newly synthesized proteins. At least some of the methyl-^{3}H-labeled bands in this region are likely to be the transiently methylated intermediates described for lipid oligosaccharides in *H.halobium* (Lechner *et al.*, 1985; Sumper, 1987).

Both sets of bands lose radiolabel upon exposure to alkaline pH; treatment of SDS-solubilized protein with 0.5 M NaOH and analysis as described for Figure 1 resulted in no radiolabeled bands except for residual label in the same pair of bands that persisted in the pattern of methyl-^{3}H-labeled bands from the taxis mutant. For the bands in the 90−135 kd range, the alkali-released radiolabel was volatile (Figure 3), a property consistent with the radioactivity being in the form of methanol, created by hydrolysis of carboxyl methyl esters. In experiments like the one illustrated in Figure 3, comparison of total radiolabel in gel slices with alkali-released, volatile radiolabel indicated that, within the variation due to differential recovery, the vast majority of label in the 90−135 kd region was volatile upon alkali treatment, while only a minority of the alkali-labile radiolabel in the 20−25 kd region was volatile. Alkali release of volatile, radiolabeled methanol provides a way to detect methyl-^{3}H-labeled esters even in protein that has incorporated intact [methyl-^{3}H]methionine during synthesis, and thus a way to determine whether methylation occurs under normal physiological conditions (Stock *et al.*, 1984). Alkali-released, volatile radioactivity in the 90−135 kd region was observed to an equivalent extent in cells labeled in the presence or absence of protein synthesis, while the bands in the 17−29 kd region contained substantially less radiolabel in cells actively synthesizing protein (Figure 3). The pattern in Figure 3 represents a maximal level of labeled material in the 17−29 kd region observed for cells labeled during protein synthesis; in several other experiments, using this and other strains, no volatile radiolabel was detected in the region.

The analytical procedure illustrated in Figure 1 was used to examine cells for changes in the intensity of methyl-^{3}H-labeled bands following sensory stimulation and adaptation. Chemostimuli resulted in distinct changes in specific bands of the 90−135 kd set (Figure 4), but no significant changes in the intensity of bands in the 17−29 kd region (data not shown). Stimulation with the attractants histidine, leucine,

a mixture of those two amino acids or with the mixture of attractants present in peptone resulted in increased methylation of specific bands, most prominently the band of lowest apparent M_r in the 90−135 kd set. Stimulation with the repellent phenol resulted in decreased methylation of specific bands, most prominently the band of lowest apparent M_r in the 90−135 kd set. These changes were evident 2.5 min after stimulation, a period over which behavioral adaptation was complete for the respective chemostimuli (M.Alam, unpublished observations). Photostimuli caused no detectable consistent changes in methyl-^{3}H-labeled bands of the 90−135 kd region (Figure 5). Labeled cells were examined 1 min after stimulation with attractant or repellent light, a time at which adaptation to either stimulus was complete. The gels for Figure 5 displayed the entire range of *H.halobium* proteins and thus resolution in the 90−135 kd region was not optimal. Parallel analyses with optimal resolution of that region provided no indication of changes in methylation induced by illumination. In some photostimulation experiments, increases were observed in the intensity and number of methyl-^{3}H-labeled bands in the 17−29 kd region as shown in Figure 5 for cells stimulated with blue light, but these increases were not a function of photoreception since they occurred not only upon illumination of phototactically competent cells (S9 and L33) but also for mutant cells unable to produce retinal and thus lacking functional photoreceptors (L07). Presumably the changes observed reflected altered levels of methylated, biosynthetic intermediates unrelated to sensory processes.

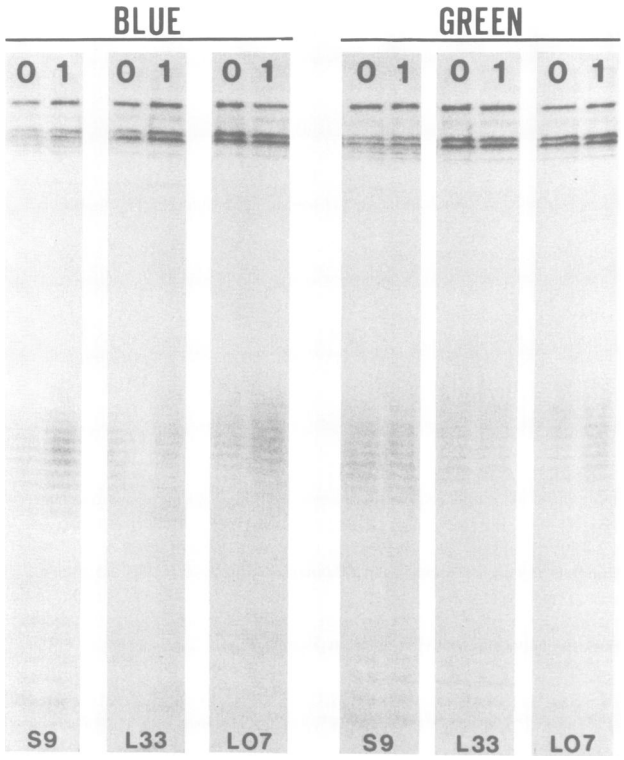

Fig. 5. No detectable, receptor-linked changes in methylation upon photostimulation. Cells of S-9, a strain containing carotenoids, bacteriorhodopsin, halorhodopsin and the sensory rhodopsins, L-33, a derivative of S-9 lacking carotenoids and bacteriorhodopsin, and L-07, a retinal$^-$ derivative of S-9, were labeled with [methyl-^{3}H]methionine for 45 min as described in the legend to Figure 1, put in cuvettes, placed in the dark for 2 min and then illuminated with repellent blue light (KG3 and UG1 filters, 80 W/m^2) with constant background illumination (KG3 and OG570 filters, 1 W/m^2) or attractant green light (KG3 and OG570 filters, 80 W/m^2). At 0 and 1 min of illumination, samples were taken into ice-cold acetone. Analysis by electrophoresis and fluorography was as for Figure 1A.

Analysis of rates of demethylation using a flow apparatus

In *E.coli*, tactic stimuli result in global changes in the activity of the methyl esterase enzyme that persist over the time course of adaptation (Toews *et al.*, 1979; Kehry *et al.*, 1984). Cellular activity of the methyl esterase can be monitored conveniently by determining the quantity of [^{3}H]methanol released from cells labeled with [methyl-^{3}H]methionine, placed in a flow apparatus and washed continually with buffer containing an excess of nonradioactive methionine (Kehry *et al.*, 1984). The amount of radiolabeled methanol produced in a unit time, detected as volatile radioactivity (Chelsky *et al.*, 1984), provides a value for the rate of cellular demethylation. Analysis in similar experiments of tactically wild-type cells of *H.halobium* revealed that release of methyl groups in a volatile form was significantly affected by chemotactic stimulation (Figure 6). No change in a low rate of release of volatile methyl groups was observed following chemostimulation of taxis mutants, Pho71 and Pho72, that, like the mutant shown in Figure 1, were incapable of chemotaxis or phototaxis and lacked almost all the methyl-^{3}H-labeled bands in the 70−135 kd region (Alam *et al.*, in preparation). As observed for *E.coli*, effects on the rate of release of volatile methyl groups were transient, persisting for periods roughly equivalent to times required for behavioral adaptation to the chemical stimuli. Precise correlations were not possible because of the effects of mixing time in the apparatus, which has its greatest influence on extending the time course of response to removal of a stimulating compound (Kehry *et al.*, 1984). The data imply a relationship between sensory behavior, specifically adaptation and transient changes in release of a volatile form of methyl groups. This is consistent with the relationship observed in the well-characterized sensory system of *E.coli*, in which the only volatile product released is methanol. However, the manner in which release of volatile methyl groups is affected by chemostimuli differs between *H.halobium* and *E.coli*. In the halobacterial species, both positive stimuli (attractant addition or repellent removal) and negative stimuli (attractant removal or repellent addition) resulted in an increased release of volatile methyl groups (Figure 6), while in the enteric bacteria positive stimuli cause a decrease and negative stimuli an increase (Toews *et al.*, 1979; Kehry *et al.* 1984). In addition, in *E.coli* equivalent-sized stimuli of the opposite sign (addition or removal of the identical concentration of attractant or repellent) produce approximately the same absolute magnitude of net change in demethylation rate, although the time courses differ (Kehry *et al.*, 1984, 1985), while for *H.halobium* the change in release of volatile methyl groups appeared to be greater following chemo-effector addition than following chemo-effector removal (Figure 6). This phenomenon was investigated by submitting radiolabeled cells in chase conditions in the flow apparatus to sequential rounds of addition and removal of peptone or phenol (Figure 7). The results revealed that the reduced amount of volatile methyl groups released following removal of the repellent phenol may reflect the reduced specific activity of the remaining

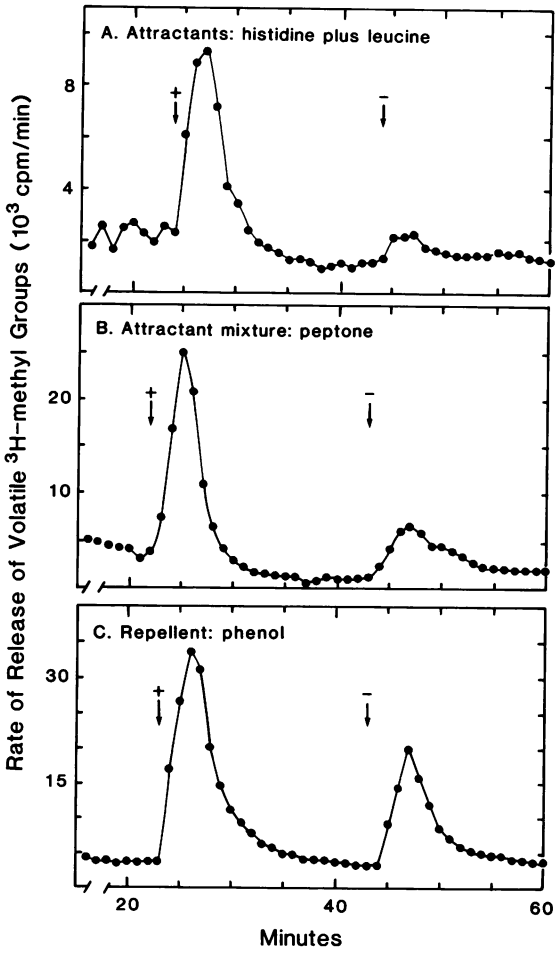

Fig. 6. Chemostimuli-induced changes in rate of release of [^{3}H]methyl groups in conditions of a nonradioactive chase. Flx15 cells were radiolabeled with [methyl-^{3}H]methionine as described in Materials and methods, washed free of unincorporated radiolabeled methionine, placed on a filter in a flow apparatus and subjected at room temperature to a continuous flow (0.4 ml/min) of buffer containing 0.1 mM, nonradioactive methionine. Fractions collected for periods of 1 min were analyzed for volatile radioactivity. Chemoeffectors were added (arrows with '+') by switching the inlet tube from buffer to buffer plus 10 mM histidine and 5 mM leucine (**panel A**), buffer plus 1% Oxoid peptone (**panel B**) or buffer plus 5 mM phenol (**panel C**), and removed (arrows with '−') by transferring the inlet tube from the solution containing active compounds back to buffer alone.

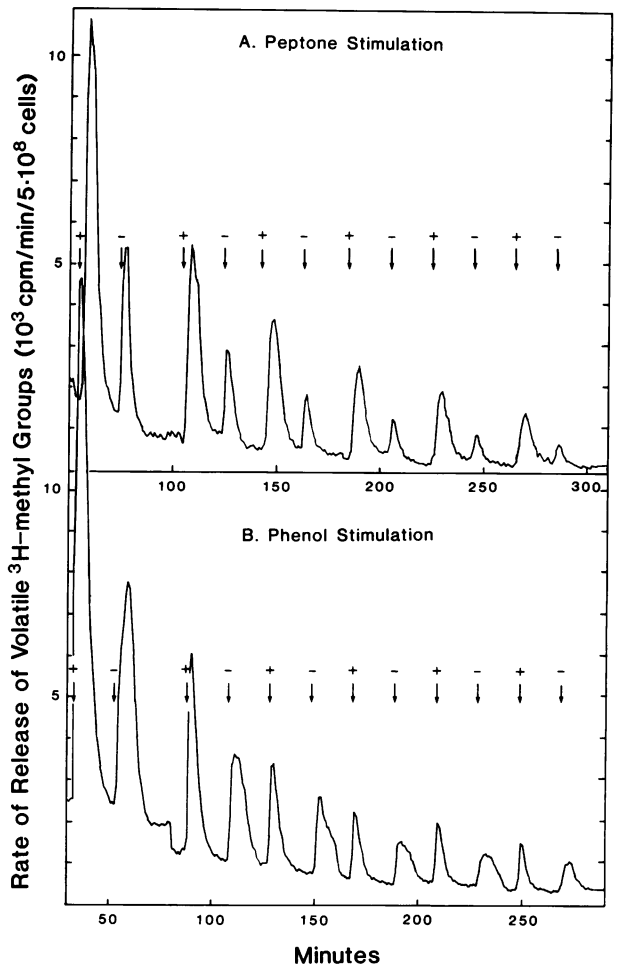

Fig. 7. Repeated stimulation of methyl-^{3}H-labeled cells in a flow apparatus. Flx15 were prepared as described in the legend to Figure 6 and 6.75×10^8 (**panel A**) or 4.65×10^8 (**panel B**) cells were placed on the filter of a flow apparatus. Addition and removal of 1% Oxoid peptone (panel A) or 5 mM phenol (panel B) was repeated for six cycles, and volatile radioactivity determined, as described in the legend to Figure 6.

methyl groups. Each stimulus in the series, whether addition or removal of phenol, resulted in less total radiolabel released than the previous stimulus (Figure 7B). This reduction was probably a function of progressive depletion of radiolabeled methyl groups by each response. In contrast, there was a clear asymmetry in the effect of addition versus removal of peptone, even when examined in sequential rounds of stimulation (Figure 7A).

The six pairs of sequential stimuli appeared to deplete most of the ^{3}H-labeled methyl groups that could be cleaved in response to tactic stimuli and thus the sum of radioactivity released by all 12 stimuli provided an estimate for the minimal cellular content of tactically active methyl groups. Assuming that the specific activity of released methyl groups was the same as that of the input [methyl-^{3}H]methionine, there were 2800 (peptone experiment) or 3500 (phenol experiment) methyl groups per cell. Values approximately two-thirds as large were calculated for the quantity of methyl-^{3}H groups released as volatile material by treatment with alkali from slices of an SDS−polyacrylamide gel corresponding to the region from 90 to 135 kd. These values for the number of tactically active methyl groups per cell are similar to the content of 2000 methyl groups calculated for the somewhat smaller *E.coli* cell (Kehry *et al.*, 1984).

Flow experiments were used to investigate the possibility that photostimuli as well as chemostimuli affected methylesterase activity. Distinct effects were observed only when the resolution of the procedure was increased by decreasing the sampling time from 60 to 18 s. In a pattern similar to that seen with chemostimuli, increases in release of volatile methyl groups were induced for a brief period after both positive and negative photostimuli (Figure 8A). The effects of photostimuli differed from those caused by chemostimuli in magnitude (~5% of the magnitude of a chemostimulus-induced release) and duration (<1 min, taking into consideration the clearing time of the flow apparatus, versus several minutes for chemostimuli). The brief period of altered rate of release of volatile methyl groups corresponds to the brief period of behavioral adaptation after photostimulation (Spudich and Stoeckenius, 1979; Hildebrand and Schimz, 1985). Pho81, a mutant strain

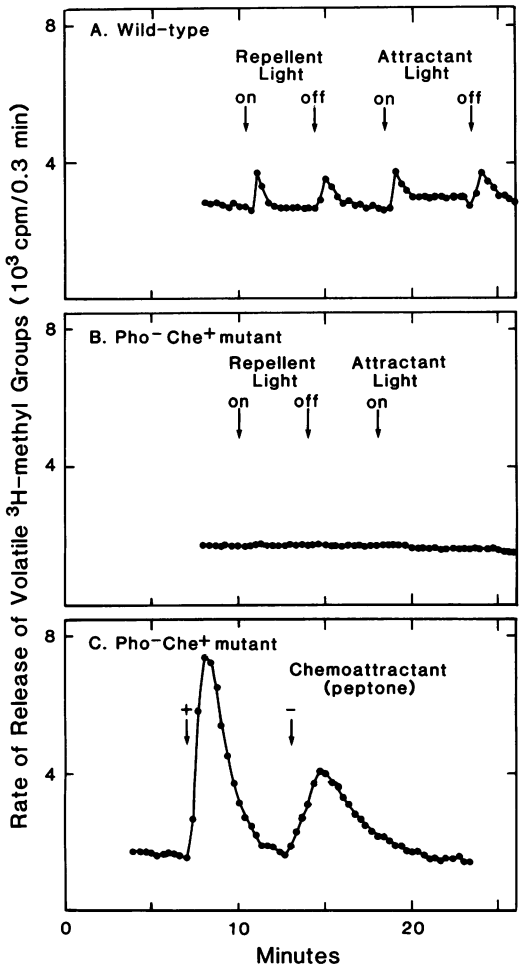

Fig. 8. Photostimulus-induced changes in rate of release of [^{3}H]methyl groups in conditions of a nonradioactive chase. The procedure was as described in the legend to Figure 6, except that the flow rate was increased to 1.5 ml/min and the fraction time was reduced to 18s. Cells of the tactically wild-type strain Flx15 (**panel A**) and the phototaxis$^-$, chemotaxis$^+$ mutant Pho81 (**panels B** and **C**) were stimulated (arrows) by turning on and off repellent blue light (KG3 BG3 filters, 8.5 W/m^2) with constant background illumination as for Figure 5, or attractant green light (KG3 and OG570 filters, 380 W/m^2) (panels A and B) or by adding and removing 1% Oxoid peptone (panel C).

unable to perform phototaxis but normal for chemotaxis (Sundberg *et al.*, 1985) exhibited no changes in release of volatile methyl groups upon photostimulation (Figure 8B), but responded normally to a chemostimulus (Figure 8C). These observations indicate that the effect of photostimulation on release of volatile methyl groups requires a functional phototactic system and imply that a change in a methyl cleaving activity and/or in a methylated substrate is related to the mechanism of adaptation to photostimuli. The quantity of volatile methyl groups released following photostimulation was small, representing only a few percent of the radiolabeled methyl groups in the cell. This minor change would be extremely difficult to detect in methyl-^{3}H-labeled bands by fluorography; thus it has not yet been possible to identify a source of the methyl groups released following photostimulation.

Discussion

The data presented here provide substantial evidence for direct involvement of methylation and demethylation in the mechanisms of chemotaxis and phototaxis by *H.halobium* and identify adaptation as the sensory process in which those reactions are likely to be involved. At this general level of description there are striking parallels with the extensively studied chemosensory system of *E.coli*. However, the sensory system of this archaebacterium exhibited variations and extensions of themes established in the study of chemotaxis by enteric bacteria.

Methyl-^{3}H-labeled macromolecules in H.halobium

The set of methyl-accepting proteins represented by electrophoretic species with apparent M_r from 90 to 135 kd, plus a single species at 70 kd, have many properties in common with the well-characterized methyl-accepting chemotaxis proteins of *E.coli*. The electrophoretic pattern of these methyl-^{3}H-labeled bands (Figure 1) is reminiscent of patterns exhibited by methyl-accepting chemotaxis proteins identified in eubacteria, although the apparent M_r values are higher than the commonly observed range of 60 000–70 000. Methyl groups are attached to the proteins of *H.halobium* in an acid-stable, alkali-labile manner consistent with the modified groups being carboxyl methyl esters. The methyl groups were continually turned over, probably by the action of enzymes analogous to the methyltransferase and methylesterase identified in eubacteria. Methyl groups were released in a volatile form from intact cells and by alkali treatment *in vitro*. A mutant strain of *H.halobium* defective in phototaxis and chemotaxis was missing almost all of the methyl-^{3}H-labeled bands in the 90–135 kd region, implying that the behavioral defect in the mutant is functionally related to the reduced level of methylation. Finally, sensory stimulation resulted in specific changes in the extent of methylation of particular electrophoretic species, as well as in transient changes in the rate of release of volatile methyl groups in a manner consistent with a mechanistic link to sensory behavior, a link analogous to the well-documented situation in the enteric bacteria.

Methylation in *H.halobium* had been analyzed previously by measuring radiolabel in slices of gels containing material from whole cells labeled *in vivo* with [methyl-^{3}H]methionine in the absence of protein synthesis or from membrane labeled *in vitro* with S-adenosyl[methyl-^{3}H]methionine. Two studies (Schimz, 1981; Bibikov *et al.*, 1982) concluded that there were methyl-accepting proteins in *H.halobium* that migrated in an SDS–polyacrylamide gel just below bovine serum albumin at an M_r of ~60 kd. We found no indication of methyl-accepting species of this apparent M_r. However, the 90–135 kd bands described in this study are likely to correspond to the methyl-^{3}H-labeled material migrating above bovine serum albumin observed by Schimz (1982). Covalent modification at multiple sites on transducer proteins of the enterics creates an array of electrophoretic forms (Hazelbauer and Harayama, 1983; Simon *et al.*, 1985). Thus the multiplicity of methyl-^{3}H-labeled bands observed in *H.halobium* (Figure 1) may reflect multiple electrophoretic forms of only a few different gene products. The electrophoretic pattern of labeled bands from *H.halobium* extends over a much greater range of apparent M_r than the range covered by modified forms of any single transducer of *E.coli*

(~7 kd), so it is likely that the pattern for *H.halobium* includes more than one gene product. Also, it is possible that not every methyl-^{3}H-labeled band in the 90–135 kd region is a form of a transducer protein. In particular, the pair of faintly labeled bands at ~97 kd persisted after alkali treatment and in the taxis mutant lacking other methylated bands in the region (Figure 1).

The set of methyl-^{3}H-labeled bands with apparent M_r from 17 000 to 29 000 exhibited features characteristic of biosynthetic intermediates. It is conceivable that a methyl-accepting protein involved in sensory mechanisms might be obscured among the variable array of methyl-^{3}H-labeled bands in this range of apparent M_r but we have no reason for believing this to be the case. Our observation that the methyl-^{3}H labeling of these bands can change by a process not related to photoreception during even a short, 1 min, photostimulation experiment means that the preliminary report of increases in methylation of species of 20 kd in *H.halobium* after illumination for 40–60 min (Bibikov *et al.*, 1982) cannot be interpreted unambiguously.

Release of volatile methyl groups monitored by flow experiments

Flow assays revealed that cells of *H.halobium* continually release a volatile form of methyl groups and that the rate of that release is affected transiently by sensory stimuli. The nature of the effects suggests strongly that the phenomenon reflects the functioning of the sensory system. Mutants examined that were unable to respond tactically to a stimulus exhibited no changes in methyl release upon exposure to an ineffective stimulus. Effects on methyl release were transient, corresponding roughly in duration and magnitude to the periods required for sensory adaptation. In these characteristics, the release of volatile methyl groups from *H.halobium* resembles release of methanol from *E.coli*. In the enteric species, methanol is produced by hydrolysis of carboxyl methyl esters on transducer proteins, catalyzed by a specific demethylase. Methanol is essentially the only volatile form of methyl groups produced in the cell (Toews and Adler, 1979). Tactic stimulation of *E.coli* causes transient changes in demethylase activity, resulting in altered rates of methanol production during the time course of adaptation (Toews *et al.*, 1979; Kehry *et al.*, 1984). Since the methyl-accepting taxis proteins of *H.halobium* have several features in common with the methyl-accepting transducers of *E.coli*, it is likely that methanol is produced by hydrolysis of carboxy methyl esters of the methyl-accepting proteins of *H.halobium*. Characterization of the volatile form of methyl groups released from *H.halobium* is in progress. However, the influence of sensory stimuli on the rate of release of volatile methyl groups must involve a feature in *H.Halobium* that does not occur in *E.coli*. In the enteric species, the period of adaptation to a positive stimulus involves a net increase in methylation of the relevant transducer and a concomitant global inhibition of demethylase activity while adaptation to a negative stimulus involves loss of methyl groups from the relevant transducer and increased demethylase activity. In *H.halobium*, changes in methylation of the putative transducers following sensory stimulation exhibit the same pattern: increase after positive stimuli and decrease after negative stimuli (Figure 4). In striking contrast, effects on the rate of release of volatile methyl groups by sensory stimuli do not exhibit the same symmetry: both positive and negative stimuli result in an increased rate of methyl release. Thus the pattern of changes in rate of release of volatile methyl groups cannot be completely accounted for by the observed changes in the extent of methylation of the methyl-accepting proteins in the 90–135 kd set. Presumably some process that is present in *H.halobium* but not in *E.coli* results in release of voltatile methyl groups at the same time that net methylation increases on the methyl-accepting proteins. It might be that methylation and demethylation occur simultaneously on different methyl-accepting proteins that overlap in electrophoretic positions in such a way that specific demethylations are observed. Conceivably the process could reflect a relatively minor alteration in the control of enzyme activity. For instance, if the methylesterase were not globally inhibited during the period of increasing net methylation, as it is in *E.coli*, then methyl group turnover might be at a higher rate until the adaptative process adjusted rates to steady-state values. Alternatively, methanol or another volatile compound derived from methyl groups could be produced from a source other than the identified methyl-accepting proteins in the 90–135 kd set. Such a reaction ought to be related to the sensory system, since, in the tactic mutants tested, lack of response to a stimulus was accompanied by lack of effect on methyl release. Free [methyl-^{3}H]methionine is probably not involved in such a reaction since the flow assays in which the methyl release was detected were performed with methyl-^{3}H-labeled cells that had been extensively washed and placed in an excess of unlabeled methionine. In such conditions it seems unlikely that there would be sufficient [methyl-^{3}H]methionine remaining to produce the amount of volatile, radiolabeled methyl groups observed in the experiments. It is interesting that in flow assays *Bacillus subtilis* exhibits an increased rate of production of volatile methyl groups after addition or after removal of a repellent (Thoelke *et al.*, 1987). The authors of that study suggest that this asymmetry could reflect the existence of a methyl-accepting intermediate between transducer and methanol; such an intermediate might account for our observations in *H.halobium*. In any case, it appears that the tactically related metabolism of methyl groups is more complex in *B.subtilis* and *H.halobium* than it is in *E.coli*.

Phototaxis and methyl-accepting proteins

Phototaxis by eubacteria does not appear to involve dedicated photoreceptors but rather detection of changes in proton-motive force that occur as photosynthetic rates are altered by changes an illumination (Harayama and Iino, 1977; Armitage, 1988). Such photosensitivity appears to occur independently of methyl-accepting proteins (Armitage, 1988). In contrast, *H.halobium* contains two sensory rhodopsins that function directly as photoreceptors (Spudich and Spudich, 1982; Takahashi *et al.*, 1985; Spudich *et al.*, 1986; Wolff *et al.*, 1986; Marwan and Oesterhelt, 1987). The existence of dedicated photoreceptors raises the possibility that methyl-accepting proteins are involved in phototaxis by this archaebacterium. Indeed, our flow assays showed that photostimulation induced transient changes in the metabolism of methyl groups, in a pattern at least in part consistent with hydrolysis of carboxyl methyl esters on a transducer protein. Thus, it is tantalizing that Pho81, the phototaxis$^-$ chemotaxis$^+$ mutant that exhibited no changes in methyl release upon photostimulation (Figure 8), lacks a specific methylated species in the 90–135 kd set and it has recently been shown that this same band can be labeled

by appropriate reduction with radioactive retinal (Spudich *et al.*, 1988). This electrophoretic species might be the source of the volatile methyl groups released upon photostimulation and may be a methyl-accepting phototaxis transducer protein.

A growing body of evidence has identified analogies and homologies among the chemosensory systems of eubacteria (Craven and Montie, 1983; Hirota, 1984; Greenberg *et al.*, 1985; Nowlin *et al.*, 1985; Ordal and Nettleton, 1985; Shapiro *et al.*, 1985). The observations presented in this study provide substantial evidence that the parallels are not restricted to the eubacterial kingdom, but extend across the wide taxonomic and evolutionary distance between eubacteria and archaebacteria (Woese, 1987). It appears that mediation of adaptation by modulation of the level of protein carboxyl methylation is a mechanism of broad taxonomic occurrence and thus of substantial antiquity. Particularly tantalizing is the evidence for involvement of carboxyl methylation in adaptation to photostimuli detected by the sensory rhodopsins of *H.halobium*. Visual rhodopsin found in eukaryotes is a photosensory molecule more closely related in function to the sensory rhodopsins of *H.halobium* than to the structurally related ion-pumping rhodopsins in the same species (Hargrave *et al.*, 1983). It will be of great interest to investigate the way in which adaptation to light stimuli, recognized by the sensory rhodopsins of the archaebacterial species, is related to the reactions of methylation and demethylation.

Materials and methods

Bacterial strains and growth conditions

Strain Flx15 is bacteriorhodopsin$^-$, halorhodopsin$^-$, sensory rhodopsins$^+$ derivative of *H.halobium*, OD2. Pho81 is a mutant derivative of Flx15 that has lost sensitivity to all photostimuli, but retains chemotactic sensitivity (Sundberg *et al.*, 1985). M402 is a chemotaxis$^-$, phototaxis$^-$ derivative of L-33 (Wagner *et al.*, 1981), selected as described by Schimz and Hildebrand (1979b). Highly motile derivatives of chemotaxis$^+$ strains were obtained by two rounds of picking cells from the perimeter of the chemotactic ring formed in semisolid agar (0.25%) plates containing complex Oxoid peptone medium, after inoculation in the center and incubation for 5 days at 37°C. Cells were grown at 40°C under limited aeration and continuous illumination in a basal salts medium containing Oxoid peptone and 0.1% arginine (Oesterhelt and Stoeckenius, 1974).

Radiolabeling with [methyl-^{3}H]methionine

L-[methyl-^{3}H]methionine (15 or 75–80 Ci/mmol) from DuPont New England Nuclear or Amersham was treated under vacuum to remove ethanol before use. Cells were grown to a density of $\sim 2.5 \times 10^8$ cells/ml, harvested by centrifugation, washed two or three times in basal salts medium plus 0.1% arginine and suspended at the same density in the same medium containing 30–100 μg/ml of puromycin. After 10 min at 37°C, [methyl-^{3}H]methionine at a specific activity of 15 Ci/mmol was added to a final concentration of 4 μM. Reactions were terminated at the desired time by mixing 1 vol of the labeled cell suspension with 9 vols ice-cold acetone. Cells attained maximal labeling of the 90–135 kd bands 40–60 min after addition of radiolabeled methionine at the indicated concentration. Protein was collected by centrifugation, washed with 50% ice-cold acetone and dried before solubilization in electrophoresis sample buffer.

SDS–PAGE and fluorography

SDS–PAGE was performed essentially by the procedure of Laemmli (1970), with modifications described by Randall and Hardy (1977). Samples were solubilized by boiling 2 min in sample buffer. Gels were stained, destained, treated with Amplify solution for 30 min, dried and analyzed by fluorography.

Determination of volatile radioactivity released by alkali treatment

Volatile radioactivity generated by alkali treatment of material displayed in SDS–polyacrylamide gels was analyzed after the gels were stained, destained and dried. The gels were sliced with a razor blade slicer (2 mm pieces, 1 cm wide), and each slice placed in 0.5-ml microfuge tube which was then placed in a 15-ml scintillation vial containing 6 ml of scintillation fluid (Rotiszint 22, Roth, Karlsruhe, FRG). Sodium hydroxide (0.1 ml of 0.5 M solution) was added to each microfuge tube, the scintillation vials stoppered and incubated at room temperature for 24 h. Volatile radioactivity, equilibrated between sample and scintillation fluid was determined by scintillation counting (Chelsky *et al.*, 1984).

Flow assays

Cells were labeled as described above except that the [methyl-^{3}H]methionine was at a specific activity of 75–80 Ci/mmol and the final concentration of methionine was 0.4–0.6 μM. The procedure was essentially that described by Kehry *et al.* (1984) except that the medium used was the *H.halobium* basal salts medium plus 0.1% arginine. The filter assembly (Gelman acrodisc, 0.2 μm) was connected at the outlet end to a fraction collector. The inlet (Luer fitting) was adapted to peristaltic-pump tubing (0.42 ml/min for 1 min samples or 1.5 ml/min for 18 s samples, Evergreen Scientific). The filter was washed by a flow of 10 ml of basal salts medium before the addition of cells. Cells were incubated for 10 min at 37°C in basal salts, 0.1% arginine, 50 μg/ml puromycin, then radiolabeled methionine was added and the incubation continued for 40 min. Labeled cells were harvested by centrifugation and washed three times with basal salts–arginine–puromycin, suspended in the same buffer plus 0.1 mM methionine and a suspension containing between 5×10^8 and 2.5×10^9 cells was pumped onto the filter. The deposited cells were subjected to a continuous flow of basal salts–arginine–puromycin–0.1 mM methionine. Fractions were collected from the time the cells were loaded. The time course of production of volatile radioactivity by unstimulated cells had the form of a multiple exponential, consistent with observations in *E.coli* (Kehry *et al.*, 1984), but the amount of radiolabel released per cell and the apparent decay times were greater for *H.halobium*. The amount of volatile radioactivity released from the cells was too great to be accounted for solely by loss of methyl groups from the radiolabeled material detected in slices of polyacrylamide gels, so it seemed likely that other molecules not retained on gels contributed to the steady state release of volatile, methyl-^{3}H-labeled material. Thus the turnover time of tactically active methyl groups could not be determined from steady state rates of methanol production. As an alternative, methyl-^{3}H-labeled cells were placed in flow apparatuses in parallel experiments and stimulated with 5 mM phenol at 33, 66, 120 or 180 min after addition of nonradioactive methionine. Volatile radioactivity released following stimulation (adjusted for the amount of unstimulated release) declined in an apparent single exponential decay over the time the cells were submitted to the chase conditions, with an apparent half-life of 230 min. Addition of attractant or repellent was accomplished by switching the inlet tube to the desired solution. Photostimuli were given by switching on or off a photographic projector (Noris 250W) directed at the cells and equipped with Schott cut-off filters KG3 and UG1 or OG570.

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