SseA is a chaperone for the SseB and SseD translocon components of the *Salmonella* pathogenicity-island-2-encoded type III secretion system

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The type III secretion system (TTSS) encoded by the *Salmonella* pathogenicity island 2 (SPI-2) is required for bacterial replication inside macrophages and for systemic infection in mice. Many TTSS secreted proteins, including effectors and components of the translocon, require chaperones which promote their stability, prevent their premature interactions or facilitate their secretion. In this study, the function of the first gene (*sseA*) of one of the SPI-2 operons (*sseA–G*) was investigated. This operon includes genes that encode translocon components (SseB, SseC and SseD), translocated proteins (SseF and SseG) and putative chaperones (SscA and SscB). *sseA* encodes a 12·5 kDa protein with a C-terminal region with the potential to form a coiled-coil structure, but no sequence similarity to other proteins. Mutation of *sseA* results in severe virulence attenuation and an intracellular replication defect. It is shown here that SseA is not a secreted protein, but is required for SPI-2-dependent translocation of two effector proteins (SifA and PipB). Furthermore, the translocon components SseB and SseD were not detected in an *sseA* mutant strain. By using a yeast two-hybrid assay and column binding experiments, it is demonstrated that SseA interacts directly with SseB and SseD. These results indicate that SseA is a chaperone for SseB and SseD. The inability of an *sseA* mutant to assemble the SPI-2 TTSS translocon accounts for its high level of virulence attenuation *in vivo*. To the authors' knowledge, this is the first chaperone described for the SPI-2 TTSS.

INTRODUCTION

Type III secretion systems (TTSSs) are specialized protein delivery systems found in many Gram-negative pathogenic bacteria. They are responsible for the secretion and translocation of effector proteins from bacteria into the host cell cytosol, where they subvert a variety of different cellular processes. Type III secretion typically involves a secreton for exporting proteins from the bacterial cell, a translocon for transferring effector proteins into host cells, various regulators that control gene transcription and protein secretion, and chaperones (Hueck, 1998). Chaperones have been identified for both effector proteins and translocon components. They are necessary to prevent degradation and promote secretion of effector proteins, and to prevent premature degradation of, and interaction between, translocon components. Generally, chaperones of the effectors specifically interact with one substrate, while chaperones of the translocators associate with two substrates (Page & Parsot, 2002). The genes encoding these chaperones are often located in the vicinity of the genes encoding their substrates (Page & Parsot, 2002). Chaperones of the TTSSs have common physiochemical characteristics, including low molecular masses (<15 kDa) and predicted amphipathic helices near their C termini (Page & Parsot, 2002).

Salmonella enterica serovar Typhimurium (*S. typhimurium*) has two TTSSs, encoded by *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2, respectively). The SPI-1 TTSS is required for cell invasion, and it induces significant intestinal secretory and inflammatory responses (Galan, 2001; Wallis & Galyov, 2000). The SPI-2 TTSS is required for intracellular replication and systemic infection (Cirillo *et al.*, 1998; Hensel *et al.*, 1995, 1998; Ochman *et al.*, 1996; Shea *et al.*, 1996).

Abbreviations: CI, competitive index; HA, haemagglutinin; Sif, *Salmonella*-induced filament; SPI-2, *Salmonella* pathogenicity island 2; *S., typhimurium Salmonella enterica* serovar Typhimurium; TTSS, type III secretion system.

On the basis of similarities of sequence and organization with genes of other bacterial pathogens, several genes located in a 9 kb region within SPI-2 (operon *sseA–G*) were initially proposed to encode secreted proteins (Hensel *et al.*, 1998). SseB, SseC and SseD are encoded by this operon and have been shown to be secreted *in vitro* onto the bacterial surface, where they remain associated, and to be necessary for translocation of effector proteins into the host cell (Beuzón *et al.*, 1999; Klein & Jones, 2001; Nikolaus *et al.*, 2001). Strains carrying mutations in *sseB*, *sseC* or *sseD* are as attenuated as those that are completely defective for secretion, and display the same intracellular phenotypes (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Klein & Jones, 2001; Yu *et al.*, 2002). SscA and SscB have been proposed to be chaperones on the basis of their similarities to the SycD and IpgC chaperones from *Yersinia* spp. and *Shigella flexneri*, respectively (Cirillo *et al.*, 1998; Hensel *et al.*, 1998). Mutation of *sseE* does not lead to a noticeable attenuation of virulence (Hensel *et al.*, 1998); SseF and SseG have been shown to be translocated into the host cell (Hansen-Wester *et al.*, 2002; Kuhle & Hensel, 2002). Strains carrying mutations in *sseF* or *sseG* are mildly attenuated and partially defective in the formation of tubular membranous structures known as *Salmonella*-induced filaments (Sifs), which are induced upon SPI-2-mediated translocation of the effector protein SifA into HeLa cells (Beuzón *et al.*, 2000; Brumell *et al.*, 2002; Garcia-del Portillo *et al.*, 1993; Guy *et al.*, 2000; Kuhle & Hensel, 2002; Stein *et al.*, 1996). The first gene of the operon encodes SseA, a small protein that contains a domain with potential to form a coiled-coil structure, but which has no significant sequence similarity to other proteins (Hensel *et al.*, 1998). An *sseA* mutant strain is strongly attenuated in virulence and severely defective in intracellular replication (Hensel *et al.*, 1998).

In this study, we have investigated the function of SseA. Unlike other proteins encoded within the same SPI-2 operon, SseA does not appear to be secreted, but is required for SPI-2-dependent translocation of two different effector proteins (SifA and PipB). Furthermore, the SPI-2 TTSS translocon components SseB and SseD were not detected in an *sseA* mutant strain, although the intracellular levels of SseC remained unaffected. We show that SseA binds to SseB and SseD. These results, together with the predicted physiochemical properties of SseA, indicate that this protein is a chaperone for SseB and SseD. Therefore, the inability of an *sseA* mutant to correctly assemble the SPI-2 TTSS translocon explains its high level of virulence attenuation *in vivo*.

METHODS

Bacterial strains and growth conditions. The *S. typhimurium* strains used in this study are listed in Table 1. Bacteria were grown in Luria–Bertani (LB) medium supplemented with carbenicillin (100 µg ml^{-1}), or chloramphenicol (35 µg ml^{-1}), as appropriate. To induce SPI-2 gene expression and SPI-2-dependent secretion, bacteria were grown in magnesium minimal medium MES (MgM/MES), containing 170 mM 2-(*N*-morpholino)ethanesulphonic

Table 1. *S. typhimurium* strains used in this study

Strain	Description*	Source/reference
12023	Wild-type	NCTC†
HH100	Δ*sseA*::*aphT* (Km^r)	Hensel *et al.* (1998)
HH102	Δ*sseB*::*aphT* (Km^r)	Hensel *et al.* (1998)
HH109	*ssaV*::*aphT* (Km^r)	Deiwick *et al.* (1999)
HH205	Δ*sseD*::*aphT* (Km^r)	Yu *et al.* (2002)

*Km^r, Kanamycin-resistant.
†National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London, UK.

acid at pH 5·0, 5 mM KCl, 7·5 mM (NH$_4$)$_2$SO$_4$, 0·5 mM K$_2$SO$_4$, 1 mM KH$_2$PO$_4$, 8 µM MgCl$_2$, 38 mM glycerol and 0·1 % Casamino acids (Beuzón *et al.*, 1999) with the corresponding antibiotics when appropriate. Bacteria were grown at 37 °C overnight with aeration.

Plasmids. The complementing plasmid p*sseA* (Hensel *et al.*, 1998) is a derivative of pACYC184 (Chang & Cohen, 1978) carrying the *sseA* gene under the control of a constitutive promoter.

The complementing plasmid p*sseBD* is a derivative of the complementing plasmid p*sseB* (Hensel *et al.*, 1998), carrying the *sseB* and *sseD* genes under the control of a constitutive promoter. A DNA fragment including the complete ORF of *sseD* with its corresponding ribosome-binding site was amplified by PCR from 12023 genomic DNA using primers SSED-F (5′-ATAGCATGCATAGTAGCGTTCAGGCGAG-3′) and SSED-R (5′-ATAGTCGACTTACCTCGTTAATGCCCGG-3′). The 640 bp PCR product, containing terminal *Sph*I and *Sal*I sites, was digested and ligated into the corresponding sites of p*sseB*, generating p*sseBD*.

Plasmid pACB C-2HA (Knodler *et al.*, 2002), a derivative of pACYC184 (Chang & Cohen, 1978) encoding an haemagglutinin (HA)-tagged version of PipB, was a kind gift from L. Knodler (Rocky Mountain Laboratories, Hamilton, MT, USA). Plasmid pFVP25.1, carrying *gfp–mut3A* under the control of the *rpsM* constitutive promoter (Valdivia & Falkow, 1996), was introduced into bacterial strains and used for fluorescence visualization where indicated.

Preparation of cell fractions. Bacterial cell densities were determined by measurement of the OD$_{600}$ value. To ensure that protein from equal numbers of cells was analysed, in all experiments protein samples were adjusted to OD$_{600}$ values such that a volume corresponding to 10 ml of a culture of OD$_{600}$ 0·6 was taken up in 100 µl of protein denaturing buffer for gel electrophoresis. Cell cultures were cooled on ice, centrifuged at 35 000 *g* for 5 min at 4 °C, and the proteins present in the supernatant were collected by trichloroacetic acid precipitation (culture supernatant). After removal of the culture medium, the cell pellets were washed with ice-cold PBS and resuspended in 0·3 ml of PBS. The suspensions were mixed gently with 0·2 ml n-hexadecane for 5 min at room temperature and centrifuged at 10 000 *g* for 10 min at room temperature (Michiels *et al.*, 1990). The organic layer was discarded, the aqueous layer mixed with 1·2 ml acetone and held at −20 °C to form a precipitate, and the cell pellet was stored at −70 °C before analysis (bacterial pellet). The acetone precipitate was centrifuged, dried and stored at −70 °C before analysis (hexadecane fraction). All fractions were dissolved in protein denaturing buffer before PAGE.

PAGE and Western analysis of proteins. Protein fractions were dissolved in the appropriate volume of protein denaturing buffer

(Beuzón *et al.*, 1999) and held at 100 °C for 5 min. Proteins were immediately separated on 12 % SDS-polyacrylamide gels (Laemmli, 1970). For Western analysis, gels were transferred to Immobilon-P membranes (Millipore) and examined using the ECL detection system under conditions recommended by the manufacturer (Amersham Life Science). Incubation of membranes with primary antibodies (rabbit anti-SseA, anti-SseB, anti-SseC, anti-SseD) was followed by incubation with horseradish peroxidase-conjugated anti-rabbit as secondary antibody.

Recombinant SseA protein purification and generation of anti-SseA polyclonal antibody. For the expression of recombinant SseA, a DNA fragment including the complete ORF of *sseA* minus the start codon was amplified by PCR from 12023 genomic DNA, using primers SSEA-T72 (5′-GGATCCATGATAAAGAA-AAAGCTGCGT-3′) and SSEA-T73 (5′-GGATCCTTACCTTTTT-GTTTTTCCTGACGG-3′). The 350 bp PCR product, containing terminal *Bam*HI sites, was digested and ligated into vector pET-15b (Novagen), generating pID834. This plasmid expresses a full-length version of SseA bearing an N-terminal hexahistidine-tag (His₆SseA), under the control of a T7 promoter. Plasmid pID834 was introduced into *Escherichia coli* strain BL21 (DE3), and SseA was expressed following standard protocols (Sambrook & Russell, 2001). For antibody production, the 14·3 kDa recombinant protein was purified under denaturing conditions by metal-chelating chromatography using the QIAexpress Protein Purification System according to the instructions of the manufacturer (Qiagen) and used as an antigen for rabbit immunization (Abcam). Antibody titres were checked by Western blot using total cell fractions of *E. coli* strain BL21 (DE3) expressing SseA.

Affinity purification of the anti-SseA antibody. For affinity purification of the anti-SseA antiserum, purified His₆SseA (20 mg ml⁻¹) was coupled to water-activated Affi-Gel 10 (Bio-Rad) in the presence of 0·1 M MOPS, pH 7·5, and 0·3 M NaCl. The slurry was then centrifuged at 400 ***g*** for 5 min at 4 °C. After removing the supernatant, the remaining reactive esters were blocked by addition of Tris/HCl to a final concentration of 50 mM at pH 7·2. It was then washed twice with 0·1 M MOPS, pH 7·5/0·3 M NaCl, and twice with elution buffer (0·1 M glycine, pH 2·5). The washed slurry was neutralized by succesive washings with 50 mM Tris/HCl (50 mM), pH 7·2, until neutral pH was reached. Anti-SseA serum (1·8 ml) and 0·2 ml of 10× TBS (120 mM Tris/HCl, pH 8·0/1·5 M NaCl) were added to the resin and incubated overnight with gentle shaking at 4 °C. The slurry was then poured into an Econo-Column chromatography column support (Bio-Rad), and both the flow-through and the washes with 10 ml of 1× TBS were collected. The purified antibody was eluted with 0·1 M glycine/HCl, pH 2·5, and neutralized by addition of 1 M Tris/HCl, pH 8·0. The affinity-purified rabbit polyclonal anti-SseA antibody was used for Western analysis at a dilution of 1:5000.

Column binding assay. The ability of Ni²⁺–NTA column-immobilized His₆SseA to bind SseB was determined following the method described by Elliott *et al.* (1999). Briefly, His₆SseA was expressed from pID834 following standard procedures (Sambrook & Russell, 2001). The bacterial pellet was resuspended in buffer containing 300 mM NaCl and 50 mM NaH₂PO₄ (pH 8·0) (column buffer) supplemented with 10 mM imidazole, and lysed by sonication. The lysate was cleared by centrifugation and incubated at 4 °C for 1 h with 1 ml of Ni²⁺–NTA beads (Pierce) with gentle mixing. The suspension was then poured into a column, the flow-through was collected and re-applied twice more, followed by three washes with 4 ml of column buffer supplemented with 20 mM imidazole. Wild-type *Salmonella*-secreted proteins corresponding to the bacterial surface protein fraction (hexadecane fraction, as described above) were concentrated from 100 ml of culture grown in minimal medium at low pH, resuspended in column buffer supplemented

with 20 mM imidazole, and passed three times through the packed column. The column was then washed three times with 4 ml of column buffer supplemented with 20 mM imidazole. Bound proteins, including the excess of His₆SseA bound to the column and any other proteins interacting with it, were eluted in 0·5 ml aliquots with column buffer supplemented with 250 mM imidazole. The resulting samples were examined by Western blotting.

Yeast two-hybrid system. The yeast two-hybrid host used in this study was PJ69-4A (*MAT***a** *trp1-901 leu2-3112 ura3-52 his3-200 gal4D gal80D LYS2*::*GAL1–HIS3 GAL2–ADE1 met2*::*GAL7–lacZ*), which has three independent reporter genes under the control of three different GAL promoters (James *et al.*, 1996). A DNA fragment encoding SseA was amplified by PCR from wild-type *S. typhimurium* strain 12023 genomic DNA using the primers SSEAY-F (5′-ATC-GAATTCATAAAGAAAAAGGCTGCG-3′) and SSEAY-R (5′-ATCC-TCGAGTTACCTTTTTGTTTTTCC-3′). The PCR product, containing terminal *Eco*RI and *Xho*I sites, was digested and ligated into the *Eco*RI and *Sal*I sites of the ADH-1-driven fusion vector pGBT9. Likewise, a DNA fragment encoding SseD was amplified by PCR from 12023 genomic DNA using the primers SSEDY-F (5′-ATCGAATTCGAAGCGAGTAACGTAGCAC-3′) and SSEDY-R (5′-ATCCTCGAGTTACCTCGTTAATGCCCG-3′), and ligated into the vector pGAD424. Constructs were co-transformed into PJ69-4A, and analysis of the transformants by growth of yeast on minimal medium to monitor both *HIS3* and *ADE1* reporter gene activation was performed as described previously (Hartland *et al.*, 2000). To measure the induction of the *lacZ* reporter gene, β-galactosidase assays of yeast strains grown in liquid culture were performed as described by Francis *et al.* (2000). The values shown in Results and Discussion for the β-galactosidase assays represent the mean±SD (Miller units) corresponding to three measurements for each of three independent cultures assayed. The host strain PJ69-4A was co-transformed with a pGAD424 empty vector and the pGBT vector expressing the SseA fusion protein, and the resulting transformants were used as a negative control for both assays. The yeast two-hybrid host strain and the fusion vectors were kindly provided by G. Frankel (Centre for Molecular Microbiology and Infection, London, UK).

Bacterial infection of HeLa cells. HeLa (clone HtTA1) cells were kindly provided by S. Méresse (Centre d'Immunologie de Marseille-Luminy, Marseille, France). Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and 2 mM glutamine at 37 °C in 5 % CO₂. HeLa cells were seeded onto glass coverslips (12 mm diameter) in 24-well plates at a density of 5×10⁵ cells per well, 24 h before infection. Bacteria were grown in LB broth for 16 h at 37 °C with shaking, diluted 1:33 in fresh LB broth and grown in the same conditions for 3·5 h. The cultures were diluted in Earle's buffered salt solution, pH 7·4, and added to the cells at an m.o.i. of approximately 100:1. The infection was allowed to proceed for 15 min at 37 °C in 5 % CO₂. The monolayers were washed once with DMEM containing FCS and 100 μg gentamicin ml⁻¹ and incubated in this medium for 1 h, after which the gentamicin concentration was decreased to 16 μg ml⁻¹. Cell monolayers were fixed at 8 h after invasion, in 3·7 % paraformaldehyde in PBS, pH 7·4, for 15 min at room temperature and then washed three times in PBS.

Antibodies and reagents. The rabbit polyclonal anti-SseA (this work), anti-SseB (Beuzón *et al.*, 1999), anti-SseC and anti-SseD (Nikolaus *et al.*, 2001) antibodies were used at a dilution of 1:10 000. The mouse mAb anti-LAMP-1 H4A3 developed by J. T. August and J. E. K. Hildreth was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa (Department of Biological Sciences, Iowa, IA, USA), and was used at a dilution of 1:2000. Anti-*Salmonella* goat polyclonal antibody CSA-1 was

purchased from Kirkegaard and Perry Laboratories (Gaithensburg, MD) and was used at a dilution of 1 : 400. The mouse mAb anti-HA 16B12 was purchased from Covance (Berkeley Antibody Company) and was used at a dilution of 1 : 1000. Texas red sulfonyl chloride-conjugated donkey anti-mouse and FITC-conjugated donkey anti-goat antibodies were purchased from Jackson Immunoresearch Laboratories and were used at a dilution of 1 : 400. Horseradish peroxidase-conjugated anti-rabbit antibody was purchased from Amersham Life Sciences and was used at a dilution of 1 : 20 000.

Virulence tests. Female BALB/c mice (20–25 g) were inoculated intraperitoneally with a 0·2 ml volume of physiological saline containing 10^5 bacteria. Bacteria were grown overnight at 37 °C in LB medium with aeration (150 r.p.m.), diluted into fresh medium (1 : 100) and grown until an OD_{550} value of between 0·35 and 0·6 was reached. Cultures were then diluted in physiological saline to a concentration of $2·5 \times 10^5$ bacteria per strain per ml, and thoroughly mixed before the infection (input). The number of colony-forming units (c.f.u.) of each strain in the input was enumerated by plating a dilution series of the inoculum and using the appropriate antibiotic to discriminate between the strains. Mice were killed 48 h after inoculation; their spleens were then removed, placed in sterile water and homogenized by mechanical disruption. Bacteria were pelleted by centrifugation at 13 000 g and resuspended in sterile water. Bacterial c.f.u. were enumerated by plating a dilution series onto LB agar and LB agar with the corresponding antibiotic. The competitive index (CI) was calculated as the ratio between the mutant and wild-type strain within the output (bacteria recovered from the host after infection), divided by their ratio within the input (Freter et al., 1981; Taylor et al., 1987).

RESULTS AND DISCUSSION

SseA is not secreted in SPI-2 *in vitro* inducing conditions

In an effort to analyse SseA expression and localization, a recombinant hexahistidine-tagged SseA protein was expressed in *E. coli*, purified and used to raise an anti-SseA antibody. The resulting antibody, when affinity-purified, was capable of detecting SseA expressed from the chromosome in the wild-type *Salmonella* strain 12023. The presence or absence of SseA was then examined by immunoblot analysis both in the wild-type and *sseA* mutant strains, in different culture fractions, after growth in MgM/MES at low pH, conditions that induce SPI-2-dependent secretion (Beuzón et al., 1999; Hansen-Wester et al., 2002; Klein & Jones, 2001). Each culture was fractionated into three samples corresponding to (a) the bacterial pellet, containing all non-secreted proteins, (b) the bacterial surface protein fraction (hexadecane fraction), where several SPI-2-secreted proteins are found, including the translocon components SseB, SseC and SseD (Beuzón et al., 1999; Hansen-Wester et al., 2002; Nikolaus et al., 2001), and (c) culture supernatant, where translocated effectors are also found (Hansen-Wester et al., 2002).

After growth at low pH to induce SPI-2-mediated secretion, SseA was detected in the bacterial pellets of the wild-type strain and the *sseA* mutant strain carrying a plasmid expressing SseA constitutively. However, it was not detected in the bacterial surface protein fraction or in the culture supernatant of either strain (Fig. 1a). In the same conditions, SseB was found on the bacterial surface of the wild-type strain, as expected (Fig. 1b). These results indicate that SseA is not a SPI-2 secreted protein.

SseA is required for Sif formation and PipB translocation

To determine whether SseA is required for translocation of SPI-2 effectors, we analysed by confocal immunofluorescence microscopy the intracellular behaviour of the *sseA* mutant strain after infection of HeLa epithelial cells. As detected by LAMP-1 labelling, the *sseA* mutant strain remained within vacuoles, but was completely deficient for the formation of Sifs. The ability to produce Sifs was restored in an *sseA* mutant strain harbouring a plasmid expressing SseA constitutively (Fig. 1c). These results suggested that the *sseA* mutant might be defective for SPI-2-mediated translocation of effectors.

We therefore examined the requirement for SseA in the translocation of an HA-tagged version of the SPI-2 effector protein PipB (Knodler et al., 2002). This protein has been shown to be translocated specifically through the SPI-2 TTSS and to be localized in the vacuolar membrane and in Sifs (Knodler et al., 2002). HeLa cells were infected with wild-type, *ssaV* mutant (a SPI-2 null mutant; Beuzón et al., 1999) or *sseA* mutant strains, each carrying a plasmid expressing the HA-tagged version of PipB, and analysed by confocal immunofluorescence microscopy using an anti-HA antibody. Translocated PipB was detected in Sifs and on the membrane of the *Salmonella*-containing vacuole when expressed in the wild-type strain, but not when expressed in either the *ssaV* or *sseA* mutant strains (Fig. 2). These results indicate that SseA is required for efficient SPI-2-dependent translocation of effectors.

Effect of the *sseA* mutation on intracellular levels of SseB, SseC and SseD

SseA has no sequence similarity with other TTSS components, but is relatively small (12·5 kDa) and has a C-terminal region predicted to form a coiled-coil structure (Hensel et al., 1998). These are all characteristics of TTSS chaperones (Page & Parsot, 2002). For these reasons, we investigated a possible role of SseA as a chaperone for the translocon components SseB, SseC and SseD, which are encoded immediately downstream of *sseA* within the same operon (Cirillo et al., 1998).

The intracellular levels of SseB, SseC and SseD were examined by immunoblot analysis using specific polyclonal antibodies, in the wild-type and the *sseA* mutant strains. As expected, the three proteins were readily detected in the wild-type strain (Fig. 3a, b, c). By contrast, SseB and SseD were not detected in the *sseA* mutant strain, while the levels of SseC remained unaffected (Fig. 3a, b, c). The presence of intracellular SseB and SseD was partially restored in an *sseA*
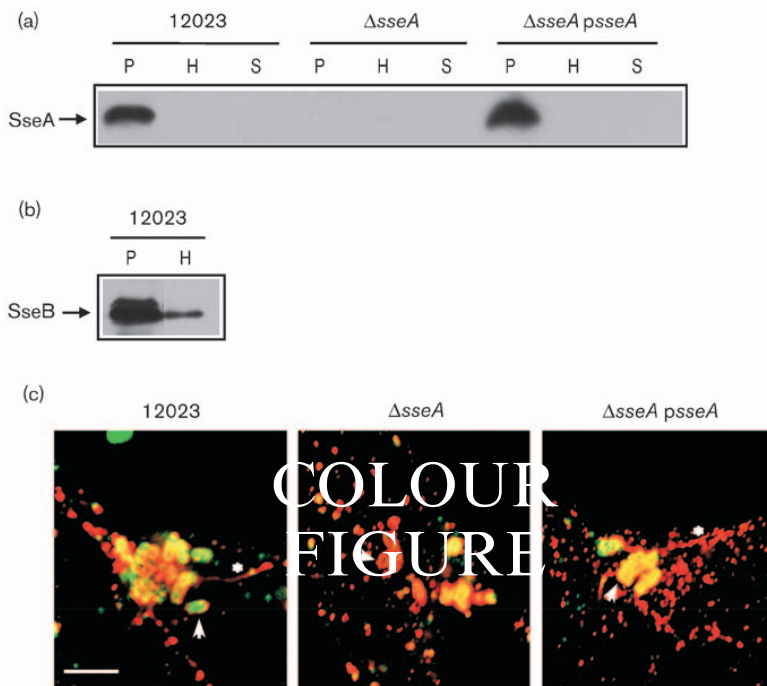
Fig. 1. (a, b) Subcellular localization of SseA in SPI-2-inducing conditions. *S. typhimurium* wild-type (12023), *sseA* mutant (Δ*sseA*) or *sseA* mutant carrying the complementing plasmid (Δ*sseA* p*sseA*) were cultured in MgM/MES at pH 5·0, and pellet (P), bacterial cell surface (H) and culture supernatant (S) fractions were analysed by immunoblotting with the anti-SseA (a) or anti-SseB (b) antibodies. (c) *S. typhimurium* bacteria carrying a mutation in *sseA* are completely deficient for the formation of Sifs, but remain enclosed within a vacuole. HeLa cells were infected with wild-type (12023), *sseA* mutant (Δ*sseA*) strains or the *sseA* mutant strain carrying the complementing plasmid (Δ*sseA* p*sseA*). Cells were fixed 8 h after bacterial invasion and examined by confocal fluorescence microscopy. Sifs (marked with asterisks) and vacuolar membranes (marked with arrows) were labelled with an anti-LAMP-1 antibody (red), while bacteria expressed green fluorescent protein constitutively (green). Bar, 5 μm.

mutant strain carrying a plasmid constitutively expressing SseA (p*sseA*), and both proteins were found at levels similar to those of the wild-type when the *sseA* mutant strain carried a plasmid expressing SseB and SseD simultaneously (p*sseBD*) (Fig. 3a, b).

However, SseB and SseD were not detected by immunoblot analysis of the corresponding bacterial surface protein fractions (hexadecane fraction) in the *sseA* mutant strain carrying either p*sseA* or p*sseBD* (Fig. 3a, b). In contrast, SseB and SseD were readily detected on the hexadecane fraction
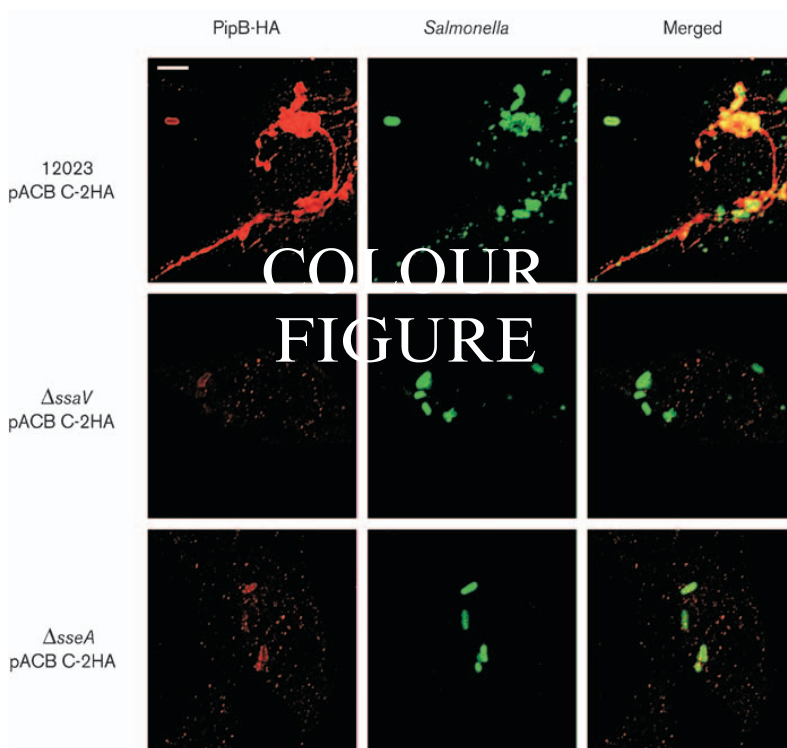

Fig. 2. SseA is required for efficient SPI-2-dependent translocation of PipB. HeLa cells were infected with wild-type (12023), *sseA* mutant (Δ*sseA*) or SPI-2 null mutant (Δ*ssaV*) strains, all carrying plasmid pACB C-2HA expressing an HA-tagged version of PipB. Cells were fixed 8 h after bacterial invasion and examined by confocal fluorescence microscopy. HA-tagged PipB was detected with an anti-HA antibody (red), while bacteria were detected with an anti-*Salmonella* antibody (green). HA-tagged PipB was detected in Sifs and on the vacuolar membrane only when expressed in wild-type bacteria. Bar, 5 μm.
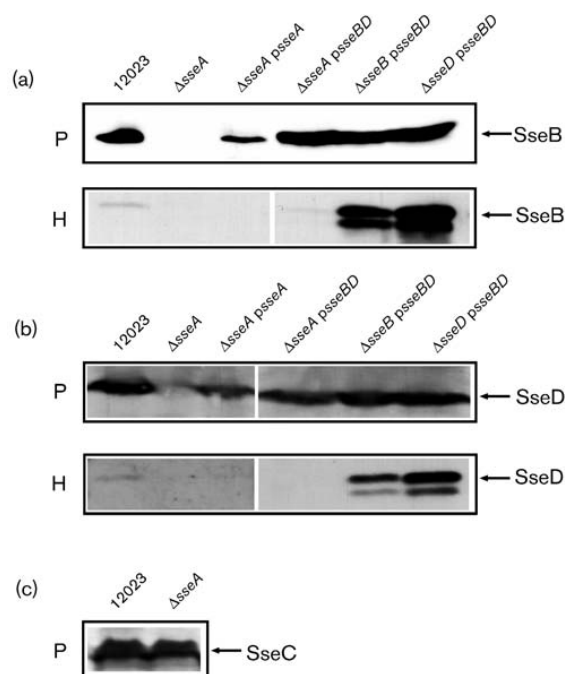
Fig. 3. SseB and SseD are not detected intracellularly in an *S. typhimurium sseA* mutant strain after growth in SPI-2-inducing conditions. The wild-type strain (12023), *sseA* mutant strain (Δ*sseA*), *sseA* mutant strain carrying either the p*sseA* complementing plasmid (Δ*sseA* p*sseA*) or the p*sseBD* complementing plasmid (Δ*sseA* p*sseBD*), and *sseB* and *sseD* mutant strains carrying the p*sseBD* complementing plasmid (Δ*sseB* p*sseBD* and Δ*sseD* p*sseBD*, respectively) were cultured in MgM/MES, pH 5·0, and the pellet (P) and hexadecane (H) fractions were analysed by immunoblotting. (a) Immunoblots probed with anti-SseB antibody. (b) Immunoblots probed with anti-SseD antibody. (c) Immunoblots probed with anti-SseC antibody.

of the *sseB* and *sseD* single mutant strains carrying p*sseBD* (Fig. 3a, b).

The effect of a mutation in *sseA* on the levels of SseB and SseD suggested that SseA might act as a chaperone for these two translocon components. The lack of effect on the intracellular levels of SseC rules out a polar effect of the *sseA* mutation since all these genes form part of the same transcriptional unit (Cirillo *et al.*, 1998). The lack of effect of the *sseA* mutation on SseC was not unexpected, as the *sscA* gene, located immediately upstream of *sseC*, is predicted to encode a chaperone similar to that (SycD) which interacts with the SseC homologue (YopB) in *Yersinia* spp. (Neyt & Cornelis, 1999).

SseA interacts with SseB in a column binding assay

To determine if an interaction occurs between SseA and SseB, we examined the ability of His_6SseA immobilized on an Ni^{2+}–NTA column to bind SseB. When a hexadecane-extracted preparation of secreted proteins (see Methods)
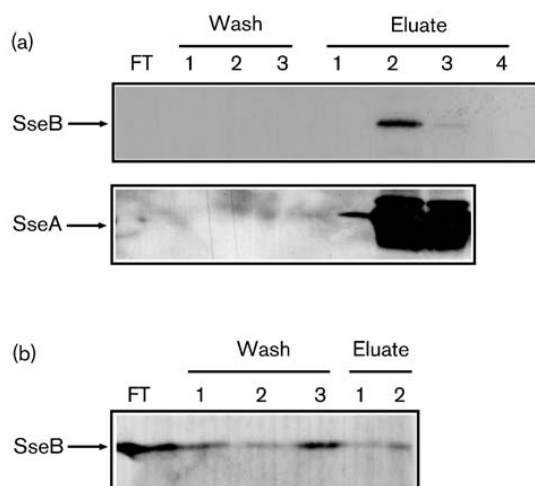

Fig. 4. Immobilized SseA binds to SseB. Bacterial surface-associated proteins were obtained from *S. typhimurium* wild-type strain (12023) cultures grown in minimal medium at low pH. Proteins were subjected to a column binding assay with immobilized His_6SseA. Flow-through (FT), washes and eluted fractions were analysed by immunoblotting with anti-SseA or anti-SseB antibodies. (a) Both His_6SseA and SseB were detected in the eluate fractions. Wash (lanes 1, 2, 3): successive fractions obtained by washing the column with buffer supplemented with 20 mM imidazole. Eluate (lanes 1, 2, 3, 4): successive fractions obtained by washing the column with buffer supplemented with 250 mM imidazole. (b) SseB was not retained in an empty Ni^{2+}–NTA column. Wash (lanes 1, 2, 3): as mentioned above. Eluate (lanes 1 and 2): fractions obtained by washing the column with buffer supplemented with 250 mM imidazole.

was applied to a column containing immobilized His_6SseA, SseB was retained on the column and could only be eluted together with His_6SseA by using a high concentration (250 mM) of imidazole (Fig. 4a). In contrast, SseB was not detected in the flow-through or wash fractions from the same column (Fig. 4a). In a control experiment, SseB was not retained on an Ni^{2+}–NTA column that was not pre-bound with His_6SseA (Fig. 4b), but instead flowed through the column with the wash buffer containing a lower concentration (20 mM) of imidazole.

We were unable to use the same method to determine if an interaction occurs between His_6SseA and SseD, because the anti-SseD antibody had a relatively high level of cross-reactivity to the large amounts of column-bound His_6SseA which detach from the column with the high-imidazole concentration eluted fractions (Fig. 4a, and data not shown).

SseA and SseD interact in the yeast two-hybrid assay

As an alternative approach to establish if an interaction occurs between SseA and SseD, the corresponding genes were ligated into yeast two-hybrid vectors to generate

chimeric proteins with the GAL4 binding and activation domains, respectively. Plasmids expressing these fusions were co-transformed into a *Saccharomyces cerevisiae* reporter strain. The ability of the SseA and SseD fusion proteins to interact with each other when expressed within the same yeast strain was confirmed by their ability to grow on minimal medium, resulting from *ADE1* and *HIS3* reporter gene activation, compared to the complete growth defect of the control strain (data not shown). Protein–protein interactions were also shown by measuring the induction of an independent reporter gene (*lacZ*), by performing β-galactosidase assays of the transformed yeast strains after growth in liquid culture. The reporter strain co-expressing the SseA and SseD fusion proteins consistently had significant β-galactosidase activity ($6 \cdot 27 \pm 0 \cdot 37$ Miller units), which was not detected in the control strain ($0 \cdot 48 \pm 0 \cdot 07$ Miller units). The direct interaction of SseA and SseD in the yeast two-hybrid assay supports the hypothesis that SseA acts as a chaperone for SseD.

Expression of SseB and SseD from a plasmid does not restore Sif formation or virulence of an *sseA* mutant

The effect of the *sseA* mutation on the levels of SseB and SseD could explain the virulence defect of the *sseA* mutant as well as its intracellular phenotypes, since SseB and SseD are necessary for translocation of SPI-2 TTSS effectors (Nikolaus *et al.*, 2001). We have shown that the absence of detectable SseB and SseD in the *sseA* mutant strain can be fully complemented by simultaneous expression of these two proteins from a plasmid (p*sseBD*). The absence of detectable SseB and SseD in the *sseA* mutant strain was partially complemented by expression of SseA from a plasmid (p*sseA*) (Fig. 3). No extracellular SseB or SseD was detected in the *sseA* mutant strain carrying either p*sseA* or p*sseBD* (Fig. 3a, b), although it is possible that some protein is secreted under these conditions but is below the level of detection by the antibodies. Alternatively, some secretion might occur in infected cells. We therefore analysed this strain for the ability to induce Sifs in cultured HeLa cells and for virulence in the mouse model of infection.

HeLa cells were infected for 8 h with wild-type, *sseA*, *sseB*, *sseD*, *sseA* p*sseA*, *sseA* p*sseBD*, *sseB* p*sseBD* or *sseD* p*sseBD* strains, then fixed and labelled with an anti-LAMP1 antibody, and examined for Sif formation. Although the expression of SseA from a plasmid was capable of restoring wild-type levels of Sif formation in an *sseA* mutant strain, expression of SseB and SseD from a plasmid was only capable of partial complementation of the *sseA* mutant (Fig. 5a). However, expression of SseB and SseD from the plasmid fully complemented the defect in Sif formation of the *sseB* and *sseD* single mutant strains (Fig. 5a).

To analyse the ability of the p*sseA* and p*sseBD* plasmids to complement the virulence defect of the *sseA* mutant strain, the *sseA* mutant strain carrying either p*sseA* or p*sseBD*, or none, was used to inoculate mice intraperitoneally in a
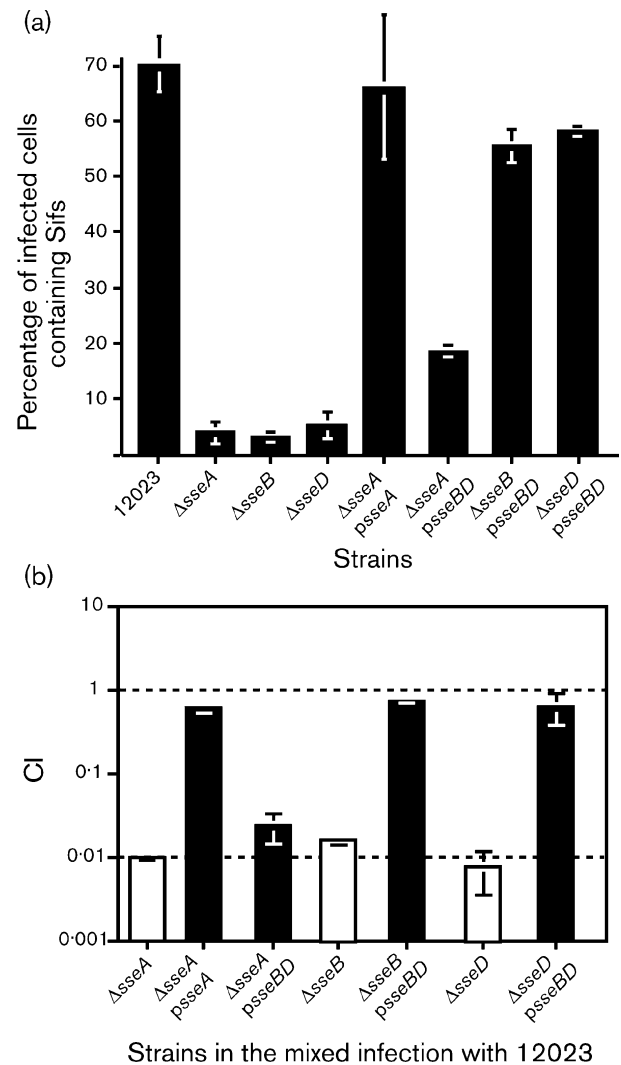

Fig. 5. Sif formation and virulence defects of *S. typhimurium* strains. (a) Sif formation was examined in HeLa cells infected with the wild-type (12023), *sseA* mutant (Δ*sseA*), *sseB* mutant (Δ*sseB*) or *sseD* mutant (Δ*sseD*) or these strains carrying the plasmids shown. Cells were fixed 8 h after bacterial invasion and examined by confocal fluorescence microscopy. Sifs were labelled with an anti-LAMP-1 antibody, while bacteria were detected with an anti-*Salmonella* antibody. The number of infected cells containing Sifs was determined by confocal microscopy examination ($n = 100$ infected cells). Each infection was carried out in triplicate and the standard deviations from the means are shown. (b) Graphical representation of the CI analysis of the wild-type strain (12023) in mixed infections with strains carrying mutations in *sseA*, *sseB* or *sseD* (open bars), and with the mutant strains carrying plasmids expressing either SseA or SseB and SseD simultaneously (solid bars).

mixed infection with the wild-type strain. Mice were killed 48 h after inoculation, and their spleens were homogenized and plated to determine bacterial load of each strain (on the basis of antibiotic resistances), and to calculate the CI. In

agreement with previous reports (Hensel *et al.*, 1998), the *sseA* mutant was highly attenuated (CI = 0·01) (Fig. 5b). However, whereas the *sseA* p*sseA* strain gave a CI close to 1·0, indicating that it is as virulent as the wild-type strain, the virulence of *sseA* p*sseBD* was only slightly higher than that of the *sseA* mutant strain (CI = 0·023). As with Sif formation, p*sseBD* was able to restore the virulence of the *sseB* and *sseD* single mutant strains back to wild-type levels (Fig. 5b).

The complete recovery of virulence and Sif formation in the *sseA* p*sseA* strain is intriguing, since SseB and SseD were not detected extracellularly on immunoblots of proteins extracted from this strain grown in SPI-2-inducing conditions (Fig. 3). Presumably, the extracellular levels of SseB and SseD required to restore SPI-2 TTSS function in infected HeLa cells are below the threshold of detection by immunoblot with the anti-SseB and anti-SseD antibodies.

RT-PCR experiments showed that mutation of *sseA* did not affect *sseB* transcript levels (data not shown). Our results indicate that SseA is required for the stability of SseB and SseD, since these proteins were not detected on immunoblots from the *sseA* mutant strain grown in SPI-2-inducing conditions (Fig. 3). However, this is not the only role of SseA in the SPI-2 TTSS, since expression of SseB and SseD simultaneously from a plasmid does not restore Sif formation or virulence in an *sseA* mutant strain (Fig. 5). One possible explanation is that SseA may act as a chaperone for yet another SPI-2 protein. Although we cannot rule out this possibility, it seems unlikely that this is the case since the phenotypes of the remainder of the proteins encoded in the *sseA–G* operon are different from those observed for the *sseA* p*sseBD* strain. Strains defective in SseF and SseG are only mildly attenuated in virulence and partially affected in Sif formation (Guy *et al.*, 2000; Hansen-Wester *et al.*, 2002; Hensel *et al.*, 1998), whereas a strain defective in SseE is not attenuated in virulence and has no other reported phenotype (Hensel *et al.*, 1998). SscA is predicted to be a chaperone for SseC on the basis of sequence similarity (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Neyt & Cornelis, 1999) and SscB is also predicted to be a chaperone (Cirillo *et al.*, 1998; Hensel *et al.*, 1998). Furthermore, type III chaperones of the translocators studied to date bind one or two substrates but not more (Page & Parsot, 2002).

However, in view of the lack of detectable extracellular SseB and SseD displayed *in vitro* by the *sseA* mutant strain carrying p*sseBD*, a direct role of SseA in secretion of SseB and SseD is possible. Alternatively, SseA could be required for the correct folding of SseB and SseD, which would be essential for the proper assembly of the translocon, and consequently for SPI-2-mediated translocation of effectors into the host cell. Based on its similarity to EspA, SseB is predicted to be a major component of the SPI-2 translocon, and to bind to itself as well as to the other components of the translocon (Delahay *et al.*, 1999; Hartland *et al.*, 2000; Hensel *et al.*, 1998). Supporting this idea, SseB (and also SseD) has a domain with the potential to form a coiled-coil

structure, which could be involved in these interactions (Hensel *et al.*, 1998). It is conceivable that chaperone binding is necessary to prevent incorrect folding of SseB and SseD or their premature association with themselves or other translocon components, events which could also account for the deficient secretion of both proteins observed in the *sseA* mutant strain carrying p*sseBD*. However, the experiments presented in this study do not discriminate between a direct role of SseA in secretion of SseB and SseD, versus a role in facilitating their correct folding which may be required for efficient secretion and function. Additional research into the interaction of SseA with SseB and SseD, as well as the interaction of SseB with itself and other proteins, will further our understanding of the role of SseA as a SPI-2 chaperone.

NOTE ADDED IN PROOF

After acceptance of this manuscript, SseA was shown to be a chaperone for SseB (Zurawski & Stein, 2003).

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