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Bacterial Virulence

Methods and Protocols

Chapter 5

Profiling of Secreted Type 3 Secretion System Substrates by *Salmonella enterica*

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Abstract

Many of *Salmonella enterica* virulence-associated phenotypes, including its ability to manipulate various host pathways are mediated by translocation of specific effector proteins via type 3 secretion systems (T3SSs) into the host cell. Culturing *Salmonella* under a defined set of stimulating conditions in vitro can mimic the physiological signals *Salmonella* senses during the infection and results in the secretion of these effectors into the growth medium. Here we describe a *Salmonella* secretion assay to identify and quantify protein substrates secreted by T3SS-1 and demonstrate how this method can be utilized to study the secretion of T3SS-1 effectors and flagellum components in different genetic backgrounds or under varying growth conditions.

Key words *Salmonella*, Secretion assay, Flagella, Type 3 secretion system (T3SS), Effectors, SPI-1, SPI-2

1 Introduction

Salmonella enterica is a gram-negative, facultative intracellular bacterial pathogen responsible for an annual 180 million infections and 300,000 deaths worldwide [1]. Typhoidal and nontyphoidal serovars can be classified into more than 2500 stereotypically distinct serovars, according to the Kauffmann–White scheme based on three groups of antigens expressed on the bacterial surface [2]. Many of the virulence determinants of *S. enterica* are secreted to the extracellular environment, into neighboring bacterial cells or directly into host cells by assorted bacterial secretion systems [3].

Type III secretion systems 1 and 2 encoded on *Salmonella* pathogenicity island (SPI) 1 and 2, respectively. T3SS-1 is associated with the early stage of infection, mediates invasion of intestinal epithelial cells and stimulation of intestinal inflammation, by the secretion of designated effectors across the host cell membrane into its cytoplasm. Currently, at least 10 effectors (SopB [SigD], SopA,

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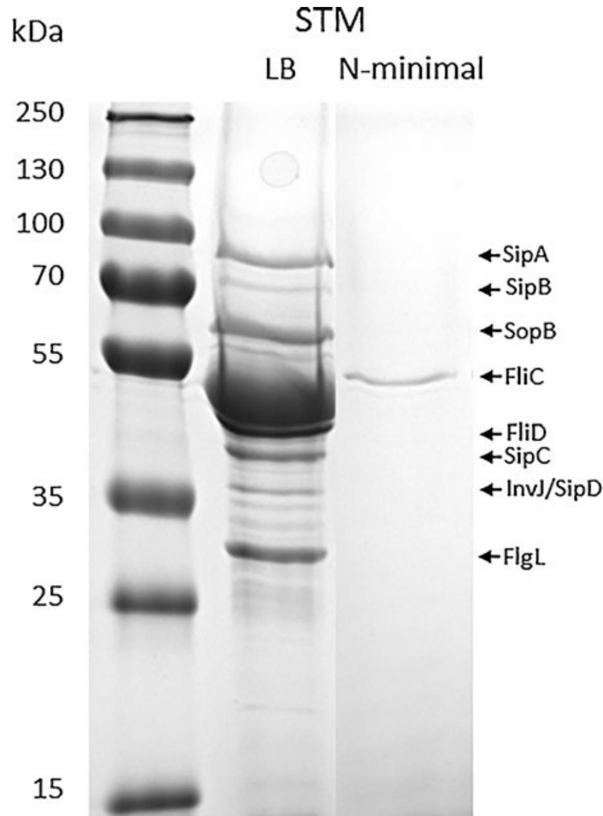

Fig. 1 The secretome of *S.* Typhimurium grown in different growth media. *S.* Typhimurium SL1344 (STM) cultures were grown in LB for 5.5 h and in N-minimal medium for 7.5 h at 37 ˚C. Cultures supernatant were filtered and precipitated by TCA. Equal amounts (25 μL) from the precipitated fractions were separated on 10% SDS–polyacrylamide gel and followed by Coomassie G-250 staining. The secreted protein profile of each culture is shown and the main proteins are indicated by arrows

SipA, SipB, SipC, SipD, SptP [StpA], SopD, SopE, and SopE2) are known to be secreted via T3SS-1, and at least 8 additional effectors (AvrA, SlrP, SspH1, SteA, SteB, SteE, SpvD, and GtgE) can be secreted by either T3SS-1 and T3SS-2 [4, 5]. T3SS-2 promotes intracellular bacterial survival and systemic infection by the secretion of about 30 effectors crossing the *Salmonella* containing vesicle (SCV) into the host cell cytoplasm [6].

Another secretion system is the flagellum, which is evolutionarily related to the T3SS and enables the bacteria to swim in liquids and swarm on surfaces [7]. The flagellar T3SS exports flagellar proteins from the bacterium cytoplasm through the growing structure from the base up, of the basal body, hook and filament during the flagellum assembly [8].

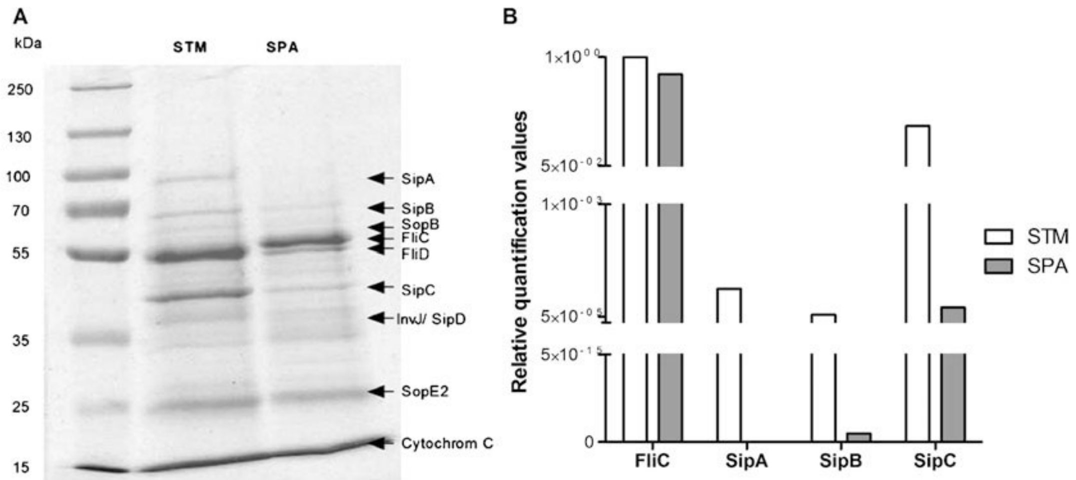

Fig. 2 *Salmonella* secretion assay of different genetic backgrounds. (**a**) *S*. Typhimurium SL1344 (STM) and *S*. Paratyphi A 45157 (SPA) cultures were grown in LB at 37 ˚C for 5.5 h, and normalized to an OD_{600} of 2.4–2.6. Exogenous human cytochrome C (8 μg) was added to the bacterial cultures as a spike-in and loading control. Culture supernatants were precipitated by TCA, and equal amounts (25 μL) from the precipitated fractions were separated on a **12%** SDS–polyacrylamide gel and stained with Coomassie G-250. This panel is reproduced from Elhadad et al. [11] with permission from the publisher (ASM Journals). (**b**) Relative (to STM FliC) quantification of FliC, SipA, SipB, and SipC (normalize to Cytochrome C) was conducted using Image J [12]

Salmonella senses environmental signals and conditions such as nutrient availability, pH, temperature, and osmolarity and regulates gene expression accordingly [9]. Under laboratory growth conditions that mimic the small intestine milieu (nutrient rich LB medium, slightly alkaline with high osmolarity), *Salmonella* upregulates the flagella and the SPI-1 gene expression and secretes T3SS-1 effectors and flagellum components to the growth medium. In contrast, under conditions that stimulate the SCV environment that are poor in phosphate, manganese, and magnesium with acidic pH (like N-minimal media), the expression and secretion of T3SS-1 effectors and flagellum component is repressed [10].

The following *Salmonella* secretion assay protocol enables to identify and quantify secreted proteins from different *Salmonella* serovars and genetic backgrounds, growing under varying growth conditions. To demonstrate this protocol, we compared the secreted proteins of *Salmonella* Typhimurium grown under SPI-1 and SPI-2 induction conditions (Fig. 1) and analyzed the secretome of *S*. Typhimurium and *Salmonella* Paratyphi A cultured under SPI-1 induction conditions (Fig. 2).

2 Materials

2.1 Growth Media

1. Luria–Bertani broth (LB). For 1 L LB broth, dissolve 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1L dH_2O.
2. N-minimal medium pH 5.8 [5 mM Kcl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 80 mM MES, 38 mM glycerol, 0.1% casamino acids, 24 μM $MgCl_2$, 337 μM PO_43^- [13].

To prepare N-minimal medium, first make and autoclave to sterilize the following stock solutions separately:

- 5× minimal salt solution. Weight 0.932 g KCl, 2.48 g $(NH_4)_2SO_4$, 0.218 g K_2SO_4, 46.66 g MES hydrate [2-(N-Morpholino)ethanesulfonic acid hydrate], add ddH_2O to a final volume of 500 mL.
- 10% casamino acids stock solution. Dissolve 50 g casamino acids in 500 mL ddH_2O.
- 25 mM $MgCl_2$. Dissolve 0.238 g of $MgCl_2$ in 100 mL ddH_2O.
- 0.1 M PO_43^-. Mix 80.2 mL of 1 M K_2HPO_4 (dissolve 17.4 g K_2HPO_4 in 100 mL dH_2O) and 19.8 mL of 1 M KH_2PO_4 (dissolve 13.6 g KH_2PO_4 in 100 mL dH_2O) and add 900 mL ddH_2O.

To prepare 100 mL of N-minimal medium, mix 20 mL of 5× minimal salts solution, 0.6 mL 50% glycerol, 1 mL of 10% casamino acids stock, 96 μL of 25 mM $MgCl_{2,}$ 337 μL of 0.1 M PO_43^-.

Adjust pH to 5.8 with HCl and filter-sterilize.

1. Cytochrome C from equine heart (1 mg/mL). Weight 1 mg Cytochrome C in 1 mL ddH_2O, store at −20 °C.
2. 10 mL sterile syringe.
3. 33 mm sterile syringe filter unit with 0.22 μm hydrophilic PVDF membrane (low protein binding).
4. Trichloroacetic acid (TCA) 6.1 N.
5. Ice-cold acetone (−20 °C).
6. SDS-PAGE sample buffer (2×). Mix 1.5 mL of 1 M Tris pH 6.8, 1.2 mL of 10% SDS, 6 mL of 50% glycerol, 1.5 mL of β-Mercaptoethanol, 0.18 mg of bromophenol blue. Aliquot into 1 mL portions and store at −20 °C.
7. 10%–12% SDS-polyacrylamide gel.
8. Coomassie Brilliant Blue G-250 gel staining dye.

3 Methods

1. Grow an overnight culture of *S.* Typhimurium (we normally use *S.* Typhimurium SL1344 strain) or *S.* Paratyphi A (we normally use *S.* Paratyphi A 45157 strain [14]) in 2 mL LB broth using 16 ×150 mm glass tubes (23 ml volume) with appropriate antibiotic selection (*see* **Note 1**). Grow the culture under aerobic conditions, on a roller drum or on an orbital shaker at 250 rpm, at 37 °C.
2. For secretion assay conducted under SPI-1 inducing conditions, subculture by transferring 100 μL of the overnight grown culture to 10 mL fresh LB medium in a 125 mL Erlenmeyer flask. Grow the subculture for 5.5 h at 37 °C with shaking (250 rpm) to reach early stationary phase (*see* **Notes 2** and **3**). Skip to **step 4**.
3. For secretion assay under SPI-2 inducing conditions, transfer 0.5 mL of the overnight culture into a test tube and pellet the bacteria in a microcentrifuge at 8500 *g* for 2 min. Remove the supernatant and wash the bacterial pellet twice with 0.5 mL of sterile N-minimal medium. Resuspend the bacterial pellet carefully and add 200 μL of the suspended washed bacteria to 10 mL of N-minimal medium in a 125 mL Erlenmeyer flask. Subculture for 4–7.5 h at 37 °C with shaking at 250 rpm (*see* **Notes 2** and **4**).
4. To stop bacterial growth, place the flasks on ice and keep the cultures cold for the entire procedure.
5. Measure OD_{600} of the cultures. Normalize all assayed colures to the same OD, using fresh growth medium to a final volume of 9 mL. Transfer to 15 mL conical centrifuge tubes.
6. To each OD-normalized culture (9 mL volume), add 9 μL of human cytochrome C (1 mg/mL) as a spike-in control. Mix gently by inverting the tube 2–3 times.
7. Pellet the bacteria by centrifugation at 20,000 *g* for 5 min at 4 °C. Carefully collect the top supernatant (about 8 mL) and transfer it to a 10 mL syringe attach to a sterile 33 mm syringe filter unit, with 0.22 μm PVDF membrane. Avoid disrupting the bacterial pellet (*see* **Note 5**).
8. Slowly and gently filter the supernatant into a clean 15 mL tube (*see* **Note 6**).
9. Transfer 7.2 mL of the filtered supernatant into a new 15 mL conical centrifuge tube for TCA precipitation. Add 800 μL of TCA to a final concentration of 10%. Gently mix by upturning the tubes 2–3 times and incubate the samples on ice for 1–3 h, or overnight if convenient.

10. To precipitate the proteins, centrifuge the samples at 20,000 *g* for 45 min at 4 °C to pellet the TCA-insoluble fraction (*see* **Note 7**).
11. Remove supernatant carefully and wash the pellet with 1.6 mL ice-cold acetone and recentrifuge for 30 min at 20,000 *g* at 4 °C.
12. Completely remove the supernatant and air-dry the pellet for 15 min at room temperature.
13. For cultures grown in LB, resuspend the pellet directly in 100 μL of SDS-PAGE sample buffer and boil for 5 min.
14. For cultures grown in N-minimal medium, resuspend the pellet directly in 60 μL of SDS-PAGE sample buffer and boil for 5 min (*see* **Notes 8** and **9**).
15. To collect the sample, briefly spin down the tubes at 300 *g* for a few seconds. Store the secreted protein fractions at −80 °C freezer until SDS-PAGE analysis.
16. For SDS-PAGE analysis, run 25 μL of the secreted fractions on 10% SDS-polyacrylamide gel.
17. Stain the gel with Coomassie G-250 and image the gel using a gel documentation system.
18. For *S. enterica* cultures grown in LB, the expected profile of the secreted flagella components and the major T3SS-1 effectors is well characterized (Figs. 1, 2 and [11]) and therefore SDS-PAGE and Coomassie staining may be sufficient for analysis. However, for profiling T3SS-2 effectors (*see* **Note 10**), or in case when the analysis of an uncharacterized protein is required, western blotting using specific antibodies is required.
19. For a relative (no absolute values) quantification of the secreted proteins, use an image analysis software such as Image J [12]. Quantify the SDS-PAGE gel densitometry bands of the desired proteins and normalize them to the spike-in loading control of the cytochrome C signal (*see* **Note 11**).

4 Notes

1. For *S.* Typhimurium SL1344 use 50 μg/mL streptomycin.
2. Do not overgrow to minimize spontaneous cell lysis of cells.
3. Under these conditions a culture of *S.* Typhimurium SL1344 reaches OD_{600} of ~4.8.
4. Under these conditions, a culture of *S.* Typhimurium SL1344 reaches OD_{600} of ~1.5.
5. If you wish to determine the expression of a specific protein in the whole bacterial fraction by western blotting, spin down 1 mL of bacterial culture at 20,000 *g* at 4 °C. Aspirate the

supernatant and resuspend in 0.2 mL of SDS-PAGE sample buffer (adjust volume according to the OD of the compared cultures) and boil for 5 min. Store at −80 °C freezer until needed.

6. Do not squeeze the remaining air via the syringe to avoid lysis of residual bacteria. This ensures that traces of whole bacteria proteins will not be carried over into the secreted fraction.
7. Mark the position of the tube in the rotor, as the pellet may not be visible.
8. If samples turn yellow (indicating acidity), add 1 μL of 1 M Tris (pH 8.8) to the sample and mix. This will bring the sample to neutral pH (blue).
9. The obtained pellet is very compact. Make sure to repeatedly pipet the sample buffer on the tube walls to collect all the precipitated protein.
10. T3SS-2 effectors are normally secreted to the medium in much lower amounts than the major T3SS-1 effectors or the flagellum components. Therefore, Western blotting or Liquid chromatography–mass spectrometry are needed to analyze T3SS-2 effectors secretion.
11. For ImageJ detailed instructions: http://www.yorku.ca/yisheng/Internal/Protocols/ImageJ.pdf

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