Vol. 20, e200024 (2023)
doi: 10.2142/biophysico.bppb-v20.0024

Biophysics and Physicobiology
https://www.jstage.jst.go.jp/browse/biophysico/

*Review Article (Invited)*

# Flagellar polymorphism-dependent bacterial swimming motility in a structured environment

Yoshiaki Kinosita[1], Yoshiyuki Sowa[2,3]

[1] *CPR, RIKEN, Wako, Saitama 351-0198, Japan*
[2] *Department of Frontier Bioscience, Hosei University, Tokyo 184-8584, Japan*
[3] *Research Center for Micro-Nano Technology, Hosei University, Tokyo 184-8584, Japan*

Received April 18, 2023; Accepted May 29, 2023;
Released online in J-STAGE as advance publication May 30, 2023
Edited by Makoto Miyata

**Most motile bacteria use supramolecular motility machinery called bacterial flagellum, which converts the chemical energy gained from ion flux into mechanical rotation. Bacterial cells sense their external environment through a two-component regulatory system consisting of a histidine kinase and response regulator. Combining these systems allows the cells to move toward favorable environments and away from their repellents. A representative example of flagellar motility is run-and-tumble swimming in *Escherichia coli*, where the counter-clockwise (CCW) rotation of a flagellar bundle propels the cell forward, and the clockwise (CW) rotation undergoes cell re-orientation (tumbling) upon switching the direction of flagellar motor rotation from CCW to CW. In this mini review, we focus on several types of chemotactic behaviors that respond to changes in flagellar shape and direction of rotation. Moreover, our single-cell analysis demonstrated back-and-forth swimming motility of an original *E. coli* strain. We propose that polymorphic flagellar changes are required to enhance bacterial movement in a structured environment as a colony spread on an agar plate.**

**Key words:** bacterial flagellum, flagellar polymorphism, chemotaxis, colony spreading, TIRFM

***Significance***

Bacteria employ motility as a survival strategy, which allows them to migrate toward and away from favorable and unfavorable environments, respectively. Flagellum-mediated motility plays a pivotal role in various biological processes that affect the survival of bacteria, including biofilm formation, pathogenicity, and symbiosis with animals. Here, we summarize the structure and function of bacterial flagella and describe how polymorphisms in flagellar filaments provide advantages for efficient motility.

## Introduction

In the 17th century, Antoni van Leeuwenhoek shed light on a miniature world using a single-lens optical microscope and observed the wriggling of small particles. He noticed the existence of life and called them animalcules (microorganisms). Currently, state-of-the-art observation techniques, such as optical microscopy, electron microscopy, and X-ray crystallography have revealed many details about these microorganisms, including the diversity of their motility mechanisms [1]. Bacterial motility can be classified either as two-dimensional (2-D) planar movement or three-

Corresponding authors: Yoshiaki Kinosita, CPR, RIKEN, Wako, Saitama 351-0198, Japan. ORCID iD: https://orcid.org/0000-0003-4521-2800, e-mail: yoshiaki.kinosita@gmail.com; Yoshiyuki Sowa, Department of Frontier Bioscience, Hosei University, 3-7-2 Kajino-cho, Koganei, Tokyo 184-8584, Japan. ORCID iD: https://orcid.org/0000-0002-1691-2018, e-mail: ysowa@hosei.ac.jp

dimensional (3-D) swimming movement. Bacterial 2-D movements are highly diverse and include twitching, gliding, and polysaccharide injection. In contrast, most 3-D swimming behaviors are driven by the bacterial flagellar motor (BFM), with the exception of certain bacteria, such as *Spiroplasma* spp. and *Synechococcus* spp. [2]. The BFM is the most extensively investigated bacterial motility system, and in this review, we highlight bacterial flagellar motility and emphasize the importance of flagellar polymorphisms for effective propagation in structured environments.

## Bacterial Flagellum

Most swimming bacteria move toward favorable environments by rotating their flagella. The flagellum comprises a motor (BFM), a hook that works as a universal joint, and a helical filament that acts as a propeller [3-5]. Here, we focused on the flagella of *Salmonella* and *Escherichia coli*, the most extensively studied bacteria (unless explicitly stated otherwise) (Fig. 1A). The flagellum is one of the most sophisticated molecular machineries in bacteria (Fig. 1B), and have more than 30 related genes known as *flg*, *flh*, *fli*, *flj*, *mot*, and *che*, which have functions dependent on their locations on the genome map [6].

The filament and hook are polymers composed of single proteins, FliC and FlgE, respectively, and the junctions between them are composed of FlgK (HAP1) and FlgL (HAP3). Elongation of the flagellar filament is achieved by the polymerization of flagellin molecules with the help of the filament cap consisting of five FliD (HAP2) subunits. At the base of the flagellum, the export apparatus composed of a transmembrane export gate complex (FlhA, FlhB, FliP, FliQ, and FliR) and ATPase complexes (FliH, FliI, and FliJ) transports flagellin molecules as well as junction and cap proteins through an axial hole approximately 2 nm in diameter. The energy source for the transport process is the protonmotive force across the cytoplasmic membrane supported by ATP hydrolysis [7-9]. The recently developed *in vitro* reconstitution of the export apparatus will allow a more detailed discussion of transport activity in response to perturbations in the near future [10].

The motor is composed of four rings: (i) the MS (membrane-supramembrane) (FliF) [11,12]; (ii) P (peptidoglycan [PG]) (FlgI); (iii) L (lipopolysaccharide) (FlgH); and (iv) C (cytoplasmic) (FliG, FliM, and FliN). L- and P-rings work as bushings and are spanned by a rod, which works as a drive shaft and connects the MS-ring and hook [13]. The MotA-MotB stator complex assembles around the MS- and C-rings that act as a rotor of the BMF, functions as the proton channel, and anchors to the PG layer to serve as a force generator of the BFM [14-17]. The interactions between the stator and rotor generate the rotational torque of the BFM through coupling ion transits. These motor structures represent gram-negative bacteria, but are highly diverse among bacterial species [18]. For example, gram-positive bacteria lack L- and P-rings; *Vibrio* sp. have extra rings called H- and T-rings, and *Spirochetes* have a distinctive ring structure known as a "collar" [19,20].

## How do the Flagella Drive the Swimming Motility of Bacteria?

Before 1973, two opposing models were proposed: (i) the continuous morphological changes in flagella, such as eukaryotic ones (or cilia), and (ii) rotation of flagella functioning as a screw [21]. In 1974, flagellar rotation was confirmed by a tethered cell assay, which showed a cell body spinning through a single flagellar filament attached to a glass surface [22-24]. Notably, the tethered cell assay was one of the first functional assays at the single-molecule level. Furthermore, the direction of the spinning cell bodies frequently switched from counterclockwise (CCW) to clockwise (CW) and vice versa, changing the switching frequency in response to the extracellular environment. These observations indicate that a reversible rotary motor rotates each flagellum at its base and that the bacterial chemotactic system controls motor switching. The chemotactic response is a characteristic of life that employs motility for survival strategies, such as climbing up to a gradient of an attractant or escaping from a repellent [25].

## Rotation and Switching Mechanism of the BFM

A fundamental question in rotary-motor studies is how a motor couples input energy with mechanical rotation. Specifically, the chemical cycles of the stator through ion transit drives the rotational movements of the rotor ring. The atomic structure of the stator complex, which consists of a MotB dimer surrounded by a MotA pentamer ring, revealed recently by cryo-electron microscopy, has provided a paradigm for the rotation and switching mechanisms of BFM [26,27]. Fig. 1B shows the proposed model of the rotation mechanism of BFM; the MotA ring can rotate around the MotB dimer coupled with influx of ions and generate torque against the PG layer since MotB molecules are anchored to it. Torque is transmitted to the C-ring associated with the interaction of FliG and MotA and, consequently, the rotation of the rod, hook, and flagellar filament. This scenario explains the motor switch from the CCW to CW direction using a simple geometric model: the MotA ring of the stator interacts with outer or inner rim of the C-ring to drive the rotor CCW or CW, respectively. The change in the interaction points between FliG and MotA is caused by a conformational change

in the C-ring induced by the binding of the response regulator CheY-P to the FliM molecule [28]. This model remains to be proven by direct observation at the single-molecule levels, and it is necessary to develop a system for the reconstitution of BFM into artificial lipid membranes, such as that developed for the $F_oF_1$ ATP synthase [29].

## Chemotaxis

Chemotactic behavior is controlled by a two-component regulatory system (Fig. 1C) [30]. The major components are methyl group-accepting chemotactic proteins (MCPs), a histidine kinase (CheA), a response regulator (CheY), and a phosphatase (CheZ). *E. coli* BFM rotates in the CCW direction without signals from the chemotactic systems. Once MCPs sense an increase in repellent chemicals (or a decrease in attractants) from the external environment, CheA undergoes autophosphorylation and phosphorylates the response regulator CheY. Phosphorylated CheY (CheY-P) can bind to FliM and FliN in the C-ring, and this binding causes motor switching from CCW to CW. CheZ dephosphorylates CheY-P and reduces CheY-P concentration, resulting in motor switching from CW to CCW. Other chemotactic components, CheB and CheR regulate the methylation level of MCPs which achieves adaptation to the environment and responses dynamically to wide range of chemical concentrations; and CheW acts as a scaffold forming minimal core units for signal transduction, a trimer of the dimer of MCPs (two CheW monomers, one CheA dimer, and six receptors).

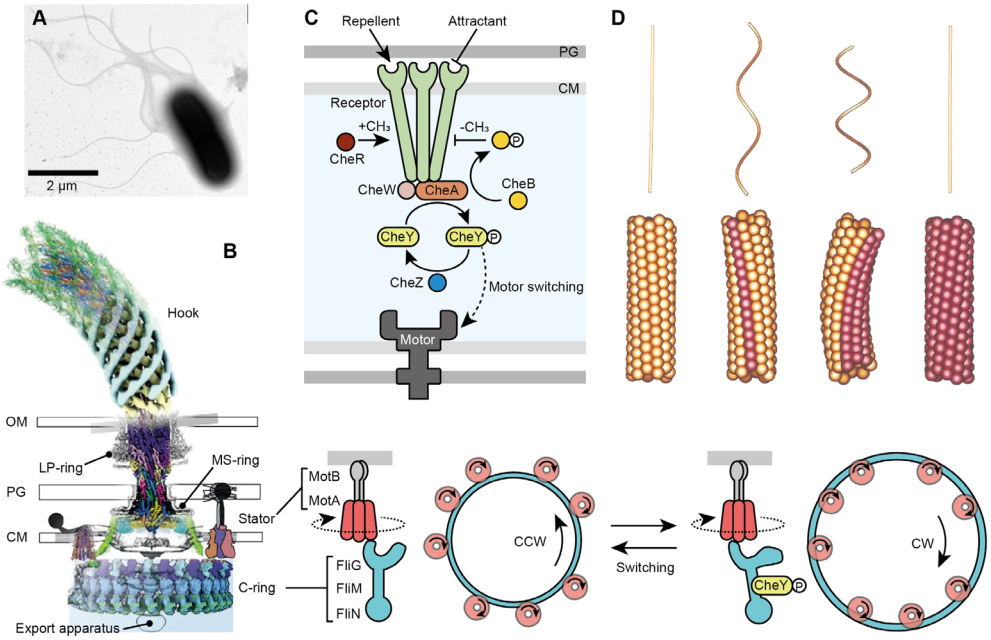


**Figure 1** Structure of bacterial flagellum. (A) Electron micrograph of *E. coli* cell. (B) Current structural and functional models of BFM. OM, outer membrane; PG, peptidoglycan layer; CM, cytoplasmic membrane. (C) Schematics of chemosensory systems. (D) Polymorphic flagellar changes depend on the number of protofilaments; the R-type protofilaments are shown in red. These figures were reproduced with permission from "***Seibutsubutsuri***, 61 (5), 2021" for Fig. 1A, "***Front. Microbiol.***, 13, 948383, 2022" for Fig. 1B, and "***Q. Rev. Biophys.***, 30 (1), 1-65, 1997" for Fig. 1D, with modifications.

## Flagellar-polymorphism-dependent Chemotactic Behaviors

As described above, the wave propagation model was one of the leading hypotheses for determining the driving force of flagellar motility. This is because flagellar filaments show polymorphisms in different helical pitches and radii [31]. Flagellar polymorphism has been demonstrated in *in vitro* copolymerization experiments [32], and helical parameters were determined by observing single flagellar filaments using dark-field microscopy [33,34]. Mechanical torsional forces from the rotation of the BFM cause flagellar polymorphism [35], as do pH and ionic strength [31]. The polymorphic mechanism of the filament was modeled by assuming two slightly different flagellin conformations, the L- and R-types, and the helical forms were thought to be arranged as a mixture of two distinct flagellin conformations (Fig.1D) [36-38]. As each filament is a tubular structure consisting of 11 protofilaments of flagellin molecules, this model predicts the occurrence of 12 different helical filament types, such as normal, curly, and coiled, when observed under a microscope [37,39-41].

Flagellated bacteria exhibit distinct chemotactic behaviors that move toward favorable environments. This type of chemotactic behavior is highly correlated with flagellation patterns. They can be broadly classified into two groups: (i) peritrichous and (ii) polar (Fig. 2A). To understand the details of the swimming patterns for Fig. 2C-E, Fig. 2B shows the relationship between the rotational direction of the motor and the helicity of the flagellar filament.

An *E. coli* cell has 5–10 left-handed flagellar filaments protruding from its cell body (peritrichous flagella), and the CCW rotation of a bundle of flagella (when viewed from the filament to the motor) propels the cell forward [42]. The cell undergoes reorientation (tumbling) upon switching the flagellar rotation from CCW to CW, leading to a change in the filament shape from the left-handed normal type to the right-handed curly type (Fig. 2C) [43]. Although *Rhizobium lupini* possesses peritrichous flagella, its swimming pattern differs slightly from that of *E. coli*. *R. lupini* cells swim forward by CW rotation of the right-handed filament, and the swimming direction is changed by the disruption of the flagellar bundle caused by different rotational rates or transient stops for each filament [44].

In the case of the polar flagellum (monotrichous), a single left-handed polar flagellum propels the *Vibrio* spp. cells forward and backward by CCW and CW rotations, respectively (Fig. 2D) [43]. Moreover, cells sometimes change their swimming direction by approximately 90° because of the buckling instability of their straight hook (this behavior is called as "flick") [45,46]. Helical-shaped bacteria, such as *Campylobacter* spp., *Helicobacter* spp., and magnetic bacteria, form polar flagella (amphitrichous) or bipolar flagella (bipolar lophotrichous). The coordination of bipolar flagellar rotation contributes to efficient swimming behavior [47].

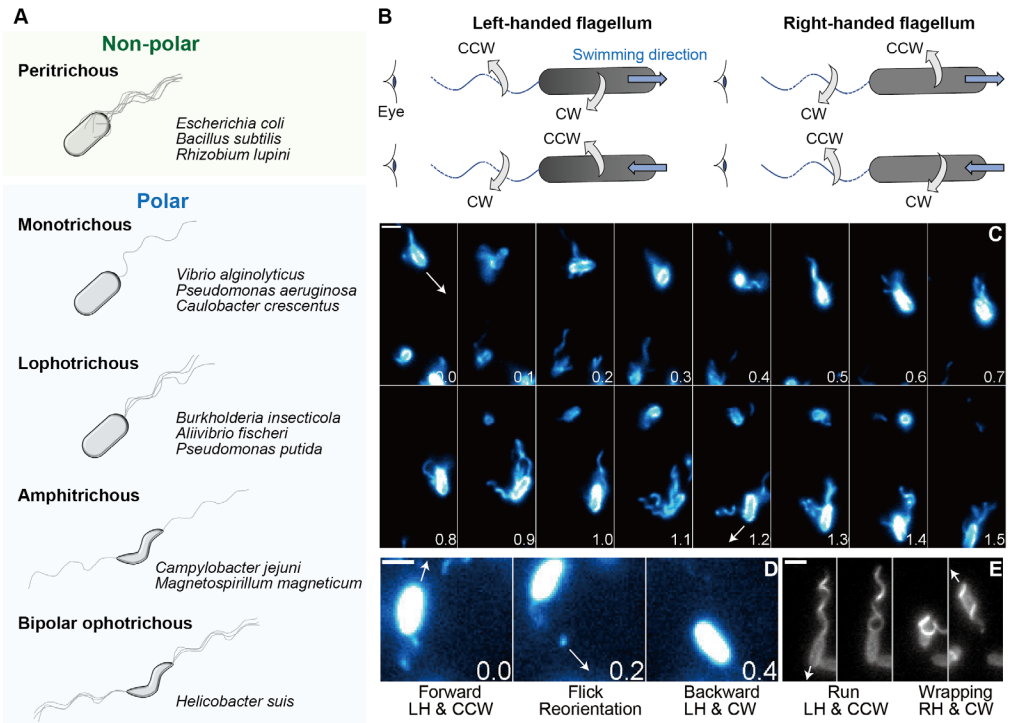


**Figure 2** Chemotactic behaviors with relation to flagellation pattern. (A) Flagellation patterns and representative bacterial species. (B) Effect of flagellar shape and rotational direction on cell swimming direction. The rotational direction of the motor was defined as viewed from the filament towards the motor. In left-handed filaments, cells move forward and backward by counterclockwise (CCW) and clockwise (CW) flagellar rotation, respectively. In right-handed filaments, cells move forward and backward by CW and CCW flagellar rotation, respectively. (C) Run-and-tumble behavior of *E. coli*: between 0 and 0.7 s, a single cell moved forward by CCW rotation of left-handed flagellar filaments; at 0.8 s, the flagella bundle was disrupted by motor switching, causing the cell to change its swimming direction (tumble). During tumbling, the flagellar filament transformed from a left-handed normal filament into a right-handed curly filament. (D) Forward-flick-backward swimming behavior of *A. fischeri*. (E) Wrapping motion of *B. insecticola* at 0.625 s intervals. A cell moves forward by the CCW rotation of the left-handed flagellar filament. The flagellar filament transformed into a right-handed coiled filament owing to motor switching from the CCW to CW direction. Consequently, the cells changed their swimming direction without reorientation of the cell body. Images in Fig. 2C-E are fluorescent images; arrows indicate the direction of swimming. Scale bars = 2 μm. These figures were reused and modified with permission from "***Seibutsubutsuri***, 61 (5), 2021" for Fig. 2C and 2D, and "***ISME J***, 12(3):838-848, 2018, Springer Nature" for Fig. 2E with modifications.

Recently, a new type of flagellar-based motility under high viscous drag conditions, flagellar-wrapping motion, was observed using fluorescence microscopy (Fig. 2E) [43,48]. Cells swim smoothly when the left-handed flagellar filament(s) rotates in the CCW direction. Once the motor switches from the CCW to the CW direction, the flagellar filaments transform from the left-handed normal form into the right-handed coiled form. Consequently, the filament wraps around the cell body and propels the cell forward via CW rotation. This motility mode was also reported in *Magnetospirillum magneticus* AMB-1 [49], *Shewanella putrefaciens* [50,51], *Pseudomonas putida* [52,53], *Pseudomonas aeruginosa* [54], *Allivibrio fischeri* [55], *Burkholderia insecticola* [55], *Campyrobacter jejuni* [47], and *Helicobacter suis* [56].

**Distinct Chemotactic Behaviors in *E. coli* K-12 ATCC10798**

Herein, we describe a distinct mode of motility initially described by Kinosita et al. [57], using the original *E. coli* K-12 strain ATCC10798, which deviates from the conventional run-and-tumble approach. This strain was first isolated in 1922 from the fecal matter of a patient with convalescent diphtheria [58]. Subsequently, many K-12 derivatives have been isolated and analyzed for their motility characteristics. Early investigations employed a soft agar plate assay as a facile screening approach to assess swimming and chemotactic abilities [59]. Given that cells with defects in either swimming or chemotaxis fail to generate chemotactic rings on soft agar plates, the extent of spreading is a good indicator of motility. However, owing to the prevalent use of positively selected strains in soft agar plate assays, a comprehensive understanding of the motility traits of the ATCC10798 strain remains elusive.

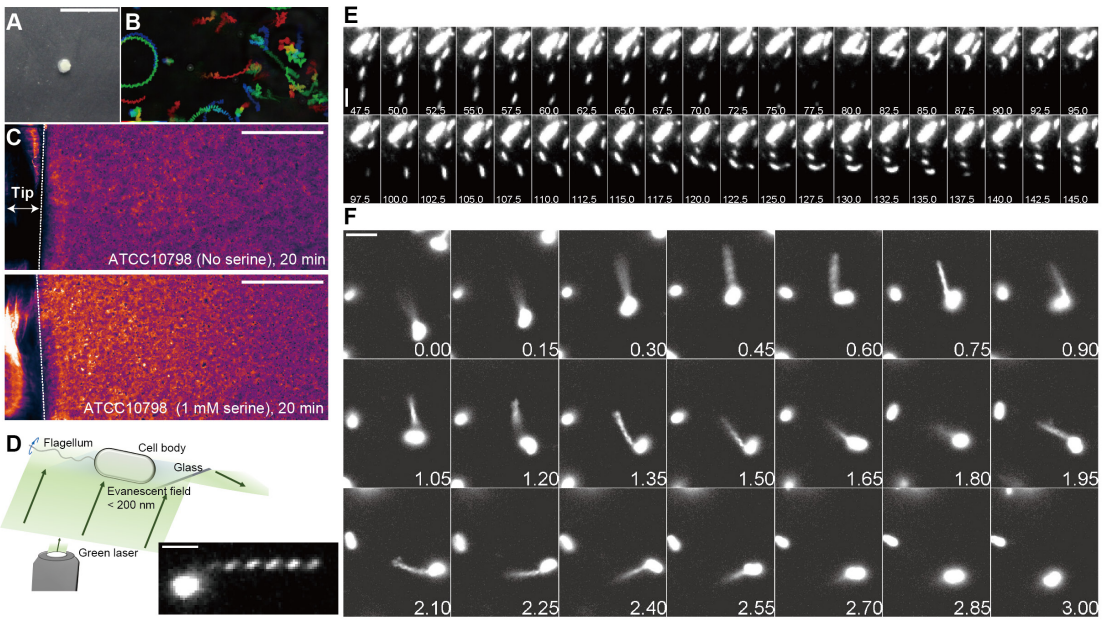


**Figure 3** Distinct chemotactic behaviors in the original *E. coli* K-12 ATCC10798. (A) Motility of the original *E. coli* K-12 strain on soft agar plates. Scale bar = 1 cm. (B) Sequential phase-contrast images taken at 50-ms intervals for 10 s were integrated using an intermittent color code: "red → yellow → green → cyan → blue." Scale bar = 20 μm. (C) Capillary assay of ATCC10798 cells. In the presence of 1 μM Serine, ATCC10798 cells gathered near the tip, denoted by orange color, indicating that the cells showed chemotactic responses. Scale bar = 200 μm. (D) Imaging of fluorescently labeled single filaments using TIRFM. A filament of ATCC10798 was oriented from the 2$^{nd}$ to the 4$^{th}$ quadrant relative to the major axis of the filament, indicating right-handed helicity. (E) Sequential TIRFM images of W3110 cells taken at 2.5-ms intervals. The orientation of the flagellar filament(s) is from the 1$^{st}$ to the 3$^{rd}$ quadrant relative to the major axis of the filament, indicating left-handed helicity of the filaments. From 47.5 to 65.0 ms, the wave of flagella propagates in a direction away from the hook end toward the flagellar tip, indicating that the flagella rotate in the CCW direction. The direction of rotation was switched at 67.5 ms, and their helicity changed from left- to right-handed within 70 ms. Scale bar = 2 μm. (F) Sequential fluorescent images of the original *E. coli* K-12 ATCC10798 cells, taken at 0.15-s intervals. From 0 to 0.9 s, the cell exhibits forward movement by CCW rotation of the right-handed flagellar filament. At 0.9 s, the motor is switched from the CCW to CW direction, resulting in a change in the swimming direction. This behavior is similar to the back-and-forth movement observed in *Vibrio alginolyticus*, rather than the run-and-tumble movement observed in the standard motility of *E. coli.* Scale bar = 5 μm. These figures were reused with permission from the "***Scientific Reports***, 28; 10(1), 15887, 2020, Springer Nature" for Fig. 3A-F, with modifications.

Initially, we assessed the motility traits of ATCC10798 cells on a soft agar plate and found that they failed to spread (Fig. 3A). Nevertheless, ATCC10798 cells swam in the liquid medium with reorientations (Fig. 3B). These results indicate that factors, other than motor rotation and switching, are required to spread colonies on soft agar plates. To identify these factors, we performed a series of experiments including (i) a tethered cell assay, (ii) a micro-capillary assay, (iii) fluorescence microscopy, (iv) genetic manipulation, and (v) an agarose assay. Our tethered cell assay showed no significant differences in motor speed or switching ability between ATCC10798 and W3110 (a standard motility strain), implying that motor properties did not affect colony spreading. Subsequently, a capillary assay was performed to evaluate the chemotactic ability of the original strain, which indicated that its chemotactic system remained active (Fig. 3C). We analyzed the flagellar dynamics of ATCC10798 cells using total internal reflection fluorescence microscopy (TIRFM) (Fig. 3D and E) [60,61]. This technique revealed that the ATCC10798 cells formed a right-handed filament with a pitch of 1.3 μm and a helix radius of 0.14 μm, indicative of a curly-type filament. Interestingly, we observed no flagellar-polymorphic changes, regardless of motor switching (Supplementary Movie 4 in [57]). Moreover, ATCC10798 cells showed back-and-forth swimming behavior, which diverged from the typical run-and-tumble behavior (Fig. 3F). Conversely, W3110 cells displayed flagellar transformation, transitioning from the left- to right-handed form via motor switching from the CCW to CW direction in a representative run-and-tumble approach (Figs. 1C and 3E).

We also investigated the cause of the absence of flagellar polymorphisms in the ATCC10798 strain. By sequencing the genes of the major components related to motility between ATCC10798 and W3110, we identified a single substitution, N87K, of FliC in ATCC10798. To examine whether FliC (N87K) caused a defect in flagellar polymorphism, we replaced the *fliC* gene of ATCC10798 with that of W3110. This replacement restored the flagellar polymorphisms, transitioning from the left- to right-handed form and vice versa, and promoted colony spreading on soft agar plates. These results suggest that flagellar polymorphisms are critical factors in colony spreading.

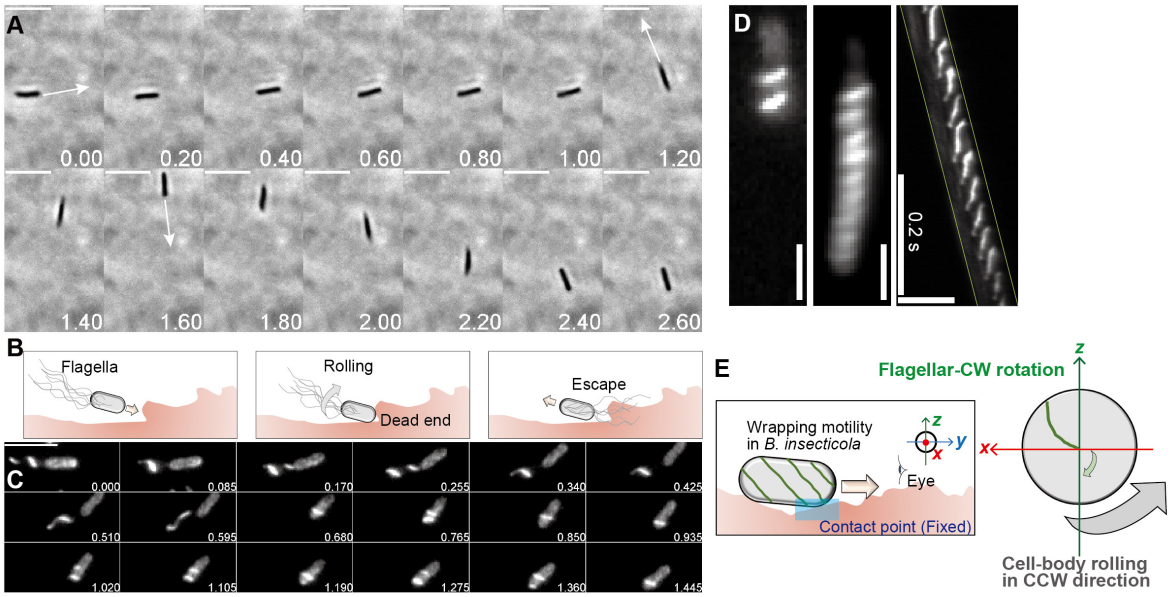


**Figure 4** Flagellar polymorphisms required for moving in a structured environment. (A) Sequential-phase contrast images of cellular migration of *E. coli* W3110 cells on 0.2% agarose. The arrows indicate the swimming direction after a reversal, with an angle change of approximately 180°. Scale bar = 5 μm. (B) Diagram showing *E. coli* escape a dead end within a structured environment. (C) Sequential fluorescence images of *Burkholderia insecticola*. From 0 to 0.5 s, the cell showed forward movement through CCW rotation of left-handed flagellar filaments before becoming trapped in a dead end. The rotational direction of the motor was then switched from CCW to CW, transforming the filament into a right-handed coiled state. This allowed continuous CW rotation to reorient the swimming direction of the cell. Scale bar = 5 μm. (D) Flagella contact the surface, and its point of contact transiently changes. Still image (*left*), sum-type projection (*middle*), and kymograph of the middle portion of the left image (*right*). The green lines indicate the edges of the cell body. Scale bars = 2 μm (left and middle) and 3 μm (right). (E) Diagram showing how the wrapping motion occurs in *B. insecticola*. (D) The kymograph illustrates that the flagellar wave propagation moves in the opposite direction of the flagellar end. This suggests that the flagellar helix instantaneously interacts with the surface and subsequently transiently changes its interaction point by continuous flagellar rotation around its cell body. These figures were reproduced with permission from "***Scientific Reports***, 28; 10(1), 15887, 2020, Springer Nature" for Fig. 4A and "***ISME J***, 12(3):838-848, 2018, Springer Nature" for Fig. 4D with modifications.

**Flagellar Polymorphism is Required to Slip through the Structured Environment**

It is widely known that bacterial colony spread on agar is associated with chemotaxis, particularly motor switching [62]. However, defects in flagellar polymorphisms inhibit colony spreading, even though motor torque and switching are functional. To examine the importance of flagellar polymorphisms in colony spreading on soft agar plates, the swimming behavior of *E. coli* cells on a semi-solid agarose pad was observed under a microscope. Regarding the agar experiments, once the ATCC10798 cells adhered to the surface, they lost the ability to swim, whereas the W3110 cells exhibited frequent attachment to the surface followed by escape via 180° reversals without reorientation of the cell body (Fig. 4A). These findings suggest that flagellar polymorphisms are essential for colony spread in structured environments. Turner et al. [63] reported the sole maneuver in structured environments: the flagellar bundle transforms from a normal to a curly state, with curly filaments forming a bundle that propels the cell forward in the direction opposite to its original swimming trajectory (Fig. 4B). Notably, this reverse motion also occurred in the Δ*cheY* strain by rolling the flagellar bundle to the opposite pole of the cell [64].

Our conclusion that flagellar polymorphism is required for motility in a structured environment agrees with previous reports that some specific point mutations in the *fliC* gene in peritrichously flagellated *Salmonella* or *E. coli* cells, cause a defect in flagellar polymorphism and support cell swimming in liquid, but not colony spreading on a soft agar plate [65,66]. This scenario, deduced from the observation of peritrichously flagellated bacteria, is consistent with the results obtained for polar-flagellated bacteria. Polar-flagellated bacteria possess multiple flagellins, such as FlaA and FlaB in *Shewanella* spp. [51,67,68], FlaJ-O in *Caulobacter crescentus* [69], and FlaA-E in *Vibrio cholera* [70], and several deletions of these flagellins did not affect swimming motility in liquid, but only affected migration on a soft agar plate. Kühn et al. [51] reported that FlaB in *S. putrefaciens* is crucial for flagellar polymorphisms and for the transition from regular swimming to wrapping motion. In contrast, *S. putrefaciens* cells that encode only the *flaA* genes exhibit no motility on agar because of deficient flagellar polymorphisms. These reports support the conclusion that flagellar polymorphisms are crucial for cell migration in a structured environment. Flagellar transformation enabled a single cell to escape the dead end by reversing its swimming direction (Fig. 4C) [55]. This phenomenon can be explained by the focal-adhesion model, such as that of gliding bacteria, by observing fluorescently labeled filaments using TIRFM (Fig. 4D and E) [71].

**Conclusion and Perspective**

In this mini review, we summarize the structure and function of bacterial flagella and discuss the importance of flagellar polymorphisms in bacterial motility in structured environments. The polymorphic transformation of flagellar filaments has been an interesting biophysical research topic for more than 50 years, and recent fluorescence imaging studies have revealed its essential role as a physiological phenomenon. An example is the forward and backward motility of the original *E. coli* K-12 strain, ATCC10798 [57]. We showed that these continuous movements work well in liquid environments but not in structured environments. At first glance, this motility is not a good survival strategy, but it may be convenient for escaping from other factors, such as phages or host immunity. Another example is the new motility wrapping of cell bodies with coiled filaments in bacteria under high-viscosity conditions. Thus, flagellar polymorphisms have different effects on bacterial survival, depending on the environment.

Novel flagellar movements may be discovered in the future; Archaeal motility is considered to be a promising candidate [61]. Archaea exhibit swimming behavior similar to that of bacteria driven by the rotation of their helical flagellum, which shows polymorphism, although its structure differs from that of the bacterial flagellum. [72,73]. Future research must expand beyond the bacterial kingdom to examine whether flagellar polymorphisms are critical factors for flagellated microorganisms to move effectively in structured environments.

**Conflict of Interest**

The authors declare there are no competing financial interests.

**Author Contributions**

Y.K and Y.S wrote the manuscript.

**Data Availability**

The evidence data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Acknowledgements**

We would like to thank Prof. Takayuki Nishizaka and Dr. Yusuke V. Morimoto for their help with the original research. This work was supported in part by the Japan Society for the Promotion Science (JSPS) Fellowship for Japan Junior Scientists (JP15J12274) and a postdoctoral fellowship for research abroad from JSPS, KAKENHI Grant Numbers JP20K22640, JP21K15062 and JP23K14161, the special postdoctoral program from RIKEN (given to Y.K.), and JSPS KAKENHI Grant Numbers JP20K06564, JP21H00410, JP21H05892, JP23K05701, and JP23H04429 (given to Y.S.).

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