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Restoration of flagellar clockwise rotation in bacterial envelopes by insertion of the chemotaxis protein CheY

(*Escherichia coli*/*Salmonella typhimurium*/signaling protein/bacterial behavior/signal transduction)

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ABSTRACT **When cells of the bacterium *Salmonella typhimurium* are incubated with penicillin and lysed in a dilute buffer, flagellated cytoplasm-free envelopes are formed. When the envelopes are tethered to glass by their flagella and then energized, some of them spin. The direction of rotation of wild-type envelopes is exclusively counterclockwise (CCW). We perturbed this system by including in the lysis medium (and hence in the envelopes) the chemotaxis protein CheY. As a result, some of the envelopes rotated exclusively clockwise (CW). The fraction of envelopes that did so increased with the concentration of CheY; at a concentration of 48 μM (pH 8), all functional envelopes spun CW. The fraction also increased with the pH of the lysis medium in the range 6.6–8.4. The results were the same in the presence or absence of intracellular Ca^{2+}. Reconstituted envelopes failed to respond to chemotactic stimuli. None of them changed the direction of their rotation. However, when the intracellular pH was lowered to 6.6 or below, envelopes that spun CW stopped rotating, while envelopes that spun CCW continued to rotate. This phenomenon was reversible. We conclude that CheY *per se*, without any additional free cytoplasmic mediators, interacts with a switch at the base of the flagellum to cause CW rotation.**

Bacterial chemotaxis is migration toward favorable chemicals (attractants) and away from unfavorable ones (repellents) (1). The unstimulated behavior of peritrichous bacteria is smooth swimming with occasional brief periods of tumbling (2, 3). This unstimulated mode of swimming is the consequence of alternating flagellar rotation: smooth swimming results from counterclockwise (CCW) rotation and tumbling results from clockwise (CW) rotation. Attractants or repellents shift the rotation to CCW or CW bias (4) and thus cause smooth swimming or tumbling, respectively. The molecular mechanism that regulates the direction of this rotation is not known.

For studying this regulation mechanism, an *in vitro* system consisting of functional cell envelopes, isolated from *Escherichia coli* and *Salmonella typhimurium* (5), was used. The internal content of these envelopes can be controlled and predetermined. The envelopes have intact cytoplasmic membrane and parts of the cell wall. They are essentially free of cytoplasm and contain instead the medium in which the bacteria, from which the envelopes were derived, were lysed. Due to the absence of cytoplasm, the flagella of the envelopes do not rotate unless an electron donor is added for respiration (5, 6). This lack of rotation in the absence of an added energy source serves as a control, carried out for every envelope, for lack of cytoplasmic remnants in it.

The flagella of envelopes prepared from wild-type *S. typhimurium* rotate exclusively CCW (7), unlike the bacteria from which the envelopes are derived, which alternate between CCW and CW rotation (8). Our previous studies indicated that a cytoplasmic constituent is required for CW rotation in wild-type motors (7). This constituent was suggested to be the *cheY* gene product (7, 9, 10). To test this hypothesis directly, we inserted the purified CheY protein into the envelopes. Enhanced levels of CheY were obtained by transcriptionally fusing the *cheY* gene to the tryptophan promoter of *Serratia marcescens* (11). In this paper, we describe the behavior of CheY-containing envelopes and show that CheY is indeed the cytoplasmic constituent that causes CW rotation.

MATERIALS AND METHODS

Chemicals. Antibodies to flagellin were a gift from the National Center for Enterobacteriaceae, Central Laboratories, Ministry of Health, Jerusalem. Penicillin G (K^+ salt), chloramphenicol, DL-lactate, and valinomycin were obtained from Sigma. Tetraethylenepentamine (Tetren) was obtained from Merck. Other chemicals were of analytical grade.

Bacteria. The strain used in this study was *S. typhimurium* ST1 wild type for chemotaxis (35). The cells were grown in nutrient broth as described (5).

Preparations. Cell envelopes were isolated by penicillin treatment and subsequent osmotic lysis as described (7) except that the lysis medium contained 100 mM KP_i and 0.1 mM Tetren at the specified pH. Tetren was used rather than EDTA because it chelates heavy metals, which are inhibitory for motility (12), but it does not bind Ca^{2+} and Mg^{2+} (13). CheY-containing envelopes were prepared similarly, except that the lysis medium also contained purified CheY at the desired concentration. (The average concentration of CheY in the envelopes is presumed to be the same as in the lysis medium.) The CheY protein was overproduced in *E. coli* CY15040 containing the pRL22 plasmid, isolated, and purified as described (11). The purity of the CheY was >99.9% as determined on $NaDodSO_4$/polyacrylamide gel electrophoresis (11).

Assays. Flagellar rotation was assayed at room temperature by the tethering technique (8) as described (14), using a flow chamber (15).

RESULTS

Dose Response of CheY-Containing Envelopes. We used functional envelopes isolated from *S. typhimurium*, since they are superior in yield and size to their *E. coli* counterparts. CheY-containing envelopes were tethered to a microscope cover glass in a flow chamber and their lactate-driven rotation was monitored. Each envelope constituted a separate determination. We took into account only envelopes

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Abbreviations: CCW, counterclockwise; CW, clockwise; MCP, methyl-accepting chemotaxis protein.

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whose rotation stopped in the absence of lactate, thus indicating lack of cytoplasmic remnants in them (cf. discussion in ref. 7). The effect of the entrapped CheY on the fraction of envelopes rotating CW is demonstrated in Fig. 1, which shows results obtained with three different batches of CheY. In the absence of CheY, all envelopes rotated CCW (≈600 determinations). However, CW-rotating envelopes were observed with increasing concentrations of CheY until at 48 μM all envelopes rotated CW. CheY had no effect in control experiments in which it was added externally up to a concentration of 8.5 μM; all rotating envelopes remained CCW (16 determinations). The above results indicate that CheY interacts directly with the switch and causes CW rotation (see *Discussion*).

Unidirectional Rotation of Tethered Envelopes. An intriguing observation related to Fig. 1 was that, at any CheY concentration, each tethered envelope rotated exclusively in one direction, either CW or CCW. To examine whether the direction of rotation in CheY-containing envelopes can be reversed, we exposed CW- or CCW-rotating envelopes to attractants or repellents, respectively. [Attractants and repellents shift the rotation in intact bacteria to CCW and CW bias, respectively (4).] Thus, we added a potent mixture of attractants, L-serine (1 mM) plus L-aspartate (0.01 mM), to CW-rotating envelopes containing either 8.5 or 48 μM CheY (pH 7–8). A repellent mixture composed of sodium acetate (30 mM), L-leucine (10 mM), and $CoCl_2$ (10 μM) at pH 7.0 was added to CCW-rotating envelopes containing CheY (CheY concentrations equivalent to that which causes 50% CW rotation at pH 7.6). None of the envelopes changed their direction of rotation in response to the stimuli (12 and 5 determinations, respectively). Similar lack of response of CCW-rotating CheY-free envelopes to repellents has been reported (7). The results are consistent with observations in whole cells that the direction of flagellar rotation in a mutant lacking all the cytoplasmic chemotaxis proteins but containing increased levels of CheY was not reversed (from CW to CCW) by serine (10).

Since it has been suggested that Ca^{2+} may be indirectly involved in determining the direction of flagellar rotation (16, 17), we examined its effect on the direction of rotation in the envelopes by preparing them in the presence of either 0.1–0.2 mM $CaCl_2$ or 0.5 mM EGTA, in addition to 8.5–23 μM CheY.

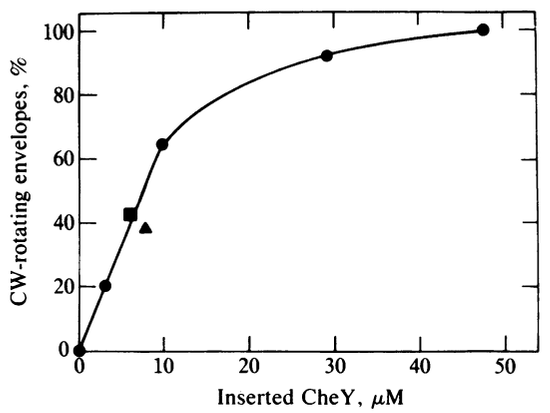

FIG. 1. Dose dependence of the fraction of CW-rotating envelopes on the amount of inserted CheY at pH 8.0. Each envelope rotated exclusively in one direction. The rotation was driven by DL-lactate (2 mM). The value of 100% represents all the rotating envelopes. The various symbols represent results obtained with three different batches of CheY. The numbers of envelopes used for each point on the graph were >600, 10, 28, 34, 28, 11, and 13 from 0–48 μM CheY, respectively. (Each envelope constituted a separate determination.) In calculating the CheY concentration, we assumed a size of 13 kDa.

Neither the presence of Ca^{2+} nor its complete absence (i.e., in the presence of EGTA) changed the distribution of CW- and CCW-rotating envelopes (16 and 11 determinations, respectively).

pH Dependence of CW Rotation. Since an increase or a decrease in intracellular pH (pH_{in}) causes an attractant or a repellent response, respectively (18, 19), we examined whether pH_{in} modifies the effect of CheY on the direction of rotation. [CheY-free envelopes rotate exclusively CCW at any given pH (7).] For this purpose, we prepared envelopes in CheY-containing lysis medium at various pH values and examined the distribution of CW- and CCW-rotating envelopes driven by lactate. As shown in Fig. 2, there was a linear correlation between the fraction of CW-rotating envelopes and the pH of the lysis medium (the presumed pH_{in}). No CW rotation was observed at pH 6.4 and below. It appears, therefore, that the effect of pH_{in} on the effectiveness of CheY is not related to the repellent-like response of a decrease in pH_{in}, which should have increased the effectiveness of CheY.

To investigate further this pH dependence and to examine the effect of gradual change of pH_{in} on rotation, we prepared CheY-containing envelopes at pH 7.0, recorded their lactate-driven rotation, and exposed them to different external pH values in the presence of lactate. [The intracellular pH in cell envelopes is equalized with the extracellular pH within 0.3–2.5 min (6).] The CheY concentration was equivalent to that which causes 50% CW rotation at pH 7.6. When the external pH was shifted from 7.0 to 8.0, the envelopes were not affected by the change in pH. When the external pH was shifted from 7.0 to 6.0, all the CW-rotating envelopes gradually stopped. The period of time for the CW-rotating envelopes to come to a halt was dependent on the externally

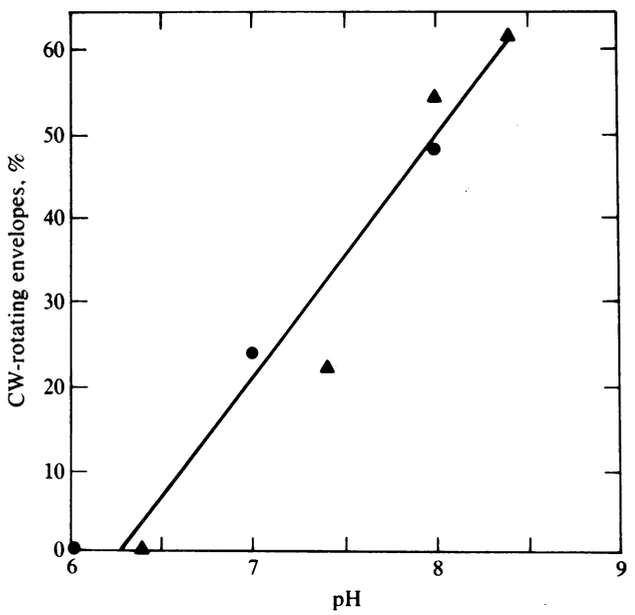

FIG. 2. The correlation between the fraction of the CW-rotating envelopes and the pH of the lysis medium. CheY-containing envelopes were prepared as described, but at various pH values of the lysis medium. Thus, the pH values shown represent the pH values of both the internal and external milieu. The rotation was driven by DL-lactate (2 mM). The results shown were obtained with two batches of CheY. The symbols used are the same as in Fig. 1. The actual CheY concentrations in the envelopes varied between 7 and 10 μM, but the values shown in the figure are normalized, according to Fig. 1, to a concentration of 7.5 μM. (The dependence of the fraction of CW-rotating envelopes on the CheY concentration is linear in the range of 7–10 μM.) At 7.5 μM, 50% of the envelopes rotate CW at pH 7.65 [the natural pH_{in} of *E. coli* (20, 21)]. All other experimental details were as in Fig. 1. The correlation coefficient for a linear fit was 0.97.

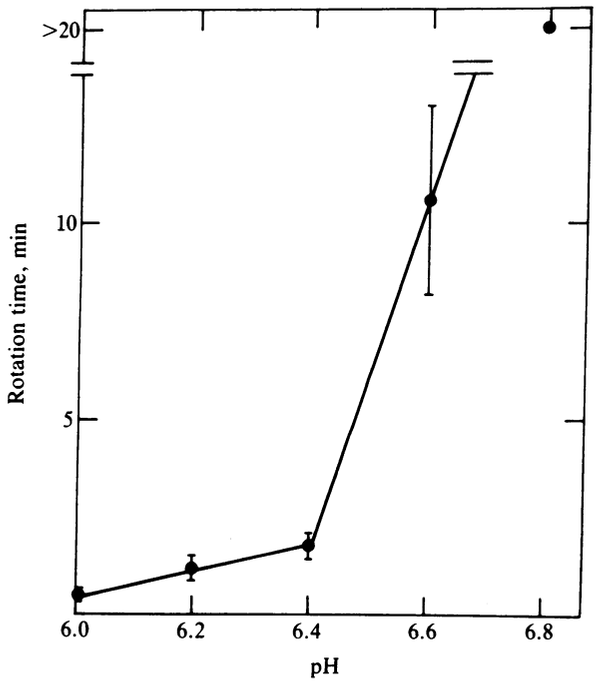

FIG. 3. Dependence of the rotation time of CW-rotating envelopes on the pH of the flow medium. The rotation time is considered as the period between the time at which the pH of the medium in the flow chamber changed and the time at which the rotation of the envelope stopped. Envelopes containing CheY at a concentration yielding (at pH 7.5) 50% CW rotation were prepared at pH 7.0 as described. (With a few batches of CheY used for this experiment, we had to use larger concentrations of CheY than those shown in Fig. 1.) See text for details. The confidence intervals are SD (5–13 determinations for each point).

imposed pH (Fig. 3) or, presumably more correctly, on the rate and extent of the decrease in pH_{in}. The phenomenon was reversible—i.e., a shift back to pH 7.0 restored the rotation in the original direction. The selective effect of the pH shift on CW-rotating envelopes is probably the reason for the absence of CW-rotating envelopes in preparations made at pH 6.4 and below (Fig. 2). CCW-rotating envelopes, in contrast, were unaffected by the shift in pH and continued to rotate in the original direction. These results indicate that pH_{in} values from ≈6.6 and below do not permit CW rotation.

Mechanistic Irreversibility of the Flagellar Motor in Envelopes. To investigate whether the cause of the unidirectional rotation in all the above experiments was a mechanistic irreversibility of the motor, we exposed the envelopes to artificially imposed proton fluxes of inverse polarity. Our rationale was that if cell envelopes had everything required for rotational reversal, they should behave like nonchemotactic mutant cells of *Streptococcus* sp., which reverse the direction of their rotation upon reversing the direction of proton flow (22). The experiment was performed as follows. After identifying a rotating envelope in the presence of 2 mM DL-lactate, the observation chamber was flushed with lactate-free medium. The rotation of the envelopes consequently stopped, and then a lactate-free medium at a different pH was flushed through the chamber. The observations following this treatment are given in the third column of Table 1. When the rotation driven by the artificially imposed ΔpH stopped, lactate was re-added at the original pH and the respiration-driven rotation was recorded (last column in the table). As shown in Table 1, an inwardly directed proton gradient rotated the flagella in the original direction. An inverted proton gradient could not rotate the flagella or could not sustain more than a few revolutions. Similar observations were reported for CheY-free envelopes (7). It therefore seems that the motor is mechanistically irreversible, independent of whether the envelopes are rotating CCW or CW.

DISCUSSION

This study provides evidence that purified CheY, included in envelopes containing buffer only, irreversibly causes CW rotation. Due to the absence of any of the original cytoplasmic constituents (both macromolecules and small molecules) in the envelopes, it is possible to conclude, with a high degree of confidence, that CW rotation is caused by CheY *per se*. This is probably accomplished by a direct interaction between CheY and the switch. This conclusion is in accordance with genetic reversion analyses, which demonstrated that mutations in the *cheY* gene could be phenotypically compensated for by mutations in the *flaAII* (*cheC*) or *flaBII* (*cheV*) genes—the switch genes (9). Similarly, Clegg and Koshland concluded from their study of intact bacteria containing increased levels of CheY that no other processing gene products are required for the interaction between CheY and the *flaAII* and *flaBII* gene products (10).

The concentrations of CheY that were effective in causing CW rotation within the envelopes are in the range of CheY concentrations in intact bacteria. Considering the molecular stoichiometry between *tar* and *cheY* gene products (24) and the number of Tar molecules in a cell of *E. coli* (25), the number of CheY molecules per bacterial cell can be estimated to be <4000. Considering the average volume of a cell envelope to be 2.3×10^{-15} liter (26), 4000 molecules correspond to 3.4 μM. This concentration is on the linear part of the curve in Fig. 1. As a matter of fact, assuming that a wild-type bacterium spends ≈90% of its time in CCW rotation, one may roughly expect to find, based on Fig. 1, 1.5 μM or 1800 CheY molecules in this bacterial cell. There is considerable evidence that the switch molecules are parts of the flagellar motor (27), and so with ≈10 flagella per cell, it

Table 1. Rotation of CheY-containing envelopes by artificially imposed ΔpH

Type of envelope*	Polarity of imposed ΔpH†	Distribution of rotations‡ driven by	
		Artificially imposed ΔpH	Respiration at end of exp.
CW	Positive	14 CW	11 CW, 3 NR
CCW	Positive	9 CCW	5 CCW, 4 NR
CW	Negative	13 NR, 2 transient CW	10 CW, 3 NR, 2 ND
CCW	Negative	18 NR, 1 transient CCW	10 CCW, 9 NR

The envelopes were treated with valinomycin (10 nmol per mg of protein; in addition, 5 μM valinomycin included in the flow medium) to avoid formation of a diffusion potential (cf. ref. 23). NR, no rotation; ND, not determined. By the term "transient" we mean that the envelopes rotated two or three cycles and then stopped.

*Direction of rotation of CheY-containing envelopes driven by lactate before the shift in pH.

†Positive ΔpH (the polarity produced by respiration) means acid outside. The magnitude of the imposed ΔpH varied between 1.1 and 2.4 pH units. The initial pH_{in} was between 7.4 and 8.4.

‡Values given are numbers of envelopes.

seems likely that the number of switch molecules per cell is of the order of 100 or less. This number is smaller than the above numbers of CheY molecules per cell by at least an order of magnitude. If this conclusion is correct, there should be a large excess of CheY molecules in the envelopes at any of the CheY concentrations shown in Fig. 1.

What does determine the amount of CheY molecules that interact with the switch? What is the mode of this interaction? What is the status of the rest of the CheY molecules in the cell (in a whole bacterium or in an envelope)?

(*i*) CheY may be mainly free in the cell. It may act there via its binding to the switch, the extent of which may be determined by the dissociation constant (≈ 7 μM according to Fig. 1). In line with this possibility, a Hill plot based on Fig. 1 and on the basic assumption that most of the CheY in the envelopes is free, yields a straight line (correlation coefficient, >0.99), with a slope indicating a Hill coefficient of 1.62 ± 0.04 (SD)—i.e., a positive cooperativity (28). On the other hand, the observation that the rotation of tethered envelopes is unidirectional is not obviously consistent with this possibility. [A similar apparent inconsistency in a sensory system was observed in the binding of GTP[γS] to the regulatory component of adenylate cyclase (29). In whole bacteria, where reversals of rotation do occur, there is no obvious inconsistency.] This unidirectionality appears to imply either that the binding of CheY to the switch in the envelopes is irreversible or that once the switch binds CheY it is locked in CW position, which persists even after CheY is detached from it. This implication is further substantiated by the results related to Figs. 2 and 3: As long as pH_{in} permitted CW rotation, CheY caused CW rotation. Changing pH_{in} to nonpermissive values caused CW rotation to stop rather than to be shifted to CCW rotation. (CCW rotation did not stop.) Furthermore, the very same envelopes, the rotation of which came to a halt as soon as pH_{in} was reduced, were the ones that restored CW rotation as soon as the original pH_{in} was restored. Apparently, when pH_{in} was lowered to nonpermissive values, CheY was locked within the switch and was unable to get off, or the switch was permanently locked in CW position. Otherwise—i.e., if CheY were released at the nonpermissive pH and rebound at the restored pH—another distribution of rotations should have been observed due to redistribution of the occupancy of the binding sites. Taking the irreversible effect of CheY on the switch together with the basic assumption that there are free CheY molecules in the cell, it should be anticipated that CCW-rotating envelopes containing nonsaturating concentrations of CheY would eventually become CW-rotating envelopes; this has not been observed (observation time up to 12 hr). It may therefore be that only a small fraction of the CheY molecules within the envelopes are active. For example, CheY may be activated by an interaction with a cytoplasmic component present in the envelopes in residual trace amounts. These considerations, when applied to whole bacteria, suggest that chemotactic stimulation may vary the ratio between the active and nonactive forms of CheY and, thus, sequentially affect the amount of CheY bound to the switch, the direction of flagellar rotation, and the chemotactic response of the bacteria.

(*ii*) CheY may be mainly bound in the cell, the binding sites being the switch and other specific locations in the membrane. The extent of CheY binding to the switch may be determined by the relative affinities of all its binding sites in the cell. Since the membrane receptors [e.g., methyl-accepting chemotaxis protein (MCP) molecules and enzymes II of the phosphotransferase system] are presumably the only chemotaxis molecules, besides the switch molecules, that reside in the envelopes, perhaps the other binding sites are on them. {The number of MCP molecules per cell [$\approx$2700 in *E. coli* (30) and significantly more in *S. typhimurium* (31)] appears to be sufficient for serving as storage sites for CheY in the cell.} In whole bacteria, based on these considerations, chemotactic stimulation may alter the affinity of, e.g., MCP to CheY and thus affect the amount of CheY that interacts with the switch.

Both alternatives comply with studies that indicated that the excitatory signaling in chemotaxis of peritrichous bacteria is by way of diffusion (17, 32), probably a diffusion of a 10- to 80-kDa polypeptide (32).

In a previous publication, we suggested that the lack of both switching ability and mechanistic reversibility[§] in envelopes containing buffer only may stem from the absence of a hypothetical cytoplasmic species—the "CW facilitator" (7). The CheY protein appeared then as a good candidate for this species. The results of this study indicate that, although CheY causes CW rotation in envelopes, it is not sufficient for conferring switching ability or mechanistic reversibility to them. Apparently, other cytoplasmic constituents are required for these functions to occur as well as for the reversibility of the CheY effect on the switch. One possibility is that the *cheZ* gene product is required for detachment of CheY from the switch or for reversing the CW position of the switch. The absence of the CheZ protein from the envelopes may thus be a reason for the irreversibility of the CheY effect on the switch and, consequently, perhaps also for the lack of both switching ability and mechanistic reversibility in the envelopes. CheZ appears to be the best candidate for this role, because it is the only cytoplasmic protein known from genetic studies both to interact with the switch (9) and to be involved in CCW rotation [mutation in the *cheZ* gene causes biased CW rotation (33)].[¶]

Whatever the mechanism for CheY activity, the results described above demonstrate that isolated and purified chemotaxis proteins can be inserted into "empty" (buffer-containing) bacterial envelopes and affect the rotation of their flagella. This seems to open the way for stepwise restoration of chemotaxis in these envelopes.

§Note that we are dealing in this study with three functions related to the flagellar motor, all of which appear to occur in bacteria but not in the envelope system: (*i*) reversibility of the direction of flagellar rotation upon changing the polarity of the protonmotive force (PMF) (denoted as "mechanistic reversibility" and dealt with in Table 1), (*ii*) switching the direction of rotation while the polarity of the PMF is kept constant ("switching ability"; dealt with in the second section under *Results*), and (*iii*) reversibility of CheY binding or of CheY effect on the switch (dealt with above).

¶Based on these data and in analogy to CheY being a CW signal, it was suggested that CheZ is a CCW signal. However, since wild-type envelopes rotate CCW in the absence of CheZ (7), it appears that there is no need to assign this role to CheZ. Furthermore, *cheZ* mutants do respond to attractants (33) although with a longer response-delay time (34).

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