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Protonmotive Force and Bacterial Sensing

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The role of the proton gradient and external pH in the motility and chemotaxis of *Bacillus subtilis* was investigated. Presence of a substantial proton gradient is not necessary for motility or chemotaxis, as long as the electrical potential is sufficient to maintain motility. Changes in the proton gradient do, however, lead to changes in swimming behavior, and these changes are mediated by two processes. One is sensitive to external pH and probably operates through a pH receptor. The second is sensitive to changes in the proton gradient. When the level of the protonmotive force is high enough to maintain motility, changes in the components of the protonmotive force are sensed by the bacteria and lead to behavioral changes, but changes in the protonmotive force are not necessary for chemotaxis.

An electrochemical proton gradient formed across the bacterial cytoplasmic membrane appears to be an energy source for many membrane-associated processes. As conceived by Mitchell (23), this protonmotive force (Δp) consists of two components, the membrane electrical potential ($\Delta\psi$) and the transmembrane proton gradient (ΔpH). Abundant evidence has accumulated to show that $\Delta\psi$ and ΔpH exist in bacteria, as well as in mitochondria and chloroplasts, and that Δp can be used as an energy source for oxidative phosphorylation, active transport, and motility (9). A major question regarding Δp and motility has been whether changes in Δp are required for chemotaxis.

The motility of chemotactic bacteria such as *Escherichia coli*, *Salmonella typhimurium*, and *Bacillus subtilis* normally consists of periods of swimming in approximately straight lines (smooth swimming) interrupted by abrupt random changes of direction (tumbling). This random pattern of swimming is, however, altered when the bacteria sense attractant or repellent gradients. When the bacteria move in a favorable direction (i.e., up an attractant gradient or down a repellent gradient), tumbling is suppressed, and they thus show a net movement toward the favorable environment (3, 16). A temporal comparison mechanism, indicating the presence of a rudimentary bacterial memory, is involved in regulation of the swimming behavior (16). These observations have been rationalized in terms of a parameter called the tumble regulator, which determines whether the bacteria tumble or swim and is sensitive to the presence of environmental stimuli (12, 13, 16).

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The energized membrane state influences both the motility and the behavior of the bacteria. Larsen et al. (14) demonstrated that bacterial motility depends on Δp and not ATP concentration. This result was extended by Manson et al. (18), who showed that a *Streptococcus* strain depleted of ATP would regain motility if either a $\Delta\psi$ or a ΔpH was artificially imposed with ionophores. Matsuura et al. (19) also showed that *B. subtilis* motility could be driven by an artificial Δp. In addition to the dependence of motility on the presence of Δp, membrane-active drugs affect swimming behavior (5, 8), as do uncouplers and inhibitors of oxidative phosphorylation (26). Other treatments which were assumed to affect Δp were also shown to affect the swimming behavior of *B. subtilis* (6, 7, 32). Szmelcman and Adler (36) used the transmembrane distribution of a lipophilic cation as a probe for $\Delta\psi$ and showed that changes in the distribution occurred when *E. coli* was subjected to gradients of attractants and repellents. Using cyanine dye fluorescence as an indicator of $\Delta\psi$, Miller and Koshland (20) showed that (i) changes in membrane potential were sensed by bacteria and altered behavior, but (ii) changes in behavior could occur without a change in membrane potential.

The role of external pH, as well as ΔpH, in swimming behavior has also been examined. Motility and chemotaxis were normal in the external pH range of 6 to 8 for *E. coli* (1, 2) and *B. subtilis* (25, 32). *E. coli* cells also migrated to neutral pH (39), and rapid changes in external pH affected the behavior of *B. subtilis* (7, 27) and were interpreted to mean that the "high energy intermediate of oxidative phosphorylation" is not the tumble regulator (27).

In addition, deJong and van der Drift dem-

onstrated that swimming behavior is affected by nigericin, an ionophore that selectively alters ΔpH (6).

These results did not, however, fully determine the separate roles of external pH and ΔpH in swimming behavior or the relationship between $\Delta\psi$ and ΔpH. Therefore, further experiments on the role of external pH and ΔpH in the regulation of swimming behavior were initiated. In the experiments described below, the roles of ΔpH, $\Delta\psi$, and external pH in swimming behavior regulation were investigated by altering the levels of these parameters in a number of ways and observing the effects on swimming behavior and cyanine dye fluorescence. The results provide a complex but self-consistent picture of the interrelationship among these variables and their effects on bacterial behavior.

MATERIALS AND METHODS

Bacteria and growth conditions. *B. subtilis* strain W168 (15) has no specific growth requirements and is normally chemotactic. More than 90% of the bacteria from a liquid culture are motile, and they show the normal random pattern of swimming (in the absence of a stimulus gradient), which consists of alternating periods of smooth swimming and tumbling. The bacteria were grown at 30°C from a spore stock in a tryptone phosphate medium containing added calcium, magnesium, and maganese chloride (4). For chemotaxis experiments, the bacteria were harvested from the growth medium in midlog phase (optical density at 590 nm, 0.3 to 0.8), washed three times, and resuspended in a modification of the chemotaxis medium of Ordal and Goldman (26), which contains glycerol, lactate, 10 mM potassium phosphate, 140 μM $CaCl_2$, 100 μM $MgCl_2$, and 100 μM $MnCl_2$ at pH 7.

Microscopy and demonstration of chemotaxis. The bacteria were observed with a Leitz dark-field microscope and tungsten illumination system as described previously (37). Chemotactic responses were observed with a variation of the temporal gradient assay of Macnab and Koshland (16). The bacteria were placed in a test tube, rapidly mixed with the agent to be tested, and then placed on a microscope slide without a cover slip. Observation began 5 to 10 s after the mixing. The times reported for smooth swimming or tumbling are the times at which 50% of the bacteria were judged to have returned to the normal random swimming pattern after the stimulus. Unstimulated tumbling frequency was measured by a videotape technique (21). These measurements were made at a low concentration of bacteria (optical density at 590 nm, 0.01; 7×10^5 bacteria per ml), which obviated problems due to the change of medium pH by metabolic processes or oxygen depletion which occur when higher concentrations of bacteria are used.

Fluorescence. Fluorescence of the cyanine dye diS-C_3-(5) (for structure and nomenclature, see Sims et al [33]) was measured on a Spex Fluorolog instrument as described previously (20) with excitation and emission wavelengths of 622 and 670 nm, respectively, and a slit band pass of 10 nm. Agents to be tested were added from ethanolic stock solutions to a bacterial suspension in a fluorimeter cuvette and rapidly mixed by a specially constructed stirring device (J. B. Miller, Ph.D. thesis, University of California, Berkeley, 1977). Observations were begun immediately upon addition. The dye was used at a concentration of 167 mM, and the bacteria were suspended at an optical density at 590 nm of 0.3 (about 2×10^8 bacteria per ml). Presence of the dye at this dye/bacteria concentration ratio does not affect the motility of the bacteria, although higher ratios do (22).

Chemicals. Nigericin (lot 189-380B-171-A) and A23187 (lot 361-X17-226) were obtained from R. J. Hosley and R. Hamill, respectively, of Eli Lilly & Co. The cyanine dye was a gift from A. S. Waggoner, Amherst College. These chemicals were kept as 1 mM stock solutions in 95% ethanol.

RESULTS

Effects of external pH on dye fluorescence and bacterial swimming behavior. The membrane potential, as recorded by the fluorescence of the cyanine dye diS-C_3-(5), was affected by the external pH of the medium in which the bacteria were suspended. The fluorescence was lowest at pH 9 (Fig. 1). As the pH was lowered from 9 to about 5, there was a steady increase, but at about pH 5 there was an abrupt two- to threefold increase in the fluorescence. The fluorescence also increased when the pH was raised from 9 to 10. Since dye fluorescence

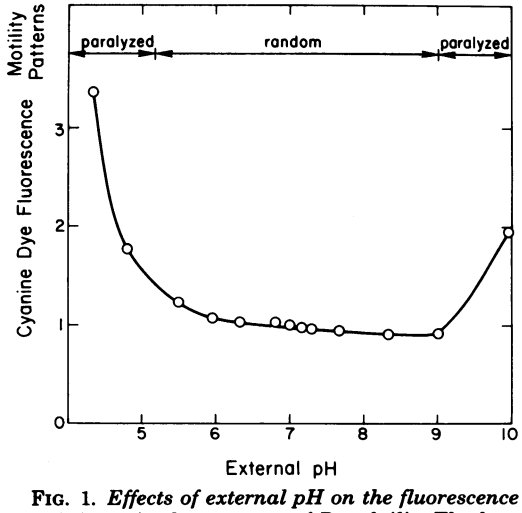

FIG. 1. *Effects of external pH on the fluorescence of diS-C_3-(5) in the presence of B. subtilis. The bacteria were prepared in chemotaxis buffer, and fluorescence was determined as described in the text. The pH of the medium was altered by adding suitable amounts of HCl or NaOH. The fluorescence intensity was measured after 5 min of equilibration at the experimental pH. Fluorescence intensity in this and subsequent figures is given in arbitrary units, where the base-line fluorescence in chemotaxis buffer at pH 7 is given the value 1.*

is inversely proportional to $\Delta\psi$, increased fluorescence indicates depolarization (20, 40). The dye fluorescence began to change immediately upon addition of acid or base (Fig. 2). The time dependence of the fluorescence change was, however, different depending on whether acid or base was added.

The swimming behavior of the bacteria was also dependent on the external pH of the medium in which they were suspended. The bacteria maintained normal motility and a normal tumbling frequency within the pH range 6 to 7.5, but outside this range they became progressively less motile. The smooth swimming response of the bacteria generated by a temporal gradient of 0 to 1 mM L-alanine occurred at any pH within this range. The response lasted for approximately 1 min at pH 6, 7, or 7.5.

Although the tumbling frequency remained normal (about 8 to 10 tumbles per min for this strain) at different constant external pH values, rapid changes in the external pH did lead to transient changes in tumbling frequency. When the pH was abruptly changed from 7.5 to 7 or from 6 to 7, the bacteria responded with a period of smooth swimming which lasted for up to 30 s. If the pH was abruptly changed from 7 to 6 or 7 to 7.5, the bacteria responded with 10 to 20 s of constant tumbling, followed by a return to the normal random swimming pattern.

The change in behavior when the pH was changed from 7 to 6 was variable. Generally, only 10 to 40% of the bacteria showed the constant tumbling, whereas the remainder showed either random behavior or even smooth swimming. In addition, the bacteria were occasionally paralyzed for 1 to 5 s after the pH change, but then recovered motility and went through the pattern of behavioral changes appropriate to the pH change made. These changes in swimming behavior when the pH was changed agree with those reported by Ordal and Goldman (27), but differ from those reported by deJong et al. (7).

Effects of nigericin on dye fluorescence and bacterial swimming behavior. Nigericin is an ionophore which, at low concentration, mediates an electroneutral exchange of protons and potassium (28), and the addition of this ionophore to the bacteria in the presence of diS-C_3-(5) affected the dye fluorescence under certain conditions (Fig. 3). When nigericin was added to the bacteria in chemotaxis buffer at pH 7.5, there was no change in the dye fluorescence. If the bacteria were in chemotaxis buffer with the pH adjusted to 6, however, addition of nigericin was followed by a biphasic change in the dye fluorescence. The fluorescence initially decreased (indicating membrane hyperpolarization) 10 to 15% over 1 min, but this decrease was followed by a rapid increase in the fluorescence (indicating membrane depolarization) that plateaued at a level 1.5 to 2 times higher than the original baseline. This change in fluorescence was not altered when 1 mM L-alanine was added simultaneously with the nigericin.

The addition of nigericin also affected the

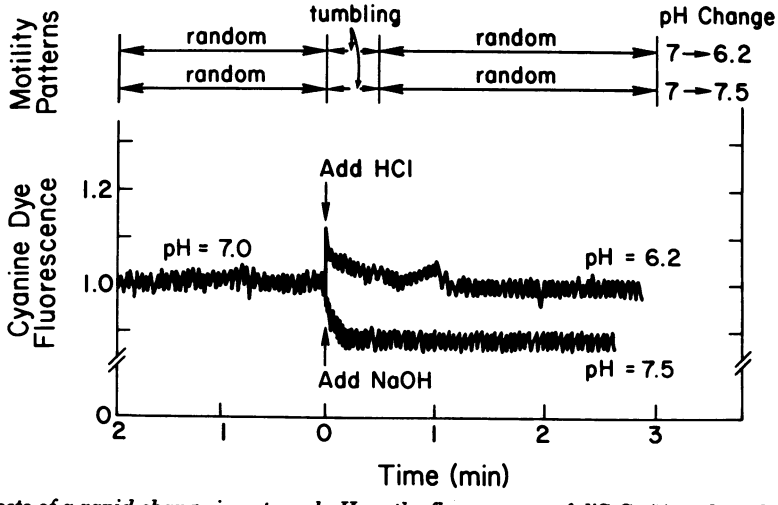

FIG. 2. *Effects of a rapid change in external pH on the fluorescence of diS-C_3-(5) and on the motility of B. subtilis. The bacteria were prepared in chemotaxis buffer and fluorescence and motility patterns were measured as described in the text. The timing of the behavioral responses is the average of 5 to 10 repetitions and is accurate to within 10 s. The bacteria were stirred at pH 7 in the presence of the dye for 5 min, and at the time indicated by the arrows 0.1 N HCl or 0.1 N NaOH was added to change the external pH to the values indicated. Fluorescence units are arbitrary as described in the legend to Fig. 1.*

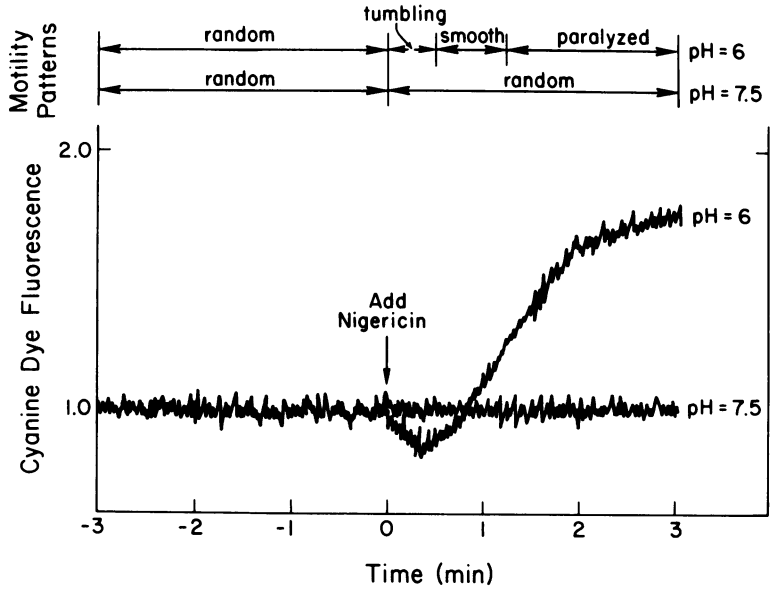

FIG. 3. *Effects of nigericin addition on fluorescence of diS-C$_3$-(5) and on motility of B. subtilis in media of different external pH's. The bacteria were prepared in chemotaxis buffer of the indicated pH (pH adjusted with either HCl or NaOH) and were stirred with the cyanine dye for several minutes. At the time indicated by the arrow, 0.1 μM nigericin was added to the bacteria, and the fluorescence and motility patterns were determined as described in the text. In some experiments, 1 mM L-alanine was added simultaneously with nigericin, but the fluorescence changes were no different than when nigericin alone was added. Fluorescence units are arbitrary as described in the legend to Fig. 1.*

swimming behavior of the bacteria under some conditions but not under others. When the bacteria were suspended at pH 6, the addition of nigericin was followed by a brief period of constant tumbling behavior which lasted for about 30 s. The tumbling was followed by a 10- to 20-s period of smooth swimming, which was in turn followed by complete paralysis. At pH 7.5, however, addition of nigericin led to no change in the bacterial swimming behavior. Simultaneous addition of the strong attractant alanine along with nigericin also led to different behavioral changes at the different pH's. At pH 6.2, the simultaneous addition of the strong attractant 1 mM alanine and nigericin did not alter the tumbling to smooth to paralysis behavioral response seen when nigericin alone was added. At pH 7.5, however, simultaneous addition of alanine and nigericin led to the 1 min of smooth swimming behavior seen when alanine alone was added.

DISCUSSION

Relationship of the ΔpH to motility and chemotaxis. These results, combined with those from other laboratories (6, 16, 27, 39), show that the presence of a ΔpH across the bacterial membrane is not necessary for motility provided the overall Δψ is sufficient to maintain motility. Assuming that *B. subtilis* is similar to *E. coli*, then at alkaline pH the ΔpH is too small to measure (11, 29, 30), and only the electrical potential remains. The bacteria, however, remain motile and have a normal tumbling frequency. Also supporting the conclusion that motility does not require a ΔpH is the finding that nigericin does not affect the motility of the bacteria when added at pH 7.5. Nigericin mediates an electroneutral exchange of protons for potassium, and thus it selectively affects ΔpH while leaving Δψ intact (28, 29). Thus, when nigericin is added to the bacteria at pH 7.5 where there is little or contribution of ΔpH to the total Δp, there is no change in the overall energy available for motility, and the bacteria remain motile. These results complement those of Manson et al. (18) and Matsuura et al. (19), who showed that bacteria can use an artificially induced Δψ or ΔpH for motility.

A proton gradient is also not necessary for chemotaxis. This follows first from the observation made in many laboratories that chemotaxis proceeds normally at pH 7.5 (1, 7, 12, 16, 27). Since there is no pH gradient at this pH, chemotaxis cannot require it. This point was reemphasized by adding nigericin as described above and showing a chemotactic response. Furthermore, because the bacteria have a normal tum-

bling frequency and unaltered response to attractants at different pH's or in the presence of nigericin at pH 7.5, the absolute level of either the pH or the ΔpH cannot be the tumble regulator. A complication of the results with nigericin is due to the potassium gradient (300 mM inside [9] versus 10 mM outside) found with these bacteria. In the presence of nigericin, the potassium gradient would apparently tend to drive protons into the cells, thus setting up an acid inside ΔpH, which is the reverse of that found physiologically. It is impossible to predict the magnitude of such an effect, but the internal buffering capacity of the cell, the presence of active potassium and proton pumps, the low concentration of nigericin used in these experiments, and the possibility that the potassium gradient is in equilibrium with the $\Delta\psi$ argue that the effect would be small. At any rate, even if such an acid inside ΔpH were set up due to nigericin addition at pH 7.5, the fact is that the bacteria remain normally motile and chemotactic, thus supporting the conclusion that these processes are independent of ΔpH.

Although ΔpH is not required for chemotaxis, treatments which alter ΔpH lead to changes in the swimming behavior of the bacteria. In general, when the external pH is changed away from neutrality (i.e., from pH 7 to 7.5 or from pH 7 to 6), the bacteria respond with a period of constant tumbling. On the other hand, when the external pH is changed toward neutrality (e.g., from pH 6.2 to 7 or from pH 7.5 to 7), the bacteria respond with a definite, but short, period of smooth swimming. These results indicate that the bacteria show positive chemotaxis to neutral pH, which is in agreement with results which show chemotaxis to neutral pH in capillary tests (39).

Another pH treatment which affects swimming behavior is the rapid addition of nigericin at pH 6.2. At this pH, the ΔpH is expected to be a substantial portion of the total Δp and the addition of nigericin would be expected to abolish ΔpH. Nigericin addition at pH 6.2 is immediately followed by a period of constant tumbling behavior. At pH 7.5, however, addition of the same concentration of nigericin does not lead to swimming behavior changes, and a change in ΔpH would also not be expected because ΔpH is negligible at alkaline pH. Thus, nigericin addition leads to behavioral changes only when it affects ΔpH. A more extensive series of experiments by deJong and van der Drift (6) with nigericin includes similar results.

Changes in external pH and the addition of nigericin thus affect tumbling frequency by two separate processes. In the first instance, the bacteria show "pH receptor taxis" in which pH 7 is the preferred external pH. Changes toward pH 7 cause smooth swimming, and changes away from pH 7 cause tumbling. This behavior would not be expected if the bacteria were responding to a change in ΔpH, because changes in pH from 7 to 7.5 or from 6 to 7 both decrease ΔpH, yet lead to different behavioral responses. Thus, it appears most likely that the bacteria have a pH receptor (or receptors) similar to receptors for other chemoeffectors. There is, however, also a behavioral response to a change in the pH gradient across the membrane, because nigericin affects swimming behavior only when it causes a significant change in ΔpH.

Response to nigericin. The behavioral responses and changes in the Δp of *B. subtilis* after nigericin addition are complex (6). The bacteria first show a period of constant tumbling, which is followed by a period of smooth swimming, and finally they are completely paralyzed. During these behavior changes, there is an initial increase in the membrane potential (decrease in cyanine dye fluorescence, which was not noted by deJong and van der Drift [6]), followed by a large depolarization (increase in fluorescence).

The apparent complexity of the response to nigericin can be explained by considering the nature of nigericin and the components of the Δp. The hypothesis proceeds as follows. At pH 6.2, the Δp has a large component due to the ΔpH and a substantial, but smaller, component due to the $\Delta\psi$ (25, 28). Upon addition of nigericin, the large component due to ΔpH is rapidly dissipated, thus lowering the total Δp. Constant tumbling results from this loss of available energy, as predicted by previous work (20, 26). During this period there is probably a brief period of increased membrane potential (indicated by the dye fluorescence), followed by a steady decrease in membrane potential. Smooth swimming can be caused by hyperpolarization, but the initial period probably coincides too closely with the change in the pH gradient, which predominates and causes tumbling. As the total Δp later falls, it causes a smooth swimming period. It has been shown that a decrease in the Δp increases the probability of counterclockwise rotation, and thus the bacteria become essentially all smooth swimming at the low values of Δp (S. Khan and R. M. Macnab, personal communication).

The final stage of the response to nigericin is paralysis, and this paralysis is due to the large depolarization indicated by the final increase in cyanine dye fluorescence. The depolarization may be due to an inability of the bacteria to compensate fully for the large decrease in the level of the Δp because of the dissipation of ΔpH,

or the final depolarization may be due to the uncoupling effects of nigericin which are seen under some conditions (28). In addition, acidification of the cytoplasm in the presence of nigericin at pH 6.2 might be significant and deleterious.

Additivity of behavioral responses to ionophores and attractants. When two treatments which alter the swimming behavior of bacteria are performed at the same time, the resulting behavioral change is often an additive combination of the separate responses (31, 34, 38). For instance, addition of an attractant can convert the tumbling response to a repellent into a smooth response of shorter duration than the response found when attractant alone was added. Similarly, addition of two attractants can lead to a response that is longer than that due to either attractant alone. The response to blue light can also be reversed when attractants are added simultaneously (17). Results such as these imply that the different treatments can be integrated by the same sensing machinery.

Such additivity has also been found for attractants and certain ionophores. Attractant responses add with the responses seen when carbonyl cyanide-*m*-chlorophenylhydrazone (24) or valinomycin (7) is added. Such simultaneous addition of ionophores and attractants does not affect the change in electrical potential due to ionophore addition (20).

In contrast to this often seen additivity of responses, however, there are some stimuli which are not additive. For instance, ribose and galactose are both attractants, but the presence of one lowers the response to the other (35). Also, other classes of attractants and repellents are not strictly additive (37). These results imply a more complex pattern for the tumble frequency regulator and also imply that a further signal from such a component then interacts with the tumble frequency regulator.

An interesting case of non-additivity is reported in this paper, namely the inability of the attractant alanine to overcome the constant tumbling behavior which follows addition of nigericin at pH 6.2. Apparently, nigericin affects the tumble frequency regulator differently than attractants and other ionophores. Nigericin must somehow uncouple the tumble frequency regulator from inputs due to attractant gradients. Either nigericin produces a very large signal to the tumble frequency regulator which overwhelms signals from attractants, or nigericin specifically uncouples the tumble regulator from attractant inputs. Lately, we have found an additional case of non-additivity with attractants and the divalent cation ionophore A23187 (Miller and Koshland, unpublished data).

Relationship of ΔpH-Δψ to the tumbling frequency regulator. The results of this work taken with those reported earlier lead to the conclusion that changes in ΔpH or Δψ are sufficient but not necessary for changes in the swimming behavior of chemotactic bacteria. In addition, the absolute levels of ΔpH and Δψ do not determine the tumbling frequency. Furthermore, these results, along with those of others (16, 19, 32), show that neither component of the Δp is absolutely required for either motility or chemotaxis, although sufficient energy must be available from one of the components to turn the flagella. The level of the electrochemical proton gradient is, therefore, not the tumbling frequency regulator which controls the behavior of bacteria and their chemotactic responses.

The Δp is required for bacterial survival in all but the most unusual environments (10). The Δp is used to drive reactions which maintain the internal ionic composition of the bacteria within ranges that allow cell growth and division and to accumulate nutrient molecules against concentration gradients. Finally, the Δp is used to maintain the motility of the bacteria, which allows them to move in space and, by chemotaxis, to accumulate in appropriate areas. It is perhaps not surprising then that major changes in the Δp are not used to regulate swimming behavior in response to attractants. Changes in the level of the electrochemical proton gradient, however, can be an important signal of a changing environment, and the bacterial sensory system can respond to such changes with an appropriate response. Thus, changes in the Δp and Δψ or ΔpH are detected like chemoeffector levels, temperature (21), or light (17). They can transmit a signal to the molecular components regulating swimming behavior and thus alter the tumbling frequency.

This work also suggests certain special features of the membrane potential. The lack of additivity in certain cases suggests a bypass mechanism for membrane potential, which might indicate that some proteins in the signalling system are gated and do not respond or overrespond at certain potential values.

Finally, the two responses to external pH and ΔpH appear to have survival value for the bacteria. First, the bacteria must maintain their internal pH within certain limits. If the external pH is significantly different than the internal pH, then a large amount of energy would be required to maintain the proper internal pH. Because the bacteria can move toward neutral pH, this problem is minimized. Second, the response of the bacteria to changes in ΔpH in the

absence of an external pH change is also significant because it allows the bacteria to respond to potentially deleterious environmental conditions which affect the energized membrane state.

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