Shigella flexneri Phagosomal Escape Is Independent of Invasion

Susanne Paetzold,† Sebastian Lourido,†‡ Bärbel Raupach, and Arturo Zychlinsky*

Department of Cellular Microbiology, Max-Planck-Institut für Infektionsbiologie, Charité Platz 1, D-10117 Berlin, Germany

Received 28 March 2007/Returned for modification 17 May 2007/Accepted 17 July 2007

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, phagosomal 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 phagosomal escape can be uncoupled in S. flexneri.

* Corresponding author. Mailing address: Department of Cellular Microbiology, Max-Planck-Institut für Infektionsbiologie, Charité Platz 1, 10117 Berlin, Germany. Phone: 49 30 28460300. Fax: 49 30 28460301. E-mail: zychlinsky@mpiib-berlin.mpg.de.
† S.P. and S.L. contributed equally to this study.
‡ Present address: Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63130.
§ Published ahead of print on 30 July 2007.
TABLE 1. Bacterial strains and characteristics

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL1344</td>
<td>Wild-type S. enterica serovar</td>
<td>7</td>
</tr>
<tr>
<td>SL1344ΔSPI-1</td>
<td>Typhimurium</td>
<td></td>
</tr>
<tr>
<td>SL1344ΔSPI-1/pSPI-1</td>
<td>Deletion of SPI-1 in SL1344 transformed with pSPI-1</td>
<td>This study</td>
</tr>
<tr>
<td>M90T</td>
<td>Wild-type S. flexneri</td>
<td>12</td>
</tr>
<tr>
<td>M90TΔSER</td>
<td>Deletion of the SER in M90T transformed with pSPI-1</td>
<td>This study</td>
</tr>
<tr>
<td>M90TΔSER/pSPI-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M90TΔlipH7.8</td>
<td>Deletion of pipaH7.8 in M90T transformed with pipaH7.8</td>
<td>This study</td>
</tr>
<tr>
<td>BS176</td>
<td>M90T cured of the large virulence plasmid</td>
<td>12</td>
</tr>
<tr>
<td>BS176/pSPI-1</td>
<td>BS176 transformed with pSPI-1</td>
<td>This study</td>
</tr>
</tbody>
</table>

Shigella serovar Typhimurium strain SL1344 and the SER (delimited by spa40 and spa1042) or pipaH7.8 from wild-type S. flexneri strain M90T to produce strains SL1344ΔSPI-1, M90TΔSER, and M90TΔlipH7.8, 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 strain SGSC_10F09 (Salmone-ella Genetic Stock Center). Two consecutive deletions produced a construct containing SPI-1 from mutS to aceA in a pBeloBAC11 vector. The gene ipaH12 was cloned under the control of its own promoter into the low-copy-number vector pWK29 (16) to create the plasmid pSPI-1. Actin was visualized with Alexa 488-phalloidin. Using confocal microscopy, the ratio 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 antisera 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-
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 with and 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.
the presence and absence of chloroquine. The noninvasive strains M90T/H9004 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 M90T/H9004/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 M90T/H9004/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 M90T/H9004/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 M90T/H9004 microorganisms detected inside a total of 71 infected HeLa cells analyzed. M90T/H9004/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 M90T/H9004/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 IpaH7.8. A report by Fernandez-Prada et al. (3) identified IpaH7.8 as the gene that facilitates S. flexneri escape from the phagosome. Since IpaH7.8 is carried in the large virulence plasmid outside the SER, we tested the effect of IpaH7.8 on phagosomal escape. 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 M90T/H9004/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 IpaH7.8. A report by Fernandez-Prada et al. (3) identified IpaH7.8 as the gene that facilitates S. flexneri escape from the phagosome. Since IpaH7.8 is carried in the large virulence plasmid outside the SER, we tested the effect of IpaH7.8 on phagosomal escape. 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 M90T/H9004/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.
phages (Fig. 4A), which was restored to wild-type levels by pipaH<sub>7.8</sub>, thus confirming that the delayed cytotoxicity phenotype was dependent on <i>ipaH</i><sub>7.8</sub>. Furthermore, in Fig. 4B we show, using the same chloroquine assay described above, that strain M90TΔipaH<sub>7.8</sub> escapes from the phagosome. Moreover, M90TΔipaH<sub>7.8</sub> also recruits polar actin tails in HeLa cells (Fig. 4C). Microscopic analysis of actin tail formation confirmed that a lack of <i>ipaH</i><sub>7.8</sub> does not affect infectivity. Comparable numbers of intracellular bacteria were detected inside HeLa cells infected with M90T (24.6 bacteria/cell), M90TΔipaH<sub>7.8</sub> (26.9 bacteria/cell), and M90TΔipaH<sub>7.8</sub>/pipaH<sub>7.8</sub> (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, M90TΔipaH<sub>7.8</sub>, and M90TΔipaH<sub>7.8</sub>/pipaH<sub>7.8</sub> being 10.1%, 7.1%, and 5.5%, respectively. These data show that <i>ipaH</i><sub>7.8</sub> does not play a role in <i>S. flexneri</i> 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 phagosomal escape factor carried outside the SER in the virulence plasmid remain to be identified.

Our findings demonstrate that SPI-1 from <i>S. enterica</i> serovar Typhimurium can functionally complement a deletion of the SER in <i>S. flexneri</i>. Surprisingly, SPI-1 allowed <i>S. flexneri</i> to escape the phagosomes of host cells, even though this function is unique to <i>Shigella</i>. 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 <i>S. flexneri</i>.

ACKNOWLEDGMENTS

We thank Michael Kolbe and Vivian Wolter for production of the SipC antibody and Ulrike Abuabed and Soo-Kyung Peuschel for excellent technical assistance. We also acknowledge the support of Volker Brinkmann.

<ref>REFERENCES</ref>