High-Resolution pH Imaging of Living Bacterial Cells To Detect Local pH Differences

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ABSTRACT Protons are utilized for various biological activities such as energy transduction and cell signaling. For construction of the bacterial flagellum, a type III export apparatus utilizes ATP and proton motive force to drive flagellar protein export, but the energy transduction mechanism remains unclear. Here, we have developed a high-resolution pH imaging system to measure local pH differences within living Salmonella enterica cells, especially in close proximity to the cytoplasmic membrane and the export apparatus. The local pH near the membrane was ca. 0.2 pH unit higher than the bulk cytoplasmic pH. However, the local pH near the export apparatus was ca. 0.1 pH unit lower than that near the membrane. This drop of local pH depended on the activities of both transmembrane export components and FliI ATPase. We propose that the export apparatus acts as an H+/-protein antiporter to couple ATP hydrolysis with H+ flow to drive protein export.

IMPORTANCE The flagellar type III export apparatus is required for construction of the bacterial flagellum beyond the cellular membranes. The export apparatus consists of a transmembrane export gate and a cytoplasmic ATPase complex. The export apparatus utilizes ATP and proton motive force as the energy source for efficient and rapid protein export during flagellar assembly, but it remains unknown how. In this study, we have developed an in vivo pH imaging system with high spatial and pH resolutions with a pH indicator probe to measure local pH near the export apparatus. We provide direct evidence suggesting that ATP hydrolysis by the ATPase complex and the following rapid protein translocation by the export gate are both linked to efficient proton translocation through the gate.

Received 13 October 2016 Accepted 4 November 2016 Published 6 December 2016

Protons (H+) are utilized for energy and signal transduction in the complex biological networks in living cells to support various biological activities (1–3). Intracellular pH homeostasis is fundamentally essential for living cells to maintain various cellular functions. It has been reported that intracellular compartments generate a local H+ gradient within the cytoplasm (4) even though the diffusion coefficient of H+ is extremely high, estimated to be on the order of 10−7 to 10−6 cm2/s (5, 6). Therefore, precise measurements of local pH around biological nanomachines are critical for understanding the role of H+ in their biological activities.

For construction of the bacterial flagellum, which is a supramolecular motility machine, 14 different flagellar proteins are transported by a type III export apparatus to the distal end of the growing flagellar structure. The type III export apparatus utilizes ATP and proton motive force (PMF) across the cytoplasmic membrane to drive flagellar protein export (7–9). The export apparatus is composed of a PMF-driven transmembrane export gate made of FlhA, FlhB, FlhO, FlhI, FlhQ, and FlhR and a cytoplasmic ATPase complex consisting of FlhI, Flh ATPase, and FljI (Fig. 1A) (10, 11). FlhI, FlhJ, and FlhR are not essential for flagellar protein export (12, 13), suggesting that PMF is the primary energy source. Interestingly, the export gate by itself utilizes Na+ as the coupling ion in addition to H+ when FlhH and FlhI are not functional. FlhA shows the H+ and Na+ channel activities, suggesting that FlhA may act as an energy transducer of the export gate, although its H+ channel activity is quite low (14).

The FlhI ATPase forms the FlhH2FlhI complex with the FlhH2 homodimer in the cytoplasm (15, 16). Since FlhH2-yellow fluorescent protein (YFP) shows rapid exchanges between the flagellar basal body (FBB) and the cytoplasmic pool in an ATP-independent manner, the FlhI2FlhI complex is proposed to act as a dynamic carrier to deliver export substrates and chaperone-substrate complexes to the export gate (17). FlhI also forms the FlhIR ring complex at the flagellar base (18, 19). FlhI binds to the center of the FlhIR ring to facilitate ATP hydrolysis by FlhI (20). The FlhIR complex associates with the FBB through interactions of FlhH with FlhA and a C ring component protein, FlhN (21–23) (Fig. 1A). The FlhH2FlhI2FlhJ complex is structurally similar to F- and V-type rotary ATPases, which couple ATP synthesis or hydrolysis with H+ translocation (16, 20, 24).

InvC is a FliI homolog of the Salmonella enterica virulence type III secretion system (T3SS) and has been shown to act as an unfoldase to induce the release of chaperone from the chaperone-substrate complex and to unfold the substrate for efficient protein export in an ATP-dependent manner (25). Recently, Erhardt et al., have shown that increased PMF is capable of bypassing the secre-
show that the local pH near the export apparatus is ca. 0.1 unit lower than the pH near the cytoplasmic membrane surface and that this small drop in pH requires the presence of Flii and export gate components.

RESULTS

pH resolution of an in vivo pH imaging system. Precise measurements of cytoplasmic pH near the export apparatus are essential for understanding the energy transduction mechanism of PMF-driven flagellar type III protein export. Therefore, we have developed a high-resolution pH imaging system that can be used for in vivo imaging, with a fluorescent protein, pHluorin(M153R), and a fluorescence optical microscope with a dual-wavelength illumination system. The fluorescence probe pHluorin is a ratiometric pH indicator whose emission intensities at a wavelength of 508 nm by the excitation at wavelengths of 410 and 470 nm show remarkable pH dependence over a pH range from 5.5 to 8.5, so that the emission intensity ratio (410/470 ratio) can be used to measure the pH around the probe (30). The M153R mutation was introduced to make this probe much more stable and brighter (31). The pHluorin(M153R) probe was excited by a xenon lamp equipped with a high-speed wavelength switcher that can switch the wavelength between 410 and 470 nm with a switching speed of less than 2 ms. Each fluorescent image was captured by an electron-multiplying charge-coupled device (EMCCD) camera (see Fig. S1A in the supplemental material). Our pH imaging system can measure pH over a range from 5.5 to 8.5 (see Fig. S1B). To estimate the pH resolution of our pH imaging system, the fluorescence intensities from purified pHluorin(M153R) solutions were measured at different protein concentrations and pH 7.0. The standard deviations of the observed pH values indicate that the accuracy of intracellular pH measurement is 0.02 unit (see Fig. S1C). Because the brightness of the pHluorin probe was 400 to 1,000 arbitrary units (AU) when expressed in living Salmonella cells, we were able to detect a pH difference in the range of 0.04 to 0.07.

pHluorin(M153R) labeling at membrane proximity. To carry out high-resolution pH imaging at membrane proximity, the pHluorin(M153R) probe must be localized near the cytoplasmic membrane. FliG is a C ring protein involved in flagellar motor rotation and directly associates with the cytoplasmic face of the FBB MS ring formed by 26 copies of the transmembrane flagellar protein FlIF (32). The pHluorin(M153R)-FliG fusion protein is very stable and functional and is localized to the flagellar base (31). It has been shown that purified pHluorin(M153R)-FliG can measure the pH over a range from 5.5 to 8.5 (see Fig. S2A in the supplemental material). We used this fusion protein as a probe to investigate whether our pH imaging system is capable of detecting the difference between the local pH near the inner surface of the cytoplasmic membrane and the pH of the bulk cytoplasm. To identify the exact location of the pHluorin(M153R) probe in the FBB, the FBBs were purified from wild-type and pHluorin(M153R)-flIG strains and observed by electron cryomicroscopy (cryo-EM) (Fig. 1B). The FBB structure containing pHluorin(M153R)-FliG showed an extra density corresponding to the pHluorin(M153R) probe inside the C ring, whose diameter is 45 nm and height is 16.5 nm (Fig. 1C and D). This indicates that pHluorin(M153R)-FliG can measure not only the local cytoplasmic pH at membrane proximity but also the local pH near the export apparatus.

FIG 1 Location of the pHluorin(M153R) probe at the flagellar base. (A) Schematic diagram of the bacterial flagellar basal body with a type III export apparatus attached. The export apparatus consists of a PMF-driven transmembrane export gate made of FlhA, FlhK, FlhO, FlhP, FlhQ, and FlhR and a cytoplasmic ATPase complex consisting of FliH, FliI, and FliJ. To measure the local pH near the gate, the pHluorin(M153R) probe was fused to the N terminus of FliG. Peri, periplasm; CM, cytoplasmic membrane; Cyto, cytoplasm. (B) Average 3D image of the FBB purified from the Salmonella HK1002 strain (wild type [WT]). A c100 rotational symmetry was enforced for the refinement of the axial section images of the wild-type FBB and the pHluorin(M153R)-FliG FBB were processed and superimposed. The location of the pHluorin(M153R) probe is indicated by arrows. The pHluorin(M153R) position looked flexible due to the flexibility of the C-terminal region of pHluorin(M153R), thereby reducing the electron density of the pHluorin(M153R) probe.

pHluorin(M153R) labeling at membrane proximity. To carry out high-resolution pH imaging at membrane proximity, the pHluorin(M153R) probe must be localized near the cytoplasmic membrane. FliG is a C ring protein involved in flagellar motor rotation and directly associates with the cytoplasmic face of the FBB MS ring formed by 26 copies of the transmembrane flagellar protein FlIF (32). The pHluorin(M153R)-FliG fusion protein is very stable and functional and is localized to the flagellar base (31). It has been shown that purified pHluorin(M153R)-FliG can measure the pH over a range from 5.5 to 8.5 (see Fig. S2A in the supplemental material). We used this fusion protein as a probe to investigate whether our pH imaging system is capable of detecting the difference between the local pH near the inner surface of the cytoplasmic membrane and the pH of the bulk cytoplasm. To identify the exact location of the pHluorin(M153R) probe in the FBB, the FBBs were purified from wild-type and pHluorin(M153R)-flIG strains and observed by electron cryomicroscopy (cryo-EM) (Fig. 1B). The FBB structure containing pHluorin(M153R)-FliG showed an extra density corresponding to the pHluorin(M153R) probe inside the C ring, whose diameter is 45 nm and height is 16.5 nm (Fig. 1C and D). This indicates that pHluorin(M153R)-FliG can measure not only the local cytoplasmic pH at membrane proximity but also the local pH near the export apparatus.
In vivo calibration of the pHluorin(M153R)-FliG probe. To test the capability of cytoplasmic pH measurements by the pHluorin(M153R)-FliG probe, we expressed pHluorin(M153R)-FliG in a Salmonella strain with deletion of the flagellar master operon flhDC and measured intracellular pH over an external range of 6.5 to 7.5 in the presence of 20 μM gramicidin and 20 mM potassium benzoate, by which the intracellular pH can be controlled to the same value as the external one. The pHluorin(M153R)-FliG probe was diffused in the cytoplasm (see Fig. S2B in the supplemental material), and the 410/470 ratio was the same as that of purified pHluorin(M153R)-FliG under each of the four pHs from 6.0 to 7.5 that we measured (see Fig. S2A). This indicates that pHluorin(M153R)-FliG can be used as a pH indicator probe to measure the local pH near the cytoplasmic membrane in living cells.

Measurements of bulk cytoplasmic pH. Cytoplasmic pH is maintained at around 7.5 over a range of external pHs from 5.5 to 8.0 (33). To carry out precise measurements of the bulk cytoplasmic pH by our pH imaging system, we transformed wild-type Salmonella cells with a plasmid encoding pHluorin(M153R) and recorded the ratiometric pH images at an external pH of 7.0. The pH was measured to be 7.34 ± 0.17 (Fig. 2A; see also Table S1). Because no MotAB complex is expressed in the ΔfliM-fliN::tetRA mutant (40), we introduced the ΔfliM-fliN::tetRA allele into the Salmonella pHluorin(M153R)-fliG strain by P22-mediated transduction. The pHluorin(M153R)-FliG probes formed fluorescent spots in the cells (Fig. 2B, upper panel), indicating that they bind to the cytoplasmic face of the MS ring and hence are localized close to the membrane. The local pH was measured to be 7.53 ± 0.20 in living Salmonella cells at an external pH of 7.0 (Fig. 2B; see also Table S1). So, the cytoplasmic pH near the membrane is about 0.2 unit higher than the bulk cytoplasmic pH (Fig. 2). To confirm this, we fused pHluorin(M153R) to a transmembrane protein (Flk) and a membrane targeting sequence (MTS) of MinD (MinDMTS), both of which are localized to the cytoplasmic membrane (41, 42).
In agreement with previous reports, these two fusions were localized to the cytoplasmic membrane (Fig. 2C and D, upper panels). The local pH values near the membrane measured by pHluorin(M153R)-fliG were 7.43 ± 0.09 and 7.52 ± 0.14, respectively (Fig. 2C and D, middle panels). The former value was close to the bulk cytoplasmic pH (7.34 ± 0.17) and the latter value was almost the same as that at the cytoplasmic face of the MS ring (7.53 ± 0.20). Since Flk has a relatively large cytoplasmic domain, these results indicate that the local pH values depend on a distance between the pHluorin(M153R) probe and the cytoplasmic membrane (Fig. 2, lower panels), suggesting the presence of a local pH gradient in the cytoplasm of living cells toward the membrane surface.

Measurements of local pH near the flagellar type III export apparatus. Next, we measured the local pH at the cytoplasmic surface of the cell membrane in the pHluorin(M153R)-fliG strain at external pH 7.0. The local pH near the export apparatus was measured to be 7.43 ± 0.24, ca. 0.1 unit lower than that near the membraneΔfliM-fliN::tetRA pHluorin(M153R)-fliG and pHluorin(M153R)-minDts strains (Fig. 3A; also see Table S1). In contrast, in the absence of the MotAB complex, which conducts H+ to generate torque for flagellar motor rotation, the local pH was 7.45 ± 0.27, almost the same as the local pH near the cytoplasmic membrane measured in the pHluorin(M153R)-fliG strain (see Table S1), indicating that the proton channel activity of the MotAB complex does not contribute to the local pH change observed in the pHluorin(M153R)-fliG fliR::Tn10 strain. The pHluorin(M153R)-fliG cells showed the export activity under the same experimental conditions (see Fig. S5, lane 3), indicating that the type III flagellar export apparatus is in an active state during vivo pH imaging. Therefore, we suggest that a small pH drop near the functional export apparatus is due to the H+ influx through the export gate.

Effects of FliI ATPase activity on local pH difference near the export apparatus. Each cell of the ΔfliH-fliI fliB(P28T) bypass mutant forms a couple of flagella even in the absence of FliiH and FliI (12). PMF consists of the H+ gradient (ΔpH) and the electric potential difference (Δφ) across the cytoplasmic membrane. Only Δφ of PMF is used for flagellar protein export by wild-type cells, whereas both ΔpH and Δφ are essential for protein export by the ΔfliH-fliI fliB(P28T) bypass mutant (34). The ΔpH component is probably required for H+ movement through the export gate in the absence of FliiH and FliI (34), raising the possibility that the hexameric ring complex of Flii ATPase may contribute to efficient H+ flow through the gate. To test this, we measured the local pH of the ΔfliH-fliI fliB(P28T) pHluorin(M153R)-fliG strain in motility buffer at external pH 7.0. The cells of this strain retained the export activity under the same experimental conditions (see Fig. S5, lane 4, in the supplemental material). The bulk cytoplasmic pH of the ΔfliH-fliI fliB(P28T) mutant was essentially the same as that of the wild type (Fig. 4A; see also Table S1). However, the local pH near the export apparatus was 0.12 unit higher in the absence of FliiH and FliI (34), raising the possibility that the proton channel activity of the MotAB complex does not contribute to the local pH change observed in the ΔfliH-fliI fliB(P28T) mutant (34). Since FliiH and FliI were expressed from the chromosomal DNA or a plasmid in the ΔfliH-fliI fliB(P28T) mutant was essentially the same as that of the pHluorin(M153R)-fliG cells (Fig. 4B; see also Table S1). When FliiH and FliI were expressed from a plasmid (pKOR1), we observed the same local pH values near the export apparatus as in the ΔfliH-fliI fliB(P28T) strain (see Table S1), indicating that ΔfliH and ΔfliI contribute to the 0.12-unit-pH drop near the export apparatus.

To test whether the ATPase activity of Flii contributes to the drop of local pH near the export apparatus, we analyzed the effect of Flii(E211Q) and Flii(E211D) mutations on the local pH near the export apparatus. The Flii(E211Q) mutation abolishes the ATPase activity of Flii but does not affect the subcellular localization of Flii-YFP (17, 22). The Flii(E211D) mutation reduces the ATPase activity of Flii by about 100-fold (29). When Flii was expressed in the ΔfliH pHluorin(M153R)-fliG cells, a decrease in the local pH by about 0.13 unit was observed in comparison with the vector control (Fig. 4; see also Table S1 in the supplemental material). However, when Flii(E211Q) or Flii(E211D) was ex-
culture medium had reached an OD_600 of ca. 1.6 to 1.8. All measurements were done at ca. 23°C. (C) Measurements of local pH of YVM1070 (ΔfliH fliI flhB(P28T)) pHluorin(M153R)-fliG (blue line) cells at external pH 7.0. The local pH was measured with more than 200 fluorescent spots. The pH distribution was fitted by a Gaussian function. The local pH distribution of YVM1049 (red line) and YVM1063 (fliB* pH-flG) cells at external pH 7.0. The local pH was measured with more than 100 fluorescent spots were counted for each strain.

FIG 4 Effect of depletion of FliH and FliI on the bulk cytoplasmic pH and local pH near the export apparatus. (A) Measurements of the bulk cytoplasmic pH of SJW1103 (wild type [WT]) and MMH0117 (ΔfliHΔfliI) cells transformed with pYVM001 at external pH 7.0 (n = 280 cells). P values were calculated using a two-tailed t test. (B) Measurements of the local pH of the ΔfliHΔfliI pH-flG (YVM1049) (red line) and YVM1063 (fliB* pH-flG) (blue line) cells at external pH 7.0. The local pH was measured with more than 200 fluorescent spots. The pH distribution was fitted by a Gaussian function. The local pH distribution of YVM1004 (pH-flG) is also shown by a black dashed line as a reference. All cells were incubated at 30°C in TB until the culture medium had reached an OD_-600 of ca. 1.6 to 1.8. All measurements were done at ca. 23°C. (C) Measurements of local pH of YVM1070 (ΔfliH pH-flG) carrying pTrc99A (V, black line), pMM1702 (WT, light blue line), pKK211 (E211Q, green line), or pMM1702(E211D) (E211D, orange line) at external pH 7.0. More than 100 fluorescent spots were counted for each strain.
**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** *Salmonella* strains and plasmids used in this study are listed in Table S2 in the supplemental material. The *fliG* gene on the chromosome is replaced by a *pHluorin(M153R)-fliG* allele using the λ Red homologous recombination system (46) as described previously (47). L broth (LB) was prepared as described before (10). T broth (TB) contained 10 g of Bacto tryptone and 5 g of NaCl per liter. Ampicillin and arabinose were added to the medium at final concentrations of 100 μg/ml and 0.02% (wt/vol), respectively.

**DNA manipulations.** DNA manipulations were carried out as described before (47, 48). DNA sequencing reactions were carried out using BigDye v3.1 as described in the manufacturer’s instructions (Applied Biosystems), and then the reaction mixtures were analyzed by a 3130 Genetic Analyzer (Applied Biosystems).

**Flagellar protein export assay.** *Salmonella* cells were grown in 5 ml of LB with shaking at 30°C until the cell density had reached an optical density at 600 nm (OD600) of ~1.0. After low-speed centrifugation, the cells were washed twice with motility buffer and incubated at 30°C for 1 h. Cultures were centrifuged to obtain cell pellets and culture supernatants. Cell pellets were resuspended in the SDS loading buffer. Proteins in the culture supernatants were precipitated by 10% trichloroacetic acid, suspended in the Tris-SDS loading buffer, and heated at 95°C for 5 min. After SDS-PAGE, immunoblotting with polyclonal anti-FliD and anti-FliC antibodies was performed as described before (10).

**Purification of pHluorin(M153R) and pHluorin(M153R)-FliG.** pHluorin(M153R) was purified from the soluble fractions of BL21 (DE3) lysates carrying pYVM007 as described before (33). pHluorin(M153R)-FliG-His was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography from the soluble fractions of BL21 (DE3) lysates carrying pYVM003 as described before (15, 31).

**Fluorescence microscopy.** *Salmonella* cells expressing pHluorin (M153R), pHluorin (M153R)-FliG, or pHluorin (M153R)-flk or pHluorin (M153R)-MinD_MTS were observed under a custom-built microscope. An optical system was built on an inverted fluorescence microscope (IX-71; Olympus) with a 150× oil immersion objective lens (UAP0150XOTIRFM; numerical aperture [NA], 1.45; Olympus) and 1.6× variable inserts and with an electron-multiplying charged couple device (EMCCD) camera (C9100-02; Hamamatsu Photonics). The pHluorin(M153R) probe was excited by a xenon lamp with two excitation filters, 400AF30 (Omea Optical) for 410-nm excitation and BP 470 – 490 (Olympus) for 470-nm excitation. A high-speed wavelength switcher (Lambda DG-4; Sutter) was used to switch between these two excitation filters, with a switching speed of less than 2 ms. Fluorescence emission was passed through a dichroic mirror (FF510-Di01-25x36; Semrock) and an emission filter (520DF40; Omega Optical). Each fluorescent image was captured by the EMCCD camera. The high-speed wavelength switcher and the EMCCD camera were controlled by MetaMorph 3.6 software (Molecular Devices).

**Determination of cytoplasmic pH using pH imaging system.** Bulk cytoplasmic pH of each *Salmonella* living cell expressing pHluorin (M153R) and local cytoplasmic pH of cells expressing pHluorin (M153R)-FliG or pHluorin (M153R)-MinD_MTS, were determined from the ratio of the fluorescence intensity in the 410-nm and 470-nm excitation wavelength images (see Fig. S7 in the supplemental material). Two fluorescence images of pHluorin (M153R) were captured by an EMCCD camera with an exposure time of 1 s for each excitation by a xenon lamp through neutral-density (ND) filters to avoid the influence of photobleaching. A set of images were analyzed with an image processing program developed based on the Igor Pro 6 (WaveMetrics) or Imagej version 1.48 (National Institutes of Health). We defined the fluorescence intensity of the cell body determined by the image profile after subtraction of the total background intensity consisting of the instrumental background and the auto-fluorescence of the cell. The instrumental background intensity was defined as the mean pixel intensity of an arbitrary 100- by 100-pixel region outside the cells. The autofluorescence intensity was defined as the mean pixel intensity of 50 wild-type cells producing no fluorescent proteins. Due to an optical resolution limit by the wavelength [a peak of emission wavelength of pHluorin (M153R) is 508 nm] and numerical aperture (NA, 1.45) of the objective lens, the spatial resolution of this system is around 214 nm. Because the pixel size of the image is 33.3 nm, we carried out smoothing of each fluorescence image by processing over 7 by 7 pixels. The pH was determined using the standard curve obtained from purified pHluorin (M153R) or purified pHluorin (M153R)-FliG (see Fig. S1B). These steps were performed separately for each image.

**Determination of the local pH around the export apparatus.** Each fluorescence image was captured with a 5-s exposure. The fluorescence intensity of a single fluorescent spot of pHluorin (M153R)-FliG was determined by an integral fluorescent intensity value after subtraction of the total background intensity consisting of the instrumental background and the autofluorescence of the cell. Background threshold was determined by fitting the intensity distribution with a two-dimensional (2D) Gaussian function using a program developed on the basis of the Igor Pro 6 software (WaveMetrics) (see Fig. S8 in the supplemental material). The local pH was determined by the 410/470 ratio of each fluorescent spot. The 410/470 ratio of purified pHluorin (M153R)-FliG-His was measured by our pH imaging system at different pH values to prepare the calibration curve shown in Fig. S2A. For in vivo calibration, SJW1368, which is a *Salmonella* fliHDC deletion strain that cannot express any flagellar genes, was transformed with pYVM008, and then, the resulting transformants were suspended in the motility buffer at various external pH values in the presence of 20 μM gramicidin and 20 mM potassium benzoate, and intracellular pH values were measured under our pH imaging system.

**Cryo-EM and image processing.** Hook-based basal bodies (HBBs) with the C ring attached were prepared from two *Salmonella* strains, HK1002 and TM041, as described previously (19). A 3-μl solution of HBB was applied onto a holey carbon grid (Quantifoil R0.6/1.3; Quantifoil Micro Tools), which had been glow discharged in a weak vacuum for 5 s immediately before use. The grids were blotted twice for 3 s and quick-frozen in liquid ethane using Vitrobot (FEI). Electron cryomicroscopy (cryo-EM) images were collected as described before (19). Defocus and astigmatism in the image were determined using CTFIND3 (49). HBB images were boxed out with BOXER (50) and aligned, classified, and averaged using the RELION2D.PY program (49). Three-dimensional (3D) image reconstruction of the wild-type and pHluorin (M153R)-labeled HBB structures was carried out using the RELION program with c100 symmetry (50).

**Electron cryotomography and subtomogram averaging.** Minicells of the *Salmonella* wild-type strain and the ΔfliH-ΔfliB (P287) bypass mutant were prepared as described previously (19). Images of the minicells were collected at the liquid-nitrogen temperature using a Titan Krios electron microscope (FEI) operated at 300 kV and with a Falcon 4k × 4k direct electron detector (FEI) as described before (19). Images were generally binned 2-fold, and 3D reconstructions were calculated using the IMOD software package (51).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01911-16/-/DCSupplemental.
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