Modulation of *Salmonella* virulence by a novel SPI-2 injectisome effector that interacts with the dystrophin-associated protein complex.

Running Title

Characterisation of a novel *Salmonella* effector

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Abstract word count: 217 (Abstract) + 149 (Importance)

Text word count: 5012

Abstract

The injectisome encoded by *Salmonella* pathogenicity island 2 (SPI-2) had been thought to translocate 28 effectors. Here, we used a proteomic approach to characterise the secretome of a clinical strain of invasive non-typhoidal *Salmonella enterica* serovar Enteritidis, that had been mutated to cause hyper-secretion of the SPI-2 injectisome effectors. Along with many known effectors, we discovered the novel SseM protein. *sseM* is widely distributed between the five subspecies of *Salmonella enterica,* is found in many clinically-relevant serovars, and is co-transcribed with *pipB2, a* SPI-2 effector gene. Translocation of SseM required a functional SPI-2 injectisome. Following expression in human cells, SseM interacted with five components of the dystrophin-associated protein complex (DAPC), namely β-2-syntrophin, utrophin/ dystrophin, α-catulin, α-dystrobrevin and β-dystrobrevin. The interaction between SseM and β-2-syntrophin and α-dystrobrevin was verified in *S.* Typhimurium-infected cells and relied on the PDZ domain of β-2-syntrophin and a sequence corresponding to a PDZ-binding motif (PBM) in SseM. A Δ*sseM* mutant strain had a small competitive advantage over the wild-type strain in the *S.* Typhimurium/mouse model of systemic disease. This phenotype was complemented by a plasmid expressing wild type SseM from *S.* Typhimurium or *S.* Enteritidis and was dependent on the PBM of SseM. Therefore, a PBM within a *Salmonella* effector mediates interactions with the DAPC and modulates systemic growth of bacteria in mice.

Importance

In *Salmonella enterica*, the injectisome machinery encoded by *Salmonella* pathogenicity island 2 (SPI-2) is conserved among the five subspecies and delivers proteins (effectors) into host cells that are required for *Salmonella* virulence. The identification and functional characterisation of SPI-2 injectisome effectors advances our understanding of the interplay between *Salmonella* and its host(s). Using an optimised method for preparing secreted proteins and a clinical isolate of the invasive non-typhoidal (iNTS) *Salmonella enterica*

serovar Enteritidis strain D24359, we identified 22 known SPI-2 injectisome effectors and one new effector - SseM. SseM modulates bacterial growth during murine infection and has a sequence corresponding to a PDZ-binding motif that is essential for interaction with the PDZ-containing host protein β-2-syntrophin and other components of the dystrophin-associated protein complex (DAPC). To our knowledge, SseM is unique among *Salmonella* effectors in containing a functional PDZ-binding motif and is the first bacterial protein to target the DAPC.

Introduction

Following entry into host cells, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) resides in membrane-bound compartments known as *Salmonella*-containing vacuoles (SCVs). Acidification and nutritional starvation of the vacuole lumen activate the two-component regulatory system SsrAB to induce expression of *Salmonella* pathogenicity island 2 (SPI-2) genes followed by the assembly of a type three secretion apparatus known as the SPI-2 injectisome [1-3]. The associated gatekeeper complex, comprising SsaL, SsaM and SpiC, enables the injectisome to secrete the translocon proteins SseBCD while preventing the premature translocation of effectors; once the translocon pore is formed on the SCV membrane, the neutral pH of host cell cytosol is sensed and the signal is transduced into the bacterial cytosol to disassociate the gatekeeper complex from the export gate component SsaV to allow translocation of effectors [4-6]. Approximately 28 such effectors are translocated into host cells [7, 8]; collectively these enable bacterial replication in host cells and suppress both innate and adaptive immune responses [1, 9-11].

Since the discovery of SPI-2 injectisome [9, 10, 12], different approaches have been explored for the identification of SPI-2 injectisome effectors. By similarity search for known effectors of other injectisomes, SspH1/2 [13]; SlrP, SifA, SseI, SseJ and SifB [14, 15]; SopD2 [16], PipB2 [17], and SseK1/2 [18] were shown to be SPI-2 injectisome effectors. Screening for SsrAB-regulated factors (*srf*) either by MudJ mutagenesis [19] or

transcriptomic analysis [20, 21] revealed SrfA-M and SseL. Both SrfH (i.e. SseI) and SseL were subsequently verified as SPI-2 effectors. Screening a transposon mini-Tn5-cycler-generated library of translational fusions between *Salmonella* chromosomal genes and *cyaA*' during cell infection, identified known effectors (SlrP, PipB2, SseJ, SrfH and AvrA) and new effectors (SteA, SteB and SteC) [22]. A gatekeeper mutant Δ*ssaL* strain that hypersecretes effectors into culture medium has been used to identify effectors by proteomic analysis: 17 known effectors and 6 new SPI-2 effectors were identified: SpvD, GtgE, GtgA, SteD, SteE and CigR [23]. Both the CyaA translocation screen and the Δ*ssaL*-based secretion screen used *S.* Typhimurium strain 12023 (i.e. ATCC14028) and its derivatives [22] [23]. In an alternative approach, Auweter et al [24] used stable isotope labelling with amino acids in cultures of *S.* Typhimurium SL1344 wt and SPI-2 null mutant strains to identify 12 effectors: SpvC/D, SopD2, SifA, SseJ, SteC, SteA, SseL, PipB2, PipB, GtgE and SteE.

S. Typhimurium strain ATCC14028 and SL1344 have been widely used in laboratories for over 50 years; the former was isolated from a chick in 1960 and the latter from cattle in 1966. Proteomic analysis has not yet been used to screen SPI-2 effectors from contemporary clinical isolates. In this study, we sought to identify SPI-2 effectors from a clinical isolate of invasive non-typhoidal *Salmonella* (iNTS) Enteritidis strain D24359 by comparing the secretomes of an isogenic hypersecretion gatekeeper mutant and a SPI-2 null mutant. We found 22 known effectors and one previously unidentified effector - SseM. SseM is present in all 5 subspecies of *S. enterica*. The postsynaptic density-95/discs large/zonula occludens-1 (PDZ) domain binding motif (PBM) of SseM mediates interaction with dystrophin-associated protein complex (DAPC) and is involved in modulation of *Salmonella* virulence.

Results

Discovery of SPI-2 injectisome effector SseM by proteomic analysis

105 To investigate the SPI-2 injectisome effector repertoire of a clinical isolate of *Salmonella*
106 *enterica* subspecies *enterica,* we exploited the hypersecretion phenotype of a gatekeeper
107 mutant. We chose an invasive non-typhoidal *Salmonella enterica* serovar Enteritidis (*S.*
108 Enteritidis) strain D24359 that was isolated from blood of a Malawian patient and is sensitive
109 to antibiotics carbenicillin, kanamycin and chloramphenicol [25] to make a Δ*spiC* single
110 mutant (an effector hypersecretion mutant) and a Δ*spiC,ssaC* SPI-2 null mutant [4]. The
111 bacterial strains were grown in SPI-2-inducing medium MgM-MES at pH 5.0 for 6 h. Then
112 the supernatant was concentrated and subjected immediately to SDS-PAGE after which a 1
113 cm gel slice was analysed by mass spectrometry (**Fig. 1A**). The resulting peptides were
114 compared to predicted protein sequences from the annotated *S.* Enteritidis strain D24359
115 sequence. From two experiments, we identified 22 known SPI-2 effectors and one previously
116 unidentified effector D24359_01053 (**Table 1 and Fig. 1B**).
117
118 D24359_01053 consists of 103 amino acids. Searching the BLAST Protein database of *S.*
119 Typhimurium LT2 showed that D24359_01053 is almost identical to STM2779: there are 95
120 identical residues in the predicted 110 amino acids of STM2779 (**sFig. 1A**). Bioinformatic
121 analysis revealed that D24359_01053/STM2779 is conserved in all five subspecies of
122 *Salmonella enterica* but not in *S. bongori*, with most being predicted to be 110 amino acids in
123 length (**sFig. 1B**). We named this effector SseM (*Salmonella* secreted effector M).
124
125 To further analyse the distribution of SseM and SseM variants among serovars of *S. enterica*
126 subspecies *enterica*, 834 complete *Salmonella* genomes with the highest assembly quality
127 were downloaded from Enterobase (https://enterobase.warwick.ac.uk/) and used to
128 construct an *sseM* database with *stm2779* as the reference sequence. The SseM protein
129 variants in the context of genomic or serovar diversity were displayed with the Grapetree
130 phylogenetic tool [26] (**Fig. 1C and D**). Most of the sequenced strains of different serovars
131 had full length SseM (**Fig. 1D**), with the N- and C-terminal regions of SseM highly conserved

(**sFig. 2**). Our analysis revealed the presence of two common pseudogene variants; *sseM*_2_pseudo, present in 111 out of 113 *S.* Typhi genomes and *sseM*_6_pseudo, present in 40 out of 126 *S.* Enteritidis genomes (**Fig. 1D**). *sseM*_2_pseudo is the result of an additional cytosine in *sseM* of *S.* Typhi, which generates a stop codon immediately after the predicted 30th residue; while the *sseM*_6_pseudo is due to the mutation of the predicted 84th codon TGG to TAG, resulting in a truncated version of SseM (**sFig. 3)**. In summary, SseM is widely distributed among the five subspecies of *Salmonella enterica* and so represents a new conserved "core" effector protein.

Expression, secretion, and translocation of SseM

sseM is located 175 nt downstream of SPI-2 effector gene *pipB2* (**Fig. 2A**). Both *pipB2* and *sseM* (*stm2779*) share the same transcriptional start site [27] and are controlled by SsrAB [20, 28]. To verify SsrAB dependence on expression of SseM and to test if *pipB2* and *sseM* were operonic or not, a rabbit polyclonal antibody against the C-terminal peptide (PYFPVVPGERETDV) of *S.* Typhimurium SseM was obtained. *S.* Typhimurium 12023 wt and derivative strains were grown in MgM-MES at pH 5.0, and proteins in whole bacterial lysates were immunoblotted. The antibody detected SseM in wt and a Δ*sseM* mutant expressing SseM from a plasmid (p*sseM*) but not in lysates derived from the Δ*sseM* mutant, demonstrating the specificity of the SseM antibody (**Fig. 2B)**. As expected, SseM was not detected in lysates derived from a *ssrA::mTn5* mutant. Furthermore, deleting the promoter of *pipB2* but not *pipB2* itself led to the loss of SseM (**Fig. 2B)**. Taken together, the data indicate that *sseM* and *pipB2* are bicistronic, with their expression activated by SsrAB.

As SseM from *S.* Typhimurium strain LT2 and most of *S. enterica* species have been annotated as a 110-residue long protein that uses TTG rather than ATG (21 nucleotides downstream of TTG), as its start codon (**sFig. 4**), we defined the actual start codon of *sseM*

by changing the ATG to ACG on plasmid p*sseM*. The resulting plasmid p$sseM^{ACG}$ was transformed into the Δ*sseM* mutant strain to check the expression of SseM by immunoblot. SseM was undetectable in the Δ*sseM* mutant carrying plasmid p$sseM^{ACG}$, indicating that *sseM* uses the ATG as its start codon to encode a 103-residue long protein (**Fig. 2C)**.

Next, we investigated SPI-2 injectisome-dependent secretion of SseM by immunoblotting. The wt and a Δ*ssaV* mutant strains were grown in MgM-MES at pH 5.0 for 4 h to assemble the SPI-2 injectisome, then the pH of medium was changed to 7.2 to allow effector secretion [5] . Although SseM was detected in bacterial lysates from both wt and Δ*ssaV* mutant strains, secreted SseM was only detected in samples prepared from the wt culture (**Fig. 2D**). This result agrees with the mass spectrometric result of *S.* Enteritidis strain D24359, demonstrating that SseM secretion is dependent on the SPI-2 injectisome.

Translocation of SseM into mammalian cells from intracellular *Salmonella* was then tested by immunoblotting. For this, HeLa cells were infected with different bacterial strains for 8 h, then translocated proteins were extracted from post-nuclear supernatant with Triton X-100 and subject to immunoblotting using the anti-SseM antibody. A small quantity of translocated SseM was detected from HeLa cells infected with wt strain, and significantly more was detected in the mutant strain carrying *sseM* on a plasmid (**Fig. 2E**). However, attempts to detect translocated SseM with the rabbit anti-SseM antibody by immunofluorescence microscopy failed. To further investigate translocation of SseM by microscopy, a plasmid expressing C-terminal HA-tagged SseM (p*sseM-HA*) was transformed into the wt or Δ*ssaV* mutant strains and these were used to infect HeLa cells. Translocated SseM-HA was detected with an anti-HA epitope antibody in cells infected by wt but not the Δ*ssaV* mutant strain (**Fig. 2F**). These results demonstrate that SseM is translocated into host cell via the SPI-2 injectisome.

SseM interacts with β-2-syntrophin and its associated proteins

To identify host cell proteins with which SseM interacts, stable HeLa cell lines expressing GFP or GFP::SseM were established, then lysates were subjected to GFP-trap immunoprecipitation followed by mass spectrometric analysis. Utrophin/dystrophin, α-catulin, α-dystrobrevin, β-dystrobrevin and four PDZ domain- containing proteins β-2-syntrophin, disks large homolog 1 (DLG1), peripheral plasma membrane protein CASK and protein lin-7 homolog C were co-purified with GFP::SseM but not by GFP alone (**Table 2**). These putative targets of GFP::SseM were also specifically co-immunoprecipitated with Flag::SseM but not by another Flag-tagged SPI-2 injectisome effector (Flag::SpvD) from transiently transfected HEK 293 cells (**Table 2**). β-2-syntrophin, utrophin/dystrophin, α-catulin, α-dystrobrevin and β-dystrobrevin are components of the dystrophin-associated protein complex (DAPC) signalosome [29, 30], and had much higher ion scores and number of peptides detected in our screenings than the other three PDZ domain-containing proteins. Therefore, further work was focussed on the interaction between SseM and β-2-syntrophin and its associated proteins.

As an independent test of the validity of the mass spectrometry results, and to check if SseM of *S.* Enteritidis (SseM^{SEN} to distinguish it from SseM of *S.* Typhimurium) also interacts with the same targets, HEK 293 cells were transiently transfected to express GFP-tagged effectors and cell lysates subjected to immunoprecipitation before immunoblotting. Like GFP::SseM, GFP:: SseM^{SEN} also interacted with β-2-syntrophin and α-dystrobrevin (**Fig. 3A**). However, GFP::SseM-HA failed to interact with β-2-syntrophin and α-dystrobrevin, indicating that the C-terminus of SseM is crucial for its interaction with the host cell targets.

Then, to test if SseM translocated from intracellular *Salmonella* interacts with the same host cell proteins, HeLa cells were infected with bacterial strains for 17.5 h. Infected cells were

213 then lysed, the lysate proteins were immunoprecipitated with the rabbit anti-SseM antibody
214 and subjected to immunoblotting. β-2-syntrophin and α-dystrobrevin were co-
215 immunoprecipitated from cells infected with wt strain or the Δ*sseM* mutant complemented
216 with plasmid p*sseM* but not the Δ*sseM* mutant strain (**Fig. 3B**). Therefore, and importantly,
217 translocated SseM interacts with β-2-syntrophin and α-dystrobrevin in physiological
218 conditions.
219
220 **A PDZ domain-binding motif of SseM and PDZ domain of β-2-syntrophin are essential**
221 **for interaction between SseM and β-2-syntrophin**
222
223 Syntrophins use their PDZ domains to interact with C-terminal PDZ domain-binding motifs
224 (PBMs) in other proteins such as the α_{1D}-adrenergic receptor (α_{1D}-AR) [29-31]. As SseM-HA
225 failed to interact with β-2-syntrophin and α-dystrobrevin, we hypothesised that SseM itself
226 might have a C-terminal PBM. Alignment of α_{1D}-AR and SseM revealed that SseM indeed
227 has a RETDV sequence at its C-terminus that corresponds to the PBM (^{568}RETDI^{572}) in α_{1D}-
228 AR (**Fig. 3C**). To test if the RETDV motif of SseM was required for its interaction with the
229 host cell targets, a set of mutated GFP-SseM variants transiently expressed in HEK 293
230 cells were immunoprecipitated with GFP-trap and subjected to immunoblotting. As shown in
231 **Fig. 3D**, GFP::SseM^{RE2A}, GFP::SseM^{T101A}, and GFP::SseM^{V103A} failed to interact with β-2-
232 syntrophin and α-dystrobrevin. Consistent with this result, translocated SseM^{V103A} also failed
233 to interact with β-2-syntrophin and α-dystrobrevin (**Fig. 3B**), demonstrating that the putative
234 PBM of SseM is required for its interaction with β-2-syntrophin and α-dystrobrevin in
235 transfected or infected cells.
236
237 To test if β-2-syntrophin mediates interaction between SseM and α-dystrobrevin, we
238 knocked out β-2-syntrophin in HEK293 cells with two different guideRNAs (g361 and g363).
239 Knockout of β-2-syntrophin abolished α-dystrobrevin co-immunoprecipitated with GFP-SseM

(**Fig. 3E**). This result suggests that SseM interacts with its host cell targets through the interaction between its PBM and the PDZ domain of β-2-syntrophin. In agreement with this hypothesis, predication of interaction between SseM and PDZ domain of β-2-syntrophin with AlphaFold Colab Multimer showed that the RETDV residues of SseM fit in the binding pocket between β-strand B (βB) and α-helix B (αB) of the β-2-syntrophin PDZ domain (**Fig. 3F**). Based on our structural predication and the structural data of other PDZ domains and PBMs [32, 33], we predicted that residues ^{125}GLGI^{128} or H176 of β-2-syntrophin (Highlighted in **sFig. 5**) are crucial for mediating its interaction with the PBM of SseM. To test this, GFP-tagged β-2-syntrophin or its variants were co-expressed with mCherry-tagged SseM or SseM^{V103A} in β-2-syntrophin knock out HEK293 cells, and cell lysates were subject to GFP-trap immunoprecipitation. Mutating GLGI to 4 As or mutating the substrate-specific residue H to Y or V of β-2-syntrophin abolished its interaction with SseM although the mutants still interacted with α-dystrobrevin (**Fig. 3G**). Taken together, the data demonstrate that the PBM of SseM and PDZ domain of β-2-syntrophin are essential for the interaction between SseM and β-2-syntrophin.

The PBM of SseM modulates *Salmonella* growth *during murine infection*

We next assessed the contribution of SseM to *Salmonella* growth in systemic tissues of mice by competitive index (CI) analysis [34], involving intraperitoneal injection of a mixed inoculum of wt and Δ*sseM* mutant strains in susceptible mice. At 3 days post inoculation, the Δ*sseM* mutant strain significantly outcompeted the *wt::Km* strain (CI=1.800 ± 0.558). The Δ*sseM* mutant strain harbouring plasmid p*sseM* failed to outcompete the *wt::Km* strain (CI= 0.842 ± 0.196) and the CI results were significantly different to that of the Δ*sseM* mutant strain *vs* *wt::Km* strain (**Fig. 4**), showing that the small fitness difference was SseM-dependent. However, SseM-HA or SseM^{V103A} did not complement the Δ*sseM* mutant strain in the mixed infection (CI= 2.137 ± 0.979, 1.394 ± 0.253, respectively). In contrast, SseM^{SEN} did

267 complement the Δ*sseM* mutant strain in the mixed infection (CI= 0.885 ± 0.215). These
268 results demonstrate that SseM modulates the growth of *Salmonella* during systemic infection,
269 and this phenotype is dependent on the PBM of SseM.
270
271 **Discussion**
272
273 In this work, we investigated the SPI-2 injectisome effector repertoire of the clinical isolate *S.*
274 Enteritidis strain D24359 and identified a previously undescribed effector, which we have
275 named SseM. Like Niemann et al [23], we exploited the property of SPI-2 gatekeeper
276 mutants to hypersecrete effectors into SPI-2-inducing culture medium, which was then
277 collected for mass spectrometry analysis. While Niemann et al [23] passed 500 ml of culture
278 supernatant through a column containing solid-phase extraction resin to prepare samples for
279 mass spectrometry analysis, we only needed to concentrate 50 ml of culture supernatant
280 using a centrifugal filter and fractionated the concentrated samples by SDS-PAGE to
281 prepare samples for mass spectrometry analysis. Our approach therefore provides an easy
282 and cheap method to prepare multiple samples for investigating the SPI-2 effector repertoire
283 from other serovars of *S.* enterica. Eighteen effectors were identified in both our study and
284 that of Niemann et al [23] and a further five unique effectors were found in each study. The
285 genes of certain effectors like *steE* and *sspH1* are not present in D24359. The congruity
286 between these two studies suggests that most, if not all, the SPI-2 repertoire has been
287 identified for these two strains. However, it remains possible that some effectors might be
288 expressed and secreted in low amounts or subject to regulatory control that is absent from *in*
289 *vitro* growth conditions and still await discovery.
290
291 Our previous analysis revealed that all serovars of *S. enterica* subspecies *enterica* have a
292 set of 'core' effectors (SseF, SseG, PipB, SteA, SifA, SteD and PipB2) [7]. Here we showed
293 that the newly identified effector SseM is not only present in all serovars of *S. enterica*

subspecies *enterica* but is also present in all other four subspecies of *S. enterica* - hence we conclude that SseM is an eighth 'core' effector.

Although SseM is annotated as a 110-residue hypothetical protein in most *Salmonella* databases (sFig. 1) we showed experimentally that *sseM* encodes a protein of 103 amino acids, which is translocated by the SPI-2 injectisome and under control of the *pipB2* promoter and the two-component regulatory system SsrAB (Fig. 2B and [20]). This is supported by RNAseq data which only revealed transcription start sites before the *pipB2* gene [27], leading us to conclude that SseM and PipB2 are encoded in the same operon.

SseM, when expressed in isolation in human cells or after translocation by intracellular *Salmonella*, interacted specifically with components of DAPC signalosome [29, 30]: β-2-syntrophin, utrophin/ dystrophin, α-catulin, α-dystrobrevin and β-dystrobrevin. Of particular interest, we identified a PDZ binding motif within SseM and found that both this and the PDZ domain of β-2-syntrophin were required to mediate the interaction between SseM and components of the DAPC signalosome. Both DAPC and DLG1 are involved in several key cellular functions that include not only cell signalling from the adrenergic receptor but also regulation of the cell's cortical cytoskeleton, cell migration and formation of focal adhesions [30, 35, 36] as well both DLG1 and DAPC regulating tight junctions of polarised epithelial cells [37, 38]. We hypothesise that via its PBM, SseM interferes with one or more of these processes. To our knowledge, SseM is unique among *Salmonella* effectors in containing a functional PBM and as a bacterial protein targeting the DAPC. Several viral oncoproteins target DLG1 to regulate viral virulence [39, 40]. It therefore now essential to investigate the biochemical consequences and physiological significance of SseM's interaction with DLG1 and DAPC components.

There are several examples of bacterial effectors whose function is mediated through short linear motifs that mediate protein-protein interactions. These include three other PBM-containing effectors (Map, OspE and NleG8) characterised in enteropathogenic *Escherichia coli* [41, 42], *Shigella flexneri* [43], *Citrobacter rodentium* and enterohemorrhagic *Escherichia coli,* respectively [44], with each effector/PBM sequence required for virulence of the corresponding pathogens [41-44]. We found that the Δ*sseM* mutant strain slightly outcompeted the wt strain in the *S.* Typhimurium/ mouse systemic infection model. This modulation of *Salmonella* growth was dependent on the functional PBM of SseM, suggesting that an interaction between SseM and the DAPC acts to restrain bacterial replication during growth in infected tissues. AvrA [45] and SteC [22, 46] are two other effectors whose absence leads to a slight growth advantage of *Salmonella*. The fact that several such mutants exist points to an important aspect of bacterial virulence that remains to be understood.

Materials and Methods

Bacterial strains and growth conditions

Bacteria were grown in Luria Bertani (LB) medium supplemented with carbenicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}) or chloramphenicol (10 μg ml^{-1}), for strains resistant to these antibiotics (Ap^r, Km^r and Cm^r, respectively). To induce SPI-2 gene expression and SPI-2 dependent secretion, bacteria were grown in MgM-MES at pH 5.0 with the corresponding antibiotics when appropriate.

The λ Red recombination system [47] was used to construct the following mutants: *S.* Enteritidis strain D24359 derivatives Δ*spiC::Km* mutant and Δ*spiCssaC::Km* mutant (Primers are listed in Supplemental Table 1), *S.* Typhimurium strain 12023 derivatives Δ*sseM::Km* mutant, Δ$pipB2^{promoter}$*::Km* mutant and Δ*pipB2::Km* mutant. When necessary, pCP20 was

used to remove the antibiotic resistance cassette. *S.* Typhimurium strain 12023 derivatives *ssrA::mTn5* mutant [12] and Δ*ssaV::aphT* mutant [2] were described previously.

Plasmids

Complementing plasmids were constructed by ligating HindIII and PstI-digested plasmid p*ssaGpr* (Ap^r) [6], a pWSK29 [48] derivative containing the DNA sequence of *ssaG* promoter, with the corresponding digested PCR products: p*sseM*, p$sseM^{ACG}$, p*sseM-HA* and p$sseM^{V103A}$ by using *S.* Typhimurium 12023 genomic DNA as PCR template, and p$sseM^{SEN}$ by using *S.* Enteritidis D24359 genomic DNA as PCR template.

PciI and NotI-digested M6pblast-GFP (Ap^r) [49] was ligated with NcoI and NotI-digested PCR products (Supplemental Table 1 for primers and corresponding gene) to construct GFP-tagged effector transfection plasmids: using *S.* Typhimurium 12023 genomic DNA as PCR template for making p*gfp::spvD*, p*gfp::sseM*, p*gfp::sseM-HA*, p$gfp::sseM^{RE2A}$, p$gfp::sseM^{T101A}$ and p$gfp::sseM^{V103A}$; using *S.* Enteritidis D24359 genomic DNA as PCR template to make p$gfp: :sseM^{SEN}$.

PCR products of *sseM* or *spvD* replaced *steD* gene of pCG36 (Km^r) to make p*flag::sseM* and p*flag::spvD*, and replaced *steD* gene of pCG189 (Ap^r) to make p*mCherry::sseM* and p$mCherry::sseM^{V103A}$, respectively.

A codon-modified form of SNTB2 gene, eliminating an internal NotI digestion site was synthesised by Invitrogen, and subcloned to PciI and NotI-digested M6pblast-GFP to make plasmid p*gfp::SNTB2*. Overlapping PCR was carried out to amplify $SNTB2^{GLGI4A}$, $SNTB2^{H176Y}$ and $SNTB2^{H176V}$; PciI and NotI-digested PCR products were cloned to plasmid M6pblast-GFP to make p$gfp::SNTB2^{GLGI4A}$, p$gfp::SNTB2^{H176Y}$ and p$gfp::SNTB2^{H176V}$.

All the plasmids constructed in this study were verified by DNA sequencing.

375

376 **Preparation of secreted proteins for mass spectrometry analysis and immunoblotting**

377 Bacteria were grown overnight in 5 ml LB broth. 1 ml culture was pelleted, washed once with
378 MgM-MES at pH 5.0, and subcultured into 50 ml of MgM-MES at pH 5.0 prior to 6h
379 incubation at 37°C, 200 rpm. Bacteria were pelleted at 10,000 × *g* for 10 min at 4°C, the
380 supernatant was filtered through a ϕ0.2 μm membrane (Acrodisc Syringer Filter, 0.2 μm
381 Supor Membrane, Low protein binding, non-pyrogenic, PALL Life Science) followed by
382 concentration to approximately 200 μl on an Amicon® Ultra-15 Centrifugal Filter with
383 Ultracel-3k membrane (UFC9003, Millipore) at 4°C. 50 μl of concentrated supernatant was
384 run approximately 1 cm into a 12% SDS-PAGE separating gel. The 1cm gel slice, stained
385 with PageBlue Protein Staining Solution (Thermo Fisher Scientific) was sent for mass
386 spectrometry analysis at the Institute of Biochemistry and Biophysics (IBB) at the Polish
387 Academy of Sciences, Warsaw, Poland. Acquired spectra were compared to our annotated
388 *S.* Enteritidis D24359 sequence using the MASCOT search engine.

389

390 For pH shift analysis, the subculture was grown for 4 h at pH 5.0 and switched to MgM-MES
391 at pH 7.2 for another 1.5 h. The whole bacterial lysate and secreted fraction were prepared
392 as described previously [5] to make 10 μl of whole bacterial lysate equal to 0.1 OD_{600} of
393 culture and 10 μl of secreted fraction equal to 0.6 OD_{600} of culture.
394 Antibodies used in this study are listed in Supplemental Table 2.

395

396

397 **Bioinformatic analysis**

398 Sequencing data for *S.* Enteritidis strain D24359 has been published previously (ENA
399 accession: ERR037572); however, no genome assembly or annotation was published. To
400 this end, we have downloaded the reads and evaluated their quality using FastQC v0.11.6.
401 The reads were determined to be quality- and adapter-trimmed. Following this, short read

assembly was performed using Unicycler v0.4.5. The resulting assembly had 668 contigs and N50 of 10,609. To improve the annotation, we have applied Ragout v2.0 with 4 reference-quality Enteritidis genomes (A1636: GCF_015241115.1, CP255: GCF_015240995.1, D7795: GCF_015240855.1, and P125109: GCA_015240635.1). This resulted in a much more contiguous assembly (2 contings, N50 4,705,460) with 200 kb (~5%) of the assembly represented as N's because of the ambiguity in the syntenic blocks.

The resulting assembly was annotated using Prokka v1.12 against a custom *Salmonella* protein database that contained 234,913 unique *Salmonella* proteins annotated using RefSeq Identical Protein Groups. The produced annotation contained 4,448 putative protein-coding genes. The predicted proteins were used as a reference during the mass spectrometry analysis. The code and files necessary to reproduce the assembly and annotation are available at the repository https://github.com/apredeus/D24359.

Protein BLAST was used to search the presence of D24359_01053 in *S. bongori*, *S. enterica* subspecies *salamae*, *arizonae, houtenae*, *indica* and several common serovars of *S. enterica* subspecies *enterica*. 'Identical Proteins' in other *S. enterica* serovars were identified and the protein sequences were aligned with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

To compare the different SseM protein sequence types among *Salmonella* serovars, the complete *Salmonella* genomes were downloaded from Enterobase by searching "Complete Genome" in the "Status" field, which represents the highest assembly quality with circular chromosomes and plasmids (https://enterobase.warwick.ac.uk/, accessed on 2023/06/30). The SISTR1 result from Enterobase were used to identify the subspecies and serovars of

the genomes. Only 834 genomes that belong to *Salmonella enterica* subspecies I were included in the analysis.

The *sseM* (*stm2779*) nucleotide sequence from *Salmonella* Typhimurium LT2 (RefSeq: GCF_000006945.2) was used as a reference. A BLAST database was constructed from the *sseM* sequence. Each of the 834 *Salmonella* genomes was queried against the *sseM* database using BLASTn v2.14.0+ [50]. The aligned DNA sequences were then extracted and translated into protein sequences using Seqkit v2.4.0 [51]. The unique SseM protein sequences were summarized and aligned using Clustalo v1.2.4 [52].

To visualise the SseM types in conjunction with the genomic diversity of the *Salmonella* genomes, an MStree of the 834 complete *Salmonella* genomes was generated on Enterobase using the cgMLST scheme with the MSTree2 algorithm [53]. The tree was visualised with Grapetree [26]. In the cgMLST scheme, clusters of genomes with fewer than 900 allele differences are uniform for serovars [53]. Therefore, in Grapetree, the nodes with fewer than 900 allele differences are collapsed into bubbles to visualize the serovars.

AlphaFold Colab Multimer [54] was used to predicate the complex between SseM and the PDZ domain (R^{114} – E^{199}) of β-2-syntrophin.

Bacterial infection, translocation analysis and co-immunoprecipitation

HeLa cells and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (Sigma) at 37°C in 5% (V/V) CO_2. Infection with *S.* Typhimurium was done as described previously [15].

For translocation assays, HeLa cells were infected for 5 h, then proteasome inhibitor MG132 (Sigma) was added to a final concentration of 10 μg/ml and cells incubated for another 3 h. For immunoblotting analysis, cells were collected, washed once with cold PBS and lysed for 15 min on ice with 50 μl of 0.1%Triton X-100 in PBS. Soluble fraction (containing translocated effectors) was separated from insoluble fraction (containing bacteria and nucleus) by centrifugation at 16,000 × *g* for 10 min at 4°C. Alternatively, cells on glass coverslips were infected as above, fixed, immuno-labelled and analysed with a Zeiss 710 confocal microscope as described [15].

To immuno-precipitate SseM, HeLa cells seeded in a ϕ15 cm petri dish were infected for 14.5 h, then MG132 was added to a final concentration of 10 μg/ml and cells incubated for another 3 h. After a PBS wash, cells were resuspended into 800 μl of buffer A (50mM Tris pH 7.5, 150mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 1mM EDTA and cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail (Roche)) and lysed for 30 min on ice. The lysate was centrifuged at 16,000 × *g* for 10 min at 4°C, supernatant was incubated with 40 μl of Protein G Agarose (Pierce) on a roller at 4°C. The pre-cleaned supernatant was then incubated with 30 μl of Protein G Agarose pre-bound with 50 μl of rabbit anti-SseM antibody for 3.5 h on the roller at 4°C. The agarose was washed 4 times with buffer A and resuspended into 30 μl of 2 × protein loading buffer.

Generation of stable cell lines and SNTB2 knockout cell lines

Lipofectamine™ 2000 (Invitrogen) was used to transfect HeLa cells with plasmid. Transfected cells were selected with blasticidin to establish stable HeLa cell lines expressing GFP or GFP::SseM.

Two different guide RNAs targeting the coding sequence 871st -891st nt (GGTGTGGATAGCTACGAACC) or 1072nd -1092nd nt (TGCTCTATGACTGTATGCCG) of

SNTB2 were cloned onto pX330 [55] with annealed oligos XJY361/362 or XJY363/364 to construct plasmids p361 [KO1] or p363 [KO2] respectively. 24 h after transfection, HEK293 cells were seeded into 96-well plates at 0.3 cells per well. Single clones were screened by immunoblotting with mouse anti-Syntrophin antibody.

Immunoprecipitation from stable cell lines or transfected cells

GFP-trap agarose (Chromotek) or anti-Flag M2 affinity gel (Sigma) were used to immuno-precipitate GFP-tagged protein or Flag-tagged protein by using buffer B (5% glycerol, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), PBS) as lysis buffer and buffer C (5% glycerol, 0.1% Triton X-100, 1 mM PMSF, PBS) as washing buffer.

One ϕ10 cm dish of HeLa stable cell line expressing GFP or GFP::SseM, or one ϕ10 cm dish of HEK293 cells transiently transfected with 3 μg plasmid DNA p*flag::sseM* or p*flag::spvD* for 16 h were used for immunoprecipitation. After 4 washes with buffer C, the beads were washed twice with PBS before sending for mass spectrometry analysis. Acquired spectra were compared to database of *Homo sapiens* (Uniprot) using the MASCOT search engine.

For immunoblotting analysis, HEK293 cells seeded in one well of 6-well plate was transfected with 1.5 μg plasmid DNA or co-transfected with 0.75 μg of each plasmid for 16 h before collecting cells for GFP-trap immunoprecipitation. After 4 washes with buffer C, the beads were then resuspended into 30 ml of 2 × protein loading buffer.

Mouse ethics statement

Animal experiments were performed in accordance with ASPA and UK Home Office regulations. The project licence for animal research (P2ED1F62A) was approved by Imperial College London Animal Welfare and Ethical Review Body (ICL AWERB). Prior to

experimentation, mice were given at least one week to acclimatise to the ICL Animal Research Facility.

Mouse mixed infection

The virulence of *S.* Typhimurium strain 12023 derivative wt*::Km* strain is indistinguishable from wild-type *S.* Typhimurium strain 12023 (J. Poh and D. W. Holden, unpublished), and was used as wild -type strain for competitive index (CI) studies. Female BALB/c mice (7-8 weeks) mice were inoculated intraperitoneally with a mixture of two strains comprising 500 colony-forming units of each strain in PBS, and the CIs were determined from spleen homogenates 72h post-inoculation as described previously [34].

Single sample T-test was used to compare the log10 CI to the hypothetical value of 0 (the value of 0 means that two strains grew equally well *in vivo*). One-Way ANOVA corrected by Dunnett's multiple comparison test was used to compare the log10 CI to that of the Δ*sseM* pWSK29/ wt*::Km* pWSK29 group.

Data and materials availability

The code and files necessary to reproduce the assembly and annotation of D24359 are found here https://github.com/apredeus/D24359. All other data are available in the main text of supplementary materials. Correspondence and requests for materials should be addressed to Xiu-Jun Yu: x.yu@imperial.ac.uk and Teresa L.M Thurston: t.thurston@imperial.ac.uk.

Acknowledgments

A Biotechnology and Biological Sciences Research Council David Phillips Fellowship BB/R011834/1 and ERC starting grant funded by the Engineering and Physical Sciences

Research Council EP/X02377X/1 to TLMT and a Wellcome Trust Investigator Award, 209411/Z/17/Z to DWH funded this work.

We thank members from the Holden, Thurston and Hinton laboratories for feedback, and Dr. Camilla Godlee for providing plasmids pCG36 and pCG189.

Competing interests

The authors declare they have no competing interests.

Figure Legends

Fig.1 Identification of SPI-2 effector SseM and conservation in *Salmonella enterica* subspecies I genomes.

(A) Schematic for identification of novel *Salmonella* secreted proteins (B) Matched peptides (red fonts) of newly identified effector, D24359_01053, from one MS analysis. (C-D) GrapeTree phylogenetic visualization of SseM distribution and protein sequence variation; branch length indicates the number of allele differences between the cgMLST types, as shown by the scale bar. The nodes with fewer than 900 allele differences were collapsed into bubbles, which are consistent with serovars. The size of each bubble is proportional to the number of genomes it represents. The bubbles that correspond to *Salmonella* serovars Typhi, Typhimurium and Enteritidis are labelled. The colour of the bubbles indicates the serovars (C) or the diverse SseM protein sequence types (D).

Fig.2 Analysis of expression, secretion and translocation of SseM. (A) Genetic organisation of *pipB2-sseM* operon and regions of deletion used in (B) are indicated with ∇. The dash dotted arrow indicates the transcript of *pipB2-sseM*. (B) Expression of SseM is controlled by the *pipB2* promoter and SsrAB. Bacterial strains were grown in MgM-MES at pH 5.0 for 6h. Bacterial lysates were analysed by immunoblotting. Intrabacterial protein DnaK and SPI-2 translocon protein SseB were used as controls. (C) Indicated bacterial lysates, including strains expressing the mutation of the predicated start codon ATG to ACG (p*sseM*^{ACG}) were analysed by immunoblotting. (D) Secretion of SseM upon pH shift. Bacterial strains were grown at pH 5.0 for 4 h, then switched pH to 7.2 for another 1.5 h before preparing samples for immunoblotting. (E) Translocation analysis by immunoblotting. HeLa cells were infected for 5 h, then proteasome inhibitor MG132 was added for another 3h before fractionation with Triton-X 100. Triton-X 100 soluble fraction (PNS) contains translocated proteins. (F) Translocation analysis by confocal microscopy. HeLa cells were infected with bacteria expressing SseM-HA for 5 h, then proteasome inhibitor MG132 was added for another 3 h before fixation. Fixed cells were labelled with antibodies to visualize *Salmonella* (red) and SseM-HA (green). Scale bar: 5 μm.

Fig.3 SseM interacts with β-2-syntrophin and α-dystrobrevin. (A) GFP-tagged protein was expressed in HEK293 cells, immunoprecipitated with GFP-trap agarose and analysed by immunoblotting. GFP::SpvD was used as negative control. (B) HeLa cells were infected with *Salmonella* for 14.5 h, then proteasome inhibitor MG132 was added for another 3 h. Cell lysates were immunoprecipitated with rabbit anti-SseM antibody before immunoblotting. (C) Alignment of SseM and C-terminus of α_{1D}-AR. Bold fonts indicate the conserved PDZ binding motif (PBM). (D) Ectopically expressed GFP-tagged SseM variants in HEK293 cells were immunoprecipitated with GFP-trap agarose and analysed by immunoblotting. (E) WT or β-2-syntrophin (SNTB2) knockout HEK293 cells (KO1 and KO2) were transfected with GFP::SseM and protein-protein interactions analysed in cell lysates by immuno-precipitation

585 and immunoblotting. GFP::SseM^{V103A} was included as an additional control. “m” indicates
586 protein marker. (F) Model of complex between SseM PBM (red) and PDZ domain of β-2-
587 syntrophin (green and grey) derived from AlphaFold Colab Multimer. α-helix B (αB) and β-
588 strand B (βB) of β-2-syntrophin PDZ domain are coloured in grey with key amino acids
589 annotated. (G) PDZ domain of β-2-syntrophin is required to interact with SseM.
590 mCherry::SseM was co-expressed with indicated GFP-tagged β-2-syntrophin (GFP::SNTB2)
591 variant in SNTB2 KO1 HEK293 cells, and subject to protein-protein interaction analysis.
592 mCherry::SseM^{V103A} was used as control.
593
594 **Fig.4 The PBM of SseM is required to downregulate *Salmonella* virulence *in vivo*.**
595 BALB/c mice were inoculated by intraperitoneal injection with equal numbers (500 cfu of
596 each of the two strains) of the indicated bacteria. Bacteria were recovered from infected
597 spleens 72 h post-inoculation, and CI values were calculated. The log10 CIs were used for
598 statistical analysis: single sample T-test was used to compare the log10 CI to the
599 hypothetical value of 0 and p value is indicated in the round bracket, one-way ANOVA
600 followed by Dunnett’s multiple comparison test was used to compare with the Δ*sseM*
601 pWSK29/*wt::Km* pWSK29 group (ns: not significant; **: p <0.01).
602
603 **sFig.1 Alignment of SseM from different subspecies and different serovars of**
604 ***Salmonella enterica*.** (A) Alignment of D24359_01053 and STM2779. Identities:
605 95/103=92%; positives: 97/103=94%. (B) Alignment of SseM protein sequences. Accession
606 numbers for non-*enterica* subspecies are highlighted in grey. EAW1721535.1: *S. enterica*
607 subsp. *indica*; WP_080161159.1: *S. enterica* subsp. *arizonae*; WP_072157437.1: *S. enterica*
608 subsp. *salamae*; WP_071651510.1: *S. enterica* subsp. *houtenae*; WP_011233152.1: serovar
609 Paratyphi A; WP_077905756.1: serovars Agona, Indiana, Goldcoast, Brancaster,
610 Senftenberg; WP_023166795.1: serovars Kentucky, Senftenberg and Gallinarum;
611 WP_077909235.1: serovars Muenchen, Litchfield, Manhattan; WP_077910820.1: serovars

612 Paratyphi B, Java; WP_077463764.1: serovars Heidelberg, Newport, Infantis, Hadar etc.
613 EDQ7330352.1: serovar Paratyphi C; HCK6744786.1: serovars Anatum, Eko, Typhi;
614 WP_014343883.1: serovars Typhimurium, Saintpaul, Paratyphi B, Berta, Bareilly;
615 WP_077945811.1: serovars Brunei, Newport; WP_016701746.1: serovar Enteritidis.
616
617 **sFig.2 Alignment of SseM variants**
618 Sequence alignment of SseM variants from Fig. 1D.
619
620 **sFig.3 Alignment of *stm2779*, *sseM_2_pseudo* and *sseM_6_pseudo*.** The extra 'C'
621 (underlined bold font) in *sseM_2_pseudo* results in a stop codon (red fonts) after the
622 predicated 30th amino acid. The mutation of the predicated 84th codon TGG to TAG (red font)
623 in *sseM_6_pseudo* results in a truncated protein missing the last 28 residues of SseM.
624
625 **sFig.4 Alignment of *stm2779* and *D24359_01053*.** Green fonts indicate the predicated
626 start codon (TTG) and actual start codon (ATG) of *stm2779*. Plasmid p*sseM*^{ACG} was
627 constructed by changing the ATG (green fonts) to ACG (red fonts) on p*sseM*.
628
629 **sFig.5 Predicted secondary structural features of β-2-syntrophin PDZ domain with**
630 **AlphFold Colab Multimer.** The secondary structure of PDZ domain (italic fonts) are
631 indicated. Mutated residues of PDZ domain of β-2-syntrophin used in Fig. 3F are highlighted
632 in green.
633

References

1. Cirillo, D.M., et al., *Macrophage-dependent induction of the Salmonella pathogenicity island 2 type III secretion system and its role in intracellular survival.* Mol Microbiol, 1998. **30**(1): p. 175-88.
2. Beuzon, C.R., et al., *pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of Salmonella typhimurium.* Mol Microbiol, 1999. **33**(4): p. 806-16.
3. Deiwick, J., et al., *Environmental regulation of Salmonella pathogenicity island 2 gene expression.* Mol Microbiol, 1999. **31**(6): p. 1759-73.
4. Yu, X.J., M. Liu, and D.W. Holden, *SsaM and SpiC interact and regulate secretion of Salmonella pathogenicity island 2 type III secretion system effectors and translocators.* Mol Microbiol, 2004. **54**(3): p. 604-19.
5. Yu, X.J., et al., *pH sensing by intracellular Salmonella induces effector translocation.* Science, 2010. **328**(5981): p. 1040-3.
6. Yu, X.J., et al., *SsaV Interacts with SsaL to Control the Translocon-to-Effector Switch in the Salmonella SPI-2 Type Three Secretion System.* mBio, 2018. **9**(5).
7. Jennings, E., T.L.M. Thurston, and D.W. Holden, *Salmonella SPI-2 Type III Secretion System Effectors: Molecular Mechanisms And Physiological Consequences.* Cell Host Microbe, 2017. **22**(2): p. 217-231.
8. Pillay, T.D., et al., *Speaking the host language: how Salmonella effector proteins manipulate the host.* Microbiology (Reading), 2023. **169**(6).
9. Hensel, M., et al., *Simultaneous identification of bacterial virulence genes by negative selection.* Science, 1995. **269**(5222): p. 400-3.
10. Ochman, H., et al., *Identification of a pathogenicity island required for Salmonella survival in host cells.* Proc Natl Acad Sci U S A, 1996. **93**(15): p. 7800-4.

659 11. Fong, W.Y., et al., *Genome-wide fitness analysis identifies genes required for in vitro*
660 *growth and macrophage infection by African and global epidemic pathovariants of*
661 *Salmonella enterica Enteritidis.* Microb Genom, 2023. **9**(5).
662 12. Shea, J.E., et al., *Identification of a virulence locus encoding a second type III*
663 *secretion system in Salmonella typhimurium.* Proc Natl Acad Sci U S A, 1996. **93**(6):
664 p. 2593-7.
665 13. Miao, E.A., et al., *Salmonella typhimurium leucine-rich repeat proteins are targeted*
666 *to the SPI1 and SPI2 type III secretion systems.* Mol Microbiol, 1999. **34**(4): p. 850-
667 64.
668 14. Miao, E.A. and S.I. Miller, *A conserved amino acid sequence directing intracellular*
669 *type III secretion by Salmonella typhimurium.* Proc Natl Acad Sci U S A, 2000.
670 **97**(13): p. 7539-44.
671 15. Beuzon, C.R., et al., *Salmonella maintains the integrity of its intracellular vacuole*
672 *through the action of SifA.* EMBO J, 2000. **19**(13): p. 3235-49.
673 16. Brumell, J.H., et al., *SopD2 is a novel type III secreted effector of Salmonella*
674 *typhimurium that targets late endocytic compartments upon delivery into host cells.*
675 Traffic, 2003. **4**(1): p. 36-48.
676 17. Knodler, L.A., et al., *Salmonella type III effectors PipB and PipB2 are targeted to*
677 *detergent-resistant microdomains on internal host cell membranes.* Mol Microbiol,
678 2003. **49**(3): p. 685-704.
679 18. Kujat Choy, S.L., et al., *SseK1 and SseK2 are novel translocated proteins of*
680 *Salmonella enterica serovar typhimurium.* Infect Immun, 2004. **72**(9): p. 5115-25.
681 19. Worley, M.J., K.H. Ching, and F. Heffron, *Salmonella SsrB activates a global*
682 *regulon of horizontally acquired genes.* Mol Microbiol, 2000. **36**(3): p. 749-61.

20. Rytkonen, A., et al., *SseL, a Salmonella deubiquitinase required for macrophage killing and virulence.* Proc Natl Acad Sci U S A, 2007. **104**(9): p. 3502-7.

21. Coombes, B.K., et al., *SseL is a salmonella-specific translocated effector integrated into the SsrB-controlled salmonella pathogenicity island 2 type III secretion system.* Infect Immun, 2007. **75**(2): p. 574-80.

22. Geddes, K., et al., *Identification of new secreted effectors in Salmonella enterica serovar Typhimurium.* Infect Immun, 2005. **73**(10): p. 6260-71.

23. Niemann, G.S., et al., *Discovery of novel secreted virulence factors from Salmonella enterica serovar Typhimurium by proteomic analysis of culture supernatants.* Infect Immun, 2011. **79**(1): p. 33-43.

24. Auweter, S.D., et al., *Quantitative mass spectrometry catalogues Salmonella pathogenicity island-2 effectors and identifies their cognate host binding partners.* J Biol Chem, 2011. **286**(27): p. 24023-35.

25. Lanzilao, L., et al., *Strain Selection for Generation of O-Antigen-Based Glycoconjugate Vaccines against Invasive Nontyphoidal Salmonella Disease.* PLoS One, 2015. **10**(10): p. e0139847.

26. Zhou, Z., et al., *GrapeTree: visualization of core genomic relationships among 100,000 bacterial pathogens.* Genome Res, 2018. **28**(9): p. 1395-1404.

27. Kroger, C., et al., *An infection-relevant transcriptomic compendium for Salmonella enterica Serovar Typhimurium.* Cell Host Microbe, 2013. **14**(6): p. 683-95.

28. Colgan, A.M., et al., *The Impact of 18 Ancestral and Horizontally-Acquired Regulatory Proteins upon the Transcriptome and sRNA Landscape of Salmonella enterica serovar Typhimurium.* PLoS Genet, 2016. **12**(8): p. e1006258.

29. Lyssand, J.S., et al., *Blood pressure is regulated by an alpha1D-adrenergic receptor/dystrophin signalosome.* J Biol Chem, 2008. **283**(27): p. 18792-800.

30. Lyssand, J.S., et al., *Alpha-dystrobrevin-1 recruits alpha-catulin to the alpha1D-adrenergic receptor/dystrophin-associated protein complex signalosome.* Proc Natl Acad Sci U S A, 2010. **107**(50): p. 21854-9.

31. Chen, Z., et al., *Syntrophins regulate alpha1D-adrenergic receptors through a PDZ domain-mediated interaction.* J Biol Chem, 2006. **281**(18): p. 12414-20.

32. Tochio, H., et al., *Solution structure of the extended neuronal nitric oxide synthase PDZ domain complexed with an associated peptide.* Nat Struct Biol, 1999. **6**(5): p. 417-21.

33. Doyle, D.A., et al., *Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ.* Cell, 1996. **85**(7): p. 1067-76.

34. Beuzon, C.R. and D.W. Holden, *Use of mixed infections with Salmonella strains to study virulence genes and their interactions in vivo.* Microbes Infect, 2001. **3**(14-15): p. 1345-52.

35. Constantin, B., *Dystrophin complex functions as a scaffold for signalling proteins.* Biochim Biophys Acta, 2014. **1838**(2): p. 635-42.

36. Bhat, S.S., R. Ali, and F.A. Khanday, *Syntrophins entangled in cytoskeletal meshwork: Helping to hold it all together.* Cell Prolif, 2019. **52**(2): p. e12562.

37. Wu, H., et al., *Subcellular targeting and cytoskeletal attachment of SAP97 to the epithelial lateral membrane.* J Cell Sci, 1998. **111 (Pt 16)**: p. 2365-76.

38. Kachinsky, A.M., S.C. Froehner, and S.L. Milgram, *A PDZ-containing scaffold related to the dystrophin complex at the basolateral membrane of epithelial cells.* J Cell Biol, 1999. **145**(2): p. 391-402.

731 39. Lee, S.S., R.S. Weiss, and R.T. Javier, *Binding of human virus oncoproteins to*
732 *hDlg/SAP97, a mammalian homolog of the Drosophila discs large tumor suppressor*
733 *protein.* Proc Natl Acad Sci U S A, 1997. **94**(13): p. 6670-5.
734 40. Javorsky, A., P.O. Humbert, and M. Kvansakul, *Viral manipulation of cell polarity*
735 *signalling.* Biochim Biophys Acta Mol Cell Res, 2023. **1870**(7): p. 119536.
736 41. Simpson, N., et al., *The enteropathogenic Escherichia coli type III secretion system*
737 *effector Map binds EBP50/NHERF1: implication for cell signalling and diarrhoea.*
738 Mol Microbiol, 2006. **60**(2): p. 349-63.
739 42. Alto, N.M., et al., *Identification of a bacterial type III effector family with G protein*
740 *mimicry functions.* Cell, 2006. **124**(1): p. 133-45.
741 43. Yi, C.R., et al., *Systematic analysis of bacterial effector-postsynaptic density 95/disc*
742 *large/zonula occludens-1 (PDZ) domain interactions demonstrates Shigella OspE*
743 *protein promotes protein kinase C activation via PDLIM proteins.* J Biol Chem, 2014.
744 **289**(43): p. 30101-13.
745 44. Popov, G., et al., *Distinct Molecular Features of NleG Type 3 Secreted Effectors*
746 *Allow for Different Roles during Citrobacter rodentium Infection in Mice.* Infect
747 Immun, 2023. **91**(1): p. e0050522.
748 45. Hardt, W.D. and J.E. Galan, *A secreted Salmonella protein with homology to an*
749 *avirulence determinant of plant pathogenic bacteria.* Proc Natl Acad Sci U S A, 1997.
750 **94**(18): p. 9887-92.
751 46. Poh, J., et al., *SteC is a Salmonella kinase required for SPI-2-dependent F-actin*
752 *remodelling.* Cell Microbiol, 2008. **10**(1): p. 20-30.
753 47. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in*
754 *Escherichia coli K-12 using PCR products.* Proc Natl Acad Sci U S A, 2000. **97**(12):
755 p. 6640-5.

48. Wang, R.F. and S.R. Kushner, *Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in Escherichia coli.* Gene, 1991. **100**: p. 195-9.

49. Randow, F. and J.E. Sale, *Retroviral transduction of DT40.* Subcell Biochem, 2006. **40**: p. 383-6.

50. Camacho, C., et al., *BLAST+: architecture and applications.* BMC bioinformatics, 2009. **10**: p. 1-9.

51. Shen, W., et al., *SeqKit: A Cross-Platform and Ultrafast Toolkit for FASTA/Q File Manipulation.* PLOS ONE, 2016. **11**(10): p. e0163962.

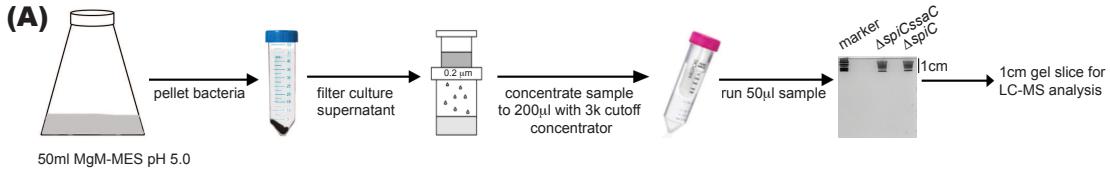
52. Sievers, F., et al., *Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega.* Mol Syst Biol, 2011. **7**: p. 539.

53. Achtman, M., et al., *EnteroBase: hierarchical clustering of 100 000s of bacterial genomes into species/subspecies and populations.* Philos Trans R Soc Lond B Biol Sci, 2022. **377**(1861): p. 20210240.

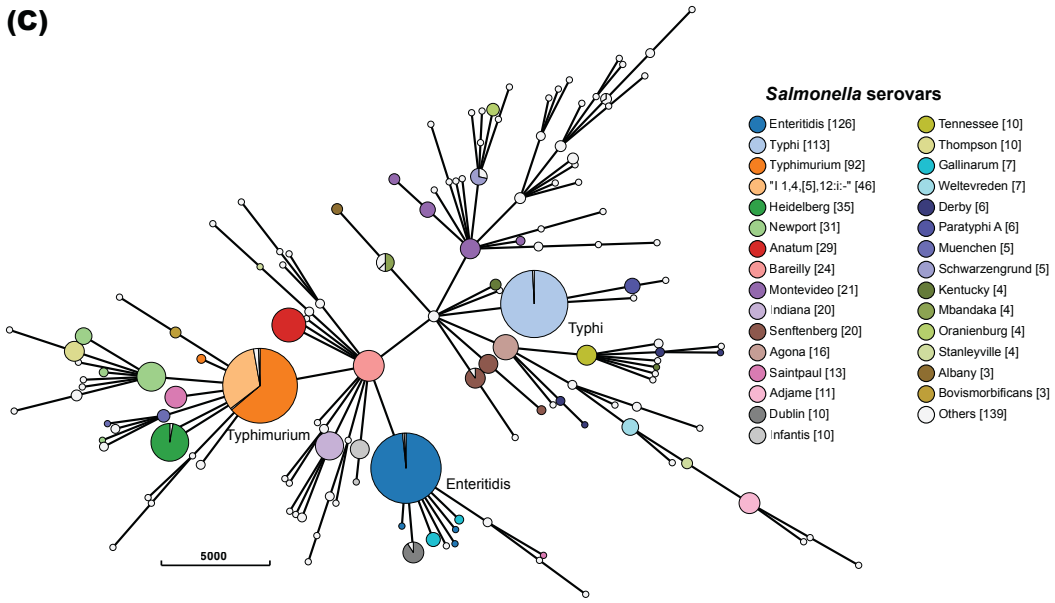
54. Jumper, J., et al., *Highly accurate protein structure prediction with AlphaFold.* Nature, 2021. **596**(7873): p. 583-589.

55. Cong, L., et al., *Multiplex genome engineering using CRISPR/Cas systems.* Science, 2013. **339**(6121): p. 819-23.

Figures and Figure Legends for Yu et al.,



(B) MPGCISACSCVVSSTPTDPKAPPKPKWIELKGYCVTCTSQEDIRPPKEGEPKQKMTPKDLVLLRGLLKDLGVTQNPWQDPTVKSTNCGLSFFPVDAGERETDV



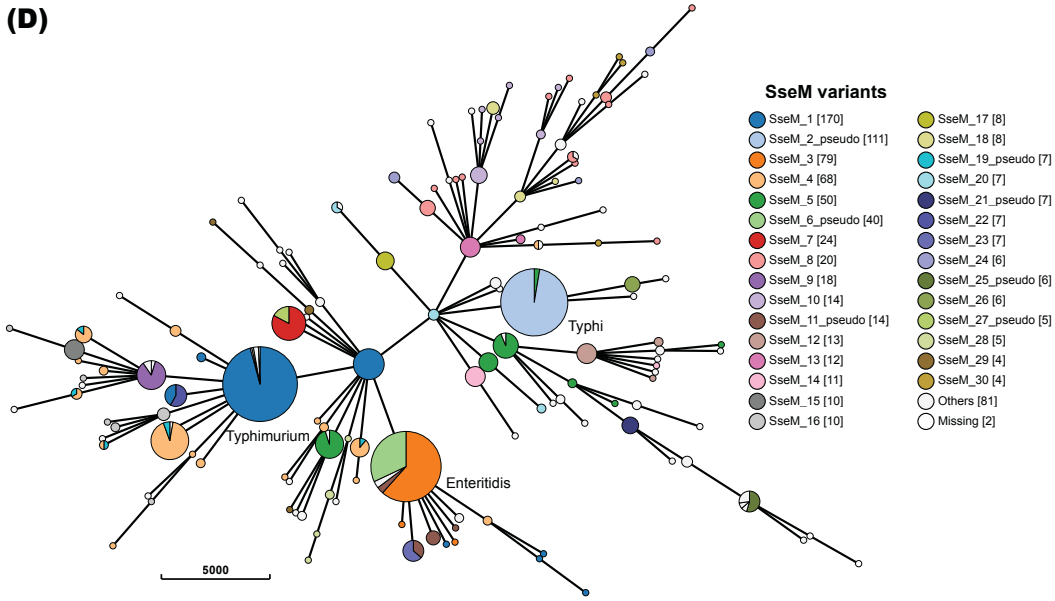


Fig.1 Identification of SPI-2 effector SseM and conservation in *Salmonella enterica* subspecies I genomes.

(A) Schematic for identification of novel *Salmonella* secreted proteins (B) Matched peptides (red fonts) of newly identified effector, D24359_01053, from one MS analysis. (C-D) GrapeTree phylogenetic visualization of SseM distribution and protein sequence variation; branch length indicates the number of allele differences between the cgMLST types, as shown by the scale bar. The nodes with fewer than 900 allele differences were collapsed into bubbles, which are consistent with serovars. The size of each bubble is proportional to the number of genomes it represents. The bubbles that correspond to *Salmonella* serovars Typhi, Typhimurium and Enteritidis are labelled. The colour of the bubbles indicates the serovars (C) or the diverse SseM protein sequence types (D).

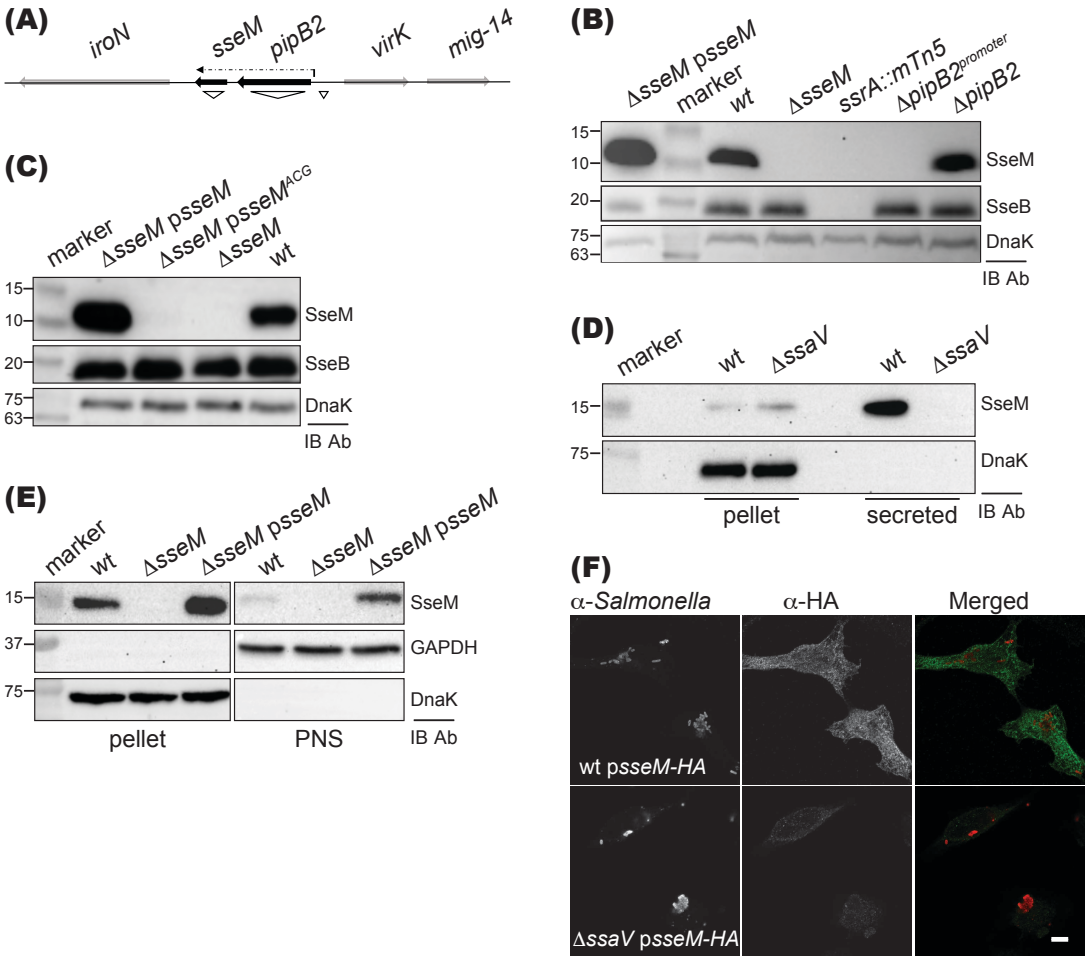


Fig.2 Analysis of expression, secretion and translocation of SseM. (A) Genetic organisation of *pipB2-sseM* operon and regions of deletion used in (B) are indicated with ∇. The dash dotted arrow indicates the transcript of *pipB2-sseM*. (B) Expression of SseM is controlled by the *pipB2* promoter and SsrAB. Bacterial strains were grown in MgM-MES at pH 5.0 for 6h. Bacterial lysates were analysed by immunoblotting. Intrabacterial protein DnaK and SPI-2 translocon protein SseB were used as controls. (C) Indicated bacterial lysates, including strains expressing the mutation of the predicated start codon ATG to ACG (p*sseM*^{ACG}) were analysed by immunoblotting. (D) Secretion of SseM upon pH shift. Bacterial strains were grown at pH 5.0 for 4 h, then switched pH to 7.2 for another 1.5 h before preparing samples for immunoblotting. (E) Translocation analysis by immunoblotting. HeLa cells were infected for 5 h, then proteasome inhibitor MG132 was added for another 3h before fractionation with Triton-X 100. Triton-X 100 soluble fraction (PNS) contains translocated proteins. (F)

Translocation analysis by confocal microscopy. HeLa cells were infected with bacteria expressing SseM-HA for 5 h, then proteasome inhibitor MG132 was added for another 3 h before fixation. Fixed cells were labelled with antibodies to visualize *Salmonella* (red) and SseM-HA (green). Scale bar: 5 μm.

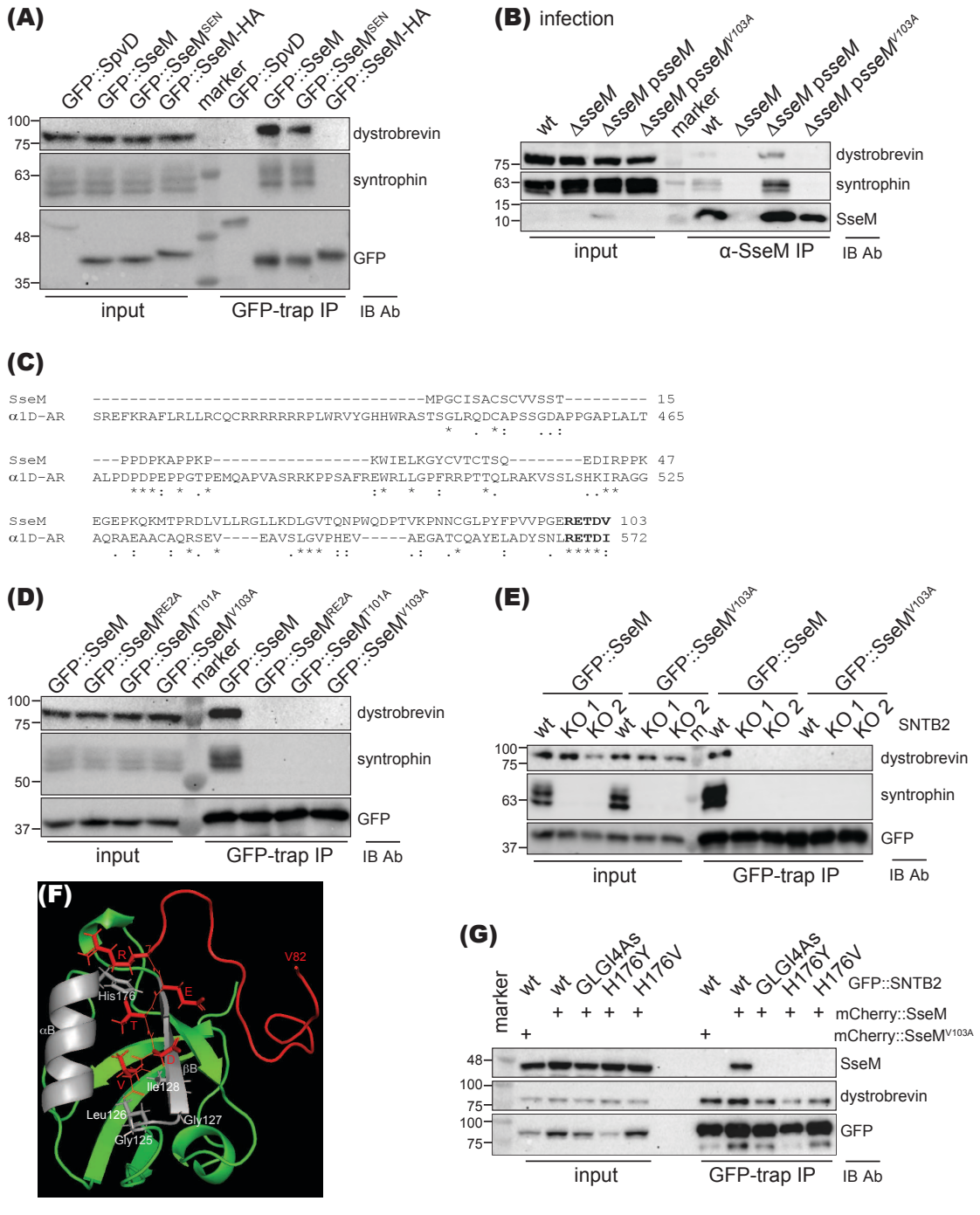


Fig.3 SseM interacts with β-2-syntrophin and α-dystrobrevin. (A) GFP-tagged protein was expressed in HEK293 cells, immunoprecipitated with GFP-trap agarose and analysed by immunoblotting. GFP::SpvD was used as negative control. (B) HeLa cells were infected with *Salmonella* for 14.5 h, then proteasome inhibitor MG132 was added for another 3 h. Cell lysates were immunoprecipitated with rabbit anti-SseM antibody before immunoblotting. (C) Alignment of SseM and C-terminus of α_{1D}-AR. Bold fonts indicate the conserved PDZ binding

41 motif (PBM). (D) Ectopically expressed GFP-tagged SseM variants in HEK293 cells were
42 immunoprecipitated with GFP-trap agarose and analysed by immunoblotting. (E) WT or β-2-
43 syntrophin (SNTB2) knockout HEK293 cells (KO1 and KO2) were transfected with GFP::SseM
44 and protein-protein interactions analysed in cell lysates by immuno-precipitation and
45 immunoblotting. GFP::SseM^{V103A} was included as an additional control. “m” indicates protein
46 marker. (F) Model of complex between SseM PBM (red) and PDZ domain of β-2-syntrophin
47 (green and grey) derived from AlphaFold Colab Multimer. α-helix B (αB) and β-strand B (βB)
48 of β-2-syntrophin PDZ domain are coloured in grey with key amino acids annotated. (G) PDZ
49 domain of β-2-syntrophin is required to interact with SseM. mCherry::SseM was co-expressed
50 with indicated GFP-tagged β-2-syntrophin (GFP::SNTB2) variant in SNTB2 KO1 HEK293 cells,
51 and subject to protein-protein interaction analysis. mCherry::SseM^{V103A} was used as control.
52

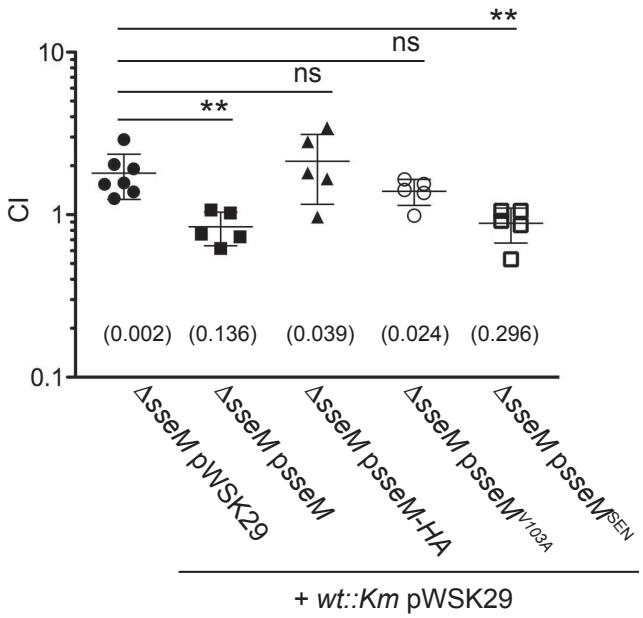


Fig.4 The PBM of SseM is required to downregulate *Salmonella* virulence *in vivo*. BALB/c mice were inoculated by intraperitoneal injection with equal numbers (500 cfu of each of the two strains) of the indicated bacteria. Bacteria were recovered from infected spleens 72 h post-inoculation, and CI values were calculated. The log10 CIs were used for statistical analysis: single sample T-test was used to compare the log10 CI to the hypothetical value of 0 and p value is indicated in the round bracket, one-way ANOVA followed by Dunnett's multiple comparison test was used to compare with the Δ*sseM* pWSK29/*wt::Km* pWSK29 group (ns: not significant; **: p <0.01).

Table 1 Identified secreted proteins with a ratio of ion scores (Δ*spiC*/Δ*spiCssaC*) >2

Protein name	Accession	Ion score (number of significant distinct peptides)					
		Exp. 1			Exp. 2		
		Δ*spiC*	Δ*spiCssaC*	ratio	Δ*spiC*	Δ*spiCssaC*	ratio
SseJ	D24359_02298	4402(20)	0	∞	1913(10)	0	∞
SseK1	D24359_04168	1783(26)	0	∞	376(10)	0	∞
SspH2	D24359_01462	1361(18)	0	∞	317(4)	0	∞
SseK3	D24359_01787	1352(14)	0	∞	172 (3)	0	∞
SifB	D24359_02265	1077(15)	0	∞	63(1)	0	∞
SsaI[a]	D24359_02075	511(4)	0	∞	0	0	
SteD	D24359_01555	408(2)	0	∞	173(2)	0	∞
SopF	D24359_01900	357(8)	0	∞	0	0	
SseF	D24359_02071	315(3)	0	∞	0	0	
SpvB	D24359_04489	308(3)	0	∞	0	0	
SseI	D24359_02786	280(5)	0	∞	35(1)	0	∞
SseG	D24359_02072	107(2)	0	∞	50(1)	0	∞
SteC	D24359_02367	7493(41)	183(2)	40.9	2598(18)	0	∞
SpvD	D24359_04491	1606(16)	46(1)	34.9	375(4)	0	∞
SifA	D24359_01885	1543(15)	63(1)	24.5	305(5)	0	∞
SpvC	D24359_04490	2320(12)	139(2)	16.7	217(3)	0	∞
AvrA	D24359_00969	1727(16)	119(2)	14.5	786(6)	39(1)	20.2
SlrP	D24359_02997	5843(41)	482(10)	12.1	2515(25)	57(1)	44.1
SopD2	D24359_02858	3510(20)	318(6)	11.0	1530(11)	112(2)	13.7
SseL	D24359_01422	6946(22)	902(11)	7.7	2395(15)	669(7)	3.6
SseM	D24359_01053	1050(7)	150(3)	7.0	1788(7)	0	∞
PipB2	D24359_01052	8069(19)	1287(11)	6.3	3078(15)	107(2)	28.8
SteA	D24359_02246	4594(19)	1023(13)	4.5	3057(16)	713(7)	4.3
PipB	D24359_02748	1152(13)	358(6)	3.2	819(9)	0	∞
SlyB[b]	D24359_02111	746(5)	271(3)	2.8	794(1)	154(1)	5.2
GtgE	D24359_02782	1433(11)	624(7)	2.3	507(4)	177(3)	2.9

TIGR00156 family protein[c]	D24359_00650	306(3)	138(2)	2.2	0	0

Note: the data shown are based on the following criteria: (1) If the identified protein is detected in both experiments, both (Δ*spiC*/Δ*spiCssaC*) ratios must be >2, ion score >100 and at least 2 peptides detected in one of the experiments. (2) If the protein is detected in only one experiment the ratio must be >2, ion score >250 and at least 2 peptides detected.

[a] SsaI is a *Salmonella* SPI-2 injectisome rod protein

[b] SlyB is an outer membrane lipoprotein

[c] D24359_00650: YgiW/YdeI family stress tolerance OB fold protein

Table 2 LC-MS/MS analysis of SseM interacting proteins

Protein	Gene	Accession	Ion score (number of significant distinct peptides)					
			Exp. 1		Exp. 2		Exp. 3	
			GFP::SseM	GFP	GFP::SseM	GFP	Flag::SseM	Flag::SpvD
β-2-syntrophin	SNTB2	Q13425	4530(19)	0	1945(18)	0	2086(15)	0
α-1-syntrophin	SNTA1	Q13424	226(3)	0	0	0	244(2)	0
β-1-syntrophin	SNTB1	Q13884	0	0	0	0	383(5)	0
utrophin	UTRN	P46939	2605(36)	0	673(15)	0	6944(71)	0
dystrophin	DMD	P11532	1301(12)	0	904(10)	0	330(8)	0
α-catulin	CTNNAL1	Q9UBT7	1702(14)	0	862(12)	0	430(6)	0
α-dystrobrevin	DTNA	Q9Y4J8	1140(16)	0	582(11)	0	871(11)	0
β-dystrobrevin	DTNB	O60941	906(14)	0	484(10)	0	1241(11)	0
Disks large homolog 1	DLG1	Q12959	1077(12)	0	585(8)	0	540(10)	0
peripheral plasma membrane protein CASK	CASK	O14936	593(9)	0	204(3)	0	44(2)	0
protein lin-7 homolog C	LIN7C	Q9NUP9	404(4)	0	71(1)	0	211(4)	0
45 kDa calcium-binding protein	SDF4	Q9BRK5	302(2)	0	136(1)	0	0	0
26S proteasome non-ATPase regulatory subunit 3	PSMD3	O43242	188(4)	0	205(2)	0	59(2)	0
heat shock cognate 71 kDa protein	HSPA8	P11142	4998(30)	1442(17)	4860(31)	1135(15)	2680(19)	881(12)

endoplasmic reticulum chaperone BiP	HSPA5	P11021	2341(23)	550(12)	2564(24)	413(7)	762(7)	235(6)
heat shock 70kDa protein	HSPA1A	P0DMV8	1462(14)	379(5)	1776(13)	314(4)	0	0
ADP/ATP translocase 2	SLC25A5	P05141	861(10)	0	0	0	0	0
Tubulin β chain	TUBB	Q5JP53	2052(17)	1988(14)	2248(16)	1618(13)	1761(13)	85(4)
Tubulin β-4B chain	TUBB4B	P68371	2172(16)	2088(12)	1898(15)	1453(13)	1428(12)	71(3)
Tubulin β-2B chain	TUBB2B	Q9BVA1	0	0	0	0	1197(11)	0
Tubulin α-1A chain	TUBA1A	Q71U36	0	0	0	0	1672(12)	0

Proteins containing PDZ domain(s) are underlined.