Supplementary material

Experimental procedures

*Generation of mutant strains and plasmids for complementation*

For the construction of mutant strains deficient in *ssaG*, *ssaH*, or *ssaI*, the 'one step' deletion method (Datsenko & Wanner, 2000) with previously described modifications (Chakravortty et al, 2002) was used. After replacement of the target gene by the *aph* cassette, FLP-mediated recombination was performed (Datsenko & Wanner, 2000) to remove the resistance gene. The resulting mutant strains were confirmed by PCR and DNA sequencing.

Plasmids for the expression of wild-type alleles of the deleted genes under control of promoter Pro *ssaG* were generated by PCR amplification of *ssaG* using primers ProSsaG-For-HindIII and SsaG-Rev-XbaI, for *ssaGH* using ProSsaG-For-HindIII and SsaH-Rev-XbaI, and for *ssaGHI* using ProSsaG-For-HindIII and SsaI-Rev-XbaI. The resulting products were digested by HindIII and XbaI and cloned into HindIII/XbaI-digested pWSK29. Confirmed plasmid constructs were introduced into mutant strains by electroporation.

For analysis of translocation, effector gene *sseJ* was chromosomally fused to the HA tag (Uzzau et al, 2001) and the mutant allele was moved into *ssaG*, *ssaH* or *ssaI* mutant strains or plasmid-complemented strains using P22 transduction according to standard methods (Maloy et al., 1996).

*Analysis of intracellular replication*

Intracellular replication was assessed by the gentamicin protection assay. RAW macrophages were infected as for immuno-fluorescence analyses, extracellular bacteria were killed by addition of gentamicin and at 2 h and 16 h after infection, host cells were washed three times with DMEM and lysed by addition of PBS containing 0.1 % Triton X-100. Serial dilutions
were made and plated onto LB agar to determine the viable number of gentamicin-protected bacteria.

Negative-staining of bacteria and labeled bacteria

Bacteria were fixed with 1 % formaldehyde and washed twice in TE-buffer. Samples were then adsorbed onto carbon film, washed in TE-buffer and stained with 2 % uranyl acetate in TE-buffer (pH 4.5). Labeled bacteria were fixed with 1 % glutaraldehyde for 15 min at RT and washed several times with TE-buffer before staining. After air-drying samples were examined in a Zeiss transmission electron microscope EM 910 at an acceleration voltage of 80 kV at calibrated magnifications.

Embedding of bacteria

Bacteria labeled for SseB or SseC or double labeled were fixed with 3 % glutaraldehyde for 1 h on ice, washed with PBS, dehydrated with a graded series of acetone and embedded in Spurr’s resin (Spurr, 1969). Ultrathin sections of about 80 nm were cut with a diamond knife and counterstained with 4 % aqueous uranyl acetate and lead citrate before examining in a Zeiss EM 910 at 80 kV.

Immuno labeling of ultrathin sections

Bacteria were fixed as described above for immuno FESEM, dehydrated with a graded series of ethanol on ice and embedded in LRWhite resin (polymerization at 50°C for 2 days). Ultrathin sections were put onto formvar-coated nickel grids and incubated on drops of the purified SseB antibody (80 µg IgG protein/ml) at 4°C overnight, samples were then washed with PBS and incubated with protein A gold particles (15 nm in diameter) for 30 min at RT, washed with PBS containing 0.01 % Tween 100, washed in distilled water and air-dried. Counter-staining was performed with 4 % aqueous uranyl acetate for 2 min before examination in the Zeiss TEM9100 at an acceleration voltage of 80 kV.
### Supplementary Table

*Oligonucleotides used in this study*

<table>
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<th>Designation</th>
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<tr>
<td>SsaG-Del-For</td>
<td>5'-GTATCCTTACGATGTATTTATTTTAAGGAAAGCATTATG&lt;br&gt;GTGTAGGCTGGAGCTGCTTC-3'</td>
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Supplementary Figure legends

Fig. S 1. Functional characterization of mutant strains deficient in \( ssaG \), \( ssaH \) or \( ssaI \). Gene-specific deletions of \( ssaG \), \( ssaH \) or \( ssaI \) were constructed. In addition, mutant strains were complemented by plasmid-borne expression of wild-type alleles of the deleted genes. A) Intracellular replication. The ratio of intracellular bacteria recovered 2 h and 16 h after infection was calculated for various strains. The graph shows the means of three independent experiments and error bars indicate the standard deviation from means. B) Translocation of HA-tagged SseJ by intracellular \( Salmonella \) wild type, various mutant strains and complemented mutant strains. Detection of intracellular \( Salmonella \) was performed by immuno-straining with rabbit anti-\( Salmonella \) O4 test sera and Cy2-conjugated secondary antibody (green). SseJ-HA was detected using rat anti-HA monoclonal antibody and Cy3-conjugated secondary antibody (red). Merged images include the phase-contrast images of the samples. Scale bars correspond to 20 \( \mu \)m.

Fig. S 2. Variable length of SPI2-T3SS appendages. \( S. \) Typhimurium WT was grown for 9 h in PCN-P media at pH 5.8 and analyzed by negative stain (A) and FESEM (B) as described for Fig. 3. Note the variable length of appendages on individual bacterial cells. Scale bars represent 25 nm in A) and 500 nm in B).

Fig. S 3. Cross-linking analysis of SseB. \( S. \) Typhimurium wild type (wt) and strains harboring mutations in various SPI2 genes were grown over night in PCN-P media at pH 7.4 to induce SPI2 expression without secretion or at pH 5.8 for induce expression and secretion. In order to sample the organization of proteins in SPI2-dependent appendages and to access different
sub-cellular compartments, we performed chemical cross-linking analyses of bacteria using DSS (disuccinimidyl suberate) or BS\textsuperscript{3} (bis[sulfosuccinimidyl]suberate). DSS is a membrane-permeable cross linker and the non membrane-permeable compound BS\textsuperscript{3} that reacts predominantly with surface-exposed structures. Subsequently, protein of total bacterial cell fractions was separated by SDS-PAGE on 12 % Tricine gels and transferred onto nitrocellulose membranes. Western blots were probed with the anti SseB antiserum and HRP-coupled secondary antibody to detect cross-linking products.

After cross-linking with BS\textsuperscript{3} of bacteria grown under SPI2-inducing conditions at neutral pH, only monomeric SseB was detected by Western blot analysis. In contrast, a major cross-linking product of SseB of 43 kDa and several products of minor abundance were detected in lysates of bacteria grown under conditions inducing secretion. The 43 kDa product was also observed in the \textit{sseC} and \textit{sseD} mutant background, indicating that SseC and SseD are not required for its formation. Therefore, this cross-linking product is most likely a dimer of secreted SseB subunits. Cross-linking with the membrane-permeable compound DSS resulted in a product of about 34 kDa present only in bacteria grown at pH 7.4 but absent in secreting bacteria. Since this product was not obtained using BS\textsuperscript{3}, the cross-linking product might represent a product of SseB and a specific chaperone for SseB, most likely SseA (Zurawski & Stein, 2003). We also analyzed the cross-linked fractions with antisera raised against SseC or SseD. It was not possible to detect specific homodimers or oligomers of SseC or SseD, or cross-linking products that indicate the formation of heterodimers of subunits (data not shown).

Fig. S 4. Effect of bafilomycin A\textsubscript{1} on phagosome acidification. Various concentrations of bafilomycin, or an equal amount of the solvent DMSO were added to RAW macrophages. After incubation for 1 h, acridine orange was added to a final concentration of 1 \textmu M and the
fluorescence of living cells was analyzed by confocal microscopy. Acridine orange yields red fluorescence in acidic compartments and green fluorescence in neutral compartments.

Fig. S 5. Effect of various concentrations of bafilomycin A₁ on intracellular replication of *Salmonella* in RAW macrophages. RAW cells were infected with *S*. Typhimurium wild type at an MOI of 10 and various concentrations of bafilomycin as indicated were added. As control, an equal amount of the solvent DMSO was added. The inhibitor was maintained throughout the experiment. At 2 h or 16 h after infection, host cells were lysed by the addition of 0.1 % Triton X-100 and intracellular bacteria were quantified by plating serial dilutions onto agar plates. Mean counts of colony-forming units (CFU) and standard deviation are given for a representative experiment.

Fig. S 6. Characterization of Pro *sseA*::GFP reporter plasmid pLS824. The construction and characteristics of reporter plasmid pLS824 have been previously described (Jantsch *et al*., 2003). Briefly, a transcriptional fusion between the promoter of *sseA* (Pro *sseA*) to GFP Mut3 was generated in the background of low copy number plasmid pWSK29 (Wang & Kushner, 1991). A) *Salmonella* wild type (WT) without plasmid, harboring plasmid pFPV25.1 for constitutive expression of GFP (const.), or pLS824, or *ssrB*-deficient strain P8G12 (*ssrB*::mTn5) harboring pLS824 were grown for 16 h in PCN or PCN-P minimal media at pH 7.4. Expression of SPI2 genes during growth in low phosphate minimal medium PCN-P but not in high phosphate minimal media (PCN) has been described (Deiwick *et al*, 1999). GFP fluorescence was measured by flow cytometry on a FACscalibur (BF). Expression of the Pro *sseA*::GFP reporter is similar and fully dependent on the function of SsrB. B) The same set of
strains was used for the infection of RAW macrophages and 16 h after infection, intracellular bacteria were recovered and labeled for *Salmonella* LPS. The GFP fluorescence of the LPS-positive population was analyzed by flow cytometry as described for Fig. 7B. Means and standard deviation are shown.
References:


