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Chemosensory Responses of *Halobacterium halobium*

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Responses of *Halobacterium halobium* cells to chemical stimuli have been shown by a capillary technique. Cells were attracted by D-glucose and several amino acids and repelled by phenol. Certain chemicals, such as acetate, benzoate, indole, and $NiSO_4$, that are known to act as repellents of *Escherichia coli* cells served as attractants for *Halobacterium*. In the presence of ethionine, sensitivity to attractants was reduced. Arsenate prevented the attraction by glucose without lowering the cellular adenosine 5'-triphosphate level. The ability for chemo-accumulation toward glucose and histidine was interfered with by the formation of photosensory systems. Light-induced motor responses and chemosensory behavior toward glucose and histidine became detectable in the late stationary growth phase only. The behavior toward acetate and indole was not connected to photobehavior in that way: both substances acted as attractants already in the late log phase. Inhibition of bacteriorhodopsin synthesis by L-nicotine allowed chemo-accumulation toward glucose and histidine already in the late logarithmic phase.

Photophobic behavior, mediated by bacteriorhodopsin in the case of light decrease (step-down response) and probably by another retinal protein in the case of light increase (step-up response), has been observed by Hildebrand and Dencher (4) and Dencher and Hildebrand, (Z. Naturforsch., in press). In addition to light-induced motor responses, sensitivity toward chemicals has now been defined for *Halobacterium halobium*. Photophobic activity and chemosensory behavior can be regarded as excitatory phenomena comparable to processes in sensory cells.

Since both properties have been discovered in *H. halobium*, this organism seems well suited for studying the relationship between different sensory systems in bacteria and their integrative properties.

This investigation deals with the first analysis of several chemicals in modulating the behavior of *H. halobium* and with the relationship between chemo-accumulation and photosensory properties.

MATERIALS AND METHODS

Bacterial strain. *H. halobium* R_1, a mutant strain lacking gas vacuoles, was used for experiments.

Chemicals. Oxoid bacteriological peptone (code L 37) was purchased from Nährboden und Chemie GmbH, Wesel, Federal Republic of Germany; L-nicotine was from Fluka, Neu-Ulm, Federal Republic of Germany; luciferin-luciferase (firefly enzyme) was from Sigma Chemical Co., Munich, Federal Republic of Germany; and disodium ATP was from Boehringer, Mannheim, Federal Republic of Germany.

All other chemicals were of analytical reagent grade.

Growth medium and culture conditions. The growth medium contained 250 g of NaCl, 20 g of $MgSO_4 \cdot 7H_2O$, 3 g of trisodium citrate$\cdot 2H_2O$, 2 g of KCl, and 10 g of peptone in 1,000 ml of water; the pH was adjusted to 6.8. Agar plates were prepared by adding 1.5% agar to the growth medium. The medium was sterilized for 20 min at 120°C. Unless otherwise noted, cells were grown in 20-ml cultures on a rotatory shaker (LSR-TK; Braun Melsungen AG, Melsungen, Federal Republic of Germany) under semiaerobic conditions (100 rpm) at 37°C and illumination of 100 to 300 $\mu W/cm^2$ (15-W fluorescent lamp) near the Erlenmeyer flasks. The generation time under these conditions was 7 to 8 h. Nicotine cells were prepared by adding nicotine to the growth medium to a final concentration of 1 mM (9).

Capillary assay. Chemosensory activity was determined by the method of Adler (1). Cultures were grown to stationary phase (usually 7 to 10 days). Cells were harvested by centrifugation (10,000 rpm for 2 min at room temperature). Bacteria were suspended in 5 mM potassium phosphate buffer containing 250 g of NaCl, 0.3 mg of $MnSO_4$, 7 mg of $CaCl_2$, 0.04 mg of $FeCl_2$, and 0.05 mg of $CuSO_4$ in 1,000 ml at pH 6.8. This solution is referred to as "assay medium"; it does not allow bacterial growth. All chemicals to be tested were dissolved in this medium, and pH values were adjusted to 6.8 with NaOH or HCl. The final concentration of bacteria was about 10^8 cells per ml of assay medium. In the assay, 0.2 ml of the cell suspension was maintained at 37°C on an Eppendorf Thermostat. A sealed glass capillary (7 cm long, 0.5-mm inside diameter) was passed quickly through a flame several times

and plunged into 0.2 ml of assay medium containing the chemical to be tested. About 1.5 cm of the capillaries became filled with solution. Control capillaries were filled with assay medium alone. After cooling, the capillaries were rinsed with water, inserted into the cell suspension, and removed after 120 min of incubation in the dark. The capillaries were rinsed, and the contents were transferred to growth medium containing no peptone. After appropriate dilution, samples were plated on agar. The plates were incubated at 37°C, and colonies were counted after 7 days.

Photophobic responses. Single cells were observed under a microscope (Leitz Ortholux). Stimuli at a wavelength of 370 nm (1 s, 0.14 $\mu W/cm^2$) to evoke a step-up response and of 565 nm (1 s, 8 $\mu W/cm^2$) to evoke a step-down response were produced with a 200-W mercury lamp connected to a monochromator (4). For each determination, 30 to 50 individual cells were examined.

ATP determination. From cell suspensions prepared for capillary assay, 1-ml samples were removed and mixed with 1 ml of cold perchloric acid (35%, vol/vol). After standing in an ice bath for 15 min, the solution was neutralized by the addition of 3 ml of cold 2 M $KHCO_3$ solution. The mixture was then centrifuged, and the supernatant was used for ATP assay by the luciferin-luciferase method on a bioluminescence analyzer (XP 2000; Skan AG, Allschwil, Switzerland) (12; K. L. Schimz and H. Holzer, Arch. Microbiol., 121:225–229, 1979).

RESULTS

Attractants and repellents. *H. halobium* was attracted by D-glucose, as well as by several L-amino acids (Table 1). One millimolar histidine, asparagine, leucine, and methionine led to an accumulation of cells in the capillaries containing these amino acids. No response could be elicited by 1 mM arginine, valine, or glutamine (data not shown).

In contrast to *Escherichia coli* (10), *Halobacterium* cells were not repelled by certain organic acids and Co^{2+} and Ni^{2+} ions. *Halobacterium* cells accumulated in response to 20 mM sodium acetate, 30 mM sodium benzoate, and 0.5 mM $NiSO_4$. No response was elicited with $CoSO_4$. Therefore, the effect of $NiSO_4$ seems to be due to the cation. Simultaneous stimulation by 20 mM acetate and 0.3 mM indole, which has been reported to be a strong repellent for *E. coli* (2), led to a high accumulation in the capillaries (Table 1). *Halobacterium* cells were repelled by 5 mM sodium phenolate (Table 1).

Optimum conditions. The optimum temperature for *H. halobium* is about 40°C. All assays were performed at 37°C. No higher temperatures were used to avoid evaporation during the incubation period. The number of cells moving into the capillaries depended on the concentration of the attractant or repellent and on the incubation time.

The time course of accumulation in 1 mM glucose is shown in Fig. 1. Incubation for 120 min was sufficient to show significant differences between cells in control capillaries containing assay medium alone and cells in glucose-containing capillaries. The cell suspensions used for all assays contained 2×10^7 to 3×10^7 cells in 0.2 ml. A total of 5.6×10^3 cells had moved into the control capillaries by the end of the experiment; 1.4×10^4 cells were found in the glucose capillaries. Maximum responses were elicited with 1 mM glucose and 0.5 mM histidine (Fig. 2).

TABLE 1. *Response of H. halobium to various chemicals after a 2-h incubation in the capillary assay*[a]

Chemical	Response (no. of cells in capillary × 10^4) in:		
	Assay medium	With 1 mM ethionine	With 10 mM potassium arsenate and 10 mM DL-lactate
Attractant			
None	0.53	0.54	0.44
1 mM glucose	1.2	0.59	0.43
20 mM sodium acetate	1.26		
20 mM sodium acetate plus 0.3 mM indole	1.75		
30 mM sodium benzoate	0.82	0.44	
1 mM L-histidine	1.26		
1 mM L-asparagine	1.24		
1 mM L-leucine	0.81		
1 mM L-methionine	0.95		
0.5 mM quinine hydrochloride	0.92		
0.5 mM $NiSO_4$	0.84		
Repellent			
5 mM sodium phenolate	0.22	0.24	0.16

[a] For details, see the text. Means of $n = 16$ measurements. The standard deviation was about 20%.

Phenol concentrations above 5 mM seriously damaged the cells. With 5 mM phenol, neither viability nor motility of the cells was altered. Normal motility was demonstrated by suspending the cells in 5 mM phenol and counting the number of bacteria that had moved into phenol-containing capillaries after 2 h. The number of bacteria found there was the same as those in control experiments with assay medium.

During incubation in the capillary assay, the cell suspension was not aerated. ATP synthesis by means of the purple membrane could not take place because incubation was performed in the dark to avoid interference of photophobic activity with chemosensory behavior.

To determine whether changes in the cellular ATP content during incubation, which could

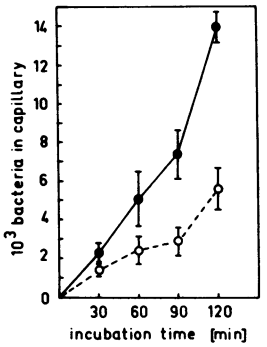

FIG. 1. *Accumulation rate of H. halobium toward glucose. Means of n = 16 measurements ± standard deviation. Symbols: ○, control without glucose; ●, 1 mM glucose in capillary.*

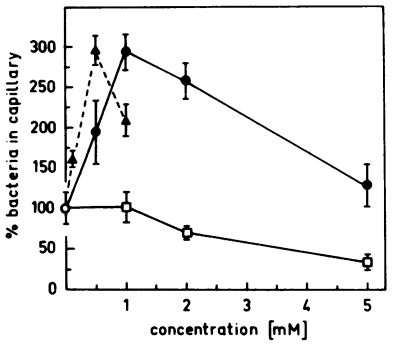

FIG. 2. *Concentration-response curves for chemosensory behavior of H. halobium after 2 h of incubation. Means of n = 16 measurements ± standard deviation. Symbols: ●, glucose; ▲, histidine; □, phenol; ○, control. A total 6.2 × 10^3 cells per capillary = 100%.*

influence the chemosensory behavior of *H. halobium*, might occur under these conditions, we measured the amount of ATP. A 2-h treatment of cells under these conditions did not significantly lower the amount of ATP. At the end of the experiment, 83% of the ATP present at the beginning was still detectable.

Inhibition studies. Stimulus transduction and adaptation in the chemosensory system of *E. coli* have been shown to depend essentially on protein methylation (3, 5). The donor for the methyl group is *S*-adenosylmethionine (11). Since ethionine instead of methionine is known to be incorporated to form *S*-adenosylethionine, the latter substance not being a methyl donor, the possible influence of ethionine on chemosensory behavior was investigated. For this purpose, cells were preincubated for 30 min in assay medium containing 1 mM ethionine. The same ethionine concentration was present in the capillaries. Cells treated in this way no longer accumulated in response to glucose and benzoate. The behavior toward phenol, however, remained unaffected (Table 1). Ethionine did not influence the photophobic responses at all.

Potassium arsenate has been reported to prevent chemosensory reactions by drastically lowering the ATP level. Motility is not inhibited, provided an oxidizable substrate is available (6). In *Halobacterium*, 10 mM potassium arsenate in the presence of 10 mM sodium DL-lactate did not reduce the amount of ATP within 2 h, but nevertheless inhibited chemo-accumulation toward glucose. No inhibition of motility was observed when in a control experiment the same concentration of arsenate was added to both the capillary and the medium.

The response to phenol was not inhibited under these conditions (Table 1). Light-induced motor responses were not impaired by arsenate.

Influence of the age of the culture. *H. halobium* cells from the logarithmic growth phase do not respond to light stimuli (4). In the stationary phase, when the purple membrane has been synthesized (9), the cells start to show photophobic behavior (Fig. 3).

To investigate whether the whole pathway for sensory transduction is synthesized simultaneously with or before the photoreceptors, we determined chemo-accumulation at different growth periods. The number of viable cells in the assay was kept constant at all times by appropriate dilution. The motility of the cells was measured by counting the percentage of cells moving into control capillaries filled with

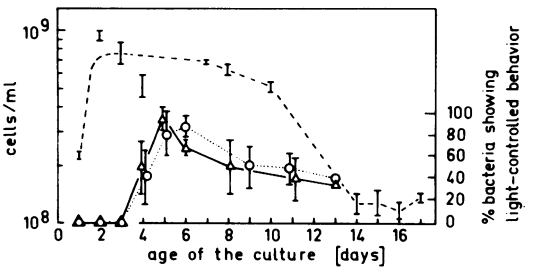

FIG. 3. *Growth curve of H. halobium and dependence of photophobic behavior on the age of the culture. Culture conditions were as follows. Cells were grown in 50 ml of medium at 37°C. On the first 2 days, shaking was at 100 rpm. From days 3 through 17, shaking was reduced to 50 rpm. Growth curve (dashed line, left ordinate): Viable cells per milliliter of culture determined by means of duplicate plating. Photophobic behavior (right ordinate): Means of determinations from n = 10 different cultures ± standard deviation, except for day 13. Symbols: △, percentage of bacteria showing step-up response at 370 nm; ○, percentage of bacteria showing step-down response at 565 nm.*

assay medium and was found to remain fairly constant for 17 days.

Cells from young cultures did not exhibit chemosensory behavior toward glucose and histidine (Fig. 4). In the late stationary phase, at about the same time that light-controlled behavior became detectable, the cells began to respond to these chemicals.

When *Halobacterium* cells are grown in the presence of 1 mM nicotine, retinal synthesis, and thereby formation of the photoreceptors, is blocked (9). Nicotine-grown cells accumulated in glucose capillaries much earlier than did cells grown in normal medium. The response to glucose could be detected already at the end of the log phase (Fig. 4). In contrast, accumulation toward sodium acetate and indole did not seem to be influenced by the presence of photosystems in that manner, for this reaction could be observed long before photophobic responses and attraction by glucose and histidine occurred.

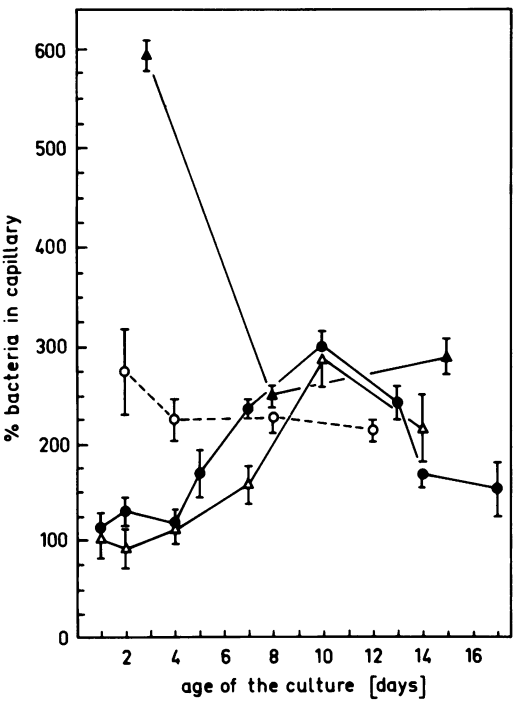

FIG. 4. *Dependence of chemosensory behavior of H. halobium on the age of the culture. Means of n = 16 measurements ± standard deviation. Symbols: ●, accumulation of normally grown cells to 1 mM glucose; △, 0.5 mM histidine; ▲, 20 mM sodium acetate plus 0.3 mM indole; ○, accumulation of cells grown in the presence of 1 mM nicotine to 1 mM glucose. All values are in reference to control measurements with the assay medium in the capillaries (6.8 × 10^3 cells per capillary = 100%).*

DISCUSSION

H. halobium responds to chemical stimuli obviously in much the same manner as other bacteria do. Optimum concentrations for glucose and amino acids do not differ widely from those found for *E. coli* and *Pseudomonas*.

The ability of the cells to maintain their ATP level during the time of incubation without oxidizable substrates, sufficient oxygen supply, or light is certainly due to the large K^+ gradient, which had been built up during growth under illumination and which allows ATP synthesis under these conditions for hours (13).

Acetate, benzoate, and indole, which are known as repellents of *E. coli* cells, act as attractants of *H. halobium*. Since a single mutation in one gene coding for a methyl-accepting protein is sufficient to reverse the chemosensory behavior of *E. coli* (7), it may not be too surprising to find chemicals attracting one species of bacteria and repelling another.

Although at this time we have no detailed knowledge of the molecular mechanism of chemoreception, sensory transduction, and adaptation, we expect to find a similar fundamental mechanism as that for *E. coli* on further investigation with *H. halobium*. Inhibition of chemoaccumulation by ethionine might indicate that methylation also plays a central role in the chemosensory pathway of halobacteria. The response to the repellent phenol is not inhibited by ethionine. As for *B. subtilis* (8), methionine does not seem to be required for response to a repellent. Photophobic reactions are not inhibited by ethionine. This may indicate that light-controlled behavior could be mediated through a mechanism similar to repellent responses or by a mechanism different from that of chemosensitivity. Ten millimolar arsenate neither lowers the ATP level nor prevents reactions to light stimuli; yet it inhibits the response to glucose. Therefore, the reason for this inhibitory effect must be different from that described for *E. coli* and *Salmonella typhimurium* (6), but for the time being we are not able to explain it.

The most striking feature in photophobic and chemosensory behavior of *H. halobium* seems to be the partial interdependence and independence among some of these sensory activities. Observation of light-induced responses and attraction by glucose and histidine occurring simultaneously in stationary cultures would lead to the conclusion that the prerequisite structures for both activities are synthesized or activated simultaneously. Therefore, it is surprising to observe that inhibition of the photoreceptor formation allows chemosensory responses to occur

much earlier. From these findings we conclude that chemosensory behavior can develop independently from photophobic behavior and that the pathway for sensory transduction and control of motor responses must already be functioning before the photoreceptors are synthesized. The late occurrence of sensitivity to glucose and histidine in normally growing cells may be explained by assuming some interference of purple membrane formation with the accessibility of these chemoreceptors or some other membrane proteins necessary for sensory transduction.

The response to acetate and indole is not influenced by the formation of photoreceptors in that manner. Chemosensory behavior, therefore, seems to operate in at least two different ways, one being influenced by the synthesis of photoreceptors and the other not being influenced.

Flagellar response to all sensory stimuli and its control may be handled by common structural elements, whereas the signals from photoreceptors and chemoreceptors might flow through different pathways. A detailed investigation of the molecular basis for signalling and flagellar response will be necessary to gain more insight into the sensory mechanism of *H. halobium*.

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