Pausing of Flagellar Rotation Is a Component of Bacterial Motility and Chemotaxis

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When bacterial cells are tethered to glass by their flagella, many of them spin. On the basis of experiments with tethered cells it has generally been thought that the motor which drives the flagellum is a two-state device, existing in either a counterclockwise or a clockwise state. Here we show that a third state of the motor is that of pausing, the duration and frequency of which are affected by chemotactic stimuli. We have recorded on video tape the rotation of tethered *Escherichia coli* and *Salmonella typhimurium* cells and analyzed the recordings frame by frame and in slow motion. Most wild-type cells paused intermittently. The addition of repellents caused an increase in the frequency and duration of the pauses. The addition of attractants sharply reduced the number of pauses. A chemotaxis mutant which lacks a large part of the chemotaxis machinery owing to a deletion of the genes from *cheA* to *cheZ* did not pause at all and did not respond to repellents by pausing. A tumbly mutant of *S. typhimurium* responded to repellents by smooth swimming and to attractants by tumbling. When tethered, these cells exhibited a normal rotational response but an inverse pausing response to chemotactic stimuli: the frequency of pauses decreased in response to repellents and increased in response to attractants. It is suggested that (i) pausing is an integral part of bacterial motility and chemotaxis, (ii) pausing is independent of the direction of flagellar rotation, and (iii) pausing may be one of the causes of tumbling.

Bacteria swim by rotating their flagella (4). The rotation is carried out by a motor embedded in the cytoplasmic membrane (7, 15). The swimming of peritrichous bacteria such as *Escherichia coli* and *Salmonella typhimurium* consists of movement in almost straight lines (runs) with occasional brief periods of tumbling (6, 17). When bacterial cells are tethered to glass by their flagella, many of them spin (26). On the basis of experiments with tethered cells it is generally accepted that the runs are caused by counterclockwise (CCW) rotation of the flagella and that the tumbles are caused by clockwise (CW) rotation (13). It is further thought that the motor is a two-state device, existing in either a CCW or a CW state (3, 16). The data collected during this study indicate that the motor is a three-state device, with pausing as the third state.

The phenomenon of pausing has been observed since 1974. Silverman and Simon reported that tethered cells of *E. coli* stopped rotating (26). At the same time Macnab and Koshland, using high-intensity dark-field microscopy, noted that free flagella of *S. typhimurium* are occasionally stationary (18). Macnab and Han have also shown that a flagellum on a given cell may pause while the other flagella on the same cell rotate (16). Larsen et al. (13) did not find a correlation between tumbling and stopping, and the phenomenon has been all but neglected. The first quantitative analysis of the pauses of tethered *E. coli* cells was made by Ravid and Eisenbach in a study which yielded a correlation between the degree of χ bacteriophage infection and incessant rotation (22). In the present study we quantified the behavior of tethered individual cells under the influence of attractants

and repellents and studied the behavior of chemotaxis mutants. These studies suggest that pausing is an integral part of the chemotaxis machinery of bacteria.

MATERIALS AND METHODS

Chemicals. Antibodies to flagellin were a gift from the National Center for Enterobacteriaceae, Central Laboratories, Ministry of Health, Jerusalem, Israel. Tetraethylenepentamine (Tetren) was obtained from Merck & Co., Inc. Other chemicals were of analytical grade.

Bacteria. The *E. coli* strains used in this study were RP437, wild type for chemotaxis (20), and RP1091 (21). The *S. typhimurium* strains were ST1, wild type for chemotaxis, and ST120 (25). The cells were grown either in a rich medium (tryptone broth for *E. coli* and nutrient broth for *S. typhimurium*) or in the H-1 minimal medium of Kaiser and Hogness (11) as previously described (8, 9). The cells were washed and resuspended in 10 mM potassium phosphate (pH 7)–0.1 mM Tetren–0.1 mM L-methionine before experimentation.

Data acquisition and analysis. Flagellar rotation was assayed by the tethering technique (26). Most of the flagella of the cells were sheared off by a blender, and the cells were subsequently tethered onto a precleaned cover glass (23) by using *flaI*-preadsorbed antibodies against flagellin as described earlier (22). Initially we cleaned the cover glass with fuming nitric acid. However, since we observed no difference in the behavior of the tethered cells when the glass had been precleaned with a dry Kimwipe, most of our studies were carried out with dry-cleaned cover glass.

Data were obtained by observing video tapes (recorded at 50 frames per s) of cells in a flow chamber (5) under a Zeiss phase-contrast microscope. The flow medium was identical to the potassium phosphate-Tetren-methionine suspending medium described above. When indicated, it was supplemented with glycerol (2 mM). The liquid medium in the field

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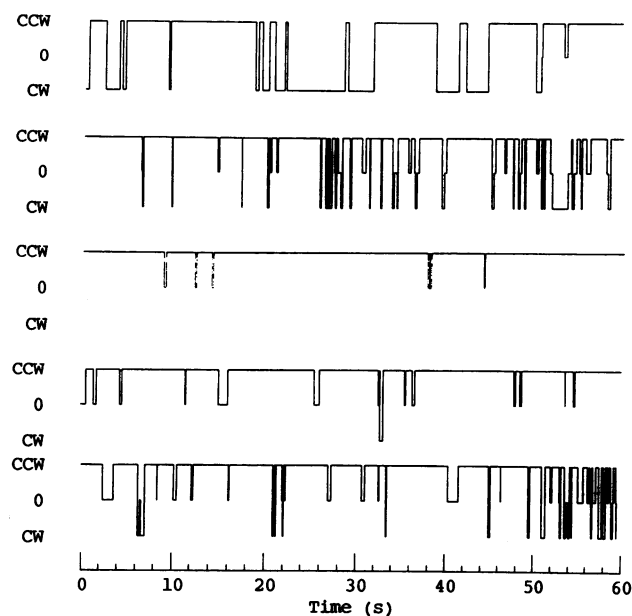
FIG. 1. Plots of the rotational states of five unstimulated cells of wild-type *E. coli* RP437. The cells in the upper two plots were in the presence of glycerol (2 mM). The cells in the lower three plots were in the absence of any external energy source. The measured rates of rotation of the cells were (from top to bottom) 11.9 ± 1.1, 6.2 ± 1.2, 4.4 ± 0.4, 4.3 ± 0.9, and 4.2 ± 0.7 Hz. The fractions of time spent pausing by each cell within the 1-min period shown in the figure were 0.7, 7.7, 1.6, 6.4, and 10.8%. The pauses of all the cells occurred at random angles.

of view of the flow chamber flowed continuously at a constant average speed of 38 ± 11 μm/s (mean ± standard deviation) measured as described by Meister and Berg (19). The cells to be analyzed were chosen as follows. All rotating cells in a given field were marked on a video screen. Of these, all the cells which did not pause at fixed angles or did not pause at frequencies higher than the rotation frequency were analyzed. Cells that stopped for many seconds were excluded. The video tape was played in slow motion (8.3 frames per s). CCW, pausing, and CW states were entered by pressing one of three keys on an Apple II Plus microcomputer. The keystrokes were input to an Applesoft BASIC program, which recorded the time of the keystroke on an internal clock within the microcomputer. Data files for individual cells were transferred to the mainframe IBM computer at the Weizmann Institute of Science. The plots were produced by using an IBM graphics package.

RESULTS

Unstimulated behavior of wild-type cells. The unstimulated behavior of five representative tethered cells of wild-type *E. coli* RP437 is shown in Fig. 1. Almost all the cells paused intermittently. The pauses were independent of the growth conditions (see Materials and Methods) and of the presence of an external energy source (the upper two cells versus the lower three cells). The fraction of time spent pausing varied from cell to cell and is given in the legend to Fig. 1. The average fraction of time spent pausing in a sample size of 12 cells was 4.8 ± 3.5%. The average frequency of pausing was 10 ± 7 pauses per min. The rotation rate of the cells varied from 4 to 13 Hz, the average being 7.6 ± 2.3 Hz. There was no apparent correlation in this sample of cells between the

rate of rotation or the fractional time of CW rotation and the fraction of time spent pausing (Fig. 2A). A rough correlation may exist between the frequency of the pauses and the average duration of a single pause (Fig. 2B).

Initially data were collected by observing video tapes frame by frame. Figure 3 shows the angles of an RP437 cell
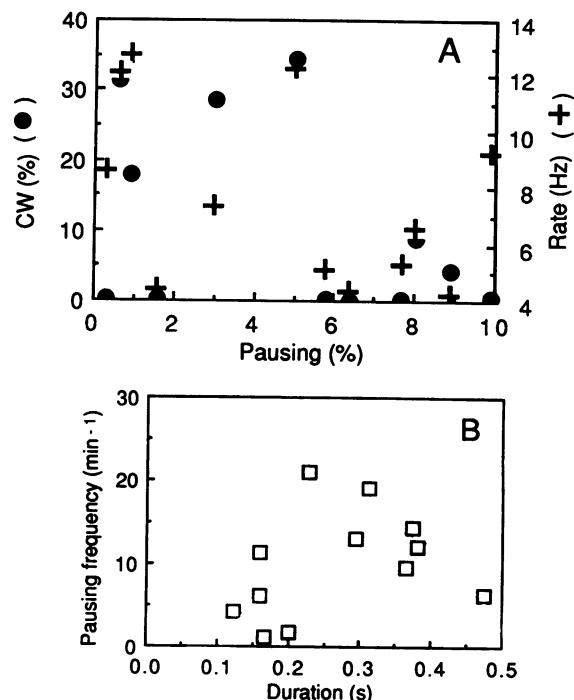

FIG. 2. Correlation between the various parameters of rotation and pausing. RP437 cells were used for the analysis, which was carried out as described in Materials and Methods. The sample size was 12 cells. The pauses of all the cells occurred at random angles. (A) Fraction of time spent rotating CW (●) and average rate of rotation (+) of the sample cells plotted against the fraction of time spent pausing. (B) Average pausing frequency of the sample cells plotted against the average duration of a pause of the same cell.
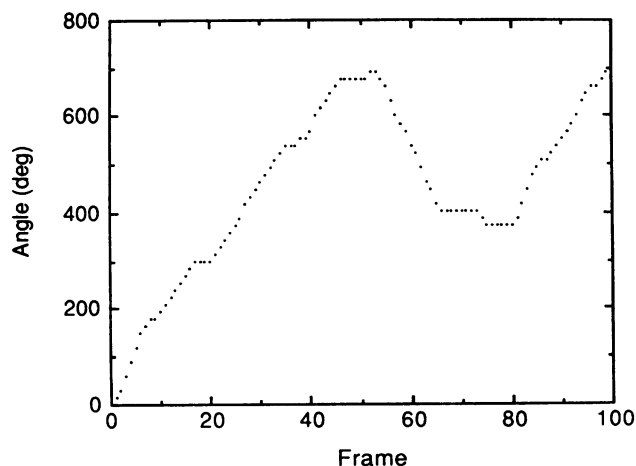

FIG. 3. Frame-by-frame analysis of an RP437 cell. The angle of rotation (in degrees) relative to the first frame analyzed is given as a function of the frame number. Each frame represents 20 ms. The entire record took place over 2 s.

in 100 consecutive frames (total time, 2 s). This particular cell was selected to illustrate the details of the pausing phenomenon. The rotation of the cell completely ceased during a pause (Fig. 3). No rapid reversals of direction of rotation (>20 ms) were observed in association with a pause. Since the duration of the pauses was usually longer than 60 ms (three frames), subsequent analyses were carried out with slow-motion tape as described in Materials and Methods.

We have observed at least two types of pauses: (i) pauses which may have resulted from interactions between the cells and the underlying glass surface and (ii) pauses which did not result from such interactions. The two types were distinguishable because the former occurred at fixed angles (or the cell repeatedly slowed down at a certain angle), whereas the latter occurred at arbitrary and apparently random angles. Pauses of the first type were observed in almost every field, but were disregarded. The data presented in this paper are for cells showing pauses of the second type only.

To examine a possible relation between pausing and the chemotaxis machinery of *E. coli*, we studied the effects of chemotactic stimuli on RP437, wild type for chemotaxis, and monitored the behavior of two chemotaxis mutants.

Stimulated behavior of wild type cells. Repellents and attractants had a significant effect on the pausing of the wild-type strain (Fig. 4). Since there were large variations in behavior from cell to cell, this figure shows detailed data on an individual cell rather than statistical data on a large number of cells. The same cell was exposed sequentially to a repellent and to attractants. In other experiments, when cells were exposed only to a repellent or only to attractants, the response was similar to that shown in Fig. 4. Upon addition of indole, a potent repellent of *E. coli* (29) and *S. typhimurium* (28), there was a sharp increase in the frequency and duration of the pauses; this increase occurred simultaneously with the well-known shift to CW bias. The fraction of time which the cell shown in Fig. 4 spent pausing increased from 5% before stimulation (time period 0 to 62 s) to 40% immediately after stimulation (62 to 87 s). When adaptation (27) occurred, the cell essentially resumed its unstimulated behavior (12% pausing in the interval between 87 and 120 s). Removal of indole resulted in almost incessant CCW rotation. Similarly, addition of the attractant mixture serine plus aspartate resulted in incessant CCW rotation. (Frame-by-frame analysis of cells in the same field revealed that the angular velocity of some cells increased in response to the addition of the attractants. The average relative change of the velocity of 13 cells was 1.8 ± 0.7.) After 6 min, removal of the attractants resulted in resumption of pauses and CW rotation (13% pausing for the period 987 to 1,020 s).

The experiment shown in Fig. 4 was carried out in the presence of an energy source (2 mM glycerol) to prevent fluctuations in the energy level as a result of metabolism of the attractants. Similar experiments were carried out (i) in the absence of glycerol and (ii) with nonmetabolizable attractants (α-aminoisobutyrate and α-methylaspartate). In both cases the same pattern of change in pausing frequency and duration as in Fig. 4 was observed (results not shown). Thus, it appears that the energy level of the cells is not directly involved in pausing. This conclusion is supported by studies of energy-depleted cells (M. Eisenbach, Y. Margolin, and A. Wolf, unpublished results).

The phenomenon of pausing was a response to all the repellents tested (Table 1). When relatively high repellent concentrations were used, the effect of repellent addition
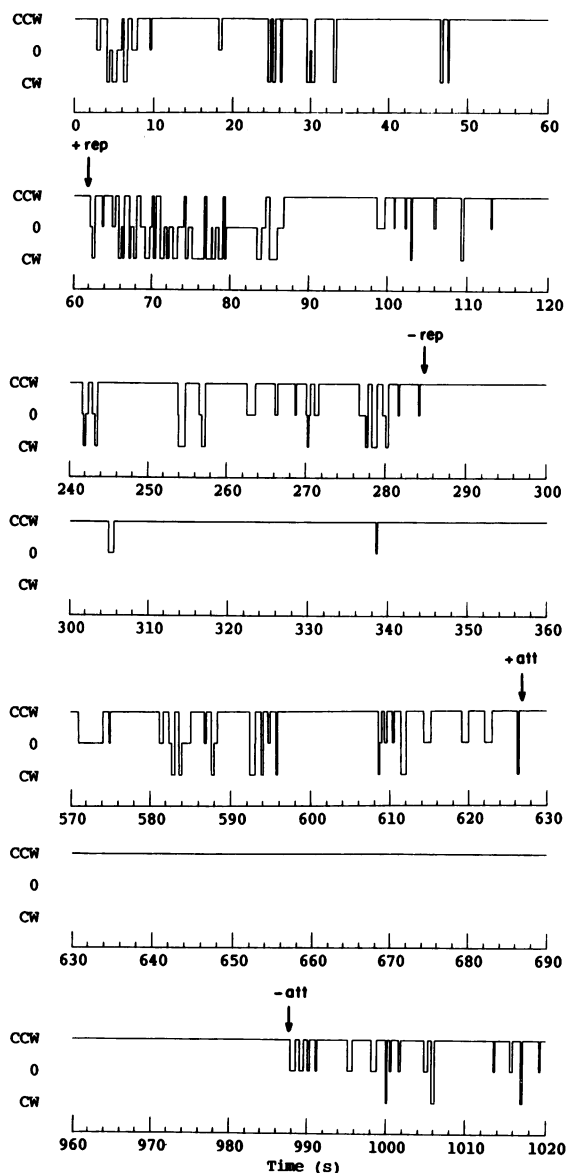

FIG. 4. Plot of the motion of a selected wild-type cell exposed to a repellent and to attractants. Cells in the flow chamber were originally in potassium phosphate-methionine-Tetren solution supplemented with glycerol (2 mM). The composition of the medium was changed by moving the input tube to another test tube containing a different medium. The time for the new medium to reach the flow chamber was determined independently to be 55 ± 14 s. Within this confidence interval, the times shown by arrows in the figure were determined from the changes in the rotational bias. The repellent (rep) was indole (0.5 mM). The attractants (att) were a mixture of L-serine (1 mM) and L-aspartate (10 μM). Data were collected continuously during a time interval of 17 min. Note that only segments of this time interval are shown.

was a complete stop of rotation. Rotation was resumed only if the repellent was removed within a few minutes.

To test whether the phenomenon of complete stopping was observed with swimming as well as with tethered cells, we added high repellent concentrations to a suspension of washed bacteria and examined the response of the cells under the microscope. Benzoate at 70 mM (pH 6.5) arrested the swimming completely. Spinning down the cells and

TABLE 1. Effects of repellents on the persistence of
rotation of tethered wild-type cells

Repellent and pH	Concn (mM)	Effect[a]
Acetate		
pH 5.5	40	Stopping
pH 6.0	10	Pausing, then stopping
Benzoate		
pH 5.0	20	Stopping
pH 6.5	70	Stopping
pH 6.7	50	Transient pausing
Indole	0.6	Transient stopping
	0.3	Transient pausing
NiSO$_4$	2	Stopping
	0.2	Transient pausing

[a] Pausing is defined here as a phenomenon in which cells alternate between brief periods (less than a few seconds) of no motion and periods of rotation. Stopping is a phenomenon in which cells cease their rotation for an extended period. Transient means that the phenomenon as a whole is temporary and is followed by the prestimulus behavior.

suspending them in benzoate-free medium restored the swimming of the cells.

Behavior of chemotaxis mutants. Table 2 includes observations with nonchemotactic mutants which represent two extremes with regard to pausing behavior. *E. coli* RP1091 is a mutant derived from RP437 and lacks a large part of the chemotaxis machinery as a result of a deletion of the genes from *cheA* to *cheZ* (21). Unlike the rotation of RP437 cells, that of RP1091 cells was completely incessant: no pauses were observed (except for some cells which paused at fixed angles and were disregarded). Addition of a repellent did not bring about pausing. A different phenomenon was observed with ST120, a switch mutant of *S. typhimurium*. Although this mutant had a classic response to stimuli with regard to the direction of rotation, it had an inverted response with regard to pausing: attractants caused more pausing, and repellents caused less pausing. The wild-type *S. typhimurium* strain, ST1, behaved like the wild-type *E. coli* strain, RP437.

The observations (i) that attractants and repellents alter the frequency of pausing in wild-type cells, (ii) that a mutant which lacks a large part of the chemotaxis machinery does not pause, and (iii) that a chemotaxis mutant has an aberrant response to chemotactic stimuli with regard to pausing suggest that the phenomenon of pausing is part of the chemotaxis machinery in bacteria.

DISCUSSION

The cells analyzed and described in this paper paused at arbitrary angles. However, many tethered cells paused at fixed angles, apparently as a result of mechanical hindrance. Applying the criteria discussed in Materials and Methods, we disregarded these cells. There did not appear to be any general difference between the behavior of cells which paused at fixed angles and those which paused at random angles. Some of the former had higher frequencies and durations of pauses, but others had comparable ones. (We suggest that the reason that pausing was previously overlooked as a factor in bacterial motility is that other investigators did not distinguish between these two types of pauses.)

Intermittent pauses, other than those due to mechanical hindrance, could, in principle, be the result of a number of factors: (i) chemotactic signaling, i.e., pausing as a consequence of a signal sent to the motor by the chemotaxis machinery; (ii) temporary exhaustion of a local pool of energy in the vicinity of the motor; (iii) an intrinsic property of the motor; (iv) an artifact of the tethering technique; (v) very rapid reversals of direction of rotation; and (vi) incomplete reversals of the flagellar motor. On the basis of the data presented above and the discussion below, we conclude that the pauses observed with tethered cells most probably result from factor (i).

Common criteria for the involvement of the chemotaxis machinery in any phenomenon are modulation of the phenomenon by chemotactic stimuli, absence of the phenomenon in mutants which lack the chemotaxis machinery, and anomalous behavior in specific chemotaxis mutants. All these criteria are fulfilled by the phenomenon of pausing: attractants eliminate pauses, whereas repellents enhance pausing (Fig. 4); different chemotaxis mutants have different degrees of unstimulated pausing (22) (Table 2); in the absence of a large part of the chemotaxis machinery there are no pauses (Table 2, RP1091); and a missense mutation in a gene which encodes a switch protein of the motor leads to an inverse pausing response (Table 2, ST120). It thus appears that the pauses at arbitrary angles result from a signal received by the motor from the chemotaxis machinery. It also appears that there may be separate signals for pausing and for changing the direction of flagellar rotation. The latter conclusion is based on the difference between the rotational and the pausing responses to stimuli (Table 2); on the lack of correlation between CW rotation and pausing in unstimulated cells (Fig. 2); and on the observation that tethered, cytoplasm-free cell envelopes (9) which do not respond to

TABLE 2. Behavior of tethered chemotaxis mutants[a]

Strain	Relevant genotype or phenotype[b]	Unstimulated behavior[c]	Effect[c] of addition of:	
			Attractants[d]	Repellent[e]
E. coli				
RP437	Wild type	0.3–9.9% P, CCW-cw	P↓, CCW↑	P↑, CCW↓
RP1091	Δ(*cheA-cheW*)	No P, CCW	None	None
S. typhimurium				
ST1	Wild type	0–8% P, CCW-cw	P↓, CCW↑	P↑, CCW↓
ST120	*flaQ* (*cheC* tumbly)	0–9% P, CW-ccw	P↑, CCW↑	P↓, CCW↓

[a] The behavior of the cells was assayed in the presence of glycerol (2 mM) as described in Materials and Methods.

[b] See reference 14 for the nomenclature of chemotaxis genes.

[c] The percentage represents the fraction of time spent pausing. Abbreviations: CCW-cw, CCW rotation with occasional brief periods of CW rotation; CW-ccw, CW rotation with occasional brief periods of CCW rotation; P, pausing, ↑ and ↓, increase or decrease, respectively.

[d] The attractants were L-serine (1 mM) and L-aspartate (10 μM).

[e] The repellent was benzoate (50 mM [pH 6.0 or 6.7]).

chemotactic stimuli by changing the direction of rotation (23, 24) do respond to repellents by stopping (M. Eisenbach, unpublished observations). The role of pausing in chemotaxis is currently being studied with a number of chemotaxis mutants.

The observations that the pauses can be eliminated by mutations or by chemotactic stimuli and that a given repellent can cause more or less pausing or have no effect, depending on the mutation (Table 2), argue against factors (ii) to (iv) as causes of pausing. If the pauses were the result of temporary energy exhaustion, an intrinsic property of the motor, or an artifact of the tethering technique, the pauses should also have been observed in strain RP1091 and chemotactic stimuli should either have no effect on the pauses or have the same effect in all strains. This was not the case (Table 2). The conclusion that the phenomenon of pausing is not correlated with the energy level is further supported by the observations that energization of the cells by glycerol had no effect on the frequency and duration of the pauses (glycerol was experimentally measured to significantly elevate the proton motive force [A. Wolf and M. Eisenbach, unpublished results]) (Fig. 1) and that there was no apparent correlation between the fraction of time spent pausing and the rate of rotation (Fig. 2). Other evidence that pausing is not merely an artifact of the tethering technique comes from the observation that on swimming cells, individual flagella which have been slowed down by external means in order to be visible in high-intensity dark-field microscopy have been seen to pause intermittently (16, 18). Furthermore, repellent concentrations which caused tethered cells to stop their rotation also caused swimming cells to come to a halt (see Results). Unstimulated swimming cells have not been observed to pause intermittently. However, this may be because pauses of different flagella are asynchronous in a given cell (16). Pausing of some flagella in a given cell in response to a low concentration of a repellent may not have been distinguished, because it occurred at almost the same time as reversal of the rotation of the flagella. As discussed below, both phenomena may lead to tumbling.

Possibility (v), i.e., that the pauses are primarily very rapid reversals of the flagella, was ruled out by our frame-by-frame analysis (Fig. 3). However, it is possible, but highly unlikely, that reversals could occur in a time interval shorter than 20 ms (the duration of a single frame on our video tape). Also, the data cannot exclude the last possibility, i.e., that the pauses are the manifestation of a defective switching process. Studies with chemotaxis mutants, aimed at answering this question, are under way.

The results with ST120 (Table 2) indicate that pausing, as well as CW rotation, may be involved in tumbling. ST120 is known to have an inverse swimming response to chemotactic stimuli (12; our unpublished observations). Nevertheless, tethered ST120 cells respond conventionally to stimuli with regard to the direction of rotation, i.e., an increased or decreased fraction of time in CCW direction in response to attractants or repellents, respectively (12) (Table 2). Khan et al. (12) have shown that the smooth swimming of this strain can derive from CW rotation of the flagella. They also suggested that tumbling must result from brief motor reversals. The data in Table 2 suggest another interpretation, according to which the pauses also are a cause of tumbling: addition of attractants to ST120 causes more pausing, with resultant tumbling, whereas addition of repellents eliminates pausing, with resultant smooth swimming. Thus, one of the phenotypes of the mutation in the switch mutant, ST120, is an inverse pausing response (but a "normal" reversal response), which leads to an inverse swimming response. If this interpretation is correct, the phenomenon of pausing should be an important determinant of the behavior of the bacteria. Studies of other switch mutants are currently in progress.

Recent studies have indicated that pausing is a common phenomenon in various species of bacteria, i.e., *Halobacterium halobium* (1), *Rhodobacter sphaeroides* (2), and *Rhizobium melitoti* (10). The flagella of the last two species do not reverse; they can only rotate in the CW direction or pause. Thus, pausing may be widespread in bacteria.

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