Quantitative insights into actin rearrangements and bacterial target site selection from *Salmonella* Typhimurium infection of micropatterned cells

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Summary

Reorganization of the host cell actin cytoskeleton is crucial during pathogen invasion. We established micropatterned cells as a standardized infection model for cell invasion to quantitatively study actin rearrangements triggered by *Salmonella* Typhimurium (S. Tm). Micropatterns of extracellular matrix proteins force cells to adopt a reproducible shape avoiding strong cell-to-cell variations, a major limitation in classical cell culture conditions. S. Tm induced F-actin-rich ruffles and invaded micropatterned cells similar to unconstrained cells. Yet, standardized conditions allowed fast and unbiased comparison of cellular changes triggered by the SipA and SopE bacterial effector proteins. Intensity measurements in defined regions revealed that the content of pre-existing F-actin remained unchanged during infection, suggesting that newly polymerized F-actin in bacteria-triggered ruffles originates from the G-actin pool. Analysing bacterial target sites, we found that bacteria did not show any preferences for the local actin cytoskeleton specificities. Rather, invasion was constrained to a specific ‘cell height’, due to flagella-mediated near-surface swimming. We found that invasion sites were similar to bacterial binding sites, indicating that S. Tm can induce a permissive invasion site wherever it binds. As micropatterned cells can be infected by many different pathogens they represent a valuable new tool for quantitative analysis of host–pathogen interactions.

Introduction

*Salmonella* Typhimurium (S. Tm) is a Gram-negative, flagellated bacterium causing annually more than a billion cases of severe gastroenteritis in humans worldwide (Mead et al., 1999). S. Tm is ingested with contaminated food and water. As shown in tissue culture models, this bacterium employs flagella-driven motility to approach the cell layer and slide along the cell layer’s surface (Misselwitz et al., 2012). This early phase of infection seems to be governed by generic physical forces acting on any particle moving along a surface. Once in close proximity to a cell, S. Tm either attaches reversibly or docks irreversibly (Misselwitz et al., 2011) by using adhesins (reversible binding) and the needle of the *Salmonella* pathogenicity island (SPI)-1 encoded type III secretion system 1 (TTSS-1) (irreversible binding) (Lara-Tejero and Galan, 2009; Misselwitz et al., 2011). Once irreversibly attached to the host cell, the bacterium injects different effector proteins through this needle. Several of these factors trigger, either directly or indirectly, the formation of pronounced F-actin-enriched membrane ruffles (Finlay et al., 1991): SopE and SopE2 act as guanine nucleotide exchange factors (GEFs) for Rac1 and Cdc42 (Hardt et al., 1998; Rudolph et al., 1999; Friebel and Hardt, 2000; Stender et al., 2000; Friebel et al., 2001; Cain et al., 2004; Patel and Galan, 2006); SipC promotes actin nucleation and bundling (Chang et al., 2005; 2007; Myeni and Zhou, 2010); SipA induces F-actin bundling (Zhou et al., 1999a,b; Gaikin et al., 2002; Higashide et al., 2002; Liic et al., 2003; McGhie et al., 2004), inhibits F-actin depolymerization and potentiates the action of SipC (McGhie et al., 2001). SopB is a phosphatidylinositol phosphatase that indirectly promotes actin rearrangements: either by producing second messengers acting on Cdc42 and/or RhoG to activate the Arp2/3 complex and to form F-actin-enriched membrane ruffles [trigger mechanism of invasion (Norris et al., 1998; Zhou et al., 2001)] or by activating RhoA resulting in activation of Myosin IIA/B and a stress-fibre-like dependent uptake of the bacteria (Hanisch et al., 2011; 2012). The induced F-actin-enriched ruffles facilitate uptake of the bacterium into the host cell (Hayward and Koronakis, 2002; Patel and Galan, 2005; 2006; Schlumberger and Hardt, 2006).

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The interaction of S. Tm with its host cells has been extensively studied in the last decades. However, due to the complexity of the host–pathogen interaction, studying invasion on the single cell level has remained challenging. Conventionally grown cells show a high variability in invasion on the single cell level has remained challenging. Therefore, we have focused on micropatterned cells as a model to study bacterial infection.

**Results**

**Micropatterned cells as a model to study bacterial infection**

To produce single cells that adopt a similar shape, morphology and physiology, epithelial HeLa cells (clone Kyoto) were grown on coverslips harbouring fibronectin-coated patterns. The coverslips were produced by coating the surface of conventional glass coverslips with poly(lysine)-g-poly(ethylene glycol) (PLL-g-PEG) and imprinting specific oxidized shapes on this surface using photolithography by deep UV light (Azioune et al., 2011). Upon incubation with fibronectin, the oxidized, ‘shaped’ regions bind the fibronectin, while the rest of the surface does not. Thus, as cells are only able to attach to the fibronectin, the micropatterns force cells into a specific shape. We used the crossbow-shape micropattern that promotes a ‘polar’ intracellular organization of the actin cytoskeleton and endocytic compartments (Thery et al., 2006; Schauer et al., 2010). As previously shown, contractile F-actin bundles formed at non-adhesive sides along the ‘bowstrings’ and cortical actin accumulated at the adhesive side along the ‘extrados’ (Fig. 1A, middle panel) of non-infected control cells. Micropatterned cells were infected with the wild-type strain of S. Tm (Table S1, between 1 and 10 bacteria per cell, constant infection inoculum of c. $6 \times 10^6$ bacteria) harbouring a plasmid constitutively expressing GFP [pM965 (Stecher et al., 2004)]. The infection was stopped at the indicated time points by fixation and compared with ‘classically’ cultured cells that were non-constrained (Fig. 1A). To quantitatively measure actin rearrangements induced in micropatterned cells, we averaged the intensity of F-actin staining from many cells at different time points projecting the Maximum Intensity Projection (MIP) of each aligned cell into an average intensity map for the different time points post infection. The average F-actin distribution is represented with a heat map, where the regions containing most F-actin are showing the brightest colour (yellow). In (B), cells were infected with a Salmonella strain lacking a functional type III secretion system 1 and constitutively expressing Invasin from Yersinia pseudotuberculosis (S. Tm* inv*). Infection in non-patterned (left panel) and patterned (middle panel) cells as well as average F-actin maps (right panel) for 5 and 20 min post infection are shown. Scale bars: 10 μm.

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average intensity map (Fig. 1A, right panel). At 5 min post infection, F-actin-enriched ruffles appeared at the extrados and bowstrings sides, intensified after 20 min of infection and disappeared between 20 and 120 min post infection, similarly to non-patterned cells (reviewed in Schlumberger and Hardt, 2006). Although strong variations were observed at the single cell level, average F-actin intensity maps revealed a clear difference between the analysed time points (Fig. 1, right panel). Indeed, average F-actin intensity maps showed that actin rearrangements peaked at about 20 min p.i., a fact that was difficult to assess in unconstrained cells. Together our data reveal that S. Tm triggers actin remodelling in micropatterned cells with similar kinetics as observed in classical cell culture conditions (reviewed in Schlumberger and Hardt, 2006; Agbor and McCormick, 2011).

Additionally, we infected micropatterned cells by bacteria that use the ‘zipper’ mechanism of host cell invasion (Isberg et al., 1987; reviewed in Steele-Mortimer et al., 2000) (Fig. 1B). The zipper mechanism is characteristic for, e.g. *Yersinia pseudotuberculosis* and *Listeria monocytogenes* that express proteins on their surface, which bind to receptors on the host cell’s surface (Cossart and Sansonetti, 2004; da Silva et al., 2012). As a result, bacteria are taken up through receptor-mediated ‘wrapping’ of the host cell membrane around the bacterium, a process involving much less actin rearrangements than is the case for the trigger mechanism. To follow the entry through the zipper mechanism we infected micropatterned cells with a *Salmonella* strain lacking a functional TTSS-1 and expressing Invasin from *Y. pseudotuberculosis* (S. Tm<sup>invG, invAsn</sup>). As expected and similar to classical cell culture conditions, average intensity maps of actin staining revealed that Invasin-coated *Salmonella* did not trigger pronounced actin rearrangements even 20 min after infection (Fig. 1B, right panel). In order to investigate if other cell types that are grown on micropatterned coverslips could be infected as well, we successfully challenged retinal pigment epithelial (RPE-1) cells with S. Tm<sup>invG</sup> and S. Tm<sup>invG, invAsn</sup> as well as 3T3 fibroblasts with *Shigella flexneri* (trigger mechanism) and *L. monocytogenes* (zipper mechanism; Fig. S1). Together these data showed that ‘standardized’ infection into micropatterned cells can be used as a model for different cell types and different invasive bacteria employing either the trigger or the zipper mechanism.

*Newly polymerized actin in membrane ruffles is recruited from the soluble G-actin pool*

Next, we addressed which intracellular actin pool feeds the *de novo* polymerization within membrane ruffles. Possibly, the actin might originate from the G-actin pool or from depolymerization of distally located F-actin. We selectively analysed crossbow-shaped, micropatterned epithelial HeLa cells, which were infected along the ‘extrados’ of the cell (Fig. S2A and B). This allowed us to monitor whether contractile F-actin bundles that accumulate along the ‘bowstring’ site of the cell were disassembled during the course of infection. To quantify changes in the local actin cytoskeleton we compared the normalized intensity of the F-actin signal along the ‘extrados’ (contractile F-actin bundles) and along the ‘bowstring’ (cortical F-actin) at different time points of infection. For this, MIPs of crossbow-shaped cells infected along the ‘extrados’ of the cell were calculated (Fig. 2A). The average F-actin fluorescence measured in the specific region was normalized to the average F-actin fluorescence measured in the cytosol (Fig. S2C) for each individual cell. Analyses were performed for the indicated time points of infection and the data were plotted for the ‘extrados’ and ‘bowstring’ regions. We found that the normalized intensity of F-actin in the ‘extrados’ region of the cell was significantly increased at 5 and 20 min of infection. This is in line with the triggering of membrane ruffles along the ‘extrados’. The signal returned to levels of non-infected cells at 2 h post infection (Fig. 2B). In contrast, the normalized intensity of F-actin in the ‘bowstring’ region did not change significantly over the time-course of infection (Fig. 2C). These data demonstrate that the amount of contractile F-actin fibres along the bowstrings remained unchanged while F-actin polymerized into ruffles along the ‘extrados’ part of the cell.

To assess F-actin levels in the ‘extrados’, we performed a similar analysis by focusing on cells, which were infected only on one side of the ‘extrados’ (Fig. S2B and C). The ‘extrados’ of these cells was divided into an ‘uninfected’ and an ‘infected’ area. We then analysed the intensity of the F-actin stain in the non-infected area. The intensity of the F-actin in the non-infected ‘extrados’ part remained constant over the time-course of infection (Fig. 2D). This was in line with the data obtained for the contractile F-actin bundles.

In order to verify if indeed depolymerization of pre-existing F-actin is dispensable for the formation of S. Tm-induced ruffles, we treated cells with the filament stabilizing agent Jasplakinolide. Jasplakinolide is known to inhibit the depolymerization of F-actin within minutes (Cramer, 1999). However, *in vivo*, high-dosed Jasplakinolide can also lower the critical concentration of actin and promote nucleation of new filaments (Bubb et al., 2000). Therefore, we treated HeLa cells either with 0.05% DMSO as a depolymerizing agent Jasplakinolide. Jasplakinolide is known to inhibit the depolymerization of F-actin within minutes (Cramer, 1999). However, *in vivo*, high-dosed Jasplakinolide can also lower the critical concentration of actin and promote nucleation of new filaments (Bubb et al., 2000). Therefore, we treated HeLa cells either with 0.05% DMSO or with increasing concentrations of Jasplakinolide for 3 min. The cells were then infected with S. Tm<sup>invG</sup> and incubated for another 5 min. The actin rearrangements induced were first studied in non-constrained cells (Fig. S3). At Jasplakinolide concentrations between 0.03 and 0.125 µM, the actin cytoskeleton of uninfected cells...
remained unchanged. However, at higher concentrations of Jasplakinolide, accumulation of aberrantly polymerized actin could be observed which intensified in a dose-dependent manner (Fig. S3). In cells treated with up to 0.125 μM of Jasplakinolide (that did not overly alter the morphology of the actin cytoskeleton), S. Tm was still able to induce pronounced actin ruffles (Fig. S3). Interestingly, this was even true at higher doses (Fig. S3). Similar results were obtained in micropatterned cells treated with 0.06 μM of Jasplakinolide (Fig. S4). We found that bacteria infected Jasplakinolide-treated cells induced pronounced actin ruffles as visualized by average intensity maps of many cells (Fig. S4B) and the significant increase of normalized intensity of F-actin in the cellular ‘extrados’ region 5 min post infection (Fig. S4C). Hence our combined data indicated that distally located pre-existing F-actin structures do not need to be disassembled in order to permit S. Tm-induced ruffling, thus favouring the conclusion that the soluble G-actin pool is the source for pathogen-triggered actin polymerization.

Net recruitment of soluble G-actin upon S. Tm-induced ruffling would predictively lead to a lower total G-actin content. To test this prediction, we used a fractionation assay based on the selective retention of cytoskeletal filaments, and release of their soluble protomers upon cell permeabilization (Sellin et al., 2012). HeLa cells were infected with S. Tm, or for comparison treated with Latrunculin B for 15 min, followed by gentle permeabilization and separation of supernatant and cell pellet fractions (Fig. S5). Latrunculin B is a G-actin-binding drug that depolymerizes F-actin. The analysis revealed that S. Tm infection causes a significant decrease in soluble actin levels (31.1 ± 1.3% of total actin in supernatant versus 43.5 ± 0.8% in control-treated cells). In contrast, a brief treatment with Latrunculin B resulted in the opposite effect (59.3 ± 3.2% of actin in supernatant). Thus, while lacking the single cell and spatial resolution of the present microscopy-based approach, a bulk biochemical assay supports the conclusion that S. Tm-triggered ruffles are formed by actin recruitment from the soluble G-actin pool.
Global actin rearrangements triggered by different S. Tm strains

The scale of actin cytoskeletal rearrangements induced by different invasive S. Tm strains in cultured cells is difficult to compare, as F-actin organization is strongly interrelated with the cell morphology. Thus, we asked if micropatterned cells allowed an unbiased quantitative comparison of actin rearrangements triggered by different strains of S. Tm which were profcient in or displayed well defined defects in TTSS-1-triggered invasion (see Supplementary Table S1). First we calculated the average intensity maps after 5 min of infection (Fig. 3, left panel). Based on these F-actin data, the cells could be divided into three groups: Whereas non-infected cells and cells infected with a Salmonella strain harbouring no TTSS (SB161, S. Tm^{invG}) or none of the four key effector proteins (M566, S. Tm^{D4}) showed almost no actin ruffles, cells infected with a strain harbouring SipA as the only of the four key effector proteins (M516, S. Tm^{SipA}) showed slight but detectable actin rearrangements. The wild-type strain (SB300, S. Tm^{inv}) and a strain harbouring only SopE out of the four key effector proteins (M701; S. Tm^{SopE}) triggered pronounced actin rearrangements (corresponding host-cell-bound bacteria are shown in Supplementary Fig. S6). Therefore, the strains induced the same actin rearrangement activity in micropatterned cells as observed earlier in non-constrained cells (Hardt et al., 1998; Zhou et al., 1999b; Ehrbar et al., 2002; Higashide et al., 2002; Perrett and Jepson, 2009), validating our model as a ‘standardized’ alternative to study actin rearrangements.

In order to statistically compare rearrangements of the actin cytoskeleton induced by different bacteria, the average intensity maps were transformed into density maps (Fig. 3B) that can be compared by a multivariate, two-sample test which was recently developed (Duong et al., 2012). Briefly, fluorescence signals from the actin staining were transformed into a cloud of co-ordinate points by segmentation. Then, co-ordinates were replaced by Gaussian functions (kernels) that were summed, revealing the underlying density of the F-actin throughout the cell (Fig. 3B). The red contours represent areas in which F-actin was most concentrated and the yellow contours represent areas with the least F-actin concentration. Thus density maps corresponded well to the average intensity maps. Density maps were compared with a novel black-box statistical test (Duong et al., 2012). P-values of pairwise comparisons confirmed the results obtained with the average intensity maps: The F-actin morphology of S. Tm^{invG} and S. Tm^{D4} did not differ significantly from non-infected cells. In contrast, S. Tm^{SipA} significantly differed from non-infected cells (P-value for significance < 0.05; see Table 1). Interestingly, the strain S. Tm^{SopE} also triggered strong actin ruffling; however,
it was evenly distributed across the entire cell. Instead it was evenly distributed across the entire cell. Rather concentration (Fig. 4A, middle panel). To our surprise, the bacteria docked at the ‘bowstring’ and the ‘extrados’ with the same efficiency. Small preferences were only observed for the edges. The latter is probably explained by the spontaneous formation of small ruffles in these regions of the micropatterned cells. Such pre-existing ruffles are known to facilitate formation of small ruffles in these regions of the micropatterned cells. The 50% probability contour that represents the smallest volume containing 50% of all observed bacteria showed that bacteria always docked at the same height of about 0.7 μm above the surface of the cover slip (see Fig. 4A, right panel).

It has been shown recently that target site selection of S. Tm on cell layers is determined by flagella-driven nearsurface swimming (Misselwitz et al., 2012). In order to test if the observed constant ‘binding height’ on single patterned cells was linked to flagella-driven motility, we compared the 3D density maps of the wild-type strain with that of different mutants: A fully motile strain harbouring no TTSS-1 (S. TmInvG, Fig. 4B) was used to investigate a possible role in binding site selection by the TTSS-1. To test flagella-driven motility, we analysed the S. Tm mutant strain S. TmInvG;flgK that lacks the TTSS-1 and the flgK gene (Fig. 4C). S. TmInvG;flgK is therefore devoid of functional flagella and is hence non-motile. 3D analysis of the cellular distribution of the three strains revealed that although the S. TmInvG strain showed a less constrained binding than the wild-type strain, most bacteria were found around 0.7 μm above the cover slide. In contrast, S. TmInvG;flgK did not show any site preference on the host cell. Instead it was evenly distributed across the entire surface of the host cell. Finally, we also analysed the strain S. TmInvG;Invasin missing a TTSS-1 but expressing flagella and Invasin from Y. pseudotuberculosis (Fig. 4D). Using this strain, we addressed the question if targeted binding at a specific height was dependent on receptor availability on the cell or if it was simply due to swimming constraints and independent of the receptor bond as well as the subsequent invasion mechanism (trigger versus

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### Table 1.

| Sample 1          | Sample 2          | P-value  | Significance |
|-------------------|-------------------|----------|--------------|
| Non-infected      | S. TmInvG (SB161) | 0.0549   | ns           |
| Non-infected      | S. TmInvG (M566)  | 0.0860   | ns           |
| Non-infected      | S. TmInvG (M516)  | 0.0001   | ****        |
| Non-infected      | S. TmInvG (M701)  | 3.223E-42 | ****        |
| Non-infected      | S. TmInvG (SB300) | 0        | ****        |
| S. TmInvG (SB161) | S. TmInvG (M566)  | 0.0852   | ns           |
| S. TmInvG (SB161) | S. TmInvG (M516)  | 0.6338   | ns           |
| S. TmInvG (SB161) | S. TmInvG (M701)  | 8.344E-18 | ****        |
| S. TmInvG (SB161) | S. TmInvG (SB300) | 9.8035E-16 | **** |
| S. TmInvG (M566)  | S. TmInvG (M516)  | 0.0033   | ****        |
| S. TmInvG (M566)  | S. TmInvG (M701)  | 8.1568E-123 | ****   |
| S. TmInvG (M566)  | S. TmInvG (SB300) | 0        | ****        |
| S. TmInvG (M516)  | S. TmInvG (M701)  | 1.2749E-21 | ****   |
| S. TmInvG (M516)  | S. TmInvG (SB300) | 0        | ****        |
| S. TmInvG (M701)  | S. TmInvG (SB300) | 3.0059E-80 | **** |

The P-values are calculated according to a density-based method as described in Duong et al. (2012).

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these rearrangements differed significantly from those induced by the wild-type strain S. Tmwt. Thus, our analysis showed that SopE is not sufficient to trigger wild-type levels of ruffling. Micropatterned cells therefore allow detailed analysis of actin rearrangements induced by the presence of different effector proteins.

In a next step, we quantified the distribution of Rac1, a direct host cell target of SopE (Hardt et al., 1998; Rudolph et al., 1999; Friebl et al., 2001). Average intensity maps of Rac1 were determined in uninfected cells and in cells 5 min post infection with the wild-type strain S. Tmwt or the ‘SopE-only’ strain S. TmSopE. Both S. Tm strains showed relocalization of Rac1 to the cell periphery in response to bacterial infection (Fig. S7A and B). Statistical analysis of the corresponding Rac1 density maps confirmed that infection with S. Tmwt or S. TmSopE modified the localization of Rac1 significantly as compared with non-infected cells (Fig. S7C). However, we did not detect any significant difference between Rac1 relocalization by S. Tmwt or S. TmSopE, suggesting that the Rac1 redistribution is mostly attributable to SopE.

S. Tm docks at a specific ‘height’ to host cells where it induces a permissive environment for invasion

Finally, we employed anisotropic micropatterned cells to investigate if local actin cytoskeleton characteristics may affect S. Tm entry at specific cellular sites. Epithelial, crossbow-shaped micropatterned HeLa cells were infected with S. Tmwt constitutively expressing GFP (pM965) and the infection was stopped at the indicated time points of infection (5, 20 or 120 min p.i.) by fixation. The cells were then aligned and MIPs of the bacteria bound to the cells were made. The MIPs revealed that the bacteria accumulated at the cell periphery without any apparent site preference around the entire cell (Fig. 4A, left panel). However, because of the high density of bacteria, MIPs may have failed to reveal subtle site preferences. Thus, we used a density-based approach to reveal areas, in which bacteria were concentrated during docking and subsequent invasion. Bacteria were automatically detected in the 3D stack images taken from each infected cell and their point co-ordinates were extracted using segmentation analysis. Again, point co-ordinates were replaced by kernels and summed, revealing density maps of bacteria, in which red contours represent areas in which bacteria were most concentrated and the yellow contours represent areas with least bacterial concentration (Fig. 4A, middle panel). To our surprise, the bacteria docked at the ‘bowstring’ and the ‘extrados’ with the same efficiency. Small preferences were only observed for the edges. The latter is probably explained by the spontaneous formation of small ruffles in these regions of the micropatterned cells. Such pre-existing ruffles are known to facilitate formation of small ruffles in these regions of the micropatterned cells. The 50% probability contour that represents the smallest volume containing 50% of all observed bacteria showed that bacteria always docked at the same height of about 0.7 μm above the surface of the cover slip (see Fig. 4A, right panel).

It has been shown recently that target site selection and availability on the cell or if it was simply due to swimming constraints and independent of the receptor bond as well as the subsequent invasion mechanism (trigger versus
The Yersinia-like S. Tm strain bound at the same specific height and with no site preference to the cells (see Fig. 4A and D). Together these results suggest that target site selection on individual host cells depends mostly on bacterial swimming.

Finally, we assessed the bacterial trafficking patterns after host cell invasion using time-course experiments. Analysing bacterial localization of S. Tm<sup>wt</sup> and S. Tm<sup>invG</sup>, Invasin at 20 min after infection, we observed that bacteria moved towards the centre of the cell (Fig. 4E). Interestingly, the preferred accumulation at cell edges was still evident at 20 min post infection. Again, we did not observe any preference for cell regions containing either contractile F-actin fibres or cortical F-actin. Within the first
20 min of infection, the bacteria remained at the same height, i.e. 0.7 μm above the surface. Only later (2 h post infection) they showed a broader density distribution and localized near the nucleus (Fig. 4E). Hence bacteria seem to invade at the same focal plane as they dock and later move towards particular juxtanuclear positions.

Discussion

The field of cellular microbiology has made tremendous progress in the last decades and the invasion mechanisms of many different pathogens have been unravelled at appreciable detail. Of particular importance for this achievement has been the use of microscopy to visualize the processes, which take place upon bacterial entry (Ehsani et al., 2009). However, due to high cell-to-cell variability in cell morphology, quantitative analysis of subtle changes and spatial distributions of proteins and bacteria have remained challenging. In this study we used for the first time micropatterned cells as a tool to study host–pathogen interactions in a quantitative way. We show that micropatterned cells are invaded by bacteria using either the zipper mechanism (Y. pseudotuberculosis, L. monocytogenes) or the trigger mechanism (Salmonella Typhimurium, S. flexneri) and that the kinetics of infection are similar to whose found in non-patterned cells. Thus, micropatterned cells represent an interesting infection model, which allows quantitative analysis of bacteria-induced changes. Furthermore, standardization of host cells allowed us to address questions in the infection process of S. Tm, which could not be answered by conventional cell culture techniques.

Upon contact with its host cell, S. Tm induces extensive actin-containing membrane ruffles (Finlay et al., 1991; reviewed in Schlumberger and Hardt, 2006). Actin, which is polymerized in these ruffles, could derive directly from the cellular G-actin pool, or alternatively, from depolymerization of pre-existing distal F-actin structures. Upon S. flexneri infection, bulk measurement of the ratio of G-actin versus total actin content has suggested that the soluble G-actin pool decreases at early time points of infection. The authors therefore concluded that newly formed actin filaments derive from de novo polymerization of the G-actin pool (Clerc and Sansonetti, 1987). However, in this study, only the total and the soluble G-actin could be measured. Therefore no spatial or single cell information of the different pools could be derived. Using micropatterned cells we studied the changes in the actin cytoskeleton during infection with S. Tm in a spatially defined and quantitative manner. Our data reveal that in epithelial HeLa cells the content of contractile F-actin fibres as well as of cortical F-actin in non-infected regions remain unaffected during the time-course of infection while pronounced F-actin structures (ruffles) were formed in the infected areas. Using a biochemical assay, we could also confirm that the total G-actin pool decreases during the time-course of infection. Therefore, our combined data indicate that newly polymerized actin in ruffles is recruited from the soluble G-actin pool.

Salmonella Typhimurium expresses several effector proteins that trigger actin rearrangements during invasion, including SopE, SopE2, SopB and SipA. A mutant harbouring neither SopE/E2 nor SopB (S. TmSipA) cannot produce ruffles (Mirold et al., 2001; Zhou et al., 2001). However, SipA was reported to interact directly with actin and to form F-actin foci (Zhou et al., 1999a,b; McGhie et al., 2001; 2004; Lilic et al., 2003). The average intensity maps and statistical comparison of cells infected with S. TmSipA or the wild-type strain demonstrated that SipA, in the absence of SopB, SopE and SopE2, induces small and local, but clearly detectable actin rearrangements. Furthermore, we found that a strain harbouring SopE, but missing SopE2, SopB and SipA (S. TmSopE2) induced strong actin rearrangements that differed from those triggered by the wild-type strain, suggesting that different effectors act together in a co-ordinated way during bacterial invasion. Micropatterned cells allow studying even very subtle actin rearrangements and quantifying them by statistical methods, opening the door to novel aspects of pathogen-triggered intracellular changes. We have here shown that this analysis is not limited to actin, but can also be employed to quantify the relocalization of other host factors such as Rac1. To the best of our knowledge, this is the first time that Rac1 relocalization upon infection could be shown in a quantitative way, paving way for a plethora of applications in the analysis of host–pathogen interactions, e.g. the role of different host cell proteins in protein trafficking during pathogen invasion. As cells can be transfected with plasmids or siRNA prior to patterning as well as treated with inhibitors or stimulators after seeding their use in host–pathogen interaction studies can also be extended to a wide array of applications. Fast and robust statistical analysis for the distribution of host proteins and/or bacteria may enable whole-genome screening for host proteins targeted by bacteria as well as bacterial effectors involved in host cell invasion and subversion. Furthermore, micropatterned cells could be employed to characterize in detail the function of candidate proteins from such screens.

Further, we investigated target site selection of S. Tm on micropatterned cells. Crossbow-shaped, micropatterned cells show a ‘standardized’ anisotropy, as several proteins and compartments are stereotypically localized (Supplementary Fig. S8A and B). In addition to the strong differences in the actin cytoskeleton along the ‘extrados’ and the ‘bowstring’ sides, several organelles are organized in a polar fashion. The front of the crossbow-shaped cell (indicated in blue in Supplementary Fig. S8A)
harbours the Golgi apparatus and multivesicular bodies/lysosomes that are orientated towards the adhesive ‘extrados’. Furthermore, Rab6-containing secretory vesicles concentrate at adhesive areas (Schauer et al., 2010). The cell rear (indicated in red in Fig. S8A) harbours the nucleus (Thery et al., 2006). It has been described earlier that the exocyst complex is important to provide new membrane for the formation of the actin ruffle induced upon S. Tm entry (Nichols and Casanova, 2010). One would therefore predict that bacteria invade preferentially in the vicinity of the exocyst complex. Surprisingly, we found no preference for S. Tm docking and entry either for the cell front/rear or for regions harbouring contractile F-actin bundles/cortical actin. However, our data showed that S. Tm bound at a specific height. This site preference was strongly dependent on the presence of functional flagella but independent of invasion mechanisms: bacteria triggered their uptake into host cells through a TTSS-1 at a similar height as bacteria using a zipper-mediated invasion mechanism, such as Y. pseudotuberculosis, which binds to integrins (Isberg et al., 1987). Our data therefore strongly suggests that physical constraints given by surface forces induced upon swimming are solely responsible for docking site selection, thus enforcing recent findings from non-constrained cell layers (see Supplementary Fig. S8B; Misselwitz et al., 2012).

Similar to host cell binding, S. Tm did not show a site preference for invasion at the ‘extrados’ or the ‘bowstring’ regions of the cell. Our data suggest that S. Tm employs swimming motility in combination with the TTSS-1 in order to induce a permissive site regardless of the underlying cytoskeletal structures. These data indicate that S. Tm can subvert any part of the anisotropic cell cortex and transform it into a permissive site for invasion. This remarkable property, together with the fact that S. Tm is able to target cells due to near-surface swimming might to some extent explain the broad host cell and species specificity observed for this pathogen.

Taken together, we could extend the current model of S. Tm invasion into HeLa cells (Fig. 5): S. Tm swims in the medium, randomly hitting the bottom of the culture dish and then sliding along the surface by flagella-driven physical forces. Due to this sliding, S. Tm encounters cells at a specific height of about 0.7 μm. Bacteria harbouring a TTSS-1 (a) dock irreversibly regardless of the underlying actin cytoskeleton (cortical F-actin or contractile F-actin bundles) and induce actin-rich ruffles from the soluble G-actin pool (indicated as blue dots) of the cell. The TTSS-1-dependent effector proteins work in a co-ordinated manner to induce ‘wild-type-like ruffles’ from the soluble G-actin pool, leaving distally located actin structures unchanged. Bacteria that do not harbour a TTSS-1 (b) bind at the same height via their fimbriae, with no preference for the ‘bowstring’ or the ‘extrados’ regions, but do not trigger actin rearrangements. At later time points of infection, the F-actin pool returns to a basal state, bacteria move to a juxtanuclear position and establish a Salmonella containing vacuole (SCV).
host–pathogen interactions. Our approach discloses a significant potential for addressing topological and quantitative questions for the interaction of bacteria, viruses and parasites with their host cells.

**Experimental procedures**

**Bacterial strains and plasmids**

The S. Typhimurium strains used were isogenic derivatives of SL1344 (SB300) of S. enterica subspecies I serovar Typhimurium (Supplementary Table S1; Hoiest and Stocker, 1981). Strains M701 [S. Typhimurium (Muller et al., 2009)], M566 [S. Typhimurium (Ewen et al., 1997)], SB161 S. Typhimurium (Kaniga et al., 1994)], M516 [S. Typhimurium (Schlumberger et al., 2007)] and M2424 [S. Typhimurium (Hoffmann et al., 2010)] have been described previously. Plasmids pM965 (Stecher et al., 2004) and pEGFP-C3/Rac1WT (Hage et al., 2009) have been described previously. pCLLA-GFP and pH355 (Invasin expression) were kindly provided by C. Jacobi and H. Rössmann respectively.

**Cell culture and bacteria growth conditions**

HeLa cells (clone Kyoto) and 3T3 fibroblasts were grown in DMEM (Gibco) supplemented with 10% FCS (Omnilab) and 50 mg l⁻¹ Streptomycin (AppliChem); retinal pigment epithelial (RPE-1) cells (Invitrogen) were grown in DMEM/F12 (Invitrogen) supplemented with 10% FCS and 2 mg l⁻¹ Streptomycin (AppliChem) at 37°C and 5% CO₂. The GFP-Rac1WT HeLa Kyoto cells were stably transfected with plasmid pEGFP-C3/Rac1WT and cultured in DMEM (Gibco) supplemented with 10% FCS (Omnilab) and 500 mg l⁻¹ Neomycin (AppliChem) at 37°C, 5% CO₂. For the infection of HeLa/RPE-1/3T3 cells, S. Typhimurium was grown in lysogenic broth (LB) supplemented with 0.3 M NaCl (Sigma) and 50 mg l⁻¹ Streptomycin (AppliChem) for 12 h at 37°C and subcultured for 4 h in S. flexneri strain M90T (Sansonetti et al., 1986) was cultured in BTCS medium. L. monocytogenes EGDe strain (Buchrieser et al., 2003) was prepared as described (Dai et al., 1997).

**Preparation of micropatterned coverslips, cell seeding, drug treatment and infection**

The micropatterned coverslips were fabricated according to Azioune et al. (2011). They were incubated for 1 h in a mix of 5 μg μl⁻¹ Fibrinogen (Invitrogen) and 0.05 μg μl⁻¹ Fibrinogen-Alexa-647 (Invitrogen). The coverslips were then washed with fresh medium and 40 000 cells per coverslip were seeded onto them. After cell attachment for 20 min and washing to remove unbound cells, adherent cells were incubated for full spreading for an additional 3.5 h prior to infection. For the experiments using Jasplakinolide (Enzo Life Sciences), cells were treated 3 min prior to infection with the indicated concentrations of Jasplakinolide dissolved in DMSO. DMSO (0.05%) was used as control that corresponded to the highest amount of DMSO present in the Jasplakinolide titration. Cells were infected with bacteria at different multiplicities of infection (to obtain between 1 and 10 bound bacteria per micropatterned cell) for the indicated time points (5 min, 20 min and 120 min for S. Typhimurium, 15 min for L. monocytogenes and S. flexneri) before fixation. Non-patterned HeLa cells were seeded on glass coverslips 1 day before infection and infected in the same way as described above.

**Fluorescence staining**

HeLa, RPE-1 or 3T3 cells were fixed with 4% (wt/vol) paraformaldehyde (PFA, Sigma) supplemented with 4% sucrose for 15 min and permeabilized with 0.1% Triton X-100 (Sigma) for 5 min. Cells were blocked with 3% BSA (PAA) supplemented with 4% sucrose (Sigma) and incubated with either TRITC Phalloidin (0.5 μg ml⁻¹, Sigma) or Alexa647 Phalloidin (3.5 μg ml⁻¹, Invitrogen) and DAPI (1 μg ml⁻¹, Sigma). The coverslips were mounted with Mowiol (VWR International).

**Image acquisition**

Microscopic acquisition of images was performed either with a Zeiss Axiolab 200M inverted microscope equipped with an Ultraview confocal head (Perkin Elmer) and a krypton-argon laser (643-RYP-A01 Melles Griot, the Netherlands) using a 100× oil immersion objective (PLAN-Apochromat Zeiss) and z intervals of 0.2 μm or with a Zeiss 200M (inversed) PALM-Microdissection widefield microscope with a 63× oil immersion objective (Zeiss) with z intervals of 0.3 μm, followed by image deconvolution.

**Image analysis**

Average F-actin intensity maps were obtained by a modified ‘Reference Cell’ analysis (Thery et al., 2006). The F-actin signals of 3D stacks of each cell were first reduced to a 2D representation using Maximum Intensity Projection of all slides of the stack. All 2D images of single cells from one condition were then assembled into one stack and aligned using the corresponding fluorescent micropattern for each cell. Then, the average intensity of each pixel over the assembled stack of aligned cells was calculated by an Average Intensity Projection. A heat map (Fire Lookup Table) was applied to the Z-projected image to facilitate examination and interpretation of the experimental results (the region with the most F-actin being displayed in yellow).

The measurement of the fluorescence ratio of the actin-stain in different cell areas was performed on cells that were only infected from the adhesive ‘extrados’ side. Maximum Intensity Projections were generated and the average intensity of the F-actin signal was measured in specific, non-overlapping regions, namely (i) the ‘extrados’ side, (ii) the ‘bowstrings’ side, (iii) the centre of the cell and (iv) the non-infected ‘extrados’ side (see Supplementary Fig. S2C). The ratio of the average F-actin fluorescence in the ‘extrados’ versus cell centre and ‘bowstrings’ versus centre were calculated for each individual cell.

To visualize 2D bacterial distributions the 3D stacks obtained by microscopy were first reduced to 2D using Maximum Intensity Projections. All 2D images of bacteria from one condition were assembled into one stack and aligned using the fluorescent micropattern of each cell. Then, a Maximum Intensity Projection of all slides of the aligned, assembled stack was performed.

Density map analysis of F-actin structures and bacteria were performed according to Schauer et al. (2010). For the analysis of F-actin, average F-actin intensity maps (see above) were segmented with the multidimensional image analysis (MIA) interface.
running under MetaMorph (Universal Imaging Corporation) based on wavelet decomposition (Racine et al., 2007). The segmentation was performed in 2D and the watershed function was applied to cut long F-actin structures into several co-ordinates. For the analysis of bacteria, the fluorescence signals recorded in the 3D stack of each cell were segmented in 3D. Fluorescent objects were detected as fluctuations that are 100-fold larger than noise. The watershed function was applied to separate bacteria in clusters. The co-ordinates of segmented bacteria were aligned relative to the centre of the corresponding micropattern.

The obtained co-ordinates from segmentation analysis (in 2D for F-actin and in 3D for bacteria) were used to calculate corresponding density maps with a non-parametric, unbinned kernel density estimator (Simonoff, 1996) programmed in the ks library (Duong, 2007, ks: Kernel smoothing, R package version 1.8.10) in the open-source R programming language (R Development Core Team; R: A Language and Environment for Statistical Computing. Vienna, Austria). In brief, each co-ordinate was replaced by a normal distribution (kernel) and summed over the entire population to be analysed. For visualizing kernel density estimates probability contours were used (Bowman, 1993; Hyndman, 1996). Contours represent the smallest area (for 2D) or volume (for 3D) in which a percentage of all structures are found. For example, the 50% contour represents the smallest area/volume in which 50% of all structures are localized. Graphical representation in three dimensions was achieved using the rgl library (Adler, D. and Murdoch, D. rgl: 3D visualization device system (OpenGL), R package version 0.84) and the misc3d library (Feng, D. and Tierney, L. misc3d: Miscellaneous 3D Plots, R package version 0.6-1). To ensure robust statistics of bacterial entry sites, bacterial co-ordinates were mirrored along the x-axis resulting in aggregated density maps for one half of a symmetric cell that was duplicated to facilitate visualization.

P-value calculations

P-values between pairwise comparisons of different conditions were calculated according to Duong et al. (2012) using a completely automatic test programmed in the ks library (Duong, 2007, ks: Kernel smoothing, R package version 1.8.10) in R (R Development Core Team; R: A Language and Environment for Statistical Computing. Vienna, Austria).

Actin partitioning in permeabilized cells

HeLa cells were infected with S. Tm (moi 200) or treated with 10 μM Latrunculin B (Calbiochem) for 15 min, washed, and subsequently permeabilized at 37°C in 10 mM phosphate/137 mM NaCl/3 mM KCl/1 mM EDTA/0.2% saponin buffer supplemented with protease inhibitor cocktail (Roche). The supernatant fraction was immediately transferred to cold acetone (67% final concentration) and precipitated at −20°C. Supernatant (S) and cell pellet (P) fractions were resuspended in equal volumes of Western blot sample buffer, loaded on 15% SDS-PAGE, and transferred onto nitrocellulose filter according to manufacturer’s recommendations (Bio-Rad). Western blot detection was performed with antibodies directed against Beta-actin (AC40, SIGMA), Op18 (anti-SLEEIQ, see Sellin et al., 2012), Calnexin (C-20, Santa Cruz Biotechnology) and appropriate secondary reagents. Autoradiogram band intensities were quantified using Alphaview SA.

Acknowledgements

Many thanks to Tarn Duong for assistance with density calculations and Csaba Balazs of the Light Microscopy Center Zürich as well as Jacques Laville and his team for help with the data handling and analysis at ETH. We thank the participants of the EMBO Microscopy course 2011 for help with S. flexneri and L. monocytogenes infection, Jost Enninga for helpful discussions and critical reading of the manuscript and Alessandro Piola for help with the figures. P.V. and W.-D.H. were supported by the Swiss National Science Foundation (310030-132997) and a grant (InfectX) to W.-D.H. from the Swiss SystemsX.ch initiative, evaluated by the Swiss National Science Foundation. M.E.S. was supported by a Swedish Research Council post-doctoral fellowship. K.S. received funding from the Fondation pour la Recherche Médicale en France and Association pour la Recherche sur le Cancer. This project was further supported by grants from Agence Nationale de la Recherche (#2010 BLAN 122902), the Centre National de la Recherche Scientifique and Institut Curie.

Conflict of interest statement

A patent has been filed on the density-based two sample test.

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(right) of the cell. In (C), cellular regions are shown that were used to measure average intensity of F-actin to quantify F-actin ratios of Fig. 2B–D. For Fig. 2D only cells were considered that showed an infected and a non-infected side of the ‘extrados’.

Fig. S3. Analysis of the effect of Jasplakinolide on \( S. Tm^m \) induced actin rearrangements in non-constrained cells. Representative Maximum Intensity Projections (MIP) of confocal stacks taken of unpatterned HeLa cells. The cells were treated either with 0.05% DMSO [corresponding to the amount present in \( S. Tm^m \)] or with increasing concentrations of Jasplakinolide for 8 min. The cells in the middle panel were infected after 3 min of pre-incubation with Jasplakinolide, for 5 min with \( S. Tm^m \) and stained with Phalloidin. The right panels show a blow ups of selected regions. The presence of actin ruffles is indicated with an arrow. Bacteria and the nucleus are shown in blue and the actin cytoskeleton in red. Scale bar: 10 \( \mu \)m.

Fig. S4. Analysis of the effect of Jasplakinolide on \( S. Tm^m \) induced actin rearrangements in micropatterned cells. In (A) representative Maximum Intensity Projections (MIP) of confocal stacks taken of patterned HeLa cells, treated with 0.06 \( \mu \)M Jasplakinolide (Jasp)) or with increasing concentrations of Jasplakinolide for 8 min. The cell in the left panel is not infected, while the cell in the right panel is infected with \( S. Tm^m \) for 5 min. The actin cytoskeleton is shown in red, the nucleus in blue and the bacteria in green. Scale bar: 10 \( \mu \)m. In (B) the Average Intensity map of the F-actin distribution for N micropatterned cells is shown. The F-actin distribution is represented with a heat map, where the regions containing most F-actin are showing the brightest colour (yellow). HeLa cells stably expressing GFP-Rac1 were infected with either the wild-type strain (\( S. Tm^m \)) or a strain harbouiring only SopE out of the four key effector proteins (\( S. Tm^{SopE} \)). In (B), the corresponding density maps are shown. Density contours are shown from 10% (red) to 90% (yellow) and represent the smallest regions that contain the percentage of analysed Rac1 structures. In (C), density maps are pairwise compared and the \( P \)-values of the comparisons are given. The \( P \)-values are calculated according to Duong et al. (2012).

Fig. S5. Partitioning of actin in permeabilized \( S. Tm^m \)-infected cells. HeLa cells were mock-treated (Co), infected with \( S. Tm \) or treated with 10 \( \mu \)M Latrunculin B (Lat B) for 15 min, washed, and gently permeabilized in an isotonic buffer at 37°C followed by immediate separation of supernatant and cell pellet fractions (see Experimental procedures for details). Western blots in (A) show the partitioning of Beta-actin between the soluble supernatant (S) and the cell pellet (P) fractions. Quantifications in (B) show the mean ± SD from densitometric analysis of replicate samples as in (A). In (C), detection of the cytosolic protein Op18 confirms efficient plasma membrane permeabilization, while detection of the ER-integral protein Calnexin reveals that cells were not dislodged or fragmented by the present treatment.

Fig. S6. Distribution of bacteria from infected cells analysed in Fig. 3. Maximum Intensity Projections of bacteria from N-infected cells analysed for their F-actin distribution in Fig. 3 at 5 min p.i. are shown. The images are ordered corresponding to the degree of observed actin rearrangement, from top (no actin rearrangements, light green) to middle (little actin rearrangement, dark green) to bottom (extensive actin rearrangements, light blue).

Fig. S7. Relocalization of Rac1 upon infection by different strains of \( S. Tm^m \). In (A), the average Rac1 distribution of N cells is represented with a heat map, where the regions containing most Rac1 are showing the brightest colour (yellow). HeLa cells stably expressing GFP-Rac1 were infected with either the wild-type strain (\( S. Tm^m \)) or a strain harbouring only SopE out of the four key effector proteins (\( S. Tm^{SopE} \)). In (B), the corresponding density maps are shown. Density contours are shown from 10% (red) to 90% (yellow) and represent the smallest regions that contain the percentage of analysed Rac1 structures. In (C), density maps are pairwise compared and the \( P \)-values of the comparisons are given. The \( P \)-values are calculated according to Duong et al. (2012).

Fig. S8. Schemes of anisotropic, crossbow-shaped cells. The scheme in (A) shows the anisotropic distribution of the actin cytoskeleton (cortical F-actin and contractile F-actin bundles), the microtubule organization centre (MTOC), the Golgi apparatus, secretory vesicles, lysosomes and the nucleus in the cell. In (B), the cell is schematically divided into two parts containing the ‘extrados’ (front of the cell, marked in blue) and the ‘bowstrings’ (rear of the cell, marked in red) and the structures and compartments concentrated in the respective parts are described. The model is based on data described in Thery et al. (2006) and Schauer et al. (2010).

Table S1. Bacterial strains used in this study.

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