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SseL Is a *Salmonella*-Specific Translocated Effector Integrated into the SsrB-Controlled *Salmonella* Pathogenicity Island 2 Type III Secretion System▽

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Bacterial pathogens use horizontal gene transfer to acquire virulence factors that influence host colonization, alter virulence traits, and ultimately shape the outcome of disease following infection. One hallmark of the host-pathogen interaction is the prokaryotic type III secretion system that translocates virulence factors into host cells during infection. *Salmonella enterica* possesses two type III secretion systems that are utilized during host colonization and intracellular replication. *Salmonella* pathogenicity island 2 (SPI2) is a genomic island containing approximately 30 contiguous genes required to assemble a functional secretion system including the two-component regulatory system called SsrA-SsrB that positively regulates transcription of the secretion apparatus. We used transcriptional profiling with DNA microarrays to search for genes that coregulate with the SPI2 type III secretion machinery in an SsrB-dependent manner. Here we report the identification of a *Salmonella*-specific translocated effector called SseL that is required for full virulence during murine typhoid-like disease. Analysis of infected macrophages using fluorescence-activated cell sorting revealed that *sseL* is induced inside cells and requires SsrB for expression. SseL is retained predominantly in the cytoplasm of infected cells following translocation by the type III system encoded in SPI2. Animal infection experiments with *sseL* mutant bacteria indicate that integration of SseL into the SsrB response regulatory system contributes to systemic virulence of this pathogen.

The gram-negative bacillus *Salmonella enterica* serovar Typhimurium infects multiple hosts by using virulence factors that promote invasiveness and intracellular survival. The genetic diversity of this pathogen has been shaped prominently by phage lysogeny and by acquisition of mobile genetic elements, many of which harbor genes that have become important for virulence during the diverse stages of host infection (3, 13, 23, 24). In the *S. enterica* species, two major virulence determinants are encoded on large pathogenicity islands called *Salmonella* pathogenicity islands 1 and 2 (SPI1 and SPI2, respectively), both of which encode a functional type III secretion system (T3SS) to translocate protein effectors into host cells during infection. The SPI1 T3SS is common to all *Salmonella* lineages and secretes effectors responsible for entry into nonphagocytic cells (16). Serotypes belonging to *S. enterica* additionally possess SPI2, which is absent from the other recognized *Salmonella* species, called *S. bongori*. SPI2 injects proteins required for intracellular survival and systemic infections of mammalian cells (19, 21, 25) and likely represents a major second phase in the evolution of *Salmonella* virulence (1). A more pervasive role for SPI2 in intestinal inflammation and early colonization events is indicated by recent data showing that SPI2 is activated in the lumen of the intestine in a manner independent of SPI1-mediated invasion (2) and contributes to disease (5, 8, 18).

SPI2 contains at least four operons encoding type III apparatus proteins, molecular chaperones, effectors, and a two-component regulatory system encoded by the genes *ssrA* and *ssrB* (4, 20, 25). This regulatory system includes a membrane-bound sensor kinase (SsrA) and the cognate response regulator (SsrB) that activates transcription of the SPI2 T3SS and effector substrates located outside of SPI2 (14, 27). To date there is no evidence for SsrB-independent expression of the SPI2 T3SS or its effectors, suggesting that over evolutionary time, *Salmonella* has integrated the expression of the SPI2 secretion machinery with effector substrates that are necessary in the host environment. As such, SsrB has emerged as a principal regulator of virulence genes in *Salmonella enterica*.

Analysis of coregulated genes using microarrays is one approach to identify genes that act in concert during a specific growth stage (9, 15, 26), yet this approach has not been utilized to identify new virulence factors in the SsrB regulon. To facilitate effector protein discovery, we analyzed the transcriptional profiles of wild-type and *ssrB* mutant *Salmonella enterica* grown under SPI2-inducing conditions to search for SsrB-dependent virulence factors. Here we present data on a virulence factor controlled by SsrB that we have named SseL (STM2287; *Salmonella* secreted effector L). SseL is an SPI2 effector that is translocated into host cells during intracellular infection and is required for full virulence during animal infections. SseL appears to be specific to some serotypes of *S. enterica* subspecies I (Typhi, Paratyphi A,

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and Choleraesuis), suggesting that its function could be important to unique aspects of *Salmonella* virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* and *Salmonella* spp. were propagated in LB broth supplemented with antibiotics as appropriate. The concentrations of antibiotics used were as follows unless otherwise stated; streptomycin, 50 μg ml^{-1}; chloramphenicol, 10 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}. *Salmonella* mutants generated in this study are isogenic derivatives of the wild-type strain SL1344 (*Salmonella enterica* serovar Typhimurium). A wild-type strain resistant to chloramphenicol (SL1344 *ushA*::*cat*) was used for competitive infections of animals and has been described elsewhere (11). *S. bongori* (serovar 66:z) was obtained from the *Salmonella* Genetic Stock Centre in Calgary, AB, Canada. *S. bongori* SARC12 containing a bacterial artificial chromosome encoding the entire *S. enterica* SPI2 genomic island (pB6) (17) was a gift from M. Hensel, Erlangen, Germany. An acidic minimal medium low in phosphate and magnesium (LPM) used for the induction of SPI2 has been described elsewhere (7), and microarray methods have been described previously (11).

Cloning and mutant construction. An unmarked, in-frame deletion mutant of STM2287 was constructed by homologous recombination. A DNA fragment encoding the open reading frame plus approximately 0.9 kb of flanking genomic DNA was amplified by PCR using the primers BKC117 (5′ CCG AGC TCA TGG AAA AAA TGG GCT GGA C 3′) and BKC120 (5′ CGG GGT ACC TTG ACA CCG CAG AAC AGG C 3′). This PCR product was cloned into pBluescript KS(+) as a SacI/KpnI fragment and then used as a template for inverse PCR using the primers BKC118 (5′ ACG CGT CGA CTG TAA GCG CCT CAT CGC TCA C 3′) and BKC119 (5′ ACG CGT CGA CTA CAG TCT CCA GTA ATG GTG A 3′). This DNA product was incubated with SalI and then self-ligated to produce a circular plasmid containing the in-frame deletion. The deletion allele was subcloned as a SacI/KpnI fragment into the suicide plasmid pRE112 (12) and transformed into *E. coli* SM10 λ *pir* to generate a donor strain for conjugation into SL1344. Merodiploid clones were selected on streptomycin and chloramphenicol; grown in liquid LB medium without antibiotic selection for 6 h; and then plated on 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, and 5% (wt/vol) sucrose and grown overnight at 25°C. Mutants with the desired deletion were confirmed by sequencing. Using the *sseL* deletion strain as parent, a wild-type *sseL* gene was reintroduced into this strain by reversion. This was accomplished by homologous recombination of the BKC117_120 PCR fragment from pRE112 as described above. An *sseL* allele tagged with tandem hemagglutinin (HA) epitopes at the carboxy terminus was generated by PCR and SL1344 genomic DNA as template. The primers 5′ ACG CGT CGA CCG ATT GCC GTC AAA GGT ATT 3′ and 5′ GGA AGA TCT CTG GAG ACT GTA TTC ATA TAT TTG CCG 3′ were used to amplify *sseL* with its native promoter from *S. enterica*. This DNA was cloned as a SalI/BglII fragment into pWSK129-*gogB*-2HA (10), in which the *gogB* allele was released as a SalI/BglII fragment. The tagged *sseL* allele and its promoter were then subcloned out of pWSK129 as a SalI/XbaI fragment and into similarly digested pWSK29 for expression studies.

Secretion assays. Overnight cultures of *Salmonella* were washed in LPM medium (pH 5.8) [5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 80 mM morpholineethanesulfonic acid, 38 mM glycerol, 0.1% Casamino Acids, 24 μM Mg^{2+}, and 337 μM PO_4^{3-}]. Bacteria were subcultured 1:50 into LPM medium and grown at 37°C with shaking to an optical density of ~0.5 at 600 nm. Bacteria were centrifuged, and the supernatant was passed through an 0.22-μm low-protein-binding HT Tuffryn membrane (Pall Life Sciences). Trichloroacetic acid was added to a final concentration of 10% (vol/vol), and the samples were incubated at 4°C overnight. Precipitated proteins were centrifuged (~20,000 × *g*) for 30 min at 4°C, washed in ice-cold acetone, and then solubilized in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (100 mM Tris-HCl [pH 6.8], 20% [vol/vol] glycerol, 4% [wt/vol] SDS, 0.002% [wt/vol] bromophenol blue, and 200 mM dithiothreitol) according to the A_{600} of the original culture. The bacterial cell fraction was solubilized in SDS sample buffer, and proteins were analyzed by SDS-PAGE and immunoblotting. The following antibodies and dilutions were used for immunoblotting: mouse anti-HA (1:1,000), mouse anti-DnaK (1:5,000), and rabbit anti-SseB or anti-SseD (1:1,500). Both primary and secondary antibodies were diluted in a blocking solution of Tris-buffered saline, Tween 20, and 5% (wt/vol) nonfat milk. Secondary antibodies conjugated to horseradish peroxidase were used at 1:5,000, and antigen-antibody complexes were detected using enhanced chemiluminescence.

Cell culture and infection of cells. HeLa and RAW264.7 cells were cultured using Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, Utah) containing 10% fetal bovine serum (FBS). The day prior to infection, cells were seeded into 24-well cell culture plates and incubated overnight in an atmosphere of 37°C and 5% CO_2. HeLa cells were infected with invasive *Salmonella* for 10 min as described previously (6), and RAW264.7 cells were infected for 25 min with stationary-phase *S. enterica* opsonized with 20% normal human serum. Following infection, the medium was aspirated, cells were washed with phosphate-buffered saline (PBS), and fresh cell culture medium containing gentamicin (100 μg ml^{-1}) was added for 1 hour, after which the gentamicin concentration was reduced to 10 μg ml^{-1} for an additional hour or for 18 h postinfection. After infected monolayers were washed with PBS, the cells were lysed in a solution of Triton X-100 (1%, vol/vol) and sodium dodecyl sulfate (0.1%, wt/vol) to release intracellular bacteria. Samples were diluted and assayed for viable counts on solid LB medium.

For derivation of bone marrow-derived macrophages (BMDM), bone marrow from the tibia and femur of uninfected 129/svJ mice was collected into 10 ml sterile Hanks' balanced salt solution (Sigma, Oakville, ON, Canada). Cells were washed twice in DMEM and seeded in 10 ml DMEM supplemented with 20% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg ml^{-1} streptomycin (Invitrogen, Burlington, ON, Canada), and 30% conditioned medium from L-929 hybridoma cells as a source of macrophage colony-stimulating factor at a concentration of 1.5×10^7 cells in 100-mm dishes. The medium was changed at day 5, and cells were allowed to differentiate at 37°C for an additional 2 days. For infection of BMDM, cells were removed from dishes and washed twice in 10 ml DMEM. Cells were seeded at 2×10^5 cells per well in DMEM plus 10% FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate in 24-well tissue culture plates 12 h prior to infection. Cells were infected at a multiplicity of infection of 100:1 for 20 min with opsonized wild-type *Salmonella* or an *ssrB* mutant containing pEGFP-*sseL* or pEGFP. Cells were washed with warmed PBS and supplied with 1 ml DMEM-50 μg ml^{-1} gentamicin for 2 h. After 2 h, cell supernatants were replaced with 1 ml DMEM-10 μg ml^{-1} gentamicin.

Flow cytometry. At 30 min and 8 h postinfection, supernatants were removed from BMDM and cells were washed three times in sterile PBS. Cells were scraped into 300 μl of cold fluorescence-activated cell sorting (FACS) buffer containing PBS, 2% FBS, 0.1% sodium azide, and 1 mM EDTA and transferred into a 96-well round-bottomed tissue culture plate. Plates were centrifuged for 3 min to pellet cells, and FACS buffer was removed. BMDM were incubated with 25 μl of biotinylated primary antibody F-480 (Serotec, Raleigh, NC) at a dilution of 1:300 in FACS buffer for 30 min at 4°C. Cells were washed twice in FACS buffer followed by incubation with streptavidin-phytoerythrin (Cedarlane Laboratories Ltd., Hornby, ON, Canada) at a dilution of 1:400 in FACS buffer for 30 min at 4°C. Cells were washed twice in FACS buffer and suspended in a final volume of 150 μl of FACS buffer for flow cytometry analysis. Samples were run on a FACSCalibur flow cytometer, with data for 10,000 events collected per sample using Cell Quest Pro software (BD Biosciences, Mississauga, ON, Canada). Data were analyzed using Flow Jo flow cytometry software (Tree Star Inc., Ashland, OR). Groups were compared using a two-tailed, unpaired *t* test.

Biochemical fractionation of host cells and translocation assay. HeLa cells were infected for 8 h as described above with *Salmonella* strains containing HA epitope-tagged *sseL*. Infected cells were scraped into cold PBS, centrifuged, and mechanically lysed through a 22-gauge needle in a buffer containing 250 mM sucrose, 3 mM imidazole (pH 7.4), and 0.5 mM EDTA. Lysates were centrifuged sequentially at 3,000 × *g* (to collect unbroken host cells, nuclei, cytoskeleton, and bacteria) and at 41,000 × *g* to collect the host cell membrane fraction (pellet) and the host cell cytosol fraction (supernatant). Each fraction was analyzed by SDS-PAGE and immunoblotting using the following antibodies: mouse anti-HA (1:1,000), mouse anti-DnaK (1:5,000), mouse anti-β-tubulin (1:5,000; clone E7; Developmental Studies Hybridoma Bank, University of Iowa), and rabbit anti-calnexin (1:5,000; Stressgen). All primary antibodies were diluted in a blocking solution of Tris-buffered saline–Tween with 5% (wt/vol) nonfat milk. Secondary antibodies conjugated to horseradish peroxidase were used at 1:5,000, and antigen-antibody complexes were detected using enhanced chemiluminescence.

Expression of GFP-tagged proteins, transfection and microscopy. SseL was fused to enhanced green fluorescent protein (GFP) for use in transfection and microscopy studies. The entire *sseL* open reading frame was amplified by PCR from SL1344 genomic DNA using the primers 5′ GGA AGA TCT GTG AGC GAT GAG GCG CTT A 3′ and 5′ ACG CGT CGA CTT ACT GGA GAC TGT ATT CAT ATA TTT GC 3′ and then cloned into pEGFP-C1 (Clontech) as a BglII/SalI fragment to generate pGFP-SseL, which produces SseL with GFP fused in frame to the amino terminus. For transfection experiments, HeLa cells were grown to ~40% confluence on glass coverslips and were transfected for 24 to 40 h using FuGENE 6 transfection reagent (Roche). At the desired time point, samples were fixed in 2.5% paraformaldehyde-PBS solution (pH 7.4) for 15 min at 37°C. Samples were washed with

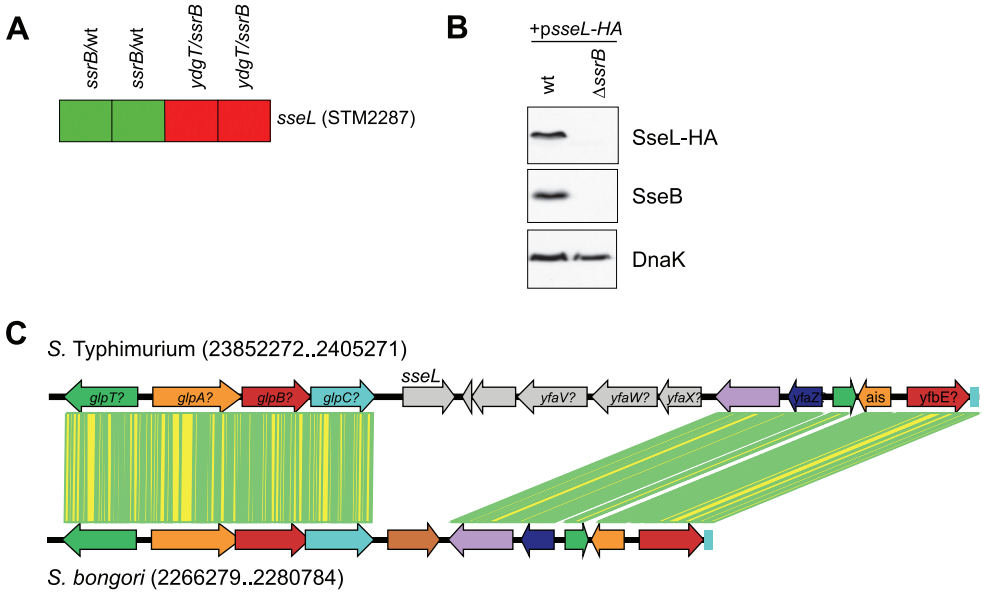

FIG. 1. Identification of *sseL*, a new member of the SsrB regulon. (A) Relative expression ratios of *sseL* between wild-type *Salmonella*, an *ssrB* mutant, and a *ydgT* mutant (11). Green indicates a negative relative expression in the *ssrB* mutant compared to wild type, and red indicates a positive relative expression in *ydgT* mutants compared to the Δ*ssrB* strain. (B) Expression of SseL requires SsrB. Wild-type and *ssrB* mutant *Salmonella* strains expressing an SseL-HA fusion protein were grown in SPI2-inducing medium. Whole-bacterial-cell lysates were examined by Western blotting for the expression of each protein. As controls, bacterial lysates were probed for the SsrB-dependent protein, SseB, and the SsrB-independent protein, DnaK. (C) The syntenic gene environment surrounding *sseL* in *S. enterica* serotype Typhimurium (top) was compared to the assembled, preannotated *S. bongori* genome downloaded from the Wellcome Trust Sanger Institute (bottom). Vertical green and yellow lines represent regions of sequence identity between the two genomes, and the absence of vertical lines indicates divergence or absence of the corresponding sequence in the *S. bongori* genome. Number ranges following the species name indicate the nucleotide positions of the genome region within the aligned window.

PBS and then blocked and permeabilized in 1% normal goat serum-0.2% saponin in PBS for 10 min. Cells were stained with Alexa 568-phalloidin (Molecular Probes and Invitrogen) and then mounted onto glass slides using ProLong Gold antifade reagent (Molecular Probes). Samples were viewed using a Zeiss Axioskop 2, and images were captured using Northern Eclipse v6.0 software.

Competitive infection of experimental animals. Animal protocols were in accordance with the Canadian Council on the Use of Laboratory Animals and were approved by the Animal Research Ethics Board at McMaster University and the University of British Columbia. For single infections, female C57BL/6 mice (Jackson Laboratories, Maine) were infected orally with ~10^6 CFU of *Salmonella* in 0.1 M HEPES (pH 8.0)-0.9% NaCl. For competitive infection experiments, two mouse strains were used that are susceptible (C57BL/6) or resistant (129/svImJ) to *Salmonella*-induced mortality. C57BL/6 mice have a nonfunctional *Nramp1*^{s} (Slc11a1) allele and have defects in controlling intracellular *Salmonella* replication, whereas 129/svImJ mice are homozygous for the *Nramp*^{r} allele and are better able to limit intracellular replication of *Salmonella*. Mice were infected orally (~1×10^6 CFU for C57BL/6 or ~1×10^8 CFU for 129/svImJ) with a mixed inoculum containing wild-type *Salmonella* resistant to chloramphenicol (*ushA*::*cat*) (11) and an unmarked mutant strain under investigation. At 72 h after infection the spleen and liver were homogenized (Polytron MR-21; Kinematic) in PBS, diluted, and plated on solid LB medium containing streptomycin for determination of total *Salmonella* CFU. Colonies were replica printed onto chloramphenicol-containing plates for enumeration of SL1344 *ushA*::*cat* bacteria. The competitive index (CI) was calculated on log-transformed CFU counts as (mutant output/wild-type output)/(mutant input/wild-type input). CI data were analyzed using a one-sample *t* test and compared to a theoretical mean of 1. A *P* value of <0.05 was considered significant.

RESULTS

SseL is a member of the SsrB regulon. The transcription factor SsrB is required for the expression of the T3SS encoded within SPI2 and for the expression of its substrate effector proteins. By analyzing coregulated genes using DNA arrays (11), we identified a gene whose expression under SPI2-inducing conditions was strongly SsrB dependent. The expression of this gene (STM2287) was 43.5-fold reduced in an *ssrB* mutant compared to wild-type *Salmonella* (Fig. 1A). STM2287 has homologues in the *S. enterica* serotypes Typhi, Paratyphi A, and Choleraesuis but no detectable homologue in *S. bongori* (www.sanger.ac.uk/Projects/Salmonella), which lacks the SPI2 genomic island. STM2287 has a low G+C content (38.7%) and does not appear to be organized within an operon (Fig. 1C). We further examined the genetic context of *sseL* within *S. enterica* serovar Typhimurium and compared this to genetic regions within *S. bongori*. As shown in Fig. 1C, *sseL* and five genes downstream of *sseL* (two hypothetical genes and the *yfaVWX* operon) are absent from the corresponding region in *S. bongori*, while the gene synteny in the regions upstream and downstream of this apparent insertion is conserved. We were unable to identify obvious genetic elements involved in the horizontal transfer of *sseL* such as inverted repeat regions, phage genes, or tRNA genes. For reasons described below we named STM2287 SseL (*Salmonella* secreted effector L), according to the established nomenclature (20, 27) for *Salmonella* effectors secreted by the SPI2-encoded type III secretion system.

To verify that expression of SseL was under the control of SsrB, we cloned the native *sseL* promoter region in front of a cloned *sseL* gene containing tandem HA epitopes at the C terminus. This construct was introduced into wild-type *Salmonella* and an *ssrB* mutant, and expression was examined under SPI2-inducing conditions (7). Expression of the SseL-HA fu-

sion protein was detected only in a wild-type background and not in the *ssrB* mutant (Fig. 1B). As a control, we probed the same bacterial cell lysates for the SsrB-regulated protein, SseB. As expected, SseB was expressed only in the presence of SsrB. Collectively, these data identified a new protein that coregulated with the SPI2 T3SS and related effector proteins in an SsrB-dependent manner.

SseL is induced inside macrophages during intracellular infection. Since SseL expression was completely SsrB dependent and SsrB is inducible in the intracellular environment (4), we examined whether SseL was also expressed during infection of macrophages. We constructed a translational fusion of the SseL protein to GFP and examined the expression of this fusion protein from the *sseL* promoter in wild-type *Salmonella* and an *ssrB* mutant using FACS. SseL expression was detected following infection of bone marrow-derived macrophages as early as 30 min postinfection (Fig. 2A), and fluorescence of the fusion protein was dependent on SsrB during infection of macrophages, since there was no fluorescence increase in cells infected with the SsrB mutant. By 8 h after infection with wild-type bacteria, the number of GFP-positive macrophages increased ~6-fold, while the number of GFP-positive cells infected with the *ssrB* mutant remained low and did not change over time (Fig. 2B). The low level of GFP at 30 min postinfection in macrophages infected with *ssrB* mutants likely originates from the initial inoculum as carryover from the overnight cultures. This background level did not reach statistical significance compared to uninfected cells, and the number of GFP-positive macrophages infected with *ssrB* mutants did not increase significantly from 30 min to 8 h postinfection, unlike macrophages infected with wild-type *S. enterica* serovar Typhimurium. These data demonstrate that the intracellular environment within macrophages delivers an appropriate signal to activate SsrB-dependent expression of SseL.

SseL is a virulence factor during systemic typhoid. We next investigated whether SseL is a virulence factor during murine typhoid. We generated a nonpolar, in-frame deletion of *sseL* in *S. enterica* serotype Typhimurium and tested the virulence of this strain in competitive infections with the isogenic parent strain. We infected mice by the oral route and calculated the competitive index in the liver and spleen 3 days after infection of both resistant and susceptible mouse lines. In susceptible mice (C57BL/6), the competitive index for Δ*sseL* in the liver and spleen was 0.23 ± 0.04 and 0.33 ± 0.06, respectively (Fig. 3A), indicating that bacteria lacking SseL had a competitive disadvantage during systemic infection. We also examined the virulence of *sseL* mutants in a mouse strain that resists lethal infection with *S. enterica* serovar Typhimurium (129/svImJ). In these mice, the virulence defect of the *sseL* mutant was also apparent, producing competitive indices in the liver and spleen that were significantly less than unity when competed against wild-type bacteria (liver and spleen CIs, 0.25 ± 0.07 and 0.30 ± 0.10, respectively) (Fig. 3B). In order to test whether the reduction in virulence of the Δ*sseL* strain was due to a specific loss of *sseL*, we replaced the *sseL* deletion allele with a wild-type copy and competed this reverted strain against the wild-type SL1344 *ushA*::*cat* strain in competitive infections of animals. In these experiments, the *sseL* reversion strain and the SL1344 *ushA*::*cat* strain competed equally as determined by CIs from the liver and spleens of infected C57BL/6 mice (Fig. 3A). These data indicated that loss of the *sseL* gene was responsible for the virulence defect of the mutant strain.

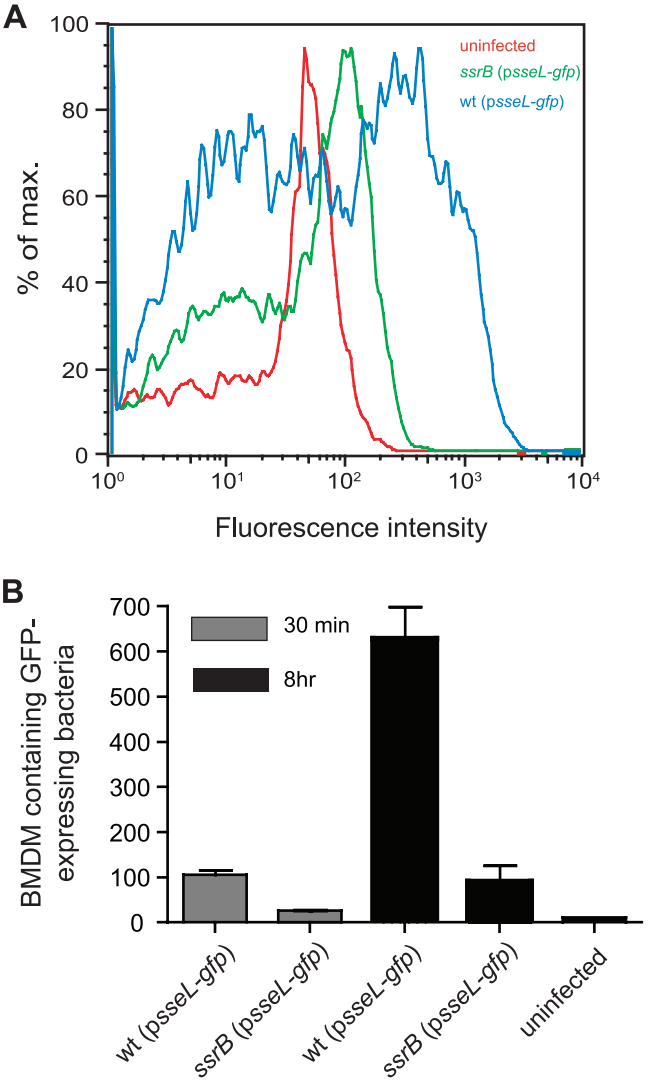

FIG. 2. SseL is expressed inside macrophages in an SsrB-dependent manner. (A) GFP expression of intracellular bacteria in BMDM. BMDM were infected with wild-type (wt) SL1344 (blue) or *ssrB*::*aphT* mutants (green), each containing p*sseL-gfp*, and harvested 30 min postinfection for flow cytometry. Fluorescence intensity of intracellular bacteria expressing GFP in F-480-positive macrophages compared with baseline fluorescence of uninfected cells (red) is represented. (B) Number of BMDM containing intracellular bacteria expressing GFP. BMDM were infected as described above and harvested 30 min (gray bars) and 8 h (black bars) postinfection for quantitative analysis of F-480/GFP-double-positive macrophages by flow cytometry. Error bars represent standard deviations of triplicate samples representative of experiments performed twice. SL1344 versus *ssrB*::*aphT*: 30 min, $P = 0.0003$; 8 h, $P = 0.0015$.

Since the ability to replicate inside macrophages is a hallmark of systemic *Salmonella* pathogenesis, we tested whether survival or replication inside macrophages was impaired by deletion of *sseL*. The number of wild-type *Salmonella* bacteria increased inside macrophages 3.0- ± 1.26-fold over 18 h as reported previously (7). In the same time period, the *sseL* mutant increased in numbers by 3.73- ± 2.35-fold, which was

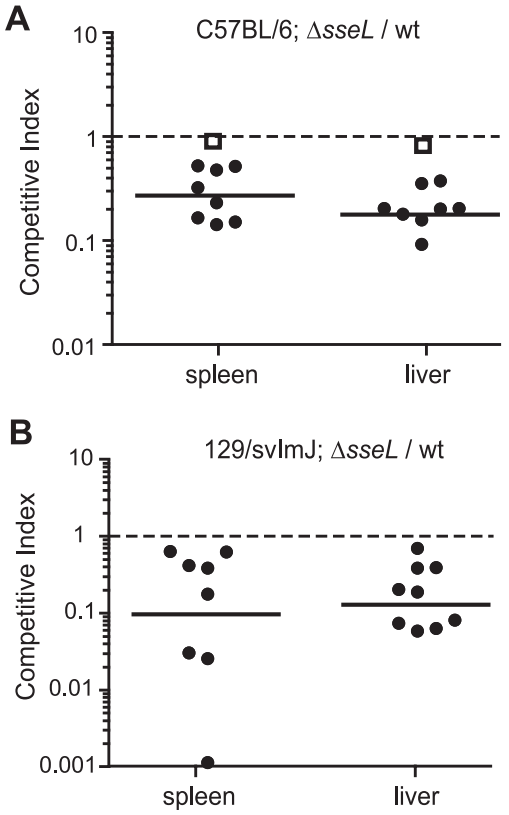

FIG. 3. SseL is required for virulence during infection of mice. Groups of susceptible (A) and resistant (B) mice were infected orally with a mixed inoculum containing wild-type (wt) *Salmonella* and the Δ*sseL* strain. The CIs for the mutant strains were determined in the spleen and liver at day 3 after infection. Each data point (filled circles) represents one animal, and horizontal bars indicate geometric means. C57BL/6 *sseL* CI, $P < 0.001$ (spleen and liver); 129/sv *sseL* CI, $P < 0.0002$ (spleen and liver). Open squares indicate the mean CI for the wild-type *sseL* reversion strain (CI for spleen, 0.93 ± 0.13, $P = 0.67$; CI for liver, 0.88 ± 0.12, $P = 0.40$).

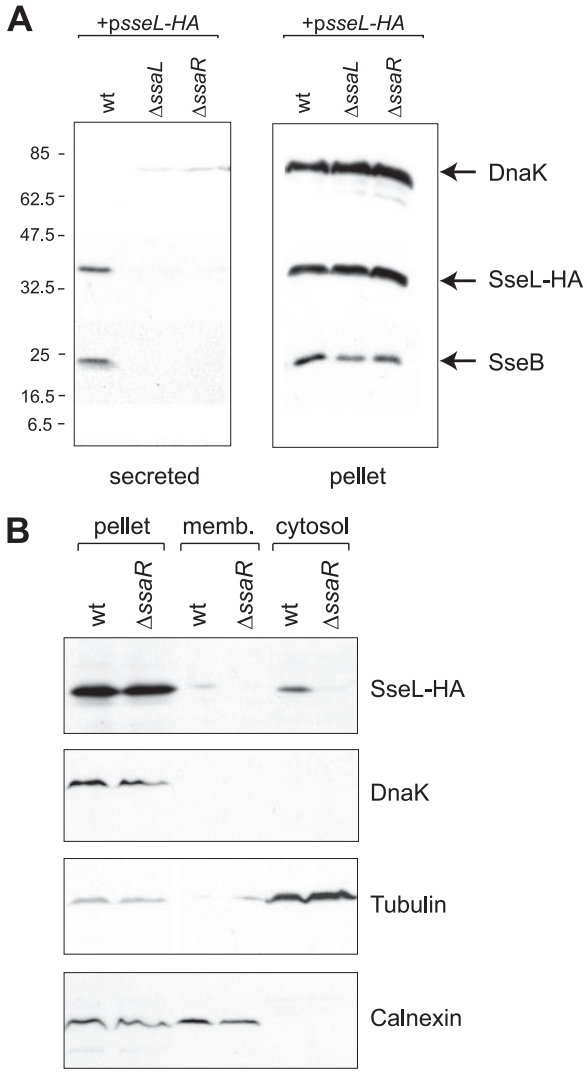

FIG. 4. SseL is secreted by the SPI2 type III secretion system and translocated into the host cytoplasm during infection. (A) Western blot analysis of bacterial cell lysates (pellet, right panel) and secreted proteins (left panel) from wild type (wt) and *ssaR* and *ssaL* mutants (7) grown in LPM medium. Protein fractions were probed for DnaK, SseL-HA, and SseB. The positions of molecular mass standards (in kilodaltons) are indicated to the left of the panel. (B) Fractionation of host cells following infection reveals translocation and retention of SseL in the host cell cytoplasm, dependent on SPI2 T3SS activity. HeLa cells were infected with either wild-type *Salmonella* or a strain defective in SPI2-mediated type III secretion (Δ*ssaR*), each expressing SseL-HA. Following infection, host cells were biochemically fractionated into pellet, membrane, and cytosol fractions and probed using antibodies to HA, DnaK (bacterial cytosolic protein), tubulin (host cell cytosolic protein), and calnexin (a host cell membrane protein).

not significantly different from the wild type. As a control, we infected macrophages with a strain deficient in SPI2-mediated type III secretion, the Δ*ssaR* strain. The number of intracellular *ssaR* mutants decreased by half (0.55 ± 0.35) over 18 h, as reported previously (7). Together, these data indicate that SseL is required for virulence during systemic infection of both susceptible and resistant animals but that this virulence attenuation appears independent of the ability to replicate inside macrophages. Rather, this protein likely contributes to another aspect of the host-pathogen interaction during infection.

SseL is translocated into host cells during infection by the T3SS encoded by SPI2. The majority of genes dependent on SsrB for expression in *Salmonella* encode components of the SPI2 T3SS or the effectors that are translocated through the apparatus needle. To test whether SseL was a new translocated effector, we first examined whether this protein was secreted in a type III-dependent manner. Whole-cell lysates and the extracellular secreted protein fractions from wild-type and SPI2 T3SS mutants expressing HA-tagged SseL were analyzed for the presence of the fusion protein following growth under SPI2-inducing conditions. SseL-HA was consistently found in the secreted protein fraction from wild-type bacteria but not from a mutant defective in one of the core apparatus genes (*ssaR*) or from a second mutant shown previously to be defective for SPI2-mediated type III secretion (*ssaL*) (7) (Fig. 4A).

We then tested whether SseL was translocated into host cells during intracellular infection. To do this, we used an approach that allowed for translocation and subcellular localization determination. We infected epithelial cells with an *S. enterica*

serovar Typhimurium strain expressing SseL-HA and separated host cell lysates into various fractions to identify where this protein was delivered during infection. As controls in our fractionation experiments, we probed the separated host cell fractions for tubulin and calnexin as markers of cytoplasmic and membrane-bound proteins, as well as DnaK, a bacterial cytoplasmic protein that is not secreted and can be used as a marker of bacterial viability in the host cell during the experiment. During intracellular infection, the majority of SseL was delivered by bacteria to the host cell cytosol in a manner dependent on the activity of the SPI2 T3SS since this delivery was blocked in a Δ*ssaR* strain (Fig. 4B). A small amount of SseL localized to the membrane fraction during host cell infection (Fig. 4B), although the abundance of membrane-localized SseL was minor compared to the cytosolic fraction. Host cell tubulin and calnexin and bacterial DnaK fractionated into their expected locations of cytosol, membrane, and pellet fractions, respectively. To further verify the predominant cytoplasmic localization of SseL, we constructed a GFP-SseL fusion protein, transfected this construct into host epithelial cells, and examined the distribution of the fluorescent protein by fluorescence microscopy. GFP-SseL was distributed throughout the cytoplasm of transfected cells (data not shown), in accord with the translocation data described above. These data established that SseL is a translocated effector during infection that localizes principally to the host cell cytosol during intracellular infection.

DISCUSSION

Salmonella pathogenesis requires not only a diverse arsenal of virulence effectors but also the appropriate transcriptional machinery to integrate the expression of virulence proteins with their delivery system during infection. *S. enterica* has evolved to integrate the expression of the SPI2 type III secretion system with the effector substrates that are translocated through this apparatus for efficient infection of host cells. This coregulation is achieved largely by SsrB, the horizontally acquired transcription factor that is inducible in the intracellular environment. The parsimonious nature of bacterial gene expression makes analysis of coregulated genes by DNA microarray a useful tool for assigning new functional relationships between genes that otherwise are not apparent. Others have used such approaches towards the identification of new flagellar genes in *Salmonella* (15) and genes likely involved in PmrA-PmrB-dependent modifications of lipopolysaccharide (26). We took advantage of the fact that the horizontally acquired transcription factor SsrB has emerged as a central player in the integrated virulence response to the intracellular environment and used transcriptional profiling as a tool for effector protein discovery. Using this approach we identified a new SsrB-dependent protein in *S. enterica* serovar Typhimurium that is translocated into host cells during infection and that promotes virulence during systemic infection of mice.

The lack of widespread distribution of *sseL* in public genomic databases suggests that this virulence factor is unique to *S. enterica*. Homologues of *sseL* were identified in three other *S. enterica* subspecies I serotypes including Typhi, Paratyphi A, and Choleraesuis. Subspecies I strains of *S. enterica* account for 99% of infections and clinical disease in humans whereas the other subspecies are commonly found in cold-blooded animals and are generally nonpathogenic in humans (1). The only other similar amino acid sequence identified by BLAST was from *Escherichia coli* and included a hypothetical gene annotated as a putative sulfatase/phosphatase (29% identity over 342 amino acids; 11% gaps). Of course, we cannot rule out the possibility that *sseL* homologues will be identified in other bacterial species as more genome data become publicly available. A comparison of the gene synteny surrounding *sseL* in *S. enterica* serotype Typhimurium and *S. bongori* revealed that *sseL* occurs in a genetic region that is different from the corresponding region in *S. bongori*. We were unable to identify the nature of this apparent insertion, as there are no obvious sequence repeats or other markers of mobile genetic elements in the vicinity of *sseL*. However, the lower G+C content of this gene (38%) compared to that of the remaining genome (~52%) is suggestive of acquisition by horizontal gene transfer at some point in the evolution of this lineage. Our analysis is in accord with previous genetic analyses of *S. enterica* and *S. bongori* strains by comparative genomic hybridization using microarrays that demonstrated the absence of the adjacent genetic elements (*yfaVWX*) from *S. bongori* (22). The stable retention of *sseL* in some subspecies I serotypes with no evidence as yet of lateral gene transfer to other organisms suggests that these serotypes might encounter a similar host environment during infection that requires SseL function for fitness. Whether the addition of *sseL* to the *S. enterica* genome leads to an immediate gain of function or whether its expression evolved over time to be transcriptionally integrated with the SPI2 regulatory cascade is unknown. However, the *sseL* promoter appears to be exquisitely fine-tuned for regulation by SsrB because the deletion of the SsrB regulator silences this gene under conditions that are otherwise activating. SseL was translocated into infected host cells by the SPI2 type III secretion system, and SseL function was shown to be required for full virulence during infections of animals. However, because the *sseL* mutant was not attenuated for replication inside macrophages in vitro, we conclude that SseL function is related to the interaction between *Salmonella* and the diverse host environment, perhaps to subvert the ensuing host response to infection or alter the phenotype of infected cells in a way that promotes host colonization.

In summary, we used transcriptional profiling using DNA microarrays to identify SseL as a new type III secreted effector in *S. enterica* serovar Typhimurium. This effector is integrated into the intracellular SsrB regulatory cascade, where it plays an important role in virulence in the mouse model of *S. enterica* serovar Typhimurium infection. The acquisition of *sseL* by SPI2-containing *S. enterica* serovar Typhimurium appears to have led to increased fitness that facilitated more efficient colonization of host animals. Over evolutionary time, this genetic element likely underwent regulatory fine-tuning to fully integrate into the SsrB regulon for maximal expression in the intracellular environment of the host. Identification of the virulence factors responsible for host disease during *Salmonella* infection provides the platform on which to examine the molecular mechanisms of the host-pathogen interaction. Further studies on this and other effectors will bring to light important insight into how these interactions alter the outcome of disease following infection.

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