The EMBO Journal Vol. 19 No. 13 pp. 3235–3249, 2000

Salmonella maintains the integrity of its intracellular vacuole through the action of SifA

Carmen R.Beuzón, Stéphane Méresse[1], Kate E.Unsworth, Javier Ruíz-Albert, Steve Garvis, Scott R.Waterman, Timothy A.Ryder[2], Emmanuel Boucrot[1] and David W.Holden[3]

Department of Infectious Diseases, Imperial College School of Medicine, Du Cane Road, London W12 0NN, [2]Electron Microscopy Unit, Queen Charlotte's and Chelsea Hospital, Goldhawk Road, London W6 0XG, UK and [1]Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Case 906, 13288 Marseille Cedex 9, France

[3]Corresponding author
e-mail: d.holden@ic.ac.uk

A method based on the Competitive Index was used to identify *Salmonella typhimurium* virulence gene interactions during systemic infections of mice. Analysis of mixed infections involving single and double mutant strains showed that OmpR, the type III secretion system of *Salmonella* pathogenicity island 2 (SPI-2) and SifA [required for the formation in epithelial cells of lysosomal glycoprotein (lgp)-containing structures, termed Sifs] are all involved in the same virulence function. *sifA* gene expression was induced after *Salmonella* entry into host cells and was dependent on the SPI-2 regulator *ssrA*. A *sifA*$^-$ mutant strain had a replication defect in macrophages, similar to that of SPI-2 and *ompR*$^-$ mutant strains. Whereas wild-type and SPI-2 mutant strains reside in vacuoles that progressively acquire lgps and the vacuolar ATPase, the majority of *sifA*$^-$ bacteria lost their vacuolar membrane and were released into the host cell cytosol. We propose that the wild-type strain, through the action of SPI-2 effectors (including SpiC), diverts the *Salmonella*-containing vacuole from the endocytic pathway, and subsequent recruitment and maintenance of vacuolar ATPase/lgp-containing membranes that enclose replicating bacteria is mediated by translocation of SifA.

Keywords: Competitive Index/SPI-2/trafficking/type III secretion/vacuole

Introduction

Systemic infection of mice by *Salmonella typhimurium* is a useful and intensively studied model of typhoid fever. Following oral inoculation, bacteria can survive the acid pH of the stomach, adhere to and invade cells of the intestinal epithelium, acquire nutrients, survive in the blood, replicate within macrophages and induce cytotoxicity (Finlay, 1994; Richters-Dahlfors *et al*., 1997). Numerous genes that contribute to *S.typhimurium* virulence have been identified in recent years (Groisman and Ochman, 1997). Although the biochemical functions of some of these genes have been elucidated, little is known about their regulation *in vivo* and how they interact during the infectious process. Many virulence genes are clustered together on 'pathogenicity islands' (PAIs), which appear to have been acquired by horizontal transfer from unknown sources. SPI-1 and SPI-2 are two *Salmonella* PAIs that encode structurally similar but functionally distinct type III secretion systems (TTSSs) which translocate virulence proteins from bacterial to host cells during the infectious cycle (Hueck, 1998). The SPI-1 encoded TTSS, called Inv/Spa, plays an important role in invasion of epithelial cells (Galán and Curtiss, 1989; Galán, 1996). Most of the genes associated with Inv/Spa are encoded within SPI-1 at 63 centisomes (cs) on the chromosome (Mills *et al*., 1995), but at least two of the secreted effector proteins are encoded elsewhere: the *sopB* gene is present on the SPI-5 pathogenicity island (Wood *et al*., 1998) and SopE is encoded by a temperate bacteriophage (Hardt *et al*., 1998).

SPI-2, located at 30 cs, encodes the second type III secretion system (Ochman *et al*., 1996; Shea *et al*., 1996). The SsrA/B two-component regulatory system of SPI-2 is required for SPI-2 gene expression (Valdivia and Falkow, 1997; Cirillo *et al*., 1998; Deiwick *et al*., 1999). Recent work has shown that in cultured host cells, transcription of *ssrA* is modulated by OmpR, a two-component regulatory system protein which responds to changes in osmolarity, pH and temperature (Lee *et al*., 2000).

The SPI-2 secretion system plays a crucial role in systemic growth of *Salmonella* in its host (Hensel *et al*., 1995; Shea *et al*., 1996) and is required for bacterial proliferation in macrophages (Ochman *et al*., 1996; Cirillo *et al*., 1998; Hensel *et al*., 1998). *Salmonella typhimurium* replicates intracellularly within a vacuole that diverts from the normal phagocytic pathway (Méresse *et al*., 1999b). The *Salmonella*-containing vacuole (SCV) rapidly loses early endocytic markers such as EEA1 and the transferrin receptor (Steele-Mortimer *et al*., 1999) and acquires some lysosomal membrane glycoproteins (lgps), but does not interact extensively with either the early endocytic pathway or mature lysosomes (Garcia del Portillo and Finlay, 1995; Rathman *et al*., 1997). After uptake into macrophages, the SCV undergoes acidification to a pH between 4.0 and 5.0 (Rathman *et al*., 1996). SPI-2 mediated secretion can be induced *in vitro* by acidic conditions, suggesting that pH could be a physiological signal for SPI-2 secretion (Beuzón *et al*., 1999; Lee *et al*., 2000). Only one SPI-2 gene (*spiC/ssaB*) has been demonstrated to encode an effector protein. SpiC is reported to inhibit fusion of SCVs with lysosomes, homotypic fusion of endosomes and transferrin recycling (Uchiya *et al*., 1999). Two leucine-rich repeat proteins, SspH-1 and SspH-2, which are encoded outside SPI-2, have also been identified as targets of this secretion system (Miao *et al*., 1999),

although their functions remain unknown. This raises the possibility that, as for the Inv/Spa system, other effectors may be encoded elsewhere on the chromosome.

To identify virulence genes that interact during infection, we have developed a method based on the Competitive Index (C.I.), in which single and double mutant strains are analysed by mixed infections of mice. In this paper we describe the application of this approach to SPI-2, *ompR* and *sifA* of *S.typhimurium.* C.I. analysis indicates that these three loci interact during systemic infection. The *sifA* gene is required for the formation in epithelial cells of lgp-containing tubular membrane structures termed Sifs (Garcia del Portillo *et al*., 1993b; Stein *et al*., 1996). We show that expression of *sifA* is strongly induced after *Salmonella* enters host cells and this induction is dependent on *ssrA*. Although Sifs are not detectable in infected macrophages, SifA function is important in these cells because a *sifA*$^-$ mutant strain has a strong replication defect in macrophages. Several hours after uptake, the majority of *sifA*$^-$ mutant bacteria lose their vacuoles and are found in the host cell cytosol. We conclude that SifA has an important role in the maintenance of the vacuolar membrane surrounding wild-type bacterial cells.

Results

Analysis of virulence gene interactions in vivo

The method we have developed for identifying virulence gene interactions relies on the additive effect of mutations in virulence genes with different functions. Because *Salmonella* virulence is multifactorial, combination of mutations in genes with different functions results in strains with increased attenuation (Baumler *et al*., 1997; Shea *et al*., 1999).

The C.I. is a sensitive measure of the relative degree of virulence attenuation of a particular mutant in mixed infection with the wild-type strain. It is defined as the ratio of the mutant strain to the wild-type in the output divided by the ratio of the two strains in the input (Freter *et al*., 1981; Taylor *et al*., 1987).

To establish if mutations in two different virulence genes have additive effects on virulence attenuation, we determined C.I. values for double mutant versus single mutant strains. In the case of two genes that contribute equally to the same virulence function (by encoding essential components of a macromolecular structure, for example), strains carrying mutations in either single gene would be expected to have the same level of attenuation as a strain carrying both mutations. Therefore, the C.I. of either single mutant versus the double mutant should be 1.0 (Figure 1, upper panel). In the case of two genes (*a* and *b*) with independent functions, the virulence attenuation of a double mutant strain will reflect the additive effect of both mutations (Figure 1, lower panel). The C.I. of a double mutant strain versus an *a*$^-$ strain will be equivalent to the C.I. of a *b*$^-$ strain versus the wild-type strain. Similarly, the C.I. of a double mutant strain versus a *b*$^-$ strain will be equivalent to the C.I. of an *a*$^-$ strain versus the wild-type strain, and all comparisons will have values of <1.0.

We have demonstrated previously that the C.I. of a *purD*$^-$ mutant strain of *S.typhimurium* (a purine auxotroph)

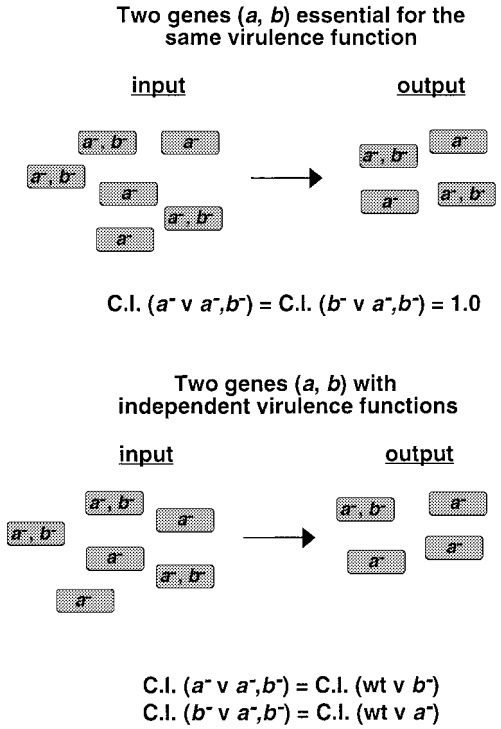


Fig. 1. Theoretical representation of Competitive Index (C.I.) analysis. C.I. is the c.f.u. ratio of double and single strains recovered from the infected animal divided by the c.f.u. ratio of double and single strains in the inoculum. Panels represent different degrees of functional relationship between two hypothetical genes (*a* and *b*) and the C.I. predicted for each case. In this case:

$$\text{C.I.} = \frac{a^-, b^-}{a^-}\ (\text{output}) \Big/ \frac{a^-, b^-}{a^-}\ (\text{input})$$

versus the wild-type strain is not statistically different from the C.I. of a *purD*$^-$, *ssaV*$^-$ double mutant strain versus an *ssaV*$^-$ single mutant (Shea *et al*., 1999), and this result was confirmed as a control for this study (Table I). The *ssaV* gene is thought to encode an inner membrane component of the SPI-2 secreton (Hensel *et al*., 1997), and is required for the secretion of SseB, which is probably a translocon component (Beuzón *et al*., 1999). When subjected to the analysis indicated in Figure 1, the C.I.s (Table I) confirm that *purD* and *ssaV* have totally unrelated functions, whereas *sseB* and *ssaV* are equally necessary for SPI-2 function.

SPI-2 and ompR interact in vivo

The OmpR/EnvZ two-component system responds to a variety of physicochemical changes in the environment (Heyde and Portalier, 1987; Thomas and Booth, 1992). Strains carrying mutations in *ompR/envZ* are highly attenuated in systemic infection of mice but mutations in genes previously known to be regulated by OmpR could not account for its virulence defect (Dorman *et al*., 1989; Chatfield *et al*., 1991). However, recently it has been shown that *ssrA* transcription is regulated by OmpR in infected host cells (Lee *et al*., 2000). Furthermore, we have found that a mutation in *ompR* reduces SseB levels during bacterial growth in host cells and certain laboratory media (results not shown). It was therefore of interest to determine if *ompR* interacts with the SPI-2 TTSS genes *in vivo*.

Table I. Competitive Index analysis of *S.typhimurium* mutants

Mixed infection[a]	C.I.[b]
Controls	
wt versus *ssaV*$^-$	0.006
wt versus *purD*$^-$	0.0005
ssaV$^-$ versus *ssaV*$^-$, *purD*$^-$	0.0038[d]
purD$^-$ versus *ssaV*$^-$, *purD*$^-$	0.019[d]
wt versus *ssaV*$^-$, *purD*$^-$	0.00006
wt versus *sseB*$^-$	0.016
ssaV$^-$ versus *ssaV*$^-$, *sseB*$^-$	1.2[c]
sseB$^-$ versus *ssaV*$^-$, *sseB*$^-$	0.82[c]
wt versus *ssaV*$^-$, *sseB*$^-$	0.014
ompR/envZ	
wt versus *ompR*$^-$	0.008
ssaV$^-$ versus *ssaV*$^-$, *ompR*$^-$	0.823[c]
ompR$^-$ versus *ssaV*$^-$, *ompR*$^-$	0.21[c]
wt versus *ssaV*$^-$, *ompR*$^-$	0.010
sifA	
wt versus *sifA*$^-$	0.012
sifA$^-$ p*sifA* versus *sifA*$^-$	0.074[d]
ssaV$^-$ versus *ssaV*$^-$, *sifA*$^-$	1.34[c]
sifA$^-$ versus *ssaV*$^-$, *sifA*$^-$	1.17[c]
wt versus *ssaV*$^-$, *sifA*$^-$	0.0019

[a]Strains used in the mixed infection experiments are referred to by the relevant genotype and are described in Table II. The *ssaV*$^-$ strain used was marked with either Km or Cm resistance cassettes to distinguish it from the competing strain.
[b]The C.I. was calculated as the output ratio of mutant or double mutant to wild-type or single mutant bacteria, divided by the input ratio. The C.I.s shown are the means of three independent infections of mice. Mice were inoculated i.p. with a mixture of two strains comprising ~5×10^4 c.f.u. of each strain. Mouse spleens were harvested after 48 h for enumeration of bacterial c.f.u. The different strains used were differentiated on the basis of antibiotic sensitivity. The C.I.s of double mutant versus single mutant strains were analysed by Student's *t*-test.
[c]Not significantly different from the C.I. of the corresponding single mutant strain versus wild-type strain.
[d]Not significantly different from 1.0 ($p < 0.05$).

The C.I. of an *ompR*$^-$ mutant strain versus the wild-type strain following intraperitoneal (i.p.) inoculation confirmed that its level of attenuation was similar to that of a typical SPI-2 mutant strain (Table I). To investigate possible interactions between *ompR* and SPI-2 genes *in vivo*, a double mutant strain was constructed carrying mutations in *ompR* and *ssaV*. The C.I. of this strain versus either single mutant strain was not significantly different from 1.0. This result implies that the virulence functions of the two genes are related, and the *in vitro* regulation of SPI-2 genes by *ompR* (Lee *et al*., 2000) also occurs *in vivo.*

SPI-2 and sifA interact in vivo

The *sifA* gene is located within the *potABCD* operon on the *Salmonella* chromosome, and was probably acquired by horizontal transfer from an unknown source (Stein *et al*., 1996). Strains carrying mutations in *sifA*, *ompR* or *envZ* are all defective for the formation of Sifs (Garcia-del Portillo *et al*., 1993b; Stein *et al*., 1996; Mills *et al*., 1998). *sifA*$^-$ mutant strains are reported to be attenuated in virulence following oral inoculation of mice, although they do not have a replication defect in epithelial cells (Stein *et al*., 1996). In competition experiments, the virulence of a strain carrying a transposon insertion in *sifA* was found to be as attenuated as an *sseB*$^-$ mutant following i.p. inoculation of mice, and this defect was complemented by introduction of the wild-type *sifA* allele on a plasmid (Table I). A *sifA*$^-$ mutation was therefore introduced to the *ssaV*$^-$ mutant strain and single and double mutant strains were analysed by C.I. The double mutant strain was no more attenuated than either single mutant (Table I). Therefore, the proteins encoded by these genes appear to be involved in the same important virulence function of *S.typhimurium* during systemic infection.

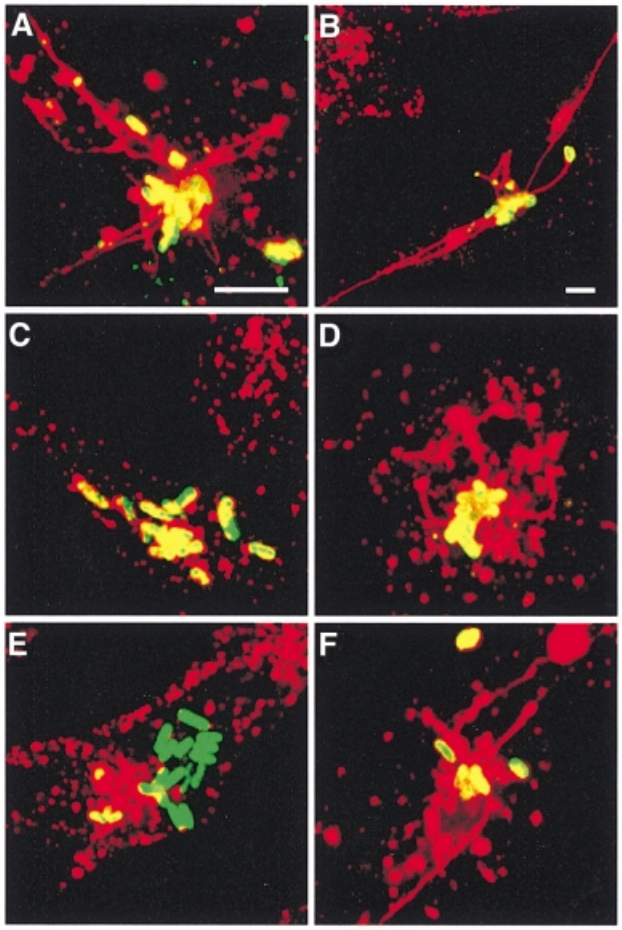
SPI-2 mutant strains fail to induce Sifs

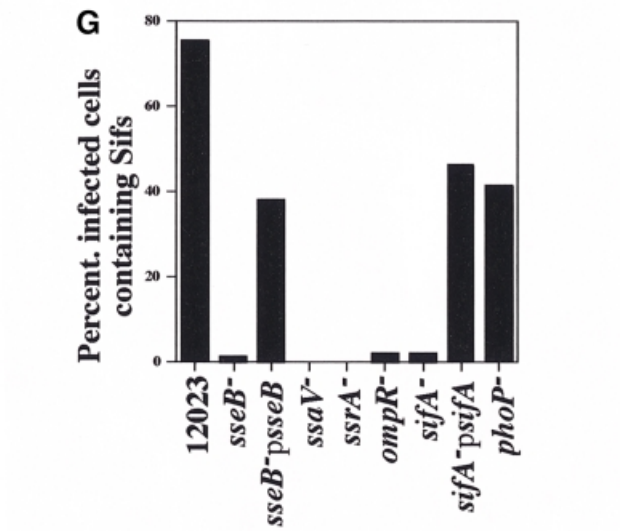
The genetic evidence linking SPI-2 and *sifA* (Table I) led us to ask if SPI-2 mutant strains could induce the formation of Sifs in epithelial cells. *Salmonella typhimurium* strains carrying a plasmid constitutively expressing green fluorescent protein (GFP) were used to infect HeLa cells, and these were subsequently stained with an anti-LAMP-1 antibody to reveal Sifs (Garcia-del Portillo *et al*., 1993b) (Figure 2). At 7 h post-invasion, >70% of cells infected with wild-type bacteria contained Sifs, whereas Sifs were detected in 1% or less of cells infected with strains carrying mutations in either *ssaV*, *ssrA* or *ompR*. Approximately 1% of cells infected with either *sseB*$^-$ or *sifA*$^-$ mutant strains contained Sifs, and the introduction of plasmids carrying functional genes to the respective mutant strains restored Sif formation to ~40% of infected cells. Similar results to those shown in Figure 2 were obtained when infected cells were examined 16 h after bacterial invasion, which shows that the onset of Sif formation is not simply delayed in SPI-2 mutants. It has been reported that *Salmonella* strains with non-auxotrophic mutations affecting replication inside host cells are incapable of inducing Sif formation (Garcia del Portillo *et al*., 1993a). Therefore, the intracellular replication defect of SPI-2 and *ompR*$^-$ mutant strains (Figure 4; Lee *et al*., 2000) could account for the lack of Sifs. A *phoP*$^-$ mutant strain was used to investigate this possibility. *phoP/Q* exerts regulatory control over at least 40 virulence genes of *Salmonella* (Miller and Mekalanos, 1990), is required for bacterial replication in macrophages, but functions independently of SPI-2 in host cells (Valdivia and Falkow, 1997; results not shown). The *phoP*$^-$ strain was nevertheless capable of inducing Sifs in ~40% of infected cells (Figure 2G). Therefore, we conclude that the failure of SPI-2 and *ompR*$^-$ mutants to induce Sifs is not simply a consequence of their replication defect, but is specifically connected with the function of these genes.

sifA expression requires the SPI-2 regulator ssrA

SPI-2 gene expression is induced following uptake of bacteria into host cells (Cirillo *et al*., 1998). The expression of some genes can be detected after 1 h (Uchiya *et al*., 1999), and maximal expression is achieved after 6 h (Cirillo *et al*., 1998). This expression is dependent on the SPI-2 two-component regulatory system SsrA/B (Cirillo *et al*., 1998; Hensel *et al*., 1998). In view of the phenotypic similarities between SPI-2 and *sifA*$^-$ mutant strains, it was of interest to know if their patterns of gene expression are similar. To analyse this, a plasmid was constructed carrying a *sifA::gfp* reporter fusion, transferred into different *S.typhimurium* strains, and expression of the fusion protein was examined during bacterial growth in HeLa cells. Wild-type bacterial cells expressed *sifA::gfp*

only when they were intracellular (Figure 3, top panel). Approximately 2% of bacterial cells expressed *sifA::gfp* as early as 1 h post-invasion. The percentage of bacterial cells expressing the fusion increased gradually thereafter, with ~90% of cells positive for GFP by 6 h post-invasion (results not shown). These kinetics are similar to those reported for SPI-2 (*ssaH::gfp)* expression (Cirillo *et al*., 1998). No *sifA::gfp* expression was detected in the *ssrA*⁻ mutant strain (Figure 3, middle panel). In *ompR*⁻ mutant strains, the number of cells expressing *sifA::gfp* was ~50% that of wild-type cells, and the overall level of expression was lower than that seen in wild-type cells, consistent with the effect of an *ompR*⁻ mutation on SPI-2 *ssaH* gene expression (Lee *et al*., 2000). As expected, the level of *sifA::gfp* expression in a *phoP*⁻ mutant strain was comparable to that of the wild-type strain (Figure 3).





A sifA mutant strain has a replication defect in macrophages

As most SPI-2 mutants have a replication defect inside host cells, and *sifA*⁻ mutants are attenuated in the systemic phase of infection, a *sifA*⁻ mutant strain was tested for replication in macrophage-like RAW cells. The *sifA*⁻ mutant strain was found to have a replication defect comparable to that of a strain carrying a mutation in *ssaV* (Figure 4). This result was surprising, since *sifA*⁻ strains are reported to be proficient for replication in HeLa cells (Stein *et al*., 1996). The introduction of the plasmid carrying a functional *sifA* gene to the *sifA*⁻ strain restored replication to wild-type levels (Figure 4E). This demonstrates that the replication defect is not due to a polar effect of the integrated transposon on the downstream *potC* gene, or an unrelated mutation. In these assays, the *ompR*⁻ mutant strain also displayed a replication defect, in agreement with a recent report (Lee *et al*., 2000).

Association of LAMP-1 with SCVs in RAW macrophages

Since *sifA* has an important role in intra-macrophage replication of *S.typhimurium* we investigated the distribution of LAMP-1 in RAW macrophages infected with different *S.typhimurium* strains. At different time points following bacterial uptake, cells were fixed and stained with an antibody against LAMP-1, then examined by confocal immunofluorescence microscopy. No structures resembling Sifs were observed at any time point in macrophages infected with wild-type bacteria. At 2 h post-uptake, LAMP-1 was associated with ~80% of wild-type, *ssaV*⁻ or *sifA*⁻ strains (Figure 5A, upper panel; Figure 5C). However, at 10 h post-uptake, LAMP-1 was present on >50% of vacuoles containing wild-type, and >70% of vacuoles containing *ssaV*⁻ bacteria, whereas <10% of the *sifA*⁻ bacteria were associated with this marker (Figure 5A, lower panel; Figure 5B). A time-course experiment

Fig. 2. Sif formation by *S.typhimurium* mutant strains in HeLa cells. Confocal immunofluorescence analysis of Sif formation in cells infected with: 12023 (**A** and **B**), *sseB*⁻ (**C**), *sseB*⁻ p*sseB* (**D**), *sifA*⁻ (**E**) and *sifA*⁻p*sifA* (**F**) strains. Cells were fixed at 7 h post-infection and stained with mouse anti-LAMP-1 and TRSC-conjugated donkey anti-mouse antibodies. In (A–E) the bacterial strains carried a plasmid expressing *gfp* constitutively. In (F), bacterial cells were detected by goat anti-*Salmonella* and FITC-conjugated donkey anti-goat antibodies. *sseB*⁻ and *sifA*⁻ mutant strains fail to induce Sifs (C and E). This defect can be complemented by the introduction of plasmids carrying the respective wild-type alleles (D and F). Bars correspond to 4 μm; the scale in (C–F) is equivalent to that of (A). (**G**) represents typical results from one of three experiments where cells (n = 50) infected by each *S.typhimurium* strain were evaluated for Sif formation at 7 h post-invasion. Values are given as percentage of infected cells containing Sifs.

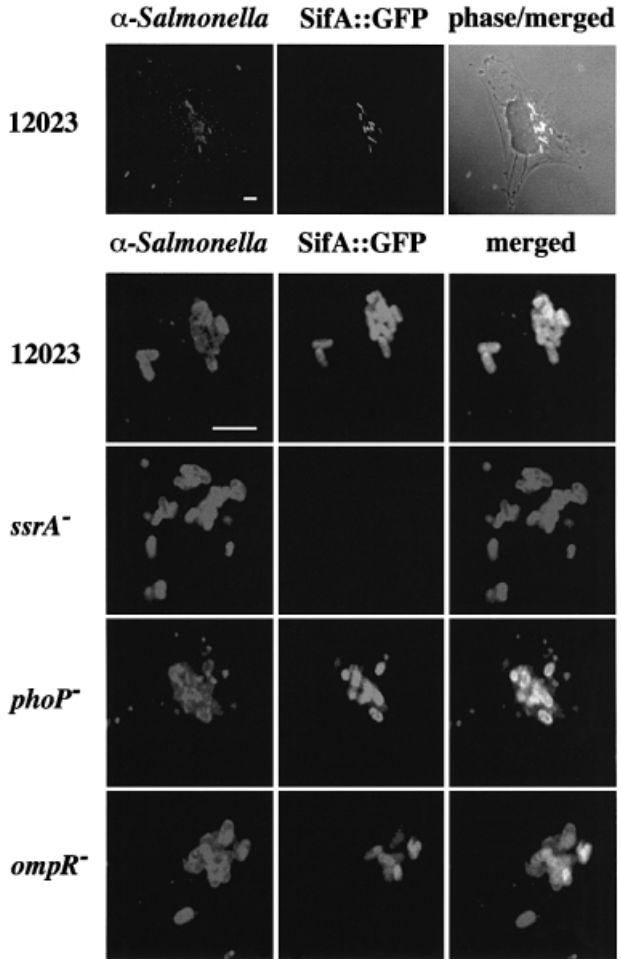


Fig. 3. *sifA*::*gfp* expression by *S.typhimurium* mutant strains in infected HeLa cells. Confocal immunofluorescence analysis in cells 6 h after invasion with different strains carrying a plasmid-borne *sifA*::*gfp* fusion (pID812). *Salmonella typhimurium* was detected with goat anti-*Salmonella*, and TRSC-conjugated donkey anti-goat antibodies. The upper panel shows three extracellular bacteria not expressing GFP, and several GFP-positive intracellular bacteria. SifA is expressed within HeLa cells in an *ssrA*-dependent manner (middle panel). SifA expression is not affected by a *phoP*$^-$ mutation but was only detected in ~50% of bacterial cells carrying the *ompR*$^-$ mutation. Bars correspond to 4 μm.

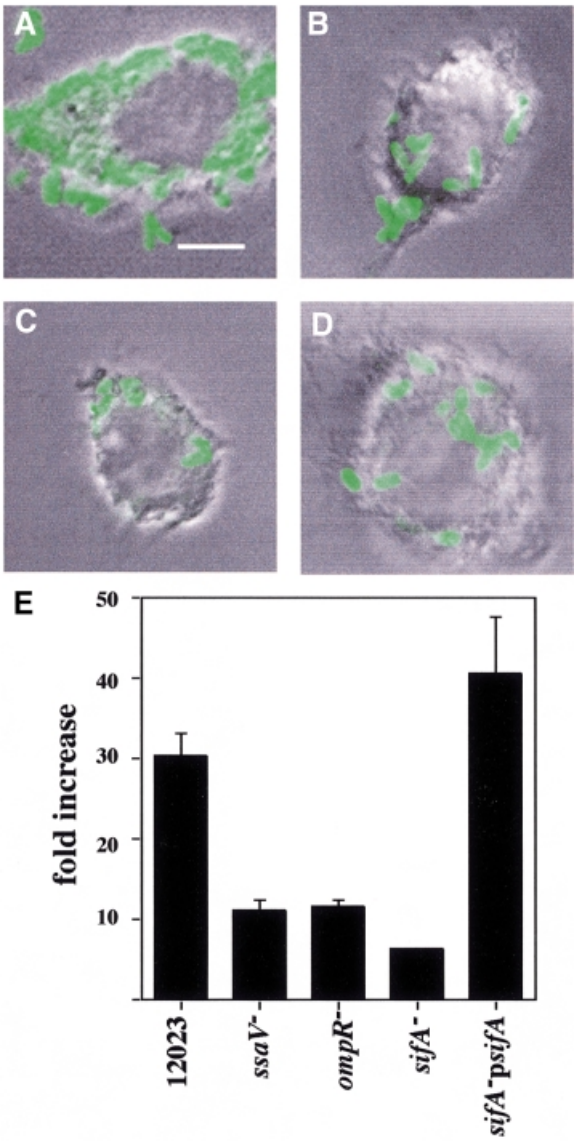


Fig. 4. A *sifA*$^-$ mutant strain is defective for replication within RAW macrophages. Opsonized bacteria were taken up by phagocytosis and at 2 and 16 h host cells were either lysed and cultured for enumeration of intracellular bacteria (gentamicin-protected), or fixed and examined by phase-contrast and confocal fluorescence microscopy. (**A**, **B**, **C** and **D**) represent typical cells infected with GFP-expressing 12023, *ssaV*$^-$, *sifA*$^-$ and *ompR*$^-$, respectively. In (**E**), the values shown represent the fold increase calculated as a ratio of the intracellular bacteria between 2 and 16 h post-uptake. Each strain was infected in triplicate and the standard errors from the means are shown. The results shown are representative of three independent experiments. Scale bar represents 4 μm.

showed that the decline in LAMP-1 association with the *sifA*$^-$ strain began ~5 h post-uptake (Figure 5C). We conclude from these results that the maintenance of LAMP-1 on vacuoles containing wild-type bacteria is dependent on the *sifA* gene.

Trafficking of S.typhimurium strains in epithelial cells

To determine if the apparent absence of lgp in the vicinity of the *sifA*$^-$ mutant cells was restricted to LAMP-1 and to macrophages, we examined the localization of another lgp, LIMP-1, also known as LAMP-3 or CD63 antigen, in infected epithelial cells. We found that LIMP-1 is a constituent of Sifs in cells infected with wild-type bacteria (Figure 6, upper panel). Furthermore, whereas LIMP-1 was present in >75% of vacuoles containing wild-type bacteria and >90% of vacuoles containing *ssaV*$^-$ bacteria, <25% of *sifA*$^-$ cells were associated with this marker (Figure 6). Similar results were obtained in HeLa cells using antibodies against LAMP-1 (Figure 7, upper panel) and LAMP-2 (not shown). LIMP-1, LAMP-1 and LAMP-2 share similar lysosomal targeting sequences, and are likely to follow the same trafficking pathway (Hunziker and Geuze, 1996). We therefore examined the acquisition of the vacuolar ATPase (vATPase), which is responsible for the acidification of endocytic vacuoles (Sato and Toyama, 1994), since its origin, function and targeting process are different to those of these lgps (Hunziker and Geuze, 1996). Interestingly, vATPase was also found to be a component of Sifs (Figure 7, lower panel), was present on vacuoles containing wild-type and

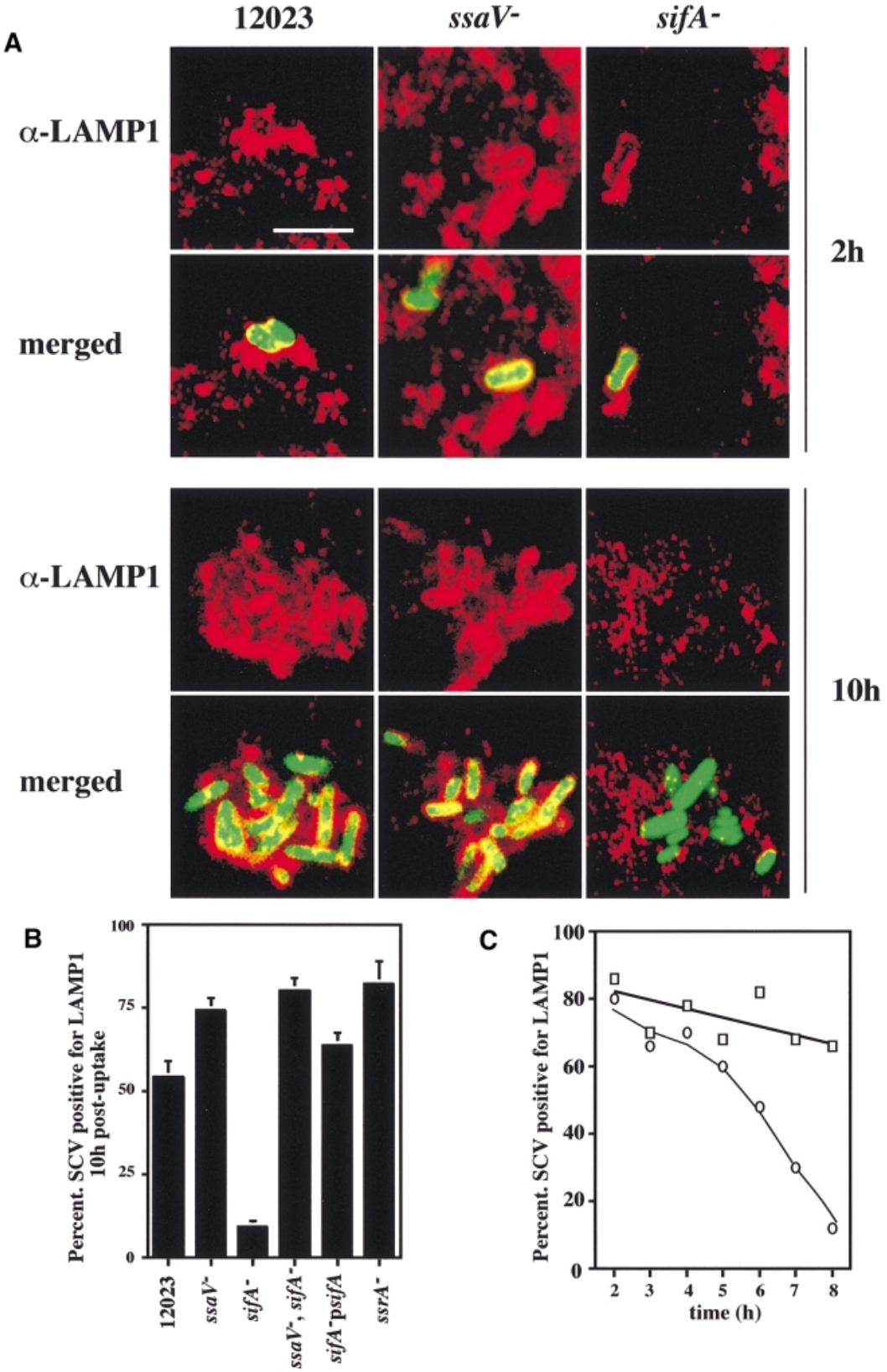


Fig. 5. The lysosomal membrane protein LAMP-1 is not associated with *sifA*⁻ mutant bacteria 10 h post-uptake in RAW macrophages. Confocal microscopic analysis was carried out on macrophages infected with wild-type (12023), *ssaV*⁻ or *sifA*⁻ strains constitutively expressing GFP, at 2 h [upper panels (**A**)] or 10 h [lower panels (A)] post-uptake. LAMP-1 was detected using a rabbit polyclonal and TRSC-conjugated donkey anti-rabbit secondary antibodies. Scale bar corresponds to 4 μm. The percentage of bacteria (n = 50) co-localizing with LAMP-1 was determined for these and two other bacterial strains 10 h after uptake. The percentage of bacteria (n = 50) co-localizing with LAMP-1 at different time points was determined for 12023 (open squares) and *sifA*⁻ mutant (open circles) strains (**C**). Results shown in (**B**) are the means ± SE of three independent experiments. For the *sifA*⁻, p*sifA* strain, bacteria were detected with goat anti-*Salmonella* and FITC-conjugated donkey anti-goat antibodies.

ssaV⁻ bacteria, but was only detected at a low frequency in association with *sifA*⁻ bacteria (Figure 7, lower panel).

Maintenance of the vacuolar membrane enclosing wild-type S.typhimurium is dependent on sifA

The lack of association of lgps and vATPase with *sifA*⁻ mutant bacteria suggested that either vacuoles containing *sifA*⁻ bacteria are non-fusogenic, as is the case for vacuoles containing some other intracellular pathogens, such as *Legionella* spp. (Sinai and Joiner, 1997), or that these bacteria are no longer enclosed by a membrane. The presence of a vacuolar membrane around *sifA*⁻ bacteria was investigated by testing the accessibility of intracellular *S.typhimurium* strains to anti-LPS antibody in RAW cells treated with the pore-forming toxin streptolysin-O from *Streptococcus pyogenes*. The rationale of this experiment is that, after plasma membrane permeabilization, bacteria present in the macrophage cytosol would be accessible to the antibody added to the extracellular medium whereas bacteria protected by a vacuole would not. Infected cells were treated with streptolysin-O, fixed and stained with antibodies in the absence of detergent. At 8 h post-uptake most wild-type and *ssaV*⁻ bacteria were protected from the anti-LPS antibody. In contrast, >55% of *sifA*⁻ bacteria were decorated with the anti-LPS antibody, indicating their presence in the cytosol (Figure 8A). Examination of intracellular bacteria under the electron microscope confirmed the presence of cytosolic *sifA*⁻ bacteria (Figure 8D), whereas wild-type, *ssaV*⁻ and *sifA*⁻, p*sifA* cells were always associated with a vacuolar membrane (Figure 8B, C and E). These results show that maintenance of a lgp/vATPase-containing membrane around wild-type bacteria requires the *sifA* gene.

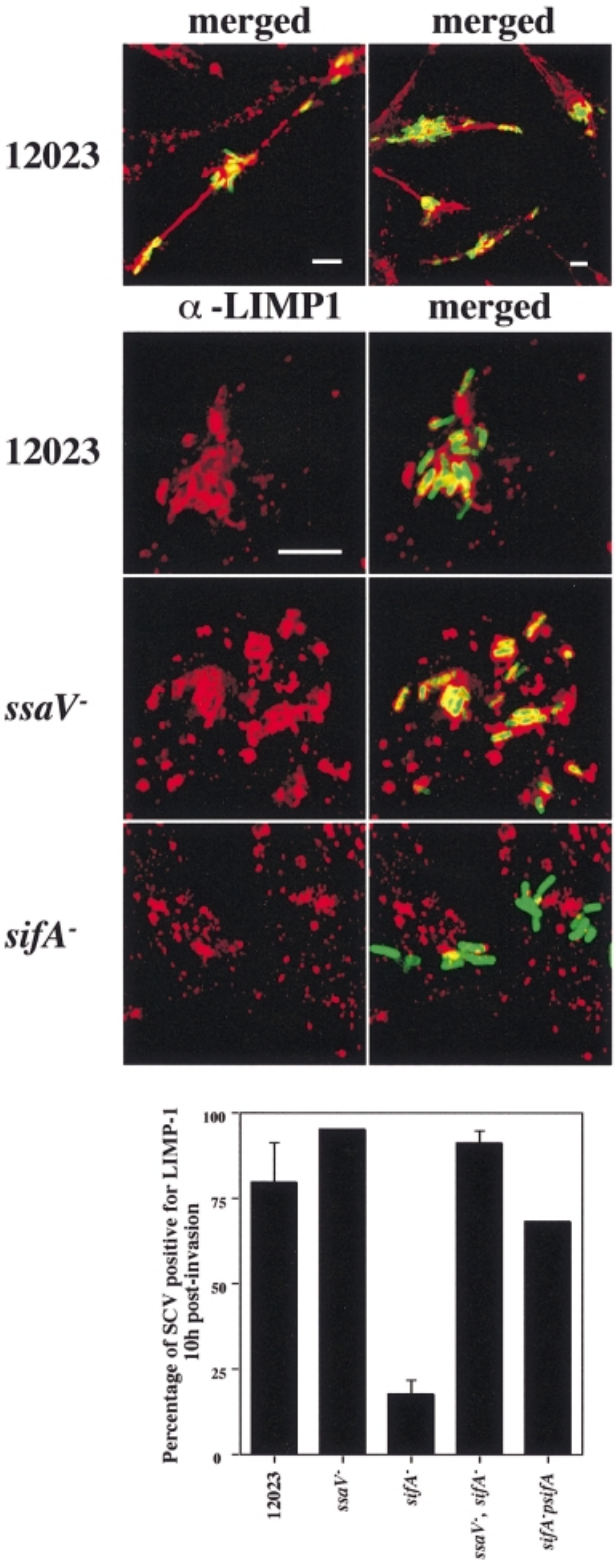


Fig. 6. LIMP-1 is a component of Sifs and is not associated with *sifA*⁻ mutant bacteria 10 h after invasion of HeLa cells. Confocal microscopic analysis was carried out on cells infected with wild-type (12023), *ssaV*⁻ or *sifA*⁻ strains constitutively expressing GFP. LIMP-1 was detected using a mouse mAb and TRSC-conjugated donkey anti-mouse secondary antibodies. Low magnification images show that LIMP-1 is a component of Sifs in cells infected with wild-type bacteria (upper panel). Higher magnification images show LIMP-1-containing SCVs for wild-type and *ssaV*⁻ mutant bacteria, but weak or no association of LIMP-1 with *sifA*⁻ mutant cells. Scale bars correspond to 4 μm. The percentage of bacteria (n = 50) co-localizing with LIMP-1 was determined for these and two other bacterial strains. Results shown are the means ± SE of three independent experiments. For the *sifA*⁻, p*sifA* strain, bacteria were detected with goat anti-*Salmonella* followed by FITC-conjugated donkey anti-goat antibodies.

SifA is similar to secreted proteins from Salmonella

The results described above show that the maintenance of the lgp/vATPase-containing membrane of the SCV is dependent on SifA, which in turn requires the SPI-2 TTSS regulator, *ssrA*. The C.I. analysis also suggests that *sifA* and *ssaV* are functionally related (Table I). One obvious explanation of these results is that SifA is a SPI-2 effector protein. BLAST and FASTA searches using the predicted amino acid sequence of *sifA* revealed that it has weak but significant similarity at the N-terminal region to three *Salmonella* proteins: SspH-2, SspH-1 and SlrP (Figure 9A). SspH-1 and SspH-2 are preferentially translocated into the host cell cytosol by the SPI-1 and SPI-2 TTSS, respectively (Miao *et al*., 1999), but their function is unknown. The *slrP* gene is present on a pathogenicity island and, like SspH-1 and SspH-2, shares similarity to other proteins secreted by TTSSs (Tsolis *et al*., 1999). However, unlike all these proteins, SifA does not contain leucine-rich repeats. Its region of similarity is confined to approximately the first 100 amino acids of

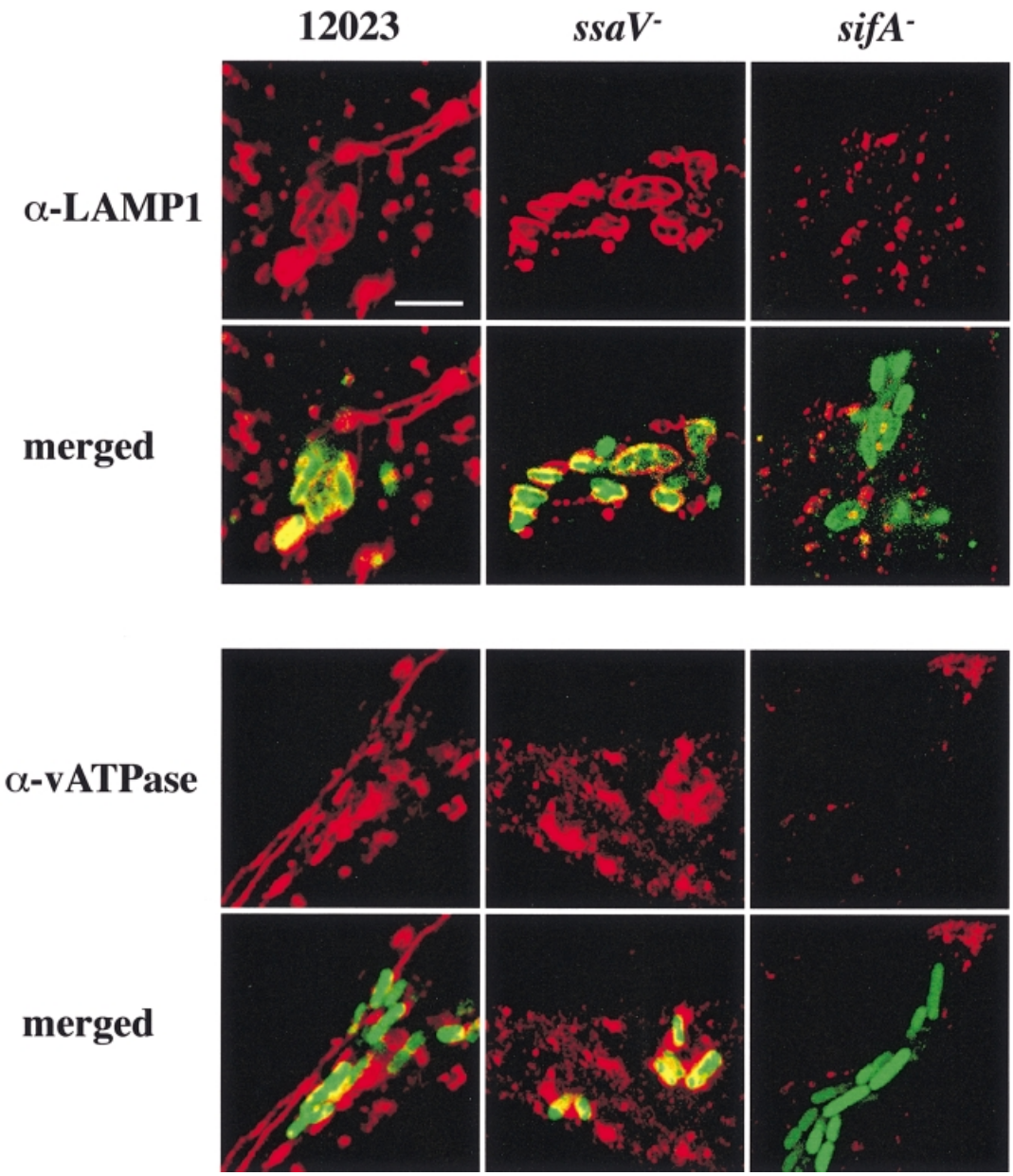


Fig. 7. LAMP-1 and vATPase are not associated with *sifA*⁻ mutant bacteria 10 h post-invasion in HeLa cells. Confocal microscopic analysis was carried out on cells infected with wild-type (12023), *ssaV*⁻ or *sifA*⁻ strains constitutively expressing GFP. LAMP-1 and vATPase were detected using mouse monoclonal antibodies, followed by TRSC-conjugated donkey anti-mouse secondary antibodies. Scale bar corresponds to 4 μm.

SspH-2, SspH-1 and SlrP: over this region, SifA is most similar (30% identical) to SspH-2, and has lower similarity to SlrP and SspH-1 (Figure 9A). The N-terminal regions of SspH-2 and SspH-1 are sufficient for their secretion and translocation (Miao *et al*., 1999), suggesting that SifA may be a secreted protein.

As standard BLAST or FASTA database searches are not always sufficient to detect subtle sequence relationships, we used a multiple sequence alignment of the N-terminal regions of SifA, SspH-1, SspH-2 and SlrP (from Trp31 to Glu106 of the SifA amino acid sequence) to build a profile that was then used to search the non-redundant sequence database (NRDB). This method allows more sensitive database searches to be carried out, by highlighting the critical residues within a set of aligned sequences. The search using this profile revealed similarity to a portion of only one protein: the N-terminal end of *Salmonella dublin* SopD, with an E value of 0.0559 (indicating that the similarity is likely to be significant) (Figure 9A). SopD is translocated into HeLa cells via the Inv/Spa TTSS, its N-terminal region being sufficient to direct translocation (Jones *et al*., 1998).

Infection of HeLa cells expressing SifA with a sifA⁻ mutant strain restores Sif formation

To investigate whether SifA is secreted into the host cell cytosol, three SifA–adenylate cyclase (CyaA) fusion proteins were constructed. The CyaA reporter system has

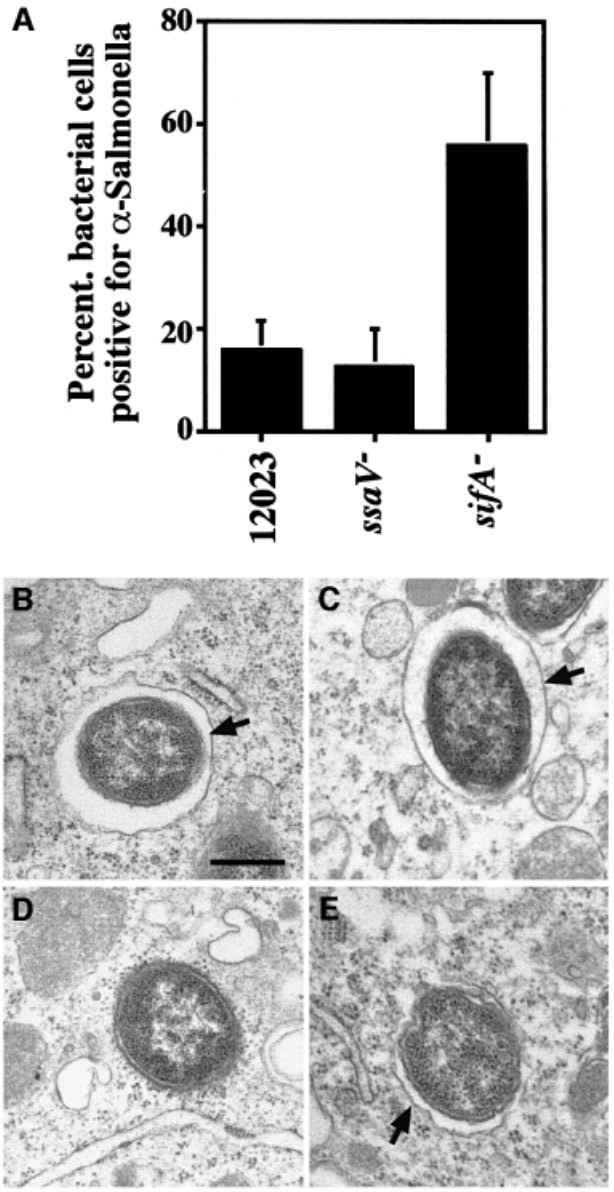


Fig. 8. Streptolysin-O permeabilization and electron microscopy of host cells infected with different bacterial strains. (**A**) RAW cells were infected with different strains expressing GFP for 8 h, then permeabilized with streptolysin-O, and incubated with anti-LPS antibody. The percentage of bacteria staining positive for the anti-LPS antibody was determined. Results shown are the means ± SE of three independent experiments. In each experiment, between 100 and 150 bacteria were scored for each strain. Electron microscopy analysis of RAW macrophages infected with either wild-type (**B**), *ssaV*⁻ (**C**), *sifA*⁻ (**D**) or *sifA*⁻, p*sifA* (**E**) strains, 10 h post-uptake. Infected cells were fixed and processed for transmission electron microscopy. Arrows in (B, C and E) point to vacuolar membranes, which are absent in (D). Scale bar corresponds to 0.5 μm.

been used previously to demonstrate translocation of SspH-1 and SspH-2 fusion proteins via the SPI-2 secretion system (Miao *et al*., 1999). The fusions contained either the N-terminal 53, 113 or 217 amino acids of SifA, which is 336 amino acids in length. The predicted sizes of the different fusion proteins were verified by immunoblotting using an anti-CyaA antibody, and their CyaA activities were demonstrated by functional complementation of an *Escherichia coli cyaA*⁻ mutant strain (Glaser *et al*., 1988). These constructs were transferred into wild-type, *ssaV*⁻ or *sifA*⁻ strains. These were then used to infect RAW macrophages, which were assayed for CyaA activity at different time points (Miao *et al*., 1999). No accumulation of cAMP (reflecting CyaA activity) was detected at 6 or 8 h post-uptake for any of the strains, despite their efficient uptake by macrophages (results not shown). This suggests that either SifA is itself not translocated into the host cell cytosol, or that following translocation, its conformation or interaction with a receptor prevents determination of CyaA activity of the fusion protein.

In view of the strong circumstantial evidence favouring an effector function for SifA, HeLa cells were transiently transfected with plasmids encoding either SifA::GFP, or the GFP as a control, then infected with different *S.typhimurium* strains. Confocal imaging showed that the GFP was localized both in the cytoplasm and the nucleus. In contrast, the SifA::GFP fusion protein was associated mainly with membranes, vacuoles containing wild-type or *sifA*⁻ strains, and Sifs. In addition, formation of Sifs was restored in SifA::GFP expressing cells infected with the *sifA*⁻ mutant strain (Figure 9B). Collectively these results suggest that SifA is translocated on to the cytosolic face of the vacuolar membrane where it mediates membrane fusion events.

Maintenance of the vacuolar membrane enclosing SPI-2 mutant strains is not dependent on sifA

We have established that *sifA* expression requires the SPI-2 regulator *ssrA*, and that SifA is required for the maintenance of the vacuolar membrane enclosing wild-type bacteria. If, as suggested by our results, SifA is secreted by the SPI-2 TTSS, an *ssaV*⁻ mutant strain [which lacks a functional secreton (Beuzón *et al*., 1999)] would be defective in SifA function and might also be expected to be released into the host cell cytosol. However, *ssaV*⁻ mutant bacteria were found within vacuoles in macrophages (Figure 8C). If SifA is not secreted by the SPI-2 TTSS, SifA could account for the maintenance of a vacuolar membrane surrounding *ssaV*⁻ bacteria. In this case, bacterial cells carrying both *sifA*⁻ and *ssaV*⁻ mutations would be released into the cytosol. However, vacuoles containing these double mutant bacteria were associated with LAMP-1 in macrophages, and with LIMP-1, LAMP-1, LAMP-2 and vATPase in HeLa cells (Figures 5 and 6 and results not shown). Furthermore, since *sifA* expression is *ssrA* dependent (Figure 3), an *ssrA*⁻ strain would also be expected to be found in the host cell cytosol. Like *ssaV*⁻ and *ssaV*⁻, *sifA*⁻ double mutant bacteria, *ssrA*⁻ cells were associated with LAMP-1 in macrophages (Figure 5). Therefore, *sifA* is not required for maintenance of the vacuolar membrane enclosing *ssaV*⁻ or *ssrA*⁻ bacteria, and the recruitment of lgp/vATPase-containing membrane to these cells must reflect a different process to that observed with the wild-type strain. The simplest explanation for these results is that the SPI-2 TTSS translocates other effectors which prevent the SCV from interacting with the endocytic pathway, prior to the action of SifA. An *ssaV*⁻ strain cannot secrete any effectors, and is enclosed by a vacuole by interactions with the endocytic pathway (Figure 10).

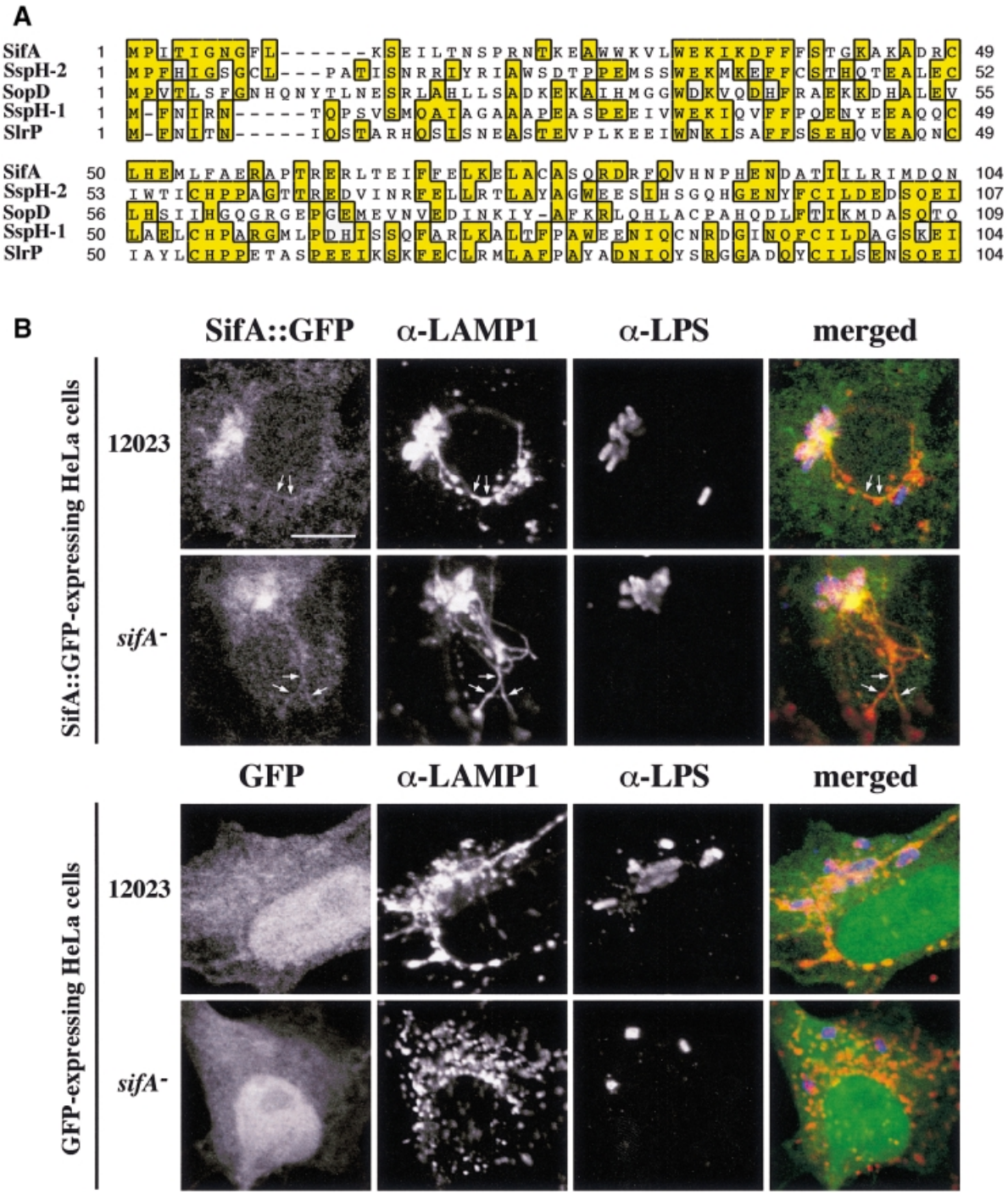


Fig. 9. (**A**) Multiple alignment of the N-terminal region of SifA with other *Salmonella* proteins. The alignment was generated by ClustalW (http://www2.ebi.ac.uk/clustalw/) and Multalin (http://www.toulouse.inra.fr /multalin.html) and edited by hand. (**B**) Formation of Sifs is restored in *sifA*⁻ mutant-infected cells expressing SifA::GFP. HeLa cells transiently expressing SifA::GFP (upper panels) or GFP as a control (lower panels) were infected with wild-type (12023) or *sifA*⁻ strains, and fixed with paraformaldehyde at 8 h post-infection. LAMP-1 and LPS were detected using TRSC- and Cy5-conjugated donkey anti-rabbit and anti-mouse secondary antibodies, respectively. Three-colour confocal microscopic analysis was performed. Scale bar represents 10 μm. Expression of SifA::GFP in HeLa cells restores the presence of a LAMP-1-enriched vacuole around *sifA*⁻ bacteria. SifA–GFP is localized on SCVs and Sifs (arrows in the top panels).

Discussion

In this paper we describe a general method for identifying virulence gene interactions *in vivo* based on the C.I. The technique involves direct comparisons between single and double mutant strains rather than combinations of different single mutants and the wild-type strain. This approach allows the relative attenuation of two mutations to be assessed directly in the same animal, avoiding host-to-host variation.

In the present work, we have used C.I. analysis to show that *ompR*, SPI-2 genes and *sifA* interact during systemic

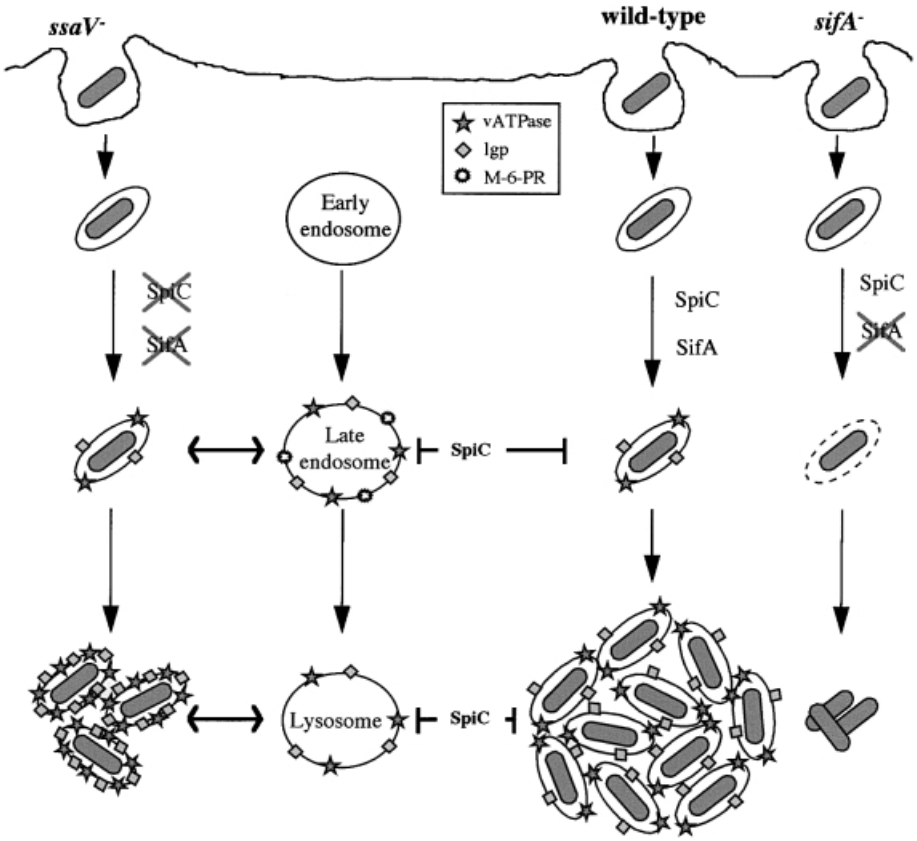


Fig. 10. Model showing the role of the SPI-2 secretion system in the maturation of the SCV.

infection of mice. A functional relationship between these loci was suggested by the finding that *ompR*⁻ mutants fail to induce Sif formation in epithelial cells (Mills *et al*., 1998), and the recent demonstration that OmpR regulates *ssrA* expression during infection of cultured macrophages (Lee *et al*., 2000). Our C.I. analysis indicates that *in vivo*, the attenuated virulence of an *ompR*⁻ mutant can be attributed entirely to its interaction with SPI-2 genes, and vice versa. This was surprising in view of the finding that a mutation in *ompR* delayed but did not eliminate induction of a SPI-2 *ssaH* gene in cultured macrophages (Lee *et al*., 2000). It is possible that OmpR exerts tighter control over SPI-2 function *in vivo* than in cultured cells; alternatively, the partial effect of an *ompR*⁻ mutation over SPI-2 gene expression may be sufficient to reduce expression below a functionally effective threshold. OmpR regulation of SPI-2 gene expression is therefore analagous to that of PhoP/Q over SPI-1 and SPI-3 genes (Pegues *et al*., 1995; Blanc-Potard and Groisman, 1997): both regulators are present in the non-pathogenic relative *E.coli*, but have extended their regulatory range in *Salmonella* to encompass virulence genes on PAIs.

In infected epithelial cells, *sifA* induces the formation of dramatic tubular membrane structures (Sifs), that are enriched in lysosomal membrane glycoproteins. However, the physiological relevance of these structures is not clear, despite the fact that the *sifA* gene is known to be important for virulence in mice (Stein *et al*., 1996). The C.I. tests confirm the importance of the *sifA* gene in *S.typhimurium* virulence, and suggest that *sifA* and SPI-2 functions are intimately connected *in vivo*. Since attenuated SPI-2 mutant strains fail to induce Sifs, and *sifA* expression is dependent on *ssrA*, this also explains the observation that *ompR*⁻ mutants fail to make Sifs (Mills *et al*., 1998). The systemic *in vivo* attenuation of the *sifA*⁻ mutant strain can be explained by its intramacrophage replication defect, which is similar to that of SPI-2 mutants. Consistent with this, the onset of *sifA* gene expression in cultured cells corresponds to that of SPI-2 genes (Cirillo *et al*., 1998).

Several lines of evidence suggest that SifA is a SPI-2 effector protein. First, the C.I. analysis implies that both loci are involved in the same function. Secondly, although *sifA* is regulated by *ssrA*, it is not present in SPI-2, which contains all the genes known to be required for secretion and translocation of SPI-2 effectors. Thirdly, *sifA* has a significantly lower G+C content than the average for the *S.typhimurium* chromosome, is flanked by repeat sequences, and is a virulence determinant specific to *Salmonella*, all of which suggest that it was acquired by horizontal transfer (Stein *et al*., 1996). A gene for an effector protein of the Inv/Spa TTSS of *Salmonella* was also acquired independently by horizontal transfer, which has led to the suggestion that this may allow bacterial pathogens to fine-tune their TTSSs (Hardt *et al*., 1998). Fourthly, SifA has weak but significant similarity to other *Salmonella* secreted proteins, and this is restricted to the regions reported to be sufficient for secretion and translocation. Finally, the recruitment of the SifA::GFP fusion protein to the SCV and on Sifs when expressed in host

cells, and its ability to rescue Sif formation in cells infected with the *sifA*⁻ mutant strain suggests that it is normally translocated from wild-type bacteria to the cytosolic face of the vacuole. The localization of SifA to host cell membranes is consistent with the hypothesis that it mediates membrane fusion events (see below). If SifA binds to a receptor in the vacuolar membrane this might also explain the failure to demonstrate translocation using SifA::Cya fusions, since this could interfere with CyaA activity of this fusion protein.

Our results show that the maintenance of a lgp/vATPase-containing membrane around wild-type but not SPI-2 mutant strains of *S.typhimurium* requires a functional *sifA* gene. We present a model (Figure 10) to account for the intracellular phenotypes of different mutant strains. The vacuole containing wild-type bacteria, through the action of the SPI-2 TTSS effector SpiC (Uchiya *et al*., 1999) and possibly other SPI-2 effectors, does not interact with late endosomes and lysosomes, and SifA actively recruits and maintains the lgp/vATPase-containing membrane. It is known that acquisition of lgps by the SCV in epithelial cells occurs by recruitment of pre-existing lgp-containing vesicles in a rab7-dependent manner, without direct interaction with lysosomes (Méresse *et al*., 1999a). Through the action of unknown SPI-2 effectors, wild-type *Salmonella* also inhibits trafficking of the NADPH oxidase to the SCV (Vázquez-Torres *et al*., 2000). These processes create an environment conducive to bacterial replication. Strains containing mutations in *ssaV* or *ssrA* do not have a functional SPI-2 secretion system, and the vacuoles acquire lgps and vATPase through interactions with late endosomes and lysosomes. A strain carrying a mutation in *sifA* retains the ability to translocate SpiC and other effectors, which prevent interactions with late stages of the endocytic pathway. However, because this strain cannot recruit or maintain lgp/vATPase-containing membrane through the action of SifA, the vacuole cannot be sustained, and the bacterial cell is released into the cytoplasm. For intracellular pathogens to replicate but remain within a membrane-bound vacuole, there must be a progressive net increase in the surface area of the vacuolar membrane. SifA is therefore likely to control directed membrane fusion events, and the lack of SifA would lead to insufficient membrane acquisition to enclose the replicating bacterial cells, which would become exposed to the cytosol.

The intra-macrophage replication defect displayed by the *sifA*⁻ mutant strain is comparable to the phenotype of *S.dublin* or *E.coli* K-12 strains carrying the *hly* gene of *Listeria monocytogenes*. These bacteria can also escape to the macrophage cytosol by secreting listeriolysin, but they fail to replicate (Gentschev *et al*., 1995). This suggests that, unlike other intracellular pathogens such as *Listeria* which can replicate in the macrophage cytosol (de Chastellier and Berche, 1994), *Salmonella* cannot exploit this environment for growth. To our knowledge, SifA is the first example of a bacterial protein responsible for the maintenance of a membrane-bound vacuole. Specificity of vesicular trafficking in the eukaryotic cell is conferred by the integral membrane vSNARE and tSNARE proteins (Rothman and Wieland, 1996), and is modulated by rab GTPases (Schimmoller *et al*., 1998). In view of the involvement of the rab7 GTPase in the biogenesis of SCVs (Méresse *et al*., 1999a) and its presence on Sifs (S.Méresse and J.-P.Gorvel, unpublished observations) it is possible that SifA acts as an activator of this membrane fusion machinery, thereby ensuring a continuous flow of membrane towards the SCV.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table II. H3P6 carries a mini-Tn*5* transposon insertion that was mapped between nucleotides 44 and 139 of the *sifA* open reading frame (ORF; 1012 nucleotides in length) and was identified but not further characterized in the original STM screen of *S.typhimurium* (Hensel *et al*., 1995). The mutation was transduced by phage P22 into strain 12023 prior to use in this study. Double mutant strains were also constructed by P22-mediated transductions, carried out as described previously (Davis *et al*., 1980). Bacteria were grown in Luria–Bertani (LB) medium supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (25 μg/ml) or chloramphenicol (50 μg/ml for plasmid-containing strains, 10 μg/ml for chromosomal integrants) as appropriate. CJD359 and CS105 were kindly provided by C.Dorman and S.Miller, respectively.

Plasmids

Plasmid p*sseB*, used to complement the Δ*sseB* mutant strain, has been described previously (Hensel *et al*., 1998). Plasmid pFPV25.1, carrying *gfpmut3A* under the control of a constitutive promoter, was introduced into bacterial strains for fluorescence visualization where indicated (Valdivia and Falkow, 1997). pID812 is a derivative of pFPV25, a vector carrying promoterless *gfpmut3A* (Valdivia and Falkow, 1997) containing a translational fusion of a fragment of *sifA* to the *gfp* gene. A fragment including 160 bp of the *sifA* promoter region and the sequence encoding the N-terminal 270 amino acids of SifA was amplified by PCR from 12023s genomic DNA using SIFA1 (5′-GGATCCGACAGTAATG-CGTTTATACGCGAAGCTCTC-3′) and SIFA2 (5′-CATATGGGTCT-TATCCGCTAGTAAAACCTCTTTTAC-3′) primers. The 1 kb PCR product, containing terminal *Bam*HI and *Nde*I sites, was digested and ligated into pFPV25, generating pID812. p*sifA* is a derivative of pACYC184 (Chang and Cohen, 1978) carrying the *sifA* gene under the control of a constitutive promoter. A DNA fragment including the complete ORF of *sifA* and its ribosomal binding site (rbs) was amplified by PCR from 12023s genomic DNA using SIFN-B (5′-GGATCCT-TACTCCAGTATAAGTGAG-3′) and SIFAT2 (5′-CTCGAGGTGAC-GTCTGAGAAAG-3′) primers. The ~1 kb PCR product, containing terminal *Bam*HI and *Xho*I sites, was digested and ligated into pACYC184 *Eco*RV–*Sal*I sites inside the Tet^r gene generating p*sifA*. All the constructions were verified by DNA sequencing.

Mouse mixed infections

Female BALB/c mice (20–25 g) were used for all infection studies and were inoculated i.p. with a 0.2 ml volume of bacterial cells suspended in physiological saline. To prepare the inocula bacteria were grown overnight at 37°C in LB broth with shaking (150 r.p.m.) and then used to inoculate fresh medium (1:100) and grown under the same conditions for 2–3 h until an OD_{550} of 0.35–0.6 was reached. Cultures were then diluted in physiological saline to a concentration of 2.5×10^5 bacteria per strain and mixed before the infection. The c.f.u. were enumerated by plating a dilution series of the inoculum, using antibiotics to distinguish between the strains. To confirm that the presence of the antibiotic did not result in lower bacterial recovery, strains carrying antibiotic resistance were plated on LB and LB with the appropriate antibiotic. Mice were killed at 48 h post-inoculation by carbon dioxide inhalation. The spleens were removed and bacteria recovered and enumerated after plating a dilution series on to LB agar and LB agar with the appropriate antibiotics (Shea *et al*., 1999).

Statistical analysis

Each C.I. value is the mean of three independent mice infections. Student's *t*-test was used to analyse the C.I.s of single mutant strains versus double mutant strains (i.e. a^- versus a^-b^-) with two null hypotheses: mean C.I. is significantly different from 1.0 and mean C.I. is significantly different from the C.I. of the single mutant versus the wild-type strain (wt versus *b*). Probabilities (p) of ≤ 0.05 were considered significant.

Table II. Strains used in this study

Name	Description	Source or reference
12023s	wild-type	NTCC
P3F4	*ssrA*::mTn*5* in 12023s	Hensel *et al*. (1995)
P3H6	*sifA*::mTn*5* in 12023s	this study
P4E3	*purD*::mTn*5* in 12023s	Hensel *et al*. (1995)
CS105	*phoP-102*::Tn*10d*Cm in 12023s	Miller *et al*. (1989)
CJD359	*ompR-1009*::Tn*10* in SL1344	Dorman *et al*. (1989)
HH102	*ΔsseB::aphT* in 12023s	Hensel *et al*. (1998)
HH109	*ssaV::aphT* in 12023s	Deiwick *et al*. (1999)
HH110	*ssaV*::Cm^r in 12023s	Shea *et al*. (1999)
HH111	*ssaV*::Cm^r, *purD*::mTn*5* in 12023s	Shea *et al*. (1999)
HH123	*ssaV*::Cm^r, *sseB::aphT* in 12023s	this study
HH172	*ssaV*::Cm^r, *sifA*::mTn*5* in 12023s	this study
HH182	*ompR-1009*::Tn*10* in 12023s	this study
HH187	*ssaV::aphT*, *ompR-1009*::Tn*10* in 12023s	this study

Sequence analysis

Sequence database similarity searches were carried out by Blast2 (http://www.ncbi. nlm.nih.gov/BLAST/), WU-Blast2 and Fasta3 (http://www2.ebi.ac.uk/blast2/ and /fasta3/) (Pearson and Lipman, 1988; Altschul *et al*., 1997). All the searches used derived amino acid sequences.

The multiple alignment shown in Figure 9A was generated by ClustalW (http://www2.ebi.ac.uk/clustalw/) and Multalin (http://www.toulouse.inra.fr/multalin.html) (Corpet, 1988; Thompson *et al*., 1994). The sequences included can be found under the SWALL accession numbers Q56061 (SifA), Q9XDN9 (SspH-1), AAF00615 (SspH-2) and Q9XCV2 (SlrP). A ClustalW multiple alignment of the region of interest was used to generate a profile by ProfileWeight. This profile was then used to search the NRDB using the program Profilesearch. Both programs are available at the Bioccelerator Web server (http://shag.embl-heidelberg.de:8000/Bic/).

Antibodies and reagents

The mouse monoclonal antibodies (mAbs) anti-LAMP-1 H3A4 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 Siences, Iowa, IA), and were used at a dilution of 1:2000. Anti-LAMP-2 mouse mAb CD3 was obtained from Dr Minoru Fukuda (The Burnam Institute, La Jolla Cancer Research Foundation, La Jolla, CA), and was used at a dilution of 1:1000, and anti-vATPase mouse mAb OSW2 was obtained from Dr Satoshi B.Sato (Kyoto University, Kyoto, Japan) and was used at a dilution of 1:2000. Anti-LAMP-1 rabbit polyclonal antibody 156 against the 11-residue cytoplasmic domain of LAMP-1 has been described previously (Steele-Mortimer *et al*., 1999), and was used at a dilution of 1:1000. Anti-*Salmonella* goat polyclonal antibody CSA-1 was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD) and was used at a dilution of 1:400. Anti-*Salmonella* LPS mouse mAb 1E6 was purchased from Biodesign International (Kennebunk, ME) and was used at a dilution of 1:10 000. Texas Red sulfonyl chloride (TRSC)-, cyanine 5 (Cy5)- and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse, anti-rabbit and anti-goat antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA), and used at a dilution of 1:400.

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.

Bacterial infection of HeLa cells

HeLa cells were seeded on to glass coverslips (12 mm diameter) in 24-well plates at a density of 5×10^4 cells per well, 24 h before infection. Bacteria were incubated for 16 h at 37°C with shaking, diluted 1:33 in fresh LB broth and incubated in the same conditions for 3.5 h. The cultures were diluted in Earle's buffered salt solution (EBSS) pH 7.4 and added to the HeLa cells at a multiplicity of infection (m.o.i.) of ~100:1. The infection was allowed to proceed for 15 min at 37°C in 5% CO_2. The monolayers were washed once with DMEM containing FCS and 100 μg/ml gentamicin and incubated in this medium for 1 h, after which the gentamicin concentration was decreased to 16 μg/ml.

Bacterial infection of macrophages and survival assays

Macrophages were seeded at a density of 4×10^5 cells per well in 24-well tissue culture plates, 24 h before use. Bacteria were cultured at 37°C with shaking until they reached an OD_{600} of 2.0. The cultures were diluted to an OD_{600} of 1.0 and opsonized in DMEM containing FCS and 10% normal mouse serum for 20 min. Bacteria were added to the monolayers at an m.o.i. ~100:1, centrifuged at 170 *g* for 5 min at room temperature and incubated for 25 min at 37°C in 5% CO_2. The macrophages were washed once with DMEM containing FCS and 100 μg/ml gentamicin and incubated in this medium for 1 h. The medium was replaced with DMEM containing FCS and 16 μg/ml gentamicin for the remainder of the experiment. 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 on to LB agar.

Immunofluorescence and electron 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. Antibodies were diluted in 10% horse serum, 1% bovine serum albumin (BSA), 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 on Mowiol. Samples were analysed using a fluorescence microscope (BX50; Olympus Optical Co., Ltd) or a confocal laser scanning microscope (LSM510, Zeiss).

For transmission electron microscopy, cell suspensions were fixed in 3% glutaraldehyde prepared in 0.1 M cacodylate buffer pH 7.3. Fixation was for 1–2 h at room temperature, after which the cells were washed in fresh buffer before post-fixing in 1% osmium tetroxide in the same buffer. The cells were encased in agar (Ryder and MacKenzie, 1981), dehydrated through a graded series of alcohols and embedded in Araldite epoxy resin. Ultrathin sections were cut on a diamond knife and stained in alcoholic uranyl acetate and lead citrate before examination in a transmission electron microscope operated at 75 kV.

Streptolysin-O permeabilization of RAW cells

Streptolysin-O was obtained from Dr S.Bhakdi (Mainz, Germany). RAW cells were grown on coverslips, infected for 8 h with different strains, then rapidly washed with ice-cold ICT buffer (50 mM HEPES–KOH pH 7.1, 4 mM $MgCl_2$, 10 mM EGTA, 8.4 mM $CaCl_2$, 78 mM KCl, 1 mM DTT, 1 mg/ml BSA). They were then incubated for 5 min at 4°C with 2 μg/ml streptolysin-O in ICT buffer, extensively washed, incubated for 5 min at 37°C in the same buffer and immediately fixed in 3.7% paraformaldehyde. Coverslips were treated for immunofluorescence by double labelling as described above but in the absence of detergent.

Mouse anti-*Salmonella* LPS was used to detect bacteria present in the cytosol. Antibody raised against the LAMP-1 cytoplasmic domain was used as control for plasma membrane permeabilization.

Expression of SifA::GFP in HeLa cells

The full *sifA* ORF was amplified from *S.typhimurium* genomic DNA by PCR using the following primers: 5′-AAAAAAGAATTCCACCATGCCGATTACTATAGGGAATGG-3′ and 5′-AAAAAACCCGGGCTAAAAAACAACATAAACAGCCGC-3′. The PCR product was subcloned into the unique *Eco*RI and *Xma*I sites of pEGF-C1 vector (Clontech Laboratories) into the same reading frame as GFP, generating *psifA*::*gfp*. Vectors encoding GFP or SifA::GFP were used to transfect HeLa cells with FuGene 6 (Boehringer Mannheim) following the manufacturer's instructions. Cells were further incubated for 24–48 h and infected with bacterial strains as described. The expressed SifA::GFP fusion protein was checked by western blot analysis using an anti-GFP mouse mAb JL-8 (Clontech) to be of the expected size (583 amino acid residues, 66 kDa).

Acknowledgements

We thank Agnès Ullmann for providing an antibody against adenylate cyclase, Antoine Danchin for *E.coli* strain TP610pVUC1, and Christoph Tang and Herb Arst for valuable suggestions. We are very grateful to Colin Gleeson for practical assistance. This project was supported by grants from the MRC (UK) to D.W.H., and from CNRS and INSERM to S.M. C.B. was supported by an EMBO Fellowship.

References

Altschul,S., Madden,T., Schaffer,A., Zhang,J., Zhang,Z., Miller,W. and Lipman,D. (1997) Gapped blast and PSI-blast: a new generation of protein database search programs. *Nucleic Acids Res.*, **25**, 3389–3402.

Baumler,A., Tsolis,R., Valentine,P., Ficht,T. and Heffron,F. (1997) Synergistic effect of mutations in *invA* and *lpfC* on the ability of *Salmonella typhimurium* to cause murine typhoid. *Infect. Immun.*, **65**, 2254–2259.

Beuzón,C., Banks,G., Deiwick,J., Hensel,M. and Holden,D.W. (1999) pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of *Salmonella typhimurium*. *Mol. Microbiol.*, **33**, 806–816.

Blanc-Potard,A. and Groisman,E. (1997) The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival. *EMBO J.*, **16**, 5376–5385.

Chang,A.C. and Cohen,S.N. (1978) Construction and characterisation of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.*, **134**, 1141–1156.

Chatfield,S., Dorman,C., Hayward,C. and Dougan,G. (1991) Role of *ompR* dependent genes in *Salmonella typhimurium* virulence: mutants deficient in both OmpC and OmpF are attenuated *in vivo*. *Infect. Immun.*, **59**, 449–452.

Cirillo,D., Valdivia,R., Monack,D. and Falkow,S. (1998) Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. *Mol. Microbiol.*, **30**, 175–188.

Corpet,F. (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.*, **16**, 10881–10890.

Davis,R.H., Botstein,D. and Roth,J.R. (1980) *Advanced Bacterial Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

de Chastellier,C. and Berche,P. (1994) Fate of *Listeria monocytogenes* in murine macrophages: evidence for simultaneous killing and survival of intracellular bacteria. *Infect. Immun.*, **62**, 543–553

Deiwick,J., Nikolaus,T., Erdogan,S. and Hensel,M. (1999) Environmental regulation of *Salmonella* pathogenicity island 2 gene expression. *Mol. Microbiol.*, **31**, 1759–1773.

Dorman,C., Chatfield,S., Higgins,C., Hayward,C. and Dougan,G. (1989) Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated in vivo. *Infect. Immun.*, **57**, 2136–2140.

Finlay,B.B. (1994) Molecular and cellular mechanisms of *Salmonella* pathogenesis *Curr. Top. Microbiol. Immunol.*, **192**, 163–185.

Freter,R., O'Brien,P. and Macsai,M. (1981) Role of chemotaxis in the association of motile bacteria with intestinal mucosa: *in vivo* studies. *Infect. Immun.*, **34**, 234–240.

Galán,J.E. (1996) Molecular genetic bases of *Salmonella* entry into host cells. *Mol. Microbiol.*, **20**, 263–271.

Galán,J.E. and Curtiss,R.,III (1989) Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. *Proc. Natl Acad. Sci. USA*, **86**, 6383–6387

Garcia-del Portillo,F. and Finlay,B.B. (1995) Targeting of *Salmonella typhimurium* to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose-6-phosphate receptors. *J. Cell Biol.*, **129**, 81–97.

Garcia-del Portillo,F., Zwick,M.B., Leung,K.Y. and Finlay,B.B. (1993a) Intracellular replication of *Salmonella* within epithelial cells is associated with filamentous structures containing lysosomal membrane glycoproteins. *Infect. Agents Dis.*, **2**, 227–231.

Garcia-del Portillo,F., Zwick,M.B., Leung,K.Y. and Finlay,B.B. (1993b) *Salmonella* induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells. *Proc. Natl Acad. Sci. USA*, **90**, 10544–10548.

Gentschev,I., Sokolovic,Z., Mollenkopf,H.J., Hess,J., Kaufmann,S.H., Kuhn,M., Krohne,G.F. and Goebel,W. (1995) *Salmonella* strain secreting active listeriolysin changes its intracellular localization. *Infect. Immun.*, **63**, 4202–4205.

Glaser,P., Ladant,D., Sezer,O., Pichot,F., Ullmann,A. and Danchin,A. (1988) The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*: cloning and expression in *Escherichia coli*. *Mol. Microbiol.*, **2**, 19–30.

Groisman,E. and Ochman,H. (1997) How *Salmonella* became a pathogen. *Trends Microbiol.*, **5**, 343–349.

Hardt,W.-D., Urlaub,H. and Galán,J.E. (1998) A substrate of the centisome 63 type III protein secretion system of *Salmonella typhimurium* is encoded by a cryptic bacteriophage. *Proc. Natl Acad. Sci. USA*, **95**, 2574–2579.

Hensel,M., Shea,J.E., Gleeson,C., Jones,M.D., Dalton,E. and Holden,D.W. (1995) Simultaneous identification of bacterial virulence genes by negative selection. *Science*, **269**, 400–403.

Hensel,M., Shea,J.E., Raupach,B., Monack,D., Falkow,S., Gleeson,C. and Holden,D.W. (1997) Functional analysis of *ssaJ* and the *ssaK/U* operon, 13 genes encoding components of the type III secretion apparatus of *Salmonella* pathogenicity island 2. *Mol. Microbiol.*, **24**, 155–167.

Hensel,M. *et al*. (1998) Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. *Mol. Microbiol.*, **30**, 163–174.

Heyde,M. and Portalier,R. (1987) Regulation of major outer membrane porin proteins of *Escherichia coli* K 12 by pH. *Mol. Gen. Genet.*, **208**, 511–517.

Hueck,C.J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.*, **62**, 379–433.

Hunziker,W. and Geuze,H.J. (1996) Intracellular trafficking of lysosomal membrane proteins. *BioEssays*, **18**, 379–389.

Jones,M.A., Wood,M.W., Mullan,P.B., Watson,P.R., Wallis,T.S. and Galyov,E.E. (1998) Secreted effector proteins of *Salmonella dublin* act in concert to induce enteritis. *Infect. Immun.*, **66**, 5799–5804.

Lee,A., Detweiler,C. and Falkow,S. (2000) OmpR regulates the two-component system SsrA–SsrB in *Salmonella* pathogenicity island 2. *J. Bacteriol.*, **182**, 771–781.

Méresse,S., Steele-Mortimer,O., Finlay,B.B. and Gorvel,J.P. (1999a) The rab7 GTPase controls the maturation of *Salmonella typhimurium*-containing vacuoles in HeLa cells. *EMBO J.*, **18**, 4394–4403.

Méresse,S., Steele-Mortimer,O., Moreno,E., Desjardins,M., Finlay,B.B. and Gorvel,J.P (1999b) Controlling the maturation of pathogen-containing vacuoles: a matter of life and death. *Nature Cell Biol.*, **1**, 183–188.

Miao,E., Scherer,C., Tsolis,R., Kingsley,R., Adams,L., Baumler,A. and Miller,S. (1999) *Salmonella typhimurium* leucine-rich repeat proteins are targeted to the SPI1 and SPI2 type III secretion systems. *Mol. Microbiol.*, **34**, 850–864.

Miller,S.I. and Mekalanos,J.J. (1990) Constitutive expression of the *phoP* regulon attenuates *Salmonella* virulence and survival within macrophages. *J. Bacteriol.*, **172**, 2485–2490.

Miller,S.I., Kukral,A.M. and Mekalanos,J.J. (1989) A two-component regulatory system (*phoP/phoQ*) controls *Salmonella typhimurium* virulence. *Proc. Natl Acad. Sci. USA*, **86**, 5054–5058.

Mills,D.M., Bajaj,V. and Lee,C.A. (1995) A 40 kb chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent

from the corresponding region of the *Escherichia coli* K-12 chromosome. *Mol. Microbiol.*, **15**, 749–759.
Mills,S., Ruschkowski,S., Stein,M. and Finlay,B. (1998) Trafficking of porin-deficient *Salmonella typhimurium* mutants inside HeLa cells: *ompR* and *envZ* mutants are defective for the formation of *Salmonella*-induced filaments. *Infect. Immun.*, **66**, 1806–1811.
Ochman,H., Soncini,F.C., Solomon,F. and Groisman,E.A. (1996) Identification of a pathogenicity island required for *Salmonella* survival in host cells. *Proc. Natl Acad. Sci. USA*, **93**, 7800–7804.
Pearson,W. and Lipman,D. (1988) Improved tools for biological sequence analysis. *Proc. Natl Acad. Sci. USA*, **85**, 2444–2448.
Pegues,D.A., Hantman,M.J., Behlau,I. and Miller,S.I. (1995) PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* invasion genes: evidence for a role in protein secretion. *Mol. Microbiol.*, **17**, 169–181.
Rathman,M., Sjaastad,M.D. and Falkow,S. (1996) Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. *Infect. Immun.*, **64**, 2765–2773.
Rathman,M., Barker,L.P. and Falkow,S. (1997) The unique trafficking pattern of *Salmonella typhimurium*-containing phagosomes in murine macrophages is independent of the mechanism of bacterial entry. *Infect. Immun.*, **65**, 1475–1485.
Richter-Dahlfors,A., Buchan,A. and Finlay,B. (1997) Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. *J. Exp. Med.*, **186**, 569–580.
Rothman,J.E. and Wieland,F.T. (1996) Protein sorting by transport vesicles. *Science*, **272**, 227–234.
Ryder,T.A. and MacKenzie,M.L. (1981) The routine preparation of seminal fluid specimens for transmission electron microscopy. *J. Clin. Pathol.*, **34**, 1006–1009.
Sato,S.B. and Toyama,S. (1994) Interference with the endosomal acidification by a monoclonal antibody directed toward the 116 (100)-kD subunit of the vacuolar type proton pump. *J. Cell Biol.*, **127**, 39–53.
Schimmoller,F., Simon,I. and Pfeffer,S.R. (1998) Rab GTPases, directors of vesicle docking. *J. Biol. Chem.*, **273**, 22161–22164.
Shea,J.E., Hensel,M., Gleeson,C. and Holden,D.W. (1996) Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. *Proc. Natl Acad. Sci. USA*, **93**, 2593–2597.
Shea,J., Beuzón,C., Gleeson,C., Mundy,R. and Holden,D.W. (1999) Influence of the *Salmonella typhimurium* pathogenicity island 2 type III secretion system on bacterial growth in the mouse. *Infect. Immun.*, **67**, 213–219.
Sinai,A.P. and Joiner,K.A. (1997) Safe haven: the cell biology of nonfusogenic pathogen vacuoles. *Annu. Rev. Microbiol.*, **51**, 415–462.
Steele-Mortimer,O., Méresse,S., Gorvel,J.P. and Finlay,B.B. (1999) Biogenesis of *Salmonella typhimurium*-containing vacuoles in epithelial cells involves interactions with the early endocytic pathways. *Cell Microbiol.*, **1**, 33–49.
Stein,M., Leung,K., Zwick,M., Garcia-del Portillo,F. and Finlay,B. (1996) Identification of a *Salmonella* virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells. *Mol. Microbiol.*, **20**, 151–164.
Taylor,R., Miller,V., Furlong,D. and Mekalanos,J. (1987) Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc. Natl Acad. Sci. USA*, **84**, 2833–2837.
Thomas,A.D. and Booth,I.R. (1992) The regulation of expression of the porin gene *ompC* by acid pH. *J. Gen. Microbiol.*, **138**, 1829–1835
Thompson,J., Higgins,D. and Gibson,T. (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673–4680.
Tsolis,R.M., Townsend,S.M., Miao,E.A., Miller,S.I., Ficht,T.A., Adams,L.G. and Baumler,A.J. (1999) Identification of a putative *Salmonella enterica* serotype *typhimurium* host range factor with homology to IpaH and YopM by signature-tagged mutagenesis. *Infect. Immun.*, **67**, 6385–6393.
Uchiya,K., Barbieri,M., Funato,K., Shah,A., Stahl,P. and Groisman,A. (1999) A *Salmonella* virulence protein that inhibits cellular trafficking. *EMBO J.*, **18**, 3924–3933.
Valdivia,R. and Falkow,S. (1997) Fluorescence-based isolation of bacterial genes expressed within host cells. *Science*, **277**, 2007–2011.
Vázquez-Torres,A., Xu,Y., Jones-Carson,J., Holden,D.W., Lucia,S.M., Dinauer,M.C., Mastroeni,P. and Fang,F.C. (2000) *Salmonella* Pathogenicity Island 2-dependent evasion of the phagocyte NADPH oxidase. *Science*, **287**, 1655–1658.
Wood,M., Jones,M., Watson,P., Hedges,S., Wallis,T. and Galyov,E. (1998) Identification of a pathogenicity island required for *Salmonella* enteropathogenicity. *Mol. Microbiol.*, **29**, 883–891.

Received April 10, 2000; revised and accepted May 10, 2000