Identification of Novel Type III Secretion System Determinants in *Shigella flexneri*

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Summary

Many Gram-negative bacteria, such as *Shigella*, *Salmonella*, *Yersinia* and pathogenic *Escherichia* spp., utilize type III secretion systems (T3SSs) to inject virulence proteins into the cytosol of eukaryotic host cells. T3SSs are complex nanomachines that assemble within and span the bacterial cellular envelope. While the structure of the type III secretion apparatus (T3SA) is relatively conserved, each pathogen delivers its own unique set of proteins. These translocated virulence proteins, referred to as effectors, modulate host cellular processes to promote bacterial replication and spread.

Effectors are traditionally defined by proteinaceous signals and are post-translationally delivered to the membrane embedded T3SA via interactions with type III secretion chaperones. In this study, the requirement of T3S chaperones in effector secretion was assessed in *Shigella flexneri*. The development of a high-throughput plate-based secretion assay enabled the side-by-side analysis of numerous effectors from mutant strains lacking known chaperones. Strikingly, in the absence of all known T3S chaperones the secretion of the majority of effectors was unaffected, suggesting that unidentified secretion factors or chaperone-independent secretion pathways may exist.

Since effector gene expression is tightly regulated, global RNA sequencing was conducted to identify novel factors involved in the type III secretion of effectors. This led to the identification of FinO and YgaC. Previously only linked to the regulation of plasmid conjugation, the RNA chaperone FinO was demonstrated to mediate the secretion of a subset of effectors that were not yet associated with any of the characterized T3S chaperones. FinO promoted effector secretion via recognition of structural motifs located in the 5’ untranslated regions of effector mRNAs in a translation-dependent manner. YgaC, a chromosomally encoded protein of unknown function promoted translation-dependent secretion of multiple effectors. The identification of mRNA-encoded secretion determinants and new chaperones involved in translation-dependent secretion point to a co-translational pathway and provide additional insights into type III effector secretion. Given the structural conservation of T3SAs and the prevalence of YgaC and FinO-domain proteins, it is possible that they may play a similar role in T3SSs of other Gram-negative pathogens.

Introduction

Type III secretion systems are major virulence factors

Type III secretion systems (T3SSs) are essential membrane-spanning complex nanomachines utilized by many Gram-negative human and plant pathogens, such as *Shigella*, *Salmonella*, *Yersinia*, *Escherichia* and *Erwinia* spp.[1,2,3,4]. T3SSs inject tens of proteins, referred to as effectors, into targeted eukaryotic cells[5]. The translocated effectors usurp host cellular processes to promote bacterial survival and spread**.** While the structural components of type III secretion apparatuses (T3SAs) are highly conserved, each pathogen translocates its own unique set of effectors into host cells, likely accounting for the variety of strategies they use to establish an infection[6]. The virulence-associated T3SA is evolutionary related and shares structural homology with motility-conferring flagellar T3SSs[7,8].

T3SAs are composed of 20-25 different structurally conserved proteins. These proteins form multiple complexes, that include the extracellular needle, the envelope-embedded basal body and associated export apparatus, and cytosolic components[9,10] (Figure 1). The basal body includes the inner rod, which anchors the protruding needle, a hollow tube that serves as a conduit for the delivery of unfolded effectors into the host cell cytosol[11]. The translocation of effectors requires the translocon, a complex of secreted proteins that form a pore within the host cell membrane and allows docking of the T3SA needle[12,13,14].

Effectors are delivered to the cytosolic surface of the membrane-embedded T3SA via components of the dynamic sorting platform through interactions with type III secretion chaperones[15,16,17,18]. Upon docking of the sorting platform, the T3SA undergoes a conformational rearrangement[19,20]. At this time, chaperone-translocon component and chaperone-effector complexes bind the T3SS-specific ATPase, which energizes their dissociation to facilitate the loading of the unfolded protein into the T3SA export apparatus[21,22]. These proteins are then transported through the narrow needle into the cytosol of host cells using energy generated by the proton motive force[23].

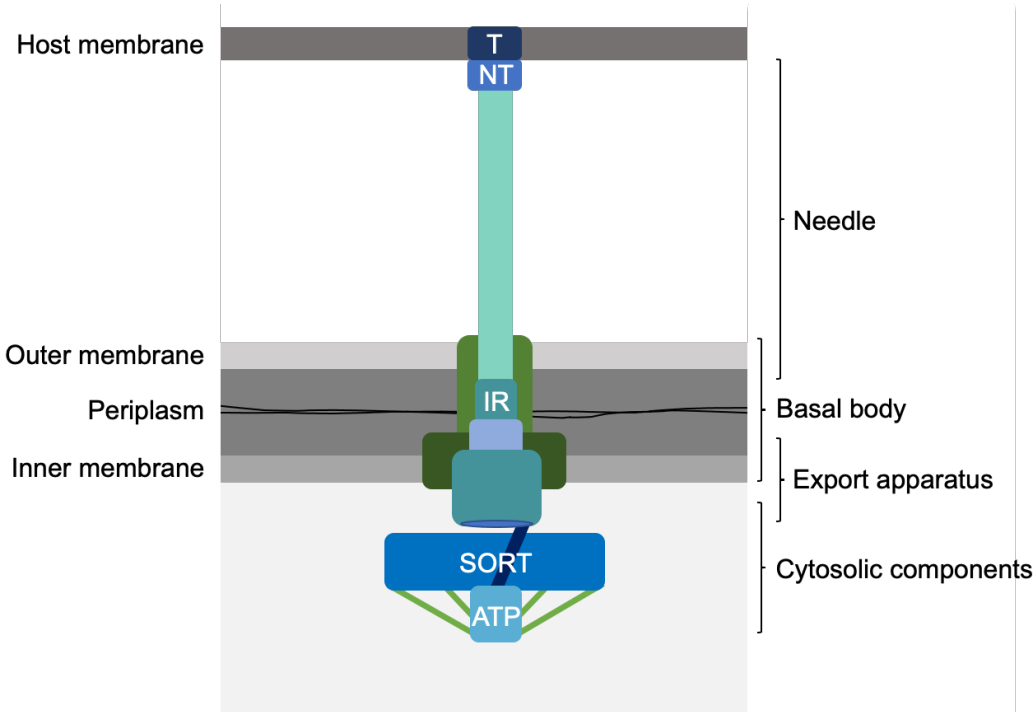

Figure 1. Structural schematic of the type III secretion apparatus. T, translocon; NT, needle tip; IR, inner rod; SORT, sorting platform; ATP, ATPase.

The multiple roles of type III secretion chaperones

Type III secretion class I and class II chaperones bind to and promote the secretion of effectors and translocon components, respectively[24,25,26]. Chaperones mediate the binding of the secretion-destined proteins to the dynamic sorting platform in a sequential manner, controlling their order of secretion. Prior to the activation of the T3SA, class II chaperones are bound to the sorting platform, ensuring that translocases are the first proteins delivered into host cells. Post secretion of the translocon components, class I chaperones are found associated with the sorting platform[15]. In the absence of their cognate chaperones, the secretion of effectors is markedly diminished or abolished.

In comparison to large molecular chaperones, T3S chaperones are small, acidic proteins. They do not assist in protein folding, but rather bind as homodimers to effector protein domains generally located within the first 50 to 100 amino acids[27,28]. In addition to promoting secretion, some chaperones stabilize their bound proteins or mask hydrophobic effector regions that function as host cell membrane targeting domains[29].

There are a few reports of effectors, that require two different chaperones for efficient secretion[30,31,32]. Lastly, post secretion of translocon components, there are examples of class II chaperones that modulate the activity of factors involved in regulating effector transcription[33,34,35,36,37].

Despite the growing evidence for the role of the sorting platform in promoting the timely secretion of effectors, T3S chaperones have only been identified to bind to and/or mediate the secretion of approximately a third of the effectors of the well-studied *Shigella* Mxi-Spa, *Salmonella* Pathogenicity Island 1 (SPI-1) or 2 (SPI-2), *Yersinia* Ysc and pathogenic *Escherichia coli* (EPEC/EHEC) Esc T3SSs (Table 1)[38-45]. These observations question the commonly held notion that effectors are targeted for secretion via interactions with their cognate chaperones and raise the intriguing possibility of the existence of new classes of chaperones and/or chaperone-independent type III secretion pathway(s).

Type III secreted proteins are defined by a set of signals

Type III secreted proteins are traditionally described as containing a bipartite secretion signal within their first 50 to 100 residues composed of an extreme amino(N)-terminal sequence followed by a downstream chaperone binding domain[27,46]. The N-terminal secretion sequence is essential for secretion and is characterized by its intrinsically disordered nature[47]. Furthermore, evidence exists that structural motifs common to chaperone-effector complexes function as general three-dimensional secretion signals[48].

mRNA-encoded sequences can also play a role in defining effectors as secreted proteins[49]. The first non-proteinaceous type III secretion signal was identified in the mRNA of *Yersinia* YopE[50]. The fusion of the native 5' untranslated region (UTR) and the first 45 nucleotides of the *yopE* coding sequence to a heterologous protein is sufficient to generate a variant recognized as a type III secreted protein. Similarly, the fusion of the 5'UTR of a subset of *Salmonella* SPI-2 effectors or the 5'UTR of *fliC,* the needle filament subunit of the *Salmonella* flagellar T3SS, is sufficient to define a reporter protein as a type III secreted substrate[51,52]. Notably, a role for these mRNA secretion signals was only revealed in the absence of their chaperone-binding domains, raising questions regarding

Table 1: Summary of effectors and chaperones of common T3SS-utilizing bacteria.

Effector	Chaperone	Effector	Chaperone	Effector	Chaperone
***Shigella spp.* (Mxi-Spa)**		***Salmonella* (SPI-1 T3SS)**		***Escherichia coli* (Esc T3SS)**	
IcsB	IpgA	SipA (SspA)	InvB	Tir	CesT
IpgD	IpgE	SopA	InvB	Map	CesT
IpaA	Spa15	SopE2	InvB	EspH	CesT
IpgB1	Spa15	SopE1	InvB	EspJ	CesT
IpgB2	Spa15	SopB	SigE	EspZ	CesT
OspB	Spa15	AvrA	-	NleA/EspI	CesT
OspC1	Spa15	SopD	-	NleB1	CesT
OspC2	Spa15	SptP	SicP	NleB2	CesT
OspC3	Spa15	SspH1	-	NleC	CesT
OspD1	Spa15	SpvC	-	NleG	CesT
OspD2	Spa15	SlrP	-	NleH1	CesT
IpaH1.4	-			NleH2	CesT
IpaH4.5	-	***Salmonella* (SPI-2 T3SS)**		EspF	CesF
IpaH7.8	-	PipB	-	EspFU	-
IpaH9.8	-	PipB2	SrcA	EspG	-
IpaJ	-	SseL	SrcA	EspG2	-
OspD3	-	SopD2	-	EspK	-
OspE1	-	SifA	-	EspL	-
OspE2	-	SseJ	-	EspL2	-
OspF	-	SseI	-	EspM	-
OspG	-	SspH2	-	EspN	-
OspI	-	SpvB	-	EspO	-
OspZ	-	SseF	SscB	EspR	-
VirA	-	SseG	SscB?	EspS	-
cIpaH2	-	SteC	-	EspT	-
cIpaH3	-	Slrp1	-	EspV	-
cIpaH4	-	GogB	-	EspW	-
cIpaH5	-	SifB	-	EspX	-
cIpaH7	-	SpiC	-	EspY	-
		SseK1	-	NleB3	-
***Yersinia* (Ysc T3SS)**		SseK2	-	NleD	-
YopE	SycE	SteA	-	NleE	-
YopJ	-	SteB	-	NleE2	-
YopH	SycH			NleF	-
YopM	-			NleH3	-
YopT	SycT			NleI	-
YpkA/YopO	SycO			NleJ	-
YopN	SycN/YscB			NleK	-
YopQ/K	-			NleL (ESPX7)	-
				Cif	-
				Efa1	-

the relative contributions of the mRNA- and protein-encoded sequences in promoting effector secretion. The existence of mRNA-encoded type III secretion signals suggests an effector secretion pathway that is linked to translation. Consistent with this hypothesis, in pulse-chase experiments, radioactively-labelled secreted *Yersinia* YopE is observed immediately after the addition of labelled methionine, but not after the following addition of unlabeled methionine (chase)[53]. In addition, in the absence of its T3S chaperone, the secretion of *Shigella* IpaA requires active translation[54]. These studies suggest the existence of post- and co-translational secretion pathways, presumably directed via distinct mRNA- and protein-encoded secretion signals.

Shigella spp. are causative agents of bacillary dysentery in humans

Shigella spp. (*S. flexneri, S. boydii, S. sonnei* and *S. dysenteriae*) are pathogenic members of the *Enterobacteriaceae* family. They are well adapted to humans and highly infectious. As few as 10 to 100 bacteria are sufficient to cause bacillary dysentery (shigellosis), a severe and often bloody diarrheal disease. Shigellosis is the leading worldwide cause of diarrheal mortality in individuals over the age of five[55,56].

Shigella spp. are professional intracytoplasmic pathogens that are transmitted via a fecal-oral route. Upon reaching the colon, they traverse the intestinal epithelium via transcytosis by microfold (M) cells. Once released, they are engulfed by resident macrophages, which subsequently undergo rapid cell death via pyroptosis[57,58]. In this way, *Shigella* gain access to and invade the basolateral surface of intestinal epithelial cells. Once inside, they rapidly escape from phagosomes and replicate to high titers within the cytosol while spreading to adjacent epithelial cells using actin-based motility[59]. Each of these steps is, at least in part, dependent on the Mxi-Spa type III secretion system.

Evidence for chaperone-independence in *Shigella* type III secretion

Shigella spp. translocate ~30 different effectors into host cells. Almost all effectors, known chaperones, as well as structural components and regulators needed to form the T3SA are encoded on a large ~220kb virulence plasmid (VP)[60]. Growth at 37°C signals host environment and induces the expression and assembly of the T3SA, which is held in an

inactive off configuration until it makes host cell contact[61-63]. *Shigella* effector expression is tightly regulated and occurs in two waves. The transcription of first wave effectors and the structural components of the T3SA are coordinately controlled by VirB[64]. Once the secretion of first wave effectors is initiated, MxiE, the transcription factor that controls the expression of the second effector wave is activated[33]. Prior to activation of the T3SA, MxiE is held in an off configuration via binding to its anti-activator, OspD1, a Spa15-chaperoned first wave effector[65] (Figure 2). Once OspD1 is secreted, MxiE is activated by the class II chaperone, IpgC, subsequently binding to defined regulatory boxes within second wave effector promoters and inducing their gene expression[66,67].

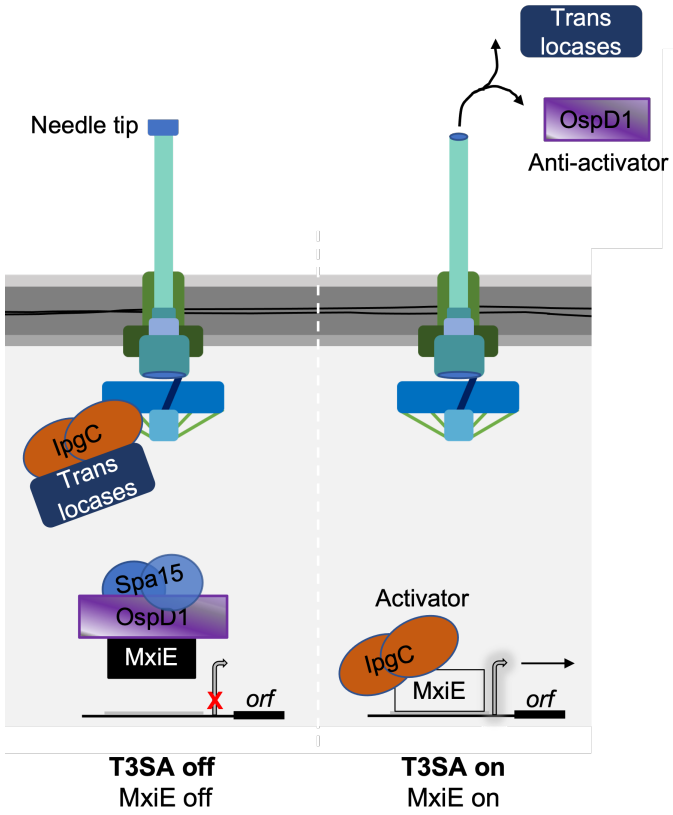

Figure 2. Schematic of the secretion-dependent regulation of MxiE activity. *orf*, open reading frame; T3SA, type III secretion apparatus.

Prior to secretion activation, almost all *Shigella* first wave effectors are held in complex with one of the class I effector chaperones, IpgA, IpgE or Spa15. IpgA and IpgE are class IA chaperones that bind to and promote the secretion of IcsB and IpgD, respectively, while Spa15, a multicargo class IB chaperone, binds to and promotes the secretion of nine effectors[54,68-72]. Prior systematic yeast-based protein interaction screens detected interactions between each of the three *Shigella* chaperones and their known substrates[73]. With exception of Spa15 and IpaH1.4, no interactions were detected between IpgA, IpgE and Spa15 and the remaining effectors, the majority of which are second wave effectors. This resulted in a hypothesis that these effectors are secreted independently of known chaperones, which was further supported by their unimpaired secretion observed in absence of the *Shigella* multicargo chaperone, Spa15.

These observations question the commonly held notion on the essential role of chaperones in effector secretion and raise the intriguing possibility of the existence of yet unidentified chaperones or chaperone-independent type III secretion pathways. This work (i) examines the requirement of known T3S chaperones in *Shigella* effector secretion and (ii) investigates the existence of uncharacterized secretion factors. Furthermore, it focusses on (iii) understanding how effectors are defined as secreted substrates allowing their recognition by the T3SA and (iv) investigates if chaperone-independent type III secretion pathways exist.

REFERENCES

1. Andrews, G. P. & Maurelli, A. T. mxiA of *Shigella flexneri* 2a, which facilitates export of invasion plasmid antigens, encodes a homolog of the low-calcium-response protein, LcrD, of *Yersinia pestis. Infect. Immun.* 60, 3287–3295 (1992).
2. Troisfontaines, P. & Cornelis, G. R. Type III secretion: more systems than you think. *Physiol. Bethesda Md* 20, 326–339 (2005).
3. Kubori, T., Sukhan, A., Aizawa, S. I. & Galán, J. E. Molecular characterization and assembly of the needle complex of the *Salmonella typhimurium* type III protein secretion system. *Proc. Natl. Acad. Sci. U. S. A.* 97, 10225–10230 (2000).
4. Daniell, S. J. *et al.* The filamentous type III secretion translocon of enteropathogenic *Escherichia coli. Cell. Microbiol.* 3, 865–871 (2001).

5. Gauthier, A., Thomas, N. A. & Finlay, B. B. Bacterial Injection Machines. *J. Biol. Chem.* 278, 25273–25276 (2003).
6. Galán, J. E., Lara-Tejero, M., Marlovits, T. C. & Wagner, S. Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. *Annu. Rev. Microbiol.* 68, 415–438 (2014).
7. Pallen, M. J., Beatson, S. A. & Bailey, C. M. Bioinformatics, genomics and evolution of non-flagellar type-III secretion systems: a Darwinian perspective. *FEMS Microbiol. Rev.* 29, 201–229 (2005).
8. Abby, S. S. & Rocha, E. P. C. The Non-Flagellar Type III Secretion System Evolved from the Bacterial Flagellum and Diversified into Host-Cell Adapted Systems. *PLoS Genet.* 8, e1002983 (2012).
9. Kubori, T. *et al.* Supramolecular structure of the S*almonella typhimurium* type III protein secretion system. *Science* 280, 602–605 (1998).
10. Diepold, A., Kudryashev, M., Delalez, N. J., Berry, R. M. & Armitage, J. P. Composition, Formation, and Regulation of the Cytosolic C-ring, a Dynamic Component of the Type III Secretion Injectisome. *PLOS Biol.* 13, e1002039 (2015).
11. Radics, J., Königsmaier, L. & Marlovits, T. C. Structure of a pathogenic type 3 secretion system in action. *Nat. Struct. Mol. Biol.* 21, 82–87 (2014).
12. Collazo, C. M. & Galán, J. E. The invasion-associated type III system of *Salmonella typhimurium* directs the translocation of Sip proteins into the host cell. *Mol. Microbiol.* 24, 747–756 (1997).
13. Blocker, A. *et al.* The tripartite type III secreton of *Shigella flexneri* inserts IpaB and IpaC into host membranes. *J. Cell Biol.* 147, 683–693 (1999).
14. Blocker, A. *et al.* Structure and composition of the *Shigella flexneri* 'needle complex', a part of its type III secreton. *Mol. Microbiol.* 39, 652–663 (2001).
15. Lara-Tejero, M., Kato, J., Wagner, S., Liu, X. & Galan, J. E. A Sorting Platform Determines the Order of Protein Secretion in Bacterial Type III Systems. *Science* 331, 1188–1191 (2011).
16. Nans, A., Kudryashev, M., Saibil, H. R. & Hayward, R. D. Structure of a bacterial type III secretion system in contact with a host membrane in situ. *Nat. Commun.* 6, 10114 (2015).
17. Diepold, A. *et al.* A dynamic and adaptive network of cytosolic interactions governs protein export by the T3SS injectisome. *Nat. Commun.* 8, 15940 (2017).
18. Makino, F. *et al.* The Architecture of the Cytoplasmic Region of Type III Secretion Systems. *Sci. Rep.* 6, 33341 (2016).
19. Hu, B., Lara-Tejero, M., Kong, Q., Galán, J. E. & Liu, J. In Situ Molecular Architecture of the *Salmonella* Type III Secretion Machine. *Cell* 168, 1065-1074.e10 (2017).

20. Marlovits, T. C. *et al.* Structural insights into the assembly of the type III secretion needle complex. *Science* 306, 1040–1042 (2004).
21. Allison, S. E. *et al.* Identification of the docking site between a type III secretion system ATPase and a chaperone for effector cargo. *J. Biol. Chem.* 289, 23734–23744 (2014).
22. Thomas, N. A. *et al.* CesT is a multi-effector chaperone and recruitment factor required for the efficient type III secretion of both LEE- and non-LEE-encoded effectors of enteropathogenic *Escherichia coli*. *Mol. Microbiol.* 57, 1762–1779 (2005).
23. Wilharm, G. *et al. Yersinia enterocolitica* type III secretion depends on the proton motive force but not on the flagellar motor components MotA and MotB. *Infect. Immun.* 72, 4004–4009 (2004).
24. Parsot, C. The various and varying roles of specific chaperones in type III secretion systems. *Curr. Opin. Microbiol.* 6, 7–14 (2003).
25. Thomas, N. A., Ma, I., Prasad, M. E. & Rafuse, C. Expanded roles for multicargo and class 1B effector chaperones in type III secretion. *J. Bacteriol.* 194, 3767–3773 (2012).
26. Ménard, R., Sansonetti, P., Parsot, C. & Vasselon, T. Extracellular association and cytoplasmic partitioning of the IpaB and IpaC invasins of *S. flexneri. Cell* 79, 515–525 (1994).
27. Sory, M. P., Boland, A., Lambermont, I. & Cornelis, G. R. Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the cyaA gene fusion approach. *Proc. Natl. Acad. Sci.* 92, 11998–12002 (1995).
28. Schesser, K., Frithz-Lindsten, E. & Wolf-Watz, H. Delineation and mutational analysis of the *Yersinia pseudotuberculosis* YopE domains which mediate translocation across bacterial and eukaryotic cellular membranes. *J. Bacteriol.* 178, 7227–7233 (1996).
29. Costa, S. C. P. & Lesser, C. F. A Multifunctional Region of the *Shigella* Type 3 Effector IpgB1 Is Important for Secretion from Bacteria and Membrane Targeting in Eukaryotic Cells. *PLoS ONE* 9, e93461 (2014).
30. Cao, H. *et al.* Novel T3SS effector EseK in *Edwardsiella piscicida* is chaperoned by EscH and EscS to express virulence. *Cell. Microbiol.* 20, (2018).
31. Day, J. B. & Plano, G. V. A complex composed of SycN and YscB functions as a specific chaperone for YopN in *Yersinia pestis*. *Mol. Microbiol.* 30, 777–788 (1998).
32. Silva-Herzog, E. *et al.* Scc1 (CP0432) and Scc4 (CP0033) function as a type III secretion chaperone for CopN of *Chlamydia pneumoniae*. *J. Bacteriol.* 193, 3490–3496 (2011).

33. Mavris, M. *et al.* Regulation of transcription by the activity of the *Shigella flexneri* type III secretion apparatus. *Mol. Microbiol.* 43, 1543–1553 (2002).
34. Bergman, T. *et al.* Analysis of the V antigen lcrGVH-yopBD operon of *Yersinia pseudotuberculosis*: evidence for a regulatory role of LcrH and LcrV. *J. Bacteriol.* 173, 1607–1616 (1991).
35. Darwin, K. H. & Miller, V. L. Type III secretion chaperone-dependent regulation: activation of virulence genes by SicA and InvF in *Salmonella typhimurium*. *EMBO J.* 20, 1850–1862 (2001).
36. Tucker, S. C. & Galán, J. E. Complex function for SicA, a *Salmonella enterica* serovar *typhimurium* type III secretion-associated chaperone. *J. Bacteriol.* 182, 2262–2268 (2000).
37. Francis, M. S., Lloyd, S. A. & Wolf-Watz, H. The type III secretion chaperone LcrH co-operates with YopD to establish a negative, regulatory loop for control of Yop synthesis in *Yersinia pseudotuberculosis*. *Mol. Microbiol.* 42, 1075–1093 (2001).
38. Letzelter, M. *et al.* The discovery of SycO highlights a new function for type III secretion effector chaperones. *EMBO J.* 25, 3223–3233 (2006).
39. Wattiau, P., Bernier, B., Deslee, P., Michiels, T. & Cornelis, G. R. Individual chaperones required for Yop secretion by Yersinia. *Proc. Natl. Acad. Sci.* 91, 10493–10497 (1994).
40. Trülzsch, K. *et al.* Analysis of chaperone-dependent Yop secretion/translocation and effector function using a mini-virulence plasmid of *Yersinia enterocolitica*. *Int. J. Med. Microbiol.* 293, 167–177 (2003).
41. Hong, K. H. & Miller, V. L. Identification of a novel *Salmonella* invasion locus homologous to *Shigella* ipgDE. *J. Bacteriol.* 180, 1793–1802 (1998).
42. Ehrbar, K., Hapfelmeier, S., Stecher, B. & Hardt, W.-D. InvB Is Required for Type III-Dependent Secretion of SopA in *Salmonella enterica* Serovar *Typhimurium*. *J. Bacteriol.* 186, 1215–1219 (2004).
43. Bronstein, P. A., Miao, E. A. & Miller, S. I. InvB Is a Type III Secretion Chaperone Specific for SspA. *J. Bacteriol.* 182, 6638–6644 (2000).
44. Ho Lee, S. & Galán, J. E. InvBIs a Type III Secretion-Associated Chaperone for the *Salmonella enterica* Effector ProteinSopE. *J. Bacteriol.* 185, 7279–7284 (2003).
45. Fu, Y. & Galán, J. E. Identification of a specific chaperone for SptP, a substrate of the centisome 63 type III secretion system of *Salmonella typhimurium*. *J. Bacteriol.* 180, 3393–3399 (1998).
46. Chen, L. *et al.* Substrate-Activated Conformational Switch on Chaperones Encodes a Targeting Signal in Type III Secretion. *Cell Rep.* 3, 709–715 (2013).

47. Buchko, G. W. *et al.* A multi-pronged search for a common structural motif in the secretion signal of *Salmonella enterica* serovar *Typhimurium* type III effector proteins. *Mol. Biosyst.* 6, 2448–2458 (2010).
48. Birtalan, S. C., Phillips, R. M. & Ghosh, P. Three-Dimensional Secretion Signals in Chaperone-Effector Complexes of Bacterial Pathogens. *Mol. Cell* 9, 971–980 (2002).
49. Anderson, D. M. & Schneewind, O. *Yersinia enterocolitica* type III secretion: an mRNA signal that couples translation and secretion of YopQ. *Mol. Microbiol.* 31, 1139–1148 (1999).
50. Anderson, D. M. & Schneewind, O. A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. *Science* 278, 1140–1143 (1997).
51. Niemann, G. S. *et al.* RNA Type III Secretion Signals That Require Hfq. *J. Bacteriol.* 195, 2119–2125 (2013).
52. Majander, K. *et al.* Extracellular secretion of polypeptides using a modified *Escherichia coli* flagellar secretion apparatus. *Nat. Biotechnol.* 23, 475–481 (2005).
53. Cheng, L. W., Anderson, D. M. & Schneewind, O. Two independent type III secretion mechanisms for YopE in *Yersinia enterocolitica*. *Mol. Microbiol.* 24, 757–765 (1997).
54. Page, A.-L., Sansonetti, P. & Parsot, C. Spa15 of *Shigella flexneri*, a third type of chaperone in the type III secretion pathway. *Mol. Microbiol.* 43, 1533–1542 (2002).
55. Ashida, H., Ogawa, M., Mimuro, H. & Sasakawa, C. *Shigella* infection of intestinal epithelium and circumvention of the host innate defense system. *Curr. Top. Microbiol. Immunol.* 337, 231–255 (2009).
56. DuPont, H. L., Levine, M. M., Hornick, R. B. & Formal, S. B. Inoculum size in shigellosis and implications for expected mode of transmission. *J. Infect. Dis.* 159, 1126–1128 (1989).
57. Schroeder, G. N. & Hilbi, H. Molecular Pathogenesis of *Shigella* spp.: Controlling Host Cell Signaling, Invasion, and Death by Type III Secretion. *Clin. Microbiol. Rev.* 21, 134–156 (2008).
58. Suzuki, T. *et al.* Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in *Shigella*-infected macrophages. *PLoS Pathog.* 3, e111 (2007).
59. Bernardini, M. L., Mounier, J., d'Hauteville, H., Coquis-Rondon, M. & Sansonetti, P. J. Identification of icsA, a plasmid locus of *Shigella flexneri* that governs bacterial intra- and intercellular spread through interaction with F-actin. *Proc. Natl. Acad. Sci. U. S. A.* 86, 3867–3871 (1989).

60. Buchrieser, C. *et al.* The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri*. *Mol. Microbiol.* 38, 760–771 (2000).
61. Tobe, T. *et al.* Temperature-regulated expression of invasion genes in *Shigella flexneri* is controlled through the transcriptional activation of the virB gene on the large plasmid. *Mol. Microbiol.* 5, 887–893 (1991).
62. Epler, C. R., Dickenson, N. E., Bullitt, E. & Picking, W. L. Ultrastructural analysis of IpaD at the tip of the nascent MxiH type III secretion apparatus of *Shigella flexneri*. *J. Mol. Biol.* 420, 29–39 (2012).
63. Mueller, C. A. *et al.* The V-antigen of *Yersinia* forms a distinct structure at the tip of injectisome needles. *Science* 310, 674–676 (2005).
64. Gall, T. L. *et al.* Analysis of virulence plasmid gene expression defines three classes of effectors in the type III secretion system of *Shigella flexneri*. *Microbiol. Read. Engl.* 151, 951–962 (2005).
65. Parsot, C. *et al.* A secreted anti-activator, OspD1, and its chaperone, Spa15, are involved in the control of transcription by the type III secretion apparatus activity in *Shigella flexneri*. *Mol. Microbiol.* 56, 1627–1635 (2005).
66. Pilonieta, M. C. & Munson, G. P. The chaperone IpgC copurifies with the virulence regulator MxiE. *J. Bacteriol.* 190, 2249–2251 (2008).
67. Bongrand, C., Sansonetti, P. J. & Parsot, C. Characterization of the Promoter, MxiE Box and 5′ UTR of Genes Controlled by the Activity of the Type III Secretion Apparatus in *Shigella flexneri*. *PLoS ONE* 7, e32862 (2012).
68. Ogawa, M., Suzuki, T., Tatsuno, I., Abe, H. & Sasakawa, C. IcsB, secreted via the type III secretion system, is chaperoned by IpgA and required at the post-invasion stage of *Shigella* pathogenicity: The role of IcsB in *Shigella* pathogenicity. *Mol. Microbiol.* 48, 913–931 (2003).
69. Niebuhr, K. *et al.* IpgD, a protein secreted by the type III secretion machinery of *Shigella flexneri*, is chaperoned by IpgE and implicated in entry focus formation. *Mol. Microbiol.* 38, 8–19 (2000).
70. van Eerde, A., Hamiaux, C., Pérez, J., Parsot, C. & Dijkstra, B. W. Structure of Spa15, a type III secretion chaperone from *Shigella flexneri* with broad specificity. *EMBO Rep.* 5, 477–483 (2004).
71. Costa, S. C. P. *et al.* A New Means To Identify Type 3 Secreted Effectors: Functionally Interchangeable Class IB Chaperones Recognize a Conserved Sequence. *mBio* 3, (2012).
72. Hachani, A. *et al.* IpgB1 and IpgB2, two homologous effectors secreted via the Mxi-Spa type III secretion apparatus, cooperate to mediate polarized cell invasion and inflammatory potential of *Shigella flexenri*. *Microbes Infect.* 10, 260–268 (2008).

73. Schmitz, A. M., Morrison, M. F., Agunwamba, A. O., Nibert, M. L. & Lesser, C. F. Protein interaction platforms: visualization of interacting proteins in yeast. *Nat. Methods* 6, 500–502 (2009).

Chapter 1:

High-Throughput Screening of Type III Secretion Determinants Reveals a Major Chaperone-Independent Pathway

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ABSTRACT

Numerous Gram-negative bacterial pathogens utilize type III secretion systems (T3SSs) to inject tens of effector proteins directly into the cytosol of host cells. Through interactions with cognate chaperones, type III effectors are defined and recruited to the sorting platform, a cytoplasmic component of these membrane-embedded nanomachines. However, notably, a comprehensive review of the literature reveals that the secretion of most type III effectors has not yet been linked to a chaperone, raising questions regarding the existence of unknown chaperones as well as the universality of chaperones in effector secretion. Here, we describe the development of the first high-throughput type III secretion (T3S) assay, a semi-automated solid plate-based assay, which enables the side-by-side comparison of secretion of over 20 *Shigella* effectors under a multitude of conditions. Strikingly, we found that the majority of *Shigella* effectors are secreted at equivalent levels by wildtype and variants of *Shigella* that no longer encode one or all known *Shigella* T3S effector chaperones. In addition, we found that *Shigella* effectors are efficiently secreted from a laboratory strain of *Escherichia coli* expressing the core *Shigella* type III secretion apparatus (T3SA), but no other *Shigella*-specific proteins. Furthermore, we observed that the sequences necessary and sufficient to define chaperone-dependent and -independent effectors are fundamentally different. Together, these findings support the existence of a major, previously unrecognized, noncanonical chaperone-independent secretion pathway, likely common to many T3SSs.

IMPORTANCE

Many bacterial pathogens use specialized nanomachines, including type III secretion systems, to directly inject virulence proteins (effectors) into host cells. Here, we present the first extensive analysis of chaperone dependence in the process of type III effector secretion, providing strong evidence for the existence of a previously unrecognized chaperone-independent pathway. This noncanonical pathway is likely common to many bacteria, as an extensive review of the literature reveals that the secretion of multiple type III effectors has not yet been knowingly linked to a chaperone. While additional studies

will be required to discern the molecular details of this pathway, its prevalence suggests that it can likely serve as a new target for the development of antimicrobial agents.

INTRODUCTION

Numerous Gram-negative bacteria, including *Shigella, Salmonella, Escherichia* and *Yersinia* species, utilize type III secretion systems (T3SSs) to inject proteins directly into host cells. T3SSs are complex nanomachines composed of 20 to 25 different proteins that form a membrane-embedded needle complex (1). Upon contact with host cells, the protein complex at the tip of the needle, the translocon, forms a pore in the host cell membrane completing the channel that function as a conduit for the delivery (translocation) of effector proteins into the host cell cytosol. These effectors proceed to usurp host cellular processes to promote bacterial replication and spread. While each pathogen encodes its own unique set of effectors, many of the structural components of their type III secretion apparatuses (T3SAs) are conserved, suggesting a common mechanism of effector recognition.

On the basis of studies primarily conducted in the 1990s, type III secreted (T3S) effectors are currently typically described as containing a bipartite secretion signal composed of an extreme amino-terminal secretion sequence followed by a chaperone-binding domain (CBD) within their first 50 to 100 residues (2-4). The N-terminal secretion sequence is not defined but rather is characterized by its intrinsically disordered nature (5), and while essential, its role in secretion remains to be discovered. In contrast, there is evidence that structural motifs common to chaperone-effector complexes serve as the three-dimensional signals that define effectors and target their recognition by the T3SA (4, 6, 7) Furthermore, through interactions with cognate chaperones, effectors are recruited to the sorting platform, the multiprotein complex that docks on the cytoplasmic surface of the membrane-embedded T3SA (8-11).

And yet, a comprehensive review of the literature reveals that over the past 20 years chaperones have only been identified for only a third (38/109) of the effectors of the well-studied *Shigella* Mxi-Spa (11/31), *Salmonella* SPI1 (6/11), *Salmonella* SPI2 (4/20),

Yersinia Ysc (4/6) as well as enteropathogenic *Escherichia coli*/enterohemorrhagic *E. coli* (EPEC/EHEC) Esc (13/41) T3SSs (12-19) (see Table S1 in the supplemental material). These observations question the commonly held notion that chaperones play essential roles in effector secretion and raise the possibility for the existence of unidentified chaperones and/or a chaperone-independent T3S pathway.

Here we describe the development of a semi-automated solid-plate-based secretion assay to study the secretion of *Shigella flexneri* effectors. This high-throughput type III secretion assay enabled the first comprehensive investigation of the roles of known and candidate T3S chaperones in mediating effector secretion. Using this assay, in addition to confirming all previously established *Shigella* effector chaperone-dependencies, we determined that the majority of *Shigella* effectors are efficiently secreted independently of all known and numerous candidate T3S chaperones. Furthermore, we found that, in contrast to chaperone-dependent effectors, the sequences that define chaperone-independent effectors are not restricted to their amino termini but rather are located throughout the effector. Together, these findings strongly suggest the existence of a major, previously unappreciated, T3S chaperone-independent type III effector secretion pathway, likely common to multiple pathogens.

RESULTS

A solid-plate-based assay increases throughput of detection of *Shigella* type III effector secretion. Upon contact with host cells, the translocon complex, positioned at the tip of the T3SA, is inserted into the host membrane. This interaction triggers a conformational change, which leads to activation of the T3SA resulting in the injection of translocon components followed by effectors into host cells. *In vitro* conditions that mimic host cell contact and trigger secretion into liquid media have been established for several pathogens (20, 21). In the case of *Shigella* species, exposure of liquid exponential-phase cultures to the dye Congo red (CR) stimulates type III secretion activation (22). However, while liquid-culture-based type III secretion assays are generally reproducible, their throughput is limited due to the number of steps involved (see Fig. S1A in the

supplemental material). To address this issue, we developed the first semiautomated solid-plate-based secretion assay. The solid-plate-based assay (Fig. 1A) is performed with the assistance of a pinning robot. In the first step, the robot, outfitted with a 96-pin tool, is used for quadruplicate (quad) spotting of equivalent volumes of saturated liquid bacterial cultures onto CR-containing solid media. Following an overnight incubation, the robot, outfitted with a 384- pin tool, is used to transfer bacteria from the first tray onto a second CR-containing solid-medium tray, over which a nitrocellulose membrane is immediately laid. During a 6-to-18-h incubation at 37°C, released proteins are absorbed onto the membrane, which is subsequently removed, washed and immunoblotted for protein(s) of interest. Using this assay, we have observed similar amounts of IpaD, a component of the *Shigella* translocon, present within each of the four spots derived from a single culture, as well as between quad spots originating from separate independent cultures. In contrast, under the same conditions, we have observed no evidence of GroEL, a highly abundant cytoplasmic protein, demonstrating that proteins deposited on the nitrocellulose membranes are not present due to bacterial lysis but rather are released from intact bacteria (Fig. S1B).

We next confirmed that the solid-plate assay monitors type III-dependent secretion and also examined the levels of effectors released via the solid and liquid secretion assays. For these studies, to directly compare the secretion of effectors, we studied the behavior of C-terminally 3xFLAG-tagged variants, each encoded downstream of a consensus Shine-Dalgarno sequence and expressed via an IPTG (isopropyl-β-D-1-thiogalactopyranoside)-inducible *lac* promoter (23, 24). Our initial studies focused on five FLAG-tagged effectors, each of which is released to nitrocellulose membranes by wildtype (WT) but not Δ*spa47 Shigella* (Fig. 1B), a strain that is secretion incompetent due to the absence of the essential T3SS ATPase (25). Notably, similar relative levels of the five effectors were observed to be secreted from WT *Shigella* via the solid-plate-based (Fig. 1B) and liquid secretion (Fig. 1C) assays, while GroEL was observed only in the pellet fractions of the liquid secretion assays, which contained intact bacterial cells (Fig. 1C). These observations confirm the validity of the solid-plate-based assay and demonstrate its functional complementarity with the conventional liquid assay.

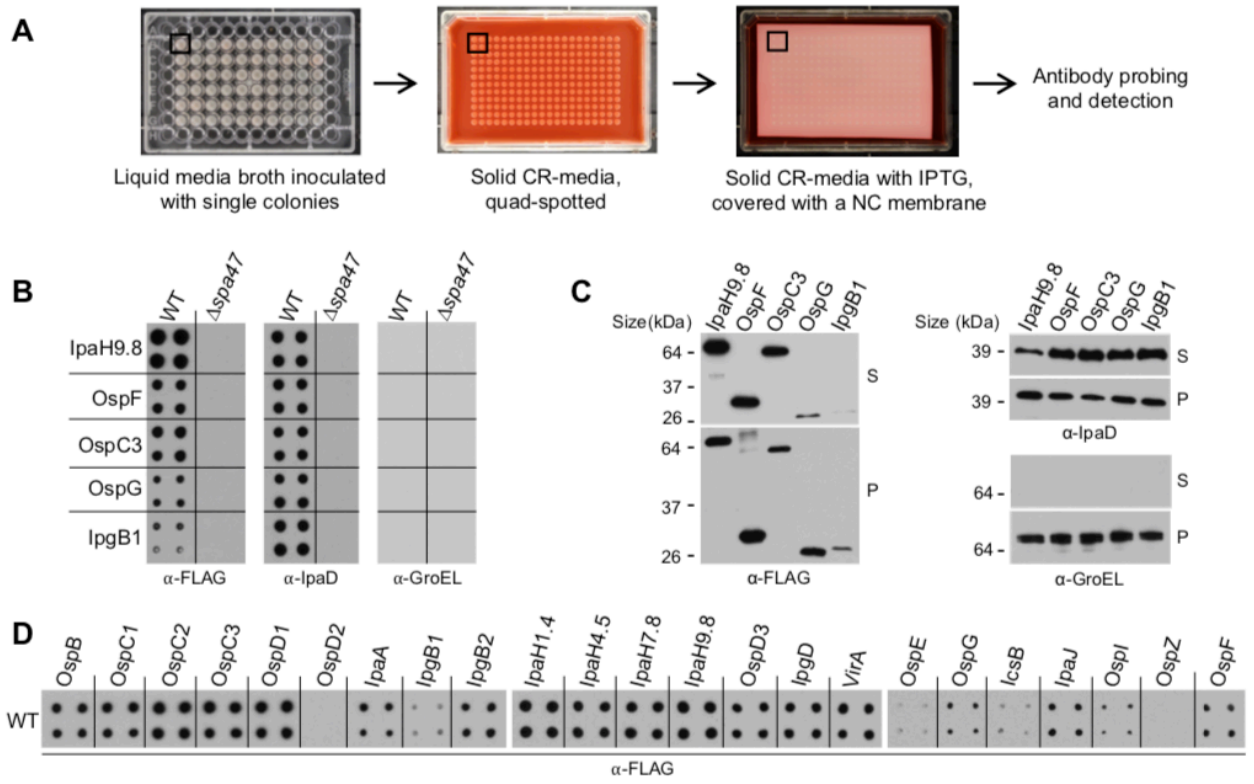

FIG 1 The solid-plate-based secretion assay reproducibly monitors *Shigella* type III effector secretion. (A) Schematic representation of the novel solid-plate-based secretion assay. Liquid cultures, grown in a 96-well-format, are quadruplicate (quad)-spotted onto solid CR-containing media using a pinning robot. After transfer of the colonies to a second plate, a nitrocellulose (NC) membrane is overlaid and the plate is then incubated at 37˚C. Membranes are removed and probed with an antibody of interest. (B) Secretion of five designated IPTG-induced FLAG-tagged effectors by wildtype (WT) and Δ*spa47 Shigella* monitored via a 6-hour solid-plate-based secretion assay. (C) Secretion of the same FLAG-tagged effectors by WT *Shigella* monitored via a 30-minute liquid secretion assays. With the exception of supernatant fractions derived from IpaH9.8, equal cell equivalents of whole cell pellet lysates (P) and precipitated supernatant fractions (S) were analyzed. Twenty-five percent of the supernatant fraction of the more abundantly secreted IpaH9.8 was examined. (D) Secretion of 23 IPTG-driven FLAG epitope-tagged effectors by WT *Shigella* monitored via a 6-hour solid-plate-based secretion assay. In each panel, all of the images shown are from the same exposure of three membranes immunoblotted with designated antibodies and are representatives of at least 3 independent experiments. CR, Congo red.

We next investigated the ability of the solid secretion assay to detect the release of 23 different FLAG-tagged effectors from WT *Shigella* (Fig. 1D). After incubating the nitrocellulose membrane-overlain plate for 6 h, a time point that enables the detection

of most effectors secreted at low levels without saturating the signals of those that are more robustly released, we observed secretion of all but two effectors. The secretion of one of these, OspZ, became detectable when a more sensitive chemiluminescence reagent was used (Fig. S2). Given our inability to detect secretion of OspD2, it was excluded from further studies. These observations provide the foundation for large-scale side-by-side comparative studies of the secretion levels of most *Shigella* effectors under different conditions.

The majority of *Shigella* effectors are efficiently secreted independently of known T3S chaperones. *Shigella* spp. encode three T3S class I effector chaperones. The class IA chaperones, IpgA and IpgE, are dedicated to the secretion of a single effector each, IcsB and IpgD, respectively (26, 27), whereas the class IB chaperone, Spa15, mediates the secretion of nine effectors, IpaA, IpgB1, IpgB2, OspB, OspC1, OspC2, OspC3, OspD1, and OspD2 (24, 28, 29) (Table 1). In prior systematic yeast two-hybrid (Y2H) and/or protein interaction platform assays, interactions were detected between each of these three chaperones and their respective 11 effectors (24). In contrast, with the exception of IpaH1.4, which interacted with Spa15, no interactions were detected between the three chaperones and 8 of the remaining 11 effectors listed in Table 1. (OspI and OspZ had not yet been discovered when these prior studies were conducted).

The observations summarized above suggested the existence of an as-of-yet-unknown chaperone(s) or the possibility that many *Shigella* effectors are secreted independently of known class I T3S chaperones. To investigate the latter, we directly compared the secretion levels of >20 FLAG-tagged *Shigella* effectors from WT, Δ*spa15,* Δ*ipgA*, and Δ*ipgE Shigella,* a feat that was not technically feasible prior to the development of our solid-plate-based secretion assay. After a 6-h incubation, as expected, we observed substantially decreased or absent secretion of each known chaperone-dependent effector from the deletion strain which lacks its cognate chaperone (Fig. 2A; see also Fig. S3A). Specifically, secretion of IcsB was not detected from Δ*ipgA Shigella*, while the secretion of each of the eight Spa15-dependent effectors from Δ*spa15 Shigella* was markedly impaired or absent. While the level of secretion of IpgD was

decreased only modestly in the absence of its chaperone IpgE, we observed no evidence of IpgD secretion from Δ*ipgE Shigella* via a liquid secretion assay (Fig. S3B). As the liquid assay monitors secretion over 30 min and the solid-plate assay over 6 h, it appears that IpgE plays a key role in mediating early secretion of IpgD.

TABLE 1 Summary of virulence plasmid encoded *Shigella* effectors and their cognate chaperones

Effector	Chaperone (class)	Effector	Chaperone (class)
IcsB	IpgA (IA)	IpaH1.4	-
IpgD	IpgE (IA)	IpaH4.5	-
IpaA	Spa15 (IB)	IpaH7.8	-
IpgB1	Spa15 (IB)	IpaH9.8	-
IpgB2	Spa15 (IB)	IpaJ	-
OspB	Spa15 (IB)	OspD3	-
OspC1	Spa15 (IB)	OspE	-
OspC2	Spa15 (IB)	OspF	-
OspC3	Spa15 (IB)	OspG	-
OspD1	Spa15 (IB)	OspI	-
OspD2	Spa15 (IB)	OspZ	-
IpaB	IpgC (II)	VirA	-
IpaC	IpgC (II)		

The remaining 11 *Shigella* effectors are secreted at essentially the same levels from WT, Δ*spa15,* Δ*ipgA* and Δ*ipgE Shigella* (Fig. 2A). Given the possibility that one or more class I chaperones might work in a functionally redundant or cooperative manner in mediating secretion, we wanted to test effector secretion from Δ*spa15*Δ*ipgE*Δ*ipgA Shigella,* a strain that lacks all three class I T3S chaperones. Only the known chaperone-dependent effectors displayed markedly decreased or absent secretion from this strain (Fig. 2B; see also Fig. S3C), further supporting the assertion that most *Shigella* effectors are secreted independently of all currently known class I T3S chaperones.

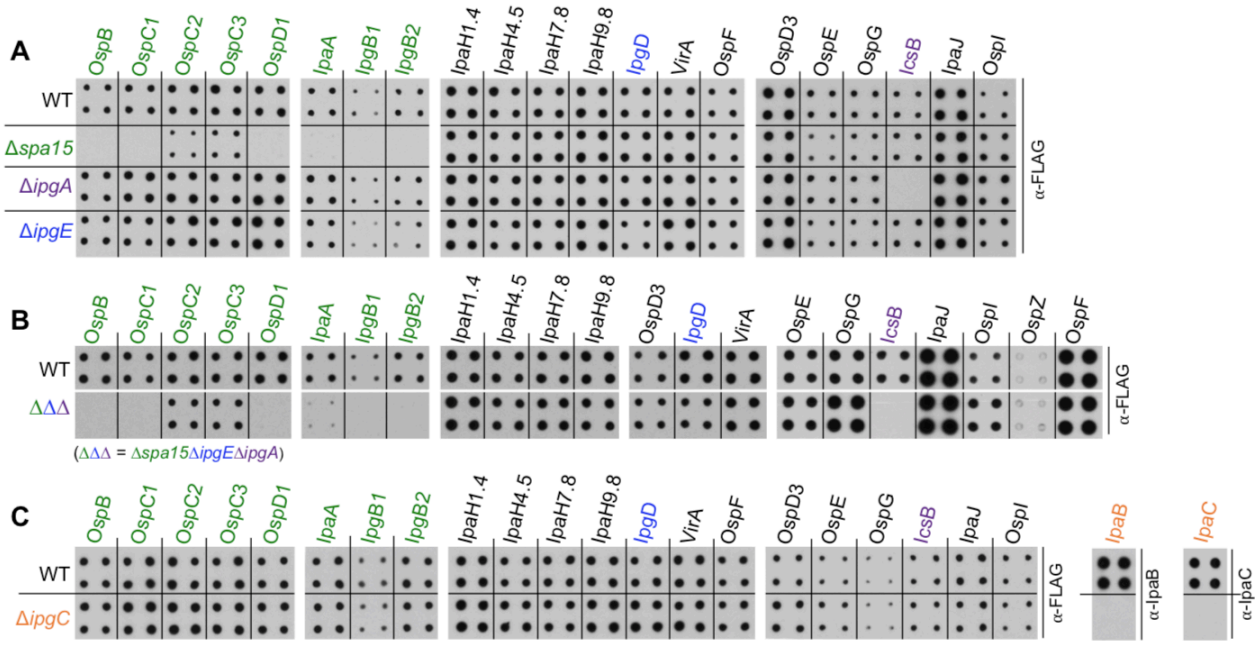

FIG 2 The majority of *Shigella* effectors are secreted independently of known class I and II T3S chaperones. Six-hour solid-plate-based secretion assays of each of the designated IPTG-induced FLAG-tagged effectors or translocon components, IpaB and IpaC, from wildtype (WT) and *Shigella* deletion strains, each of which no longer encodes one (A) or all known class I chaperones (IpgA, IpgE and Spa15) (B) or the single *Shigella* class II chaperone (IpgC) (C). Nitrocellulose membranes were probed with indicated antibodies. The images shown in each panel are from a single assay of three solid-plate overlays, each treated in the same manner. In (A, B), the images shown on the right are longer exposures than those of the left and middle panels, as they are images of effectors that are secreted at lower levels. Blots shown are representative of at least 3 independent experiments.

Next, although the secretion of effectors has never been directly linked to a class II chaperone, we tested the secretion of *Shigella* effectors in the absence of its sole class II T3S chaperone, IpgC. As expected, the absence of IpgC had no effect on the secretion levels of effectors but resulted in decreased levels of secretion of the IpaB and IpaC translocon components, (Fig. 2C; see also Fig. S3D), whose secretion is known to be dependent on IpgC (30). These findings demonstrate that the majority of *Shigella* effectors are efficiently secreted via a pathway independent of all known T3S chaperones.

Chaperone-independent effectors are efficiently secreted by *E. coli* bacteria that express a functional *Shigella* T3SA. All of the proteins needed to form the *Shigella* T3SA, most of its effectors, and all of its T3S chaperones are encoded on a large ~220kb virulence plasmid (VP) (31). Laboratory strains of *E. coli* that carry this plasmid invade and replicate within infected epithelial cells at even higher titers than WT *Shigella* (32), suggesting that it encodes all of the proteins involved in effector secretion. Additionally, the VP encodes >25 proteins of unknown function, one or more of which could potentially be a previously unidentified T3S chaperone. To investigate this possibility, we considered generating strains that no longer encode each of these proteins. However, given the possibility that two or more of these proteins might work in a functionally redundant manner, we used the following strategy to generate a means to study effector secretion in the absence of all proteins of unknown function. Using recombineering (33), we introduced the *mxi-spa* operons, which encode all structural components of the T3SA and two class I chaperones (IpgE and Spa15), into the chromosome of the nonpathogenic laboratory strain *E. coli* DH10β. The introduction of these operons, plus a plasmid that conditionally expresses the *mxi-spa* transcriptional regulator, VirB, resulted in the generation of mT3SA_*E. coli*, a type III secretion-competent strain, which contains only 8% of the *Shigella* virulence plasmid DNA and none of the chromosomally encoded *Shigella* pathogenicity islands (Fig. 3A).

mT3SA_*E. coli,* unlike WT *Shigella* and the chaperone deletion strains, encodes no effectors or translocon components. Thus, we first needed to establish that effector secretion remains chaperone dependent in the absence of competition for access to the T3SA. Thus, we compared the levels of Spa15-dependent effectors secreted by mT3SA_*E. coli* and mT3SAΔ*15*_*E. coli,* a strain that lacks the chaperone Spa15. Notably, the secretion of each was markedly diminished in the absence of Spa15 (Fig. 3B), establishing the relevancy of the use of this strain to study the secretory behavior of the putative chaperone-independent effectors. We next studied the secretion of our collection of FLAG-tagged *Shigella* effectors expressed in mT3SA_*E. coli* (Fig. 3C). As expected, we observe no evidence of IcsB secretion from mT3SA_*E. coli* due to the absence of its cognate chaperone IpgA. Strikingly, all the remaining effectors not only were efficiently

secreted from mT3SA_*E. coli* but also were secreted at the same relative levels as were observed from WT *Shigella* (Fig. 1D) under the same experimental conditions. These observations demonstrate that none of the *Shigella*-specific proteins encoded outside the *mxi-spa* operons play a role in mediating effector secretion, thus strongly supporting the existence of a common chaperone-independent secretion pathway. However, they do not rule out the seemingly less likely existence of an as-yet-to-be-discovered new class of T3S chaperones, which would be the first to not be restricted to a single pathogen species but rather to be common to nonpathogenic *E. coli* and *Shigella.*

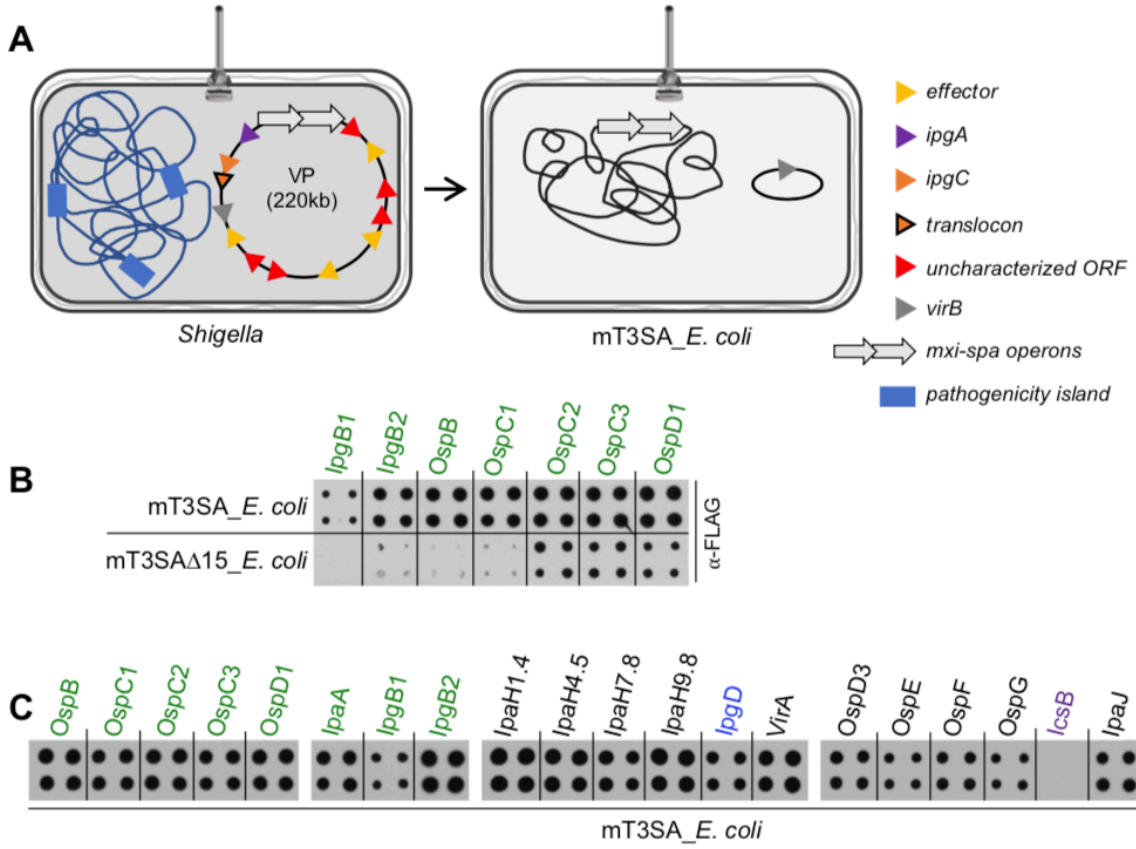

FIG 3 *Shigella* effectors are efficiently secreted by a core T3SA in *E. coli*. (A) Schematic representation of WT *Shigella* versus mT3SA_*E. coli.* The genetic elements transferred from *Shigella* into mT3SA_*E. coli* include VirB, a major T3SS transcriptional regulator, and the *mxi-spa* operons, which encode all the core structural components of the *Shigella* T3SA. (B) Secretion of designated Spa15-dependent IPTG-induced FLAG-tagged effectors from mT3SA and mT3SA∆*15* in *E. coli* (C) and of 22 IPTG-induced FLAG-tagged effectors from mT3SA_*E. coli* monitored via a 6-hour solid-plate assay. Nitrocellulose membranes were probed with α-FLAG antibody. In (C) images shown are from the same exposure of three membranes. Blots shown are representative of at least 3 independent experiments. T3SA, type III secretion apparatus.

We next wanted to investigate whether any of the proteins present in mT3SA_*E. coli* might serve in the recruitment of chaperone-independent effectors to the machine. However, it was not possible to monitor effector secretion in their absence, as almost all of the proteins encoded within the well-studied *mxi-spa* operons (Table S2) are essential for secretion. Thus, to gain insights regarding how chaperone-independent effectors are recruited to the T3SA, we conducted an extensive yeast two-hybrid screen for binary interactions between effectors and cytoplasmic components of the T3SA. Specifically, we systematically tested for interactions between 17 effectors and 16 *mxi-spa* encoded proteins. The latter included components of the sorting platform (MxiK, MxiN, Spa33 and Spa47), the export apparatus (MxiA, Spa9, Spa13, Spa24, Spa29, Spa40), the basal body (MxiG) as well as regulators (Spa32, MxiC, MxiE and MxiL) (31) and the multicargo chaperone Spa15. In the case of MxiG and MxiA, we screened for interactions involving their predicted cytosolic domains (34, 35). No interactions, other than the previously observed interactions between Spa15 and its cognate effectors (24) were detected (Table S3), suggesting that cytosolically exposed T3SA proteins are not involved in the direct recruitment of either chaperone-dependent or chaperone-independent effectors to the T3SA.

Chaperone-dependent and -independent effectors are defined by fundamentally different determinants. T3S effectors are commonly described as containing a bipartite secretion signal composed of an N-terminal secretion sequence followed by a downstream chaperone-binding domain (CBD). These effector domains have primarily been identified by studying the secretory behavior of heterologous proteins fused to N-terminal effector fragments (2, 36). As a first step towards comparing the sequences that define chaperone-dependent and chaperone-independent effectors as secreted proteins, we studied the secretion of a heterologous mammalian protein that is normally not secreted, MyoD (37), fused to the first 50, 100, or 200 amino acids of two chaperone-dependent (OspD1 and OspB) and chaperone-independent (VirA and OspF) effectors (Fig. 4A). As expected, fusion of the first 50 residues of the two chaperone-dependent effectors, the sequences that contain their secretion sequences and previously mapped

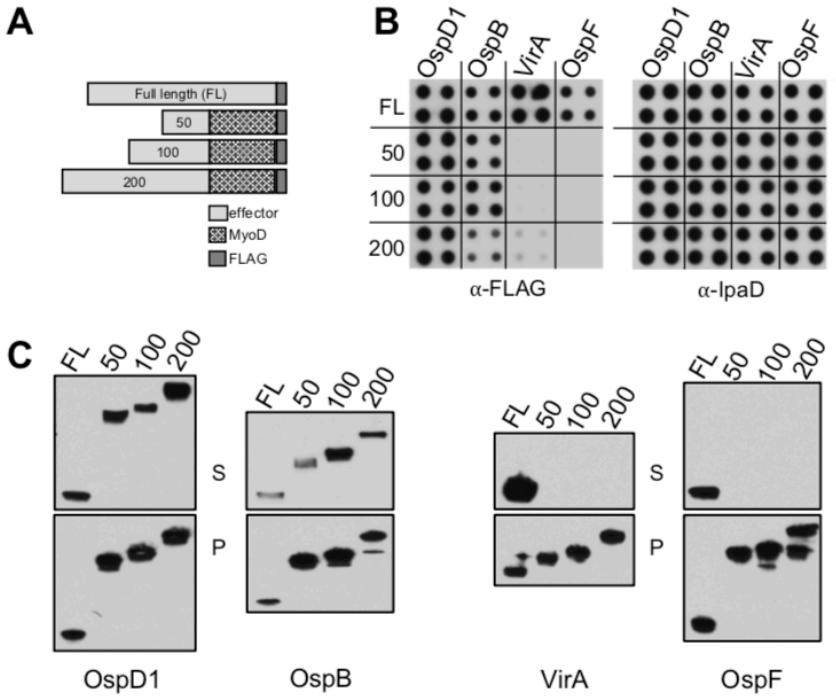

FIG 4 Secretion signals of chaperone-independent effectors are not limited to their amino termini. (A) Schematic representation of effector fusion proteins. (B, C) Secretion of designated IPTG-induced chaperone-dependent (OspD1 and OspB) and -independent (VirA and OspF) FLAG-tagged effector proteins from WT *Shigella* via a 6-hour solid (B) or 30-minute liquid (C) secretion assay. Equal cell equivalents of whole cell pellet lysates (P) and precipitated supernatant fractions (S) were separated by SDS-PAGE and immunoblotted with α-IpaD (B) or α-FLAG (B, C) antibodies. Blots shown are representatives of 3 independent experiments.

CBDs (38), is sufficient to generate secreted variants of MyoD-FLAG a assayed via either the solid-plate-based (Fig. 4B) or liquid (Fig. 4C) secretion assay. In fact, fusion to just the first 50 residues resulted in MyoD variants that were secreted at levels equivalent to those seen with each of the corresponding FLAG-tagged full-length effectors. In contrast, MyoD was not secreted when fused to the first 50, 100 or 200 amino acids of the tested chaperone-independent effectors, OspF and VirA, demonstrating that their N-termini are not sufficient to mediate secretion. The absence of secretion was not due to T3SS inactivity, as equivalent levels of IpaD were secreted by each of the MyoD-expressing strains. It was also not due to instability of the fusion proteins, as they were present at roughly equivalent levels in the bacterial pellet fractions (Fig. 4C). Thus, the sequences

of the chaperone-dependent and -independent effectors needed to mediate the recognition of MyoD as a type III secreted protein are substantially different.

We next compared the sequences of the same chaperone-dependent (OspD1 and OspB) and -independent (VirA and OspF) effectors that are necessary for their secretion using a scanning deletion mutagenesis strategy. Depending on the size of the effector, we generated 50 to 100 amino acid deletions, smaller deletions for OspD1, OspB, and OspF and larger ones for VirA (Fig. S4A). In each case, we kept the first 50 residues intact, in order to not perturb potential N-terminal secretion sequences. In the case of the chaperone-dependent effectors, we also examined variants that no longer contain the 11 amino acids that correspond to their previously mapped CBDs (38,39). Via both, the solid-plate-based (Fig. 5A) and liquid (Fig. 5B) secretion assays, we again observed

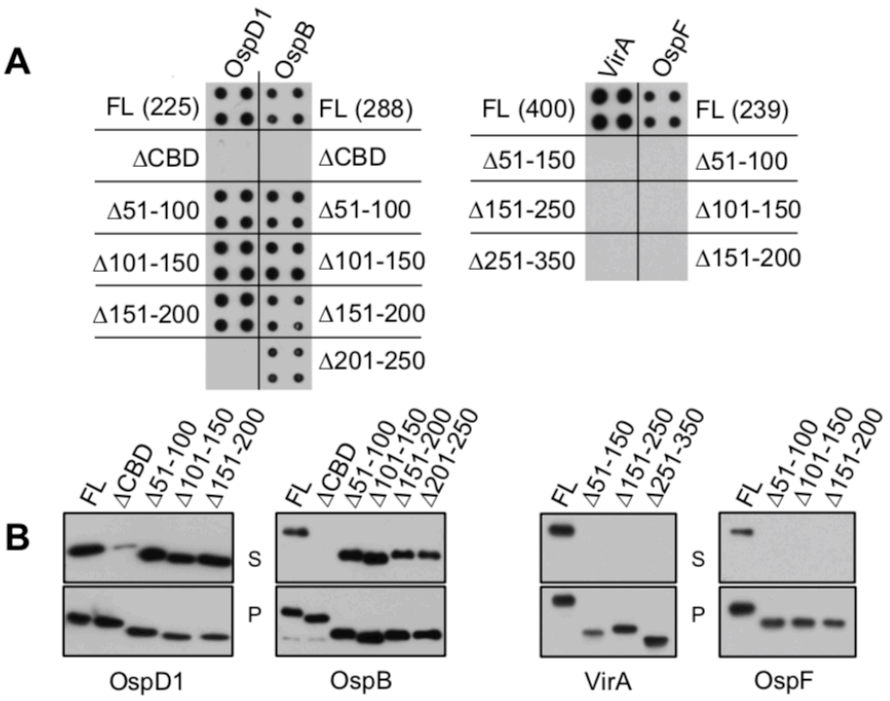

FIG 5 The sequences required for the secretion of chaperone-dependent and -independent effectors are fundamentally different. Secretion of designated deletion variants of chaperone-dependent (OspD1, OspB) and -independent (VirA, OspF) effectors monitored via a 6-hour solid-plate based (A) or 30-minute liquid (B) secretion assay. Equal cell equivalents of whole cell pellet lysates (P) and precipitated supernatant fractions (S) were separated by SDS-PAGE and immunoblotted with α-FLAG antibody. Blots shown are representatives of at least 3 independent experiments. FL, full-length.

fundamental differences in the sequences necessary to define chaperone-dependent and -independent effectors as secreted proteins. The only residues identified to play a role in defining chaperone-dependent effectors were those of the CBD, which is part of the bipartite secretion signal. In contrast, none of the mutated variants of the chaperone-independent effectors (VirA or OspF) were secreted despite being present at relatively equivalent levels in the total (Fig. 5B) and soluble fractions of bacterial lysates (Fig. S4B). Furthermore, their lack of secretion was not due to inhibition of T3SS activity, as equivalent levels of secreted IpaD were observed under all conditions (Fig. S4C).

Additional studies are needed to further refine the sequences essential for defining chaperone-independent effectors as secreted substrates. Nevertheless, these studies provided a clear demonstration that the sequences necessary and sufficient to define at least these chaperone-dependent and -independent effectors as secreted substrates are fundamentally different, thus providing further support for the existence of at least two distinct type III effector secretion pathways.

DISCUSSION

In this report, we describe the development of a solid-plate-based secretion assay that enables, for the first time, the side-by-side concurrent analysis of secretion of >20 different *Shigella* effectors under multiple conditions. Remarkably, despite extensive evidence that effectors are both defined as secreted proteins (4, 6, 7) and recruited to the T3SA sorting platform via interactions with cognate chaperones (11), we found that the majority of *Shigella* effectors are efficiently secreted independently of all known T3S chaperones. Furthermore, we demonstrate that chaperone-independent effectors are efficiently secreted via mT3SA_*E. coli,* a laboratory strain of *E. coli,* which contains the operons needed to form a functional *Shigella* T3SA but none of the virulence-associated genes located within 92% of the *Shigella* virulence plasmid DNA nor within its chromosomal pathogenicity islands. This last observation suggests that if the secretion of chaperone-independent effectors is mediated via other, as-yet-unknown proteins, they are likely encoded in chromosomal regions conserved between *Shigella* and non-pathogenic *E.*

coli DH10β and hence constitute a new class of T3S chaperones. Moreover, our observations strongly support the existence of two different modes of effector recognition, as the sequences that are necessary and sufficient to define chaperone-dependent and -independent effectors are fundamentally different. Thus, we propose that the chaperone-independent effectors are secreted via a previously unrecognized noncanonical secretion pathway.

A series of recent elegant imaging studies demonstrated that the membrane-embedded portion of the T3SA is static, exhibiting few structural changes between its resting and secreting states (40). In contrast, dynamic changes are observed at its cytosolic surface (9, 41, 42), where effectors are recruited and loaded into the export apparatus. These changes presumably reflect docking of the sorting platform, as it delivers effectors from the bacterial cytosol to the membrane-localized T3SA (8, 9, 34). These observations raise numerous questions regarding how chaperone-independent effectors are recruited to the T3SA. First, are they directly or indirectly recruited to the sorting platform? The latter seems more likely, as we observe no evidence of direct binding of effectors to components of the sorting platform via the Y2H assay. Similarly, other groups have not reported evidence of the chaperone-independent binding of effectors to the sorting platform (11). Alternatively, given previously reported observations that not all membrane-embedded T3SAs have associated sorting platforms (8, 9, 43), might chaperone-independent effectors be directly recruited by the export apparatus?

While our studies to systematically examine the roles of chaperones in mediating the secretion of a large complement of T3S effectors resulted in the discovery of widespread chaperone-independence, a review of the literature suggests that chaperone-independence is likely not an uncommon occurrence. For example, chaperones have only been identified for only a third (38/109) of known effectors of the well-studied *Shigella*, *Salmonella, Yersinia*, or pathogenic *E. coli* T3SSs (12-19). In addition, as we observed for a few *Shigella* chaperone-dependent effectors, *Yersinia* YopE and YopH are inefficiently secreted in the absence of their cognate chaperones (38, 44), suggesting that under select conditions, even those effectors that bind chaperones might be secreted via a chaperone-independent pathway.

In summary, here, using a high-throughput semiautomated solid-plate-based *Shigella* secretion assay, we present evidence for the existence of a common noncanonical chaperone-independent type III secretion pathway. Future studies are needed to dissect the molecular details of this pathway. Nevertheless, given its prevalence, it offers a new and exciting target for the development of novel therapeutic agents in this emerging era of widespread antibiotic resistance.

MATERIALS AND METHODS

Strains, plasmids, and oligonucleotides are summarized in Tables S4 and S5.

Plasmid Construction.

Effector-FLAG expression plasmids. The *plac* (IPTG-inducible) effector 3xFLAG tagged expression plasmids were generated as previously described (24, 45).

Effector-MyoD-FLAG expression plasmids. The first 150, 300 and 600 basepairs of the genes encoding OspD1, OspB, OspF and VirA were PCR-amplified from their corresponding pDSW206 expression plasmids using a 5' oligonucleotide that binds to the vector upstream of the 5' flanking *attB* site (DSW206 F) plus a gene/location-specific 3' oligonucleotide, i.e., OspB_50 R. The amplified gene fragments were introduced into pDNR221 or pDNR223 via Gateway BP reactions to generate entry plasmids. After the gene fragment inserts were sequence-verified, each was transferred into pDSW206-ccdB-MyoD-FLAG via a Gateway LR reaction.

Effector deletion expression plasmids. Each gene deletion was generated via overlap (SOEing) PCR using the following strategy: (1) Two first round PCRs were conducted using the corresponding full-length gene-specific pDSW206 plasmid as a template. The upstream fragment was amplified using DSW206 F plus a gene/location-specific reverse oligonucleotide, i.e., OspB_51_100_3 while the downstream fragment was amplified using a gene/location specific forward oligonucleotide, i.e. OspB_51_100_5 plus RrnB R, an oligonucleotide that binds downstream of the *attB* site. (2) The two first round

fragments were then used as templates with Univ5 and RrnB R oligonucleotides to generate fragments that contain the desired deletions flanked by *attB* sites. The amplified fragments were introduced into pDNR221 via BP reactions. After the gene inserts were sequence-verified, each was transferred into pDSW206-ccdB-FLAG via a Gateway LR reaction.

Yeast expression plasmids. Each yeast expression plasmid was generated via Gateway recombination, as previously described (24). ORFs encoding the proteins listed in Table S4 were PCR-amplified in a closed (stop codon containing) conformation. Those that contain Shine-Dalgarno sequences were generated using the semi-nested PCR strategy described above for OspI and OspZ. Those that do not were amplified via a single round of PCR. The amplified fragments were then introduced into either pDNR221 or pDNR223 to generate Gateway entry clones. In the case of OspF (K134A), a synthetic gBlock fragment (IDT, Skokie, Illinois) with flanking *attB* sites was introduced into pDNR223. After the gene inserts were sequence-verified, effectors were introduced into pAD-ccdB, while components of the T3SA into pBD-ccdB via Gateway LR reactions.

Strain Construction.

***Shigella* deletion strains.** Each of the single deletion strains (Table S4), except for Δ*ipgC Shigella*, was generated in *S. flexneri* 2457T via λ Red recombination (46) using the oligonucleotides described in Table S5. In each case, the KAN^R cassette was resolved using the FLP recombinase. The strain Δ*spa15*Δ*ipgE*Δ*ipgA Shigella* was generated by first removing *ipgE* from Δ*spa15 Shigella* to generate Δ*spa15*Δ*ipgE::*KAN *Shigella.* After resolution of the KAN^R cassette, *ipgA* was then deleted from the strain to generate Δ*spa15*Δ*ipgE*Δ*ipgA::* KAN *Shigella.*

Generation of mT3SA_*E. coli* and mT3SAΔ*15_E. coli*. mT3SA_*E. coli* was generated using a modified version of the strategy previously described (33, 37). First a capture vector was generated that is designed to capture the region of virulence plasmid (VP) DNA present between the VirB promoter site located upstream of IpgD and Spa40. This was done by modifying pLLX13-*ipaJ-bla-spa40,* the original capture vector developed to

capture the region of the virulence plasmid encoded between IpaJ and Spa40. Specifically, semi-nested PCR was used to generate the fragment of DNA present between *icsB* and *ipgD.* Gibson assembly was then used to swap this fragment with the original targeting sequence 1 of pLLX13-*ipaJ-bla-spa40*. After the integrity of this new capture vector, pLLX13-*icsB/ipgD-bla-spa40*, was confirmed via PCR, sequence analysis and restriction digestion, it was transformed into *E. coli* DH10β that carry a version of the *Shigella* virulence plasmid (VP *ΔipgD*::KAN) plus the λ Red recombinase. Homologous recombination was then used to introduce the desired region of VP DNA into the capture vector, thus generating pmT3SA. After the integrity of pmT3SA was confirmed by whole-plasmid sequencing it was transformed into *E. coli* DH10β, which have a landing pad (LP) integration site at the *atp1/gidB* locus (47). After which the landing pad recombination system (48) was used to introduce the region of captured DNA onto the *E. coli* chromosome to generate mT3SA∆*ipgD*::KAN_*E. coli*. The integrity of mT3SA∆*ipgD*::KAN_*E. coli* was confirmed by PCR after which the KAN^R cassette was removed to generate mT3SA_*E. coli.* The λ Red recombination system was then used to remove *spa15* to generate mT3SA∆*spa15::*KAN_*E. coli.* The KAN^R cassette was resolved to generate mT3SA∆*15_E. coli*.

Liquid T3S assay. Liquid secretion assays were performed as previously described (24). Overnight cultures grown in trypticase soy (TCS) broth were diluted 1:100 into 2 ml of TCS broth and incubated for 100 minutes, at which time 1 mM IPTG was added to the cultures. After another 45 minutes of incubation, the OD_{600} of each bacterial culture was measured. Equivalent numbers of bacteria from each culture were pelleted, resuspended in 2 ml of phosphate-buffered saline (PBS) plus 10 μM Congo red (Sigma) and incubated for 30 minutes. All incubations were carried out at 37˚C with aeration. Bacterial cultures were then centrifuged and the cell pellets were resuspended in loading dye (40% glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol). After an additional centrifugation step, to remove remaining intact bacteria, proteins in the supernatant fractions were trichloroacetic acid (TCA) precipitated [10% (vol/vol)] and resuspended in loading dye. Equal cell equivalents of supernatant and pellet fractions

were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and immunoblotted with α-FLAG (Sigma, F1804, 1:10,000), α-IpaD (1:40,000) or α-GroEL (Sigma, G6532 1:100,000) antibodies. The α-IpaD antibody is a generous gift from Wendy Picking, University of Kansas, Lawrence, KS.

Solid-plate-based T3S assay. A 96-well plate (Corning) containing TCS broth was inoculated with the designated strains and incubated with agitation for 6-18 hours on a plate shaker. A BM3-BC pinning robot (S&P Robotics Inc., Toronto, Canada) outfitted with a 96-pin tool was then used to transfer equal volumes of saturated cultures onto solid trays (Nunc) that contain solid TCS media (Sigma) plus 10 μM Congo red (CR). Each colony was spotted in quadruplicate. After an overnight incubation, the BM3-BC pinning robot outfitted with a 384-pin tool was used to transfer bacteria to a solid media tray containing TCS media plus CR and 1mM IPTG onto which a pre-cut nitrocellulose membrane (Pierce) was immediately laid. All incubations were carried out at 37°C. After another 6-18 hour incubation, the overlaid membrane was removed, washed with buffer (Tris-buffered saline, 0.1% Tween 20) to eliminate attached cells and then probed with one of the following antibodies: α-FLAG, α-IpaB (1:20,000), α-IpaC (1:40,000), α-IpaD or anti-GroEL. The α-IpaB/C/D antibodies are generous gifts from Wendy Picking, University of Kansas, Lawrence, KS. Secretion was quantified using ImageJ (49) and secreted protein amount relative to WT were summarized in heatmaps using Matrix3png (50).

Solubility test. Overnight cultures of WT *S. flexneri* 2457T grown in TCS broth were diluted 1:100 into 9ml of TCS broth. After a 100 minute incubation, 1 mM IPTG was added to the cultures. After another 45 minutes, based on the OD_{600} readings, equivalent numbers of bacteria from each culture were pelleted. All incubations were carried out at 37˚C with aeration. The bacterial pellets were resuspended in 2 ml of PBS containing protease inhibitor cocktail (Sigma), sonicated on ice for 1 min for 2 cycles. The lysed cells were centrifuged and cell equivalent volumes of supernatant and pellet fractions were

separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with α-FLAG and α-DnaK (Abcam, ab69617, 1:10,000).

Y2H assay. The Y2H pAD, pBD expression plasmids were introduced into MaV103 or MaV203, respectively. The Y2H assays were performed in a 96-well format as previously described (24,51). In this case, selection was conducted on medium lacking leucine, tryptophan and histidine, plus 30-50 mM 3-amino-1,2,4-triazole. Growth was scored after 3 days of incubation at 30˚C.

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AUTHOR CONTRIBUTIONS

N.H.E. and C.F.L. conceived and designed the experiments, N.H.E., A.Z.R. and J.E.R. conducted the experiments, N.H.E. and C.F.L. analyzed the data and wrote the manuscript.

REFERENCES

1. Galan, J. E., Lara-Tejero, M., Marlovits, T. C., and Wagner, S. (2014) Bacterial Type III Secretion Systems: Specialized Nanomachines for Protein Delivery into Target Cells. *Annu Rev Microbiol* 68, 415-438
2. Sory, M. P., Boland, A., Lambermont, I., and Cornelis, G. R. (1995) Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the cyaA gene fusion approach. *Proc Natl Acad Sci U S A* 92, 11998-12002

3. Schesser, K., Frithz-Lindsten, E., and Wolf-Watz, H. (1996) Delineation and mutational analysis of the *Yersinia pseudotuberculosis* YopE domains which mediate translocation across bacterial and eukaryotic cellular membranes. *J Bacteriol* 178, 7227-7233
4. Chen, L., Ai, X., Portaliou, A. G., Minetti, C. A., Remeta, D. P., Economou, A., and Kalodimos, C. G. (2013) Substrate-activated conformational switch on chaperones encodes a targeting signal in type III secretion. *Cell Rep* 3, 709-715
5. Buchko, G. W., Niemann, G., Baker, E. S., Belov, M. E., Smith, R. D., Heffron, F., Adkins, J. N., and McDermott, J. E. (2010) A multi-pronged search for a common structural motif in the secretion signal of *Salmonella enterica* serovar *Typhimurium* type III effector proteins. *Mol Biosyst* 6, 2448-2458
6. Lilic, M., Vujanac, M., and Stebbins, C. E. (2006) A common structural motif in the binding of virulence factors to bacterial secretion chaperones. *Mol Cell* 21, 653-664
7. Birtalan, S., Phillips, R., and Ghosh, P. (2002) Three-dimensional secretion signals in chaperone-effector complexes of bacterial pathogens. *Mol Cell*
8. Diepold, A., Kudryashev, M., Delalez, N. J., Berry, R. M., and Armitage, J. P. (2015) Composition, formation, and regulation of the cytosolic c-ring, a dynamic component of the type III secretion injectisome. *PLoS Biol* 13, e1002039
9. Diepold, A., Sezgin, E., Huseyin, M., Mortimer, T., Eggeling, C., and Armitage, J. P. (2017) A dynamic and adaptive network of cytosolic interactions governs protein export by the T3SS injectisome. *Nat Commun* 8, 15940
10. Parsot, C. (2003) The various and varying roles of specific chaperones in type III secretion systems. *Current Opinion in Microbiology* 6, 7-14
11. Lara-Tejero, M., Kato, J., Wagner, S., Liu, X., and Galan, J. E. (2011) A sorting platform determines the order of protein secretion in bacterial type III systems. *Science* 331, 1188-1191
12. Letzelter, M., Sorg, I., Mota, L. J., Meyer, S., Stalder, J., Feldman, M., Kuhn, M., Callebaut, I., and Cornelis, G. R. (2006) The discovery of SycO highlights a new function for type III secretion effector chaperones. *EMBO J* 25, 3223-3233
13. Wattiau, P., Bernier, B., Deslee, P., Michiels, T., and Cornelis, G. R. (1994) Individual chaperones required for Yop secretion by *Yersinia*. *Proc Natl Acad Sci U S A* 91, 10493-10497
14. Trulzsch, K., Roggenkamp, A., Aepfelbacher, M., Wilharm, G., Ruckdeschel, K., and Heesemann, J. (2003) Analysis of chaperone-dependent Yop secretion/translocation and effector function using a mini-virulence plasmid of *Yersinia enterocolitica*. *Int J Med Microbiol* 293, 167-177
15. Hong, K. H., and Miller, V. L. (1998) Identification of a novel *Salmonella* invasion locus homologous to *Shigella* ipgDE. *J Bacteriol* 180, 1793-1802

16. Ehrbar, K., Hapfelmeier, S., Stecher, B., and Hardt, W. D. (2004) InvB is required for type III-dependent secretion of SopA in *Salmonella enterica* serovar *Typhimurium*. *J Bacteriol* 186, 1215-1219
17. Bronstein, P. A., Miao, E. A., and Miller, S. I. (2000) InvB is a type III secretion chaperone specific for SspA. *J Bacteriol* 182, 6638-6644
18. Lee, S. H., and Galan, J. E. (2003) InvB is a type III secretion-associated chaperone for the *Salmonella enterica* effector protein SopE. *J Bacteriol* 185, 7279-7284
19. Fu, Y., and Galan, J. E. (1998) Identification of a specific chaperone for SptP, a substrate of the centisome 63 type III secretion system of *Salmonella typhimurium*. *J Bacteriol* 180, 3393-3399
20. Michiels, T., Wattiau, P., Brasseur, R., Ruysschaert, J. M., and Cornelis, G. (1990) Secretion of Yop proteins by *Yersiniae*. *Infect Immun* 58, 2840-2849
21. Kaniga, K., Tucker, S., Trollinger, D., and Galan, J. E. (1995) Homologs of the *Shigella* IpaB and IpaC invasins are required for *Salmonella* typhimurium entry into cultured epithelial cells. *J Bacteriol* 177, 3965-3971
22. Bahrani, F. K., Sansonetti, P. J., and Parsot, C. (1997) Secretion of Ipa proteins by *Shigella flexneri*: inducer molecules and kinetics of activation. *Infect Immun* 65, 4005-4010
23. Weiss, D. S., Chen, J. C., Ghigo, J. M., Boyd, D., and Beckwith, J. (1999) Localization of FtsI (PBP3) to the septal ring requires its membrane anchor, the Z ring, FtsA, FtsQ, and FtsL. *J Bacteriol* 181, 508-520
24. Schmitz, A. M., Morrison, M. F., Agunwamba, A. O., Nibert, M. L., and Lesser, C. F. (2009) Protein interaction platforms: visualization of interacting proteins in yeast. *Nat Methods* 6, 500-502
25. Venkatesan, M. M., Buysse, J. M., and Oaks, E. V. (1992) Surface presentation of *Shigella flexneri* invasion plasmid antigens requires the products of the spa locus. *J Bacteriol* 174, 1990-2001
26. Ogawa, M., Suzuki, T., Tatsuno, I., Abe, H., and Sasakawa, C. (2003) IcsB, secreted via the type III secretion system, is chaperoned by IpgA and required at the post-invasion stage of *Shigella* pathogenicity. *Mol Microbiol* 48, 913-931
27. Niebuhr, K., Jouihri, N., Allaoui, A., Gounon, P., Sansonetti, P. J., and Parsot, C. (2000) IpgD, a protein secreted by the type III secretion machinery of *Shigella flexneri*, is chaperoned by IpgE and implicated in entry focus formation. *Mol Microbiol* 38, 8-19
28. Page, A. L., Sansonetti, P., and Parsot, C. (2002) Spa15 of *Shigella flexneri*, a third type of chaperone in the type III secretion pathway. *Mol Microbiol* 43, 1533-1542

29. Hachani, A., Biskri, L., Rossi, G., Marty, A., Menard, R., Sansonetti, P., Parsot, C., Van Nhieu, G. T., Bernardini, M. L., and Allaoui, A. (2008) IpgB1 and IpgB2, two homologous effectors secreted via the Mxi-Spa type III secretion apparatus, cooperate to mediate polarized cell invasion and inflammatory potential of *Shigella flexenri*. *Microbes Infect* 10, 260-268
30. Menard, R., Sansonetti, P., Parsot, C., and Vasselon, T. (1994) Extracellular association and cytoplasmic partitioning of the IpaB and IpaC invasins of *S. flexneri*. *Cell* 79, 515-525
31. Buchrieser, C., Glaser, P., Rusniok, C., Nedjari, H., D'Hauteville, H., Kunst, F., Sansonetti, P., and Parsot, C. (2000) The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri*. *Molecular Microbiology*
32. Sansonetti, P. J., Ryter, A., Clerc, P., Maurelli, A. T., and Mounier, J. (1986) Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. *Infect Immun* 51, 461-469
33. Reeves, A. Z., and Lesser, C. F. (2016) Transfer of Large Contiguous DNA Fragments onto a Low Copy Plasmid or into the Bacterial Chromosome. *Bio Protoc* 6
34. Hu, B., Morado, D. R., Margolin, W., Rohde, J. R., Arizmendi, O., Picking, W. L., Picking, W. D., and Liu, J. (2015) Visualization of the type III secretion sorting platform of *Shigella flexneri*. *Proc Natl Acad Sci U S A* 112, 1047-1052
35. Abrusci, P., Vergara-Irigaray, M., Johnson, S., Beeby, M. D., Hendrixson, D. R., Roversi, P., Friede, M. E., Deane, J. E., Jensen, G. J., Tang, C. M., and Lea, S. M. (2013) Architecture of the major component of the type III secretion system export apparatus. *Nat Struct Mol Biol* 20, 99-104
36. Charpentier, X., and Oswald, E. (2004) Identification of the secretion and translocation domain of the enteropathogenic and enterohemorrhagic *Escherichia coli* effector Cif, using TEM-1 beta-lactamase as a new fluorescence-based reporter. *J Bacteriol* 186, 5486-5495
37. Reeves, A. Z., Spears, W. E., Du, J., Tan, K. Y., Wagers, A. J., and Lesser, C. F. (2015) Engineering *Escherichia coli* into a protein delivery system for mammalian cells. *ACS Synth Biol* 4, 644-654
38. Costa, S. C., Schmitz, A. M., Jahufar, F. F., Boyd, J. D., Cho, M. Y., Glicksman, M. A., and Lesser, C. F. (2012) A New Means To Identify Type 3 Secreted Effectors: Functionally Interchangeable Class IB Chaperones Recognize a Conserved Sequence. *MBio* 3
39. Costa, S. C., and Lesser, C. F. (2014) A multifunctional region of the *Shigella* type 3 effector IpgB1 is important for secretion from bacteria and membrane targeting in eukaryotic cells. *PLoS One* 9, e93461

40. Nans, A., Kudryashev, M., Saibil, H. R., and Hayward, R. D. (2015) Structure of a bacterial type III secretion system in contact with a host membrane in situ. *Nat Commun* 6, 10114
41. Makino, F., Shen, D., Kajimura, N., Kawamoto, A., Pissaridou, P., Oswin, H., Pain, M., Murillo, I., Namba, K., and Blocker, A. J. (2016) The Architecture of the Cytoplasmic Region of Type III Secretion Systems. *Sci Rep* 6, 33341
42. Hu, B., Lara-Tejero, M., Kong, Q., Galan, J. E., and Liu, J. (2017) In Situ Molecular Architecture of the *Salmonella* Type III Secretion Machine. *Cell* 168, 1065-1074 e1010
43. Zhang, Y., Lara-Tejero, M., Bewersdorf, J., and Galan, J. E. (2017) Visualization and characterization of individual type III protein secretion machines in live bacteria. *Proc Natl Acad Sci U S A* 114, 6098-6103
44. Lloyd, S. A., Norman, M., Rosqvist, R., and Wolf-Watz, H. (2001) *Yersinia* YopE is targeted for type III secretion by N-terminal, not mRNA, signals. *Mol Microbiol* 39, 520-531
45. Mou, X. S., S; Du, J; Reeves, AZ; Lesser, CF. (2018, in press) A novel synthetic bottom-up approach reveals the complex interplay of *Shigella* effectors in regulation of epithelial cell death. *Proc Natl Acad Sci U S A*
46. Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 97, 6640-6645
47. Du, J., Reeves, A. Z., Klein, J. A., Twedt, D. J., Knodler, L. A., and Lesser, C. F. (2016) The type III secretion system apparatus determines the intracellular niche of bacterial pathogens. *Proc Natl Acad Sci U S A* 113, 4794-4799
48. Kuhlman, T. E., and Cox, E. C. (2010) Site-specific chromosomal integration of large synthetic constructs. *Nucleic Acids Res* 38, e92
49. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9, 671-675
50. Pavlidis, P., and Noble, W. S. (2003) Matrix2png: a utility for visualizing matrix data. *Bioinformatics* 19, 295-296
51. Walhout, A. J., and Vidal, M. (2001) High-throughput yeast two-hybrid assays for large-scale protein interaction mapping. *Methods* 24, 297-306

SUPPLEMENTAL MATERIAL

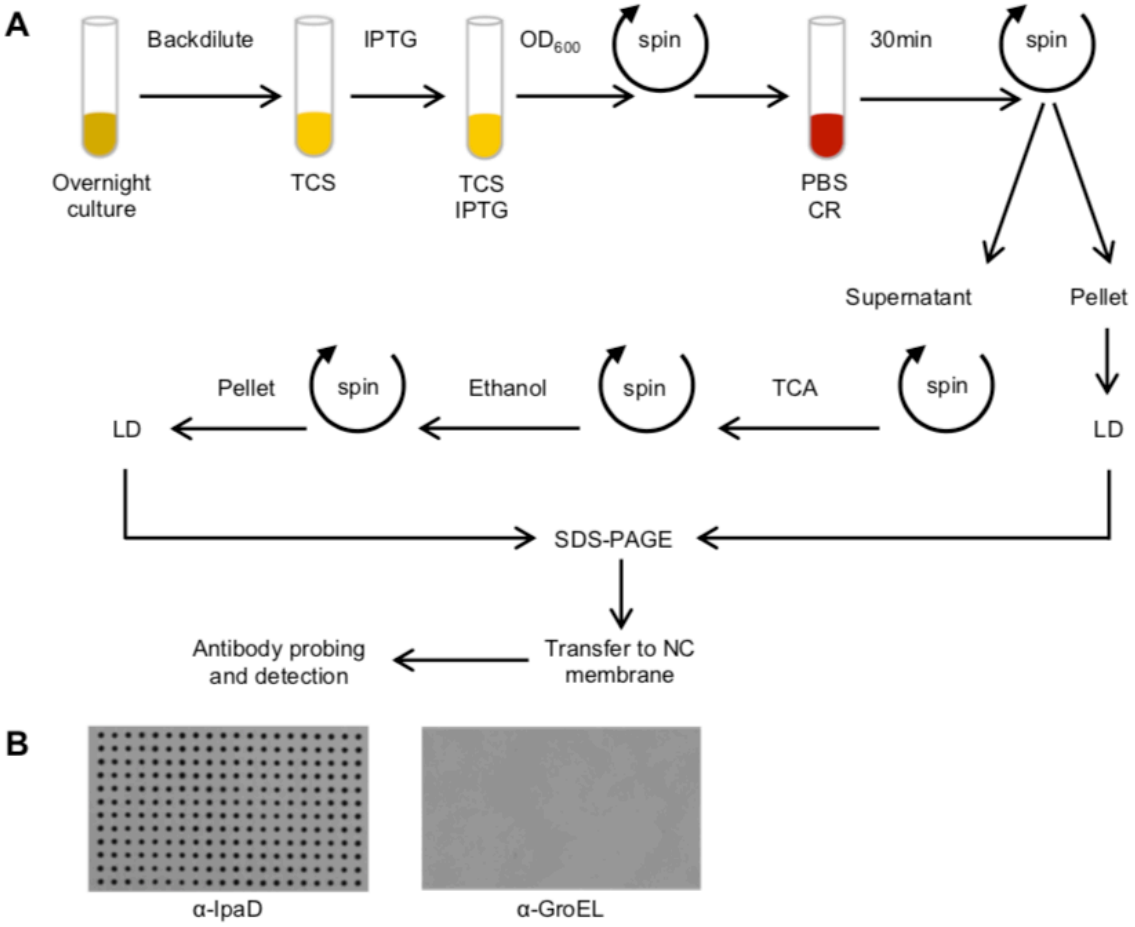

FIG S1 *Shigella* type III effector secretion monitored by the liquid and the novel solid plate-based secretion assay. (A) Schematic representation of the liquid secretion assay. Proteins are released by bacteria into CR-supplemented buffer, precipitated, separated via SDS-PAGE and immunoblotted with antibodies of interest. (B) Immunoblots obtained from a solid-plate assay of WT *Shigella*. Sixty independent colonies were pinned, overlaid membranes were incubated for 6 hours, removed and probed with α-IpaD and α-GroEL antibodies. Blots shown are representative of at least 3 independent experiments. CR, Congo red; LD, loading dye; OD, optical density; PBS, phosphate-buffered saline.

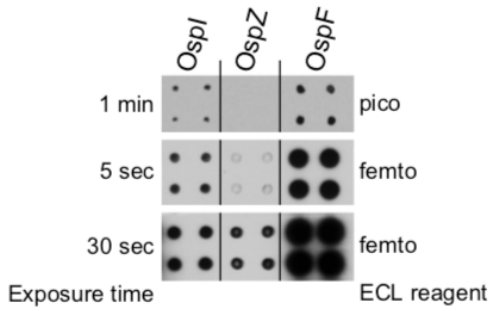

FIG S2 Detection of OspZ secretion from *Shigella*. Secretion of IPTG-induced FLAG-tagged OspI, OspZ and OspF from WT *Shigella*, monitored via a 6-hour solid plate-based assay. The blot was probed with α-FLAG antibody and exposed with different chemiluminescence reagents that detect pico- (pico) or femtogram (femto) protein amounts for indicated time points. Blots shown are representative of 3 independent experiments. ECL, enhanced chemiluminescence.

FIG S3 The majority of *Shigella* effectors are secreted independently of class I and II T3S chaperones. (A, C, D) Six-hour solid-plate secretion assays of each of the designated IPTG-induced FLAG-tagged effectors or translocon components, IpaB and IpaC from wildtype (WT) and *Shigella* deletion strains, each of which no longer encodes one (A) or all class I chaperones (IpgA, IpgE and Spa15) (C) or the single *Shigella* class II chaperone (IpgC) (D). Nitrocellulose membranes were probed with indicated antibodies. The intensities of each quad were quantified using ImageJ. Heatmaps were generated that depict the ratio of each protein secreted from the designated strain as compared to WT *Shigella.* The heatmaps shown are representative of at least 3 independent experiments. (B) Secretion of IPTG-induced FLAG-tagged IpgD from WT, Δ*spa15*, Δ*ipgA* and Δ*ipgE Shigella* monitored via a 30-minute liquid assay. Equal cell equivalents of proteins in the precipitated supernatant fractions (S) and whole cell pellet lysates (P) were separated by SDS-PAGE and immunoblotted with α-FLAG antibody. Blots shown are representative of at least 3 independent experiments. T3S, type III secretion.

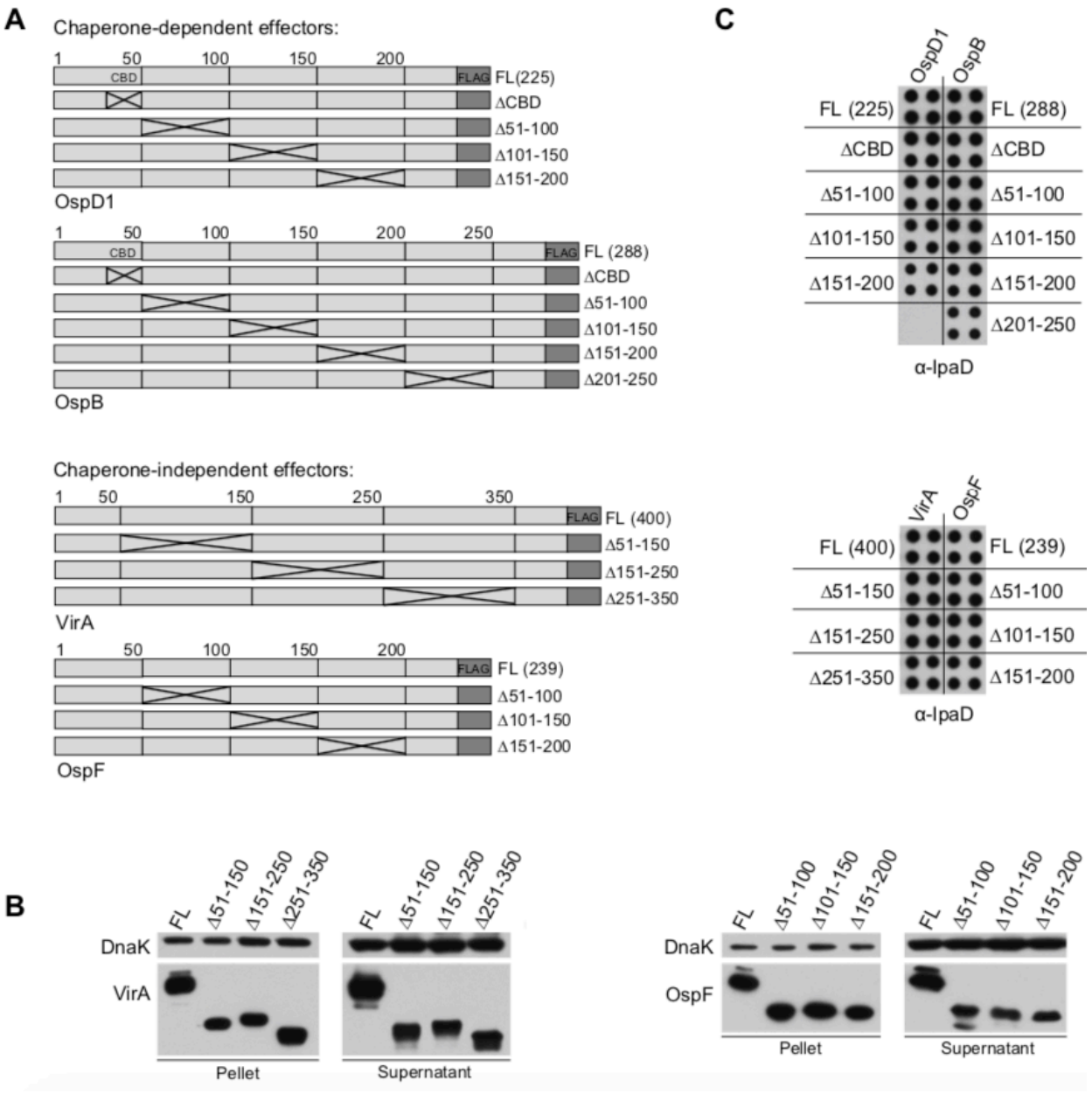

FIG S4 Expression of effector variants does not impact T3SA activity. (A) Schematic representation of chaperone-dependent (OspD1, OspB) and -independent (OspF, VirA) FLAG-tagged effector variants carrying designated deletions. (B) Immunoblots of sonicated fractions of WT *Shigella* expressing each of the designated effector variants. Equal cell equivalents of proteins in the pellet and supernatant fractions were separated by SDS-PAGE and immunoblotted with α-FLAG and α-DnaK antibodies. Blots shown are representatives of at least 2 independent experiments. (C) Immunoblots of 6-hour solid secretion assays of WT *Shigella* expressing each of the designated effector variants probed with α-IpaD antibody. Blots shown are representatives of at least 3 independent experiments. CBD, chaperone-binding domain; FL, full-length.

TABLE S1 Summary of effectors and their cognate chaperones

Effector	Chaperone	Class
***Shigella* (Mxi-Spa T3SS)**		
Virulence-plasmid-encoded		
IpaA	Spa15	IB
IpgB1	Spa15	IB
IpgB2	Spa15	IB
OspB	Spa15	IB
OspC1	Spa15	IB
OspC2	Spa15	IB
OspC3	Spa15	IB
OspD1	Spa15	IB
OspD2	Spa15	IB
IcsB	IpgA	IA
IpgD	IpgE	IA
IpaH1.4	-	-
IpaH4.5	-	-
IpaH7.8	-	-
IpaH9.8	-	-
IpaJ	-	-
OspD3	-	-
OspE1	-	-
OspE2	-	-
OspF	-	-
OspG	-	-
OspI	-	-
OspZ	-	-
VirA	-	-
Chromosomally-encoded		
IpaH_1	-	-
IpaH_2	-	-
IpaH_3	-	-
IpaH_4	-	-
IpaH_5	-	-
IpaH_6	-	-
IpaH_7	-	-
***Yersinia* (Ysc T3SS)**		
YopE	SycE	IA
YopJ	-	-
YopH	sycH	IA
YopM	-	-
YopT	SycT	IA
YpkA/YopO	SycO	IA
***Salmonella* (SPI1 T3SS)**		
SipA (SspA)	InvB	IB
SopA	InvB	IB
SopE2	InvB	IB
SopE1	InvB	IB
SopB	SigE	IA
AvrA	-	-
SopD	-	-
SptP	SicP	IA

SspH1	-	-
SpvC	-	-
SlrP	-	-
***Salmonella* (SPI2 T3SS)**		
PipB	-	-
PipB2	SrcA	IB
SseL	SrcA	IB
SopD2	-	-
SifA	-	-
SseJ	-	-
SseI	-	-
SspH2	-	-
SpvB	-	-
SseF	SscB	IA?
SseG	SscB?	?
SteC	-	-
Slrp1	-	-
GogB	-	-
SifB	-	-
SpiC	-	-
SseK1	-	-
SseK2	-	-
SteA	-	-
SteB	-	-
***Escherichia coli* (Esc T3SS)**		
Tir	CesT	IB
Map	CesT	IB
EspH	CesT	IB
EspJ	CesT	IB
EspZ	CesT	IB
NleA/EspI	CesT	IB
NleB1	CesT	IB
NleB2	CesT	IB
NleC	CesT	IB
NleG	CesT	IB
NleH1	CesT	IB
NleH2	CesT	IB
EspF	CesF	IA
EspFU	-	-
EspG	-	-
EspG2	-	-
EspK	-	-
EspL	-	-
EspL2	-	-
EspM	-	-
EspN	-	-
EspO	-	-
EspR	-	-
EspS	-	-
EspT	-	-
EspV	-	-
EspW	-	-
EspX	-	-

EspY	-	-
NleB3	-	-
NleD	-	-
NleE	-	-
NleE2	-	-
NleF	-	-
NleH3	-	-
NleI	-	-
NleJ	-	-
NleK	-	-
NleL (ESPX7)	-	-
Cif	-	-
Efa1	-	-

TABLE S2 Proteins encoded in *mxi-spa* operons

Protein	**Function** (1-3)	**Location** (3,4)
Spa15	class I chaperone	cytoplasm
IpgE	class I chaperone	cytoplasm
MxiE	transcription factor	cytoplasm
MxiK	sorting platform	cytoplasm
MxiN	sorting platform	cytoplasm
Spa33	sorting platform	cytoplasm
Spa47	ATPase	cytoplasm
Spa13	ATPase-associated stalk	cytoplasm
MxiC	gatekeeper	cytoplasm
Spa9	export apparatus	cytoplasmic membrane
Spa24	export apparatus	cytoplasmic membrane
Spa29	export apparatus	cytoplasmic membrane
Spa40	export apparatus	cytoplasmic membrane
MxiA	export apparatus	cytoplasmic membrane with large
MxiG	needle complex inner rings	cytoplasmic membrane with large
MxiJ	needle complex inner rings	cytoplasmic membrane
MxiI	inner rod	inside of basal body
MxiD	needle complex outer rings	outer membrane
MxiH	needle filament	external
Spa32	ruler protein, needle-length control	inside of basal body
IpgF	peptidoglycase	n/a
MxiM	pilotin, assembly of outer rings	outer membrane
MxiL	postulated regulator, secreted	n/a

Table S3. Summary of Y2H studies.

GAL4 binding domain (BD) fusions	GAL4 activation domain (AD) fusions																
	IpaA	**IpaH1.4**	**IpaH4.5**	**IpaH7.8**	**IpaH9.8**	**IpgB1**	**OspB**	**OspC1**	**OspC2**	**OspC3**	**OspD1**	**OspD2**	**OspD3**	**OspE**	**OspF(K134A)**	**OspG**	**OspZ**
IpgF	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
MxiA(318-686)	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
MxiC	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
MxiE	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
MxiG(1-126)	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
MxiK	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
MxiL	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
MxiN	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Spa9	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Spa13	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Spa24	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Spa29	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Spa32	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Spa33	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Spa40	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Spa47	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Spa15	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No

TABLE S4 Strain and plasmid summary

Strain/Plasmid	Characteristics	Source
Strain:		
Shigella flexneri 2457T serotype 2a	wildtype	(5)
Δ*ipgA::*KAN *Shigella*	Δ*ipgA*::FRT-KAN^R-FRT	This study
Δ*ipgA Shigella*	Δ*ipgA*::FRT	This study
Δ*ipgE::*KAN *Shigella*	Δ*ipgE*::FRT-KAN^R-FRT	This study
Δ*ipgE Shigella*	Δ*ipgE*::FRT	This study
Δ*spa15 Shigella*	Δ*spa15*::FRT	(6)
Δ*spa15*Δ*ipgE::*KAN *Shigella*	Δ*spa15*Δ*ipgE*::FRT-KAN^R-FRT	This study
Δ*spa15*Δ*ipgE Shigella*	Δ*spa15*Δ*ipgE*::FRT	
Δ*spa15*Δ*ipgE*Δ*ipgA::*KAN *Shigella*	Δ*spa15*Δ*ipgE*Δ*ipgA*::FRT-KAN^R-FRT	This study
Δ*spa47::*KAN *Shigella*	Δ*spa47*::FRT-KAN^R-FRT	This study
Δ*spa47 Shigella*	Δ*spa47*::FRT	This study
Shigella flexneri M90T serotype 5a	wildtype	(7)
Δ*ipgC::*TET *Shigella* M90T	Δ*ipgC*::FRT-TET^R-FRT	(8)
Escherichia coli DH10β		Invitrogen
E.coli DH10β *atp/gidB::*LP	*atp/gidB::*Landing Pad cassette, TET^R	Addgene (83036)
mT3SAΔ*ipgD::*KAN_*E.coli*	mT3SA (intergenic region of icsB and IpgDΔ*ipgD*::FRT-KAN^R-FRT -*ipgE* thru *spa40*, ~18kb) integrated in chromosome	This study
mT3SA_*E.coli*	mT3SA (intergenic region of icsB and IpgDΔipgD::FRT-*ipgE* thru *spa40*, ~18kb) integrated in chromosome	This study
mT3SAΔ*15::*KAN_*E.coli*	mT3SA (intergenic region of icsB and IpgDΔipgD::FRT-*ipgE* thru *mxiA*Δ*spa15*::FRT-KAN^R-FRT thru *spa40*) integrated in chromosome	This study
mT3SAΔ*15_E.coli*	mT3SA (intergenic region of icsB and IpgDΔipgD::FRT-*ipgE* thru *mxiA*Δ*spa15*::FRT thru *spa40*) integrated in chromosome	This study
Plasmid:		
pCP20	Temperature sensitive, AMP^R, FLP recombinase	(9)
pKD46	Temperature sensitive, λ Red recombinase, AMP^R	(9)
pLLX13-ipaJ-bla-spa40	T3SS capture vector, AMP^R, Tet^R, incP ori	(10)
pLLX13-icsB/ipgD-bla-spa40	T3SS capture vector to generate pmT3SA, AMP^R, TET^R	This study

pmT3SA	pLLX13 that carries T3SA genes from pVP Δ*ipgD*::FRT-KAN^R-FRT (*icsB*/*ipgD*Δ*ipgD*::FRT-Kan^R-FRT thru *spa40*), incP ori, TET^R, KAN^R	This study
pTKRED	Temperature sensitive, λ Red recombinase and I-SceI endonuclease, SPEC^R	(11)
Δ*ipgD*::KAN VP	Δ*ipgD*::FRT-KAN^R-FRT virulence plasmid from *S. flexneri* 2457T carrying deletion of *ipgD*	(12)
Entry plasmids		
pDNR221	Gateway® donor vector, pUC ori, KAN^R	Invitrogen
pENTR221-ospB(50)	Entry clone, 1-150bp of *ospB*, open configuration	This study
pENTR221-ospB(100)	Entry clone, 1- 300bp of *ospB*, open configuration	This study
pENTR221-ospB(200)	Entry clone, 1-600bp of *ospB*, open configuration	This study
pENTR221-ospBΔ51-100	Entry clone, with 151-300bp deletion in *ospB*, open configuration	This study
pENTR221-ospBΔ101-150	Entry clone, with 301-450bp deletion in *ospB*, open configuration	This study
pENTR221-ospBΔ151-200	Entry clone, with 451-600bp deletion in *ospB*, open configuration	This study
pENTR221-ospBΔ201-250	Entry clone, with 601-750bp deletion in *ospB*, open configuration	This study
pENTR221-ospD1(50)	Entry clone, 1-150bp of *ospD1*, open configuration	This study
pENTR221-ospD1(100)	Entry clone, 1- 300bp of *ospD1*, open configuration	This study
pENTR223-ospD1(200)	Entry clone, 1-600bp of *ospD1*, open configuration	This study
pENTR221-ospD1Δ51-100	Entry clone, with 151-300bp deletion in *ospD1*, open configuration	This study
pENTR221-ospD1Δ101-150	Entry clone, with 301-450bp deletion in *ospD1*, open configuration	This study
pENTR221-ospD1Δ151-200	Entry clone, with 451-600bp deletion in *ospD1*, open configuration	This study
pENTR221-ospF(50)	Entry clone, 1-150bp of *ospF*, open configuration	This study
pENTR221-ospF(100)	Entry clone, 1- 300bp of *ospF*, open configuration	This study
pENTR221-ospF(200)	Entry clone, 1-600bp of *ospF*, open configuration	This study
pENTR221-ospFΔ51-100	Entry clone, with 151-300bp deletion in *ospF*, open configuration	This study
pENTR221-ospFΔ101-150	Entry clone, with 301-450bp deletion in *ospF*, open configuration	This study
pENTR221-ospFΔ151-200	Entry clone, with 451-600bp deletion in *ospF*, open configuration	This study
pENTR221-ospI	Entry clone, open and closed configuration	(13)
pENTR221-ospZ	Entry clone, open and closed configuration	(13)

pENTR221-mxiL	Entry clone, closed configuration	This study
pENTR221-spa33	Entry clone, yeast codon optimized, closed configuration	This study
pENTR221-virA(50)	Entry clone, 1-150bp of *virA*, open configuration	This study
pENTR221-virAΔ51-150	Entry clone, with 151-450bp deletion in *virA*, open configuration	This study
pENTR221-virAΔ151-250	Entry clone, with 451-750bp deletion in *virA*, open configuration	This study
pENTR221-virAΔ251-350	Entry clone, with 751-1050bp deletion in *virA*, open configuration	This study
pDNR223	Gateway donor vector, pUC ori, $SPEC^R$	Invitrogen
pENTR223-ipgF	Entry clone, closed configuration	This study
pDNR223-mxiA(318-386)	Entry clone, 952-2061 bp of *mxiA*, closed configuration	This study
pENTR223-mxiC	Entry clone, closed configuration	This study
pENTR223-mxiE	Entry clone, closed configuration	This study
pENTR223-mxiG(1-126)	Entry clone, 1-378 bp of *mxiG*, closed configuration	This study
pENTR223-mxiK	Entry clone, closed configuration	This study
pENTR223-mxiN	Entry clone, closed configuration	This study
pENTR223-ospF(K134A)	Entry clone, OspF K134A, closed configuration	This study
pENTR223-spa9	Entry clone, closed configuration	This study
pENTR223-spa13	Entry clone, closed configuration	This study
pENTR223-spa24	Entry clone, closed configuration	This study
pENTR223-spa29	Entry clone, closed configuration	This study
pENTR223-spa32	Entry clone, closed configuration	This study
pENTR223-spa40	Entry clone, closed configuration	This study
pENTR223-spa47	Entry clone, closed configuration	This study
pENTR223-virA(100)	Entry clone, 1- 300bp of *virA*, open configuration	This study
pENTR223-virA(200)	Entry clone, 1-600bp of *virA*, open configuration	This study
Effector-FLAG expression plasmids		
pDSW206-ccdB-FLAG	Destination vector, 3xFLAG, p*lac* (IPTG), low copy (ColE1 ori), AMP^R	(6)
pDSW206-IcsB-FLAG	Expression clone, effector ORF, 3xFLAG, p*lac* (IPTG), low copy (ColE1 ori), AMP^R	(6)
pDSW206-ipaA-FLAG	"	(6)
pDSW206-ipaJ-FLAG	"	(6)
pDSW206-ipaH1.4-FLAG	"	(6)
pDSW206-ipaH4.5-FLAG	"	(6)
pDSW206-ipaH7.8-FLAG	"	(6)
pDSW206-ipaH9.8-FLAG	"	(6)
pDSW206-ipgB1-FLAG	"	(6)
pDSW206-ipgB2-FLAG	"	(6)
pDSW206-ipgD-FLAG	"	(6)

pDSW206-ospB-FLAG	"	(6)
pDSW206-ospC1-FLAG	"	(6)
pDSW206-ospC2-FLAG	"	(6)
pDSW206-ospC3-FLAG	"	(6)
pDSW206-ospD1-FLAG	"	(6)
pDSW206-ospD2-FLAG	"	(6)
pDSW206-ospD3-FLAG	"	(6)
pDSW206-ospE-FLAG	"	(6)
pDSW206-ospF-FLAG	"	(6)
pDSW206-ospG-FLAG	"	(6)
pDSW206-ospI-FLAG	"	(13)
pDSW206-ospZ-FLAG	"	(13)
pDSW206-virA-FLAG	"	(6)
pNG162-virB	Expression clone, p*lac* (IPTG), *virB*, low copy (SC101 ori), $SPEC^R$	(10)
Effector-MyoD-FLAG expression plasmids		
pDSW206-ccdB-myoD-FLAG	Destination vector, p*lac* (IPTG), *myoD*-3xFLAG, low copy (ColE1 ori), AMP^R	
pDSW206-ospB(50)-myoD-FLAG	expression clone, *effector*(bp)-*myoD*-3xFLAG, low copy (ColE1 ori), AMP^R	This study
pDSW206-ospB(100)-myoD-FLAG	"	This study
pDSW206-ospB(200)-myoD-FLAG	"	This study
pDSW206-ospD1(50)-myoD-FLAG	"	This study
pDSW206-ospD1(100)-myoD-FLAG	"	This study
pDSW206-ospD1(200)-myoD-FLAG	"	This study
pDSW206-ospF(50)-myoD-FLAG	"	This study
pDSW206-ospF(100)-myoD-FLAG	"	This study
pDSW206-ospF(200)-myoD-FLAG	"	This study
pDSW206-virA(50)-myoD-FLAG	"	This study
pDSW206-virA(100)-myoD-FLAG	"	This study
pDSW206-virA(200)-myoD-FLAG	"	This study
Effector deletion expression plasmids		
pDSW206-ospBΔcbd-FLAG	Expression clone, *effector*Δdeletion-3xFLAG, p*lac* (IPTG), low copy (ColE1 ori), AMP^R	(14)
pDSW206-ospBΔ51-100-FLAG	"	This study
pDSW206-ospBΔ101-150-FLAG	"	This study
pDSW206-ospBΔ151-200-FLAG	"	This study
pDSW206-ospD1Δcbd-FLAG	"	(14)
pDSW206-ospD1Δ51-100-FLAG	"	This study
pDSW206-ospD1Δ101-150-FLAG	"	This study
pDSW206-ospD1Δ151-200-FLAG	"	This study
pDSW206-ospFΔ51-100-FLAG	"	This study

pDSW206-ospFΔ101-150-FLAG	“	This study
pDSW206-ospFΔ151-200-FLAG	“	This study
pDSW206-virAΔ51-150-FLAG	“	This study
pDSW206-virAΔ151-250-FLAG	“	This study
pDSW206-virAΔ251-350-FLAG	“	This study
Y2H expression plasmids		
pAD-ccdB	Destination vector, GAL4AD-fusion, low copy (cen), LEU, AMP^R	(15)
pAD-ipaA	Expression vector, GAL4AD-fusion, low copy (cen), LEU, AMP^R	(6)
pAD-ipaH1.4	“	(6)
pAD-ipaH4.5	“	(6)
pAD-ipaH7.8	“	(6)
pAD-ipaH9.8	“	(6)
pAD-ipgB1	“	(6)
pAD-ospB	“	(6)
pAD-ospC1	“	(6)
pAD-ospC2	“	(6)
pAD-ospC3	“	(6)
pAD-ospD1	“	(6)
pAD-ospD2	“	(6)
pAD-ospD3	“	(6)
pAD-ospE	“	(6)
pAD-ospF(K134A)	“	This study
pAD-ospG	“	(6)
pAD-ospZ	“	This study
pBD-ccdB	Destination vector, GAL4BD-fusion, low copy (cen), TRP, KAN^R	(15)
pBD-ipgF	Expression vector, GAL4BD-fusion, low copy (cen), TRP, KAN^R	This study
pBD-mxiA(318-386)	“	This study
pBD-mxiC	“	This study
pBD-mxiE	“	This study
pBD-mxiG(1-126)	“	This study
pBD-mxiK	“	This study
pBD-mxiL	“	This study
pBD-mxiN	“	This study
pBD-spa9	“	This study
pBD-spa13	“	This study
pBD-spa15	“	(12)
pBD-spa24	“	This study
pBD-spa29	“	This study

pBD-spa32	“	This study
pBD-spa33	“	This study
pBD-spa40	“	This study
pBD-spa47	“	This study

TABLE S5 Oligonucleotide summary

Oligo	Sequence, 5′–3′
5’ Oligos to generate deletion strains	
IpgA_UP	CTCTCATTCTAATATATAGAAGGCCATAGAAATGTGTCGCGTGTA
IpgE_5 Wanner	GGTGAAAGGGTATTCGTCATTTGTATAAGAGGAATATATGGTGTA
Spa15_UP	TGTATTAAAGACTATTTAGTGAGGTTTAAATATGAGTAACGTGTA
Spa47_5 Wanner	CTTATAATCAATGAGCTATACAAAATTGCTCACTCAATTAGTGTA
3’ Oligos to generate deletion strains	
IpgA_DN	TGTTTAGAATTTGCATGATACCCCCTATATGTTAGTTCACCATAT
IpgE_3 Wanner	TAAATACGAAACGGGACATTAATACCCCTTCATTCTTCGCACATA
Spa15_DN	ATTGAGTGAGCAATTTTGTATAGCTCATTGATTATAAGACCATAT
Spa47_3 Wanner	TTTTAAAACCTTATCTAATTGTTTCACCAATAAGCTCCATCATATG
5’ Oligos to PCR *Shigella* ORFs into Gateway entry vectors	
MxiL F GW	CGAAGGAGATAGAACCATGATTAATCAAATAAATGCAAGC
MxiA_952 F GW	CGAGGGGACAACTTTGTACAAAAAAGTTGGCATGGTCGTAGAAA
MxiC F GW	CGAAGGAGATAGAACCATGCTTGATGTTAAAAATAC
MxiE F GW	CGAAGGAGATAGAACC ATGAGTAAATATAAAGGTCTAAATAC
MxiG F GW	CGAGGGGACAACTTTGTACAAAAAAGTTGGCATGTCTGAGGCAA
MxiK F GW	CGAAGGAGATAGAACCATGATAAGAATGGATGGAATTTATA
MxiL F GW	CGAAGGAGATAGAACCATGATTAATCAAATAAATGCAAGC
MxiN F GW	CGAAGGAGATAGAACCATGAAGGTATGCAATATGCAAAAAG
OspI F GW	CGAAGGAGATAGAACCATGATTAATGGGGTGTCGTTACA
OspZ F GW	CGAAGGAGATAGAACCATGATTAGTCCCATCAAGAATATTA
Spa9 F GW	CGAGGGGACAACTTTGTACAAAAAAGTTGGCGTGTCTGATATAG
Spa13 F GW	CGAAGGAGATAGAACCATGGAGGCATTAGATAAAAGGATTA
Spa24 F GW	CGAGGGGACAACTTTGTACAAAAAAGTTGGCATGCTGAGTGACA
Spa29 F GW	CGAGGGGACAACTTTGTACAAAAAAGTTGGCATGGACATTTCAA
Spa32 F GW	CGAAGGAGATAGAACCATGGCATTAGATAATATAAA
Spa33 F GW	CGAAGGAGATAGAACCATGTGTGGGGATTGGGTAATTCGTA
Spa40 F GW	CGAAGGAGATAGAACCATGGCAAATAAAACAGAAAAGCCGA
Spa47 F GW	CGAAGGAGATAGAACCATGAGCTATACAAAATTGCTCACTC
Univ5	GGGGACAACTTTGTACAAAAAAGTTGGCGAAGGAGATAGAACCA
3’ Oligos to PCR *Shigella* ORFs into Gateway entry vectors	
MxiL R GW	GGGGACAACTTTGTACAAGAAAGTTGGTTACCATGTCGAATCAT
MxiA R GW	GGGGACAACTTTGTACAAGAAAGTTGGCTAAATAGTCTTTAATAC

MxiC R GW	GGGGACAACTTTGTACAAGAAAGTTGGTTATCTAGAAAGCTCTTT
MxiE R GW	GGGGACAACTTTGTACAAGAAAGTTGGTTAAATTTTTTCATTTATT
MxiG_378 R GW	GGGGACAACTTTGTACAAGAAAGTTGGCTACGAGTGGTTCTTAT
MxiK R GW	GGGGACAACTTTGTACAAGAAAGTTGGTCATAGGCATGATGTCT
MxiL R GW	GGGGACAACTTTGTACAAGAAAGTTGGTTACCATGTCGAATCAT
MxiN R GW	GGGGACAACTTTGTACAAGAAAGTTGGTTAATCATTAACAGGATT
OspI R GW	GGGGACAACTTTGTACAAGAAAGTTGGGCAAAGCCTCTTACTTT
OspZ R GW	GGGGACAACTTTGTACAAGAAAGTTGGATAGACTTTAATCTCTG
Spa9 R GW	GGGGACAACTTTGTACAAGAAAGTTGGTCAAACCCCACTCTTAA
Spa13 R GW	GGGGACAACTTTGTACAAGAAAGTTGGTTATCTAATGCCATACTT
Spa24 R GW	GGGGACAACTTTGTACAAGAAAGTTGGCTAAGCAGGAATATTGA
Spa29 R GW	GGGGACAACTTTGTACAAGAAAGTTGGTTATCTAACAAATAGATT
Spa32 R GW	GGGGACAACTTTGTACAAGAAAGTTGGTTAGCATTCTTCTTCACT
Spa33 R GW	GGGGACAACTTTGTACAAGAAAGTTGGTTACTCCTTTACCATCCA
Spa40 R GW	GGGGACAACTTTGTACAAGAAAGTTGGTTAATGAGTGTTTTCAAC
Spa47 R GW	GGGGACAACTTTGTACAAGAAAGTTGGTCTAATTGTTTCACCAAT

Oligos used to generate MyoD fusion protein Gateway compatible destination vectors

OspB_50 R	GGGGACAACTTTGTACAAGAAAGTTGGTTTCTCTCCTAAAAAAAC
OspB_100 R	GGGGACAACTTTGTACAAGAAAGTTGGACTCTGTGGTAGCCATG
OspB_200 R	GGGGACAACTTTGTACAAGAAAGTTGGAGCAGGGGCATTGTTAA
OspD1_50 R	GGGGACAACTTTGTACAAGAAAGTTGGTTCTTCATTGATGGCGT
OspD1_100 R	GGGGACAACTTTGTACAAGAAAGTTGGGTATTTACGCGCTGCAT
OspD1_200 R	GGGGACAACTTTGTACAAGAAAGTTGGTGTAAGTTTTATCCCACT
OspF_50 R	GGGGACAACTTTGTACAAGAAAGTTGGATAGTACGCTGGGTATT
OspF_100 R	GGGGACAACTTTGTACAAGAAAGTTGGCCCAACAAAATCCCCCT
OspF_200 R	GGGGACAACTTTGTACAAGAAAGTTGGAACATCTGACGCCGGAT
VirA_50 R	GGGGACAACTTTGTACAAGAAAGTTGGTTCGTGTGGAGAATATA
VirA_100 R	GGGGACAACTTTGTACAAGAAAGTTGGAAAAACCGAACATATGC
VirA_200 R	GGGGACAACTTTGTACAAGAAAGTTGGTATTTTTGTACTATTGCT
DSW206 F	GACATCATAACGGTTCTGGC
RrnB R	GAAGAGCTCGTTTGTAGAAACGCAAAAAGGCC

Oligos used to generate scanning deletion constructs

OspB_51_100_5	TGGTTTTTTTAGGAGAGAAAGAACCAATAGTAATAAATAA
OspB_51_100_3	TTATTTATTACTATTGGTTCTTTCTCTCCTAAAAAAACCA
OspB_101_150_5	AATCATGGCTACCACAGAGTCAATTAGGCCTTGGTTCGGA
OspB_101_150_3	TCCGAACCAAGGCCTAATTGACTCTGTGGTAGCCATGATT
OspB_151_200_5	ACGGTAGTCCTGGTTCTCATGAAAGTCTTTCTTGTATCCT
OspB_151_200_3	AGGATACAAGAAAGACTTTCATGAGAACCAGGACTACCGT
OspB_201_250_5	ATTTTAACAATGCCCCTGCTCAAGAGCTTTTTCCCTACTC
OspB_201_250_3	GAGTAGGGAAAAAGCTCTTGAGCAGGGGCATTGTTAAAAT
OspD1_51_100_5	CCCACGCCATCAATGAAGAAAGTGAATCGCTGTTGGCAGC
OspD1_51_100_3	GCTGCCAACAGCGATTCACTTTCTTCATTGATGGCGTGGG

OspD1_101_150_5	CCGATGCAGCGCGTAAATACTTTGATTTATCACCAAAAGA
OspD1_101_150_3	TCTTTTGGTGATAAATCAAAGTATTTACGCGCTGCATCGG
OspD1_151_200_5	ATGGAGATTTTATTAAAACTGAAATAGCAGACAGACTTAA
OspD1_151_200_3	TTAAGTCTGTCTGCTATTTCAGTTTTAATAAAATCTCCAT
OspF_50_150_5	GGCAATACCCAGCGTACTATGACAAGTTTCATATTAGTAT
OspF_50_150_3	ATACTAATATGAAACTTGTCATAGTACGCTGGGTATTGCC
OspF_100_150_5	GTAAGGGGGATTTTGTTGGGGCTCAGTTTACGCTATATGT
OspF100_150_3	ACATATAGCGTAAACTGAGCCCCAACAAAATCCCCCTTAC
OspF150_200_5	AATCTCGTGTGGGGATAGGAAGACCTGAAGACTGGAAATA
OspF150_200_3	TATTTCCAGTCTTCAGGTCTTCCTATCCCCACACGAGATT
VirA_50_150_5	GCATATATTCTCCACACGAAGCACCAGAAGTTATTGAAAC
VirA_50_150_3	GTTTCAATAACTTCTGGTGCTTCGTGTGGAGAATATATGC
VirA_150_250_5	ATTTTAAAACTCACAAGCCTGACCTATGTTTTCAAACAGA
VirA_150_250_3	TCTGTTTGAAAACATAGGTCAGGCTTGTGAGTTTTAAAAT
VirA_250_350_5	ATTCAAACTTCCCCCAACAATCGCACATAACATGTAATGG
VirA_250_350_3	CCATTACATGTTATGTGCGATTGTTGGGGGAAGTTTGAAT

Oligos used to generate pmT3SA

minT3 1F	CGAGTACGGCCCCAAGGTCCAAACGGTGATTAATTTTTTAGTTG
minT3 2F	CCCTATATTACCCTGTTATCCCTAGCGTAACTATCGATCTCGAGT
minT3 3F	AATAGGCCGAAATCGGCAAGGATCCCTATATTACCCTGTTATCC
minT3 GBS R	TATACTTTAGATTTTAATTAAACGCGTTTAATTATCCTCAGTCATA

REFERENCES

1. Galan, J. E., Lara-Tejero, M., Marlovits, T. C., and Wagner, S. (2014) Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. *Annu Rev Microbiol* 68, 415-438
2. Cherradi, Y., Hachani, A., and Allaoui, A. (2014) Spa13 of *Shigella flexneri* has a dual role: chaperone escort and export gate-activator switch of the type III secretion system. *Microbiology* 160, 130-141
3. Zahrl, D., Wagner, M., Bischof, K., Bayer, M., Zavecz, B., Beranek, A., Ruckenstuhl, C., Zarfel, G. E., and Koraimann, G. (2005) Peptidoglycan degradation by specialized lytic transglycosylases associated with type III and type IV secretion systems. *Microbiology* 151, 3455-3467
4. Hu, B., Morado, D. R., Margolin, W., Rohde, J. R., Arizmendi, O., Picking, W. L., Picking, W. D., and Liu, J. (2015) Visualization of the type III secretion sorting platform of *Shigella flexneri*. *Proc Natl Acad Sci U S A* 112, 1047-1052
5. Labrec, E. H., Schneider, H., Magnani, T. J., and Formal, S. B. (1964) Epithelial Cell Penetration as an Essential Step in the Pathogenesis of Bacillary Dysentery. *J Bacteriol* 88, 1503-1518

6. Schmitz, A. M., Morrison, M. F., Agunwamba, A. O., Nibert, M. L., and Lesser, C. F. (2009) Protein interaction platforms: visualization of interacting proteins in yeast. *Nat Methods* 6, 500-502
7. Onodera, N. T., Ryu, J., Durbic, T., Nislow, C., Archibald, J. M., and Rohde, J. R. (2012) Genome sequence of *Shigella flexneri* serotype 5a strain M90T Sm. *J Bacteriol* 194, 3022
8. Sidik, S., Kottwitz, H., Benjamin, J., Ryu, J., Jarrar, A., Garduno, R., and Rohde, J. R. (2014) A *Shigella flexneri* virulence plasmid encoded factor controls production of outer membrane vesicles. *G3 (Bethesda)* 4, 2493-2503
9. Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 97, 6640-6645
10. Reeves, A. Z., Spears, W. E., Du, J., Tan, K. Y., Wagers, A. J., and Lesser, C. F. (2015) Engineering *Escherichia coli* into a protein delivery system for mammalian cells. *ACS Synth Biol* 4, 644-654
11. Kuhlman, T. E., and Cox, E. C. (2010) Site-specific chromosomal integration of large synthetic constructs. *Nucleic Acids Res* 38, e92
12. Du, J., Reeves, A. Z., Klein, J. A., Twedt, D. J., Knodler, L. A., and Lesser, C. F. (2016) The type III secretion system apparatus determines the intracellular niche of bacterial pathogens. *Proc Natl Acad Sci U S A* 113, 4794-4799
13. Mou, X. S., S; Du, J; Reeves, AZ; Lesser, CF. (2018, in press) A novel synthetic bottom-up approach reveals the complex interplay of *Shigella* effectors in regulation of epithelial cell death. . *Proc Natl Acad Sci U S A*
14. Costa, S. C., and Lesser, C. F. (2014) A multifunctional region of the *Shigella* type 3 effector IpgB1 is important for secretion from bacteria and membrane targeting in eukaryotic cells. *PLoS One* 9, e93461
15. Walhout, A. J., and Vidal, M. (2001) High-throughput yeast two-hybrid assays for large-scale protein interaction mapping. *Methods* 24, 297-306

Chapter 2:

The RNA Chaperone FinO Mediates Type III Effector Secretion in *Shigella flexneri*

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SUMMARY

Many Gram-negative bacterial pathogens utilize type III secretion systems to inject proteins, referred to as effectors, into the cytosol of host cells to promote bacterial survival. Effectors are conventionally defined by proteinaceous signals and are post-translationally delivered to the membrane embedded type III secretion apparatus via interactions with cognate type III secretion chaperones. However, the secretion of most *Shigella* effectors has not been linked to a chaperone. Here, we report that the RNA chaperone FinO, well-known for its regulatory role in bacterial plasmid conjugation, is involved in type III effector secretion. Unlike known type III secretion chaperones, which recognize protein domains, FinO post-transcriptionally promotes effector secretion via the recognition of structural motifs located within the 5' untranslated region of their mRNAs. Furthermore, FinO-dependent effector secretion is mediated via a translation-dependent pathway. Thus, this study reveals a new mechanism of type III effector secretion in *Shigella flexneri* that may apply to other T3SS-utilizing pathogens.

INTRODUCTION

The virulence of many Gram-negative bacterial pathogens, including *Shigella, Salmonella* and *Yersinia* spp., is dependent on their type III secretion systems (T3SSs)[1,2,3]. These T3SSs function by directly injecting (translocating) tens of proteins, referred to as effectors, from the bacterial cytosol into host cells. T3SSs are structurally conserved nanomachines composed of several multiprotein complexes, including a membrane-embedded export apparatus and a protruding needle, which docks onto host cells[4]. Each pathogen translocates its own unique set of effectors. Effectors are conventionally defined by a bipartite secretion signal, located within their first 50 to 100 amino acids. This secretion signal is composed of an amino(N)-terminal sequence and a downstream domain, which is recognized and bound by cognate type III secretion chaperones[5,6,7]. T3S chaperones are small acidic proteins that, unlike molecular chaperones, do not assist in protein folding, but rather maintain bound effectors in a secretion-competent state[8,9,10].

Furthermore, few reports of RNA determinants exist that define effectors as secreted substrates[11,12,13].

Effectors are delivered to the type III secretion apparatus (T3SA) with the help of the sorting platform, a dynamic protein complex that docks onto the cytosolic surface of the machine and determines the order of effector secretion[14]. Traditionally, the secretion of effectors is modeled to depend on T3S chaperones, which recruit effectors to the sorting platform. Bound effector-chaperone complexes are recognized and dissociated by the T3SA-specific ATPase, which loads the unfolded effectors into the export apparatus of the machine, subsequently resulting in their secretion[15]. However, the secretion of many effectors has not yet been linked to a cognate chaperone. While this may reflect limited investigations in this area, it has been established that the majority of *Shigella flexneri* effectors do not directly bind to nor require one of its characterized chaperones for their secretion[16,17].

Shigella effector gene expression is tightly regulated and occurs in two waves. The genes encoding first wave effectors are transcribed coordinately with the operons that encode components of the T3SA[18]. The majority of these effectors, which are synthesized prior to the assembly and activation of the T3SA, are held in a secretion competent state, in complex with one of the three characterized class I chaperones, IpgA, IpgE or Spa15[19,20,21]. Upon contact, these chaperone-dependent effectors are rapidly injected into host cells. In contrast, second wave effectors, which are transcribed post-secretion activation, are almost exclusively secreted via a pathway independent of IpgA, IpgE, and Spa15[17,22]. These observations suggest that their secretion is mediated via a yet unidentified T3S chaperone or a chaperone-independent pathway.

Reasoning that yet unidentified factors, involved in the secretion of second wave effectors, are under a similar transcriptional regulation, we compared the *Shigella* transcriptomes pre- and post-activation of the T3SA. Post-secretion activation, a substantial enrichment in *finO* transcript was observed. The RNA chaperone FinO is encoded on a large ~220kb *Shigella* virulence plasmid, which also harbors the genetic material for the production of the T3SA[23]. This transcriptomic response was surprising, as FinO had previously only been linked to plasmid conjugation, the type IV secretion

system-mediated transfer of genetic material from one bacterial cell to another[24,25,26], a system, not present on the *Shigella* virulence plasmid[23]. Further analyses led to the discovery that FinO promotes the secretion of at least a subset of second wave effectors via the recognition of mRNA-encoded determinants present within their 5' untranslated regions (5'UTRs). Furthermore, the secretion of FinO-dependent effectors was demonstrated to require active translation. These results demonstrate a role for FinO in promoting translation-dependent effector secretion by possibly utilizing a co-translational type III secretion pathway and support the role of mRNA determinants in defining effectors as secreted substrates.

RESULTS

Shigella type III secretion system activation is associated with increased RNA chaperone FinO gene expression

T3SSs translocate effectors into host cells. However, for T3SSs of some bacteria, *in vitro* conditions that trigger the release of effectors into the surrounding media have been identified. For example, in the case of *Shigella* spp., growth at 37°C induces the expression and assembly of the T3SA[27], which, in the absence of host cells, is activated upon exposure to the dye Congo red[28,29].

The operons that encode the structural components of the T3SA, all its transcriptional regulators, chaperones and the vast majority of effectors are encoded on a large *Shigella* virulence plasmid (VP)[23]. The transcription of the *Shigella* T3SA structural genes as well as first wave effectors, is controlled by VirB, while the expression of second wave effectors is regulated by MxiE[27,22]. MxiE is only activate once the secretion of the first set of effectors has begun. Reasoning, that factors involved in the secretion of second wave effectors would exhibit similar gene expression patterns, the transcriptomes of *Shigella* pre- and post-exposure to Congo red were compared using RNA sequencing (RNAseq) analysis (Figure 1A).

Accordingly, transcripts of MxiE-regulated genes were substantially enriched post-exposure to Congo red (Tables S1 – S4, Figure 1B). However, we also observed global

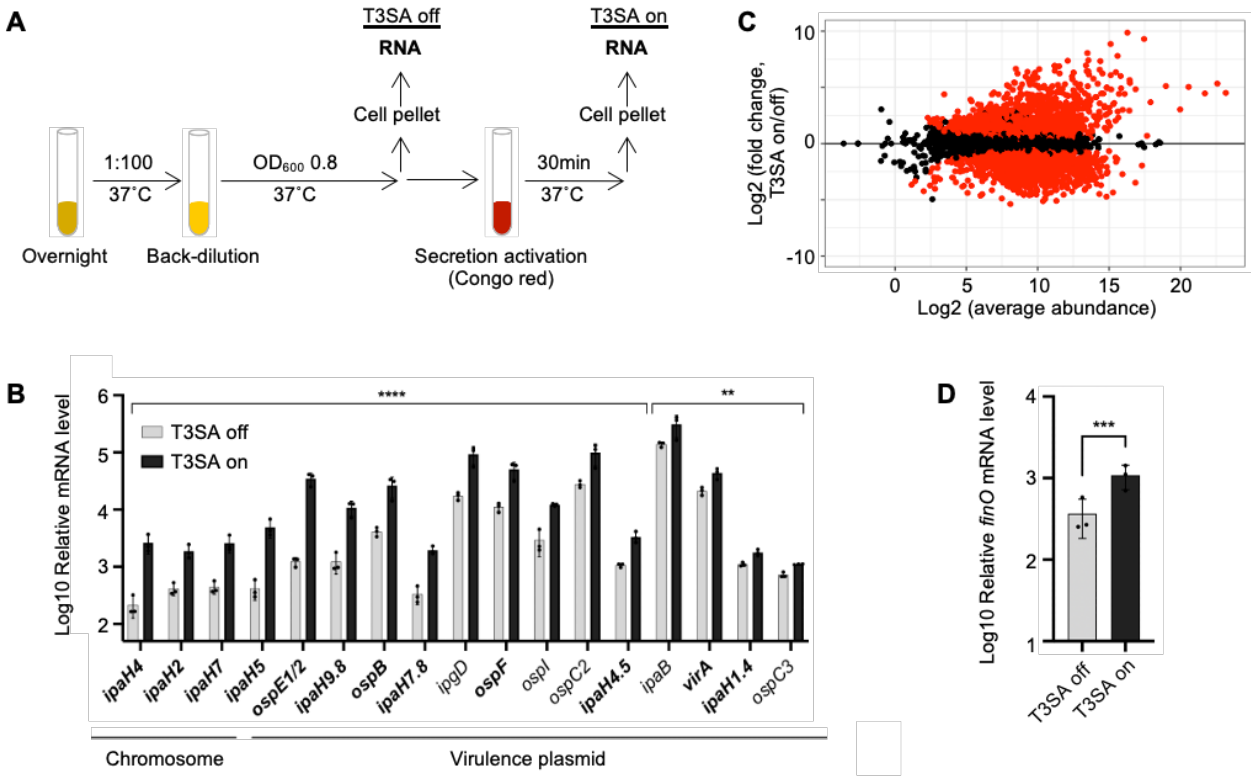

Figure 1. *Shigella* type III secretion system activation is associated with increased RNA chaperone FinO gene expression. (A) Schematic of the protocol used to obtain *Shigella* RNA samples pre- and post-activation of the type III secretion apparatus (T3SA). Equal amounts of bacterial cell were harvested pre- and post-incubation with phosphate-buffered saline supplemented with Congo red (CR), followed by RNA extraction, sequencing and analysis. (B) Bar plot of type III effector genes whose transcripts are substantially enriched post-activation of the *Shigella* T3SA (post-CR) in comparison to pre-exposure to CR (pre-CR). MxiE-regulated effectors are shown in bold. (C) MA plot of transcripts that map to *Shigella* chromosomal genes post-activation of the *Shigella* T3SA. Each dot represents a chromosomally annotated ORF (GenBank accession number AE014073.1), red indicates significant differential expression, genes complying with an applied threshold of log2 fold changes >0.53 and adjusted pValue < 0.05 were considered statistically significant hits. (D) Bar plot of relative expression levels of *finO* detected pre- and post-activation of the T3SA. (B - D) The values shown are based on data from three biological replicates for each condition. Statistical significance in each was determined by a two-sided Wald test with the Benjamini-Hochberg correction for multiple hypothesis testing. ****p-value < 0.0001, ***p-value < 0.0002 and **p-value < 0.01.

changes in the transcript levels of many chromosomally encoded genes (Figure 1C). This likely reflects the exposure of the bacteria to the Congo red containing buffer, as a prior study, which compared the RNA profiles of wildtype *Shigella* and a strain with a constitutively active T3SA in absence of Congo red, did not reveal such a global response[30]. Nevertheless, given that Congo red induced the expression of MxiE-

regulated effectors, we continued our analyses, focusing exclusively on transcripts that map to the large T3SA-encoding VP. In addition to transcripts of T3SS-related genes, mRNA levels corresponding to seven VP-encoded genes were substantially enriched post-secretion activation (Table 1). These genes code for three proteins involved in the

Table 1: **Substantially enriched VP-gene transcripts post secretion activation.**

Gene[a]	Gene product[a] and function	Log2 FC[b]	MxiE-Box
T3SS:			
ospE1	T3S effector	5.61	Yes
ospE2	T3S effector	3.7	Yes
ipaH9.8	T3S effector	3.07	Yes
ospB	T3S effector	2.62	Yes
ipaH7.8	T3S effector	2.51	Yes
ipgF	Periplasmic muramidase	2.37	No
ipgD	T3S effector	2.36	No
ospF	T3S effector	2.15	Yes
ospI	T3S effector	2.02	N/A
ospC2	T3S effector	1.8	No
ipgA	T3S chaperone	1.68	No
ipaH4.5	T3S effector	1.59	Yes
ipaB	T3S effector	1.13	No
ipgE	T3S chaperone	1.09	No
virA	T3S effector	1.03	Yes
ipaH1.4	T3S effector	0.68	Yes
ospC3	T3S effector	0.62	No
Miscellaneous:			
phoN1	Periplasmic non-specific acid phosphatase	3.9	N/A
stbA	Plasmid partition protein, plasmid maintenance	2.77	N/A
ccdA	Antitoxin, plasmid maintenance	2.61	N/A
phoN2/apy	Periplasmic phosphatase apyrase	1.63	Yes
sepA	Extracellular serine protease, promotes invasion of host cells	1.57	N/A
finO	RNA chaperone, regulates bacterial conjugation	1.52	N/A
tap	Leader peptide RepL, plasmid replication	1.33	N/A

[a]*Shigella flexneri* 2a str. 301 plasmid (GenBank accession number *NC_004851.1*) was used as reference sequence. [b]Genes are arranged based on their classification in order of descending log2 foldchange (Log2 FC) (post-/pre-Congo red) values. Only genes considered differentially expressed are shown (adjusted p-value ≤ 0.05). (Genes encoding IS-elements and hypothetical proteins are excluded from this table.). T3SS, type III secretion system

maintenance or replication of the VP; SepA, an autotransporter that promotes *Shigella* invasion into epithelial cells, two periplasmic acid phosphatases of unknown function, and the fertility inhibition protein FinO (Figure 1D)[31,32]. This latter result was surprising, as FinO has only been established to regulate the expression of the plasmid conjugation machinery, yet only remnants of such a system are found on the *Shigella* VP or elsewhere in its genome[23,26,33,34]. In addition, the VP has been repeatedly shown to be non-conjugative[35,36]. Given the growing evidence for the involvement of RNA chaperones in regulating aspects of bacterial virulence[37,38,39,40,41], we proceeded to investigate a role for FinO in *Shigella* pathogenesis and type III effector secretion.

FinO is involved in post-invasion steps in *Shigella* pathogenesis

Shigella spp. are intracellular pathogens that are transmitted via a fecal-oral route. Upon reaching the colon, *Shigella* traverse the intestinal epithelium to gain access to the basolateral surface of intestinal epithelial cells, which they selectively invade[42]. Within the cytosol of infected cells, they establish a replicative niche and use actin-based motility to spread from cell-to-cell[43]. Each of these steps is, at least in part, dependent on the Mxi-Spa type III secretion system.

To begin to assess whether FinO is involved in *Shigella* pathogenesis, we compared the ability of wildtype (WT) and Δ*finO* mutant *Shigella* to form plaques within infected host cell monolayers. Plaques are regions of dead cells lysed when *Shigella* reach high intracellular levels and spread to neighboring cells. The number and size of observed plaques reflect the ability of *Shigella* to initially invade and then spread from cell to cell, respectively. Two days post-infection, monolayers of mouse embryonic fibroblasts (MEFs) infected with WT or Δ*finO* *Shigella* strains exhibited an equivalent number of plaques, demonstrating that FinO is not involved in the initial invasion of host cells, a process dependent on a functional T3SA and several first wave effectors (Figures 2A and 2B). However, monolayers infected with Δ*finO* displayed significantly smaller plaques (Figures 2A and 2C), revealing a role for FinO in the pathogenesis post-invasion of host cells. This finding could reflect a decrease in the ability of *Shigella* to grow within cells or spread from one cell to another. However, the former is likely not the case; if anything, a

modest increase in levels of intracellular Δ*finO* mutant bacteria, as compared to WT *Shigella,* is observed in infected HeLa cells at one- and three-hours post-infection (Figure 2D).

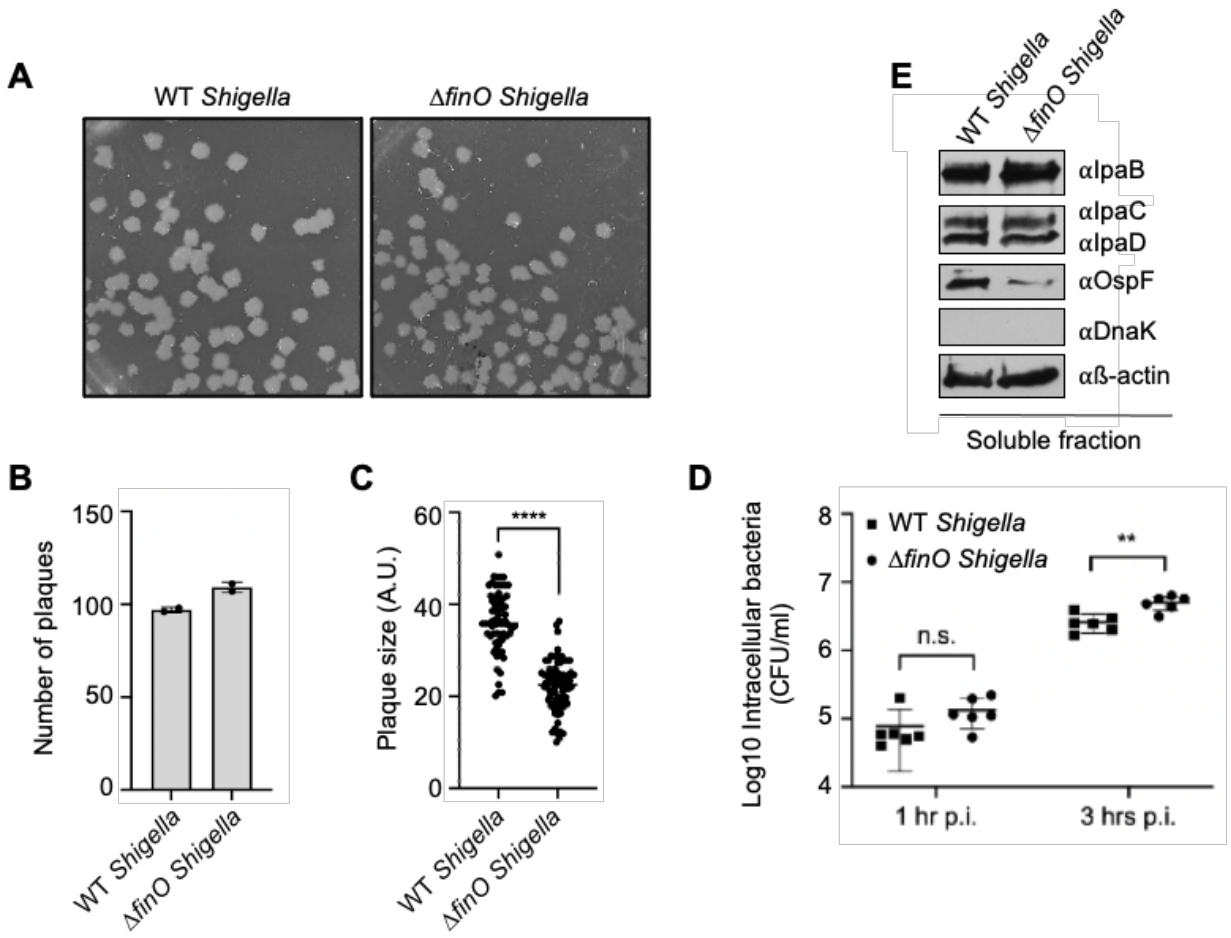

Figure 2. FinO is involved in post-invasion steps in *Shigella* pathogenesis. (A, B, C) Plaque assays to assess the ability of WT and Δ*finO Shigella* to invade and spread from cell-to-cell within infected monolayers of mouse embryonic fibroblasts (MEFs). Assays were performed twice, each time with two biological replicates. (A) Representative images of MEF monolayers obtained 48 hours post-infection with designated strains. (B) Box plot of total number of plaques/well and (C) Scatter plot of measured sizes of a total of 69-82 plaques formed within cell monolayer infected with each strain per well of representative plaque assay pictured in (A), data are mean ± SD, ****p-value < 0.0001, Student's t-test. (D) Box plot of bacterial colony forming units (CFU)/ml isolated from HeLa cells one and three hour/s post-infection with WT or Δ*finO Shigella*, two biological replicates with three technical repeats of each were tested. (E) Translocation assays to compare levels of effectors present within the soluble fractions of HeLa cells 3-hours post-infection with WT or Δ*finO Shigella.* Cell lysates were subjected to denaturing SDS-PAGE before immunoblotting with antibodies that recognize IpaB, IpaC, IpaD, OspF, DnaK (bacterial lysis control) and β-actin (loading control). The immunoblot shown is representative of two experimental repeats with two biological replicates.

Next, we investigated a role for FinO in the translocation of components of the T3SA, initially focusing on IpaB, IpaC, and IpaD, the first proteins delivered into host cells. IpaB and IpaD are components of the needle tip complex, which holds the T3SA in an inactive off configuration pre-host cell contact. IpaB and IpaC are translocases that form a pore complex in the host cell membrane, a crucial step before effector translocation can occur. Three hours post-infection, equivalent IpaB, IpaC, and IpaD levels were observed in the soluble fractions of HeLa cells infected with WT or Δ*finO Shigella* (Figure 2E). In contrast to IpaB, IpaC, and IpaD, the level of OspF, a second wave effector, was decreased in the cytosol of infected cells. These findings suggest that FinO does not regulate the initial expression or activity of the T3SA but acts later to promote the translocation of, at least, OspF into host cells. As plaques formed within infected host cell monolayers by WT and Δ*ospF Shigella* are similar in number and size[44], it seems likely that FinO plays a role in the secretion of additional effectors.

FinO promotes the secretion of multiple second wave effectors

We next investigated a role for FinO in regulating the secretion of effectors. After confirming that WT and Δ*finO Shigella* exhibit similar *in vitro* growth kinetics (Figure S1), we compared the levels of IpaB, IpaC, and IpaD present within bacteria when the strains were grown under conditions that induce the expression of the T3SA (Figure 3A). Consistent with the results of translocation assays (Figure 2E), equivalent levels of all three proteins were observed in bacterial cell lysates. Similarly, thirty minutes post-exposure of the bacteria to Congo red, comparable levels of IpaB, IpaC, and IpaD were observed in the supernatants of both strains. These observations again demonstrate that FinO is not involved in the expression, assembly, or initial activation of the T3SA.

We then proceeded to test whether FinO is involved in the secretion of effectors. For these studies, we used FLAG-tagged effectors that were under the control of their endogenous promoters, when expressed from a low copy number plasmid (Figure 3B). Thirty minutes post-exposure to Congo red, equivalent levels of IpgD and OspC2, two VirB-regulated, first wave chaperone-dependent effectors, were observed in the supernatants of WT and Δ*finO Shigella* (Figure 3C), demonstrating that FinO is not

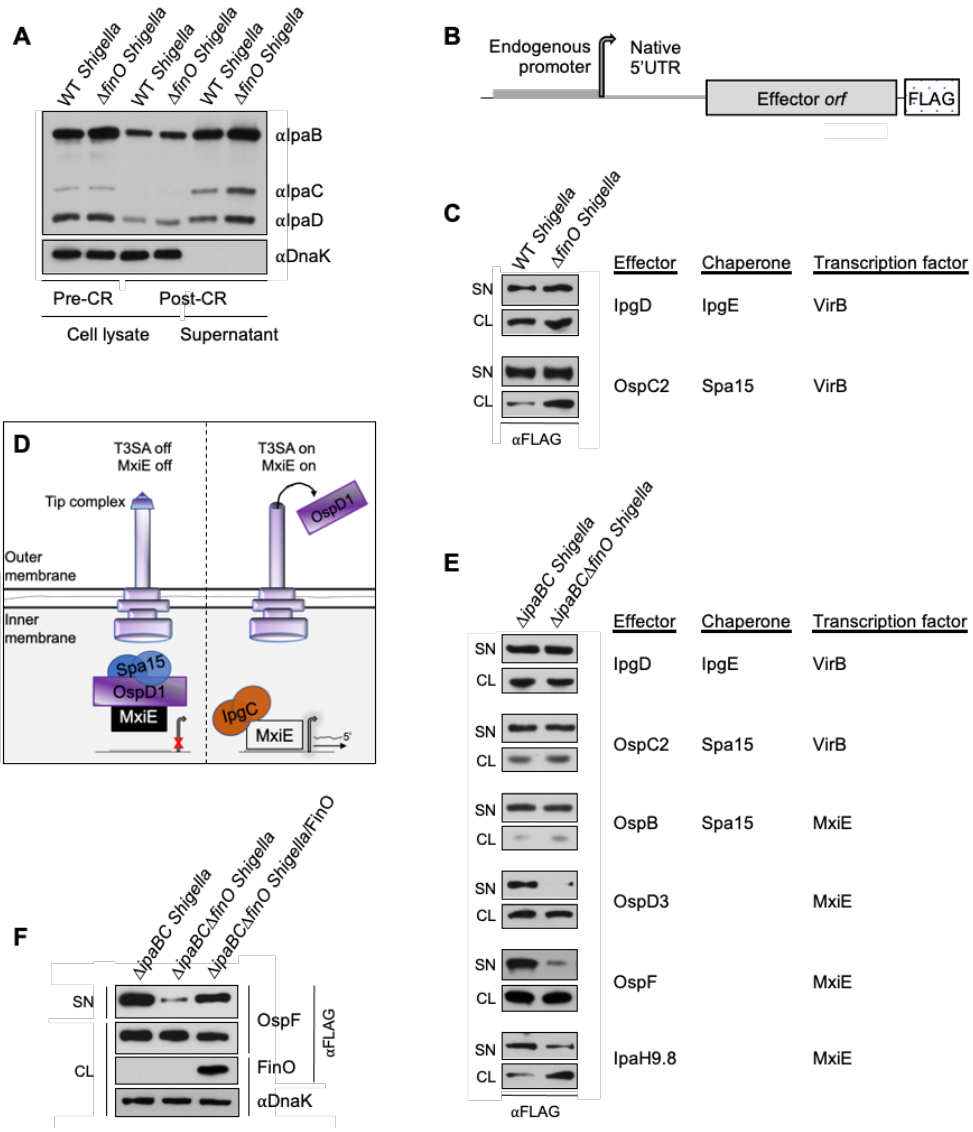

Figure 3. FinO promotes the secretion of multiple second wave effectors. (A, C, E, F) Secretion assays. Each strain was grown under conditions that induce T3SS expression. Bacteria were pelleted and transferred to phosphate-buffered saline plus Congo red (CR). Cell equivalents of bacterial cell lysates (CL) and supernatant fractions (SN) were subjected to denaturing SDS-PAGE before immunoblotting with designated antibodies. The blots shown are representative of at least three experimental repeats. (A) Secretion of IpaB, IpaC and IpaD from WT and Δ*finO Shigella.* Immunoblots of culture supernatant fractions and bacterial cell lysates obtained pre- and 30 minutes post-exposure to CR. (B) Schematic of constructs used to study FLAG-epitope tagged effectors, when gene expression is under the control of their endogenous promoters, resulting in effector transcripts with unique native 5'untranslated regions (5'UTRs). (C) Secretion of endogenously expressed, chaperone-dependent effectors by WT and Δ*finO Shigella*. Immunoblots of culture supernatants and cell lysates of chaperoned effectors obtained post-exposure to CR. (D) Schematic of the secretion-dependent regulation of MxiE activity in *Shigella* pre- and post-activation of the type III secretion apparatus (T3SA). (E) Secretion of endogenously expressed FLAG-tagged effectors by Δ*ipaBC* and Δ*ipaBC*Δ*finO Shigella*. Immunoblots of culture supernatants and cell lysates obtained post-exposure to CR. (F) Secretion of endogenously expressed OspF-FLAG by Δ*ipaBC* and Δ*ipaBC*Δ*finO Shigella*, in the presence/absence of a low copy number plasmid that conditionally expresses FinO-FLAG via an IPTG-inducible promoter.

involved in their secretion. Under these experimental conditions, we were unable to study the secretion of MxiE-regulated effectors, as they are poorly expressed (Figure S2A). Hence, we sought to identify conditions under which we could monitor the *in vitro* secretion of these second wave MxiE-regulated effectors. Prior to secretion activation, MxiE is held in an inactive conformation in complex with OspD1, a first wave effector (Figure 3D)[45]. Once OspD1 is secreted, MxiE is free to bind its activator, IpgC, the class II chaperone that, prior to secretion activation, is held in complex with IpaB and IpaC[46]. *Shigella* strains that lack needle tip IpaB exhibit leaky secretion[47]. This leads to OspD1 release and the liberation of IpgC, resulting in the induction of MxiE-regulated gene expression. Consistent with these observations, *Shigella* that lack both IpaB and IpaC (Δ*ipaBC Shigella)* express significant levels of OspB, OspD3, OspF, and IpaH9.8 (Figure S2B). Having established the conditions to monitor the *in vitro* expression of second wave effectors, we investigated a role for FinO in their secretion by comparing the levels of each released into the supernatants of Δ*ipaBC* and Δ*ipaBC*Δ*finO* mutant *Shigella* strains (Figure 3E). Consistent with the earlier observation, equivalent levels of IpgD and OspC2 were secreted by both *Shigella* strains. The absence of FinO had no effect on the secretion of OspB, a chaperone-dependent second wave effector. In contrast, the Δ*ipaBC*Δ*finO* mutant strain secreted dramatically decreased levels of OspF, OspD3, and IpaH9.8, three second wave, putative chaperone-independent, effectors. This observation was entirely attributable to FinO, as OspF secretion from Δ*ipaBC*Δ*finO* *Shigella* was fully restored with the introduction of a FinO-FLAG expression plasmid (Figure 3F), demonstrating that FinO is involved in promoting the secretion of a subset of MxiE-regulated effectors.

FinO does not regulate MxiE activity

RNA-binding proteins are emerging as major post-transcriptional regulators of the activity of virulence-related genes, including those involved in T3SSs. For example, CsrA, HfQ or the FinO-domain protein, ProQ target hundreds of *Salmonella* transcripts, including ones that control the expression of its T3SSs[40,41,48]. We hence investigated whether FinO controls the activity of MxiE-regulated genes by comparing the luciferase production of

WT, Δ*mxiE,* and Δ*finO Shigella,* which carry a plasmid that expresses the luciferase-producing *Photorhabdus luminscens luxCDABE* operon under the control of a MxiE box-containing promoter (Figure 4A)[49]. When grown at 37°C in Congo red-containing media, all three strains displayed comparable growth kinetics (Figure 4B). As expected, minimal bioluminescence was observed in the absence of MxiE. In contrast, WT and Δ*finO Shigella* exhibited similar patterns of bioluminescence, suggesting that FinO does not regulate MxiE activity and hence the transcription of OspD3, OspF, and IpaH9.8. Thus, we next investigated whether FinO acts post-transcriptionally to control effector secretion.

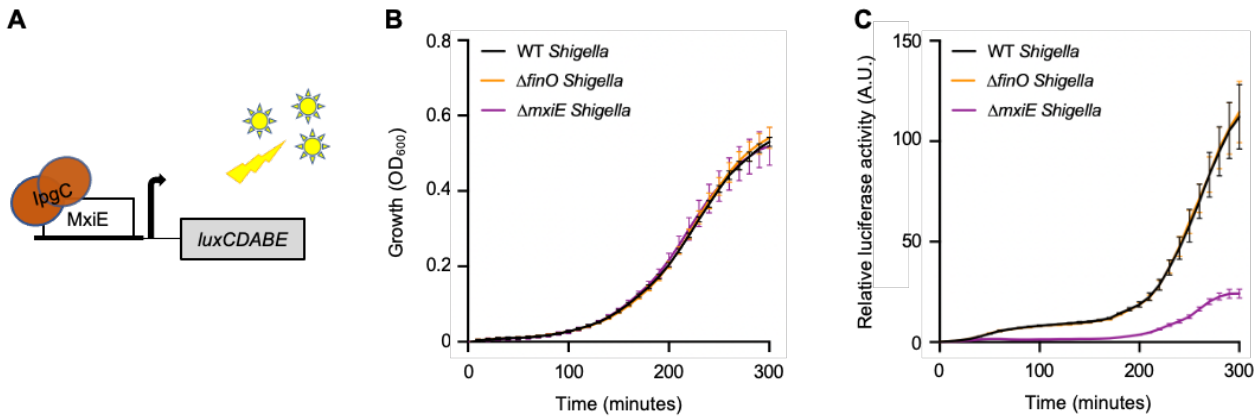

Figure 4. FinO does not regulate MxiE activity. (A) Schematic of MxiE-luc reporter, whereby the *luxCDABE* operon is expressed under the control of a MxiE-regulated promoter. (B) Growth of designated strains that contain the MxiE-luc reporter plasmid and detected (C) Bioluminescence. Bacteria cultivated in tryptic soy broth supplemented with Congo red in 96-well plates. OD_{600} and emitted light were measured every 10 minutes. Data shown are representative of three biological replicates using six technical replicates each time.

FinO-mediated secretion is dependent on effector transcript 5'UTR

The RNA chaperone FinO is best characterized for its role as negative regulator of plasmid conjugation, the transfer of genetic material between bacterial cells. FinO post-transcriptionally inhibits the translation of TraJ, the conjugational master regulator, in part, via the recognition and direct binding of stem loop structures located within the 5'UTR of *traJ* mRNA[50,33,51,52]. That is why we next tested whether the 5'UTRs of effector mRNAs are involved in the FinO-mediated effector secretion pathway. For these studies, we

tested the secretion of FLAG-epitope tagged effectors expressed from an isopropyl β-d-1-thiogalactopyranoside (IPTG)-inducible *lac* promoter. In these constructs, each native effector 5'UTR is replaced with an identical vector-derived synthetic sequence, that includes a consensus ribosome binding site sequence (Figure 5A). Strikingly, the replacement of the native 5'UTRs of each of the three effectors resulted in their secretion via a pathway that no longer depended on FinO, i.e., equivalent levels of OspD3, OspF and IpaH9.8 were secreted by Δ*ipaBC* and Δ*finO*Δ*ipaBC Shigella* (Figure 5B). This result is in stark contrast to the FinO-dependent secretion of these effectors observed when they are expressed from their endogenous promoters that generate native effector transcripts (Figure 3E). These observations suggest that, while signal determinants contained within effector coding sequences are capable of ensuring their secretion, under native conditions, FinO-dependent effector secretion is dictated by mRNA signals present within the 5'UTR of effector transcripts. The *ospF* 5'UTR consists of 21 nucleotides and is predicted to form a stable stem loop structure (ΔG = -3.6 kcal/mol), a conformation in which the ribosome binding site (RBS) and start codon are contained within a base-paired duplex (Figure 5C). To investigate whether mutations predicted to weaken or abolish the formation of this structure influences OspF secretion, we introduced mutations in nucleotides located directly upstream of the translational start site and downstream of the ribosome binding site. (Figures 5D and S3). The introduction of a single mutation at position -1, predicted to decrease the stability of the stem loop (ΔG = -0.1 kcal/mol), resulted in a ~50% secretion reduction, while a triple mutation, predicted to prevent secondary structure formation, diminished OspF secretion by ~90% (Figures 5E and 5F). These mutations minimally altered the levels of OspF present in the bacterial cell lysates, suggesting that they directly impair secretion rather than impact mRNA stability or the rate of translation.

FinO-dependent type III effector secretion requires active translation

Effectors are conventionally described as being secreted via a post-translational pathway. However, there is evidence that under certain conditions, i.e., in the absence of their

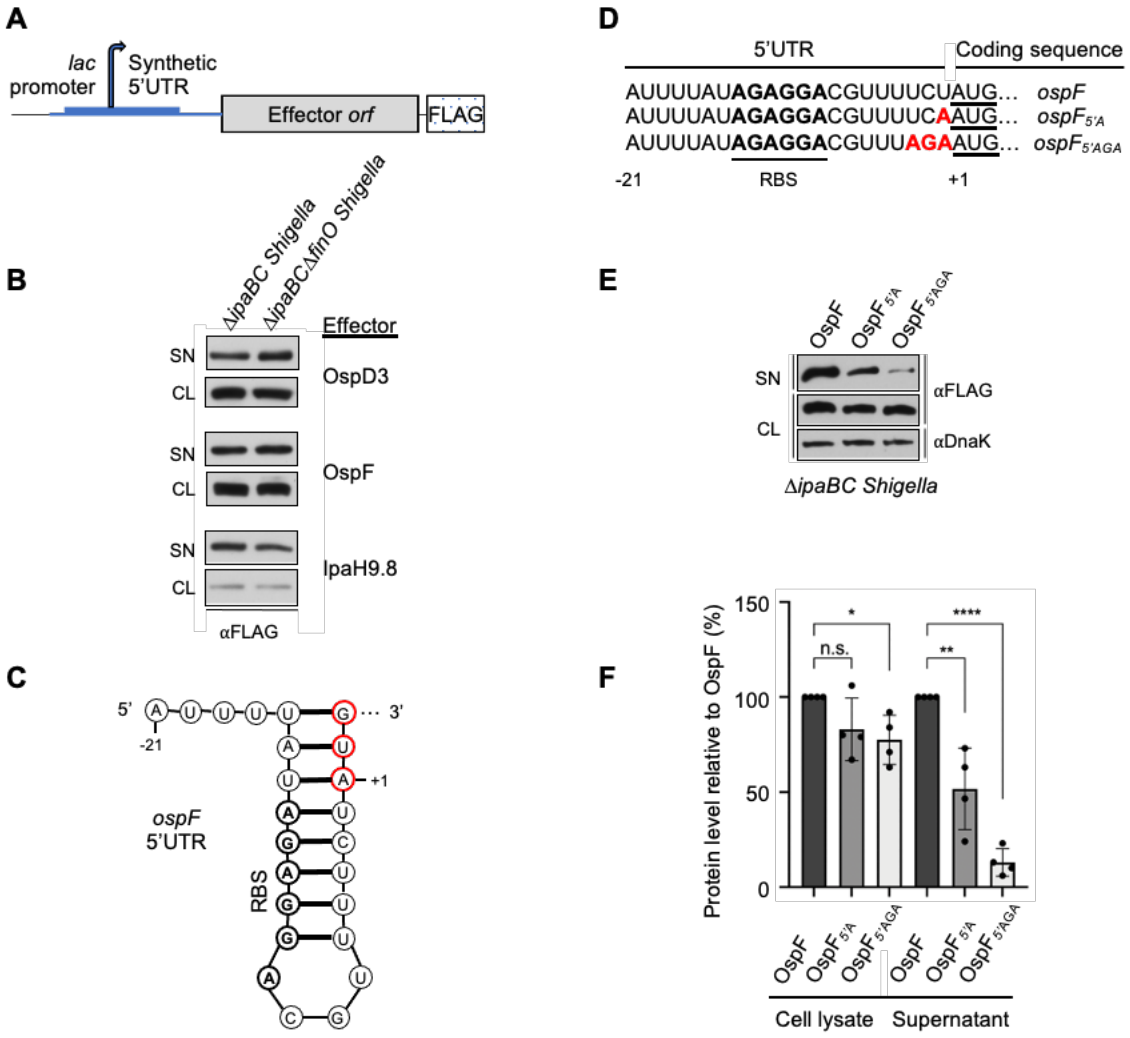

Figure 5. FinO-mediated secretion is dependent on effector transcript 5'UTR. (A) Schematic of constructs used to study FLAG-tagged effectors expressed under the control of an IPTG-inducible p*lac* promoter. (B, E) Secretion assays performed as described in Fig. 3. Representative images of at least three independent repeats. (B) Secretion of IPTG-controlled FLAG-tagged effectors from Δ*ipaBC* and Δ*ipaBC*Δ*finO Shigella*. Immunoblots of culture supernatants (SN) and cell lysates (CL) obtained post-exposure to Congo red (CR). (C) Predicted secondary structure of *ospF* 5'untranslated region (5'UTR) (https://rna.urmc.rochester.edu), ribosome binding site highlighted in bold, nucleotides of the start codon circled in red. (D) Sequences of WT and mutant *ospF* 5'UTR variants, introduced mutations notated in red, ribosome binding site (RBS) in bold and start codon nucleotides underlined. (E) Secretion of unchanged FLAG-tagged OspF effector protein from Δ*ipaBC Shigella*, native and variant *ospF* transcripts (shown in D) were expressed from the same endogenous promoter. Immunoblots of SN and CL obtained 30 min post-exposure to CR are displayed; detected protein level of $OspF_{5'A}$ and $OspF_{5'AGA}$ was quantified and relative to OspF visualized in a (F) Bar plot; representative images of four independent repeats; data are mean ± SD, n.s., not significant, ****p-value < 0.0001, **p-value < 0.005 and *p-value < 0.02, Student's t-test.

cognate chaperones, the secretion of some takes place via a translation-dependent pathway[53]. In this case, their secretion is inhibited in the presence of chloramphenicol, an antibiotic that blocks *de novo* protein synthesis[54,55]. Our finding that FinO-mediated type III secretion requires effector transcript 5'UTR, suggests that their secretion is coupled to translation. To test this hypothesis, we compared the secretion levels of natively expressed OspD3, OspF, and IpaH9.8 in the presence and absence of chloramphenicol (Figure 6A). To ensure that these MxiE-regulated effectors were expressed prior to the addition of chloramphenicol, we examined their secretion from Δ*ipaBC Shigella*. Strikingly, when expressed from native promoters, the secretion of each was completely blocked in the presence of chloramphenicol (Figure 6B). Given that the replacement of the native effector 5'UTR with a synthetic sequence results in a FinO-independent T3S pathway, we investigated whether the secretion of IPTG-driven effectors still requires active translation. Interestingly, when non-native 5'UTRs were existent, OspF and OspD3 were secreted via a pathway that did not require active translation. In contrast, the secretion of IpaH9.8 remained chloramphenicol-sensitive, suggesting a secretion pathway still linked to translation. Taken together, this data provides evidence, that *Shigella* FinO serves as RNA chaperone in a translation-dependent type III secretion pathway.

DISCUSSION

T3SSs mediate the translocation of tens of different effectors into the cytosol of host cells. While the structures of these complex nanomachines are conserved, each delivers a unique set of effector proteins. A significant advance in this area was the discovery of the sorting platform, a dynamic complex of proteins that determines the effector secretion hierarchy by mediating their delivery to the T3SA via interactions with designated chaperones. However, chaperones have not yet been identified for the majority of effectors, raising questions regarding the existence of currently unrecognized chaperones or chaperone-independent secretion pathways. Here, we report that the RNA chaperone FinO promotes the secretion of at least three *Shigella* effectors, whose secretion is not mediated by any of the three well-characterized T3S chaperones, IpgA, IpgE or Spa15.

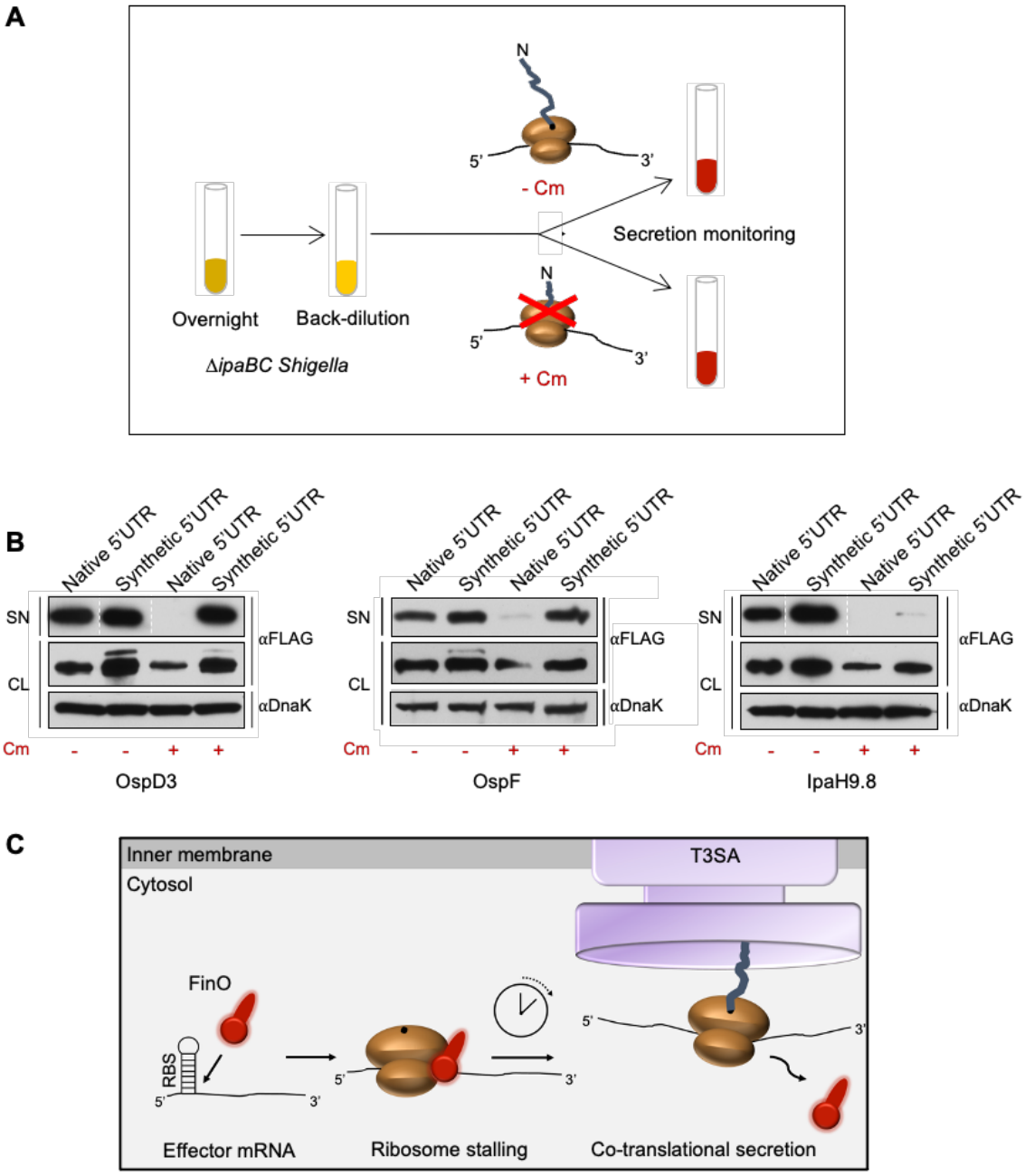

Figure 6. FinO-dependent type III effector secretion requires active translation. (A) Schematic of protocol used to study the role of translation in effector secretion. (B) Secretion of FLAG-epitope tagged OspD3, OspF and IpaH9.8 in presence or absence of chloramphenicol (Cm) from *ΔipaBC Shigella.* Each effector is expressed under the control of its endogenous promotor (native 5'UTR in transcript, Fig. 3B) or the IPTG-inducible *lac* promoter (synthetic 5'UTR in transcript, in Fig. 5A). Representative images of two independent repeats, effectors were analyzed on the same immunoblot under identical conditions, images were cropped for better side-by-side presentation of each individual effector, indicated by dashed line. (C) Schematic of proposed co-translational type III effector secretion pathway promoted by RNA chaperone FinO; RBS, ribosome binding site; T3SA, type III secretion apparatus.

This is a newly identified role for FinO, as prior studies have only focused on its function as post-transcriptional regulator of bacterial plasmid conjugation[33], during which FinO recognizes stem loop secondary structures located in the 5'UTR of *traJ* mRNA and its complementary sequence, the non-coding small RNA FinP[51], promoting the formation of a stable mRNA-sRNA duplex. Analogous, the 5'UTR of effector mRNA was identified to play a role during FinO-mediated type III secretion. In the case of OspF, we determined that a predicted stem loop within this region likely serves as a structural secretion signal, as mutations that disrupt this structure resulted in a dramatic secretion decrease. While we have not yet demonstrated a direct binding interaction between FinO and this element, FinO has previously been established to directly bind to the stem loops of its RNA targets[51].

Interestingly, while mutations in the 5'UTR of *ospF* mRNA disrupted a predicted stem loop and reduced the secretion of the encoded protein, the replacement with a synthetic sequence resulted in unimpaired effector secretion via a FinO-independent pathway. Strikingly, this observation is reminiscent of the findings that lead to the heated debate between the Schneewind and Wolf-Watz laboratories regarding the existence of mRNA-encoded secretion signal sequences. The Schneewind lab, which was the first to propose the presence of mRNA-encoded signals, identified a role for effector mRNA in the secretion of *Yersinia* YopE[11]. Using a reductionist-based approach, they demonstrated that fusion of the native 5'UTR and the first 45 nucleotides of the *yopE* coding sequence to a reporter protein is sufficient to generate a type III secreted variant. Furthermore, they found that frame-shift mutations that completely changed the N-terminal amino acid sequence had a minimal effect on the secretion of the reporter protein. In direct contrast, the Wolf-Watz lab reported that the same frame-shift mutations in full-length YopE result in poorly secreted variants, demonstrating the importance of the N-terminal amino acid sequence in their experimental system[56]. In this case, YopE was expressed from an arabinose-inducible promoter, and its native 5'UTR was replaced with a synthetic vector-derived sequence. Based on our studies, we propose that the Wolf-Watz lab likely was unable to detect a role for an mRNA-encoded secretion signal due to the replacement of the endogenous promoter and 5'UTR with non-native elements.

Furthermore, together these studies support the concept that multiple determinants define effectors. We propose that these determinants are not redundant, but rather provide a means by which the hierarchy of effector secretion is maintained. In support of this hypothesis, decreased levels of OspF were translocated into host cells in the absence of FinO.

How might RNA chaperones, like FinO, post-transcriptionally regulate the secretion of effectors? Regulatory elements that control translation are often located within the 5'UTR of mRNAs. For example, the ribosome binding and translational start sites of *Shigella ospF* are buried within a stem loop structure predicted to form within its 5'UTR. Since FinO binds to and destabilizes stem loops of its established RNA targets, it likely acts in a similar manner to modulate structures within the 5'UTRs of FinO-dependent T3S effectors. We hypothesize that FinO promotes the binding of the ribosome binding site sequence of effector 5'UTR to its complementary sequence, the 16S ribosomal RNA[57], promoting the initiation of translation. However, rather than inducing effector translation, FinO binding would lead to ribosome stalling and translation arrest, which would provide time for the ribosome-mRNA complex to travel to the export gate of the T3SA and enable co-translational effector secretion (Figure 6C). Future studies will focus on discerning whether FinO controls the secretion of effectors via direct mRNA interactions or in conjunction with sRNAs and will assess if components of the sorting platform are involved.

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AUTHOR CONTRIBUTIONS

N.H.E. and C.F.L conceived and designed the experiments. N.H.E. and C.Y.L conducted the experiments. N.H.E. performed RNAseq and carried out the bioinformatic analysis. N.H.E. and C.F.L analyzed the data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Cammie F. Lesser (clesser@mgh.harvard.edu).

Materials availability Materials, including strains and plasmids, are available upon request from the authors.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All bacterial strains, plasmids and oligonucleotides are summarized in detail in Tables S5-7.

Bacterial strains and growth conditions. In this study, the wildtype and mutant variants of *Shigella flexneri* serotype 2a strain 2457T were used[58]. Bacteria were grown at 37˚C

and cultured in liquid TCS (trypticase soy) broth under constant aeration or on solid TCS agar plates supplemented with 10μM Congo red (CR). All plasmid cloning steps were carried out in *Escherichia coli* DH5α, which were grown at 37˚C in liquid Luria-Bertani (LB) broth under constant aeration or on solid LB agar plates. Where noted, antibiotics were used at the following concentrations: 100μg/ml ampicillin (Amp), 50μg/ml kanamycin (Kan), 100μg/ml spectinomycin (Spec).

Eukaryotic cell lines and growth conditions. HeLa cells, obtained from ATCC, and mouse embryonic fibroblasts (MEFs), a generous gift from Dr. Marcia Goldberg, were grown at 37˚C in 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

METHOD DETAILS

Generation of an IPTG-inducible FinO expression plasmid. An Isopropyl β-d-1-thiogalactopyranoside (IPTG)-inducible FinO-FLAG expression plasmid was generated using the Gateway (Invitrogen) based recombination system. The FinO coding sequence lacking its stop codon (open configuration) was PCR-amplified from *Shigella* such that flanking *attB* sites were introduced at both ends and a consensus Shine-Dalgarno sequence upstream engineered upstream of its start codon. After introduction into pDNR221 via a Gateway™ BP reaction, the sequence of the *gene insert of the entry clone* was verified by Sanger sequencing. A Gateway LR reaction was performed to introduce *finO* into pDSW206-ccdB-FLAG, a p*lac* destination plasmid with an in-frame 3xFLAG epitope-tag sequence located at the 3' end of the Gateway cassette. The resulting plasmid was verified by restriction digestion analyses.

Generation of endogenous effector expression plasmids. Plasmids for endogenous expression of effectors IpgD, OspB, OspD3, OspF and IpaH9.8 were generated via Gateway™ cloning as previously described[59]. First, fragments of DNA containing gene *ORFs*, in an open configuration, plus 200 – 407bp of upstream nucleotides were PCR

amplified from *Shigella* virulence plasmid DNA using oligos described in Table S4 and flanking *attB* sites at both ends were introduced. Upstream gene regions were chosen to include regulatory elements of promoter regions, such as the previously mapped MxiE-binding site[60]. In the case of *ospD3*, which is in an operon with *ospC1*, a >2kb fragment of DNA was amplified, containing both gene *ORF*s plus an endogenous promoter region. Plasmids for endogenous expression of stem loop disrupted OspF were generated with gBlock gene fragments obtained from IDT. Received gBlocks contained the *ospF* ORF in an open configuration plus a 96bp upstream region, which harbors the unchanged promoter sequence with its MxiE-binding site, plus the native 5' untranslated region (UTR) with an unchanged Shine-Dalgarno sequence, but carried a single (position 21, T to A, referred to 5'A) or three nucleotide alterations (positions 19-21, TCT to AGA, referred to 5'AGA) introduced directly upstream of the start codon. Each synthetic gBlock had flanking *attB* sites at both ends. Each PCR or gBlock fragment was introduced into pDNR221. After the inserts were sequence-verified, they were transferred into pCMD136-ccdB-FLAG, a low-copy number plasmid (SC101 origin, copy number 1-5) engineered to contain a Gateway cassette followed by an in-frame 3xFLAG epitope-tag sequence. All generated endogenous expression plasmids were verified by restriction digests.

Generation of luciferase reporter plasmid. pMxiE-*luc* was generated by replacing the PLhrtO promoter in pMM534 with the endogenous promoter region of IpaH7.8, that harbors a regulatory MxiE-box in its promoter[60,61]. The *ipaH7.8* promoter was amplified by PCR from pTSAR1Ud2.4s[62] with pMxiE-*luc* Fwd/Rev oligonucleotides, listed in Table S7, that add *XhoI* and *KpnI* restriction sites and a consensus Shine-Dalgarno sequence[62]. The digested PCR product was ligated with the XhoI/KpnI cut pMM534 fragment using T4 DNA ligase (New England Biolabs). The new construct (pMxiE-*luc*) was sequence-verified by Sanger sequencing. The plasmid pTSAR1Ud2.4s was a generous gift from Dr. François-Xavier Campbell-Valois, University of Ottawa, Canada. The plasmid pMM534 was obtained from Addgene (#112530).

Deletion strain construction. The deletion strains used in this study were generated via the λ Red recombination system[63] using oligonucleotides described in Table S7. The Δ*ipaBC*Δ*finO Shigella* strain was generated after first removing the *ipaBC* locus from wildtype (WT) *Shigella* resulting in the generation of Δ*ipaBC::*KAN *Shigella.* After resolution of the Kan^R cassette, which is flanked by FRT (FLIP recognition target) sites, using the FLP recombinase encoded on pCP20, *finO* was then deleted from the strain to generate Δ*ipaBC*Δ*finO::*KAN *Shigella.* The Kan^R cassettes of the Δ*finO* and Δ*mxiE Shigella* strains were also resolved. Generated gene deletion strains were verified by PCR using gene- and Kan^R cassette-specific oligonucleotides.

Liquid secretion assay. Liquid secretion assays were performed as previously described[16]. In brief, overnight cultures grown in TCS broth were diluted into 2 ml of TCS broth and incubated at 37°C under constant aeration until mid-log growth phase was reached. When the proteins of interest were not under an endogenous promoter, but under the transcriptional control of a p*lac* promoter, gene expression was induced for 45 minutes with 1 mM IPTG when the diluted cultures reached an OD_{600} of ~0.4. OD_{600} of each bacterial culture was measured and equivalent numbers of bacteria from each culture was pelleted, resuspended in 2 ml of phosphate-buffered saline (PBS) supplemented with 10 μM Congo red (Sigma) and incubated for an additional 30 minutes. Bacterial cultures were centrifuged and the cell pellets resuspended in loading dye. After an additional centrifugation step to remove remaining intact bacteria, proteins in the supernatant fractions were TCA (trichloroacetic acid)-precipitated [10% (vol/vol)] and resuspended in loading dye. Proteins from supernatant fractions and cell lysates were separated via SDS-PAGE and immunoblotted with designated antibodies.

Chloramphenicol assay. Cultures were treated similarly to a liquid secretion assay, with the difference that mid-log phase grown Δ*ipaBC Shigella* cultures were split in two and the antibiotic chloramphenicol (30μg/ml, Cm) was added directly to one half, whereas the other half was treated equally in absence of it. After five minutes, the cells were briefly

centrifuged and resuspended in PBS-CR supplemented with or without Cm. Supernatant fractions and cell lysates were prepared as described above.

Growth and Luciferase assay. Overnight cultures of designated strains with or without the luciferase reporter plasmid, pMxiE-*luc*, were diluted to an optical density 600nm (OD_{600}) of 0.02 in 200µl TCS broth in 96-well plate wells (Corning) and incubated in a plate reader (SpectraMax i3x, Molecular Devices) with periodic shaking and constant incubation temperature at 37˚C. Optical densities and bioluminescence measurement (absorbance, all wavelengths) were measured every ten minutes for 5 or 10 hours. Six technical replicates were studied each time. Data was analyzed and the absorbance at 600nm and relative luciferase activity (arbitrary unit) over time was plotted using GraphPad Prism.

Plaque assay was performed as previously described[64]. In brief, one day prior to infection, $7x10^5$ mouse embryonic fibroblasts (MEFs)/well were seeded in six-well plates (Corning). The following day, confluent monolayers were infected with mid-log phase grown bacteria at a multiplicity of infection (MOI) of 0.002. Bacteria was centrifuged onto the cells (800x*g,* 10 minutes), followed by an incubation at 37˚C in 5% CO_2. After one hour, the media was replaced with fresh DMEM containing 0.5% agarose, 10% FBS, 0.45% glucose and gentamicin (25µg/ml). After 48 hours of incubation, an additional overlay (DMEM, 0.7% agarose, 10% FBS, 0.45% glucose, gentamicin (25µg/ml) and 0.1% neutral red) was applied. After an additional incubation for four hours, the plates were imaged in high resolution using a photo scanner (Epson Perfection 4990). ImageJ was used to quantify the formed plaque area and data was plotted using GraphPad Prism.

Gentamicin protection assay. HeLa cells were seeded at a density of 4×10^4 per well in a 96-well plate. The following day, WT and Δ*finO Shigella* overnight cultures were back diluted 1:100 into TCS broth and incubated at 37C for 2 hours until mid-log phase was reached, upon which same cell equivalents were suspended in DMEM. Confluent HeLa cell monolayers were washed twice with pre-warmed DMEM and infected, at a multiplicity

of infection (MOI) of 100, with the designated bacterial strains, using two biological replicates with three technical repeats of each. Infection is carried out at 37C with 5% CO_2. Bacteria was centrifuged onto the HeLa cells at 1,000 rpm for 10 minutes and incubated for 30 minutes. Media was removed and HeLa cells were washed three times with DMEM, supplemented with gentamicin (50µg/ml). After which, fresh DMEM plus gentamicin was added to each well. One- and three-hour-infection durations were assessed. At the end of each time point, infection was stopped and bacteria from within the HeLa cytosol isolated. HeLa cells were washed 3 times with phosphate-buffered saline (PBS) and then permeabilized for 15 minutes at 37C with 1% Triton X-100 in PBS. Cell lysate containing intact cytoplasmic bacteria was collected and serial dilutions of each were plated to determine colony forming units (CFU) for each time point.

Translocation assay was performed as previously described[65]. In brief, $6x10^5$ HeLa cells seeded per well in six-well plates were infected at a multiplicity of infection (MOI) of 30 with WT or Δ*finO Shigella*. Bacteria was centrifuged onto the cells (800x*g*, 10 minutes) followed by an incubation for one hour at 37˚C in 5% CO_2, at which point gentamicin (50µg/ml) was added to each well. After two additional hours, each well was washed three times with pre-warmed PBS. Cells were incubated on ice for 15 minutes in 250µl ice cold radioimmunoprecipitation assay (RIPA) buffer (25mM Tris, pH 8, 150mM NaCl, 0.1% SDS, 1% NP-40 plus cOmplete™ cocktail protease inhibitors (Roche) to lyse mammalian but not bacterial cell membranes. A sterile cell scraper was used to remove attached cells and the lysates were centrifuged to separate the soluble (supernatant) fraction from the insoluble (pellet) fractions. After an additional centrifugation step, proteins from the supernatant fraction were separated via SDS-PAGE and immunoblotted with designated antibodies.

Western blot. Protein lysates and supernatant fractions from liquid secretion and translocation assays were heated for 5 min at 95°C before being separated via SDS-PAGE, after which a transfer onto nitrocellulose membranes occured. Membranes were blocked in 5% milk in 1x TBST for 1 hour at room temperature and then probed with

1:10000 α-FLAG (Sigma Aldrich), 1:1000 α-OspF, 1:20,000 α-IpaB, 1:20,000 α-IpaC, 1:40000 α-IpaD, 1:10,000 α-DnaK (Abcam) or 1:50,000 β-actin (Abcam) primary antibodies. The α-OspF antibody was a gift from Dr. Anthony Maurelli, University of Florida, Gainesville, USA. The α-IpaB, IpaC and IpaD antibodies were gifts from Dr. Wendy Picking, University of Kansas, Lawrence, USA. Probed membranes were incubated with 1:10000 α-mouse- or -rabbit-HRP secondary antibodies (Jackson ImmunoResearch Laboratories). Blots were treated with a chemiluminescent substrate and exposed to a film. Developed films were scanned using a photo scanner (Epson Perfection 4990) and protein levels quantified using ImageJ.

Secondary structure analysis of effector mRNA. Secondary structure analysis of the 5'UTR and first 30 nucleotides of the coding sequence of *ospF* mRNA were performed using the RNAstructure tool (v.6.0.1)[66]. The RNA sequence was generated by transcribing the corresponding DNA sequence of the *Shigella* virulence plasmid using the Transcription and Translation Tool (Attotron Biosensor Corporation).

RNA extraction, library preparation and sequencing. Three biological replicate cultures of wildtype *Shigella flexneri* serotype 2a strain 2457T were grown overnight at 37°C in TCS broth under constant aeration. Cultures were back diluted 1:100 into fresh media and grown under the same conditions until they reached mid-log phase growth (OD_{600} of 0.8). Each culture was divided in two and 800µl of each were pelleted, half of the pellets was washed in PBS and immediately placed on dry ice. The other half was resuspended in PBS-CR and incubated for another 30 minutes at 37°C under constant aeration (as described in liquid secretion assay). Bacterial cell equivalents were pelleted, washed in PBS and immediately placed on dry ice. RNA was extracted from each pellet using hot phenol-chloroform described in detail in Lorenz *et al*.[67] and total RNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA quality was evaluated using an Agilent Bioanalyzer 2100 and the Agilent RNA 6000 Nano kit (Santa Clara). Ribosomal RNA (rRNA) was depleted using the Ribo Zero rRNA removal kit for Gram-negative bacteria (Illumina) and cDNA libraries were

generated with a NEBNext® Ultra™ directional RNA library preparation kit for the Illumina system using NEBNext® multiplex oligonucleotides for Illumina index primer set 1 (New England Biolabs). The size distribution of the generated library was monitored using an Agilent 2200 TapeStation high-sensitivity D1000 ScreenTape system. High-throughput sequencing, as well as Bioanalyzer and TapeStation analyses were carried out at the Biopolymers Facility at Harvard Medical School, where cDNA libraries were pooled and sequenced in paired-end mode (2x 75bps) using a mid-output NextSeq 500 flow cell (Illumina).

High-performance computing and bioinformatic transcriptomic analysis. An average of 15 million reads per sample and condition were obtained. Data and differential expression analyses were performed using a Linux-based high-performance computing platform (O2, Harvard Medical School) and R/Rstudio with loaded DESeq2 and ggplot2 packages. Sequencing depth and RNA composition were taken into account when transcript counts were DESeq2-normalized using a median of ratios method (Tables S1 and S3). An average of 88% of the sequencing reads were mapped to the *Shigella* chromosome (GenBank accession number *AE014073.1*) and 7% to the virulence plasmid pCP301 (GenBank accession number NC_004851.1). Chromosomal and virulence plasmid transcript counts are available in Table S1 and S2. Genes exhibiting an applied threshold of log2 foldchanges >0.53 and adjP (adjusted p-Value) <0.05 were considered statistically significant hits (Tables S2 and S4).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were carried out in R/RStudio using the DeSeq2 package. A two-sided Wald test with the Benjamini-Hochberg correction for multiple hypothesis was applied for differential gene expression analyses. Statistical differences between two means were tested using the unpaired Student's t-test with GraphPad Prism.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse α-β-actin	Abcam	Cat# ab49900
Mouse α-FLAG	Sigma Aldrich	Cat# F1804
Mouse α-DnaK	Abcam	CAT# ab69617
Rabbit α-IpaB	Gift from Wendy Picking	N/A
Rabbit α-IpaC	Gift from Wendy Picking	N/A
Rabbit α-IpaD	Gift from Wendy Picking	N/A
Rabbit α-OspF	Gift from Anthony Maurelli	N/A
Goat α-mouse-HRP	Jackson ImmunoResearch Laboratories	Cat# 115-035-003
Goat α-rabbit-HRP	Jackson ImmunoResearch Laboratories	Cat# 111-035-144
Bacterial and Virus Strains		
Escherichia coli DH5α	Laboratory strain collection	N/A
Wildtype (WT) *Shigella flexneri* 2457T serotype 2a	Laboratory strain collection	N/A
Δ*finO::*KAN *Shigella flexneri 2457T serotype 2a*	This study	N/A
Δ*finO Shigella flexneri 2457T serotype 2a*	This study	N/A
Δ*ipaBC Shigella flexneri 2457T serotype 2a*	Laboratory strain collection	N/A
Δ*ipaBC*Δ*finO::*KAN *Shigella flexneri 2457T serotype 2a*	This study	N/A
Δ*mxiE Shigella flexneri 2457T serotype 2a*	Laboratory strain collection	N/A
Chemicals, Peptides and Recombinant Proteins		
Congo red	Sigma Aldrich	Cat# C6277
Ethanol 200 proof	Thermo Fisher Scientific	Cat# 04-355-450
Ethanol 190 proof	Thermo Fisher Scientific	Cat# 04-355-722
Methanol	Thermo Fisher Scientific	Cat# A412P4
Trichloroacetic acid (TCA)	Sigma Aldrich	Cat# T0699
Neutral red	Millipore Sigma	Cat# N2889
Nitrocellulose membrane	Thermo Fisher Scientific	Cat# PI-88018
Tween 20	Thermo Fisher Scientific	Cat# BP337-100
Acrylamide:bisacrylamide 29:1 (40% polyacrylamide)	Thermo Fisher Scientific	Cat# BP1408-1
Gentamicin sulfate	Sigma Aldrich	Cat# G3632
Ampicillin sodium salt	Sigma Aldrich	Cat# A9518
Chloramphenicol	Sigma Aldrich	Cat# C0378
Kanamycin sulfate	Sigma Aldrich	Cat# K1377
Spectinomycin dihydrochloride pentahydrate	Thermo Fisher Scientific	Cat# 50-213-657
Tetramethylethylenediamine (TEMED)	Thermo Fisher Scientific	Cat# PI17919
Ammonium persulfate (APS)	Sigma Aldrich	Cat# A9164

Acid phenol:chloroform pH 4.5	Thermo Fisher Scientific	Cat# AM9722
Phenol:chloroform:isoamyl alcohol	Thermo Fisher Scientific	Cat# 15593049
Chloroform:isoamyl alcohol 24:1	Sigma Aldrich	Cat# C0549-1PT
Eukaryotic Cell Lines		
Mouse embryonic fibroblasts	Gift of Marcia Goldberg	N/A
HeLa CCL-2	American Type Culture Collection (ATCC)	N/A
Critical Commercial Assays		
Turbo DNA-free kit	Ambion	Cat# AM1907
Ribo Zero rRNA removal kit for Gram-negative bacteria	Illumina	Cat# MRZGN126
NEBNext® Ultra™ directional RNA library preparation kit	Illumina	Cat# E7420
NEBNext® multiplex oligonucleotides for Illumina index primer set 1	New England Biolabs	Cat# E7335S
West Pico PLUS Chemiluminescent Substrate	Thermo Fisher Scientific	Cat# 34580
Gateway™ BP Clonase Enzyme Mix	Thermo Fisher Scientific	Cat# 11789021
Gateway™ LR Clonase Enzyme Mix	Thermo Fisher Scientific	Cat# 11791100
cOmplete™ cocktail protease inhibitors	Millipore Sigma	Cat# 11697498001
Oligonucleotides		
See Table S7 for all oligonucleotides used in this study.		
Recombinant DNA		
pCP20	Laboratory strain collection	[63]
pDNR221	Laboratory strain collection	Invitrogen
pENTR221-*finO* (open configuration)	This manuscript	N/A
pENTR221-endP-*ipgD* (open configuration)	This manuscript	N/A
pENTR221-endP-*ospB* (open configuration)	This manuscript	N/A
pENTR221-endP-*ospC1-ospD3* (open configuration)	This manuscript	N/A
pENTR221-endP-*ospF* (open configuration)	This manuscript	N/A
pENTR221-endP-$ospF_{5'A}$ (open configuration)	This manuscript	N/A
pENTR221-endP-$ospF_{5'AGA}$ (open configuration)	This manuscript	N/A
pENTR221-endP-*ipaH9.8* (open configuration)	This manuscript	N/A
pCMD136-ccdb-FLAG	Laboratory strain collection	[59]
pCMD136-endP-*ipgD*-FLAG	This manuscript	N/A
pCMD136-endP-*ospB*-FLAG	This manuscript	N/A
pCMD136-endP-*ospC2*-FLAG	Laboratory strain collection	[59]
pCMD136-endP-*ospC1-ospD3*-FLAG	This manuscript	N/A
pCMD136-endP-*ospF*-FLAG	This manuscript	N/A
pCMD136-endP-$ospF_{5'A}$-FLAG	This manuscript	N/A
pCMD136-endP-$ospF_{5'AGA}$-FLAG	This manuscript	N/A

pCMD136-endP-*ipaH9.8*-FLAG	This manuscript	N/A
pDSW206-ccdB-FLAG	Laboratory strain collection	[16]
pDSW206-*finO*-FLAG	This manuscript	N/A
pDSW206-*ipgD*-FLAG	Laboratory strain collection	[16]
pDSW206-*ospB*-FLAG	Laboratory strain collection	[16]
pDSW206-*ospC2*-FLAG	Laboratory strain collection	[16]
pDSW206-*ospD3*-FLAG	Laboratory strain collection	[16]
pDSW206-*ospF*-FLAG	Laboratory strain collection	[16]
pDSW206-*ipaH9.8*-FLAG	Laboratory strain collection	[16]
pTSAR1Ud2.4s	Gift from François-Xavier Campbell-Valois	[62]
pMM534	Addgene	Cat# 112530
pMxiE-*luc*	This manuscript	N/A
Software and Algorithms		
ImageJ (1.52q)	National Institutes of Health	https://imagej.nih.gov/ij/
GraphPad Prism 8 (v9.0.1)	Graphpad Software	https://www.graphpad.com/scientific-software/prism/
O2 High Performance Compute Cluster	Harvard Medical School	https://it.hms.harvard.edu/our-services/research-computing
R/Rstudio	R project	https://www.r-project.org
Bowtie2 (v2.3.4.3)	[68]	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
ggplot2 (v3.1.0)	[69]	https://ggplot2.tidyverse.org.
DESeq2 (v1.31.18)	[70]	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
Transcription and Translation Tool	Attotron Biosensor Corporation	http://biomodel.uah.es/en/lab/cybertory/analysis/trans.htm
RNAstructure Web Server (v6.0.1)	[66]	https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html

REFERENCES

1. Andrews, G. P. & Maurelli, A. T. mxiA of *Shigella flexneri* 2a, which facilitates export of invasion plasmid antigens, encodes a homolog of the low-calcium-response protein, LcrD, of *Yersinia pestis*. *Infect. Immun.* 60, 3287–3295 (1992).
2. Kubori, T. *et al.* Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* 280, 602–605 (1998).
3. Troisfontaines, P. & Cornelis, G. R. Type III secretion: more systems than you think. *Physiol. Bethesda Md* 20, 326–339 (2005).

4. Galán, J. E., Lara-Tejero, M., Marlovits, T. C. & Wagner, S. Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. *Annu. Rev. Microbiol.* 68, 415–438 (2014).
5. Sory, M. P., Boland, A., Lambermont, I. & Cornelis, G. R. Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the cyaA gene fusion approach. *Proc. Natl. Acad. Sci.* 92, 11998–12002 (1995).
6. Schesser, K., Frithz-Lindsten, E. & Wolf-Watz, H. Delineation and mutational analysis of the *Yersinia pseudotuberculosis* YopE domains which mediate translocation across bacterial and eukaryotic cellular membranes. *J. Bacteriol.* 178, 7227–7233 (1996).
7. Buchko, G. W. *et al.* A multi-pronged search for a common structural motif in the secretion signal of *Salmonella enterica* serovar *Typhimurium* type III effector proteins. *Mol. Biosyst.* 6, 2448–2458 (2010).
8. Bennett, J. C. Q. & Hughes, C. From flagellum assembly to virulence: the extended family of type III export chaperones. *Trends Microbiol.* 8, 202–204 (2000).
9. Parsot, C. The various and varying roles of specific chaperones in type III secretion systems. *Curr. Opin. Microbiol.* 6, 7–14 (2003).
10. Thomas, N. A., Ma, I., Prasad, M. E. & Rafuse, C. Expanded roles for multicargo and class 1B effector chaperones in type III secretion. *J. Bacteriol.* 194, 3767–3773 (2012).
11. Anderson, D. M. & Schneewind, O. A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. *Science* 278, 1140–1143 (1997).
12. Anderson, D. M. & Schneewind, O. *Yersinia enterocolitica* type III secretion: an mRNA signal that couples translation and secretion of YopQ. *Mol. Microbiol.* 31, 1139–1148 (1999).
13. Niemann, G. S. *et al.* RNA Type III Secretion Signals That Require Hfq. *J. Bacteriol.* 195, 2119–2125 (2013).
14. Lara-Tejero, M., Kato, J., Wagner, S., Liu, X. & Galan, J. E. A Sorting Platform Determines the Order of Protein Secretion in Bacterial Type III Systems. *Science* 331, 1188–1191 (2011).
15. Allison, S. E. *et al.* Identification of the docking site between a type III secretion system ATPase and a chaperone for effector cargo. *J. Biol. Chem.* 289, 23734–23744 (2014).
16. Schmitz, A. M., Morrison, M. F., Agunwamba, A. O., Nibert, M. L. & Lesser, C. F. Protein interaction platforms: visualization of interacting proteins in yeast. *Nat. Methods* 6, 500–502 (2009).

17. Ernst, N. H., Reeves, A. Z., Ramseyer, J. E. & Lesser, C. F. High-Throughput Screening of Type III Secretion Determinants Reveals a Major Chaperone-Independent Pathway. *mBio* 9, (2018).
18. Gall, T. L. *et al.* Analysis of virulence plasmid gene expression defines three classes of effectors in the type III secretion system of *Shigella flexneri*. *Microbiol. Read. Engl.* 151, 951–962 (2005).
19. Ogawa, M., Suzuki, T., Tatsuno, I., Abe, H. & Sasakawa, C. IcsB, secreted via the type III secretion system, is chaperoned by IpgA and required at the post-invasion stage of *Shigella* pathogenicity: The role of IcsB in *Shigella* pathogenicity. *Mol. Microbiol.* 48, 913–931 (2003).
20. Niebuhr, K. *et al.* IpgD, a protein secreted by the type III secretion machinery of *Shigella flexneri*, is chaperoned by IpgE and implicated in entry focus formation. *Mol. Microbiol.* 38, 8–19 (2000).
21. van Eerde, A., Hamiaux, C., Pérez, J., Parsot, C. & Dijkstra, B. W. Structure of Spa15, a type III secretion chaperone from *Shigella flexneri* with broad specificity. *EMBO Rep.* 5, 477–483 (2004).
22. Mavris, M. *et al.* Regulation of transcription by the activity of the *Shigella flexneri* type III secretion apparatus. *Mol. Microbiol.* 43, 1543–1553 (2002).
23. Buchrieser, C. *et al.* The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri*. *Mol. Microbiol.* 38, 760–771 (2000).
24. Lederberg, J. & Tatum, E. L. Gene recombination in *Escherichia coli*. *Nature* 158, 558 (1946).
25. Finnegan, D. J. & Willetts, N. S. Two classes of Flac mutants insensitive to transfer inhibition by an F-like R factor. *Mol. Gen. Genet. MGG* 111, 256–264 (1971).
26. Lawley, T. D., Klimke, W. A., Gubbins, M. J. & Frost, L. S. F factor conjugation is a true type IV secretion system. *FEMS Microbiol. Lett.* 224, 1–15 (2003).
27. Tobe, T. *et al.* Temperature-regulated expression of invasion genes in *Shigella flexneri* is controlled through the transcriptional activation of the virB gene on the large plasmid. *Mol. Microbiol.* 5, 887–893 (1991).
28. Qadri, F. *et al.* Congo red binding and salt aggregation as indicators of virulence in *Shigella* species. *J. Clin. Microbiol.* 26, 1343–1348 (1988).
29. Bahrani, F. K., Sansonetti, P. J. & Parsot, C. Secretion of Ipa proteins by *Shigella flexneri*: inducer molecules and kinetics of activation. *Infect. Immun.* 65, 4005–4010 (1997).
30. Silué, N., Marcantonio, E. & Campbell-Valois, F.-X. RNA-Seq analysis of the T3SA regulon in *Shigella flexneri* reveals two new chromosomal genes upregulated in the on-state. *Methods San Diego Calif* 176, 71–81 (2020).

31. Benjelloun-Touimi, Z., Sansonetti, P. J. & Parsot, C. SepA, the major extracellular protein of *Shigella flexneri*: autonomous secretion and involvement in tissue invasion. *Mol. Microbiol.* 17, 123–135 (1995).
32. Páez-Espino, A. D., Chavarría, M. & de Lorenzo, V. The two paralogue phoN (phosphinothricin acetyl transferase) genes of Pseudomonas putida encode functionally different proteins: The two phoN genes of P. putida. *Environ. Microbiol.* 17, 3330–3340 (2015).
33. Arthur, D. C. *et al.* FinO is an RNA chaperone that facilitates sense-antisense RNA interactions. *EMBO J.* 22, 6346–6355 (2003).
34. van Biesen, T. & Frost, L. S. The FinO protein of IncF plasmids binds FinP antisense RNA and its target, traJ mRNA, and promotes duplex formation. *Mol. Microbiol.* 14, 427–436 (1994).
35. Sansonetti, P. J., Kopecko, D. J. & Formal, S. B. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. *Infect. Immun.* 35, 852–860 (1982).
36. Parajuli, P., Rajput, M. I. & Verma, N. K. Plasmids of *Shigella flexneri* serotype 1c strain Y394 provide advantages to bacteria in the host. *BMC Microbiol.* 19, 86 (2019).
37. Gebhardt, M. J., Kambara, T. K., Ramsey, K. M. & Dove, S. L. Widespread targeting of nascent transcripts by RsmA in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci.* 117, 10520–10529 (2020).
38. Chao, Y. & Vogel, J. The role of Hfq in bacterial pathogens. *Curr. Opin. Microbiol.* 13, 24–33 (2010).
39. Holmqvist, E., Li, L., Bischler, T., Barquist, L. & Vogel, J. Global Maps of ProQ Binding In Vivo Reveal Target Recognition via RNA Structure and Stability Control at mRNA 3′ Ends. *Mol. Cell* 70, 971-982.e6 (2018).
40. Westermann, A. J. *et al.* The Major RNA-Binding Protein ProQ Impacts Virulence Gene Expression in *Salmonella enterica* Serovar *Typhimurium*. *mBio* 10, (2019).
41. Sittka, A., Pfeiffer, V., Tedin, K. & Vogel, J. The RNA chaperone Hfq is essential for the virulence of *Salmonella typhimurium*. *Mol. Microbiol.* 63, 193–217 (2007).
42. Ashida, H., Ogawa, M., Mimuro, H. & Sasakawa, C. *Shigella* infection of intestinal epithelium and circumvention of the host innate defense system. *Curr. Top. Microbiol. Immunol.* 337, 231–255 (2009).
43. Bernardini, M. L., Mounier, J., d'Hauteville, H., Coquis-Rondon, M. & Sansonetti, P. J. Identification of icsA, a plasmid locus of *Shigella flexneri* that governs bacterial intra- and intercellular spread through interaction with F-actin. *Proc. Natl. Acad. Sci. U. S. A.* 86, 3867–3871 (1989).
44. Zurawski, D. V., Mitsuhata, C., Mumy, K. L., McCormick, B. A. & Maurelli, A. T. OspF and OspC1 Are *Shigella flexneri* Type III Secretion System Effectors That

Are Required for Postinvasion Aspects of Virulence. *Infect. Immun.* 74, 5964–5976 (2006).

45. Parsot, C. *et al.* A secreted anti-activator, OspD1, and its chaperone, Spa15, are involved in the control of transcription by the type III secretion apparatus activity in *Shigella flexneri*. *Mol. Microbiol.* 56, 1627–1635 (2005).
46. Pilonieta, M. C. & Munson, G. P. The chaperone IpgC copurifies with the virulence regulator MxiE. *J. Bacteriol.* 190, 2249–2251 (2008).
47. Ménard, R., Sansonetti, P. & Parsot, C. The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD. *EMBO J.* 13, 5293–5302 (1994).
48. Martínez, L. C. *et al.* Integration of a complex regulatory cascade involving the SirA/BarA and Csr global regulatory systems that controls expression of the *Salmonella* SPI-1 and SPI-2 virulence regulons through HilD: Activation of SPI-1 and SPI-2 regulons by the SirA regulatory cascade. *Mol. Microbiol.* 80, 1637–1656 (2011).
49. Craney, A. *et al.* A synthetic luxCDABE gene cluster optimized for expression in high-GC bacteria. *Nucleic Acids Res.* 35, e46 (2007).
50. Ghetu, A. F. *et al.* The FinO repressor of bacterial conjugation contains two RNA binding regions. *Biochemistry* 38, 14036–14044 (1999).
51. Arthur, D. C. *et al.* Mapping interactions between the RNA chaperone FinO and its RNA targets. *Nucleic Acids Res.* 39, 4450–4463 (2011).
52. Mark Glover, J. N. *et al.* The FinO family of bacterial RNA chaperones. *Plasmid* 78, 79–87 (2015).
53. Page, A.-L., Sansonetti, P. & Parsot, C. Spa15 of *Shigella flexneri*, a third type of chaperone in the type III secretion pathway. *Mol. Microbiol.* 43, 1533–1542 (2002).
54. Jardetzky, O. Studies on the mechanism of action of chloramphenicol. I. The conformation of chloramphenicol in solution. *J. Biol. Chem.* 238, 2498–2508 (1963).
55. Wolfe, A. D. & Hahn, F. E. Mode of Action of Chloramphenicol. Effects of Chloramphenicol upon a Ribosomal Amino Acid Polymerization System and its Binding to Bacterial Ribosome. *Biochim. Biophys. Acta* 95, 146–155 (1965).
56. Lloyd, S. A., Norman, M., Rosqvist, R. & Wolf-Watz, H. Yersinia YopE is targeted for type III secretion by N-terminal, not mRNA, signals. *Mol. Microbiol.* 39, 520–531 (2001).
57. Shine, J. & Dalgarno, L. The 3’-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proc. Natl. Acad. Sci. U. S. A.* 71, 1342–1346 (1974).

58. Labrec, E. H., Schneider, H., Magnani, T. J. & Formal, S. B. Epithelial Cell Penetration as an Essential Step in the Pathogenesis of Bacillary Dysenthery. *J. Bacteriol.* 88, 1503–1518 (1964).
59. Mou, X., Souter, S., Du, J., Reeves, A. Z. & Lesser, C. F. Synthetic bottom-up approach reveals the complex interplay of *Shigella* effectors in regulation of epithelial cell death. *Proc. Natl. Acad. Sci.* 115, 6452–6457 (2018).
60. Bongrand, C., Sansonetti, P. J. & Parsot, C. Characterization of the Promoter, MxiE Box and 5′ UTR of Genes Controlled by the Activity of the Type III Secretion Apparatus in *Shigella flexneri*. *PLoS ONE* 7, e32862 (2012).
61. Mimee, M. *et al.* An ingestible bacterial-electronic system to monitor gastrointestinal health. *Science* 360, 915–918 (2018).
62. Campbell-Valois, F.-X. *et al.* A Fluorescent Reporter Reveals On/Off Regulation of the *Shigella* Type III Secretion Apparatus during Entry and Cell-to-Cell Spread. *Cell Host Microbe* 15, 177–189 (2014).
63. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci.* 97, 6640–6645 (2000).
64. Duncan-Lowey, J. K., Wiscovitch, A. L., Wood, T. E., Goldberg, M. B. & Russo, B. C. *Shigella flexneri* Disruption of Cellular Tension Promotes Intercellular Spread. *Cell Rep.* 33, 108409 (2020).
65. Slagowski, N. L., Kramer, R. W., Morrison, M. F., LaBaer, J. & Lesser, C. F. A functional genomic yeast screen to identify pathogenic bacterial proteins. *PLoS Pathog.* 4, e9 (2008).
66. Reuter, J. S. & Mathews, D. H. RNAstructure: software for RNA secondary structure prediction and analysis. *BMC Bioinformatics* 11, 129 (2010).
67. Lorenz, C., Dougherty, T. J. & Lory, S. Transcriptional Responses of *Pseudomonas aeruginosa* to Inhibition of Lipoprotein Transport by a Small Molecule Inhibitor. *J. Bacteriol.* 202, e00452-20 (2020).
68. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359 (2012).
69. Hadley Wickham. *ggplot2: Elegant Graphics for Data Analysis*. (Springer-Verlag New York, 2016).
70. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550 (2014).

SUPPLEMENTAL FIGURES

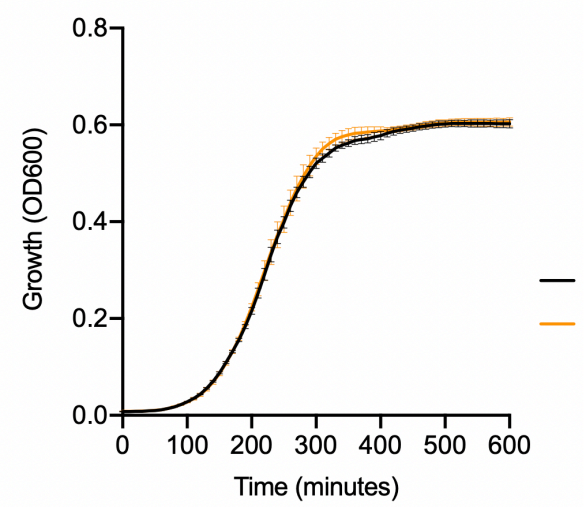

Figure S1. FinO has no effect on *Shigella in vitro* growth. Optical density 600nm (OD_{600}) measurements of wildtype (WT) and Δ*finO Shigella* grown in TCS media obtained every 10 minutes for 10 hours, data shown is representative of three biological repeats using six technical replicates each time, data are mean ± SEM.

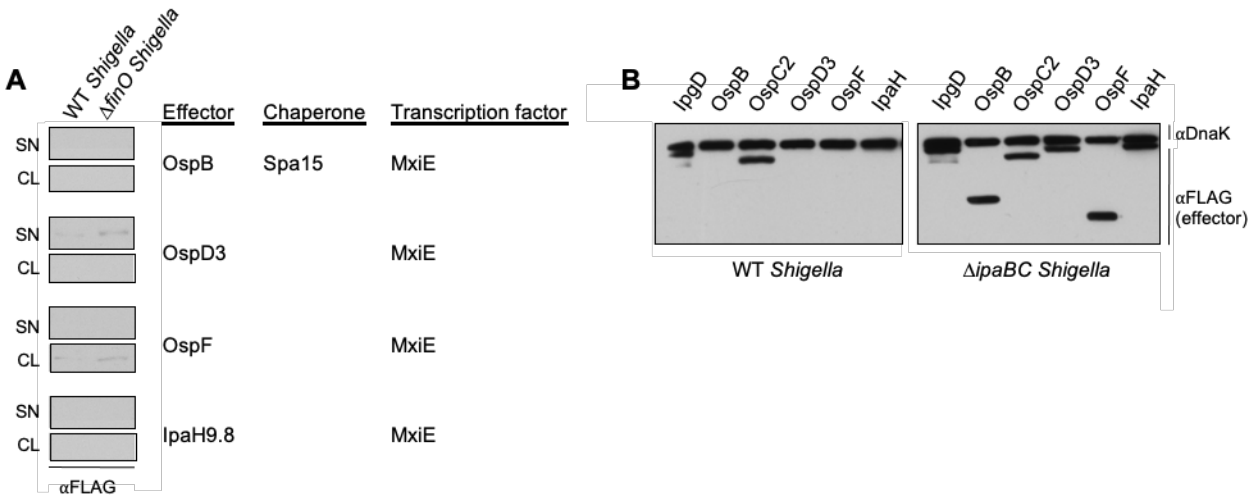

Figure S2. Increased MxiE-regulated late effector production in leaky Δ*ipaBC Shigella*. (A) Secretion of MxiE-regulated FLAG-tagged effectors from WT and Δ*finO Shigella,* when under the control of their native promoters*,* assay was conducted as described in Fig. 3*.* Culture supernatants (SN) and cell lysates (CL) obtained post-exposure to Congo red are shown. Immunoblots are representative of at least three experimental repeats. (B) Immunoblots of bacterial cell lysates of mid-log phase grown WT and Δ*ipaBC Shigella*, that endogenously express the designated effectors from a low copy number plasmid, blots were probed with antibodies that recognize FLAG-epitope tag and DnaK (loading control). Blots shown are representative of at least three experimental repeats.

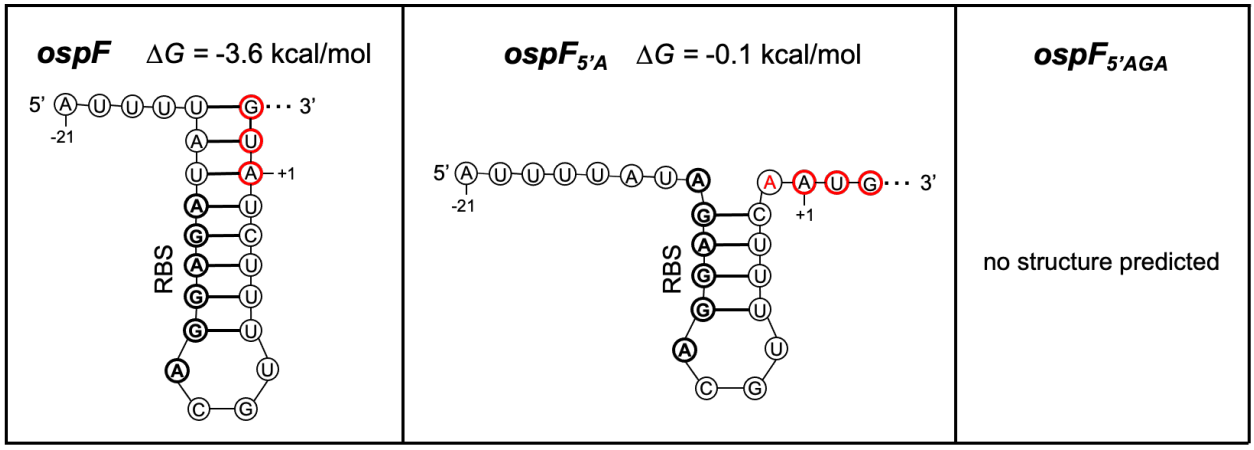

Figure S3. Native *ospF* mRNA 5'UTR is predicted to form a stem-loop. Predicted secondary structures of native (*ospF*) and modified effector 5'UTR, when a single (*ospF*$_{5'A}$) or three (*ospF*$_{5'AGA}$) point mutations are introduced. RNAstructure tool (v.6.0.1)[1] was used to identify predicted RNA secondary structures. Introduced modification highlighted in red; 5'UTR, 5' untranslated region; RBS, ribosome binding site (in bold); Start, translational start site (in red).

SUPPLEMENTAL TABLES

Table S1: VP-gene transcripts pre- and post-secretion activation.

Table S2: Differences in virulence plasmid gene transcripts.

Table S3: Chromosomal gene transcripts pre- and post-secretion activation.

Table S4: Differences in chromosomal gene transcripts.

Tables S1 – S4 are available upon request.

Table S5: Bacterial strains used in this study

Name	Characteristics	Source
WT *Shigella*	*Shigella flexneri* 2457T serotype 2a, wildtype	Laboratory strain collection
Δ*finO*::KAN *Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*finO*::FRT-*KanR*-FRT	This study
Δ*finO Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*finO*::FRT	This study
Δ*ipaBC Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*ipaBipaC*::FRT	Laboratory strain collection
Δ*ipaBC*Δ*finO*::KAN *Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*ipaBipaC*::FRT, ΔfinO::FRT-*KanR*-FRT	This study
Δ*mxiE Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*mxiE*::FRT	Laboratory strain collection

Table S6: Plasmids used in this study

Name	Characteristics	Source
pCP20	Temperature sensitive, Amp^R, FLP recombinase	[2]
pKD46	Temperature sensitive, λ Red recombinase, Amp^R	[2]
pDNR221	Gateway® donor vector, pUC ori, Kan^R, Cm^R	Invitrogen
pENTR221-*finO*	Entry vector, open and closed configuration, Kan^R	This study
pENTR221-endP-*ipgD*	Entry vector, open configuration with 313bp upstream endogenous promoter region, Kan^R	This study
pENTR221-endP-*ospB*	Entry vector, open configuration with 250bp upstream endogenous promoter region, Kan^R	This study
pENTR221-endP-*ospC1-ospD3*	Entry vector, open configuration with 2217bp upstream endogenous promoter region, Kan^R	This study
pENTR221-endP-*ospF*	Entry vector, open configuration with 200bp upstream endogenous promoter region, Kan^R	This study
pENTR221-endP-*ipaH7.8*	Entry vector, open configuration with 407bp upstream endogenous promoter region, Kan^R	This study
pENTR221-endP-*ipaH9.8*	Entry vector, open configuration with 295bp upstream endogenous promoter region, Kan^R	This study
pCMD136-ccdb-FLAG	Destination vector containing a gateway cassette and 3xFLAG, very low copy (pSC101 ori), $Spec^R$, Cm^R	[3]
pCMD136-endP-*ipgD*-FLAG	Endogenous promoter expression plasmid, $Spec^R$	This study
pCMD136-endP-*ospB*-FLAG	Endogenous promoter expression plasmid, $Spec^R$	This study
pCMD136-endP-*ospC2*-FLAG	Endogenous promoter expression plasmid, $Spec^R$	[3]
pCMD136-endP-*ospC1-ospD3*-FLAG	Endogenous promoter expression plasmid, $Spec^R$	This study
pCMD136-endP-*ospF*-FLAG	Endogenous promoter expression plasmid, $Spec^R$	This study
pCMD136-endP-*ipaH7.8*-FLAG	Endogenous promoter expression plasmid, $Spec^R$	This study
pCMD136-endP-*ipaH9.8*-FLAG	Endogenous promoter expression plasmid, $Spec^R$	This study
pDSW206-ccdB-FLAG	Destination vector containing a gateway cassette and 3xFLAG, p*lac* (IPTG), low copy (ColE1 origin), Amp^R, Cm^R	[4]
pDSW206-*finO*-FLAG	IPTG-inducible expression plasmid, Amp^R	This study
pTSAR1Ud2.4s	GFP reporter plasmid, MxiE-promoter, pUC18 backbone, ColE1 origin, Amp^R	[5]
pMM534	pZE2-PLhrtO-luxCDABE, *luxCDABE*, constitutive promoter PLhrtO, ColE1 origin, KanR	[6]
pMxiE-*luc*	Luciferase reporter plasmid, *luxCDABE*, MxiE-promoter, pMM534 backbone, Kan^R	This study

Table S7: Oligonucleotides used in this study

Name	Characteristics	Notes
Oligonucleotides to generate deletion strains:		
finO 5W	GAGCAGAAGCGACCGGTACTGACA CTGAAGCGGAAGACAGAGGGAACA GCGTGTAGGCTGGAGCTGCTTC	F: to generate Δ*finO Shigella*. Underlined: homology to Kan^R cassette
finO 3W	ATCAAGCACGGCCTGAAGTTCTGC CTTTATCCGGTTCTGGCGACGAATT TGGTCCATATGAATATCCTCCTTAG	R: to generate Δ*finO Shigella.* Underlined: homology to Kan^R cassette
Oligos to confirm deletion strains:		
Kan_F	GATGATCTGGACGAAGAGCATCAG	F: to verify Δ*finO Shigella* strains, part of set 1
FinO_174DN	CGCCAGACCTGGCCGTTCCG	R: to verify Δ*finO Shigella* strains, part of set 1
FinO 400up	CTGACGTGGTGTGAAACGCGC	F: to verify Δ*finO Shigella* strains, part of set 2
FinO Rev	GCTTCCTTTCGGAAGCGGAAG	R: to verify Δ*finO Shigella* strains, part of set 2
Oligos to PCR *ORF*s into Gateway entry vectors:		
5' universal	GGGGACAACTTTGTACAAAAAAGTT GGC**GAAGGAGATAGAACC**ATG	F: To generate pENTR221 clones that require a Shine-Dalgarno (SD) sequence. Underlined: AttB1 site; Bold: SD
5GW_finO	C**GAAGGAGATAGAACC**ATGACAGA GCAGAAGCGACCGGTAC	F: To generate pENTR221-*finO* (open/closed). Bold: SD
3GW_finO_NS	GGGGACAACTTTGTACAAGAAAGTT GGTTTCTCATCAAGCACGGCCTGA AGTTCTGCC	R: To generate pENTR221-*finO* (open). Underlined: AttB2 site
Oligos to PCR endogenous promoter-*ORF* fragments into Gateway entry vectors:		
ipgD_endP_GW	GGGGACAACTTTGTACAAA AAAGTTGGCACTTTATTAACTCTCC ATTAC	F: to generate pENTR221-endP-ipgD. Underlined: AttB1 site
ipgD_rev_NS	GGGGACAACTTTGTACAAGAAAGTT GGTACAAATGACGAATACCCTTTCA C	R: to generate pENTR221-endP-ipgD. Underlined: AttB2 site
ospB_endP_GW	GGGGACAACTTTGTACAAA AAAGTTGGCGGATGATCTCCACGG TACTTTAGG	F: to generate pENTR221-endP-ospB. Underlined: AttB1 site
ospB_rev_NS	GGGGACAACTTTGTACAAGAAAGTT GGATCCAGTTCTTTATTAATAATAAA AGTC	R: to generate pENTR221-endP-ospB. Underlined: AttB2 site
ospC1_endP_GW	GGGGACAACTTTGTACAAAAAAGTT GGCTAAGGCGATTTTGAACTCATAA GGG	F: to generate pENTR221-endP-ospC1-ospD3. Underlined: AttB1 site
ospD3_rev_NS	GGGGACAACTTTGTACAAGAAAGTT GGGCTTTTTATATTCTTCATAATTTC TAGAAAA	R: to generate pENTR221-endP-ospC1-ospD3. Underlined: AttB2 site

ospF_endP_GW	GGGGACAACTTTGTACAAAAAAGTTGGCCAAGGGCTACCGCAACCGGGAGCGC	F: to generate pENTR221-endP-ospF. Underlined: AttB1 site
ospF_rev_NS	GGGGACAACTTTGTACAAGAAAGTTGGCTCTATCATCAAACGATAAAATGGT	R: to generate pENTR221-endP-ospF. Underlined: AttB2 site
ipaH7.8_endP_GW	GGGGACAACTTTGTACAAAAAAGTTGGC AGTCATACAAGGACTTCATTCGCGG	F: to generate pENTR221-endP-ipaH7.8. Underlined: AttB1 site
ipaH7_8_rev_NS	GGGGACAACTTTGTACAAGAAAGTTGGTGAATGGTGCAGTCGTGA	R: to generate pENTR221-endP-ipaH7.8. Underlined: AttB2 site
ipaH9.8_endP_GW	GGGGACAACTTTGTACAAAAAAGTTGGCGTTGCCTTGGAAAGTTGATCTGACC	F: to generate pENTR221-endP-ipaH9.8. Underlined: AttB1 site
ipaH9_8_rev_NS	GGGGACAACTTTGTACAAGAAAGTTGGTGAATGGTGCAGTTGTGAGCCGT	R: to generate pENTR221-endP-ipaH9.8. Underlined: AttB2 site
Oligos to generate pMxiE-*luc* reporter plasmid:		
pMxiE-luc Fwd	CAATGGTACC**TTTCTCCTCTTTA**GAATAAATATTTAAAATTGTAT	F: to generate MxiE-regulated luciferase expression plasmid. Underlined: XhoI restriction site
pMxiE-luc Rev	CAATCTCGAGCCCTCGTATTAAATGTGTAT	R: to generate MxiE-regulated luciferase expression plasmid. Underlined: KpnI restriction site

REFERENCES

1. Reuter, J. S. & Mathews, D. H. RNAstructure: software for RNA secondary structure prediction and analysis. *BMC Bioinformatics* **11**, 129 (2010).
2. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci.* **97**, 6640–6645 (2000).
3. Mou, X., Souter, S., Du, J., Reeves, A. Z. & Lesser, C. F. Synthetic bottom-up approach reveals the complex interplay of *Shigella* effectors in regulation of epithelial cell death. *Proc. Natl. Acad. Sci.* **115**, 6452–6457 (2018).
4. Schmitz, A. M., Morrison, M. F., Agunwamba, A. O., Nibert, M. L. & Lesser, C. F. Protein interaction platforms: visualization of interacting proteins in yeast. *Nat. Methods* **6**, 500–502 (2009).
5. Campbell-Valois, F., Schnupf, P. & Sansonetti, P. Design of a Transcription-based Secretion Activity Reporter (TSAR) for the Type III Secretion Apparatus of *Shigella flexneri* and Uses Thereof. *BIO-Protoc.* **4**, (2014).
6. Mimee, M. *et al.* An ingestible bacterial-electronic system to monitor gastrointestinal health. *Science* **360**, 915–918 (2018).

Chapter 3:

YgaC Promotes Type III Effector Secretion in *Shigella flexneri*

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SUMMARY

Many Gram-negative bacteria utilize type III secretion systems to inject effectors into the cytosol of eukaryotic host cells to promote bacterial replication and spread. The secretion of effectors is mediated via interactions with designated type III secretion chaperones, small acidic proteins that play a crucial role in their post-translational recruitment to the membrane embedded type III secretion apparatus. However, the secretion of many *Shigella* effectors has not been linked to a chaperone, suggesting that unidentified chaperones might exist. Considering the tight, secretion-dependent gene regulation of effectors, we applied RNA sequencing to identify novel type III secretion factors that display similar transcriptional regulation. Here, we report that YgaC, a chromosomally encoded, low molecular weight protein of unknown function, promotes the translation-dependent secretion of multiple effectors, suggesting that it may function as a chaperone in a co-translational type III secretion pathway.

INTRODUCTION

Many Gram-negative bacteria, including *Shigella*, *Salmonella*, *Yersinia* and pathogenic *Escherichia* species, utilize syringe-like type III secretion systems (T3SSs) to directly inject proteins into the cytosol of eukaryotic host cells. T3SSs are complex nanomachines that assemble within and span the bacterial cellular envelope. They are kept in an inactive off state until the needle tip of the machine docks onto the host cell[1,2]. While the structural components of type III secretion apparatuses (T3SAs) are relatively conserved, each pathogen delivers its own unique set of proteins[3]. These translocated proteins, referred to as effectors, modulate host cellular processes to promote bacterial replication and spread. The secretion of many effectors is dependent on type III secretion chaperones, low molecular weight (~15kDa), acidic proteins that directly bind as homodimers to domains within the N-termini of effectors[4,5,6]. Three effector chaperones are described in *Shigella* spp. The two designated class IA chaperones, IpgA and IpgE, mediate the secretion of IcsB or IpgD, respectively, while the multicargo class IB chaperone, Spa15, promotes secretion of nine effectors[7,8,9,10].

The transcription of *Shigella* effectors is tightly regulated and occurs in two waves[11,12]. First wave effectors are controlled by VirB, the transcription factor that also regulates the expression of the operons which encode the structural components of the T3SA[13]. Prior to secretion activation, the majority of these effectors are stored and held in a secretion-competent state in complex with IpgA, IpgE or Spa15. The expression of the second *Shigella* effector wave is mediated by MxiE, a transcription factor that is activated after the first wave effector, OspD1, is secreted[14]. Prior to its secretion, OspD1 binds to and inhibits MxiE. Upon OspD1 release, MxiE is activated by IpgC, a class II T3S chaperone, which is only free to bind post-secretion of the translocon components, IpaB and IpaC[15,16]. Intriguingly, the secretion of most second wave *Shigella* effectors is not dependent on any of the characterized chaperones[17], raising the question of the existence of yet unidentified chaperones.

Reasoning that yet unidentified factors involved in the secretion of second wave effectors are under a similar transcriptional regulation, the transcriptomes of wildtype and constitutively secreting *Shigella flexneri* were compared using RNA sequencing (RNAseq), which led to the identification of YgaC. Initial effector secretion was found to not be impacted by YgaC. However, late secretion of almost every tested effector was impaired in its absence. In contrast to conventional T3S chaperones that mediate effector secretion in a post-translational manner, the secretion of YgaC-dependent effectors was found to require active translation, suggesting that it might serve as a novel chaperone in a co-translational type III secretion pathway. YgaC is widely distributed and highly conserved in Enterobacteriaceae, which raises the intriguing possibility that it might be involved in the secretion of effectors in other Gram-negative bacterial pathogens.

RESULTS

Shigella type III secretion activation is associated with transcript enrichment of uncharacterized chromosomal genes

The transcription of most putative chaperone-independent effectors is controlled by MxiE, a regulator that is only active once the initial secretion of effectors has begun[12]. To identify factors involved in the secretion of these effectors, we reasoned that they would display

a similar pattern of gene regulation. The *Shigella* transcriptomes were compared and gene transcripts identified that were enriched when the *Shigella* T3SA was in an active configuration (Figures 1a and 1b). Wildtype (WT) and Δ*ipaBC Shigella* were grown in liquid broth, under conditions in which the T3SA of WT *Shigella* is assembled but not active. In contrast, under the same conditions, the Δ*ipaBC Shigella* T3SA is active, constitutively secreting effectors due to the absence of IpaB, an essential component of the needle tip complex which holds the T3SA in an off configuration[18]. As expected, when the T3SA is active, the transcripts of most MxiE-regulated effectors were significantly upregulated (Figure 1c), while the other genes encoded on the large T3SA-encoding *Shigella* virulence plasmid (VP) were expressed at similar levels under both conditions (Table 1 and Supplementary Table 1). Notably, the transcripts of ~5% of chromosomally encoded genes were substantially enriched under secreting conditions (Supplementary Table 2), including those corresponding to MxiE-regulated effectors and few hypothetical proteins (Figures 1d and 1e and Table 2).

Table 1 | Summary of significantly upregulated virulence plasmid genes.

Gene name[a]	Gene product[a] and function	Log2 FC[b]	MxiE-Box
ipaH7.8	T3S effector	6.78	Yes
ospE1	T3S effector	6.37	Yes
ipaH9.8	T3S effector	5.61	Yes
ipaH1.4	T3S effector	4.98	Yes
ospE2	T3S effector	4.93	Yes
ospB	T3S effector	4.25	Yes
ipaH4.5	T3S effector	3.90	Yes
phoN2/apy	Periplasmic phosphatase apyrase	2.87	Yes
ospC1	T3S effector	2.42	Yes
ospF	T3S effector	2.16	Yes
virA	T3S effector	2.06	Yes
ospD2	T3S effector	1.54	No
mob9	Plasmid mobilization protein	1.50	Unknown
ipgB1	T3S effector	0.97	No

[a]Genome used as reference sequence is *Shigella flexneri* 2a str. 301 plasmid (GenBank accession number *NC_004851.1*). [b]Genes are arranged in descending order in relation to Log2 fold change (Log2 FC) values of Δ*ipaBC*/WT *Shigella* comparison. Only values considered differentially expressed are shown (adjusted p-value ≤ 0.05). T3S, type III secretion. (Genes encoding IS-elements and hypothetical proteins were excluded from this table.)

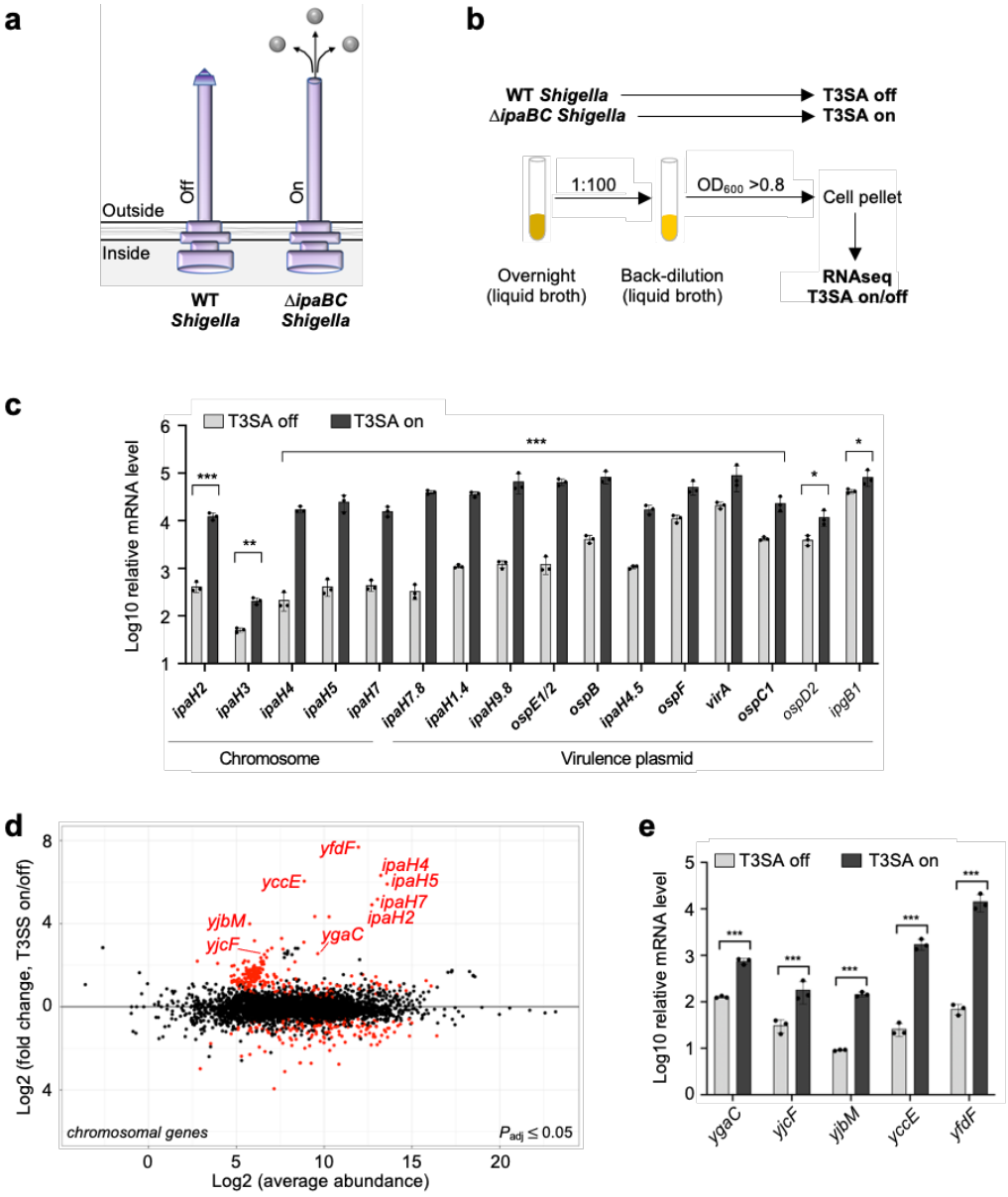

Figure 1 | *Shigella* type III secretion activation is associated with transcript enrichment of uncharacterized chromosomal genes. (**a**) Schematic of differences in the type III secretion apparatus (T3SA) status of wildtype (WT, off state) and Δ*ipaBC* (on state) *Shigella*. (**b**) Schematic of the protocol used to obtain WT and Δ*ipaBC Shigella* samples for RNA sequencing (RNAseq) analysis. Equal cell amounts of mid-log phase grown bacteria were harvested and whole transcriptomes analyzed. (**c**) Relative expression levels of chromosomal and virulence plasmid encoded *Shigella* effector genes measured when the T3SA was in off/on state; MxiE-regulated effectors are shown in bold. (**d**) MA plot of RNAseq data to display substantial differences of *Shigella* chromosomal genes in Δ*ipaBC* when compared to WT *Shigella*. Each dot represents a chromosomally annotated gene (GenBank accession number AE014073.1), red indicates significant differential expression, genes complying with an applied threshold of log2 fold change >0.53 and adjusted p-value < 0.05 were considered statistically significant. (**e**) Relative expression levels of uncharacterized chromosomal genes measured with the T3SA status in off/on state. Transcript counts in (**c**) and (**e**) were normalized using a median of ratios method, adjusted p-values of differences in transcript count were determined when a differential expression analysis with the DESeq2 package in R/RStudio was performed. ***, <0.000001; **, <0.0001; *, <0.01.

Table 2 | Summary of the 20 most significantly upregulated chromosomal genes.

Gene ID[a]	Gene name[a]	Function	MxiE-Box	Log2FC[b]
S2558	*yfdF*	Uncharacterized	Predicted[19]	7.54
S1947	*ipaH_4*	T3S effector	Known	6.16
S1074	*yccE*	Uncharacterized	Predicted[19]	5.94
S2119	*ipaH_5*	T3S effector	Known	5.74
S2782	*ipaH_7*	T3S effector	Known	5.07
S0934	*ipaH_2*	T3S effector	Known	4.82
S2360	*preT*	Dihydropyrimidine dehydrogenase	Unknown	4.28
S3573	*yjbM*	Uncharacterized	Unknown	3.89
S2885	*ygaC*	Uncharacterized	Not predicted[19]	2.54
S3593	*yjcF*	Uncharacterized	Unknown	2.43
S1443	*emrK*	Multidrug resistance protein K	Unknown	2.18
S2362	*mglC*	Methyl-galactoside transport system permease protein	Unknown	2.04
S1268	*ipaH_3*	T3S effector	Known	1.97
S3201	*yeeS*	Putative RADC family DNA repair protein	Unknown	1.27
S0590	*gltA*	Citrate synthase	Unknown	1.25
S0611	*kdpC*	Potassium-transporting ATPase ATP-binding subunit	Unknown	1.12
S3779	*fdoH*	Formate dehydrogenase-O iron-sulfur subunit	Unknown	1.07
S4430	*prlA*	Protein translocase subunit SecY	Unknown	1.02
S3778	*fdoG*	Formate dehydrogenase-O major subunit	Unknown	0.99
S0115	*lpdA*	Dihydrolipoyl dehydrogenase	Unknown	0.60

[a]Genome used as reference sequence is *Shigella flexneri* 2a str. 301 (GenBank accession number *AE014073.1*). [b]Genes are arranged in descending order in relation to Log2 fold change (Log2 FC) values of Δ*ipaBC*/WT *Shigella* comparison. Only values considered differentially expressed are shown (adjusted p-value ≤ 0.05). Known type III secretion (T3S) effectors are in bold, uncharacterized genes of interest are underlined. (Genes encoding IS-elements were excluded from this table.)

YgaC is involved in type III effector secretion

Next, a role for the hypothetical products of *yfdF, yccE, ygaC, yjbM* and *yjcF*, five of the most up-regulated chromosomally encoded genes, in type III effector secretion was investigated. After establishing that the absence of each had no effect on *Shigella* growth (Figure 2a), the level of secreted IpaB was assessed (Figure 2b). As a component of the translocon apparatus, IpaB is one of the first proteins to be secreted. IpaB was secreted at equivalent levels by WT *Shigella* and each of the deletion strains, suggesting that none

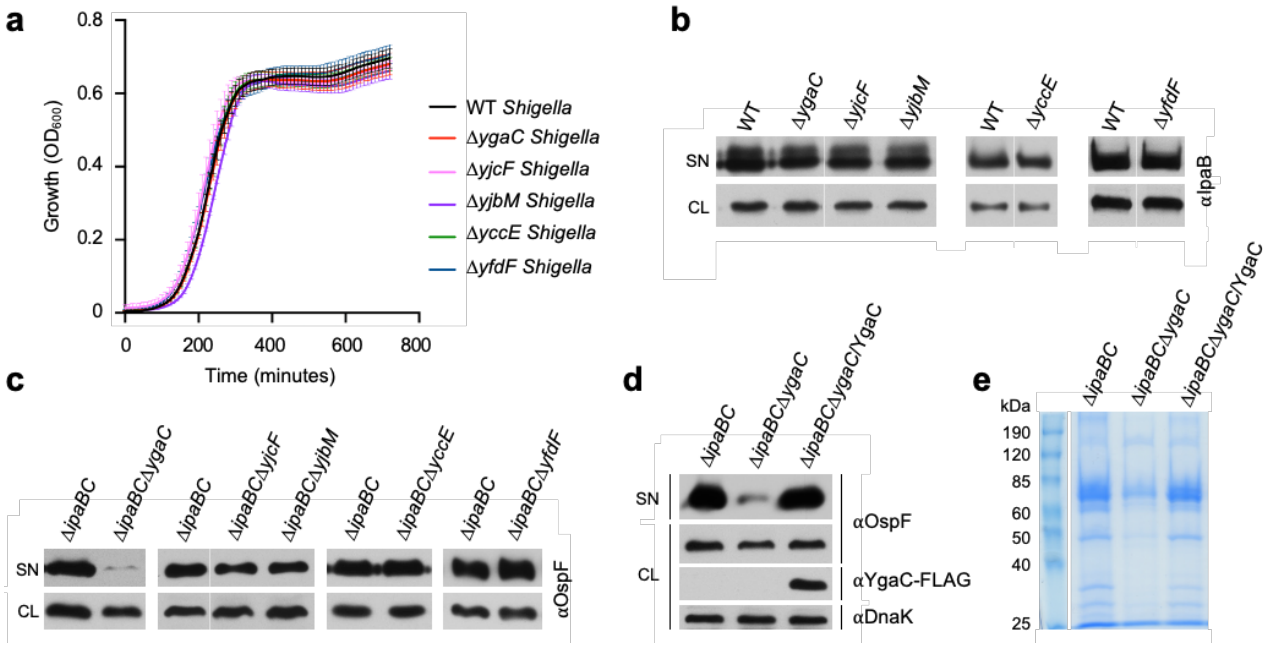

Figure 2 | YgaC is involved in type III effector secretion. (**a**) Optical density 600nm (OD_{600}) measurements of wildtype (WT) *Shigella* and candidate gene knockout strains grown in tryptic soy (TCS) broth obtained every 10 minutes for 12 hours, data shown is representative of three biological repeats using six technical replicates each time; data are mean ± SD. (**b**) Expression and secretion of IpaB from WT *Shigella* and candidate gene knockout strains when grown under conditions that induce expression of the type III secretion system. Cell equivalents of bacterial cell lysates (CL) and supernatant fractions (SN) were obtained thirty minutes post-exposure to phosphate-buffered saline supplemented with Congo red. (**c**) Leaky secretion of endogenously expressed OspF released within 2.5 hours into TCS broth by Δ*ipaBC Shigella* and candidate gene knockout strains or (**d**) by Δ*ipaBC*Δ*ygaC Shigella* that was complemented with an IPTG-inducible YgaC-FLAG expression plasmid. CL and/or SN were subjected to denaturing SDS-PAGE before (**e**) Coomassie Blue staining or immunoblotting with designated antibodies (b, c, d). Blots shown are representative of at least three experimental repeats.

of these upregulated gene products play a role in the regulation of expression or the assembly of the T3SA.

To assess a role in the secretion of second wave effectors, Δ*ipaBC Shigella* strains were generated that no longer encode *yfdF, yccE, ygaC, yjbM* or *yjcF* and the secretion level of OspF, a MxiE-regulated, putative chaperone-independent effector[17] was monitored. The absence of *ygaC* resulted in decreased OspF secretion (Figure 2c), that was fully restored with the introduction of a *ygaC-FLAG* expression plasmid into Δ*ipaBC*Δ*ygaC Shigella* (Figure 2d). To begin to assess whether YgaC plays a role in the

secretion of additional effectors, we examined the secreted Coomassie-stained supernatant fractions of Δ*ipaBC* and Δ*ipaBC*Δ*ygaC Shigella.* Strikingly, markedly decreased levels of secreted proteins were observed in the absence of *ygaC*, which was fully restored with the expression of *ygaC-FLAG* (Figure 2e). These observations suggest that YgaC is involved in mediating *Shigella* type III effector secretion.

YgaC promotes late type III secretion of multiple effectors

To further explore to what extent effector secretion is dependent on YgaC, the solid plate-based secretion assay[17] was used to monitor the secretion of eighteen different effectors from *Shigella*. Each FLAG-epitope tagged effector was under the control of its endogenous promoter, when expressed from a low copy-number plasmid. Under these conditions, multiple effectors, including both chaperone-dependent and chaperone-independent, were secreted at markedly decreased levels from Δ*ipaBC*Δ*ygaC* as compared to Δ*ipaBC Shigella* (Figures 3a and 3b).

Next, the secretion of eleven of these effectors was assessed using a liquid secretion assay (Figure 3c). This assay enables one to not only examine levels of secreted proteins, but also of those present within the bacteria. The absence of *ygaC* had variable effects on the effector level that remained within the bacteria. Again, the absence of *ygaC* led to markedly decreased secretion of the majority of both chaperone-independent and chaperone- dependent effectors.

Notably, no substantial differences in effector secretion levels were observed from WT *Shigella* lacking *ygaC* (Supplementary Figures 1a and 1b). Furthermore, YgaC-FLAG could not be detected when expressed from its endogenous promoter in WT *Shigella* (Supplementary Figures 1c and 1d). In contrast, YgaC-FLAG was observed in Δ*ipaBC Shigella*, suggesting that its production is linked to late secretion conditions. Furthermore, a decrease in detected intracellular YgaC-FLAG protein level was noted upon exposure of the bacterial cells to Congo red, the dye used for the *in vitro* activation of the *Shigella* T3SA[20]. These observations suggest that YgaC promotes the late secretion of multiple chaperone-dependent and -independent *Shigella* effectors and proposes that it serves as a novel T3S chaperone.

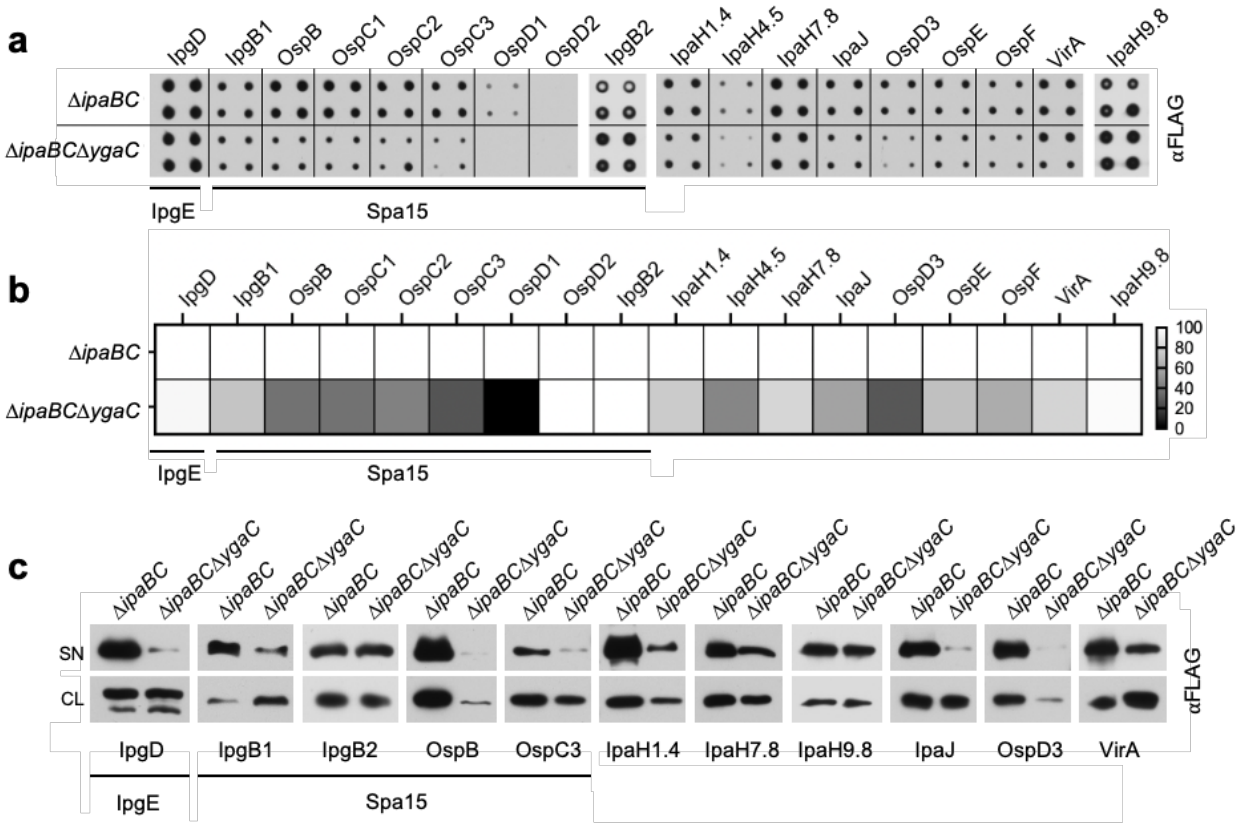

Figure 3 | YgaC promotes the secretion of multiple effectors. (**a**) Representative images of plate secretion assays of Δ*ipaBC* and Δ*ipaBC*Δ*ygaC Shigella* strains that harbor designated low-copy number effector plasmids, under the control of their endogenous promoters. Membranes were incubated for 3 hours on top of bacteria grown on tryptic soy agar media supplemented with Congo red and immunoblotted with αFLAG antibody. Data of three experimental repeats were quantified and plotted in a (**b**) Heatmap, demonstrating the detected secretion level from Δ*ipaBC*Δ*ygaC* relative to Δ*ipaBC Shigella*. (**c**) Cell equivalents of bacterial cell lysates (CL) and supernatant fractions (SN), obtained after 2.5 hours of growth in TCS broth from Δ*ipaBC* and Δ*ipaBC*Δ*ygaC Shigella* that harbor plasmids for the endogenous expression of designated effectors, were subjected to denaturing SDS-PAGE before immunoblotting with αFLAG antibody.

Secretion of YgaC-dependent effectors requires active translation

Type III effector secretion is mediated in a post-translational manner with the help of designated T3S chaperones[21]. However, evidence for translation-dependent type III effector secretion exist[9,22]. To test if YgaC-mediated effector secretion is part of a post- or co-translational pathway, *in vitro* secretion assays in presence or absence of chloramphenicol were performed (Figures 4a and 4b). Secretion linked to translation is inhibited in the presence of this antibiotic, which blocks the process of *de novo* protein

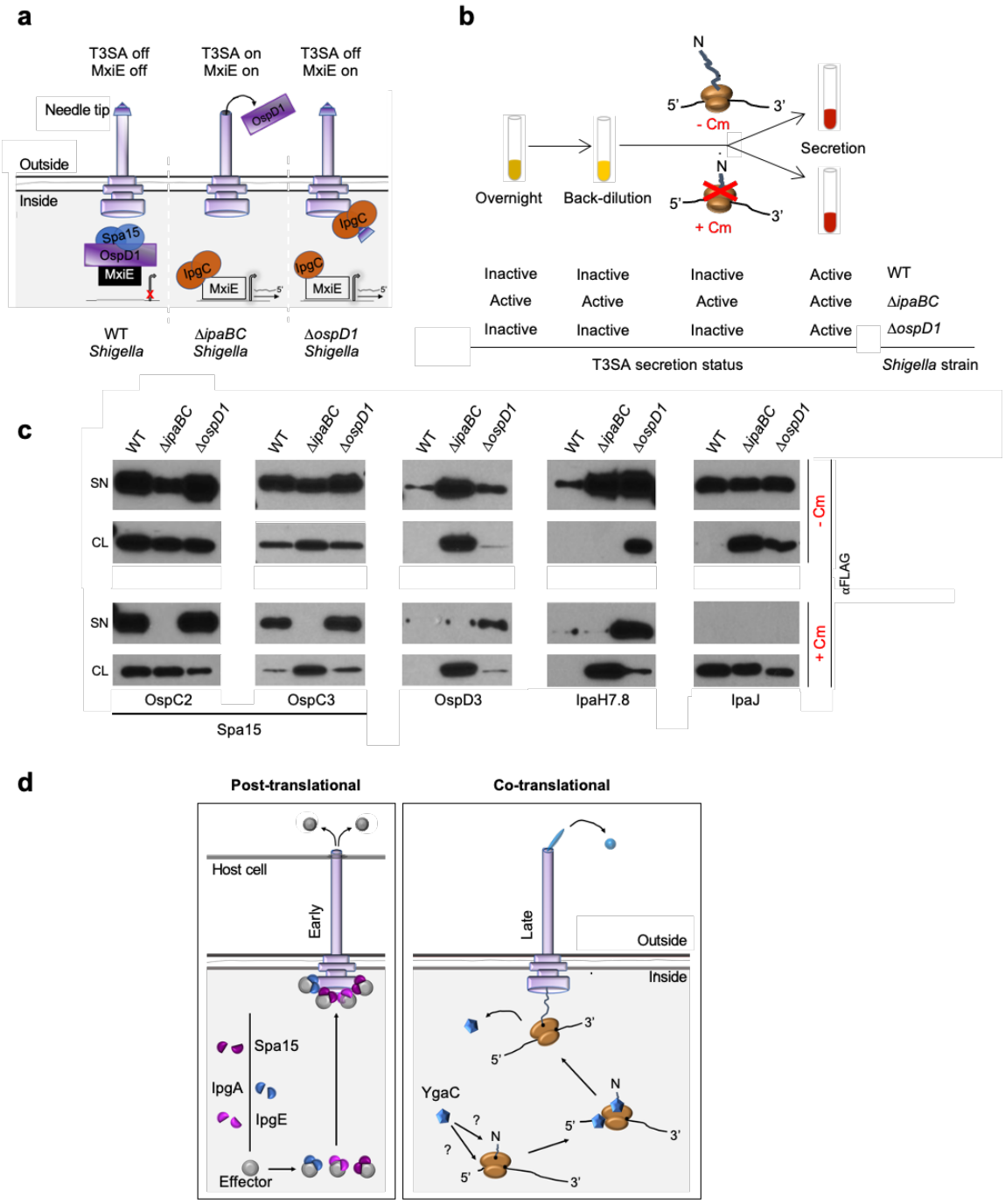

Figure 4 | Secretion of YgaC-dependent effectors requires active translation. (**a**) Schematic of the secretion-dependent regulation of MxiE activity in wildtype (WT), Δ*ospD1* and leaky Δ*ipaBC Shigella*. T3SA, type III secretion apparatus. (**b**) Schematic of protocol used to study the role of translation during effector secretion. Cm, chloramphenicol. (**c**) Secretion of FLAG-epitope tagged effectors thirty minutes post incubation in PBS-CR in presence or absence of Cm from WT, Δ*ipaBC* or Δ*ospD1 Shigella.* Each effector is expressed under the control of its endogenous promotor. Cell equivalents of bacterial cell lysates (CL) and supernatant fractions (SN) were subjected to denaturing SDS-PAGE before immunoblotting with αFLAG antibody. Representative images of three independent repeats. (**d**) Schematic of proposed type III secretion pathways.

synthesis[23,24]. For the simultaneous analysis of VirB- and MxiE-regulated effectors under these conditions, the Δ*ospD1 Shigella* strain was included, which lacks the negative regulator of MxiE, allowing expression of second wave effectors at a time when the T3SA is in an off state. Under these conditions, secretion of pre-made effector proteins can be studied. The secretion of all tested YgaC-dependent effectors by Δ*ipaBC Shigella* was observed to require active translation (Figure 4c), which may suggest that YgaC promotes late secretion of the majority of *Shigella* effectors via a co-translational pathway (Figure 4d).

DISCUSSION

This study identified YgaC as a novel *Shigella* type III secretion factor. Its absence from WT *Shigella* has no effect on the initial secretion of effectors. However, the absence of *ygaC* from constitutively secreting Δ*ipaBC Shigella* results in the impaired secretion of most effectors, which suggests that it is involved in the process of late type III effector secretion.

YgaC is a low molecular weight (13 kDa) protein, that is similar in size to characterized T3S chaperones[25], and is highly conserved in Enterobacteriaceae (Supplementary Table 4 and Supplementary Figure 2). Intriguingly, T3S chaperones and *Salmonella typhimurium* YgaC, which is almost identical to its *Shigella* homolog, share structural homology[26]. T3S chaperones are acidic proteins that typically bind as homodimers to defined effector protein regions to promote their secretion[21]. However, no direct interaction of YgaC with itself or any tested effector was detected via an extensive yeast-two hybrid screen (Supplementary Table 3). Furthermore, YgaC displays basic features (pI 7.9), a typical attribute of RNA chaperones, as it allows the interaction with negatively charged RNA molecules[27]. Intriguingly, in studies that prevent *de novo* protein synthesis, the secretion of YgaC-dependent effectors was observed to require active translation. These observations suggest that YgaC may be involved in a co-translational effector secretion pathway, in which it likely functions as a new class of T3S chaperone.

Future studies will focus on deciphering the exact mechanism of effector recognition and will investigate the role of YgaC in other T3SS-utilizing pathogens.

MATERIALS AND METHODS

Strains, plasmids and oligonucleotides are summarized in Supplementary Tables 5-7.

Plasmid Construction.

Endogenous YgaC-FLAG expression plasmid. Plasmid for endogenous expression of carboxy-terminally 3xFLAG epitope-tagged YgaC was generated via Gateway™ cloning, as described earlier[28]. In brief, the fragment of DNA containing *ygaC*, in an open configuration (without a stop codon), plus 253bp of upstream nucleotides were PCR amplified from *Shigella* chromosomal DNA adding flanking *attB* sites at both ends and introduced into pDNR221-ccdB, sequence verified and transferred into pCMD136-ccdB-FLAG, an expression plasmid containing a Gateway cassette followed by an in-frame 3xFLAG epitope-tag sequence. Generated plasmids were verified using restriction digest.

IPTG-inducible YgaC-FLAG expression plasmid. An IPTG-inducible YgaC-FLAG expression plasmid was generated using the Gateway™ based cloning strategy. Gene in open (without stop codon) and closed (with stop codon) configurations were PCR-amplified that introduced flanking *attB* sites at both ends and a consensus Shine-Dalgarno sequence upstream of the start codon. After introduction into a pDNR221-ccdB vector via a BP Gateway reaction, the sequence of the gene insert of the entry clone was verified using Sanger sequencing. A Gateway LR reaction was performed to transfer the *ygaC* gene into pDSW206-ccdB-FLAG, a p*lac* destination plasmid with an in-frame 3xFLAG epitope-tag sequence located at the 3' end of the Gateway cassette. The generated expression plasmid was verified by restriction digest.

Yeast expression plasmids. Each yeast expression plasmid was generated via Gateway™ cloning. ORFs encoding the proteins listed in Supplementary Table 3 were

PCR-amplified in a closed (stop codon containing) conformation and then introduced into either pDNR221-ccdB or pDNR223-ccdB to generate Gateway entry clones. After the gene inserts were sequence-verified, they were transferred into high copy number yeast expression plasmid, *ygaC* was introduced into pGBKT7-GW[29] and all other gene ORFs into pGADT7-GW[29] via Gateway LR reactions.

Strain Construction.

***Shigella* deletion strains.** Each of the single deletion strains (Supplementary Table 5), was generated in *S. flexneri* 2457T via λ Red recombination[30] and verified using oligonucleotides described in Supplementary Table 7.

Liquid assays. Liquid secretion assays were performed as previously described[10]. In brief, overnight cultures grown in tryptic soy (TCS) broth were diluted 1:100 into 2 ml of TCS broth and incubated for two hours. The optical density (OD_{600}) of each bacterial culture was assessed. Equivalent numbers of bacteria from each culture were pelleted, resuspended in 2 ml of phosphate-buffered saline (PBS) plus 10 μM Congo red (Sigma) and incubated for 30 minutes. All incubations were carried out at 37˚C with constant aeration. Bacterial cultures were centrifuged and the cell pellets were resuspended in loading dye. After an additional centrifugation step, to remove remaining intact bacteria, proteins in the supernatant fractions were trichloroacetic acid (TCA) precipitated [10% (vol/vol)] and resuspended in loading dye. Equal cell equivalents of supernatant and pellet fractions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes and immunoblotted with αFLAG (Sigma, 1:10,000), αIpaB (1:20,000), αOspF (1:1000) or αDnaK (Abcam, 10,000) antibodies. The αIpaB antibody was a generous gift from Wendy Picking, University of Kansas, Lawrence, KS. The αOspF antibody was a gift from Dr. Anthony Maurelli, University of Florida, Gainesville, USA.

Chloramphenicol assays. Cultures were treated similarly to a liquid secretion assay, with the following adaptations: WT, Δ*ipaBC* and Δ*ospD1 Shigella* strains were back

diluted into 5ml media. Cultures were split in two, when mid-log growth phase was reached followed by the addition of chloramphenicol (30µg/ml, Cm) to one half. The other half of the culture was treated equally in absence of Cm. After five minutes, the cells were briefly centrifuged and resuspended in PBS-CR supplemented with or without Cm and incubated under constant aeration, for 30 minutes at 37C. Supernatant fractions and cell lysates were prepared and analyzed as described above.

Leaky secretion assays. Secretion assays with Δ*ipaBC Shigella* were conducted in absence of PBS-CR, if not noted otherwise. All incubations were carried out at 37˚C with constant aeration. Overnight cultures grown in tryptic soy (TCS) broth were diluted 1:100 into 2 ml of fresh TCS broth and incubated for 2.5 hours. The optical density (OD_{600}) of each bacterial culture was assessed. Bacterial cultures were centrifuged and the cell pellets were resuspended in loading dye. After an additional centrifugation step, proteins in the supernatant fractions were TCA-precipitated, resuspended in loading dye and analyzed as described in 'Liquid secretion assay'. Overall leaky secretion was assessed, when SDS-PAGE separated supernatant fractions were stained with Coomassie Blue (GelCode Blue, ThermoFisher).

Plate-based secretion assay. Solid secretion assays were performed as previously described[17]. In brief, a 96-well plate (Corning) containing TCS broth was inoculated with the designated strains and incubated with agitation for 4 hours on a plate shaker. A BM3-BC pinning robot (S&P Robotics Inc., Toronto, Canada) outfitted with a 96-pin tool was then used to transfer equal volumes of saturated cultures onto solid trays (Nunc) that contain solid TCS media (Sigma) plus 10 µM CR. Each colony was spotted in quadruplicate. All incubations were carried out at 37°C. All incubations were carried out at 37°C. After an overnight incubation, the BM3-BC pinning robot outfitted with a 384-pin tool was used to transfer bacteria to a solid media tray containing TCS media plus CR onto which a pre-cut nitrocellulose membrane (Pierce) was immediately laid. After 3 hours, the overlaid membrane was removed, washed and probed with αFLAG antibody.

The secreted protein amount was quantified using ImageJ[31] and plotted using GraphPad Prism.

Y2H assay. The yeast expression plasmids were introduced into MaV103 or MaV203, respectively. The yeast-two hybrid (Y2H) assays were performed in a 96-well format as previously described[32,10]. In this case, selection was conducted on medium lacking leucine, tryptophan and histidine, plus 40 mM (3-AT) 3-amino-1,2,4-triazole. Growth was scored after 3 days of incubation at 30˚C.

RNA sequencing (RNAseq). RNA extraction, library preparation and sequencing were performed as previously described[33], with the following alterations. Three biological replicate cultures of WT and Δ*ipaBC Shigella flexneri*, grown overnight at 37C in TCS broth under constant aeration were diluted 1:100 into fresh media and grown under the same conditions until mid-log phase (OD_{600} of 0.8) was reached. Bacterial cell pellets of 800µl culture of each were harvested, briefly washed and immediately shock-frozen. Total RNA of each cell pellet was extracted using hot phenol-chloroform as previously described[34], their concentrations were next measured using a NanoDrop spectrophoto - meter (Thermo Fisher Scientific). The RNA quality was evaluated using an Agilent Bioanalyzer 2100 and the Agilent RNA 6000 Nano kit (Santa Clara). Ribosomal RNA (rRNA) was depleted using the Ribo Zero rRNA removal kit for Gram-negative bacteria (Illumina). The cDNA libraries were generated with a NEBNext® Ultra™ directional RNA library preparation kit for the Illumina system using NEBNext® multiplex oligonucleotides for Illumina index primer set 1 (New England Biolabs). The size distribution of the generated library was evaluated using an Agilent 2200 TapeStation high-sensitivity D1000 ScreenTape system. High-throughput sequencing, as well as Bioanalyzer and TapeStation analyses were carried out at the Biopolymers Facility at Harvard Medical School, where cDNA libraries were pooled and sequenced in paired-end mode (2x 75bps) using a mid-output NextSeq 500 flow cell (Illumina). An average of 16 million reads per sample and condition were obtained. High-performance computing and bioinformatic transcriptomic analysis was applied, as previously described[33]. Data and differential

expression analysis were performed using a Linux-based high-performance computing platform (O2, Harvard Medical School) and R/Rstudio with the DESeq2 and ggplot2 packages. The sequencing reads were mapped to the *Shigella* chromosome (GenBank accession number *AE014073.1*) and virulence plasmid pCP301 (GenBank accession number NC_004851.1). Genes complying with an applied threshold of LCE >0.53 and adjusted p-value < 0.05 were considered statistically significant hits.

Quantification and statistical analysis. Statistical analysis was carried out in R/RStudio using the package DeSeq2, where a two-sided Wald test with the Benjamini-Hochberg correction for multiple hypothesis was applied during differential gene expression analysis. Statistical differences between two means were tested by an unpaired student's t-test with GraphPad Prism.

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AUTHOR CONTRIBUTIONS

N.H.E. and C.F.L conceived and designed the experiments. N.H.E. and C.Y.L conducted the experiments. N.H.E. performed RNAseq and carried out bioinformatic analysis. N.H.E. and C.F.L. analyzed the data and wrote the manuscript.

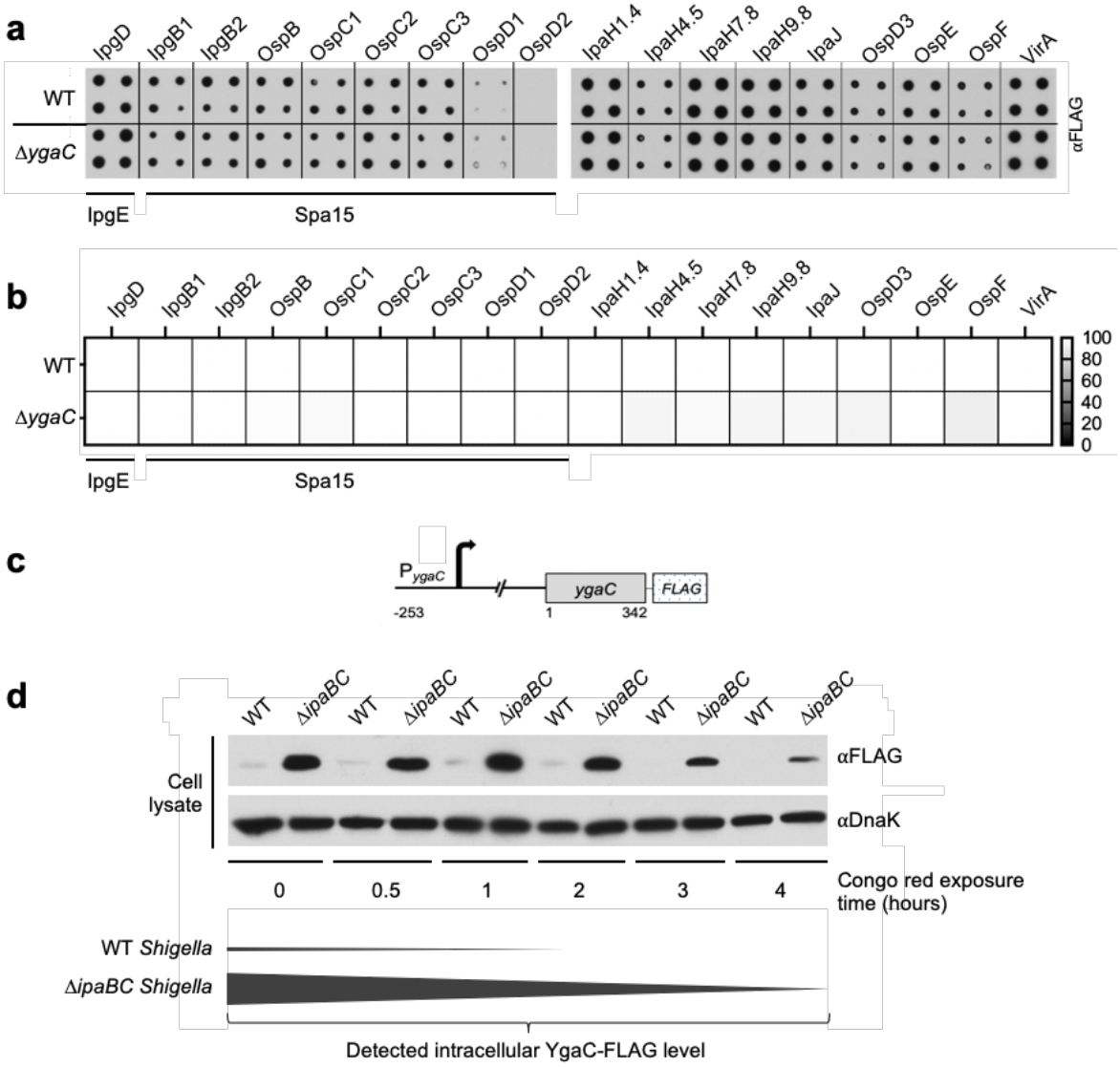
REFERENCES

1. Blocker, A. *et al.* Structure and composition of the *Shigella flexneri* 'needle complex', a part of its type III secreton. *Mol. Microbiol.* 39, 652–663 (2001).
2. Blocker, A. *et al.* The tripartite type III secreton of *Shigella flexneri* inserts IpaB and IpaC into host membranes. *J. Cell Biol.* 147, 683–693 (1999).
3. Galán, J. E., Lara-Tejero, M., Marlovits, T. C. & Wagner, S. Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. *Annu. Rev. Microbiol.* 68, 415–438 (2014).
4. Luo, Y. *et al.* Structural and biochemical characterization of the type III secretion chaperones CesT and SigE. *Nat. Struct. Biol.* 8, 1031–1036 (2001).
5. Bronstein, P. A., Miao, E. A. & Miller, S. I. InvB Is a Type III Secretion Chaperone Specific for SspA. *J. Bacteriol.* 182, 6638–6644 (2000).
6. Costa, S. C. P. *et al.* A New Means To Identify Type 3 Secreted Effectors: Functionally Interchangeable Class IB Chaperones Recognize a Conserved Sequence. *mBio* 3, (2012).
7. Niebuhr, K. *et al.* IpgD, a protein secreted by the type III secretion machinery of *Shigella flexneri*, is chaperoned by IpgE and implicated in entry focus formation. *Mol. Microbiol.* 38, 8–19 (2000).
8. Ogawa, M., Suzuki, T., Tatsuno, I., Abe, H. & Sasakawa, C. IcsB, secreted via the type III secretion system, is chaperoned by IpgA and required at the post-invasion stage of *Shigella* pathogenicity: The role of IcsB in *Shigella* pathogenicity. *Mol. Microbiol.* 48, 913–931 (2003).
9. Page, A.-L., Sansonetti, P. & Parsot, C. Spa15 of *Shigella flexneri,* a third type of chaperone in the type III secretion pathway. *Mol. Microbiol.* 43, 1533–1542 (2002).
10. Schmitz, A. M., Morrison, M. F., Agunwamba, A. O., Nibert, M. L. & Lesser, C. F. Protein interaction platforms: visualization of interacting proteins in yeast. *Nat. Methods* 6, 500–502 (2009).
11. Schroeder, G. N. & Hilbi, H. Molecular Pathogenesis of *Shigella* spp.: Controlling Host Cell Signaling, Invasion, and Death by Type III Secretion. *Clin. Microbiol. Rev.* 21, 134–156 (2008).
12. Mavris, M. *et al.* Regulation of transcription by the activity of the *Shigella flexneri* type III secretion apparatus. *Mol. Microbiol.* 43, 1543–1553 (2002).
13. Gall, T. L. *et al.* Analysis of virulence plasmid gene expression defines three classes of effectors in the type III secretion system of *Shigella flexneri*. *Microbiol. Read. Engl.* 151, 951–962 (2005).
14. Parsot, C. *et al.* A secreted anti-activator, OspD1, and its chaperone, Spa15, are involved in the control of transcription by the type III secretion apparatus activity in *Shigella flexneri*. *Mol. Microbiol.* 56, 1627–1635 (2005).

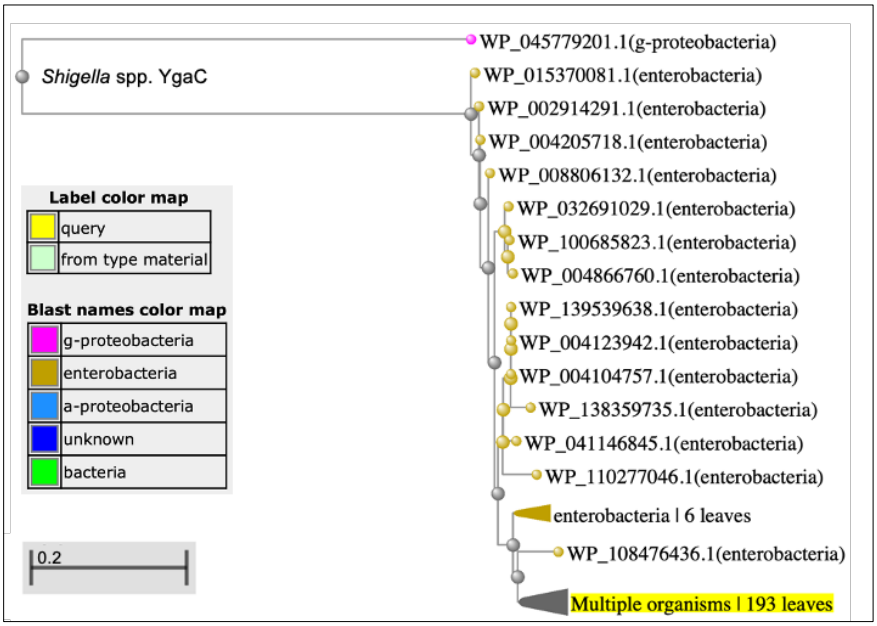
15. Pilonieta, M. C. & Munson, G. P. The chaperone IpgC copurifies with the virulence regulator MxiE. *J. Bacteriol.* 190, 2249–2251 (2008).
16. Lunelli, M., Lokareddy, R. K., Zychlinsky, A. & Kolbe, M. IpaB-IpgC interaction defines binding motif for type III secretion translocator. *Proc. Natl. Acad. Sci. U. S. A.* 106, 9661–9666 (2009).
17. Ernst, N. H., Reeves, A. Z., Ramseyer, J. E. & Lesser, C. F. High-Throughput Screening of Type III Secretion Determinants Reveals a Major Chaperone-Independent Pathway. *mBio* 9, (2018).
18. Ménard, R., Sansonetti, P. & Parsot, C. The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD. *EMBO J.* 13, 5293–5302 (1994).
19. Silué, N., Marcantonio, E. & Campbell-Valois, F.-X. RNA-Seq analysis of the T3SA regulon in *Shigella flexneri* reveals two new chromosomal genes upregulated in the on-state. *Methods San Diego Calif* 176, 71–81 (2020).
20. Qadri, F. *et al.* Congo red binding and salt aggregation as indicators of virulence in *Shigella* species. *J. Clin. Microbiol.* 26, 1343–1348 (1988).
21. Wattiau, P., Woestyn, S. & Cornelis, G. R. Customized secretion chaperones in pathogenic bacteria. *Mol. Microbiol.* 20, 255–262 (1996).
22. Karlinsey, J. E., Lonner, J., Brown, K. L. & Hughes, K. T. Translation/secretion coupling by type III secretion systems. *Cell* 102, 487–497 (2000).
23. Jardetzky, O. Studies on the mechanism of action of chloramphenicol. I. The conformation of chlioramphenicol in solution. *J. Biol. Chem.* 238, 2498–2508 (1963).
24. Wolfe, A. D. & Hahn, F. E. Mode of Action of Chloramphenicol. Effects of Chloramphenicol
upon a Ribosomal Amino Acid Polymerization System and its Binding to Bacterial Ribosome.
Biochim. Biophys. Acta 95, 146–155 (1965).
25. Stebbins, C. E. & Galán, J. E. Maintenance of an unfolded polypeptide by a cognate chaperone in bacterial type III secretion. *Nature* 414, 77–81 (2001).
26. Aramini, J.M., Swapna, G.V.T., Ramelot, T.A., & Ho, C.K. Solution NMR structure of the putative cytoplasmic protein *ygaC* from *Salmonella typhimurium.* Northeast Structural Genomics target StR72. https://www.rcsb.org/structure/2g7j (2006).
27. Semrad, K. Proteins with RNA Chaperone Activity: A World of Diverse Proteins with a Common Task - Impediment of RNA Misfolding. *Biochem. Res. Int.* 2011, 1–11 (2011).
28. Mou, X., Souter, S., Du, J., Reeves, A. Z. & Lesser, C. F. Synthetic bottom-up approach reveals the complex interplay of *Shigella* effectors in regulation of epithelial cell death. *Proc. Natl. Acad. Sci.* 115, 6452–6457 (2018).

29. Lu, Q. *et al.* Arabidopsis homolog of the yeast TREX-2 mRNA export complex: components and anchoring nucleoporin. *Plant J. Cell Mol. Biol.* 61, 259–270 (2010).
30. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci.* 97, 6640–6645 (2000).
31. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675 (2012).
32. Walhout, A. J. M. & Vidal, M. High-Throughput Yeast Two-Hybrid Assays for Large-Scale Protein Interaction Mapping. *Methods* 24, 297–306 (2001).
33. Ernst, N. H., Lin, C. Y. & Lesser, C. F. The RNA chaperone FinO Mediates Type III Effector Secretion in *Shigella flexneri*. (2021).
34. Lorenz, C., Dougherty, T. J. & Lory, S. Transcriptional Responses of *Pseudomonas aeruginosa* to Inhibition of Lipoprotein Transport by a Small Molecule Inhibitor. *J. Bacteriol.* 202, e00452-20 (2020).

SUPPLEMENTARY FIGURES



Supplementary Figure 1 | YgaC-FLAG is not detectable in wildtype *Shigella*.
(**a**) Representative images of plate secretion assays of wildtype (WT) and Δ*ygaC Shigella* strains that harbor designated low-copy number effector plasmids, under the control of their endogenous promoters. Membranes were incubated for 3 hours on top of bacteria grown on tryptic soy agar media supplemented with Congo red and immunoblotted with αFLAG antibody. Data of three experimental repeats was quantified and plotted in a (**b**) heatmap, demonstrating the secretion level from Δ*ygaC* relative to WT *Shigella*. (**c**) Schematic of construct used to study endogenous YgaC-FLAG when expressed from its native promoter. (**d**) Immunoblots of WT and Δ*ipaBC Shigella* cell lysates, that harbor the endogenous YgaC-FLAG expression plasmid, cells were harvested before and after growth in liquid broth supplemented with Congo red. Blots were probed with antibodies that recognize FLAG-epitope tagged YgaC or DnaK (loading control).



Supplementary Figure 2 | Enterobacterial YgaC is widely distributed. Distance tree (fast minimum evolution tree) of bacterial YgaC-like proteins, the tree was generated using BLAST pairwise alignments (RefSeq)[1], with an applied maximum sequence difference of 0.9.

SUPPLEMENTARY TABLES

Supplementary Table 1 | Differences in virulence plasmid gene transcripts.

Supplementary Table 2 | Differences in chromosomal gene transcripts.

Supplementary Tables 1 and 2 are available upon request.

Supplementary Table 3 | Proteins tested for binary interaction with YgaC via Y2H

T3S effectors:	T3SA components:	RNA chaperones:
OspB	MxiA (cyto)	CsrA
OspC1	MxiC	Hfq
OspC2	MxiG (cyto)	Molecular co-/chaperones:
OspC3	MxiH	DnaJ
OspD1	MxiK	DnaK
OspD2	MxiL	GroEL
OspD3	MxiN	GroES
OspE2	Spa9	Trigger factor

OspF (K134A)	Spa13	SecB
OspG	Spa24	Ribosomal proteins:
OspZ	Spa29	RpsB
IpaA	Spa32	RplW
IpaB	Spa33	Miscellaneous:
IpaC	Spa40	IpgF
IpaD	Spa47	MxiE
IpaH1.4	T3S chaperones:	YgaC
IpaH4.5	IpgA	
IpaH7.8	IpgC	
IpaH9.8 (C337A)	IpgE	
IpgB1 (Q131A)	Spa15	

Supplementary Table 4 | Multiple sequence alignments of YgaC-like bacterial proteins. T3SA, type III secretion apparatus, cyto, cytosolic region of the protein. (available upon request).

Supplementary Table 5 | Bacterial strains used in this study.

Name	Characteristics	Source
WT *Shigella*	*Shigella flexneri* 2457T serotype 2a, wildtype	Laboratory strain collection
WT *Shigella* M90T	*Shigella flexneri* M90T serotype 5a, wildtype	Gift from Marcia Goldberg
Δ*ipaBC Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*ipaBipaC*::FRT	Laboratory strain collection
Δ*ospD1 Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*ospD1*::FRT	Laboratory strain collection
Δ*ygaC Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*ygaC*::FRT-*KanR*-FRT	This study
Δ*yccE Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*yccE*::FRT-*KanR*-FRT	This study
Δ*yfdF Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*yfdF*::FRT-*KanR*-FRT	This study
Δ*yjbM Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*yjbM*::FRT-*KanR*-FRT	This study
Δ*yjcF Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*yjcF*::FRT-*KanR*-FRT	This study
Δ*ipaBC*Δ*ygaC Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*ipaBipaC*::FRT, Δ*ygaC*::FRT-*KanR*-FRT	This study
Δ*ipaBC*Δ*yccE Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*ipaBipaC*::FRT, Δ*yccE*::FRT-*KanR*-FRT	This study
Δ*ipaBC*Δ*yfdF Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*ipaBipaC*::FRT, Δ*yfdF*::FRT-*KanR*-FRT	This study
Δ*ipaBC*Δ*yjbM Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*ipaBipaC*::FRT, Δ*yjbM*::FRT-*KanR*-FRT	This study

Δ*ipaBC*Δ*yjcF Shigella*	*Shigella flexneri* 2457T serotype 2a, Δ*ipaBipaC*::FRT, Δ*yjcF*::FRT-*KanR*-FRT	This study

Supplementary Table 6 | Plasmids used in this study.

Name	Characteristics	Source
pKD46	Temperature sensitive, λ Red recombinase, AmpR	Laboratory strain collection
pDNR221-ccdb	Gateway donor vector, pUC ori, KanR, CmR	Invitrogen
pENTR221-ygaC	Entry vector, open configuration, KanR	This study
pENTR221-endP_ygaC	Entry vector, open configuration, KanR, *ygaC* plus 253bp upstream region	This study
pCMD136-ccdb-flag	Destination vector containing a gateway cassette and 3xFLAG, very low copy (pSC101 ori), SpecR, CmR	Laboratory strain collection
pCMD136-endP-ipgD-flag	Endogenous promoter expression plasmid, SpecR	Laboratory strain collection
pCMD136-endP-ospB-flag	Endogenous promoter expression plasmid, SpecR	Laboratory strain collection
pCMD136-endP-ospC2-flag	Endogenous promoter expression plasmid, SpecR	Laboratory strain collection
pCMD136-endP-ospC1-ospD3-flag	Endogenous promoter expression plasmid, SpecR	Laboratory strain collection
pCMD136-endP-ospF-flag	Endogenous promoter expression plasmid, SpecR	Laboratory strain collection
pCMD136-endP-ipaH7.8-flag	Endogenous promoter expression plasmid, SpecR	Laboratory strain collection
pCMD136-endP-ipaH9.8-flag	Endogenous promoter expression plasmid, SpecR	Laboratory strain collection
pCMD136-endP-ospC1-flag	Endogenous promoter expression plasmid, SpecR	This study
pCMD136-endP-ospC3-flag	Endogenous promoter expression plasmid, SpecR	This study
pCMD136-endP-ospD1-flag	Endogenous promoter expression plasmid, SpecR	This study
pCMD136-endP_ospC1-flag	Endogenous promoter expression plasmid, SpecR	This study
pCMD136-endP_ipgB1-flag	Endogenous promoter expression plasmid, SpecR	This study
pCMD136-endP_ipgB2-flag	Endogenous promoter expression plasmid, SpecR	This study
pCMD136-endP_ospE-flag	Endogenous promoter expression plasmid, SpecR	This study
pCMD136-endP_ipaH4.5-flag	Endogenous promoter expression plasmid, SpecR	This study
pCMD136-endP_ipaH7.8-flag	Endogenous promoter expression plasmid, SpecR	This study
pAM238-endP_ipaJ-flag	Endogenous promoter expression plasmid, SpecR	This study
pCMD136-endP_ospE1-ipaH1.4-flag	Endogenous promoter expression plasmid, SpecR	This study
pCMD136-endP_ygaC-flag	Endogenous promoter expression plasmid, SpecR	This study
pDSW206-ccdB-flag	Destination vector containing a gateway cassette and 3xFLAG, p*lac* (IPTG), low copy (ColE1 ori), AmpR, CmR	Laboratory strain collection
pDSW206-ygaC-flag	IPTG-inducible expression plasmid, AmpR	This study
pGBKT7-GW	Yeast expression vector containing a gateway cassette, GAL4BD-fusion, high copy (2μ ori) TRP, NeoR/KanR	Addgene (#61703)
pGBKT7-ygaC	Yeast expression vector containing a gateway cassette, GAL4BD-fusion, high copy (2μ ori) TRP, NeoR/KanR	This study
pGADT-GW	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	Addgene (#61702)
pGADT-OspB	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-OspC1	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study

pGADT-OspC2	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-OspC3	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-OspD1	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-OspD2	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-OspD3	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-OspE2	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-OspF (K134A)	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-OspG	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-OspZ	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-IpaA	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-IpaB	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-IpaC	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-IpaD	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-IpaH1.4	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-IpaH4.5	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-IpaH7.8	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-IpaH9.8 (C337A)	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-IpgB1 (Q131A)	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-MxiA(cyto)	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-MxiC	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-MxiG(cyto)	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-MxiH	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-MxiK	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-MxiL	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-MxiN	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-Spa9	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-Spa13	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-Spa24	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-Spa29	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-Spa32	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-Spa33	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-Spa40	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-Spa47	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-IpgA	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study

pGADT-IpgC	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-IpgE	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-Spa15	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-CsrA	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-Hfq	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-DnaJ	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-DnaK	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-GroEL	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-GroES	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-TF	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-SecB	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-RpsB (S2)	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-RplW (L23)	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-IpgF	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-MxiE	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study
pGADT-YgaC	Yeast expression vector containing a gateway cassette, GAL4AD-fusion, high copy (2μ ori) LEU, CmR	This study

Supplementary Table 7 | Oligonucleotides used in this study.

Name	Characteristics	Notes
Oligos to generate deletion strains:		
yfdF_5W	TATATTTGTAACAAATCAATCAACATGGAATAAAATCATGgtgtaggctggagctgcttc	Fwd. Underlined: homolgy to Kan^R cassette
yfdF_3W	GTTGAAAAATCAAAAAACTGGAAAAATAATTTTAAATATCggtccatatgaatatcctcc	Rev. Underlined: homolgy to Kan^R cassette
yccE 5W,	AATATACATGGAATTAGTTGCACTGCAAATAATTATTTGAAACAGGCCTGGgtgtaggctggagctgcttc	Fwd. Underlined: homolgy to Kan^R cassette
yccE 3W,	ATAACGATTTAAATAGTCAAGTTGCGCATTCATTTCCTTATTTTTTAACggtccatatgaatatcctccttag	Rev. Underlined: homolgy to Kan^R cassette
ygaC 5W,	TTACGACCAGACGAGGTGGCGCGCGTACTTGAAAAAGTCGGTTTTACTGTCGgtgtaggctggagctgcttc	Fwd. Underlined: homolgy to Kan^R cassette
ygaC 3W,	TTCGCCAAATAAACCATTCAAATAACGTTCAAGCGCAACACGCGAACTAAggtccatatgaatatcctccttag	Rev. Underlined: homolgy to Kan^R cassette
yjbM 5W,	GTCAATAAGTATACTGACGATTGCACTGATGAGGATTTAAACGATCGTGACgtgtaggctggagctgcttc	Fwd. Underlined: homolgy to Kan^R cassette
yjbM 3W,	CGATTTAACCCCGAACAAGTCATAAATTAATTTAGAAGTGTCTGTAAGggtccatatgaatatcctccttag	Rev. Underlined: homolgy to Kan^R cassette
yjcF 5W,	CGCTATAATGGTTTAAATAATATGTTTTTCCCTCTTTGCCAGATTAACGATAACgtgtaggctggagctgcttc	Fwd. Underlined: homolgy to Kan^R cassette
yjcF 3W,	GTGTTGCATAATATCTGCAGTAATCTTCATAGCGACTGCATTAATATTATCggtccatatgaatatcctccttag	Rev. Underlined: homolgy to Kan^R cassette
Oligos to confirm deletion strains:		
Kan_F	GATGATCTGGACGAAGAGCATCAG	Fwd. To verifiy deletion strains

Ygac 100up	CTGAGGTTCATCGCGGC	Fwd. To verifiy Δ*ygaC Shigella* strains
YccE 100up	CAGGTGAAAAAGGGTGAGCG	Fwd. To verifiy Δ*yccE Shigella* strains
YjcF 200up	CTCTCCCCTTCCCCGGTC	Fwd. To verifiy Δ*yjcF Shigella* strains
YjbM 60up	CGCATAATTTATGGCATGCACG	Fwd. To verifiy Δ*yjbM Shigella* strains
S2558_up	CCTGGCTATTGTGCGCTTATAC	Fwd. To verifiy Δ*yfdF Shigella* strains
Ygac Rev	CTTCCTCGATTATTGCCGCC	Rev. To verifiy Δ*ygaC Shigella* strains
YccE Rev	GCTAAACAGAATATCTGCGGCG	Rev. To verifiy Δ*yccE Shigella* strains
YjcF Rev	GCACCTTTAACAGGTTAGCTCG	Rev. To verifiy Δ*yjcF Shigella* strains
YjbM Rev	CTCCCTGCTAATAGCATTGATAGAG	Rev. To verifiy Δ*yjbM Shigella* strains
S2558_dn	CCGACAATGACCCGGACGTAATGATG	Rev. To verifiy Δ*yfdF Shigella* strains
Oligos to generate Gateway entry clones:		
5' universal	GGGGACAACTTTGTACAAAAAAGTTGGC**GAAGGAGATAGAACC**ATG	Fwd. Underlined: AttB1 site. Bold: Shine Dalgarno sequence (SD)
3GW_ygaC_endP	GGGGACAACTTTGTACAAGAAAGTTGGCGTGGCGATCTTGTTAGTGGTGGGCGC	Rev. Underlined: AttB2 site
5GW_ygaC_253	GGGGACAACTTTGTACAAAAAAGTTGGCGGACTTCAATACAGATTCGAGGCTG	Fwd. To generate entry clone. Underlined: AttB1 site
5GW_ygaC_IPTG2	**CGAAGGAGATAGAACC**ATGTATTTACGACCAGACGAGGTGGCG	Fwd.To generate entry clone. Bold: Shine Dalgarno sequence (SD)
3GW_ygaC_IPTG	GGGGACAACTTTGTACAAGAAAGTTGG**TTA**ACTGGCTTCGCCAAATAAACCATTC	Rev. To generate entry clone (closed confirmation). Underlined: AttB2 site. Bold: stop codon
3GW_ygaC_NS	GGGGACAACTTTGTACAAGAAAGTTGGACTGGCTTCGCCAAATAAACCATTC	Rev. To generate entry clone (open confirmation)

REFERENCE

1. States, D. J. & Gish, W. Combined use of sequence similarity and codon bias for coding region identification. *J. Comput. Biol. J. Comput. Mol. Cell Biol.* 1, 39–50 (1994).

General Discussion

Many *Shigella* type III effectors are secreted in the absence of all characterized chaperones. Type III secretion systems (T3SSs) are utilized by many Gram-negative pathogens to deliver proteins across their cell envelope into the cytosol of host cells. T3SSs are essential virulence determinants that promote bacterial survival and spread[1]. The highly conserved structure of the components of type III secretion apparatuses (T3SAs) suggests a common mechanism of substrate recognition and secretion. Conventionally defined by proteinaceous signals, effectors are post-translationally delivered to the membrane embedded T3SA mediated via interactions with designated T3S chaperones[2,3].

In the first part of this study, an automated plate-based secretion assay was developed. This assay was used to compare the secretion of multiple *Shigella* effectors from a variety of strains. Using this platform, the T3S chaperone requirement for the secretion of *Shigella* effectors was assessed. Surprisingly, the secretion of a significant number of effectors (12 of 22) was found to be unimpaired from strains that lack any or all of its known T3S chaperones, IpgA, IpgE and Spa15. These observations suggested the existence of additional uncharacterized chaperones and/or chaperone-independent secretion pathways.

The T3SA is assembled and held in an off configuration until it contacts host cell membranes. Strikingly, many of the putative chaperone-independent effectors are only expressed once the status of the T3SA is in an on state and secretion is activated. Based on this observation, whole transcriptomic analyses were conducted to identify genes that exhibit similar patterns of expression, reasoning that they might encode proteins involved in the secretion of these effectors. These studies led to the identification of FinO and YgaC as putative novel T3S chaperones.

YgaC is involved in the secretion of effectors from an activated type III secretion system. YgaC, unlike the virulence plasmid-encoded structural components of the *Shigella* T3SA, its chaperones, regulators and most effectors, is a chromosomally-encoded protein[4]. YgaC has no effect on the initial secretion of *Shigella* effectors. However, the absence of YgaC from a constitutively secreting strain resulted in the

impaired secretion of most effectors. YgaC is highly conserved and widely distributed throughout enterobacterial genera and can be found in many T3SS-utilizing pathogens, raising a possibility that it is more broadly involved in mediating effector secretion. Future studies will further explore the role of and the mechanism by which YgaC promotes effector secretion.

FinO mediates the secretion of a subset of *Shigella* effectors via the recognition of their 5'UTRs. The RNA chaperone, FinO was initially identified as the negative regulator of plasmid conjugation, the type IV secretion system-mediated transfer of genetic material from one bacterial cell to another[5,6,7]. FinO post-transcriptionally inhibits the translation of *traJ,* the transcription factor of genes encoding components of the conjugation machine[8]. FinO recognizes and binds to stem loop secondary structures located in the *traJ* 5' untranslated region (UTR) and its complementary sequence, the non-coding sRNA FinP[9] (Figure 1). FinO binding stabilizes FinP by preventing its degradation from RNases. Additionally, FinO binding leads to the destabilization of the stem loop structures in *traJ* and FinP promoting the formation of a stable mRNA-sRNA duplex, in which the *traJ* ribosomal binding site is buried, preventing its translation and ultimately leading to its degradation[10].

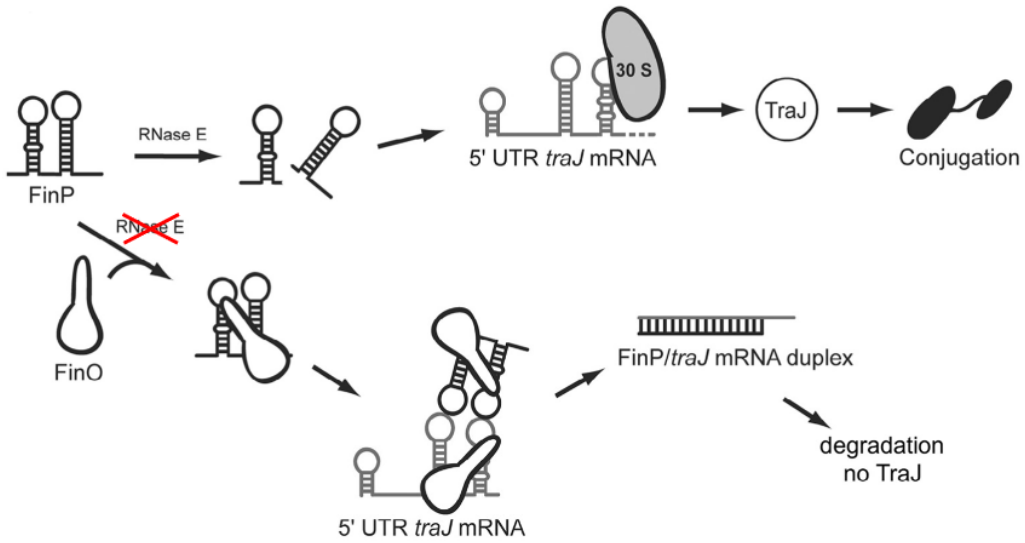


Figure 1. Schematic overview of the regulation of *traJ* by the RNA chaperone FinO; mRNA, messenger ribonucleic acid. Taken from Glover *et al*.[11], modified.

Shigella FinO is encoded on the large 220 kB virulence plasmid, which only encodes remnants of the conjugation machinery[4,12]. Surprisingly, my studies uncovered that the function of FinO is not limited to the regulation of bacterial conjugation, as they identified that FinO is involved in mediating the secretion of OspF, IpaH9.8 and OspD3, three putative chaperone-independent effectors. FinO promotes their secretion via the recognition of information encoded within their 5'UTRs. Similar to its characterized mRNA target, *traJ*, the 5'UTRs of *ospF*, *ipaH9.8* and *ospD3* mRNAs are each predicted to form secondary stem loop structures that sequester their ribosome binding site (RBS) and/or translational start site (Figure 2).

The 5'UTR of *ospF* is only 21 nucleotides in length and predicted to form a single stem loop. The introduction of a single point mutation predicted to disrupt this structure had a minor effect on the intrabacterial levels of OspF, but resulted in a dramatic decrease in the amount secreted. This finding suggests that the stem loop within the 5'UTR of *ospF* functions as a type III secretion signal. The existence of similar stem loops within *ipaH9.8* and *ospD3* are suggestive of a FinO-specific secretion signal which will be the subject of future studies.

Insights into the longstanding debate regarding the existence of a *Yersinia* YopE mRNA-encoded type III secretion signal. The FinO requirement for OspF, OspD3 and IpaH9.8 secretion is eliminated when they are expressed via an IPTG-inducible promoter. In this setting, their native 5'UTR is replaced with a synthetic sequence. The unimpaired secretion of this construct is in stark contrast to the impaired secretion observed when mutations predicted to disrupt FinO binding are introduced into the native 5'UTR of *ospF*. These discordant observations are reminiscent of those that led to the heated unresolved debate of the Schneewind and Wolf-Watz laboratories regarding the existence of an mRNA-encoded secretion signal within the transcript of YopE, a *Yersinia* type III secretion effector. The Schneewind lab was the first to propose the existence of structural mRNA-encoded type III secretion signals[14]. They found that the fusion of the first 45 nucleotides of *yopE* to a reporter protein was sufficient to generate a type III secreted variant. Furthermore, they demonstrated that frame-shift mutations that markedly altered the

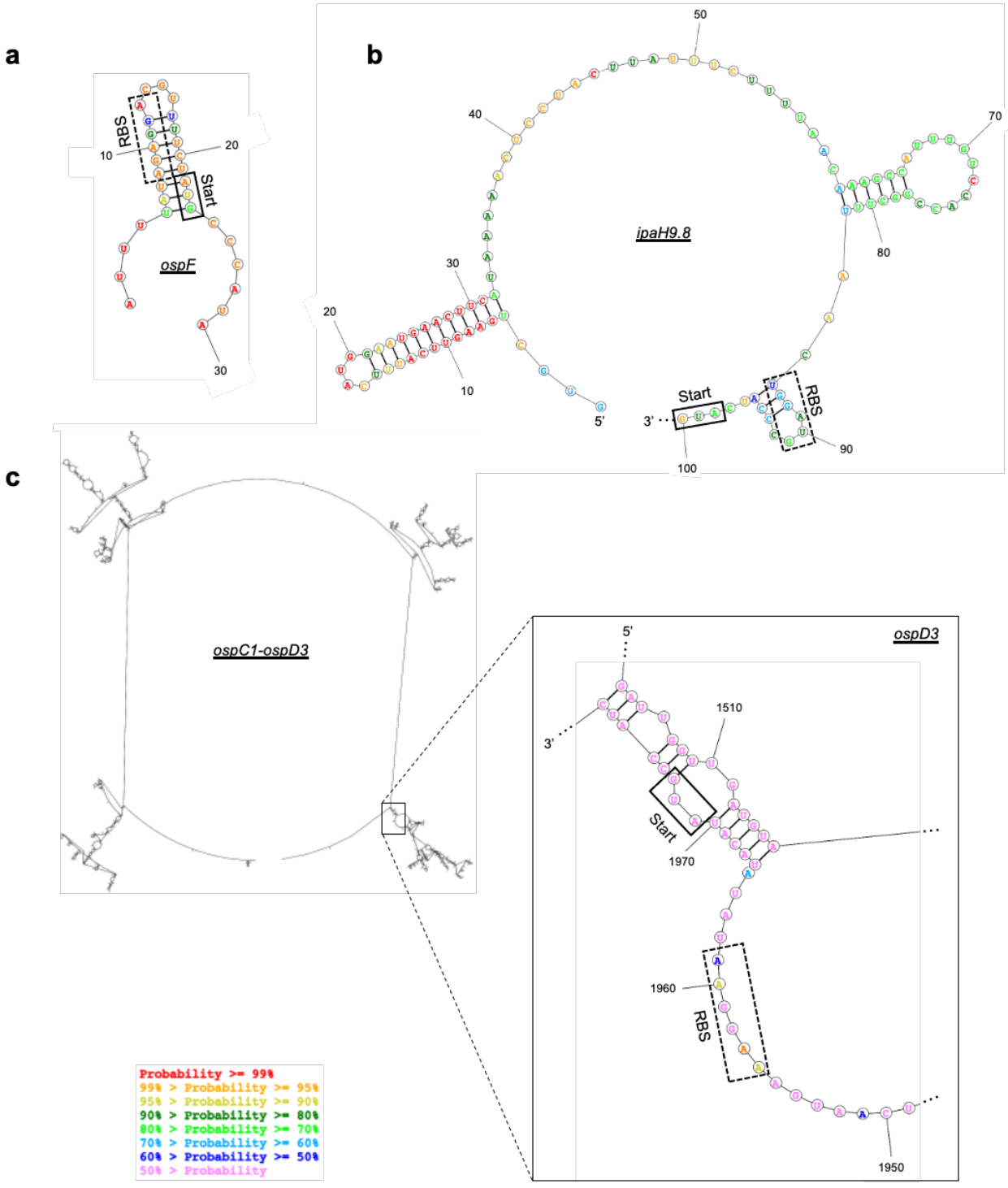


Figure 2. Secondary structure predictions of *ospF*, *ipaH9.8 and ospD3* transcripts. Predicted secondary structures of non-coding and coding mRNA sequences of (**a**) *ospF*, (**b**) *ipaH9.8* and (**c**) *ospD3* (part of *ospC1-ospD3* transcript). RBS, ribosome binding site (dashed lined black box); Start, translational start site (solid lined black box). RNAstructure tool (v.6.0.1) was used to identify predicted RNA secondary structures[13].

coding sequence of the N-terminal amino acids encoded by *yopE* nucleotides had no effect on the secretion of the hybrid protein. In contrast, the Wolf-Watz lab found that frame-shift mutations in the first 50 nucleotides of *yopE* resulted in unimpaired secretion of full-length effector[15]. Notably, the Schneewind lab YopE-reporter was expressed via the endogenous *yopE* promoter, while the Wolf-Watz studied the fate of YopE expressed from an arabinose-inducible promoter. Thus, the Schneewind lab studied the consequences of the frame-shift mutations within the context of the native 5'UTR, while the Wolf-Watz lab was studying a variant of YopE whereby its native 5'UTR is replaced with a synthetic sequence.

Based on my observations and those of the Schneewind and Wolf-Watz labs, I propose that mRNA-encoded type III secretion sequences play a dominant role in defining effectors as secreted proteins, as mutations that disrupt the stem loops within effector 5'UTR impair secretion. However, when expressed via heterologous promoters that generate synthetic, non-native 5'UTR sequences, effectors are secreted due to the existence of additional signals encoded within the effector open reading frame sequence. Whether or not their efficient secretion is due to differences in their mode of transcription and/or their altered 5'UTR remains to be determined. Nevertheless, under native expression conditions, mRNA-encoded signals are crucial determinants that control effector secretion.

Evidence for the involvement of RNA chaperones in type III effector secretion. In addition to my observations with *Shigella* OspF, OspD3 and IpaH9.8 and those of the Schneewind lab with YopE, the mRNA of several *Salmonella* Pathogenicity Island 2 (SPI-2) T3S effectors have been demonstrated to encode type III secretion determinants. In this case, the RNA chaperone Hfq was found to directly bind to a sequence motif within the 5'UTR of an effector transcript and its absence resulted in decreased effector secretion[16]. FinO and Hfq RNA chaperones are widely distributed among Proteobacteria, which raises the possibility that they, and potentially other RNA-binding proteins, are broadly involved in mediating effector secretion.

Evidence for co- and post-translational effector type III secretion pathways. These studies suggest that multiple signals exist that act together to define effectors as secreted substrates, including those identified within their protein and mRNA sequences. These sequences likely regulate the timing and/or efficiency of delivery of effectors into host cells. Accordingly, in the absence of FinO, decreased levels of OspF were delivered into host cells. Multiple signals may also function as checkpoints to ensure that only destined effectors are secreted into host cells.

The nature of the secretion signals likely determine the secretion pathway. Via interactions with traditional T3S chaperones, proteinaceous signals promote the post-translational secretion of premade effectors. These signals allow the rapid delivery of stored effectors upon secretion activation. In contrast to mRNA-encoded signals, which presumably direct the secretion of effectors via a co-translational pathway mediated by RNA chaperones, such as FinO and Hfq. It is interesting that FinO-dependent effectors are each transcribed after activation of the T3SA. Given that bacterial transcription-translation is a mostly coupled process[17,18], I speculate that they are secreted as the they are being translated. Intriguingly, the secretion of endogenously expressed OspF depends on the activity of ribosomes, yet the replacement of its native 5'UTR with a synthetic sequence abolishes this requirement. This observation demonstrates that, at least, some effectors encode multiple signals enabling their delivery via different pathways. Similar to my observations, *Shigella* IpaA secretion was also demonstrated to occur via different pathways, determined by the availability of the T3S chaperone Spa15[19]. Under conditions that prevent active translation, robust Spa15-mediated secretion of stored IpaA was observed. In contrast to its Spa15-independent secretion, which was delayed and required active translation.

A model for how FinO promotes the co-translational secretion of effectors. Regulatory elements that control translation are often located within the 5'UTR of mRNAs. For example, the ribosome binding and translational start sites of *Shigella ospF* are buried within a stem loop structure predicted to form within its 5'UTR. Since FinO binds to and destabilizes stem loops of its RNA targets, it likely acts in a similar manner to modulate

structures within the 5'UTRs of FinO-dependent effectors. I hypothesize that FinO promotes the binding of the RBS Shine-Dalgarno sequence of effector 5'UTR to its complementary sequence, the 16S ribosomal RNA[20] promoting translational initiation. However, rather than inducing effector translation, FinO binding leads to translation arrest. This ribosome stalling provides time for the ribosomal-mRNA complex to travel to the secretion machinery (Figure 3). This process is reminiscent of the co-translational common secretory (Sec) pathway, in which an elongation arrest is induced due to the binding of the signal recognition particle to the substrate's amino-terminal signal peptide[21]. Whether additional proteins (e. g. components of the sorting platform) or RNAs (e.g. sRNAs) are involved in the targeting and/or anchoring of the putative FinO-mRNA-ribosome complex to the T3SA will be the subject of future investigations.

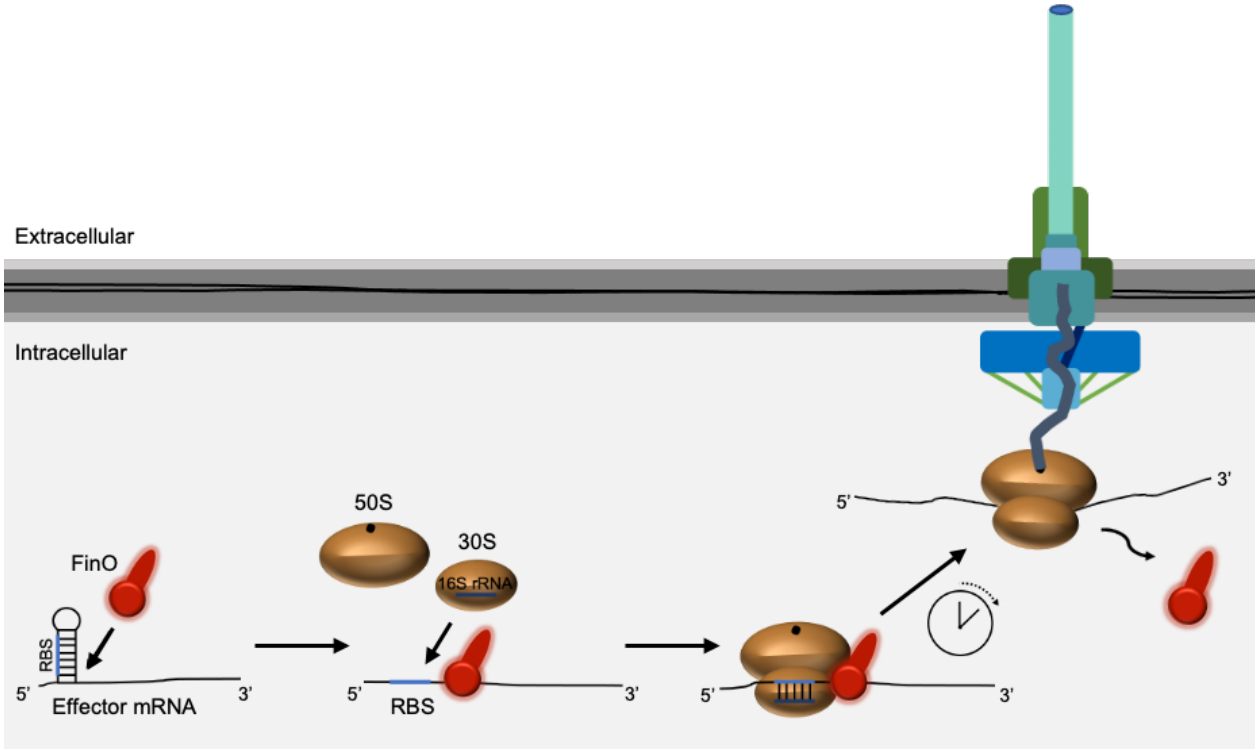


Figure 3. Model of FinO-mediated type III effector secretion. RBS, ribosome binding site; mRNA, messenger ribonucleic acid; rRNA, ribosomal ribonucleic acid.

Concluding remarks

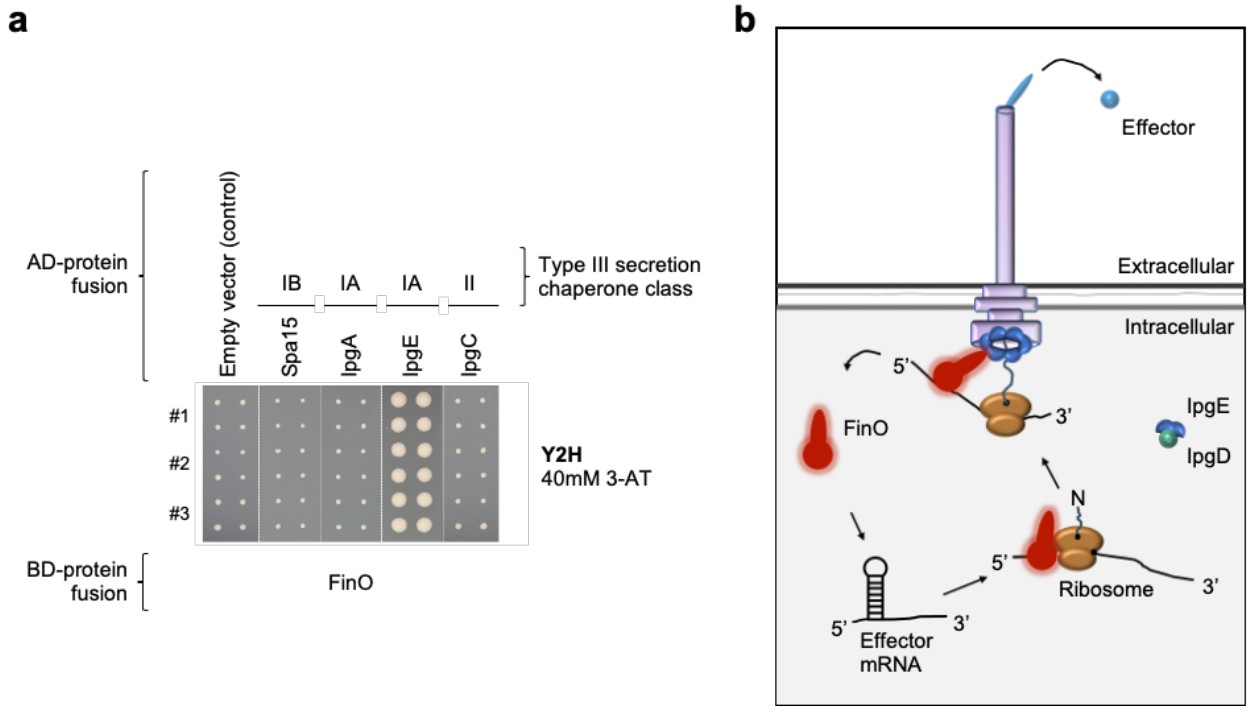
The studies described in this thesis revealed important new insights into the secretion of effectors by the *Shigella* T3SS. First, they have established that many effectors are secreted via a pathway independent of conventional type III secretion chaperones. Second, they have led to the identification of potentially two new classes of T3S chaperones, represented by FinO and YgaC. Third, they have identified an mRNA-encoded structural type III secretion signal within an effector 5' untranslated region. Lastly, they support the existence of post- and co-translational type III effector secretion pathways. Given the prevalence of FinO and YgaC and the structural conservation of T3SAs, it is likely that these findings will prove relevant to T3SSs of other Gram-negative pathogens.

REFERENCES

1. Galán, J. E., Lara-Tejero, M., Marlovits, T. C. & Wagner, S. Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. *Annu. Rev. Microbiol.* 68, 415–438 (2014).
2. Arnold, R., Jehl, A. & Rattei, T. Targeting effectors: the molecular recognition of Type III secreted proteins. *Microbes Infect.* 12, 346–358 (2010).
3. Wattiau, P., Woestyn, S. & Cornelis, G. R. Customized secretion chaperones in pathogenic bacteria. *Mol. Microbiol.* 20, 255–262 (1996).
4. Buchrieser, C. *et al.* The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri*. *Mol. Microbiol.* 38, 760–771 (2000).
5. Lederberg, J. & Tatum, E. L. Gene recombination in *Escherichia coli. Nature* 158, 558 (1946).
6. Finnegan, D. J. & Willetts, N. S. Two classes of Flac mutants insensitive to transfer inhibition by an F-like R factor. *Mol. Gen. Genet. MGG* 111, 256–264 (1971).
7. Lawley, T. D., Klimke, W. A., Gubbins, M. J. & Frost, L. S. F factor conjugation is a true type IV secretion system. *FEMS Microbiol. Lett.* 224, 1–15 (2003).
8. van Biesen, T. & Frost, L. S. The FinO protein of IncF plasmids binds FinP antisense RNA and its target, traJ mRNA, and promotes duplex formation. *Mol. Microbiol.* 14, 427–436 (1994).
9. Arthur, D. C. *et al.* Mapping interactions between the RNA chaperone FinO and its RNA targets. *Nucleic Acids Res.* 39, 4450–4463 (2011).

10. Arthur, D. C. *et al.* FinO is an RNA chaperone that facilitates sense-antisense RNA interactions. *EMBO J.* 22, 6346–6355 (2003).
11. Mark Glover, J. N. *et al.* The FinO family of bacterial RNA chaperones. *Plasmid* 78, 79–87 (2015).
12. Sansonetti, P. J., Kopecko, D. J. & Formal, S. B. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. *Infect. Immun.* 35, 852–860 (1982).
13. Reuter, J. S. & Mathews, D. H. RNAstructure: software for RNA secondary structure prediction and analysis. *BMC Bioinformatics* 11, 129 (2010).
14. Anderson, D. M. & Schneewind, O. A mRNA signal for the type III secretion of Yop proteins by *Yersinia enterocolitica*. *Science* 278, 1140–1143 (1997).
15. Lloyd, S. A., Norman, M., Rosqvist, R. & Wolf-Watz, H. Yersinia YopE is targeted for type III secretion by N-terminal, not mRNA, signals. *Mol. Microbiol.* 39, 520–531 (2001).
16. Niemann, G. S. *et al.* RNA Type III Secretion Signals That Require Hfq. *J. Bacteriol.* 195, 2119–2125 (2013).
17. Miller, O. L., Hamkalo, B. A. & Thomas, C. A. Visualization of bacterial genes in action. *Science* 169, 392–395 (1970).
18. Kohler, R., Mooney, R. A., Mills, D. J., Landick, R. & Cramer, P. Architecture of a transcribing-translating expressome. *Science* 356, 194–197 (2017).
19. Page, A.-L., Sansonetti, P. & Parsot, C. Spa15 of *Shigella flexneri*, a third type of chaperone in the type III secretion pathway. *Mol. Microbiol.* 43, 1533–1542 (2002).
20. Shine, J. & Dalgarno, L. The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proc. Natl. Acad. Sci. U. S. A.* 71, 1342–1346 (1974).
21. Jan, C. H., Williams, C. C. & Weissman, J. S. Principles of ER cotranslational translocation revealed by proximity-specific ribosome profiling. *Science* 346, 1257521–1257521 (2014).

Appendix



Appendix Figure | Type III secretion chaperone IpgE directly binds to FinO and is hypothesized to be involved in the FinO-mediated type III effector secretion process. (**a**) Yeast two-hybrid interaction studies of FinO with known *Shigella* T3S chaperones (40mM 3-AT); growth was scored after 3 days of incubation at 30˚C; experiment was performed three independent times using three biological replicates. (**b**) Schematic of proposed involvement of hexameric IpgE[1,2] in a translation-dependent FinO-mediated type III secretion pathway. AD, activation domain; BD, binding domain; mRNA, messenger ribonucleic acid; N, amino; 3-AT, 3-amino-1,2,4-triazole

REFERENCES

1. Roblin, P., Dewitte, F., Villeret, V., Biondi, E. G. & Bompard, C. A *Salmonella* Type Three Secretion Effector/Chaperone Complex Adopts a Hexameric Ring-Like Structure. *J. Bacteriol.* 197, 688–698 (2015).
2. Lara-Tejero, M., Kato, J., Wagner, S., Liu, X. & Galan, J. E. A Sorting Platform Determines the Order of Protein Secretion in Bacterial Type III Systems. *Science* 331, 1188–1191 (2011).

Appendix Table: Proteins tested for binary interaction with FinO via Y2H.

T3S effector	PPI	T3SS-related protein	PPI	T3SS-unrelated protein	PPI
Shigella spp.:		T3SA components:		RNA chaperones:	
OspB	No	MxiA(cyto)	No	CsrA	No
OspC1	No	MXiC	No	FinO	No
OspC2	No	MxiG(cyto)	No	HfQ	No
OspC3	No	MxiH	No	YgaC	No
OspD1	No	MxiK	No	Molecular co-/chaperones:	
OspD2	No	MxiL	No	DnaJ	No
OspD3	No	MxiN	No	DnaK	No
OspE2	No	Spa9	No	GroEL	No
OspF (K134A)	No	Spa13	No	GroES	No
OspG	No	Spa24	No	Trigger factor	No
OspZ	No	Spa29	No	SecB	No
IpaA	No	Spa32	No	Ribosomal proteins:	
IpaB	No	Spa33	No	RpsB (S2)	No
IpaC	No	Spa40	No	RplW (L23)	No
IpaD	No	Spa47	No		
IpaH1.4	No	T3S chaperones:			
IpaH4.5	No	IpgA	No		
IpaH7.8	No	IpgC	No		
IpaH9.8 (C337A)	No	IpgE	Yes		
IpgB1 (Q131A)	No	Spa15	No		
EPEC:		Miscellaneous:			
EspT	No	IpgF	No		
NleC (E184A)	No	MxiE (AB)	No		
NleD (E134A)	No				
Salmonella spp.:					
SopE2 (D124A)	No				
Yersinia spp.:					
YopE144	No				
YopJ (C172A)	No				
YopM	No				
YopT (R209A)	No				

List of Abbreviations

AD	activation domain
BD	binding domain
CFU	colony forming units
CL	cell lysate
Cm	chloramphenicol
C	carboxy
CR	Congo red
FR	forward reverse (closed confirmation)
Fwd	forward
IPTG	isopropyl-β-D-1-thiogalactopyranoside
Kan	kanamycin
MEF	mouse embryonic fibroblasts
N	amino
NS	non-stop (open confirmation)
n.s.	not significant
PBS	phosphate-buffered saline
RBP	RNA-binding protein
RBS	ribosome binding site
Rev	reverse
mRNA	messenger ribonucleic acid
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SN	supernatant
TCA	trichloroacetic acid
TCS	tryptic soy
T3SA	type III secretion apparatus
T3SS	type III secretion system
VP	virulence plasmid
Y2H	Yeast-two hybrid
3-AT	3-amino-1,2,4-triazole
5’UTR	5’untranslated region