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Shigella flexneri Phagosomal Escape Is Independent of Invasion▽

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Infections with *Salmonella enterica* serovar Typhimurium and *Shigella flexneri* result in mucosal inflammation in response to epithelial cell invasion and macrophage cytotoxicity. These processes are mediated by type III secretion systems encoded in homologous virulence loci in the two species, namely, *Salmonella* pathogenicity island 1 (SPI-1), carried in the genome, and the *Shigella* entry region (SER), carried in a large virulence plasmid. Here we show that SPI-1 can functionally complement a deletion of SER in *S. flexneri*, restoring invasion of epithelial cells, macrophage cytotoxicity, and phagosomal escape. Furthermore, *S. flexneri* phagosomal escape requires the SER and another gene(s) carried on the large virulence plasmid. We demonstrate that the processes of invasion and phagosomal escape can be uncoupled in *S. flexneri*.

Salmonella enterica serovar Typhimurium and *Shigella flexneri* are the etiological agents of gastroenteritis and bacillary dysentery, respectively. The symptoms of these diseases are caused by mucosal inflammation in response to bacterial invasion of epithelial cells and induction of macrophage apoptosis (5, 6, 17). These processes are triggered by the delivery of multiple bacterial effector proteins into host cells via type III secretion systems (TTSS). In *S. enterica* serovar Typhimurium, the structural proteins of the TTSS, as well as many of its regulators and effector proteins required for the intestinal phase of the disease, are encoded by a region of the chromosome called *Salmonella* pathogenicity island 1 (SPI-1) (8). A homologous region is carried in a 30-kb locus in the large virulence plasmid of *S. flexneri.* We refer to this locus as the *Shigella* entry region (SER) (8, 11). Despite these similarities, infections with *S. enterica* serovar Typhimurium and *S. flexneri* differ soon after their uptake into host cells. While *S. enterica* serovar Typhimurium replicates inside modified phagosomes (15), *S. flexneri* ruptures the phagosomal membrane and gains access to the cytoplasm (13). In addition to invasion, the SER is thought to carry genes required for phagosome escape, because noninvasive *S. flexneri* mutants also fail to escape from the phagosome in macrophages.

In this study, we demonstrate that SPI-1, carried on a single-copy plasmid, can complement a deletion in the SER for epithelial cell invasion, phagosome escape, and induction of macrophage apoptosis. Surprisingly, an *S. flexneri* strain lacking the entire large virulence plasmid and complemented with SPI-1 is invasive but fails to escape the phagosome and does not replicate inside epithelial cells, indicating that phagosome escape requires genes carried in the large virulence plasmid outside the SER. This is the first report demonstrating that invasion and phagosome escape can be uncoupled in *S. flexneri*.

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MATERIALS AND METHODS

Bacterial strains, tissue culture, and growth conditions. The bacterial strains used in this study and their characteristics are listed in Table 1. *Salmonella* and *Shigella* strains were grown at 37°C in Luria-Bertani broth and Trypticase soy broth, respectively, and supplemented with ampicillin (100 μg/ml), kanamycin (25 μg/ml), and/or chloramphenicol (12.5 μg/ml) when indicated. HeLa cells, a human epithelial cell line, and RawB cells, a murine macrophage-like cell line, were grown at 37°C in a humidified 5% CO_2 atmosphere in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 100 U-100 μg of penicillin-streptomycin per ml. Bone marrow-derived macrophages were prepared as described elsewhere (14). Briefly, bone marrow cells from C57BL/6 mice (Charles River) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 5% horse serum, 10 mM HEPES, 1 mM pyruvate, 10 mM L-glutamine, and 20% macrophage colony-stimulating factor-conditioned medium collected from an L929 macrophage colony-stimulating factor-producing cell line and incubated in a humidified 7% CO_2 atmosphere at 37°C.

Invasion, intracellular growth, and cytotoxicity assays. Overnight cultures of *Salmonella* strains were grown without aeration at 37°C and used directly for infections. *Shigella* strains were grown with aeration at 37°C, and mid-logarithmic-phase cultures were used for infections. HeLa cells and macrophages were seeded at 1×10^5 cells per well in 24-well plates and at 5×10^4 cells per well in 96-well plates and grown for 24 h at 37°C. Prior to infection, the growth medium was aspirated, the cells were washed twice with phosphate-buffered saline (PBS), and serum-free medium was added. Bacterial inocula were prepared in PBS and centrifuged onto the cells (2,000 rpm, 10 min, 37°C), and infected cultures were incubated for 20 min at 37°C. Cultures were washed three times with PBS, and fresh medium containing 100 μg/ml gentamicin was added. To test for epithelial cell invasion and intracellular growth, HeLa cells were infected with *Salmonella* at a multiplicity of infection (MOI) of 10:1 or with *Shigella* strains at an MOI of 100:1 and assayed in a gentamicin protection assay (4). At the indicated time points, the medium was removed, and the cells were washed with PBS and lysed with 0.1% Triton X-100. The numbers of viable bacteria were obtained by plating dilutions of the lysates on tryptic soy agar plates. Colonies were counted after overnight incubation of the plates at 37°C. For macrophage cytotoxicity assays, cells were infected with *Shigella* as described above. Cytotoxicity was quantified by measuring the release of lactate dehydrogenase (LDH) from infected cells, using a CytoTox 96 kit (Promega) following the manufacturer's instructions.

Chloroquine assay. Phagosomal escape was evaluated with a modified chloroquine resistance assay (4). Briefly, RawB cells were seeded 24 h prior to infection at 2.5×10^5 cells per well in 24-well plates. Cells were infected at an MOI of 10:1 as described above. At 30 min postinfection, cells were washed with PBS, and fresh medium containing 100 mM HEPES and 100 μg/ml gentamicin, with or without 2.5 mg/ml chloroquine (Sigma), was added to each well. At 2 hours postinfection, cells were washed twice with PBS and lysed in 0.1% Triton X-100, and the CFU per well were determined. For all assays described above, the standard error was calculated based on at least three independent determinations.

Plasmids and knockout strains. We used the method described by Datsenko and Wanner (2) to delete SPI-1 (delimited by *mutS* and *sitA*) from wild-type *S.*

TABLE 1. Bacterial strains and characteristics

Strain	Description	Source or reference
SL1344	Wild-type *S. enterica* serovar Typhimurium	7
SL1344ΔSPI-1	Deletion of SPI-1 in SL1344	This study
SL1344ΔSPI-1/pSPI-1	SL1344ΔSPI-1 transformed with pSPI-1	This study
M90T	Wild-type *S. flexneri*	12
M90TΔSER	Deletion of the SER in M90T	This study
M90TΔSER/pSPI-1	M90TΔSER transformed with pSPI-1	This study
M90TΔipaH$_{7.8}$	Deletion of *ipaH*$_{7.8}$ in M90T	This study
M90TΔipaH$_{7.8}$/pipaH$_{7.8}$	M90TΔipaH$_{7.8}$ transformed with pipaH$_{7.8}$	This study
BS176	M90T cured of the large virulence plasmid	12
BS176/pSPI-1	BS176 transformed with pSPI-1	This study

enterica serovar Typhimurium strain SL1344 and the SER (delimited by *virB* and *spa40*) or *ipaH*$_{7.8}$ from wild-type *S. flexneri* strain M90T to produce strains SL1344ΔSPI-1, M90TΔSER, and M90TΔipaH$_{7.8}$, respectively. A single-copy plasmid harboring SPI-1 (pSPI-1) was used to complement deletions of SPI-1 in *S. enterica* serovar Typhimurium and of the SER in *S. flexneri* to generate strains SL1344ΔSPI-1/pSPI-1 and M90TΔSER/pSPI-1, respectively. To construct pSPI-1, regions upstream and downstream of SPI-1 were deleted from the bacterial artificial chromosome harbored by *Escherichia coli*/SGSC_10F09 (Salmonella Genetic Stock Center). Two consecutive deletions produced a construct containing SPI-1 from *mutS* to *sitA* in a pBeloBAC11 vector. The gene *ipaH*$_{7.8}$ was cloned under the control of its own promoter into the low-copy-number vector pWSK29 (16) to create the plasmid pipaH$_{7.8}$ and transformed into M90TΔipaH$_{7.8}$ to produce strain M90TΔipaH$_{7.8}$/pipaH$_{7.8}$.

Immunoblots. Proteins from supernatants of stationary-phase cultures of different strains were precipitated with ice-cold acetone, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotted with sera from rabbits immunized with recombinant SipC. The blots were incubated with horseradish peroxidase-labeled anti-rabbit antibodies and developed with an ECL Western blotting detection kit (Amersham).

Immunofluorescence microscopy. HeLa cells were seeded on 12-mm glass coverslips 24 h prior to infection. Cells were infected with *Shigella* as described above, fixed in 3.7% paraformaldehyde at 3 h postinfection, and permeabilized with 0.1% Triton X-100. Filamentous actin was visualized with Alexa 594-phalloidin (Molecular Probes). Bacteria were stained with rabbit anti-*Shigella* serotype 5A serum and secondary goat anti-rabbit antibodies conjugated with cyan-2. For quantification of actin tail formation, *Shigella*-infected cells were fixed at 5 h postinfection. To distinguish intracellular from extracellular bacteria, external bacteria were labeled using rabbit anti-*Shigella* serotype 5A serum and secondary goat anti-rabbit antibodies conjugated with cyan-5. After permeabilization, intracellular microorganisms were stained with rabbit anti-*Shigella* serotype 5A serum and secondary cyan-3-conjugated goat anti-rabbit antibodies. Actin was visualized with Alexa 488-phalloidin. Using confocal microscopy, the ratio of polar actin tails to intracellular bacteria was determined. Since the number of intracellular bacteria varied with the invasive capacity of the tested *Shigella* strains, we analyzed an average of 50 (48 to 71) infected cells per strain.

RESULTS AND DISCUSSION

SPI-1 is functional in *S. flexneri*. Strains transformed with pSPI-1 secrete SipC, a *Salmonella* effector protein encoded in SPI-1 and secreted through the TTSS (Fig. 1A). Proteins from supernatants of stationary-phase cultures of different strains were precipitated and immunoblotted with anti-SipC antiserum as described in Materials and Methods. Both *Salmonella* and *Shigella* strains complemented with pSPI-1 secreted SipC as efficiently as wild-type *Salmonella*.

To assess the functional complementation of pSPI-1 in *Salmonella* and *Shigella* strains, deletion strains were assayed in a gentamicin protection assay with HeLa human epithelial cells. Deletion of either SPI-1 in *S. enterica* serovar Typhimurium or the SER in *S. flexneri* led to an almost 1,000-fold decrease in invasiveness (Fig. 1B). SL1344ΔSPI-1/pSPI-1 was as invasive as wild-type SL1344, showing that the plasmid-borne SPI-1 is fully functional. Interestingly, complementation of M90TΔSER with pSPI-1 restored invasion to almost wild-type levels. The slightly lower invasion efficiency seen with M90TΔSER/pSPI-1 might reflect differences in regulation of SPI-1 and the SER. Other studies previously showed that *S. enterica* serovar Typhimurium SipB only partially complements a deletion of the *S. flexneri* effector protein IpaB (5), supporting the hypothesis that despite functional similarities the two proteins may have different efficiencies.

To determine the role of the remaining large virulence plasmid genes in *Shigella* virulence, we transformed BS176, a derivative of M90T cured of the large virulence plasmid (12), with pSPI-1 to generate strain BS176/pSPI-1. Interestingly, despite an initial invasion rate in HeLa cells similar to that of M90TΔSER/pSPI-1, BS176/pSPI-1 failed to grow intracellularly (Fig. 2A). In contrast to the 30-fold increase in growth of M90TΔSER/pSPI-1 between 2 and 6 h postinfection ($P = 0.0003$; unpaired t test), BS176/pSPI-1 counts were not statis-

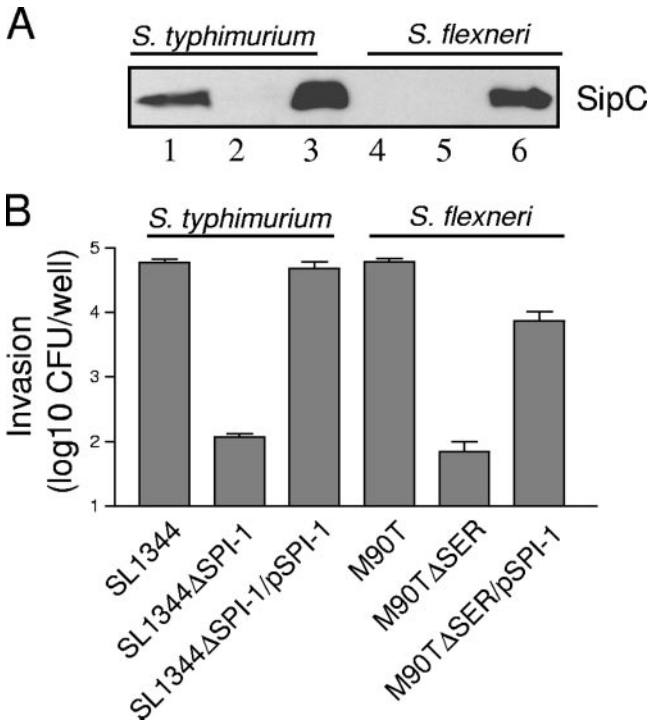

FIG. 1. Plasmid-borne SPI-1 can complement a SPI-1 deletion in *S. enterica* serovar Typhimurium and an SER deletion in *S. flexneri*. (A) The culture supernatants of strains SL1344 (lane 1), SL1344ΔSPI-1 (lane 2), SL1344ΔSPI-1/pSPI-1 (lane 3), M90T (lane 4), M90TΔSER (lane 5), and M90TΔSER/pSPI-1 (lane 6) were precipitated, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and probed with an anti-SipC serum. The data show that SipC is efficiently secreted from *Shigella* and *Salmonella* strains complemented with pSPI-1. (B) Invasiveness of epithelial cells by SPI-1 and SER deletion strains is rescued by pSPI-1. HeLa cells were infected with the strains described above at an MOI of 10:1 for *Salmonella* and 100:1 for *Shigella* strains. Intracellular bacteria were quantified at 4 h postinfection in a gentamicin protection assay. Values represent the means of measurements for triplicate samples of a representative experiment, and error bars indicate standard deviations.

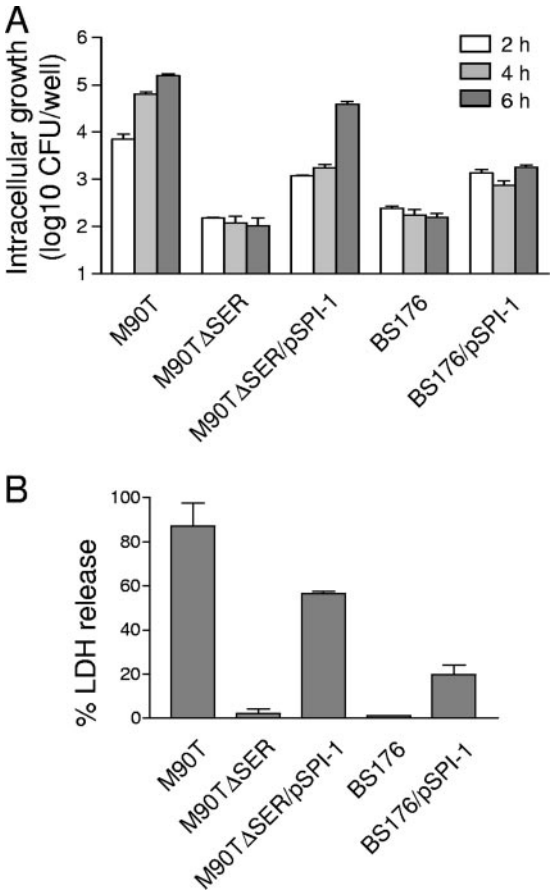

FIG. 2. Intracellular growth and macrophage cytotoxicity require invasion as well as other functions encoded in the large virulence plasmid outside the SER. (A) BS176, an *S. flexneri* strain cured of the large virulence plasmid, invades but cannot grow inside HeLa cells when complemented with pSPI-1. HeLa cells were infected at an MOI of 100:1, and intracellular bacteria were quantified at 2, 4, and 6 h postinfection in a gentamicin protection assay. (B) BS176/pSPI-1 fails to induce cytotoxicity to the same level as that by M90TΔSER/pSPI-1 in bone marrow-derived macrophages. Macrophages were infected at an MOI of 100:1, and cytotoxicity was measured by LDH release at 4 h postinfection. Values represent the means of measurements for triplicate samples of a representative experiment, and error bars indicate standard deviations.

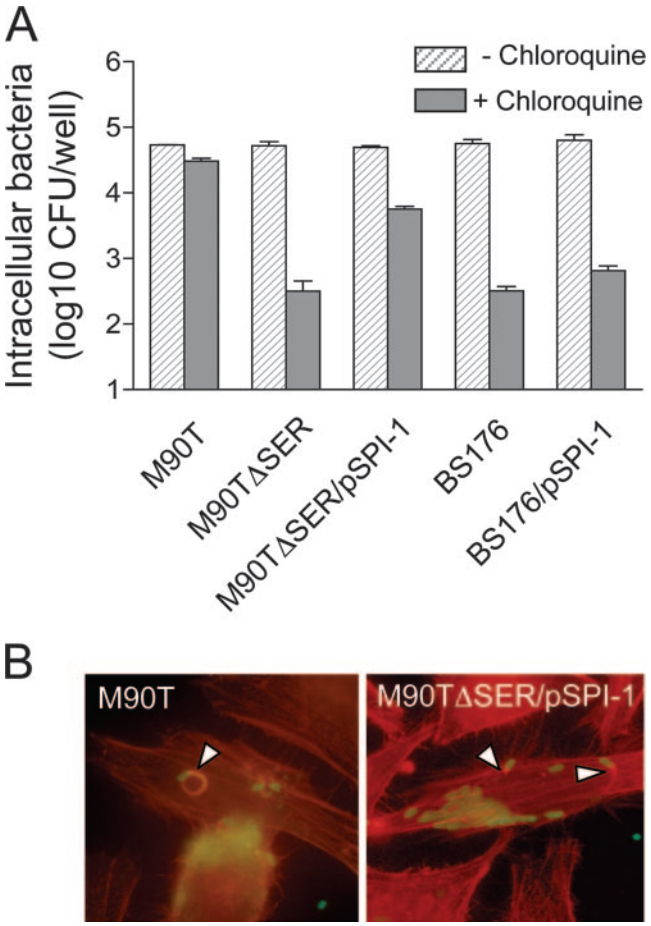

FIG. 3. A gene(s) in the large virulence plasmid outside the SER is required for *S. flexneri* phagosome escape. (A) pSPI-1 complements an SER deletion in M90T, allowing bacteria to escape the phagosomes of infected RawB macrophages. RawB cells were infected at an MOI of 10:1 and incubated in the presence of gentamicin, with or without chloroquine, as indicated in the figure. At 2 hours postinfection, the cells were lysed and the surviving bacteria were enumerated. CFU for wells containing only gentamicin represent total intracellular bacteria. The presence of intracellular bacteria that survive chloroquine indicate a cytoplasmic localization. Values represent the means of measurements for triplicate samples of a representative experiment, and error bars indicate standard deviations. (B) HeLa cells were infected with the indicated *Shigella* strains at an MOI of 100:1 and fixed at 3 h postinfection. Actin was detected with phalloidin (red), and bacteria were detected with an anti-lipopolysaccharide antibody (green). Bacteria that escape the phagosome polymerize actin tails (arrowheads).

tically different in the same period ($P = 0.06$; unpaired t test). Intracellular replication was correlated with the induction of cytotoxicity in bone marrow-derived macrophages, as assayed by LDH release (Fig. 2B). Complementation with pSPI-1 restored cytotoxicity to different levels in M90TΔSER and BS176. The partial complementation of M90TΔSER/pSPI-1 is in agreement with the partial complementation seen for invasion. Curiously, BS176/pSPI-1 released only low levels of LDH. Since the induction of apoptosis requires phagosomal escape (18), these data suggest that this phenotype as well as the lack of intracellular growth in epithelial cells indicates that BS176/pSPI1 is trapped in a vacuole.

SPI-1 enables *Shigella* phagosome escape and intracellular motility. Phagosome escape was evaluated in a chloroquine resistance assay (4). Simultaneously, we also monitored the release of LDH to confirm that increases in sensitivity to antibiotics did not represent lysis of the host cell (data not shown). As expected, macrophages phagocytosed both invasive and noninvasive bacteria efficiently, as reflected by similar colony counts recovered from cultures incubated only with gentamicin (Fig. 3A, hatched bars). In this assay, the accumulation of chloroquine in phagosomes at bactericidal concentrations selects for bacteria that escape the vacuole. Hence, similar colony counts for cultures treated with chloroquine and gentamicin and with gentamicin alone indicate phagosomal escape, while lower counts for cultures treated with chloroquine and gentamicin than for cultures incubated only with gentamicin indicate bacterial phagosomal localization. Wild-type *Shigella* escapes the phagosome, as reflected by similar counts in

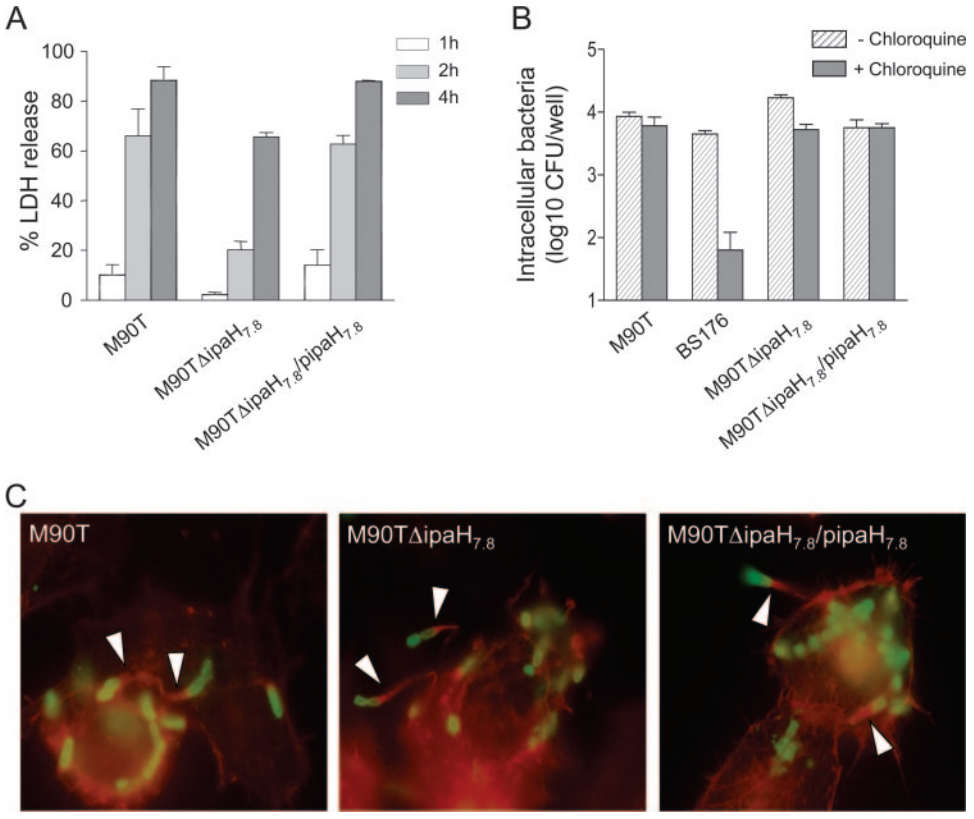

FIG. 4. $ipaH_{7.8}$ is not required for phagosome escape. (A) *S. flexneri* deleted in $ipaH_{7.8}$ is less cytotoxic to macrophages than are wild-type strains. RawB cells were infected at an MOI of 100:1, and cytotoxicity was measured by LDH release. (B) *S. flexneri* lacking $ipaH_{7.8}$ escapes the phagosome and is resistant to chloroquine. RawB macrophages were infected as described in the legend to Fig. 3 and plated for CFU counts at 2 h postinfection. Values in panels A and B represent the means of measurements for triplicate samples of a representative experiment, and error bars indicate standard deviations. (C) *S. flexneri* lacking $ipaH_{7.8}$ shows polar actin polymerization similar to that of the wild type. HeLa cells infected with *Shigella* strains at an MOI of 100:1 were fixed at 3 h postinfection and analyzed as described in the legend to Fig. 3.

the presence and absence of chloroquine. The noninvasive strains M90TΔSER and BS176 were unable to escape the phagosome and were killed by chloroquine. This phenotype was rescued, by >1 log, by the presence of pSPI-1 in M90TΔSER/pSPI-1. However, in accordance with our previous observations, BS176/pSPI-1 was trapped in the phagosome and killed similarly to the noninvasive strains (Fig. 3A, gray bars).

Phagosomal escape is a prerequisite for polar actin recruitment and intracellular motility of *S. flexneri* (1, 9, 10). To further test the ability of M90TΔSER/pSPI-1 to escape the phagosome, we analyzed HeLa cells infected with the different strains by immunofluorescence microscopy. We observed polar actin tails formed by the wild-type *Shigella* strain as a control and by M90TΔSER/pSPI-1 (Fig. 3B), which directly demonstrates the ability of these strains to escape the phagosome. The percentage of actin tails formed inside infected cells was quantified by microscopy and showed that SPI-1 is functional in *Shigella*. For wild-type *Shigella*, 10.1% of the internalized bacteria showed polar actin recruitment, and an average of 24.6 bacteria/cell were detected. In contrast, we did not observe a single actin tail associated with any of the intracellular M90TΔSER microorganisms detected inside a total of 71 infected HeLa cells analyzed. M90TΔSER/pSPI-1, however, could escape from phagosomes and initiate actin recruitment. As expected from the results obtained from gentamicin protection assays, the average number of intracellular bacteria was slightly reduced (8.5 bacteria/cell) compared to that of the wild type, but 2% of the intracellular bacteria showed polar actin tail formation. At this point, it is not clear why there is a difference in the numbers of bacteria that recruited actin in wild-type *Shigella* and M90TΔSER/pSPI-1. These results, however, strongly suggest that phagosome escape requires either the SER or SPI-1, since deletion of the SER leads to a lack of escape and complementation with pSPI-1 restores it. Furthermore, since pSPI-1 restores invasion but not phagosomal escape to BS176, it is clear that a gene or genes outside the SER and carried in the large virulence plasmid are also required for this function.

***Shigella* phagosomal escape does not depend on $IpaH_{7.8}$.** A report by Fernandez-Prada et al. (3) identified $ipaH_{7.8}$ as the gene that facilitates *S. flexneri* escape from the phagosome. Since $ipaH_{7.8}$ is carried in the large virulence plasmid outside the SER, we tested the effect of $IpaH_{7.8}$ on phagosome escape in the $ipaH_{7.8}$ deletion strain M90TΔipaH$_{7.8}$. In addition, $ipaH_{7.8}$ was cloned to create the plasmid pipaH$_{7.8}$ and transformed into M90TΔipaH$_{7.8}$ to produce strain M90TΔipaH$_{7.8}$/pipaH$_{7.8}$. Deletion of $ipaH_{7.8}$ had no effect on invasion or replication of the strain in HeLa cells (data not shown). However, M90TΔipaH$_{7.8}$ had delayed cytotoxicity in RawB macro-

phages (Fig. 4A), which was restored to wild-type levels by pipa$H_{7.8}$, thus confirming that the delayed cytotoxicity phenotype was dependent on *ipaH*$_{7.8}$. Furthermore, in Fig. 4B we show, using the same chloroquine assay described above, that strain M90TΔipa$H_{7.8}$ escapes from the phagosome. Moreover, M90TΔipa$H_{7.8}$ also recruits polar actin tails in HeLa cells (Fig. 4C). Microscopic analysis of actin tail formation confirmed that a lack of *ipaH*$_{7.8}$ does not affect infectivity. Comparable numbers of intracellular bacteria were detected inside HeLa cells infected with M90T (24.6 bacteria/cell), M90TΔipa$H_{7.8}$ (26.9 bacteria/cell), and M90TΔipa$H_{7.8}$/pipa$H_{7.8}$ (24.6 bacteria/cell). The numbers of actin tails formed per internalized bacterium were similar for the three strains under comparison, with the average percentages for M90T, M90TΔipa$H_{7.8}$, and M90TΔipa$H_{7.8}$/pipa$H_{7.8}$ being 10.1%, 7.1%, and 5.5%, respectively. These data show that *ipaH*$_{7.8}$ does not play a role in *S. flexneri* escape from the phagosome. The discrepancy between our results and those published previously (3) might be due to differences in the deleted strains or in the interpretation of the data. We feel confident that actin recruitment is a reliable reporter for intracytoplasmic location. This issue could be addressed by directly comparing both strains in the same experimental setup. Thus, the gene or genes for the required phagosome escape factor carried outside the SER in the virulence plasmid remain to be identified.

Our findings demonstrate that SPI-1 from *S. enterica* serovar Typhimurium can functionally complement a deletion of the SER in *S. flexneri*. Surprisingly, SPI-1 allowed *S. flexneri* to escape the phagosomes of host cells, even though this function is unique to *Shigella*. The results presented here indicate that phagosome escape, although it requires invasion, also needs another gene or genes present in the large virulence plasmid. Although we cannot rule out the involvement of the SER in phagosome escape, the data suggest for the first time that the processes of invasion and phagosome escape can be uncoupled in *S. flexneri*.

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