SseF and SseG are translocated effectors of the type III secretion system of Salmonella pathogenicity island 2 that modulate aggregation of endosomal compartments

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Summary
The type III secretion system encoded by Salmonella pathogenicity island 2 (SPI 2) is important for intracellular proliferation in infected host cells. Intracellular Salmonella use this system to translocate a set of effector proteins into the host cell. We studied the role of SseF and SseG, two SPI 2-encoded proteins. SseF and SseG are not required for translocation of effector proteins such as SseJ, encoded by genes outside of SPI 2. Rather, both proteins are translocated and interact with phagosomal membranes after translocation. In infected epithelial cells the formation of Salmonella-induced filaments, endosomal aggregates rich in lysosomal glycoproteins, is dependent on the function of SPI 2. We observed that, in mutant strains deficient for sseF or sseG, the formation of aggregated endosomes can take place, but the composition of the structures is different from those observed in cells infected with Salmonella wild type. These observations indicate that SseF and SseG modulate the aggregation of host endosomes.

Introduction
Pathogenic bacteria have evolved a variety of strategies to modulate the function of eukaryotic cells during pathogenesis. One of these sophisticated mechanisms is the delivery of proteins into the host which subsequently interfere with host cell functions. For this function various Gram-negative pathogens employ type III secretion systems (TTSS). Type III secretion systems translocate, in a contact-dependent manner, effector proteins into eukaryotic host cells (for review, see Hueck, 1998).

Type III secretion systems have a central role in the pathogenesis of infections with Salmonella enterica. Salmonella enterica is an important pathogen of humans and animals responsible for diseases ranging from localized gastrointestinal infections to systemic, life-threatening bacteraemia such as typhoid fever. Invasion of eukaryotic cells and intracellular survival and proliferation within host cells are key features of S. enterica pathogenesis. Both virulence traits are linked to the function of two TTSS utilized by S. enterica for interactions with host cells. Extracellular Salmonella are able to invade non-phagocytic cells such as cells of the intestinal epithelium. The TTSS encoded by genes clustered within Salmonella pathogenicity island 1 (SPI 1) translocate a group of proteins required for invasion. Salmonella is also able to survive within phagocytes and to proliferate in infected cells. A large set of functions is required for this phenotype, and it was observed that a second TTSS encoded by SPI 2 is important for the intracellular phenotype (for review, see Hensel, 2000). Mutants deficient in SPI 2 are highly attenuated in systemic pathogenesis and show a reduced intracellular replication. Work by different groups revealed that the function of SPI 2 is required for the modulation of intracellular processes such as exclusion of the NADPH-oxidase from Salmonella-containing vesicles (SCV) (Vazquez-Torres et al., 2000), avoidance of nitrosative damage by exclusion of iNOS from SCV (Chakravortty et al., 2002), and a global interference with cellular trafficking (Uchiya et al., 1999). A remarkable phenotype of Salmonella-infected epithelial cells is the formation of filamentous aggregates of endosomes containing lysosomal glycoproteins (Igp) (Garcia-del Portillo et al., 1993) termed Salmonella-induced filaments (SIF) (Stein et al., 1996). Work by several groups recently demonstrated that SIF formation is dependent on the function of the TTSS of SPI 2 (Beuzon et al., 2000; Guy et al., 2000; Brumell et al., 2001a).

Although the role of the TTSS encoded by SPI 2 for the various phenotypes has been demonstrated, the contribution to a specific cellular phenotype has not been established for most of the translocated effector proteins. Several proteins were identified that are encoded by the SPI 2 locus and secreted by the TTSS of SPI 2 under in vitro conditions. SseB, SseC and SseD are secreted and
predominantly located on the surface of the bacterial cell (Beuzon et al., 1999; Klein and Jones, 2001; Nikolaus et al., 2001). These proteins also function as translocon for the translocation of effector proteins by intracellular Salmonella (Nikolaus et al., 2001). sseF and sseG are further genes clustered with sseBCD in the SPI 2 locus and have been proposed as putative effectors. SseF and SseG do not exhibit significant sequence similarity to known proteins and also lack a conserved N-terminal domain that is required for translocation of a group of SPI 2 effector proteins termed ‘Salmonella translocated effectors’ or STE (Miao and Miller, 2000). Analysis of mutant strains in sseF or sseG revealed that both genes have only minor contribution to systemic pathogenesis and intracellular proliferation (Hensel et al., 1998). We have recently been able to detect in vitro secretion of epitope-tagged derivatives of SseF and SseG (Hansen-West et al., 2002). Work by Guy et al., 2000 suggested that SseF and SseG are required for the SIF phenotype in Salmonella-infected epithelial cells, i.e. the formation of endosomal aggregates rich in lgp such as LAMP-1. Based on these observations, we analysed the role of SseF and SseG for the function of the TTSS of SPI 2 and the contribution of both proteins to the cellular microbiology of Salmonella. In contrast to other SPI 2-encoded secreted proteins, SseF and SseG do not function as translocator, but are translocated effectors. We observed that both proteins interact with endosomal compartments and modulate the formation of aggregates of endosomes.

Results

SseF and SseG are not required for secretion of substrate proteins

We analysed the effect of mutations in sseF and sseG on the secretion of SPI 2 substrate proteins in vitro. Bacterial cultures were grown under conditions that induce the expression of SPI 2 genes and trigger secretion of substrate proteins by the TTSS of SPI 2. Under these experimental conditions, mutant strains in sseF, sseG, or sscBsseFG, but not the ssaV mutant strain, were capable of secreting SseB (Fig. 1), as well as SseC and SseD (data not shown). As observed before, SPI 2 proteins secreted under in vitro conditions by wild-type S. Typhimurium as well as by mutant strains in sseF, sseG, or sscBsseFG were located on the bacterial surface and also present in the culture supernatant.

We next analysed the contribution of sseF and sseG to intracellular replication of S. Typhimurium in macrophages (Fig. 2). In accordance with a previous study (Hensel et al., 1998), individual mutations in sseF or sseG resulted in a reduction of intracellular proliferation. We observed that strain MvP373 harbouring a deletion of sscB, sseF and sseG showed a reduction of intracellular replication similar to that of strains with individual mutations. This observation indicates that SseF and SseG have no synergistic effect on the intracellular proliferation.

SseF and SseG are not required for translocation of effector proteins of SPI 2

We have previously described the use of the epitope tag M45 derived from the adenoviral protein E4-6/7 (Obert et al., 1994) to follow synthesis and secretion of putative substrate proteins of the SPI 2-encoded TTSS (Hansen-
secreted an epitope-tagged derivative of SseJ. SseJ-M45 is translocation of SseJ as a member of the STE family using (Miao and Miller, 2000). In this study, we analysed the members of the STE family as translocated effectors. 

Translocation of SseJ-M45 (and other STE proteins, data not shown) was only observed in a subset of infected RAW 264.7 cells. No translocation of SseJ-M45 was observed by strains harbouring mutations in sseB, sseC, or sseV (data not shown), indicating that a functional TTSS of SPI 2, as well as translocon formation, is required for SseJ-M45 translocation. Translocation of the tagged protein was observed in host cells infected by strains defective in the TTSS encoded by SPI 1. Translocation of SseJ-M45 by strains harbouring mutations in sseF and sseG was similar to that of the wild-type strain. These observations indicate that SseF and SseG are not acting as a translocator for further substrate proteins in vivo, and that their function is distinct from those of SseB, SseC and SseD.

SseF and SseG are effector proteins translocated by intracellular Salmonella

Because our previous experiments established that SseF and SseG are secreted proteins that are not required for the translocation of STE proteins, we hypothesized that SseF and SseG are further effector proteins that are translocated into the host cell. Translocation of M45 epitope-tagged derivatives of SseF and SseG by intracellular S. Typhimurium was analysed in comparison to epitope-tagged SseJ, a known Salmonella translocated effector. After infection of RAW 264.7 macrophages with wild-type S. Typhimurium harbouring plasmids for the expression of epitope-tagged SseJ, SseF or SseG, these proteins are translocated by intracellular bacteria (Fig. 3). Translocation of SseF-M45 or SseG-M45 was not detected in host cells infected with the ssaV strain harbouring plasmids for the expression of the fusion constructs, thus dependent on the function of the TTSS of SPI 2. No effect was observed by a mutation in invG ablating the function of the SPI 1-encoded TTSS (data not shown). The translocated proteins were frequently co-localized with LAMP-1, a lgp present in membranes of late endosomes and lysosomes (Fig. 3).

The kinetics of translocation of SPI 2 effector proteins were analysed. The proportion of infected RAW 264.7 cells positive for translocated SseF-M45 or SseG-M45 at various time-points after infection is shown in Fig. 4. Translocation of SseF or SseG was not detectable before 4 h after infection, but 10 h after infection 57% or 40% of the infected cells were positive for translocated SseF-M45 or SseG-M45 respectively. Translocation of both proteins showed similar kinetics. The fluorescence signals for SseG-M45 were generally weaker compared to SseF-M45, either indicating that the amounts of translocated SseG-M45 were lower than those of SseF-M45, or that the epitope tag in SseG-M45 was less accessible to antibody binding.

Fig. 2. Role of SseF and SseG for intracellular proliferation of S. Typhimurium. RAW 264.7 macrophages were infected with S. Typhimurium wild type (WT) and various mutant strains at a multiplicity of infection of about 1. Non-internalized bacteria were removed by washing followed by addition of gentamicin to kill remaining bacteria. At 2 h and 12 h after infection, macrophages were lysed and the number of intracellular cfu was determined by plating serial dilutions on Muller-Hinton plates. Intracellular replication is expressed as the increase in intracellular cfu from 2 h to 12 h after infection. Experiments were performed in triplicate on three independent occasions.

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SseF and SseJ are associated with host cell membranes after translocation

In order to determine the subcellular localization of SseF, SseG and SseJ after translocation in more detail, fractionation of host cells was performed. RAW 264.7 cells were infected with S. Typhimurium harbouring plasmids for the expression of SseJ-M45, SseF-M45 or SseG-M45 fusion proteins as indicated. Sixteen hours after infection, the cells were harvested and fractionated. This procedure yielded a debris fraction containing bacteria, nuclei and cytoskeleton, a host cell cytosolic fraction and a host cell membrane fraction. These fractions were subjected to Western blot analyses and probed for the presence of M45-tagged effector proteins as well as for the gp91<sub>phox</sub> subunit of the NADPH-oxidase and β-tubulin as specific markers of the host cell membrane and cytosolic fractions respectively (Fig. 5). In the background of wild-type S. Typhimurium, both SseF-M45 and SseJ-M45 were present in the membrane fraction. However, no translocation was observed in the background of the ssaV mutant strain, and the M45-tagged proteins were only detected in the debris fraction. We have not been able to detect significant amounts of translocated SseG-M45 in any fraction. These results indicate that translocated SseF and SseJ are located in, or associated with, host cell membrane compartments.

SseF and SseG interact with the endosomal system

Immunostaining of SseF-M45, SseG-M45 and SseJ-M45 in infected RAW 264.7 cells indicated that the translocated proteins were associated with the SCV but also co-localized with membrane compartments distant from the
SCV (Fig. 3). However, the pattern of staining of SseF, SseG or SseJ was different from those of translocated M45-tagged SPI 1 effector proteins, such as SopE (Hardt et al., 1998) or SopE2 (Stender et al., 2000) that appear to be freely diffusible in the cytoplasm. Microscopic analyses suggested that translocated SPI 2 effectors SseF, SseG and SseJ were not equally distributed in the host cell cytoplasm, but rather present in certain regions or organelles of the host cell. Previous studies established that Salmonella resides inside membrane-bound vesicles that contain late endosomal/lysosomal membrane proteins such as the glycoprotein LAMP-1. Staining for LAMP-1 indicated that translocated SPI 2 effectors SseF, SseG and SseJ co-localize with this membrane marker (Fig. 3). To study the subcellular localization in more detail, we next infected the epithelial cell line HeLa with Salmonella strains harbouring plasmids for expression of epitope-tagged proteins. We analysed the co-localization of translocated SseF-M45 and SseG-M45 with host cell markers in HeLa cells (Fig. 6). Intracellular S. Typhimurium induce the formation of filamentous aggregates of endosomes in infected epithelial cells referred to as Salmonella-induced filaments or SIF (Garcia-del Portillo et al., 1993). This phenomenon was also observed under our experimental conditions, as the formation of tubular compartments rich in LAMP-1 was induced after infection. Detection of translocated SseF-M45 or SseG-M45 in infected epithelial cells revealed a distinct filamentous staining pattern for both proteins that co-localized with LAMP-1 (Fig. 6). We also observed that SseJ-M45 translocated by wild-type S. Typhimurium was targeted to the same structures (Fig. 7). These staining patterns were reminiscent of the pattern of SiaF-GFP in transfected cells (Beuzon et al., 2000; Brumell et al., 2001a). Similar patterns of staining were observed for SseF-M45 or SseG-M45 and LAMP-1 (Fig. 6), LAMP-2, LAMP-3 and V-ATPase (data not shown). Our observations indicate that translocated SseF-M45 and SseG-M45 are preferentially localized within endosomal compartments, in the membranes, or associated with the membranes, of these compartments. Interestingly, a recent study indicated a similar association with endosomal compartments for the translocated SPI 2 effector PipB (Knodler et al., 2002).

Fig. 4. Kinetics of translocation of SseF and SseG. RAW 264.7 cells were infected with S. Typhimurium wild type harbouring plasmids for the expression of SseF-M45 (filled symbols) or SseG-M45 (open symbols). At various time-points after infection, cells were fixed and processed for immunostaining of S. Typhimurium and M45-tagged effect or proteins. For each time-point, 100 infected cells were randomly selected and the proportion of infected host cells showing signals for translocated effector proteins was quantified. The mean values (± standard deviation) of three independent experiments are shown.

Fig. 5. Localization of translocated SseF-M45 and SseJ-M45. RAW 264.7 cells were infected with wild-type S. Typhimurium (A), or wild-type S. Typhimurium harbouring plasmid p2095 (sseF::M45) or p2129 (sseJ::M45) (B) or the ssaV strain harbouring p2095 or p2129 (C) and subjected to cell fractionation 16 h after infection as described in Experimental procedures. Fractions were subjected to SDS-PAGE and immunoblotting for the M45 epitope tag, a membrane fraction marker (gp91phox) or a cytosolic marker (β-tubulin).

SseF and SseG modulate the formation of Salmonella-induced filaments

Various reports have established that the function of the SPI 2 secretion system is required for the induction of SIF formation (Beuzon et al., 2000; Guy et al., 2000; Brumell et al., 2001a). Furthermore, Guy et al., 2000 observed that SIF formation is absent in strains harbouring mutations in sseF or sseG. We performed further analyses with mutant strains in sseF, sseG and a strain harbouring a deletion of sscBsseFG. Our observations confirm that the formation of filamentous aggregates of endosomes that are rich in lip such as LAMP-1 is largely reduced in epithelial cells infected with these mutant strains. However, we observed that certain filamentous aggregates are still formed in...
epithelial cells infected with the sseF or sseG mutant strains. These filamentous aggregates were readily detectable by staining for translocated effectors using confocal microscopy (Fig. 7), but difficult to detect by epifluorescence microscopy. In contrast to the appearance of SIF in cells infected with S. Typhimurium wild type, these structures are only poorly stained for LAMP-1, and a punctuate rather than continuous distribution of LAMP-1 was observed. Detection of SseJ-M45 (Fig. 7) and SseF-M45 (data not shown) indicated that translocated SPI 2 effectors are present in these filamentous structures and distributed continuously within the structures. The filamentous structures observed after infection with sseF or sseG mutant strains were termed ‘pseudo-SIF’.

The formation of SIF and pseudo-SIF structures was quantified in HeLa cells infected with wild-type S. Typhimurium, various mutant strains (Fig. 8). Host cells infected with wild-type Salmonella predominantly formed SIF. In accordance with a previous report (Beuzon et al., 2000), we found that neither SIF nor pseudo-SIF were formed by ssaV or sifA-deficient strains. Cells infected with sseF, sseG or sscBsseFG mutant strains formed SIF with highly reduced frequency, but about 35% of the infected cells showed formation of pseudo-SIF structures. Formation of SIF structures was fully restored in the sseF or sscBsseFG strains by complementation with a plasmid expressing sscBsseF::M45 or sscB:ssseFG::M45, respectively, indicating that epitope-tagged effectors have biological functions similar to the wild-type proteins.

Immunofluorescence analyses indicated the SIF structures formed in S. Typhimurium-infected HeLa cells contained LAMP-1, LAMP-2, LAMP-3 and V-ATPase, but were negative for cathepsin D (late endosome/lysosome marker), mannose-6-phosphate receptor, CD71 (transferrin receptor, surface marker), EEA1 (early endosomal antigen), LBPA (lyso-bisphosphatidic acid, lysosomal marker), or mitochondrial membranes as indicated by staining of infected HeLa cells with ‘Mitotracker’ (Molecular Probes). For a more detailed characterization of pseudo-SIF structures, we analysed the co-localization of various markers with translocated SseJ in the background of strains harbouring mutations in sseF, sseG or sscBsseFG. Under these conditions, the discontinuous, punctuated distribution observed for LAMP-1 was also observed for LAMP-2, LAMP-3 and V-ATPase. Immunostaining for the markers cathepsin D, mannose-6-phosphate receptor, CD71, LBPA did not reveal co-localization of these proteins with the pseudo-SIF structures (data not shown). We propose that pseudo-SIF also represent aggregates of endosomal vesicles, but that the composition of these vesicles is different to those of SIF formed in cells infected with wild-type Salmonella.

Discussion

The TTSS encoded by SPI 2 is required for the intracellular pathogenesis of Salmonella and mediates translocation of a set of effector proteins by intraphagosomal Salmonella into the host cell. SseF and SseG are two
Fig. 7. Effect of mutations in **sseF** and **sseG** on formation of *Salmonella*-induced filaments. *S. Typhimurium* wild type (WT), or mutant strains deficient in **sseF** or **sseG** harbouring plasmid p2129 for the expression of **sseJ::M45** were used for the infection of HeLa cells. At 16 h after infection, HeLa cells were fixed and immunostaining for *Salmonella*, the M45 tag and LAMP-1 was performed as described in the legend to Fig. 3. Violet staining indicates co-localization of translocated SseJ-M45 with LAMP-1. Arrows indicate representative patterns of *Salmonella*-induced filaments (SIF) in HeLa cells infected with *S. Typhimurium* wild type, or SIF-like structures, termed pseudo-SIF, in cells infected with **sseF** or **sseG** mutant strains. Pseudo-SIF are characterized by a punctuated distribution of LAMP-1 staining in contrast to SIF that exhibit a continuous distribution of LAMP-1 and SseJ-M45.
novel translocated effector proteins of the TTSS of SPI 2 and encoded by genes within the SPI 2 locus. In this study, we described the characteristics of the translocation of SseF and SseG, as well as of SseJ, a recently identified member of the STE family of SPI 2 effectors encoded by genes outside of the SPI 2 locus (Miao et al., 2000). We demonstrated that SseF and SseG are not required for the secretion and translocation of other substrate proteins of the TTSS of SPI 2, thus are not part of the translocator that is composed of SseBCD, other products of SPI 2 genes. We observed that SseF and SseG are translocated dependent on the function of the TTSS of SPI 2. These observations indicate that translocated effector proteins are also encoded by genes within the SPI 2 locus in addition to those encoded by genes on individual loci outside of SPI 2. Interestingly, after translocation, members of both groups are associated with endosomal membranes. In accordance with a previous study (Guy et al., 2000), we found that aggregation of endosomal compartments that are rich in lysosomal glycoproteins (Igp) referred to as SIF is reduced in sseF or sseG mutant strains. Closer examination revealed that formation of filamentous structures was induced by these mutant strains. However, the composition of these filaments appears to be different from those of the filamentous structures induced by wild-type Salmonella. A previous study (Brumell et al., 2001b) and the data reported here demonstrate that SIF induced by wild-type Salmonella predominantly show a continuous distribution of Igp. Less frequently, events of a discontinuous, or punctuate distribution of the markers were observed that we refer to as pseudo-SIF. Staining of translocated effectors in pseudo-SIF revealed that these structures are also filamentous aggregates that have a lower content of Igp. Despite the use of numerous host cell markers, we have not been able to detect characteristic marker proteins that are present in the pseudo-SIF structures. Double labelling for Igp and the translocated effector SseJ demonstrated that SIF formation is highly reduced in cells infected with sseF or sseG mutants, but that the frequency of pseudo-SIF formation is increased. Based on these observations we propose that SseF and SseG are translocated effectors that interact with endosomal compartments and are required for the modulation of endosome aggregation. Various effector proteins are translocated by intracellular Salmonella by means of the SPI 2-encoded TTSS. These effectors may have different contributions to the modulation of intracellular trafficking and the induction of endosomal aggregates. SifA appears to be absolutely essential for the induction of endosomal aggregates and maintenance of the SCV (Beuzon et al., 2000; Brumell et al., 2001a). The S. Typhimurium wild type and mutant strains deficient in sseF or sseG were enclosed by Igp-containing membranes to the same extent, indicating that SseF and SseG are not required for the maintenance of the SCV as observed for SifA and that their function is distinct from that of SifA (data not shown). However, our data indicate that SseF and SseG are further effectors that modulate the composition of the endosomal aggregates, for example by mediating the fusion of a subset of endosomes rich in Igp such as late endosomes and lysosomes. Further work has to reveal the nature of these compartments and the molecular mechanisms by which SseF and SseG modulate these fusion events.

The three SPI 2 effector proteins studied in this work are all targeted to endosomal compartments after translocation. A similar observation has recently been made for PipB, another effector protein of the SPI 2-encoded TTSS that is encoded by a gene within SPI 5 (Knodler et al., 2002). Furthermore, an association with Igp-containing endosomal compartment was also observed for SifA, another effector of the SPI 2 system. In a study by Beuzon et al., 2000, a SifA-GFP fusion protein was expressed from plasmids in transfected cells. However, the localization of SifA after translocation by intracellular Salmonella has not been reported.
also SifA, show a subcellular localization that is different from those of other effector proteins. The subcellular localizations of several effector proteins translocated by TTSS of various pathogens have been studied by immunofluorescence. Several effectors of the SPI 1 system are located in the cytoplasm of infected host cells and interact with cytosolic target proteins, as observed for SopE and SopE2 (Hardt et al., 1998; Stender et al., 2000). Furthermore, the translocation into the host cell cytoplasm was described for Yersinia spp. effectors YopE (Rosqvist et al., 1994), and YopH (Persson et al., 1999), whereas targeting of YopA (YopO) to the inner surface of the cytoplasmic membrane was observed after translocation (Hakansson et al., 1996). Targeting of translocated effector to the nucleus has been reported for Yersinia spp. YopM (Skryzypek et al., 1998) and Tir of EPEC is inserted into the host cell cytoplasmic membrane to form a receptor for intimin after translocation into the cytoplasm (Kenny et al., 1997). The preferential targeting to endosomal compartments appears to be a unique characteristic of SPI 2 effector proteins.

In summary, we have demonstrated that SPI 2-encoded SseF and SseG are translocated effector proteins that are associated with endosomal compartments. Endosomal aggregates induced by S. Typhimurium wild type and mutant strains in sseF or sseG have a different composition, indicating that SseF and SseG modulate the aggregation of host cell endosomes with SCV. Further work has to reveal the mechanisms by which these SPI 2 effectors interfere with normal vesicular trafficking in order to enable the intracellular survival and replication of S. Typhimurium.

### Experimental procedures

#### Bacterial strains and culture conditions

*S. enterica* serotype Typhimurium (S. Typhimurium) strain NCTC 12023 was used as wild-type strain throughout this study and for the construction of mutant strains. Strains used in this study are listed in Table 1. For infection experiments, bacterial strains were grown in Luria–Bertani (LB) broth containing 50 μg ml⁻¹ carbenicillin if required for the maintenance of plasmids. Bacterial cultures were grown in glass test tubes with agitation.

#### Strain construction

Plasmids were introduced into *S. Typhimurium* by electroporation or by P22 transduction according to standard procedure (Maloy et al., 1996). Strain MvP373 with a replacement of sscBsseFG by aph were constructed by the Red deletion technique (Datsenko and Wanner, 2000). Primers sscB-red-del (5' - CTGACGTAAATCATTATCACGTGAAAATAACAATCAATAGGTG - 3') and sseG-red-del (5' - CTTCAGCTGGAGCTGCTTC - 3') were used to amplify the aph gene from pKD4 (Datsenko and Wanner, 2000). The resulting PCR product was purified and used to transform *S. Typhimurium* to kanamycin resistance as previously described (Hansen-Wester and Hensel, 2002).

#### Protein secretion in vitro

Preparation and analyses of proteins secreted by the SPI 2-encoded TTSS in vitro was performed as described before (Nikolaus et al., 2001). Briefly, S. Typhimurium wild type and various mutant strains were grown with agitation for 16 h at 37°C in 200 ml PCN-P media adjusted to pH 5.8. Bacteria were pelleted by centrifugation and the culture supernatant was passed through a filter membrane (0.2 μm pore size) to remove residual bacteria. Protein secreted into the culture media was concentrated by precipitation with 10% TCA. Secreted protein located on the bacterial cell surface was detected by vigorous mixing of a concentrated bacterial suspension and concentrated by TCA precipitation.

#### Cell culture

The murine monocyte cell line RAW 264.7 (passage numbers 4–20) and the human epithelial cell line HeLa (passage number 14–20) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, antibiotics, and glucose.

| Strain or plasmid | Relevant characteristics | Reference |
|-------------------|--------------------------|-----------|
| **S. enterica strains** | | |
| *S. Typhimurium* NCTC 12023 | wild type | identical to ATCC 14028 |
| P3B12 | invG-mTn5 | (Deiwick et al., 1998) |
| P2D6 | ssaV-mTn5 | (Shea et al., 1996) |
| P3H6 | sifA-mTn5 | (Beuzon et al., 2000) |
| HH104 | sseC-aphT | (Hensel et al., 1998) |
| HH107 | δsseF-aphT | (Hensel et al., 1998) |
| HH108 | δsseG-aphT | (Hensel et al., 1998) |
| NP:ssaV | ssaV-aphT | (Deiwick et al., 1998) |
| MvP373 | δsscB sseFG::aph | this study |
| **Plasmids** | | |
| p2095 | Pro_{ssa} sscB sseFG::M45 in pWSK29 | (Hansen-Wester et al., 2002) |
| p2096 | Pro_{ssa} sscB sseFG::M45 in pWSK29 | (Hansen-Wester et al., 2002) |
| p2129 | Pro_{ssa} sseJ::M45 in pWSK29 | (Hansen-Wester et al., 2002) |
were cultured in Dulbecco’s modified Eagle medium (DMEM, Invitrogen) containing 10% heat-inactivated fetal calf serum (FCS, Invitrogen) and 2 mM glutamine at 37°C in an atmosphere of 5% CO₂. Cells were cultured in the absence of antibiotics.

**Bacterial infection of macrophages and survival assay**

For infection of RAW 264.7 cells, *S. Typhimurium* strains were grown to stationary phase. The OD₆₀₀ of the cultures was adjusted with LB to 0.2 and the bacteria were washed once with phosphate-buffered saline (PBS). They were diluted in DMEM containing FCS and glutamine and added to the cells seeded in 24-well tissue culture plates at a multiplicity of infection of about 1. The bacteria were centrifuged onto the cells at 500 g for 5 min and incubated for 25 min at 37°C in an atmosphere of 5% CO₂. After infection, the macrophages were washed three times with PBS and incubated for 1 h in medium containing FCS, glutamine and 10 μg ml⁻¹ gentamicin (Sigma). The medium was replaced with medium containing FCS, glutamine and 10 μg ml⁻¹ gentamicin for the remainder of the experiment.

For enumeration of intracellular bacteria, macrophages were washed three times with PBS and lysed with 1% Triton X-100 for 10 min at RT, and serial dilutions were plated on Muller-Hinton agar plates.

**Bacterial infection of HeLa cells**

For infection of HeLa cells, *S. Typhimurium* strains were grown at 37°C with agitation to stationary phase. The cultures were then diluted 1:30 with fresh LB broth and incubated for further 3.5 h at 37°C with agitation to reach late logarithmic phase. The OD₆₀₀ of the cultures was adjusted with LB to 0.2 and the bacteria were washed once with PBS. Subsequently, the inocula were diluted in DMEM containing FCS and glutamine and added to the epithelial cells at a multiplicity of infection (MOI) of about five. The bacteria were centrifuged onto the cells at 500 g for 5 min, incubated for 25 min at 37°C in an atmosphere of 5% CO₂ and further processed as described for infection of macrophages.

**Subcellular fractionation of infected RAW 264.7 cells**

RAW 264.7 cells were seeded 24 h before infection in 80 cm² flasks at a density of ~1.3 x 10⁷ cells/flask and infected at an MOI of 10 as described above. Sixteen hours after infection cells were washed twice with PBS, scraped into 2 ml of cold 50 mM Tris-HCl, pH 7.5 containing 0.2% saponin and 5% protease inhibitor cocktail (20 mM AEBSF, 10 mM EDTA, 1.3 mM bestatin, 140 μM E-64, 10 μM leupeptin, 3 μM aprotinin, Sigma) and incubated for 5–10 min on ice. The lysate was centrifuged at 4°C for 5 min at 16 000 g, and the supernatant representing the cytosolic fraction was transferred to a new tube. The pellet was washed twice with cold PBS, resuspended in 2 ml of cold 50 mM Tris-HCl, pH 7.5 containing 0.1% Triton X-100 and 5% protease inhibitor cocktail and incubated for 15 min on ice. The suspension was centrifuged at 4°C for 5 min at 16 000 g, and the supernatant representing the membrane fraction was transferred to a new tube. The pellet containing the debris fraction was washed twice with cold PBS.

Proteins in the cytosolic and membrane fractions were precipitated with 10% TCA for at least 1 h on ice, collected by centrifugation at 4°C for 10 min at 20 000 g and washed twice with PBS. Proteins of the various fractions were dissolved in appropriate volumes of SDS-PAGE sample buffer and boiled for 10 min. If required, samples of the debris fraction were centrifuged for 15 min at 11 340 g to remove DNA.

**SDS-PAGE and Western blot**

Samples obtained from *in vitro* secretion experiments or cell fractionation experiments were separated by SDS-PAGE on 10 or 12% tricine gels according to Schägger and Jagow (1987). Subsequently, proteins were transferred onto 0.45 μm nitrocellulose membranes (Schleicher and Schuell) by semi-dry transfer and membranes were blocked with 5% BSA in TBS-T at 4°C overnight. M45-tagged proteins were detected by incubation with the anti-M45 antibody at a dilution of 1:100 in 0.5% BSA in TBS-T for 2 h. For detection, anti-mouse-HRP (Amersham) was used at a dilution of 1:10 000 followed by chemiluminescent detection using the ECL system (Amersham).

**Immunofluorescence**

For immunofluorescence analyses, the cells were grown in 24-well tissue culture plates on glass coverslips. After infection and incubation for different time periods, the cells were fixed with 3% para-formaldehyde in PBS for 15 min at room temperature and then washed three times with PBS. The antibodies were diluted in a blocking solution consisting of 10% goat normal serum, 1% bovine serum albumin (BSA) and 0.1% saponin (Sigma) in PBS. The coverslips were incubated with various antibodies as detailed below, and washed three times with PBS after each incubation step. The coverslips were mounted on Fluoprep (bioMérieux) and sealed with Entellan (Merck). Samples were analysed using a confocal laser scanning microscope (Leica TCS-NT).

**Antibodies**

If not otherwise stated, all antibody incubations were performed for 1 h at room temperature. Monoclonal antibodies against the M45 epitope of the adenoviral E4-6/7 protein (Obert et al., 1994) were used for detection of translocated fusion proteins. For immunostaining, samples were incubated with hybridoma culture supernatants at dilutions of 1:2 to 1:10 for 3 h at RT. The αM45 antibodies were detected using a goat anti-mouse Cy5 conjugate (Jackson) in a dilution of 1:200.

The S. Typhimurium was detected using rabbit α*Salmonella* O4-test sera (Difco) (dilution 1:1000) as primary antibody and goat anti-rabbit Cy2 conjugate (Jackson, dilution 1:1000). Rabbit anti Sendai recombinant SeeB have been described (Beuzon et al., 1999).

Host cell proteins were detected with rat anti-murine LAMP-1 (DHB, H4A3, dilution 1:1000), mouse anti-human LAMP-1 (DHB, H4A3, dilution 1:1000), mouse anti-human LAMP-2 (DHB, H4B4, dilution 1:1000), mouse anti-human LAMP-3 (DHB, H5C6, dilution 1:1000) or mouse anti-human V-ATPase (kindly provided by Dr Sato, Kyoto, Japan, dilution 1:2000), mouse anti-human transferrin receptor (Roche, dilution 1:500), mouse anti-human mannose-6-phosphate receptor (ABR, dilution 1:50), rabbit anti-EEA1 (ABR, dilution 1:1000), rabbit anti-

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human cathepsin D (Oncogene, dilution 1 : 100), mouse anti-LBPA (kindly provided by Dr J. Gruenberg, Geneva, dilution 1 : 1000) as primary antibodies. For cathepsin D staining, cells were fixed with ice-cold methanol for 10 min and antibody was diluted in blocking solution without saponin. For Western blotting, mouse anti-gp91phox (BD Bioscience, dilution 1 : 10 000) and mouse anti-β-tubulin (DSHB, E7, dilution 1 : 1000) were used as primary antibodies. Goat anti-rat Cy3 conjugate (Jackson), goat anti-rabbit Cy3 conjugate (Jackson), or goat anti-mouse Cy3 conjugate (Jackson) were used as secondary antibodies at a dilution of 1 : 1000.

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