Identification of a novel *Salmonella* type III effector by quantitative secretome profiling

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Identification of a novel *Salmonella* effector SopF

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Abstract

*Salmonella enterica* serovar Typhimurium is arguably one of the most studied bacterial pathogens and successful infection requires the delivery of its virulence factors (effectors) directly into host cells via the type III secretion systems (T3SSs). Central to *Salmonella* pathogenesis, these effector proteins have been subjected to extensive studies over the years. Nevertheless, whether additional effectors exist remains unclear. Here we report the identification of a novel *Salmonella* T3SS effector STM1239 (which we renamed SopF) via quantitative secretome profiling. Immunoblotting and β-lactamase reporter assays confirmed the secretion and translocation of SopF in a T3SS-dependent manner. Moreover, ectopic expression of SopF caused significant toxicity in yeast cells. Importantly, genetic ablation of *sopF* led to *Salmonella* strains defective in intracellular replication within macrophages and the mutant were also markedly attenuated in a mouse model of infection. Our study underscores the utility of quantitative secretome profiling in identifying novel virulence factors for bacterial pathogens.
Introduction

*Salmonella enterica* serovars are intracellular bacterial pathogens that can cause a wide spectrum of diseases, ranging from intestinal inflammation to life-threatening typhoid fever as a systemic infection. Central to *Salmonella* pathogenesis are the two type III secretion systems (T3SSs) encoded on two distinct *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and -2)\(^1\). By delivering virulence factors (also termed effector proteins) via its T3SSs, *Salmonella* has evolved the mechanisms to subvert various host cellular processes including immune responses, cytoskeleton rearrangement and vesicular trafficking\(^2,3\). Notably, the effectors translocated via SPI-1 T3SS are largely involved in facilitating bacterial invasion of host cells\(^4\), whereas SPI-2 T3SS-delivered effectors are mostly associated with promoting intracellular survival and replication\(^5,6\).

The core components of T3SSs are strikingly conserved among different Gram-negative bacteria including both animal and plant pathogens\(^7,8\), however, the full repertoire of their T3SS effectors show remarkable strain specificity and plasticity, thus leading to distinct disease manifestations of individual pathogens.

Therefore, a fundamental interest in bacterial pathogenesis is the discovery and characterization of these effector proteins encoded by a given pathogen. As one of the most studied bacterial model pathogens, *Salmonella enterica* serovar Typhimurium (referred as *S. Typhimurium* thereafter) encodes over 60 effector proteins, most of which have already been identified over the years by various biochemical, genetic, and bioinformatic approaches\(^9,10\). In the last decade, mass spectrometry (MS)-based proteomics has evolved into a highly sensitive and quantitative tool to examine complex
protein mixtures. With appropriate designs of control groups (e.g., T3SS-deficient bacterial strains), proteomic profiling of bacterial secretome can achieve both high-throughput and high accuracy in identifying novel T3SS effectors. Niemann et al. and Auweter et al. examined the secretome of Salmonella that was cultured under SPI-2-inducing conditions and identified several novel effector proteins as well as many known substrates of T3SS\textsuperscript{11,12}. In addition, similar strategies of secretome profiling by using quantitative proteomics have been successfully applied to further expand the effector repertoire of other bacterial pathogens including enteropathogenic Escherichia coli (EPEC)\textsuperscript{13}, Citrobacter rodentium\textsuperscript{14} and Bacillus cereus\textsuperscript{15}.

Despite such significant progresses, a proteomic catalogue of S. Typhimurium effector proteins of SPI-1 T3SS remains unknown. Herein we report the identification of a novel S. Typhimurium T3SS effector STM1239 (that we renamed SopF) by quantitative secretome profiling under SPI-1 inducing conditions. Multiple lines of evidence firmly established the in-vitro secretion of SopF and its translocation into infected epithelial cells in a T3SS-dependent manner. Notably, genetic ablation of Salmonella sopF led to significantly decreased proliferation in macrophages as well as attenuated virulence in a murine infection model. Our work highlights the utility of quantitative secretome profiling in identifying novel bacterial virulence factors.
Materials and Methods

Bacterial strains and growth conditions

The *Salmonella enterica* serovar Typhimurium strain SL1344 was described previously\textsuperscript{16}. All bacterial strains were maintained at -80°C in Luria-Bertani (LB) broth supplemented with 25% (v/v) glycerol. The frozen strains were routinely grown on LB plates with 1.5% agar at 37°C, unless stated otherwise. A single colony was picked and then inoculated into 3 mL of LB medium. Then the overnight culture was diluted 1:20 into 3 mL of LB broth (supplemented with 0.3 M NaCl to induce the expression of SPI-1 T3SS\textsuperscript{17}). The bacteria were harvested for infection assays when they grew to the mid-exponential phase (OD\textsubscript{600} ≈ 0.9). Antibiotics were added as required with the following final concentrations (streptomycin, 30 μg/mL; ampicillin, 50 μg/mL; kanamycin, 50 μg/mL).

Experimental design and statistical rationale

To identify secreted effector proteins under SPI-1 inducing conditions by quantitative proteomics, we used an SPI-1 T3SS-deficient strain (ΔinvA) as a negative control. For the preparation of *Salmonella* secretome samples, 1 mL of bacterial culture (OD\textsubscript{600} ≈ 0.9) was centrifuged at 10,000 × g for 5 min. The culture supernatants containing T3SS effectors secreted by *Salmonella* were collected and passed through a 0.22 μm membrane filter. Trichloroacetic acid (TCA) precipitation was then carried out at 4°C overnight. Protein pellets were collected by centrifuging at 15,000 × g for 15 min at 4°C and washed twice with cold acetone\textsuperscript{18}. The precipitates were boiled in the SDS-PAGE sample buffer containing 60 mM Tris-HCl (pH 6.8), 1.7% (w/v) SDS, 6%
(v/v) glycerol, 100 mM dithiothreitol (DTT), and 0.002% (w/v) bromophenol blue for 5 min. Then the secretome samples were further fractionated by 10% SDS-PAGE and subjected to in-gel digestion as previously reported\textsuperscript{19}. Briefly, gel slices were cut into 1 mm\textsuperscript{3} cubes and destained with 50% acetonitrile (ACN) in 50 mM triethyl ammonium bicarbonate (TEAB). After dehydration by ACN, proteins were digested in a buffer containing 1.2 ng/\mu L trypsin and 50 mM TEAB (10% ACN) for 16 h at 37°C. The tryptic peptides were extracted from gel cubes twice with 50% ACN and 5% formic acid (FA) for 20 min at 37°C. The resulting peptides were pooled and vacuum dried for stable isotopic labeling reactions.

The dimethyl labeling reactions were performed as described previously\textsuperscript{20}. Peptide samples were resuspended in 100 mM TEAB, and then 0.6 M NaBH\textsubscript{3}CN were added. Peptide samples from each group were labeled with either 4% deuterated formaldehyde (CD\textsubscript{2}O) or formaldehyde (CH\textsubscript{2}O), respectively. After incubation for 1 h at room temperature, the labeling reactions were quenched with 1% (v/v) ammonia solution followed by the addition of 5% FA. The differentially labeled samples were gently mixed and then dried again by vacuum centrifugation. The samples were stored at -20°C prior to further LC-MS/MS analyses. In total, three biological replicates of secretome samples from each strain were analyzed.

Nanoflow reversed-phase LC separation was carried out on an EASY-nLC 1200 system (Thermo Scientific). The capillary column (75 \mu m \times 150 mm) with a laser-pulled electrospray tip (Model P-2000, Sutter instruments) was home-packed with 5 \mu m, 100 Å Magic C18AQ silica-based particles (Michrom BioResources Inc., Auburn,
CA). The mobile phase was comprised of solvent A (97% H₂O, 3% ACN, and 0.1% FA) and solvent B (100% ACN and 0.1% FA). The LC separation was carried out with the following gradient: solvent B was started at 7% for 3 min, and then raised to 40% in 40 min; subsequently, solvent B was rapidly increased to 90% in 2 min and maintained for 10 min before 100% solvent A was used for column equilibration. Eluted fractions from the capillary column were electrosprayed directly onto a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Scientific) for MS/MS analyses in a data-dependent acquisition mode. One full MS scan (m/z 350-1500) was acquired by the Orbitrap mass analyzer and subsequently the top 10 most intense ions were fragmented by collision-induced dissociation (CID) in the ion trap with the following parameters: ≥ +2 precursor ion charge, 2 Da precursor ion isolation window, and 35% normalized collision energy. Dynamic exclusion was set with repeat duration of 30 s and exclusion duration of 12 s.

Raw MS files were processed by MaxQuant (version 1.5.4.1) and searched against the S. Typhimurium LT2 protein database (strain LT2 / SGSC1412 / ATCC 700720, 11/7/2011, 5,199 sequences) downloaded from UniProt (http://www.uniprot.org/). The precursor mass tolerance was set at 20 ppm and the fragment mass tolerance was set at 0.8 Da. Trypsin was selected as the digestive enzyme with a maximum of two missed cleavages. Dimethyl (K, N-term) and dimethyl (D₄K, D₄N-term) were set as fixed modifications for light- and heavy-labeled samples, respectively. Oxidation (M) was set as a variable modification. The peptide and protein identifications were filtered to achieve a false discovery rate (FDR) < 1%. Intensity values obtained from MaxQuant
for identified proteins were further processed by using Perseus. The missing values were replaced with random numbers from a normal distribution (width = 0.3, shift = 1.8). Filtered proteins with normalized ratios (L/H) > 2.0 or < 0.5 and p-values <0.05 (Student’s t-test) were considered to be statistically significant.

**Molecular cloning and construction of bacterial mutants**

*Salmonella sopF*, *invA*, and *ssaV* deletion mutants (ΔsopF, ΔinvA, and ΔssaV) were generated by homologous recombination as previously described. In addition, the lambda red recombination system was used to construct the ΔsopF strain chromosomally expressing the kanamycin resistance gene (ΔsopF::kan). Briefly, the chromosomal sequence of *sopF* was stably replaced with a kanamycin marker, and successful deletion or insertion of the target gene was confirmed by PCR analyses. For constructing bacterial strains that harbor a plasmid-borne *sopF* gene in the WT or ΔinvA backgrounds, the *sopF* fragment was amplified and inserted into an arabinose-inducible pBAD-based plasmid (low copy number) with a C-terminal 3×FLAG tag. For the dual-luciferase reporter assays, the opening reading frame (ORF) of *sopF* was cloned into the mammalian expression vector pcDNA3.

**Western blot analyses**

The *Salmonella* strains expressing 3×FLAG-tagged effectors were used for preparing bacterial secretome samples as described above. The protein samples were further separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Immunoblotting analyses were carried out with primary antibodies specific for FLAG (Cwbio, China) (1:2,000) and horseradish peroxidase (HRP)-conjugated
secondary antibodies (Cwbio, China) (1:5,000). The blots were scanned with the Tanon-5200 Image System (Tanon, China).

**Gentamicin protection assays with epithelial cells and macrophages**

Both HeLa and murine macrophage RAW264.7 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco) under an atmosphere of 5% CO₂ at 37°C. Mammalian cells were seeded in 24-well plates at a density of 5 × 10⁵ cells/well before infection. *Salmonella* invasion was performed when cell monolayers reached 70-85% confluence. Infection of HeLa cells was carried out for 45 min in Hanks’ balanced salt solution (HBSS) with a multiplicity of infection (MOI) of 10 (a higher MOI of 100 was used for immunoblotting analyses of translocated effectors in host cytosol). For macrophage infection, bacteria were opsonized at 37°C for 20 min and then added to a RAW264.7 macrophage monolayer at an MOI of 10 (the infection was allowed for 15 min). After infection, cell monolayers were washed with pre-warmed HBSS and incubated further for 1 h in pre-warmed DMEM supplemented with 100 μg/mL gentamicin to kill extracellular bacteria. Subsequently, cells were washed again with HBSS, and fresh DMEM supplemented with 10 μg/mL gentamicin was added. At indicated time points, cells were washed extensively with PBS and lysed in 20 mM Tris-HCl (pH 7.6) buffer containing 150 mM NaCl and 1% Triton X-100. Samples were serially diluted and plated on selective medium to count intracellular bacteria by colony-forming units (CFU) assays. The extent of replication was then determined by dividing the number of intracellular bacteria at different time points by the number at 1 hpi.
For Western blot analysis of SopF translocation, infected cell lysates were first centrifuged at 600 × g for 5 min to remove nuclei and cell debris, and then the post-nuclear supernatants were centrifuged at 4000 × g to separate intracellular bacteria from host cytosol.

**β-lactamase reporter assays**

β-lactamase reporter assays were performed as previously described. SopF fused with β-lactamase TEM-1 was expressed in either WT *Salmonella*, ΔinvA or ΔssaV backgrounds. The resulting strains were used to infect HeLa cells as described above. At 2 h post infection, a small molecule substrate of β-lactamase (CCF2) was added into the cell culture medium and further incubated for 2 h at room temperature. Translocation of effectors together with the fused TEM would result in the cleavage of CCF2 and emission of blue instead of green fluorescence. The infected cells were visually inspected under an inverted confocal microscope (LSM 700, Zeiss, Germany) and the cells emitting blue fluorescence were considered as positive for effector translocation.

**Yeast toxicity assays**

The ORF of sopF was cloned into a yeast expression vector pYES downstream of an inducible *GAL1* promoter, where the expression of the target gene can be repressed by the addition of glucose and induced by the addition of galactose. The plasmids were next transformed into the yeast strain W303 by using a standard lithium acetate method. Colonies on the selective medium were PCR checked to confirm the successful transformation. The transformants were grown in SD/-Ura media containing
2% glucose overnight and plated onto the selective medium with either 2% galactose or glucose in 5-fold serial dilutions prior to incubation at 30°C for 2-3 days. For all assays, growth of yeast strains was compared to control strains harboring an empty vector.

**Competitive infection assays in a mouse model**

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Chinese Association for Laboratory Animal Sciences (CALAS) and approved by the ethics committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China). Wild-type S. Typhimurium and ΔsopF mutant strains with kanamycin resistance were cultured in LB to the exponential growth phase (OD₆₀₀ ≈ 0.9) before competitive infection.

Fifteen female C57BL/6 mice of 6 weeks old were infected orally with a mixed inoculum (~ 1 × 10⁷ CFU/mouse) of bacterial cells containing an equivalent number of each test strain in 0.1 M HEPES buffer (pH 8.0) with 0.9% (w/v) NaCl. Mice were sacrificed at 24 h post infection. The cecum, spleen, and liver were harvested and homogenated for the determination of bacterial loads by plating on LB plates with or without kanamycin (30 μg/mL) simultaneously. The competitive index (CI) was calculated from CFU assays as: \((ΔsopF \text{ mutant/wild type})_{\text{output}}/(ΔsopF \text{ mutant/wild type})_{\text{input}}\). The competitive infection data were analyzed by using a one-sample t test.
Results

Quantitative profiling of *S. Typhimurium* secretome identified a putative T3SS effector

*Salmonella* Typhimurium is arguably one of the best characterized models for bacterial pathogens and many of its T3SS effectors have been uncovered over the years by various approaches. Nevertheless, a comprehensive catalogue of SPI-1 T3SS effectors has been lacking, which hampers a full understanding of SPI-1 functions (i.e., certain functions are unassigned). In this study, we set out to identify additional putative effectors in *S. Typhimurium* by using quantitative proteomics. Bacteria were grown in hyperosmotic LB media to induce the expression of SPI-1 T3SS, and the proteins from the culture supernatants were precipitated and digested by trypsin (Fig. 1A). The resulting peptides were further subjected to light or heavy dimethyl labeling prior to quantitative LC-MS/MS analyses. To distinguish background proteins that are abundantly present independently of T3SS, we analyzed in parallel the culture supernatant from a *Salmonella* strain deficient in SPI-1 T3SS (*Δ*inv*A* mutant)\(^{25}\). In total, 145 bacterial proteins (with at least two unique peptides per protein) were identified in the WT and *Δ*inv*A* strains (Table S1). As expected, flagella proteins (e.g., FliD and FlgK) top the list and are the most abundant proteins identified in the secretome samples. In addition, we also found abundant presence of non-secreted proteins such as the outer membrane protein OmpA and molecular chaperon GroEL, indicative of unavoidable cell lysis during bacterial culturing. As their presence is independent of bacterial T3SS, these proteins tend to have comparable levels between the WT and *Δ*inv*A* samples,
yielding light-to-heavy ratios close to 1.0 (see OmpA in Fig. 1B). The next set of proteins of high levels in the list are SipA (a known SPI-1 effector) and the components of T3SS translocons (SipB, SipC and SipD), which all have relatively large ratios due to their preferential enrichment in the WT samples (see SipA in Fig. 1B). Differential analyses with the whole list of proteins led to the identification of most known SPI-1 effectors with ratios larger than 2, suggesting the robustness and specificity of our secretome profiling (Fig. 2A). A complete list of identified SPI-1 effectors and some effectors that can be translocated by both SPI-1 and SPI-2 T3SSs (e.g., SlrP) is provided as Table 1.

Intriguingly, we found a putative cytoplasmic protein STM1239 bearing a ratio similar to those of known SPI-1 effectors (Fig 2A, red). In fact, STM1239 can only be detected in the WT but not ΔinvA samples (Fig. 2B). In total, six unique peptides of this protein were identified (see supplementary Table S1) and Fig. 2C shows a representative MS/MS spectrum of the peptide NVVEDVPLFPESR, which carries a nice series of y-type fragment ions from y7 to y12. Taken together, our secretome profiling data suggest that _Salmonella STM1239_ (which we renamed sopF) encodes a putative T3SS effector.

**A functional SPI-1 T3SS is required for the secretion and translocation of _Salmonella SopF_**

SopF has a predicted molecular mass of 42.3 kDa. BLAST analyses reveal that it is highly conserved among different serovars of _Salmonella enterica_. S. Typhimurium
SopF shares >95% sequence identity with its orthologues in *S. Typhi*, *S. Newport* and *S. Dublin* (Fig. S1A). In addition, the sequence of SopF shares 65.9% identity to that of S5N8P0 in *Salmonella bongori* N268-08, indicating presence of homologs among different *Salmonella* species as well.

To further confirm our proteomic data by immunoblotting assays, we generated *Salmonella* strains expressing plasmid-borne SopF-3 × FLAG in the WT or SPI-1 T3SS-deficient (ΔinvA) backgrounds. SopD, a known SPI-1 effector\(^\text{26}\), was chosen as a positive control because it showed similar levels of secretion as SopF in the culture supernatants (suggested by our proteomic data). We cultured various bacterial strains under SPI-1-inducing conditions and probed the levels of SopF and SopD in the secretome samples with a FLAG-specific antibody. Similar to SopD, SopF was only detected in the culture supernatants prepared from WT bacteria but not the SPI-1 T3SS-deficient strains harboring a SopF-expressing plasmid, although this protein was expressed at similar levels in both strains when the bacterial lysates were probed with FLAG antibodies (Fig. 3A). Consistent with our proteomic data, therefore, these immunoblotting results demonstrate that SopF can be secreted into the bacterial culture supernatant under SPI-1-inducing conditions in a T3SS-dependent manner.

Furthermore, we examined whether SopF can be translocated into host cells during bacterial infection. We infected HeLa cells with WT, SPI-1 T3SS-deficient (ΔinvA) and SPI-2 T3SS-deficient (ΔssaV) strains carrying epitope-tagged effectors. At 2 h (for SPI-1) or 6 h (for SPI-2) post infection (hpi), infected host cells were lysed and fractionated into two fractions containing host cytosol and bacterial pellets, respectively. Then
individual samples were subjected to immunoblotting analyses to determine the levels
of SopF and two controls (SopD and a known SPI-2 effector SseF) with FLAG-specific
antibodies. Western blotting data show that SopF can be readily detected in the host
cytosol and the fraction containing intracellular bacteria from cells infected by WT and
ΔssaV strains but not the ΔinvA mutant harboring a SopF-expressing plasmid (Fig. 3B).
In control experiments, the detection of SopD in both cellular fractions was dependent
on a functional SPI-1 T3SS whereas SseF was only absent in the cytosol of host cells
infected by the ΔssaV mutant lacking a SPI-2 T3SS (Fig. 3B). Taken together, these
data indicate that SopF can be delivered into host epithelial cells during *Salmonella*
infection and its delivery is strictly and specifically dependent on a functional SPI-1
T3SS.

To further establish *Salmonella* SopF as a *bona fide* substrate of T3SS, we utilized
the β-lactamase reporter system to monitor effector translocation into infected host
cells27. A small molecule CCF2, once located intracellularly, can emit green
fluorescence upon processing by cellular esterases. If further cleaved by β-lactamase,
it results in a compound emitting blue fluorescence. Therefore, this molecule can serve
as a sensitive intracellular sensor for β-lactamase (and hence effectors when fusion
proteins are expressed). Indeed, we found emission of blue fluorescence in HeLa cells
that were infected by the WT and ΔssaV strains harboring a SopF-TEM expressing
plasmid (Fig. 3C), indicating the delivery of SopF into host cells. In contrast, only green
fluorescence was observed for HeLa cells infected with the SPI-1 T3SS-deficient ΔinvA
mutant expressing the same SopF fusion protein. Likewise, host cells infected by
*Salmonella* strains expressing SopD-TEM exhibited similar emission of fluorescence. In contrast, no detectable levels of blue fluorescent signals could be observed in the ΔssaV strain harboring an SseF-expressing plasmid or in the WT strain carrying a vector-borne OmpA-TEM. Collectively, these data unequivocally established that SopF is a *bona fide* effector protein of *Salmonella* SPI-1 T3SS.

**Ectopic expression of *Salmonella* SopF interfered with yeast growth**

To probe the potential functional roles of SopF as a newly identified T3SS effector, we first set to determine whether ectopic expression of SopF in host cells results in any notable cellular phenotypes. *Saccharomyces cerevisiae* has been widely used as a model system to carry out initial characterization of bacterial effectors\(^{28}\). For instance, a large panel of *Legionella pneumophila* effectors has been screened for host toxicity upon ectopic expression in yeast, leading to identification of many effectors targeting conserved mammalian pathways\(^{29,30}\). Here we introduced into yeast an expression plasmid that allows galactose-inducible production of His-tagged SopF. Compared to the control strain harboring the empty vector, the SopF-expressing yeast strain exhibited significant growth inhibition when grown on selective media containing 2% galactose (Fig. 4A). Immunoblotting analyses further show that the expression of SopF coincided with toxicity and grow inhibition in yeast (Fig. 4B). In contrast, no growth defect was observed when glucose-containing media were used regardless of the presence of SopF-carrying plasmids. These data indicate that ectopic expression of SopF inhibited yeast growth likely due to its targeting of highly conserved cellular...
Deletion of sopF led to a replication defect of Salmonella in RAW264.7 macrophages but did not affect bacterial virulence during infection of HeLa cells

As we established that SopF can be translocated into host cells during Salmonella infection and its ectopic expression inhibits yeast growth, next we sought to investigate its potential contribution to bacterial virulence. We generated a sopF deletion strain (ΔsopF) and assayed its invasion of host epithelial cells as well as intracellular replication rates by CFU counting. HeLa infection assays suggest that ΔsopF mutant invaded host cells equally well as the WT bacteria (with invasion rates normalized to 100%) (Fig. 5A). Furthermore, we found that the intracellular multiplication rate of the Salmonella strain lacking sopF did not differ significantly from that of the WT bacteria (Fig. 5B).

Due to the absence of virulence phenotypes in non-phagocytic epithelial cells, next we assayed bacterial replication within phagocytes such as RAW264.7 macrophage cells. Notably, deletion of sopF resulted in a significant growth defect of intracellular Salmonella at 8 hpi (Fig. 5C). Compared to the WT bacteria, Salmonella lacking sopF exhibited an even larger drop in the fold of replication at later time points (i.e., 16 or 24 hpi). In addition, complementation of the ΔsopF mutant with a plasmid-encoded copy of sopF restored its intracellular multiplication to the levels of the WT bacteria. Collectively, these results demonstrate that SopF promotes intracellular replication of Salmonella in macrophage cells, while its loss did not compromise bacterial virulence.
during infection of HeLa cells.

**SopF contributes to *Salmonella* fitness in a mouse model of infection**

Since the deletion of *sopF* led to a growth defect within infected RAW264.7 macrophages, we further investigated its potential contribution to *in vivo* infection using C57BL/6 mice as a more physiologically relevant host. *Salmonella sopF* was in-frame deleted and stably replaced with a kanamycin resistance gene. C57BL/6 mice were orally co-infected with an equivalent mixture of the WT strain and Δ*sopF* mutant. As shown in Fig. 5D, the geometric mean competitive index for Δ*sopF* mutant in the liver, spleen and cecum was 0.53, 0.42 and 0.59, respectively, indicating that the Δ*sopF* mutant was significantly out-competed by the WT strain during systemic infection. Given the lack of prominent virulence attenuation during infection of HeLa cells, virulence phenotypes associated with the Δ*sopF* mutant are likely to be dependent on the context of infection.

**Discussion**

*Salmonella enterica* serovar Typhimurium is a Gram-negative bacterial pathogen that utilizes T3SSs to deliver effector proteins into host cells, thus facilitating its initial internalization and subsequent intracellular proliferation. Because of their critical roles in bacterial pathogenesis, T3SS effectors have been extensively discovered and catalogued by using an array of genetic, biochemical and bioinformatic approaches during the past decades. Nevertheless, the sheer number of uncharacterized open
reading frames in the genome suggests the potential presence of unknown effectors. In this study, we applied quantitative proteomics to investigate the secretome of *S. Typhimurium* SL1344 under SPI-1 inducing conditions. In comparison to a T3SS-deficient strain, differential proteomic analyses successfully identified 11 known effectors, including 9 SPI-1 effectors and one effector (SlrP) translocated by both SPI-1 and SPI-2 T3SSs. Importantly, our comprehensive secretome data also uncovered a novel *Salmonella* effector SopF. By using immunoblotting and β-lactamase reporter assays we provided compelling evidence that SopF can be secreted into the culture medium and translocated into host epithelial cells during infection in a SPI-1 T3SS-dependent manner.

SopF has a predicted domain DUF3626 (Domain of Unknown Function) spanning amino acids 205 to 341 (Fig. S1B). This domain DUF3626 is specifically found in bacteria. SopF is encoded in the SPI-11 island, which is 14 kb in length and locates next to the Gifsy-1 prophage. Interestingly, a number of genes in this island have been reported to be involved in bacterial virulence, such as *pagC*, *pagD* and *msgA* (Fig. S1B). Located downstream of *sopF*, these genes have a similar GC content as *sopF* and were found to be important for *Salmonella* survival inside macrophages. Furthermore, *pagC* and *pagD* are regulated by the PhoP/PhoQ two-component system, which is located upstream of *sopF* and regulates a bundle of virulence proteins. Therefore, it would also be interesting to determine whether PhoPQ plays any regulatory role in the expression of *sopF*.

Recently, Brown *et al* found evidence that the expression of STM1239/SopF was
regulated by the SPI-1 regulator HilA\textsuperscript{34}, indicating SopF as a potential effector. In addition, Carol et al mapped the direct regulatory networks of seven Salmonella transcriptional factors. Notably, they found positive regulation of sopF by InvF, a transcriptional regulator of SPI-1 T3SS genes such as sopB, sicA and sopE\textsuperscript{35}. Taken together, these findings are consistent with our discovery that Salmonella sopF encodes a novel effector protein of SPI-1 T3SS. Interestingly, Fiskin et al provided evidence of ubiquitinated SopF in Salmonella-infected host cells\textsuperscript{36} As Salmonella lacks a complete ubiquitination system, post-translational modification of SopF must have occurred in mammalian cells by hijacking the host ubiquitination machinery, implying the translocation of this protein during bacterial infection. Furthermore, we found that ectopic expression of SopF is toxic in yeast, indicating that it may target some conserved host processes.

Though deletion of sopF did not result in marked difference in Salmonella virulence during infection of host epithelial cells, we found that SopF promoted bacterial intracellular proliferation within infected RAW264.7 macrophages. Importantly, competitive infection in the C57BL/6 mice demonstrates that SopF was required for the full virulence of S. Typhimurium in vivo. Differences in virulence phenotypes obtained with various infection models may be attributed to some specific host factors that restrict pathogen proliferation. Similarly, Salmonella ΔsifA mutant was found to be defective in intracellular replication within macrophages\textsuperscript{37} while they grew well in infected HeLa cells\textsuperscript{38}.

In conclusion, we exploited quantitative proteomic profiling to comprehensively
identify secreted *Salmonella* proteins under SPI-1 inducing conditions. Comparative analyses of bacterial secretome from the WT and T3SS-deficient strains led us to unveil SopF as a novel effector. We verified its secretion and translocation in a T3SS-dependent manner. Though it did not affect bacterial invasion or survival in epithelial cells, SopF exhibited significant toxicity when expressed in yeast and also contributed to full *Salmonella* virulence in both RAW264.7 macrophages and animal models. Future work will be focused on elucidating the physiological functions of this newly identified effector in the context of *Salmonella* pathogenesis.

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**Data Availability**

The proteomics data reported in this manuscript have been deposited to the iProx database (URL: http://www.iprox.org/page/HMV006.html) with the accession number IPX0000941000.
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Figure 1. (A) A quantitative proteomic workflow for \textit{S. Typhimurium} secretome analysis. \textit{S. Typhimurium} wild type and \textit{ΔinvA} strains were grown in hyperosmotic LB and proteins in culture supernatants were precipitated by the addition of trichloroacetic acid (TCA). Samples were further fractionated by SDS-PAGE and in-gel digested prior to dimethyl labeling and LC-MS/MS analyses. Reactions with deuterated formaldehyde yield heavy-labeled peptides whereas light-labeled peptides were generated by treatment with regular formaldehyde. (B) Representative mass spectra of the triply protonated peptide STLKPEGQQALDQLYSQLSNLDPK from OmpA (left), a nonspecific background protein and the triply protonated peptide IDVTAHATAEAVTNASESK from SipA (right), a known T3SS effector. Peptides with light and heavy labels are indicated by open and filled triangles, respectively.

Figure 2. Proteomic identification of \textit{Salmonella} SopF as a putative T3SS effector. (A) Distribution of protein quantification ratios in a decreasing order. The black bars represent previously known or confirmed T3SS effector proteins, the light grey bars indicate the components of translocon (a translocation channel that forms a pore in the host cell membrane and allows passage of effector proteins), the red bar indicates the novel effector SopF identified in this study, and the white bars denote non-secreted proteins. (B) A representative mass spectrum of the doubly protonated peptide NVVEDVPLFPESR from SopF. (C) A representative MS/MS spectrum of the dimethyl labeled peptide NVVEDVPLFPESR under collision-induced dissociation (CID).

Figure 3. \textit{In vitro} secretion of \textit{Salmonella} SopF and its translocation during infection. (A) Immunoblotting analyses of secretome samples and cell lysates from the wild-type bacteria and \textit{ΔinvA} mutant containing pSopF-FLAG. SopD, a known SPI-1 T3SS
effector, was used as a positive control. (B) Immunoblotting analyses of translocated SopF during *Salmonella* infection. HeLa cells were infected by WT, ΔinvA and ΔssaV strains harboring pSopF-FLAG at an MOI of 100. Proteins from bacterial pellets and host cytosol were probed by immunoblotting assays. (C) Validation of SopF translocation by β–lactamase reporter assays. HeLa cells were infected by WT, ΔinvA and ΔssaV strains expressing a plasmid-encoded TEM fused to the effector of interest at an MOI of 100. Cells emitting blue fluorescence indicate successful translocation of the fusion protein and green cells indicated no translocation. SopD and SseF were used as positive controls for SPI-1 and SPI-2 effectors respectively. Bars, 15 μm.

**Figure 4.** Production of *Salmonella* SopF in yeast caused growth defects. (A) 5-fold serial dilutions of yeast strains were spotted onto selective plates containing 2% glucose or galactose respectively. A yeast strain harboring an empty vector was used as a negative control. (B) Immunoblotting analyses of SopF-His expression in the yeast strains tested above. 3-phosphoglycerate kinase (PGK) was used as a loading control.

**Figure 5.** Functional characterization of *Salmonella* strains lacking sopF (ΔsopF). (A & B) HeLa cells were infected by WT and ΔsopF strains, and then bacterial invasion rates and intracellular growth were determined by CFU assays. Data were obtained from three independent experiments. (C) RAW264.7 macrophages were infected by WT, ΔsopF, ΔsopF+pSopF and SPI-2-deficient (ΔssaV) strains, and then intracellular growth was determined by gentamicin protection assays. Data were obtained from three independent experiments. (D) Mixed infection of *Salmonella* WT and sopF deletion strains with kanamycin resistance in a murine infection model. Fifteen female C57BL/6
mice were infected orally with equivalent amounts of each strain for 24 h. The competitive indexes (CI) were determined in liver, spleen and cecum lysates following oral infection. Each point represents data from a single mouse and the averaged CI is shown as a solid line. **p<0.01, ***p<0.001.
Table 1  A list of *Salmonella* T3SS effector proteins identified from quantitative analyses of bacterial secretome.

| Protein ID | Protein description                        | Unique peptides | Sequence coverage (%) | Average ratio |
|------------|-------------------------------------------|-----------------|-----------------------|---------------|
| Q7CQD4     | Guanine nucleotide exchange factor SopE2   | 4               | 22.1                  | High\(^1\)   |
| Q8ZNR3     | Secreted effector protein SopA            | 21              | 40.3                  | 19.0          |
| Q9XCV2     | Leucine-rich repeat protein SlrP           | 14              | 25.2                  | High          |
| Q56019     | Cell invasion protein SipB                | 47              | 67.1                  | 120.4         |
| O30916     | Inositol phosphate phosphatase SopB       | 32              | 61.0                  | 54.0          |
| O52623     | Guanine nucleotide exchange factor SopE   | 12              | 55.8                  | 26.2          |
| P40613     | Surface presentation of antigens protein SpaN | 22        | 64.3                  | 29.0          |
| Q56026     | Cell invasion protein SipD                | 24              | 78.4                  | 18.4          |
| Q56027     | Cell invasion protein SipA                | 67              | 88.6                  | 101.0         |
| Q56020     | Cell invasion protein SipC                | 45              | 86.6                  | 13.7          |
| P40722     | Sop effector protein SopD                 | 6               | 19.9                  | 5.3           |
| P74873     | Effector protein SptP                     | 22              | 49.9                  | 4.7           |
| Q8ZPY9     | Putative cytoplasmic protein STM1239 (SopF)| 6               | 18.2                  | High          |

\(^1\) Average ratios were calculated from the intensities of light-labeled peptides divided by those of heavy-labeled peptides. “High” indicates that the proteins were only detected for the light-labeled peptides.
Figure 1.

A

WT

$\Delta$invA

TCA

Trypsin

LC-MS/MS

B

STLKPEGQALDLQLYSLSNLDPK

OmpA

IDVTAHATEAVTNASSESK

SipA

Relative abundance

m/z

1:1
Figure 2.

A. SopE2, SirP, GtgE, SipB, SipA, SopB (Not detected in the T3SS-deficient mutant)

- **Known/confirmed T3SS effectors**
- **Translocon components**
- **SopF**
- **SopD, SipC**
- **SpIP**
- **Non-secreted proteins**

B. NVVEDVPLFPESR

- Relative abundance
- m/z 764.90

C. Dimethyl - NVVEDVPLFPESR

- Relative abundance
- m/z 764.90
- [M+2H]^{2+}
Figure 3.

A

| Bacterial lysates | SopF  | SopD |
|-------------------|-------|------|
| WT                | ![Image](#) | ![Image](#) |
| ΔinvA             | ![Image](#) | ![Image](#) |

| Culture supernatants | WT  | ΔinvA  |
|----------------------|-----|--------|
|                     | ![Image](#) | ![Image](#) |
| ΔinvA                | ![Image](#) | ![Image](#) |

IB: α-FLAG

B

| Bacterial pellets | Host cytosol |
|-------------------|--------------|
| WT                | ΔinvA ΔssaV |
|                   | WT ΔinvA ΔssaV |

| SopF            |
|-----------------|
| ![Image](#)     |

| DnaK            |
|-----------------|
| ![Image](#)     |

| Bacterial pellets | Host cytosol |
|-------------------|--------------|
| WT                | ΔinvA |
|                   | WT ΔinvA |

| SopD            |
|-----------------|
| ![Image](#)     |

| DnaK            |
|-----------------|
| ![Image](#)     |

| Bacterial pellets | Host cytosol |
|-------------------|--------------|
| WT                | ΔssaV |
|                   | WT ΔssaV |

| SseF            |
|-----------------|
| ![Image](#)     |

| DnaK            |
|-----------------|
| ![Image](#)     |

IB: α-FLAG
Figure 4.

A

Vector

SopF

Glucose

Vector

SopF

Galactose

B

|       | Vector | SopF |
|-------|--------|------|
| Glu   | Glu    |      |
| Gal   | Gal    |      |

IB: α-His

PGK

IB: α-PGK
Figure 5.