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Signal transduction in *Halobacterium* depends on fumarate

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The isolation of a straight-swimming mutant of *Halobacterium halobium* is reported which has a defect in switching the rotational sense of its flagellar motor. Cells of this mutant strain could be complemented with an extract from wild-type cells by mild sonication and resealing of the cells in fresh medium. The switch factor responsible for restoration of wild-type behaviour was isolated from membrane vesicle preparations. Its chemical nature is proposed to be that of fumarate on the basis of chemical, chromatographic and mass spectrometric analysis. Since the switch factor (fumarate) was released from a membrane-bound state by heat and was accumulated into mutant cells that lack this compound, it is proposed that a membrane-bound protein exists which specifically binds the switch factor. Both the switch factor and fumarate cause stimulus-induced responses in cells at the level of one or few molecules.

Key words: fumarate/flagellar motor/*Halobacterium*/photophobic response/signal transduction

Introduction

Halobacteria orient in response to a variety of environmental stimuli to find optimal growth conditions. Most prominent attractant stimuli are light and chemicals providing the cells with metabolic energy by photosynthesis, respiration or fermentation (Schimz and Hildebrand, 1988; Spudich and Bogomolni, 1988; Oesterhelt and Tittor, 1989). *Halobacteria* have polarly inserted and motor-driven flagellar filaments which form bundles visible in the light microscope (Alam and Oesterhelt, 1984). Clockwise rotation causes the cell to swim forward and a switch to counter-clockwise rotation reverses the direction of swimming. The cell spends equal times in both modes and thus carries out a random walk unless a stimulus prolongs or shortens single runs. This is the basis for the orientational behaviour of the cells (Hildebrand and Dencher, 1975; Hildebrand and Schimz, 1985; Stoeckenius *et al.*, 1988).

Attractant stimuli cause inhibition and repellent stimuli activation of the flagellar motor switch (Spudich and Stoeckenius, 1979). A quantitative model for the photophobic signal transduction chain that is activated after blue light application has been established. It links stimulus size and average response time of the motor switch by a simple equation (Marwan and Oesterhelt, 1987). Further analysis of the signal chain excluded membrane potential changes as an integral part and demonstrated single photon effectiveness (Oesterhelt and Marwan, 1987; Marwan *et al.*, 1988). Also methyl-accepting proteins have been invoked in both chemosensing and photosensing (Schimz, 1981; Alam *et al.*, 1988). Integration of sensory input through chemo- and photoreceptors and potentially through others, e.g. oxygen sensors and thermosensors, occurs and finally all pathways merge into a linear chain of events that regulates the flagellar motor switch.

Many molecules have been invoked in the signalling network, including reception, amplification, integration and adaptation. However, the only component of the halobacterial sensory system isolated so far is sensory rhodopsin, a retinal-containing photoreceptor protein (Schegk and Oesterhelt, 1988). The identification and isolation of more components will depend on a combination of genetic and biochemical methods together with quantitative behavioural analysis. We therefore isolated a mutant unable to switch its flagellar motor and developed a complementation assay by which somatic curing of the mutational defect is measured. This approach allowed the isolation of a small molecule, the switch factor, which restores wild-type behaviour in the mutant and is functionally replaced by fumarate.

Results

Isolation and characterization of a straight-swimming and photophobic-negative mutant (switch mutant)

Halobacterium halobium strain M407 was subjected to mutagenesis by nitrosoguanidine (Oesterhelt and Krippahl, 1983) and blue light-blind mutants were enriched in one end of a capillary flashed with blue light to repel the majority of wild-type cells (Sundberg *et al.*, 1985). Single colonies from the cell mixture were checked for a negative phenotype in the photophobic response. Mutant strain M415 was characterized by a smooth swimming behaviour without spontaneous reversals within the observation time of 2 h. This mode of swimming could be caused either by a monopolarly flagellated cell or a bipolarly flagellated cell rotating its two flagellar motors in opposite directions (Alam and Oesterhelt, 1984). These alternatives could be distinguished by analysis of cells tethered via their flagella to the microscopic slide. The rotational direction of the cell then reflects that of the flagellar motor and, as a result, 98% were shown to turn their flagellar motor clockwise and only 2% counter-clockwise. This is a clear demonstration that the cells were monopolarly flagellated and practically no reversal occurs within the life time of the cells.

The swimming behaviour of M415 cells upon blue light stimulation was recorded and analysed with a computer-assisted motion analysis system (W.Marwan and D.Oesterhelt, in preparation). Three types of behaviour could be distinguished: no response (~90%), a stop response and a reversal (Figure 1A–C respectively). For comparision, Figure 1D shows the response of wild-type cells. A reversal of the swimming direction of the cells occurs when the

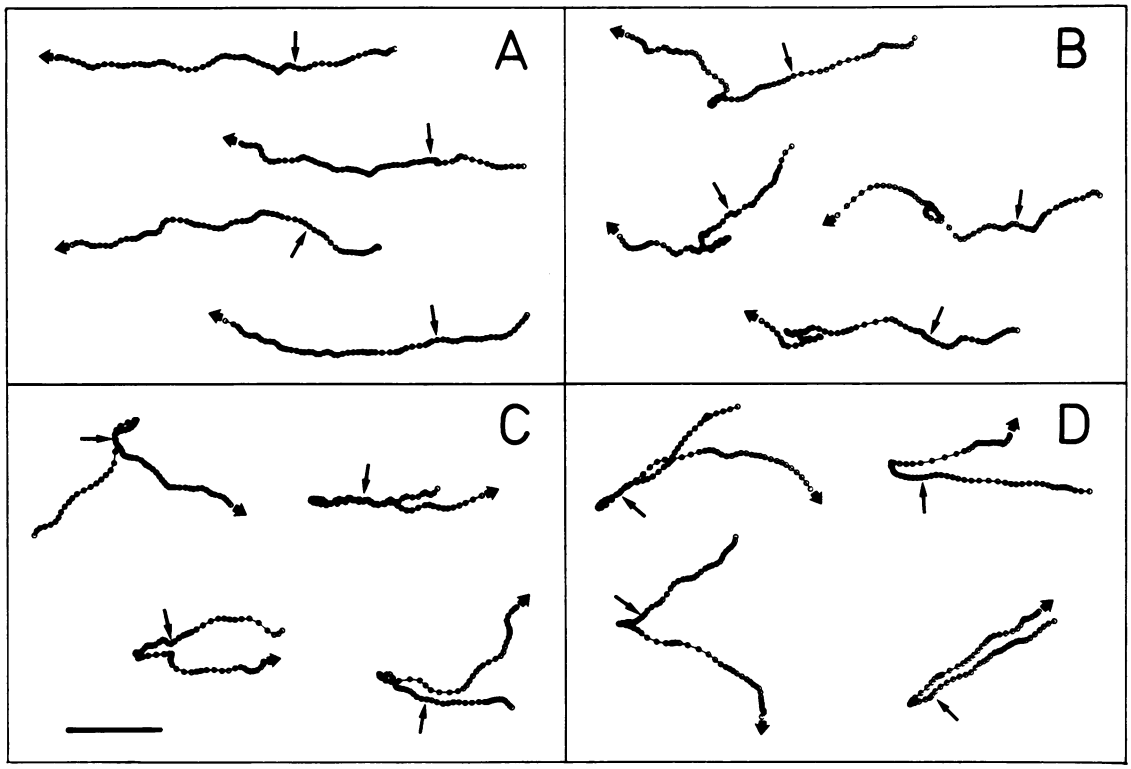

Fig. 1. Typical swimming paths of mutant cells of strain M415 (**A**–**C**) and wild-type Flx37 cells (**D**) upon blue light stimulation as recorded by a computer-assisted motion analysis system. Three types of response can be distinguished in M415 cells: (**A**) no response, (**B**) stop response and (**C**) reversal. Direction of the cell movement and the onset of the light stimulus are indicated by arrows. The centroids of the cells are displayed at 67 ms time intervals as open circles. The bar represents 5 μm.

Table I. Response of different clones of M415 to blue light

Subclone	1 (1.5 days)	2 (3 days)	3 (3 days)	4 (1.5 days)	5 (3 days)	6 (3 days)	7 (3 days)	8 (1.5 days)	9 (1.5 days)
% reacting cells	1	2	2	3	5	6	13	18	45

Cells of mutant strain M415 were cloned and material from single colonies taken into liquid culture (35 ml) to the stationary phase (5 days). Growth was continued after inoculating 35 ml of fresh medium with 1.5% of a stationary culture for the times indicated in parentheses. Before measurement the cell suspension was diluted 1:10 with fresh medium. The photophobic response of 100 cells from each subclone was measured as described in Materials and methods.

flagellar motor switches its rotational sense (Alam and Oesterhelt, 1984). If a cell carries out a stop response, the movement in the original direction is resumed afterwards and the rotation of the flagellar motor is the same as before the stop. Direct evidence for this behaviour was obtained by visual inspection of individual cells.

Mutant cells of a 3 day old culture were analysed for responses to saturating blue light flashes in more detail. The fraction of reactive cells (stop responses and reversals) was constant over 3 years of continuous cultivation without cloning, indicating the stability of the genotype. The fraction of reactive cells depended, however, on the age of the culture and was higher (~20%) on the second day of cultivation but minimal on the third and subsquent days (≤10%). Reactive cells of a culture responded to each light stimulus. Representative values from 12 cells in three different cultures were 102 photophobic responses to 104 stimulus applications. This means that two subpopulations in M415 cultures exist which are heterogeneous in their light responses. Without light stimulation, cells of either subpopulation never showed spontaneous reversals, but slower swimming or a stop lasting no longer than a fraction of a second occurred occasionally. This is reminiscent of the pausing and the velocity fluctuation of the *Escherichia coli* motor recently described (Eisenbach *et al.*, 1989).

The heterogeneity of the M415 phenotype could be linked to transient differences of genotypes by cloning. As summarized in Table I, cell populations from different clones showed different ratios of reactive and non-reactive cells with a minimal percentage of 1% reactive cells. Upon continuous cultivation of the clones these genetic differences disappeared and populations ended at the average level of ~10% reactive cells in 3 day old cultures. A similar phenomenon was also described by Hildebrand and Schimz (1987). These authors found wild-type cell cultures derived from single colonies with transiently different spontaneous reversal frequencies.

Reconstitution of the photophobic response in mutant cells

The majority of M415 cells (85–90%, or more) were unable to respond to light and were thus defective in their signal transduction chain. This strain could be used for identification of the missing component which caused the PHO^- phenotype since the cells could be cured by transfer of a

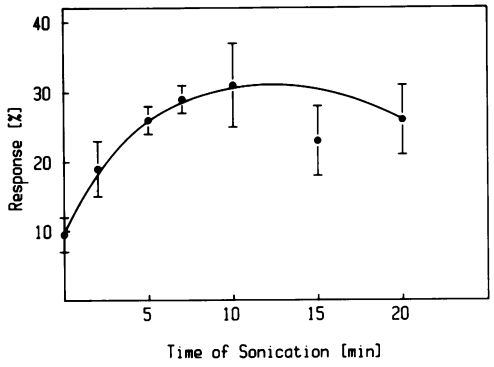

Fig. 2. Extent of reconstitution of the photophobic response as a function of sonication time. Cells of strain M415 were treated with ultrasound in the presence of a saturating concentration of switch factor for various times. The complementation rate was estimated as described in Materials and methods. For each data point, 60–180 cells were tested. Without ultrasonic treatment no elevated response level within 60 min incubation was observed.

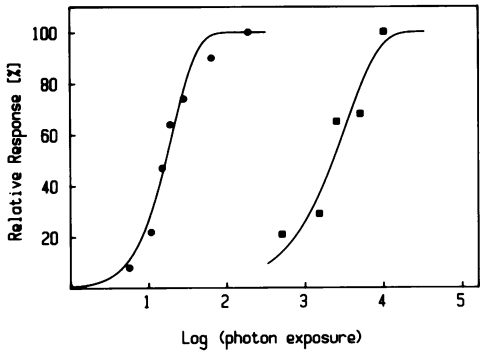

Fig. 3. Dependence of the photophobic response of complemented cells of the mutant strain M415 (■) and the untreated parent strain M407 (●) on photon exposure. The complementation of the cells was carried out in the presence of 3 U switch factor as described in Materials and methods. A relative response of 100% is defined as the maximum fraction of cells reacting upon light stimulation. This fraction was 100% for wild-type cells and 34% for the M415 cells. The solid lines represent computed Poisson stimulus–response curves. The *n*-values of 1 for complemented M415 cells and 2 for M407 cells yielded the best fit. Stimulus light was applied as 20 ms or 2 s pulses to M407 or M415 cells respectively. The log *F* of 1 equals a photon exposure of 1.1×10^{-5} mol/m^2.

soluble molecular component released by heat treatment of wild-type membrane vesicle fractions (see purification and characterization of the switch factor) in a complementation experiment. The substance that reconstitutes motor switching will be called the switch factor.

The halobacterial cell has a plasma membrane that is mechanically stabilized only by a regular layer of surface glycoproteins (for review see Baumeister *et al.*, 1989) which needs Mg^{2+} ions for stability. The cells become mechanically labile when suspended in a Mg^{2+}-free EDTA-containing salt solution. M415 cells were treated this way and were then permeabilized by weak sonication in the presence of switch factor. In control experiments fluorescein was shown to enter the cells upon this treatment. The mutant cells were allowed to reseal after permeabilization by incubation in fresh medium and were then subjected to light stimulation. Complemented mutant cells showed either stop responses or reversals upon blue light stimulation but not every cell became reactive after treatment with wild-type

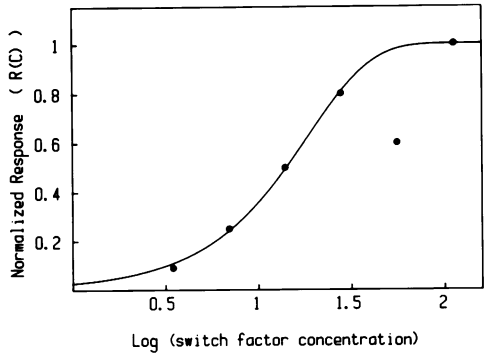

Fig. 4. Dose–response curve for the reconstitution of the photophobic response in M415. The complementation efficiency was measured as a function of the concentration of purified switch factor. For each data point, 6–10 experiments involving 30 cells each were evaluated. The normalized response was defined as $R(c) = (R - R_0)/(R_{max} - R_0)$, where R_0 is the response of the control without added factor, R the response at a given factor concentration and R_{max} is the response at saturating factor concentration. For this experiment $R_0 = 0.05$ and $R_{max} = 0.25$. The line represents the dose–response curve calculated for an *n*-value of 1.75 according to the Poisson distribution. The data were fitted to the experimental data as described by W.Marwan and D.Oesterhelt (in preparation).

extract. The percentage of complemented cells as a function of sonication time is shown in Figure 2. Permeabilization and complementation balanced with cell damage and disintegration. This was concluded from the microscopic observation of increasing numbers of cells deformed or lacking parts of their cytoplasmic content. Thus, cell disintegration limited the time of sonication, and an optimum at which ~30% of cells responded to light stimuli was found. Controls were carried out by stepwise dilution of the switch factor solution until complementation of the mutant cells was no longer observed. Independent of the fact that the yield of complemented cells was not 100%, the experiment could be used to assay quantitatively the substance responsible for complementation. The mutant cells were found to contain ~10^5 times less switch factor than wild-type cells did (see below).

The light sensitivity of the complemented cells was measured. As shown in Figure 3 by the stimulus–response curves, the sensitivity of complemented cells was about two orders of magnitude less than that of wild-type cells. This is due to the fact that mutant cells were complemented with limiting amounts of switch factor (see below and Discussion).

Quantitative assay for the switch factor concentration

The switch factor concentration could be assayed and quantitated through dose–response curves recorded at light saturation in a standard test where the percentage of complemented cells was plotted against the logarithm of factor concentration. In this assay a mixture of M415 cell suspension and factor solution was sonicated and the cells responding to blue light stimulation were counted. The factor solution was diluted in logarithmic steps until 50% of the maximal effectiveness per unit volume was reached. The inverse of this dilution was defined as the number of activity units present in the undiluted factor solution. By this method the activity of extracts could be determined exactly over several orders of magnitude (Figure 4).

Independent of knowledge on the chemical nature and the concentration of the switch factor, its effectiveness could be analysed by Poisson statistics. The information obtained

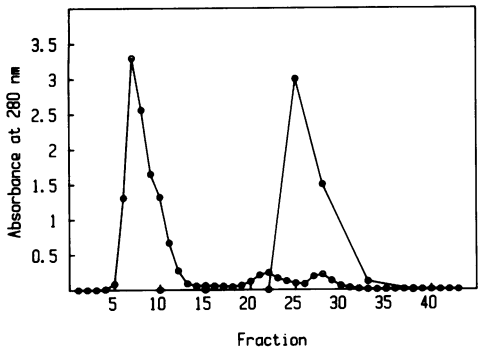

Fig. 5. Gel filtration of the supernatant of heat-treated cell envelope vesicles on a Biogel P-2 column with 3 M ammonium acetate as elution buffer. The absorption at 280 nm (○——○) and the relative activity of the switch factor (●——●) as determined from dose–response curves after appropriate dilution of the samples were measured. The maximum activity shown in the figure corresponds to 3×10^8 units.

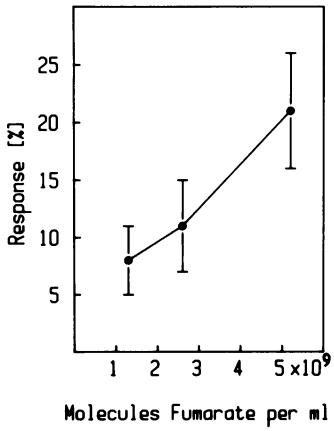

Fig. 6. Complementation of M415 cells with fumarate. Cell density was 10^9/ml and the complementation was carried out as described in Materials and methods.

included the number of molecules of the active substance which at minimum can reconstitute a photophobic response (Hegemann and Marwan, 1988; Marwan *et al.*, 1988). The increase from 10% reacting mutant cells without added factor to 30% reacting cells at saturating factor concentrations was defined as an increase in probability of complementation of 1. A plot of the logarithm of factor concentration (inverse of the dilution) versus this probability (0–1) fitted the theoretical line of $n = 1.75$ in Figure 4, i.e. at minimum, a single molecule of the factor was able to reconstitute the photophobic response. Although this does not mean that every molecule provokes a response, it shows that maximal sensitivity was developed by the cellular system.

The high sensitivity could be confirmed by a different approach. Here, the number of mutant cells that would be complemented by the extract from a given number of wild-type cells was estimated. A ratio of 20 000 complemented mutant cells per wild-type cell was found. At an average cell volume of 1.4 fl (Hartmann *et al.*, 1977) this corresponds to a minimal concentration of the factor of 30 μM in the cell. The apparent discrepancy between a high cellular concentration and a stochastic efficacy is explained by the fact that the factor is bound to the membrane. Membrane binding is concluded from the fact that only negligible factor concentrations are found in membrane

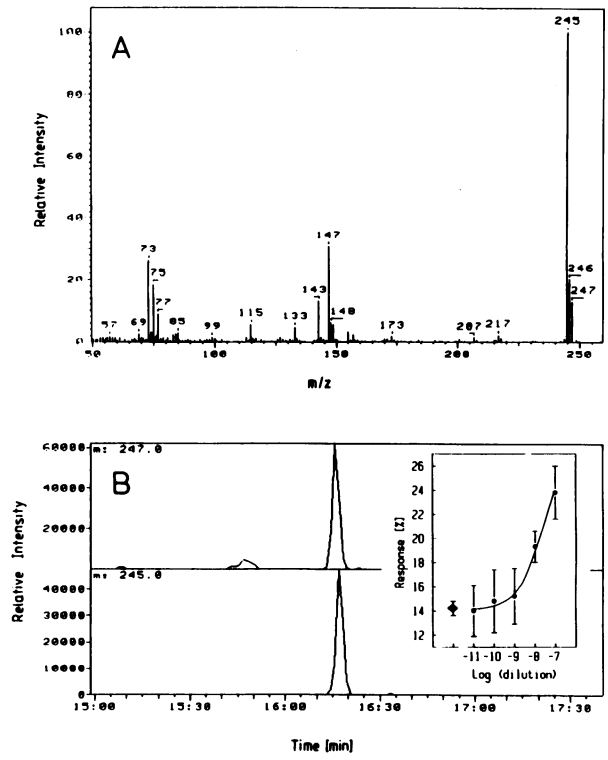

Fig. 7. Quantitative mass spectrometric determination of fumarate in a switch factor preparation (**A**) Mass spectrum of TMS-fumarate; (**B**) intensities of the ions *m/z* 247 (TMS-d_2-fumarate) and *m/z* 245 (TMS-fumarate) measured by GC–MS. The inset in (B) shows the dose–response curve from which the switch factor concentration was determined. The curve was measured by computer-assisted motion analysis. The rhombus indicates the response of the control without added switch factor.

vesicle suspensions prior to heat treatment, which then releases the factor from a presumptive binding protein (see below). This is confirmed by an experiment where at a given and limiting factor concentration and constant volume, increasing numbers of mutant cells were added for complementation. A decrease of reactive cells from 30 to 7% upon increasing the cell concentration (10^8 cells/ml) by a factor of 10 indicated that the factor is collected by the cells as would be predicted by the assumption that binding occurs within the cell.

Purification and characterization of the switch factor and its functional substitution by fumarate

Wild-type cells were homogenized by ultrasonic disruption and the cell envelopes separated by centrifugation from the supernatant, which had no detectable activity. Upon heat treatment the envelope vesicle suspension released the switch factor into the supernatant as indicated by a rise of activity by seven orders of magnitude (not shown). Gel chromatography of the switch factor solution on Biogel-P-2 in 3 M ammonium acetate revealed a mol. wt of the active substance of < 1000 (Figure 5). Cycles of lyophilization of the active fractions and dissolution in water preserved activity and removed salt.

The lyophilized material was subjected to TLC and treated according to chemical group analysis. Material from the main spot seen by iodine vapour stain reacted with $FeCl_3$ and permanganate and was lacking UV absorption above 250 nm. This indicated an unsaturated carboxylic acid. In

Table II. Spontaneous stop responses after complementation of M415 cells with switch factor or fumarate

Sample	Method	Stops	Stops/min	No. of cells observed
Control	visual observation	3	0.02	75
Switch factor complemented	visual observation	10	0.20	25
Control	motion analysis	3	0.04	795
Fumarate complemented	motion analysis	15	0.23	794

M415 cells were complemented with 4.8×10^6 units of switch factor or 50 mM fumarate and their behaviour was analysed by visual observation or computer-assisted motion analysis. Only stops but no reversals were observed in this experiment. Each single cell was observed for periods of 5 s (motion analysis) or 2 min (visual observation) and the reactions per min are listed. Note that the stops observed in the control are extremely short compared to those of complemented cells. In the motion analysis experiment only cells that moved at least 10 μm during the observation period were evaluated. A stop was indicated by the computer when a cell reduced its velocity to 10% of its average velocity for a period of at least 1 s. The data show that the switch factor introduces stop responses with an error probability of $\alpha \ll 1\%$ ($t = 3.16$). Fumarate allows spontaneous stops with an error probability of $\alpha \ll 0.1\%$ ($t = 4.09$).

view of the fact that the unknown substance occurred in the upper micromolar range within the cell we guessed it to be the citric acid cycle intermediate fumaric acid and examined this substance for its ability to replace the factor in the reconstitution assay. As Figure 6 shows, fumaric acid, but not maleic acid, reconstituted the photophobic response, even when only a few molecules per cell were added to the complementation batch.

The ability of fumarate to reconstitute the photophobic response does not necessarily prove that fumarate is the switch factor since fumarate might be converted into switch factor after it entered the cell. Therefore the switch factor preparation was tested for both fumarate with mass spectrometry and the switch factor with the activity assay, to allow a quantitative comparison. Fumaric acid was analysed by the GC-MS-MID technique. The mass spectrum of trimethylsilylated fumaric acid (mol. wt 260) (Figure 7A) shows an intensive fragment at *m/z* 245 ($M^+ - CH_3$) which was used for quantitative measurement using deutero fumaric acid (*m/z* 247) as internal standard. A known amount (100 ng) of deutero fumaric acid was added to the switch factor preparation and the ratio of deuterated (*m/z* 247) and non-deuterated (*m/z* 245) fumaric acid was measured. The gas chromatogram in Figure 7(B) shows the presence of ~115 ng of fumaric acid after isotope correction. This is ~40% of the fumaric acid derivative content expected by the measurement of the switch factor activity (Figure 7B, inset) and is evidence that at least a considerable part of the biological activity was contributed by fumarate. In the same preparation of switch factor, no succinate or malate was found.

Spontaneous stops of mutant cells after complementation with switch factor and fumarate

The straight-swimming mutant M415 has lost completely the ability to execute spontaneous reversals, and in addition the

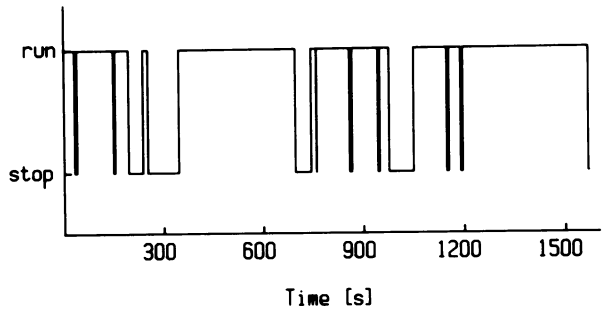

Fig. 8. Swimming pattern of a single cell complemented with fumarate (50 mM). Swimming periods and periods of spontaneous stopping were recorded over 25 min.

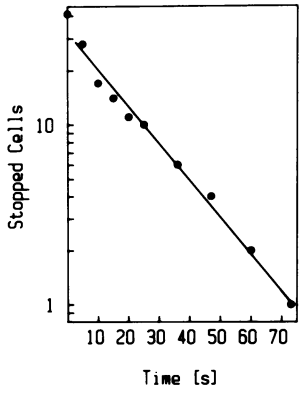

Fig. 9. Semilogarithmic plot of stopped complemented cells of strain M415 as a function of time after a spontaneous stopping at $t = 0$. The stop periods of 42 cells observed in experiments as in Figure 8 were grouped into time intervals of 5 s (up to 25 s) and 10 s (other data points).

huge majority of the cells does not respond to light. In the experiments described so far, low concentrations of switch factor or fumarate were used in complementation experiments to demonstrate effectiveness and to quantitate activity. Under these conditions only light-induced stops and reversals occurred (cf. Figure 1).

Complementation of M415 cells with higher concentrations of switch factor or fumarate allowed the detection of spontaneous stops of the cells as well (Table II). Unlike M415 cells before complementation (including those cells responding to light), high switch factor (or fumarate) concentrations caused durations of spontaneous stops from a few seconds to more than a minute in the same cell. In addition to spontaneous stops, complemented cells also showed occasional spontaneous reversals in the dark but less frequently than in light. A typical pattern of movement of a fumarate-complemented cell executing spontaneous stops is shown in Figure 8. Figure 9 presents the fractions of cells which after a stop did not resume their motion as function of time elapsed after the stop. The exponential nature of the time course suggests that the change from the stop to the running mode was a stochastic event occurring with a constant probability per unit of time (cf. Marwan and Oesterhelt, 1987).

Some of these cells that showed spontaneous stopping after complementation with fumarate were able to reverse the direction of rotation of their flagellar bundle when stimulated with light. In a representative experiment where 24 of these cells were stimulated with light, all of them responded and

five cells even showed reversal of the swimming direction. Hence the decision whether stopping or reversing occurred seemed to depend on the amount of factor released from the membrane (our unpublished results, see Discussion).

An interesting question concerns the problem of whether spontaneous and light-induced switching are mediated by the same compound. The finding that spontaneous stops occurred after complementation with switch factor allows no discrimination of both possibilities since the preparation might be contaminated with a second active substance. However, the observation that fumarate (analytical grade) caused spontaneous stops clearly indicated that both spontaneous and light-induced motor switching depended either on fumarate itself or on compounds originating from fumarate within the cells.

Discussion

Our results demonstrate the existence of a small molecule which allows not only light-induced but also spontaneous motor switching events in a straight-swimming mutant of *H.halobium*. Because of this function we call the substance 'switch factor'. The name and the function do not imply the necessity of direct molecular interaction of the substance with the flagellar motor. The switch factor must, however, be part of that section of the signal transduction chain which lies between the motor and the site of integration of light, of chemical and possibly of other stimuli. Fumarate can functionally replace the switch factor. The mass spectrometric analysis in the presence of deuterated fumarate as internal standard showed that the amount of fumarate and the minimum amount of active molecules present in the extract were in the same range. The lack of quantitative agreement could be either due to low accuracy of factor determination or to the possibility that in addition to fumarate another active substance might be present in our factor preparations. Good candidates would be derivatives of fumaric acid, such as mono(thio)ester, which are metabolically interconvertable with fumaric acid during the time of curing the complemented mutant cells. It should be stressed, however, that on TLC plates the active substance co-migrated with fumaric acid and had the same chemical characteristics as fumaric acid.

The switch factor is released from the halobacterial membranes upon heating. This shows that it is not covalently bound and thus points to an equilibrium between a membrane-bound and soluble state of the switch factor. The effectiveness of the factor was shown to be in the range of one to a few molecules. For principle reasons dose–response curves (e.g. Figure 4) had to be recorded using not a single cell but many cells. Since the effectiveness of the factor at a given concentration in individual cells might be different after complementation, the dose–response curve could show statistical broadening which then gives n-values that are too small. For this reason it might be that not one, but a few molecules are required to provoke a response and, in consequence, the factor concentrations calculated on the basis of dose–response curves are minimum concentrations.

On the basis of single molecule effectiveness a minimal concentration of 2×10^4 switch factor molecules per cell, corresponding to 30 μM, is found. Since the steady-state concentration of fumaric acid in the cytoplasm is only ~50 molecules/cell (our unpublished results), very clearly a binding capacity of at least 2×10^4 molecules must exist in the membrane. The observation that both heat denaturation of membrane vesicle fractions as well as light stimuli of intact cells release the switch factor from the membrane (W.Marwan and D.Oesterhelt, in preparation) immediately suggests the existence of a fumarate (switch factor) specific binding protein in the membrane. The existence of such a protein, called here fumarate (factor) binding protein (FBP), is further corroborated by the observation that mutant cells collect the switch factor during the complementation experiment and that even small amounts of fumarate introduced into mutant cells are not degraded by metabolic reactions. This further suggests that the mutant has mainly unliganded FBP molecules. A sketch of a minimal model fitting our results is shown in Figure 10. It suggests that a release of the factor from the binding protein (FBP) may be caused by receptor activation and followed by several reaction steps before the motor receives the information, finally causing a reversal. In the event that such steps also involve soluble cytoplasmic components, a set of independent, straight-swimming mutants should allow the isolation and identification of these molecules by the same complementation assay described here.

The mutant cells have ~10^5 times less switch factor molecules bound to the membrane than wild-type cells. Assuming Poissonian distributions of molecule numbers, the mutant cells should have none, one, two or a few liganded FBP molecules before complementation. This provides an explanation for the occurrence of the active subpopulation of the mutant cells. It consists of cells with just enough liganded FBP molecules to produce a successful signal. In fact, very high photon exposures are required to cause a reaction in these cells. The same range is found for reactivity of previously non-reactive mutant cells complemented with limiting amounts of switch factor, as shown in Figure 3. Thus, the two parameters, photon exposure producing maximal numbers of photoproduct molecules initiating the signal chain and the number of liganded FBP molecules, which must experience specific molecular interaction to release a switch factor molecule, are decisive for the production of an effective signal which at minimum might consist of a single molecule.

High amounts of switch factor added for complementation cause not only light-induced but also spontaneous events in mutant cells. We explain this by the assumption that increasing fumarate occupancy of FBP introduced by complementation or by metabolism establishes a stationary state between bound and free fumarate subjected to stochastic fluctuations and spontaneous switching events are the

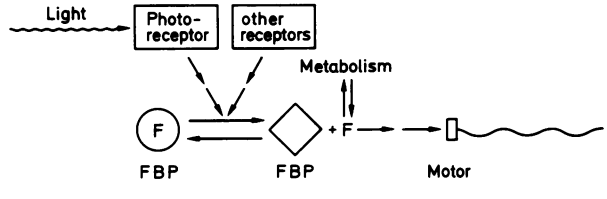

Fig. 10. Sketch of a minimal model of the signal transduction chain. Only elements necessary for a consistent explanation of the results described are shown. Two arrows indicate an unknown number of reaction steps. F = fumarate or switch factor, FBP = fumarate-binding protein.

consequence. At the same time a disorder of fumarate metabolism of the M415 cells must be postulated.

Increasing concentrations of switch factor saturate first the light response, but before causing spontaneous reversals an increasing number of stops are seen, something that was never reported for the wild-type. The cells have polar inserted flagellar bundles consisting of individual right-handed filaments, each very likely driven by an individual flagellar motor (our unpublished results). The filaments associate tightly and rotate in both directions without the phenomenon of bundle dissociation typical for the tumbling mode of *E.coli*. In wild-type cells a mechanical co-operation due to a certain stiffness of the filaments may cause the flip from the clockwise mode of all filaments in the bundle to the counter-clockwise mode and vice versa. At intermediate concentrations of the switch factor artificially introduced into the mutant cells, the co-operative change of all filaments from clockwise to counter-clockwise does not occur. If part of the flagellar filaments are rotating clockwise and the other part counter-clockwise a dissociation of the flagellar bundle would be the consequence and might be visualized by dark-field microscopy. The alternative possibility that stopping of the flagellar rotation occurs during the transition between one rotational sense to another in wild-type cells and that this stopping period is extended in complemented cells, cannot yet be ruled out. In this context it should be noted that stopping of individual flagellar motors has been described in *E.coli* (Lapidus *et al.*, 1988).

The potential of complementation analysis and identification of fumarate as an integral part of the halobacterial signal transduction chain will promote the elucidation of the molecular events in signalling, specifically in that part which concerns the transduction of chemical information into a mechanical response. We have shown that fumarate plays an essential role in signal transduction of *Halobacterium*. Future experiments will reveal whether fumarate is involved in eukaryotic or eubacterial signal chains as well.

Materials and methods

Bacterial strains and culture conditions

Halobacterium halobium cells of strains M407 (*SWI*$^+$ *SOPI*$^+$ *SOPII*$^+$, *BOP*$^-$ *HOP*$^+$, selected by M.Alam as highly motile cell type; see also Marwan and Oesterhelt, 1987), M415 (*SWI*$^-$ *SOPI*$^+$ *SOPII*$^+$, *BOP*$^-$ *HOP*$^+$ derived from M407, see below) and Flx37 (*SWI*$^+$ *SOPI*$^+$ *SOPII*$^+$ *BOP*$^-$ *HO*-deficient; Spudich and Spudich, 1982) were grown under standard conditions in the dark (Oesterhelt and Krippahl, 1983). Cultures were inoculated with 1.5% of a 5 day old stationary culture and grown in 2 l Erlenmeyer flasks (700 ml medium) for isolation of the switch factor or in 100 ml flasks (35 ml medium) for the reconstitution assay.

Preparation of tethered cells

Cells with sheared flagellar bundles were tethered to siliconized coverslips according to Manson *et al.* (1980). For shearing the flagellar bundles, glass beads (3 g, 125–200 μm, Serva, Heidelberg, FRG) were added to 4 ml of a 2 day old cell culture and shaken on a vortex mixer for 2 min. The cells were immotile after this treatment when checked under the microscope indicating that the cells had lost their flagellar filaments. The sheared flagella were separated from the cells by centrifugation in an Eppendorf cap (6000 r.p.m., 3 min), the cells washed twice with basal salt solution (BS, standard growth medium without peptone) and resuspended in an equal volume of BS. The suspension was added to the edge of a siliconized coverslip mounted on a microscopic slide with shreds of a coverslip as spacer. The specimen was inverted and stored in a humid chamber for 30 min at room temperature. Cells not adsorbed to the cover slip were then removed by sucking 0.15 ml growth medium through the space between slide and cover slip. After sealing the edges with a paraffin:vaseline mixture (2:1) and further incubation for 30 min at 40°C the motion of the tethered cells was analysed under the microscope.

Isolation of a switch mutant

Cells of strain M407 were mutagenized by treatment with 1-methyl-3-nitro-1-nitrosoguanidine according to the method described by Oesterhelt and Krippahl (1983), washed and resuspended in basal salt solution to a final density of 10^9 cells/ml. Sterile glass capillaries, sealed at one end and filled with growth medium, were immersed with the open end into the bacterial suspension. The capillaries were irradiated at intervals (1 s light, 5 s dark) for 1 h at room temperature with blue light from a 150 W halogen lamp filtered through a BG3 filter (Schott, Mainz, FRG). Capillaries were cut in the middle and the suspension from the distal end was plated on agar. After incubation at 40°C for 10 days, material from single colonies was cultured in liquid medium (35 ml) for 2 days and then cells screened for their response to light. Cells not responding are called PHO-negative phenotypes. Cells swimming continuously straight without ever stopping or reversing are called SWI (switch)-negative phenotype.

Purification of the switch factor

Cells from 8.4 l of a 60 h culture were harvested by centrifugation (9000 *g*, 20 min, 10°C), washed with 2.1 l washing buffer (4.3 M NaCl, 10 mM Tris pH 7.4), resuspended in 40 ml disruption buffer (1 M KC1, 25 mM potassium phosphate pH 7.5) and disrupted by ultrasonic treatment for 5 min on ice (Branson cell disruptor B15, USA). After addition of 80 ml ice-cold KT buffer (3.5 M KCl, 10 mM Tris pH 7.4), debris and unbroken cells were removed by centrifugation (10 000 *g*, 10 min, 10°C) and the membrane vesicles in the supernatant pelleted by ultracentrifugation at 200 000 *g* for 1 h at 10°C. The vesicles were resuspended in 4 ml washing buffer, aliquoted and stored at −28°C after shock freezing in liquid nitrogen.

Aliquots of 0.5 ml vesicle suspension were mixed with an equal volume of half-concentrated washing buffer in an Eppendorf cap and kept in a water-bath at 100°C for 1 min. After cooling on ice and centrifugation at 64 000 *g* for 15 min (Airfuge, Beckman, USA) the supernatant was applied to a Biogel P-2 column (Biorad, USA) which was equilibrated with 3 M ammonium acetate. Fractions of 1.5 ml were collected and tested for UV absorption (280 nm) and activity in the reconstitution assay (see below). The three consecutive fractions which showed maximal activity were pooled and lyophilized.

Complementation assay

A culture of M415 cells was grown for 3 days at 40°C and incubated overnight at room temperature without shaking. Aliquots of 1 ml cell suspension were pelleted in an Eppendorf centrifuge for 3 min at 6000 r.p.m. at room temperature, gently resuspended in 0.5 ml complementation buffer (4.3 M NaCl, 10 mM Tris pH 7.4, 1 mM EDTA) and incubated for at least 30 min at room temperature. For complementation, 50 μl of this cell suspension and 50 μl of the test solution diluted with KT buffer were mixed in a small test tube (35 × 6 mm, no. 2775/1, Assistent, Sondheim, Rhön, FRG) and exposed to a weak ultrasound treatment for 5 min (Eltrosonic Contact Cleaner, power output 5 W, Eltrosonic, Wiesbaden, FRG). The suspension was then diluted with 0.5 ml standard medium and incubated for at least 20 min at room temperature before complementation for photophobic responses was tested within the next hour. When the complementation rate was to be assayed by computer-assisted motion analysis, cells were resuspended in 250 μl complementation buffer which then contained 2 mM EDTA. After sonication 200 μl instead of 500 μl standard medium were added.

Quantitation of photophobic response

For estimation of the percentage of complemented cells, 5 μl of the cell suspension were put onto a slide and covered with a slip which were both pre-cleaned with acetone, rinsed twice with bidistilled water and air dried. Before measurement, the specimen was incubated for 5 min on a thermostatted stage (40°C) and then measured at the same temperature. For the microscopical set-up see Marwan and Oesterhelt (1987). Cells were observed in constant orange light (120 W/m^2), produced from a 100 W halogen lamp with an OG570 filter (3 mm) (Schott, FRG). Stimulation was with a saturating blue light pulse of 2 s duration (11 mmol m^{-2} s^{-2}, see Discussion). The stimulating light came from a 100 W mercury lamp (HBO 100 W, Osram, FRG) and was filtered through a BG3 filter (1 mm). Out of each complementation batch, 30 cells were stimulated and the number of cells showing a photophobic response was counted. Results are expressed as the percentage of reacting cells. If not indicated otherwise, each cell was stimulated only once. Statistical treatment of the data was carried out according to Weber (1986). Error bars indicate the standard error of the mean.

Computer-assisted motion analysis

The data acquisition and evaluation by computer-assisted motion analysis was carried out as described (W.Marwan and D.Oesterhelt, in preparation). Briefly, the motion of swimming cells was recorded at a frame rate of 10 Hz. The time-lapse positions of individual cells were calculated as centroids shown by circles in Figure 1. Tracks of cells were then evaluated for deviation from a straight line by determining the angle between the position at the actual time and positions 400 ms before and after this time. The value of the cosine of this angle was taken as a measure for deciding whether stops or reversals (spontaneously or stimulated) occurred. Cos $\beta = 0.8$ ($\beta = 37°$) was chosen as a deciding value. Only cells which moved with an average speed of at least 2.6 μm/s were used for analysis. This automatic procedure was checked by visual inspection of a statistically relevant number of individual tracks.

Mass spectrometry

For mass spectrometric analysis, the supernatant obtained after heat treatment of vesicles was filtered through a Centricon 10 filter to remove all components with a mol. wt >10 kd. The minimal switch factor concentration in the filtrate was estimated by the complementation assay. To an aliquot of this solution which contained at least 290 ng switch factor (calculated for the mol. wt of fumaric acid), 100 ng of deutero fumaric acid (MSD-Isotopes, Merck Inc. Montreal, Canada) was added. The mixture was acidified with 1 N HCl, extracted with ether and the organic phase was evaporated to dryness. Trimethylsilylation was carried out by reaction with bis-trimethylsilyltrifluoroacetamide (BSTFA, Pierce, USA) for 15 min at 120°C. The samples were analysed by the GC-MS-MID technique. A HR-GC 5160 gas chromatograph (Carlo Erba, Milan, Italy) was coupled with a Varian-Finnigan mass spectrometer datasystem CH7A-SS300. Gas-chromatographic separation was achieved on a fused silica capillary (Durabond 1, 20 m × 0.32 mm, J & W Scientific, Cordova, CA, USA) using helium (2 ml/min) as the carrier gas. A temperature program with 3 min at 70°C, followed by a gradient of 5°C/min was applied.

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