

Rhizobium meliloti Swims by Unidirectional, Intermittent Rotation of Right-Handed Flagellar Helices

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The 5 to 10 peritrichously inserted complex flagella of *Rhizobium meliloti* MVII-1 were found to form right-handed flagellar bundles. Bacteria swam at speeds up to 60 $\mu\text{m/s}$, their random three-dimensional walk consisting of straight runs and quick directional changes (turns) without the vigorous angular motion (tumbling) seen in swimming *Escherichia coli* cells. Observations of *R. meliloti* cells tethered by a single flagellar filament revealed that flagellar rotation was exclusively clockwise, interrupted by very brief stops (shorter than 0.1 s), typically every 1 to 2 s. Swimming bacteria responded to chemotactic stimuli by extending their runs, and tethered bacteria responded by prolonged intervals of clockwise rotation. Moreover, the motility tracks of a generally nonchemotactic ("smooth") mutant consisted of long runs without sharp turns, and tethered mutant cells showed continuous clockwise rotation without detectable stops. These observations suggested that the runs of swimming cells correspond to clockwise flagellar rotation, and the turns correspond to the brief rotation stops. We propose that single rotating flagella (depending on their insertion point on the rod-shaped bacterial surface) can reorient a swimming cell whenever the majority of flagellar motors stop.

Mechanisms of flagellar rotation and chemotaxis in bacteria have been extensively studied in a few species, notably *Escherichia coli* and *Salmonella typhimurium* (for reviews see references 7 and 17). These bacteria swim by the clockwise (CW) and counterclockwise (CCW) rotation of their helical filaments; frequent switches between the two modes of rotation enable them to change direction. Tactic stimuli alter the switching frequency, thereby biasing the overall movement in a favorable direction. Intrinsic to this concept (at least in these species) are polymorphic changes of the flagellar filament (10): during propulsion of the cell (CCW rotation) the left-handed helical waveform is stabilized by the viscous torsional resistance of the medium; CW rotation induces a polymorphic transition to right-handedness (progressing from the flagellar base), which leads to "tumbling" and reorientation of the cell. Hence, the physical properties of the flagellar filament are an important factor in bacterial swimming behavior (20).

Two types of flagellar filaments, termed plain and complex, have been observed. Plain filaments are found in the majority of swimming bacteria studied, including *E. coli* and *S. typhimurium*. Complex flagella have been observed in certain soil bacteria, such as *Pseudomonas rhodos* 9-6 (23), *Rhizobium lupini* H13-3 (21, 22) and *Rhizobium meliloti* MVII-1 and 2011 (8, 14). Plain and complex flagella differ in their physicochemical properties and their fine structure. Complex filaments are more fragile (and, by implication, more rigid), are more resistant to heat depolymerization, have a higher content of hydrophobic amino acids, and, by electron microscopy, have a conspicuous surface structure that is dominated by a set of three-start helical ridges. Two- and three-dimensional reconstructions of highly resolved electron micrographs (27; S. Trachtenberg, D. J. DeRosier, and R. M. Macnab, *J. Mol. Biol.*, in press) suggest that the complex filament structure differs from the plain filament structure by pairwise perturbations of the flagellin subunits

to generate the prominent three-start helix. Together with unique tubular features seen in the filament, the helical ridges may account for the brittleness and rigidity of the complex filament (Trachtenberg et al., in press).

We have previously shown (8) that *Rhizobium* strains with peritrichously inserted complex flagella swim efficiently in viscous media and are capable of responding to chemotactic stimuli. In view of the distinctive features of the complex flagellar filaments, *R. meliloti* MVII-1 was chosen for a study of flagellar gross morphology, flagellar rotation, and swimming patterns in the presence and absence of tactic stimuli. The results reported here show that complex filaments form right-handed helices. Cells of *R. meliloti* swim and change direction by an alternation between CW rotation and brief stops of flagellar rotation. They respond to tactic stimuli by extending the duration of CW rotation and, hence, the length of unidirectional "runs."

MATERIALS AND METHODS

Bacterial strains. *R. meliloti* wild-type strain MVII-1 (12) and the high-motility mutant 10406 (producing about twice as many flagella as the parent) have been previously described (14). The "smoothly" swimming strain 10463 was selected, on swarm plates containing 0.1 mM L-proline, as a generally nonchemotactic mutant (15) of *R. meliloti* MVII-1.

Media and growth conditions. Bacteria were grown in RB minimal medium supplemented with vitamins (8) and 0.2% mannitol as the sole carbon source in a rotary shaker bath (G76; New Brunswick Scientific Co., New Brunswick, N.J.) at 30°C. Swarm plates containing RB salts, L-proline (0.1 mM), and agar (0.3%; Difco Laboratories, Detroit, Mich.) were inoculated in the center and incubated at 30°C for 2 days. Motile cells were picked from the periphery of growth.

Antibody preparation. Complex flagellar filaments were detached from bacteria grown on plates and purified by differential centrifugation as previously described (14). Antibody against purified whole filaments was prepared from rabbits as described (21). Any somatic antibodies were removed from the serum by "depletion" (6) using flagella-free *R. meliloti* cells obtained after mechanical detachment

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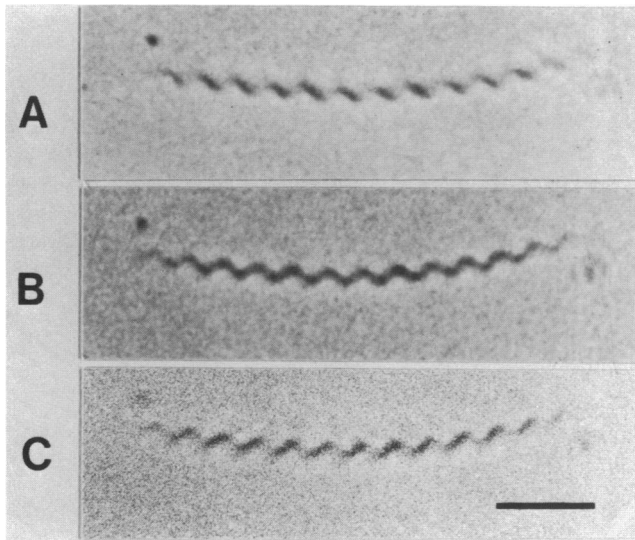


FIG. 1. A set of micrographs focusing on (A) the top, (B) the middle, and (C) the bottom of a bundle of *R. meliloti* RU10406 flagellar filaments. Bar, 5 μm .

of the flagellar filaments (14). The depleted antiserum was stored at -20°C .

Observation of tethered bacteria. Suspensions of motile *R. meliloti* cells ($4 \times 10^8/\text{ml}$) in buffer (10 mM potassium phosphate, pH 7.0, 1 mM magnesium sulfate) were passed 15 times through a 26-gauge needle to shear flagellar filaments. This avoids attachment to glass by more than one filament, which would prevent rotation. Cells were separated from detached filaments by centrifugation and then suspended in buffer to 3×10^8 cells per ml. A small chamber, consisting of a microscope slide and a cover slip positioned on two fixed cover slips, was used for the tethering experiments. After the slide was coated with dilute (1:300) anti-flagella antibody, the chamber was filled with 30 μl of cell suspension and kept at 28°C for 10 min to allow for antibody-mediated adherence of flagellar stubs to the slide surface. The chamber was then carefully rinsed with buffer to remove nonattached cells (9). Bacteria attached to the slide by single flagella were observed through a Zeiss Standard 14 phase-contrast microscope (Zeiss, Oberkochen, Federal Republic of Germany). The observer thus looks from the rotating cell body towards the tethered flagellum. Frequencies of starts and stops and rotation rates were determined from video recordings (Sony SL-C7E) of the tethered cells.

Observation of detached flagellar bundles and of swimming bacteria. Bundles of flagellar filaments were prepared by mixing purified flagella (100 mg/ml; 14) suspended in 10 mM sodium chloride–10 mM potassium phosphate (pH 7) buffer with an equal volume of 1% Methocel 65HG (Fluka, Neu-Ulm, Federal Republic of Germany) dissolved in the same buffer (24). Helical bundles were examined by phase-contrast light microscopy using a Leitz Ortholux II microscope (Leitz, Wetzlar, Federal Republic of Germany) equipped with a 60-W halogen lamp, an oil-immersion phase-contrast condenser (NA 0.9), and an NPL Fluotar objective ($\times 100$, NA 1.32). Micrographs were taken with an automatic Leitz Vario-Orthomat camera at 400-fold magnification.

Observation of swimming bacteria. Swimming bacteria (3×10^7 cells in 80 μl) were placed in a Neubauer chamber equipped with an optical grating (50 by 50 μm) and examined by phase-contrast microscopy. Cells were chemotactically

stimulated by the addition of 20 μl of attractant. The path of swimming cells was videorecorded and traced on transparent foil mounted on the video screen. Distances were determined by reference to the grating of the chamber. All experiments were performed at 28°C in a controlled-temperature room.

RESULTS

Handedness of flagellar filaments. The handedness of the flagellar helix can be determined by dark-field light microscopy of swimming bacteria (16, 20) or by phase-contrast microscopy of detached flagellar bundles (24). Attempts to apply the former method to swimming cells of *R. meliloti* were not successful, since the filaments were too short to extend sufficiently beyond the light halo of the cell for inspection (R. Schmitt and R. M. Macnab, unpublished data). We therefore determined the helical handedness of bundles of detached complex flagella by the method of Shimada et al. (24). Figure 1 shows a sequence of phase-contrast micrographs focussing on (A) the top, (B) the middle, and (C) the bottom of a flagellar bundle and demonstrates that the complex flagellar filaments of *R. meliloti* 10406 have a right-handed helix. Under the near-physiological conditions used (10 mM salts, pH 7), the helical period was 1.8 μm and the amplitude was 0.3 μm . These values compare to 1.56 and 0.29 μm , respectively, determined by electron microscopy of the negatively stained filaments (pH 4.5; 14).

Behavior of swimming cells. Tracks of free-swimming cells were video recorded and traced on transparent foils mounted on a video screen (Fig. 2). *R. meliloti* MVII-1 wild type swam at speeds up to 40 $\mu\text{m}/\text{s}$ (8); the high-motility derivative 10406 (which overproduces flagella; 14) attained speeds up to 60 $\mu\text{m}/\text{s}$. Unidirectional swimming (runs) alternated

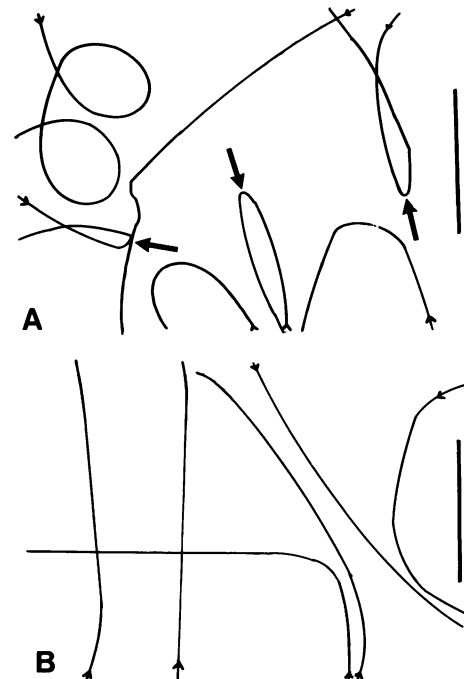


FIG. 2. Motility tracks of (A) *R. meliloti* RU10406 (wild type) and (B) the general nonchemotactic (smooth) mutant RU10463. Arrowheads on the tracks indicate start and direction of swimming; arrows indicate turns. Bar, 100 μm .

with sudden directional changes (turns) marked by arrows in Fig. 2A), typically every 1 to 2 s. The turns were quick, and cells did not exhibit the vigorous angular motion (tumbling) observed in species with plain flagella like *E. coli* and *S. typhimurium* (5, 20). The wide-circle tracks also seen in Fig. 2A do not represent turns, but reflect the frictional resistance of counter-rotating cells that swim close to the glass surface, a phenomenon also observed with other motile bacteria (26). It was found that the direction of curved trajectories was CW when the cell swam close to the upper glass surface and CCW when it swam close to the lower glass surface (see Fig. 2A). Thus, by inference *R. meliloti* cells are propelled by CW-rotating flagellar motors, a notion in accord with the data from tethered cells (below). *R. meliloti* 10463, isolated as a generally nonchemotactic mutant, was found to swim unidirectionally for up to 700 μm without any turns (Fig. 2B). This strain resembles the smooth mutants of *E. coli*, which rotate their flagella exclusively in the CCW sense (4, 15). Like these, *R. meliloti* 10463 has lost the device that generates directional changes of swimming.

Chemotaxis in *E. coli* and *S. typhimurium* occurs by variation of the tumbling frequency (5, 19). Increasing attractant concentrations cause cells to tumble less frequently and, hence, to make longer runs. We have previously shown by capillary assays that amino acids like L-proline, L-phenylalanine, and L-glutamate are attractants (in this order of strength) of *R. meliloti* cells (8). To examine the swimming behavior of *R. meliloti* under tactic stimuli, temporal gradients (19) of attractant were applied. Various concentrations of the three amino acids were added to a motility chamber containing swimming *R. meliloti* 10406 cells in buffer, and the average lengths of unidirectional runs were determined 0.5 to 5 min after stimulation. During this period of time the response was at maximum (data not shown). Stimulation by 10^{-2} M L-proline, L-phenylalanine, or L-glutamate increased the mean run length from 90 μm to 350, 220, or 140 μm ,

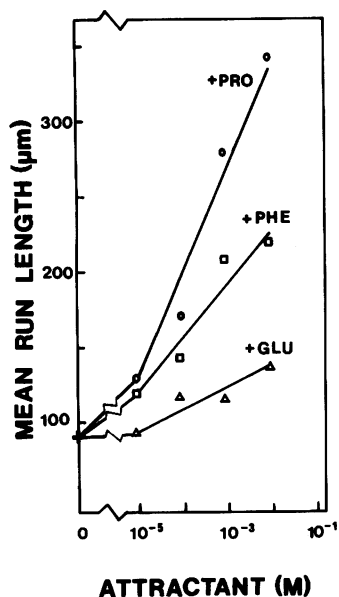


FIG. 3. Averaged lengths of runs of swimming *R. meliloti* RU10406 cells as a function of the type and concentration of a chemotactic attractant. Temporal gradients (19) of proline (\circ), phenylalanine (\square), and glutamate (\triangle) were applied by switching from buffer to the indicated concentration of the attractant. Each point represents the mean of 60 independent runs measured 0.5 to 5 min after stimulation.

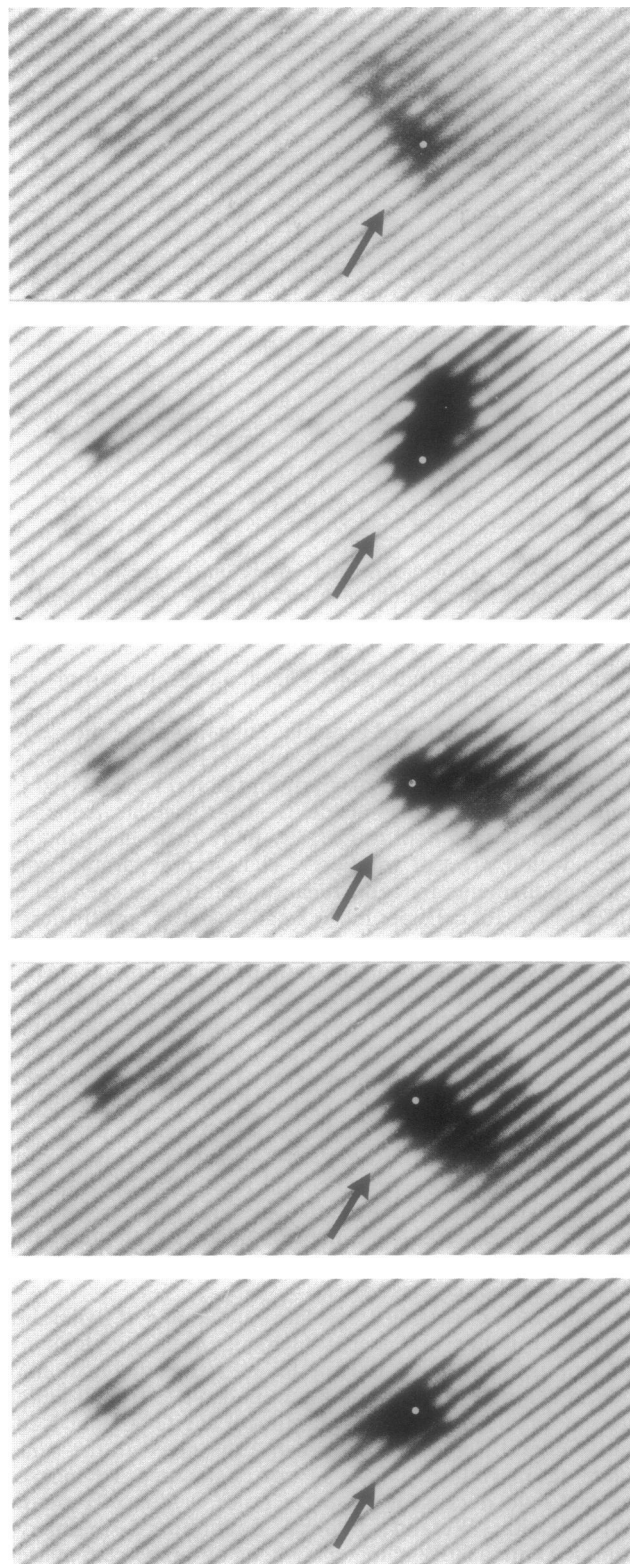


FIG. 4. Rotation of an *R. meliloti* RU10406 cell (arrow) tethered by one flagellum to the microscope slide (below the cell). The rotation was recorded through a Zeiss phase-contrast microscope onto video tape and then transferred to film. Five sequential phases are shown. The arrow mounted in a fixed position and the spot at the left serve as directional references. The white dot marks the apparent rotation axis of the cell.

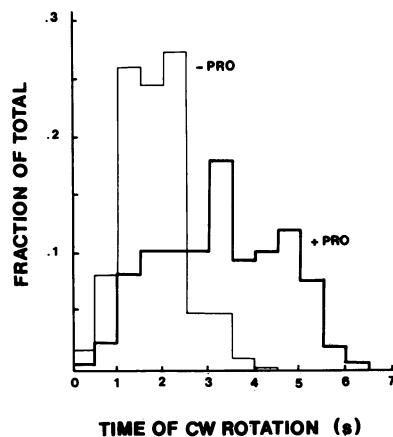


FIG. 5. Proportion of intervals of CW rotation of tethered cells in the presence (thick line) and absence (thin line) of attractant (1 mM L-proline). Measurements were taken 0.5 to 5 min after stimulation. The histogram was composed from 80 independent determinations pooled into 0.5-s increments (using eight tethered cells). Numbers of determinations within an increment are plotted as a fraction of the total number of determinations either with or without L-proline.

respectively (Fig. 3). Thus, depending on the strength of the tactic stimulus, *R. meliloti* reacted by a corresponding increase in the lengths of runs. This behavior compares with the tactic response of the well-studied enterobacteria (5, 19), and it was of interest to elucidate the mode by which *R. meliloti* cells temporarily prevent turns in their swimming paths.

Behavior of tethered cells. *R. meliloti* cells tethered by single flagella to the microscope slide by anti-flagella antibody (25) rotated exclusively in the CW sense as viewed from above, corresponding to CW rotation of the flagella on free-swimming cells. This is documented by the series of photographs of a single tethered bacterium shown in Fig. 4. Many tethered cells were inspected: all exhibited CW rotation at constant angular velocity (absolute speed varying from cell to cell), with very brief intermittent stops (<0.1 s). These stops could easily be observed by eye, but were difficult to resolve by video recording at 30 frames per s (they often appeared as "slow downs" between intervals of constant rotational speed).

In an attempt to relate the rotation pattern of single flagella to the behavior of the swimming cell, we have timed and averaged 10 intervals of interrupted CW rotation for each of eight tethered cells in the presence and absence of a chemotactic attractant (8). Slow-motion video recordings were used for this analysis. The interval values were pooled at 0.5-s increments, and each pool was plotted as a fraction of total measurements. The resulting distributions (Fig. 5) indicate a mean of 1.8 s for intervals of CW rotation in nonstimulated cells and a mean of 3.3 s for cells stimulated with 1 mM L-proline (8). These results strongly suggest that *R. meliloti* responds to attractants by extending the time of CW rotation, i.e., by suppressing stops between intervals of CW rotation.

We have thus correlated CW rotation of the right-handed flagellar filaments of *R. meliloti* MVII with runs and the brief stops of flagellar rotation with turns of the swimming cells. This interpretation was supported by the behavior of tethered cells of the generally nonchemotactic smooth *R. meliloti* strain 10463 (Fig. 2B), which showed continuous CW rotation for up to 7 min without any detectable stop.

DISCUSSION

The peritrichously inserted complex flagella of *R. meliloti* form right-handed helical bundles that rotate only in the CW sense. Intervals of CW rotation (typically 1 to 2 s) alternate with brief stops (<0.1 s). CW rotation was correlated with forward swimming (runs), and the stops were correlated with sudden directional changes (turns) of swimming cells. This correlation was confirmed by the observed swimming and rotation patterns of the smooth mutant strain 10463. Tactic stimulation of wild-type *R. meliloti* induced prolonged intervals of CW rotation and, correspondingly, longer runs. The overall swimming behavior of *R. meliloti* thus resembles that of bacteria like *E. coli*, but turns were briefer and without the vigorous angular motion (tumbling) characteristic of the latter (5, 15). This slight behavioral difference is thought to be a consequence of the distinctive CW-stop rotation pattern of the complex flagella as opposed to the CCW-CW switching of the polymorphic plain flagella (20).

Handedness of the flagellar helix, sense of flagellar rotation, and consequent conversion of torque into thrust are interrelated (20). Most flagellated bacteria studied have left-handed filaments, swim by CCW rotation, and reorient by CCW-CW switching. The few known exceptions with right-handed flagellar helices are two species of halophilic archaeobacteria (1, 2), *Caulobacter crescentus* (13), and *Rhodobacter sphaeroides* (3). Cells of the former three species appear to swim forward and backward by alternating between CW and CCW rotation of their (rigid) right-handed flagellum (*C. crescentus*) or flagellar bundles (halophiles). This mode of swimming does not require turns of the cell body, quite distinct from the tumbling of *S. typhimurium* cells produced by the CW rotation of polymorphic filaments (20). The rotation pattern of the single *R. sphaeroides* flagellum, which involves unidirectional CW rotation and periodical stopping (3), is the pattern most similar to that of *R. meliloti* flagellar rotation. However, there are at least three differences between the two systems: (i) *R. sphaeroides* is driven by a single plain flagellum, whereas *R. meliloti* has 5 to 10 peritrichously inserted complex flagella (8); (ii) the *R. sphaeroides* flagellar filament assumes a curious coiled structure during stops (facilitating, by implication, Brownian motion of the cell; 3), whereas the complex filaments of *R. meliloti* are brittle and presumably rigid (14; Trachtenberg et al., in press) with little if any structural polymorphism; and (iii) in *R. sphaeroides* cells the velocity of flagellar rotation is variable and intermittent stops last up to several seconds (3), whereas in *R. meliloti* the rotational velocity of single flagella appears to be constant and stops are very brief (less than 0.1 s).

The major difference between these various systems of bacterial swimming pertains to the mode by which directional changes are attained and controlled. This is the crucial step in the "biased random walk" of swimming cells. Hence, in any tactic response, nature has obviously tried a number of mechanisms that are now working in various bacterial species. In addition to tumbling, forward-and-backward alternation, and Brownian motion, we propose here that the turns characteristic of swimming *R. meliloti* cells represent still another mechanism. This is borne out by two observations. (i) First, efficient swimming with complex filaments requires either peritrichous (*R. meliloti*, *R. lupini* H13-3) or subpolar (*P. rhodos* 9-6) flagellation with more than one flagellum per cell. Cells of the mutant strain *P. rhodos* B9, bearing a single complex flagellum, show rapid "spinning" but no translational motion (23). (ii) Second, switching

between CCW and CW rotation of individual flagella on a single *S. typhimurium* cell is asynchronous and, hence, independently controlled (11, 18). The "voting hypothesis" of Ishihara et al. (11) claims that a functional bundle is formed whenever one half or more of the motors rotate in the CCW sense.

We propose here a model for the swimming behavior of *R. meliloti* cells, which adapts the voting hypothesis. These cells possess 5 to 10 peritrichously inserted complex flagella (8). We assume individual control of flagellar rotation (asynchrony) and that cells only swim forward when the majority are in CW rotation and a bundle is formed. If less than half of the motors rotate, while the others stop, single flagella in CW rotation are still capable of conferring thrust to the cell. Provided these individually rotating flagella are inserted subpolarly or laterally, the cell will change direction owing to its rod-shaped geometry, similar to the *P. rhodos* mutant strain B9 (23). As is discussed by Ishihara et al. (11), the voting hypothesis may be too simple an assumption for *E. coli*, since the fit of data (swimming/tumble periods versus observed time of individual flagella spent in CCW or CW rotation) is not very good. Although the results with tethered *R. meliloti* cells tend to be more favorable (longer periods of CW rotation, briefer stops), the present data are still too crude for computing bias correlations of the flagellar motors (11) in support of the model. Brownian motion may also be operative in producing directional changes (3); however, the briefness and extreme reorientation seen in many turns argue against this being the major force in changing the direction of swimming *R. meliloti* cells.

The proposed model also accounts for the observed response to tactic stimuli, which increase the length of runs and the duration of CW rotation. If the bias of individual flagellar motors towards CW rotation increases with the stimulus (Fig. 3), the probability of individual flagellar filaments forming a functional bundle is higher (majority of filaments in CW rotation), a condition that results in longer runs. Its distinctive features make *R. meliloti* an attractive organism for the study of motility and chemotaxis. It will be interesting to see whether the principal mechanisms found in other bacteria (7, 17) are applicable or whether peculiarities of the *R. meliloti* system open new insights into the functioning and control of the flagellar motor.

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