Expression and secretion of Salmonella Pathogenicity Island-2 virulence genes in response to acidification exhibit differential requirements of a functional type III secretion apparatus and SsaL

Brian K. Coombes 1, Nat F. Brown 1, Yanet Valdez 1, John H. Brumell 1†, B. Brett Finlay 1,2,*

Michael Smith Laboratories 1 and Departments of Biochemistry and Molecular Biology, Microbiology and Immunology 2, University of British Columbia, Vancouver, B.C.

V6T 1Z3 Canada

* Correspondence to:

Dr. B. Brett Finlay, Ph.D.
Biotechnology Laboratory
237-6174 University Boulevard
University of British Columbia
Vancouver, British Columbia, Canada
V6T 1Z3
Phone: (604)-822-2210
Fax: (604)-822-9830
E-mail: bfinlay@interchange.ubc.ca

Running title: SPI-2 secretion activity is required for translocon expression

†Current address: Infection, Immunity, Injury and Repair Program, Hospital for Sick Children, Toronto, Ontario, M5G 1X8, Canada and Department of Molecular and Medical Genetics, University of Toronto
Summary

*Salmonella* pathogenicity island (SPI) –2 encodes a type III secretion system pivotal to the intracellular survival of *Salmonella* and consequently for virulence in mammals. SPI-2 encodes virulence factors (called effectors) that are secreted and translocated into the host cell, the type III secretion apparatus responsible for the delivery of these effectors and a two-component regulatory system that regulates intracellular expression of SPI-2. *Salmonella* SPI-2 secretion activity appears to be induced in response to acidification of the vacuole in which it resides and replicates. Here we show that the expression of the SPI-2 proteins, SseB and SseD (filament and pore forming components of the secretion apparatus, respectively) in response to acidification requires an intact secretion system and SsaL, a *Salmonella* homologue of SepL, a regulator required for type III-dependent secretion of translocators but not effectors in attaching and effacing gastrointestinal pathogens. In addition, we show that the expression of SPI-2-encoded effectors is acid-regulated but can be uncoupled from the expression of filament and translocon components, thus showing a differential requirement of SsaL for expression. The secretion and translocation of SPI-2-encoded effectors requires SsaL, but SsaL is dispensable for the secretion of SPI-2 effectors encoded in other pathogenicity loci, suggesting a secretion regulation function for SsaL.

Further, we demonstrate that the differential expression of adjacent genes within the *sseA* operon (*sseD, sseE*) occurs at the transcriptional level. These data indicate that a *Salmonella* SPI-2 activation state is achieved by an acid-regulated response that requires a functional SPI-2 secretion system that includes SsaL. These data also suggest the existence of a previously unrecognized regulatory element within SPI-2 for the ‘effector operon’ region downstream of *sseD* that might demarcate the expression of translocators and effectors.
Introduction

Intracellular survival of *S. enterica* serovar Typhimurium (*S. Typhimurium*) requires a set of virulence proteins encoded in the chromosomal pathogenicity island designated *Salmonella* Pathogenicity Island (SPI)-2 (1,2). SPI-2 is a 25 kb virulence locus that encodes a type III secretion system (TTSS) and related proteins, called effectors which are substrates of this secretion apparatus. Infection of macrophages by *Salmonella* activates the SPI-2 virulence locus, which allow *Salmonella* to establish a replicative vacuole – called the *Salmonella*-containing vacuole (SCV) – inside host cells. SPI-2-dependent activities are responsible for SCV maturation along the endosomal pathway to prevent bacterial degradation in phagolysosomes, for interfering with trafficking of NADPH oxidase-containing vesicles to the SCV (3), remodeling of host cell microfilaments (4,5) and microtubule networks (6,7), both of which play a role in maintaining the integrity of the SCV membrane and directing SCV traffic. As such, the regulation of SPI-2 is integral to *Salmonella* pathogenesis.

SPI-2 TTSS substrates share a similar temporal pattern of expression within host cells and are activated in response to environmental cues presumably sensed by the bacteria in the SCV lumen. Within SPI-2 these substrates include effectors, and SseB (a component of the oligomeric filament structure of the type III apparatus), and SseC and SseD (the pore-forming translocation complex, or translocon) (8). To date, only three SPI-2-encoded effectors, SseF, SseG (9,10) and probably SsaB (SpiC) (11), are documented to be translocated into host cells in an SPI-2-dependent fashion although SPI-2 substrates are also encoded outside SPI-2 in other pathogenicity islands (12) and in lysogenic prophages (13) that are not genetically linked to SPI-2. The coordinated expression of SPI-2 secretion substrates is controlled by the SPI-2-encoded two-component regulatory system SsrA/SsrB (1,14) and the upstream regulators OmpR/EnvZ which modulate the expression of the *ssrAB* operon (15). Once activated, SsrB acts on multiple promoters in SPI-2 and in various regions of the genome (16,17) to induce synthesis of SPI-2 translocator and effector substrates. Several open reading frames (ORFs) encoded within SPI-2 remain uncharacterized including *ssaL*, encoding a protein with homology to the locus of enterocyte effacement...
(LEE)-encoded sepL of the attaching and effacing pathogens enterohemorrhagic E. coli (EHEC) (18),
enteropathogenic E. coli (EPEC) and Citrobacter rodentium (19). We recently identified sepL in a
 genetic screen and showed it is required for type III secretion in C. rodentium of the translocon complex,
EspA, –B, and –D, but not for the secretion of the LEE-encoded effector, Tir (20). Interestingly, ssaL and
sepL contain no other homologues in any other pathogenic bacteria with type III secretion systems
indicating that they probably are not part of the TTSS core complex, which is generally well-conserved
among type III-containing pathogens (21).

Previous work on the environmental cues within the host cell that activate SPI-2 gene expression
and type III secretion has implicated Mg\(^{2+}\) and PO\(_4\)\(^{3-}\) ion limitation and pH (15,17,22,23). Since the pH of
the SCV falls below 5.5 within 20 minutes after its formation inside infected host cells (24), it is possible
that this environmental cue plays a prominent regulatory role in SPI-2 induction. In vitro conditions
resembling the intracellular environment of the SCV such as low pH of minimal medium activate the
secretion activity of SPI-2 (22,25), but less is known about how acidic pH regulates SPI-2 gene
expression. In two previous studies, the pH of minimal medium did not have an effect on the expression
of SPI-2 genes (22) or on a SPI-2 substrate encoded within a prophage outside of SPI-2 (17). However, it
was also reported that acidic pH of minimal medium activated the ssrA promoter in wild-type Salmonella
(15), highlighting an apparent discrepancy given that SsrA and SsrB are required for SPI-2 gene
expression.

To examine the way in which Salmonella responds to acidification of the vacuole in which it
resides, we analysed the expression and secretion of SPI-2 effectors and translocators under pH
conditions that mimic those of the SCV. We demonstrate that expression and secretion of both
translocators and effectors occurs only in acidic minimal medium and not in minimal medium at neutral
pH. A Salmonella strain with a mutation in ssaR (a conserved type III apparatus component) is deficient
in not only the secretion of effectors and translocators but also in the expression of translocators at acidic
pH. A Salmonella strain deficient in ssaL, an uncharacterized SPI-2 ORF with homology to the secretion
regulator sepL of attaching and effacing pathogens, displays a phenotype distinct to the ssaR mutant in
that it restricts the expression and secretion of translocators encoded within SPI-2, yet is dispensable for
secretion of SPI-2 effectors encoded in other pathogenicity loci. We also demonstrate that after
acidification, a functional type III secretion system and SsaL are required for optimal activation of the
sseA promoter which controls the expression of the SPI-2 filament and pore-forming components, but
show that expression of the downstream effectors is differentially regulated with respect to type III
secretion and SsaL.
Experimental Procedures

Bacterial strains and mutant construction

*S. Typhimurium* strain SL1344 was used as the wild-type strain throughout this study and all mutants used in these experiments are isogenic derivatives of SL1344. Strains used in this work are listed in Table 1. *S. Typhimurium* and *Escherichia coli* strains were routinely cultured in LB broth supplemented with the appropriate antibiotics to maintain plasmids as required. A non-polar, unmarked, in-frame deletion of *ssaL* was generated by allelic exchange from a counter-selectable suicide vector expressing SacB (26). First, the *ssaL* open reading frame and approximately 1 kb of flanking genomic DNA upstream and downstream of *ssaL*, were amplified by PCR using the oligonucleotide primers BKC45 (5’ CCA GAG TAT CGG CAA TTG CTT 3’) and BKC46 (5’ AAC AGC CTC ACT CAT CGA CAT 3’) to generate a 3038 bp DNA fragment. This fragment was cloned into pCR2.1 (Invitrogen) to generate a plasmid template that was amplified by inverse PCR using BKC47 (5’ ACG CGT CGA CCA TCG CTA CCT CTT TTA TCT TCA C 3’) and BKC48 (5’ ACG CGT CGA CAA GTC GGT TTT ATT CTG ATA CCT GGC 3’, *SalI* sites underlined). This PCR product was digested with *SalI* and then ligated to generate an internal, in-frame deletion allele of *ssaL* that eliminated the coding region for amino acids 9-333, which was confirmed by DNA sequencing. The *ssaL* deletion allele was cloned into the unique *XbaI* and *SacI* sites of pRE112 (27) and transformed into *E. coli* SM10 λpir (28) to generate a donor strain for conjugation. pRE112-ΔssaL was conjugated into *S. Typhimurium* SL1344 and merodiploid colonies were isolated, grown for 6 h in LB broth without antibiotic selection, diluted and then plated onto agar containing 1% (w/v) tryptone, 0.5 % (w/v) yeast extract, 5% (w/v) sucrose and incubated at 30°C overnight. Sucrose-resistant colonies were selected and the presence of the *ssaL* deletion was confirmed by PCR, restriction enzyme analysis, and DNA sequencing.

Plasmid construction
To generate a wild-type allele of *ssaL* under the control of its native promoter, SL1344 chromosomal DNA was amplified by PCR using primers BKC69 (5' CCG CTC GAG ACA TCT CGG GGA GAA CCA TGA A 3'; *Xho*I site underlined) and BKC70 (5' AGG AAG CTT ATG ATG AGC CAG AAA GCC AA 3'; *Hind*III site underlined), which included the *ssaL* stop codon. The resulting fragment was digested with *Xho*I and *Hind*III and cloned into the unique *Xho*I/*Hind*III sites of pWSK29. To generate wild-type alleles of *sseA-D* under the control of a constitutive promoter, chromosomal DNA was amplified by PCR using primers *sseA*-F (5' ATG GGA TCC TGT ATA TGG AGG GGA ATG ATG 3'; *Bam*HI site underlined) and *sseD*-R (5' ATG GTC GAC TTA CCT CGT TAA TGC CCG GAG 3'; *Sal*I site underlined). The resulting 3487-bp fragment was cloned into pWSK129 as a unique *Bam*HI/*Sal*I fragment under the control of the *lacZα* promoter. An epitope-tagged version of SsaL was created by fusing a double hemagglutinin tag (2HA) to the carboxyl terminus of SsaL. SsaL and its native promoter were amplified with the primers BKC71 (5' ACG CGT CGA CAC ATC TCG GGG AGA ACC ATG AA 3') and BKC72 (5' CGG GAT CCG AAT AAA ACC TGA TTT ATC TTT ACT TCA CG 3'), which eliminated the *ssaL* stop codon. The resulting PCR product was digested with *Sal*I/*Bam*HI and cloned into the *Sal*I/*Bgl*II sites of pBKC-HA, to generate the *ssaL*-2HA fusion, which was then moved into pWSK29 to generate an ampicillin-resistant clone for expression in *Salmonella*. A transcriptional fusion of the *sseA* promoter to an artificial operon consisting of *tnpR* and *lacZ* was constructed by PCR amplifying the promoter region of *sseA* using oligos *psseA*-f (5' ATA CTC GAG CGT ATT CTT GAT TTT CAT CGG TG 3'; *Xho*I site underlined) and *psseA*-r (5' ATA CAA TTG CCC TTT CAG CAA GCT GTT GAC 3'; *Mfe*I site underlined) and cloning the product into pIVET5n cut with *Xho*I and *Mfe*I. The resulting plasmid was integrated into the *Salmonella* chromosome by homologous recombination. Similar transcriptional fusions to *lacZ* were created but fused at *sseD* (oligonucleotides 5' ATG CAA TTG CCC TTT CAG CAA GCT GTA ATA CCA GTG CTA CGT 3' and 5' ATG CTC GAG ACC GGC ATA TTT GAA ACC GTG 3') and *sseE* (oligonucleotides 5' ATG GAA TTC ACC ATT GCT CTA TTT CTT GTA C 3' and 5' ATG CTC GAG ACC GGC ATA TTT GAA ACC GTG 3'). All constructs were confirmed by DNA
sequencing and transformed into the appropriate *Salmonella* strains by either electroporation or
conjugation. Plasmid pssrAB was kindly provided by Dr. M. Hensel, Erlangen, Germany. Plasmids
encoding the *sepL* gene and upstream regulatory region from enteropathogenic and enterohemorrhagic *E.
coli* were kindly provided by Dr. W. Deng, University of British Columbia. A list of plasmids used in this
work is outlined in Table 1.

Recombinant SPI-2 proteins and generation of antibodies

Polyclonal antibodies to SseB, SseD, SseE and SseG were generated by repeated immunization of New
Zealand white rabbits with 1 mg recombinant glutathione S-transferase (GST) fusions of each protein.
Each GST fusion was constructed by PCR amplification of the effector gene from genomic DNA of *S.
Typhimurium* SL1344 using the following primers: SseB, forward 5′-GCA GGA TCC ATG TCT TCA
GGA AAC ATC TTA TGG-3′, reverse 5′-CGT GTC GAC TCA TGA GTA CGT TTT CTG CGC TAT-
3′; SseD, forward 5′-GCA GGA TCC ATG GAA GCG AGT AAC GTA GCA CTG-3′, reverse 5′-CGT
GTC GAC TTA CCT CGT TAA TGC CCG GAG TAT-3′; SseE, forward 5′-GCA GGA TCC ATG
GTG CAA GAA ATA GAG CAA TGG-3′, reverse 5′-CGT GTC GAC TTA AAA ACG TCG CTG
GAT AAG ATG-3′; SseG, forward 5′-GCA GGA TCC ATG AAA CCT GTT AGC CCA AAT GCT-3′,
reverse 5′-CGT GTC GAC TTA CTC CGG CGC ACG TTG TTC TGG-3′. After digestion with *BamHI*
and *SalI* (sites underlined above), the PCR product was cloned into pGEX6P-1 (Pharmacia Biotech). This
plasmid was transformed into the BL21 strain of *E. coli* and overexpression of recombinant fusion
proteins was accomplished with 1 mM IPTG for 3 h (1h for expression of SseG). SseB was purified using
glutathione-coupled beads (Sigma) according to standard protocols (29). Purification of SseD, SseE and
SseG was performed using the sarkosyl lysis procedure described by Frangioni and Neel (30). Raw
antisera were affinity-purified using Sepharose beads (Pharmacia) with covalently coupled antigen. Non-
specific cross-reactivity of the antibodies was minimized by incubation with acetone powders of the BL21
strain that was used to overexpress the recombinant fusion proteins.
Cell culture

HeLa cells were maintained in Dulbecco’s Modified Eagle medium (DMEM; Hyclone, Logan, Utah) supplemented with 10% fetal calf serum (FCS). For immunofluorescence studies, HeLa cells were seeded onto 1 cm sterile glass coverslips in 24-well tissue culture dishes and incubated for approximately 18 h prior to infection. For gentamicin protection assays, RAW 264.7 murine macrophages were used between passages 10-15 and maintained in DMEM supplemented with 10% FCS. RAW 264.7 cells were seeded in 24-well culture dishes 18 h prior to infection. All cell lines were cultured at 37 °C in 5% CO₂.

Analysis of Salmonella mutants in mice

Female BALB/c mice (6-8 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed in sterilized, filter-top cages under specific pathogen-free conditions at the University of British Columbia Animal Facility. The protocols used here were in direct accordance with animal care guidelines as outlined by the University of British Columbia’s Animal Care Committee and the Canadian Council on the Use of Laboratory Animals. Salmonella cultures were grown overnight in LB broth and then diluted in phosphate-buffered saline to give approximately $1.6 \times 10^5$ cfu/ml. Groups of mice (n=5) were infected by intraperitoneal injection with 0.3 ml of diluted bacterial suspension, followed by daily monitoring throughout the study period. Mice that showed signs of extreme distress were euthanized.

Gentamicin protection assays

RAW 264.7 cells were infected with opsonized stationary phase bacteria as described previously (31). At 2 and 21 h after infection, gentamicin-treated cells were washed with PBS and then lysed in 0.25 ml of 1% Triton X-100, 0.1% SDS in PBS. Lysates were diluted in PBS and plated onto LB agar followed by incubation at 37° C. Colonies were enumerated and expressed as colony forming units (cfu) /ml. The fold increase in the number of intracellular bacteria was determined by dividing the cfu values at 21 h by the cfu values at 2 h post infection for each condition.
**In vitro secretion assays**

*In vitro* culture conditions were developed based on minimal medium used to induce expression and secretion of SPI-2 genes (8,22). *Salmonella* strains were grown overnight in LB broth, washed twice in low phosphate, low magnesium-containing medium (LPM) and then inoculated at a 1:50 dilution in 3 ml of LPM medium at either pH 7.0 or pH 5.8. The composition of LPM medium was: 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 38 mM glycerol (0.3% v/v), 0.1% casamino acids, 8 µM MgCl₂, 337 µM PO₄³⁻, 100 mM Tris-HCl (for titration to pH 7.0), or 80 mM 2-[N-morpholino]ethanesulfonic acid (for titration to pH 5.8). Cultures were grown at 37°C with shaking for 4-6 h after which the optical density at 600 nm was measured. Bacteria were collected by centrifugation for 2 minutes at 12,000 rpm (4°C). The supernatant was passed through a 0.22 µm filter and then precipitated with trichloroacetic acid (10% final concentration, v/v) at 4°C for 4-16 h.

**Analysis of secreted proteins**

The TCA-insoluble fraction from above was collected by centrifugation, washed with ice-cold acetone and solubilized with a volume of 2× SDS-sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.002% bromophenol blue and 200 mM dithiothreitol) adjusted according to the OD₆₀₀ of the original culture. When necessary, solubilized secreted proteins were neutralized with an appropriate volume of non-titrated Tris. The bacterial pellet fraction from above was also dissolved in a volume of 2× SDS-sample buffer adjusted according to the OD₆₀₀ of the original culture. Proteins from equivalent numbers of bacterial cells, as determined by OD₆₀₀ readings, were separated on 10% or 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and then blocked in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) and 5% (w/v) powdered non-fat milk for 1 h at room temperature. Blots were incubated with the following primary antibodies in TBST + 5% non-fat milk: rabbit affinity-purified antibodies raised against recombinant SseB, SseD, SseG and SseE (1:1500), mouse anti-HA monoclonal antibody (1:2000; Covance), mouse anti-DnaK monoclonal antibody (1:3500;
Stressgen). Secondary antibodies conjugated to horseradish peroxidase were used at a 1:5000 dilution in TBST for 1 h at room temperature. Antibody complexes were detected using enhanced chemiluminescence (Amersham Biosciences).

Chemiluminescent β-galactosidase assays

sseA promoter activity was examined using transcriptional fusions to lacZ and a chemiluminescence assay described by Kehres et al. (32). Approximately 1 kb of chromosomal DNA upstream of the sseA start codon was transcriptionally fused to the lacZ gene and was used to generate a single copy chromosomal fusion, P_{sseA}:lacZ, in wild-type Salmonella and in various SPI-2 mutants by homologous recombination as described above. Similar lacZ reporter strains were constructed but terminated at different regions of the sseA operon, including sseD and sseE to generate P_{sseD}:lacZ and P_{sseE}:lacZ. LacZ reporter strains were cultured overnight in LB medium, then washed twice in LPM medium and inoculated into fresh LPM medium at either pH 7.0 or pH 5.8 to give an optical density reading of approximately 0.05 at 600 nm. Cultures were incubated with shaking at 37°C for 3-8 h and samples were removed every hour for enumeration of colony forming units and for β-galactosidase activity assays. For β-galactosidase assays, 0.2 ml of the culture was removed and the bacteria were pelleted by centrifugation. The supernatant was discarded and the bacterial pellet was stored at –20 °C until the end of the experiment. Each pellet fraction was resuspended in 0.2 ml of PBS and then 50 µl of chloroform was added to lyse the bacteria. 2 µl of the lysate was transferred to a well of a black microtitre plate containing 100 µl of Galacto-Star substrate mix (Applied Biosystems, Bedford, MA). Reactions were incubated for 60 min at room temperature and then plates were read by using the luminescence detection function of the Spectrafluor Plus (TECAN, Austria). Light emission was expressed as relative light units (RLU) per 10⁶ bacteria based on cfu determinations derived from matched bacterial cultures. Each condition was performed in quadruplicate and averaged.
Results

Salmonella requires low pH and an intact SPI-2 type III secretion apparatus for secretion of filament and translocon components.

To examine the molecular basis of SPI-2 gene expression we performed in vitro secretion assays to study the SPI-2 translocon. We first established and optimised in vitro conditions for SPI-2 secretion assays using minimal medium previously reported to induce the expression of SPI-2 genes and affinity purified antibodies against SseB and SseD. Using this optimised procedure we were able to reproducibly isolate SPI-2-secreted protein from 1.5 ml of non-stationary culture without the need to extract the bacterial surface with hexadecane or mechanical shearing, which had previously been required to detect sufficient levels of SPI-2 translocon components. Wild-type Salmonella and a Salmonella invA::Kan mutant that has a generalized defect in SPI-1-mediated type III secretion were then tested for the ability to secrete the filament component, SseB and the translocon component, SseD, when the bacteria were grown in low phosphate, low magnesium-containing medium (LPM) at pH 7.0 or pH 5.8. In secreted protein fractions, SseB and SseD were detected from wild-type and SPI-1 mutant bacterial cultures grown only in LPM medium at pH 5.8 and not in LPM medium at pH 7.0 (Figure 1A). SseB and SseD secretion required SsaR, a conserved component of type III secretion systems since an ssaR mutant did not secrete these molecules under these same conditions. To control for the presence of cytosolic proteins in secreted protein fractions due to bacterial lysis, we probed each fraction with an antibody against the cytoplasmic molecule, DnaK, which was negative in all cases.

Expression of filament and translocon components requires acidic pH and an intact SPI-2 type III secretion apparatus.

Because the ssaR mutant did not secrete SseB and SseD into the SPI-2 secreted fraction, we tested whole bacterial cell lysates for the expression of these molecules in LPM medium at pH 5.8. Interestingly, the ΔssaR strain, was deficient in cellular pools of both SseB and SseD in LPM medium at acidic pH (Figure
1B). Based on these data, we hypothesized that a functional SPI-2 type III secretion system was necessary for full expression of SseB and SseD. Since minimal medium at neutral pH does not support SPI-2 type III secretion (22), Salmonella cells grown in LPM medium at pH 7.0 should show reduced expression of translocators. To test this, we cultured Salmonella to mid log phase in LPM medium at pH 7.0 and then probed whole bacterial cell lysates for SseB and SseD. As shown in Figure 2B, Salmonella grown in LPM medium at pH 7.0 showed little to no SseB or SseD protein in whole cell lysates, indicating a pH-dependent control of translocon expression. The growth kinetics of Salmonella in LPM medium at pH 5.8 or 7.0 was similar over a 24-hour growth period (Figure 1D) and all protein gel lanes were loaded with protein from equivalent numbers of bacteria.

Salmonella cultures grown to stationary phase show altered SPI-2 gene expression

Some reports examining SseB expression from wild-type stationary phase cultures demonstrated that SseB accumulation was pH-independent in minimal medium (8,22). Because we found very limited SseB and SseD expression from Salmonella grown in LPM at pH 7.0, we sought to confirm that wild-type Salmonella grown to stationary phase no longer exhibit pH-regulated SPI-2 gene expression. Wild-type Salmonella and ΔssaL and ΔssaR mutants were grown to stationary phase by overnight growth in LPM medium at either neutral or acidic pH, following previously published methods (22,23). Whole bacterial cell fractions were then probed for the presence of SseB. Consistent with previous reports, wild-type Salmonella grown under these conditions expressed SseB when grown in both neutral and acidic minimal medium (Figure 1C). The SPI-2 mutants tested in these experiments did not express SseB in neutral LPM medium but did express a small amount of SseB when grown in LPM medium at acidic pH. Based on these data, we hypothesize that Salmonella grown to stationary phase demonstrate decreased fidelity of SPI-2 regulation, possibly due to an independent regulatory mechanism not encoded within SPI-2, or because of acidification by the bacteria of the medium during prolonged culture to stationary phase.
The SepL homologue, SsaL, is required for translocator secretion and expression.

Our laboratory has shown that the LEE-encoded secretion regulator SepL is required for secretion of the translocon components EspA, EspB and EspD, but not for secretion of the effector molecule, Tir in attaching and effacing pathogens (20). sepL has a single homologue in sequence databases, called ssaL, which is an uncharacterised ORF within SPI-2. To test whether SsaL is required for secretion of SPI-2 translocators, we constructed an in-frame, unmarked deletion in the ssaL gene of SPI-2 and performed expression and secretion assays for SseB and SseD. SsaL was required for secretion (see Figure 3) and full expression (Fig 1B) of both SseB and SseD. Together, these results demonstrate that a functional SPI-2 TTSS, including SsaL, is required for the expression of the SPI-2 translocon.

Complementation studies in the ΔssaL strain

To rule out a possible inability of the ΔssaL strain to respond to environmental stimuli, such as low magnesium, that might limit SPI-2 expression and subsequent SseB and SseD accumulation, we overexpressed in a ΔssaL mutant background the SPI-2 two-component regulator, SsrA/SsrB, from a low-copy plasmid, which was previously shown to decrease the environmental constraints imposed by various media on SPI-2 gene expression in Salmonella (23). Transformation of ΔssaL with pssrAB did not restore cytoplasmic pools of SseB and SseD in the ΔssaL strain (Figure 2A), suggesting that environmental effects unique to the ΔssaL strain are unlikely. As a control for the function of this plasmid, we expressed pssrAB in wild-type Salmonella and confirmed SseB accumulation in both low and high Mg2+-containing medium as reported in the original publication (23) (data not shown). Further, the presence of pssrAB and high magnesium did not compensate for the lack of SsaL in terms of SseB expression (data not shown).

We then tested the ability of the ΔssaL strain to express and secrete SseB when complemented with a plasmid that expresses SsaL from its native promoter. In this series of experiments, complementation of the ssaL mutant with a wild-type ssaL allele restored both expression and secretion of SseB to a level comparable to the wild-type strain harbouring the same complementation plasmid (Figure 2B). As a
control in this series of experiments, we tested SseB expression and secretion in a *Salmonella* mutant with an inactive allele of *ssrB*, the transcriptional activator component of the SsrA/SsrB SPI-2 two-component system. As expected, the *ssrB* mutant strain neither expressed nor secreted SseB under conditions that induced SseB expression and secretion from wild-type cells and from the complemented *ssaL* mutant (Figure 2B). To test the possibility that SepL from enterohemorrhagic *E. coli* might complement the ΔssaL strain, we expressed *sepL* from EHEC in both wild-type *Salmonella* and the ΔssaL strain and measured the expression and secretion of SseB. Expression of SepL in wild-type *Salmonella* lead to an increase in the amount of secreted SseB, which is consistent with its role as a secretion regulator in EHEC and EPEC (20) (Figure 2C). Interestingly, expression of SepL in the ΔssaL strain lead to an increase in the amount of SseB in whole bacteria lysates, but could not restore secretion of SseB (Figure 2C). These data suggest that while SepL can complement some SsaL activity involved in expression of SseB, SsaL performs an additional function in controlling subsequent secretion that cannot be compensated for by the presence of SepL.

Cytoplasmic SseB is not labile in the absence of SPI-2-dependent type III secretion.

One possible explanation for the lack of SseB and SseD in *Salmonella* cells grown in LPM medium at pH 7.0, and in ΔssaL and ΔssaR mutants at pH 5.8 is that these type III secretion substrates could be degraded in the absence of secretion. To test this possibility, we constructed *Salmonella* strains that expressed SseB along with its chaperone, SseA (33,34), from a constitutive promoter and then tested these strains for SseB using expression and secretion assays. As shown in Figure 3A, *Salmonella* strains containing either an empty plasmid or the *sseB* expression construct did not secrete SseB in LPM at pH 7.0 as expected from the above experiments. However, when these same strains expressed SseB from the constitutive *lac* promoter, SseB was detected in the secreted protein fraction from wild-type *Salmonella* grown in LPM medium at pH 5.8 but not from the *ssaR* or *ssaL* mutants (Fig 3A), thereby confirming that both SsaR and SsaL are required for translocator secretion. We also tested whole bacterial lysates from
the same strains for the presence of SseB. As shown in Figure 3B, SseB accumulated in bacteria grown
in LPM medium at pH 7.0 when SseB was expressed from the constitutive lac promoter, indicating that in
the absence of SPI-2-dependent type III secretion, intracellular SseB is not degraded in the bacterial
cytosol. In LPM medium at pH 5.8, both the ΔssaL and ΔssaR strains expressing constitutive SseB also
accumulated non-secreted, intracellular pools of SseB with no visible breakdown products (Figure 3B).
These data demonstrate that intracellular SseB is not inherently labile in the absence of SPI-2-dependent
type III secretion and is stably maintained in the cytoplasm of type III secretion mutants and when
Salmonella is subjected to environmental conditions that restrict SPI-2 secretion activity. It was also
noted that in wild-type Salmonella which over-expressed SseA –B, -C, and –D, the levels of SseB
secreted into the culture supernatant was lower than for non-transformed wild-type cells (Figure 3A). It is
possible that this resulted from the increased competition for the SPI-2 apparatus by three over-expressed
type III secretion substrates (SseBCD), or possibly due to titration by overexpressed SseC or SseD of an
essential endogenous chaperone required for SseB secretion.

The SPI-2 regulator SsaL is required for Salmonella virulence in vitro and in vivo.

To validate the significance of SsaL for Salmonella disease, we tested the ability of the ssaL mutant strain
to replicate in macrophages and for virulence in vivo. The ΔssaL strain did not replicate in the murine
macrophage cell line RAW 264.7 (Figure 4A). The numbers of intracellular wild-type Salmonella
increased approximately 3.5-fold over 21 h whereas the ssaL deletion strain showed a net decrease of
intracellular bacteria over the same time period. The numbers of intracellular bacteria at 2 h was similar
for both wild-type and the ssaL mutant indicating that invasion was not affected (data not shown). The
level of attenuation was similar to that of a Salmonella strain deleted for an essential SPI-2 chaperone
(sseA) or a conserved type III apparatus component (ssaR). The ΔssaL strain was also attenuated for
intracellular replication in HeLa cells, which was evident at time points longer than 8 h post-infection
(data not shown). The intracellular replication defect in the ΔssaL strain was due to the loss of ssaL since
complementation of the allele on a plasmid under the control of its native promoter restored intracellular growth (Figure 4A).

Since the ability to cause a lethal systemic infection in mice is predicated on the ability of *Salmonella* to replicate within macrophages, we tested whether the ΔssaL strain was attenuated for virulence in this model. As shown in Figure 4B, wild-type *Salmonella* caused a lethal infection in mice whereas the ΔssaL strain did not. The ΔssaR strain was also attenuated in the mouse model of systemic infection, as expected. Complementation of the ssaL mutant with a wild-type ssaL allele restored virulence *in vivo* (Figure 4B).

**SsaL requires an acidic minimal medium and the SPI-2 regulator, SsrB for expression.**

To further characterize the expression requirements for SsaL, we constructed a plasmid that expressed SsaL with a C-terminal HA epitope and expressed this construct in wild-type *Salmonella* and in ΔssaR and ΔssaL mutants. Expression of SsaL did not occur in minimal medium at neutral pH, consistent with the expression patterns observed for other SPI-2-encoded molecules (Figure 5A). However, upon acidification of the medium, expression of SsaL was induced in wild-type *Salmonella* and in ΔssaR and ΔssaL mutants (Figure 5A), indicating that SPI-2 type III secretion activity is not required for SsaL production. SsaL-HA was also not a substrate for SPI-2-dependent type III secretion (Figure 5A). To test whether expression of the ssaL gene is regulated by the SsrA/SsrB system in response to low pH, we expressed SsaL-HA in a *Salmonella* mutant with an inactive allele of ssrB. Expression of SsaL was completely abolished in the absence of SsrB (Figure 5B), indicating that the role for SsaL in expression/secretion of the filament and pore-forming components of SPI-2 is downstream from the activation of SsrA/SsrB.

**SsaL is required for secretion and translocation of SPI-2-encoded effectors but not for effectors encoded outside of SPI-2.**
Because the ssaL homologue, sepL, found in LEE-containing enteropathogens is dispensable for type III secretion of effectors but not translocon components (20), we tested whether a ΔssaL strain showed a similar phenotype. We performed secretion assays as described in the Materials and Methods and probed bacterial cell fractions and secreted protein fractions for the SPI-2-encoded molecules, SseG and SseE.

SseG is a bona fide SPI-2-dependent type III secreted effector (9,35), whereas SseE has not been previously characterized as an effector molecule. As demonstrated in Figure 6A, none of the Salmonella strains expressed SseG in LPM medium at pH 7.0, which is consistent with the regulatory pattern observed for SseB and –D. SseE was observed in very limited amounts in LPM medium at pH 7.0. However, growth of Salmonella in LPM medium at pH 5.8 induced the expression of both SseG and SseE to similar levels in wild-type cells and in the SPI-2 mutants (ΔssaR and ΔssaL), and a SPI-1 mutant (invA::Kan) (Fig 6A). Wild-type bacteria and SPI-1 mutant bacteria secreted SseG into the culture supernatant whereas the ΔssaR and ΔssaL strains did not secrete SseG under acidic conditions shown previously to induce SPI-2 secretion (Fig 6B). SseE was expressed under SPI-2-inducing conditions in all the strain backgrounds tested, but was not recovered in detectable amounts from the concentrated secreted fraction of either wild-type bacteria or SPI-1 mutant bacteria (Fig 6B). To further examine other SPI-2 effectors that are encoded in other pathogenicity loci outside of SPI-2, we tested the secretion of PipB, a SPI-2 effector that localizes to the membrane portion of Salmonella-induced filaments (Sif) and vacuoles containing bacteria (12), and SopD2, a SPI-2 effector that localizes to late endosomes following translocation into host cells (36). Neither PipB (Figure 6C) or SopD2 (data not shown) was secreted by the ΔssaR strain, yet these proteins were detected in secreted protein fractions from the ΔssaL strain, indicating a phenotypic difference between a general SPI-2 secretion mutant (ΔssaR) and a mutant lacking SsaL. To verify that expression of PipB in these strains did not affect expression of SseB or SseD, we tested whole cell lysates from strains expressing PipB. As expected, expression of PipB did not restore the expression of SseB or SseD in any of the strains tested (Figure 6D). To examine the effect of ssaL deletion on the translocation of PipB into host cells, we used immunofluorescence to examine
whether this strain could translocate epitope-tagged PipB into epithelial cells. As expected, PipB was not translocated into host cells by the ΔssaL strain (data not shown), thus confirming its defect for assembling a translocation filament and translocon pore.

The sseA promoter demonstrates pH-dependent activation in LPM medium.

The above experiments examining SseB expressed from a constitutive lac promoter (Figure 3) demonstrated that SseB was not degraded in the absence of SPI-2-dependent type III secretion. To test whether this pattern of expression was reflected at the transcriptional level, we expressed from the bacterial chromosome a single copy transcriptional fusion of the sseA promoter fused to lacZ (P_{sseA}::lacZ) and measured β-galactosidase activity in various strains grown in LPM medium that was titrated to neutral or acidic pH. sseA lies immediately upstream of sseB and is part of the same transcriptional unit. As shown in Figure 7A, β-galactosidase activity was significantly lower in wild-type Salmonella grown in LPM medium at pH 7.0, compared to the same reporter strain grown in LPM medium at pH 5.8 at all time points tested. Together with the SseB stability data from Figure 3, these results suggest that the lack of SseB and SseD in Salmonella grown in LPM medium at neutral pH is due to decreased activity of the sseA promoter.

Mutations in ssaR and ssaL have a negative effect on the transcriptional activation of the sseA promoter.

To test further whether the decreased amounts of SseB and SseD in the ΔssaL and ΔssaR mutant backgrounds was reflected at the transcriptional level, we constructed the chromosomal integration of the P_{sseA}::lacZ reporter in ΔssaL and ΔssaR mutant backgrounds and performed time course experiments using the P_{sseA}::lacZ reporter strains grown in LPM medium at pH 5.8 and pH 7.0. Viable counts were also performed on the same bacterial cultures such that β-galactosidase activity was normalized to bacterial counts at each time point. Consistent with our protein expression data, sseA induction was reduced
significantly in ssaL and ssaR mutant backgrounds at low pH compared with wild-type cells (Figure 7B).

Also consistent with the protein data, β-galactosidase activity from cultures grown at neutral pH were
significantly lower than those at pH 5.8 and did not differ between wild-type Salmonella and the ΔssaR
and ΔssaL mutants (Figure 7C). Taken together, these data verify the requirement for (i) acidification and
(ii) SPI-2 secretion activity including SsaL for activation of the sseA promoter.

Transcriptional activity of genes downstream of the pore-forming translocon genes can be
uncoupled from the requirement of type III secretion and SsaL.

The expression experiments shown in Figure 6 demonstrated that while the expression of effectors
downstream of the filament and pore-forming genes (SseB and SseD) was also pH-dependent, the ΔssaR
and ΔssaL strains expressed similar amounts of SseE and SseG compared to wild-type Salmonella, which
contrasts with the expression pattern for the upstream genes. To examine this, we constructed single-
copy chromosomal transcriptional fusions of lacZ to the sseD gene and the immediately downstream
gene, sseE, and measured β-galactosidase activity from wild-type, ΔssaR and ΔssaL reporter strains. SseD
is the second component of the pore-forming translocon (8). As shown in Figure 8, β-galactosidase
activity from Salmonella strains with the P_{sseD}::lacZ reporter was comparable to the activity for the
P_{sseA}::lacZ fusion in terms of magnitude and demonstrated lower activity in the ΔssaR and ΔssaL mutants
compared to wild-type. β-galactosidase activity from the P_{sseE}::lacZ reporter were not significantly
different between wild-type Salmonella or the ΔssaR and ΔssaL mutants, thus confirming the expression
patterns observed previously for SseE. β-galactosidase activity from the P_{sseE}::lacZ reporter strains was
approximately 3-fold higher than either the P_{sseD}::lacZ or P_{sseA}::lacZ reporters in all strains at all time
points tested (Figure 8). Together with the protein expression data, these data suggest that expression of
the genes downstream of the filament (sseB) and pore-forming component of the SPI-2 translocon (sseD)
can be uncoupled from the expression of the upstream genes with respect to the requirement of type III
secretion and the presence of SsaL.
Discussion

We have identified a regulatory mechanism for the SPI-2 translocon components SseB and SseD that is responsive to acidic pH and requires a functional SPI-2 TTSS. A *Salmonella* strain with a mutation in the SPI-2-encoded molecule, SsaL, and a second *Salmonella* strain with a mutation in a conserved SPI-2 type III secretion apparatus component displayed a similar phenotype for translocator expression and secretion, but differed in their competency for secretion of SPI-2 effectors encoded outside of SPI-2. This pattern of expression was reflected at the transcriptional level and was reproducible using both environmental and genetic approaches to block SPI-2-dependent type III secretion.

It is known that the two-component regulatory system comprised of SsrA/SsrB positively regulates SPI-2-encoded genes. SsrA is the putative sensor kinase component and SsrB is the transcriptional activator that acts on promoters in SPI-2 and in other regions of the genome. Using transcriptional fusions to *gfp*, Lee and colleagues demonstrated that the promoter controlling *ssrAB* was activated in acidic minimal medium but not in minimal medium at neutral pH (15). We therefore reasoned that genes controlled by SsrA/SsrB (such as the *sseA* operon) should also demonstrate acid-induced activation. We found that components of the SPI-2 filament (SseB), translocon (SseD), and effectors (SseG, SseE) required acidified minimal medium for expression and accumulation within wild-type bacteria. It was of interest to find that SseE was localized to bacterial cells but was not found in the secreted fraction from any of the *Salmonella* strains tested. Although there are currently no published data on SseE, based on its localization within *Salmonella* cells we hypothesize that it could play a chaperone role or other accessory role for SPI-2-dependent type III secretion. The lack of an *sseE* homologue in any pathogenic bacterium suggests it might play a role unique to the function of SPI-2.

As mentioned, a previous report demonstrated that activation of the *ssrA* promoter required minimal medium at acidic pH (15). However, two other studies investigating SPI-2 gene expression (22,23) reported that the expression of SPI-2 genes was pH-independent. These data are difficult to reconcile with each other given that SsrA/SsrB is a requisite activator of SPI-2 gene expression. Our results are in agreement with the former study (15) and show that activation of the *sseA* promoter and
expression of both translocon components (SseB, SseD) and effectors (SseG, SseE) requires acidic pH.

This pH-inducible activation was SsrB-dependent, since no SPI-2 protein accumulation or secretion was seen in an ssrB mutant. Given that the SCV acidifies to below pH 5.5 within 20 minutes after uptake into bone marrow-derived macrophages (24), it’s likely that an acidic pH is a major environmental cue determining not only activation of SPI-2 secretion, but also inducing the expression of SPI-2-encoded genes. We suspect that the bacterial growth stage represents an important contribution to the regulation of SPI-2, since we found that growth of Salmonella to stationary phase in neutral minimal medium – conditions which were used in previous studies that did not find pH-dependent SPI-2 gene expression – caused accumulation of SseB in wild-type Salmonella but not in ssaL or ssaR mutants. Whether this represents a stress response by the bacteria will require additional experimentation. It should be noted that in our expression and secretion experiments, all bacterial cultures were tested during exponential-phase growth, after 4 to 6 h of incubation in minimal medium. The sensitivity of our expression and secretion assays allowed us to perform these experiments before the bacterial cultures reached saturation in stationary phase and allowed us to detect SPI-2 secreted proteins from culture volumes of less than two ml without the need to extract the bacterial surface by chemical or mechanical means – conditions which were previously reported as being necessary to detect SPI-2 secreted proteins in vitro (8,22).

The data presented here are consistent with the hypothesis that the pH-dependent secretion of a putative transcriptional repressor may play a role in controlling SPI-2 gene expression and that SsaL might be involved in this process. In such a model, repression of the translocon promoter upstream of sseA would occur in the absence of SPI-2 type III secretion, which is inhibited during bacterial growth in neutral minimal medium or disabled in SPI-2 apparatus mutants. Following activation of SPI-2-dependent type III secretion, as when bacteria encounter an acidified intracellular environment, the active secretion of a putative repressor would lower its cytoplasmic concentration and de-repress the translocon promoter, facilitating SsrB-mediated expression of SseB, –C, and –D. Given that type III secretion systems have shown a hierarchal secretion of regulators, translocators and effectors (37), it is possible that a SPI-2 TTSS intermediate exists in which the apparatus is permissive for secretion of regulatory
controllers prior to engaging translocators and effector substrates. This transitional structure would represent a secretion-competent but not translocation-competent intermediate in which substrates could be secreted into the SCV lumen prior to formation of the filament and translocon pore. Given that *ssaL* mutants constrain the expression of the SPI-2 translocon it is possible that SsaL is involved in such a transitional secretion complex. This hypothesis is supported by data from Cirillo and colleagues (38), who showed that a *Salmonella sseB* mutant (that can still secrete, but not translocate, SPI-2 substrates) could still activate promoters controlling the SPI-2 molecules, SpiA (SsaC), SscB and SsrA. This group also reported that SPI-2 gene expression did not require an intact secretion apparatus. However, this conclusion may be limited insofar as the expression studies were performed with a limited number of SPI-2 mutants containing plasmid-based transcriptional fusions to GFP, whereas it has been observed that SPI-2 gene expression can be improperly regulated when SPI-2 genes are expressed from medium copy plasmids from their native promoters [B. Coombes, B. Finlay, unpublished data and (9)]. It is possible that an increased copy number of SPI-2 promoters in these instances could titrate out a putative repressor molecule leading to the observed gene expression patterns from plasmid-based studies. While it is possible that SsaL is part of the core type III apparatus, genetic and phenotypic evidence might suggest a more complex role. First, SsaL has only one homologue (SepL) in type III secretion systems from other pathogens, whereas it has been recognized that the core structure of the type III apparatus is generally well conserved (21,39). Second, SepL from attaching and effacing gastrointestinal pathogens has been shown to regulate the transition from translocon to effector secretion (20) and is dispensable for secretion of type III effectors. Third, SepL from EHEC can moderately complement an *ssaL* mutation in *Salmonella* for expression of SseB, but not for secretion, suggesting a bifunctional role for SsaL in SPI-2, and lastly, an *ssaL* mutant retains the ability to secrete non-SPI-2-encoded effectors, while a generalized secretion mutant (*ΔssaR*) does not.

Interestingly, Day and Lee (40) recently hypothesized that a SPI-1-encoded protein called OrgC functions as a SPI-1-secreted repressor of SPI-1 virulence genes. This hypothesis was based on
phenotypic studies using an orgC deletion mutant and by comparing the gene synteny of orgC to that of
virulence genes in other pathogens. In particular, the position of orgC in the SPI-1 operon,

\[ \text{prgHIJKorgABC} \]

corresponds to the position of a transcriptional repressor, lcrQ in the Yersinia virulence
plasmid (40). While the hypothesis that OrgC is a transcriptional repressor of SPI-1 was not formally
tested by Day and Lee, it remains plausible that a corresponding mechanism exists to repress SPI-2 gene
expression until the appropriate intracellular environmental cue(s) is sensed.

It was of interest that SPI-2-encoded effectors and translocators were repressed in a similar
fashion in minimal medium at neutral pH, but there was no difference in SseE or SseG expression
between wild-type, ssaR and ssaL mutants in acidic minimal medium. It is possible that SPI-2-encoded
effectors downstream of sseD are subject to different regulatory control compared to the translocators,
despite being present in the same putative operon. Transcription fusions of \( \text{lacZ} \) to various regions of this
operon, including sseD and the immediately downstream gene, sseE confirmed that the expression of
upstream translocators and downstream ‘effectors’ has different requirements with respect to type III
secretion and the presence of SsaL. These data also suggest the presence of a previously unrecognised
regulatory element that might act specifically on genes downstream of the translocon, since the activity of
the \( \text{P}_{\text{sseE}::\text{lacZ}} \) reporter was significantly greater than two upstream reporters. Indeed, there is already
evidence that SPI-2 genes in the same transcriptional unit contain distinct promoters that are differentially
activated. For example, Feng and colleagues (41) recently reported that unlike many two-component
regulatory systems, regulation of the sensor kinase SsrA is partially uncoupled from regulation of the
response regulator SsrB in SPI-2, owing to their distinct promoters. Emerging evidence implies that
combinatorial pair-wise interactions of different response regulators can interact at the same promoter,
suggesting an increase in the complexity of prokaryotic transcriptional regulation akin to that seen in
eukaryotic systems (42-44). Since SPI-2 gene regulation is integral to \( \text{Salmonella} \) pathogenesis, it is
reasonable to speculate that virulence gene regulation in \( \text{Salmonella} \) is under complex regulatory control
and insight into this process will lead to gains in understanding how intracellular \( \text{Salmonella} \) interact with
host cells to cause disease.
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Figure Legends

Figure 1. Expression and SPI-2 secretion of translocators is acid-inducible and requires SsaL and a functional SPI-2 type III secretion system. (A). Analysis of SPI-2 secreted proteins. Bacterial cultures were grown in low-phosphate, low-magnesium (LPM) medium at neutral or acidic pH and SseB (top panel) and SseD (middle panel) were detected in the secreted protein fraction by Western blot analysis. Secreted protein fractions were also probed for an intracellular marker, DnaK (bottom panel). (B) Detection of SseB and SseD in *Salmonella* lysates. Whole bacterial cell lysates from the cultures from (A) were subjected to Western blot analysis for SseB and SseD. Lysates were also probed for DnaK (bottom panel). (C). Detection of SseB from stationary phase *Salmonella* cultures. Bacterial cultures were grown for 16 h to stationary phase in the indicated media and subjected to Western blot analysis for SseB in whole cell lysates. Expression and secretion assays were repeated five times with similar results. All proteins fractions were normalized to OD$_{600}$ readings for each culture, and (D) differences between the growth of cultures in acidic or neutral minimal medium were not detected.

Figure 2. Complementation studies of ΔssaL. (A) Effect of overexpression of SsrA/B. ΔssaL *Salmonella* containing the plasmid, pssrAB were grown in LPM medium (pH 5.8) and protein from equal numbers of cfu were subjected to Western analysis for SseB (top panel), SseD (middle panel) and DnaK (bottom panel). (B) Complementation of SseB expression by re-introduction of a wild-type ssaL allele. Wild-type *Salmonella* and a ΔssaL strain each containing pssaL were grown in LPM medium (pH 5.8) and protein from equal numbers of bacteria were subjected to Western analysis for SseB in the whole bacterial fraction (pellet) and the secreted protein fraction. As a control, protein samples were probed for the bacterial cytoplasmic molecule, DnaK. (C) The SsaL homologue, SepL from EHEC, moderately restores expression of SseB in whole bacterial lysates (pellet) but does not restore secretion of SseB. The sepL gene from EHEC was expressed in wild-type *Salmonella* and the ΔssaL mutant by growing cultures in
LPM medium at pH 7.0 or 5.8. SseB in whole bacterial lysates and in secreted proteins fractions was detected by Western blot.

Figure 3. SseB is stable in the cytoplasm in the absence of SPI-2 type III secretion. Wild-type *Salmonella* and the ΔssaL and ΔssaR strains expressing SseA, -B, -C, and –D from the constitutive lac promoter (psseA-D) were grown in LPM medium at neutral and acidic pH. Each strain containing an empty plasmid (pWSK129) was also cultured under identical conditions. SseB was detected in the secreted protein fraction (A) and the whole bacterial lysate (B) from each of the strains by Western blotting. Lanes contain proteins from equal numbers of bacteria as determined by OD600 readings.

Figure 4. *ssaL* is required for *Salmonella* virulence *in vitro* and *in vivo*. (A). Gentamicin protection assays. RAW264.7 murine macrophages were infected with wild-type *Salmonella* and the SPI-2 mutants shown and the numbers of intracellular bacteria were enumerated at 2 h and 20 h after infection. Data are presented as the mean fold increase in intracellular bacteria with standard deviation from three separate experiments. (B). Survival plots for Balb/c mice (n=5) infected with wild-type *S. Typhimurium* (black squares), ΔssaR *S. Typhimurium* (black diamonds), ΔssaL *S. Typhimurium* (black triangles), and the complemented ΔssaL strain (black circles). Percentage of the mice surviving on each day after infection is shown. Replication and virulence experiments were repeated three times.

Figure 5. Expression of *ssaL* requires acidic pH and the SPI-2 regulator, SsrB. (A) *Salmonella* strains that expressed SsaL-HA or that were transformed with empty plasmid were grown in LPM medium at pH 7.0 and 5.8 and cell fractions were probed for the presence of SsaL-HA. SsaL-HA was not detected in LPM (pH 7.0) medium in any of the strains tested, but was induced upon acidification of the minimal medium (upper panel). SsaL-HA was not detected in the secreted fraction from any of the strains tested (lower panel). (B) Expression of *ssaL* is SsrB-dependent. A *Salmonella* mutant with an inactive allele of
ssrB was tested for expression of epitope-tagged ssaL. Whereas wild-type Salmonella expressed SsaL-HA under acidic conditions, SsaL-HA expression was abolished in the absence of SsrB.

Figure 6. SPI-2-encoded effectors are expressed in acidic minimal medium and require SsaL for secretion but secretion of non-SPI-2-encoded effectors is retained. Wild-type Salmonella and ssaL, ssaR, and invA mutants were grown in LPM medium at neutral and acidic pH and tested for presence of SseG and SseE in whole bacterial lysates (A), and in secreted protein fractions (B) As a control, each fraction was also probed for DnaK. SPI-2 effectors encoded outside of the SPI-2 pathogenicity island including PipB (C) were also examined for their expression and secretion from various Salmonella strains. (D) Expression of PipB does not restore the expression of SseB or SseD in whole bacterial lysates.

Figure 7. Activation of the sseA promoter requires acidic minimal medium and a functional SPI-2 type III secretion system. (A). Wild-type Salmonella with a single chromosomal integration of a P_{sseA}::lacZ reporter fusion was grown in LPM medium at pH 7.0 and pH 5.8 for the times indicated in the Figure. β-galactosidase activity and bacterial counts were measured at each time point and data are expressed as the relative light units of β-galactosidase activity per 1 × 10^6 cfu. Data are the means with standard errors from triplicate determinations. (B). SL1344 P_{sseA}::lacZ (closed squares), ΔssaR P_{sseA}::lacZ (open circles) and ΔssaL P_{sseA}::lacZ (closed diamonds) strains were grown in LPM medium at pH 5.8 and β-galactosidase activity was measured at the indicated time points. Data are the means with standard errors from quadruplicate determinations. *, p<0.01 compared to wild-type strain. (C). Salmonella reporter strains were cultured in LPM medium at neutral pH and β-galactosidase activity was measured as described above. Data are the means with standard errors from quadruplicate determinations and the experiments were repeated at least three times.
Figure 8. Differential regulation and activity of sseD and sseE::lacZ reporters. Single-copy chromosomal integrations of the reporters, P_{sseD}::lacZ (solid lines) and P_{sseE}::lacZ (dashed lines) were constructed in wild-type Salmonella (SL1344) and in the ΔssaR and ΔssaL mutants. β-galactosidase activity was measured at the indicated time points from whole bacterial lysates and expressed as relative light units of β-galactosidase activity per 1 × 10^6 cfu. Data are the means with standard errors from quadruplicate determinations. p<0.05 for wt P_{sseD}::lacZ compared to ΔssaR P_{sseD}::lacZ or ΔssaL P_{sseD}::lacZ; p<0.01 for wt P_{sseD}::lacZ compared to wt P_{sseE}::lacZ.
| Strain or plasmid | Genotype or Description | Source or Reference |
|-------------------|-------------------------|---------------------|
| **Strains**       |                         |                     |
| *S. enterica* sv. Typhimurium |                         |                     |
| SL1344            | wild-type, Sm<sup>R</sup> | ATCC               |
| SL1344 ΔssaR      | SL1344 *ssaR*, unmarked deletion, Sm<sup>R</sup> | (45)               |
| SL1344 ΔssaL      | SL1344 *ssaL*, unmarked in-frame deletion, Sm<sup>R</sup> | this work           |
| SL1344 ΔsseA      | SL1344 *sseA*, unmarked in-frame deletion, Sm<sup>R</sup> | (31)               |
| SB103             | SL1344 *invA::kan*, Sm<sup>R</sup> | (46)               |
| SL1344 ΔpipB      | SL1344 *pipB*, unmarked in-frame deletion, Sm<sup>R</sup> | (12)               |
| SL1344 ΔsifA      | SL1344 *sifA*, unmarked in-frame deletion, Sm<sup>R</sup> | (47)               |
| SL1344 *ssrB::Kan* | SL1344 *ssrB::kan*, Sm<sup>R</sup>, transduced by P22 from 14028s | (12)               |
| NB3               | SL1344 *PseA::*lacZ*, Sm<sup>R</sup>, Amp<sup>R</sup> | this work           |
| NB4               | SL1344 *ssaR*, *PseA::*lacZ*, Sm<sup>R</sup>, Amp<sup>R</sup> | this work           |
| NB5               | SL1344 *ssaL*, *PseA::*lacZ*, Sm<sup>R</sup>, Amp<sup>R</sup> | this work           |
| NB17              | SL1344 *PseD::*lacZ*, Sm<sup>R</sup>, Amp<sup>R</sup> | this work           |
| NB19              | SL1344 *ssaR*, *PseD::*lacZ*, Sm<sup>R</sup>, Amp<sup>R</sup> | this work           |
| NB21              | SL1344 *ssaL*, *PseD::*lacZ*, Sm<sup>R</sup>, Amp<sup>R</sup> | this work           |
| NB18              | SL1344 *PseE::*lacZ*, Sm<sup>R</sup>, Amp<sup>R</sup> | this work           |
| NB20              | SL1344 *ssaR*, *PseE::*lacZ*, Sm<sup>R</sup>, Amp<sup>R</sup> | this work           |
| NB22              | SL1344 *ssaL*, *PseE::*lacZ*, Sm<sup>R</sup>, Amp<sup>R</sup> | this work           |
| **Escherichia coli** |                         |                     |
| DH5α              | general cloning strain  | our collection      |
| DH5α λpir         | for propagation of ρ-dependent plasmid | our collection      |
| SM10 λpir         | *thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km λpir* | (28)               |
| **Plasmids**      |                         |                     |
| PCR2.1            | Amp<sup>R</sup>, Kan<sup>R</sup>, general cloning plasmid | Invitrogen          |
| pWSK29            | pSC101 ori f<sup>1</sup> *ori lac2α*, Amp<sup>R</sup>, low copy vector | (48)               |
| pWSK129           | pSC101 *ori f<sup>1</sup> ori lacZα*, Km<sup>R</sup>, low copy vector | (48)               |
| pBKC-2HA          | pWSK129-based vector, contains 2HA (*BglII/HindIII*) | our collection      |
| pssrAB            | *ssrAB* cloned into pWSK29 | (23)               |
| pssaL             | produces SsaL from native promoter in pWSK29, Amp<sup>R</sup> | this work           |
| pssaL-2HA         | produces SsaL with C-terminal tandem HA fusion, Amp<sup>R</sup> | this work           |
| pRE112            | *oriT oriV sacB1*, Cm<sup>R</sup>, counterselectable suicide vector | (27)               |
| pIVET-sseA        | *sseA* promoter fused to *lacZ*, cloned into pIVET5n, Amp<sup>R</sup> | this work           |
| pRE112ΔssaL       | pRE112 carrying deletion allele of *ssaL* corresponding | this work           |
|                   | to amino acids 9 through 333, Cm<sup>R</sup> |                     |
| pssE-A<sub>Dc</sub> | 3487-bp fragment of *sseA* through *sseD* cloned into | this work           |
| pRE112ΔssaL       | pWSK129 under lac promoter |                     |
| pRE112ΔssaL       | pRE112 carrying deletion allele of *ssaL* corresponding | this work           |
|                   | to amino acids 9 through 333, Cm<sup>R</sup> |                     |
| pssE-A<sub>Dc</sub> | 3487-bp fragment of *sseA* through *sseD* cloned into | this work           |
| pRE112ΔssaL       | pWSK129 under lac promoter |                     |
| pRE112ΔssaL       | pRE112 carrying deletion allele of *ssaL* corresponding | this work           |
|                   | to amino acids 9 through 333, Cm<sup>R</sup> |                     |
| pssE-A<sub>Dc</sub> | 3487-bp fragment of *sseA* through *sseD* cloned into | this work           |
| pRE112ΔssaL       | pWSK129 under lac promoter |                     |
| pRE112ΔssaL       | pRE112 carrying deletion allele of *ssaL* corresponding | this work           |
|                   | to amino acids 9 through 333, Cm<sup>R</sup> |                     |
| **Other**         |                         |                     |
| Sm, streptomycin; Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin | |
A  Secreted

B  Whole cell lysate

C  Whole cell lysate (stationary phase)

D  Optical density

Coombes et al. Figure 1
A Whole cell lysate

|        | wt | ΔssaR | ΔssaL | ΔssaL (pssrAB) | invA::Kan |
|--------|----|-------|-------|---------------|-----------|
| SseB   |    |       |       |               |           |
| SseD   |    |       |       |               |           |
| DnaK   |    |       |       |               |           |

B

pellet

|        | wt | wt (pEHEC-sepL) | ΔssaL | ΔssaL (pEHEC-sepL) | ΔssaL (pEHEC-sepL) | ΔssaL (pssrAB) | ssrB::Kan |
|--------|----|----------------|-------|------------------|------------------|---------------|-----------|
| SseB   |    |                |       |                  |                  |               |           |
| secreted |    |                |       |                  |                  |               |           |
| DnaK   |    |                |       |                  |                  |               |           |

C

pellet

|        | wt | wt (pEHEC-sepL) | ΔssaL | ΔssaL (pEHEC-sepL) | ΔssaL (pEHEC-sepL) | ΔssaL (pEHEC-sepL) | ΔssaL (pEHEC-sepL) | ΔssaL (pssrAB) | ssrB::Kan |
|--------|----|----------------|-------|------------------|------------------|---------------|---------------|---------------|-----------|
| SseB   |    |                |       |                  |                  |               |               |               |           |
| secreted |    |                |       |                  |                  |               |               |               |           |
| DnaK   |    |                |       |                  |                  |               |               |               |           |

pH 7.0

pH 5.8

pellet

secreted

Coombes et al. Figure 2
A

Secreted

LPM (pH 7.0)  LPM (pH 5.8)

SseB

DnaK

B

Whole cell lysate

LPM (pH 7.0)  LPM (pH 5.8)

SseB

DnaK
A

LPM (pH 7.0)  LPM (pH 5.8)

pellet

α-HA

secreted

α-HA

LPM (pH 7.0)  LPM (pH 5.8)

pellet

DnaK

B

Whole cell lysate

pH 7.0  pH 5.8

α-HA

DnaK
A

SL1344 $\text{P}_{\text{sseA}}::\text{lacZ}$ fusion

B

C

Coombes et al. Figure 7
Coombes et al. Figure 8
Expression and secretion of salmonella pathogenicity island-2 virulence genes in response to acidification exhibit differential requirements of a functional type III secretion apparatus and SsaL

Brian K. Coombes, Nat F. Brown, Yanet Valdez, John H. Brumell and B. Brett Finlay

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