Salmonella invades fibroblasts by multiple routes differing from the entry into epithelial cells

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Running title: Varied Salmonella invasion routes in fibroblasts
Fibroblasts are ubiquitous cells essential to tissue homeostasis. Despite their non-phagocytic nature, fibroblasts restrain replication of intracellular bacterial pathogens as *Salmonella enterica* serovar Typhimurium. The extent at which the entry route of the pathogen determines this intracellular response is unknown. Here, we analyzed *S. Typhimurium* invasion in fibroblasts obtained from diverse origins, including primary cultures and stable non-transformed cell lines derived from normal tissues. Features distinct to the invasion of epithelial cells were found in all fibroblasts tested. In some fibroblasts, bacteria lacking the type-III secretion system encoded in the *Salmonella* pathogenicity island 1 displayed significant invasion rates and induced the formation of lamellipodia and filopodia at the fibroblast-bacteria contact site. Other bacterial invasion traits observed in fibroblasts were the requirement of phosphatidylinositol 3-kinase, MAP-kinase MEK1, actin filaments and microtubules. RNA interference studies showed that different Rho-family GTPases are targeted by *S. Typhimurium* to enter into distinct fibroblasts. Rac1 and Cdc42 knockdown affected invasion of normal rat kidney fibroblasts whereas none of the GTPases tested (Rac1, Cdc42, RhoA, or RhoG) was essential for invasion of immortalized human foreskin fibroblasts. Collectively, these data reveal a marked diversity in the modes used by *S. Typhimurium* to enter into fibroblasts.
INTRODUCTION

Salmonella enterica serovar Typhimurium (hereafter referred as S. Typhimurium) is an intracellular bacterial pathogen that causes pathologies ranging from gastroenteritis to systemic disease (35). Upon oral ingestion, S. Typhimurium preferentially associates to M cells located in the Peyer’s patches of the distal ileum (47). Bacteria also cross the epithelial barrier via invasion of enterocytes or upon being captured by dendritic cells located in the intestinal lamina propria (77).

S. Typhimurium invasion of eukaryotic cells has been extensively characterized in cultured epithelial cells and is mediated by a specialized type III secretion system encoded in the Salmonella pathogenicity island 1 (SPI1-TTSS) (22). Mutants devoid of this secretion system are severely affected in the capacity to penetrate the intestinal epithelium of calves and streptomycin-treated mice, two extensively used animal models of S. Typhimurium enterocolitis [reviewed in (34, 83)]. The SPI1-TTSS is composed by more than 30 proteins, including dedicated regulators, chaperons, structural proteins of the secretion apparatus and secreted effector proteins (23). Delivery of SPI1 effector proteins to the host cell cytosol triggers a profound rearrangement of the actin cytoskeleton in both epithelial cell and macrophages (24, 61, 67, 80). A consequence of these changes is the induction of membrane ruffling and macropinocytosis at the site of Salmonella-epithelial cell contact (20, 25, 38, 61).

Membrane ruffling occurs subsequently to the activation of the Rho-family GTPases Cdc42 and Rac1 by bacterial effector proteins that mimic eukaryotic guanine nucleotide exchange factors (GEFs), as SopE and SopE2 (36, 74). S. Typhimurium injects into epithelial cells other SPI-1 effectors with capacity to modulate actin dynamics such as the phosphoinositide phosphatase SopB, also known as SigD. Based on the high affinity to lipids of pleckstrin...
homology (PH) domains present in most GEFs, it has been proposed that Cdc42 and Rac1 activity could be modulated via endogenous GEFs in response to changes in phosphoinositides promoted by SopB (84). In fact, recent data have shown that SopB/SigD stimulates SGEF, a RhoG-specific exchange factor (60). Other SPI1-TTSS-secreted proteins contributing to membrane ruffling and bacterial uptake are SipA and SipC, which bind to actin and induce bundling of filamentous actin (39, 56, 85, 86). Once the invasion process has been completed, S. Typhimurium down-regulates Cdc42 and Rac1 via another SPI1-TTSS effector protein, SptP, a GTPase-activating protein (21). Important host factors implicated in S. Typhimurium invasion of epithelial cells include the N-WASP- and Scar/WAVE-Arp2/3 complexes, which transmit the signal from active Cdc42 or Rac1 to the actin cytoskeleton (69, 80). A recent study revealed that S. Typhimurium invasion can also occur in the absence of functional N-WASP/WAVE activators (33). Other host factors linked to S. Typhimurium invasion include the SNARE protein VAMP8/endobrevin, the focal-adhesion kinase (FAK), the scaffolding protein p130Cas, and the multidomain protein IQGAP1 (8, 16, 68).

To date, several studies support the existence of varied S. Typhimurium invasion routes in non-phagocytic cells. Thus, bacteria activate the Arp2/3 complex to enter into polarized and non-polarized epithelial cells but Rac1, and not Cdc42, is targeted by the bacteria to invade the apical membrane of polarized cells (14, 15). Conversely, bacterial invasion of the basolateral membrane relies on a toxin B-sensitive GTPase distinct from Cdc42, Rac1, RhoA and RhoG (15). Early studies performed in fibroblasts and epithelial cells also reported an apparent dispensability of the GTPases Ras, RhoA and Rac1 for induction of membrane ruffling (49). More recently, a novel Arp2/3 complex activator named WASH was shown to promote a S. Typhimurium invasion route independently of membrane ruffling (33).
Our lab has focused in the last years in the characterization of the S. Typhimurium lifestyle inside fibroblasts (79). Evidence for S. Typhimurium infection of this host cell type in vivo has not been provided. However, fibroblasts are ubiquitous non-phagocytic cells that have a long life span in the connective tissue, which make them attractive targets to be colonized by bacteria during infection. Indeed, cell tropism to fibroblasts has been shown to occur in some viral and protozoan latent infections (7, 70). Interestingly, S. Typhimurium wild-type bacteria do not proliferate inside cultured fibroblasts and this response is orchestrated by bacterial functions as the PhoP-PhoQ two component system, the virulence plasmid-encoded regulator SpvR and the alternative sigma factor RpoS (27, 79). Lack of any of these bacterial regulators leads to exacerbated intracellular bacterial growth (10). Considering this unique intracellular response, we reasoned that the mode of entry of S. Typhimurium into fibroblasts could differ at some extent from that of epithelial cells, in which wild-type bacteria proliferate extensively (2, 71). Using primary fibroblast cultures and cell lines derived from non-transformed fibroblasts of diverse origin, here we show that S. Typhimurium invasion of fibroblasts substantially differ from the entry routes previously described in epithelial cells. In addition, we show evidence for a marked heterogeneity of mechanisms used by S. Typhimurium to invade fibroblasts.
MATERIALS AND METHODS

Bacterial strains and growth conditions. The S. Typhimurium wild-type virulent strain SL1344 (40) and the isogenic derivate strains SB169 (sipB::aphT), SB220 (sipC::aphT), MD0173 (invG::aphT), MD1662 (invA/pRI203), MD1663 (invA/pIL14), MvP602 (ΔSPI-4::aphT), and MvP603 (ΔSPI-4::aphT ΔinvG) have been described elsewhere (28, 30, 50, 64).

The pRI203 and pIL14 plasmids express the invasin protein (Inv) from Yersinia pseudotuberculosis (46) and the afimbrial adhesin AFA-I from uropathogenic E. coli (53), respectively. The MD706 (ΔSPI-1::KanR) strain, derived from SL1344, was constructed for this study using one-step inactivation with PCR products obtained from plasmid pKD4 as template (17). The primers used were: SPII-KOF [5’-CTA CCG CAA TCG GTA ACG CGC AAT TAT CGT CAG GTA CAG CAG GGT TAT GTG TGT AGG CTG GAG CTG CTT CG-3’] and SPII-KOR [5’-TAT GGC CTT ATA AGG CTT GCA GTC TTT CAT GGC CAG CAA GTA ACG TCT GAT CAT ATG AAT ATC CTC CTT AG-3’]. This mutant MD706 (ΔSPI-1::KanR) lacks a region of ~34 kb of SPI-1 encompassing from sprB to the invH gene.

The Escherichia coli strains MC1061 (18) and DH5α (laboratory stock) were used for comparison in the adherence/invasion assays. Bacteria were grown routinely in Luria broth (LB) at 37°C. When appropriate, kanamycin 30 µg/ml or ampicillin (50 µg/ml) were added to the growth media.

Fibroblast and epithelial cell lines. Cell lines derived from normal tissues included NRK-49F normal rat kidney fibroblasts (ATCC CRL-1570) and 3T3-Swiss embryo mouse fibroblasts (ATCC CCL-92). Primary mouse intestinal fibroblasts (MIF) were isolated from
C57BL/10 mice following the method of Strong et al., described for human intestinal fibroblasts (75). Human telomerase reverse transcriptase (hTERT)-immortalized BJ-5ta fibroblasts (ATCC CRL-4001), derived from BJ normal human foreskin primary fibroblasts (ATCC CRL-2522), were also used. HeLa (ATCC CCL-2) and Henle-407 (ATCC CCL-6) epithelial cells were used in invasion assays for comparison. NRK-49F fibroblasts were propagated in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% of fetal bovine serum (FBS) (v/v) and 4 mM L-glutamine. DMEM-10% FBS was used to propagate 3T3-Swiss fibroblasts and Henle-407 epithelial cells. Primary MIF fibroblasts were grown in DMEM medium containing 10% FBS, 4 mM L-glutamine and 2.5% of [N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid), HEPES] (v/v). Minimum essential medium Eagle (MEM) containing 10% FBS was used to grow HeLa cells. BJ-5ta fibroblasts were propagated in a 4:1 ratio of DMEM:medium 199 containing 10% FBS, 1 mM sodium pyruvate and 4 mM L-glutamine.

**Bacterial infection assays.** Bacteria were grown 18 h overnight without aeration (non-shaking) in LB medium at 37°C. Fibroblasts and epithelial cells were seeded in 24-well plates to reach a density of ~5-8 x 10^4 cells/well at the time of infection. Unless otherwise indicated, bacteria were used at a multiplicity of infection (MOI) of 10:1 (bacteria:eukaryotic cell) and incubated with cultured cells for 20 min. After extensive washing with Hank’s buffered saline solution (HBSS), cell-associated bacteria were enumerated upon lysis of the fibroblasts in a solution containing phosphate-buffered saline (PBS) pH 7.4, 1% Triton X-100 (v/v) and 0.1% sodium dodecyl sulphate (SDS) (w/v). To determine invasion rates, the cells were infected as above and washed repeatedly with a pre-warmed HBSS solution. Infected cells were then incubated in fresh culture medium containing 100 µg/ml gentamicin until 2 h post-infection.
At this time, the culture cells were lysed as above in a PBS pH 7.4 solution containing 1% Triton X-100, 0.1% SDS. The number of viable intracellular bacteria was determined by plating as previously described (10).

**Invasion inhibition assays.** The requirement of specific host functions for *S. Typhimurium* entry into fibroblasts or epithelial cells was assessed with inhibitors at the following concentrations: i) 25 µM LY294002 (CalBiochem, Darmstadt, Germany) or 100 nM wortmannin (Sigma, St. Louis, MO) to block phosphatidylinositol-3-kinase (PI3K); and, ii) 50 µM PD98059 (CalBiochem, Darmstadt, Germany) to block MEK1 kinase. These inhibitors were added for 2 h (LY294002, wortmannin) or 1 h (PD98059) prior to the infection. Filamentous actin was disrupted by incubating the fibroblasts with 1 µg/ml cytochalasin-D (Sigma, St. Louis, MO) for 20 min prior to bacterial infection. Microtubules were disrupted by treating the cells with 1-20 µg/ml nocodazole (Sigma, St. Louis, MO) for 1 h before bacterial infection. The effect on invasiveness was determined by counting of viable intracellular bacteria as described above.

**Gene silencing by RNAi.** Silencing of the Rho-family GTPases Rac1, Cdc42, RhoA and RhoG gene expression was achieved using synthetic SMART pools (Dharmacon, Inc., Chicago, IL), each comprising four proprietary siRNA sequences. siRNA were transfected into NRK-49F rat kidney fibroblasts, immortalized BJ-5ta human foreskin fibroblasts and Henle-407 human epithelial cells using HiPerfect Transfection Reagent (Qiagen GmbH, Hilden, Germany). The silencing efficiency of these RNAi constructs for their targeted mRNA was tested by quantitative RT-PCR 48 and 72 h after transfection on total RNA template isolated using the SV total RNA isolation system kit (Promega, Madison, WI). RNA samples were used to generate cDNA by High-Capacity cDNA Archive kit (Applied Biosystems,
Foster City, CA) according to the manufacturer protocol. Quantitative RT-PCR was performed using a real-time detection system with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and specific primers for rat (r-) or human (h-) Rho-family GTPases: r-Cdc42 (5’-TTC CCG TCG GAG TAT GTA CC-3’ and 5’-CAG GCA CCC ACT TTT CTT TC-3’); r-Rac1 (5’-AGT TAC ACG ACC AAT GCG TTC ACC ACC-3’ and 5’-AAT GAT GCA CTC ACA AGG C-3’ and 5’-CTT CTG GAG TCC ATT TTT CTG G-3’); r-RhoA (5’-TGT GGC AGA TAT TGA AGT GGA C-3’ and 5’-CTT CTG GAG TCC ATT TTT CTG G-3’); r-RhoG (5’-ACA A CT AAT GCC TTC CCC AAG-3’ and 5’-AAC AGA TGA CGA AGA CGT TGG-3’); h-Cdc42 (5’-CCT TTC TTG CTT GTT GGG ACT C-3’ and 5’-CTC CAC ATA CTT GAC AGC CTT C-3’); h-Rac1 (5’-GTC CCA ACA CTC CCA TCA TCC-3’ and 5’-ACA GCA CCA ATC TCC TTA GCC-3’); h-RhoA (5’-GCA GGT AGA GTT GGC TTT GTG-3’ and 5’-GAC TTC TGG GGT CCA CTT TTC-3’); and, h-RhoG (5’-TGC CTG CTC ATC TGC TAC A C-3’ and 5’-ACG AAA ACG TTG GTC TGA GG-3’). mRNA levels were normalized to GTPase mRNA in untransfected cells. RNAi knockdown efficiency and specificity were also assessed at the protein level by Western assays of cell lysates prepared at 48 and 72 h post transfection. GTPase protein levels were determined with mouse monoclonal antibodies anti-Cdc42 (clone 44, BD Transduction Laboratories); anti-Rac1 (clone 23A8, Upstate Biotechnology); and anti-RhoA (clone 26C4, Santa Cruz Biotechnology, Inc.). Rabbit polyclonal antibody anti-RhoG (Santa Cruz Biotechnology, Inc.) was also used. Protein depletion by RNAi was normalized to endogenous levels of tubulin using mouse monoclonal anti-α-tubulin antibody (clone DM1A, Sigma, St. Louis, MO).

**Immuno-fluorescence microscopy.** Cell monolayers were fixed in 3% (w/v) paraformaldehyde pH 7.4 for 10 min at 37°C. After washing in PBS pH 7.4, cells were
permeabilized in a solution containing 0.1% (w/v) saponin (Sigma, St. Louis, MO) and 1% (v/v) goat serum (Invitrogen, Carlsbad, CA). The immuno-staining was performed as previously described (26). Primary antibodies and dilutions used included: polyclonal rabbit anti-
*Salmonella* lipopolysaccharide (LPS), 1:2,000 (Difco, Detroit, MI), monoclonal mouse anti-*Salmonella* LPS, 1:200 (clone MLK33, gift of Dr. J.M. Slauch, University of Illinois, Urbana-Champaign, USA) and polyclonal rabbit anti-*E. coli*, 1:200 (gift of Dr. M. Vicente, CNB, Madrid, Spain). Secondary goat antibodies anti-rabbit or anti-mouse conjugated to either Alexa-Fluor 594 or Alexa-Fluor 488 (Molecular Probes, Eugene, OR) were used at a dilution of 1:1,000. Cy5-conjugated secondary antibody anti-rabbit (Jackson ImmunoResearch Lab., West Grove, PA) was used at a dilution of 1:500. For differential staining of inside versus outside bacteria (in-out staining), cells were fixed as above and stained as previously described (11). Briefly, extracellular bacteria were stained in non-permeabilized cells with either polyclonal rabbit anti-*Salmonella* LPS or anti-*E.coli* antibodies followed by anti-rabbit Cy5-conjugated as secondary antibody. Upon permeabilization, intracellular bacteria were stained with either monoclonal mouse anti-*Salmonella* LPS antibody or rabbit polyclonal anti-*E.coli* antibody followed by anti-mouse or anti-rabbit Alexa 594 as secondary antibodies. Filamentous actin was stained with 1 µg/ml phalloidin conjugated to Alexa 488 (Molecular Probes, Eugene, OR). In other experiments, 1 µg/ml phalloidin conjugated to Alexa 594 (Molecular Probes, Eugene, OR) was also used. When required, nuclei were stained with 0.5 µg/ml DAPI (4′-6′-diamino-2-phenylindole) for 5 min. Cells were examined using a Bio-Rad 2100 Radiance System attached to a Zeiss Axiovert 200 microscope.

**Scanning electron microscopy.** Coverslips containing infected cell monolayers were fixed for 4 h at 4°C in a solution containing 2.5 % (w/v) glutaraldehyde and 200 mM HEPES pH 7.2
and further rinsed in HEPES 200 mM pH 7.2 buffer. The samples were then dehydrated with a graded series of acetone and subjected to critical-point drying with CO₂. The samples were further coated with gold and graphite and examined in a JEOL scanning electron microscopy JM-6400 at a voltage of 40 Kv.

**Statistical analysis.** Data were analyzed with the GraphPad Prism 5.0 software (GraphPad Inc., San Diego, CA) using one-way analysis of variance (ANOVA) with a Tukey’s post-test or Student’s t-test. A p value lower than 0.05 was considered significant.
RESULTS

S. Typhimurium entry into normal rat kidney fibroblasts (NRK-49F) occurs by two alternative routes. S. Typhimurium invades epithelial cells using the specialized type-III secretion system encoded in the Salmonella-pathogenicity island 1 (SPI1-TTSS) (22). To determine the requirement of SPI1-TTSS in fibroblast invasion, normal rat kidney fibroblasts (NRK-49F) were infected with the S. Typhimurium wild-type strain SL1344 and isogenic mutants defective in SipB or SipC, two SPI1-encoded invasion proteins. sipB and sipC mutants displayed a significant capacity to invade NRK-49F fibroblasts (~30% of the rate of the wild-type strain), much higher than the observed in HeLa epithelial cells (~1% of the rate of the wild-type strain) (Fig. 1A). This phenotype was confirmed with an isogenic mutant lacking the entire SPI-1 pathogenicity island, MD706 (ΔSPI-1::KanR), which displayed an invasion rate of ~18% compared to the wild-type parental strain (Fig. 1A). The entry exhibited by a motile non-pathogenic Escherichia coli strain MC1061 was found to be 10-fold lower than that of the ΔSPI-1 mutant (Fig. 1A). The significant capacity of the ΔSPI-1 mutant to invade NRK-49F fibroblasts was also confirmed in other S. Typhimurium genetic backgrounds as 14028s and LT2 (data not shown).

S. Typhimurium adhesion and penetration of polarized epithelial cell barriers is promoted by the concerted action of the co-regulated type I and type III secretion systems encoded in the Salmonella-pathogenicity islands 4 (SPI-4) and 1 (SPI-1) (29, 51, 54, 58). To test whether SPI4 was involved in bacterial entry into fibroblasts, we used ΔSPI-4 and ΔSPI-4invG isogenic strains, obtained from Prof. M. Hensel. InvG is an outer membrane protein absolutely essential for the correct assembly of the SPI-1 TTSS apparatus and invG mutants are defective in type III secretion (23). Invasion assays in NRK-49F fibroblasts discarded a contribution of
SPI-4 in this infection model (Fig. 1B). Lack of SPI-4 has also no effect in the entry rate of the "invG" mutant (Fig. 1B). We also asked whether flagella, which facilitates invasiveness in tissue culture cells, activates innate immune responses, and promotes anti-apoptotic processes in epithelial cells (81), was essential for bacterial entry into NRK-49F fibroblasts. Invasion assays performed with flagellin-defective mutants in both wild-type and ΔSPI-1 backgrounds discarded such possibility (data not shown). Based on these observations, we next aimed to dissect the contribution of cytoskeletal proteins in the invasion of fibroblasts. This was assessed with microtubule- or actin-destabilizing drugs as nocodazole or cytochalasin-D. Both drugs reduced significantly the entry of wild-type and ΔSPI-1 bacteria into NRK-49F fibroblasts (Fig. 1C). The effect of nocodazole was found more pronounced in the case of the ΔSPI-1 mutant (Fig. 1C). We next examined by microscopy the morphological alterations occurring in NRK-49F fibroblast during bacterial uptake. Prominent membrane ruffling, macropinocytosis and massive actin rearrangement were phenomena observed exclusively during invasion of wild-type bacteria (Fig. 2A). Unexpectedly, invading ΔSPI-1 bacteria were found associated to lamellipodia-like structures at the bacteria-host cell contact site (Fig. 2B). Although discrete accumulations of actin were visualized surrounding motile E. coli bacteria ingested by the fibroblast, no lamellipodia were observed in this case (Fig. 2B). Scanning electron microscopy confirmed the ability of the S. Typhimurium ΔSPI-1 mutant to trigger the formation of lamellipodia and filopodia in the fibroblast-bacteria contact area (Fig. 2C). Taken together, these data demonstrate that entry of S. Typhimurium into NRK-49F fibroblasts proceeds by two distinctive routes differing in the involvement of the SPI1-TTSS system, the requirement of microtubules and the extent at which the host cell membrane is remodeled during the bacterial uptake process.
S. Typhimurium entry into fibroblasts derived from others sources. To define whether the mode of entry displayed by the S. Typhimurium ΔSPI-1 mutant in NRK-49F fibroblasts was a generalized phenomenon, the behavior of this strain was examined in fibroblasts obtained from other sources. Fibroblasts obtained from normal tissues as the 3T3-Swiss mouse embryo fibroblasts and primary fibroblasts obtained from mouse intestine (MIF), were used. Adhesion and invasion rates among the different bacteria and fibroblasts used were determined by counting both viable intracellular bacteria and cell-associated bacteria. In addition to the E. coli motile strain MC1061, a second E. coli strain displaying poor motility, DH5α, was also included to assess whether these latter bacteria could be engulfed at some extent by the fibroblast. As noticed in pilot experiments, the most optimal infection conditions in these fibroblasts were found to be 30 min for S. Typhimurium wild-type bacteria and 60 min for the S. Typhimurium ΔSPI1 mutant and E. coli strains. In contrast to NRK-49F fibroblasts, the invasion rate displayed by the S. Typhimurium ΔSPI1 mutant in 3T3-Swiss fibroblasts was not different to that shown by the E. coli motile strain MC1061 (Fig. 3A). In MIF intestinal fibroblasts the ΔSPI1 mutant exhibited a higher invasion rate than E. coli MC1061, although the difference was not statistically significant (Fig. 3A). On the other hand, the entry of ΔSPI1 mutant bacteria into 3T3-Swiss and MIF fibroblasts was significantly higher rate than that of E. coli DH5α, which was negligible (Fig. 3A). Indeed, in most cases no viable intracellular E. coli DH5α was recovered from the fibroblasts. Interestingly, the adherence of S. Typhimurium ΔSPI-1 and the motile E. coli MC1061 strain was found lower (40-60%) than that shown by wild-type bacteria in 3T3-Swiss and MIF fibroblasts (Fig. 3B). Because such difference was not evident in NRK-49F fibroblasts (Fig. 3B), the diminished adherence to the fibroblast could be a factor contributing to the decreased invasiveness of the ΔSPI-1 mutant observed in these
other fibroblasts. Taken together, these data show that the S. Typhimurium SPI1-TTSS-independent invasion route is not a generalized phenomenon and that occur only in fibroblasts obtained from certain sources.

**S. Typhimurium entry into telomerase-immortalized human foreskin fibroblasts.**

Considering the variety of results obtained in non-transformed fibroblast cell lines derived from normal tissues and primary fibroblasts (Fig. 3), we decided to optimize a new fibroblast infection model. The purpose was to bypass the disadvantages linked to the usage of normal non-transformed cell lines, stabilized by undefined mutational processes overcoming cellular aging, or primary cultures displaying limited passage capability. To this aim, we used human telomerase reverse transcriptase (hTERT)-immortalized BJ-5ta cells derived from normal human foreskin fibroblasts (73). The expression of hTERT in normal cells extends life span while maintaining a normal ‘non-transformed’ phenotype (6, 48). Interestingly, microscopy analysis of human foreskin BJ-5ta fibroblasts infected with S. Typhimurium wild-type bacteria revealed that actin rearrangement in the bacteria-fibroblast contact area was not as pronounced as in normal rat kidney NRK-49F fibroblasts (Fig. 4A, B). This discrete accumulation of actin in the bacteria-fibroblast contact area was also common in the mouse intestinal primary fibroblasts MIF (data not shown). Macropinocytosis was another phenomenon rarely observed in BJ-5ta or MIF fibroblasts infected with wild-type bacteria (data not shown). The invasion rates of S. Typhimurium wild-type and ΔSPI-1 strains in the immortalized human foreskin BJ-5ta fibroblasts, determined as the ratio of intracellular versus cell-associated bacteria, were 37% and 6%, respectively (Fig. 4C). For comparison, the rates calculated for the E. coli strains MC1061 and DH5α accounted for values of 45% and 35%, respectively (Fig. 4C).
Interestingly, the invasion rate displayed by the ΔSPI-1 mutant in the immortalized BJ-5ta fibroblasts was higher than in 3T3-Swiss and MIF fibroblasts (Fig. 3A). Strikingly, the difference in invasiveness of BJ-5ta fibroblasts found between the ΔSPI-1 and the motile E. coli MC1061 (6% and 0.45%, respectively) correlated with a higher adhesion rate of the E. coli MC1061 strain (see Fig. 4C). This unexpected result suggested that, at least in the immortalized BJ-5ta fibroblasts, bacterial adherence may not directly influence the efficiency of the entry process. To test this assumption, we evaluated in the immortalized BJ-5ta fibroblasts the entry rate of S. Typhimurium invA isogenic strains expressing the invasin protein from Yersinia pseudotuberculosis (46) or the afimbrial adhesin AFA-I from pathogenic E. coli (53). Since InvA is an essential structural protein of the S. Typhimurium SPI-1-encoded type III secretion system (23), these two strains should invade the immortalized BJ-5ta fibroblasts via the SPI-1 independent route and, in addition, by ligand-receptor interactions following the expression of the Inv protein of Y. pseudotuberculosis. Only if bacterial adherence positively influences entry into these fibroblasts, an increase in bacterial invasion should also be detected in the invA strain expressing the AFA-I adhesin. Despite the notable increase in adherence promoted by the AFA-I adhesin (Fig. 5), the invasion rate increased only upon expression of the invasin of Y. pseudotuberculosis (Fig. 5).

Collectively, these data reveal that, as it was observed in NRK-49F fibroblasts, S. Typhimurium invades the immortalized human foreskin BJ-5ta fibroblasts by SPI1-TTSS-dependent and independent routes. The data obtained in these immortalized fibroblasts also permit to separate mechanistically the adhesion and invasion processes.

Phosphatidylinositol 3-kinase (PI3K) and the MAP kinase MEK1 are required for efficient bacterial entry into fibroblasts. Phosphatidylinositol 3-kinase (PI3K) and MAP
kinase MEK1 are host functions targeted by many bacterial pathogens that invade non-phagocytic cells by the ‘zipper’ mechanism. In this entry mode, host cell membrane surface receptors are activated independently of type-III secretion systems (13, 63). In agreement with that, *S. Typhimurium* invasion of epithelial cells is known to be insensitive to PI3K or MEK1 kinase inhibitors (57, 65, 72, 76, 78). Based in the results obtained in NRK-49F and BJ-5ta fibroblasts, consistent with the existence of two alternative routes differing in the contribution of the SPI1-TTSS system, we aimed to analyze whether PI3K and MEK1 were kinases required for bacterial entry into these fibroblasts. Two PI3K inhibitors, wortmannin and LY294002, were used together with PD90589, a MEK1 inhibitor. PI3K inhibition caused a significant reduction in the invasion of NRK-49F fibroblasts by both wild-type and ΔSPI-1 strains while no obvious effect was observed upon MEK1 inhibition (Fig. 6A). When tested in the immortalized foreskin human fibroblasts BJ-5ta, inhibition of both PI3K and MEK1 reduced the invasiveness of *S. Typhimurium* wild type and ΔSPI-1 strains (Fig. 6A). Interestingly, these drugs also affected the uptake of the *E. coli* motile strain MC1061 at an extent similar to that observed for the *S. Typhimurium* ΔSPI-1 mutant (Fig. 6A). This fact may reflect some similarities in the mechanisms that operate during entry of the ΔSPI-1 mutant and the uptake of the *E. coli* MC1061 strain. Control experiments performed in HeLa epithelial cells confirmed that neither PI3K nor MEK1 are required for entry of *S. Typhimurium* wild-type bacteria (Fig. 6B). No comparative invasion assay with ΔSPI-1 or *E. coli* MC1061 strains was possible due to the low number of gentamicin-protected bacteria recovered from untreated HeLa epithelial cells (data not shown). Altogether, these data demonstrate that PI3K is a kinase targeted by *S. Typhimurium* to trigger uptake by fibroblasts. In the case of MEK1, this kinase seems to play a relevant role in the SPI1-independent entry mode and only in certain fibroblasts.
Role of Rho-family GTPases in *S. Typhimurium* entry into fibroblasts. *S. Typhimurium* targets preferentially the Rho-family GTPases Rac1 and Cdc42 to trigger the cytoskeletal remodeling required for uptake by epithelial cells (11, 36, 61, 74). Cdc42 is also exploited by *S. Typhimurium* to induce nuclear responses as the induction of the proinflammatory cytokine IL-8 (60). Interestingly, recent RNA interference (RNAi) studies have shown that membrane ruffling and bacterial internalization into epithelial cells could depend on the GTPases Rac1 and RhoG, with Cdc42 dispensable for these events (60). To get insights on the GTPases targeted by *S. Typhimurium* to enter into fibroblasts, we applied RNAi in cells responding differently to bacterial infection at the level of membrane ruffling and macropinocytosis, such as the NRK-49F and the immortalized BJ-5ta fibroblasts (see Fig. 4). GTPases targeted by this RNAi treatment included Rac1, Cdc42, RhoA and RhoG. The level and specificity of the interference was assessed by qRT-PCR (data not shown) and western analyses (see below). Both *S. Typhimurium* wild-type and ΔSPI-1 strains were used in the different assays.

Interference of Rac1 and Cdc42 was accompanied by a significant decrease, ~50%, in the invasion rate of wild-type bacteria in NRK-49F fibroblasts (Fig. 7 A, B). Of note, this effect was observed even under conditions in which knockdown of Rac1 was not totally efficient (Fig. 7 A). A slightly lower, although still statistically significant, effect on wild-type bacteria invasion was also observed upon depletion of the GTPase RhoA (Fig. 7 A, B). By contrast, no appreciable effect in bacterial internalization was evident in RhoG-depleted NRK-49F fibroblasts. A similar requirement for GTPases Rac1, Cdc42 and to lesser extent of RhoA, was observed for entry of the ΔSPI-1 mutant (Fig. 7 A, B). Taken together, these data indicate that *S. Typhimurium* engage preferentially Rac1 and Cdc42 to promote entry into NRK-49F fibroblasts by the SPI1-TTSS-dependent or the independent invasion routes. Microscopy...
analyses supported the RNAi data. Thus, knockdown of either Rac1 or Cdc42 abrogated the
capacity of wild-type bacteria to induce membrane ruffling in NRK-49F fibroblasts (Fig. 7 C),
with only a partial defect on membrane ruffling observed in RhoA-depleted fibroblasts and no
changes upon depletion of the RhoG GTPase (Fig. 7 C).

Unexpectedly, depletion of Rac1, Cdc42, RhoA or RhoG in the human foreskin fibroblasts
BJ-5ta caused no major effect in internalization of either wild-type or ΔSPI-1 strains (Fig. 8 A,
B). Because as occurred in NRK-49F rat fibroblasts, Rac1 knockdown was not very efficient
in BJ-5ta fibroblasts (Fig. 8A), it is not possible to discard a putative contribution of this
particular GTPase in bacterial entry. This ‘generalized’ lack of effect contrasted however with
the data obtained upon treatment of Henle-407 human epithelial cells with the same set of
RNAi specific for human GTPases. In contrast to a recent study with the same epithelial cell
line (60), our RNAi data indicated that S. Typhimurium could target Cdc42 to promote entry
(Fig. 9 A,B). To recall that the similar level of Rac1 interference obtained in BJ-5ta human
fibroblasts and Henle-407 human epithelial cells had different consequences in bacterial
invasion in these two cell lines, with a negative effect observed only in the case of epithelial
cells (compare Figs. 8 and 9). Taken together, these data argue for a variety of entry
mechanisms that can be differentiated not only between fibroblast and epithelial cells, but also
among fibroblasts of distinct origins.
Salmonella invasion of eukaryotic cells has been extensively studied in epithelial cells and macrophages with relatively few analyses in other cell types. Prominent membrane ruffling and macropinocytosis triggered by SPI1 effectors occur in most cases (61, 62), but exceptions to this association of events are also known. Thus, a mutant lacking the SPI1 effectors SopE and SopE2 was shown to invade the fibroblast-like COS-7 cells in the absence of membrane ruffling (74). A very recent report by Hänisch et al. (33) has also demonstrated that activation of the Arp2/3-complex by S. Typhimurium and bacterial uptake can occur in fibroblasts devoid of functional WASP and WAVE proteins, which are central components linking Cdc42 and Rac1 function to rearrangement of the actin cytoskeleton. These authors, which used the SV40 virus-transformed human lung fibroblast cell line VA-13, were able to separate the membrane ruffling event from bacterial invasion, being the former dependent in WASP and WAVE connection with the Arp2/3 complex and the latter associated to the role of a new Arp2/3 complex activator named WASH (33). In this scenario, wild-type bacteria could be potentially stimulating the function of three different positive regulators of the Arp2/3-complex. Our study, based in the usage of non-transformed fibroblasts derived from different sources, extends these observations to reveal that wild-type S. Typhimurium invades primary fibroblasts isolated from mouse intestine or immortalized foreskin human fibroblasts in the absence of prominent membrane ruffling and macropinocytosis (Fig. 4 and data not shown). These moderate membrane alterations occurring in non-transformed fibroblasts in response to wild-type Salmonella could reflect of the particular anatomy of these cells. Fibroblasts are normally ‘embedded’ into the connective tissue and this fact may impede these cells to undergo rapid and intense changes in the membrane surface in response to invading bacteria as
those observed in *S. Typhimurium*-infected epithelial cells facing a luminal compartment (34, 35, 83).

Another aspect uncovered by our study was a mode of entry into fibroblasts that takes place independently of the SPI1-TTSS system. This observation has precedents in previous studies performed in epithelial cells. An early study claimed a SPI1-TTSS-independent invasion route in Chinese-hamster-ovary (CHO) epithelial cells (42) and a recent work, based in the use of a three-dimensional organotypic model of human colonic epithelium, proved the dispensability of the SPI1-TTSS system for invasion of epithelial cells (41). Other findings include the capacity of *S. Typhimurium* strains deficient in SPI1 effector proteins for invading bovine small intestine explants (66) and the efficient translocation exhibited by a *S. Typhimurium* SPI1-defective mutant in an M cell in-vitro model (55). Altogether, these observations suggest that *S. Typhimurium* may use multiple mechanisms to induce internalization by epithelial cells. The data obtained here with non-transformed fibroblasts support this concept of varied modes of bacterial entry. This assumption is further sustained by the visualization of phenomena as filopodia and lamellipodia induced by ΔSPI-1 mutant bacteria that are intimately apposed to the fibroblast surface (Fig. 2A, 2B). To our knowledge, this is the first report of host cell surface remodeling promoted during bacterial entry by a *S. Typhimurium* strain lacking the SPI1-TTSS apparatus.

The analysis of the cytoskeletal proteins required for *S. Typhimurium* invasion of fibroblasts unequivocally showed a requirement of microtubules for bacterial entry, which was more apparent in the case of the ΔSPI-1 mutant. This finding was unexpected given the dispensability of microtubules shown for the invasion of epithelial cells by this pathogen (20). On the other hand, microtubules and microtubule motors are used by some intracellular pathogens to invade epithelial cells and modulate the intracellular vacuolar niche (37, 82).
Most of these pathogens also exploit actin cytoskeleton dynamics to promote entry (5, 82). Exceptions are certain strains of *Campylobacter jejuni* and *Citrobacter freundii*, which enter epithelial cells via a microtubule-dependent but actin-independent process (59). The fact that actin and microtubules are required for *S. Typhimurium* entry into fibroblasts suggests that in some cases the pathogen may trigger remodeling of both cytoskeleton networks, as *Shigella flexneri* does in epithelial cells (82). Another distinct trait found in our study was the requirement of kinases PI3K and MEK1 for *S. Typhimurium* invasion of fibroblasts, known to be dispensable for entry into epithelial cells (57, 72, 76, 78). Upon contact with the fibroblast, *S. Typhimurium* may then employ mechanisms similar to those described for *Listeria monocytogenes* (45), group A and group B streptococci (9, 65), *C. jejuni* (43), *Helicobacter pylori* (52), and *Chlamydia pneumoniae* (12), which subvert PI3K and MEK1 signaling subsequently to bacterial ligand-host surface receptor interactions. Further work is required to sustain the apparent capacity of *S. Typhimurium* to exploit ligand-receptor interactions for invasion. In this line, the diminished uptake of the *E. coli* motile strain MC1061 in fibroblasts treated with PI3K and MEK1 inhibitors suggest that fibroblasts may be equipped with a large variety of receptors capable of promoting cytoskeletal changes upon stimulation with different bacterial products, some of them even present in non-pathogenic *E. coli*. The behavior in NRK-49F and BJ-5ta fibroblasts of this *E. coli* motile strain MC1061 compared to that of the *S. Typhimurium* ΔSPI-1 mutant has however some clear distinctions. Examples are the different invasion rates found in NRK-49F and BJ-5ta fibroblasts (Figs. 1A and 4C), and the limited alterations of the actin cytoskeleton associated with the uptake of the *E. coli* MC1061 bacteria (Fig. 2B). These evidences support the idea of a mode of bacteria entry used by *S. Typhimurium* independently of the SPII-TTSS, which may rely in a pathway partially similar to that stimulated by non-pathogenic *E. coli*. Based on the differences in entry displayed by
these two strains in some fibroblasts (NRK-49F, BJ-5ta) but not in others (3T3-Swiss, MIF). We favor the hypothesis that distinct fibroblast receptors could be stimulated by surface structures of these two bacteria. These distinct ligand-receptor interactions, which may induce common targets as the kinases PI3K and/or MEK1, could ultimately dictate the extent at which the actin/microtubules are remodeled and the ultimate efficiency of the entry process.

The assays performed with a mutant devoid of either the SiiE adhesin encoded in SPI-4 (31) or the flagella discarded any involvement of these surface structures. However, many *S. enterica* serovars are known to encode multiple fimbriae (44). Major *S. Typhi* fimbriae were recently shown to be dispensable for adhesion and invasion of cultured epithelial cells (4). Conversely, the *S. Typhimurium* fimbriae Lpf and Fim influence invasion in several cultured epithelial cell lines (3). FimH-dependent internalization of *S. Typhimurium* into dendritic cells has also recently been shown (32). Further investigations focused on a putative contribution of these structures to fibroblast invasion needs therefore to be performed. Experiments are currently in progress in our group to select for invasion-defective mutants in a ∆SPI-1 genetic background.

Of interest in our study was also to define whether the bacterial adhesion process was directing the subsequent entry into the fibroblast. Because the motile *E. coli* strain MC1061 was counted in relatively high numbers as cell-associated bacteria, it was possible that the entry could be just a ‘consequence’ of the ability of the bacteria to adhere to the fibroblast surface and a rather unspecific ‘phagocytic’ capacity of the fibroblast. The assays performed in the BJ-5ta with *invA* strains devoid of a functional SPI1-TTSS system but expressing either the invasin (Inv) protein of *Y. pseudotuberculosis* or the afimbrial adhesin AFA-I discarded such correlation between adherence and invasion. Based on these observations, we favor the idea that the differential entry of the *S. Typhimurium* ∆SPI-1 and *E. coli* MC1061 strains in
fibroblasts as NRK-49F and BJ-5ta occur upon distinct bacterial-host receptor interactions. Interesting aspects to address in future investigations include the infection phase in which *S. Typhimurium* ‘wild-type’ bacteria could invade host cells independently of the SPI1-TTSS and the benefit for the pathogen of such mode of entry. Equally appealing is the possibility that such SPI1-TTSS independent entry mode occurs stochastically as part of the inherent heterogeneity of the bacterial population that colonize the host tissues.

We lastly aimed to decipher the panel of GTPases of the Rho-family targeted by *S. Typhimurium* to invade fibroblasts. The role of Rac1, Cdc2, RhoA, and RhoG was assessed in NRK49-F and BJ-5ta fibroblasts, exhibiting distinct ‘intensity’ of both ruffling and macropinocytosis (Fig. 4). Our data suggest that the set of Rho-GTPases subverted by *S. Typhimurium* to trigger internalization might vary depending of the type of fibroblast tested. Massive membrane ruffling and macropinocytosis (NRK-49F fibroblasts) correlated with engagement of Cdc42, Rac1 and, to a lesser extent, of RhoA. To the contrary, *S. Typhimurium* entry in telomerase-immortalized BJ-5ta fibroblasts displaying much less cytoskeleton reorganization occurred in the absence of Cdc42, RhoA, and RhoG or with reduced Rac1 levels (Fig. 8). These results suggest that other yet-unknown GTPase(s) of the Rho-family may direct entry of *S. Typhimurium* into these immortalized non-transformed fibroblasts. Other Rho-GTPases such as Wrch-1, RhoD, and Rif are capable of modulating cytoskeleton dynamics and promoting filopodia formation (19). Rac2 and Rac3 have also been reported to induce lamellipodia formation, and RhoB and RhoC can promote stress fibers assembly (1). It is also possible that no Rho-GTPase be absolutely essential for *S. Typhimurium* entry into these immortalized non-transformed BJ-5ta fibroblasts. This assumption would at some extent reconcile with the early study of Jones et al. showing that membrane ruffling induced by *S. Typhimurium* was independent of Ras, Rac1 and RhoA (49). However, these authors reported
major membrane ruffling structures visible upon Salmonella invasion in both 3T3-Swiss fibroblasts and MDCK epithelial cells, a situation clearly different to that observed in immortalized BJ-5ta fibroblasts. Finally, our RNAi analysis unequivocally linked Cdc42 to the entry of S. Typhimurium into the human epithelial cell line Henle-407 (Fig. 9). We are uncertain about the basis of this different outcome compared to the study of Patel and Galán, which claimed that RhoG, and not Cdc42, is targeted by S. Typhimurium to invade the same human epithelial cell line (60). The different method used to grow the bacteria may explain such discrepancy. Regardless of this fact, our data unravel as main conclusion a marked versatility in the type of Rho-GTPases that are targeted by S. Typhimurium depending on the origin and type of non-phagocytic cell that is infected. Subsequent studies based in the analysis of the phosphorylation status of these GTPases in the different fibroblasts examined here and in the absence or presence of bacteria, should reinforce the conclusions derived from RNAi interference data. Our findings also open new questions regarding the bacterial effectors and mechanisms sustaining the entry of wild-type bacteria into fibroblasts in the absence of a prominent membrane ruffling and macropinocytosis. Thus, it would be interest to address in future studies whether this mode of entry requires key activators of the Arp2/3-complex as N-WASP and WAVE. Considering the data of Hanisch et al. in the VA-13 transformed fibroblasts (33), it is tempting to postulate that N-WASP and WAVE of immortalized BJ-5ta may not be targeted by wild-type bacteria during the invasion process as are in epithelial cells. Further work directed to analyze these aspects should provide new insights on the heterogeneity of S. Typhimurium invasion routes uncovered in fibroblasts. Given the striking differences observed, we favor future studies with primary ‘normal’ cells non-transformed and not displaying limited passage capability exemplified by the telomerase-immortalized human foreskin fibroblasts described here. It would be of interest to develop similar models with
epithelial cells since up to now most of the studies have been performed with immortal cancer cells derived from malignant tumors.
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**FIG. 1.** *Salmonella* invasion of NRK-49F normal rat kidney fibroblasts show features distinct to the invasion of epithelial cells. (A) Invasion rates estimated by gentamicin-survival assays and made relative to the rate of the wild-type bacteria. Incubation time with bacteria was of 20 min. Viable intracellular bacteria were enumerated at 2h post-infection and accounted, in the case of wild-type bacteria, for 1.04% and 3.60% of the inoculum in NRK-49F fibroblasts and HeLa epithelial cells, respectively. The *S. Typhimurium* strains used were: SL1344 (wild-type), SB169 (*sipB*), SB220 (*sipC*) and MD706 (ΔSPI-1). The *E. coli* motile strain MC1061 was included for comparison. (B) Dispensability of the type I secretion system encoded in the *Salmonella* pathogenicity island 4 (SPI-4) for bacterial entry into NRK-49F fibroblasts. The *S. Typhimurium* strains used included MD176 (*invG*); MvP602 (ΔSPI-4), and MvP603 (ΔSPI-4 *invG*). (C) Inhibitory effect of nocodazole and cytochalasin on the invasion of NRK-49F fibroblasts by *S. Typhimurium* wild-type and ΔSPI-1 strains. The invasion rate of the ΔSPI-1 strain (16% of that of wild-type strain) was normalized to 100% to highlight the differences in the drug-treated fibroblasts. Data are the means and standard deviation from three independent experiments.
FIG. 2. *S. Typhimurium* entry into NRK-49F fibroblasts occurs via two modes differing in the extent of actin rearrangement and macropinocytosis. Extracellular (blue) and intracellular (red) bacteria were differentiated by the ‘in-out’ staining procedure (see Material and Methods). Actin was labelled with FITC-phalloidin (green). Entry of *S. Typhimurium* wild-type and ΔSPI-1 bacteria was compared to that of the *E. coli* MC1061 motile strain. (A) Low magnification images showing prominent membrane ruffling and macropinocytosis induced by *S. Typhimurium* wild-type bacteria in NRK-49F fibroblasts. Note that unlike the few *E.coli* MC1061 observed associated to the fibroblasts, some of the *S. Typhimurium* ΔSPI-1 bacteria were associated to lamellipodia-like membrane extensions. Bar: 10 µm; (B) Magnifications of the areas marked in panel A. Arrows indicate a large and actin-rich lamellipodium contacting a surface-located ΔSPI-1 bacterium and discrete actin accumulations surrounding two *E. coli* MC1061 bacteria located intracellularly. Bar: 5 µm; (C) Scanning electron microscope images of NRK-49F fibroblasts infected for 20 min with *S. Typhimurium* wild-type or ΔSPI-1 bacteria. Note the prominent membrane ruffling in the area where wild-type bacteria locate. Filopodia and lamellipodia were observed associated to ΔSPI-1 bacteria located onto the fibroblast surface. The ΔSPI-1 mutant was also occasionally visualized ‘retracting’ the fibroblast surface underneath invading bacteria (arrows).
FIG. 3. Requirement of the type III secretion system encoded in SPI-1 (SPI1-TTSS) for entry into diverse fibroblast cells. The cell lines used included: NRK-49F (normal rat kidney fibroblasts), 3T3-Swiss (disaggregated mouse embryo) and MIF (mouse intestinal primary fibroblasts). (A) Invasion rates of S. Typhimurium SL1344 (wild-type), MD706 (∆SPI-1), and the E. coli strains MC1061 (motile) and DH5α (non-motile) expressed as the ratio of intracellular bacteria versus total number of fibroblast-associated bacteria counted before gentamicin treatment. Using this parameter, the invasiveness of the ∆SPI1-mutant relative to wild-type was 28% (NRK-49F), 4.6% (3T3-Swiss) and 9.2% (MIF). (B) Bacterial adhesion to the different fibroblast cell lines used. Number of cell-associated bacteria was made relative to that of wild-type strain in each of the fibroblast cell lines used. Data are the means and standard deviation from three independent experiments. (*) p = 0.01-0.05; (**) p = 0.001-0.01; n.s.= not significant. Student t-test (panel A) and one-way ANOVA with Tukey’s post-test (panel B).
FIG. 4. The extent of membrane ruffling induced by *S. Typhimurium* in fibroblasts varies depending on the source of fibroblast used. (A) Actin distribution in NRK-49F normal rat kidney fibroblasts and telomerase-immortalised human foreskin BJ-5ta fibroblasts infected with *S. Typhimurium* SL1344 (wild-type) strain for 20 min. Note that the ruffles are more prominent in the infected NRK-49F fibroblasts. Actin was labelled with Alexa-594-phalloidin (red), bacteria with primary anti-rabbit *Salmonella* followed by secondary Alexa-488-conjugated goat anti-rabbit antibody (green), and the nuclei with Dapi (blue). Bar = 10 µm; (B) Enlargement of areas marked with arrows in panel A. Bar = 5 µm.; (C) Invasion rates and adherence properties of the *S. Typhimurium* ∆SPI-1 mutant and the *E. coli* strains MC1061 and DH5α in the telomerase-immortalised human foreskin BJ-5ta fibroblasts. Data are the means and standard deviation from three independent experiments. (*) $p = 0.01-0.05$; (**) $p = 0.001-0.01$; n.s.= not significant. Student *t*-test.
FIG. 5. Increased adherence to BJ-5ta immortalized human foreskin fibroblasts mediated by the afimbrial adhesin AFA-I is not followed by a higher invasion rate in the SPI1-independent entry mode. Shown are absolute numbers of fibroblast-associated bacteria counted before gentamicin treatment and those of intracellular bacteria surviving gentamicin treatment. Infection was for 20 min (wild-type) or 60 min (rest of the strains). The *S. Typhimurium* strains used included: SL1344 (wild-type), MD706 (∆SPI-1), MD1662 (*invA / inv*+) and MD1663 (*invA / afa*+). Note that the expression of the invasin protein (Inv) from *Y. pseudotuberculosis* increases bacterial entry while the enhanced adherence promoted by the afimbrial adhesin AFA-I (compared to the ∆SPI-1 strain) does not. Data are the means and standard deviation from three independent experiments. (*) $p = 0.01-0.05$; n.s.= not significant. Student *t*-test.

FIG. 6. Phosphatidylinositol 3-kinase (PI3K) and the MAP kinase MEK1 are involved at different extent in the entry into fibroblasts of *S. Typhimurium* SL1344 (wild-type), MD706 (∆SPI-1) and the *E. coli* motile strain MC1061. The role of these two kinases was assessed in invasion assays using 100 nM wortmannin (WOR), 25 µM LY294002 (PI3K inhibitors), or 50 µM PD98059 (MEK1 inhibitor). (A) Effect of the drugs in bacterial entry into normal rat kidney fibroblasts NRK-49F and immortalized human foreskin fibroblasts BJ-5ta; (B) control assay performed in HeLa human epithelial cells. Note the dispensability of these two kinases in HeLa epithelial cells and their differential requirement for bacterial entry into fibroblasts depending on the fibroblast and the bacterial strain used. Data are the mean and the standard deviation of three independent experiments. (*) $p = 0.01-0.05$; (**) $p = 0.001-0.01$; (***$p < 0.001$; n.s.= not significant. Student *t*-test.
FIG. 7. Requirement of Rho-family GTPases for entry of S. Typhimurium SL1344 (wild-type) and MD706 (ΔSPI-1) strains into NRK-49F normal rat kidney fibroblasts. (A) Levels of Rac1, Cdc42, RhoA, and RhoG upon incubation for 72 h in the presence of respective RNAi. The level of α-tubulin was determined as control; (B) Invasion rate of wild-type and ΔSPI-1 bacteria in RNAi-treated NRK-49F fibroblasts. Incubation time with bacteria was of 20 min (wild-type) and 40 min (ΔSPI-1). Viable intracellular bacteria were counted at 2 h post-infection. Shown are the percentages of interfered-NRK-49F normal rat kidney fibroblasts either exhibiting membrane ruffling in response to the infection with wild-type bacteria or containing intracellular bacteria upon an infection with the ΔSPI-1 mutant. The analysis in the ΔSPI-1 mutant was performed with ‘in-out’ staining. Data are the mean and the standard deviation of three independent experiments. [(*) $P = 0.01-0.05$; (**) $P = 0.001-0.01$; (***) $P < 0.001$; (n.s. = not significant), one-way ANOVA analysis with Tukey’s post-test]; (C) Microscopy analysis showing the capacity of wild-type bacteria to induce membrane ruffling in NRK-49F fibroblasts depleted of RhoA or RhoG. Bar = 10 µm.
FIG. 8. RNAi interference on Rac1, Cdc42, RhoA, or RhoG does not substantially affect the invasion of BJ-5ta telomerase-immortalised human foreskin fibroblasts by S. Typhimurium SL1344 (wild-type) or MD706 (ΔSPI-1) strains. (A) Levels of Rac1, Cdc42, RhoA, and RhoG upon incubation for 72 h in the presence of respective RNAi. The level of α-tubulin was determined as control. (B) Invasion rate of wild-type and ΔSPI-1 bacteria in RNAi-treated BJ-5ta human foreskin fibroblasts. Incubation time with bacteria was of 20 min (wild-type) and 60 min (ΔSPI-1). Viable intracellular bacteria were counted at 2h post-infection. Data were analysed by one-way ANOVA with Tukey’s post-test. Shown are the mean and the standard deviation of three independent experiments. No significant differences were found.

FIG. 9. Rac1 and Cdc42 are targeted by S. Typhimurium SL1344 (wild-type) to invade Henle-407 human epithelial cells. A) Levels of Rac1, Cdc42, RhoA, and RhoG upon incubation for 72 h in the presence of respective RNAi. The level of α-tubulin was determined as control; (B) Invasion rate of wild-type bacteria in RNAi-treated Henle-407 human epithelial cells. Incubation time with bacteria was 20 min and viable intracellular bacteria were counted at 2h post-infection. Data are the mean and the standard deviation of three independent experiments. [(*) *P* = 0.01-0.05; (*** *) *P*<0.001; (n.s. = not significant), one-way ANOVA analysis with Tukey’s post-test]. Note that although Rac1 and RhoG are not totally depleted by the RNAi treatment, a significant effect on invasion in observed in only the case of Rac1; (C) Representative microscopy images depicting the involvement of Cdc42 in the membrane ruffling triggered by wild-type bacteria in these epithelial cells. Bar = 10 µm.