

Rapid Preparation of Unsheathed Bacterial Flagella

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[Abstract] The flagellum is required for bacterial swimming and swarming motility. In the biphasic *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), the flagellar filament is build up by two distinct monomeric subunits, flagellin FliC and FljB. *S. Typhimurium* has the ability to switch between two flagellins, FliC and FljB, in a phase-variable manner. The switch to FliC is called phase H1 and considered important for bacterial growth and survival in the spleen in a murine infection model of typhoid fever. Flagellin is secreted as monomeric subunits, but the majority of flagellin is polymerized upon secretion as the flagellar filament. *Salmonella* flagellin has traditionally been isolated through a process involving multiple steps of centrifugation and acid treatment. Here, we delineate a simplified protocol for preparing *Salmonella*'s flagellin for analytical purpose to determine the amount of flagellin without the aid of antibodies. The growth conditions used were stationary phase, logarithmic phase and a low oxygen and high salt condition mimicking the gastrointestinal tract. Flagellin expression of other source organisms, such as other serovars of *Salmonella enterica* and *Escherichia coli*, including flagellar phase- or genetic variants can be analysed. Flagellin expression analysis complements flagella-associated phenotype analysis such as swimming and swarming behaviour.

Materials and Reagents

1. Bacterial strains (*S. Typhimurium*)
2. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
3. 100% trichloroacetic acid (Sigma-Aldrich, catalog number: T6399)
4. β -Mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
5. Trizma® base (powder) (Sigma-Aldrich, catalog number: T1503)
6. 1 M HCl solution (Sigma-Aldrich, catalog number: H1758)
7. Glycerol 1 L (Sigma-Aldrich, catalog number: 49781)
8. Sodium dodecyl sulfate - 500 g (Sigma, catalog number: L4390)
9. Tryptone (BD-catalog number: 211705)
10. Yeast extract (BD, catalog number: 212750)
11. Agar (BD, catalog number: 281230)
12. Bromophenol blue (Shelton Scientific, catalog number: IB74040)
13. Glycerol stock for one vial (see Recipes)
14. LB medium (see Recipes)
15. 1 M Tris (see Recipes)
16. 2x SDS sample buffer (see Recipes)

Equipment

1. Falcon tubes (50 ml and 15 ml) (TPP, catalog numbers: 91050 and 91115)
2. Filter flasks (pore size 0.22 μ m diameter) (TPP, catalog number: 99500)
3. Eppendorf tubes (1.5 ml) (Sarstedt, catalog number: 72.690.001)
4. Incubator (adjusted to 37 °C) (Mettler, model: ULE500)
5. Rotary shaker (INFORS-HT, model: Minitron)
6. Spectrophotometer (Hitachi, model: U1100)
7. Thermoblock (PEQLAB, model: 1202)
8. Vortex mixer (VWR International, model: 1719)
9. Luer lock tips (BD PLASTIKPAK, catalog number: 302187)
10. Needle (0.51 mm outer diameter) (BD Microlance sterile, model 25 g x 5/8" x 100)
11. Sterile petri dishes (90 mm) (Sartorius, catalog number: 14-555-735)
12. Centrifuge (Heraeus Holding, model: PICO17)
13. Microcentrifuge (NeoLab, model: Microcentrifuge II)
14. pH meter (Hanna Instruments, model: pH211)

Procedure

A. Preparation of the pre-culture (to be used for all subsequent culture conditions)

Day 0

Streak bacterial strains from -80 °C glycerol stock [equal to 87% (v/v) glycerol in LB] on LB agar plates for single colonies. Use LB agar plates containing appropriate antibiotics, if required, and incubate overnight at 37 °C (approximately 16 to 24 h).

Day 1

1. Take one bacterial colony and inoculate in 10 ml liquid LB medium (in a 50 ml Falcon tube).
2. Incubate the culture overnight at 37 °C with shaking at 150 rpm. Put the lid loose on the tube. Add appropriate antibiotics, if required.

B. Preparation of inoculum

Day 2

1. Measure the optical density of the 1:10 diluted pre-culture in a spectrophotometer and calculate the optical density of the pre-culture. Adjust the OD₆₀₀ of the pre-culture to OD₆₀₀=1 for a 1 ml volume which corresponds to approximately 5×10^8 CFU.
2. Add 100 μ l of the adjusted pre-culture to 10 ml LB medium (1:100 dilution).

C. Culture conditions

Standard growth condition to logarithmic and stationary phase in LB medium

Add 100 µl of wild type, normalized test and control inoculum to 10 ml LB medium in a 50 ml Falcon tube (a 1:100 dilution of the inoculum). Add appropriate antibiotics and inducer if required.

1. Logarithmic phase growth condition
 - a. Incubate the samples for 3 h at 37 °C with shaking at 150 rpm. Put the lid loose on the tube. The OD₆₀₀ will be approximately 0.6-0.8.
 - b. Normalize each sample to OD₆₀₀ = 0.6. Shear off flagella as described under D.
2. Stationary phase growth condition
 - a. Incubate the samples for 16 to 24 h at 37 °C with shaking at 150 rpm. Put the lid loose on the tube.
 - b. Normalize each sample to OD₆₀₀ = 0.6. Shear off flagella as described under D.

S. Typhimurium cell culture invasion condition

3. Low oxygen, high salt growth condition.
 - a. Add 100 µl wild type, normalized test and control inoculum to 10 ml LB medium with high salt concentration in a 50 ml Falcon tube (a 1:100 dilution of the inoculum). Add appropriate antibiotics and inducer, if required.
 - b. Incubate the samples at 37 °C with shaking at 150 rpm until OD₆₀₀ = 0.6-0.7 (takes approximately 4.5 h). Put the lid loose on the tube.
 - c. Normalize the OD₆₀₀ of the samples to the wild type OD₆₀₀ using LB medium with high salt concentration. Shear off flagella as described under D.

D. Shearing off cell-associated flagellin (flagella)

1. Perform the following steps on ice.
2. Place 5 ml of each normalized sample in an individual sterile petri dish plate.
3. Lift the inoculum up from the sterile petri dish plate with a sterile Luer lock tip (syringe). Place the sterile needle on the Luer lock tip and eject the cell suspension back in the petri dish plate. Work on a clean bench as this procedure creates aerosol.
4. Repeat the previous step 10 times with the same material to shear off cell-associated flagella.
5. Take 1 ml of cell suspension and transfer to an Eppendorf tube. Centrifuge at 17,700 x g for 10 min at 4 °C.
6. Transfer 800 µl of the supernatant to a new Eppendorf tube. The supernatant contains sheared-off flagella and minor amounts of secreted flagellin. Proceed with E, for flagellar preparation.
7. Remove the rest of the supernatant. The bacterial pellet is used for analysis of cell-associated protein as described under E, for cell associated protein preparation.

E. Sample preparation

For flagellar preparation

1. Centrifuge at 17,700 x g for 10 min at 4 °C.
2. Transfer 750 µl to a new Eppendorf tube.
3. Add 250 µl of cold trichloroacetic acid solution and mix gently.
4. Place the sample for at least 1 h on ice or in a -20 °C freezer.
5. Centrifuge at 17,700 x g for 40 min at 4 °C, remove the supernatant and remove the residual liquid (eventually through repeated centrifugation).

For cell associated protein preparation

1. Centrifuge 17,700 x g for 1 min at 4 °C.
2. Discard the rest of the supernatant and save the bacterial pellet.

Preparation of cell associated protein and flagellin samples for loading on protein gel

1. Prepare the sample buffer with 90 (v/v)% 1x SDS sample pre-buffer and 10 (v/v)% β-mercaptoethanol.
2. Resuspend the flagellar protein preparation in 45 µl sample buffer and 5 µl Tris-HCl (pH = 9).
3. Resuspend the bacterial pellet in 100 µl sample buffer.
4. Vortex the samples.
5. Heat up the samples 5 min at 95 °C.
6. Vortex the samples.
7. Spin down with a microcentrifuge for few seconds.

The samples can be used immediately or stored at -20 °C for further utilization.

Representative data

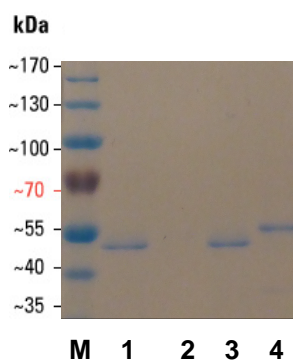


Figure 1. Coomassie brilliant blue stained 12% SDS PAGE of flagellar preparations from *Salmonella enterica* serovar Typhimurium UMR1 wild type and isogenic flagellin mutants. M. PageRuler prestained protein ladder 1. Wild type: ATCC14028-1s Nal^r; 2. UMR1 *fliC5050::MudJ fljB5001::MudCm^r*; 3. UMR1 *fljB5001::MudCm^r*; 4. UMR1 *fliC5050::MudJ*. Molecular weight of flagellin: FliC: 52 kDa, FljB: 53 kDa

Notes

1. Bacterial strains and controls.

This protocol has been developed using the wild type strain *S. enterica* serovar Typhimurium UMR1 (ATCC14028-1s NaI^r). In this case, control strains are isogenic *fljB fljC*, *fljB* and *fliC* mutants. These strains are available upon request from the corresponding author.

2. Medium volumes and culture conditions.

These factors can be subjected to change. Growth medium, medium volume and time of incubation can be adapted to the research question.

3. Samples are normalized to OD₆₀₀ = 1 in step C1b, 2b and 3c in order to enable growth culture independent estimation of flagellin expression.

4. The cell associated protein samples.

The cell associated protein samples will be used to assess whether equal amounts of cells have been used for flagellar preparation.

5. Precipitation of flagellar preparation samples.

The samples can be stored at -20 °C after adding trichloroacetic acid solution to the flagellin sample.

6. A supplementary centrifugation of 5 min at 17,700 x g between step C and D can be used to separate polymerized and monomeric secreted flagellin.

Recipes

1. Glycerol stock for one vial

250 µl 87% glycerol

750 µl bacterial overnight culture

2. LB medium (1 L)

10 g tryptone

5 g yeast extract

10 g NaCl

Variant: with high salt concentration 17.5 g NaCl (0.3 M NaCl)

Fill up to 1 L with demineralized water and adjust the medium to pH = 7

Sterilize by autoclaving or filtration

3. 1 M Tris (adjusted to pH = 9)

Add 12.14 g of Trizma[®] to deionized water for a total volume of 100 ml

Adjust the pH to 9 with 1 M HCl solution

4. 2x SDS sample buffer

25 ml 0.5 M Trizma-base (pH = 6.8)

20 ml glycerol 87%

4 g SDS

0.2 g Bromophenol-blue

45 ml H₂O

Before utilization, add 10% β-mercaptoethanol to 90% of 1x sample buffer

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