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Supplementary Information for

A novel synthetic bottom-up approach reveals the complex interplay of *Shigella* effectors in regulation of epithelial cell death.

Mou X, Souter S, Du J, Reeves AZ and Lesser CF

Cammie Lesser
Email: CLESSER@mgh.harvard.edu

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Supplementary Information Text

SI Materials and Methods

Bacterial strains. All bacterial strains are listed in Table S1. For each of the assays described below, the bacteria were grown using the following protocol. All incubations were carried out at 37°C with aeration in TCS (trypticase soy) broth. On the day of the assays, overnight cultures of mT3.1_*E. coli* and *Shigella* were back diluted at 1:40 and 1:100, respectively. When indicated, 1mM IPTG was added 1 hr post back dilution. After back dilution, the OD_{600} of each bacterial culture was measured. Based on these readings, equivalent numbers of bacteria from each culture were pelleted and used for the relevant assays.

Generation of mT3.1_*E. coli.* All primers and plasmids are listed in Table S2 and S3, respectively. pmT3SS.1 was generated using a modified version of the strategy described previously (1). Briefly, a DNA fragment that shares homology with *orfs131a* and *b* was PCR-amplified from the *Shigella* virulence plasmid and introduced in place of targeting sequence 2 (TS2) into pLLX13-*ipaJ-bla-spa40* via conventional cloning. The resulting new capture vector, pLLX13-*ipaJ-bla-orf131*, was digested with *MluI* and *PmeI* and introduced into VP_*E. coli* that carry pKD46 to generate pmT3SS.1 via homologous recombination. The resulting colonies were confirmed by colony PCR and next-generation sequencing. Once sequence verified, pmT3SS.1 and pNG162-VirB were introduced into DH10ß to generate mT3.1_*E. coli.*

Generation of IPTG-inducible expression plasmids. The IPTG-inducible expression plasmids for OspZ, OspI and OspD2_C79A were generated using the Gateway™ based cloning strategy described previously (2). Briefly, gene was amplified via PCR to introduce flanking *attB* sites as well as a consensus Shine-Dalgarno sequence upstream of each start codon. A stop codon was not included. The amplified fragments were introduced into pDNR221 to generate entry clones and once sequence verified transferred into pDSW206-ccdB-FLAG,

a Gateway-compatible destination plasmid (2) with an in-frame 3xFLAG tag and an *rrnB* terminator following a Gateway cassette.

Generation of Endogenous expression plasmids. These plasmids were also generated with Gateway™ cloning. First, fragments of DNA containing *ospC2, ospC3, ospD1, ospD2* and *virA* plus up to 466 of upstream nucleotides were PCR amplified from *Shigella* virulence plasmid DNA. In the case of *ipgB1*, which is encoded within an operon, a fragment of DNA containing *icsB, ipgA*, *ipgB1* plus 500 nucleotides located upstream of *icsB* was amplified. Fragments containing *ospC3* and *ospD2* were amplified both with and without stop codons. The other fragments were only amplified without a stop codon. Each fragment was introduced into pDNR221, sequence verified and transferred into pCMD136-ccdB-FLAG, a low-copy number plasmid engineered to contain a Gateway cassette followed by an in-frame 3xFLAG tag and an *rrnB* terminator.

Generation of *Shigella* deletion strains. The Δ*ospD2,* Δ*ospC3,* Δ*virA,* Δ*virA*Δ *ospD2 S. flexneri* strains were generated via homologous recombination using the strategy described previously (3). To generate Δ*virA*Δ*ospD2 Shigella,* the KAN^R cassette was removed from Δ*virA* using FLP recombinase (pCP20) before deleting *ospD2*.

Secretion assays. Secretion assays were conducted as previously described (4) with the following modifications. Briefly, 2.5 hrs post back dilution, an equivalent number of bacteria of each strain (normalized according to OD_{600} readings) was pelleted and designated as “bacteria”. An additional normalized aliquot of bacteria was pelleted and re-suspended in Phosphate-buffered saline (PBS) + 10 μM Congo red (Millipore Sigma #C6277) to activate the T3SA. After 30 min, the bacteria were centrifuged. The proteins in the supernatant fraction were precipitated in 10% (v/v) trichloroacetic acid. The proteins in the bacteria and supernatant fractions were separated by SDS/PAGE and analyzed by silver staining (for SepA) and immunoblotting using antibodies that recognize IpaB, IpaC and IpaD (Wendy Picking, University of Kansas), VirA (Marcia Goldberg,

Massachusetts General Hospital), OspF (Anthony Maurelli, University of Florida), FLAG (Millipore Sigma #F1804) and GroEL (Millipore Sigma # G6532).

Infection conditions. One day prior to infection, HeLa cells were seeded in tissue culture plate wells, as defined below, containing high-glucose DMEM media (Thermo Fisher Scientific #11965) plus 10% fetal bovine serum (FBS, Atlanta Biologicals # S11150). The following day, the cells were infected with bacteria in low-glucose DMEM media (Thermo Fisher Scientific #11054) plus 1% FBS. The plates were centrifuged at 800 x *g* for 10 min to promote bacteria-cell contact and when indicated 1 mM IPTG was added to cell culture media. After the indicated time of incubation, each well was washed twice with pre-warmed Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific #14025076) to remove excessive bacteria and then incubated in high-glucose DMEM plus 50 µg/ml gentamicin and 10% FBS.

Inside/outside microscopy. As previously described (4), HeLa cells were seeded in 6-well plates (3×10^5 cells/well) containing acetone-washed coverslips (Corning). Cells were infected as described in the *infection conditions* section at an MOI of 100. At 1 hr post infection, the coverslips were washed 5 times with PBS, and fixed with 3.7% (wt/vol) paraformaldehyde in PBS for 20 min. Extracellular bacteria were labeled with rabbit anti-*E. coli* (Abcam #ab137967) or anti-*Shigella* (Abcam #ab65282) polyclonal antibodies, followed by labeling with anti-rabbit Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific, #A11008). Cells were then permeabilized with 0.5% Triton X-100 in PBS for 20 min and stained with DAPI. Coverslips were mounted with mounting medium on glass slides and then examined with a 40x objective on a Nikon TE2000 florescent microscope with Chroma Technology filters. Bacteria labeled blue but not green were defined as intracellular and those labeled both were defined as extracellular. HeLa cells containing intracellular bacteria were defined as infected.

Cytotoxicity assay. HeLa cells seeded in 96-well plates (2 x 10^4 cells/well) were infected as described in the *infection conditions* section at an MOI of 100. At designated time points, cells were incubated with 16.2 μM Hoechst 33342 (Fisher Scientific #5117) plus 3 μM propidium iodide (Millipore Sigma # P4170) for 30 min before being imaged with a 4× objective on a Nikon TE2000 florescent microscope with Chroma Technology filters. Images were captured by iVision-Mac (BioVision Technologies) and the stained cell nuclei were identified and quantified using CellProfiler 2.0 (5). Cell nuclei labeled only blue were defined as live and those labeled both blue and red as dead. When indicated, Z-VAD-FMK (10 μM, Millipore Sigma #219007), ALLN (10uM, Millipore Sigma #208719), or MDL28170 (100uM, Millipore Sigma #208722) was used.

Translocation assays. As previously reported (6), HeLa cells seeded in 6-well plates (6 x 10^5 cells/well) were infected as described in the *infection conditions* section at an MOI of 30 for 60 min before addition of gentamicin to the media. At designated time points after washing each well 3 times with pre-warmed PBS, cells were incubated for 15 min on ice in 250 μl ice cold radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris, pH 8, 150 mM NaCl, 0.1% SDS, 1% NP-40 plus cOmplete™ cocktail protease inhibitors (Millipore Sigma #11836170001)) to lyse mammalian but not bacterial cells. A scraper was used to remove lysed cells from the plate and the mixture was centrifuged. The pellet fraction, containing intact bacteria was resuspended in Laemmli sample buffer while the supernatant was centrifuged a second time and the resulting supernatant was designated soluble fraction. Proteins in both fractions were separated via SDS/PAGE and analyzed by immunoblotting with antibodies against IpaB, IpaD, VirA, OspF, FLAG and β-actin (Abcam #ab49900).

Quantification of secreted IL-18. HeLa cells seeded in 96-well plates (2 x 10^4 cells/well) were infected as described in the *infection conditions* section at an MOI of 100. At designated time points, culture media from each well was transferred to a new multi-well plate. The levels of IL-18 in media were quantified

with an IL-18 Human ELISA Kit (Thermo Fisher Scientific, #BMS267-2) following manufacturer's instruction.

Gentamicin protection assays were performed as previously described(4). Briefly, HeLa cells seeded in 6-well plates (6×10^5 cells/well) were infected as described in the *infection conditions* section at an MOI of 100. At designated time points, after washing 3 times with pre-warmed HBSS, the infected cells were lysed with 0.2% (wt/vol) sodium deoxycholate. Serial dilutions of each well were plated to determine intracellular colony forming units (CFU).

Alignment of protein sequences was performed by LALIGN server (7) with the following settings: alignment method = global, all other parameters = default.

Statistical analysis. All data are expressed as mean ± standard deviation (SD). One-way ANOVA with Tukey post hoc test was performed for all experiments. $P < 0.05$ was considered statistically significant.

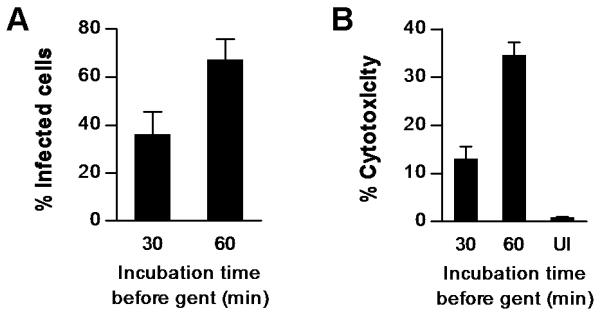

Fig. S1. Optimization of mT3.1_ *E. coli* infection conditions. (*A, B*) HeLa cells were infected with mT3.1_ *E. coli* at an MOI of 100 for 30 or 60 min before addition of gentamicin to the media. *(A)* Percentage of HeLa cells that contain intracellular bacteria at 1 hr post-infection, determined by inside/outside microscopy assay as described in Fig. 1. *(B)* Quantification of PI positive cells (% cytotoxicity) at 2.5 hrs post infection, as described in Fig. 2. UI = uninfected.

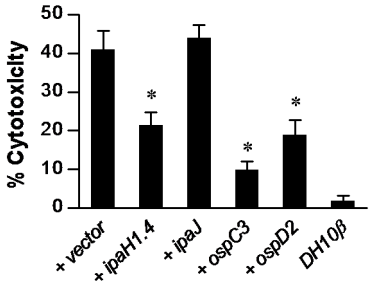

Fig. S2. Low throughput confirmatory studies of effectors that inhibit mT3.1_*E. coli*-triggered epithelial cell death. HeLa cells were infected with mT3.1_ *E. coli* at an MOI of 100 for 60 min before addition of gentamicin to the media. **P* < 0.05 as compared to (+) vector control. All data are the mean +/- SD of at least two experimental repeats, each with two technical replicates (n≥4).

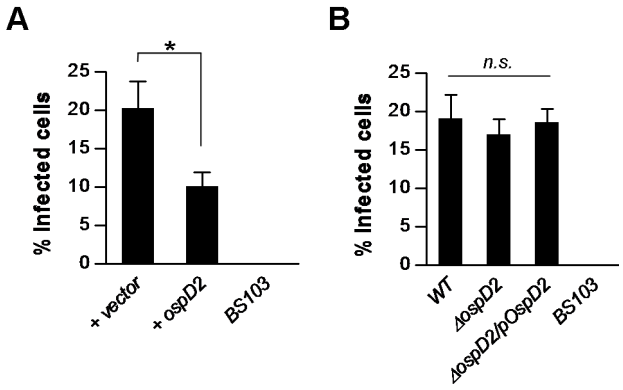

Fig. S3. Premature OspD2 expression inhibits epithelial host cell invasion. (A, B) HeLa cells were infected with the designated *Shigella* strains at an MOI of 100 for 20 min before addition of gentamicin to the media. BS103 lack the entire large *Shigella* virulence plasmid, which encodes its type III secretion system. Percentage of HeLa cells that contain intracellular bacteria was determined by inside/outside microscopy assay as described in Fig. 1, at 1 hr post-infection. Data are expressed as the mean +/- SD of three experimental repeats. *$P < 0.05$ as indicated.

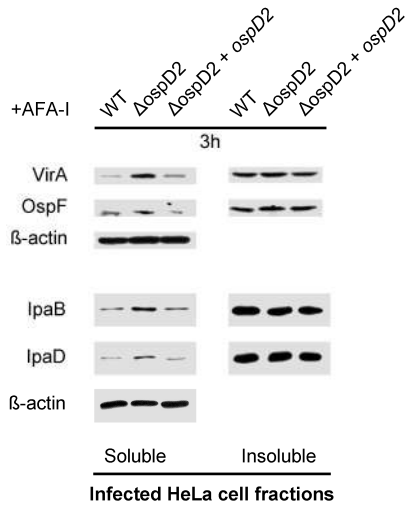

Fig S4. OspD2 acts post-invasion of host cells to inhibit T3SA activity. HeLa cells infected at an MOI of 30 with *Shigella* strains expressing AFA-I. Sixty minutes after the start of the infection, gentamicin was added to kill extracellular bacteria and IPTG was added to induce expression of OspD2-FLAG. Three hours later the cells were treated with RIPA buffer to lyse mammalian but not bacterial cell membranes. The soluble and insoluble (bacterial containing) fractions were separated and immunoblotted with designated antibodies. β-actin is the loading control for the soluble fraction. Each immunoblot is representative of at least three experimental repeats.

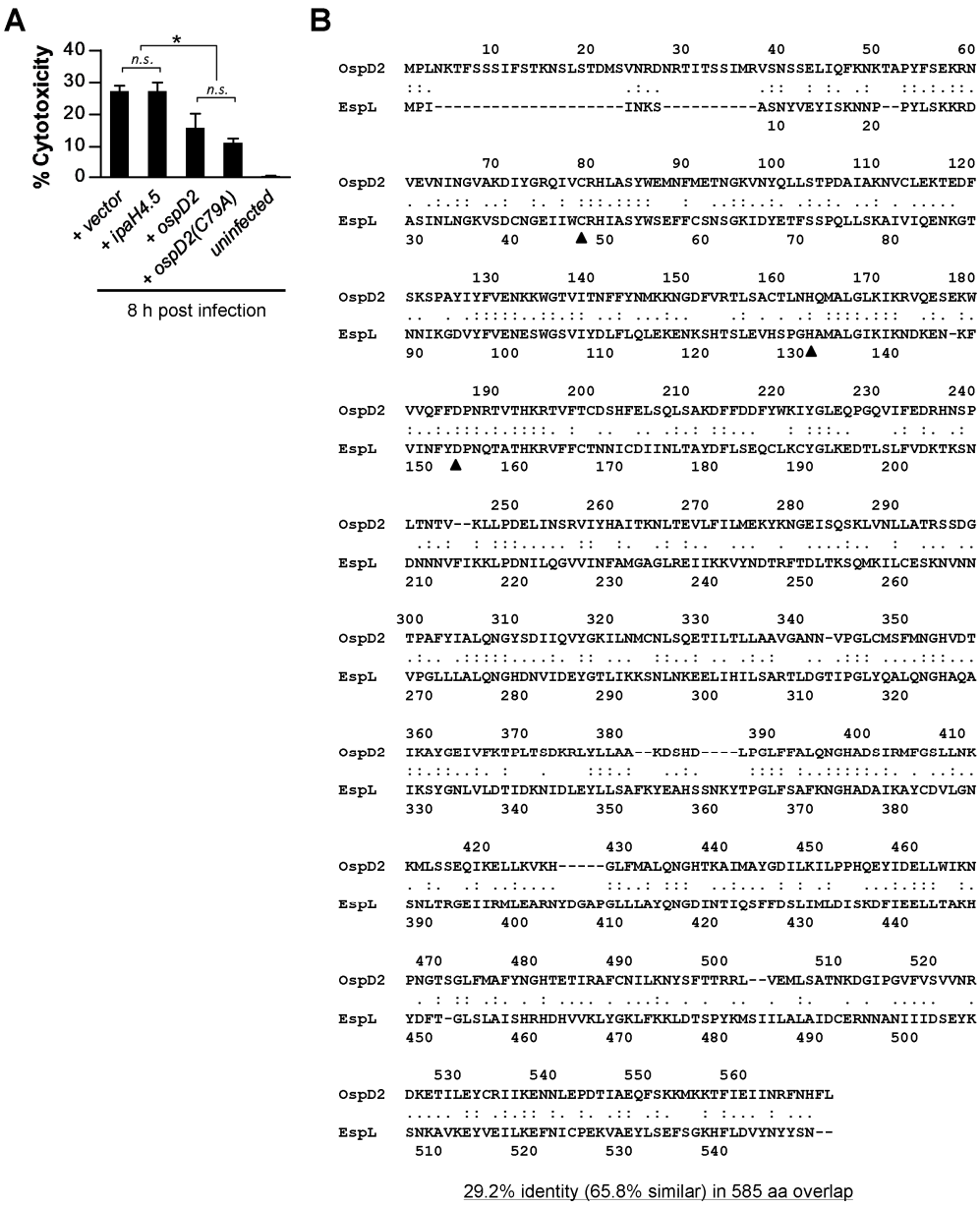

Fig. S5. The putative OspD2 cysteine protease domain does not play a role in suppressing *Shigella*-triggered epithelial cell death. (A) HeLa cells infected with each strain at an MOI of 100 for 20 min before addition of gentamicin to media. Expression of the designated FLAG-tagged effectors was induced 30 min post-invasion of host cells. At 8 hours post-infection, cells were labeled with Hoechst and PI and % cytotoxicity quantified as described in Fig. 2. **P* < 0.05 as compared to WT. All data are expressed as the mean +/- SD of at least two experimental repeats, each with two technical replicates (n≥4). (B) Protein

alignment of OspD2 and EspL was performed as described in SI methods. Single dots = similarity, double dots = identity, triangle = catalytic triad.

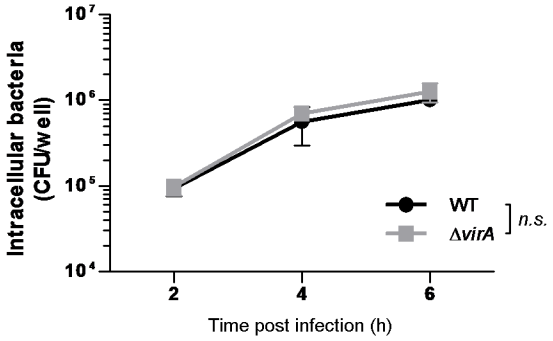

Fig. S6. Δ*virA Shigella* are not impaired in intracellular replication within infected epithelial cells. Quantification of bacteria within HeLa cells infected with WT or *ΔvirA Shigella* using the gentamicin protection assay (SI Methods). Cells were infected at an MOI of 100 for 20 min before addition of gentamicin. Data are expressed as the mean +/- SD of two experimental repeats (n=2).

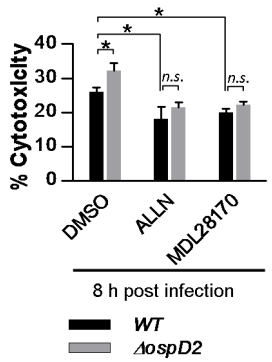

Fig. S7. Calpain inhibitors suppress WT and Δ*ospD2 Shigella* triggered epithelial cell death. HeLa cells pretreated with ALLN, MDL28170 or DMSO (vehicle control) for 3 hours were infected with WT or Δ*ospD2 Shigella* at an MOI of 100 for 20 min at which point gentamicin was added to the media. At 8 hours post infection, cells were labeled with Hoechst and PI and % cytotoxicity quantified as described in Fig. 2. All data are expressed as the mean +/- SD of at least two experimental repeats, each with two technical replicates (n≥4). **P* < 0.05 as indicated.

Table S1 | Bacterial strains used in this study.

Name	Description	Source
Shigella (or WT)	*S. flexneri* 2457T	(8)
BS103	*S. flexneri* 2457T: virulence plasmid cured	(9)
E. coli (or DH10ß)	*E. coli* DH10ß	Thermo Fisher Scientific
VP_*E. coli*	*E. coli*: pVPΔ*ipaJ*::KAN	(10)
mT3_*E. coli*	*E. coli*: pmT3SS + pNG162-virB	(10)
mT3.1_*E. coli*	*E. coli*: pmT3SS.1 + pNG162-virB	this study
mT3.1_*E. coli* + *ipaH1.4*	mT3.1_*E. coli*: pDSW206-ipaH1.4-FLAG	this study
mT3.1_*E. coli* + *ipaH4.5*	mT3.1_*E. coli*: pDSW206-ipaH4.5-FLAG	this study
mT3.1_*E. coli* + *ipaH7.8*	mT3.1_*E. coli*: pDSW206-ipaH7.8-FLAG	this study
mT3.1_*E. coli* + *ipaH9.8*	mT3.1_*E. coli*: pDSW206-ipaH9.8-FLAG	this study
mT3.1_*E. coli* + *ipaJ*	mT3.1_*E. coli*: pDSW206-ipaJ-FLAG	this study
mT3.1_*E. coli* + *ipgB2*	mT3.1_*E. coli*: pDSW206-ipgB2-FLAG	this study
mT3.1_*E. coli* + *ospB*	mT3.1_*E. coli*: pDSW206-ospB-FLAG	this study
mT3.1_*E. coli* + *ospC1*	mT3.1_*E. coli*: pDSW206-ospC1-FLAG	this study
mT3.1_*E. coli* + *ospC2*	mT3.1_*E. coli*: pDSW206-ospC2-FLAG	this study
mT3.1_*E. coli* + *ospC3*	mT3.1_*E. coli*: pDSW206-ospC3-FLAG	this study
mT3.1_*E. coli* + *ospD1*	mT3.1_*E. coli*: pDSW206-ospD1-FLAG	this study
mT3.1_*E. coli* + *ospD2*	mT3.1_*E. coli*: pDSW206-ospD2-FLAG	this study
mT3.1_*E. coli* + *ospD3*	mT3.1_*E. coli*: pDSW206-ospD3-FLAG	this study
mT3.1_*E. coli* + *ospE*	mT3.1_*E. coli*: pDSW206-ospE-FLAG	this study
mT3.1_*E. coli* + *ospF*	mT3.1_*E. coli*: pDSW206-ospF-FLAG	this study
mT3.1_*E. coli* + *ospG*	mT3.1_*E. coli*: pDSW206-ospG-FLAG	this study
mT3.1_*E. coli* + *ospI*	mT3.1_*E. coli*: pDSW206-ospI-FLAG	this study
mT3.1_*E. coli* + *ospZ*	mT3.1_*E. coli*: pDSW206-ospZ-FLAG	this study
mT3.1_*E. coli* + *virA*	mT3.1_*E. coli*: pDSW206-virA-FLAG	this study
mT3.1_*E. coli* + *icsB*	mT3.1_*E. coli*: pDSW206-icsB-FLAG	this study
mT3.1_*E. coli* + *ipaA*	mT3.1_*E. coli*: pDSW206-ipaA-FLAG	this study

mT3.1_*E. coli* + *ipgB1*	mT3.1_*E. coli*: pDSW206-ipgB1-FLAG	this study
mT3.1_*E. coli* + *ipgD*	mT3.1_*E. coli*: pDSW206-ipgD-FLAG	this study
Shigella + *ospD2*	*Shigella*: pDSW206-ospD2-FLAG	this study
Shigella + *ospD2*_untagged	*Shigella*: pDSW206-ospD2	this study
Shigella + *ipaH4.5*	*Shigella*: pDSW206-ipaH4.5-FLAG	this study
Shigella + *ospD2_C79A*	*Shigella*: pDSW206-ospD2_C79A-FLAG	this study
Shigella + endP_*ipaJ*	*Shigella*: pAM238-endP-ipaJ-FLAG	this study
Shigella + endP_*ospC2*	*Shigella*: pCMD136-endP-ospC2-FLAG	this study
Shigella + endP_*ospC3*	*Shigella*: pCMD136-endP-ospC3-FLAG	this study
Shigella + endP_*ospD1*	*Shigella*: pCMD136-endP-ospD1-FLAG	this study
Shigella + endP_*ospD2*	*Shigella*: pCMD136-endP-ospD2-FLAG	this study
Shigella + endP_*ipgB1*	*Shigella*: pCMD136-endP-IcsB-IpgA-IpgB1-FLAG	this study
Δ*ospD2*	*Shigella* Δ*ospD2*	this study
Δ*ospD2*/pOspD2	*Shigella* Δ*ospD2*: pCMD136-endP-ospD2	this study
WT/pAfaI	*Shigella*: pAfaI	this study
Δ*ospD2*/pAfaI	*Shigella* Δ*ospD2*: pAfaI	this study
Δ*ospD2*/pOspD2/pAfaI	*Shigella* Δ*ospD2*/pOspD2: pAfaI	this study
WT/pAfaI + *ipaH7.8*	WT/pAfaI: pDSW206-ipaH7.8-FLAG	this study
Δ*ospD2*/pAfaI + *ipaH7.8*	Δ*ospD2*/pAfaI: pDSW206-ipaH7.8-FLAG	this study
Δ*ospD2*/pOspD2/pAfaI + *ipaH7.8*	Δ*ospD2*/pOspD2/pAfaII: pDSW206-ipaH7.8-FLAG	this study
Δ*ospC3*	*Shigella* Δ*ospC3*	this study
Δ*ospC3*/pOspC3	*Shigella* Δ*ospC3*/pOspC3: pCMD136-endP-ospC3	this study
Δ*virA*	*Shigella* Δ*virA*	this study
Δ*virA*/pVirA	*Shigella* Δ*virA*/pVirA: pCMD136-endP-virA-FLAG	this study
Δ*virA*Δ*ospD2*	*Shigella* Δ*virA*Δ*ospD2*::FRT-KAN^{R}-FRT	this study
Δ*virA*Δ*ospD2*/pVirA	*Shigella* Δ*virA*Δ*ospD2*: pCMD136-endP-virA-FLAG	this study
Δ*virA*Δ*ospD2*/pOspD2	*Shigella* Δ*virA*Δ*ospD2*: pCMD136-endP-ospD2	this study

Table S2 | Primers used in this study. F = forward primer; R = reverse primer.

Name	Sequence	Description
Orf131a/b CV GBS F	ATTTTCACCGTTTTTTGTTTAAACGTTAACTCT AGAGGG**CCCGACTCATATTGCAATTG**	F. To generate pLLX13-ipaJ-bla-orf131. Underlined: pme I site; Bold: annealing to VP
Orf131a/b CV GBS R	TCTGGTACCCTGCAGGAGCTCTTGGCTTCAG GGATGAGGCGCCATC**AGTAAACATATCAAA AACAGGC**	R. To generate pLLX13-ipaJ-bla-orf131. Underlined: sac I site; Bold: annealing to VP
ospI_fwd_GW	C**GAAGGAGATAGAACC**ATGATTAATGGGGT GTCGTTACA	F. To generate pDNR221-ospI (open). Bold: Shine Dalgarno sequence (SD)
ospI_rev_NS	GGGGACAACTTTGTACAAGAAAGTTGGGCAA AGCCTCTTACTTTTCC	R. To generate pDNR221-ospI (open). Underlined: AttB2 site
ospZ_5GW_SD	C**GAAGGAGATAGAACC**ATGATTAGTCCCAT CAAGAATATTA	F. To generate pDNR221-ospZ (open). Bold: SD; Forward
ospZ_rev_NS	GGGGACAACTTTGTACAAGAAAGTTGGATAG ACTTTAATCTCTGGCG	R. To generate pDNR221-ospZ (open). Underlined: AttB2 site
5' universal	GGGGACAACTTTGTACAAAAAAGTTGGC**GAA GGAGATAGAACC**ATG	F. To generate pDNR221 clones that require SD. Underlined: AttB1 site; Bold: SD
ospC2_endP_GW	GGGGACAACTTTGTACAAAAAAGTTGGCCGC ACCACGCCTTCAGTAGCGGAGC	F. To generate pDNR221-endP-ospC2 (open). Underlined: attB1 site
ospC2_rev_NS	GGGGACAACTTTGTACAAGAAAGTTGGCCTC CCAAATCGTTTGCC	R. To generate pDNR221-endP-ospC2 (open). Underlined: attB2 site
ospC3_endP-GW	GGGGACAACTTTGTACAAAAAAGTTGGCTTG GGCCACGCAGGCCCCTAGCTGG	F. To generate pDNR221-endP-ospC3 (open/closed). Underlined: attB1 site
ospC3_rev_NS	GGGGACAACTTTGTACAAGAAAGTTGGTATC TCAAATCGTTTGCCTAATATTG	R. To generate pDNR221-endP-ospC3 (open). Underlined: attB2 site
ospC3_rev_FR	GGGGACAACTTTGTACAAGAAAGTTGGTTAT ATCTCAAATCGTTTGCCTAATATTG	R. To generate pDNR221-endP-ospC3 (closed). Underlined: attB2 site
ospD1_endP-GW	GGGGACAACTTTGTACAAAAAAGTTGGCTGG AGACGGTTAGCCCACTTCCCGG	F. To generate pDNR221-endP-ospD1 (open). Underlined: attB1 site
ospD1_rev_NS	GGGGACAACTTTGTACAAGAAAGTTGGAAAC AACTCTTGTATTTTGTCAGAAATAA	R. To generate pDNR221-endP-ospD1 (open). Underlined: attB2 site

ospD2_endP-GW	GGGGACAACTTTGTACAAAAAAGTTGGCAGAAGATGCCAGCTACACCACCTTG	F. To generate pDNR221-endP-ospD2 (open/closed). Underlined: attB1 site
ospD2_NS_GW	GGGGACAACTTTGTACAAGAAAGTTGGTAAAAAATGATTAAATCTATTTATTATTTCA	R. To generate all ospD2 open clones. Underlined: attB2 site
ospD2_rev_FR	GGGGACAACTTTGTACAAGAAAGTTGGTTATAAAAAATGATTAAATCTATTTATTATTTCA	R. To generate pDNR221-endP-ospD2 (closed). Underlined: attB2 site
icsB_endP-GW	GGGGACAACTTTGTACAAAAAAGTTGGCTTAATTTGTATTGCTTTGACGGTATACAG	F. To generate pDNR221-endP-ipgB1 (open). Underlined: attB1 site
ipgB_rev_NS	GGGGACAACTTTGTACAAGAAAGTTGGATTTGTATTGCTTTGACGGTATACAG	R. To generate pDNR221-endP-ipgB1 (open). Underlined: attB2 site
virA_endP_GW	GGGGACAACTTTGTACAAAAAAGTTGGCAACCACTTTGTTTTTCTATACACCC	F. To generate pDNR221-endP-virA (open). Underlined: attB1 site
virA_rev_NS	GGGGACAACTTTGTACAAGAAAGTTGGAACATCAGGAGATATGATGGCAA	R. To generate pDNR221-endP-virA (open). Underlined: attB2 site
pm128	CAATAGGAAGCTAGGTGACGGGCAACTATTTGTCTTCCATATA	R. To generate pDNR221-ospD2_C79A (open). Underlined: introduced mutation codon
pm127	TATATGGAAGACAAATAGTTGCCCGTCACCTAGCTTCCTATTG	F. To generate pDNR221-ospD2_C79A (open). Underlined: introduced mutation codon
ospD2 5W	TAGTATTATAACATCTCTATGGTTGTCTTCTATGCCGTTAGTGTAGGCTGGAGCTGCTTC	F. To generate ΔospD2. Underlined: annealing to pKD4
ospD2 3W	AACAGGTAATTACACAATTTAAATTACAGTCTCATAAAAACATATGAATATCCTCCTTAG	R. To generate ΔospD2. Underlined: annealing to pKD4
ospC3 5W	TGAAAAACATTTAGATCACTGTGCTAATACTGTGAAAAACGTGTAGGCTGGAGCTGCTTC	F. To generate ΔospC3. Underlined: annealing to pKD4
ospC3_3W_2	GACGACATTATTATTTGGCCGAGCTTTTAGGTCATATCTCCATATGAATATCCTCCTTAG	R. To generate ΔospC3. Underlined: annealing to pKD4
pm132	ATTAATAGGAAAATACATCAGGAGAAATCAAATGCAGACAGTGTAGGCTGGAGCTGCTTC	F. To generate ΔvirA. Underlined: annealing to pKD4
pm133	TTTACAGTCTGGCAGCCAATATAATATTGGCTTAAACATCCATATGAATATCCTCCTTAG	R. To generate ΔvirA. Underlined: annealing to pKD4

Table 3 | Plasmids used in this study.

Name	Description	Source
pVPΔ*ipaJ*::KAN	*S. flexneri* 2457T virulence plasmid with Δ*ipaJ*::FRT-KAN^R-FRT	(10)
pmT3SS	pLLX13 that carries T3SA genes from pVPΔ*ipaJ* (*ipaJ* thru *spa40*), incP ori, TET^R, KAN^R	(10)
pLLX13-*ipaJ-bla-spa40*	capture vector for making pmT3SS, incP ori, TET^R, KAN^R, AMP^R	(10)
pmT3SS.1	pLLX13 that carries T3SA genes from pVPΔ*ipaJ* (*ipaJ* thru *orf131b*), incP ori, TET^R, KAN^R	this study
pLLX13-*ipaJ-bla-orf131b*	capture vector for making pmT3SS.1, incP ori, TET^R, KAN^R, AMP^R	this study
pKD46	Temperature-sensitive plasmid; contains arabinose-inducible phage red recombinase gene, AMP^R	(3)
pNG162-virB	IPTG-inducible expression plasmid, pSC101 ori, SPEC^R	(10)
pAfaI	constitutive expression plasmid, p15A ori, CM^R	(11)
pDSW206-ccdB-FLAG	IPTG-inducible expression plasmid containing a gateway cassette and a 3x FLAG tag, ColE1 ori, AMP^R, CM^R	(2)
pDSW206	vector control, ColE1 ori, AMP^R	(2)
pDSW206-ipaH1.4-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ipaH4.5-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ipaH7.8-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ipaH9.8-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ipaJ-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ipgB2-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ospB-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ospC1-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ospC2-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ospC3-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ospD1-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ospD2-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ospD3-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ospE-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ospF-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ospG-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-virA-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-icsB-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)

pDSW206-ipaA-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ipgB1-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDSW206-ipgD-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	(2)
pDNR221	pDONR™221. Gateway entry plasmid containing a gateway cassette, pUC ori, KAN^R, CM^R	Thermo Fisher Scientific
pDNR221-ospI (open)	Gateway entry plasmid containing ospI ORF without a stop codon, pUC ori, KAN^R	this study
pDNR221-ospZ (open)	Gateway entry plasmid containing OspZ NS (without a stop codon), pUC ori, KAN^R	this study
pDNR221-ospD2_C79A (open)	Gateway entry plasmid containing ospD2 (C79A) NS, pUC ori, KAN^R	this study
pDNR223-ospD2 (closed)	Gateway entry plasmid containing ospD2 with a stop codon, pUC ori, $Spec^R$	(2)
pDSW206-ospI-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	this study
pDSW206-ospZ-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	this study
pDSW206-ospD2_C79A-FLAG	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	this study
pDSW206-ospD2	IPTG-inducible expression plasmid, ColE1 ori, AMP^R	this study
pDNR221-endP-ospC2 (open)	Gateway entry plasmid containing ospC2 NS and upstream 450 bp, pUC ori, KAN^R	this study
pDNR221-endP-ospC3 (open)	Gateway entry plasmid containing ospC3 NS and upstream 466 bp, pUC ori, KAN^R	this study
pDNR221-endP-ospD1 (open)	Gateway entry plasmid containing ospD1 ORF NS and upstream 424 bp, pUC ori, KAN^R	this study
pDNR221-endP-ospD2 (open)	Gateway entry plasmid containing ospD2 ORF NS and upstream 430 bp, pUC ori, KAN^R	this study
pDNR221-endP-icsB-ipgA-ipgB1 (open)	Gateway entry plasmid containing icsB-ipgA-ipgB1 ORF NS and upstream 500 bp, pUC ori, KAN^R	this study
pDNR221-endP-ospD2 (closed)	Gateway entry plasmid containing ospD2 and upstream 430 bp, pUC ori, KAN^R	this study
pDNR221-endP-ospC3 (closed)	Gateway entry plasmid containing ospC3 and upstream 466 bp, pUC ori, KAN^R	this study
pDNR221-endP-virA (open)	Gateway entry plasmid containing virA without a stop codon and upstream 252 bp, pUC ori, KAN^R	this study
pCMD136	Used for vector control, pSC101 ori, $SPEC^R$	Ann Hochschild Lab

pCMD136-ccdB-FLAG	Containing a gateway cassette and a 3x FLAG tag, pSC101 ori, $SPEC^R$, CM^R	this study
pCMD136-endP-ospC2-FLAG	Endogenous promoter expression plasmid, pSC101 ori, $SPEC^R$	this study
pCMD136-endP-ospC3-FLAG	Endogenous promoter expression plasmid, pSC101 ori, $SPEC^R$	this study
pCMD136-endP-ospD1-FLAG	Endogenous promoter expression plasmid, pSC101 ori, $SPEC^R$	this study
pCMD136-endP-ospD2-FLAG	Endogenous promoter expression plasmid, pSC101 ori, $SPEC^R$	this study
pCMD136-endP-icsB-ipgA-ipgB1-FLAG	Endogenous promoter expression plasmid, pSC101 ori, $SPEC^R$	this study
pOspD2 (pCMD136-endP-ospD2)	Endogenous promoter expression plasmid, pSC101 ori, $SPEC^R$	this study
pOspC3 (pCMD136-endP-ospC3)	Endogenous promoter expression plasmid, pSC101 ori, $SPEC^R$	this study
pVirA (pCMD136-endP-virA-FLAG)	Endogenous promoter expression plasmid, pSC101 ori, $SPEC^R$	this study
pAM238-endP-ipaJ-FLAG	Endogenous promoter expression plasmid, pSC101 ori, $SPEC^R$	(6)

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