Salmonella Pathogenicity Island 2-Encoded Proteins SseC and SseD Are Essential for Virulence and Are Substrates of the Type III Secretion System

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Survival of Salmonella enterica serovar Typhimurium within host phagocytic cells is a critical step in establishing systemic infection in mice. Genes within Salmonella pathogenicity island 2 (SPI-2) encode a type III secretion system that is required for establishment of systemic infection. Several proteins encoded by SPI-2 have homology to type III secreted proteins from enteropathogenic Escherichia coli and Yersinia and, based on that homology, are predicted to be secreted through the SPI-2 type III secretion system. We have investigated the roles of two of these proteins, SseC and SseD. We demonstrate here that the SseD protein is required for systemic Salmonella infection of the mouse, and we confirmed the virulence requirement for the SseC protein. Experiments were performed, using cellular fractionation and immunoblotting, to identify the subcellular location of the SseC and SseD proteins. Both proteins were found to localize predominantly to the bacterial cell membrane. In addition, our work revealed that SseC and SseD are exposed to the extracellular environment and are loosely associated with the bacterial membrane. Furthermore, localization of SseC and SseD to the bacterial membrane was found to require a functional SPI-2 type III secretion system. Collectively, these results indicate that the SseC and SseD proteins are secreted by the SPI-2 type III secretion system to the bacterial membrane in order to perform their virulence functions.

Salmonella enterica serovar Typhimurium is a causative agent of gastroenteritis in humans (20). This pathogen also causes systemic infection in mice, which serves as an experimental model for the study of human typhoid fever. Many of the steps leading to establishment of systemic Salmonella infection have been identified. Upon colonization of the small intestine by Salmonella, the bacteria invade M cells of Peyer’s patches, penetrate into the environment of the lymphoid follicle, and are subsequently taken up by macrophages (12). The ability of Salmonella to survive within this host phagocytic cell is believed to be a critical element in establishment of systemic infection (2). Following entry into the lymphatic system, Salmonella disseminates to the liver and spleen, where unchecked growth causes death of the host (12).

Many genes required for the various stages of Salmonella pathogenesis have been identified. A number of these virulence genes are found clustered in the chromosome in regions called pathogenicity islands. Two of these pathogenicity islands encode type III secretion systems (TTSS), and their function in Salmonella virulence is being extensively studied. Salmonella pathogenicity island 1 (SPI-1) contains genes required for bacterial invasion of M cells and epithelial cells (see reference 4 for a review). A second island, Salmonella pathogenicity island 2 (SPI-2), is essential for systemic virulence and is believed to enable the bacteria to survive within macrophages.

Genes carried within SPI-2 were originally identified in a signature-tagged transposon mutagenesis screen designed to locate genes required for survival of the pathogen within mice (8). Analysis of several mutations within SPI-2 has indicated that the genes on the pathogenicity island are required for bacterial survival within the macrophage. Strains carrying mutations in SPI-2 genes are attenuated for virulence when inoculated by the oral, intraperitoneal, or intravenous route, indicating that the genes are required for stages of infection after invasion of the intestinal mucosa (3, 7, 9, 10, 18, 22). SPI-2 mutants have a reduced rate of replication in the murine liver and spleen (21) and replication defects in primary murine macrophages and in macrophage cell lines have also been reported (3, 10, 18). Experiments looking at the expression of SPI-2 genes indicate that they are induced several hours after uptake by macrophages (3) and are induced in vitro by starvation conditions that presumably mimic the environment of the phagosomal compartment of a macrophage (1, 5).

Based on homology to type III secretion proteins in other organisms, genes within SPI-2 are predicted to encode secreted, structural, regulatory, or chaperone proteins. However, the function of only a handful of genes in SPI-2 has been established by experimental approaches. Two proteins, SpiC and SseB, are secreted outside of the bacterium by the TTSS (1, 24). SpiC is translocated across the phagosomal membrane and into the cytosol of Salmonella-infected J774 cells. Within the host cell, SpiC appears to function by interfering with membrane trafficking. The SseB protein is secreted to the bacterial cell surface, where it is loosely associated with the membrane. It was suggested that SseB may be part of a structure through which proteins are translocated, much like its homolog in enteropathogenic Escherichia coli (EPEC), EspA, which is a component of a filamentous secretion organelle. In addition, two proteins encoded by genes located outside of SPI-2, SspH1 and SspH2, are secreted through this TTSS (16).

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These two proteins contribute to the pathogenesis of salmonellosis in calves.

SseC and SseD are two additional SPI-2 proteins that are predicted to be secreted by *Salmonella* based on homology to secreted proteins in *EPEC* and *Yersinia*. SseC is 24% identical and 48% similar to EspD of *EPEC* (10). The sseC::aphT mutation was previously described (13). The sseC::aphT fragment was removed from pGEM-T Easy by digestion with *Eag*I and cloned into the *Eag*I site of pBDJ200. This plasmid was introduced into *S. enterica* SL1344 carrying pBDJ200. Selection for bacteria containing double crossovers of the mutation into the chromosome was done as previously described (13), and the presence of the mutation in the *Salmonella* chromosome was confirmed by PCR. The strain with the defined mutation in *sseC* was designated JK21. This mutation does not affect the expression of *sseD*, the gene immediately downstream of *sseC*, as determined by Western blot analysis of the strain for production of SseD (data not shown).

The sseD mutant was constructed by PCR amplifying *sseD* from the *S. enterica* chromosome using primers *sseD1* (5′-GAAAGCGCAATGATGTGCCG) and *sseD2* (5′-CTGCCATGAGGCGTTAACCC). The 2-kb product was cloned into pGEM-T Easy (resulting in plasmid pJK51), and a filled-in *BamHI* fragment containing the *aphT* cassette was cloned into the 42-bp deletion within *sseD* created by digestion with *EcoRV*. The sseD::aphT fragment was cloned into pBDJ129 as above, followed by selection for allelic exchange into the *S. enterica* chromosome. The strain with the defined mutation in *sseD* was named JK22.

The sseV::cam mutation of strain HH110 and the sseT::mTn5 mutation of strain P9B7 were moved into *S. enterica* serovar Typhimurium SL1344 by P22-mediated transduction and designated JK20 and JK14, respectively.

Construction of plasmids. Plasmids expressing SseC and SseD were used to complement JK21 and JK22. Plasmids expressing SseC and SseD from the *lac* promoter in pGEM-T Easy were unstable in *Salmonella* (data not shown); therefore, the *sseC* and *sseD* genes were cloned into the low-copy vector pACYC184 and expressed from the uninducible promoter of the tetracycline resistance gene. The *sseC* sequence was removed from pJK53 by digestion with *SalI* and *SphI* and cloned into the same sites within the Tet* gene of pACYC184. The *sseD* sequence was cloned from pJK51 into pACYC184 using the same procedure. Expression of SseC and SseD from these plasmids was confirmed by Western blot (data not shown).

Mouse virulence. BALB/c mice, 6 to 8 weeks old (Harlan Sprague), were used for all animal infection experiments. The mice were housed and maintained by the University of Iowa Department of Laboratory Animal Medicine. Bacteria were prepared for the experiment by being grown in LB overnight at 37°C and then diluted 1:100 into fresh medium and grown to an optical density at 600 nm (OD<sub>600</sub>) of 0.4 to 0.5. Cultures were diluted in phosphate-buffered saline and injected intraperitoneally into mice in a volume of 100 µl. Appropriate dilutions

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
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</tr>
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<td>ColE1 ori, lacF’, Amp’</td>
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<td>pJK51</td>
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<td>This work</td>
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<td>sseC in pGEM-T Easy, Amp’</td>
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<td>pJK76</td>
<td>sseC in pACYC184, Cm’</td>
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<tr>
<td>pJK77</td>
<td>sseD in pACYC184, Cm’</td>
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<sup>a</sup> Amp’, ampicillin resistant; Kan’, kanamycin resistant; Cm’, chloramphenicol resistant; Str’, streptomycin resistant.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this work are listed in Table 1. Liquid cultures of bacteria were grown in Lennox broth (LB) (Difco) or minimal medium with limiting magnesium (pH 5.0) (MgM) (1) as indicated. Antibiotics were added at the following concentrations: ampicillin, 100 µg ml<sup>−1</sup>; kanamycin, 25 µg ml<sup>−1</sup>; chloramphenicol, 20 µg ml<sup>−1</sup>. **Construction of mutants.** The *sseC* mutant was constructed by PCR amplification of *sseC* from the *S. enterica* chromosome using primers *sseC1* (5′-GTGCT ACGTACTGCTGCCGT) and *sseC2* (5′-CAGTATGATGCGCCGAAAAC G). The 2-kb PCR product was cloned into the vector pGEM-T Easy (Promega, Madison, Wis.), resulting in plasmid pJK53. A kanamycin resistance gene (*aphT*) lacking a transcriptional terminator was excised from *pUC*KD (13) with *BamHI*, filled in, and cloned into the Nhel site within *sseC*. The *aphT* gene cassette was inserted into *sseC* so that transcription of *sseC* and *aphT* was oriented in the same direction. Such gene insertions have been found by our laboratory to have minimal transcriptional effects on downstream genes (13). The *sseC::aphT* fragment was removed from pGEM-T Easy by digestion with *Eag*I and cloned into the *Eag*I site of pBDJ129. This plasmid was introduced into *S. enterica* SL1344 carrying pBDJ200. Selection for bacteria containing double crossovers of the mutation into the chromosome was done as previously described (13), and the presence of the mutation in the *Salmonella* chromosome was confirmed by PCR. The strain with the defined mutation in *sseC* was designated JK21. This mutation does not affect the expression of *sseD*, the gene immediately downstream of *sseC*, as determined by Western blot analysis of the strain for production of SseD (data not shown).

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of the cultures were plated on LB plates to quantitate the inoculum dose. Survival of infected mice was monitored for 21 days.

**Purification of protein and generation of antibodies.** Recombinant SseC and SseD proteins were generated by constructing protein fusions to the maltose binding protein (MBP) using the pMAL-c2 vector (New England Biolabs, Beverly, Mass.). To clone into the fusion vector, sseC and sseD were PCR amplified with primers sseC3 (5′-GATCATATATTTAATGTTGGGTGATCGCCAC-3′) plus sseC4 (5′-GACTTGTCTAGATTAAGCGCGATAGCCAG-3′) and sseD3 (5′-GATCATATATTTAATGTTGGGTGATCGCCAC-3′) plus sseD4 (5′-GATCGTAAAGCTCTACTTATATGATAGCC-3′) and cloned into pGEM-T Easy (Promega, Madison, Wis.). Purified SseC or SseD protein was run on all gels as an internal size standard. Protein ladder (Gibco-BRL), SseC ran at 62 kDa and SseD ran at 19 kDa.

Proteins were transferred to nitrocellulose software. The relative intensity of specific bands was quantitated with FujiFilm

**Western blotting.** Proteins were separated by sodium dodecyl sulfate-PAGE (12.5% polyacrylamide). The equivalent of 10 μl of an OD₆₀₀ = 0.4 culture of each fraction was loaded into a well. Proteins were transferred to nitrocellulose by electroblotting and incubated overnight with a 1:10,000 dilution of SseC or SseD primary antibody. Alkaline phosphatase-conjugated rabbit anti-sheep immunoglobulin G (Jackson ImmunoResearch, West Grove, Pa.) was used as a secondary antibody. Detection was carried out using the Immun-Star chemiluminescent protein detection system (Bio-Rad) followed by imaging with Kodak BioMax film or the FujiFilm LAS-1000 luminescent imager and accompanying software. The relative intensity of specific bands was quantitated with FujiFilm Imagent Gauge 3.12 software. As estimated by running the Benchmark preinstalled protein ladder (Gibco-BRL), SseC ran at 62 kDa and SseD ran at 19 kDa. Purified SseC or SseD protein was run on all gels as an internal size standard.

### RESULTS

**SseD is required for virulence.** The virulence requirement of several SPI-2 genes predicted to encode secreted effector proteins has been determined (10); however, the virulence requirement of the gene encoding SseD is unknown. To determine the role of SseD in virulence, we constructed a chromosomal mutation in sseD (called JK22) that has minimal polar effects on downstream genes and measured its virulence in mice. We also constructed a chromosomal mutation in sseC (called JK21) and tested its virulence in mice to confirm its phenotype. We infected groups of five mice intraperitoneally with at least 10⁷ CFU of JK21, JK21 complemented with plasmid pJK76, JK22, or JK22 complemented with plasmid pJK77 and monitored survival of the mice for 21 days. Five mice were infected with 100 CFU (about four times the 50% lethal dose [LD₅₀]) (19) of the wild-type strain SL1344 as a control for the virulence of the strains. The control mice infected with SL1344 died within 5 days. The mice infected with JK21 carrying pJK76 died within 3 days, and those infected with JK22 carrying pJK77 all died within 5 days (Table 2).

**Table 2. Mouse virulence of sseC and sseD mutants**

<table>
<thead>
<tr>
<th>Strain (genotype)</th>
<th>Plasmid (genotype)</th>
<th>Inoculum (CFU)</th>
<th>Survival (no. surviving/total no.)</th>
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<tr>
<td>SL1344 (wild type)</td>
<td></td>
<td>1.0 x 10⁷</td>
<td>0/5</td>
</tr>
<tr>
<td>JK21 (sseC::aphT)</td>
<td></td>
<td>2.0 x 10⁵</td>
<td>5/5</td>
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<tr>
<td>JK21 (sseC::aphT)</td>
<td>pJK76 (sseC⁺)</td>
<td>1.1 x 10⁵</td>
<td>0/5</td>
</tr>
<tr>
<td>JK22 (sseD::aphT)</td>
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<td>2.5 x 10⁵</td>
<td>5/5</td>
</tr>
<tr>
<td>JK22 (sseD::aphT)</td>
<td>pJK77 (sseD⁺)</td>
<td>1.3 x 10⁵</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* Mice were inoculated intraperitoneally with the indicated number of bacteria, and survival was determined after 21 days.

**SseD is induced by growth in minimal medium.** The growth conditions for induction of SPI-2 gene expression and protein localization have been previously established. In vivo, SPI-2 genes are induced by growth inside macrophages for several hours (3). In vitro, these genes are induced by growth in minimal medium with limiting divalent cations (1, 5). Furthermore, localization of SseB to the cell surface occurs as a result of growth under acidic conditions (1). We performed experiments to determine whether we could induce protein expression by growth under the same conditions. A culture of the wild-type Salmonella strain SL1344 was grown overnight in LB, washed in phosphate-buffered saline, and induced for 6 h in MgM. Samples were collected after overnight growth in LB and after 6 h of induction in MgM. Western blotting of these samples with SseD antiserum indicated that SseD was expressed and that the LD₅₀ of the sseD mutant was increased 7,800-fold to >2 x 10⁶ and that the LD₅₀ of the sseD mutant was increased more than 10,000-fold to >2.5 x 10⁶ CFU. In addition, the experiment indicated that each mutant became significantly more virulent after the introduction of a plasmid carrying the appropriate single intact gene, suggesting that the chromosomal mutations were nonpolar.

**SseC and SseD are localized to the bacterial membrane.** We next sought to determine where SseC and SseD are localized in the bacterium and whether they are secreted into the culture medium under in vitro growth conditions. The wild-type strain SL 1344 was grown in MgM (pH 5.0), and proteins from the culture supernatant, bacterial membranes, and cytoplasm/
periplasm were collected as described in Materials and Methods. Coomassie blue staining of these fractions, following sodium dodecyl sulfate-PAGE, revealed the presence of many proteins in each fraction except for the supernatant fraction (data not shown). Western blotting of these fractions with SseC (Fig. 2a) or SseD (Fig. 2b) antiserum indicated that neither protein was secreted into the culture supernatant under these growth conditions (lane 1). The majority of SseC and SseD was found in the membrane fraction (lane 3), although some protein (more for SseC than SseD) could be detected in the soluble fraction (lane 5). As a control for specificity of the antiserum, immunoblots of protein fractions isolated from SseC (JK21) and sseD (JK22) mutants did not detect protein at the size of SseC or SseD (lanes 2, 4, and 6). As a control for the purity of the fractions, the activity of the membrane protein NADH dehydrogenase was measured. We found that 100% of its activity was in the membrane fraction, demonstrating that no detectable levels of membrane proteins are contaminating the soluble fraction.

SseC and SseD are not localized properly in secretion apparatus mutants. Next, we examined whether the localization of SseC and SseD is affected by mutations in other SPI-2 genes. SsaT and SsaV are predicted to form part of the type III secretion apparatus. A transposon insertion in ssaT results in the loss of mouse virulence (9), indicating that ssaT and/or the downstream gene, ssaU, is important in SPI-2 function. A nonpolar mutation in ssaV also reduces Salmonella virulence, indicating that ssaV is also important in SPI-2 function (21). We analyzed membrane and soluble protein fractions collected from wild-type (SL1344) cells and the sseD (JK22), ssaT (JK14), and ssaV (JK21) mutant strains for the presence of SseC by immunoblotting with SseC antiserum and determining the relative concentrations of each band as described in Materials and Methods (Fig. 3a). We consistently observed that the SseC protein from soluble fractions migrated more quickly in PAGE than did the SseC protein from membrane fractions. The reason for this finding is unclear, but one possible explanation is that when SseC is removed from its usual membrane association, its mobility is altered. Significant amounts of SseC were found in the membrane fraction of the wild type (113.4 relative units) and the sseD mutant (95.4 relative units) (lanes 1 and 3). The supernatant fraction of the parent strain had 0.0 relative unit of SseC, but the sseD mutant had 18.0 relative units of SseC in the soluble fraction. Interestingly, the majority of the SseC protein was found in the soluble fraction of the ssaT and ssaV mutants (87.0 and 78.5 relative units, respectively) (lanes 6 and 8). No SseC protein above background could be detected in the membrane of the ssaT mutant (0.0 relative unit), and we detected 8.6 relative units in the membrane of the ssaV mutant.

A similar set of experiments was performed to examine SseD localization using SseD antiserum (Fig. 3b). SseD was found in the membrane fraction of wild-type (107.4 relative units) and sseC mutant (69.1 relative units) cells (lanes 1 and 3), while little protein above background was detected in the membrane fractions of the apparatus mutants ssaT and ssaV (7.0 and 9.0 relative units, respectively) (lanes 5 and 7). There was not a comparable increase in the level of SseD protein above background in the soluble fraction, which may be due to degradation of SseD when it is not properly localized. These data indicate that a functional TTSS is required for proper localization of SseC and SseD to the bacterial membrane.

SseC and SseD are secreted to the bacterial cell surface. Our cell fractionation studies indicate that SseC and SseD are localized to the bacterial membrane. As one approach to determine whether SseC and SseD are exposed to the extracellular medium, we treated whole bacteria with protease K as described in Materials and Methods. We found that SseC and SseD were degraded by protease K treatment, but to different extents. SseC was completely degraded by protease K...
treatment, while a small percentage of SseD was still detectable in the membrane after proteinase K treatment (Fig. 4a). Other workers have reported that SseB can be found on the bacterial cell surface under certain growth conditions, in a location where it can be stripped from the cell by treatment with xylene (1). This finding was interpreted to mean that the protein is loosely associated with the bacterial membrane. We used this same experimental technique to determine whether SseC and SseD have a similar association with the membrane. Our experiments revealed that about 50% of the SseC protein in the membrane could be extracted from the cell by xylene (Fig. 4b). SseD could also be extracted from the membrane by xylene, but to a lesser extent (Fig. 4b). These experimental results indicate that SseC and SseD are exposed to the extracellular environment, where they are loosely associated with the bacterial membrane.

Experiments were performed to determine whether SseC or SseD was secreted in vivo after infection of J774 cells. We were unable to detect SseD in J774 cells after 6 h of infection in Western blot experiments (data not shown). Likewise, secretion of SseC into J774 cells was not detected by immunofluorescence microscopy (data not shown).

**DISCUSSION**

In the work presented here, we demonstrate that sseD is required for the mouse virulence phenotype of *Salmonella*. An infectious dose of 7,800 or 10,000 times the LD$_{50}$ of the wild-type strain was not sufficient to cause lethal infection by sseC and sseD mutants, respectively. This adds to the list of SPI-2 genes that are known to be essential for virulence. The virulence requirement of several predicted effector proteins has been determined in other studies. Hensel et al. demonstrated that mice infected with sseC, sseB, or sseA mutants survived a dose of up to $10^6$ CFU, while mutants with mutations in sseF and sseG were less attenuated but still showed a defect in virulence (10). In addition, the LD$_{50}$ of a spIC (ssaB) mutant was reported to be greater than $3.6 \times 10^6$ CFU (24).

Because of their role in virulence and their homology to type III secreted effector proteins in other bacteria, we have examined aspects of SseC and SseD localization in greater detail. We have found that SseC and SseD are induced by growth in minimal medium with limiting magnesium, as has been reported for other SPI-2 proteins. However, even after growth in MgM, both proteins are still expressed at low levels. A large amount of cell culture (10 ml) was required to detect SseC and SseD expression by Western blotting. This requirement may

![FIG. 3. Localization of SseC and SseD in SPI-2 mutants. (a) Cell membrane (m) and soluble (s) protein fractions from SL1344 (wt), JK22 (sseD), JK14 (ssaT), or JK20 (ssaV) were probed with SseC antiserum. (b) Fractions from SL1344 (wt), JK21 (sseC), JK14 (ssaT), or JK20 (ssaV) were probed with SseD antiserum. Relative units of SseC protein in panel a, as measured by Fujifilm ImageGauge, were as follows: lane 1, 113.4; lane 2, 0.0; lane 3, 95.4; lane 4, 18.0; lane 5, 0.0; lane 6, 87.0; lane 7, 8.6; lane 8, 78.5. Relative units of SseD protein in panel b, as measured by Fujifilm ImageGauge, were as follows: lane 1, 107.4; lane 2, 1.0; lane 3, 69.1; lane 4, 1.0; lane 5, 7.0; lane 6, 1.0; lane 7, 9.0; lane 8, 1.0.](http://iai.asm.org/)

![FIG. 4. SseC and SseD are exposed to the extracellular medium. (a) SL1344 cell cultures grown in MgM (pH 5) were either incubated with proteinase K or incubated under identical conditions without the addition of proteinase K. Membrane fractions from untreated cells (lane 1) and proteinase K-treated cells (lane 2) were probed with SseC and SseD antisera. (b) SL1344 cell cultures grown in MgM (pH 5) were treated with xylene. Proteins stripped away from the cell by xylene (lane 1) and residual membrane proteins isolated from the treated cell pellet (lane 2) were detected using SseC and SseD antisera.](http://iai.asm.org/)
indicate that the in vitro growth conditions being used are still not optimal for induction of these proteins or, alternatively, that the bacteria require only low levels of expression of these proteins for their function.

A focus of our work was to determine the cellular localization of SseC and SseD. We found that both proteins are located in the bacterial membrane after growth in MgM. SseC was associated rather loosely with the cell and could be completely digested with proteinase K and extracted by treatment with xylene. SseD was also susceptible to these treatments, but to a lesser extent. These observations indicate that SseC and SseD are secreted to the bacterial cell surface during growth in MgM and are similar to findings made about the localization of SseB (11). In addition, localization of SseC and SseD to the bacterial membrane required a functional TTSS.

We think it is important to emphasize that SseC and SseD are type III secreted proteins; they are secreted to the bacterial membrane, and this secretion relies on a functional type III secretion apparatus. This differs slightly from what is often observed with proteins secreted from other TTSSs, because secretion into the growth medium can often be detected. For example, the TTSSs of several organisms, including Yersinia, EPEC, Shigella, and Salmonella (SPI-1), secrete proteins into the culture supernatant (11). Secretion of proteins into the culture supernatant by the SPI-2 TTSS has not been reported, perhaps suggesting that the in vitro growth conditions that induce expression are still not optimized for protein secretion.

The SPI-2 genes differ from many of the other TTSS genes that have been studied in that they are induced and exert their effects within a macrophage. These properties seem to suggest that the SPI-2 TTSS of internalized Salmonella mediates secretion of proteins into the phagosomal compartment and/or translocation of proteins across the phagosomal membrane into the host cytosol. Some aspect of this intracellular environment is not being re-created by growth in liquid culture to signal protein secretion into the growth medium, and perhaps this signal is provided only by a macrophage after phagocytosis.

Proteins that are secreted by TTSSs generally function in one of two ways: as effector proteins that are translocated into the host cell and alter host cell processes or as translocators that are necessary for injecting effector proteins into the host cell. With the finding that SseC and SseD are secreted, these proteins become candidates for either effectors or translocators. Techniques used to detect translocation of effector proteins into host cells have included fractionation and immunoblotting of infected host cells, immunofluorescence microscopy, and measurement of intracellular enzyme activity of cytA fusions to secreted proteins (16, 24). We used the first two techniques mentioned above to search for the presence of SseC and SseD within cells, but those experiments were unsuccessful in detecting the proteins. Techniques with greater sensitivity may be able to detect SseC or SseD within host cells. Alternatively, it may be that the proteins are not translocated into host cells but instead act as translocators similar to their homolog, YopB, from Yersinia (6).

We now know of four proteins encoded by SPI-2 that are secreted by Salmonella: SseB, SseC, SseD, and SpIC. SpIC is an effector protein that is translocated into Salmonella-infected macrophages and interferes with normal membrane trafficking in the host cell, including phagosome-lysosome fusion (24). SseB expression is induced by growth in minimal media like several other SPI-2 proteins, and it is secreted to the surface of Salmonella after acid induction (1). In a separate study, it was found that SseB is necessary for Salmonella to prevent NADPH oxidase localization and oxyradical formation at the phagosomal membrane of murine macrophages (25). However, at this point it is unclear whether this effect is due to the direct activity of SseB within the macrophage or whether SseB is required for translocating other effector proteins into the macrophage. It has also been observed that our sseD mutant is defective in NADPH oxidase assembly (A. Gallois, personal communication). These new findings serve as further evidence that Salmonella survives within macrophages due, in part, to its ability to interfere with the generation of reactive oxygen intermediates. Further experiments to determine whether SseD is directly responsible for the defect in NADPH oxidase assembly or whether a more general defect in type III protein translocation is the cause will be performed in the future.

In this study, we have demonstrated that SseC and SseD are required for systemic infection of mice by Salmonella and that both proteins are secreted to the bacterial surface in a type III-dependent fashion. Continued work to investigate these other SPI-2 secreted proteins will be critical in elucidating the mechanism by which SPI-2 mediates Salmonella survival within macrophages.

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