Intracellular survival of *Salmonella enterica* serovar Typhi in human macrophages is independent of *Salmonella* pathogenicity island (SPI)-2

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For successful infection, *Salmonella enterica* secretes and injects effector proteins into host cells by two distinct type three secretion systems (T3SSs) located on *Salmonella* pathogenicity islands (SPIs)-1 and -2. The SPI-2 T3SS is involved in intracellular survival of *S. enterica* serovar Typhimurium and systemic disease. As little is known regarding the function of the SPI-2 T3SS from *S. enterica* serovar Typhi, the aetiological agent of typhoid fever, we investigated its role for survival in human macrophages. Mutations in the translocon (*sseB*), basal secretion apparatus (*ssaR*) and regulator (*ssrB*) did not result in any reduction in survival under many of the conditions tested. Similar results were obtained with another *S. Typhi* strain or by using human primary cells. Results were corroborated based on complete deletion of the SPI-2 T3SS. Surprisingly, the data suggest that the SPI-2 T3SS of *S. Typhi* is not required for survival in human macrophages.

INTRODUCTION

Salmonella enterica is an important pathogen of animals and humans, which typically become infected by ingestion of contaminated food or water. There are two major groups of *S. enterica* that cause disease in humans: the systemic (typhoid fever) group and the non-typhoidal salmonellae group. Typhoid fever is mainly caused by *S. enterica* serovar Typhi (*S. Typhi*), a host-adapted serovar that is specific for humans. Non-typhoidal salmonellae are generally associated with gastroenteritis, which rarely develops into an invasive infection. *S. enterica* serovar Typhimurium is frequently associated with gastroenteritis in humans, whereas this serovar causes a systemic typhoid-like disease in susceptible mice. *S. Typhimurium* infection in susceptible mice is usually used as a model for *S. Typhi* infection in humans, as these serovars share many virulence factors, including *Salmonella* pathogenicity islands (SPIs) (McClelland *et al.*, 2001; Parkhill *et al.*, 2001; Sabbagh *et al.*, 2010).

S. Typhimurium encodes two distinct type three secretion systems (T3SSs), located on SPI-1 and SPI-2. The SPI-1

T3SS is involved in invasion of epithelial cells (Galán & Curtiss, 1989), whereas the SPI-2 T3SS is essential for survival and replication in host cells (Ochman *et al.*, 1996; Shea *et al.*, 1996). The SPI-2 T3SS corresponds to a 25 kb fragment organized in four operons: regulatory, structural I, structural II and effector/chaperone (see Supplementary Fig. S1, available with the online version of this paper, and Kuhle & Hensel, 2004). The two-component regulatory system SsrA/SsrB is responsible for the transcriptional regulation of SPI-2 operons as well as the regulation of effector genes located outside SPI-2 (reviewed by Haraga *et al.*, 2008). The T3SS needle complex comprises basal component proteins in the inner membrane (including SsaR), proto-channel proteins, outer membrane proteins, proteins forming a hollow tube generating the needle and outer ring proteins (including SseB), which form the translocon that traverses the host vacuolar membrane (Supplementary Fig. S1). This SPI-2 T3SS injects at least 20 effector proteins across the vacuolar membrane into the host cytosol (Haraga *et al.*, 2008). These effectors interact with the host machinery and are required for modification of the vacuole and modulation of intracellular processes (Brumell *et al.*, 2001; Waterman & Holden, 2003; Yu *et al.*, 2004), which will allow the bacteria to survive and replicate in a membrane-bound structure called the *Salmonella*-containing vacuole. Intracellular induction of SPI-2 genes was initially observed by using reporter assays (Valdivia & Falkow, 1997; Cirillo *et al.*, 1998; Pfeifer *et al.*, 1999) and was confirmed by microscopy (Kuhle & Hensel, 2002), microarray data (Eriksson *et al.*, 2003) and Western blotting (Coombes *et al.*, 2007).

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Abbreviations: CI, competitive index; IFN- γ , gamma interferon; MDMs, monocyte-derived macrophages; PBMCs, peripheral blood mononuclear cells; SPI, *Salmonella* pathogenicity island; T3SS, type three secretion system.

Two supplementary figures, showing the SPI-2 T3SS and the mutants generated during this study, are available with the online version of this paper.

Survival and replication of *S. Typhimurium* within cultured cells correlate with systemic survival and persistence in animal models, as mutants that fail to replicate in cultured cells are avirulent in animals (Fields *et al.*, 1986; Leung & Finlay, 1991; Shea *et al.*, 1999). Survival within macrophages is therefore an essential step for *Salmonella* pathogenesis. Two major systems are implicated in this process: (1) the two-component regulatory system PhoPQ and its regulon (Groisman *et al.*, 1989; Miller *et al.*, 1989; Garvis *et al.*, 2001) and (2) the SPI-2 T3SS (Shea *et al.*, 1996; Cirillo *et al.*, 1998; Hensel *et al.*, 1998). The intracellular proliferation defect of SPI-2 mutants was observed in primary macrophages and several macrophage-like cell lines as well as in epithelial cell lines (Ochman *et al.*, 1996; Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Beuzón *et al.*, 2002). *S. Typhimurium* strains deficient in SPI-2 are highly attenuated in the murine systemic infection model (Shea *et al.*, 1996; Hensel *et al.*, 1998; Chan *et al.*, 2005).

The intracellular survival defect can be observed in most individual mutants, such as *sseB*, *ssaR* and *ssrB*. The *sseB* mutant of *S. Typhimurium* has a growth defect in murine macrophages (peritoneal, RAW264.7 and J774A.1), and in epithelial cells (HEp-2) but was not recovered from liver or spleen of infected BALB/c mice (Cirillo *et al.*, 1998; Hensel *et al.*, 1998). SseB is a translocon constituent involved in pore formation in the host membrane for delivery of effector proteins. An *sseB* mutant does not secrete SseD and SseC, two other translocon proteins (Chakravorty *et al.*, 2005), and SseB is required for the translocation of effector proteins by intracellular *Salmonella* (Nikolaus *et al.*, 2001). Also, *ssaR* mutants of *S. Typhimurium* are defective in intracellular replication in murine macrophages (RAW264.7), are attenuated in BALB/c mice and are unable to secrete SPI-2 effectors (Shea *et al.*, 1996; Coombes *et al.*, 2004). Mutants of the two-component system SsrAB are unable to survive within murine macrophages (RAW264.7, J774A.1), are impaired in epithelial cell replication (HEp-2) and are avirulent in mice (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Ochman *et al.*, 1996; Shea *et al.*, 1996).

Moreover, SPI-2 has also been shown to interfere with antigen presentation (Cheminay *et al.*, 2005; Tobar *et al.*, 2006; Halici *et al.*, 2008), cytokine production (Uchiya *et al.*, 2004) and cytotoxicity (Monack *et al.*, 2001; Paesold *et al.*, 2002). The critical role of the SPI-2 T3SS for *S. Typhimurium* is therefore well established. Although most SPI-2 genes were upregulated following *S. Typhi* uptake by human macrophages (Faucher *et al.*, 2006), the specific role of the SPI-2 T3SS of *S. Typhi* has not yet been reported. In this study, we targeted specific components of the secretion system, such as the translocon (*sseB*), the basal secretion apparatus (*ssaR*) and the regulator (*ssrB*) in order to investigate the contribution of the SPI-2 T3SS during interaction of *S. Typhi* with human macrophages.

METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown overnight statically (low aeration) in Luria–Bertani (LB) broth, unless indicated otherwise. When required, antibiotics or supplements were added at the following concentrations: 50 µg ampicillin ml⁻¹, 34 µg chloramphenicol ml⁻¹, 50 µg nalidixic acid ml⁻¹ or 50 µg diamino-pimelic acid ml⁻¹.

Construction of SPI-2 mutants. Deletion of *sseB*, *ssaR* and the entire T3SS of SPI-2 [$\Delta(ssaU-ssrB)$] were generated by allelic exchange as described previously for the *ssrB* deletion (Faucher *et al.*, 2009) by using the overlap-extension PCR method (Basso *et al.*, 2002). Primers are listed in Table 2, and detailed information on the deletions is presented in Supplementary Fig. S2. Mutations were confirmed by PCR. All mutants have growth curves in LB broth similar to that of the wild-type strain (data not shown).

SseB antibodies and secretion assays. The amplified *sseB* gene of *S. Typhi* ISP1820 was cloned into pET-21a vector (pSIF128) and SseB proteins were purified by using Ni-nitrilotriacetic acid agarose beads (Qiagen) and polyclonal antibodies against SseB were raised in rabbits (GeneScript). For secretion assays, bacteria were grown in a low phosphate and magnesium medium (LPM; pH 5.8), corresponding to SPI-2-inducing conditions (Coombes *et al.*, 2004). Bacteria were pelleted, and supernatant was collected and precipitated as described previously (Faucher *et al.*, 2009). Samples were dissolved in an appropriate volume of SDS-PAGE loading buffer, according to the OD₆₀₀ value of the original culture. Proteins were separated on 10 or 15 % (w/v) SDS-polyacrylamide gels and then transferred onto PVDF membranes and incubated with rabbit anti-GroEL (Sigma) or rabbit affinity-purified antibodies raised against recombinant SseB. Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) was used as the secondary antibody and was detected with the ECL plus Western blotting detection reagent (GE Healthcare).

Infection of human cultured macrophages. The human monocyte cell line THP-1 (ATCC TIB-202) was maintained in RPMI 1640 (Wisent) containing 10 % (v/v) heat-inactivated fetal calf serum (Invitrogen), 25 mM HEPES (Wisent), 2 mM L-glutamine, 1 mM sodium pyruvate and 1 % modified Eagle's medium with non-essential amino acids (Wisent). A stock culture of these cells was maintained as monocyte-like, non-adherent cells at 37 °C in an atmosphere containing 5 % (v/v) CO₂. For macrophage infection, cells were seeded at 5 × 10⁵ cells per well in 24-well tissue-culture dishes and were differentiated by addition of 10⁻⁷ M phorbol 12-myristate 13-acetate for 48 h. Macrophage infection was performed as described previously (Daigle *et al.*, 2001) unless specified otherwise. Briefly, bacteria were grown overnight without shaking in LB broth, usually reaching an OD₆₀₀ of 0.6. Bacteria were added to the cell monolayer at an m.o.i. of 10:1, and were centrifuged for 5 min at 800g to synchronize bacterial uptake. After incubation for 20 min at 37 °C, extracellular bacteria were removed by washing cells three times with prewarmed PBS, pH 7.4, and the infected monolayers were either lysed (T₀) or incubated for 2 h with medium containing 100 µg gentamicin ml⁻¹ (Wisent) to kill extracellular bacteria, and then with 12 µg gentamicin ml⁻¹ for the remainder of the experiment. Cells were lysed by addition of 1 ml 0.1 % (w/v) sodium deoxycholate in PBS per well, and the number of surviving bacteria was determined as c.f.u. by plating on LB agar. Survival (fold increase) was defined as the number of bacteria recovered 24 h after infection divided by the number of bacteria detected 2 h after infection. Results are expressed as the mean ± SEM of replicate experiments. The non-parametric Mann–Whitney test was used for statistical analysis. Where indicated, bacteria grown to stationary phase in LB broth were diluted in PBS or

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Characteristic	Reference or source
Strains		
S. Typhi		
ISP1820	Wild-type <i>S. Typhi</i>	Hone <i>et al.</i> (1991)
DEF093	ISP1820 $\Delta sseB$	This study
DEF148	ISP1820 $\Delta ssaR$	This study
DEF149	ISP1820 $\Delta ssrB$	Faucher <i>et al.</i> (2009)
χ 8521	ISP1820 $\Delta phoP$	R. Curtiss III, Arizona State University (ASU)
Ty2	Wild-type <i>S. Typhi</i>	R. Curtiss III, ASU
DEF473	Ty2 $\Delta sseB$	This study
DEF566	ISP1820 Nal^r	This study
DEF574	ISP1820 $\Delta(ssaU-ssrB)$	This study
χ 4455	ISP1820 Δasd	R. Curtiss III, ASU
DEF010	χ 4455 gfp^+ (pHY11)	This study
DEF478	DEF010 $\Delta sseB$	This study
S. Typhimurium		
χ 3339	Mouse-passaged isolate of SL1344 <i>rpsL hisG</i>	Gulig & Curtiss (1987)
DEF458	χ 3339 $\Delta sseB$	This study
Escherichia coli		
MGN-617	SM10 λ pir <i>asd thi thr leu tonA lacY supE recA</i> RP4 2-Tc::Mu[λ pir] $\Delta asdA4$	Kaniga <i>et al.</i> (1998)
BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻)</i> , with DE3, a prophage carrying the T7 RNA <i>pol</i> gene	Novagen
Plasmids		
pET-21a	IPTG-inducible T7 RNA-polymerase-dependent expression vector, 6 × His-Tag at C terminus, Ap ^r	Novagen
pHY11	pYA810- <i>gfp</i>	H. Y. Kang, Pusan National University; Srinivasan <i>et al.</i> (1995)
pMEG-375	<i>sacRB mobRP4 oriR6K. Cm^r Ap^r</i>	R. Curtiss III, ASU
pSIF040	pMEG-375 with flanking region of <i>sseB</i> used for <i>sseB</i> deletion	This study
pSIF073	pMEG-375 with flanking region of <i>ssaR</i> used for <i>ssaR</i> deletion	This study
pSIF128	pET-21a with <i>sseB</i>	This study
pSIF187	pMEG-375 with flanking region of <i>ssaU</i> and <i>ssrB</i> used for entire SPI-2 deletion	This study

Table 2. Primers used in this study

Primer	Sequence (5'–3')*
sseB-BF	CGGGATCCAATACGCACCACGGCTTTT
sseB-BR	CCCCGGTGGACCCTACAACAGGCACATGACAC
sseB-EF	TTGTAGGGTCCACCGGGGCTTGAGCATTAAAG
sseB-ER	GCTCTAGAAATGGGACAGGCTCTTAGTTAGCAC
ssaR-BF	CGGGATCCTGCTGAGCGTCAACAGATTTATCC
ssaR-BR	ATTTCGTAACGTCCCCCGTTTAAAGTTGTCG
ssaR-EF	ACGGGGGACGTTACGAATTGGAGAGCATGGTTG
ssaR-ER	GCTCTAGAAGCAGAAACCCCGCCATATC
sseBF- <i>NdeI</i>	GGAATTCCATATGTCTTCAGGAAACATCTTATG
sseBR- <i>XhoI</i>	CCGCTCGAGTGAATACGTTTTCTGCGCTATCA
ssaU-R	CGGGATCCAATACGCTATCTGGTGCTTG
ssaU-Fover	TGTTTCGACTGCAGCCTTGTTACGTATGG
ssrB-Fover	AAGGCTGCAGTCGAAACACATCGGATGAAT
ssrB-R	GCTCTAGAAGGCGTAAGGCTCATCAAAAT

*Restriction enzyme sites are underlined. Letters in italics denote overlapping sequences.

opsonized for 20 min in 10% normal human serum before being added to macrophages at an m.o.i of 10:1. Activation of macrophages was achieved by addition of 100 U gamma interferon (IFN- γ) ml⁻¹ (PBL Biomedical Laboratories) in the medium 48 h prior to infection. The addition of IFN- γ was maintained throughout the infection.

For competitive index (CI) experiments in macrophages (Segura *et al.*, 2004), a spontaneous nalidixic acid-resistant (Nal^r) *S. Typhi* ISP1820 strain was used as the wild-type strain (DEF566). This Nal^r strain showed no intracellular attenuation compared with strain ISP1820 when both were used in a CI experiment in THP-1 macrophages (data not shown). The wild-type and mutant strains used for competition experiments were separately grown overnight (static) in LB broth, and a 1:1 mixture of the two cultures was added at an m.o.i. of 50:1 to the THP-1 cell monolayer, as described above. The numbers of viable intracellular bacteria were determined 2 and 24 h after infection by plating on LB agar and on LB agar with antibiotic. The CI for bacterial survival is defined as the mutant to wild-type ratio of bacteria recovered 24 h post-infection divided by the equivalent ratio of bacteria recovered 2 h post-infection. Statistical significance was evaluated by using Student's two-tailed *t*-test by comparing ratios (c.f.u. mutant/c.f.u. wild-type) at 24 and 2 h (Santiviago *et al.*, 2009).

Infection of human monocyte-derived macrophages (MDMs).

MDMs were obtained by using previously published protocols (Baron-Bodo *et al.*, 2005; Romieu-Mourez *et al.*, 2006; Goubau *et al.*, 2009). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation from fresh apheresis with informed and institutionally approved consent forms from healthy donors. PBMCs were resuspended at 10⁶ cells ml⁻¹ in ISCOVE medium (Wisent) supplemented with 2% human serum (Wisent), antibiotics and 700 U ml⁻¹ recombinant human GM-CSF (a generous gift from Cingene Corporation). On day 7, adherent MDMs were harvested after three washes with PBS and gentle cell scraping and were identified based on assessment of CD14, CD64 and HLA-DR. MDMs were transferred to a 24-well tissue culture plate and further incubated for 3 days (to day 10). Infection of MDMs with *S. Typhi* was performed as described for THP-1 macrophages.

Microscopy of infected macrophages. THP-1 cells were seeded on to glass coverslips (12 mm diameter) in 24-well plates. The infection was then carried out using GFP-expressing strains. For fluorescence microscopy, cell monolayers were washed in PBS and then fixed in 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature. Coverslips were washed three times in PBS and mounted on Geltol (Immunon Thermo). Samples were analysed using a fluorescence microscope (Eclipse E600; Nikon).

RESULTS

SPI-2 mutants

S. Typhi ISP1820 isogenic strains harbouring mutations in genes encoding distinct components of the SPI-2 T3SS were constructed by allelic exchange (Supplementary Figs S1 and S2). Deletion of *sseB*, encoding a secreted protein, was selected to investigate the effect of a mutation in the translocon. Deletion of *ssaR* was used to target the basal apparatus component of the needle complex and deletion of *ssrB* was used to evaluate the effect of a mutation in the regulatory system of the SPI-2 T3SS. To confirm that the SPI-2 mutants (*ssaR* and *ssrB*) of *S. Typhi* were non-functional, detection of the SseB protein in the wild-type

strain and SPI-2 mutants was monitored by using SPI-2-inducing conditions and anti-SseB antibodies. As expected, SseB was secreted in the culture supernatant of the wild-type strain, but not in the supernatant of the SPI-2 mutants (Fig. 1). In the bacterial pellet, the SseB protein was only detected in the wild-type and the *ssaR* mutant (Fig. 1). The *sseB* mutant was used as a control for the specificity of the antibody and GroEL was used as a control for bacterial cytoplasm leakage and was not detected in the culture supernatant. The data confirmed that the SPI-2 mutants were non-functional for secretion.

Phenotype of SPI-2 mutants within human macrophages

To evaluate bacterial survival within macrophages, THP-1 macrophages were infected with the wild-type strain and the isogenic SPI-2 mutants were grown at low aeration by using a gentamicin protection assay. The number of bacteria present at different times was determined by c.f.u. counts. Surprisingly, none of the specific deletion mutations in SPI-2-T3SS-encoding genes affected bacterial uptake or survival, as the number of bacteria recovered at different times after infection was similar to that of the wild-type strain (Fig. 2). As there were no differences observed between the different SPI-2 mutants, the *sseB* mutant was selected for further experiments.

The role of *S. Typhi* SPI-2 in survival in human macrophages was then assessed more specifically at different times after infection. *S. Typhi* had already begun to replicate 2 h after infection and continued for up to 3 days after infection (Fig. 3). A growth pattern similar to that for the wild-type strain was observed for the *sseB* mutant (Fig. 3). *S. Typhi* constitutively expressing green fluorescent protein (DEF010) and its *sseB* isogenic mutant (DEF478) were used to visualize bacteria within macrophages by fluorescence microscopy. Initially, many macrophages were infected with a single bacterium or a few bacteria (Fig. 3b). At 24 h post-infection, infected macrophages contained many bacterial clusters, suggesting that the bacteria had replicated (Fig. 3b). Interestingly, the

Fig. 1. *In vitro* secretion of SseB in SPI-2 mutants. Wild-type *S. Typhi* ISP1820 strain (Wt), and isogenic *sseB*, *ssaR* and *ssrB* deletion mutant strains were grown under SPI-2-inducing conditions (LPM; pH 5.8). Proteins in the supernatant (S) and bacterial pellet (P) were harvested and examined by Western blotting to detect SseB and GroEL.

Fig. 2. Effect of mutations in SPI-2 during interactions with human macrophages. THP-1 macrophages were infected with wild-type (ISP1820), *sseB*, *ssaR* or *ssrB* deletion mutant strains and the number of intracellular bacteria were determined at different times after infection. Data presented are the mean $\pm$ SEM of at least three independent experiments performed in duplicate.

infected cell monolayer was mainly intact, even 96 h post-infection (Fig. 3b) and only a few cells were detached.

To evaluate the usefulness of our infection model, an isogenic strain carrying a *phoP* deletion, a global transcriptional activator involved in *Salmonella* virulence and intracellular survival within macrophages (Miller *et al.*, 1989), was used as a control. The isogenic *phoP* mutant showed significantly reduced survival (Fig. 4), suggesting that the THP-1 macrophages are efficient for bacterial killing.

We next investigated if the absence of survival defects for an SPI-2 mutant was unique to the *S. Typhi* strain ISP1820. An *sseB* deletion was generated in *S. Typhi* strain Ty2 and no significant difference in intracellular survival between

the wild-type strain and the SPI-2 mutant was observed (Fig. 4). As a positive control, the role of SPI-2 of *S. Typhimurium* in human macrophages was then investigated. An *sseB* mutant of *S. Typhimurium* strain SL1344 was constructed and its contribution to survival in macrophages was evaluated. The *sseB* deletion in *S. Typhimurium* significantly reduced intracellular survival of the SPI-2 mutant in THP-1 macrophages (Fig. 4).

Influence of growth conditions

Salmonella can enter macrophages by several endocytic processes, which can be affected by the bacterial growth conditions prior to infection. Bacteria were grown to late exponential phase under low aeration (static), and were grown to stationary phase with or without opsonization. Regardless of the growth conditions prior to infection, there was no difference between the wild-type and the *sseB* mutant (Fig. 5). The uptake level of bacteria grown in different conditions was also similar. The number of bacteria recovered after 24 h of infection was always higher than the number of bacteria recovered after 2 h (fold increase >1) when bacteria were grown to late exponential phase without agitation (Figs 2–5). However, when *S. Typhi* uptake was investigated by using bacteria grown with shaking to stationary phase, with or without opsonization, a constant number of bacteria between 2 and 24 h after infection (fold increase of $\cong 1$) was observed (Fig. 5).

Activation of macrophages

IFN- γ is a critical cytokine that plays a central role in host defence against intracellular pathogens, including *Salmonella*

Fig. 3. Time course of *S. Typhi* survival in human macrophages. THP-1 macrophages were infected with *S. Typhi* ISP1820 and its isogenic *sseB* mutant. (a) The number of intracellular bacteria were determined at different times after infection. Data presented are the mean $\pm$ SEM of three independent experiments performed in duplicate. *P*-values are indicated for late time points. (b) Fluorescence microscopy of fixed macrophages at different times after infection with bacteria constitutively expressing the green fluorescent protein (original magnification, $\times 1000$).

Fig. 4. Role of SPI-2 in intracellular survival in different strains of *Salmonella*. THP-1 macrophages were infected with *S. Typhi* ISP1820 (Wt) and its isogenic *sseB* and *phoP* mutants, *S. Typhi* strain Ty2 and its isogenic *sseB* mutant, and *S. Typhimurium* SL1344 and its isogenic *sseB* mutant. Intracellular survival was determined by comparing the number of bacteria 24 h post-infection with the number of bacteria at 2 h (fold increase). Data presented are the mean $\pm$ SEM of at least three independent experiments performed in duplicate. Mutants demonstrating a significant ($P < 0.05$) difference compared with the isogenic wild-type strain are indicated (*).

(Muotiala & Mäkelä, 1993; Rosenberg *et al.*, 2002). As *Salmonella* may encounter activated macrophages during its life cycle inside the host, we investigated the role of SPI-2 during infection of activated macrophages. THP-1 macrophages were primed with IFN- γ for 48 h and then infected

Fig. 5. Effect of growth conditions, opsonization or macrophage activation on intracellular survival of *S. Typhi*. THP-1 macrophages were infected with *S. Typhi* ISP1820 (Wt) and its isogenic *sseB* mutant grown in LB: cultures were overnight static (late exponential growth), overnight shaking (stationary phase) with or without opsonization with human serum (opsonized), or by using activated macrophages primed with IFN- γ . Intracellular survival was determined by comparing the number of bacteria 24 h post-infection with the number of bacteria at 2 h (fold increase). Data presented are the mean $\pm$ SEM of at least three independent experiments performed in duplicate. Exact P -values are also indicated.

with wild-type *S. Typhi* and its isogenic *sseB* SPI-2 mutant. Following IFN- γ treatment, a small number of bacteria were recovered from macrophages (Fig. 5), although there was no significant difference between the wild-type and the SPI-2 mutant in the IFN- γ -primed macrophages.

Mixed infection assay of human macrophages

Survival of the wild-type strain and its isogenic mutants was evaluated simultaneously by using the CI assay. The CIs for the *sseB* and *phoP* mutants within THP-1 cells after mixed infections with the wild-type strain were 1.19 ($P = 0.50$) and 0.27 ($P = 0.0048$), respectively. These results are in agreement with our data from individual strains during infection and confirm that SPI-2 does not affect survival in human macrophages and that the PhoPQ regulon is of importance.

Infection of human MDMs

It has been shown that macrophages from immortalized cell lines allow moderate growth of an SPI-2 mutant of *S. Typhimurium* compared with a greater level of growth defect in primary cells (Hensel *et al.*, 1998; Helaine *et al.*, 2010). Human MDMs were infected with the wild-type *S. Typhi* strain and the isogenic *sseB* mutant. The number of bacteria recovered decreased over time, suggesting a defect in survival; however, the number of recovered bacteria was similar for both the wild-type strain and the SPI-2 isogenic mutant (Fig. 6).

Complete deletion of the SPI-2 T3SS

To validate and understand the surprising results obtained with the different SPI-2 mutants, a 25 kb deletion of the entire T3SS (*ssaU*–*ssrB*) was generated and tested for survival in THP-1 macrophages. Deletion of the T3SS of SPI-2 did not affect bacterial uptake or survival, as the

Fig. 6. Role of *S. Typhi* SPI-2 in human MDMs. Cells were infected with *S. Typhi* ISP1820 and its isogenic *sseB* mutant and intracellular bacteria were determined at different times after infection. Representative mean $\pm$ SEM of a duplicate MDM experiment from a single donor are shown. P -values are indicated.

number of bacteria recovered at different times after infection was similar to that of the wild-type strain (Fig. 7).

DISCUSSION

As the ability to survive and replicate inside macrophages is tightly associated with the *S. Typhimurium* SPI-2 T3SS and with virulence (Fields *et al.*, 1986), and since very little information regarding the involvement of *S. Typhi* SPI-2 T3SS in this process is available, the role of the *S. Typhi* SPI-2 T3SS inside human macrophages was investigated. Mutants corresponding to specific components of the secretion system were tested for intracellular survival, including a translocon mutant (*sseB*), an apparatus mutant (*ssaR*) and a transcriptional regulator mutant (*ssrB*), the last of these being required for expression of SPI-2 genes. These SPI-2 mutants were previously shown to be defective in intracellular replication of *S. Typhimurium* (Cirillo *et al.*, 1998; Hensel *et al.*, 1998). Moreover, any mutations in *SseB* lead to structural alterations that abolish translocon function, resulting in replication defects in macrophages (Hölzer & Hensel, 2010). *SseB* was not secreted in the *ssaR* and *ssrB* mutants, confirming that the SPI-2 T3SS is not functional (Fig. 1). Surprisingly, mutations in either *sseB* (translocon), *ssaR* (basal component) or *ssrB* (regulator), all of which abrogate SPI-2 T3SS function or expression, did not affect *S. Typhi* uptake and survival in human cells. Similar results were obtained during competition assays, when the entire SPI-2 T3SS (Δ *ssaU-ssrB*) was deleted (Fig. 7), or when macrophages derived from human monocytic cells were used (Fig. 6), confirming that intracellular survival of *S. Typhi* inside human macrophages is independent of the SPI-2 T3SS. The number of intracellular bacteria corresponding to the *sseB* SPI-2 mutant increased inside macrophages with a pattern

similar to that of the wild-type strain (Fig. 3). Despite not demonstrating a key role for intracellular survival, the SPI-2 genes of *S. Typhi* are highly induced inside human macrophages (Faucher *et al.*, 2006), suggesting that the difference may not be at the transcriptional level.

Moreover, no defect in survival was observed for an *sseB* SPI-2 mutant of *S. Typhi* strain Ty2, suggesting that the absence of a phenotype for an SPI-2 mutation in *S. Typhi* was not strain-specific (Fig. 4). However, a *phoP* mutant of *S. Typhi* known to be involved in intracellular replication of *S. Typhimurium* (Groisman *et al.*, 1989; Miller *et al.*, 1989) was defective in this model, as was an *sseB* mutant of *S. Typhimurium* (Fig. 4).

As bacterial growth conditions and opsonization affect uptake and have a significant influence on bacterial survival, replication and virulence gene expression in *S. Typhimurium* (Hensel *et al.*, 1997, 1998; Drecktrah *et al.*, 2006), we investigated the influence of growth condition on *S. Typhi* survival in macrophages. Growth to late exponential phase under low aeration promotes SPI-1 expression and T3SS-mediated invasion of *S. Typhimurium* (Lee *et al.*, 1992), while aerated stationary phase bacterial cultures or opsonization promote phagocytosis and low cytotoxicity (Hensel *et al.*, 1998; Lundberg *et al.*, 1999; Drecktrah *et al.*, 2006). Aerated stationary phase or opsonized bacteria are then usually used to study the effect of SPI-2 mutants, as the replication defect of *S. Typhimurium* is overemphasized (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; see also Fig. 5). Under all of these conditions, *S. Typhi* survived and no differences were observed between the wild-type strain and the *sseB* SPI-2 mutant (Fig. 5). Moreover, there was no significant difference observed in the uptake levels of *S. Typhi* between the different growth conditions or with or without opsonization. The presence of the Vi capsule, specific to *S. Typhi*, may be responsible for the lack of difference in bacterial uptake under different growth conditions. Interestingly, an increase in the number of bacteria recovered 24 h post-infection (ratio at 24 h:2 h >1) was only observed when *S. Typhi* was grown to late exponential phase. It will be interesting to investigate further the mechanisms of entry in macrophages of *S. Typhi*, as they may be related to its survival.

Activated macrophages play a central role in host defence and have been shown to increase phagocytic and killing activities. Survival of *S. Typhi* in human macrophages primed with IFN- γ was greatly reduced, but no significant difference was observed between the wild-type and the SPI-2 mutant. Similarly, no differences between the wild-type and the SPI-2 mutant were observed during survival in whole human blood (data not shown) or in invasion and survival of human epithelial cells (data not shown). It is possible that SPI-2 effectors may be translocated by the SPI-1 T3SS when the SPI-2 T3SS is mutated, but a double SPI-1 (*invA*)/SPI-2 (*ssaR*) mutant in *S. Typhi* survives and replicates to a level similar to that of the isogenic wild-type strain in THP-1 macrophages (data not shown). Thus, in

Fig. 7. Effect of complete deletion of the SPI-2 T3SS locus in *S. Typhi* during interactions with human macrophages. THP-1 macrophages were infected with the wild-type (ISP1820) and its isogenic mutant harbouring a complete deletion of the SPI-2 T3SS (*ssaU-ssrB*) and intracellular bacteria were determined at different times after infection. Data presented are the mean $\pm$ SEM of three independent experiments performed in duplicate. *P*-values are indicated.

contrast with *S. Typhimurium*, the SPI-2 T3SS of *S. Typhi* is not critical for intracellular survival in human macrophages.

S. Typhimurium and *S. Typhi* are closely related serovars, sharing 90 % of their DNA (McClelland *et al.*, 2001). There are no major genomic differences between the SPI-2 T3SS of *S. Typhimurium* and *S. Typhi* that may explain these phenotypic differences (Sabbagh *et al.*, 2010). Phylogenetic analyses suggest a probable difference in functionality between these serovars, as variations in translocon genes *sseC* and *sseD* and in effectors *sseF* and *sifA* were observed (Eswarappa *et al.*, 2008). The major difference between these serovars seems to be in the diversity of the effector contents, which may contribute to host adaptation. Many SPI-2 effectors characterized in *S. Typhimurium* are missing in *S. Typhi* (*sseI*, *gogB*, *spvB*, *spvC*, *sseK1*, *sseK2* and *sseK3*) or are pseudogenes (*sopD2* and *sseJ*). Several of these effectors mediate long-term systemic infection in mice (Lawley *et al.*, 2006; McLaughlin *et al.*, 2009; Ruiz-Albert *et al.*, 2002). As some effectors are not present in *S. Typhi*, this emphasizes that there are differences in the virulence mechanisms responsible for systemic disease caused by *S. Typhimurium* in susceptible mice compared with those dictating systemic disease caused by *S. Typhi* in humans. The majority of the studies on maturation and trafficking of *S. Typhimurium* effectors were performed with epithelial cells, and when macrophages were used, they were usually of murine origin. Thus, it is premature to determine or hypothesise whether the absence of a survival defect is related to a difference in the cell type and/or the host source or to a genetic difference between *S. Typhi* and *S. Typhimurium*.

It is thus possible that the host plays an essential role and may be able to discriminate between these serovars. Indeed, mutations in homologous genes that attenuate *S. Typhimurium* in mice have given different results for *S. Typhi* when administered to humans as a potential vaccine (Garmory *et al.*, 2002). Moreover, differences in survival in human macrophages between the two serovars were previously observed, with a higher rate of survival for *S. Typhi* (Alpuche-Aranda *et al.*, 1994; Ishibashi & Arai, 1995; Schwan *et al.*, 2000), similar to what we observed here. This may be because *S. Typhi* is less cytotoxic for human cells (Schwan *et al.*, 2000; Fig. 3 and data not shown). *S. Typhi* increases rapidly and no lag phase is observed in THP-1 macrophages (Figs 2, 3 and 7). Differential survival between these serovars was also detected in susceptible mice, where *S. Typhi* was rapidly eliminated (Xu *et al.*, 2009). The use of *S. Typhimurium* in mice as a surrogate for *S. Typhi* in humans has many real advantages but may also be misleading.

As SPI-2 is present in *S. Typhi*, it is unlikely that there is no evolutionary advantage in carrying it. It could be used during infection of other cell types, such as dendritic or natural killer cells. The SPI-2 T3SS may be critical when the bacteria are in a specific human niche with high

immunological cell content, such as the spleen. The SPI-2 T3SS may be required to modulate the host immune system to establish long-term asymptomatic infection, which will not necessarily translate into defects in intracellular survival. Further investigation is needed to clarify the role of the *S. Typhi* SPI-2 T3SS in human cells.

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