Chapter 24

Ion Selectivity of the Flagellar Motors Derived from the Alkaliphilic *Bacillus* and *Paenibacillus* Species

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Abstract

Many bacteria can swim using their flagella, which are filamentous organelles that extend from the cell surface. The flagellar motor is energized by either a proton (H^+) or sodium ion (Na^+) as the motive force. MotAB-type stators use protons, whereas MotPS- and PomAB-type stators use Na^+ as the coupling ions. Recently, alkaliphilic *Bacillus alcalophilus* was shown to use potassium ions (K^+) and rubidium ions (Rb^+) for flagellar rotation, and the flagellar motor from *Paenibacillus* sp. TCA-20 uses divalent cations such as magnesium ions (Mg^{2+}), calcium ions (Ca^{2+}), and strontium ions (Sr^{2+}) for coupling. In this chapter, we focus on how to identify the coupling ions for flagellar rotation of alkaliphilic *Bacillus* and *Paenibacillus* species.

Key words Flagellar motor, Stator, Alkaliphiles, *Bacillus*, *Paenibacillus*, Divalent cation

1 Introduction

The bacterial flagellar motor, which is embedded in the cell envelope, consists of three parts, the filament, hook, and basal body, and is generally powered by an electrochemical gradient of protons (H^+), sodium (Na^+), potassium (K^+), rubidium (Rb^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), or strontium (Sr^{2+}) across the cytoplasmic membrane [1–5]. Torque is generated by the electrostatic interaction at the rotor (FliG) and stator interface. *Bacillus subtilis* and *Shewanella oneidensis* MR-1 employ H^+-coupled MotAB and Na^+-coupled MotPS/PomAB stators to generate the torque required for flagellar rotation [2, 6, 7].

Before 2008, the Mot complexes were believed to contain channels that used either H^+ or Na^+, with some bacteria having only one type and others having two distinct types with different ion-coupling [7, 8]. However, in 2008, alkaliphilic *B. clausii* KSM-K16 was identified as the first bacterium with a single stator-rotor unit that uses both H^+ and Na^+ for ion-coupling, based on external pH [9]. Subsequent findings have shown that alkaliphilic *B. alcalophilus* AV1934 uses Na^+, K^+, and Rb^+ as coupling ions for flagellar rotation

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[1], suggesting that coupling ions other than H^+ and Na^+ can power the bacterial flagellar motor, including Ca^{2+}, which are abundant in nature. Subsequently, *Paenibacillus* sp. TCA20 has been shown to have a novel bacterial flagellar stator that can use Mg^{2+}, Ca^{2+}, and Sr^{2+} as coupling cations for flagellar rotation [3].

In this chapter, we introduce the methods for measuring motility and intracellular ion concentrations to elucidate the coupling ion selectivity of the flagellar motor stator. Here, we focus on this motility organ in alkaliphilic *Bacillus* and *Paenibacillus* species.

2 Materials

Ultra-pure water is used in the present study.

2.1 Motility Analysis

1. Tris medium (pH 7.7): Mix 3.63 g of Trizma base and 1.47 g of citric acid monohydrate in 930 mL of deionized water. After autoclaving, cool to 55 °C and add 10 mL of 10% (w/v) Yeast Extract, 10 mL of trace elements [10], and 50 mL of 1 M D-(+)-Glucose (*see* **Notes 1** and **2**).
2. Motility assay buffer: 30 mM Trizma base and 5 mM D-(+)-Glucose are mixed, adding the necessary amount of NaCl, KCl, or RbCl (*see* **Notes 1** and **2**).
3. Inhibitors of the flagellar motor: Proton-coupled and Na^+- and K^+-coupled flagellar motors are inhibited by the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and Ethylisopropyl amiloride 3-Amino-N-(aminoiminomethyl)-6-chloro-5-[ethyl(1-methylethyl)amino]-2-pyrazinecarboxamide) (EIPA), respectively. Both inhibitors are dissolved in dimethyl sulfoxide (DMSO). The prepared inhibitor solution is added to the appropriate concentration into the motility buffer.
4. Microscope with a microscope stage automatic temperature control system adjusted at 32 °C and recorded in high definition with a digital color camera.
5. The speed of each individual swimming cell was analyzed by 2D movement measurement capture using 2D–PTV software (Digimo Co., Ltd.).
6. The diameters of the colonies formed on the surface of soft agar medium were measured using a Vernier caliper.

2.2 Measurement of Intracellular K^+ Concentrations

1. LBK medium: LBK medium (pH 7.5) contains 10 g of Tryptone, 5 g of Yeast Extract, and 6 g of KCl per liter of deionized water. The pH is adjusted with 4 M KOH. Thereafter, the medium is sterilized.
2. A defined medium for *Escherichia coli* TK2420: The composition of a defined medium (pH 7.0) contains 4.9 g of Na_2HPO_4, 2.34 g of NaH_2PO_4, 0.29 g of Trisodium citrate, 1 g of

$(NH_4)_2SO_4$, 1 mL of 6 mM $FeSO_4$, 0.1 g of $MgSO_4$, and 1 mL of 1 M KH_2PO_4 per liter of deionized water. After autoclaving, cool to 55 °C and add 10 mL of 1 M glucose, 1 mL of 1000× thiamine (stock solution is 1 mg/mL), and various amounts of KCl. TK2420 will grow on 50 mM KCl but not on 5–10 mM KCl.

3. 0.5 M sucrose solution: The solution contains 171.1 g of sucrose per liter of deionized water. Thereafter, the solution is sterilized.
4. Growth was monitored by measuring the absorbance at A600 using a spectrophotometer.
5. The K^+ concentration in solution was determined using a flame photometer.

2.3 Measurement of Intracellular Mg^{2+} Concentrations

1. 2×TY medium: 2×TY medium contains 16 g of Tryptone and 10 g of Yeast Extract per liter of deionized water. Thereafter, the medium is sterilized.
2. 0.3 M sucrose solution: The solution contains 102.7 g of sucrose per liter of deionized water. Thereafter, the solution is sterilized.
3. The Mg^{2+} concentration in solution was determined by MG Metallogenics Mg^{2+} measurement LS (Metallogenics Co., Ltd., Chiba, Japan) using spectrophotometer.

3 Methods

3.1 Swimming Velocity Analysis in a Liquid Medium

1. Highly motile cells in the late logarithmic phase were harvested by filtration on OMNIPORE membrane filters (0.45 μm) and washed three times with 2 mL of motility analysis buffer (*see* **Notes 3** and **4**). For swimming speed assays with inhibitors, the cells were resuspended in motility assay buffer at the indicated values of pH and monovalent cation content for the assay. (CCCP is used as an inhibitor for a proton-coupled motor and EIPA is used as an inhibitor for a Na^+- and K^+-coupled motor)
2. Cells were suspended in 1 mL of the same buffer and incubated at growth temperature for 10 min.
3. For the measurement of swimming speed, cell motility was observed under a dark-field microscope. Videos of approximately 10 s were recorded from five suspensions (*see* **Note 5**).
4. The speed of each individual cell that was swimming (not tumbling) for more than 10 s was analyzed by 2D movement measurement capture (*see* **Notes 6** and **7**).
5. The swimming speed of each cell is calculated at least 1 s or more of recorded data (*see* **Note 8**). All results are the average of three independent experiments where the speed of 30 different cells was measured.

3.2 Swarming Analysis on Soft Agar Medium

1. To observe motility, bacterial cells were inoculated on the surface of Tris media containing 0.25–0.3% Noble agar (Difco) (*see* **Note 9**).
2. Plates were incubated under static culture conditions using stationary phase bacteria at a temperature suitable for growth (*see* **Notes 10** and **11**).
3. After incubation, the diameters of the colonies formed on the surface of the Tris soft agar medium were measured using a Vernier caliper. All results are the average of three independent experiments.

3.3 Measurement of Intracellular K^+ Concentration

1. A potassium uptake-deficient (Δ(*kdpABC*) *trk*Δ1 Δ*trkA*) mutant strain of *E. coli* TK2420 was used as a host for these experiments. A plasmid carrying the cloned stator gene of interest was transformed into TK2420.
2. The transformant was grown in LBK medium at 37 °C overnight and then the culture was inoculated into 50 mL of TK2420 medium [11] containing 10 mM, 25 mM, or 50 mM KCl at an absorbance of 0.01 at 600 nm (A_{600}) and grown aerobically at 37 °C. Growth was monitored by measuring the absorbance at A_{600} (*see* **Notes 12–14**).
3. The cells were harvested by centrifugation (3000 × *g*, 10 min, 25 °C).
4. The cells were washed by resuspending them in a 300 mM sucrose solution and then harvested by centrifugation (3000 × *g*, 10 min, 25 °C).
5. The cells were resuspended in 5 mL of 0.5 M sucrose solution.
6. The protein concentration was measured by the Lowry method using 100 μL of the cell suspension (*see* **Notes 15** and **16**).
7. The rest of the suspension was harvested by centrifugation (3000 × *g*, 10 min, 25 °C) and resuspended in 10 mL of 10% (w/v) TCA solution.
8. The cell suspension was shaken at 200 rpm.
9. Cells were harvested by centrifugation (10,000 × *g*, 5 min, 25 °C).
10. The supernatants were transferred to a new test tube and then diluted by 10-fold or 100-fold.
11. The K^+ concentration of the stock and the 10-fold diluted and 100-fold diluted solutions were determined using a flame photometer calibrated with standard K^+ solutions of known concentration (*see* **Note 17**).
12. The protein concentration was determined by the Lowry method with lysozyme as a standard [12]. The intracellular concentration was calculated with an assumed cell volume of 3 μL/mg cell protein [13, 14].

3.4 Measurement of Intracellular Mg^{2+} Concentration

1. A major magnesium uptake- and flagellar stator-deficient (*lys3 trpC2* Δ*motAB*Δ*motPS* Δ*ykoK* Δ*yfjQ*) mutant strain of *B. subtilis* ΔABPSΔKQ was used as a host for these experiments. A plasmid carrying cloned stator genes of interest was transformed into ΔABPSΔKQ. Wild-type and ΔABPSΔKQ strains of *B. subtilis* were used for positive and negative controls.
2. The transformant was grown in LBK medium at 37 °C overnight and then the culture was inoculated into 50 mL of 2×TY medium containing 0 mM, 1 mM, 2.5 mM, 5 mM, or 10 mM $MgCl_2$ at an absorbance of 0.01 at 600 nm (A_{600}) and was grown aerobically at 37 °C. Growth was monitored by measuring the absorbance at A_{600} (*see* **Notes 12–14**).
3. The cells in the late logarithmic growth phase were harvested by centrifugation (3000 × *g*, 10 min, 25 °C).
4. The cells were resuspended in 0.3 M sucrose solution and then harvested by centrifugation (3000 × *g*, 10 min, 25 °C).
5. The cells were resuspended in 5 mL of 300 mM sucrose solution.
6. The protein concentration was measured by the Lowry method [12] using 100 μL of the cell suspension (*see* **Notes 15** and **16**).
7. The rest of the suspension was harvested by centrifugation (3000 × *g*, 10 min, 25 °C) and resuspended in 10 mL of 0.1 M HCl.
8. The cell suspension was shaken at 200 rpm.
9. The cells were harvested by centrifugation (10,000 × *g*, 5 min, 25 °C).
10. The supernatants were transferred to a new test tube and then diluted by 10-fold or 100-fold.
11. The Mg^{2+} concentration of the prepared sample of the stock and 10-fold diluted solution and a 100-fold diluted solution was measured by MG Metallogenics Mg^{2+} measurement LS (Metallogenics Co., Ltd., Chiba, Japan) that was calibrated with standard Mg^{2+} solutions of known concentrations. (*see* **Note 17**).
12. Intracellular Mg^{2+} was calculated by assuming that a *B. subtilis* cell was a cylinder (radius, 0.4 μm; length, 4 μm).

4 Notes

1. Tris medium was not subjected to pH adjustment. When preparing the pH in the alkaline range, both NaOH and KOH cannot be used for pH adjustment. Instead, N-Methyl D-glucamine was added to adjust the pH in the alkaline range.
2. Some bacteria, for example *B. subtilis*, show some inhibition of motility in a Tris buffer. If the tested bacteria do not show

vigorous motility in the Tris buffer, we recommend using other buffer suitable for optimum pH.

3. Some bacteria show slow or no motility after the collection of the cells because of the physical shock of the filtration and centrifugation. In this case, it is suitable to perform dilution with a wide mouth tip. If the bacterial concentration is low, then buffer substitution under microscopic observation is the best method.
4. When the turbidity of the late logarithmic cells (A_{600}) does not reach 1.0, it was necessary to concentrate the cells by two to five times because the motility observation required a sufficient amount of motile cells.
5. Motility observed under the microscope was performed using the hanging drop method. A number of bacteria were present in the intermediate layer, but the depth of focus changed quickly. Therefore, it was sometimes difficult to track each swimming cell. We recommend that swimming observation be performed by focusing near the slide glass or cover glass surface.
6. The tumbling frequency of *B. subtilis* and its derivatives is drastically increased by $CaCl_2$ and it is very difficult to measure the linear swimming velocity of each strain.
7. Bacteria that were swimming relatively straight were chosen because tracking cells that change direction resulted in variation of swimming speed.
8. The distribution of the swimming velocity for all of the cells was determined. Subsequently, 90 bacterial cells were selected to be used in the swimming velocity measurement based on the standard deviation of this distribution.
9. For best results, soft agar medium plates should be prepared freshly. Plates cannot be stored more than 1 week even if humidity is controlled.
10. The motility of the cells was observed on the surface of the soft agar medium.
11. The bacteria on the soft agar plate were spread in a relatively symmetrical circle. However, after a prolonged incubation, the spread of the bacteria was no longer circular. Therefore, this assay should be performed using stationary growth phase cells.
12. LBK medium contains KCl and is suitable for the culture of the TK2420 strain that is defective in the major potassium uptake system.
13. The function of the flagellar motor was extremely reduced in the stationary phase. Therefore, for the measurement of the intracellular concentration of the coupling ion of the flagellar motor, it is desirable to use cells in the late logarithmic growth phase.
14. It is possible to calculate the number of bacterial cells from the turbidity at the time of harvest.

15. Washing the cells with the sucrose solution removed the salt from the medium so that only the internal potassium concentration was measured. For consistency, the protein concentration was measured under the same conditions.
16. To calculate the number of cells, it was necessary to remove flagellar fiber and cilia from the cell body with a waring blender. Otherwise, the amount of protein content per cell is overestimated because of the large amount of flagellar fiber and cilia present on the cells.
17. Equipment and commercial kits differ in the ranges that they can measure ion concentration. Therefore, it was necessary to prepare the samples to a suitable concentration for the method of measurement.

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