pH Sensing by Intracellular *Salmonella* Induces Effector Translocation

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One sentence summary: A physiological signal triggering intracellular *Salmonella* virulence protein delivery across the vacuolar membrane is shown to be cytosolic pH, which causes disassembly of a regulatory complex.

Salmonella enterica **is an important intracellular bacterial pathogen of humans and animals. It replicates within host cell vacuoles by delivering virulence (effector) proteins through a vacuolar membrane pore made by the SPI-2 type III secretion system (T3SS). T3SS assembly follows vacuole acidification, but when bacteria are grown at low pH effector secretion is negligible. Here we found that effector secretion was activated at low pH from mutant strains lacking a complex of SPI-2-encoded proteins SsaM, SpiC and SsaL. Exposure of wild-type bacteria to pH 7.2 after growth at pH 5.0 caused dissociation and degradation of SsaM/SpiC/SsaL complexes and effector secretion. In infected cells, loss of the pH 7.2 signal by acidification of host cell cytosol prevented complex degradation and effector translocation. Thus, intravacuolar** ***Salmonella*** **senses host cytosolic pH, resulting in degradation of regulatory complex proteins and effector translocation.**

Type III secretion systems (T3SSs) are assembled in the cell envelope of many Gram-negative bacterial pathogens. They secrete three classes of proteins: subunits of a surface-exposed needle-like structure; translocon proteins, which form a pore in host membranes; and effectors, which pass through the needle channel and translocon pore into the host cell, where they manipulate cellular processes and promote bacterial virulence (*1*). Translocon pore assembly must precede effector translocation and it is thought that upon assembly of the pore, bacteria sense host cell signals to activate effector secretion. The physiological signals and mechanisms underlying transition from translocon to effector secretion are not understood.

Assembly of the *Salmonella* pathogenicity island 2 (SPI-2) T3SS in vivo occurs following acidification of *Salmonella*-containing vacuoles (SCVs) to ~ pH 5.0 (*2, 3;* Fig. 1A). Bacteria grown at pH 5.0 in vitro secrete translocon proteins but negligible levels of effectors (*4*). SPI-2-encoded SsaL is a member of the YopN/InvE/MxiC/SepL family of regulatory proteins found throughout T3SSs of animal pathogens (*5*) and is required for secretion of the translocon proteins SseB and SseD (*6*). To study regulation of the translocon to effector switch, we examined protein secretion from an *S. enterica* serovar Typhimurium (*S*. Typhimurium) *ssaL* mutant after growth at pH 5.0 in vitro. This strain displayed enhanced secretion of chromosome-expressed double haemagglutinin (2HA)-tagged SPI-2 T3SS effector SseJ (Fig. 1B), and chromosome or plasmid-expressed SseF (fig. S1) and did not secrete translocon proteins SseB or SseC (Fig. 1, B and C). The secretion pattern for SseJ-2HA and translocon proteins was restored to wild-type by introduction of a plasmid expressing SsaL-2HA (Fig. 1C). There was no detectable secretion of SseB or effectors by an *ssaV* mutant strain, which has a non-functional SPI-2 T3SS (Fig. 1B). The *ssaL* mutant phenotype is very similar to that of strains lacking SPI-2-encoded SpiC or SsaM (*4*) (Fig. 1B). Thus SsaL, SsaM and SpiC are all required for secretion of translocon proteins, and suppress the secretion of effectors under conditions that simulate the vacuolar environment (Fig. 1A).

SpiC and SsaM form a complex (*4*). To determine if SsaL interacts with the SpiC/SsaM complex when produced at physiological levels, we constructed a bacterial strain

in which chromosomal copies of *spiC*, *ssaM* and *ssaL* were replaced with versions expressing epitope-tagged proteins (SpiC-2HA, T7-SsaM and SsaL-3Flag). This strain (wt-3tag) was indistinguishable from the wild-type strain in terms of intracellular replication and SPI-2 T3SS effector-dependent tubule formation in infected cells, indicating a functional T3SS (fig. S2). The wt-3tag strain and an isogenic mutant lacking endogenous or epitope-tagged SpiC (*spiC)* were grown at pH 5.0 and whole cell lysates were immuno-precipitated with an anti-T7 antibody. SsaL-3Flag was co-precipitated from the wt-3tag strain but not from the *spiC* mutant (Fig. 1D). Co-immune precipitation and pull-down experiments also showed that the C-terminal 18 amino acids of SsaM were required for interaction with SpiC (*4*) and SsaL (Fig. 2A). Thus, SsaL, SsaM and SpiC form a complex when bacteria are grown at pH 5.0 (Fig. 1A).

To determine if an intact complex is necessary to suppress effector secretion at pH 5.0, we constructed three small deletion mutants in an N-terminal region of SsaL corresponding to the chaperone-binding domain of YopN (fig. S3), necessary for its interaction with SycN and YscB (*7*). These mutants, and a truncated SsaM protein lacking its 18 C-terminal amino acids, all prevented formation of the ternary complex (Fig. 2, A and B) and caused the same phenotype as individual null mutants: no detectable translocon secretion and greatly enhanced effector secretion at pH 5.0 (Fig. 2, C and D). Thus, an intact ternary complex is necessary to promote translocon secretion and to suppress effector secretion at pH 5.0.

The *ssaM*, *spiC* and *ssaL* mutant phenotypes raised the possibility that their corresponding proteins might regulate the secretion switch in infected cells, once the translocon pore has been assembled. The translocon spans the membrane separating the vacuole lumen (pH ~5.0) (*8*) from the cytosol (pH 7.2). We tested whether a shift in ambient pH from 5.0 to 7.2 enhanced SPI-2 effector secretion by wild-type bacteria in vitro. Bacterial strains expressing either SseJ-2HA or SseF-2HA were grown at pH 5.0 for 4 h to activate the SPI-2 T3SS, and then exposed to pH 7.2 or 5.0. Secretion of these and other effectors was enhanced by an order of magnitude when wild-type bacteria were subjected to pH shift (Fig. 3A, fig. S4A). The overall amounts of each secreted protein were similar to those secreted by the *spiC* mutant strain at pH 5.0 or after shift to pH 7.2, and there was no detectable secretion by the *ssaV* mutant strain (Fig. 3A). In contrast, intra-bacterial and secreted levels of SseB were reduced by approximately 50% following pH shift (fig. S4A).

Inhibition of protein synthesis showed that the pH shift enhanced the secretion of preformed effectors, suggesting that it acted on the T3SS apparatus or associated proteins (fig. S4B). Enhanced secretion of SseF-2HA was detected at pH 6.4 and reached maximal levels at pH 6.8 (fig. S4C). Thus, wild-type bacteria respond to an extracellular pH shift from 5.0 to 7.2 by reducing SseB levels and triggering effector secretion.

To determine if host cytosolic pH (pHcyt) acted as a signal for the secretion switch, digitonin was used to selectively permeabilize plasma membranes of infected epithelial cells exposed to external media at different pH (pHe). After digitonin permeabilization, pHcyt was reduced by pHe 6.0, but SCV luminal pH remained within the normal range (fig. S5). Secreted SseB was detected at similar levels in untreated and permeabilized cells at pHe 6.0 (Fig. 3B). Translocation of SseF-2HA was detected in over 90% of infected permeabilized cells at pHe 7.2 (Fig. 3C), but in less than 13% of infected cells at pHe 6.0 (Fig. 3C). This inhibition was reversed if pHe was subsequently raised to 7.2 (Fig. 3C). Thus, we have observed a pH-dependent, reversible inhibition of effector translocation, which suggests that near-neutral host cytosolic pH is necessary for effector delivery across the vacuolar membrane.

We next investigated SsaL/SsaM/SpiC complex integrity in bacteria following pH shift. The wt-3tag strain was grown at pH 5.0 for 4 h and then exposed to pH 7.2 for 1 h. Because substrate recognition in T3SSs involves proteins associated with the secretion machinery in the bacterial inner membrane (*1*), bacterial cells were lysed and separated into membrane and cytosolic fractions. Membrane-associated SpiC-2HA was immuno-precipitated and immunoblots probed to detect SsaL-3Flag and T7-SsaM. As expected, both proteins were co-precipitated at pH 5.0, but neither was detected when SpiC-2HA was precipitated from membranes of cells shifted to pH 7.2 (Fig. 4A). When T7-SsaM was immuno-precipitated from membranes recovered from bacteria at pH 5.0, both SsaL-3Flag and SpiC-2HA were co-precipitated, but not following shift of bacteria to pH 7.2 (Fig. 4A). Thus, an increase in extracellular pH leads to dissociation of the membrane-bound SsaL/SsaM/SpiC complex.

We were unable to detect secretion or translocation of SsaM, SpiC (*4*) or SsaL (fig. S6). To study the stability of SsaL and SsaM following pH shift, the wt-3tag strain grown at pH 5.0 was exposed to either pH 5.0 or pH 7.2 in the presence of tetracycline to inhibit further protein synthesis, and protein levels were examined. Whereas the levels of DnaK and

a functional 3Flag-tagged SsaN (an essential component of the SPI-2 T3SS) were relatively stable over this period at either pH, levels of both SsaL-3Flag and T7-SsaM declined. However the rate of decline was much greater in bacteria following exposure to pH 7.2 compared to pH 5.0 (Fig. 4B). To determine if intrabacterial levels of SsaL and SsaM were affected following translocon pore assembly in vivo, HeLa cells were infected for 6 h with the wt-3tag strain or by a mutant version lacking all three translocon proteins. The levels of SsaL-3Flag and T7-SsaM in intracellular wt-3tag bacteria were consistently lower (by 36.7 ± 1.3% and 33.7 ± 3.6%, respectively) compared to their levels in the strain which could not assemble a translocon pore (Fig. 4C), but had an otherwise functional T3SS (fig. S7). Furthermore, loss of SsaL-3Flag and T7-SsaM was prevented by reducing pHcyt and induced following a subsequent increase in pHcyt (causing a loss of 57.3 ± 0.6% and 60.2 ± 1.9%, respectively) (Fig. 4C). Thus, in infected cells, loss of SsaL and SsaM requires near-neutral pHcyt and formation of the translocon. The translocon pore provides a means by which intravacuolar bacteria could sense pHcyt, leading to dissociation and degradation of regulatory complex components, triggering effector delivery (fig. S8).

Acidic ambient pH suppresses effector secretion but is necessary for translocon protein secretion (*3, 4, 9*). The SsaL/SsaM/SpiC regulatory complex has a critical role in this process by acting as a 'gatekeeper', enabling translocon protein secretion while suppressing effector secretion at pH 5.0. This complex is likely to interact with the cytoplasmic region of basal body of the secretion apparatus, and to respond to an unidentified pH sensor. The sensor is unlikely to be part of the translocon, because the translocon deletion mutant displayed wild-type levels of effector secretion upon pH upshift (fig. S7). The sensor might be the needle subunit itself, which has been implicated in signalling the translocator to effector switch in *Shigella* (*10*), and Yop secretion by *Yersinia* (*11*). Another possibility is that translocon pore assembly changes the pH gradient within the needle channel and that the sensor is located towards the base of the secretion apparatus. Changes in pH from mildly acidic to neutral can have dramatic effects on protein folding: for example some bacterial toxins refold following their translocation from acidic endosomes to the host cell cytosol in a partially unfolded state (*12*). The SPI-2 T3SS pH sensor might thus undergo a conformational change on exposure to neutral pH and transduce a dissociation signal to the SsaL/SsaM/SpiC complex.

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Figure legends.

Fig. 1 SsaL is required for translocon protein secretion, suppresses effector secretion and interacts with SsaM and SpiC. (A) Model of the SPI-2 T3SS spanning the inner and outer membranes of the bacterial cell and connected to a translocon pore formed in the vacuolar membrane. Translocon proteins must be secreted before effectors can be translocated. SsaV is thought to be located in the inner membrane and is essential for the function of the secretion system. **(B)** Wild-type (wt), *ssaV, spiC*, *ssaM* or *ssaL* deletion mutant strains expressing 2HA-tagged SseJ from chromosome were grown in minimal medium pH 5.0, and secreted and bacterial-associated (lysate) proteins were examined by immunoblotting to detect the HA epitope, SseB and DnaK. **(C)** A plasmid expressing SsaL-2HA was introduced into the *ssaL* deletion mutant, and secreted levels of SseJ-2HA, SseB and SseC were compared with the *ssaL* mutant by immunoblotting. **(D)** Interaction between SpiC-2HA, T7-SsaM and SsaL-3Flag. In the wt-3tag strain, *spiC*, *ssaM* and *ssaL* are replaced with versions expressing epitope-tagged proteins. This and an isogenic strain lacking SpiC (*spiC)* were grown in minimal medium pH 5.0 and whole cell lysates were immuno-precipitated with an anti-T7 antibody. The presence of the three proteins was detected in input samples (input) and following immunoprecipitation (output), by immunoblotting.

Fig. 2 Phenotypes of SsaL and SsaM variants that block ternary complex formation. **(A)** The SsaL/SpiC/SsaM complex requires the C-terminal 18 amino acids of SsaM. A strain expressing SsaL-3Flag, GST-SpiC, and either SsaM-2HA or a non-functional version lacking its C-terminal 18 amino acids ($SsaM_{104}$-2HA) (*4*), were grown in minimal medium pH 5.0 and whole lysates were used for GST pull-down (GST-SpiC) or immunoprecipitation (SsaL-3Flag, SsaM-2HA and $SsaM_{104}$-2HA). **(B)** Plasmids encoding T7-SsaM and 2HA-tagged SsaL or mutant variants were introduced into an *ssaL* deletion mutant. Whole bacterial lysates were immunoprecipitated with anti-HA antibody. SsaL-2HA and T7-SsaM were detected in input samples (input) and following immunoprecipitation (output) by immunoblotting. **(C)** The *ssaL* deletion strain expressing SseB and SseJ-2HA, and SsaL or mutant variants from a plasmid, were grown in minimal medium pH 5.0 for 5 h. Secreted fractions were analysed by immunoblot for SseB and SseJ-2HA. **(D)** The wild-type strain, an *ssaM* mutant and the mutant with or without a plasmid expressing SsaM-2HA or $SsaM_{104}$-2HA were grown at pH 5.0 and analysed as for (**C**) above.

Fig. 3 Effect of pH on effector secretion and translocation. **(A)** Bacterial strains were grown in minimal medium pH 5.0 for 4 h then exposed to pH 5.0 or 7.2 for 90 min. Secreted and bacterial-associated (lysate) 2HA-tagged effectors and DnaK were examined by immunoblotting. **(B)** HeLa cells were infected with wild-type or *ssaV* mutant *Salmonella* for 3.5 h to allow expression of the SPI-2 T3SS, then some samples were permeabilised with digitonin and exposed to pHe 6.0. Cells were fixed 2.5 h later and immunolabeled to detect *Salmonella* and secreted SseB. **(C)** HeLa cells were infected for 3.5 h with wild-type *Salmonella* expressing SseF-2HA, then permeabilised with digitonin and exposed to pH 6.0 or 7.2 for a further 2.5 h. In one sample, pHe was changed from 6.0 to 7.2, 1 h before fixation. Fixed cells were immunolabeled to detect *Salmonella,* LAMP-1 and SseF-2HA. In **(B)** and **(C),** values below the images represent the percentage of cells where secreted SseB or translocated SseF-2HA was detected, ± standard error of three experiments. (*n* >100 cells per experiment). Scale bars = 2 μm.

Fig. 4 Effect of pH on the SsaL/SsaM/SpiC complex. (A) The wt-3tag strain was grown in minimal medium at pH 5.0 for 4 h, then exposed to pH 5.0 or 7.2 for 1 h, then membrane-associated and cytosolic SpiC-2HA and membrane-associated T7-SsaM were immuno-precipitated (IP). Proteins were detected in input samples (input) and following immuno-precipitation (output), by immunoblotting. **(B)** The wt-3tag strain, and a strain in which the chromosomal copy of *ssaN* is replaced with a functional version carrying a 3Flag epitope (lower panel), were subjected to pH shift in the presence of tetracycline. Samples were removed at various times and lysates analysed by immunoblot. **(C)** HeLa cells were infected with the wt-3tag strain (wt) or an isogenic translocon mutant (*sseA-D*). At 3.5 h post-invasion, some wt-infected cells were treated with digitonin (wt + dig), and exposed to pHe 6.0 for 2.5 h (6.0) or pHe 6.0 for 1.5 h, then changed to pHe 7.2 for another 1 h (6.0 →7.2). Cells were lysed at 6 h post-invasion and analysed by immunoblot.

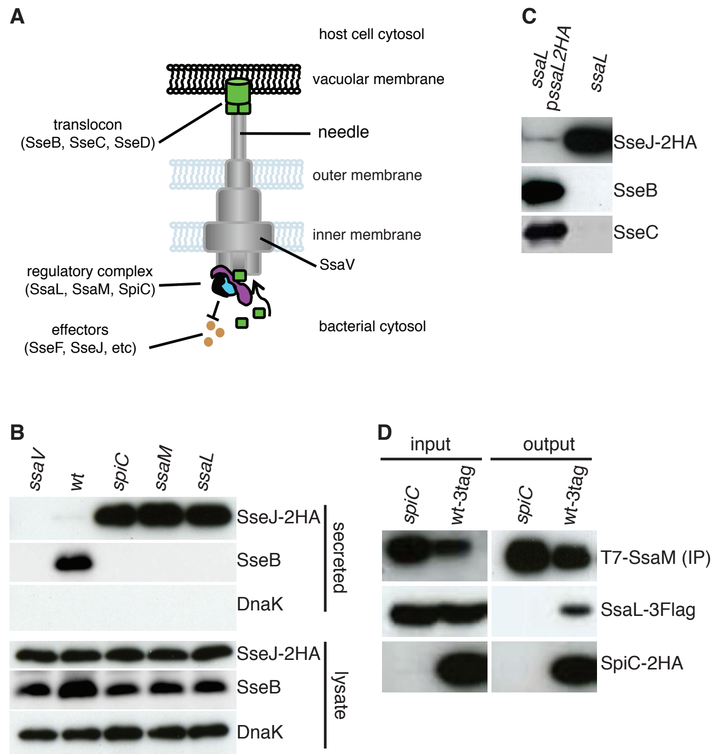

Fig.1

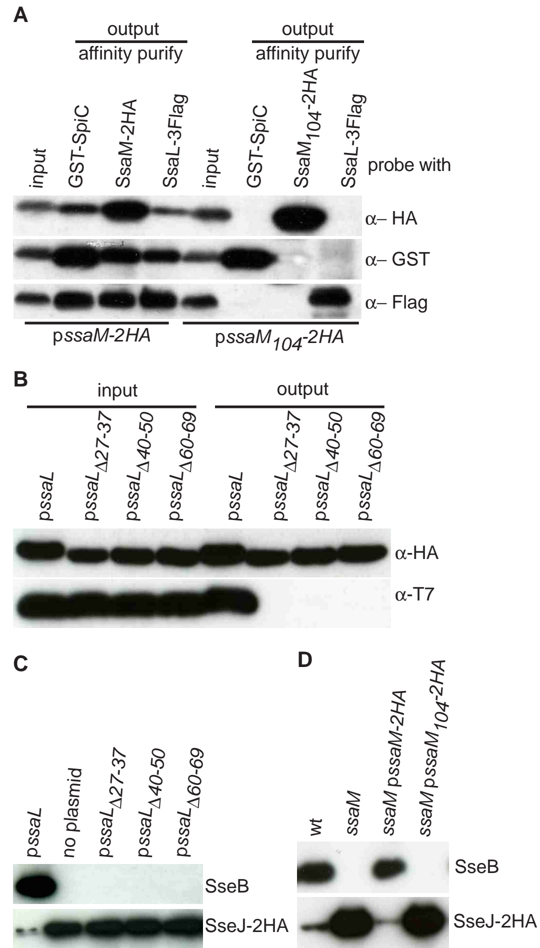

Fig. 2

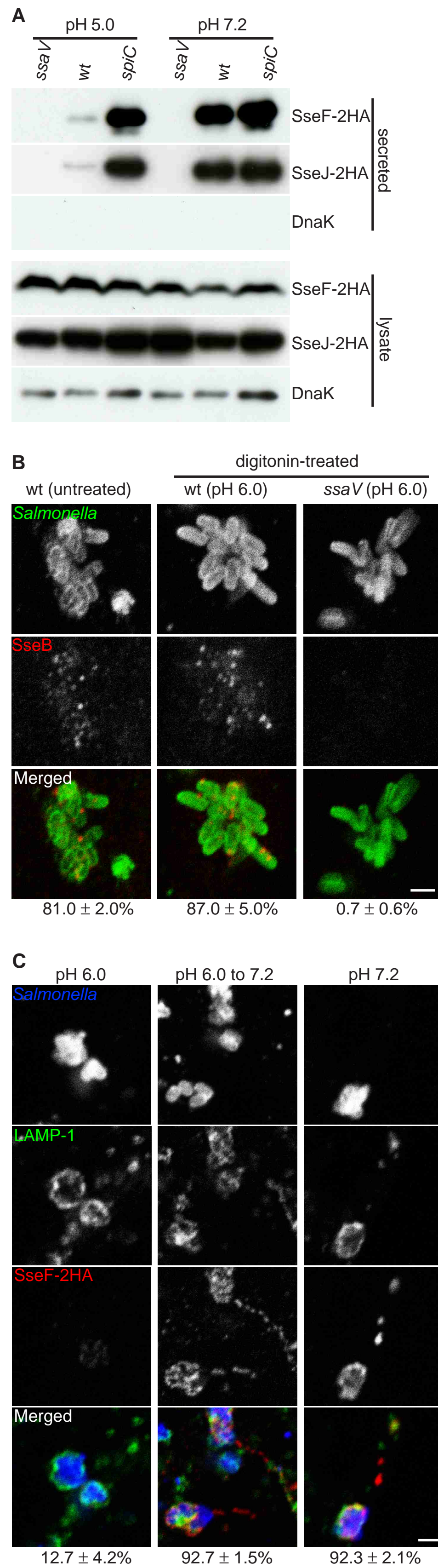

Fig.3

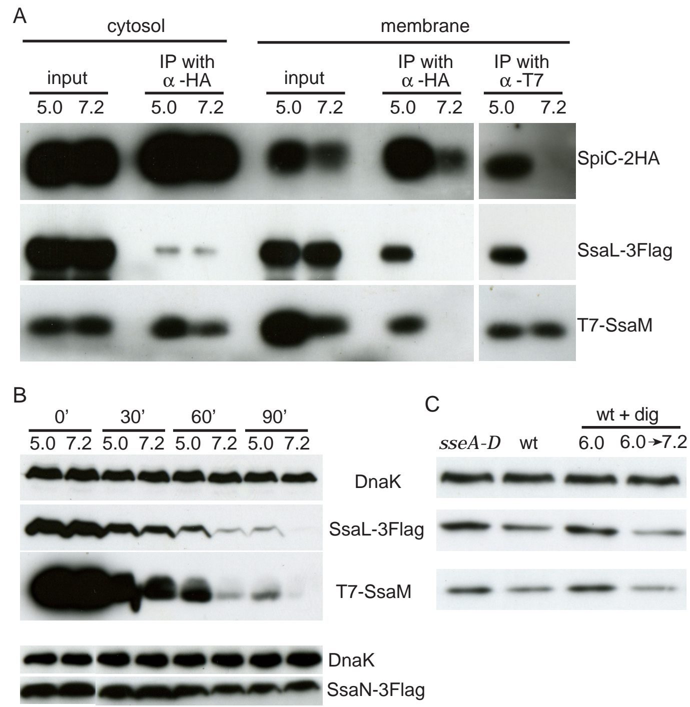

Fig.4

Supporting Online Material for

pH Sensing by Intracellular *Salmonella* Induces Effector Translocation

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Materials and Methods

Bacterial strains, plasmids and antibodies. DNA allelic exchange (*1*) was used to construct the *S*. Typhimurium *ssaL* mutant. The bacterial strains expressing SseF-2HA, SseJ-2HA or SsaN-3FLAG from the chromosome, the *spiCssaM* mutant expressing SsaL-3Flag (Fig.2) and the *sseA-D* mutant with deletion of translocon protein and chaperone genes were constructed by combination of the λ Red recombinase method (*2, 3*) and phage transduction (*4*). The *spiCssaM* mutant was used to construct the wt-3tag strain by allelic exchange. Plasmids expressing SsaL-2HA, SsaL variants or SseF-2HA under the control of the SPI-2 *sseA* promoter were constructed by standard techniques using the low-copy-number plasmid pWSK29 (*5*) as a vector. The following strains and plasmids were described previously: chromosomal *sseL-2HA* (*6*), chromosomal *steC-2HA* (*7*), *ssaV* mutant (*8*), strains expressing SseJ-2HA from the chromosome and plasmids p*gstspiC*, p*ssaM-2HA* and p$ssaM_{104}$*-2HA* (*9*). Plasmid-expressed effector SseF-2HA was labeled SseF-2HA (P).

The following antibodies were used for immunoprecipitation, immunoblotting and immunofluorescence labeling: rabbit polyclonal anti-SseB and anti-SseC (*9*), rat anti-HA (3F10, Roche), mouse anti-HA (HA.11, Covance), mouse anti-T7 (Novagen), mouse anti-Flag (M2, Sigma), mouse anti-LAMP-1 (H4A3, Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa), rabbit anti-LAMP-1 (931A) (*10*), goat anti-*Salmonella* (CSA-1, Kirkegaard and Perry Laboratories), rabbit anti-GST (Covance), rabbit anti-DnaK (*11*). The rabbit anti-*Salmonella* i-H serum (Murex Biotech Limited, UK) was used for detecting FliC. Rhodamine Red X-conjugated donkey anti-mouse or anti-rabbit antibody, donkey anti-rabbit cyanine 2 (Cy2), and Cy5 or Cy2-conjugated donkey anti-goat antibody were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Horseradish peroxidase (HRP)-conjugated donkey

anti-rabbit and sheep anti-mouse antibodies were purchased from Amersham Life Sciences.

pH shift and protein secretion assay. Bacterial strains were grown overnight in LB broth with or without antibiotic, and sub-cultured in MgM-MES medium pH 5.0 for 4 h to assemble and activate SPI-2 T3SS. For the pH shift experiments, bacterial cells were collected at room temperature by centrifugation, re-suspended into pre-warmed MgM-MES at the required pH and incubated for 1.5 h. To prevent protein synthesis, bacteria were re-suspended in MgM-MES medium containing 50 μg/ml chloramphenicol or 20 μg/ml tetracycline and incubated for different times. Secreted and bacteria-associated (lysate) proteins were then prepared and loaded for SDS-PAGE according to equivalent OD_{600} of culture as described (*9*).

Fractionation and protein-protein interaction assays. Bacterial cells collected from MgM-MES cultures were sonicated in ice cold PBS containing 1 mM PMSF, and unbroken cells and debris were removed by centrifugation (10 min at 16,000 *g*). The supernatant (whole lysate) was mixed with Triton X-100 to a final concentration at 0.2% and subjected to pull-down with immobilized glutathione beads (Pierce) according to the manufacturer's instructions, or immuno-precipitation. For fractionation assays, the supernatant was subjected to ultracentrifugation for 1 h at 185,500 *g* to separate membrane and cytosolic fractions. The membrane pellet was washed twice with PBS-PMSF, and resuspended into a volume of PBS-PMSF equal to that of the cytosolic fraction. To immunoprecipitate epitope-tagged proteins from the membrane and cytosolic fractions, Triton X-100 was added to a final concentration at 0.2%. Before incubating with antibody, the whole lysate and different fractions were pre-cleaned with protein G-immobilized beads (Pierce) for 1 h at 4°C. The antibody was incubated with pre-cleaned lysate or fraction for 2 h, then with protein G-

immobilized beads for 1.5 h. The beads were washed four times with PBS-PMSF-0.2% Triton X-100, resuspended into 2 × sample buffer, and analysed by SDS-PAGE.

pH manipulation of HeLa cell cytoplasm. To manipulate cytoplasmic pH, HeLa cells were permeabilised with 25 μg/ml digitonin in KHM (110 mM KOAc, 20 mM Hepes pH 7.2, 2 mM MgOAc) for 5 mins on ice (*12*), then incubated in ISB (5 mM NaCl, 140 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM D-glucose, 50 mM MES buffered to different pH). To verify that cytoplasmic pH changed in response to external pH following digitonin treatment, HeLa cells were preloaded with the pH-sensitive fluorescent probe 2', 7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Invitrogen) to a final concentration of 2 μM. After 20 min incubation at 37^0 C, cells were washed twice and treated with digitonin, before resuspension in ISB and incubation for 15 mins at 37^0 C. A total of 5×10^4 cells in each sample were then analysed on a FACS Calibur cytometer (Becton Dickinson) for fluorescence intensities in channels FL-1 and FL-3 (*13*). A calibration curve was also produced using 10 μM nigericin as described (*13*). Data were analysed with FlowJo 8.6.3 software. The emission ratio (FL-1/FL-3) was calculated for each pHe value.

pH measurements of *Salmonella*-containing vacuoles. Measurements of pH_{SCV} were made as described (*14*), with the following modifications. HeLa cells were grown on 35 mm glass bottomed dishes (Matek) in DMEM containing 10% fetal calf serum (FCS) at a concentration of 1.5-6 x 10^5. The medium was replaced after 12 h with DMEM/FCS containing dextran-coupled fluourescein (488 nm) (300 μg/ml) and dextran-coupled Alexa Fluor 647 (647 nm) (200 μg/ml) and incubated for a further 12 h. The cells were then

infected with dsRED-expressing wild-type *S*. Typhimurium strain 12023 in the same medium using standard protocols (12). 3.5 h post-invasion some samples were exposed to 25 μg/ml digitonin (for 5 mins on ice followed by 2 washes in the absence of digitonin) in KHM buffer. After 2.5 h incubation in ISB at pH 6.0, cells were imaged by confocal microscopy. Three-dimensional images of SCVs were selected and quantification of the mean fluorescent ratios 488 nm/647 nm was determined using Volocity software (Perkin Elmer). A standard curve for each experiment was generated to determine the pH value of the SCVs as described (*14*). Groupings of SCVs within 0.5 pH units were generated and the percentages of SCVs in each binned group were calculated. Microscopy was performed at the Facility for Imaging by Light Microscopy (FILM) at Imperial College London.

Bacterial infection of HeLa cells, immunofluorescence, immunoblotting and replication assays. HeLa cells were infected, fixed, permeabilized with saponin (except for one sample shown in fig. S6B where 0.1% Triton X-100 was used), immunolabeled as described (*9, 15*), and analysed using a confocal laser scanning microscope (LSM 510, Zeiss). Translocation of SseF-2HA expressed from plasmid was not detected at 3.5 h post-invasion, but was clearly visible at 4 h (data not shown). Therefore, for digitonin treatment, cells were infected for 3.5 h, then extracellular medium was changed to KHM containing 25 μg/ml digitonin and incubated for 5 mins on ice, followed by washing in KHM to remove excess digitonin. Infected cells were then incubated in ISB at pH 6.0 or 7.2 for 2.5 h, followed by fixation in 3% PFA. In one digitonin-treated sample, pHe was changed from 6.0 to 7.2 1 h before fixation. Immunoblot analysis was performed as described previously (*9*). Band intensities were quantified using Image J software. Intracellular bacterial replication assays were performed as previously described (*16*).

Supplementary Figure legends

Supplementary Figure 1. Hypersecretion of the SPI-2 T3SS effector SseF by *ssaL, spiC* and *ssaM* deletion mutants of *S*. Typhimurium at pH 5.0. In one strain, SseF-2HA is expressed in place of endogenous protein from the chromosome, and in the other it is expressed from a plasmid (SseF-2HA (P)). Strains were grown in minimal medium pH 5.0, and secreted and bacterial-associated (lysate) proteins were examined by immunoblotting to detect the HA epitope and DnaK.

Supplementary Figure 2. Phenotypes of the wt-3tag strain. (A) HeLa cells were infected with the wild-type (wt) or wt-3tag strain for 8 h, then fixed and labeled for LAMP-1 (red) and bacteria (green). Scale bar = 5 μM. **(B)** Intracellular replication of wt, *ssaV* mutant and wt-3tag strains in HeLa cells and RAW264.7 macrophages. The fold increase represents the increase in cfu obtained after lysing host cells at 2 h and 16 h.

Supplementary Figure 3 Alignment of N-terminal regions of SsaL and YopN. The amino acids deleted in the three variants assayed in Fig. 2B and C are indicated by solid lines above the sequence. *, identical residues; :, conserved residues; ., semi-conserved residues. The chaperone-binding domain of YopN extends from residues 32-76, and wraps around the SycN-YscB heterodimer in a horseshoe conformation (*17*).

Supplementary Figure 4 Effect of chloramphenicol and pH on effector and SseB secretion. **(A)** Wild-type *Salmonella* expressing 2HA epitope-tagged SPI-2 T3SS effectors SseL or SteC, or the translocon protein SseB, all from the chromosome, were examined as in Fig 3A. **(B)** Wild-type bacteria expressing SseF-2HA from a plasmid were grown in minimal medium pH 5.0 for 4 h then re-suspended in the same medium with (+) or without (-) chloramphenicol (Cm) at pH 7.2 or pH 5.0. Samples were harvested after 5, 15, 30 or 45 min and secreted and bacterial-associated (lysate) fractions were subjected to immunoblotting using an antibody to detect the HA epitope. **(C)** Immunoblot of SseF-2HA secreted after growth in minimal medium pH 5.0 for 4 h, followed by a 90 min incubation in the same medium buffered to the indicated pH. An anti-*Salmonella* i-H serum was used as a control to detect secreted FliC.

Supplementary Figure 5 Intracellular pH manipulation. **(A)** Exposure of HeLa cells to different extracellular pH values in the presence of digitonin changes cytoplasmic pH. HeLa cells were loaded with BCECF-AM for 20 min, then incubated in ISB at different pH values following exposure to 25 μg/ml digitonin or 10 μM nigericin. After 15 min, cells were analysed by laser scanning cytometry with excitation at 488 nm and emission at 530 nm (green; FL-1) and 640 nm (red; FL-3). The FL-1: FL-3 ratio was calculated; in the presence of digitonin or nigericin, intracellular BCECF becomes increasingly protonated by lower extracellular pH (pHe), causing an increase in red fluorescence (*13*). **(B)** Measurement of pH_{SCV}. HeLa cells were incubated with dextran-coupled fluourescein (488 nm) and Alexa Fluor (647 nm) for 12 h, then infected with dsRED-expressing wild-type *S*. Typhimurium. 3.5 h post-invasion some samples were permeabilised with digitonin or treated with 20 μM nigericin (to provide a positive control) and exposed to ISB at pH 6.0. After 2.5 h

incubation, cells were imaged by confocal microscopy. Three-dimensional images of SCVs were selected and quantification of the mean fluorescent ratios 488 nm/647 nm was determined. pH values were generated by reference to a standard curve. Groupings of SCVs within 0.5 pH units were generated and the percentages of SCVs in each binned group were calculated.

Supplementary Figure 6 Analysis of secretion and translocation of SsaL-2HA. (A) Bacterial strain *ssaL* p*ssaL-2HA* was grown in minimal medium pH 5.0 for 4 h. The medium was then changed to pH 5.0 or 7.2 for 1.5 h. Bacterial associated (lysate) and secreted fractions were analysed by immunoblot using anti-HA and SseB antibodies. **(B)** HeLa cells were infected for 6 h with the above strains and with a strain expressing SseF-2HA from a plasmid, then fixed and immunolabeled for HA-tagged proteins (red) and bacteria (green), after permeabilization with either saponin (to detect translocated protein) or triton x-100 (to detect intrabacterial protein) (*9*). Scale bar = 2 μM.

Supplementary Figure 7 Deletion of translocon proteins and their chaperones does not affect enhanced secretion of SseF-2HA upon pH upshift. The wild-type strain and a deletion mutant encompassing the *sseA-sseD* region of SPI-2 expressing SseF-2HA from a plasmid were grown in minimal medium pH 5.0 for 4 h. The medium was then changed to pH 5.0 or 7.2 for 1.5 h. Bacterial associated (lysate) and secreted fractions were analysed by immunoblot using anti-HA antibody.

Supplementary Figure 8 Model for control of effector translocation by the SPI-2 T3SS. (i) Following uptake into host cells, acidification of the vacuole lumen induces assembly of

the secretion apparatus. (ii) Membrane-associated SsaL/SsaM/SpiC regulatory complex (in purple, black and blue, respectively) prevents premature secretion of effectors (in brown). Translocon proteins (in green), connected to the T3SS apparatus, form a pore in the vacuolar membrane. (iii) The pore enables a component(s) of the T3SS to sense the elevated pH of the host cell cytosol, and a signal is transduced to the SsaL/SsaM/SpiC complex, which dissociates. (iv) Relief of effector secretion suppression enables their translocation.

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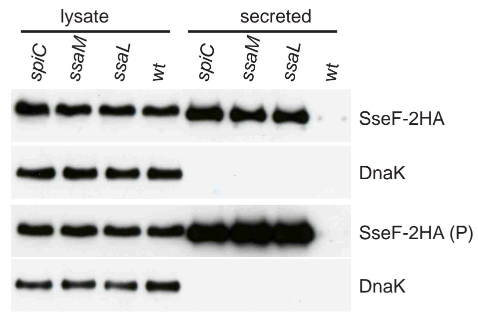
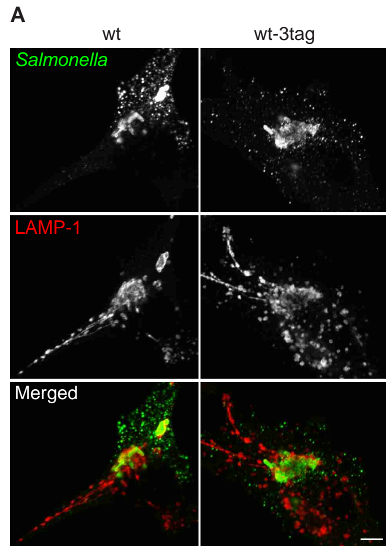


Suppl. Fig. 1

A



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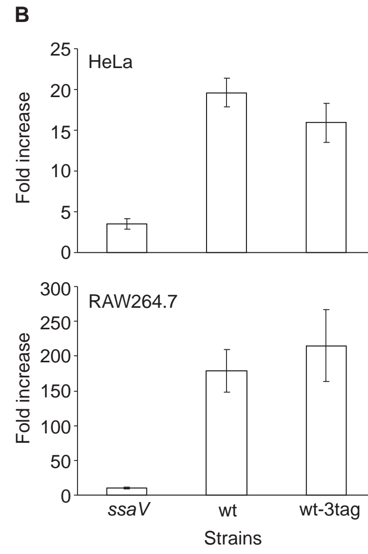

Suppl. Fig. 2

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                                       Δ27-37      Δ40-50
                                    ____________ ___________
SsaL MVKIKEVAMN---IKINEIKMTPPTAFTP--GQVIEE-QEVISPSMLALQELQETTGAAL 54
YopN MTTLHNLSYGNTPLRNEHPEIASSQIVNQTLGQFRGESVQIVSGTLQSIADMAEEV-TFV 59
     *..::::: .   :: :. :::..  ..   **.  *  :::* :: :: :: * . : :

            Δ60-69
          __________
SsaL YETMEEIGMALSGKLRENYK 74
YopN FSERKELSLDKR-KLSD--- 75
     :.  :*:.:    ** :
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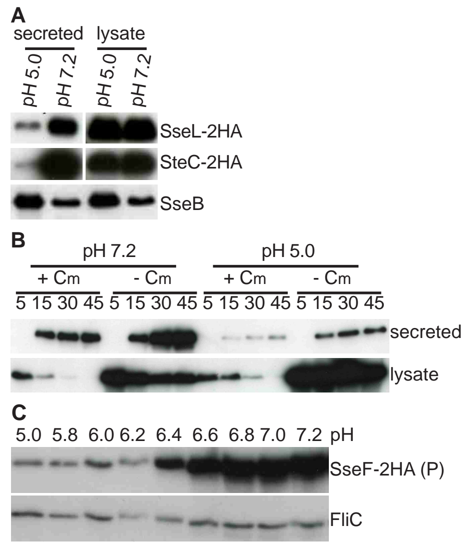
Suppl. Fig. 3



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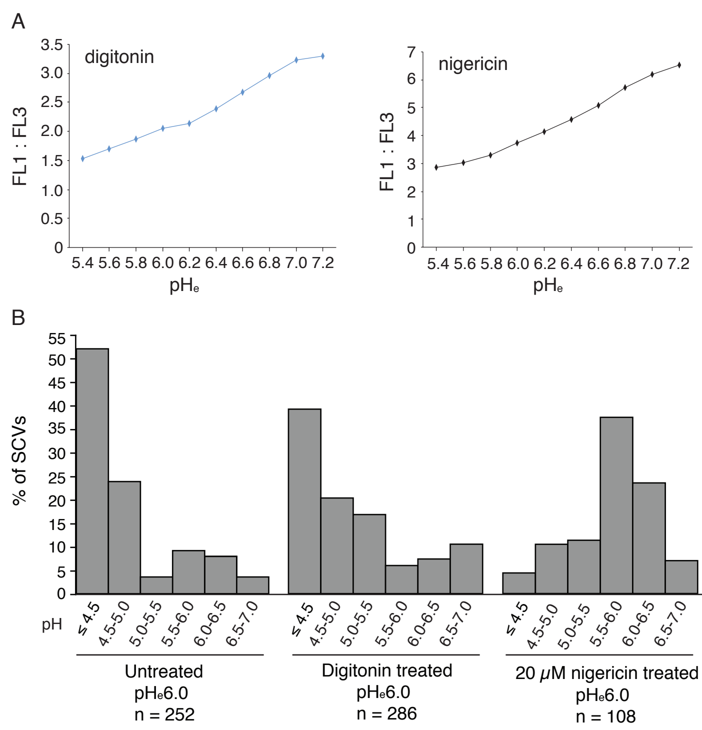

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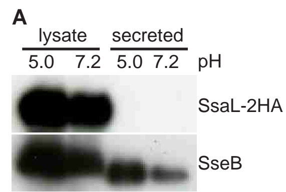

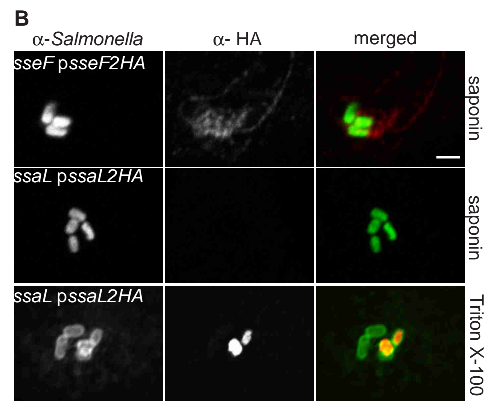

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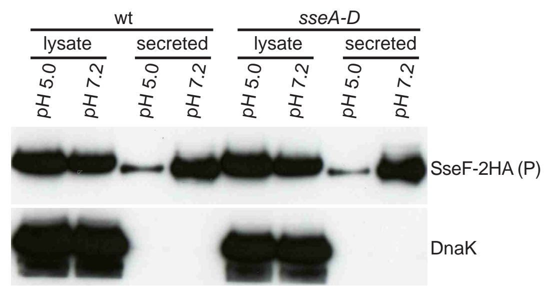

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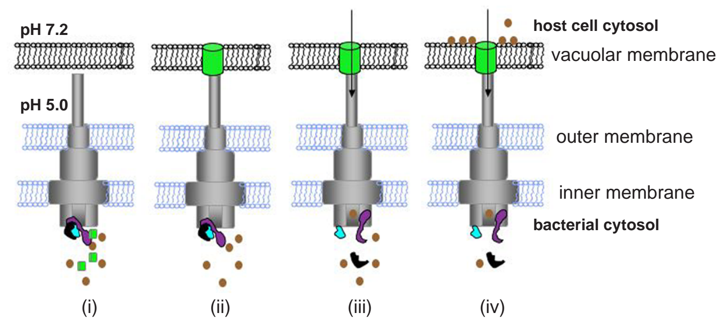

Suppl. Fig. 8