The Bimodal Lifestyle of Intracellular Salmonella in Epithelial Cells: Replication in the Cytosol Obscures Defects in Vacuolar Replication

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Abstract

Salmonella enterica serovar Typhimurium invades and proliferates within epithelial cells. Intracellular bacteria replicate within a membrane-bound vacuole known as the Salmonella-containing vacuole. However, this bacterium can also replicate efficiently in the cytosol of epithelial cells and net intracellular growth is a product of both vacuolar and cytosolic replication. Here we have used semi-quantitative single-cell analyses to investigate the contribution of each of these replicative niches to intracellular proliferation in cultured epithelial cells. We show that cytosolic replication can account for the majority of net replication even though it occurs in less than 20% of infected cells. Consequently, assays for net growth in a population of infected cells, for example by recovery of colony forming units, are not good indicators of vacuolar replication. We also show that the Salmonella Type III Secretion System 2, which is required for SCV biogenesis, is not required for cytosolic replication. Altogether this study illustrates the value of single cell analyses when studying intracellular pathogens.

Introduction

Salmonella enterica serovar Typhimurium (Salmonella Typhimurium) is a facultative intracellular pathogen, which is a common cause of gastroenteritis in humans. The ability of Salmonella to establish its intracellular niche is dependent on two Type Three Secretion Systems (T3SS). T3SS1, encoded by Salmonella Pathogenicity Island 1 (SPI) 1, is required for efficient invasion of nonphagocytic cells. In contrast, the SPI2-encoded T3SS2 is induced following internalization of Salmonella into host cells and is required for post-invasion processes. Together T3SS1 and T3SS2 translocate over 30 effector proteins into the host cell where they interact with a variety of targets [1].

In epithelial cells Salmonella Typhimurium has a bimodal lifestyle, replicating in a membrane-bound compartment known as the Salmonella-containing vacuole (SCV) [2] as well as in the cytosol [3,4,5,6,7,20,22,23,24,25]. The SCV is a modified phagosome, characterized by the presence of lysosomal membrane proteins, low pH and sustained dynamic interactions with the endocytic and biosynthetic pathways [8,9,10,11,12,13]. Early maturation of the SCV involves acquisition of lysosomal membrane proteins, such as lysosomal-associated membrane protein-1 (LAMP1) within 1–2 h following invasion [14], and movement from the cell periphery to a juxtanuclear location [15,16]. Bacterial replication is initiated following LAMP1 acquisition [14,17,18,19] and is accompanied by the appearance of dynamic membrane tubules that extend from the surface of the SCV [20,21]. Effectors translocated by the SPI2-encoded T3SS2, are essential for maturation of the replicative SCV. Bacteria lacking a functional T3SS2, and therefore unable to translocate any effectors, remain within an immature SCV, which is LAMP1-positive but does not form membrane tubules and is defective at juxtanuclear positioning [15]. These mutants also have an intracellular replication defect, in both macrophages and epithelial cells, although in epithelial cells the defect is not apparent during the initial replication phase [3,4,5,6,7,20,22,23,24,25].

While the SCV has been extensively studied, little is known about how Salmonella adapt to and/or modify the cytosolic niche. Using a polarized epithelial cell model Knodler et al showed that cytosolic Salmonella Typhimurium replicate to higher numbers than vacuolar bacteria, a phenotype dubbed “hyper-replication” [6]. This study also showed that these two intracellular populations of bacteria are transcriptionally distinct: the intravacuolar bacteria are SPI2-induced whereas the cytosolic bacteria are SPI1-induced and flagellated. Epithelial cells containing hyper-replicating SPI1-induced Salmonella undergo inflammatory cell death, marked by loss of plasma membrane integrity and activation of caspase 1 and caspase 3/7. Ultimately these cells are extruded from monolayers, both in vivo and in vitro, and the invasion-pruned Salmonella are released into the extracellular milieu [6].

Here we have investigated whether cytosolic replication of Salmonella contributes significantly to net growth in HeLa cells,
which are commonly used to study Salmonella-host cell interactions in vitro. In addition, we have compared the requirement for T3SS2 in cytosolic vs. vacuolar replication. Since cytosolic Salmonella are SPI1-induced, and do not express detectable levels of SPI2 genes [6], it seems probable that SPI2 is not required for hyper-replication, although this has not been directly demonstrated. If T3SS2 is not required for cytosolic replication this could explain why bacteria lacking T3SS2 have a delayed replication defect in epithelial cells [3,4,6,7,22,26], since cytosolic replication could potentially obscure defects in vacuolar replication. We used microscopy-based approaches, in both fixed and living cells, to assess the replication over time of both the vacuolar and cytosolic populations of Salmonella in individual epithelial cells. Our results show that, although cytosolic Salmonella Typhimurium occur in a minority of infected epithelial cells, the hyper-replication of these bacteria accounts for a significant proportion of net bacterial replication. Furthermore, cytosolic replication is SPI2-independent and can obscure replication defects in vacuolar bacteria.

Results

Analysis of Intracellular Replication of Salmonella

Following internalization into epithelial cells Salmonella Typhimurium replicates within the SCV but also in the cytosol [3,4,5,6,7]. However, the relative contribution of the two distinct intracellular populations to net replication remains undefined. To address this question we analyzed intracellular replication in cultured epithelial cells by both the standard gentamicin protection assay, which measures net replication, and a microscopy-based technique to follow bacterial replication in single cells. Many studies have shown robust replication of wild type (WT) Salmonella Typhimurium in epithelial cells (~20–30 fold over 8 h of infection, Fig. 1A) [3,5,15,20,23,25,27,28]. However, although the SPI2-encoded T3SS2 is required for vacuole biogenesis, we saw no defect in net replication for a SPI2 deletion mutant (ASPI2) over this time period. In contrast, at 16 h post infection (p.i.), there was a significant reduction in the amounts of recoverable intracellular bacteria for the SPI2 mutant. We next used standard fluorescence microscopy to examine the numbers of bacteria in individual cells at these times. For ease of detection, particularly in the subsequent live cell experiments, we used bacteria constitutively expressing the fluorescent protein mCherry (mCherry Salmonella). HeLa cells infected with mCherry Salmonella were fixed at 2, 8 and 16 h.p.i. and intracellular bacteria then enumerated by fluorescence microscopy (Fig. 1B and 1C). As expected, since the SPI2-encoded T3SS2 is not required for invasion, no difference was seen in the numbers of WT or SPI2 mutant intracellular bacteria at the earliest time point (2 h p.i.). The mean number of bacteria per infected cell was 5 ± 4 and 5 ± 3 (mean ± SD) for the WT and mutant respectively. In contrast, at 8 h p.i., following the onset of replication, the numbers of bacteria per cell varied dramatically. In some cells the bacteria occupied the entire cytoplasm of the cell and could not be accurately enumerated (TNTC, >100 bacteria), whereas at the other extreme many cells contained less than 10 bacteria. To facilitate analysis, we separated cells into three groups based on the numbers of intracellular bacteria; low (1–20 bacteria), moderate (20–100 bacteria) and high (>100 bacteria) (Fig. 1B). We then compared the frequency of each phenotype in cells infected with either WT Salmonella or the SPI2 mutant. At 8 h p.i. cells falling into the high (>100 bacteria) group accounted for 10 ± 4 (mean ± SD) % of WT infected cells compared to 7 ± 1% of the SPI2 mutant infected cells. In comparison, the moderate (20–100 bacteria per cells) group was more frequent in WT infected cells (29 ± 17%) compared to those infected with the SPI2 mutant (6 ± 6%). At 16 h this effect was enhanced with 75 ± 5% of WT infected cells containing 20–100 bacteria per cell compared to 14 ± 5% of SPI2 mutant infected cells. In contrast, cells containing >100 bacteria made up 8 ± 5% or 5 ± 3% of the population for WT infected and SPI2 mutant infected cells respectively. These results indicate that the SPI2-encoded T3SS2 is required for development of the subpopulation of cells that contain 20–100 bacteria but not the subpopulation that contains >100 bacteria.

Live Cell Imaging Defines a Role for SPI2 Early in Infection

To further investigate the role of SPI2 in infected HeLa cells we used a live cell imaging analysis method in which the increase in area occupied by mCherry fluorescent Salmonella over time approximates bacterial replication [29]. Infected HeLa cells were imaged from ~2 to 8 h p.i. (Fig. 2A, Movies S1 and S2) with images taken at 10 min intervals. Post-acquisition analysis was then carried out to estimate the net change in area occupied by mCherry Salmonella, obtained by dividing the pixel area occupied by intracellular bacteria at each time point by the area occupied at ~2 h p.i. (ΔArea; see Materials and Methods section for details). The data are shown as a scatter plot in which data points have been binned into three groups representing low (ΔArea <2), moderate (ΔArea >2–<10) and high (ΔArea ≥10) replication (Fig. 2B). After 8 hours of infection, the percentage of cells containing large numbers of bacteria was slightly higher for WT (16±0%, mean ± SD) compared to SPI2 mutant infected cells (9±1%) although this difference was not seen in the previous experiment using fixed cells (Fig. 1B and C). However, whereas the majority of cells infected with WT bacteria fell into the moderate replication group, 60±2% the majority of cells infected with the SPI2 mutant fell into the low replication group, 66±3%. Thus, with the noted exception at 8 h p.i., these live cell data are consistent with the data shown in Figure 1, and confirm a requirement for SPI2 in replication of a subpopulation of bacteria i.e. those falling in the moderate replication group.

Cytosolic Replication in HeLa Cells is SPI2-independent

We next carried out experiments to determine the intracellular location of replicating Salmonella. To differentiate between vacuolar and cytosolic bacteria HeLa cells were stained for the lysosomal transmembrane protein LAMP1, which accumulates on the membrane of SCVs. At 8 h p.i. most infected cells contained mCherry Salmonella enclosed within LAMP1-positive vacuoles (Fig. 3A) although in cells containing large numbers of Salmonella (>100) the majority of bacteria were LAMP1-negative. To confirm that the LAMP1-negative Salmonella were cytosolic we used digitonin permeabilization to selectively permeabilize the plasma membrane without affecting the integrity of intracellular membranes including that of the SCV. This selective permeabilization provides access for anti-LPS antibodies to label cytosolic but not vacuolar Salmonella. At 6 h p.i. ~15% of infected cells contained mCherry Salmonella that stained with anti-LPS antibody (Fig. 3B). No difference could be detected in the frequency of cytosolic WT and SPI2 mutant bacteria in HeLa cells (Fig. 3, and data not shown), indicating that cytosolic replication is SPI2-independent.

Replication in the SCV, but Not in the Cytosol, is SPI2 Dependent

To confirm that cytosolic replication is SPI2 independent we adapted the live-cell imaging approach to compare the rates of bacterial replication in the cytosol and the SCV. To differentiate
between vacuolar and cytosolic bacteria, we used fluorescent dextran, a fluid phase marker that efficiently labels the endocytic pathway and accumulates within the Salmonella containing vacuole (SCV) [13]. Alexa488-Dextran was internalized into the HeLa cells overnight and the cells then infected with mCherry Salmonella. To increase the number of infected cells, which could be simultaneously imaged in each experiment, nine overlapping fields were imaged and later stitched together for image analysis. In this way we imaged a total of 47 infected cells; 19 WT-infected and 28 SPI2 mutant-infected (Fig. 4). At the initiation of imaging (2–3 h p.i.) the number of bacteria per infected cell was 3±1 (mean ± SD) for WT and 5±1 for the mutant. Dextran-negative (cytosolic) bacteria were observed in 4 out of 19 WT infected cells compared to 3 out of 28 mutant infected cells. Cytosolic replication was first
observed between 4 and 6 h p.i. for both WT and mutant bacteria and resulted in 20–100 fold increases in bacterial numbers, beyond which increases could not be accurately measured (Fig. 4A and Movies S3, S4, S5, S6, compare vacuolar bacteria shown in Movie S3 and S4 with cytosolic bacteria shown in Movie S5 and S6). One WT-infected cell in this group detached from the dish during imaging after a 6 fold increase in intracellular bacteria. Replication of dextran positive (vacuolar) bacteria, which was also initiated by 4–5 h p.i., was only apparent when plotted on a different scale (Fig. 4B). By 9 h p.i. WT vacuolar bacteria increased by ~3 fold with the largest observed increase in a single cell being 6 fold. In comparison, the vacuolar SPI2 mutant bacteria increased by only ~1.4 fold and the largest observed increase was 2.3 fold. These live cell experiments confirm that SPI2 is required for Salmonella replication in the vacuole but not for cytosolic replication.

Low Magnification Live Cell Imaging of Salmonella-infected HeLa Cells Provides Novel Insight into Intracellular Populations

A major limitation of live cell imaging experiments, such as those described above is that only a few cells can be imaged at one time. To overcome this limitation we refined the multiple-field live cell imaging approach, using low magnification (20X objective) and imaging 20 overlapping fields, to allow simultaneous imaging of ~500 cells. For these experiments we used Salmonella constitutively expressing GFP (GFP Salmonella) and propidium iodide was added to the tissue culture media following infection, to allow identification of cells with compromised plasma membrane. Imaging was initiated 2.5–3 h p.i. and images were then acquired at 20 min intervals until 18 h p.i. Representative time-lapse movies (Movies S7 and S8) from one of three independent experiments reveal ongoing cell division as well as cells rounding up and detaching, with the later being particularly apparent at the later time points. GFP Salmonella can be observed in many HeLa cells, although at early time points the infected cells are not always easy to identify since there are few bacteria per cell. In contrast, cells containing hyper-replicating bacteria are readily identified from 4 h p.i. and by 8 h p.i. 11±3% (mean ± SD, n = 3) of WT-infected and 17±3% of SPI2 mutant-infected cells fell into this category (Movies S7 and S8 respectively, and still images from the movies shown in Fig. 5C and 5D with selected regions in Fig. 5A and 5B). Although some of these cells were observed detaching from the monolayer, the percentage of infected cells containing hyper-replicating bacteria did not change significantly between 8 and 16 h for either WT-infected or SPI2 mutant-infected cells.

As previously reported epithelial cells containing hyper-replicating bacteria undergo inflammatory death, characterized by activation of caspases 1 and 3/7 as well as loss of plasma membrane integrity [6]. Here we also observed that from 8 h p.i. cells containing hyper-replicating bacteria also stained with PI, indicating loss of membrane integrity (Fig. 5 and Movies S7 and S8).
Although the cells containing hyper-replicating bacteria are very conspicuous in these live cell experiments, the majority of infected cells (80–90%) contain bacteria undergoing limited replication. Very few of these cells stained with PI during the course of the experiment indicating that the plasma membrane is intact and the infected cells remain viable. Intriguingly, some of these cells do round up and detach from the monolayer although the significance of this is unclear. Comparison of movies S7 and S8, which show WT- and SPI2-mutant-infected cells respectively, reveals that intermediate replication occurs frequently in WT-infected cells but not in cells infected with the mutant (See also Fig. 5). Since this reproduces the data shown in Fig. 1 and Fig. 2 we have not quantified these populations in these experiments.

These low-magnification live cell experiments demonstrate conclusively that the SPI2 encoded T3SS is not required for cytosolic replication of *Salmonella*. In addition, they reveal the potential for replication of *Salmonella* in the cytosol.
hyper-replicating bacteria to equal or even outnumber the vacuolar population in infected HeLa cells.

**Discussion**

Historically the SCV has been considered the primary site of replication for intracellular Salmonella. However, it is now clear that this facultative intracellular pathogen has a distinct bimodal lifestyle in epithelial cells [6]. Here we have analyzed the contributions of vacuolar and cytosolic replication to net intracellular growth. Our data shows that, although the majority of infected HeLa cells contain vacuolar Salmonella these bacteria replicate rather inefficiently, ~3 fold over 8 h, and can not account for the net levels of intracellular replication in these cells. In contrast, while less than 20% of infected cells contained cytosolic bacteria this population replicated 40 fold or more so that the epithelial cells were rapidly filled with bacteria. Thus, in epithelial cells, net replication of intracellular Salmonella is a reflection of both cytosolic and vacuolar replication.

Although the requirement for the SPI2-encoded T3SS2 in macrophages has been unequivocally demonstrated [30,31,32,33], the role of this important virulence determinant in epithelial cells has been less well established [3,5,22,28]. While many studies have shown that SCV biogenesis is dependent on the T3SS2, mutants lacking the ability to translocate any T3SS2 effectors often show little or no defect in replication during the first 8 h following invasion when compared to 16 h p.i. where they are significantly defective in replication [3,5]. But those experiments primarily used gentamicin protection to quantify intracellular bacteria. Our results show that a defect in vacuolar replication is likely obscured by T3SS2-independent cytosolic replication when net replication is assayed at earlier time points during infection (i.e. 8 h). Then is the growth defect apparent at later time points? One possibility is that cells containing hyper-replicating bacteria more easily detach from the monolayer. In this case a standard gentamicin-assay, in which monolayers of infected cells are solubilized in detergent, so that recoverable cfus can be estimated by plating, would underestimate hyper-replicating bacteria. This may well be an issue for the gentamicin assay, or other protocols that involve rinsing of monolayers. However, our live cell experiments involve no rinsing steps after the change to low calcium-containing media, though an important concern is that involves rinsing of monolayers. However, our live cell experiments involve no rinsing steps after the change to low calcium-containing media, though an important concern is that cells are unable to survive or grow following calcium restoration.

Infection of Cultured Epithelial Cells with Salmonella

HeLa cells were seeded on plastic (Costar) or glass-bottom (Sensoplate; Grenier Bio-One) 24 well tissue culture plates or glass coverslips, 18-24 h before infections. Cells were infected with SPI1-induced bacteria as described previously [27]. Briefly, cultures grown overnight in LB-Miller broth with shaking (250 rpm) at 37°C were subcultured (1:33) in 10 ml of fresh LB-Miller and grown shaking at 37°C to late log phase (3.5 h). Salmonella were then collected by centrifugation and resuspended in Hank's buffered saline solution. HeLa cells were immediately infected at an MOI of ~50:1 for 10 minutes at 37°C, followed by three brief
rinses in PBS and incubated in GM for 20 min at 37°C. Cells were then incubated in GM containing 50 μg/ml gentamicin for 1 h, to kill extracellular bacteria, followed by GM containing 10 μg/ml gentamicin for the remainder of the infection. To determine the number of viable intracellular bacteria, HeLa cells were lysed in 0.2% sodium deoxycholate in PBS at 1.5 and 8 h post infection and 10 fold serial dilutions plated on LB agar. Serial plating was also used to estimate the number of bacteria in the innoculum.

Immunofluorescence Staining

HeLa cells were fixed in 2.5% paraformaldehyde for 10 min at 37°C. All subsequent steps were carried out at room temperature. Cells were blocked and permeabilized in PBS containing 10% (v/v) normal goat serum (NGS) and 0.1% (w/v) saponin (SS-PBS) for 10 min. They were then incubated with mouse monoclonal anti-LAMP-1 antibody (1:1,000, clone H4A3, Developmental Studies Hybridoma Bank) for 45–60 min in SS-PBS, washed 3 times in PBS, 0.1% (w/v) saponin, incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:800, Life Technologies) in SS-PBS for 45 min. Finally the cells were washed and mounted on glass slides using ProLong Gold antifade reagent containing the nuclear counterstain DAPI (Life Technologies).

Differential Digitonin Permeabilization Assay

To determine whether intracellular bacteria were vacuolar or cytosolic we used a differential digitonin permeabilization assay [6]. Briefly, HeLa cells were infected with mCherry Salmonella (WT or ΔSPI2 mutant). At 6 h p.i. cells were washed three times in KHM buffer [110 mM potassium acetate, 20 mM Hepes, 2 mM MgCl₂ (pH 7.3)], incubated with 150 μg/mL digitonin (Sigma) in KHM buffer for 1 minute, then immediately washed twice with KHM buffer. This and all subsequent steps were carried out at room temperature unless noted. To label cytosolic bacteria and the cytosolic face of the Golgi cells were then incubated in rabbit polyclonal anti-Salmonella lipopolysaccharide (LPS) antibody (1:200; Difco) and mouse anti-human GM130 monoclonal antibody (1:200; BD Transduction Laboratories) for 12 min at 37°C. Cells were washed twice with PBS and then fixed in 2.5% paraformaldehyde for 10 min at 37°C. Fixed cells were permeabilized in SS-PBS for 15 min followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 647-conjugated goat anti-mouse IgG at 1:400 for 45 mins. For each experiment, two coverslips were used as a permeabilization control to ensure that the plasma membrane, but not endomembranes, was permeabilized. For this, digitin-treated cells were incubated with rabbit polyclonal antibody directed against the cytoplasmic tail of calnexin (1:200; Stressgen, Enzo Life Sciences) and a mouse monoclonal antibody directed against rat luminal protein disulphide isomerase (1:200; Thermo Scientific). Finally, coverslips were washed in PBS and mounted on glass slides as above.

Fixed Cell Imaging

Cells were visualized and enumerated using inverted Nikon microscopes, Eclipse TE2000 or Ti-E, fitted with a Plan Apo 60X 1.4 NA oil immersion lens and B-2/E/C or Y-2/E/C filter blocks (Nikon Instruments). To determine the number of bacteria per cell, 150 infected cells were enumerated at each time point. Confocal images were acquired on an LSM 710 controlled by the software package ZEN (Zeiss) fitted with a Plan-Apochromat 63X 1.40 NA oil immersion lens. Pixel sizes were 0.75–0.77 μm and z-steps for optical sections were 32 μm. Excitation and bandpass cut offs were as follows: DAPI, excitation 405 nm, emission 410–495 nm; Alexa Fluor 488, excitation 488 nm, emission 493–561 nm; mCherry, excitation 561 nm, emission 578–696 nm.

Live Cell Imaging

HeLa cells grown on glass-bottom 24 well plates were infected with mCherry Salmonella or GFP Salmonella. When Dextran Alexa Fluor 488 (10,000 MW, Molecular Probes/Invitrogen) was used to identify vacuolar bacteria it was internalized overnight (0.2 mg/ml) prior to infection [13]. To stain HeLa cells with compromised plasma membranes, propidium iodide (1 μg/ml) was added 1.5 h p.i and maintained for the remainder of the infection. Live cell imaging experiments were performed using a spinning disc confocal system [6] consisting of a CSU10 spinning disc confocal (Yokogawa) with a custom AO1F-shuttered diode laser launch for excitation (Prairie Technologies), fitted to a Ti-E inverted microscope with Perfect Focus System (Nikon Instruments) controlled by Metamorph v 7.7.0 (Molecular Devices) or by widefield with the same instrument fitted with an HQ2 interline CCD camera (Photometrics) and excitation with an EXFO metal halide light source (Lumen Dynamics). Cells were maintained at 37°C with 5% CO₂ throughout imaging using a stage top incubation system (Pathology Devices). Excitation and filters were as follows: Alexa Fluor 488, excitation 488 nm, emission 525/50 nm; mCherry, excitation 561 nm, emission 600/45 nm. Filter blocks and lightpath for widefield imaging were as follows: GFP, B-2/E/C; propidium iodide (PI), Y-2/E/C; and phase contrast with phase plate Ph1 with a centered NCB filtered halogen light source focused with an ELWD condenser configured for Kohler illumination (Nikon Instruments).

For low magnification imaging of Salmonella replication, using a Plan Fluor 20X 0.5 NA ELWD non-immersion phase contrast objective lens (Nikon Instruments), nine fields, selected systematically in a non-overlapping 3x3 grid, were imaged sequentially (single optical sections) for Alexa Fluor 488 and mCherry at 10 min intervals. Alternatively, twenty overlapping fields selected systematically were imaged sequentially (widefield) for GFP, PI and phase contrast at 20 min intervals.

All post-acquisition analysis was carried out using ImageJ software (W.S. Rasbands, National Institutes of Health, Bethesda, MD). To measure the area occupied by Salmonella within individual HeLa cells, a region-of-interest was selected by thresholding on the red channel so as to include all fluorescent mCherry Salmonella. To determine the fold-change in area (ΔArea) for each infected HeLa cell, the area occupied by Salmonella at 8 h 20 min p.i. was normalized to the area occupied by Salmonella at 2 h 20 min h.p.i. Overlapping fields were stitched together using the Stitch Grid of Images plugin from the ImageJ derivative, Fiji v1.43-1.46a [36].

For high magnification imaging of Salmonella replication, using a Plan Apo 60X 1.4 NA oil immersion lens (Nikon Instruments) fitted with a temperature regulated heating collar (Bioptechs), nine overlapping fields (total of 5–10 infected cells) were imaged sequentially (single optical sections) for Alexa Fluor 488 and mCherry at 10 min intervals. Overlapping fields were stitched together as given above. Individual Salmonella were enumerated for each infected cell at each time point time and normalized to the number at the initial time-point.

Statistics

Statistical tests, including unpaired Student’s t-test for Gaussian distributions, Mann-Whitney for non-parametric distributions and ANOVA and 2-way ANOVA for multiple category Gaussian distributions, were performed using Prism (Graphpad).
Supporting Information

Movie S1  Live-cell imaging of Salmonella Typhimurium WT replication in HeLa cells at 20X magnification. Internalized 488-dextran was used to identify vacuolar Salmonella. Left panel shows mCherry Salmonella artificially colored to reveal both low (blue/purple) and high (yellow/white) intensity bacteria. Right panels show an overlay of mCherry Salmonella (red) and Dextran-488 (green). Compare dextran positive vacuolar bacteria to dextran negative cytosolic bacteria. Times post infection are indicated. (MOV)

Movie S2  Live-cell imaging of Salmonella Typhimurium SPI2 mutant replication in HeLa cells at 20X magnification. Internalized 488-dextran was used to identify vacuolar Salmonella. Left panel shows mCherry Salmonella artificially colored to reveal both low (blue/purple) and high (yellow/white) intensity bacteria. Right panels show an overlay of mCherry Salmonella (red) and Dextran-488 (green). Compare dextran positive vacuolar bacteria to dextran negative cytosolic bacteria. Times post infection are indicated. (MOV)

Movie S3  Live-cell imaging of Salmonella Typhimurium WT replication in HeLa cells at 60X magnification, showing vacuolar replication. Internalized 488-dextran was used to identify vacuolar Salmonella. Left panel shows mCherry Salmonella artificially colored to reveal both low (blue/purple) and high (yellow/white) intensity bacteria. Right panels show an overlay of mCherry Salmonella (red) and Dextran-488 (green). Compare dextran positive vacuolar bacteria to dextran negative cytosolic bacteria. Times post infection are indicated. (MOV)

Movie S4  Live-cell imaging of Salmonella Typhimurium SPI2 mutant replication in HeLa cells at 60X magnification, showing vacuolar replication. Internalized 488-dextran was used to identify vacuolar Salmonella. Left panel shows mCherry Salmonella artificially colored to reveal both low (blue/purple) and high (yellow/white) intensity bacteria. Right panels show an overlay of mCherry Salmonella (red) and Dextran-488 (green). Compare dextran positive vacuolar bacteria to dextran negative cytosolic bacteria. Times post infection are indicated. (MOV)

Movie S5  Live-cell imaging of Salmonella Typhimurium WT replication in HeLa cells at 60X magnification, showing cytosolic replication. Internalized 488-dextran was used to identify vacuolar Salmonella. Left panel shows mCherry Salmonella artificially colored to reveal both low (blue/purple) and high (yellow/white) intensity bacteria. Right panels show an overlay of mCherry Salmonella (red) and Dextran-488 (green). Compare dextran positive vacuolar bacteria to dextran negative cytosolic bacteria. Times post infection are indicated. (MOV)

Movie S6  Live-cell imaging of Salmonella Typhimurium SPI2 mutant replication in HeLa cells at 60X magnification, showing cytosolic replication. Internalized 488-dextran was used to identify vacuolar Salmonella. Left panel shows mCherry Salmonella artificially colored to reveal both low (blue/purple) and high (yellow/white) intensity bacteria. Right panels show an overlay of mCherry Salmonella (red) and Dextran-488 (green). Compare dextran positive vacuolar bacteria to dextran negative cytosolic bacteria. Times post infection are indicated. (MOV)

Movie S7  WT replication at 20X magnification over multiple fields of view. Propidium iodide was used to identify HeLa cells with compromised plasma membranes. Each frame contains an overlay of GFP Salmonella (green), propidium iodide (red) and phase contrast (gray). Times post infection are indicated. (MOV)

Movie S8  SPI2 mutant replication at 20X magnification over multiple fields of view. Propidium iodide was used to identify HeLa cells with compromised plasma membranes. Each frame contains an overlay of GFP Salmonella (green), propidium iodide (red) and phase contrast (gray). Times post infection are indicated. (MOV)

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Author Contributions
Conceived and designed the experiments: PMK SW OSM. Performed the experiments: PMK SW. Analyzed the data: PMK SW OSM. Contributed reagents/materials/analysis tools: PMK SW OSM. Wrote the paper: PMK SW OSM.

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