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Exchange of rotor components in functioning bacterial flagellar motor

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ABSTRACT

The bacterial flagellar motor is a rotary motor driven by the electrochemical potential of a coupling ion. The interaction between a rotor and stator units is thought to generate torque. The overall structure of flagellar motor has been thought to be static, however, it was recently proved that stators are exchanged in a rotating motor. Understanding the dynamics of rotor components in functioning motor is important for the clarifying of working mechanism of bacterial flagellar motor. In this study, we focused on the dynamics and the turnover of rotor components in a functioning flagellar motor. Expression systems for GFP-FliN, FliM-GFP, and GFP-FliG were constructed, and each GFP-fusion was functionally incorporated into the flagellar motor. To investigate whether the rotor components are exchanged in a rotating motor, we performed fluorescence recovery after photobleaching experiments using total internal reflection fluorescence microscopy. After photobleaching, in a tethered cell producing GFP-FliN or FliM-GFP, the recovery of fluorescence at the rotational center was observed. However, in a cell producing GFP-FliG, no recovery of fluorescence was observed. The transition phase of fluorescence intensity after full or partially photobleaching allowed the turnover of FliN subunits to be calculated as 0.0007 s^{-1}, meaning that FliN would be exchanged in tens of minutes. These novel findings indicate that a bacterial flagellar motor is not a static structure even in functioning state. This is the first report for the exchange of rotor components in a functioning bacterial flagellar motor.

1. Introduction

The bacterial flagellum is a supramolecular complex and functions as rotary motor driven by the flux of H^+ or Na^+ [1–5]. A feature of the torque-speed relationships is common between H^+- and Na^+-driven motors [6–8]. Flagellum consists of a basal body (rotary motor), a helical filament (propeller) and a hook connecting them. The motor consists of a rotor and some stator units; a stator unit is thought to be a torque generator that converts the energy of ion flux into mechanical power.

The stator part consists of several units, at least 11 in *Escherichia coli* [9]. Stator unit is thought to consist of 4 molecules of A-subunits and 2 molecules of B-subunits [10]. The B subunit contains a large periplasmic domain that has a putative peptidoglycan-binding domain, and the assembly of stator units to the motor requires this domain [11]. In *Salmonella*, a crystal structure of this domain has been solved and a model for stator assembly was proposed [12]. The basal body of Gram-negative bacteria consists of a rod and several rings, termed the L-, P-, MS-, and C-ring (Fig. 1A) [2]. During flagellar assembly, 26 copies of FliF multimerizes to form the MS-ring and 26 copies of FliG are thought to attach to the MS-ring. 34 copies of FliM and more than 100 copies of FliN assemble to form the main structure of the C-ring. FliG is thought to interact with charged residues in the cytoplasmic domain of the stator protein MotA to generate torque [13]. FliM has a sequence for binding the chemotaxis signaling molecule, phosphorylated CheY, in its N-terminal domain [14]. By binding of phosphorylated CheY, rotational direction of motor is changed from counter-clockwise (CCW) to clockwise (CW). FliN interacts with the C-terminal domain of FliM, which shows sequence homology to FliN [15]. FliG, FliM and FliN are involved in the formation of flagellar structure, the rotation of motor, and the switching of rotational direction [2].

The overall structure of flagellar motor has been investigated well. The flagellar structure has been thought to be static after its assembly is completed. However, it was recently proved that stator of flagellar motor is dynamic [5,16,17]. Understanding the dynamics of components in functioning state is important for the clarifying of working mechanism of supramolecular machines. Therefore, we have focused on the dynamics and turnover of rotor subunits, and demonstrated that the rotor components, FliN and FliM, are exchanged in a functioning motor. This is the first evidence for the exchange of rotor components in a functioning bacterial flagellar motor.

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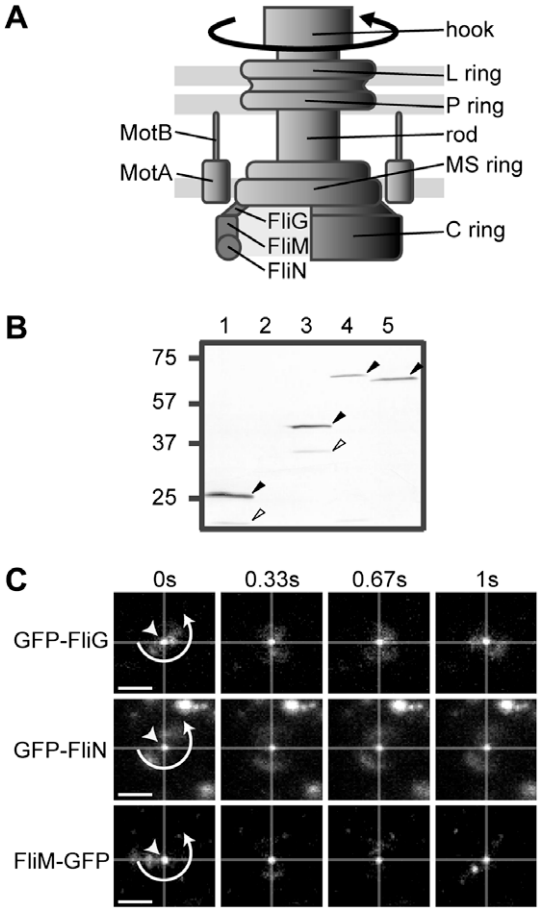

Fig. 1. (A) Diagram of *E. coli* flagellar motor. (B) Immunoblots for GFP-fusions from whole-cell extracts. Molecular mass values (kD) were shown on the left side of the panel. Black arrowheads correspond to mature GFP or GFP-fusions. White arrowheads indicate the bands smaller than the expected mature size. Lane 1–3 indicate the bands from EFS021 producing GFP, wild-type FliN and GFP-FliN, respectively. Lane 4 indicates the band from EFS011 cells producing FliM-GFP. Lane 5 indicates the band from DFB225 cells producing GFP-FliG. (C) Sequential images of tethered cells producing GFP-fusions. Fluorescent images were shown every 1/3 s. Top, DFB225 cell producing GFP-FliG; middle, EFS021 cell producing GFP-FliN; bottom, EFS011 cell producing FliM-GFP. White arrowheads and circular arrows indicate fluorescent spots localizing at the rotational center and the rotational direction of tethered cell, respectively. Scale bar, 1.6 μm.

2. Materials and methods

2.1. Bacterial strains and plasmids

Bacterial strains and plasmids were shown in Supplementary materials. LB broth (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl) was used for culture growth, transformations, and plasmid isolation. T broth (1% bactotryptone, 0.5% NaCl) was used for cell growth in motility assay and in the observation by fluorescence microscopy. Arabinose was added to T broth to be 0.002%, 0.005%, and 0.0005% to produce GFP-FliN, FliM-GFP, and GFP-FliG, respectively.

2.2. Cell preparation for the observation by fluorescence microscopy

Overnight cultures were inoculated into T broth (1/100 volume) containing appropriate concentration of arabinose and cells were grown for 4.5–5 h at 30 °C. For the observation of GFP-FliN, EFS021 strain (Δ*fliN*) harboring pFSGN3 (*gfp-fliN*) and pYS11 (*fliC-sticky*), and EFS022 (Δ*fliN fliC-sticky*) strain harboring pFSGN3 were used. For the observation of FliM-GFP, EFS011 (Δ*fli*M) strain harboring pFS6003m (*fliM-gfp*) and pFSST1 (*fliC-sticky*), and EFS012 (Δ*fli*M *fliC-sticky*) strain harboring pFS6003m were used. For the observation of GFP-FliG, DFB225 strain (Δ*fliG*) harboring pTH2100 (*gfp-fliG*) and pYS11 was used.

To shorten flagellar filaments, cells were passed through a narrow polystyrene tube connected between two syringes more than 40 times. Cells were harvested by centrifugation and resuspended in 85NaMB (10 mM potassium phosphate (pH 7.0), 85 mM NaCl, 0.1 mM EDTA). The cell suspension was incubated for 10 min at room temperature. The cell suspension was loaded into the space between a coverslip and a microscope slide with a spacer to stuck the cells on a coverslip spontaneously. Additional 85NaMB was loaded to remove the unattached cells, and the sample was incubated for 10 min at room temperature. Cells were observed under the Olympus IX71 based Total Internal Reflection Fluorescence (TIRF) microscopy [18] and images were recorded at 30.25 frames/s using EMCCD camera (DU860-BV, Andor Technology, UK).

2.3. FRAP experiment by TIRF microscopy

Fluorescence Recovery After Photobleaching (FRAP) experiment were performed using TIRF microscopy. By removing ND filter (13%) from the optical axis, tethered cells were irradiated with high intensity evanescent light and the fluorescent spot at the rotational center, which locates near glass surface (∼50 nm), was completely or partially photobleached.

2.4. Measurement of fluorescence intensity

To estimate the fluorescence intensity of a fluorescent spot, two-dimensional Gaussian function was applied to the images of single fluorescent spot using self-made program constructed by Labview ver. 8.6 (National Instruments, USA). We defined the peak value from Gaussian fit as fluorescence intensity of each spot.

2.5. Swimming speed of the cell producing GFP-fusion

Cell cultures were diluted with 85NaMB containing 20 mM serine, to suppress the change of rotational direction of motor. Swimming behavior of cells was recorded on videotape and the swimming speed was calculated on the monitor.

2.6. Measurement of rotational speed and CCW bias of motor

Cell suspension was loaded into the space between coverslips with a spacer, and was incubated for 10 min. Additional 85NaMB was loaded to remove the unattached cells on the coverslip. The suspension of polystyrene beads (φ = 0.5 μm) was loaded and sample slide was incubated for 10 min to attach bead to flagellar filament. Additional 85NaMB was loaded to remove the unattached beads. The phase contrast image of bead on flagella filament was recorded by high speed CCD camera (IPX-VGA210LMCN, IMPERX, USA) at 1250 frames/s. Image of bead was fitted by two-dimensional Gaussian function to estimate the position of bead. The angular velocity and CCW bias were calculated using self-made programs constructed by Labview ver. 8.6.

2.7. Immunoblotting for the GFP-fusions

The cells were harvested by centrifugation and condensed by sterilized water (optical density at 660 nm, 20). Cell suspensions were diluted by the same volume of 5× SDS loading buffer containing β-mercaptoethanol and were boiled at 100 °C for 5 min. The proteins were separated by SDS–PAGE and immunoblotting was performed using anti-GFP antibody (Clontech, USA). Bands were