Growth and killing of a *Salmonella enterica* serovar Typhimurium *sifA* mutant strain in the cytosol of different host cell lines

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Intracellular pathogens have developed different mechanisms which enable their survival and replication within the host cells. Some survive and replicate within a membrane-bound vacuole modified by the bacteria to support microbial growth (e.g. *Salmonella enterica* serovar Typhimurium), whereas others escape from the vacuole into the host cell cytosol, where they proliferate (e.g. *Listeria monocytogenes*). In this study a *Salmonella* strain carrying a mutation in *sifA* which is released from the vacuole was used to analyse *Salmonella* survival and replication within the cytosol of several cell lines. It was found that *Salmonella* replicates within the cytosol of epithelial cells at a higher rate than that achieved when replicating within the vacuole, but is defective for replication in the cytosol of fibroblasts or macrophages. Using an *aroC purD* double mutant strain which does not replicate within host cells, it was shown that *Salmonella* encounters a killing activity within the cytosol of macrophages. Furthermore, *in vitro* experiments using cytosol extracted from either infected or uninfected macrophages suggested that this activity is activated upon *Salmonella* infection.

INTRODUCTION

Some intracellular pathogens, such as *Salmonella enterica* serovar Typhimurium, survive and replicate within mammalian cells in a membrane-bound vacuole, modified by the bacteria to support their growth (Méresse *et al.*, 1999). Other pathogens escape from the vacuole into the host cell cytosol, where they proliferate. Although recent studies have significantly increased our understanding of the processes involved in bacterial proliferation inside a vacuolar compartment, little is known about the conditions that bacteria encounter in the host cell cytoplasm.

Several studies have analysed bacterial replication inside the host cell cytosol, in an attempt to determine whether the cytosol is inhibitory or permissive for the growth of bacteria that normally remain in the vacuole, such as *Salmonella*, or extracellular bacteria such as *Bacillus*

subtilis and non-pathogenic *Escherichia coli* (Bielecki *et al.*, 1990; Gentschev *et al.*, 1995; Goebel & Kuhn, 2000; Goetz *et al.*, 2001).

Two approaches have been used to get bacteria into the host cell cytosol. First, bacterial strains have been engineered to express and secrete listeriolysin, a protein partly responsible for the escape of *Listeria monocytogenes* into the host cell cytosol (Bielecki *et al.*, 1990; Gentschev *et al.*, 1995). Only a limited proportion of the bacterial population was released into the cytosol by this method, probably because other proteins, in addition to listeriolysin, are also required for the efficient release of *Listeria* from the vacuole. In macrophages, listeriolysin-expressing *B. subtilis* and non-pathogenic *E. coli* seemed capable of replication within the macrophage cytosol, whereas no growth was detected for *Salmonella dublin* (reviewed by Goebel & Kuhn, 2000). A second approach relies on the delivery of individual bacteria directly into the host cell cytosol by microinjection (Goetz *et al.*, 2001). Although this method guarantees the delivery to the cytosol of every bacterial cell, it may inflict mechanical damage upon the bacteria that could affect their ability to survive and replicate.

Furthermore, as bacteria are grown *in vitro* prior to their microinjection, the growth conditions used may have an effect on the set of bacterial genes expressed at the moment of their introduction into the cytosol, and therefore on the bacterial response. This second method has been applied to deliver *S. typhimurium*, *Yersinia enterocolitica* and non-pathogenic *E. coli* into the cytosol of epithelial cells, where none displayed any replication (Goetz *et al.*, 2001).

We have identified the *Salmonella* gene *sifA* as necessary to maintain the integrity of the *Salmonella*-containing vacuole (SCV) (Beuzón *et al.*, 2000). Bacteria carrying a mutation in this gene are released into the host cell cytosol several hours after uptake by macrophages (Beuzón *et al.*, 2000). SifA is secreted by a type III secretion system (TTSS), encoded in the *Salmonella* pathogenicity island 2 (SPI-2) (Brumell *et al.*, 2002a; Hansen-Wester *et al.*, 2002). The use of a *sifA* mutant and wild-type strains allows us to compare the replication of isogenic strains that differ in their intracellular sublocalization, and therefore to address the question of the ability of *Salmonella* to replicate within the cytosol of different host cell types.

We find that in epithelial cells *Salmonella* can replicate much more proficiently in the cytosol than when enclosed in a vacuole. However, bacterial replication is strongly inhibited when the bacteria are released into the cytosol of fibroblasts or macrophages. Using an *aroC purD* double mutant strain which is incapable of replication in host cells (Fields *et al.*, 1986), we show that the bacteria encounter a killing activity within the cytosol of macrophages. *In vitro* experiments using cytosol extracted from either infected or uninfected macrophages suggest that this killing activity is activated upon *Salmonella* infection.

METHODS

Bacterial strains and growth conditions. The *S. typhimurium* strains used in this study are listed in Table 1. Bacteria were grown at 37 °C with aeration in Luria–Bertani (LB) medium supplemented with ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), tetracycline (25 μg ml^{-1}) or chloramphenicol (35 μg ml^{-1}), as appropriate.

Plasmids. Plasmid p*sifA*, used to complement the *sifA*::mTn5 mutation, has been described before (Beuzón *et al.*, 2000). Plasmid pFVP25.1, carrying *gfpmut3A* under the control of a constitutive promoter, was introduced into bacterial strains

for fluorescence visualization where indicated (Valdivia & Falkow, 1997).

Cell culture. RAW 264.7 cells were obtained from ECACC (ECACC 91062702). HeLa (clone HtTA1) cells and Swiss 3T3 murine fibroblast cells were kindly provided by S. Méresse (Centre d'Immunologie de Marseille-Luminy, Marseille, France) and E. Caron (Imperial College, London, UK) respectively. Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine at 37 °C in 5% CO$_2$.

Bacterial infection of HeLa cells and Swiss 3T3 fibroblasts, and survival assays. Host cells were seeded onto glass coverslips (12 mm diameter) in 24-well plates at a density of 5×10^5 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 cells at an m.o.i. of approximately 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 gentamicin ml^{-1} and incubated in this medium for 1 h, after which the gentamicin concentration was decreased to 16 μg ml^{-1}. For enumeration of intracellular bacteria (gentamicin-protected), cells were washed three times with PBS, lysed with 0·1% Triton X-100 for 10 min, and dilution series were plated onto LB agar, at different time points after bacterial entry. For microscopic examination, cell monolayers were fixed in 3·7% para-formaldehyde (PFA) in phosphate-buffered saline (PBS) pH 7·4 for 15 min at room temperature, and washed three times in PBS.

Bacterial infection of RAW 264.7 macrophages and survival assays. Macrophages were seeded at a density of 5×10^5 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. of 100:1, centrifuged at 170 *g* for 5 min at room temperature and incubated for 25 min at 37 °C in 5% CO$_2$. These conditions render bacteria non-invasive and thus avoid cytotoxic effect in macrophages. Differences in the way of entry have no significant effect on SCV trafficking or on the intracellular fate of *Salmonella* (Buchmeier & Heffron, 1991; Rathman *et al.*, 1997; S. G. Garvis & D. W. Holden, unpublished results). The macrophages were washed once with DMEM containing FCS and 100 μg gentamicin ml^{-1} and incubated in this medium for 1 h. The medium was replaced with DMEM containing FCS and 16 μg gentamicin ml^{-1} for the remainder of the experiment. For enumeration of intracellular bacteria, macrophages were washed three times with PBS, lysed with 0·1% Triton X-100 for 10 min and a

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

Name	Description	Source or reference
12023	Wild-type	NTCC
HH109	*ssaV*::*aphT* (Km^r) in 12023	Deiwick *et al.* (1999)
P3H6	*sifA*::mTn5 (Km^r) in 12023	Beuzón *et al.* (2000)
HH208	Δ*aroC purD*::Tn*10* (Tet^r) in 12023s	Ruiz-Albert *et al.* (2002)
HH209	Δ*aro purD*::Tn*10* (Tet^r) *sifA*::mTn5 (Km^r) in 12023s	Ruiz-Albert *et al.* (2002)

dilution series was plated onto LB agar. For microscopic examination, cell monolayers were fixed in 3·7 % PFA in PBS pH 7·4 for 15 min at room temperature, and washed three times in PBS.

Preparation of spleen-derived cell suspensions. Mice were inoculated intraperitoneally (i.p.) with 10^5 c.f.u. per mouse (wild-type strain) or 10^6 c.f.u. per mouse (*ssaV* or *sifA* mutant strains), as described previously (Beuzón *et al.*, 2000). Spleens were removed aseptically 3 days after inoculation, and placed in 2 ml ice-cold PBS. Cell suspensions were obtained as described previously (Salcedo *et al.*, 2001). Briefly, cell suspensions were obtained by gentle mechanical disruption with a bent needle, filtered through a 70 μm nylon cell strainer (Becton Dickinson), and centrifuged at 400 *g* for 5 min. Red blood cells were subjected to an ammonium chloride lysis and the rest of the cells were fixed in 1 % PFA for 10 min on ice, washed twice and resuspended in PBS.

Antibodies and reagents. The mouse monoclonal antibody anti-LAMP-1 H3A4 developed by J. T. August and J. E. K. Hildreth was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa (Iowa City, IA, USA), and was used at a dilution of 1:2000 for LAMP-1 staining in HeLa cells. Anti-LAMP-1 rabbit polyclonal antibody 156 against the 11 amino acid residues of the cytoplasmic domain of LAMP-1 has been described previously (Steele-Mortimer *et al.*, 1999); it was used at a dilution of 1:1000 for LAMP-1 staining in Swiss 3T3 fibroblasts. Texas red sulfonyl chloride (TRSC)- and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse, anti-rabbit and anti-goat antibodies were purchased from Jackson Immunoresearch Laboratories, and used at a dilution of 1:200.

Immunofluorescence and electron microscopy. For immunofluorescence, cell monolayers were fixed for 15 min at room temperature in 3·7 % PFA in PBS pH 7·4, and washed three times in PBS. Antibodies were diluted in 10 % horse serum, 1 % bovine serum albumin, 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 Company) or a confocal laser scanning microscope (LSM510, Zeiss).

For transmission electron microscopy of infected HeLa cells, cell suspensions were fixed in 3 % glutaraldehyde prepared in 0·1 M sodium cacodylate 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 & 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.

For transmission electron microscopy of splenocytes, spleens were fixed in 2·5 % glutaraldehyde and 4 % PFA in PBS on ice for 1 h, rinsed in 0·1 M sodium cacodylate pH 7·3 and post-fixed in 1 % osmium tetroxide in the same buffer at room temperature for 1 h. After rinsing in buffer, 1 % tannic acid was added for 30 min. Cells were rinsed for 5 min in 1 % sodium sulfate and then dehydrated in an ethanol series followed by propylene oxide, adding 1 % uranyl acetate at the

30 % stage, embedded in Epon/Araldite 502 and finally polymerized at 60 °C for 24 h. Sections were cut on a Leica Ultracut ultramicrotome at 60 nm using a Diatome knife, contrasted with uranyl acetate and lead citrate, and examined in a Philips CM100 transmission electron microscope.

For immuno-electron microscopy, HeLa cells were infected with either wild-type or *sifA* mutant strains. At 10 h after bacterial entry, cells were fixed with 8 % PFA in PBS for 5 min at room temperature, scraped, pelleted at 100 *g* for 3 min, and further fixed for 1 h on ice. Cells were rinsed in PBS three times without disturbing the pellet, and infiltrated with 2·3 M sucrose in PBS three times for 5 min at room temperature. The samples were then frozen by immersion in liquid nitrogen, where they were stored until sectioning.

For labelling, 70 nm sections were cut on a Leica FCS ultramicrotome and transferred to copper grids on PBS containing 0·02 M glycine, blocked with 5 % FCS for 30 min and incubated with mouse monoclonal anti-LAMP-1 primary antibody at a 1:50 dilution, for 1 h. Sections were rinsed three times in PBS for 5 min and incubated for 1 h with goat f(ab′)2 anti-mouse IgG secondary antibody conjugated to 10 nm gold particles, at a 1:20 dilution, for 1 h. Sections were rinsed twice in PBS for 2 min, fixed in 2·5 % glutaraldehyde in PBS for 5 min, and rinsed in water. Sections were counterstained with uranyl acetate in methyl cellulose for 10 min on ice, picked up on copper loops to air-dry, and examined in a Philips CM100 transmission electron microscope.

Cytosol extraction and growth assays. RAW macrophages (2×10^8 cells) were washed in ice-cold PBS and scraped into 100 ml ice-cold PBS. Samples were centrifuged at 400 *g* for 5 min to collect the cells, which were then resuspended in 1 ml ice-cold PMEE (35 mM PIPES pH 7·4, 5 mM $MgSO_4$, 1 mM EGTA, 0·5 mM EDTA and 250 mM sucrose). Samples were passed through a 21 G needle several times, until more than 80 % of the cells were lysed. The lysates were cleared of nuclei and other cellular debris by centrifugation at 400 *g* for 5 min at 4 °C. Cleared lysates were then centrifuged at 150 000 *g* for 1 h at 4 °C, onto a 30 % sucrose layer. The supernatant of this centrifugation (cytosol) was either used directly for growth assays or frozen by immersion of tubes in liquid nitrogen. To obtain cytosol from *Salmonella*-infected macrophages, the cells were first infected with *aroC purD* double mutant bacteria expressing green fluorescent protein (GFP) at an m.o.i. of approximately 100:1. After 4 h, cells were processed as described above. Samples were taken from each preparation to confirm that at least 50 % of the cells were infected. For growth assays, 10^3 c.f.u. of exponentially growing wild-type bacteria were added to a 100 μl aliquot of cytosol extract and incubated at 37 °C for 8 h. All cytosol extractions were diluted to a final protein concentration of 50 μg ml^{-1} in PMEE before use. Aliquots of 10 μl were removed immediately after adding the bacteria and 8 h later, diluted, and plated onto LB plates to enumerate bacteria.

RESULTS AND DISCUSSION

Replication of *Salmonella* in the cytosol of epithelial cells

We have shown that *S. typhimurium sifA* mutant bacteria are gradually released into the cytosol of macrophages several hours after uptake. The loss of the vacuolar membrane surrounding *sifA* mutant bacteria correlates with a decrease of association between the bacteria and LAMP-1 (Beuzón *et al.*, 2000), a lysosomal
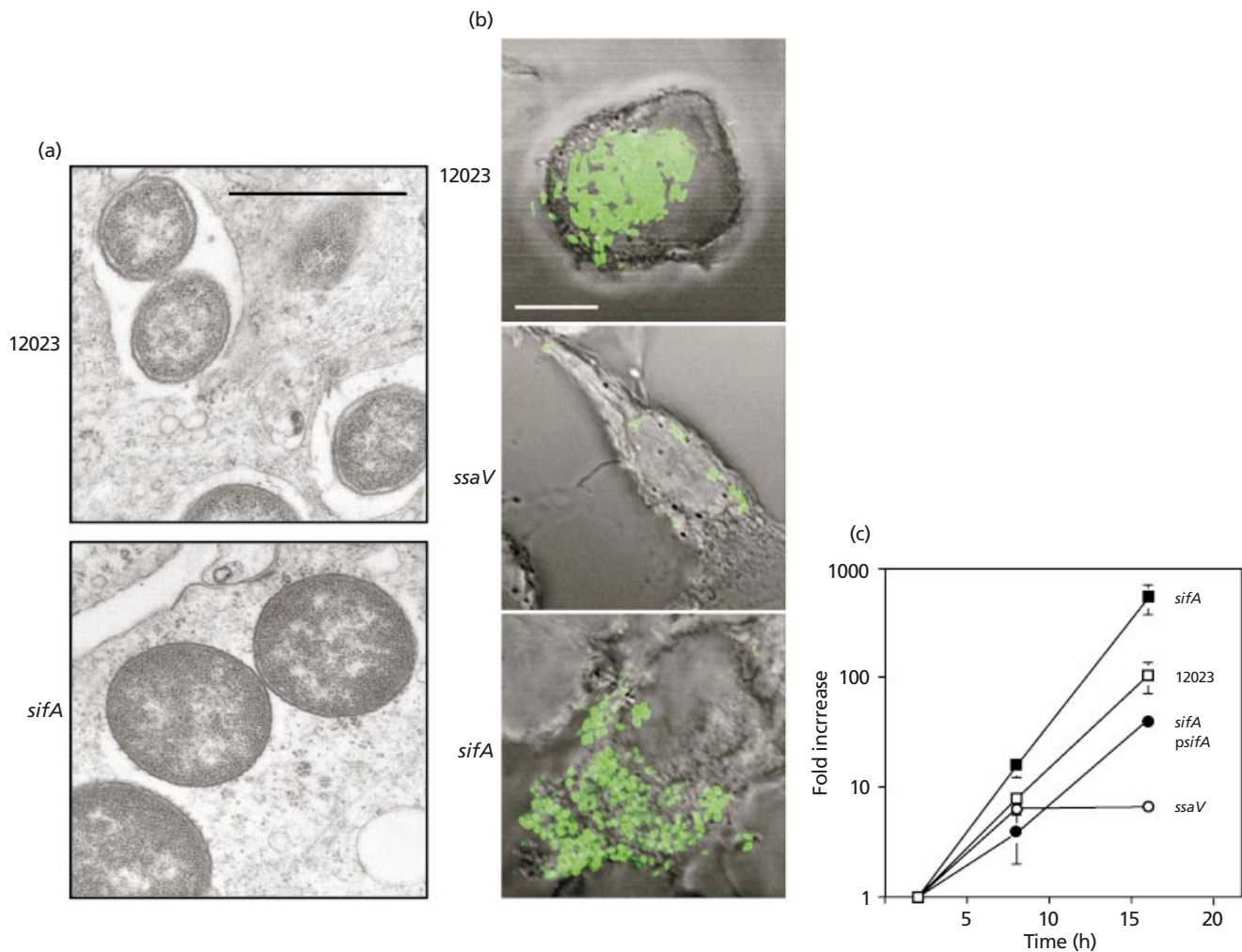
(a)

(b)

12023

12023

ssaV

sifA

sifA

sifA

(c)

Fig. 1. *S. typhimurium* bacteria carrying a mutation in *sifA* are released into the host cell cytosol, and replicate efficiently in HeLa cells. (a) Electron micrographs of HeLa cells infected with either wild-type (12023) or *sifA* mutant (P3H6) strains. Bar, 1 μm. (b, c) Replication assays were carried out in HeLa cells for wild-type (12023), *ssaV* mutant (HH109) and *sifA* mutant (P3H6) strains, and the *sifA* mutant strain carrying the *sifA*-complementing plasmid p*sifA*. At 2, 8 or 16 h after bacterial entry, cells were lysed and cultured for enumeration of intracellular bacteria (gentamicin-protected) (c); at 16 h after bacterial entry, cells were fixed and examined by phase-contrast and confocal fluorescence microscopy (b). Bar, 5 μm. The values shown in (c) represent the fold increase calculated as the ratio of the number of intracellular bacteria at 8 or 16 h, to the number at 2 h after bacterial entry. Each infection was carried out in triplicate and the standard errors from the means are shown. All strains expressed GFP constitutively.

membrane glycoprotein (lgp) that associates with the *Salmonella*-containing vacuole (SCV) (García-del Portillo & Finlay, 1995). At 10 h after invasion of HeLa cells, *sifA* mutant bacteria were also found to associate with several lgps and the vacuolar ATPase (vATPase) at a lower level than wild-type bacteria (Beuzón *et al.*, 2000), suggesting that the release of *sifA* mutant bacteria into the cytosol also occurs in epithelial cells. To confirm this, HeLa cells were examined by electron microscopy 10 h after infection with either wild-type or *sifA* mutant bacteria. Whereas vacuolar membranes are clearly visible around wild-type bacteria, no membrane could be seen surrounding the majority of *sifA* mutant bacteria (Fig. 1a), indicating that, as in macrophages, loss of SifA changes the intracellular localization of *S. typhimurium*

from the vacuole to the host cell cytosol. Therefore, *sifA* mutant bacteria can be used as a tool to investigate *S. typhimurium* replication in the cytosol of different cell lines.

A *sifA* mutant strain was reported to have replication levels comparable to, or even higher than, the wild-type strain in different epithelial cell lines (Stein *et al.*, 1996). To study this phenomenon in more detail, the number of intracellular bacteria was determined by plating lysates of infected cells at different times after bacterial entry. A strain carrying a mutation in *ssaV*, a gene essential for SPI-2 TTSS-mediated protein secretion (Beuzón *et al.*, 1999), and required for replication in HeLa cells (Cirillo *et al.*, 1998; Ruiz-Albert *et al.*, 2002), was included as a
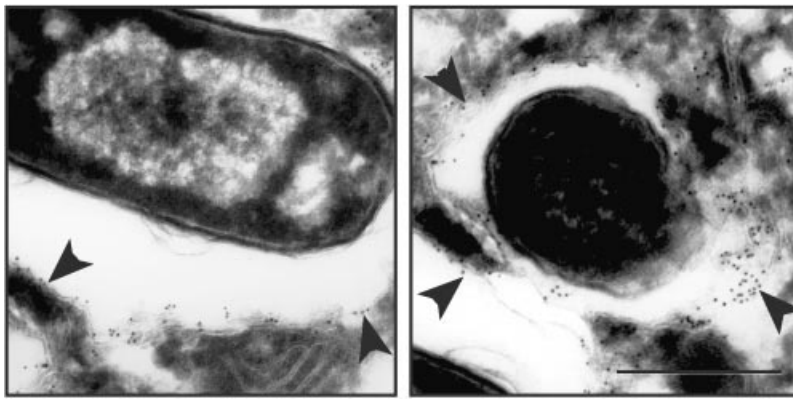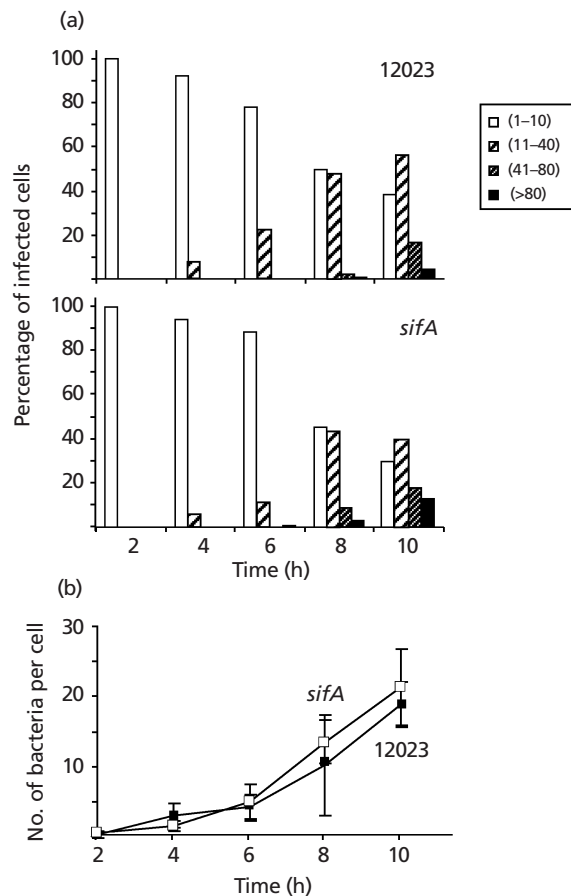
Fig. 3. Analysis of bacterial replication by microscopic examination of infected cells. HeLa cells infected with either the wild-type (12023) or the *sifA* (P3H6) mutant strain were fixed at the indicated time points after bacterial entry. The number of bacteria per infected cell was determined by confocal microscopic examination. All strains expressed GFP constitutively. (a) Values correspond to the number of infected cells ($n = 100$) containing either 1–10 bacteria, 11–40 bacteria, 41–80 bacteria or more than 80 bacteria per infected cell, and are the results from three independent experiments. (b) Values are expressed as mean number of bacteria per infected cell ($n = 100$ infected cells) $\pm$ SE, and are the result of three independent experiments. With the exception of the 2 h time point, only cells displaying bacterial replication (more than 10 bacteria per cell) were considered.

control in the assays. All strains examined carried a plasmid expressing GFP constitutively, for microscopic visualization of bacteria. Replication of the *sifA* mutant strain was comparable to that of the wild-type strain 8 h after bacterial entry, and approximately five times higher 16 h after entry; microscopic examination of the infected cells was consistent with these results (Fig. 1b). This increase in replication compared to the wild-type strain disappeared when the *sifA* mutant strain was complemented by expression of the *sifA* wild-type allele from a plasmid (p*sifA*) (Fig. 1c). This plasmid is sufficient to restore a vacuolar membrane to mutant bacteria (Beuzón *et al.*, 2000).

It is noteworthy that the replication of the *ssaV* mutant strain at 8 h after bacterial entry was equivalent to that of the wild-type strain, but was 10 times lower at 16 h (Fig. 1b, c). This is consistent with results of Brumell *et al.* (2001), who found that SPI-2 is not required for replication in HeLa cells up to 6 h after bacterial entry.

The presence of large numbers of *sifA* mutant bacteria in the cytosol of HeLa cells 10 h after entry could be explained by either an increased replication inside the vacuole followed by release into the cytosol, or release from the vacuole followed by an increased replication rate within the cytosol. To differentiate between these two possibilities, an experiment was performed in which bacterial numbers per infected cell were counted by microscopy at different time points throughout the infection. To distinguish between vacuolar and cytosolic bacteria, we used LAMP-1 as a marker for the presence of the vacuolar membrane. Immunogold labelling of ultrathin sections of HeLa cells infected with wild-type bacteria with a monoclonal anti-LAMP-1 antibody showed that LAMP-1 is localized on the SCV membrane (Fig. 2), confirming the suitability of LAMP-1 as a marker for the presence of the SCV membrane.

We first counted the proportion of HeLa cells infected with either wild-type or *sifA* mutant strains ($n = 100$ infected cells) that contained either 1–10, 11–40, 41–80 or more than 80 bacteria per cell. As expected for both strains, replication was detected 4 h after bacterial entry, as shown by the appearance of cells containing between

11 and 40 bacteria per cell. However, only a minority of the cells (5–10 %) displayed replication at this time (Fig. 3a). Wild-type bacterial replication increased steadily up to 10 h, although at this time point, 30–40 % of the infected cells still contained only 1–10 bacteria per cell (Fig. 3a). After 10 h bacterial numbers were too high to allow accurate enumeration by microscopy. The replication of the *sifA* mutant strain was lower than that of the wild-type at 4 h and 6 h. However, its replication was equal to that of the wild-type by 8 h, and was higher by 10 h, as seen by the increase in the number of infected cells containing more than 80 bacteria (Fig. 3a). Representation of these results as the mean number of bacteria per infected cell shows overall bacterial replication over time, which is in general agreement with the results of the replication assay (Fig. 3b), although the differences observed between wild-type and *sifA* mutant strains at 10 h are less marked, since the exact number of bacteria per cell in cells containing more than 80 bacteria cannot be determined accurately and the data were therefore not included in the analysis.

After establishing and validating microscopic examination of bacterial numbers as a reliable method to assess replication, we applied it to the study of replication of vacuolar versus cytosolic bacteria in cells infected with the *sifA* mutant strain, as determined by LAMP-1 association. Fig. 4(a) shows representative examples of wild-type bacteria associated with LAMP-1, and *sifA* mutant bacteria not associated with LAMP-1. For these experiments, cells containing more than 80 bacteria were not considered as it was not possible to determine the level of LAMP-1 association accurately. In cells infected with the wild-type strain only an extremely small number of bacteria were found not to be associated with LAMP-1, and this number did not increase during the time of the infection, whereas the population of LAMP-1-positive bacteria showed an increase that closely matched the overall replication (Fig. 4b). In contrast, a constant low number of LAMP-1 positive *sifA* mutant bacteria were observed throughout the time of infection, whereas the number of LAMP-1 negative bacteria increased markedly from 6 h onwards (Fig. 4b). These results indicate that the replication of the *sifA* mutant strain takes place once the bacteria have reached the cytosol of the host cell, after being released from the vacuole.

It is interesting to note that not all *sifA* mutant bacteria lost their vacuolar membrane, and that there was no net increase in this population. This may reflect a trafficking defect displayed by a small proportion of SCVs, irrespective of bacterial genotype.

A striking characteristic of the cytosolic bacteria was their unusually large size. Although these bacteria maintain a normal bacillus shape, approximately 75 % of them were 2–4 μm long, almost double the usual length of the wild-type, which ranges from 1 to 2 μm (data not shown). Such large bacteria were never found in cells infected with wild-type bacteria (*n* > 100 infected cells). A representative example can be seen in Fig. 1(a),
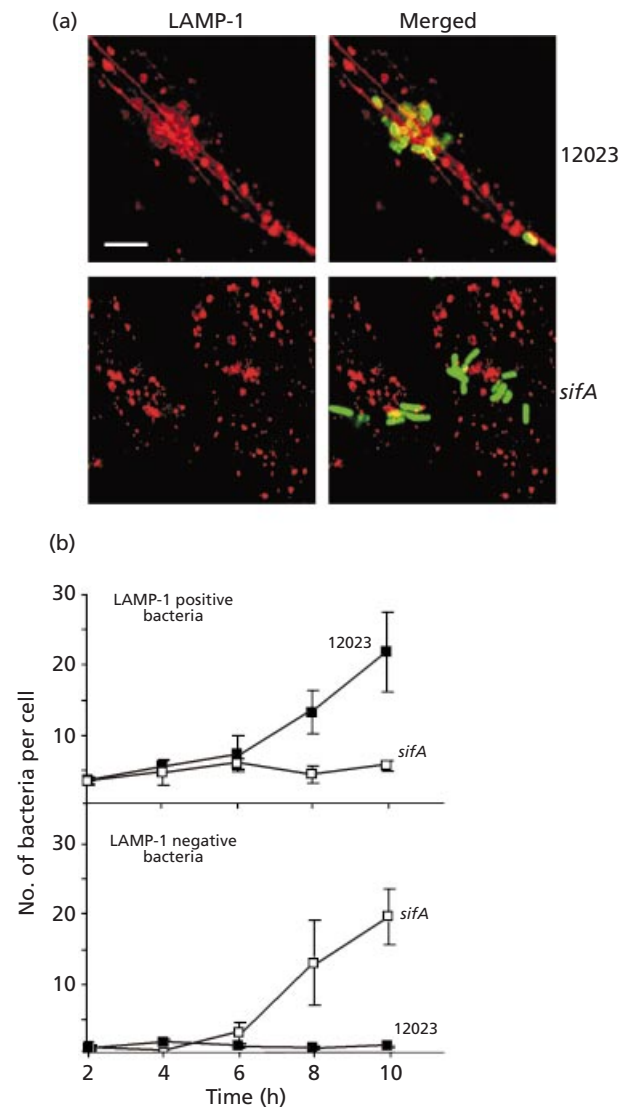

Fig. 4. Bacteria carrying a mutation in *sifA* replicate efficiently in the cytosol of HeLa cells. (a) Confocal immunofluorescence micrographs of HeLa cells infected with either the wild-type (12023) or the *sifA* mutant (P3H6) strain. Cells were fixed 10 h after bacterial entry and labelled using mouse monoclonal anti-LAMP-1 primary antibody and a TRSC-conjugated donkey anti-mouse secondary antibody to detect LAMP-1 (red). All strains expressed GFP constitutively (green). Bar, 5 μm. (b) Cells infected with either the wild-type (12023) or the *sifA* mutant strain (P3H6) were fixed at the indicated time points after bacterial entry. The number of wild-type (■) or *sifA* mutant (□) bacteria per cell, either associating or not associating with LAMP-1, was quantified by microscopic analysis. Values are expressed as mean number of bacteria ± SE for each population, per infected cell (*n* = 100 infected cells), at each time point, and are the result of three independent experiments. With the exception of the 2 h time point, only cells containing between 11 and 80 bacteria per cell were considered.

where cross-sections of both wild-type and cytosolic *sifA* mutant bacteria are shown in electron micrographs at the same magnification.

Together, these results indicate that *S. typhimurium* is capable of replicating within the cytosol of human
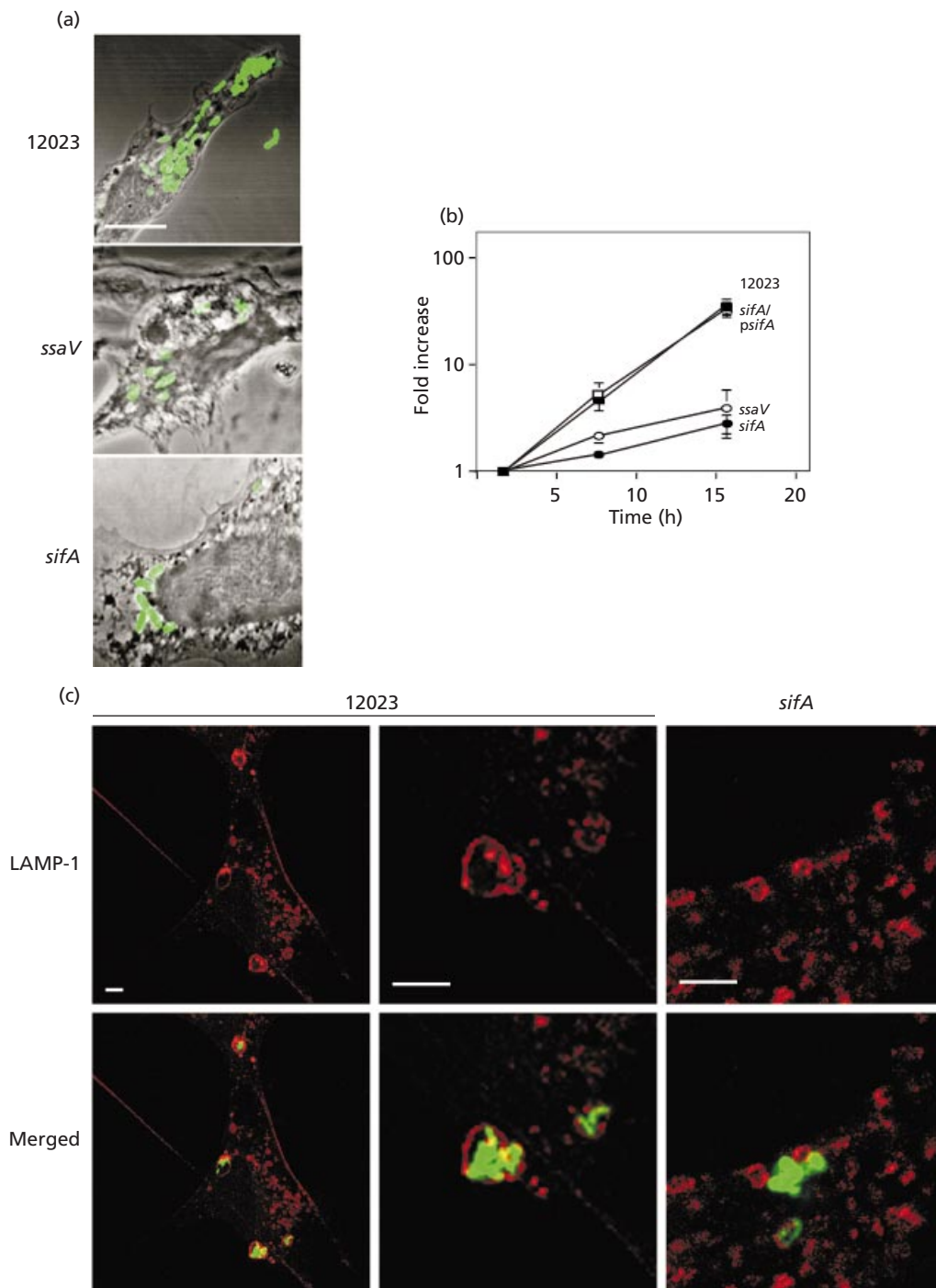
(a)

12023

ssaV

sifA

(b)

(c)

12023   *sifA*

LAMP-1

Merged

Fig. 5. Bacteria carrying a mutation in *sifA* are defective for replication within Swiss 3T3 fibroblasts. (a, b) Replication assays were carried out in Swiss 3T3 fibroblasts for wild-type (12023), *ssaV* mutant (HH109) and *sifA* mutant (P3H6) strains, or the *sifA* mutant strain carrying the *sifA*-complementing plasmid p*sifA*. (a) Cells were fixed at 16 h and examined by phase-contrast and confocal immunofluorescence microscopy. Bar, 5 µm. (b) Cells were lysed and the intracellular bacteria (gentamicin-protected) enumerated at 2, 8 and 16 h after bacterial entry. The values show the bacterial fold increase calculated as a ratio of the number of the intracellular bacteria at 8 or 16 h, to the number at 2 h. Each infection was carried out in triplicate and the standard errors from the means are shown. (c) Confocal microscopic analysis of Swiss 3T3 fibroblasts infected with either wild-type (12023) or *sifA* mutant (P3H6), and fixed 10 h after bacterial entry. LAMP-1 (red) was detected using a rabbit polyclonal anti-LAMP-1 and a TRSC-conjugated donkey anti-rabbit as primary and secondary antibodies, respectively. All strains expressed GFP constitutively. Bars, 5 µm.

epithelial cells. These results are in contrast to the results obtained by direct microinjection of *Salmonella* into epithelial cells (Goetz *et al.*, 2001). There are several possible explanations for these differences. We cannot rule out the possibility that the *sifA* mutation may confer a replication advantage upon bacteria in the cytosol. However, it seems more likely that as a consequence of microinjection, bacteria delivered into the cytosol are not capable of replication, either because of mechanical damage affecting the integrity of the bacterial cell, or because the appropriate set of genes that allow bacterial replication within the cytosol is only activated upon normal bacterial entry and passage through the vacuole.

Salmonella replication in the cytosol of non-permissive cell lines is impaired

The ability of the *sifA* mutant strain to replicate in the cytosol of epithelial cells is in contrast to its replication defect in tissue-cultured macrophages (Beuzón *et al.*, 2000; Brumell *et al.*, 2001). The *sifA* mutant strain also displayed a severe replication defect in mouse Swiss 3T3 fibroblasts, equivalent to that of the *ssaV* mutant strain, and approximately a tenth of the replication displayed by the wild-type strain (Fig. 5a, b). This cell line has been shown to be restrictive for *Salmonella* replication (Cano *et al.*, 2001; García-del Portillo, 2001; Martínez-Moya *et al.*, 1998). The presence of the SCV membrane surrounding intracellular bacteria was assessed using LAMP-1 association with either wild-type or *sifA* mutant bacteria as a marker for the vacuolar membrane, 10 h after bacterial entry. Whereas more than 60% of the wild-type bacteria were clearly associated with LAMP-1, less than 5% of the *sifA* bacteria were found to associate with the membrane marker (Fig. 5c). As observed in epithelial cells, in both macrophages and fibroblasts a high proportion of unusually large bacteria could be detected in the LAMP-1-negative population of *sifA* mutant bacteria (data not shown).

We have previously shown that the *sifA* mutant strain displays an attenuation of virulence similar to that of SPI-2 TTSS null secretion mutants when injected i.p. into the mouse (Beuzón *et al.*, 2000). We have also shown that the majority of the *sifA* mutant bacteria are accessible to anti-LPS antibody in non-permeabilized preparations of splenic macrophages obtained from heavily infected mice, suggesting that the vacuolar membrane enclosing these bacteria has been lost, or its integrity compromised (Salcedo *et al.*, 2001). To determine if *sifA* mutant bacteria are mostly localized in the cytosol of splenic macrophages *in vivo*, ultrathin sections of spleens were obtained from mice, 48 h after i.p. inoculation with wild-type or *sifA* mutant bacteria. Electron micrographs showed that, whereas most of the wild-type bacteria were clearly surrounded by a vacuolar membrane, the majority of the *sifA* mutant bacteria appeared to be free within the cytosol (Fig. 6a). Microscopic analysis of the number of bacteria per infected splenocyte was carried out to estimate in-

tracellular replication within the spleen (Salcedo *et al.*, 2001). Although mice had to be inoculated with 10 times more bacteria for the mutant strains than for the wild-type to detect significant numbers of bacteria per spleen, the *sifA* mutant strain still displayed a decrease in intracellular replication similar to that of an *ssaV* mutant strain, approximately three times lower than the replication of the wild-type strain within the spleen (Fig. 6b), supporting the results obtained in tissue-cultured macrophages.

We have previously shown that *sifA* mutant bacteria are mostly cytosolic 10 h after uptake by macrophages, and that this correlates with the progressive loss of the vacuolar membrane, beginning approximately 6 h after bacterial uptake (Beuzón *et al.*, 2000). This finding, together with the results obtained in the present work with epithelial cells, fibroblasts and splenocytes, indicates that the overall differences in replication are caused by differences in the ability of the bacteria to replicate within the cytosol of these host cells. Our results also suggest that the virulence attenuation of the *sifA* mutant strain is at least partly caused by its release into the cytosol of splenic macrophages, where *S. typhimurium* fails to replicate.

Evidence of *Salmonella* killing in the cytosol of macrophages

Although the *sifA* mutant strain has a replication defect in macrophages, we have consistently observed a small net increase in the number of intracellular mutant bacteria between 2 and 16 h (Beuzón *et al.*, 2000; and data not shown). To test if bacterial death is occurring simultaneously with bacterial replication, we followed the same approach as used by Buchmeier & Libby (1997). To estimate bacterial death in the absence of bacterial replication, we used an auxotrophic double mutant strain (*aroC purD*), which is unable to grow in cultured macrophages (Fields *et al.*, 1986). When assayed in time-course replication assays, the *aroC purD* mutant strain, which remains within a vacuole throughout the course of the infection (Ruiz-Albert *et al.*, 2002), displayed a fivefold decrease in numbers between 2 and 16 h, in close agreement with the results reported by Buchmeier & Libby (1997) (Fig. 7a). An *aroC purD sifA* triple mutant strain, which is released into the host cell cytosol at the same rate as a *sifA* mutant strain (Ruiz-Albert *et al.*, 2002), also displayed a decrease in bacterial numbers (Fig. 7a). Furthermore, the growth defect of the *aroC purD* double mutant strain, but not that of the *aroC purD sifA* triple mutant, could be completely restored by supplementing the macrophage culture medium with appropriate metabolites to supplement the auxotrophy (data not shown). These results indicate that a fraction of the cytosolic population of *Salmonella* is being actively killed by an antimicrobial activity. However, in contrast with what happens to vacuole-enclosed *Salmonella*, the low net bacterial growth in the cytosol of macrophages suggests that bacterial replication, as well as survival, is reduced in this environment. Similar replication assays using the auxo-
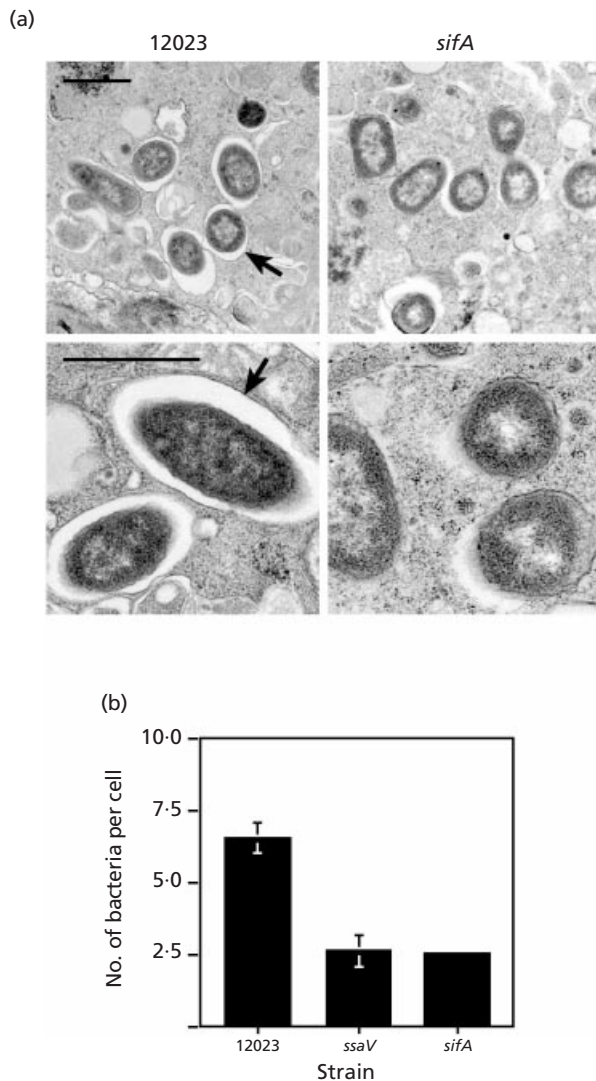
(a)

(b)

Fig. 6. *sifA* mutant bacteria are defective for intracellular replication, and are released into the host cell cytosol of splenic macrophages *in vivo*. Mice were inoculated i.p. with either 10^5 c.f.u. of the wild-type (12023) or 10^6 c.f.u of the *sifA* mutant (P3H6) per mouse, spleens harvested after 3 days, and splenocytes fixed and processed for (a) electron microscopy or (b) confocal microscopy. (a) Arrows indicate vacuolar membranes. Bars, 1 μm. (b) The number of bacteria per infected cell was determined by confocal microscopic examination ($n = 100$ infected cells). All strains expressed GFP constitutively. The values show the mean number of intracellular bacteria. Each infection was carried out in triplicate and the standard errors from the means are shown.

trophic strains in HeLa cells did not reveal any evidence of an antimicrobial activity (data not shown).

To date only one cytosolic macrophage antimicrobial peptide, ubiquicidin, has been reported (Hiemstra *et al.*, 1999). Ubiquicidin was purified from the cytosol of macrophages activated by the addition of interferon-γ, and displays antimicrobial activity *in vitro* against *S. typhimurium* as well as *Y. enterocolitica*, *E. coli*, *Staphylococcus aureus* and *L. monocytogenes* (Hiemstra
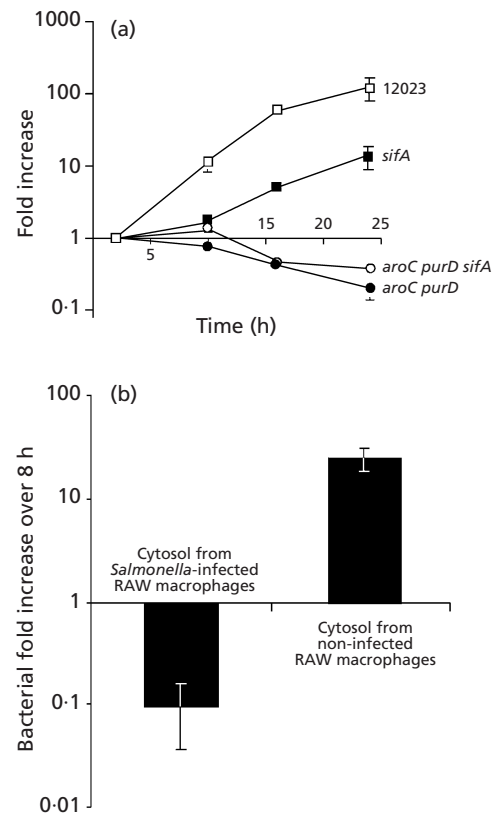
(a)

(b)

Fig. 7. The cytosol of *Salmonella*-infected macrophages has antimicrobial activity. (a) Replication assays were carried out for wild-type (12023), *aroC purD* double mutant (HH208), *sifA* mutant (P3H6), or *aroC purD sifA* triple mutant (HH209) strains in RAW macrophages. At 2, 8, 16 and 24 h after bacterial entry, cells were lysed and cultured for enumeration of intracellular bacteria (gentamicin-protected). The values represent the fold increase calculated as a ratio between the number of intracellular bacteria at 8, 16 or 24 h, to the number at 2 h after bacterial entry. Each strain was infected in triplicate and the standard errors from the means are shown. (b) Growth assays were carried out for the wild-type (12023) strain growing for 8 h in cytosol extracts from either *Salmonella*-infected or uninfected macrophages. Aliquots were taken immediately after adding the bacteria to the cytosol and 8 h after, and cultured for bacterial enumeration. The values shown represent the fold increase calculated as a ratio of the intracellular bacteria between 0 and 8 h. Each sample was assayed in triplicate; the results from three independent experiments were combined and are expressed as means ± SE.

et al., 1999). Using a similar approach, we extracted cytosol from either *Salmonella*-infected or uninfected macrophages to perform *in vitro* growth assays. When cytosol isolated from uninfected macrophages was used as growth medium for wild-type or *sifA* mutant strains, a 10–100-fold increase in bacterial counts was measured over 8 h. However, if the cytosol added was obtained from *Salmonella*-infected macrophages, the viable counts decreased by 10–100 fold (Fig. 7b and data not shown). These results are consistent with the need for macrophage activation to detect the antimicrobial activity in the cytosol that was reported previously (Hiemstra *et al.*, 1999), and suggest that *Salmonella*

infection can induce such an activity. Preliminary results have shown that antimicrobial activity induced in the cytosol of *Salmonella*-infected macrophages is also effective *in vitro* against *Staph. aureus*, *E. coli* and *L. monocytogenes* (C. R. Beuzón, A. Alyahya & D. W. Holden, unpublished results). Although the results obtained so far suggest that the bacterial killing detected in tissue culture macrophages infected with *Salmonella sifA* mutant could be accounted for by ubiquicidin, further research is necessary to determine if that is the case.

It is interesting to consider the apparent contradiction between the different replication efficiencies of *Salmonella* and *Listeria* in the cytosol of macrophages, and their equal sensitivity *in vitro* to the antimicrobial activity activated by interferon-γ addition or by *Salmonella* infection. One possible explanation is that *Listeria* is only sensitive to this activity(s) *in vitro*, because it responds to conditions within the vacuole by expressing proteins that allow it to survive and replicate within the cytosol of macrophages. An alternative explanation could be that *Salmonella*, but not *Listeria*, infection triggers the onset of this activity(s). This would explain how an extracellular soil micro-organism such as *B. subtilis* could replicate in the cytosol of macrophages if its uptake does not trigger the onset of this defence mechanism(s). Further work will be necessary to reveal the mechanism(s) that allow cytosolic pathogens to evade this activity(s).

In summary, our results indicate that when *S. typhimurium* is released into the cytosol of epithelial cells it is able to replicate, whereas in macrophage cytosol it cannot replicate and encounters an anti-*Salmonella* killing activity. Presumably, this constitutes a strong selective pressure for the maintenance of the integrity of the *Salmonella*-containing vacuole.

NOTE ADDED IN PROOF

After submission of this manuscript, similar findings on replication of a *sifA* mutant in epithelial cell cytosol were reported by Brumell *et al*. (2002b).

ACKNOWLEDGEMENTS

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