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Direction of Flagellar Rotation in Bacterial Cell Envelopes

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Cell envelopes with functional flagella, isolated from wild-type strains of *Escherichia coli* and *Salmonella typhimurium* by formation of spheroplasts with penicillin and subsequent osmotic lysis, demonstrate counterclockwise (CCW)-biased rotation when energized with an electron donor for respiration, DL-lactate. Since the direction of flagellar rotation in bacteria is central to the expression of chemotaxis, we studied the cause of this bias. Our main observations were: (i) spheroplasts acquired a clockwise (CW) bias if instead of being lysed they were further incubated with penicillin; (ii) repellents temporarily caused CW rotation of tethered bacteria and spheroplasts but not of their derived cell envelopes; (iii) deenergizing CW-rotating *cheV* bacteria by KCN or arsenate treatment caused CCW bias; (iv) cell envelopes isolated from CW-rotating *cheC* and *cheV* mutants retained the CW bias, unlike envelopes isolated from *cheB* and *cheZ* mutants, which upon cytoplasmic release lost this bias and acquired CCW bias; and (v) an inwardly directed, artificially induced proton current rotated tethered envelopes in CCW direction, but an outwardly directed current was unable to rotate the envelopes. It is concluded that (i) a cytoplasmic constituent is required for the expression of CW rotation (or repression of CCW rotation) in strains which are not defective in the switch; (ii) in the absence of this cytoplasmic constituent, the motor is not reversible in such strains, and it probably is mechanically constricted so as to permit CCW sense of rotation only; (iii) the requirement of CW rotation for ATP is not at the level of the motor or the switch but at one of the preceding functional steps of the chemotaxis machinery; (iv) the *cheC* and *cheV* gene products are associated with the cytoplasmic membrane; and (v) direct interaction between the switch-motor system and the repellent sensors is improbable.

Bacteria swim by rotating their flagella (5, 39). In the case of peritrichous bacteria, counterclockwise (CCW) rotation leads to smooth swimming and clockwise (CW) rotation leads to tumbling (23). Chemical stimuli affect the direction of flagellar rotation and, consequently, also the swimming mode of the bacteria (6, 23, 27, 28).

A subcellular system consisting of cell envelopes devoid of cytoplasm, but with functional flagella, was recently isolated from *Escherichia coli* and *Salmonella typhimurium* (10). The isolation procedure included two main steps: (i) formation of osmotically sensitive cells (spheroplasts) by a relatively short incubation period with penicillin and (ii) osmotic lysis of these spheroplasts. Upon the addition of an electron donor for respiration, the flagella of these cell envelopes rotated. It was reported, however, that unlike the flagella of intact wild-type bacteria, which rotated in both directions, those of the cell envelopes rotated almost exclusively in one direction, CCW (10). The cause of this biased rotation was not determined in that report. Since control of the direction of flagellar rotation is the mechanism of chemotaxis in bacteria, investigation of this question is important. The purposes of this study were to investigate the cause of the loss of CW rotation and to determine whether the envelopes can be made to rotate in this direction.

MATERIALS AND METHODS

Bacteria. The *E. coli* strain used in this study was RP487 (14), a K-12 derivative and wild type for chemotaxis. It was a gift from J. Adler, University of Wisconsin-Madison. *S. typhimurium* strains ST1 (wild type for chemotaxis), ST450 (*cheB*), ST120 (*cheC* 70), and ST171 (*cheZ*) (3) were a gift from D. E. Koshland, Jr., University of California-Berkeley, and strain MY1 (*cheV*) (8) was a gift from R. M. Macnab, Yale University, New Haven, Conn. The cells were grown in tryptone broth (*E. coli*) or nutrient broth (*S. typhimurium*) as previously described (10).

Preparations. Cell envelopes were isolated by penicillin treatment and subsequent osmotic lysis as previously described (10), with one exception: the termination of the incubation period with penicillin was determined when the outer membrane of ~3% of the spheroplast population had partially been detached (under the phase microscope, these cells appear to have "wings" at their edges [see Fig. 1 in reference 16]). This assured that the majority of the cell population was ready for lysis. (In contrast to our claim in a previous communication [10], a hole in the cell wall could not be observed by the phase microscope.) The lysis medium contained 50 mM KP_i (pH 6.6) and 0.1 mM EDTA. Since *S*-adenosylmethionine and ATP in the lysis medium had no effect on the sense of rotation (10), they were not included. The number of rotating cell envelopes was dependent on the preparation and varied between 0.1% and 1.0% of the total number of cell envelopes. This estimate does not include cell envelopes that were too faint, small, fast, or round to allow their direction of rotation to be determined with confidence. Spheroplasts were prepared by the same procedure as that for cell envelopes, excluding the step of osmotic lysis: after 38 to 44 min of incubation with the penicillin-containing medium, chloramphenicol was added (final concentration, 50 μg/ml), and the incubation was then continued for various periods of time.

Observation of flagellar rotation. Cells were tethered to a microscope cover glass (commercially precleaned and without further acid cleaning) in a thermostated (30°C) flow chamber by means of antibody to flagellin as previously described (10). (The source of the cover glass may be crucial

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for observing rotation of tethered envelopes. Cover glasses of a certain manufacturer totally inhibited rotation. We used Chance Proper cover glasses). The rotation of tethered envelopes was recorded by a video system connected to a Zeiss phase-contrast microscope as previously described (35).

Swimming behavior. The cells were observed in the same thermostated chamber as above. Swimming behavior was recorded and then analyzed at slow motion. Temporal assays (28) were performed as previously described (14, 40). Tracks of swimming were viewed and photographed with a Zeiss dark-field microscope.

Electron microscopy. Samples for negative staining and thin sections were prepared as previously described (10) and viewed with a JEOL transmission electron microscope (JEM-7).

RESULTS

Loss of CW rotation in cell envelopes could result from any of the following causes (or combinations thereof): penicillin treatment per se, morphological changes in the cell wall, reduced proton-motive force (PMF) in the cell envelopes (18) (the PMF is the driving force for flagellar rotation in bacteria [10, 13, 19, 22, 29, 31, 41]), and loss of a cytoplasmic component involved in generating this sense of rotation (or in repressing the opposite sense of rotation). We have examined these possibilities one by one.

CCW bias is not the consequence of penicillin treatment. Table 1 includes observations with *E. coli* cells before and after penicillin treatment. Penicillin treatment per se caused CW bias of rotation, part of which was the consequence of incubation with the hypertonic Penassay medium (Difco Laboratories). Since this rotational bias is in the opposite sense of rotation to that observed with envelopes, penicillin treatment is not the cause of the CCW bias.

TABLE 1. Direction of rotation of tethered cells treated with penicillin

Treatment of cells[a]	Flow medium	Cell proportion[b]	Sample size (no. of cells)
Untreated	KP_i-EDTA-Mg^{2+}-met[c]	85% CCW, 15% CCW-cw	67
Incubation for 44 min in penicillin-less broth[d]	Penicillin-less broth	51% CCW, 22% CW, 27% random	82
Incubation for 44 min in penicillin-containing broth[e]	Penicillin-containing broth	58% CW-ccw, 42% CW	12[f]

[a] The strain used was *E. coli* RP487.

[b] CCW-cw, CCW rotation with occasional, brief periods of CW rotation; CW-ccw, CW rotation with occasional, brief periods of CCW rotation.

[c] Final concentrations: 10 mM KP_i (pH 7.0), 0.1 mM EDTA, 5 mM $MgSO_4$, 0.1 mM L-methionine (met).

[d] Broth was 75% (vol/vol) Penassay broth and 25% tryptone broth.

[e] Penicillin concentration was 750 U/ml.

[f] This relatively small number of spheroplasts reflects the difficulty of tethering these cells (see the text).

CCW bias is not the consequence of mechanical damage or morphological changes in the cell wall. To investigate the effect of cell wall damage on flagellar rotation, we used the spheroplast system from which envelopes are prepared. These spheroplasts differ from intact bacteria by virtue of structural and permeability changes caused by the damage to the cell wall. Also, although the damage to the cell wall in spheroplasts and envelopes is basically the same, spheroplasts still possess the cytoplasm, whereas envelopes do not. (These spheroplasts, unlike regular penicillin spheroplasts [16, 24, 44], were incubated with penicillin only until a crack in the cell wall was formed. Consequently they, as well as the cell envelopes prepared from them, had the same shape as intact bacteria [10].) Chloramphenicol was added to prevent further swelling of the cells (10, 44), and the behavior of the spheroplasts was watched for extended incubation periods. The cells became spherical, and their outer membrane became partially removed (Fig. 1). The effects of these changes on the swimming mode of the spheroplasts are shown in Fig. 2 and 3. The swimming speed (Fig. 2A), as well as the number of swimming cells (Fig. 2B), decreased, but the swimming mode (Fig. 2B and 3) was unaffected for as long as 4 h after the addition of penicillin, and then the cells progressively tumbled more (expressed in Fig. 2B as loss of wild-type-like swimming; no smooth swimming was observed). Although flagellated spheroplasts were observable during the entire period of incubation tested (Fig. 1), their relative number declined with time (Fig. 2B). The loss of motility was the consequence of flagellar loss: a linear correlation between the fraction of motile cells and the fraction of cells with three or more flagella was observed (correlation coefficient, 0.995).

Flagellar rotation in these spheroplasts was not monitored because the total number of cells that could be tethered to the glass was markedly reduced by penicillin treatment (last column of Table 1). Cells could not be tethered at all after an additional 1 h of incubation with chloramphenicol. Spheroplasts that were tethered immediately after the addition of chloramphenicol (after 44 min of incubation with penicillin) either stopped their rotation (ca. 75% of the cells) or detached from the glass (or disintegrated) within 50 min of incubation with chloramphenicol. Every spheroplast had a CW bias for 10 to 15 min before its rotation stopped. This observation, together with the observation that there was no major change in the swimming mode (Fig. 2 and 3) during the period in which most mechanical damage occurred, indicates that the CCW bias is not the consequence of mechanical damage to the cell wall.

CCW bias is not the consequence of a reduced PMF. It has been shown that a reduced PMF in bacteria causes a reduced speed and smooth swimming (equivalent to CCW bias of flagellar rotation) (18). To examine whether this could be the cause of the bias in cell envelopes, we studied a *cheV* mutant of *S. typhimurium* (8). This strain has two properties that make it suitable for the purpose: (i) CW-biased rotation as a consequence of a defective gene product that is presumably associated with the cytoplasmic membrane (8, 33) and thus may not be lost during envelope isolation, and (ii) sensitivity to a reduced PMF. Reduction of the PMF indeed caused CCW bias in *cheV* bacteria, whereas *cheV* cell envelopes had a CW bias of rotation (Table 2). The effect of arsenate on the direction of rotation was probably due, in part, to its causing a decrease in the ATP level of the bacteria (38). (In similar experiments with a *cheC* mutant, the direction of rotation of the bacteria was insensitive to a reduced PMF: arsenate [L. V. Gofshtein and M. Eisenbach, unpublished

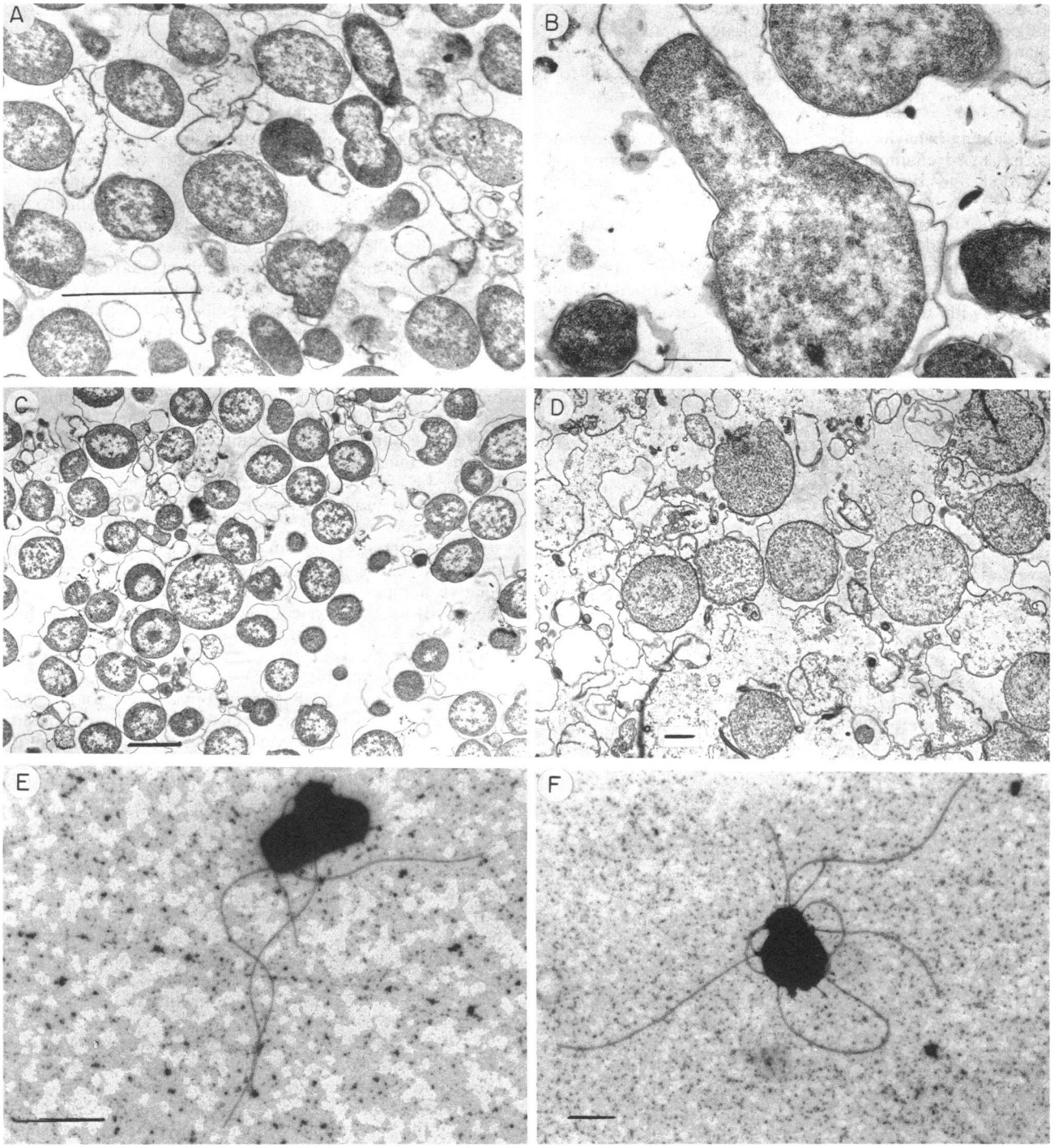

FIG. 1. Electron micrographs of penicillin spheroplasts from *E. coli*. (A) to (D), Thin sections through pellets of spheroplasts; (E) and (F) negatively stained electron micrographs. In all cases, chloramphenicol was added 44 min after the addition of penicillin. The cells were fixed 44 min (A) and (B), 75 min (C), 4 h (D), 44 min (E), and 5.75 h (F) after the addition of penicillin. Bars, 2 μm, except for micrograph B, in which it represents 0.5 μm.

data], KCN, and 2,4-dinitrophenol slowed rotation to a final halt, without affecting the direction, which remained CW. Similar observations of retention of CW rotation in *cheC* mutants have been described [18, 26]. 2,4-Dinitrophenol (5 mM) stopped the cells within ca. 2 min, KCN (1 mM) stopped them within ca. 15 min, and arsenate (10 mM) stopped them within ca. 30 min. Thus, despite the reduced average speed of rotation in cell envelopes (see Table 3), which may be an indication of a reduced PMF, a reduced PMF is not the cause of biased rotation. Furthermore, individual CCW-rotating cell envelopes (of various strains) were observed rotating as fast as CW-rotating bacteria. (The

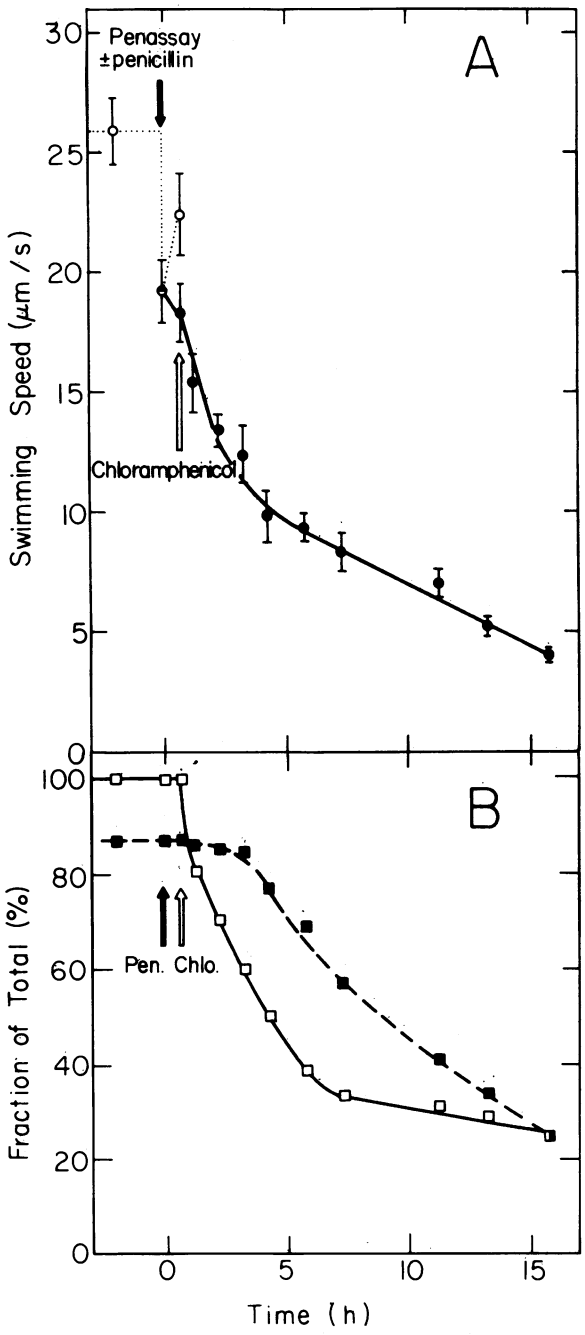

FIG. 2. Motility of penicillin-treated cells. (A) Swimming speed: the values shown are the mean of 10 to 20 motile cells ± the standard error at each time period. Cells were diluted (1:4) into penicillin-less Penassay broth (○) or penicillin-containing (10^3 U/ml) Penassay broth (●) at zero time. Chloramphenicol (50 μg/ml) was added to the penicillin-containing Penassay broth at t = 44 min. (B) Swimming pattern: the conditions were the same as in part (A). □, Proportion of motile cells per total number of cells examined (typically 20 per datum point); ■, proportion of cells with wild-type-like swimming per total number of motile cells. The temperature was 30°C.

maximal measurable speed of a CCW-rotating envelope was 9 Hz. Faster-rotating cell envelopes were observed, but their speed could not be determined.)

CCW bias is the consequence of cytoplasm loss. The absence of a cytoplasmic component required for CW rotation is the only possibility remaining from those mentioned above. To directly test this point, we studied cell envelopes prepared from *che* mutants having the opposite sense of rotation to that of wild-type envelopes. (*che* mutants [2, 33] have biased rotation of their flagella: either predominantly CW rotation [tumbling mutants] or predominantly CCW rotation [smoothly moving mutants] [3, 32].) The working hypothesis was that envelopes prepared from mutants whose defective gene product is in the cytoplasm should be similar to those prepared from the wild-type parent and have, therefore, CCW bias (the defective gene product presumably being released with the rest of the cytoplasm). Envelopes prepared from mutants whose defective gene product resides in the cytoplasmic membrane should contain the defective gene product and may (but not necessarily) retain the CW bias.

Defects in only four known *che* genes may lead to CW bias: *cheB*, *cheZ*, *cheC*, and *cheV* (33). The products of the first two genes are located predominantly in cytoplasm (37). Those of the *cheC* and *cheV* genes have not been determined but are presumably associated with the cytoplasmic membrane (33). The majority of cell envelopes prepared from *cheB* and *cheZ* mutants indeed acquired a CCW bias of rotation, as did envelopes from wild-type bacteria (Table 3). *cheC* and *cheV* mutants, however, retained their CW bias even after losing their cytoplasm. (Although cytoplasm-free envelopes are easily distinguishable under the phase microscope from cytoplasm-containing cells [10], control experiments were performed to ensure the lack of cytoplasmic remnants. At the end of an experiment, lactate [the electron donor for respiration] was eliminated from the flow medium, which continuously flowed through the observation chamber. The cell envelopes stopped rotating, whereas cells containing cytoplasm had internal energy reserves that permitted rotation [10]. The readdition of lactate restored flagellar rotation. Despite the above, we cannot exclude the possibility that soluble components still were bound to the membrane of the envelopes. This, nevertheless, should not affect the observation and conclusion reached above: if these remnants were a dominant factor, all mutants should have behaved the same.)

Can CCW-biased envelopes be made to rotate CW? Our observations (Table 3) indicated that cell envelopes can, in principle, rotate CW. To examine this further we (i) studied the rotation of envelopes exposed to PMFs of reverse polarity and (ii) studied their response to repellents.

Table 4 includes results with wild-type cell envelopes that were exposed to an artificially imposed ΔpH (internal pH − external pH) or $\Delta\psi$ (membrane potential) or both. All envelopes that were included in this table had rotated CCW in the presence of lactate and had stopped their rotation upon lactate removal from the medium. The following types of behavior could be distinguished. Of 31 envelopes (31 separate experiments) that were exposed to a positive PMF (external pH > internal pH [experiments 1 to 6] or $\Delta\Psi > 0$ [negative outside, positive inside; experiment 7] or both [experiment 8 and 9]), i.e., a PMF of the opposite polarity of that produced by lactate-driven respiration, 24 envelopes did not rotate, 2 rotated CCW, 2 rotated CW, and 3 made no more than half of a CW rotation. Control experiments have been done to ensure that the loss of motility was not the consequence of the pH itself, but rather of the inverted ΔpH: flagellar rotation (CCW), driven by lactate, was observed in the entire pH range of Table 4 (5.5 to 8.8), provided that the internal and external pHs were identical. If the exposure to

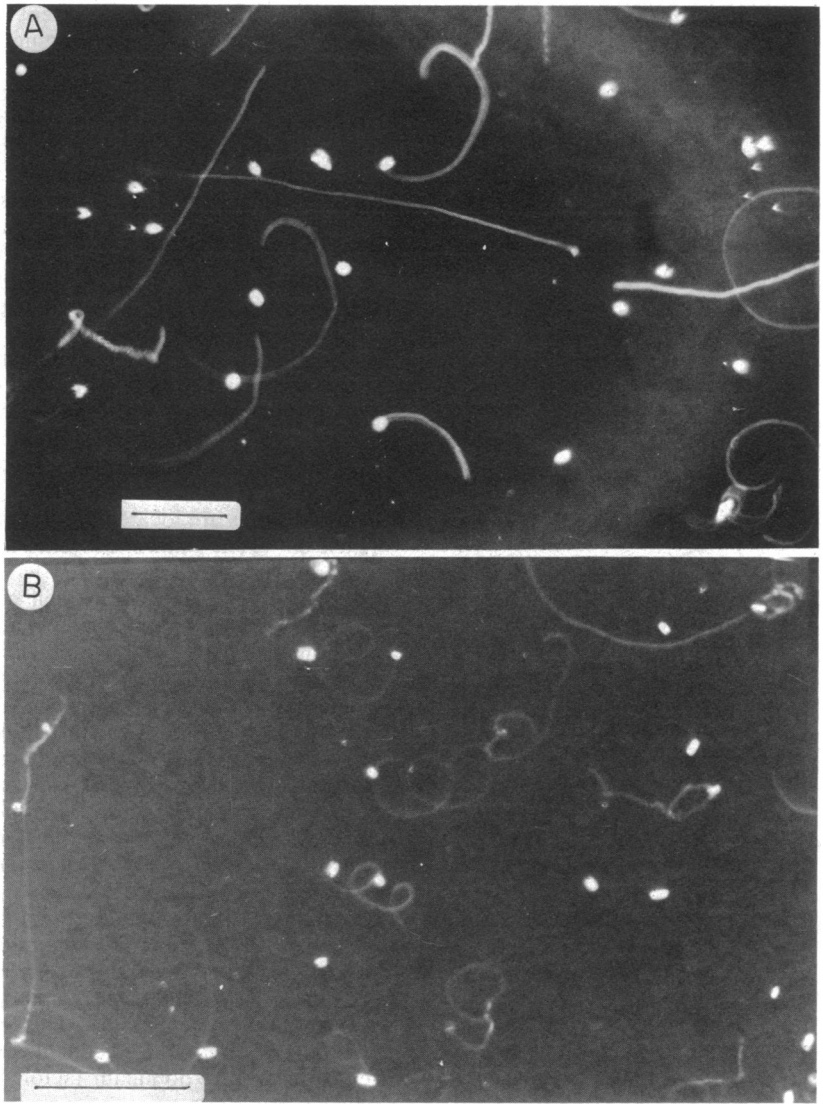

FIG. 3. Tracks made by swimming spheroplasts after 44 min with penicillin (A) and after 5 h with penicillin (B). Bars, 40 μm; exposure time, 8 s.

the inverse gradient of protons was short enough (experiments 3, 7, and 9) or if the gradient was not too large (experiment 1), the envelopes restored their rotation at the end of the experiment when lactate was added (under the original conditions). Prolonged exposure to large gradients of positive PMF prevented subsequent rotation in the presence of lactate. This, however, was not the case with a negative PMF, i.e., PMF of the same polarity as that produced by respiration. Of 21 envelopes exposed to a ΔpH of at least 2 pH units, 15 rotated CCW and only 6 did not rotate (experiments 11 and 12). Five of the nonrotating envelopes had become stuck and did not rotate even after the addition of lactate. These results indicate that most of the envelopes can be made to rotate CCW only.

Since the response of bacteria to repellents is CW flagellar rotation and concomitant tumbling, we compared the responses of intact bacteria, spheroplasts, and cell envelopes. The responses of intact bacteria and spheroplasts (all suspended in broth) to 0.5 mM indole (potent repellent of *E. coli* [43] and *S. typhimurium* [42]) were tumbling for 29 ± 13 s (±standard deviation) and 33 ± 4 s, respectively. Even spheroplasts as late as 4 h after penicillin addition still tumbled for 35 ± 4 s in response to indole. Cell envelopes, in contrast, were not affected by the addition of indole and retained their CCW rotation (Table 5).

The mechanism of response to indole is unknown, and no receptor has been identified. We have therefore tried two other repellents with a relatively well-understood mechanism of response: acetate at low pH and benzoate (20, 25, 36). The cell envelopes responded to neither repellent, whereas intact bacteria responded extensively (Table 5).

DISCUSSION

Cytoplasmic constituent is required for CW rotation in strains which are not defective in the switch. We presented evidence that the CCW bias of cell envelopes was not the consequence of penicillin treatment (Table 1), morphological changes in the cell wall (Fig. 2), or a reduced PMF (Table 2).

TABLE 2. Rotation of tethered *cheV* cells and cell envelopes

Preparation[a]	Cell proportion	Sample size (no. of cells)	Average speed ± SD (Hz)[b]
Intact cells	15% CW, 85% vibrating	82	4.2 ± 1.4
Intact cells plus KCN[c]	79% CCW, 21% vibrating	37	3.2 ± 1.0
Intact cells in arsenate[d]	83% CCW, 17% vibrating	41	3.7 ± 1.8
Cell envelopes	100% CW	6[e]	2.5 ± 1.9

[a] The flow medium for tethered bacteria (intact cells) was 10 mM KP_i (pH 6.6) plus 0.1 mM EDTA plus 0.1 mM L-methionine. The flow medium for tethered cell envelopes was the same as that for intact bacteria plus 80 mM sucrose and 20 mM sodium DL-lactate.

[b] Vibrating cells were not taken into account in calculating the average speed.

[c] Cells were subjected to KCN (1 mM) by including it with chloramphenicol (100 μg/ml) in the flow medium (18).

[d] Tethered bacteria were first subjected to a flow medium consisting of 10 mM KP_i (pH 7.0), 0.1 mM EDTA, and 0.1 mM L-methionine and then to a similar flow medium containing 10 mM potassium arsenate buffer (pH 7.0) instead of KP_i buffer. The effect of arsenate was reversible upon changing to the original KP_i-containing flow medium.

[e] Each envelope constituted a separate experiment.

We showed that CW-rotating cells acquired a CCW sense of rotation upon the release of their cytoplasm, provided that they did not have a defective membranal protein that caused CW bias (Table 3). The acquisition of CCW rotation could be the consequence of loss of a cytoplasmic component which is required for either expression of CW rotation or repression of CCW rotation. This component could be a *che* gene product (e.g., *cheA*, *cheW*, or *cheY* genes in which mutations lead to CCW rotation [33]), an unidentified low-molecular-weight material, or an ion. The component will hereafter be called CW facilitator. The possibility that remnants of cytoplasmic constituents were still in the envelopes cannot be excluded, but this is not very likely in view of the controls of energy depletion (see above) and the tests for cytoplasm release (10). The ultimate proof would be the restoration of CW rotation by the readdition of cytoplasmic constituents into the envelopes. Toward this goal, a technique of inserting macromolecules into cell envelopes by means of fusion with liposomes has already been developed (11, 24a).

***cheC* and *cheV* gene products are associated with the cytoplasmic membrane.** The *cheC* and *cheV* gene products are considered to be the switch that controls the direction of flagellar rotation (8, 9, 33). A mutation in these genes may lead to CW or CCW bias, paralysis, or lack of flagella. If the presumption that these gene products are located in the cytoplasmic membrane (8, 33) is correct and if a mutation in these genes leads to the production of impaired proteins that cannot properly respond to controlling signals (33), one would expect to find the same bias in the mutants and in their derived envelopes. Since the *cheC*- or *cheV*-linked CW rotation was indeed retained with the cell envelopes (Table 3), we may conclude that the proteins produced by these genes are associated with the cytoplasmic membrane. The function of the *cheC* and *cheV* gene products in these mutants is probably so impaired that the mutants are unaffected by the loss of the cytoplasmic CW facilitator, and CCW rotation no longer dominates.

Requirement of CW rotation for ATP is not at the level of the motor or switch. It is well established that lowering the cellular ATP level by arsenate causes wild-type cells to become smooth swimmers, i.e., CCW-rotating cells (1, 21, 38). This requirement for ATP is for *S*-adenosylmethionine synthesis and for some other, yet unidentified, reaction needed for CW rotation (21, 38; L. V. Gofshtein and M. Eisenbach, unpublished data). Our observation that envelopes from an arsenate-sensitive strain (Table 2) rotate CW, despite the absence of ATP, seems to indicate that the requirement for ATP is not at the level of the motor or switch but rather at one of the preceding functional steps of the chemotaxis machinery. Furthermore, the arsenate-sensitive

TABLE 3. Flagellar rotation in chemotaxis mutants and their derived cell envelopes[a]

Strain	Relevant genotype	Location of gene product[c]	Flagellar rotation[b] in: Intact bacteria: Cell proportion[d]	Intact bacteria: Average speed of rotation[e] ± SD (Hz)	Cell envelopes: Cell proportion[f]	Cell envelopes: Average speed of rotation ± SD (Hz)
ST1	Wild type		20% CCW, 80% CCW-cw	4.9 ± 1.7	100% CCW	3.2 ± 1.7
ST450	*cheB*	Cytoplasm[g]	16% CW-ccw, 84% CW	5.0 ± 2.2	82% CCW,[h] 18% CW	3.0 ± 1.5
ST120	*cheC*	Presumably CM	100% CW	4.6 ± 2.2	33% CW-ccw, 67% CW	2.6 ± 1.0
MY1	*cheV*	Presumably CM	16% CW[i]	4.2 ± 1.4	100% CW	2.5 ± 1.9
ST171	*cheZ*	Cytoplasm[g]	17% CW-ccw, 83% CW	7.7 ± 3.7	86% CCW, 14% CW	3.2 ± 2.1

[a] The conditions and experimental details are the same as those for Table 2.

[b] CCW-cw, CCW rotation with occasional, brief periods of CW rotation; CW-ccw, CW rotation with occasional, brief periods of CCW rotation.

[c] CM, cytoplasmic membrane (33, 37).

[d] The sample size was typically 20 cells per strain (=100%).

[e] There was no difference between the average speeds of rotation in either direction for intact bacteria or cell envelopes.

[f] The sample sizes were 26 cells for ST1 and typically 10 cells for each *che* mutant (excluding *cheV*; Table 2).

[g] Although predominantly located in cytoplasm, some of this gene product was found to be associated with the cytoplasmic membrane (37).

[h] The change in direction of rotation occurred at the lysis step. Penicillin-treated spheroplasts of the mutant retained their CW bias.

[i] The remaining 84% of the cells exhibited angular vibrations. These were not included in the average speed. Similar observations were reported for a *cheC* mutant of *E. coli* (35). Vibrating cell envelopes of any strain were not taken into account.

TABLE 4. Rotation of ST1 cell envelopes driven by artificially imposed PMF

Expt.	Change in flow medium[a]		Valinomycin present[b]	Length of treatment[c] (min)	Cell distribution[d] driven by:	
	Buffer	pH			PMF (artificially imposed)	Lactate (at end of expt)
1	$NaP_i \rightarrow NaP_i$ (100 mM)	6.3→7.3	−	7–10	3 NR	3 CCW
2	$NaP_i \rightarrow NaP_i$ (100 mM)	6.3→8.3	−	7	1 NR	1 CCW→NR[e]
3	$NaP_i \rightarrow NaP_i$ (100 mM)	6.3→8.3	−	2–3	3 NR	3 CCW
4	$KP_i \rightarrow KP_i$ (50 mM)	6.8→8.8	−	7	3 NR	3 NR
5	$KP_i \rightarrow KP_i$ (50 mM)	6.4→8.4	−	7	4 NR	3 NR, 1 ND
6	$KP_i \rightarrow KP_i$ (50 mM)	5.8→8.8	−	7	1 CCW, 4 CW,[f] 4 NR	2 CCW, 1 CCW→NR, 3 NR, 3 DET
7	$NaP_i \rightarrow KP_i$ (100 mM)	6.3→6.3	+	2–3	1 NR	1 CCW
8	$NaP_i \rightarrow KP_i$ (100 mM)	6.3→8.3	+	7–10	1 CCW, 1 CW, 2 NR	1 CCW, 2 NR, 1 DET
9	$NaP_i \rightarrow KP_i$ (100 mM)	6.3→8.3	+	2–3	3 NR	2 CCW, 1 DET
10	$KP_i \rightarrow KP_i$ (50 mM)	7.8→6.8	−	7–10	5 NR	4 CCW, 1 NR
11	$KP_i \rightarrow KP_i$ (50 mM)	7.8→5.8	−	7–10	6 CCW, 2 NR	7 CCW, 1 NR
12	$KP_i \rightarrow KP_i$ (50 mM)	8.4→5.5	−	7–10	9 CCW, 4 NR	9 CCW, 4 NR

[a] The initial flow medium was the lysis medium (internal medium) of the envelopes. Both the initial flow medium and the final flow medium (external medium) contained 0.1 mM EDTA in addition to the buffer. The solution in the flow chamber was fully exchanged within 30 s.

[b] Valinomycin was either not present (−) or applied to the cells before the tethering at a concentration of 10 nmol/mg of protein (+). Valinomycin was also included in the flow medium, when indicated, at a concentration of 5 μM (practically saturated aqueous solution of valinomycin).

[c] Length of treatment was the period between the insertion of the second flow medium and the reinsertion of the original medium.

[d] All cells included in the table rotated CCW in the presence of 2 mM DL-lactate. The chamber was then flushed with the lactate-free original medium, the rotation of the cells was stopped, and the second flow medium was flushed through the chamber. At the end of the mentioned period, lactate-containing original flow medium was flushed through the chamber. Each cell envelope constituted a separate experiment. DET, Detached; ND, not determined; NR, no rotation.

[e] There were a few slow CCW rotations followed by a final halt.

[f] One CW cell rotated for a few seconds and then stopped. The other three appeared to attempt CW rotation but did not accomplish more than half of a rotation.

components are probably cytoplasmic and are, therefore, missing in the envelopes.

Direct interaction between the switch-motor system and the repellent sensors is improbable. As reviewed recently (25), repellents may be divided into two categories: those like acetate and benzoate that work by reducing the internal pH and those like indole that work by another mechanism, possibly at the membrane level. Although the involvement of methyl-accepting chemotaxis proteins in the response to at least some repellents has been shown, the possibility of a direct interaction between the repellent sensors and the switch or motor has remained open (25). Cell envelopes contain the switch (Table 3) and motor. They presumably also contain the other required components for a direct interaction, all located at the cytoplasmic membrane: the repellent sensor(s), the internal pH sensor, and the methyl-accepting chemotaxis proteins. If indeed a direct interaction occurs between the repellent sensor and the switch or motor (an interaction that also does not require the intervention of the cytoplasmic CW facilitator), the response to repellents should occur also in cell envelopes. The lack of such a response in cell envelopes (Table 5) seems to indicate that there is no such direct interaction, unless some cofactor missing in the cell envelopes is needed for such an interaction. This conclusion is in line with recent studies that argue against linkage by direct and the motor in methyl-accepting chemotaxis protein-interaction between the membrane (12).

Motor is irreversible if cytoplasm is absent. For the purpose of determining how the proton current is coupled to flagellar rotation, Berg and co-workers applied an artificially imposed PMF of either sign to starved, intact *Streptococcus* cells and examined the direction of flagellar rotation (7, 17, 30). They found that in a strain which is insensitive to changes in cytoplasmic pH, i.e., a mutant in which the gear shift of the flagellar motor is presumably locked in one position, the sense of rotation depends on the polarity of the imposed PMF (7, 17). Other strains, however, do respond to downward shifts in internal pH by prolonged CW rotation (20, 36; Table 5), and this complicates the problem of determining cf. how the proton current is coupled to flagellar rotation. Such

TABLE 5. Flagellar rotation response of intact bacteria and cell envelopes to repellents[a]

Repellent	Flagellar rotation[b] of intact bacteria		Response time ± SD of intact bacteria	Flagellar rotation of cell envelopes
	Before	After		Before and after
Acetate (40 mM; pH 5.5)	14% CCW, 86% CCW-cw	100% CW	69 ± 15 min	100% CCW
Benzoate (40 mM; pH 7.0)	25% CCW, 75% CCW-cw	34% CW-ccw, 66% CW	151 ± 15 s	100% CCW
Indole (0.3 mM; pH 7.0)	13% CCW, 87% CCW-cw	9% CCW, 18% CW-ccw, 73% CW	30 ± 7 s	100% CCW

[a] Tethered cells of *S. typhimurium* ST1 were used for this experiment. The flow media were the same as those described in Table 3 except for their pH.

[b] The percentages given are the cell proportions in each sense of rotation before and after the addition of the repellent; 100% was typically 15 to 20 cells. CW-ccw, CW rotation with occasional, brief periods of CCW rotation; CCW-cw, CCW rotation with occasional, brief periods of CW rotation.

strains rotate CW even when protons move inward because of a chemotactic response to a downward shift in cytoplasmic pH (7, 17). We have circumvented this complication by using cell envelopes in which the motor alone can be studied without interference by the chemotaxis machinery (see above). Our observations with the envelopes were indeed different from those of Berg and collaborators with intact bacteria. Naturally oriented proton current (inwardly directed) drove the tethered envelopes in the CCW sense of rotation (Table 4), as one would expect. In the case of a proton current of inverse polarity, one of three different situations may be anticipated: (i) the gear shift of the motor is locked in one position, and thus, the motor will rotate CW (as in the case of the mutant described above [7]); (ii) the gear shift is functional and coupled to the motor, and thus, the motor will not reverse (as it does not reverse in the case of intact, wild-type bacteria [7, 17, 30]) and will rotate CCW; and (iii) the motor is mechanically constricted so as to permit CCW rotation only, possibly because of the absence of the cytoplasmic CW facilitator (see above), and thus, the motor will not rotate. The final alternative is the correct one for the majority of the cells (Table 4). It appears that in the absence of the CW facilitator, the motor of most wild-type bacteria may be mechanically unable to rotate other than CCW: the motor seems to be restricted to CCW rotation, and only the CW facilitator can presumably remove the restriction. An extensive reversed proton flow (in magnitude or time) seems to severely and irreversibly damage the motor; after such damage, it cannot rotate even under an inwardly directed proton current (Table 4). (A few cells appeared to rotate a fraction of a CW rotation, but soon after they became irreversibly stuck.)

These results seem to indicate that the motor itself is not a reversible unit. However, the complex between the switch-motor unit and the CW facilitator may possibly be reversible. One may speculate that an appropriate candidate for the CW facilitator could be the *cheY* gene product: a mutation in this gene causes an extreme CCW bias, and its product directly interacts with the *cheC* gene product, the switch protein (34). Thus, the complex (motor-switch–*cheY* gene product) may constitute a reversible unit (or part of a unit), in which the motor is mechanically derestricted. This hypothesis may, in principle, be tested by insertion of the *cheY* gene product into cell envelopes and subsequent examination for rotation, driven by an outwardly directed proton current.

Absence of swimming motility in envelopes. It was reported that cell envelopes do not swim despite their flagellar rotation (10). Our studies with spheroplasts, reported here, exclude the possibility that this lack of movement is the consequence of insufficient number of flagella per cell. Three flagella seemed to be the minimal average number required for motility: an excellent linear correlation between the fraction of motile cells and the fraction of cells with a given number of flagella was observed only for ≥ 3 flagella; other numbers yielded poorer correlation coefficients and y-intercepts different from zero. Cell envelopes have larger numbers of flagella per cell than are required for motility: 4 to 10 in *E. coli* cell envelopes and 12 to 18 in *S. typhimurium* cell envelopes (10). For comparison, the average minimal number of flagella necessary for motility of untreated *E. coli* (4) and *Salmonella* cells (15) was found to be three and four, respectively, although individual motile *Salmonella* cells with less than three flagella have been observed (R. M. Macnab, personal communication). Other previously discussed possible causes for lack of envelope motility (10), i.e., insufficient speed of flagellar rotation, or insufficient thrust for vectorial movement are also excluded by this study. The thrust should be similar in both cell envelopes and spheroplasts, and only the spheroplasts swim. It appears, therefore, that the cause of the lack of motility of envelopes is either cytoplasm loss or, more likely, an insufficient number of vigorously rotating flagella per cell. Since the fraction of vigorously rotating, tethered envelopes is small, the fraction of envelopes with most of their flagella rotating vigorously (enough to enable swimming motility) is probably very much smaller.

Other potential uses for spheroplasts. The spheroplast preparation may have a significance beyond being a step in the procedure for isolation of cell envelopes. The combination of accessibility of the cytoplasmic membrane and maintenance of a functional chemotaxis apparatus makes this system important for studying the mechanism of chemotaxis. For example, liposomes could be used to insert molecules of interest into the spheroplasts and to study their effect on chemotactic behavior. Similarly, agents that modify the membrane could be examined for their effect on chemotactic behavior.

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