Molecular Microbiology (2002) **44**(3), 645–661

Complementary activities of SseJ and SifA regulate dynamics of the *Salmonella typhimurium* vacuolar membrane

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Summary

The *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system (TTSS) of *Salmonella typhimurium* is required for bacterial replication within host cells. It acts by translocating effector proteins across the membrane of the *Salmonella*-containing vacuole (SCV). The SifA effector is required to maintain the integrity of the SCV membrane, and for the formation in epithelial cells of *Salmonella*-induced filaments (Sifs), which are tubular extensions of SCVs. We have investigated the role in *S. typhimurium* virulence of the putative SPI-2 effector genes *sifB, srfJ*, *sseJ* and *ssel.* An *S. typhimurium* strain carrying a mutation in *sseJ* was mildly attenuated for systemic virulence in mice, but strains carrying mutations in either *srfJ*, *ssel* or *sifB* had very little or no detectable virulence defect after intraperitoneal inoculation. Expression of SseJ in HeLa cells resulted in the formation of globular membranous compartments (GMCs), the composition of which appears to be similar to that of SCV membranes and Sifs. The formation of GMCs was dependent on the serine residue of the predicted acyltransferase/lipase active site of SseJ. Transiently expressed SseJ also inhibited Sif formation by wild-type bacteria, and was found to associate with Sifs, SCV membranes and simultaneously expressed SifA. Intracellular vacuoles containing *sseJ* mutant bacteria appeared normal but, in contrast to a *sifA* mutant, a *sifA sseJ* double mutant strain did not lose its vacuolar membrane, indicating that loss of vacuolar membrane around *sifA* mutant bacteria requires the action of SseJ. Collectively, these results suggest that the combined action of SseJ and SifA regulate dynamics of the SCV membrane in infected cells.

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Introduction

Salmonella enterica serovar Typhimurium (*S. typhimurium*) causes a systemic disease in mice resembling typhoid fever. This occurs as a result of bacterial replication within membrane-bound compartments in macrophages of the spleen and liver (Carter and Collins, 1974; Richter-Dahlfors *et al*., 1997; Salcedo *et al*., 2001). Many bacterial genes have been identified that are required for intracellular replication of *S. typhimurium* (Fields *et al*., 1986). Some are located in *Salmonella* pathogenicity island 2 (SPI-2), which encodes a type III secretion system (TTSS) (Hensel *et al*., 1995; Ochman *et al*., 1996; Shea *et al*., 1996).

A distinguishing feature of TTSSs is that they couple bacterial secretion with the injection of effector proteins across host cell membranes. This is accomplished by the formation of a cylindrical, needle-like organelle (secreton) that exports proteins across the two membranes of the bacterial cell, and a pore in the host cell membrane (translocon), which allows transfer of effectors into the target cell (Cornelis and Van Gijsegem, 2000). In some systems, the secreton and translocon appear to be connected by other proteins (Knutton *et al*., 1998; Sekiya *et al*., 2001). Structural elements of the secreton and translocon are conserved to varying degrees among different pathogenic bacteria, but effector functions are distinct and confer functional specificity on each individual system.

Salmonella typhimurium has two functionally independent TTSSs, both encoded by pathogenicity islands. The *Salmonella* pathogenicity island 1 (SPI-1) encoded TTSS injects effector proteins across the plasma membrane of host cells; this results in localized actin polymerization, membrane ruffling and bacterial invasion (Galán, 2001). It also induces significant intestinal secretory and inflammatory responses (Wallis and Galyov, 2000). The SPI-2 TTSS is required for bacterial replication within host cells and systemic infection of mice (Hensel *et al*., 1995; Ochman *et al*., 1996; Cirillo *et al*., 1998; Hensel *et al*., 1998). SPI-2 TTSS gene expression is controlled by the

OmpR-EnvZ (Lee *et al*., 2000) and SPI-2 encoded SsrA-SsrB two-component regulatory systems (Valdivia and Falkow, 1997; Deiwick *et al*., 1999). This secretion system is activated after entry of bacteria into host cells (Valdivia and Falkow, 1997; Cirillo *et al*., 1998;Beuzón *et al*., 2000) and translocates effector proteins across the vacuolar membrane (Miao *et al*., 1999; Uchiya *et al*., 1999; Miao and Miller, 2000).

The SPI-2 encoded effector protein SpiC, has been reported to inhibit interactions between the *Salmonella*-containing vacuole (SCV) and late endosomes and lysosomes (Uchiya *et al*., 1999). Another effector, SifA, is encoded outside SPI-2 (Stein *et al*., 1996) and is required to maintain the integrity of the SCV membrane (Beuzón *et al*., 2000). In epithelial cells, SifA induces the formation of tubular membrane structures (Sifs), which appear to be extensions of SCVs (García-del Portillo *et al*., 1993; Stein *et al*., 1996). Sifs contain proteins, such as lysosomal membrane glycoproteins (lgps) (García-del Portillo *et al*., 1993) and the vacuolar ATPase (Beuzón *et al*., 2000), which are also found in the SCV membrane (García-del Portillo and Finlay, 1995; Beuzón *et al*., 2000).

Through the action of unknown effectors, the SPI-2 TTSS also enables *Salmonella* to avoid the respiratory burst by inhibiting trafficking to the SCV of NADPH oxidase subunits (Vazquez-Torres *et al*., 2000; Gallois *et al*., 2001). Additional functions associated with the SPI-2 TTSS include the induction of a delayed apoptotic-like cell death in macrophages (van der Velden *et al*., 2000), and the assembly of an F-actin meshwork in the vicinity of the SCV, which also helps to maintain vacuolar membrane integrity (Méresse *et al*., 2001).

Recently, several genes related to the SPI-2 TTSS have been identified in *S. typhimurium*. Worley and colleagues (Worley *et al*., (2000) identified a global regulon of genes under the control of SsrA-SsrB, many of them located outside SPI-2. A conserved N-terminal amino acid sequence of approximately 150 residues has been identified in several proteins, also encoded outside SPI-2, including SifA and a related protein, SifB (Beuzón *et al*., 2000; Guy *et al*., 2000; Miao and Miller, 2000; Brumell *et al*., 2001a). This sequence was shown to be required for translocation of five of these proteins to the host cell cytosol via either the SPI-2 TTSS (SspH2, SseI, SseJ), or both SPI-1 and SPI-2 TTSSs (SspH1, SlrP) (Miao and Miller, 2000). Apart from SifA and SpiC, the functions of the other putative effectors described above are currently unknown. A strain carrying mutations in both *sspH1* and *sspH2* was attenuated for calf enteropathogenesis but did not have a virulence defect in mice (Miao *et al*., 1999). *slrP* is not regulated by SsrA-SsrB (Miao and Miller, 2000), and is required for colonization of Peyer's patches but not spleens of mice (Tsolis *et al*., 1999).

In this study, we have investigated the role of *sifB, srfJ*, *sseJ* and *sseI* during systemic infection of mice. SifB is similar to SifA over the length of the protein (Miao and Miller, 2000). SrfJ is similar to human lysosomal glucosyl ceramidase, suggesting that it may alter the host cell ceramide signal transduction pathway (Worley *et al*., 2000). The deduced amino acid sequence of *sseI* (originally referred to as *srfH* by Worley and colleagues; Worley *et al*., 2000) does not suggest a function for the protein but, like *sifB, srfJ* and *sseJ*, this gene is expressed in an SsrA-SsrB-dependent manner (Miao and Miller, 2000; Worley *et al*., 2000).

SseJ shares a high level of similarity with several proteins belonging to the 'GDSL' family of lipolytic enzymes, exemplified by an *Aeromonas hydrophila* acyltransferase/lipase (Upton and Buckley, 1995). This similarity begins at amino acid 142 of SseJ, precisely where the conserved N-terminal translocation signal described for putative effectors of SPI-1 and SPI-2 TTSSs finishes (Miao and Miller, 2000). SseJ has the three residues (Ser-Asp-His) that constitute the catalytic triad characterized in the *A. hydrophila* enzyme, within three out of five conserved blocks of sequence present in SseJ and all proteins belonging to this family (Brumlik and Buckley, 1996). These include additional key residues such as a second serine flanking the GDSL signature pattern (Robertson *et al*., 1994).

We found that *S. typhimurium* strains carrying single mutations in either *sseJ* or *srfJ* were mildly attenuated for systemic virulence, whereas mutations in either *sseI* or *sifB* conferred no detectable virulence defect. Expression of SseJ in HeLa cells resulted in the formation of large globular structures with characteristics of SCV membranes and Sifs. Ectopically expressed SseJ also reduced Sif formation by wild-type bacteria, and was found to associate with the remaining Sifs, SCV membranes, and simultaneously expressed SifA. In contrast to a *sifA* mutant, a *sifA sseJ* double mutant strain did not lose its vacuolar membrane, indicating that loss of vacuolar membrane around *sifA* mutant bacteria (Beuzón *et al*., 2000) requires the action of SseJ. Collectively, our results indicate that the combined action of SseJ and SifA regulate dynamics of the SCV membrane in infected cells.

Results

Effects of mutations in sifB, srfJ, sseJ *and* sseI *on virulence and intracellular replication*

To investigate the roles in virulence of *sifB, srfJ*, *sseJ* and *sseI,* single mutant strains were constructed which carry either disruptions in *srfJ*, *sseJ* or *sseI,* or a deletion of *sifB*. These were subjected to virulence tests by comparing

Table 1. Competitive Index (CI) analysis of *Salmonella typhimurium* mutant strains.

Mixed infection[a]	CI[b]
wt v *sseJ*⁻	0.53 ± 0.08[c]
wt v *sseI*⁻	1.04 ± 0.05[d]
wt v *sifB*⁻	0.94 ± 0.12[d]
wt v *srfJ*⁻	0.77 ± 0.04[c]
wt v *sifA*⁻	0.012 ± 0.0006
wt v *sseJ*⁻ p*sseJ*	1.13 ± 0.20[d]

a. Strains used in the mixed infection experiments are referred to by the relevant genotype and are described in Table 2.
b. The CI was calculated as the output ratio of mutant to wild-type bacteria, divided by the input ratio. The CIs shown are the means ± standard errors of at least three independent infections of mice. Mice were inoculated i.p. with a mixture of two strains comprising ~5 × 10^4 cfu of each strain. Mouse spleens were harvested after 48 h for enumeration of bacterial cfu. The different strains used were differentiated on the basis of antibiotic sensitivity. The same method was used to confirm the stability of each mutation after the infection.
c. Significantly different to 1.0.
d. Not significantly different to 1.0.

them with the wild-type parental strain in mixed infections in mice. A competitive index (CI), which provides a value for the relative degree of virulence attenuation, was determined for each mutant after recovering bacteria from infected spleens, 48 h after intraperitoneal (i.p.) inoculation. The *sifB* and *sseI* mutant strains had no detectable virulence defect in the systemic phase of infection, whereas the *srfJ* and *sseJ* mutants had very slight and moderate virulence defects respectively (Table 1). In the case of *sseJ*, a CI of 0.47 ± 0.1 was obtained using a second mutant strain in which the entire coding region was deleted. Virulence was fully restored by the introduction of a plasmid expressing *sseJ* (Table 1). Therefore, the attenuated phenotype can be attributed to a null mutation in *sseJ* rather than a polar effect or secondary mutation in this strain. In view of these results, further studies were focused on SseJ.

A hallmark of *S. typhimurium* pathogenesis is its ability to replicate inside host cells. Therefore, intracellular growth of the *sseJ* mutant was compared with that of the wild-type strain and an *ssaV* mutant. *ssaV* encodes an essential component of the SPI-2 TTSS, the mutant strain being completely defective for SPI-2 mediated secretion (Beuzón *et al.*, 1999). Replication assays were performed over 16 h in epithelial (HeLa) cells, a macrophage-like cell line (RAW 264.7), and in murine-elicited peritoneal macrophages. The *ssaV* mutant displayed a replication or survival defect in all three cell types, but the growth of the *sseJ* mutant was indistinguishable from that of the wild-type strain in HeLa and RAW cells. Only in the more restrictive growth environment within elicited peritoneal macrophages was a small but reproducible net survival defect detected (Fig. 1).

SseJ expression within HeLa cells induces membranous structures with characteristics of SCVs and Sifs

To gain further insight into the function of SseJ, an epitope-tagged version of the protein (myc::SseJ) was

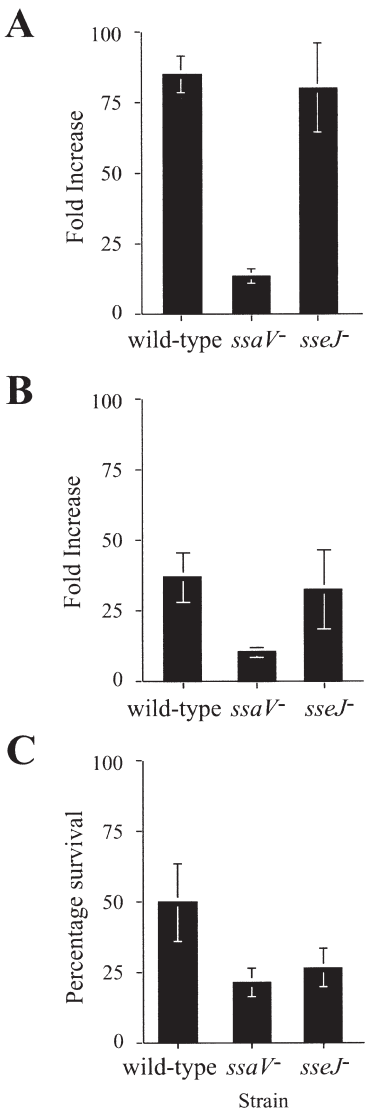

Fig. 1. Replication and survival of an *sseJ* mutant strain within host cells. Replication assays were carried out for wild-type (12023), *ssaV* mutant (HH109) and *sseJ* mutant (HH200) strains. For macrophages, opsonized stationary phase bacteria were taken up by phagocytosis, and at 2 and 16 h, macrophages were lysed and cultured for enumeration of intracellular bacteria (gentamicin-protected). HeLa cells were infected with exponentially grown bacteria and processed as above. The values shown represent the fold increase calculated as a ratio of the intracellular bacteria between 2 and 16 h after bacterial entry. Each strain was infected in triplicate and the standard errors from the means are shown. The results shown are representative of three independent experiments.
A. Replication assay within RAW macrophages.
B. Replication assay within HeLa cells.
C. Replication assay within murine-elicited peritoneal macrophages.

constructed and expressed in HeLa cells after transfection. The distribution of this protein was examined first in relation to that of LAMP-1, a lysosomal membrane glycoprotein (lgp) and a well established marker of the SCV membrane and Sifs (García-del Portillo and Finlay, 1995; Beuzón *et al*., 2000). Confocal immunofluorescence microscopy of untransfected cells using an anti-LAMP-1 antibody revealed characteristic punctate labelling throughout the cell (Fig. 2A). In contrast, in cells expressing myc::SseJ, unusual structures associated with both LAMP-1 and myc::SseJ were observed. The number and size of these structures correlated with the level of myc::SseJ expression. In cells containing the lowest detectable level of myc::SseJ expression, LAMP-1 labelling was punctate but, as the level of myc::SseJ increased, the punctate labelling was replaced by large aggregates of LAMP-1, in most cases enclosed by what appeared (in the XY plane of the confocal microscope) to

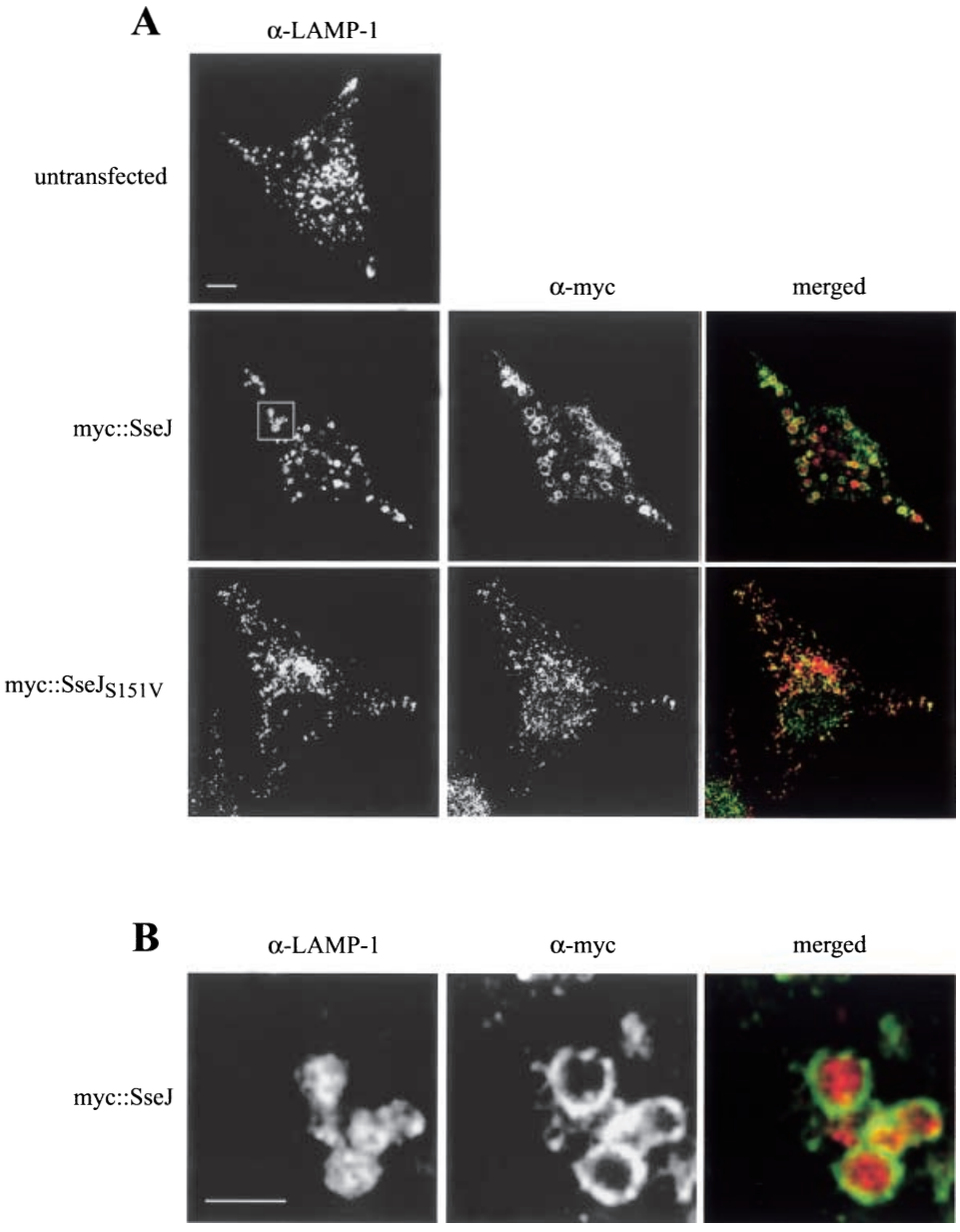

Fig. 2. Ectopically expressed SseJ redistributes and associates with LAMP-1.
A. Confocal immunofluorescence analysis of HeLa cells either untransfected, or transfected with a plasmid expressing myc::SseJ or myc::SseJ$_{S151V}$. The cells were fixed 24 h after DNA addition and labelled using mouse monoclonal and TRSC-conjugated donkey anti-mouse secondary antibodies to detect LAMP-1 (red in merged images), and a rabbit polyclonal and FITC-conjugated donkey anti-rabbit secondary antibodies to detect the myc epitope tag (green in merged images). Scale bar represents 5 μm.
B. Close-up of cells expressing myc::SseJ (boxed area in A). Scale bar represents 1 μm.

be myc::SseJ-positive rings of up to 1 μm in diameter (Fig. 2B). When analysed by a stacked series of confocal images, these compartments were shown to have globular structure and seemed to result from aggregation of LAMP-1 vesicles (results not shown). These compartments were not visible under the phase-contrast microscope. For convenience, we refer to them as globular membranous compartments (GMCs). GMCs were not a consequence of expression of the myc epitope, because similar structures were observed in HeLa cells transfected with a green fluorescent protein (GFP)::SseJ chimera (results not shown), and were specific to SseJ, as no abnormal structures and typical LAMP-1 labelling were observed in cells transfected with a myc::SifB protein (results not shown).

To determine if the formation of GMCs is dependent on the predicted acyltransferase/lipase activity of SseJ, a mutant version of the protein was constructed by substituting the predicted serine of the catalytic triad with valine ($SseJ_{S151V}$). Replacement of the active site serine in the GDSL motif of the lipases of both *A. hydrophila* and *Vibrio mimicus* leads to loss of enzyme activity (Hilton and Buckley, 1991; Shaw *et al*., 1994). Expression of myc::$SseJ_{S151V}$ in HeLa cells had no noticeable effect on the distribution of LAMP-1, and no GMCs were formed. However, this mutant protein colocalized extensively with LAMP-1, indicating that the myc::SseJ protein may be targeted to LAMP-1-containing vesicles independently of its acyltransferase/lipase activity (Fig. 2A).

To investigate the composition of the GMCs in more detail, we examined transfected cells for several other cellular markers. The lgp LIMP-1 (also known as LAMP-3 and CD63 antigen) shares the same lysosomal targetting sequence as LAMP-1 (Hunziker and Geuze, 1996). The vacuolar proton ATPase (vATPase), which acidifies endocytic vacuoles, has a different origin and targeting process, but both LIMP-1 and vATPase accumulate on SCV membranes (Steele-Mortimer *et al*., 1999; Beuzón *et al*., 2000). In cells expressing myc::SseJ, the distribution of both proteins was altered dramatically, and both accumulated within GMCs (Fig. 3A, upper and middle panels, and data not shown). Cholesterol has also been found recently to associate with Sifs and SCVs (Brumell *et al*., 2001b). In untransfected cells, cholesterol (as revealed by filipin staining), was mainly distributed in the plasma membrane and small perinuclear vesicles (data not shown). However, in cells expressing myc::SseJ this was replaced by a nearly exclusive localization in GMCs (Fig. 3A, lower panel). As for LAMP-1, redistribution of LIMP-1, vATPase and cholesterol was dependent on the active site serine residue of wild-type SseJ (data not shown). Recent work has shown that intracellular *S. typhimurium* induces actin polymerization in a SPI-2 dependent manner (Méresse *et al*., 2001). Surprisingly, Texas red-phalloidin staining of myc::SseJ-transfected cells showed that the GMCs also frequently contained F-actin (Fig 4A and B). Therefore, the interior of these compartments appears to be a complex aggregate of membranes and component(s) of the cytoskeleton.

Other subcellular compartments appeared to be unaffected by overexpression of SseJ. The distribution in transfected cells of the lysosomal marker cathepsin D, and several Golgi markers (p115, p14Kβ, GM130 and Giantin), was indistinguishable from that in untransfected cells, although occasional colocalization between myc::SseJ and cathepsin D was observed (Fig. 3B and results not shown). Therefore, overexpression of myc::SseJ in HeLa cells results in the formation of unusual membranous compartments, with a composition that is characteristic of SCV membranes and Sifs.

Effects of SseJ expression in infected cells and in cells expressing SifA

In view of these results, it was of interest to know if ectopic expression of myc::SseJ in infected cells would alter the development of SCVs and Sifs. HeLa cells were first transfected with myc::SseJ, infected for 10 h with wild-type *S. typhimurium,* then fixed and labelled with α-LAMP-1 antibody. Sif formation was inhibited by the presence of ectopically expressed SseJ, and this inhibition required the active site serine (Fig. 5A). Sifs that were detected in myc::SseJ transfected, infected cells had significantly less LAMP-1 and aberrant morphology, whereas Sifs in $SseJ_{S151V}$ transfected, infected cells appeared normal (Fig. 5B). Ectopically expressed myc::SseJ colocalized with LAMP-1 on SCV membranes and Sifs, and this association was not dependent on the active site serine (Fig 5B and C, and results not shown). GMCs were also observed in infected cells (Fig. 5C). Ectopic expression of myc::SseJ significantly inhibited intracellular replication of wild-type *S. typhimurium*. Bacterial replication was also inhibited by expression of myc::$SseJ_{S151V}$ or myc::SifB, but to a lesser extent than by myc::SseJ (Fig. 5D).

As both SseJ and SifA localized to Sifs and SCV membranes (Fig 5B and C) (Beuzón *et al*., 2000), this raised the possibility that the two proteins might associate when expressed in the same cell. It has been shown previously that transfection of HeLa cells with SifA::GFP leads to the formation of large LAMP-1-containing compartments, and structures resembling Sifs (Brumell *et al*., 2001a). When HeLa cells were simultaneously transfected with plasmids expressing both myc::SseJ and SifA::GFP, GMC-like structures were formed. Interestingly, SifA::GFP was found within these structures, and no Sifs were observed (Fig. 6). Therefore, SifA and SseJ associate with the same structures when expressed in the same cell, but their lack

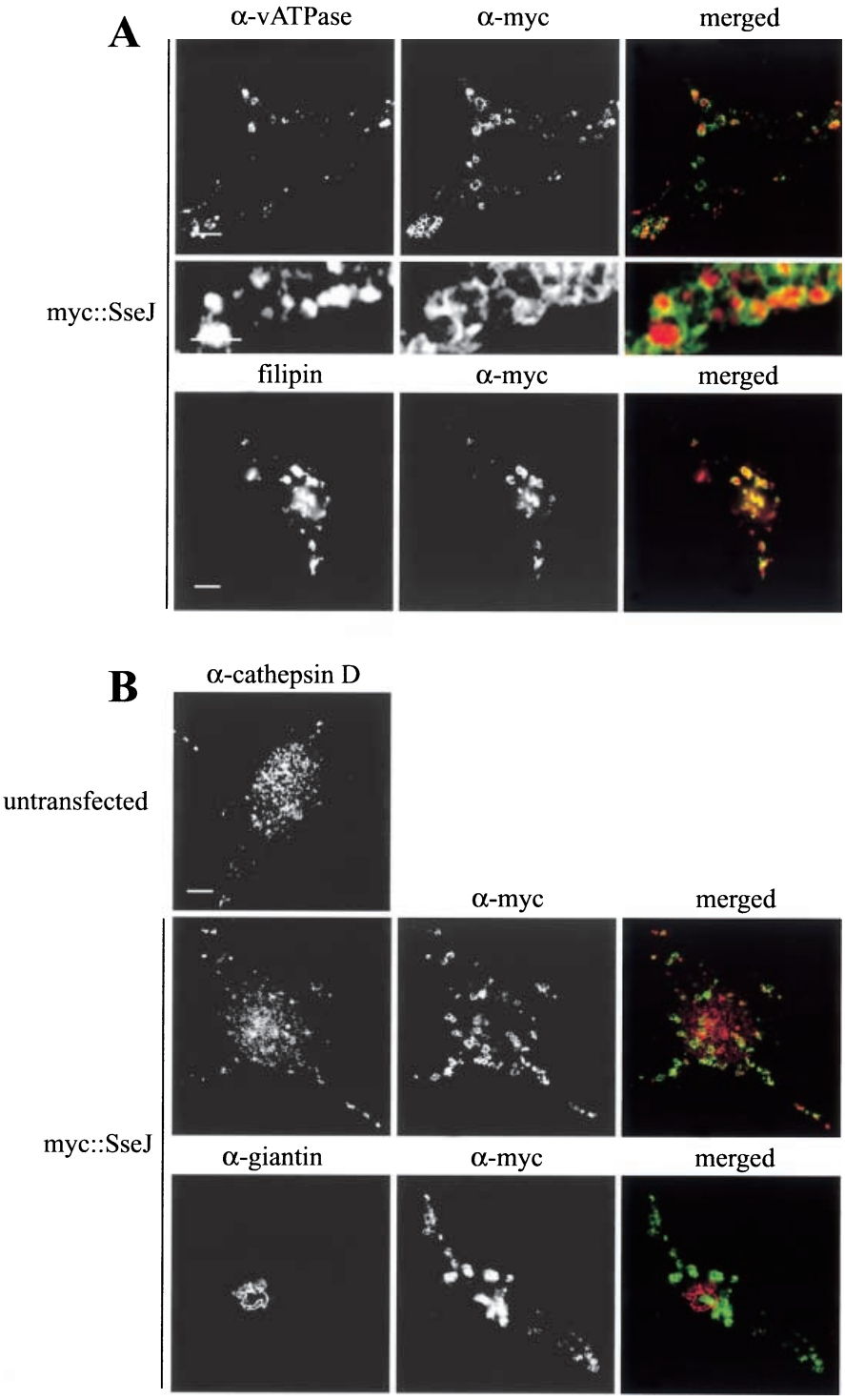

Fig. 3. SseJ redistributes and associates with markers characteristic of the SCV.
A. Confocal immunofluorescence analysis of HeLa cells transfected with a plasmid expressing myc::SseJ (green in merged images). The cells were fixed 24 h after DNA addition and labelled using a rabbit polyclonal and FITC-conjugated donkey anti-rabbit secondary antibodies to detect the myc epitope tag, and a mouse monoclonal and TRSC-conjugated donkey anti-mouse secondary antibodies to detect vATPase (upper and middle panels), or the fluorescent cholesterol-binding drug filipin (lower panel) (both red in merged images). Scale bar represents 5 μm. Middle panel shows vATPase association with myc::SseJ. Scale bar represents 1 μm.
B. Confocal immunofluorescence analysis of HeLa cells either untransfected or transfected with a plasmid expressing myc::SseJ (green in merged images). The cells were fixed 24 h after DNA addition and labelled using a mouse monoclonal and a FITC-conjugated donkey anti-mouse secondary antibodies to detect the myc epitope tag, and a rabbit polyclonal and TRSC-conjugated donkey anti-rabbit secondary antibodies to detect cathepsin D (upper and middle panels), or rabbit polyclonal and TRSC-conjugated donkey anti-rabbit secondary antibodies to detect giantin (lower panel) (both red in merged images). Scale bar represents 5 μm.

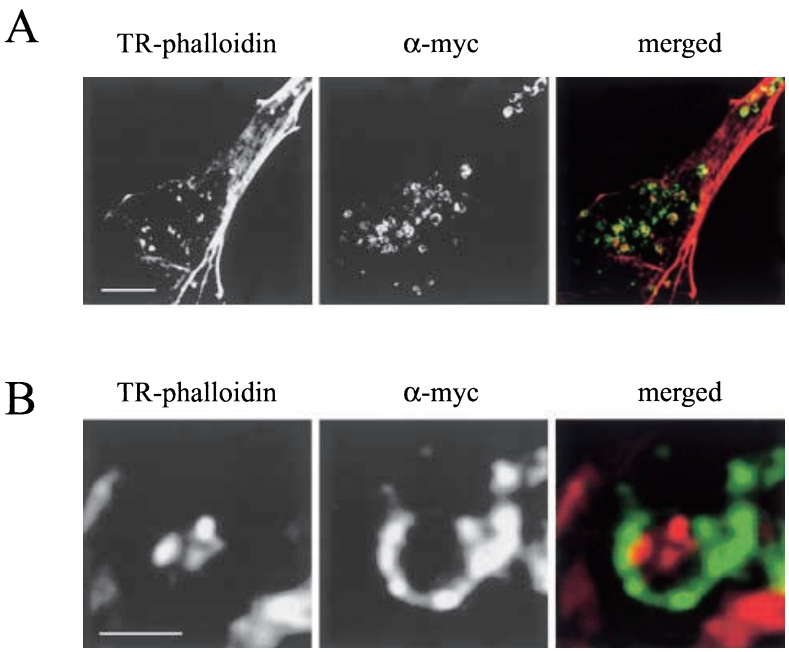

Fig. 4. Association between SseJ and F-actin in HeLa cells expressing SseJ.
A. Confocal immunofluorescence analysis of HeLa cells transfected with a plasmid expressing myc::SseJ. The cells were fixed 24 h after DNA addition and labelled using a rabbit polyclonal and FITC-conjugated donkey anti-rabbit secondary antibodies to detect the myc epitope tag (green in merged images), and a mouse monoclonal and TRSC-conjugated phalloidin to detect actin (red in merged images). Scale bar represents 5 μm.
B. Close-up confocal image showing SseJ association with actin. Scale bar represents 1 μm.

of pixel-to-pixel colocalization suggests that the two effectors do not interact directly with each other.

Analysis of SCV membrane dynamics

The results described above suggest that the function of SseJ, like that of SifA, is related to the control of SCV membrane dynamics. However, no significant differences were observed between wild-type and *sseJ* mutant bacteria with respect to LAMP–1 association with SCVs in HeLa cells and macrophages, or Sif formation in HeLa cells, at different time-points (data not shown).

Therefore, to gain some insight into SCV membrane dynamics, we first investigated the role of SifA in more detail. *sifA* mutant bacteria gradually lose their vacuolar membranes several hours after bacterial entry into host cells, indicating that SifA is required to maintain the integrity of the vacuolar membrane (Beuzón *et al*., 2000). It is possible that SifA is required for recruitment and/or fusion of membrane vesicles to the SCV, which would lead to a depletion of LAMP-1 vesicles from elsewhere in the cell. Therefore, the distribution of LAMP-1 was re-examined in HeLa cells containing wild-type, *ssaV* or *sifA* mutant bacteria at different time-points. A redistribution of LAMP-1 was only observed in cells infected with wild-type bacteria, beginning approximately 6 h after bacterial entry. By 10 h, the typical uniform punctate distribution of LAMP-1 vesicles throughout the cell mostly disappeared and was replaced by a strong accumulation of LAMP-1 in the vicinity of the bacterial microcolony (Fig. 7). At this time-point, the distribution of LAMP-1-containing vesicles appeared normal in cells infected with either *sifA* or *ssaV* mutant bacteria, although as shown previously (Beuzón *et al*., 2000) only *ssaV* mutants were associated with LAMP-1-containing SCV membranes (Fig. 7). These results are consistent with the hypothesis that SifA is involved in recruiting membrane vesicles from other parts of the cell towards SCVs.

There are at least two possible explanations for the loss of the vacuolar membrane around *sifA* mutant bacteria. First, bacterial replication may itself be sufficient to cause their release into the host cell cytosol if insufficient membrane is recruited. Alternatively, the bacteria could possess an activity that destabilizes the SCV membrane and is opposed by SifA in wild-type SCVs. If the *sifA* mutant strain loses its vacuolar membrane because it cannot recruit sufficient membrane to enclose replicating bacterial cells, then combining a *sifA* mutation with additional, unrelated mutations which inhibit intracellular bacterial replication, would be predicted to result in a strain which undergoes less vacuolar membrane loss than a strain carrying only a single *sifA* mutation. It has been shown previously that *sifA* mutant strains are not deficient for replication in HeLa cells (Stein *et al*., 1996), but replicate poorly in macrophages (Beuzón *et al*., 2000; Brumell *et al*., 2001a). We therefore introduced auxotrophic *aroC* and *purD* mutations (which completely prevented intracellular replication of wild-type *S. typhimurium* (results not shown)) into a *sifA* mutant, and compared the numbers of SCVs associated with LAMP-1 (as a marker for the SCV membrane) 10 h after bacterial uptake into host cells. Although no replication of the *sifA aroC purD* triple mutant occurred in HeLa cells and macrophages (results not

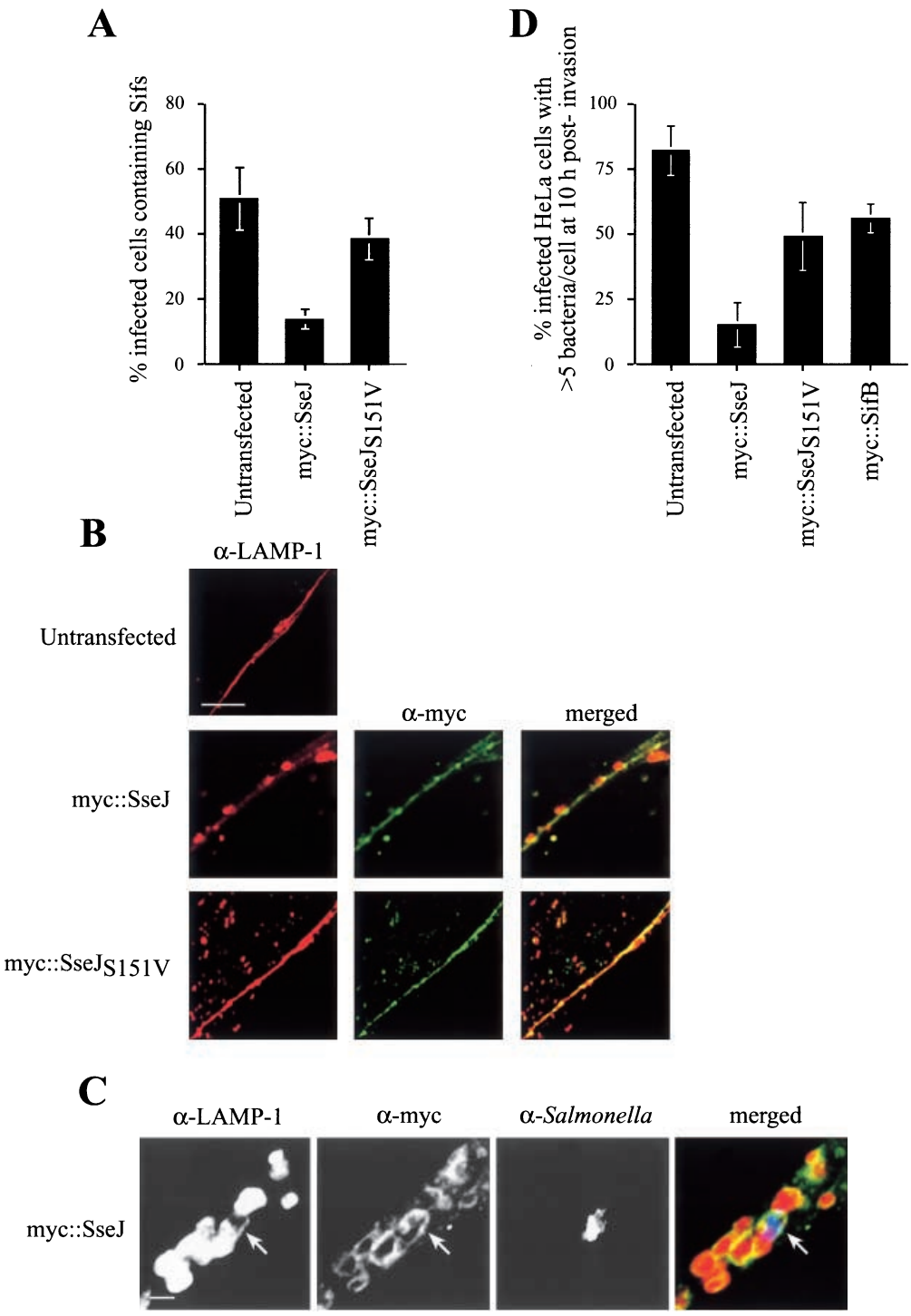

Fig. 5. Ectopically expressed SseJ in HeLa cells associates with Sifs and SCVs, and inhibits Sif formation and bacterial replication.
A. Sif formation in HeLa cells transiently expressing myc::SseJ or myc:SseJ$_{S151V}$, and infected with wild-type bacteria. The cells were fixed 10 h after bacterial invasion and labelled using a mouse monoclonal and TRSC-conjugated donkey anti-mouse secondary antibodies to detect LAMP-1, a rabbit polyclonal and FITC-conjugated donkey anti-rabbit secondary antibodies to detect the myc epitope tag, and a goat polyclonal and a Cy5-conjugated donkey anti-goat to detect bacteria.
B. Confocal immunofluorescence analysis of Sifs and colocalization with myc::SseJ or myc::SseJ$_{S151V}$ as revealed by LAMP-1 staining, 10 h after bacterial invasion. Scale bar represents 5 μm.
C. Confocal image showing SCV colocalization with myc::SseJ (green in merged image) as revealed by LAMP-1 staining (red in merged image), 10 h after bacterial invasion. Arrowhead indicates colocalization between LAMP-1 and SseJ on SCV. Scale bar represents 1 μm.
D. Quantification of bacteria within HeLa cells expressing myc::SseJ, myc:SseJ$_{S151V}$, or myc::SifB, 10 h after invasion by wild-type bacteria.

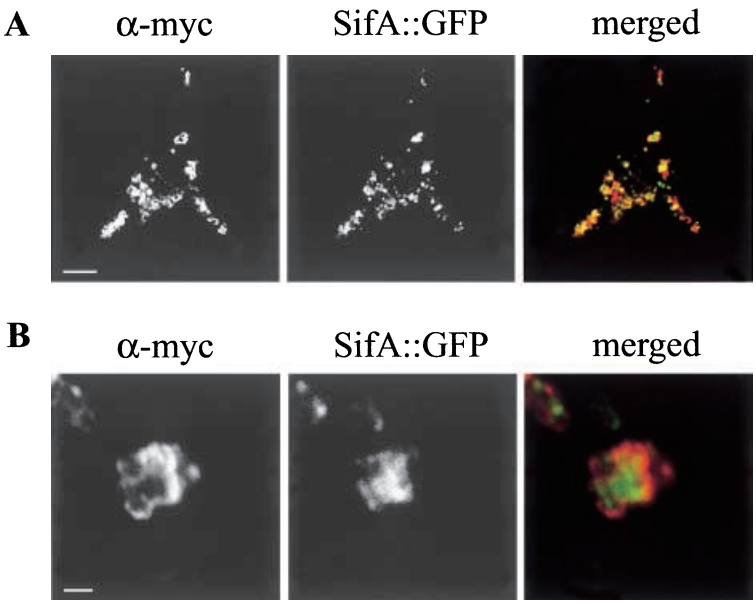

Fig. 6. Association between myc::SseJ and SifA::GFP in HeLa cells.
A. Confocal immunofluorescence analysis of HeLa cells simultaneously transfected with plasmids expressing myc::SseJ and SifA::GFP (red and green, respectively, in merged image). The cells were fixed 24 h after DNA addition and labelled using a rabbit polyclonal and TRSC-conjugated donkey anti-rabbit secondary antibodies to detect the myc epitope tag. Scale bar represents 5 μm.
B. Close up confocal image showing SseJ association with SifA. Scale bar represents 1 μm.

shown), the loss of LAMP-1 labelling was not significantly different to that of the *sifA* single mutant in either host cell type (Fig. 8A). Therefore, bacterial replication does not seem to play a significant role in the loss of vacuolar membrane around *sifA* mutant bacteria.

We then determined if the loss of vacuolar membrane associated with mutation of *sifA* requires bacterial protein synthesis. HeLa cells were infected with the *sifA* mutant and then treated with tetracycline at 6 h post uptake, when onset of loss of the vacuolar membrane occurs (Beuzón *et al*., 2000). Cells were fixed at 10 h and labelled for LAMP-1. Whereas only 27 ± 11.3% of *sifA* mutant bacteria were associated with LAMP-1 at 10 h, this increased to 82 ± 13.7% after exposure to tetracycline, a level similar to that of wild-type bacteria that had not been exposed to the antibiotic (Fig. 8B). Similar results were obtained in RAW macrophages (Fig. 8B). Therefore, loss of the vacuolar membrane around *sifA* mutant bacteria does require bacterial protein synthesis.

Loss of vacuolar membrane around sifA *mutant bacteria requires SseJ*

As myc::SseJ inhibited Sif formation in transfected, infected cells, this suggested that SifA and SseJ might have opposing effects on *Salmonella*-directed membrane dynamics. To investigate if SseJ accounts for the loss of vacuolar membrane around *sifA* mutant bacteria, we analysed the effect of the *sseJ* mutation in the absence of SifA. We constructed a *sifA sseJ* double mutant strain, and compared its level of association with LAMP-1 with that of a *sifA* single mutant in HeLa cells at 10 h after bacterial entry. In contrast to the *sifA* mutant, the majority of *sifA sseJ* double mutant bacteria remained within LAMP-1-positive compartments, although the overall percentage of bacteria associated with the marker was slightly lower than for wild-type or *sseJ* mutant bacteria (Fig. 9). An increased association of the double mutant with LAMP-1 over the *sifA* mutant was also observed in RAW macrophages (results not shown). These results show that a functional SseJ protein contributes to the loss of the *Salmonella* vacuolar membrane in the absence of SifA.

The presence of a vacuolar membrane around the *sifA sseJ* double mutant raised the possibility that SseJ compensates for the absence of SifA, both in terms of loss of vacuolar membrane, and for the strong virulence defect of the *sifA* mutant (Table 1). To analyse this possibility, the virulence of the *sifA sseJ* double mutant strain was compared with that of the *sifA* single mutant by mixed infection (Beuzón *et al*., 2001). The resulting COI (0.75 ± 0.004) is significantly different from 1.0 ($P = 0.002$); therefore, the addition of the *sseJ* mutation to a strain carrying a *sifA* mutation not only fails to compensate for its virulence defect, but actually increases its level of attenuation slightly.

Discussion

In this study, we have investigated the function of the SseJ effector protein of the TTSS encoded by SPI-2. Many candidate effector proteins for this secretion system have been proposed (Hensel *et al*., 1998; Miao and Miller, 2000; Worley *et al*., 2000) but only SpiC and SifA have been demonstrated to act as effectors and have been functionally characterized to some degree. SpiC is an inhibitor of various aspects of endocytic trafficking (Uchiya *et al*., 1999), and SifA is required to maintain the integrity

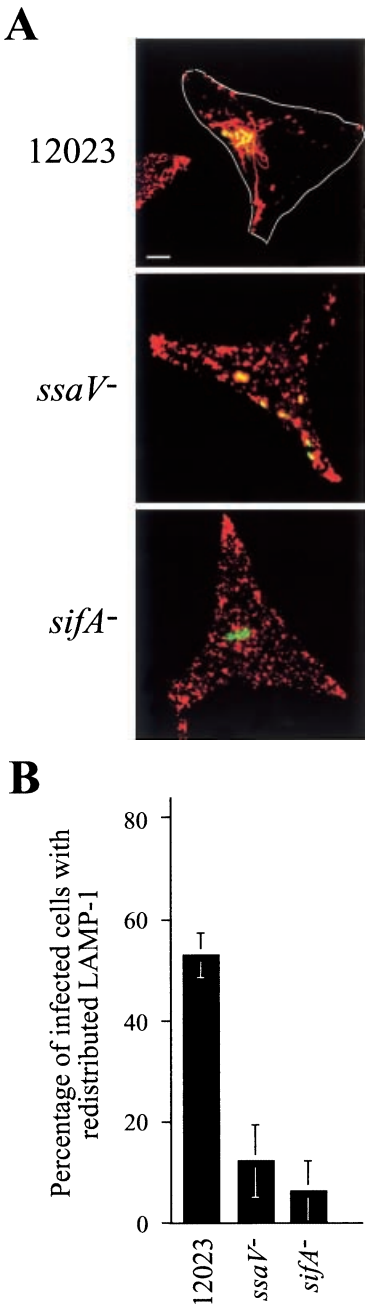

Fig. 7. Wild-type bacteria redistribute LAMP-1 in HeLa cells through the action of SifA.
A. Confocal immunofluorescence analysis of HeLa cells infected with wild-type strain (12023), *ssaV* mutant (HH109) and *sifA* (P3H6) strains. The cells were fixed 10 h after bacterial entry and labelled using a mouse monoclonal and TRSC-conjugated donkey anti-mouse secondary antibodies to detect LAMP-1 (red). All strains expressed GFP constitutively. For clarity, the wild-type infected cell (upper panel) has been outlined. Scale bar represents 5 μm.
B. Percentage of infected HeLa cells ($n = 50$) displaying redistributed LAMP-1. Results shown are the means ± standard errors of three independent experiments.

of the SCV membrane (Beuzón *et al*., 2000). Although strains carrying mutations in *sifB* and *sseI* had no detectable virulence defect by CI tests following i.p. inoculation of mice, it is possible that they contribute to SPI-2 TTSS function in ways that are not detectable by this assay. For example, they could function during stages of infection before colonization of the spleen, such as in intestinal epithelial cells or in Peyer's patches (Cirillo *et al*., 1998). Alternatively they could be required for pathogenesis of other *S. typhimurium* hosts, may have genetically redundant roles or make no significant contribution to *S. typhimurium* virulence.

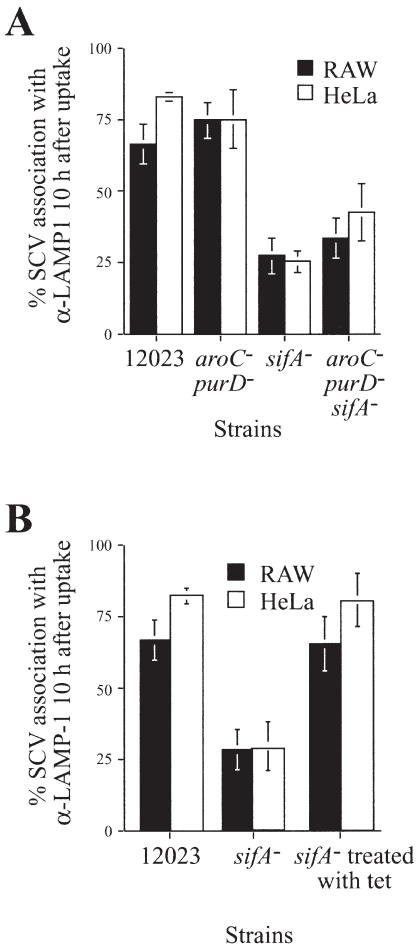

Fig. 8. Loss of vacuolar membrane from *sifA* mutant bacteria in HeLa cells requires bacterial protein synthesis but is not dependent on bacterial replication.
A. Percentage of bacteria ($n = 50$) associated with LAMP-1 in RAW macrophages (filled bars) and HeLa cells (empty bars) infected with wild-type (12023), *aroC purD* (HH208), *sifA* (P3H6) or *aroC purD sifA* (HH209) strains, 10 h after entry.
B. Percentage of bacteria ($n = 50$) associated with LAMP-1 in RAW macrophages (filled bars) and HeLa cells (empty bars) infected with wild-type (12023), *sifA* (P3H6) and *sifA* (P3H6), treated with tetracycline 6 h after uptake and determined 10 h after entry.
Results shown are the means ± standard errors of three independent experiments.

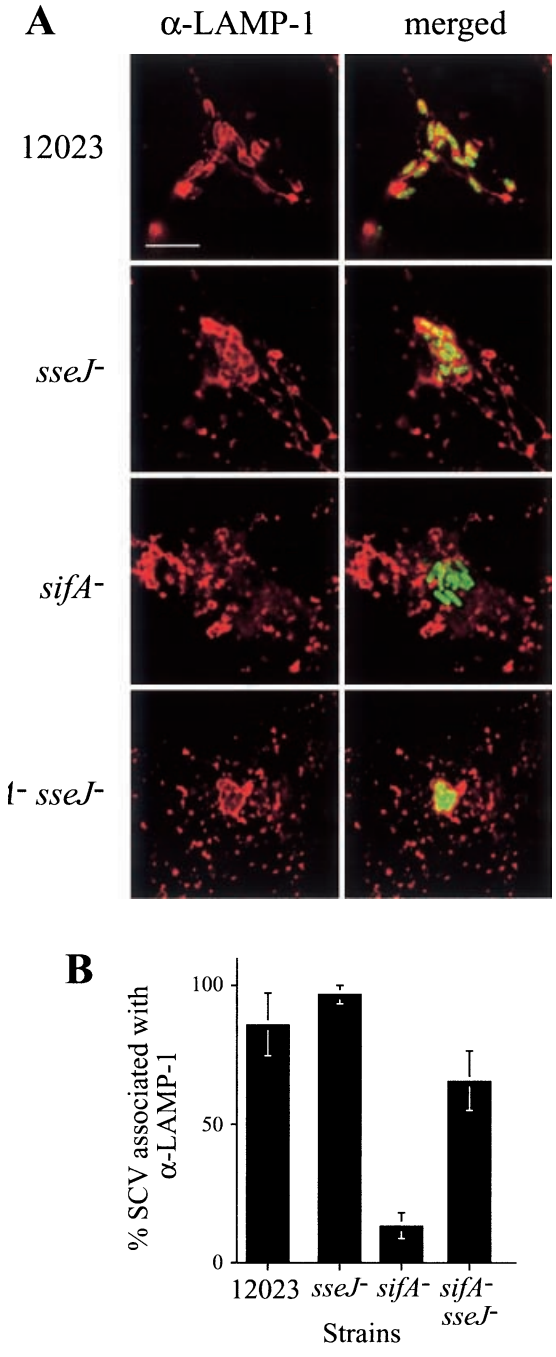

Fig. 9. The majority of *sifA sseJ* double mutant bacteria retain a vacuolar membrane.
A. Confocal microscopic analysis was carried out on HeLa cells infected with wild-type (12023), *sseJ* (HH200), *sifA* (P3H6) or *sifA sseJ* (HH202) strains constitutively expressing GFP, 10 h after entry. LAMP-1 (red) was detected using a mouse monoclonal and TRSC-conjugated donkey anti-mouse secondary antibodies. Scale bar represents 5 μm.
B. The percentage of bacteria ($n = 50$) associated with LAMP-1 was determined for these bacterial strains 10 h after entry in HeLa cells. Results shown are the means ± standard errors of three independent experiments.

This study was focused on SseJ because the corresponding mutant had a detectable virulence and macrophage-survival defect. The sequence similarity shows that SseJ is a member of the 'GDSL' family of acyltransferases/lipases (Upton and Buckley, 1995; Miao and Miller, 2000), and its function was explored further by expression in epithelial cells, and examining its effects on markers of SCV membranes. Its effects were also compared with those of a mutant version containing a single amino acid substitution of the predicted nucleophilic serine of the catalytic triad, to abolish its enzymatic activity. Expression of the wild-type, but not the mutant form of this protein, resulted in the formation of novel globular structures (GMCs), whose composition appears to be similar to that of Sifs and SCV membranes. The distribution of Golgi and lysosomal markers appeared to be normal, suggesting that overexpression of SseJ did not perturb the biosynthetic or terminal endocytic machinery of these cells.

GMCs contain F-actin and membranes enriched in cholesterol, vATPase, LAMP-1 and LIMP-1. These structures are globular, up to 1 μm in diameter, and are surrounded by SseJ, which itself seems to be membrane-associated. Further detailed analysis of the organization of these structures will require electron microscopy and possibly biochemical fractionation. In view of their composition, GMCs may be partly of late-endosomal origin. The presence of F-actin in GMCs raises the possibility that SPI-2-dependent actin assembly on the SCV (Méresse *et al*., 2001) could be an inherent property of specific membranes recruited through the action of the SPI-2 TTSS, rather than a result of F-actin nucleation on the SCV membrane induced by a SPI-2 effector protein. The absolute dependence of GMCs on the serine residue of the putative active site strongly suggests that they arise by acyltransferase/lipase activity of this protein. Determination of the intracellular substrate of SseJ will help to elucidate the biogenesis of GMCs and, more importantly, to understand its role in regulating membrane dynamics of the SCV.

Although GMCs are unlikely to have any physiological significance (as no structures resembling these compartments have been observed in cells infected with wild-type *S. typhimurium*), their similar composition to SCV membranes and Sifs; (García-del Portillo *et al*., 1993; García-del Portillo and Finlay, 1995; Beuzón *et al*., 2000) is noteworthy. The fact that transfection of HeLa cells with a vector encoding SifA leads to extensive vacuolation of LAMP-1$^+$, vATPase$^+$ and cathepsin D$^-$ compartments (Brumell *et al*., 2001a) suggests that SseJ and SifA may target the same endocytic compartments but act on them differently. This observation, together with the effects of ectopic expression of SseJ on SCVs and Sifs in infected

cells, led us to speculate that SseJ and SifA might have related functions.

We have shown previously that an *S. typhimurium ssaV* mutant, which is unable to secrete any proteins (including SifA) through the SPI-2 TTSS, does not lose its vacuolar membrane (Beuzón *et al*., 2000). As the SPI-2 effector SpiC is thought to inhibit interactions between SCVs and endosomal compartments (Uchiya *et al*., 1999), we proposed that the vacuolar membrane around an *ssaV* mutant could, in contrast with that of the wild-type strain, be derived from interactions with the late endocytic pathway (Beuzón *et al*., 2000). Two findings have led us to revise this view. First, analysis of the trafficking of a SPI-2 TTSS null mutant in macrophages showed that this strain had a low level of association with late endosomes and lysosomes, similar to that of wild-type bacteria (Garvis *et al*., 2001). Hence, it seems unlikely that a SPI-2 null mutant would have greater access to these endocytic compartments than wild-type bacteria. Second, we show here that loss of vacuolar membrane from *sifA* mutant bacteria requires the action of SseJ. Therefore, the presence of SCV membrane around *ssaV* mutant bacteria could be explained simply by an inability to translocate SseJ.

Although the *sifA sseJ* double mutant retained a vacuolar membrane, we can rule out the possibility that the *sseJ* mutation has simply compensated for the loss of *sifA*, because the *sifA sseJ* double mutant was severely attenuated in virulence compared to wild-type bacteria, and was slightly more attenuated than a *sifA* mutant. Therefore, although the double mutant strain resides within a vacuole, the properties of this vacuole must be abnormal, and restrictive for bacterial growth.

What is the role of SseJ in relation to the SPI-2 TTSS and intracellular replication of *S. typhimurium*? To replicate intracellularly yet remain within a vacuole, *S. typhimurium* must ensure a net recruitment of membrane to accommodate the growing bacterial population. Intracellular *S. typhimurium* cells are closely associated with vacuolar membrane and often appear to be in distinct vacuoles (for example, see Fig. 9A). Therefore, the controlled expansion and separation of the vacuolar membrane are likely to be important activities regulated by *S. typhimurium*. Although we have not identified its (presumably lipid) substrate, the amino acid sequence and differential effects of the wild-type and mutant proteins in host cells indicate that SseJ is an acyltransferase/lipase. SseJ localizes to the SCV membrane and is required for the loss of this membrane around *sifA* mutant bacteria. SifA is required for the redistribution of LAMP-1 containing vesicles to the vicinity of the bacterial microcolony (Fig. 7), in which a proportion is presumably incorporated into the SCV membrane. The SCV membrane is likely to be a highly dynamic structure, undergoing fusion with selected vesicular compartments, and also modification, budding and scission. SseJ could be required for modification of SCV membrane lipid(s), to facilitate one or more of these processes. The lack of a dramatic intracellular phenotype for the *sseJ* single mutant indicates that its function is likely to be highly localized, specific and to involve other proteins, which may compensate in part for its absence in the *sseJ* mutant strain.

Experimental procedures

Bacterial strains and growth conditions

The *Salmonella typhimurium* strains used in this study are listed in Table 2. Bacteria were grown in Luria–Bertani (LB) medium supplemented with ampicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), chloramphenicol (35 μg ml^{-1} for plasmid-containing strains, 10 μg ml^{-1} for chromosomal integrants) and tetracycline (15 μg ml^{-1}), for strains resistant

Table 2. *S. typhimurium* strains used in this study.

Name	Description	Source or reference
12023s	Wild-type	NTCC
12023 Nal^r	Nalidixic acid-resistant spontaneous mutant	Shea *et al*. (1999)
12023 Sm^r	Streptomycin-resistant spontaneous mutant	Shea *et al*. (1999)
12023 Nal^r Sm^r	Nalidixic acid-resistant, streptomycin-resistant spontaneous mutant	This study
HH109	*ssaV*::*aphT* (Km^r) in 12023s	Deiwick *et al*. (1999)
P3H6	*sifA*::mTn*5* (Km^r) in 12023s	Beuzón *et al*. (2000)
TT311	*purD*::Tn*10* (Tet^r) in LT2	John Roth
ST030	Δ*aroC* in 12023	Microscience Ltd
HH197	Δ*sifB*::*cat* (Cm^r) in 12023 Nal^r Sm^r	This study
HH199	*sseI*::pGP704 (Amp^r) in 12023 Nal^r	This study
HH200	*sseJ*:: pGP704 (Amp^r) in 12023 Nal^r	This study
HH201	*srfJ*:: pGP704 (Amp^r) in 12023 Nal^r	This study
HH202	*sseJ*:: pGP704 (Amp^r) *sifA*::mTn*5* (Km^r) in 12023 Nal^r	This study
HH208	Δ*aroC purD*:: Tn*10* (Tet^r) in 12023s	This study
HH209	Δ*aroC purD*:: Tn*10* (Tet^r) *sifA*::mTn*5* (Km^r) in 12023s	This study
HH210	Δ*sseJ* in 12023s	This study

to these antibiotics (Amp^r, Km^r, Cm^r, and Tet^r respectively). Insertion mutations in the *sseI*, *sseJ* and *srfJ* genes were obtained by site-directed chromosomal integration of the suicide plasmid pGP704 containing a short portion of the 5′-region of the corresponding coding sequence, as described previously (Miller and Mekalanos, 1988). The fragments of *sseI*, *sseJ* and *srfJ* ligated into pGP704 did not extend beyond the regions encoding the signal for translocation, and were obtained by polymerase chain reaction (PCR) from *S. typhimurium* strain 12023 genomic DNA, using the primers SSEI-1/–2, SSEJ-1/–2 and SRFJ-1/–2 respectively (Table 3). Mutations were transduced by phage P22 as described previously (Davis *et al*., 1980) into nalidixic acid-resistant strain 12023 (Shea *et al*., 1999) before use in this study. The stability of the mutations was verified after each infection experiment by plating on LB, and LB with the appropriate antibiotic. A deletion mutant for the *sseJ* gene was obtained following the method described by Datsenko and Wanner (2000), using SSEJ-CAT1 and SSEJ-CAT2 as primers. A non-polar deletion mutation in *sifB* was obtained by allelic exchange. The entire *sifB* gene and its flanking regions were amplified by PCR from *S. typhimurium* strain 12023 genomic DNA, using the primers SIFB-2 and SIFB-3. The 1.7 kb PCR product was ligated into pCRII-TOPO (Invitrogen), generating plasmid pID829. This plasmid was used as a template for an inverse PCR using the primers SIFB-6 and SIFB-7. The 5.1 kb PCR product, containing terminal *Sma*I sites, was digested and re-ligated to generate plasmid pID830. The *Sma*I fragment of pSL1 (Lukomski *et al*., 1996) containing a chloramphenicol resistance cassette without promoter or terminator sequences was ligated into the *Sma*I site of plasmid pID830, to generate plasmid pID831. This plasmid was digested with *Bam*HI and *Not*I, and ligated into the *Bgl*II–*Not*I sites of the suicide vector pKAS32 (Skorupski and Taylor, 1996) to generate plasmid pID832. The resulting plasmid pID832 was transferred by conjugation from *Escherichia coli* S17–1λpir to *S. typhimurium*, and exconjugants were selected as described previously (Shea *et al*., 1999).

Double and triple mutant strains were constructed by P22-mediated transductions (Davis *et al*., 1980).

Plasmids

Plasmid pFVP25.1, carrying *gfpmut3A* under the control of the *rpsM* constitutive promoter (Valdivia and Falkow, 1997), was introduced into bacterial strains and used for fluorescence visualization when indicated. The complementing plasmid p*sseJ* is a derivative of pACYC184 (Chang and Cohen, 1978) carrying the *sseJ* gene under the control of a constitutive promoter. A DNA fragment including the complete open reading frame (ORF) of *sseJ* and its ribosomal binding site (rbs) was amplified using PCR from 12023s genomic DNA using SSEJ-C1 and SSEJ-C2 primers. The 1.2 kb PCR product, containing terminal *Bam*HI and *Sal*I sites, was digested and ligated into pACYC184 within the Tet^R gene, generating p*sseJ*. The expression plasmid p*myc*::*sseJ* was constructed as follows: a DNA fragment including the complete ORF of *sseJ* was amplified using PCR from genomic DNA using SSEJ3-AB and SSEJ5-AB primers. The 1.2 kb PCR product, containing terminal *Bam*HI and *Eco*RI sites, was digested and ligated into the plasmid pRK5-myc (Lamarche *et al*., 1996), within the same reading frame as that of the c-myc epitope tag of the plasmid, generating p*myc::sseJ*. This plasmid expresses a full-length version of SseJ bearing an N-terminal fusion to the c-myc epitope tag. Following the same strategy, p*myc*::*sifB* was constructed using SIFB3-AB and SIFB5-AB primers.

The expression plasmid p*myc*::*sseJ*$_{S151V}$ was constructed as follows: two PCRs with genomic DNA as template were performed, using SSEJ5-AB with SSEJ3-S151V and SSEJ3-AB with SSEJ5-S151V as primers. The products of the two PCRs were used as templates for another PCR, using SSEJ3-AB and SSEJ5-AB as primers. The resulting PCR

Table 3. Oligonucleotide primers used in this study.

Name	Nucleotide sequence (5′–3′)
SSEJ-C1	ATGGATCCGTAAGGAGGACACTATGCC
SSEJ-C2	AAGTCGACTTTTGCTCAAGGCGTACCG
SSEJ-GFPC1	ACGAATTCGCCATTGAGTGTTGGACAGG
SSEJ-GFPC2	CCGGGATCCTTATTCAGTGGAATAATGATGAGC
SRFJ-1	GCGTCTAGAACTCATCTCTTCCGATCCG
SRFJ-2	CACGAGCTCATGATATCGGCCCAGTCGG
SSEI-1	GCGTCTAGATTGGAAGCGGATGTCTTCC
SSEI-2	CACGAGCTCTCCACTCCCGGTTCTGTTG
SSEJ-1	GCGTCTAGACATTGAGTGTTGGACAGGG
SSEJ-2	CACGAGCTCGAGTCAGACAAGCTGTCGC
SIFB-2	GGATCCTTTGAGCCTCCTCGCAGG
SIFB-3	CTCGAGATGCCACCTTCCAGTTACG
SIFB-6	CCCGGGTGTGACTGGAAGGAACAG
SIFB-7	CCCGGGAGGAGCGTTGAGAGATAGC
SIFB5-AB	CTCGGATCCATGCCAATTACTATCGGGAGAGGA
SIFB3-AB	CTCGAATTCTCAACTCTGATGAGCCTCAAT
SSEJ5-AB	CTCGGATCCATGCCATTGAGTGTTGGACAGGGT
SSEJ3-AB	CTCGAATTCTTATTCAGTGGAATAATGATGAGC
SSEJ5-S151V	TTTTGGCGACGTCTTGTCTGACTCCTTAG
SSEJ3-S151V	GGAGTCAGACAAGACGTCGCCAAAAAATA
SSEJ-CAT1	TTGCTAAAGCGTGTTTAATAAAGTAAGGAGGACACTATGCCATTGAGTGTGTAGGCTGGAGCTGCTTC
SSEJ-CAT2	TTATTCAGTGGAATAATGATGAGCTATAAAACTTTCTAACATTATGGCCATATGAATATCCTCCTTAGT

product was digested and ligated into the plasmid pRK5-myc, into the same reading frame as the c-myc epitope tag coding sequence included in the plasmid, generating p*myc*::*sseJ*$_{S151V}$. This plasmid expresses a full-length version of SseJ bearing an N-terminal fusion to the c-myc epitope tag, and where the AGC codon encoding serine residue 151 of SseJ has been replaced with a GTC codon, encoding valine. Expression plasmid p*gfp*::*sseJ* was constructed as follows: a DNA fragment including the complete ORF of *sseJ* was amplified using PCR from 12023s genomic DNA using SSEJ-GFPC1 and SSEJ-GFPC2 primers. The 1.2 kb PCR product, containing terminal *Eco*RI and *Bam*HI sites, was digested and ligated into the plasmid pEGFP-C1 (Clontech) into the same reading frame as the *gfpmut1* coding sequence included in the plasmid, generating p*gfp*::*sseJ*. This plasmid expresses a full-length version of SseJ bearing an N-terminal fusion to the green fluorescent protein (GFP). All the constructions were verified by DNA sequencing.

Mouse mixed infections

Female BALB/c mice (20–25 g) were used for Competitive Index studies (CIs and COIs). At least three mice were inoculated i.p. with a mixture of two strains comprising $\approx 5 \times 10^4$ colony-forming units (cfus) of each strain in physiological saline, and the CIs or COIs were determined from spleen homogenates 48 h post inoculation as described previously (Beuzón *et al*., 2001).

Statistical analysis

Each CI or COI value is the mean of at least three independent mice infections. Student's *t*-test was used to analyse the CIs or COIs of single mutant strains versus the wild-type strain (wt v a^-) with the null hypothesis: mean is equal to 1.0. Probabilities (*P*) of 0.05 or less were considered significant.

Antibodies and reagents

The mouse monoclonal anti-c-myc antibody 9E10 was provided by E. Caron (London University College) and was used at a dilution of 1:200. Rabbit polyclonal anti-c-myc antibody PRB-150C was purchased from Babco Laboratories (Richmond, CA) and was used at a dilution of 1:100. The mouse monoclonal antibodies anti-LAMP-1 H4A3 and anti-CD63 H5C6, developed by J. T. August and J. E. K. Hildreth, were 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, IA), and were used at a dilution of 1:1000. Anti-vATPase mouse monoclonal antibody OSW2 was obtained from Dr S. B. Sato (Kyoto University, Kyoto, Japan) and was used at a dilution of 1:1000. Anti-LAMP-1 rabbit polyclonal antibody 156 (Steele-Mortimer *et al*., 1999) was used at a dilution of 1:1000. 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. Anti-cathepsin D rabbit polyclonal antibody RC242 was purchased from Scripps Laboratories (San Diego, CA) and was used at a dilution of 1:1000. The rabbit polyclonal antibodies anti-GM130, anti-P115, anti-P14Kβ and antigiantin were obtained from Dr A. De Matteis (Consorzio Mario Negri SUD, Chieti, Italy) and used at dilutions of 1:1000, 1:500, 1:2000 and 1:2000 respectively. Texas red sulphonyl chloride (TRSC)- and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse and anti-rabbit antibodies, and cyanine 5 (Cy5)-conjugated donkey anti-goat antibody were purchased from Jackson Immunoresearch Laboratories (West Grove, PA), and used at a dilution of 1:400. TRSC-conjugated phalloidin was purchased from Molecular Probes and used at a dilution of 1:50. The fluorescent cholesterol binding drug filipin was obtained from Dr D. Young (Imperial College, London, UK) and used at a concentration of 50 μg ml^{-1}.

Cell culture

RAW 264.7 cells were obtained from ECACC (ECACC 91062702). HeLa (clone HtTA1) cells were kindly provided by Dr H. Bujard (Heidelberg, Germany). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine at 37°C in 5% CO_2. Peritoneal cells were harvested 4 days after BALB/c mice were inoculated i.p. with 5 mM periodate (De Groote *et al*., 1997). Cells were plated at a density of 5.5×10^5 cells per well in 24-well microtitre dishes and allowed to adhere for 2 h. Non-adherent cells were flushed out with pre-warmed RPMI containing 10% FCS. The adherent macrophages were incubated for a further 48 h before infection.

Bacterial infection of cultured cells and replication assays

HeLa cells were infected with exponential phase *S. typhimurium* as described previously (Beuzón *et al*., 2000). Macrophages were infected with opsonized, stationary phase *S. typhimurium* as described previously (Beuzón *et al*., 2000). To follow a synchronized population of bacteria, host cells were washed after 15 min (HeLa cells) or 30 min (macrophages) of exposure to *S. typhimurium* and subsequently incubated in medium containing gentamicin to kill extracellular bacteria. For enumeration of intracellular bacteria, macrophages were washed three times with phosphate-buffered saline (PBS), lysed with 0.1% Triton X-100 for 10 min and a dilution series was plated onto LB agar. Tetracycline was used at a final concentration of 15 μg ml^{-1} when indicated, and was shown to prevent bacterial replication, but did not affect bacterial viability over the course of the experiment.

Immunofluorescence microscopy

For immunofluorescence, cell monolayers were fixed in 3.7% paraformaldehyde in PBS, pH 7.4, for 15 min at room temperature and washed three times in PBS, except when using the anti-cathepsin D polyclonal antibody when cells were fixed as described by Brumell and colleagues (Brumell *et al*.,

2001b). Antibodies were diluted in 10% horse serum; 0.1% saponin in PBS. Coverslips were washed twice in PBS containing 0.1% saponin, incubated for 30 min with primary antibodies, washed twice with 0.1% saponin in PBS and incubated for 30 min with secondary antibodies. Coverslips were washed twice in 0.1% saponin in PBS, once in PBS and once in H_2O, and mounted in Mowiol. Samples were analysed using a fluorescence microscope (BX50; Olympus Optical) or a confocal laser scanning microscope (LSM 510, Zeiss).

Transfection of HeLa cells

HeLa cells were transiently transfected by the calcium phosphate DNA precipitation method (Matthias *et al*., 1982), using 2 μg of plasmid DNA per 60 mm-diameter Petri dish, with each dish containing 6×10^5 cells. Then, 24 h after DNA addition, coverslips were collected and washed three times with PBS, and fixed for immunofluorescence microscopy. For the double transient transfection, equivalent amounts of plasmid DNA from p*myc*::*sseJ* and p*sifA*::*gfp* (Beuzón *et al*., 2000) were mixed and used for transfection as described. Bacterial infection of transfected HeLa cells was performed as described above, 14 h after DNA addition, and cells were fixed 24 h after DNA addition (10 h after infection).

Acknowledgements

We are very grateful to Lalita Ramakrishnan for suggesting the use of auxotrophic mutants to investigate the relationship between vacuolar membrane loss and bacterial replication. We would like to thank Steve Garvis for help with the tetracycline experiments, and Mei Liu for practical assistance. We also thank Stéphane Méresse for plasmid psifA::gfp, Josep Casadesús for strain TT311, Microscience Ltd for strain ST030 and Kate Unsworth for helpful comments on the manuscript. This work was supported by grants from the MRC to David Holden, and from the BBSRC to Edouard Galyov. Abigail Blakey was supported by a BBSRC postgraduate studentship.

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