SpiC Is Required for Translocation of *Salmonella* Pathogenicity Island 2 Effectors and Secretion of Translocon Proteins SseB and SseC

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The *Salmonella* pathogenicity island 2 (SPI2) type III secretion system (TTSS) promotes *Salmonella enterica* serovar Typhimurium virulence for mice and increased survival and replication within eukaryotic cells. After phagocytosis, *Salmonella* serovar Typhimurium assembles the SPI2 TTSS to translocate over a dozen effector proteins across the phagosome membrane. SpiC has been previously shown to be a translocated effector with a large contribution to virulence (K. Uchiya, M. A. Barbieri, K. Funato, A. H. Shah, P. D. Stahl, and E. A. Groisman, EMBO J. 18:3924-3933, 1999). This report demonstrates by competitive index that the virulence phenotype of a *spiC* mutant is equivalent to that of a secretion component mutant. In addition, translocation of SPI2 effector proteins was shown to require SpiC. Thus, the severe virulence phenotype resulting from deletion of *spiC* is likely due to the inability to translocate all SPI2 effectors. SpiC was also required to secrete translocon proteins SseB and SseC but not translocated effector SseJ, indicating that lack of assembly of the translocon explains the *spiC* mutant phenotype.

Type III secretion systems (TTSS) promote the virulence of gram-negative bacteria for eukaryotic hosts by delivering bacterial proteins directly from the bacterial cytosol into eukaryotic cells (17). These secretion systems are composed of cytoplasmic, membrane-spanning, and secreted proteins. The cytoplasmic proteins consist of those that have homology to the flagellar energy apparatus, which powers the secretion of all substrate proteins, and chaperone proteins. Chaperones are typically small acidic proteins that are required for the secretion of specific subsets of TTSS substrate proteins. The TTSS membrane-spanning proteins have homology to the components of the flagellar basal body and collectively form the needle complex, which spans both the bacterial inner and outer membranes and which is required for the secretion of all substrate proteins. The TTSS secreted proteins can be subdivided into two classes, commonly known as translocon and effector proteins. Effector proteins are translocated into the eukaryotic cytoplasm, where they typically alter a specific function or pathway of the cell's physiology. In contrast, translocon proteins are hypothesized to connect the needle complex and the eukaryotic cell and to insert into the eukaryotic cell membrane to allow the delivery of effector proteins. Additionally, translocon proteins may exert effects on the eukaryotic cell themselves, possibly by interacting with membrane-associated proteins (31).

Despite the basic conservation of the structure and function of different TTSS, they can have remarkably distinct effects on eukaryotic cells. The differences in function between TTSS are presumed to be a result of the total activities of the effector and, to some extent, translocon proteins (5, 14, 16, 31). Since the translocon proteins are necessary for effector delivery, TTSS apparatus and translocon mutants typically display identical and significant defects in their virulence phenotypes. In contrast, bacteria with mutated single translocated effectors can have relatively minor measurable virulence defects, often due to redundancy in effector function (11, 13, 18–20, 24, 42).

Salmonellae are gram-negative bacteria that cause gastroenteritis and typhoid fever, a systemic illness characterized by fever and bacterial replication in lymphoid tissue rich in phagocytic cells (25). *Salmonella enterica* serovar Typhimurium is a broad-host-range pathogen that can infect a variety of animal hosts with different disease outcomes. In immunocompetent humans, *Salmonella* serovar Typhimurium infection typically results in self-limited gastroenteritis, whereas, in specific inbred mouse strains, *Salmonella* serovar Typhimurium can cause a systemic disease similar to human typhoid fever. While typhoid fever is caused by the human-specific pathogen *S. enterica* serovar Typhi, the limitation of experimental models for this pathogen has led to the widespread study of *Salmonella* serovar Typhimurium in *Nramp* mutant mice as a model for systemic disease. The ability of *Salmonella* serovar Typhimurium to cause systemic disease in inbred mice is correlated with its ability to survive and replicate within host cells, particularly macrophages. One important factor in this intracellular survival and replication is the TTSS encoded on *Salmonella* pathogenicity island 2 (SPI2) (28, 34) (Fig. 1). Most TTSS, including the *Salmonella* SPI1 system, which promotes invasion and enteric disease, translocate bacterial proteins across the eukaryotic cell plasma membrane (17). The SPI2 TTSS is relatively unique in that its expression is largely limited to intracellular bacteria (6, 39), and salmonellae use the SPI2 TTSS to translocate proteins across the phagosomal membrane (24).

Several proteins have recently been demonstrated to be se-

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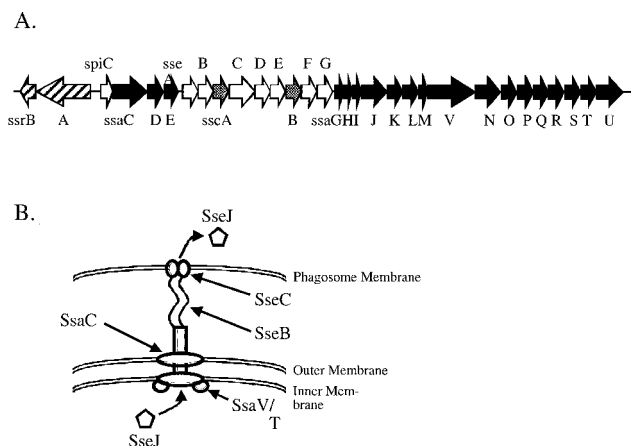
A.

B.

FIG. 1. Genetic organization of SPI2 and putative model of the structure of the SPI2 TTSS with associated translocon. (A) Genes encoding the secretion apparatus (*ssa*), putative secreted substrate proteins (*sse*), putative chaperone proteins (*ssc*), and regulatory proteins (*ssr*) (black, white, shaded, and hatched arrows, respectively). (B) Predicted function and localization of various proteins of the SPI2 TTSS. Assignment is based predominantly on homology to other characterized TTSS proteins. SseB, SseC, and SseD are secreted by the SPI2 TTSS and form a complex on the bacterial cell surface required for the translocation of known SPI2 effector proteins such as SseJ (26). Based on its homology to EspA, SseB may form a filament connecting the SPI2 TTSS to the phagosomal membrane, allowing the insertion of SseC in the phagosomal membrane. SsaC is predicted to form the outer membrane ring of the SPI2 TTSS based on its homology to InvG and other related proteins, while SsaT and SsaV are predicted to function in a more proximal aspect of the SPI2 TTSS.

creted by the SPI2 TTSS in vitro when *Salmonella* serovar Typhimurium is grown under SPI2-inducing conditions (1, 21, 26). These include SseB, SseC, and SseD, all of which are required for the translocation of known SPI2 effector proteins into infected mammalian cells. These proteins can be extracted from the bacterial cell surface and are believed to form a macromolecular structure that functions as the translocon (26). SseB has homology to EspA from enterohemorrhagic *Escherichia coli* and enteropathogenic *E. coli* (15). EspA forms a filament on the bacterial cell surface which connects the needle complex to the infected mammalian cell (7, 22, 32). Although SseB has not been shown to form such a filament, it has been visualized on the cell surface by immunofluorescence and is required for the surface association of SseC and SseD (26). SseC and SseD have homology to YopB and YopD, respectively, and thus probably function to insert into the phagosomal membrane to facilitate protein translocation (15).

In addition to the secreted translocon proteins, several other proteins have recently been shown to be translocated into infected mammalian cells. These include a family of TTSS effector proteins, such as SspH2 and SseJ, that share a conserved amino-terminal sequence that is required for translocation by SPI2 (3, 23, 24). All of the genes encoding these effector proteins are located independently outside of SPI2. One of these proteins, SifA, is required for the formation of lysosomal glycoprotein-containing filaments and for the maintenance of the phagosomal membrane (2, 37). SifA was shown to promote intracellular survival within macrophages and to be required for systemic infection in susceptible mice (2, 4). In

addition to the effectors encoded outside of SPI2, SpiC (SsaB), a protein encoded within SPI2, has been shown to be translocated into mammalian cells (38). SpiC interferes with endosomal trafficking upon expression in mammalian cells and in in vitro assays. Furthermore, *Salmonella* serovar Typhimurium *spiC* mutants also demonstrate increased trafficking to vacuolar compartments containing lysosomal markers within infected macrophages (38). Strikingly, *Salmonella* serovar Typhimurium *spiC* mutants demonstrate significant defects in both virulence for mice and intracellular survival. Such a significant virulence phenotype is unusual for an effector and is actually similar to that resulting from mutation of the SPI2 TTSS.

Therefore, experiments were performed to further define the virulence phenotype of *spiC* mutants and the role of SpiC in type III secretion.

MATERIALS AND METHODS

Bacterial strains, eukaryotic cell lines, and growth conditions. Bacterial strains and plasmids used are listed in Table 1. *Salmonella* serovar Typhimurium was grown to stationary phase in Luria broth (LB) with aeration for all infection and virulence assays. The composition of minimal media has been described previously (26). Murine monocyte cell line RAW264.7 was cultured in Dulbecco's modified Eagle medium (Gibco BRL) containing 10% fetal calf serum (FCS) (Gibco BRL) and 2 mM glutamine at 37°C in 5% CO_2.

Construction of deletion mutants. Deletion of *sifA* was accomplished by using an allelic-exchange plasmid. To construct this plasmid, DNA flanking both the 5′ and 3′ ends of *sifA* was amplified from *Salmonella* serovar Typhimurium chromosomal DNA by PCR using *Pfu* Turbo polymerase (Stratagene). The 5′ end was amplified with primers 5′-GGTTATCTCAATGAATTCCTGCTGTGG-3′ and 5′-GCGGGTACCGTCCGCTTTTGCTTTGCCAG-3′, and the 3′ end was amplified with primers 5′-GCGGGTACCCGCTCAGAACAACAAAGCGG C-3′ and 5′-GCGTCTAGACCAATAAAACGGTCGCCAGC-3′. The flanking ends were sequentially cloned into allelic-exchange vector pKAS32, yielding plasmid pJAF21. pJAF21 was then introduced into strain CS401 by conjugation and allelic exchange selecting for streptomycin resistance as previously described (29). The resulting deletion strain, JAF57, was verified by diagnostic PCR using *Taq* polymerase (Qiagen). The method described by Datsenko and Wanner (8) was used to delete *spiC*. Briefly, primers were designed to amplify a kanamycin resistance gene flanked by Flp recognition target sites from plasmid pKD4. The primers, designed to also be homologous to DNA flanking *spiC*, were 5′-TT GTGAGCGAATTTGATAGAAACTCCCATTTATGTCTGAGGTGTAGGC TGGAGCTGCTTC-3′ and 5′-AATTAAGATTAAACGTTTATTTACTACCATTTTATACCCCCATATG AATATCCTCCTTAG-3′. Purified PCR product was then transformed into *Salmonella* serovar Typhimurium harboring the λ Red recombinase on plasmid pKD46, which contains a temperature-sensitive origin of replication, by selecting for kanamycin resistance. Kanamycin-resistant strains were then cured of pKD46 by incubation at 37°C. The inserted kanamycin resistance gene was subsequently removed by transforming the strain with plasmid pCP20, which expresses the FLP recombinase, resulting in an in-frame deletion of *spiC*, producing strain JAF204. The kanamycin-sensitive, *spiC* deletion strain was finally cured of the pCP20 plasmid.

Construction of HA-tagged SseJ. Plasmid pCAS61 was constructed by annealing primers 5′-TCGACTATCCTTATGATGTTCCTGATTATGCATAAC-3′ and 5′-TCGAGTTATGCATAATCAGGAACATCATAAGGATAG-3′, which encode the hemagglutinin (HA) epitope tag sequence, and inserting the product into plasmid pWSK29 digested with *Sal*I and *Xho*I. The *sseJ* open reading frame and promoter region were amplified with primers 5′-CGCGAATTCGTCAGA TAATATGTACCAGGC-3′ and 5′-CGCGTCGACTTCAGTGGAATAATGA TGAGC-3′ by PCR using *Pfu* Turbo. Plasmid pCAS61 and the PCR product were digested with *Eco*RI and *Sal*I and ligated together, yielding plasmid pJAF111.

Generation of epitope-tagged SpiC. PCR was performed using the Expand High Fidelity system (Roche) in order to minimize the error rate of the amplification procedure. Approximately 100 ng of genomic DNA of wild-type *Salmonella* serovar Typhimurium was used as a template for amplification with oligonucleotides ProA-For-*Eco*RI (5′-GCTGAATTCATCTCGGATAGAACGG-3′) and SsaB-Rev-*Sma*I (5′-ATGCCCGGGTACCCCACCCGAATAAAG-3′). The product was digested with *Eco*RI and *Sma*I and ligated with *Eco*RI- and *Eco*RV-

TABLE 1. Strains and plasmids used in this study

Designation	Description	Reference or source
Plasmids		
pEM25	*sspH1-cyaA* in pMJH20, Ap^r	24
pEM30	*sspH2-cyaA* in pMJH20, Ap^r	24
pEM80	*sseI-cyaA* in pMJH20, Ap^r	23
pEM82	*sseJ-cyaA* in pMJH20, Ap^r	23
pKAS32	*rpsL* suicide vector for allelic exchange	35
pJAF21	Δ*sifA* in pKAS32	This study
pWSK29	Low-copy-number plasmid	40
pCAS61	pWSK29 with HA epitope tag	This study
pJAF111	*sseJ*-HA in pCAS61, Ap^r	This study
pWSK30	Low-copy-number plasmid	40
p2062	M45 epitope tag in pSK+	12
p2129	*sseJ*-M45 in pWSK29, Ap^r	12
p2127	*spiC*-M45 in pWSK30, Ap^r	This study
pEG9127	*spiC* in pBAC108L, Cm^r	38
pKD46	Plasmid expressing λ Red recombinase	8
pKD4	Plasmid with FRT-flanked^a kanamycin resistance gene	8
pCP20	Plasmid expressing the FLP recombinase	8
Salmonella serovar Typhimurium strains		
NCTC 12023	Wild type, identical to ATCC 14028s	ATCC
P3F4	NCTC 12023, *ssrA*::mTn5 Km^r	34
P7G2	NCTC 12023, *ssaC*::mTn5 Km^r	34
HH109	NCTC 12023, *ssaV*::aphT Km^r	9
EG10128	ATCC 14028s, *spiC*::aphT Km^r	38
CS050	ATCC 14028s, *phoN*::Tn10d-Kan	S. I. Miller laboratory
CS401	ATCC 14028s, *phoN2 zxx*::6251 Tn10d-Cm, Strep^r	24
CS600	ATCC 14028s, *phoN*::Tn10d-Kan, Strep^r	This study
EM232	CS401, *ssaT*::mTn5 Km^r	24
JAF204	CS401, Δ*spiC*	This study
JAF57	CS401, Δ*sifA*	This study

^{a} FRT, Flp recognition target.

digested plasmid p2062 to generate a gene fusion to the M45 coding sequence. The resulting construct was digested with *Eco*RI and *Xba*I, and the insert consisting of promoter *ssaB* and *ssaB*::M45 was subcloned in pWSK30 to generate plasmid p2127.

Bacterial infection of macrophages. *Salmonella* serovar Typhimurium strains were grown to stationary phase in LB containing the necessary antibiotics, diluted in Dulbecco's modified Eagle medium containing FCS and glutamine, and added to RAW264.7 cells seeded in 24-well tissue culture plates. The bacteria were centrifuged onto the cells at 500 × *g* for 5 min and incubated for 25 min at 37°C in 5% CO$_2$. After infection, the macrophages were washed three times with phosphate-buffered saline (PBS) and incubated for 1 h in medium containing FCS, glutamine, and 100 μg of gentamicin (Sigma)/ml. The cells were then incubated with medium containing FCS, glutamine, and 10 μg of gentamicin/ml for the remainder of the experiment.

Translocation assay. Determination of SspH1 and SspH2 translocation by the SPI2 type III secretion system was performed as previously described (24). Briefly, wild-type and mutant *Salmonella* serovar Typhimurium cells expressing either *sspH1-cyaA* or *sspH2-cyaA* from a constitutive *lac* promoter on a low-copy-number plasmid were grown to stationary phase in LB. RAW264.7 cells were plated at 5 × 10^5 cells per well in 24-well dishes and infected with bacteria for 1 h at a multiplicity of infection of 10. Seven hours postinfection, the RAW264.7 cells were lysed in 0.1 N HCl and boiled for 5 min. Cellular cyclic AMP (cAMP) was then quantitated with the Direct cAMP Correlate enzyme immunoassay kit (Assay Designs, Ann Arbor, Mich.), and total protein concentration was quantitated with the Bradford assay. Cellular cAMP was then normalized for total protein content. All experiments were performed at least twice in triplicate.

Immunofluorescence detection and microscopy. RAW264.7 cells, seeded on coverslips, were infected with *Salmonella* serovar Typhimurium at a multiplicity of infection of 2. Sixteen hours postinfection, the cells were fixed in 3% paraformaldehyde in PBS for 15 min and then washed three times with PBS. The coverslips were incubated with the appropriate antibodies diluted in a blocking solution consisting of 10% goat serum, 1% bovine serum albumin, and 0.1% saponin (Sigma) in PBS. After each incubation, the coverslips were washed three times with PBS. After staining, the coverslips were mounted on Fluoroprep

(bioMèrieux) and sealed with Entellan (Merck). Samples were analyzed using a fluorescence microscope (Zeiss Axiophot) and the Metavue imaging software (Universal Imaging Corporation). The M45 epitope was detected with a mouse monoclonal antibody at a 1:5 dilution (27), and bacteria were detected with Bacto rabbit *Salmonella* anti-O test sera (Difco) at a 1:1,000 dilution. Secondary detection was performed with goat anti-mouse Cy3 (Jackson) at 1:500 and goat anti-rabbit Cy2 (Jackson) at 1:2,000.

Determination of mouse CIs. Wild-type and mutant *Salmonella* serovar Typhimurium were grown to stationary phase in LB and used to infect BALB/c mice. Each mutant strain was marked with chloramphenicol resistance, while the wild type was marked with kanamycin resistance. Wild-type and mutant bacteria were diluted, and roughly equal numbers were mixed. An aliquot of the inoculum was then plated on selective media, and the numbers of wild-type and mutant bacteria were quantitated. A total of 10^5 bacteria in a volume of 0.2 ml were injected intraperitoneally into each mouse. In each experiment, four mice were infected with each mutant *Salmonella* serovar Typhimurium strain. Two days after infection, the mice were sacrificed and the infected spleens were removed and homogenized in sterile saline. Wild-type and mutant bacteria in each infected organ were then quantitated by serial dilution and plating on selective media. The competitive index (CI) was calculated by dividing the number of mutant bacteria isolated from infected animals by the number of wild-type bacteria recovered. This value was then corrected by the initial ratio of mutant to wild-type bacteria used to infect each animal. All experiments were performed at least twice (total of eight mice).

Preparation of surface-localized proteins. Surface-localized proteins were prepared as described previously (12). Basically, for preparation of the detached fraction, bacteria were precultured in LB for 8 h at 37°C. Bacteria were washed once in minimal medium, and equal amounts of bacteria, as adjusted by optical density at 600 nm (OD$_{600}$), were used to inoculate cultures in minimal media at neutral or acidic pH. Cultures (400 ml) in 1-liter glass flasks without baffles were incubated overnight with agitation of 200 rpm at 37°C. Bacteria were pelleted by centrifugation at 6,000 × *g* for 15 min and resuspended in 20 ml of PBS. This suspension was agitated at maximum speed in 50-ml centrifuge tubes (Falcon) on a vortex mixer (Vortex Genie 2; Scientific Industries) for 60 s. Bacterial cells

were pelleted by centrifugation at $10,000 \times g$ for 10 min, and the supernatant was passed through a 0.2-μm-pore-size filter to remove residual bacteria. Protein in the supernatant fraction was recovered by precipitation with trichloroacetic acid (10% [wt/vol], final concentration) on ice for 1 h and centrifugation for 1 h at $10,000 \times g$. The pellet was washed twice with 15 ml of acetone and recovered by centrifugation at $10,000 \times g$ for 30 min. The final pellet was air dried and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris-HCl [pH 6.8], 4% SDS, 2% β-mercaptoethanol, 12.5% glycerol, 0.01% bromophenol blue). The final concentration of protein in the detached fraction was adjusted by the cell density of the original culture (OD_{600} of a 100-ml culture $\times$ 100 = volume of the sample buffer in microliters).

Preparation of secreted proteins. Bacteria were pregrown for 12 h in LB at 37°C with shaking. Cells were washed once in sterile saline and inoculated into 40 ml of minimal medium at acidic pH at a 1/100 dilution. Cultures were grown at 37°C for 6 h. Bacteria were then removed by centrifugation at $125,000 \times g$ for 90 min. Proteins in the culture supernatant were then precipitated in 10% trichloroacetic acid on ice for 1 h. Precipitated proteins were collected by centrifugation at $75,000 \times g$ for 1 h. Protein pellets were washed twice in ice-cold acetone and allowed to dry. Samples were resuspended in SDS-PAGE sample buffer, of which 20 μl was analyzed by SDS-PAGE.

Subcellular fractionation. A culture of *Salmonella* serovar Typhimurium carrying p2127 was grown in low-phosphate minimal medium at pH 5.8 for 16 h at 37°C in a glass flask without baffles. The culture was centrifuged, washed in PBS, and resuspended in PBS. In the presence of a protease inhibitor cocktail (Sigma) the suspension was passed two times through a French press (82.7 MPa). To remove nonlysed bacteria, the solution was centrifuged at $400 \times g$ for 30 min at 4°C. After centrifugation at $100,000 \times g$ for 1 h at 4°C, the pellet (membrane fraction) was washed with double-distilled H_2O, and the supernatant (soluble fraction) was centrifuged again at $100,000 \times g$. The membrane pellet was resuspended in 10 mM Tris-HCl, pH 8.0, with a syringe needle (0.45 by 25 mm) and solubilized with 1% *N*-lauroylsarcosine (Sarkosyl) in 10 mM EDTA, pH 8.0, for 10 min on ice. The Sarkosyl-soluble and Sarkosyl-insoluble fractions were separated by centrifugation for 2 h at $100,000 \times g$. The pellet containing the Sarkosyl-insoluble outer membrane fraction was resuspended in 10 mM Tris-HCl, pH 8.0, and centrifuged again to remove the excess of Sarkosyl. The proteins of the soluble fraction and the Sarkosyl-soluble inner membrane fraction were precipitated with acetone.

Production of antisera. Polyclonal antiserum against OmpA of *S. enterica* serovar Typhimurium was raised in BALB/c mice. Outer membrane proteins were prepared by Sarkosyl extraction as described above. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. A protein of 33 kDa corresponding to the molecular weight of OmpA was recovered and confirmed by N-terminal amino acid sequencing. The nitrocellulose fragment with OmpA was dissolved in dimethyl sulfoxide and used for immunization according to standard procedures (13a).

Protein analysis. Cell-associated- and secreted-protein profiles of various *Salmonella* serovar Typhimurium strains were routinely analyzed by SDS-PAGE on Tricine gels (8 or 12%) as described by Schägger and von Jagow (30). For analysis by Western blotting, proteins were transferred onto nitrocellulose membranes (BA85; Schleicher and Schuell) by the discontinuous semidry blotting procedure. SseB and SseC were detected with polyclonal antibodies raised against recombinant proteins (1, 26). SseJ-HA was detected with a monoclonal anti-HA antibody (Covance), and SpiC-M45 was detected with a monoclonal anti-M45 antibody (27).

RESULTS

SpiC and SsaT mutants compete equally after intraperitoneal injection of BALB/c mice. Uchiya et al. demonstrated that a *spiC*::*aphT* allele resulted in dramatic attenuation of *Salmonella* serovar Typhimurium virulence for BALB/c mice, with an 50% lethal dose of $>3.6 \times 10^6$ (38). This level of attenuation is similar to that observed previously for SPI2 TTSS apparatus mutants (34). Uchiya et al. also demonstrated that the strain with *spiC*::*aphT* was equivalent to a SPI2 TTSS apparatus mutant in ability to survive and replicate within macrophages (38). These results suggest that *spiC* mutants could be altered in the delivery of SPI2 effectors to eukaryotic cells. To further establish that *spiC* mutants display virulence defects identical to

TABLE 2. Virulence of SPI2 mutant *Salmonella* serovar Typhimurium for mice

Genotype of:		Log$_{10}$ CI^a
Strain 1	Strain 2	
Wild type^b	Wild type^c	0.00 ± 0.09
ssaT	Wild type^c	-2.32 ± 0.18
$\Delta spiC$	Wild type^c	-2.31 ± 0.22
$\Delta spiC$	*ssaT*	-0.04 ± 0.04

^{a} Ratio of the number of strain 1 to strain 2 bacteria recovered from infected spleens removed 2 days after the intraperitoneal injection of 10^5 total bacteria into mice. The mean log$_{10}$ CIs from the infection of at least eight mice are reported with corresponding standard deviations.
^{b} Wild-type chloramphenicol-resistant strain CS401, parent strain for both the *ssaT* and $\Delta spiC$ strains.
^{c} Wild-type kanamycin-resistant strain CS600, isogenic to CS401.

those of SPI2 TTSS apparatus mutants within mouse tissues, a strain with an in-frame nonpolar deletion of *spiC* was constructed. The resulting strain, JAF204, and SPI2 TTSS mutant strain EM232 were then tested for the ability to compete with an isogenic wild-type strain upon intraperitoneal injection of BALB/c mice (33).

Table 2 reports the mean CIs resulting from the infection of at least eight mice for each strain. The CI represents the ratio of the number of mutant to wild-type bacteria recovered from infected spleens 48 h after the intraperitoneal inoculation of 10^5 bacteria. Like other SPI2 TTSS mutants, *ssaT* mutant strain EM232 was significantly outcompeted by the wild-type strain (2, 33). Based on homology to other TTSS and flagellar proteins, SsaT should be a component of the cytoplasmic energy apparatus that is required for type III secretion (17). *spiC* deletion strain JAF204 exhibited the same CI as the *ssaT* mutant, suggesting that the *spiC* mutant was attenuated to the same degree as the secretion mutant. To verify this result, the *spiC* deletion mutant and the *ssaT* mutant were assayed for competition against each other. Consistent with the previous results, equal numbers of *spiC* and *ssaT* mutants were recovered from infected spleens. These results suggested that SpiC could function, like SsaT, as part of the TTSS delivery apparatus.

SpiC is required for the translocation of SPI2 effector proteins into infected mammalian cells. A variety of SPI2 effector proteins have been routinely assayed for translocation by construction of gene fusions to *Bordetella pertussis cyaA*, which encodes a toxin that catalyzes the formation of cAMP only in the presence of eukaryotic cytoplasmic protein calmodulin (36). To determine if SpiC is required for the delivery of SPI2 effector proteins into the cytoplasm of infected mammalian cells, plasmids pEM25, pEM30, and pEM82 encoding CyaA fusions to SspH1, SspH2, and SseJ, respectively, were introduced into *spiC* deletion strain JAF204 and into *sifA* deletion strain JAF57. SifA is another SPI2 effector protein which has previously been shown to play a significant role in *Salmonella* serovar Typhimurium virulence, and its mutation should not necessarily alter the translocation of other effectors (2).

Production of cAMP was assayed 8 h after infection of cultured RAW264.7 macrophages and is shown in Fig. 2. As previously demonstrated, wild-type strain CS401 harboring pEM25, pEM30, and pEM82 induces production of cAMP but the *ssaT* mutant strain does not (24). The lack of cAMP pro-
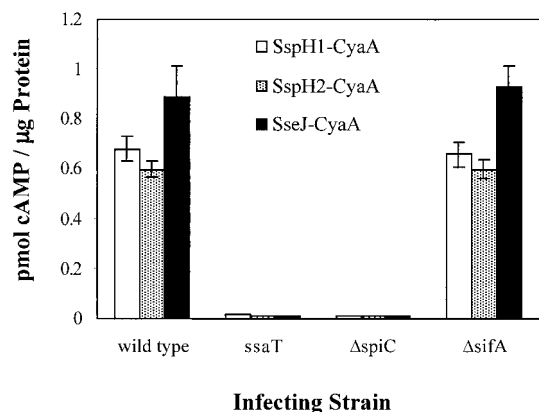
FIG. 2. Effect of deletion of *spiC* and *sifA* on the SPI2-dependent translocation of SspH1-CyaA and SspH2-CyaA. Wild-type *Salmonella* serovar Typhimurium strain CS401, *ssaT* mutant strain EM232, ΔspiC mutant strain JAF204, and ΔsifA mutant strain JAF57, each expressing *sspH1-cyaA* (pEM25), *sspH2-cyaA* (pEM30), and *sseJ-cyaA* (pEM82), were grown to stationary phase and used to infect RAW264.7 cells. Eight hours postinfection, infected macrophages were lysed and cellular cAMP levels were quantitated. Cellular cAMP content was normalized for total protein concentration. Error bars represent 1 standard deviation above and below the mean of experiments performed in triplicate.

duction in the *ssaT* mutant-infected cells is not attributable to decreased viability of this mutant, for no intracellular replication difference can be detected within 8 h of infection (data not shown). Infection with *sifA* deletion strain JAF57 resulted in nearly wild-type levels of cAMP production, indicating that SifA is not required for the translocation of SspH1, SspH2, and SseJ into macrophages. SpiC, however, was shown to be required for the translocation of SspH1, SspH2, and SseJ, because infection of macrophages with strain JAF204, harboring pEM25, pEM30, and pEM82, did not result in production of cAMP. SpiC was also shown to be required for the translocation of a CyaA fusion to SPI2 effector protein SseI (data not shown).

Further experiments were performed to establish that SpiC is essential for translocation of SPI2 effectors. Translocation of SPI2 effector SseJ was assayed by indirect immunofluorescence after bacterial infection of RAW264.7 cells. SseJ containing a carboxy-terminal M45 epitope of the adenovirus E4-6/7 protein has previously been shown to be expressed and secreted by the SPI2 TTSS under certain in vitro growth conditions (12). To determine if M45 epitope-tagged SseJ could be visualized upon translocation, the plasmid encoding SseJ-M45, p2129, was introduced into wild-type strain NCTC 12023, *ssaV* mutant strain HH109, and *spiC* mutant strain EG10128. SsaV encodes a component of the SPI2 TTSS apparatus and has previously been shown to be required for TTSS function (26). RAW264.7 cells were infected with the transformed *Salmonella* serovar Typhimurium strains and were fixed and stained for *Salmonella* and SseJ-M45 16 h postinfection. Because the *Salmonella* cells are not permeabilized in the procedure, SseJ retained within the bacteria is not detected and any intracellular M45 staining indicates SseJ-M45 translocation from the bacteria. SseJ-M45 was demonstrated to be translocated into infected RAW264.7 cells from intracellular wild-type bacteria, as shown by the diffuse red staining in Fig. 3. In cells infected with the *ssaV* and

spiC mutants, however, no SseJ-M45 was detected, indicating that these two strains are defective in SseJ translocation. Therefore, SpiC is required for the translocation of at least several, and likely all, SPI2 effectors, suggesting that a lack of translocation of a number of SPI2 effectors contributes to the severe virulence phenotype associated with deletion of *spiC*.

SpiC-M45 is not translocated into macrophages by intracellular *Salmonella* serovar Typhimurium. The observation that SpiC is required for the translocation of SPI2 effectors suggests that SpiC itself may not, in fact, be an effector protein. To independently assess SpiC translocation, a *spiC* fusion to the M45 epitope tag coding sequence was generated on a low-copy-number plasmid. RAW267.4 cells were infected with wild-type *Salmonella* serovar Typhimurium strain NCTC 12023 harboring SpiC-M45 plasmid p2127, and translocation was assayed by indirect immunofluorescence 16 h after infection. As shown in Fig. 3, although SseJ-M45 was detected within macrophages infected with wild-type *Salmonella* serovar Typhimurium harboring p2129, SpiC-M45 was not observed within cells infected with wild-type bacteria harboring p2127. This result indicates that SpiC is likely not translocated by the SPI2 TTSS, though it is possible that it is translocated in an amount that cannot be detected or that, despite its small size, it is processed.

SpiC is limited to the cytoplasm under in vitro growth conditions that result in the secretion of translocon component SseB to the cell surface. The facts that SpiC has previously been demonstrated to be translocated into infected macrophages and that it is required for the translocation of other effectors suggest that it may be a component of the translocon. Some translocon proteins have been shown to be translocated in small amounts into eukaryotic cells under certain conditions (10). SPI2 translocon proteins SseB, SseC, and SseD have previously been shown to localize to the bacterial cell surface in vitro when *Salmonella* serovar Typhimurium is grown under SPI2-inducing conditions (1, 21, 26). Presumably, these proteins are secreted by the SPI2 secretion apparatus and subsequently form a macromolecular structure on the cell surface to facilitate the translocation of SPI2 effector proteins across the phagosomal membrane of an infected host cell.

To determine if SpiC also localizes to the cell surface, plasmid p2127, encoding SpiC-M45, was introduced into wild-type and mutant *Salmonella* serovar Typhimurium and the resultant strains were grown in SPI2-inducing conditions. After growth to early stationary phase, both the surface-associated and secreted fractions were collected and examined. As shown in Fig. 4, translocon component SseB could be detected both on the cell surface (detached fraction) and in the culture supernatant of wild-type *Salmonella* serovar Typhimurium. This localization was dependent on a functional SPI2 secretion apparatus, for SseB was not secreted or localized to the cell surface by the *ssaV* mutant. SpiC, however, was not detectable either in the culture supernatant or on the cell surface of wild-type *Salmonella* serovar Typhimurium although SpiC was detected in the whole-cell fraction.

Since SpiC was not detected on either the cell surface or in the culture supernatant, the subcellular localization of SpiC was further addressed. Wild-type *Salmonella* serovar Typhimurium harboring p2127 was grown under SPI2-inducing conditions and subsequently fractionated into total membrane,
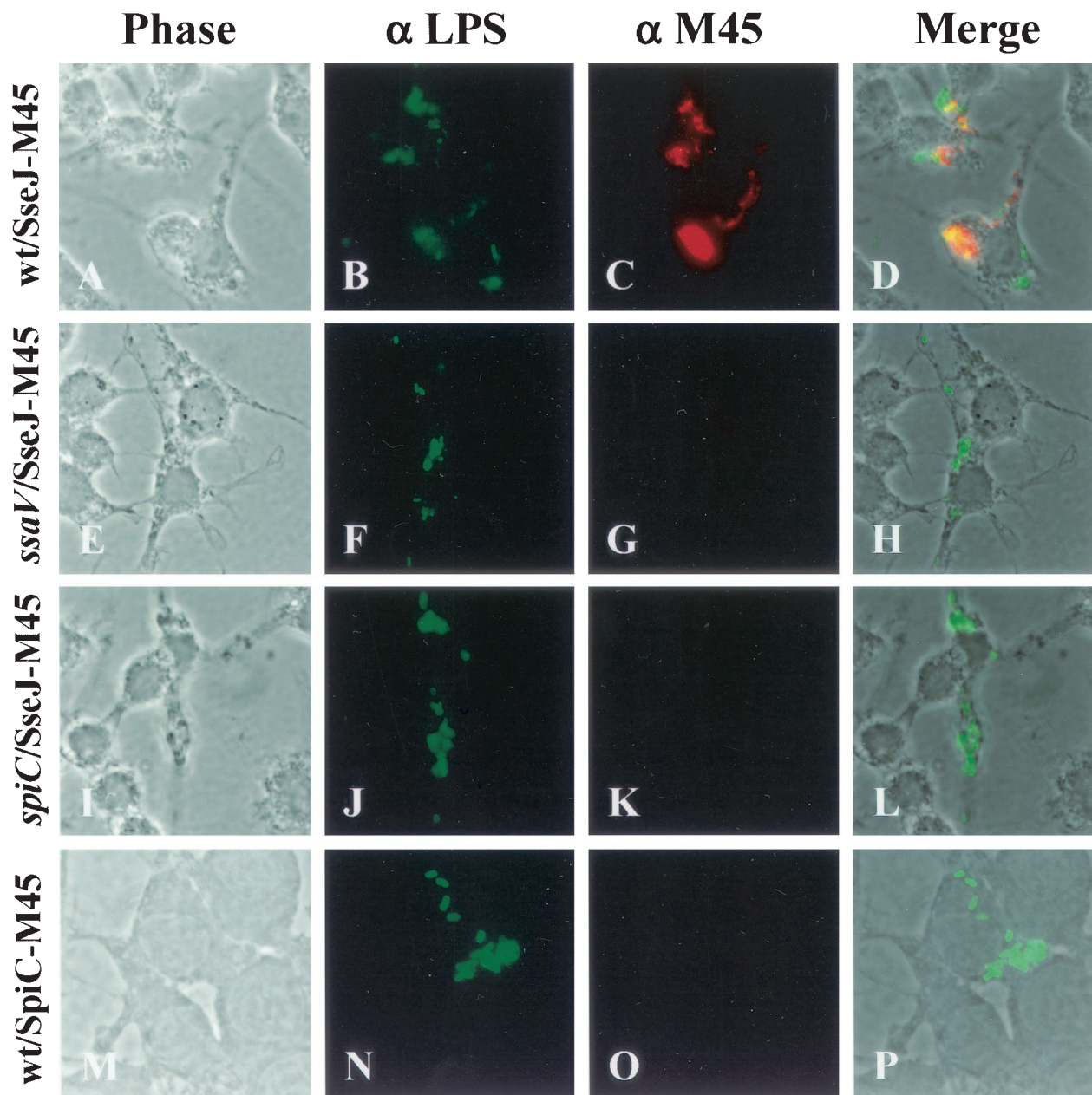
FIG. 3. Translocation of epitope-tagged SPI2 effector proteins. RAW264.7 cells were infected with wild-type *Salmonella* serovar Typhimurium strain NCTC 12023 expressing SseJ-M45 (A to D), *ssaV* mutant strain HH109 expressing SseJ-M45 (E to H), *spiC* mutant strain EG10128 expressing SseJ-M45 (I to L), and wild-type strain NCTC 12023 expressing SpiC-M45 (M to P). Sixteen hours postinfection, cells were fixed, permeabilized, and stained for bacteria (α LPS, green) and epitopes (α M45, red). Stained cells were then examined by fluorescence microscopy and photomicrographs were taken of phase contrast, green fluorescence, and red fluorescence. Merged images, in which yellow represents colocalization of green and red fluorescence, were generated with Metavue imaging software (Universal Imaging Corporation).

soluble, inner membrane, and outer membrane fractions. As shown in Fig. 5, SpiC-M45 could be detected only in the soluble fraction. The purity of the soluble and two membrane fractions was demonstrated by probing for the β subunit of ATP synthase and OmpA. Taken together, these results indicate that SpiC is present in the cytoplasm when SseB is localized to the cell surface. This suggests that SpiC is limited to the cytoplasm though it remains possible that it could be secreted under different bacterial growth conditions.

SpiC is required for the secretion and surface localization of translocon components SseB and SseC. Since *spiC* mutants are defective in translocation and since SpiC is a cytoplasmic protein, it could function as part of the SPI2 type III secretion apparatus. To test this hypothesis, the *spiC* mutant was analyzed for the surface localization of SseB and SseC, translocon components whose surface localization is dependent on the SPI2 TTSS apparatus. Bacteria were grown in SPI2-inducing conditions, and the surface-associated proteins were extracted
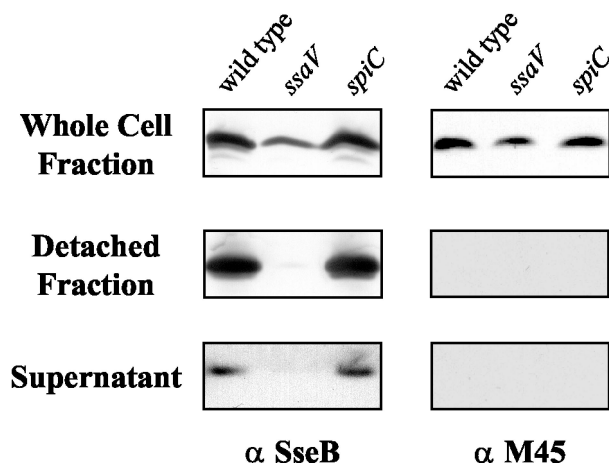
FIG. 4. Analysis of secretion of SpiC-M45. Wild-type *Salmonella* serovar Typhimurium strain NCTC 12023, *ssaV* mutant strain HH109, and *spiC* mutant strain EG10128, each harboring plasmid p2127, were grown in minimal medium at pH 5.8 to induce expression and secretion of SPI2 substrate proteins. Total cell fractions and secreted protein from the culture supernatant or detached from the cell surface were prepared as described in Materials and Methods. Equal amounts of bacterial cells as adjusted according to the OD_{600} were analyzed for the total-cell fraction. Detached-protein and supernatant fractions were prepared from equal volumes of cultures grown to similar cell densities. SseB and SpiC-M45 were detected with a polyclonal antiserum raised against recombinant SseB (1) and monoclonal antibodies against the M45 epitope (27).
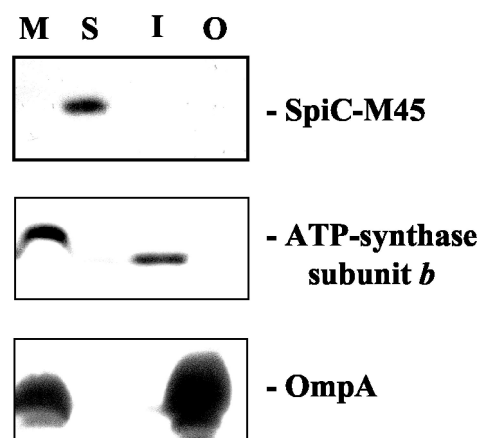

FIG. 5. Subcellular localization of SpiC-M45. Wild-type *Salmonella* serovar Typhimurium strain NCTC 12023 carrying p2127 was grown under conditions inducing the expression and secretion of SPI2 substrate proteins. Bacteria were harvested and subjected to subcellular fractionation as described in Materials and Methods. The various fractions (M, total membrane fraction; S, soluble fraction; I, inner membrane fraction; O, outer membrane fraction) were subjected to SDS-PAGE on 12% Tricine gels; protein was transferred onto nitrocellulose membranes, and the M45 epitope was detected with monoclonal antibodies and an ECL kit (Amersham). The purity of the various fractions was confirmed by Western blotting using antibodies against subunit β of ATP synthase and OmpA as tracers for the inner membrane and outer membrane fractions, respectively.

and subjected to analysis. As a control, wild-type bacteria and *ssaC* mutant bacteria were also grown similarly and analyzed. SsaC has homology to InvG from the SPI1 TTSS, which forms the outer membrane ring of the needle complex, and such mutants are completely defective in type III secretion (17). As shown in Fig. 6, SseB and SseC were detected in the detached fraction from the wild-type strain but not in the *ssaC* mutant strain. Although wild-type levels of SseB and SseC were produced in the cytoplasm, neither SseB nor SseC could be detected from the detached fraction of the *spiC* mutant, indicating that SpiC is required for the surface association of these translocon proteins. This phenotype was specifically due to the loss of SpiC, for SseB and SseC were detected on the cell surface of the *spiC* mutant complemented by a plasmid-borne wild-type allele of *spiC*. This indicated that SpiC functions in the SPI2 TTSS to promote translocon assembly, and the absence of a functional translocon explains the inability of the *spiC* mutant to translocate SPI2 effectors.

SpiC could function to maintain stability of the translocon structure on the bacterial surface. A lack of stability of the translocon structure could result in the release of translocon components into the bacterial supernatant. Therefore, to address this possibility, secretion of translocon proteins SseB and SseC into the culture supernatant in the *spiC* deletion mutant was assessed. Upon growth of wild-type and mutant *Salmonella* serovar Typhimurium in SPI2-inducing conditions, secreted proteins were precipitated from the culture supernatant. As shown in Fig. 7, SseB and SseC are secreted into the culture supernatant from wild-type, but not *ssaT* mutant, bacteria. Although *spiC* deletion strain JAF204 produces wild-type levels of SseB and SseC proteins, none are secreted into the

culture supernatant. Therefore, the lack of surface localization of SseB and SseC in the *spiC* mutant is specifically due to a lack of secretion of SseB and SseC and not to their release into the supernatant.

SpiC mutants do not have a generalized TTSS secretion defect. Several SPI2 effector and translocon components have recently been demonstrated to be secreted into the culture supernatant under certain in vitro growth conditions (12). One
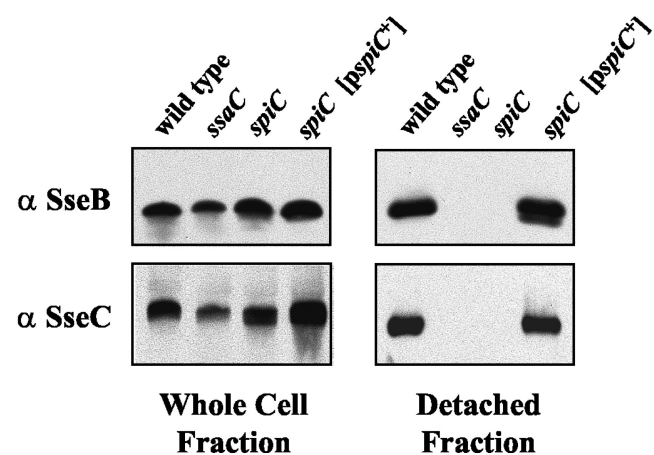

FIG. 6. Effect of mutation of SPI2 genes on the surface association of SPI2 TTSS translocon proteins SseB and SseC. Wild-type *Salmonella* serovar Typhimurium strain NCTC 12023, *ssaC* mutant strain P7G2, *spiC* mutant strain EG10128, and *spiC* mutant strain EG10128 harboring pEG9127 were grown in minimal media at pH 5.8 to induce the expression of the SPI2 TTSS and the secretion of substrate proteins. Total-cell fractions and detached-protein fractions were prepared and analyzed as described in Materials and Methods.
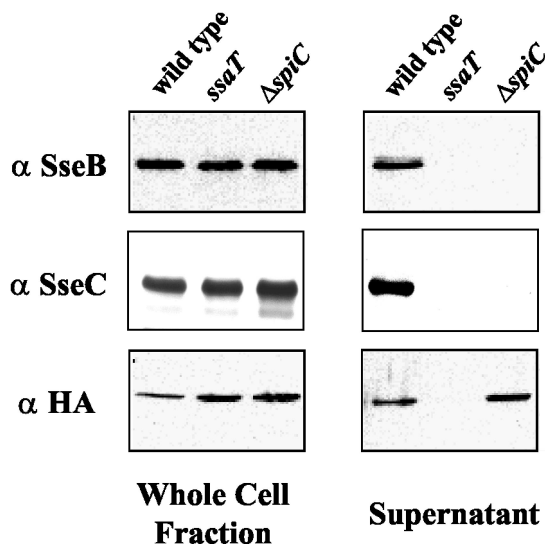
FIG. 7. Secretion of SPI2 substrate proteins. Wild-type *Salmonella* serovar Typhimurium strain CS401, *ssaT* mutant strain EM232, and Δ*spiC* mutant strain JAF204, each harboring HA epitope-tagged SseJ (pJAF111), were grown to early stationary phase in minimal media at pH 5.8 to induce the synthesis and secretion of SPI2 substrate proteins. Equal amounts of whole-cell lysates and secreted-protein fractions were prepared as described in Materials and Methods. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to immunodetection using the specified antibodies.

of the more abundant of the translocated effectors secreted into the cultured supernatant is SseJ, which in this work was demonstrated to require SpiC for translocation into the eukaryotic cell cytoplasm after bacterial infection of macrophages and epithelial cells. Therefore, to test if *spiC* mutants had a generalized type III secretion defect, an allele of *sseJ* with the coding sequence for a carboxy-terminal HA epitope tag was constructed so that secretion of SseJ into the culture supernatant could be assayed. As in the allele of *spiC* fused to the M45 coding sequence, expression of SseJ-HA was driven by the native *sseJ* promoter. The plasmid-encoded HA epitope-tagged SseJ (pJAF111) was introduced into wild-type and mutant *Salmonella* serovar Typhimurium. As shown in Fig. 7, SseJ-HA is secreted into the culture supernatant by wild-type but not *ssaT* mutant bacteria. Surprisingly, the *spiC* deletion mutant secreted SseJ at levels equivalent to those seen in wild-type bacteria. This result indicated that SpiC is required for the specific secretion of SseB and SseC by the SPI2 TTSS but not for the secretion of all substrate proteins. Therefore, SpiC appears to function as a cytoplasmic protein that is required for type III secretion of SseB and SseC and subsequent assembly of the translocon but not the secretion of translocated effectors. This indicates that SpiC is not required for the assembly of the type III cytoplasmic machinery or the needle complex.

DISCUSSION

This work provides further elucidation and definition of the role of SPI2 TTSS protein SpiC. A nonpolar deletion *spiC* mutant was constructed and was demonstrated to produce a mouse virulence phenotype equivalent to that produced by

mutation of the SPI2 TTSS apparatus. This result was explained by the observation that SpiC is a cytoplasmic protein required for translocation of SPI2 effector proteins. Furthermore, SpiC was demonstrated to be required for the surface localization of translocon proteins SseB and SseC. SpiC was also shown to not be required for type III secretion of effector protein SseJ into the culture supernatant in vitro. Therefore, SpiC is essential for assembly of the translocon and delivery of effector proteins, indicating that the *spiC* virulence phenotypes are most likely the result of an inability to translocate all SPI2 effector proteins.

The lack of translocation of SPI2 effectors displayed by the Δ*spiC* mutant was not explained by an inability to replicate and survive within infected macrophages. Translocation of cyclase fusions to SPI2 effectors was assayed before an intracellular replication defect could be measured, as equal numbers of wild-type and SPI2 mutant bacteria were recovered at the time that translocation was assayed (data not shown). In contrast, a *sifA* mutant which is defective in intracellular replication could translocate a variety of effectors. The inability of the Δ*spiC* strain to translocate SPI2 effectors was also not due to polar effects on downstream genes, for this mutant was later shown to be able to secrete SseJ-HA into the culture supernatant, a function requiring genes downstream of *spiC*. These results indicate that, similar to other TTSS, the SPI2 translocon is not required for the in vitro secretion of other TTSS substrate proteins. Therefore, secretion of SseJ into the culture supernatant was observed in the absence of SpiC although, within macrophages, the *spiC* mutants were unable to translocate SseJ. Furthermore, the translocation defect was not limited to the strain with *spiC* deleted or to the cyclase assay, for the strain with the *spiC::aphT* allele was also defective for translocation of M45 epitope-tagged SseJ as determined by indirect immunofluorescence. This indicates that SpiC is not required for type III secretion and serves a more specialized function related to the translocon.

Despite its requirement for translocation of SPI2 effectors, an M45 epitope-tagged SpiC was not observed to be translocated to the cytoplasm of infected macrophages. Indeed, SpiC appears to be limited to the bacterial cytoplasm and is not secreted or localized to the cell surface when bacteria are grown in vitro under conditions that lead to the surface localization of translocon proteins SseB and SseC and the secretion of effector protein SseJ. These results are in conflict with those reported by Uchiya et al., who identified SpiC as a translocated effector protein that functions in bacterial virulence to inhibit phagolysosomal fusion. This result was further buttressed by the fact that purified SpiC could inhibit vesicle fusion in vitro. Uchiya et al. initially demonstrated SpiC translocation by indirect immunofluorescence (38). Their results using polyclonal antibodies raised against SpiC are in direct conflict with the results here using monoclonal antibodies against M45 epitope-tagged SpiC. The lack of translocation or secretion of M45-tagged SpiC was unlikely to be the result of an inability of this protein to fold or function correctly, for the *spiC-M45* allele was able to complement a *spiC* mutant and restore the secretion and surface localization of SseB. Though it seems unlikely, the possibility exists that the M45 epitope is processed from SpiC and that, once processed, SpiC is free to be secreted and translocated.

The immunofluorescence studies in this work utilized detergents that do not permeabilize the bacteria. Protein retained within the bacteria was thus not detected in this analysis. Therefore, in the images presented here, only proteins present on the bacterial cell surface or delivered into eukaryotic cells would be detected. By this procedure, SseJ-M45 was detected within the macrophage cytoplasm upon translocation from intracellular bacteria, while no SpiC-M45 was detected, indicating that this protein was not translocated. In contrast, Uchiya et al. performed their immunofluorescence experiments using detergents that permeabilize the bacterial cell, such that bacterial cytoplasmic protein was stained (38). A review of their images indicated that most of the SpiC staining is associated with the bacteria, representing untranslocated protein. Thus, it is possible that the extrabacterial SpiC staining reflects leakage of SpiC from the permeabilized bacteria.

Regardless of whether SpiC is delivered to the cytosol of eukaryotic cells, its virulence phenotype is clearly a result of an inability to assemble a functional translocon that contains SseB and SseC. Thus, what is the role of SpiC in TTSS function? Since SpiC is required for translocon assembly, it is tempting to speculate that SpiC may itself be a component of the translocon, functioning perhaps to either anchor other translocon components to the cell surface or escort these proteins to this location. However, such proteins are usually secreted and are not usually required for the secretion of other translocon components such as SseB and SseC. Though SpiC was only detected in the bacterial cytoplasm, it is possible that a limited quantity is secreted or that it is secreted under conditions different than those used here. If SpiC is a component of the translocon, it is possible that small amounts may be directed into eukaryotic cells, for translocation of some translocon proteins into eukaryotic cells has been observed (10). The results presented here, however, suggest that SpiC is limited to the bacterial cytoplasm and is not directed either to the bacterial cell surface or into eukaryotic cells. Thus, acting within the cytoplasm, SpiC may function as a chaperone for, or regulator of, SseB and SseC secretion. In support of this hypothesis, SpiC is a small acidic protein (pI 5.4) that is required for the secretion of only a subset of proteins. Encoded immediately upstream of SseC, however, is SscA, a protein that has significant homology with *Yersinia* type III secretion chaperone LcrH. Since LcrH is required for the secretion of the SseC homolog, YopB, SscA may serve as the chaperone for SseC (41). Therefore, it is impossible at this time to easily classify SpiC. The experiments described here indicate that SpiC is a cytoplasmic protein that promotes SseB and SseC secretion and that all virulence phenotypes resulting from the loss of *spiC* are not due to the loss of a single effector but rather are due to the loss of all SPI2 effector proteins.

ACKNOWLEDGMENTS

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