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PROTOCOL

Detecting *Salmonella* Type II flagella production by transmission electron microscopy and immunocytochemistry

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The bacterial flagellum is an appendage structure that provides a means for motility to promote survival in fluctuating environments. For the intracellular pathogen *Salmonella enterica* serovar Typhimurium to survive within macrophages, flagellar gene expression must be tightly regulated, and thus, is controlled at multiple levels, including DNA recombination, transcription, post-transcription, protein synthesis, and assembly within host cells. To understand the contribution of flagella to *Salmonella* pathogenesis within the host, it is critical to detect flagella production within macrophages via microscopy. In this paper, we describe two methods for detecting bacterial flagella by microscopy both *in vitro* and *in vivo* infection models.

Keywords: *Salmonella* Typhimurium, Type II flagella

Overview

Bacteria utilize flagella to approach chemoattractants and to escape from chemorepellents. In flagellated bacteria, movement is initiated by the rotation of the flagella and the direction of the movement is determined by the torque of the motor that is embedded in the bacterial membrane (Samatey *et al.*, 2001). Although flagellar rotation is powered by proton motive force generated across the membrane, bacteria also possess an independent ATPase complex that is required for the export of the flagellar components. More than 40 different proteins are involved in flagellar assembly, making it one of the most complex structures in bacteria (Chevance and Hughes, 2008). The flagellar structure can be divided into three parts: the basal body, hook, and filament. Among them, the flagellar filament is polymerized by tens of thousands of a single protein, flagellin. Flagellin, like other flagellar components, is exported through a channel in the flagellar apparatus via an independent ATPase complex (e.g. Type III secretion system), and is then polymerized into a helical filament (Chevance and Hughes, 2008).

Salmonella enterica serovar Typhimurium (hereinafter referred as *S.* Typhimurium) is a Gram-negative pathogen that survives within macrophages and causes a typhoid-like disease in mice (Garcia-del Portillo, 2001). *S.* Typhimurium has peritrichous flagella that is required for survival inside host by promoting the adherence and invasion of *Salmonella* to host cells (Schmitt *et al.*, 2001). *S.* Typhimurium possesses two different genetic loci, *fliC* and *fljB* genes encoding Type I and Type II flagellins respectively (Renault *et al.*, 2017). In the genome, the *fljB* gene is followed by the *fljA* gene, which encodes the FliC repressor, and expression of the *fljBA* operon is dependent on a promoter region that lies between two hix sequences. When this promoter region of the *fljB* gene is flipped by a site-specific inversion event occurred between the two hix sequences (Nanassy and Hughes, 1998), expression of the *fljBA* genes is turned off, and FliC Type I flagellin is produced instead. Although switching between these two flagellin types seems to be tightly regulated, the nature of the signal mediating this inversion is currently unknown.

It was reported that production of type I and II flagella is upregulated at the late stage of infection inside epithelial cells (Hautefort *et al.*, 2008; Knodler *et al.*, 2010), but strongly downregulated during replication within macrophage phagosomes (Eriksson *et al.*, 2003; Choi *et al.*, 2017). Then again, it was also reported that flagella are required for *Salmonella*'s escape from oncotic macrophages (Sano *et al.*, 2007) and that Type I flagella have a selective advantage in a mouse model of typhoid fever but have no such advantage in the invasion of epithelial cells (Ikeda *et al.*, 2001). These findings indicate that *Salmonella* flagella production might be differentially regulated depending on flagellar types and also spatiotemporally controlled at different stages of infection. To understand the complexity of flagellar regulation and its contribution to *Salmonella* pathogenesis, it is critical to directly observe a specific type of flagella within host cells. However, it is currently challenging to detect serotype-specific flagella to access the effect of each flagellar type on *Salmonella* pathogenesis for following reasons: i) production and supply of many previously reported serotype-specific antibodies have been discontinued and ii) most of commercially available antibodies for detecting *Salmonella* flagellin have cross-reactivity to both types of flagella (de Vries *et al.*, 1998; Choi *et al.*, 2017) possibly because the N- and C-terminal flagellin domains of type I and II flagella are highly conserved (type I and II flagellins

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from *S.* Typhimurium 14028s have an overall 76.83% identity but the N- and C-terminal domains are 98.5% identical to each other). Therefore, it is required to produce a serotype-specific antibody for detecting a specific type of flagella inside eukaryotic cells.

In this paper, we described two detailed methods for detecting *Salmonella* flagella; one by transmission electron microscopy (TEM) to measure the average length or number of flagella produced in each bacterium and the other by confocal microscopy combined with immunocytochemistry (Griffiths, 1993) to detect Type II flagella production inside host cells. For the second purpose, Type II flagella-specific antibodies were produced using *in vitro* synthesized FljB-specific peptides.

Applications

To visualize the intact flagella on bacterial cells grown in a medium that mimics the environment within a macrophage phagosome, we used transmission electron microscopy combined with a negative staining technique to observe the specimen against a stained background by electron dense heavy metals (Brenner and Horne, 1959; Hayat, 2000). Two electron dense reagents, phosphotungstic acid (PTA; $H_3PW_{12}O_{40}$) and uranyl acetate (UA; $C_4H_6O_6U$), were used to stain the background and introduce contrast of the specimen when we observe the specimen using electron microscopy. Phosphotungstic acid is an anionic heavy metal. When neutralized at pH 7.4, the phosphotungstic anion has a net negative charge and does not directly stain the membrane, protein, and nucleic acid of the bacterium due to electric charge repulsion (Hayat, 2000). Therefore, PTA staining is expected to stain only the background and generate high contrast of the microscopic image because it does not stain the bacterium and flagella, which will remain electron transparent. By contrast, uranyl acetate is a cationic heavy metal and thus uranyl cations bind to carboxylic groups in proteins and to phosphate groups in nucleic acids or lipids (Cattini and Davies, 1984; Robin Harris and Horne, 1994). This chemical property of uranyl acetate that interacts with negative charged groups in the surface of specimen produce a certain degree of positive staining of the specimen itself (Hayat, 2000). Because PTA and UA have different properties in chemical and electric charges, these reagents produce quite different the staining results in terms of contrast and staining depth (Ohi *et al.*, 2004). To compare staining profiles, we first grew wild- type, a *fljB fliC* mutant *Salmonella* lacking both Types I and II flagellins, and a hyper-flagellated *Salmonella* strain (*fljB* C1362G [Choi *et al.*, 2017]) in Mg^{2+}-depleting N-minimal liquid medium and then stained with UA (Fig. 1A–C) and PTA (Fig. 1D–F). Both wild-type and the *fljB fliC* mutant *Salmonella* did not produce flagella when grown in Mg^{2+}- depleting N-minimal liquid medium, supporting the idea that *Salmonella* decreases flagella production under the phagosome-mimicking condition (Eriksson *et al.*, 2003; Choi *et al.*, 2017). Interestingly, in the hyperflagellated strain, the flagella filaments stained by UA

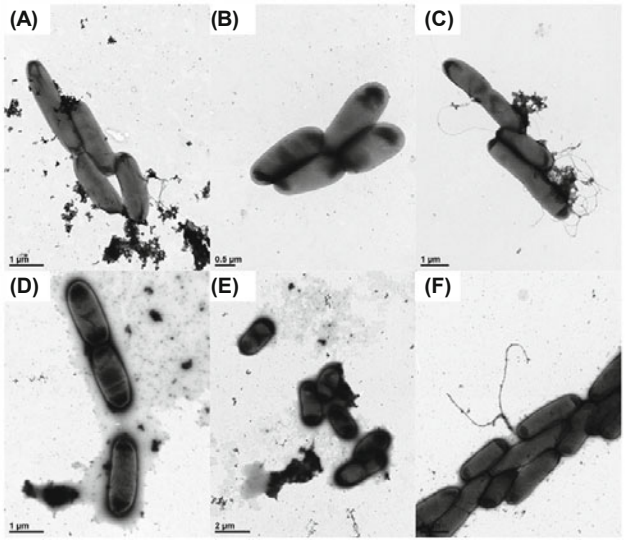

Fig. 1. (A–F) Transmission electron microscopy (TEM) images of wild-type *Salmonella* (A and D), a *fljB fliC* mutant lacking both Type 1 and II flagellins (B and E), and a *fljB* C1362G mutant overproducing Type II flagellin under low magnesium conditions (C and F). Negative staining of *Salmonella* strains with uranyl acetate (UA; A–C) and phosphotungstic acid (PTA; D–F). Bacterial strains were grown in N-minimal medium with low Mg^{2+} (0.01 mM) for 5 h.

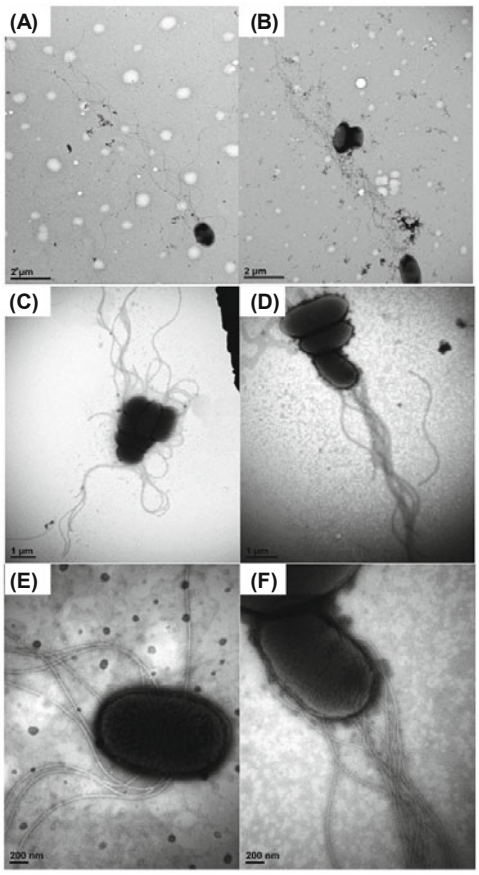

Fig. 2. (A–F) Transmission electron microscopy (TEM) images of wild-type *Salmonella* (A, C, and E) and the *fljB* C1362G mutant (B, D, and F) in LB medium. Negative staining of *Salmonella* strains with uranyl acetate (UA; A–B) and phosphotungstic acid (PTA; C–F). (E and F) Magnified TEM images of the listed strains stained by PTA. Bacterial strains were grown in LB medium for 5 h.

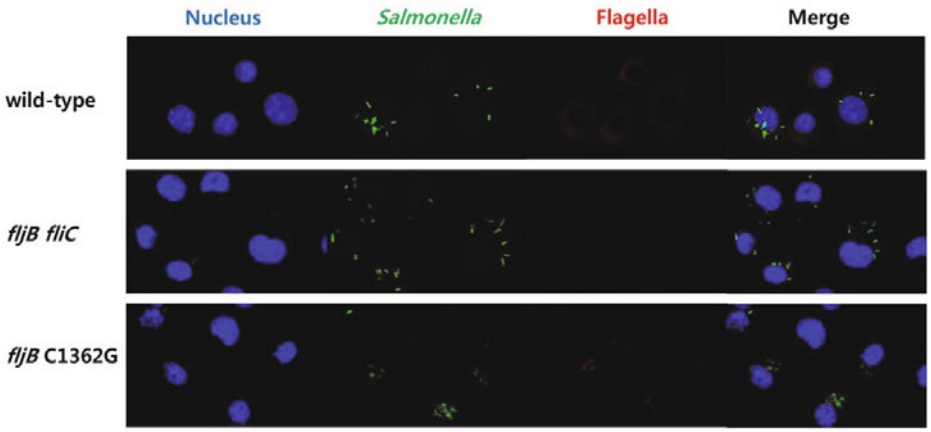

Fig. 3. Confocal laser scanning microscopy images of Type II flagella produced from the *Salmonella* strains listed above inside J774A.1 macrophages.

are surrounded by electron- dense precipitates that make it difficult to measure the length or number of flagella (Fig. 1C). The similar precipitates were also detected in UA-stained flagella from bacteria grown in LB liquid medium (Fig. 2A and B) and reported elsewhere (Kim *et al.*, 2010). The PTA-stained flagella did not have such precipitates (Figs. 1D–F, 2C, and 2D) and could be clearly detected even at a higher magnification (Fig. 2E and F). The PTA-stained flagella provide a sharper contrast to the background (Figs. 1 and 2) possibly because the negative charged surface of the flagellum does not bind to PTA. This suggests that PTA staining is more suitable for detecting flagella from *Salmonella* grown in liquid medium. Please note that PTA-stained bacteria have wrinkled surfaces (Fig. 2E and F) that appear to be caused by a disruptive effect of PTA on the biological membranes (De Carlo and Harris, 2011).

To observe *Salmonella* flagella production inside macrophages, we used immunocytochemistry, which can be used to detect specific antigens with an antibody and visualize the protein or organelle of interest. In this study, a Type II flagellin-specific antibody was used to detect flagella production inside macrophages. To improve the contrast between the object and background, we counterstained the host nucleus with DAPI and observed the entire sample by confocal laser scanning microscopy (CLSM). As shown in Fig. 3, we observed that bacteria expressing GFP were located at different planes inside host cells when we acquired a series of image sections at 500-nm intervals and rendered the image in three dimensions. To accurately locate the signals from the flagella and GFP-expressing *Salmonella*, the sample was observed in a series of focal planes, and the images were optimized as shown in Fig. 4.

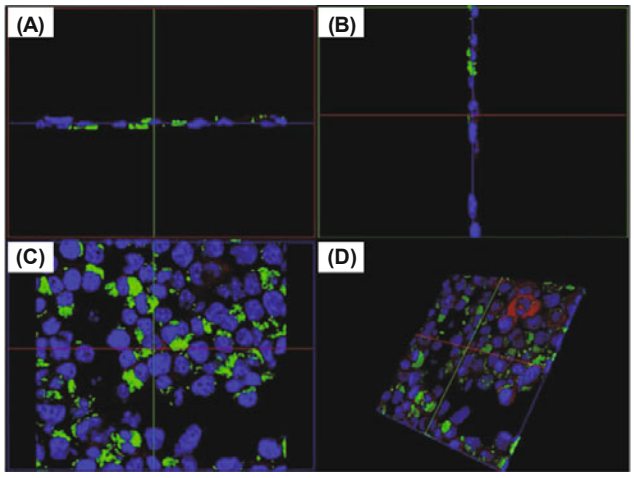

Fig. 4. 3D reconstructed images from immunocytochemistry using Z-stack. To colocalize signals from intracellular *Salmonella* and Type II flagella, we captured images on the different focal planes via Z-stack. (A) X-axis based image, (B) Y-axis based image, (C) Z-axis based image, and (D) the 3D image that was reconstructed from several focal planes are shown.

Methods

Preparation of bacterial strains

In this experiment, we used *Salmonella* enterica serovar Typhimurium 14028s and its derivative strains: a *fljB fliC* mutant lacking both *Salmonella* Type I and Type II flagellins, which was constructed by a one-step gene inactivation method (Datsenko and Wanner, 2000; Choi *et al.*, 2017) and a *fljB* C1362G substitution mutant that is hyper-flagellated in N-minimal medium containing 0.01 mM Mg^{2+} or inside macrophages, which was constructed by a fusaric acid-based anti-selection method (Maloy and Nunn, 1981; Choi *et al.*, 2017).

Bacterial culture for TEM

All *Salmonella* strains were pre-cultured overnight in Luria-Bertani (LB) medium. To mimic the environment within macrophage phagosomes, bacteria were grown in Mg^{2+}-depleted medium as follows: 1 ml of the overnight culture was washed twice with N-minimal medium without Mg^{2+}. Then, 0.1 ml of the culture was transferred to 10 ml of N-minimal medium containing 0.01 mM Mg^{2+} and grown for 5 h to an optical density at 600 nm of approximately 0.5 (OD_{600} = 0.5).

TEM with negative staining of bacterial flagella

To stain the bacterial flagella, a 0.01 ml droplet of the bacterial culture was loaded on a carbon-coated grid. We first ensured that there were a sufficient number of bacteria in the culture. Practically, if the number of bacteria was not sufficient, it was concentrated to allow detection of as many

flagellated bacteria as possible. To increase the density of the bacterial culture, the culture was allowed to stand for 30 min so that the bacteria slowly settle to the bottom of the tube. Although the bacterial culture could be concentrated by low-speed centrifugation (< 2,500 rpm), high-speed centrifugation tends to decrease the proportion of bacteria with intact flagella. The settled bacteria were transferred then onto the carbon-coated grid for 2 min and gently washed the applied bacteria with distilled water. The water was removed from the edge of the spotted cells with filter paper. Then, the bacterial flagella were negatively stained as described previously (De Carlo and Harris, 2011). Here, we used two staining reagents for negative staining, uranyl acetate (UA) and phosphotungstic acid (PTA). Because UA and PTA have different characteristics, the staining time was dependent on the reagent. For UA, ~10 sec of staining was sufficient to observe the number of flagella per bacterium. By contrast, for PTA, only 1–2 sec of staining was sufficient to stain the outlines of the flagella structure. After staining with UA or PTA, the sample was rinsed twice with distilled water and dried before examining the grid by TEM. In principle, negative staining is a procedure that stains the background of the grid but not the bacteria. However, if a sample is stained for a long time, the UA and PTA reagents will stain both the background and the specimen. Therefore, a longer exposure to UA or PTA could be used as a positive staining method for bacterial flagella.

Preparation of bacterial strains for macrophage infection

We used wild-type *Salmonella* expressing GFP for macrophage infection to colocalize signals from the bacterial body and attached flagella and to search for intact flagellar structures. Bacterial cells were cultured in LB medium containing 0.05 mg/ml ampicillin to maintain pfpv25.1, the constitutively GFP-expressing plasmid (Valdivia and Falkow, 1996). The OD_{600} of the overnight culture was measured, which was typically > 2.0, and the bacteria were diluted to an OD_{600} of 1 by adding fresh LB medium. An OD_{600} of 1 corresponds to approximately $0.9 – 1.0 \times 10^9$ bacteria/ml. The diluted bacterial culture was washed twice with Dulbecco's phosphate-buffered saline (DPBS) and resuspended in 1 ml of Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). To determine the multiplicity of infection (MOI), the bacteria were serially diluted in DPBS and plated on LB solid medium.

Preparation of macrophages

J774A.1 macrophage-like cells were used to infect *Salmonella*. J774A.1 macrophages were maintained in a monolayer culture at 60–70% confluence. A frozen vial of J774A.1 cells from a liquid nitrogen container was thawed for 1–2 min in a 37°C water with agitation and then mixed with 9 ml DMEM containing 10% FBS to dilute the dimethyl sulfoxide (DMSO) in the cryoprotectant solution. After centrifugation at 1,500 rpm for 10 min, the supernatant was removed and the macrophages were transferred to a 25T flask. When the culture reached 80% confluence, the macrophages were transferred to a 75T flask after washing the detached macrophages with DPBS. Before seeding, the number of macrophages was counted using a hemocytometer. To distinguish dead cells from live cells, 10 μl of the cells was mixed with 10 μl of trypan blue reagent and applied to the hemocytometer. After counting live cells, $3–5 \times 10^5$ live cells/ml were seeded in a 24-well plate and grown at 37°C in a 5% CO_2 incubator.

Salmonella infection to macrophages

To detect the expression of bacterial flagella in a host environment, we performed a gentamicin protection assay (Mandell, 1973). A seeded plate with J774A.1 macrophage-like cells was grown overnight, and then washed with DPBS. Then, 1 ml of *Salmonella* culture, prepared as described above, was added to each well at a multiplicity of infection (MOI) of 100 and centrifuged at 1,500 rpm for 10 min. The plate was incubated in a 5% CO_2 incubator for 20 min for *Salmonella* to invade the J774A.1 cells and then washed each well three times with DPBS to remove excess bacteria. DMEM containing 10% FBS and gentamicin (120 μg/ml) was added and incubated for 1 h to kill extracellular bacteria. Even though gentamicin selectively kills extracellular bacteria, exposure to a high concentration of gentamycin for a long time induces pinocytosis in macrophages, which might affect the replication of the intracellular bacteria (Drevets *et al.*, 1994). Thus, after incubation with a high concentration of gentamicin for 1 h, the medium was replaced with DMEM containing 10% FBS and a low concentration of gentamicin (12 μg/ml), and the plate was incubated for 9 h in a 5% CO_2 incubator.

Immunocytochemistry for microscopy

Immunocytochemistry is a technique used to directly observe the cell organelles and proteins by visualizing with an antibody conjugated to a fluorophore. Unlike other microscopy-based methods, the advantage of immunocytochemistry is that it could be used to observe intact flagella within host cells. In this experiment, we detected *Salmonella* Type II flagella inside macrophages using a primary polyclonal antibody against FljB flagellin and a secondary antibody conjugated to a Texas-Red fluorophore. At each step, the samples were rinsed with Tris-HCl buffered saline (TBS; pH = 8.0). Immunocytochemistry includes three steps, including fixation, permeation, and detection. In the fixation step, we used pre-chilled 4% paraformaldehyde dissolved in PBS to fix the samples at 4°C for less than 30 min. The sample was then rinsed three times thoroughly with TBS. In the permeation step, the sample was treated with 0.2% Triton X-100 at 4°C for 5–10 min to permeabilize the membrane and allow the antibodies to across the membrane. During immunocytochemistry, the samples were kept at 4°C, and the primary antibody was incubated between 1 h and overnight. The sample was first preincubated with 1% BSA to inhibit nonspecific interactions. After preincubation, each well was incubated with primary antibody in a solution containing 1% BSA for more than 1 h. Then, the sample was incubated with the secondary antibody in the dark. For the counter-staining, the antibody solution was removed from the slide, and the appropriate amount of fluoroshield mounting medium with DAPI was added to the slide. Then, the coverslip was mounted and pressed slightly to immerse the medium. To

prevent the sample from oxidizing, the sample was sealed with transparent nail polish.

Materials

Reagents

- 2% Uranyl acetate (UA) (EMS 22400, CAS 541-09-3)
- 1% Phosphotungstic acid (PTA) (EMS 19500, CAS 12501-23-4)
- LB broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) (MP 3002-032)
- LB agar plate (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% Bacto-agar) (Duchefa Biochemie, 9002-18-0)
- Dulbecco's modified Eagle medium (DMEM) (Welgene, LM 001-05)
- Fetal bovine serum (FBS) (Welgene, S001-07)
- Bovine serum albumin (BSA) (Sigma, A9647; CAS 9048-46-8)
- Phosphate buffered saline (PBS) (Biosesang, PR 2007-100-00)
- Tris-HCl buffered saline (TBS) (Biosesang, TR 2008-100-00)
- Dulbecco's phosphate buffered saline (DPBS) (Welgene, LB 001-02)
- 100× antibiotic-antimycotic solution (Welgene, LS 203-01)
- 4% Paraformaldehyde (CAS 30525-89-4)
- Triton X-100 (CAS 9002-93-1)
- Polyclonal anti-FljB antibody (Abclone, raised by injection of a mixture of peptides): C-DAAKNGDYEVNVATD (246-260 aa, 16 mer) and C-KTEVQELKDTPA (281-292 aa, 13 mer). Two peptide sequences were chosen from the *fljB*-specific sequences that are non-homologous to the *fliC* gene.
- Secondary antibody conjugated to Texas-Red (Santa Cruz, SC-2780)
- Fluoroshield mounting medium with DAPI (Abcam, ab-104139)
- Ampicillin (Biosesang, A1043; CAS 69-52-3)
- Distilled water
- Nail polish

Equipment

- Grid
- Transmission electron microscope, 80 kV (JEOL, JEM1010)
- SPX8 STED confocal laser scanning microscope (Leica)
- Centrifuge (Labocene)
- Four-well cell culture slides (SPL 30114)
- Coverslips 24 × 60 mm (Marienfeld 0101242)
- CO_2 incubator (Thermo Fisher)

Protocols

Negative staining of flagella for transmission electron microscopy

A. Strain preparation

1. Inoculate a single colony into LB medium for an overnight culture.
2. Transfer 1 ml of the overnight culture to a new 1.5 ml tube and wash the bacterial cells twice with N-minimal medium without Mg^{2+}.
3. Resuspend the cells in 1 ml of N-minimal medium without Mg^{2+} and inoculate the overnight culture in the N-minimal medium containing 0.01 mM $MgCl_2$ at a 1:100 dilution.
4. Grow the culture at 37°C with shaking for 5 h.
5. Transfer 3 ml of the culture to a new 15 ml tube.

B. Negative staining

1. Let the 15 ml tube stand for 30 min to allow the bacteria to settle to the bottom of the tube.
2. Transfer 10 ml of the settled bacterial cells from the bottom of the tube to a carbon-coated grid using a pipette and allow to stand for 2 min. Pipette gently to avoid deflagellation.
3. Absorb the liquid medium from the edge of the grid with a filter paper and rinse the sample with the same volume of distilled water.
4. Stain the grid with the reagent. For UA staining, prepare a droplet of UA solution and immerse the grid using a tweezer for ~10 sec. For PTA staining, immerse the grid in a droplet of PTA solution for < 2 sec.
5. After staining, quickly rinse the sample with distilled water twice as described in step 3.
6. Before inserting the grid into the TEM, air dry the grid.

Immunocytochemistry

A. Macrophage preparation.

Note: carry out all steps under aseptic conditions

1. Thaw a vial containing J774A.1 macrophage-like cells in a 37°C water bath with agitation for approximately 2 min.
2. Wipe the vial with 70% ethanol to prevent contamination.
3. Transfer the vial contents to a 15 ml conical tube containing 9 ml of DMEM with 10% FBS at 37°C and centrifuge the 15 ml tube at 1,500 rpm for 5–10 min.
4. Discard the supernatant and resuspend the pellet in 1 ml of DMEM containing 10% FBS.
5. Transfer the cell suspension to a 25T flask and incubate the flask at 37°C in humidity and 5% CO_2 until the culture reaches 80% confluence.

B. Macrophage culture on cell culture slides.

i) Preculture of J774A.1 macrophage-like cells.

1. Wash the adhered J774A.1 cells with DPBS three times.
2. Add 5 ml of DMEM containing 10% FBS and harvest the cells using a scraper. Centrifuge the cells at 1,500 rpm for 5–10 min.
3. Discard the supernatant and resuspend cells in 10 ml of DMEM containing 10% FBS and 1× antibiotic-antimycotic solution.
4. Transfer 1 ml of the cells to a 75T cell culture flask that was preincubated with 19 ml of DMEM containing 10% FBS and 1× antibiotic-antimycotic solution.
5. Incubate the flask in a 5% CO_2 incubator until 80% confluence is reached.

ii) Seed the cells in 4-well cell culture slides.

1. Wash the J774A.1 cells with DPBS three times.

2. Harvest cells using a scraper in 20 ml of DMEM containing 10% FBS and antibiotic-antimycotic and transfer the cells to a 50 ml tube. Centrifuge the cells at 1,500 rpm for 5–10 min.
3. Discard the supernatant and resuspend the cells in 20 ml of DMEM containing 10% FBS and antibiotic-antimycotic solution.
4. Count the number of cells per ml with a hemocytometer and dilute the cells to ~3×10^5 cells/ml.
5. Seed 1 ml of the cells into 4-well cell culture slide.
6. Incubate the slide in a 5% CO_2 incubator at 37°C for 18 h.

C. Preparation of bacteria.

1. Streak frozen stocks of *S.* Typhimurium strains with 10% skim milk on LB agar plates containing 50 μg/ml ampicillin.
   Note: The strains described above harbor the pFPV25.1 plasmid, which expresses the *gfp* gene from the *rpsM* promoter (Valdivia and Falkow, 1996).
2. Inoculate a single colony into 3 ml of LB medium containing 50 μg/ml ampicillin and incubate with shaking overnight.
3. Dilute the overnight culture by adding 7 ml of DPBS to reach an OD_{600} of ~1.0. (The OD_{600} of 1.0 contains approximately 9×10^8 bacteria/ml.)
4. Transfer 1 ml of the bacterial cells to a 1.5 ml tube and wash twice with DPBS.
5. Dilute the washed cells to an MOI of 100 with DMEM containing 10% FBS.

D. Infection of macrophages with bacteria.

1. Rinse the J774A.1 cells on the 4-well cell culture slides with DPBS.
2. Add 1 ml of the diluted culture to the chambers of the 4-well cell culture slides.
3. To facilitate contact between *Salmonella* and the macrophages, centrifuge the cell culture slides at 1,500 rpm for 5–10 min.
4. Incubate the slides in a 5% CO_2 incubator at 37°C for 1 h.
5. Wash the chambers of the 4-well culture slide with DPBS twice.
6. Add 1 ml of DMEM containing 10% FBS and 120 μg/ml gentamicin to the chambers to kill extracellular bacteria and incubate the slides in a 5% CO_2 incubator at 37°C for 1 h.
7. Repeat step 5 to wash the sample and add 1 ml of DMEM containing 10% FBS and 12 μg/ml gentamicin to the chambers.
8. Incubate the slides in a 5% CO_2 incubator at 37°C for 9 h.

E. Immunocytochemistry and microscopy.

1. Rinse the sample briefly with 1 ml of DPBS.
2. Add 500 μl of pre-chilled 4% paraformaldehyde to the chambers and incubate at 4°C for 30 min.
3. Wash the sample with DPBS twice and add 500 μl of 0.2% Triton X-100 to the chambers to permeabilize cells. Incubate at room temperature for 5–10 min.
4. Wash the sample with DPBS and add 1 ml of 1% BSA to the chambers to prevent nonspecific interactions and incubate the sample at 4°C for 1 h.
5. Wash the sample with DPBS twice. Add 1 ml of primary antibody with 1% BSA and incubate at 4°C for 1 h.
   Note: The dilution range for the primary antibody is between 1:200 and 1:400.
   Note: In the case of co-staining, add two types of primary antibodies that are appropriately diluted.
6. Wash the sample with DPBS and add 1 ml of the secondary antibody with 1% BSA and incubate the slides at 4°C for 1 h in a light-shielded box. From this point on, minimize the exposure to light.
   Note: The dilution range for the secondary antibody is between 1:400 and 1:800.
7. Wash the sample with DPBS and then add 1 ml of 1% BSA and disassemble the glass slide from the chamber and remove the solution.
8. After drying the glass slide, counterstain with fluoroshield mounting medium containing DAPI. Mount fluoroshield mounting medium containing DAPI on the slide and dry for 1 min.
9. Place the coverslip on the glass slide and press the coverslip slightly to eliminate any bubbles between the coverslip and the glass slide.
10. Seal the sample using nail polish.
11. Store the sample at 4°C in the dark.
12. Examine the sample using a confocal microscope at the appropriate excitation and emission wavelengths.

Expected results

Wild-type *Salmonella* decreases expression of Type II flagella inside macrophages or in low Mg^{2+} medium that mimics an environment inside a macrophage phagosome (Figs. 1 and 3). The decrease in Type II flagella production is mediated by *mgtC* leader 113, a regulatory RNA that is highly produced inside macrophages or in low Mg^{2+} medium (Choi *et al.*, 2017). Because the *fljB* C1362G substitution prevents the interaction between *fljB* mRNA and *mgtC* leader 113 RNA, the *fljB* C1362G mutant is not affect by the small RNA and is hyperflagellated inside macrophages or in low Mg^{2+} medium (Choi *et al.*, 2017) (Figs. 1 and 3). By contrast, wild-type and the *fljB* C1362G mutant *Salmonella* were similarly flagellated in LB medium, a condition that represses expression of the small RNA (Fig. 2). As a control experiment, the *fljB fliC* mutant lacking both the Type I and II flagellins does not produce flagella all tested conditions (Figs. 1 and 3).

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Conflict of Interest

The authors declare no conflict of interest.

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