Supplementary Materials

Engineering the type III secretion system in non-replicating bacterial minicells for antigen delivery

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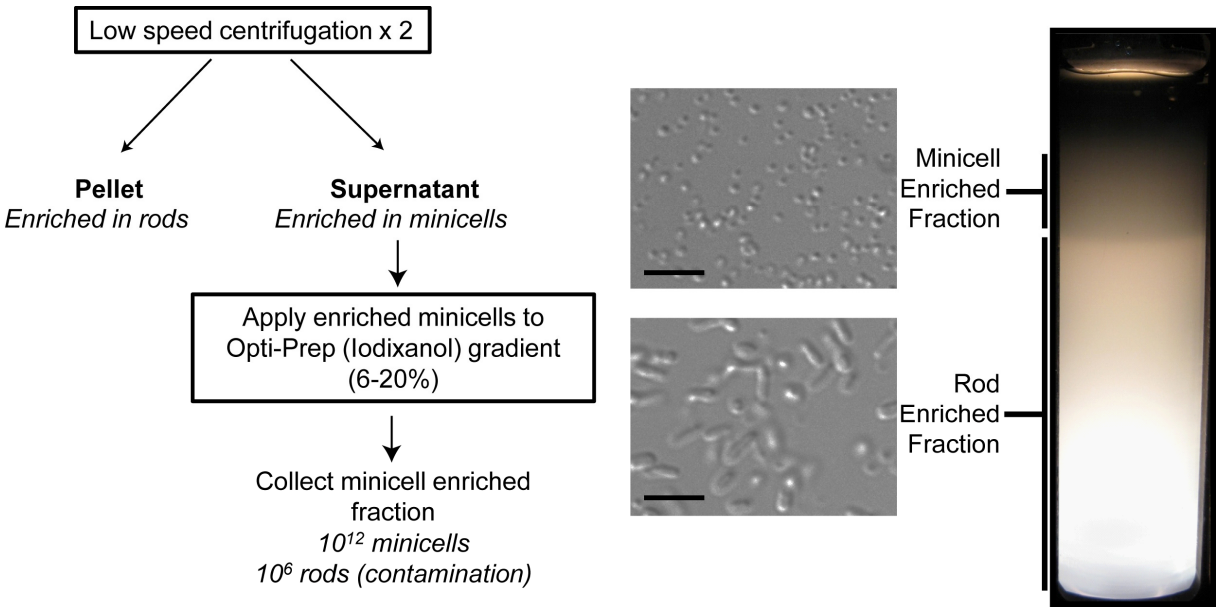
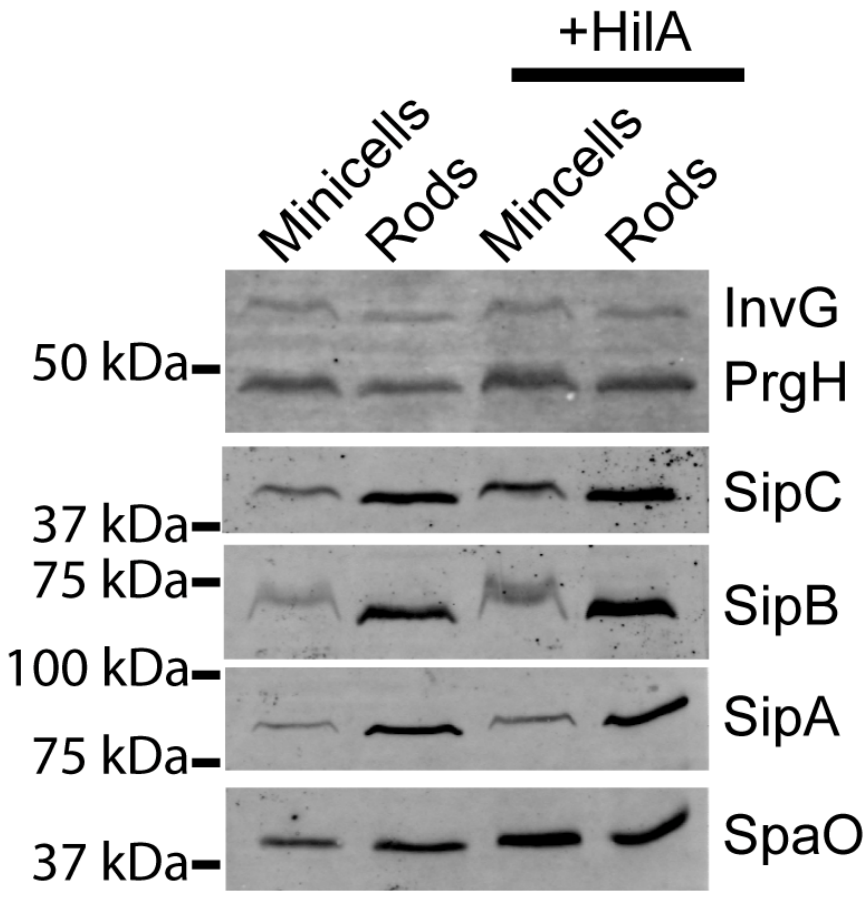
Supplementary Figure S1. Diagram of the minicell isolation protocol



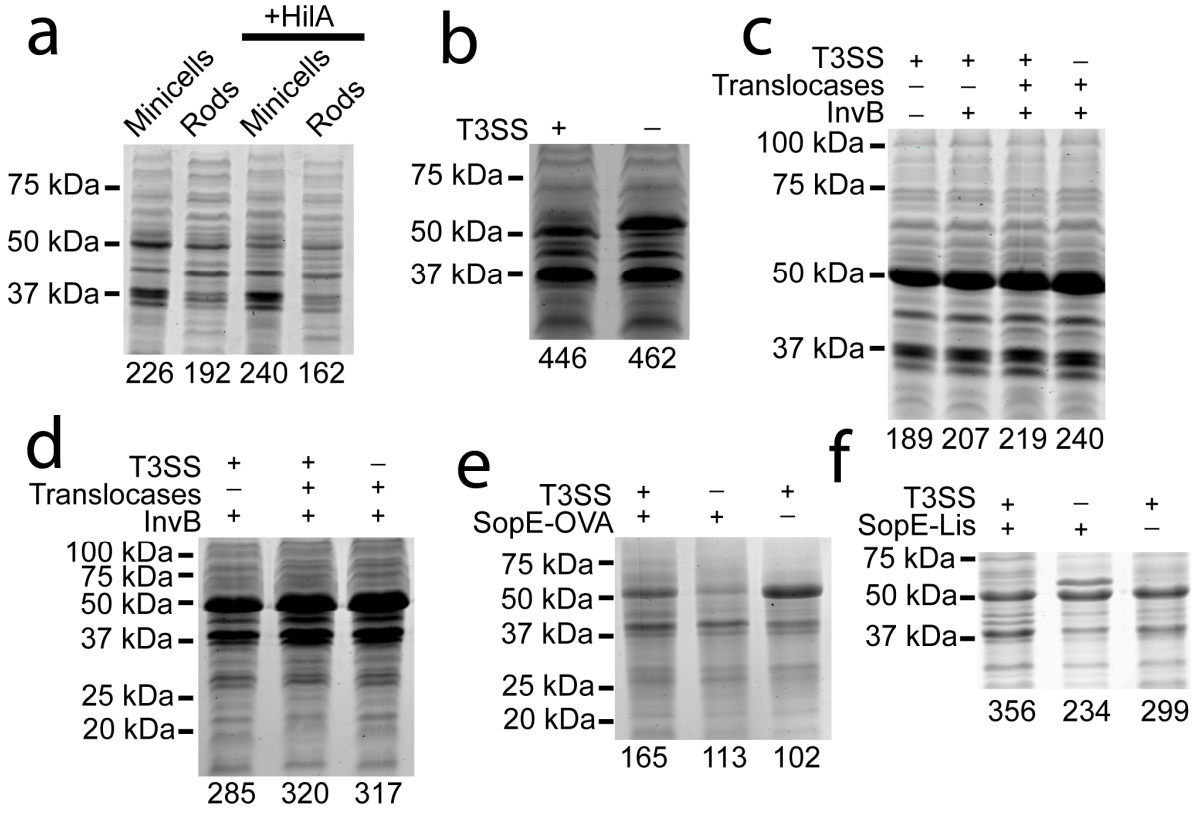
DIC images obtained from the indicated fractions of a 5-20% iodixanol gradient are shown. Scale bar: 2 µm.

Supplementary Figure S2. Comparison of the levels of selected SPI-1 T3SS-associated proteins in minicells and bacterial rods from strains with or without overexpression of the SPI-1 T3SS transcriptional regulator HilA.



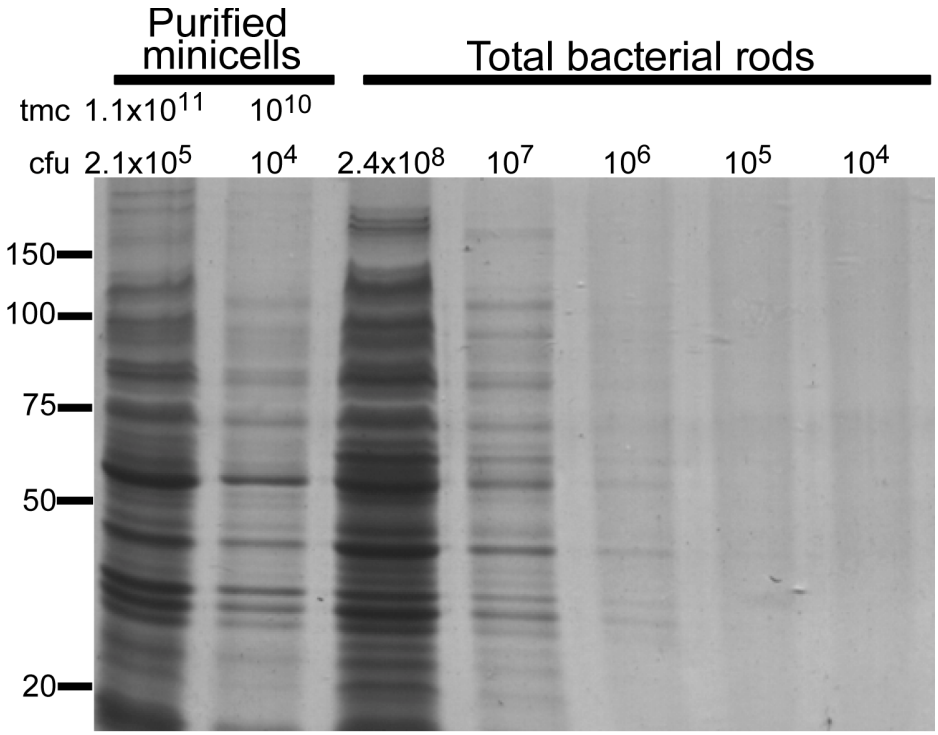
Equal amount of protein (standardized based on the values shown in Supplementary Fig. S3a) from minicell or bacterial rod lysates were analyzed by Western blot with antibodies directed to the indicated proteins.

Supplementary Figure S3. Protein content used to standardize loading in the Western blot analyzes of the different samples utilized in this study.



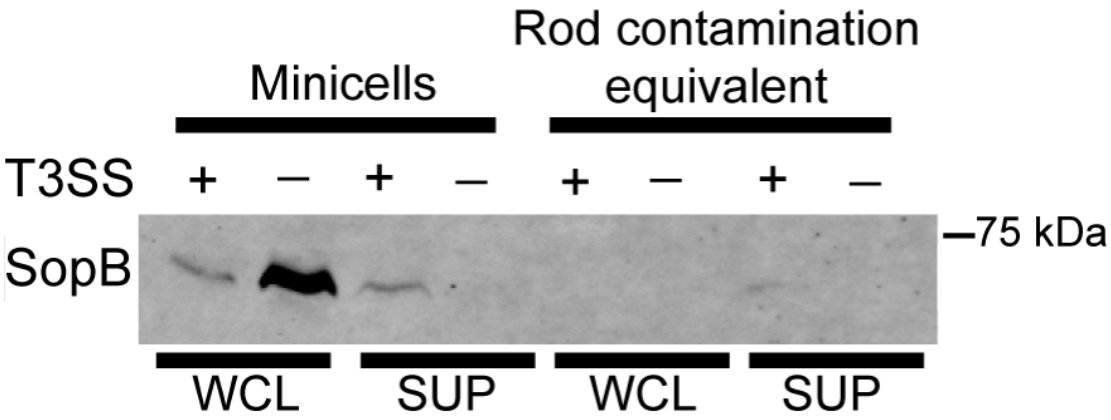
Lysates from the indicated samples were separated by SDS-PAGE, stained with Coomassie blue, and the gels scanned on an Odyssey infrared imaging system (LI-COR Biosciences). Values below each lane correspond to the integrated intensity of the entire lane. Samples in panel (**a**) correspond to experiments shown in **Fig. 1c**, samples in panel (**b**) corresponds to experiments shown in **Fig. 2c**, samples in panel (**c**) correspond to experiments shown in **Fig. 3b**, samples in panel (**d**) correspond to experiments in **Fig.3d**, samples in panel (**e**) correspond to experiments in **Fig. 4a**, and samples in panel (**f**) correspond to experiments shown in **Fig. 4d**.

Supplementary Figure S4. Comparison of lysates prepared from minicells and rods at the indicated concentrations.



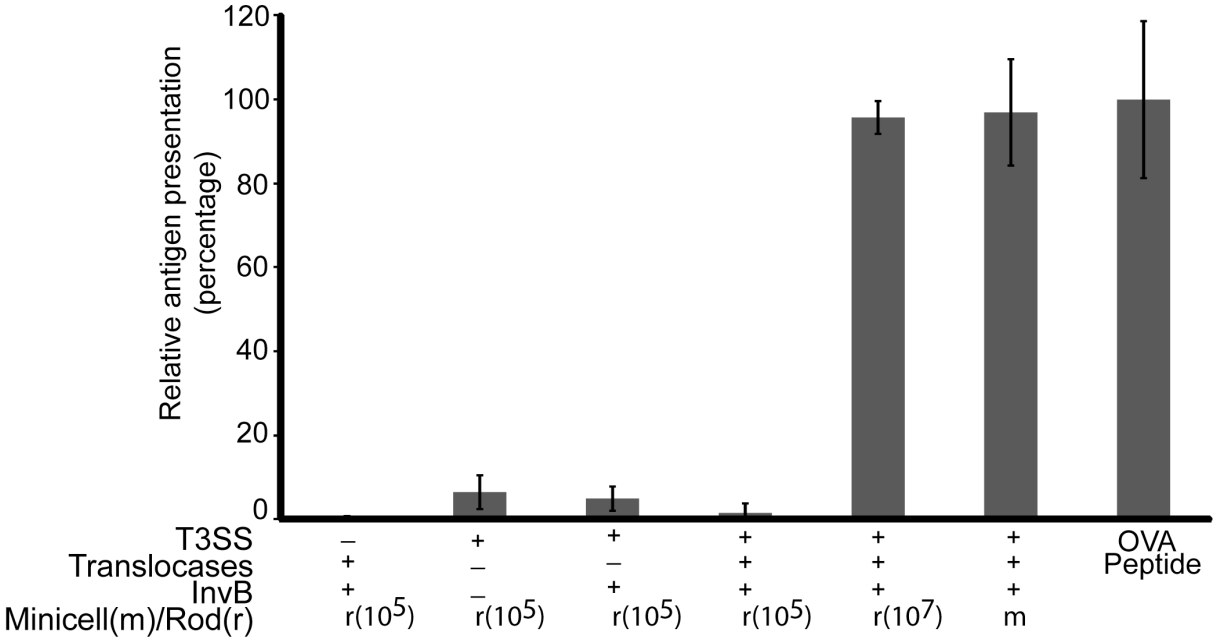
The number of minicells is indicated as total minicell count (tmc) and the number of rods is indicated as colony forming units (cfu). These results indicate that the potential contribution of rod contaminants in the minicell samples to the total amount of protein is negligible.

Supplementary Figure S5. Relative contribution of rod-derived material to the purified minicell preparation



Comparison of the levels of SopB in whole cell lysates (wcl) and supernatants (sup) obtained from minicell preparations and from an equivalent number of rods present as contaminants in the minicell preparation. These results indicate that the rod contamination does not significantly contribute to the signal observed in the minicell preparation.

Supplementary Figure S6. Bacterial rod cells in equivalent numbers to those present in minicell preparations used in the antigen presentation assay did not elicit a response.



RMA cells (C57BL/6 mouse hybridomas) were pulsed for 3 hours with equivalent number of wild type or T3SS-defective (Δ*invA*) *S.* Typhimurium cells (i. e. 10^5) to those present in the minicell preparation. In addition, RMA cells were pulsed with the minimal number of bacterial rods (10^7) necessary to stimulate a response in this assay as well as with a minicell preparation (10^{11}) for comparison. After pulsing, RMA cells were fixed, and used as APCs in a B3Z T-cell activation assay as described in experimental procedures. Values represent the levels of antigen presentation based on the β-galactosidase activity detected in the B3Z-T cell hybridoma reporter and are normalized relative to the values of the OVA peptide positive control, which was considered 100 %. The values are the mean ± standard deviation of three independent experiments.

Supplementary Table S1. Spectral counts obtained by LC-MS/MS analysis of T3SS-associated proteins in minicell and rod samples from Δ*minD S.* Typhimurium

	Spectral Counts						Relative Abundance (fold difference)[1]	
	Minicell Biological Replicates			Rod Biological Replicates				
T3SS Protein	1	2	3	1	2	3	minicells vs rods	Adjusted values[2]
AvrA	0.5	0.6	1.3	0.6	1.0	8.1	-1.9	-0.1
HilA	9.3	7.0	2.7	21.1	23.3	29.7	-3.6	-1.8
HilD	0.5	1.2	0.7	0.6	1.0	4.2	-1.4	0.4
IacP	0.7	1.0	0.5	4.3	1.5	2.5	-3.0	-1.3
InvA	2.3	2.3	1.8	2.0	1.3	4.6	1.1	-0.9
InvB	38.0	21.9	27.0	69.1	57.0	43.5	-1.9	-0.1
InvC	7.7	6.3	4.7	7.5	9.3	16.6	-1.5	-3.5
InvE	0.7	0.8	0.7	0.6	0.7	4.2	-1.2	0.6
InvF	0.5	0.6	0.5	3.5	2.0	3.5	-5.2	-3.4
InvG	48.0	34.4	40.3	52.0	65.3	69.0	-1.5	-3.5
InvH	5.0	3.1	9.0	9.8	6.0	7.1	-1.3	-3.3
InvJ	2.0	1.6	2.0	11.9	5.3	20.5	-5.4	-3.6
OrgA	3.0	0.8	2.3	7.8	4.0	2.8	-2.1	-0.3
OrgB	16.3	8.2	14.7	19.7	19.0	20.2	-1.5	0.3
PrgH	22.7	19.9	17.0	35.9	28.7	32.2	-1.6	-3.6
PrgI	11.3	9.8	7.0	9.5	6.3	9.9	1.1	-0.9
PrgJ	1.7	1.6	1.3	3.8	1.5	1.8	-1.3	-3.3
PrgK	27.0	23.4	25.0	37.3	24.0	21.6	-1.0	-3.1
SicA	38.0	26.2	28.7	111.6	89.7	66.2	-2.8	-1.0
SicP	0.5	0.6	1.2	9.0	7.0	2.1	-4.4	-2.6
SigE	18.0	16.8	16.7	83.3	47.0	28.7	-2.6	-0.8
SipA	21.3	11.7	49.7	162.5	118.3	173.0	-5.8	-4.0
SipB	151.7	54.7	114.7	445.0	395.7	273.5	-3.3	-1.5
SipC	66.0	28.5	93.0	105.0	92.7	168.1	-2.0	-0.2
SipD	7.0	3.5	13.0	61.0	46.7	64.8	-7.7	-5.9
SlrP	2.7	0.6	1.0	20.0	8.0	10.3	-9.9	-8.1
SopA	4.0	6.3	8.3	26.0	10.0	21.9	-2.6	-0.8
SopB	75.3	44.2	93.3	306.5	214.7	170.5	-3.0	-1.2
SopD	0.5	0.6	1.7	17.3	2.7	6.0	-5.7	-3.9
SopE	31.0	13.7	19.7	79.2	78.0	26.9	-2.3	-0.5
SopE2	2.7	1.2	0.5	22.8	22.0	2.8	-8.7	-6.9
SpaO	11.7	10.9	9.0	11.9	14.3	12.4	-1.2	0.6
SpaS	5.7	3.5	3.7	4.9	6.7	5.3	-1.3	-3.3
SptP	6.3	4.7	6.7	103.5	47.0	53.1	-10.5	-8.7

[1]: Relative abundance was determined by comparing the average spectral counts of rod and minicell samples
[2]: Values have been adjusted to account for the different ratio of membrane vs cytoplasmic content in the minicells (see Methods)

Supplementary Table S2. Spectral counts obtained by LC-MS/MS analysis of T3SS-associated proteins in minicell and rod samples from Δ*minD S.* Typhimurium overexpressing the T3SS positive transcription regulator HilA.

	Spectral Counts				Relative Abundance (Fold Difference)[1]		
Protein	Minicells	Rods	Minicells (+HilA)	Rods (+HilA)	Minicells (+HilA) versus minicells	Minicells (+HilA) versus rods	Minicells (+HilA) versus rods (+HilA)
AvrA	0.9	3.2	0.7	2.2	-1.4	-4.9	-3.3
HilA	1.3	25.7	86.1	175.2	67.5	3.4	-2.0
HilD	0.5	4.0	0.7	3.2	1.2	-6.1	-4.9
IacP	0.5	0.7	0.7	2.2	1.2	-1.0	-3.3
InvA	4.4	6.0	52.2	68.8	12.0	8.7	-1.3
InvB	24.0	52.7	65.3	77.4	2.7	-0.8	-1.2
InvC	3.3	11.0	45.7	65.6	14.0	4.2	-1.4
InvE	1.1	0.7	2.6	3.2	2.4	3.9	-1.2
InvF	0.5	1.5	5.2	2.2	9.6	3.5	2.4
InvG	10.2	20.7	30.0	46.2	3.0	1.5	-1.5
InvH	9.8	10.0	20.9	21.5	2.1	2.1	-1.0
InvJ	3.6	32.7	1.3	18.3	-2.8	-25.0	-14.0
OrgA	2.9	6.7	18.3	21.5	6.3	2.7	-1.2
OrgB	13.1	13.0	57.4	25.8	4.4	4.4	2.2
PrgH	19.3	26.3	97.9	108.6	5.1	3.7	-1.1
PrgI	0.5	0.5	0.7	10.8	1.2	1.3	-16.5
PrgJ	0.5	0.5	2.6	4.3	4.8	5.2	-1.6
PrgK	18.9	17.3	113.5	86.0	6.0	6.6	1.3
SicA	29.1	53.3	57.4	92.5	2.0	-0.9	-1.6
SicP	0.5	3.7	7.8	14.0	14.4	2.1	-1.8
SigE	18.2	23.3	41.8	57.0	2.3	1.8	-1.4
SipA	29.4	165.3	61.3	220.4	2.1	-2.7	-3.6
SipB	109.7	253.3	693.0	478.4	6.3	2.7	1.4
SipC	83.9	150.7	390.2	250.5	4.6	2.6	1.6
SipD	10.9	71.3	67.9	103.2	6.2	-1.1	-1.5
SlrP	0.9	6.3	2.6	33.3	2.9	-2.4	-12.8
SopA	8.0	23.3	47.0	91.4	5.9	2.0	-1.9
SopB	70.9	152.7	197.1	369.8	2.8	-0.8	-1.9
SopD	0.5	4.3	23.5	64.5	43.1	5.4	-2.7
SopE	18.9	18.7	63.9	51.6	3.4	3.4	1.2
SopE2	0.5	3.7	7.8	22.6	14.4	2.1	-2.9
SpaO	10.5	14.7	35.2	29.0	3.3	2.4	1.2
SpaP	0.9	1.7	28.7	6.5	31.6	17.2	4.5
SpaS	5.1	4.0	2.6	5.4	-1.9	-1.5	-2.1
SptP	2.5	30.3	5.2	179.5	2.1	0.2	-34.4

[1]: Relative abundance was determined by comparing the average spectral counts of rod and minicell samples

Supplementary Table S3. Bacterial strains used in this study:

Strain	Relevant genotype	References or sources
S. Typhimurium		
SL1344	wild-type *rpsL hisG*	[47]
SB136	*invA*::*aphT*	[48]
SB1780	*minD::cat*	(this study)
SB1781	*minD::cat invA*::*aphT*	P22HT*int* [SB1780] =>SB136 (this study)
SB1771	*minD::cat sipD3xF*	P22HT*int* [SB1780] =>SB1630 [31](this study)
SB1774	*invA::aphT minD::cat sipD3xF*	P22HT*int* [SB136] => SB1771 (this study)
SB2105	*sipAM45 3xFspaO*	(this study)
SB1790	*minD::cat sipAM45 3xFspaO*	P22HT*int* [SB1780] =>SB2105(this study)
SB1400	Δ*asd*	[49]
SB1777	*minD::cat* Δ*asd*	P22HT*int* [SB1780] =>SB1400(this study)
SB1788	*minD::cat invA*::*aphT* Δ*asd*	P22HT*int* [SB136] =>SB1777 (this study)

Supplementary Table S4. Plasmids used in this study:

Plasmid Name	Relevant information	References or sources
pSB3504	P^{rhaBAD}, Spectinomycin^R, *hilA*	[50]
pSB2811	pBAD24-*sopB3xF-sigE*	[51]
pSB3506	pBAD24-*sopE-OVA3xF*	(this study)
pSB3507	pBAD24-*sopE-OVA3xF invB*	(this study)
pSB3508	pBAD24-*sopE-OVA-3xF invB invE sicA sipB sipC sipD*	(this study)
pSB3510	pBAD24- *sopE-m45-LLO-p60 invB invE sicA sipB sipC sipD*	(this study)

Supplementary Table S5. Antibodies used in this study

Antibody	**Source**	**Catalog #**	**Dilution**
Anti-Flag (M2)	Sigma	F3165	1:10,000
Anti-M45	Galan laboratory		1:500
Anti-SipB	Galan laboratory		1:2,000
Anti-SipC	Galan laboratory		1:500
Anti-Needle Complex	Galan laboratory		1:5,000
Anti-mouse Dylight 680	Pierce	PI35518	1:10,000
Anti-rabbit Dylight 800	Pierce	PI35571	1:10,000
Anti-mouse HRP	Sigma	A4416	1:10,000

Supplementary Methods:

Mass spectrometry data analysis

All raw data from the MS/MS scans was processed and searched against the *Salmonella* database with MASCOT (Matrix Science Ltd. London, UK). The peptide and protein assignments from MASCOT were filtered to retain only those identifications with MASCOT scores above extensive homology. Relative protein abundance was determined by the spectral counting method [44]. Experiments were carried out in triplicate with three biological replicates for each experimental condition unless noted otherwise. Data between technical replicates in a single biological sample were normalized and spectral counts for each technical replicate in individual biological replicates were averaged. All biological replicates with a spectral count of less than one were arbitrarily assigned a value of 0.5 to reflect the limits of detection. Spectral counts were logarithmically transformed to obtain a binomial distribution then statistically evaluated using a paired, two-tailed *t*-test to determine significant differences between protein relative abundance [45] in minicell versus bacterial rod samples. Due to their different ratio of membrane vs. cytosol, minicells were found to be relatively enriched in membrane proteins (median 2.0 fold) and depleted in cytoplasmic proteins (median -1.8 fold) [note: prediction of protein subcellular localization was carried out with PSORT (http://www.psort.org)]. Therefore, the relative abundance of membrane and cytoplasmic proteins was corrected to reflect these differences. The correction factor introduced was -2.0 for predicted membrane proteins and +1.8 for predicted cytoplasmic proteins. For comparison of abundance of T3SS proteins in minicells and rods obtained from a parental strain overexpressing *hilA*, all samples were obtained from cultures grown in the presence of 0.02% rhamnose.

Antigen presentation assay

RMA cells (C57BL/6 mouse hybridomas) or bone marrow-derived dendritic cells (BMDCs) obtained from C57BL/6 mice were used as antigen-presenting cells after incubation with minicells isolated from various parental *S.* Typhimurium strains. Mouse BMDC were prepared as described previously [46]. Approximately 10^{11} minicells were used to deliver antigen to approximately 10^7 RMA cells in one well of a 6 well dish in 2 ml of Dulbecco's modified Eagle's medium (DMEM) at 37ºC for 3 hours. Alternatively, 10^{10} minicells were incubated with 10^6 BMDCs per well in a 24 well plate. For these experiments, the Δ*asd* mutation (21) was introduced into all the *S.* Typhimurium minicell-generating strains. The Δ*asd* mutation renders *S.* Typhimurium auxotrophic for L-diaminopimelic acid (DAP), which is absent in host tissues. Consequently, any small number of bacterial rods remaining in the minicell preparation will undergo rapid "DAP-less" death shortly after infection and will not contribute to the immune response. In addition to DMEM the cells were also incubated in the presence of 0.2% arabinose to induce expression from plasmids contained within the minicell. After incubation, the cells were washed three times in PBS and then fixed in 1% paraformaldehyde either for 20 min at room temperature or overnight at 4ºC. After fixation of the antigen presenting cells, the cells were washed and then plated at a concentration of $2x10^5$ in triplicate in 100 µl DMEM cultures in a 96-well dish. To each well $2x10^5$ OVA-specific B3Z T-cell hybridoma cells were then added. This transgenic cell line expresses a T-cell receptor specific for the MHC class I immunodominant peptide of OVA, SIINFEKL. The activation of the TCR of the B3Z cells was determined by an NF-AT-dependent transcriptional reporter consisting of the IL-2 promoter

fused to LacZ [35]. After incubation for approximately 19 hours, the B3Z T cells were washed, lysed, and β-galactosidase production was measured using a chromogenic substrate, chlorophenolred-β-D-galactopyranoside (CPRG, CALBIOCHEM). Addition of the OVA peptide SIINFEKL (1μM, ABBIOTEC) served as a positive control and all results were normalized relative to the values obtained with the SIINFEKL peptide.

Adoptive transfer immunization

Spleens were removed from OT-1 (CD45.2/C57BL/6) mice, homogenized using a $1mm^2$ mesh metal strainer, and red blood cells were lysed with a hypotonic buffer (0.15M NH_4CL and 1mM $NaHCO_3$, pH 7.4). A total of $1\text{-}2x10^7$ splenocytes were then injected in the tail vein of C57BL/6 (CD45.1) mice and one day later, mice were immunized by intraperitoneal administration of $1\text{-}2$ $x10^{10}$ minicells purified from different *S.* Typhimurium parental strains. Minicells were pulsed for 3 hours at 37ºC in L broth containing 0.2% arabinose prior to immunization to allow SopE-OVA antigen expression. For these experiments, the Δ*asd* mutation (21) was introduced into all the *S.* Typhimurium minicell-generating strains to avoid replication of the very small number of contaminating rod cells. Three weeks after the initial immunization, mice were boosted by i.p. administration of $1x10^7$ plaque forming units (PFUs) of recombinant Vesicular stomatitis virus expressing OVA (VSV-OVA) (22) generously provided by Leo Lefrançois (University of Connecticut Health Center, Farmington, Conn.). Five days after boosting, immunized mice were sacrificed and the spleens were removed and splenocytes prepared as described above.

Supplementary References

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