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Control of Tumbling in Bacterial Chemotaxis by Divalent Cation

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Chemotaxis is migration of organisms to higher concentrations of attractant or lower concentrations of repellent. Understanding the switch that controls whether the flagella rotate counterclockwise for swimming or clockwise for tumbling (thrashing about without making much forward progress) is central to understanding chemotaxis of peritrichous bacteria, since chemotaxis results from selective suppression of tumbles. Depletion of divalent cation by chelating agents in the presence of A23187, an ionophore that conveys divalent cation across membrane, causes incessant tumbling in *Bacillus subtilis*. Small additions of $MgCl_2$ prevent this tumbling. In this tumbling condition, the bacteria, which normally swim extensively when given attractant, do not respond even to 10 mM alanine, a strong attractant. $MnCl_2$, by contrast to other divalent cations, increases tumbling in the absence of A23187, and the effect is potentiated by the ionophore. Permeant cations, including tetraphenylarsonium ion and triphenylmethylphosphonium ion, cause permanent swimming, even in the presence of A23187 and chelating agents. We propose that divalent cation, probably Mg^{2+} ion, binds to the switch to cause swimming and that, in the absence of divalent cation at the switch, the bacterium tumbles.

Peritrichously flagellated bacteria alternately swim smoothly and tumble (thrash about without making much forward progress) (5). To swim (or tumble) the flagella rotate counterclockwise (or clockwise) (11). By suppressing tumbles when headed toward higher concentrations of attractant (5), these bacteria "bias" an otherwise "random walk" and thus accumulate in regions of high concentration of attractant, a process called chemotaxis. Logically, there must exist a switch that governs direction of rotation of the flagella. How this switch is controlled and how the control is affected by attractant binding to chemoreceptors are not understood.

Recently, Ordal and Goldman (15, 16) obtained evidence that decrease of $\sim$, the high energy state of the membrane interpreted as electrochemical gradient of H^+ ion (chemiosmotic hypothesis), causes tumbling and increase of $\sim$ causes swimming in *Bacillus subtilis*. However, since all effects on behavior are transitory, but on physiology are permanent (e.g., trifluoromethoxy-carbonylcyanidephenylhydrazone [FCCP], an uncoupler of oxidative phosphorylation and a repellent, gives sustained increase in respiration) (15), the level of $\sim$ cannot directly control this switch. Similarly, when *Paramecium* hits a barrier, its membrane depolarizes due to influx of Ca^{2+} ion, and the cilia change direction so that the protozoan backs up. Soon afterward, K^+ ion rushes out to restore the membrane potential, but reversed motion continues until the Ca^{2+} ion has been pumped out (7, 12, 13). Assuming basic similarity of underlying mechanism of behavioral control in *Paramecium* and *Bacillus*, Ordal and Goldman (15) proposed that concentration of an ion might control setting of the switch. In the present study, I have explored the effects of divalent cations and A23187, an ionophore that conveys divalent cations across membranes (8, 19), on behavior and conclude that local concentration of a divalent cation at the switch might control its setting.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* strains OI8 and OI151, have been described (15). Strain OI8 swims, although erratically, under the conditions of these experiments (state 4 tumbling frequency, see below), whereas strain OI151 tumbles mostly (state 2 tumbling frequency).

Media. Minimal sorbitol growth medium and nutrient broth with sporulation salts have been described (15). Chemotaxis medium included 10 mM potassium phosphate, pH 7, 5 mM sodium lactate, 0.05% glycerol, 0.3 mM ammonium sulfate, and chelating agent (usually ethylenediaminetetraacetic

acid [EDTA]). To remove any divalent cations, both the potassium phosphate and ammonium sulfate were treated with 2% (wt/vol) Chelex-100 resin (Bio-Rad), previously treated with 1 M HCl and then 1 M ammonium hydroxide, and then extensively washed with distilled water.

Chemicals. The antibiotic A23187 was a gift from Robert L. Hamill, Lilly Research Laboratories, Indianapolis, Inc., to whom I am grateful. It was dissolved at 3.33 μM in chemotaxis medium, without chelating agent or divalent cation, and frozen in 3-ml samples, one of which was unfrozen for a 1-day experiment. For some experiments, it was stored in the dark in the freezer as a 2 mM solution in chloroform, diluted to 3.33 μM in buffer, and removed from chloroform by bubbling air through the solution.

Microscopic observation of bacteria. One-tenth milliliter of bacteria was inoculated from a stationary-phase tryptone broth culture into 5 ml of minimal sorbitol medium and grown into mid-exponential phase. The culture was made 5 mM in sodium lactate and 0.05% in glycerol and grown for 15 min longer. Bacteria were filtered on membrane filters (Millipore Corp.), washed, suspended in chemotaxis medium at 10 Klett units/ml (filter 66), about 3.4×10^7 bacteria/ml, and placed in borosilicate disposable test tubes (18 by 150 mm) (usually 2 ml of bacterial suspension). Except for the experiment of Fig. 3, these bacteria were used in all behavioral experiments reported in this article.

In a typical experiment, 0.1 ml of bacteria was placed in a borosilicate disposable test tube (13 by 100 mm), and at time zero 0.1 ml of A23187 was added. As a function of time 9-μl samples were withdrawn using a Rainen adjustable micropipette and placed as a drop on a cleaned microscope slide. Observations were usually made at about 20 or 30 s after placement of the drop. When effect of attractant or repellent was assessed, it was added by injection from a 1-μl microcapillary as described (15). In some instances, as many as 10 drops were put on the slide at the same time. Where possible, experiments were done blind (contents of bacterial sample or injected reagent unknown until after the experiment).

In carrying out experiments to determine the Mg content of bacteria treated with A23187 (see below), samples were placed on microscope slides and observed. It was found that A23187 was effective in causing tumbling at considerably lower concentrations for these bacteria compared with those prepared by filtration (see above). For instance, 0.026 μM A23187 caused state 3 of tumbling frequency (see below) in cells prepared for measurement of Mg (large volumes; polypropylene centrifuge bottles), but 0.42 μM A23187 caused state 3 of tumbling frequency in bacteria prepared by filtration. Furthermore, bacteria prepared in large volumes and by centrifugation and put in 10 μM EDTA and 1.67 μM A23187 tumbled, whereas those prepared by filtration swam. From the results presented in this paper, it is apparent that small samples prepared by filtration are contaminated to some extent by divalent cation, so that higher levels of chelating agent and a higher concentration of ionophore to leach cation from cells are needed to observe the same effects as seen in cells prepared by centrifugation in polypropylene bottles in large volumes.

To assess tumbling frequency, seven states were defined. In state 1, exemplified by Fig. 1b of reference 15, bacteria only tumbled (clearly distinct from nonmotile) and did not swim at all. In state 7, they only swam and absolutely no abrupt directional change or tumbling was detected. In state 4, exemplified by Fig. 1a of reference 15, the bacteria swam erratically, with about 1 s at the most of smooth swimming before abrupt changes of direction. Bacteria in state 3 tumbled decidedly more than in state 4, and in state 2 they mostly tumbled, with only a few making any forward progress. Conversely, in state 5 the motion was decidedly smoother than in state 4, and in state 6 only occasional abrupt changes of direction could be seen.

Atomic absorption spectrophotometry. To certify that Mg was depleted from bacteria in the presence of A23187 and EDTA, atomic absorption spectrophotometry was used. A 200-ml amount of strain OI8 was grown in minimal sorbitol medium to 32.5 Klett units (filter 66), supplemented with 0.05% glycerol and 5 mM sodium lactate, and grown to 40 Klett units. Bacteria were washed three times and suspended at 5 Klett units in 200 ml of chemotaxis medium, of which potassium phosphate and ammonium sulfate had been treated with Chelex, including EDTA. A23187 dissolved in absolute ethanol was added (normally a 2,000-fold dilution). After 4 to 7 min the cultures were centrifuged, suspended in 5 ml of distilled water, and recentrifuged. The pellets were incubated with 0.033 ml of concentrated HNO_3 for several hours (17) and then supplemented with the supernatant of the previous centrifugation. The mixture was centrifuged. The supernatant was made 0.1% in La (using lanthanum nitrate) and was diluted in serial 10-fold dilutions using 0.1 M HNO_3 and 0.1% La (as nitrate). Atomic absorption at 285 nm in a compressed air-acetylene flame was measured in the experimental samples and in a set of standards using a Perkin-Elmer model 303 atomic absorption spectrophotometer. The Mg content was determined by interpolation (17).

RESULTS

Effect of A23187. Although *B. subtilis* strain OI8 normally swims erratically (tumbling frequency state 4 as explained in Materials and Methods), it reacted to A23187 by transient swimming, as it would to attractants. However, permanent changes in tumbling frequency ensued (Fig. 1; Table 1). Fairly quickly after addition of A23187, the bacteria reached a state of only tumbling (Fig. 1; Table 1). The propensity to tumble was correlated with loss of internal Mg from these bacteria (Table 1). At contents of Mg of 15% or more, the bacteria swam normally or more than normally (Table 1). At 1.67 μM A23187 and 0.1 μM EDTA, at least 90% of the bacteria were viable through at

least 6 min of incubation. Onset of this tumbling state was postponed if 20 μM $MgCl_2$ was present (Fig. 1). A lower concentration of EDTA, 10 μM, had an opposite effect: it promoted a greater degree of swimming than the bacteria in the absence of A23187 showed (Table 2) (however, see Materials and Methods). Increasing the EDTA concentration from 0.01 to 0.1 mM caused the bacteria to tumble (Fig. 1).

Other chelating agents besides EDTA were used (Table 2). *trans*-1-2-diaminocyclohexanetetraacetate (CDTA), whose apparent stability constants at pH 7 for divalent cations are even higher than those for EDTA (CDTA: $10^{6.31}$ for Mg^{2+} ion, $10^{8.51}$ for Ca^{2+} ion; EDTA: $10^{5.4}$ for Mg^{2+} ion, $10^{7.3}$ for Ca^{2+} ion; reference 6), had the same effect as EDTA. However, ethyleneglycol - bis(β - aminoethylether) - *N,N'* - tetraacetate (EGTA), which preferably binds Ca^{2+} ion to Mg^{2+} ion (apparent stability constants of about $10^{6.8}$ for Ca^{2+} ion and $10^{1.0}$ for Mg^{2+} ion; reference 18), promoted swimming rather than tumbling, even at 1 mM (Table 2).

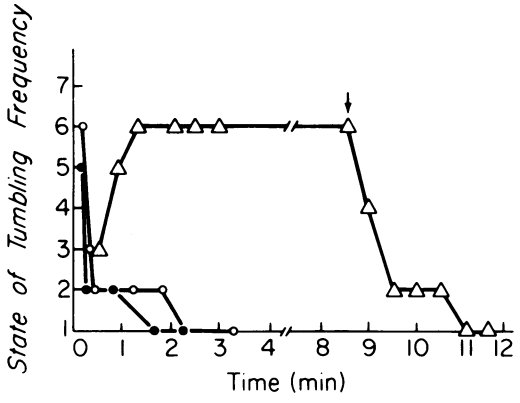

FIG. 1. *Effect of A23187 on tumbling frequency. At time zero, 1.67 μM A23187 was added to bacteria and tumbling frequency was noted as a function of time (see Materials and Methods). In some experiments presence of $MgCl_2$ caused bacteria to reach state 2 tumbling frequency, rather than state 1. At the time noted by the arrow, the culture in 10 μM EDTA was made 0.1 mM in EDTA. Symbols are as follows: (●) 0.1 mM EDTA; (○) 0.1 mM EDTA, 20 μM $MgCl_2$; (△) 10 μM EDTA.*

TABLE 1. *Effect of A23187 on behavior*[a]

Concn of A23187 (μM)	State of tumbling frequency	Behavior after addition of 10 mM alanine	% remaining (Mg) total
1.67	1	No effect	1.6
0.83	1	No effect	2.5
0.42	1	Slight swimming	2.7
0.21	1	Some swimming	3.2
0.10	1.5	More swimming	4.1
0.052	2	Much swimming	8.0
0.026	3	Much swimming	7.1
0.013	4	Much swimming	14.3
0.0065	5	Much swimming	46.9
	4	Much swimming	100.0

[a] At 3 or 4 min after addition of A23187, EDTA was at 0.1 mM. Bacteria were prepared for atomic absorption spectrophotometry as 200-ml samples by centrifugation (see Materials and Methods), rather than by filtration.

Divalent cation, as expected, reversed the effect of chelating agents (Table 3). Based on results of calculations presented in Table 3 from the apparent stability constant at pH 7.0 of EDTA for Mg^{2+} ion, the increased degree of tumbling did not occur at or above a free concentration of Mg^{2+} ion of 2 to 3 μM. When 0.1 mM divalent cation ($MgCl_2$ or $CaCl_2$) was added to bacteria after about 5 min in A23187 and 0.1

TABLE 2. *Effect of chelating agents on tumbling frequency (A23187 present)*[a]

Agent	Concn (mM)	State of tumbling frequency
EDTA	0.1	1
EDTA	0.01	6
CDTA	0.1	1
EGTA	1	6
Pyrophosphate	1	6
Pyrophosphate	10	1
EDTA[b]	1	1
EDTA	0.1	
EGTA	1	6
EDTA	0.01	
EGTA	10	4
EDTA	0.01	

[a] 1.67 μM.

[b] Brace indicates simultaneous presence of both chelating agents.

TABLE 3. *Effect of $MgCl_2$ on tumbling frequency (A23187 present)*[a]

Total[b] $MgCl_2$ (μM)	Calculated[c] free $MgCl_2$ (μM)	State of tumbling frequency
		1
20	0.94	2
40	2.4	4
60	5.3	5
80	9.6	6
100	18.3	5

[a] 1.67 μM A23187 and 0.1 mM EDTA.

[b] Neglecting contribution of Mg^{2+} in bacteria, which was found to be 0.78 μM by atomic absorption spectrophotometry (see Materials and Methods).

[c] Calculated from equation: (free metal) = (total metal)/[1 + k (free chelating agent)], where k is apparent stability constant at pH 7 (6). See text.

mM EDTA or CDTA, they began swimming and within 15 s were indistinguishable in their behavior from bacteria incubated with A23187, chelating agent, and divalent cation from the start. It should be noted that addition of 0.1 mM divalent cation [$CaCl_2$, $MgCl_2$, $Sr(NO_3)_2$, $CoCl_2$, $BaCl_2$] to bacteria in the absence of A23187 caused transient tumbling.

Bacteria incubated for a couple minutes in A23187 and 0.1 mM EDTA or CDTA did not respond by swimming to 10 mM alanine (see Table 1 also), the most powerful attractant with a threshold in the capillary assay of 3 nM. However, when the bacteria were incubated in A23187, 0.1 mM EDTA, and 20 μM $MgCl_2$, then they did show some swimming when 10 mM alanine was added. Repellent, however, was active in causing swimming bacteria to tumble: thus, 30 nM FCCP, an uncoupler of oxidative phosphorylation and a repellent of *B. subtilis* (15), caused bacteria in 1.67 μM A23187 and 10 μM EDTA to tumble transiently. Two-tenths mM $MgCl_2$ did not prevent this tumbling from occurring. However, 10 mM alanine added simultaneously with 0.1 μM FCCP prevented tumbling from occurring.

In the absence of A23187, tumbling frequency was state 4 or close to it and was not affected by identity of chelating agent (EDTA, EGTA, or CDTA) or by the presence or absence of divalent cation (Ca^{2+} ion or Mg^{2+} ion). From a survey of a great number of ions, only one was found that permanently affected tumbling frequency at low concentrations (10 to 100 μM), Mn^{2+} ion, either as chloride or carbonate (Fig. 2). The Mg content of bacteria in 3.16 μM EDTA and 10 μM $MnCl_2$ was 75% of normal (state 3 tumbling frequency) and in 3.16 μM EDTA and 0.1 mM $MnCl_2$ it was 21% of normal (state 2 tumbling frequency), as measured by atomic absorption spectrophotometry. A23187 potentiated the increase in tumbling frequency caused by Mn^{2+} ion (Fig. 2). It should be noted that the presence or absence of $MgCl_2$ or $CaCl_2$ in the absence of A23187 did not appreciably affect response to 0.1 μM FCCP (tumbling) or to 32 μM asparagine (swimming). Finally, A23187 had no effect on internal concentration of *S*-adenosylmethionine (G. W. Ordal, unpublished data).

Effect of permeant cations: tetraphenylarsonium and triphenylmethylphosphonium ions. Besides dibenzyldimethylammonium ion (see below), tetraphenylarsonium and triphenylmethylphosphonium ions are remarkable in being the only compounds so far known that cause *B. subtilis* to swim permanently. Bacteria become completely smooth swimming (state 7) either immediately for the former or after a lag for the latter (Fig. 3). Dibenzyldimethylammonium ion, which requires the permeant anion tetraphenylboron as carrier to traverse membranes (9), has no effect on strain OI151 at 320 μM unless 3.2 μM tetraphenylboron is pres-

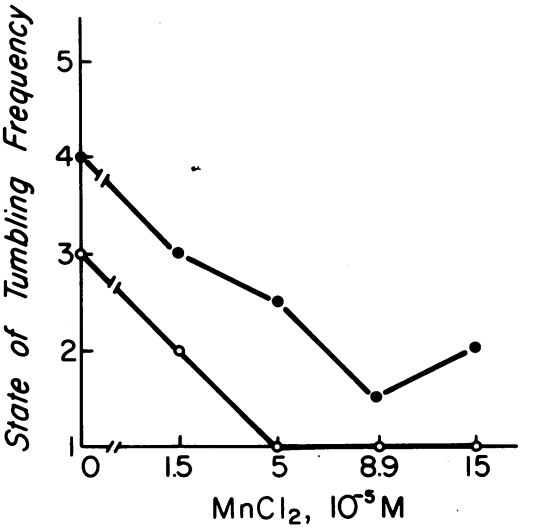

FIG. 2. *Effect of $MnCl_2$ on tumbling frequency. Experiment carried out blind (see Materials and Methods). A23187 was 1.67 μM and EDTA, the only chelating agent present, was 3.2 μM. Symbols are as follows: (○) A23187 present; (●) A23187 absent.*

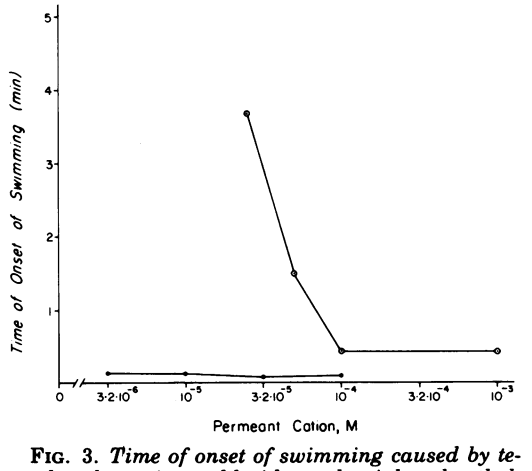

FIG. 3. *Time of onset of swimming caused by tetraphenylarsonium chloride and triphenylmethylphosphonium bromide. Bacteria were grown in nutrient broth, washed, and suspended in the chemotaxis buffer described in reference 15. In a blind experiment, 1 μl of reagent was squirted into 9 μl of bacteria on a microscope slide, and time till onset of smooth swimming (state 7) was noted. Symbols are as follows: (●) tetraphenylarsonium ion; (⊙) triphenylmethylphosphonium ion.*

ent. Although tetraphenylboron is a repellent (causes transient tumbling), it has only a brief effect at 3.2 μM (16). A 0.32 mM concentration of dibenzyldimethylammonium ion added to strain OI151 in 3.2 μM tetraphenylboron causes smooth swimming in about 3 to 6 min. If either reagent is raised threefold in concentration, the bacteria become mostly nonmotile. Either reagent alone does not cause swimming.

Besides affecting behavior in normal chemotaxis buffer, tetraphenylarsonium ion influences behavior caused by A23187 and EDTA and by repellent (FCCP). As described above, bacteria (strain OI8) in 1.67 μM A23187 and 0.1 mM EDTA tumble (state 1). Even strong attractant, such as 10 mM alanine, does not disturb this tumbling. However, 3.2 μM tetraphenylarsonium ion causes substantial swimming (state 5) within a minute of addition, and 10 μM, within 25 s. Moreover, 3.2 μM tetraphenylarsonium ion severely curtails degree of tumbling elicited by addition of 0.1 μM FCCP, and 10 μM completely abolishes it; i.e., the bacteria remain smooth swimmers (state 7).

DISCUSSION

The present study supports the suggestion made by Ordal and Goldman (15) (see above) that ions are involved in determining behavior of *B. subtilis*. In this study I have sought to identify reagents having permanent effects on tumbling frequency in contrast to those having transitory (chemotactic) effects. Because bacterial cells have become adapted to survival in diverse environments, they are good at insulating themselves from fluctuations in conditions. Accordingly, merely adding ions to their environment is insufficient to be assured that the internal concentration will change appreciably. Therefore, I have used A23187, an ionophore capable of transferring divalent cations like Ca^{2+} ion, Mn^{2+} ion, and Mg^{2+} ion, but not monovalent cations, across membranes (19). This capability is a consequence of its ability to bring divalent cation from aqueous to organic phases (19). Hence, A23187 acts to equilibrate concentrations of divalent cation in aqueous compartments divided by membranes.

The experiments presented in this paper suggest that Mg^{2+} ion (or possibly another divalent cation, although the fact that 1 mM EGTA, which binds Ca^{2+} ion but not Mg^{2+} ion, causes swimming, not tumbling, in the presence of A23187 indicates that Ca^{2+} ion is probably not this key ion) binds to the switch that controls rotational direction of the flagella. When bound with Mg^{2+} ion, this switch is presumed to cause the flagella to rotate counterclockwise for swimming. When deprived of Mg^{2+} ion, this switch then allows clockwise rotation of flagella for tumbling. Increase in tumbling frequency caused by incubation with Mn^{2+} ion is probably due to its action in causing loss of Mg from the bacteria (see Results and reference 20), although it may also (i) hinder access of Mg^{2+} ion to the switch or (ii) bind to the switch as a competitive inhibitor of Mg^{2+} ion. Attractant would seem to cause increased access of Mg^{2+} ion to the switch since deprivation of divalent cation by A23187 and EDTA or CDTA results in loss of ability of attractant to cause swimming.

In this context it may seem paradoxical that bacteria incubated in 10 μM EDTA and A23187 swim more than normally, whereas bacteria incubated in 100 μM EDTA and A23187 tumble incessantly (Fig. 1; Table 2). As noted in Materials and Methods, bacteria prepared in small volumes by filtration likely contain some contaminating Mg^{2+} ion since bacteria prepared in large volumes using centrifugation in polypropylene bottles tumble (state 1), not swim, in the presence of 10 μM EDTA and 1.67 μM A23187. However, the question remains as to why bacteria in 1.67 μM A23187, 0.1 mM EDTA, and 60 μM $MgCl_2$ (Table 3) or in 0.0065 μM A23187, 0.1 mM EDTA, and only endogenous Mg of the bacterium (Table 1) swim more than bacteria untreated with A23187. This increase in swimming might be due to A23187 itself, to A23187-catalyzed leakage of Mg^{2+} ion from cytoplasm to the switch, or to another ion, such as Ca^{2+} ion or Mn^{2+} ion, whose presence antagonizes Mg^{2+} ion binding to the switch. However, when all divalent cation is withheld by using higher concentrations of EDTA or CDTA in the presence of A23187, tumbling, unrelieved even by strong attractant, ensues.

Sensitivity of bacteria to attractants and repellents in the presence of A23187 deserves comment. Table 1 indicates that, as the concentration of A23187 is reduced, the bacteria can respond more to stimulation by 10 mM alanine, although they are still tumblers. The uncoupler of oxidative phosphorylation, FCCP, causes bacteria incubated in 10 μM EDTA and 1.67 μM A23187 to tumble; however, simultaneous addition of alanine maintains the bacteria in the swimming state. These results show that, given sufficient divalent cation to avoid incessant tumbling, the resources of the bacterium, when invoked by attractants and repellents, are powerful enough to regulate ionic concentrations at the switch despite any leakage of divalent cation to it mediated by A23187.

The permeant cations strongly influence behavior. Because of their hydrophobic character, these ions—tetraphenylarsonium ion, triphen-

ylmethylphosphonium ion, and dibenzyldimethylammonium ion—presumably traverse membrane (9) to gain access to the switch and lock it in the swimming mode. This argument is strengthened by the fact that tetraphenylarsonium ion can cause swimming by bacteria in 1.67 μM A23187 and 0.1 mM EDTA, something that even strong attractant cannot do, and can prevent tumbling caused by repellent.

Because of lack of sensitivity of duration of swimming caused by attractants or tumbling caused by repellents to ionic content of the medium (absence of A23187), it is not likely that cation migration across the plasma membrane is important in regulating behavior in *B. subtilis* as it is in *Paramecium*. Rather, it would seem that the concentration of ions governing setting of the switch would be a local concentration, possibly within the membrane, not equilibrated with the general cytoplasmic concentration (or that of the external medium) and that ionic fluxes occur between this locale and the fairly homeostatic cytoplasm.

Since the presence of methionine enhances the ability of *B. subtilis* to recover from swimming induced by attractant (18) and is required for tumbling in enteric bacteria (1, 3, 16), a derivative of methionine via *S*-adenosylmethionine (2, 4), possibly a methylated protein (10), might be involved in regulating ionic concentration at the switch.

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