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# Minimal Requirements for Rotation of Bacterial Flagella

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An in vitro system of cell envelopes from *Salmonella typhimurium* with functional flagella was used to determine the minimal requirements for flagellar rotation. Rotation in the absence of cytoplasmic constituents could be driven either by respiration or by an artificially imposed chemical gradient of protons. No specific ionic requirements other than protons (or hydroxyls) were found for the motor function.

The flagellar motor in bacteria is a biological device that converts electrochemical energy into mechanical energy. Evidence has been published that the energy source for the motor is the proton motive force (6, 9, 12, 13; for a review see reference 3), not ATP (10, 19) as with eucaryotes (4, 16). The evidence, though well established, was obtained from studies with intact bacteria, in which nonrelated processes might interfere with the observations. Such processes should be minimal in cell envelopes, where cytoplasm is absent (5). It has also been shown that flagellar rotation in intact bacteria does not require any external ions other than protons or hydroxyls (8, 12, 13). In addition, it was suggested that $K^+$ is not required internally because reduction (by monactin) of the internal $K^+$ concentration from 640 to 12 mM did not impair swimming (12). Possible requirements for internal $K^+$ (below the already determined limit of 12 mM) or for other internal ions can be revealed with the envelope preparation, in which the internal content is defined and predetermined. It was the purpose of this study to use such bacterial envelopes to determine the minimal requirements for flagellar rotation.

Flagellated cell envelopes were isolated from *Salmonella typhimurium* ST1, which was received from D. E. Koshland, Jr. The bacteria were grown as described previously (5), and envelopes were isolated by a procedure consisting of penicillin treatment to form spheroplasts and subsequent osmotic lysis (by a 200- to 500-fold dilution of the medium). Before osmotic lysis, the spheroplasts were blended for 30 s to shear off the majority of their flagella (5).

The internal medium of the cell envelopes was the medium in which their spheroplast precursors were osmotically lysed (hereafter called lysis medium). We therefore prepared envelopes in minimal lysis media that contained only buffer plus a small amount of EDTA to chelate traces of unidentified heavy metals that inhibit motility (1). The envelopes were tethered by means of antibody to flagellin (18) to a microscope cover glass in a thermostated flow chamber. Their rotation was recorded by a video system connected to a Zeiss phase-contrast microscope (14). Flagellar rotation in the same direction and with similar average speed was observed in all the lysis media examined, provided that a respiratory energy source (D,L-lactate) was included in the flow medium (Table 1). Elimination of the energy source from the flow medium halted rotation; adding it back restored rotation. This served as a control that was carried out for every single envelope, for lack of cytoplasmic remnants in the envelope. (Cells containing cytoplasm had internal energy reserves that permitted rotation.) Although we cannot exclude completely the possibility that a few of the envelopes might have contained remnants of cytoplasmic proteins (e.g., adsorbed to the membrane), it seems unlikely that residual cytoplasmic ions or other small molecules were playing a role unless extremely low concentrations (≥ 200-fold lower than the internal concentration) were sufficient (cf. reference 5 for tests for lack of cytoplasm in the envelopes). This conclusion, i.e., the presumed absence of internal or external substrates for any nonrelated process, taken together with the observation that $K^+$ could be substituted for $Na^+$ and phosphate for PIPES [piperazine-*N*,*N*′-bis(ethanesulfonic acid)] as the only internal ions, without any effect on motor rotation, indicate that there are no specific requirements other than protons (or hydroxyls) for motor function. Proton (or hydroxyl) flux seems to be the only way to drive the flagellar motor, in agreement with previous studies with intact bacteria (see reference 3 for a review).

If this conclusion is correct, the flagella of the cell envelopes should be made to rotate by an artificially imposed chemical gradient of protons (ΔpH). (Such experiments have been successfully carried out with intact bacteria [3, 6, 7, 9, 11–13].) Cell envelopes containing 100 mM $KP_i$ (pH 7.0) and 0.1 mM $K^+$ EDTA were tethered in the flow chamber mentioned above. The experimental procedure consisted of the following steps. First, respiration-linked rotation was monitored while flushing the flow chamber with a medium identical to the lysis medium (pH 7.0) but also containing D,L-lactate (2 mM). Subsequently, the chamber was flushed with a lactate-free medium (flow rate, 20 μl/

TABLE 1. Effect of the internal ionic composition on the rotation of tethered cell envelopes

| Lysis medium composition[a] | Energy source in the flow medium[b] | Avg rotation rate (Hz) ± SD[c] | No. of determinations[d] |
|---|---|---|---|
| $KP_i$ + $K^+$EDTA | $K^+$D,L-lactate | 2.9 ± 1.8 | 56 |
| $NaP_i$ + $Na^+$EDTA | $Na^+$D,L-lactate | 2.6 ± 1.6 | 54 |
| $K^+$PIPES + $K^+$EDTA | $K^+$D,L-lactate | 2.6 ± 3.5[e] | 7 |

[a] The concentrations used were 50 mM buffer at pH 6.8 ($P_i$ or PIPES and 0.1 mM EDTA. Some of the experiments included 100 mM $KP_i$ or $NaP_i$ at pH 7.0.

[b] The flow medium (prewarmed to 30°C and flowing at a rate of 0.15 ml/min [0.3-ml flow chamber] or 0.02 ml/min [4.4-μl chamber] throughout the experiment) was identical to the lysis medium. It also contained, where indicated, the mentioned energy source (2 mM).

[c] The average does not include envelopes that were too faint, small, or fast to allow the determination of their rotation speed with confidence. Each rotating cell envelope was examined for the absence of cytoplasmic remnants as described in the text. The rotation of all the cell envelopes was in the counterclockwise direction (cf. reference 15). Temperature = 30°C.

[d] Each determination was a separate experiment.

[e] This rather large standard deviation is the result of one cell envelope that rotated at 9.7 Hz.

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TABLE 2. ΔpH-driven rotation of tethered cell envelopes

| Maximal respiration-linked rotation rate (Hz) ± SD[a] | No. of determinations[b] | ΔpH-driven rotation | | | |
|---|---|---|---|---|---|
| | | Apparent ΔpH[c] | Maximal rotation rate (Hz) ± SD | Relative rotation rate (%)[d] | Duration of rotation (min) ± SD |
| 5.0 ± 0.1 | 4 | 0.5 | 1.1 ± 0.6 | 22 | 0.30 ± 0.05 |
| 2.1 ± 1.5 | 6 | 1.0 | 1.6 ± 0.9 | 76 | 1.2 ± 0.5 |
| 2.9 ± 2.1 | 7 | 1.5 | 2.4 ± 1.2 | 83 | 1.4 ± 0.4 |
| 3.7 ± 2.1 | 5 | 2.0 | 3.6 ± 2.2 | 97 | 2.5 ± 0.6 |

[a] The given rotation rate is the average of the maximal rate of the same cell envelopes used for the ΔpH-driven rotation. For experimental procedures see the text. The respiration-linked rotation rate was determined at pH 7.0 and 21°C.
[b] Each determination was a separate experiment.
[c] Internal pH was 7.0. ΔpH is defined here as $pH_{in} - pH_{out}$.
[d] The maximal rotation rate relative to the maximal respiration-linked rotation rate of the same envelopes.

min; the content of the flow chamber was fully replaced within 13 s). The rotation of the envelopes stopped within 20 to 25 s. A pH differential was then imposed by flushing the chamber with the same medium but at a pH lower than 7.0 to yield the desirable ΔpH value, and a ΔpH-driven transient rotation was observed. Finally, respiration-linked rotation was restored by flushing the chamber with a D,L-lactate-containing flow medium (pH 7.0). A pH jump of 0.5 pH units (equivalent to 29 mV at 21°C) was sufficient to cause transient rotation of the tethered envelopes (Table 2). However, to gain the maximal rate of rotation, larger imposed ΔpH values were required. Treating the cells with valinomycin (10 nmol/mg of protein and including 5 μM in the flow medium) to avoid formation of a diffusion potential (cf. reference 3) did not alter the observations. For example, the relative rotation rate in the presence of valinomycin, driven by ΔpH = 0.5, was 26 ± 6% of the respiration-linked rotation, and the duration of the rotation was 0.33 ± 0.07 min. To demonstrate the dependence of the rotation rate on the magnitude of ΔpH when all other factors that affect the rotation rate are kept constant (e.g., size and shape of the rotating cell [2]), the rotation rate of a single envelope is shown in Fig. 1A. A linear correlation ($|r| > 0.99$) between the rate and magnitude of the ΔpH was found in the range tested (cf. references 9, 11, and 13 for similar dependence in intact gram-positive bacteria; similar studies with gram-negative bacteria have not been published). In the presence of valinomycin, a linear correlation was observed also ($|r| > 0.99$; not shown in the figure).

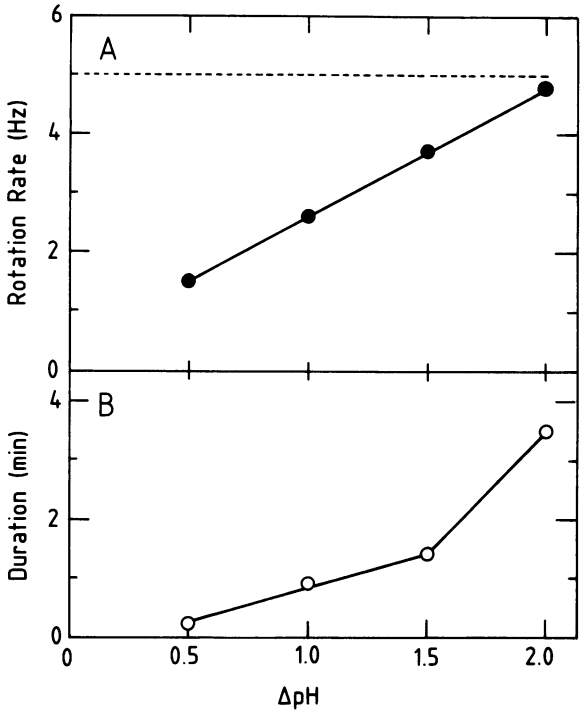


FIG. 1. Angular velocity (A,●) and duration (B,○) of ΔpH-driven rotation of a single cell envelope. The internal composition of the envelope was 100 mM $KP_i$ (pH 7.0) and 0.1 mM EDTA. The ΔpH was imposed by flushing (20 μl/min) an identical solution at appropriately lower pH (the ΔpH values in the figure should be considered as apparent values). The content of the flow chamber was fully replaced within 13 s. Between each pH jump, the rate of respiration-driven rotation (with 2 mM D,L-lactate as an electron donor) was measured at pH 7.0. When the full rate of respiration-linked rotation was restored, D,L-lactate was dropped from the medium. The rotation consequently stopped, and a new pH jump was applied. The dashed line represents the maximal rate of rotation driven by D,L-lactate. This rate was constant throughout the pH range of the experiment. Temperature = 21°C.

It seems from the data of Table 2 and Fig. 1A that the threshold for flagellar rotation in the envelopes is smaller than 29 mV (ca. 10 mV in the presence of valinomycin; data not shown) at 21°C (in agreement with the threshold values in intact gram-postivie bacteria, where values of 30 to 35 mV for *Bacillus subtilis* [9, 17] and 8 to 18 mV for *Streptococcus* species [3] were found) and that full saturation rate is achieved at apparent ΔpH = 2 (116 mV at 21°C). However, the values obtained at apparent ΔpH = 1 (58 mV) were not far from saturation (Table 2). (These values are comparable with those found for intact *B. subtilis*, 70 mV [9] or 100 mV [17].)

Although the values in Table 2 are for internal pH 7.0, similar results were obtained with envelopes in which the internal pH was 7.8 (not shown). Control experiments of respiration-linked rotation that were carried out in the whole pH range tested and at identical pH values internally and externally showed that the rotation rate in this pH range was independent of the pH.

The transient nature of the rotation was presumably a consequence of the gradual dissipation of the artificially imposed ΔpH to below the threshold value for rotation. This interpretation is supported by observations that the duration of the rotation was dependent on the magnitude of the ΔpH gradient (Table 2 and Fig. 1B; cf. references 3, 6, and 17) and that the decrease in the rotation speed with the presumed dissipation of the ΔpH was gradual.

This study thus endorses and extends other studies with intact, primarily gram-positive bacteria to envelopes of gram-negative bacteria in demonstrating that a proton (or hydroxyl) current per se, rather than another proton motive force-dependent process, drives the flagellar motor. It fur-

ther shows that no specific cytoplasmic components are required for rotation.

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