sciS, an *icmF* Homolog in *Salmonella enterica* Serovar Typhimurium, Limits Intracellular Replication and Decreases Virulence

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***Salmonella enterica* serovar Typhimurium utilizes macrophages to disseminate from the intestine to deeper tissues within the body. While *S. enterica* serovar Typhimurium has been shown to kill its host macrophage, it can persist intracellularly beyond 18 h postinfection. To identify factors involved in late stages of infection, we screened a transposon library made in *S. enterica* serovar Typhimurium for the ability to persist in J774 macrophages at 24 h postinfection. Through this screen, we identified a gene, *sciS*, found to be homologous to *icmF* in *Legionella pneumophila*. *icmF*, which is required for intracellular multiplication, is conserved in several gram-negative pathogens, and its homolog appears to have been acquired horizontally in *S. enterica* serovar Typhimurium. We found that an *sciS* mutant displayed increased intracellular numbers in J774 macrophages when compared to the wild-type strain at 24 h postinfection. *sciS* was maximally transcribed at 27 h postinfection and is repressed by SsrB, an activator of genes required for promoting intracellular survival. Finally, we demonstrate that an *sciS* mutant is hypervirulent in mice when administered intragastrically. Taken together, these data indicate a role for SciS in controlling intracellular bacterial levels at later stages of infection and attenuating virulence in a murine host**

Salmonella enterica serovar Typhimurium is an enteric pathogen that causes a self-limiting gastroenteritis in humans and a systemic typhoid-like disease in mice. Macrophages are an essential vehicle for the pathogenesis of *S. enterica* serovar Typhimurium, which utilizes them to disseminate from the intestine to the spleen and liver. *S. enterica* serovar Typhimurium survives inside and eventually kills its host macrophage. Much has been published on how *S. enterica* serovar Typhimurium manipulates the macrophage phagosome to facilitate survival (15, 19, 25, 37, 41) as well as how it kills macrophages (2, 5, 17, 20, 27, 39), but little is known about the factors that allow it to persist intracellularly beyond 24 h. This study provides evidence that a horizontally acquired gene, *sciS*, limits intracellular replication of *S. enterica* serovar Typhimurium in macrophages at late stages of infection.

Pathogenicity islands, which are horizontally acquired pieces of DNA that confer virulence traits, are especially crucial for the interaction of *S. enterica* serovar Typhimurium with eukaryotic host cells. *S. enterica* serovar Typhimurium contains two highly studied pathogenicity islands on its chromosome—*Salmonella* pathogenicity islands 1 and 2 (SPI1 and -2)—which encode separate type III secretion systems (TTSS) that facilitate invasion and survival, respectively. In the absence of SPI1, infected macrophages are not killed at early time points (1 to 6 h postinfection) (23, 39). A second killing pathway, mediated by SPI2, results in host cell death at 18 to 24 h postinfection (39). Despite this second killing pathway, bacteria can still persist inside intact macrophages beyond 24 h. To identify factors involved in the long-term persistence of *S. enterica* serovar Typhimurium in macrophages, we isolated mutants

with enhanced survival in macrophages at 24 h postinfection. One of the mutants identified was *sciS*, a homolog of *icmF* that is contained in several gram-negative pathogens.

icmF was first studied in *L. pneumophila*, where it is part of the Dot/Icm cluster of genes that form a type IV secretion system (T4SS) required for host cell killing and intracellular multiplication (29, 34). Three recent studies have shown *icmF* to be critical for allowing *L. pneumophila* to replicate in its own modified vacuole by maintaining an intact T4SS. *IcmF* was shown to be “partially required” for replication in human macrophages and essential for intracellular growth in amoeba (44). Another study showed that DotU and IcmF are required for the formation of replicative vacuoles and the translocation of the T4SS substrate, SidC (40). Additionally, DotU and IcmF serve to prevent degradation of type IV secretion components, indicating a role in stabilizing the T4SS (35).

A conserved cluster of 15 genes surrounding *icmF* in *Vibrio cholerae* has been designated IcmF-associated homologous proteins (IAHP) (9). Gram-negative pathogens containing the *icmF* homolog have from 6 to 14 of the 15 genes in this cluster, but there is some variability in the composition and arrangement between the species. *icmF* in *V. cholerae* is induced under in vivo conditions as measured in a rabbit ileal loop model (8). An *icmF* insertion mutant showed a nearly twofold increase in interleukin-8 mRNA levels in *V. cholerae*-infected intestinal epithelial cells when compared with the wild type (32). The same insertion mutant showed reduced motility, increased adherence to epithelial cells, and a higher conjugation frequency, leading to speculation that it is involved in bacterial cell surface reorganization (7). The importance of *icmF* homologs is highlighted by their conservation in nine different gram-negative pathogenic species (9); however, their exact function remains unclear. Most bacteria that contain an *icmF* homolog are pathogenic and maintain close contact with eukaryotic cells. Therefore, it is likely that *icmF* homologs

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TABLE 1. *S. enterica* serovar Typhimurium strains used in this study

Strain	Description	Reference or source
ATCC 14028	Wild type	ATCC
DP101	14028 <i>invA::cat</i>	This study
DP103	14028 Δ <i>sciS</i>	This study
DP114	DP103 + pEGFP	This study
DP137	14028 <i>sciS-lacZ</i> transcriptional fusion	This study
DP148	14028 + pEGFP	This study
MJW129	14028 <i>ssrB::cat</i>	38

and associated proteins play an important role in bacterial pathogenesis.

The *icmF* homolog in *S. enterica* serovar Typhimurium, *sciS*, named for salmonella centisome 7 island, is located within a 44-kb genomic island, which contains 9 of the 15 IAHP genes. A deletion of the entire island causes a defect in the ability of *S. enterica* serovar Typhimurium to enter Hep-2 cells (12). The only individual genes studied in this island constitute the *Salmonella* atypical fimbrial (*saf*) operon that was not required for mouse virulence (11). The remaining open reading frames in this island have not been characterized, but several encode putative proteins with homology to known virulence proteins.

In this study, we identified *sciS* in a transposon mutant screen and investigated its role in the long-term persistence of *S. enterica* serovar Typhimurium inside macrophages. We determined that *SciS* limits intracellular growth in macrophages only at late stages of infection and attenuates the lethality of *S. enterica* serovar Typhimurium in a murine host. Together, these data constitute a unique example of a horizontally acquired, temporally regulated gene that controls *S. enterica* serovar Typhimurium virulence in mice.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA manipulations. All strains were grown in Luria-Bertani (LB) broth overnight at 37°C. Antibiotics, when added, were used at the following concentrations: chloramphenicol (Cm), 30 µg/ml; kanamycin (Kan), 60 µg/ml; carbenicillin (Carb), 100 µg/ml; and tetracycline (Tet), 20 µg/ml. All strains and plasmids used or constructed in this study are listed in Table 1. The construction of the *sciS* nonpolar deletion (DP103) was performed using the pMAK705 temperature-sensitive plasmid-based system (1). A fragment containing the mutation in *sciS*, plus at least 2 kb of flanking DNA on either side was cloned into suicide plasmid pMAK705. The two fragments were amplified from the chromosome using primers TCTAGAGCATCCAGCTTCATGAATGTCGATGAG and CTGCAGGATACTCAACTGGTCGGCCAC for fragment 1 and primers CTGCAGCAGATCGCTTCGCTGCTGACC and AAGCTTGCA CATAGTCGCGCTGGTCCG for fragment 2. Fragment 1 contains an N-terminal XbaI restriction site and a C-terminal PstI restriction site and ends 1,413 bp into the *SciS* open reading frame. Fragment 2 contains an N-terminal PstI restriction site and a C-terminal HindIII site and begins 2,959 bp into the 3,871-bp *SciS* open reading frame. These two fragments were cloned separately into pCRBlunt (Invitrogen, Carlsbad, CA), excised, and ligated together into pMAK705, creating a 1,545-bp deletion in *sciS*. The resulting plasmid, pMAKf12, was transformed into 14028 at 30°C on LB-Cam plates. Resulting colonies were then selected for growth on Cam plates at the nonpermissive temperature 42°C, which forces the integration of the plasmid into the chromosome via homologous recombination. To select the rare double crossover event, ampicillin enrichment was used to remove those colonies that still contained the plasmid. Any chloramphenicol-sensitive bacteria remaining were then screened via PCR for the correct mutation. The resulting strain, DP103, contains a 1,546-bp nonpolar deletion spanning bp 1413 to 2959 in the 3,870-bp *sciS* open reading frame.

The *sciS* chromosomal *lacZ* fusion (DP137) was also made using pMAK705, but the resulting cointegrate was not resolved, leaving an *sciS* upstream fragment

transcriptionally fused to *lacZ* and an intact *sciS* gene. A DNA fragment covering the promoter region of *sciS* was amplified from the chromosome via PCR using the primers GGTACCCCTGTCAGAGGACAGACTCC and GGATCCCGCGCCCGACAAACCAGAC. The *sciS* promoter fragment spanning positions -1000 through +95 was ligated into the suicide plasmid pSC433(pMAK705::lacZ), generating plasmid pDP136(*sciS::lacZ*). This plasmid was electroporated into 14028, and the resulting colonies were grown at 42°C to select for single crossovers into the chromosome. The resulting chromosomal construct was transferred into a new 14028 background by P22 transduction. The resulting strain was confirmed via PCR before use in intracellular β -galactosidase assays.

Eukaryotic cells and infection procedure. The murine macrophage cell line, J774A0.1 (American Type Culture Collection [ATCC], Manassas, Va.), was maintained in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL, Rockville, Md.) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 0.2 mM MEM sodium pyruvate (Gibco-BRL), and 0.1 mM nonessential amino acids (Gibco-BRL). J774 macrophages in tissue culture plates were infected with overnight cultures grown rolling at 37°C. Prior to infection, cultures were adjusted to an optical density at 600 nm (OD₆₀₀) of 1.0 and resuspended in phosphate-buffered saline (PBS). After the addition of bacteria, the plates were centrifuged at 25°C for 5 min and placed in a 37°C incubator with CO₂ for 25 min. Cells were then washed three times with PBS to remove extracellular bacteria, and fresh DMEM containing 100 µg/ml gentamicin was added for 1 h at 37°C to kill extracellular bacteria. The plates were washed once with PBS and then incubated with 20 µg/ml gentamicin at 37°C.

Selection and screen for persistent mutants in J774 macrophages. A mutant bank was generated in DP101 (*invA::cam*) using MudJ transposon mutagenesis with P22 (21). The bank was plated on large M9 minimal media agar plates to reduce the number of auxotrophs. Approximately 50,000 mutants were generated on 12 separate plates. Each plate was scraped, and the bacteria resuspended in 2 ml of LB with 10% glycerol and frozen at -80°C. An overnight culture was grown from each pool and J774 macrophages in 6-well plates were infected (as described above) at a multiplicity of infection (MOI) of 1 bacterium to 10 macrophages. After 24 h of infection, the 12 infected wells were washed with PBS and the remaining macrophages were lysed with 1% Triton X-100 (Sigma Chemical, St. Louis, MO). These lysates were used to start overnight cultures, and 12 new wells were infected. This process was repeated three times to enrich for mutants that remained inside the macrophage. Four of the 12 pools showed a sharp increase in bacterial numbers after three passages. Individual mutants from these pools were scored for cytotoxicity on J774 macrophages in 96-well plates. Three candidate mutants from each of the four pools were selected and their MudJ insertions were sequenced by inverse PCR, and the results are listed in Table 2.

Cytotoxicity assays. These assays were performed as previously described by van der Velden et al. (39). Briefly, J774 macrophages seeded in 96-well plates were infected with overnight stationary-phase cultures of *S. enterica* serovar Typhimurium at an MOI of approximately 50. At the indicated time points, the supernatant was removed and used to determine the release of cytoplasmic lactate dehydrogenase (LDH) using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). Cytotoxicity was determined for each strain

TABLE 2. MudJ transposon insertions from selection for persistent mutants

Pool no.	Gene description	STM gene no. ^b
4	ND ^a	ND
4	<i>mutH</i>	3005
4	<i>mutH</i> (sibling)	3005
6	<i>pduO</i>	2050
6	<i>pduO</i> (sibling)	2050
6	<i>pduO</i> (sibling)	2050
9	<i>ycfO</i> putative glycosyl hydrolase	1209
9	<i>sciS</i>	0285
9	<i>umuD</i> homolog	1998
11	<i>mutH</i>	3005
11	<i>mutH</i> (sibling)	3005
11	ND	ND

^a ND, no data as the insertion was not retransduced or sequenced.

^b Numbers assigned to genes in the published sequence from *S. enterica* serovar Typhimurium strain LT2 by the Washington University, St. Louis, sequencing project.

by calculating the LDH released as a percentage of the maximal release from lysed macrophages.

Microscopy. J774 macrophages were grown on glass coverslips placed in 6-well plates prior to infection with *Salmonella* strains containing the plasmid pEGFP. Cells were infected at an MOI of 80 or 10. After 24 h of infection, cells were washed with PBS and fixed with 3.6% paraformaldehyde in PBS for 15 min at 25°C. The coverslips were then stained with either 4',6-diamidino-2-phenylindole (DAPI; Sigma Chemical) alone or with FM4-64 (Molecular Probes, Eugene, OR) and 4N,6N-diamidino-2-phenylindole at 25°C for 1 h. Ten fields were randomly chosen for each coverslip, and images were taken using an Applied Precision Image Restoration System (Advanced Precision Instruments, Issaquah, WA). All images were taken at 60×. Image stacks of 5 steps spaced 0.2 microns apart were taken and deconvolved. Selected images were saved in TIFF format and imported into Adobe Photoshop to be formatted for publication. The number of bacteria per infected macrophage was counted double blind for each field.

Intracellular β -galactosidase assays. Activity of β -galactosidase was assayed as described previously (26). To measure the β -galactosidase activity from intracellular bacteria, macrophages were grown in 6-well plates and infected with DP137 at an MOI of 50, as described above. Cells were collected from 3 wells and lysed with 1% Triton X-100 at the indicated time points. Lysates were serially diluted and plated to determine the number of intracellular bacteria. β -Galactosidase activity was expressed in Miller units per 10^9 bacteria.

Screen for regulators of *SciS*. A modified tetracycline-inducible transposon (T-POP) was constructed by replacing the original *Tn10* ends with *Tn5* ends (30). The T-POP transposon used the primers 5'-CAGCTGTCTCTTATACACATC TCCATTAAGGTTACCATCAGGA-3' and 5'-CAGCTGTCTCTTATACAC ATCTGTGATCTCGGGAAGCGTGGTGA-3'. The resulting fragment was cloned into the pCR2.1-TOPO (Invitrogen) to yield a vector containing the *Tn5* T-POP. The transposon was excised from the vector with *PvuII* and purified. This *Tn5* T-POP transposon was used to mutagenize the *sciS::lacZ* strain (DP137). A complex containing the *Tn5* T-POP transposon and purified transposase was electroporated into electrocompetent DP137. Transposition of the transposon then occurred randomly within the bacterial chromosome. Bacteria containing transposon insertions were then selected by plating on tetracycline and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) plates. Those mutations that caused a color change from DP137 (light blue) were transduced into DP137 and retested for color change. Chromosomal DNA was purified from those colonies that retained a color change and the transposon insertion site was sequenced.

Real-time PCR analysis. Total RNA from *S. enterica* serovar Typhimurium-infected J774 macrophages (MOI of 50) was isolated using hot phenol extraction and cleaned with an RNeasy Mini column (QIAGEN, Valencia, CA) and used as a template to make cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR was performed on the cDNA using primers to *sciS* and *gyrB* as a control. *rpoD* was initially used as a control in our real-time PCR assays, as this gene has been established as a steadily transcribed housekeeping gene for use as an internal control (33). However, the primers to this gene seem to cross-react with eukaryotic cDNA, so *rpoD* could not be used as an internal control for real-time PCR performed on cDNA from infected macrophages. *gyrB* was suggested as a housekeeping gene to use as an internal control (personal communication with Ferric Fang). Primers to *gyrB* were tested in real-time PCR experiments on cDNA from bacterial cultures in parallel with *rpoD* primers and both genes were transcribed equally in all conditions tested. Each real-time PCR was run in quadruplicate and values were expressed as fold induction to *gyrB*. The fold induction of wild-type-infected macrophages at 5 h was set to 1. Values are the average of three separate experiments.

Mouse studies. Mice were inoculated intragastrically with 200 μ l of an overnight culture of either 14028 or $\Delta sciS$ that was resuspended and diluted in PBS. The following doses were given to groups of 4 mice: 3.1×10^4 , 3.1×10^5 , 3.1×10^6 for 14028 and 3.3×10^4 , 3.3×10^5 , 3.3×10^6 for $\Delta sciS$. This experiment was repeated with groups of 8 mice with the following doses: 3.64×10^4 , 3.64×10^5 , 3.64×10^6 for 14028 and 3.70×10^4 , 3.70×10^5 , 3.70×10^6 for $\Delta sciS$. Mice were monitored for 28 days for survival, and 50% lethal dose (LD_{50}) values were calculated according to the method of Reed and Muench (31).

RESULTS

A selection and screen for mutants that persist in macrophages identified *sciS*. To investigate the contribution of *S. enterica* serovar Typhimurium genes at late stages of infection, MudJ transposon mutants that survived in J774 macrophages

FIG. 1. An *sciS* mutant displays increased intracellular numbers at late stages of infection in J774 macrophages but shows no difference in cytotoxicity. (A) Cells were infected with 14028 (diamonds) and DP103 (squares) at an MOI of 35. At the indicated time points, the macrophages were lysed with 1% Triton X-100 and intracellular bacterial numbers were determined. Each data point is the average of three individual wells. The asterisks indicate a *P* value of <0.10 as measured by a Student's *t* test. (B) Cytotoxicity to J774 macrophages by 14028 or *sciS* was quantified by measuring LDH release at 6 and 24 h postinfection. The differences between the strains in panel B were not statistically significant. Data from panels A and B are the average of three independent experiments with each time point measured in triplicate. Error bars indicate the standard deviations of the mean. w/t, wild type.

for 24 h without causing host cell lysis were selected. The bacteria used to infect the macrophages were from a bank of 50,000 MudJ transposon mutants divided into 12 separate pools. To prevent early killing of the macrophages, mutants were generated in a strain deleted for *invA*. The SPI1 TTSS has been shown to induce rapid apoptosis in macrophages, and *InvA* is required for its formation (24, 38). J774 macrophages were infected at an MOI of 0.1, and at 25 min postinfection gentamicin was added to the extracellular media to eliminate those bacteria that didn't invade or escaped their host cell via lysis. By infecting at an MOI of 0.1, the majority of infected macrophages would contain a homogeneous population of rep-

FIG. 2. An *sciS* mutant is more numerous in J774 macrophages at 24 h postinfection. These images are representative fields from experiments performed at two separate MOIs. (A and B) Macrophages were infected on glass coverslips in 6-well plates for 24 h at an MOI of 80 and stained with DAPI (DNA stain; blue). (C and D) Macrophages were infected on glass coverslips in 6-well plates for 24 h at an MOI of 10 and stained with DAPI and FM4-64 (membrane stain; red). Macrophages were infected with 14028 (A and C) and $\Delta sciS$ (B and D). (E) The average number of bacteria per infected cell for 10 individual fields ($P < 0.01$, Student's *t* test). Data from the graph in panel E represent results from three separate experiments. w/t, wild type.

licating bacteria derived from a single progenitor. Following 24 h of infection, the remaining macrophages were lysed with 1% Triton X-100 and the intracellular bacteria were recovered, used to start an overnight culture and infect new macrophages.

This process of serial passaging was repeated three times, enriching the pools for persistent mutants. Four of the original 12 pools showed an increase in bacteria recovered from infected macrophages after three passages. Individual mutants

FIG. 3. *sciS* is maximally expressed in host macrophages at 27 h postinfection. J774 macrophages were infected with DP137 at an MOI of 50. Intracellular β -galactosidase activity was determined at the indicated time points. Miller units are expressed per 10^9 intracellular bacteria. In vitro cultures of DP137 showed β -galactosidase activity below background. Data shown represent arithmetic means of three independent experiments. Error bars indicate the standard deviations of the mean.

from each of these four “enriched” pools were then isolated and screened in J774 macrophages for persistence. Three MudJ insertions from each pool were sequenced giving a total of 12 individual sequences (Table 2). These sequences included *sciS* as well as genes involved in DNA repair and propanediol metabolism. Based on its homology to a known virulence gene in *L. pneumophila* and its location in a genomic island, *sciS* was chosen for further characterization.

An *sciS* mutant is more numerous in J774 macrophages at 24 h postinfection. Surprisingly, none of the mutants isolated had measurable defects in cytotoxicity on J774 macrophages (data not shown) as measured by LDH release and crystal violet staining (39). Because the mutants were cytolytically normal, their persistence suggested that they had differences in intracellular numbers that led to their selection. Mutations that caused reduced intracellular replication could lead to an inability to break out of the phagosome, thus leading to a persistent state that would allow their selection. Conversely, mutations that cause overgrowth could be preferentially selected due to a higher representation of this population. To determine the effect of an *sciS* mutation on intracellular bacterial numbers we constructed a nonpolar deletion of *sciS* (DP103) and infected J774 macrophages to determine its effect on intracellular replication. We used two methods to enumerate intracellular bacteria: (i) quantitation of intracellular bacteria and (ii) visual inspection of bacteria per infected macrophage via microscopy. The first method is standard for measuring intracellular replication and involves lysing macrophages at 24 h postinfection and plating intracellular bacteria. The wild-type strain and DP103 had similar intracellular numbers of viable bacteria at 6 h postinfection. However, *Salmonella* 14028-infected macrophages contained two- to threefold less CFU than DP103-infected macrophages past 24 h postinfection (Fig. 1A). DP103 was also retested for cytotoxicity on J774 macrophages and showed no significant difference from wild type at 6 or 24 h postinfection (Fig. 1B). To more directly

visualize what was occurring in individual host cells, wide-field deconvolution fluorescence microscopy was used to enumerate intracellular bacteria. For these studies, 14028 and DP103 were transformed with the green fluorescent protein (GFP)-containing plasmid pEGFP, resulting in strains DP148 and DP114, respectively. J774 macrophages were infected with either DP148 or DP114 for 24 h, fixed, and then stained with DAPI (DNA; blue) and FM4-64 (membrane; red). Microscopy experiments were then performed using MOIs of 10 and 80 (Fig. 2A to D). In each experiment, the number of bacteria per infected cell was determined double blind for each strain in 10 separate fields (Fig. 2E). The results show that macrophages infected with an *sciS* mutation at an MOI of 10 had 63% more intracellular bacteria than the wild type at 24 h postinfection, while an MOI of 80 showed a 94% increase. All experimental MOIs in this study fall within the range of 10 to 80 established with the microscopy results. Differences in intracellular bacterial numbers were not evident between the strains at 6 h postinfection, as macrophages infected with an *sciS* mutation showed 7.9 bacteria per infected cell versus 8.4 for wild type at an MOI of 80 (data not shown). However, the *sciS* mutant had significantly increased intracellular bacterial numbers when measured at 24 h postinfection, correlating well with the selection procedure.

***sciS* is maximally expressed in host macrophages at 27 h postinfection.** The difference in intracellular numbers at late but not early time points suggested that *sciS* might not be expressed until late during the infection process. To investigate the temporal expression of *sciS* inside cells, we constructed an *sciS* promoter fusion to *lacZ* (DP137). Macrophages were infected with DP137 and β -galactosidase activity was measured at seven time points between 10 and 34 h postinfection (Fig. 3). In vitro, *sciS* was transcribed at undetectable levels above background; it was also minimally transcribed inside macrophages before 10 h postinfection (data not shown). *sciS* induction is markedly different than that of SPI2 genes as expression levels for seven SPI2 genes encoding effectors or structural proteins have either peaked or leveled off at 8 h postinfection (6). We found that *sciS* was maximally transcribed inside macrophages at 27 h postinfection. The timing of *sciS* transcription correlates well with the finding that an *sciS* mutant selected at 24 h postinfection shows increased intracellular numbers at the same time but not at 6 h postinfection. To verify this finding, we isolated total RNA from 14028-infected macrophages at 5 and 24 h postinfection. We measured *sciS* mRNA levels using quantitative real-time PCR. We found that *sciS* mRNA levels were sevenfold higher at 24 h than at 5 h postinfection, in agreement with our previous observations (Fig. 4).

SsrB negatively regulates *SciS*. The late transcription of *sciS* in host cells raised questions as to how it was regulated. To identify genes that affected expression of *sciS*, DP137 (*sciS*::*lacZ* promoter fusion) was mutagenized with a modified T-POP transposon. The 6,000 colonies from the mutagenesis procedure were screened for differences in transcription of *sciS* from the parental strain. The parental strain (DP137) appears light blue on an LB plate containing X-Gal, so those colonies that were white or dark blue were selected for further analysis. A dark blue colony was isolated containing a transposon insertion within *ssrB*. It was predicted to be a negative regulator of *sciS*, as the transposon insertion within *ssrB* leads

FIG. 4. *sciS* is more highly transcribed at 24 h postinfection and is negatively regulated by SsrB. *sciS* RNA levels were quantified via real-time PCR with total RNA isolated from wild-type- and Δ ssrB-infected J774 macrophages (MOI = 50). Values were normalized to the levels of *gyrB* RNA and subsequently to the 5-h time point from the wild-type-infected J774s. *sciS* is 7.5-fold more highly transcribed at 24 h postinfection than at 5 h. An *ssrB* mutant shows a 5.5-fold increase in *sciS* message at 5 h postinfection over the wild-type strain. Data shown represent arithmetic means of three independent experiments. Error bars indicate the standard deviations of the mean. w/t, wild type.

to increased expression of *sciS*. To verify this, real-time PCR was performed on total RNA isolated from macrophages infected with either an *ssrB* mutant or wild-type *S. enterica* serovar Typhimurium at 5 h postinfection. There were not sufficient numbers of intracellular bacteria at 24 h postinfection to provide the RNA necessary to do real-time PCR, as an *ssrAB* mutation impairs intracellular replication in macrophages (6). Real-time PCR showed a fivefold increase in *sciS* transcription in an *ssrB* mutant when compared to wild type at 5 h postinfection, confirming that SsrB negatively regulates *sciS* (Fig. 4). However, it is not known if SsrB directly or indirectly regulates *sciS*.

A *sciS* mutant is hypervirulent in mice. Having shown that *sciS* is important in *S. enterica* serovar Typhimurium pathogenesis of tissue culture macrophages, we tested its contribution to *S. enterica* serovar Typhimurium virulence in an in vivo mouse model. Groups of eight BALB/c mice were injected intragastrically with either 14028 or DP103(Δ *sciS*) and monitored for survival over a 28-day period. Mice began dying at approximately the same day, regardless of the inoculating strain; however, only half of the wild-type inoculated mice had died by day 9 while all of the DP103 inoculated mice died by day 11 (Fig. 5). The LD₅₀ calculated for DP103 was eightfold lower than that of 14028, indicating that the mutant was hypervirulent. This experiment was also done with groups of 4 mice, showing a ninefold reduction in LD₅₀ for DP103 (data not shown). This was an unexpected result, given that only two other mutations in *S. enterica* serovar Typhimurium (*grvA*, *pcgL*) have shown an increase in virulence (18, 28).

DISCUSSION

To identify genes associated with the later stages of *S. enterica* serovar Typhimurium infection of macrophages, mutants

were selected that persisted inside macrophages at 24 h postinfection. This selection isolated a gene, *sciS*, which limits replication in host macrophages and decreases virulence in mice. The increase in bacterial numbers seen with an *sciS* mutant at 24 h postinfection was not observed in macrophages at 6 h postinfection or in epithelial cells at any time. In addition to *sciS*, several other genes were identified in this study that were primarily involved in DNA repair and propanediol metabolism. These genes were most likely selected because the mutations attenuated bacterial growth, preventing them from lysing their host cells.

There are homologs of *sciS* in several other gram-negative pathogens, but the first identified and best characterized is *icmF*

FIG. 5. An *sciS* mutant is hypervirulent in BALB/c mice when administered intragastrically. Groups of 8 mice were inoculated with either 14028 or DP103 and monitored for 28 days. The graphed results are from the groups inoculated with the 10⁶ doses.

in *L. pneumophila*. Recently, *icmF* was isolated in a screen for mutants that have a growth defect because of an inability to lyse out of their host macrophage at the end of an infection cycle (40). It is interesting that *icmF* was isolated in much the same way that *sciS* was identified by enriching pools of mutagenized bacteria in macrophages with gentamicin in the extracellular media. Previous studies have implicated *icmF* as being partially required for survival in human macrophages, which would seem to contradict the phenotype for *sciS* reported in this study. However, differences in the way the multiplication assays are performed can explain this discrepancy. Multiplication assays performed in *L. pneumophila* typically measure numbers of bacteria in the supernatant, while replication assays in *S. enterica* serovar Typhimurium typically measure only the bacteria residing intracellularly. A delay or inability of bacteria to escape their host phagosome would result in a decreased bacterial load in the supernatant. It is tempting to speculate that *sciS* could play this role in *S. enterica* serovar Typhimurium, and this would help explain the increased bacterial loads in infected macrophages. While there is an abundance of studies on *S. enterica* serovar Typhimurium-induced host cell killing, egress of the bacteria from the phagosome and events during the latter stages of infection of macrophages have not been studied extensively.

sciS was induced only in host cells and its expression peaked at 27 h postinfection, correlating well with the selection procedure and the increased bacterial loads seen only at 24 h of infection. Interestingly, *sciS* transcription is negatively regulated by SsrB, a two-component regulator that activates salmonella pathogenicity island 2 (SPI2) genes and additional genes outside of SPI2 that are primarily involved in promoting systemic infection of the host (4, 6, 13, 41, 43). Soon after uptake of *S. enterica* serovar Typhimurium in macrophages, SsrB is expressed and induces several proteins that facilitate intracellular replication (10, 36, 42). *ssrB* RNA levels are reduced later during infection of macrophages (J. Rue, unpublished data), leading to the derepression of *sciS* at 24 h postinfection. The early expression of SsrB and its subsequent downregulation possibly could help explain the delayed expression of *SciS*. Previously reported SsrB-regulated genes are activated, making this the first evidence for SsrB-mediated repression. However, it isn't clear if repression of *sciS* by SsrB is direct or if there are intermediate factors involved. SsrB facilitates *S. enterica* serovar Typhimurium survival and replication inside macrophages following uptake, and perhaps its subsequent downregulation allows *sciS* to be expressed, limiting the bacteria from overgrowing their host cell or allowing for escape from the vacuole.

To our knowledge, *sciS* is the first example of a gene involved in limiting intracellular replication of *S. enterica* serovar Typhimurium in macrophages. Previously, several genes were identified that prevent overgrowth of *S. enterica* serovar Typhimurium in fibroblasts (3). The master regulator PhoP-PhoQ had the most profound effect on controlling replication, which was unexpected because in other cell types, PhoP-PhoQ is associated with promoting survival and replication. These differences in replication were measured at 24 h postinfection, paralleling what was seen with *sciS* in macrophages. While *S. enterica* serovar Typhimurium replicates more efficiently in macrophages than in fibroblasts, our results provide evidence

that *sciS* is involved in attenuating bacterial growth in host macrophages at later stages of infection.

It is important for pathogens to limit their effects upon the cells they infect in order to achieve a balance with their host. An example of this phenomenon on a cellular level of *S. enterica* serovar Typhimurium pathogenesis is demonstrated with its invasion process. *S. enterica* serovar Typhimurium injects SopE and SopE2 into epithelial cells to activate Cdc42 and Rac1 which induces ruffling and promotes uptake of the bacteria into host cells (16). Another secreted protein, SptP, reverses these effects to restore a normal actin cytoskeleton once the bacterium is inside of the cell (14). These proteins are delivered simultaneously in equal amounts; however, the SptP protein is degraded much more slowly than SopE, allowing it to reverse the effects of SopE (22). It is interesting that this process is temporally regulated, as a process set in motion by the bacterium can be purposely modulated at a later time. This is a recurring theme in pathogenesis and one that is further illustrated with *sciS*.

The attenuation of deleterious effects upon a host cell can be further extended to an animal model, as a *sciS* deletion showed an eightfold increase in lethality with a mouse model. This hypervirulent phenotype in mice was initially a surprise, given that almost all known *S. enterica* serovar Typhimurium mutations reduce virulence. Only two other *S. enterica* serovar Typhimurium mutations have been shown to increase virulence in mice (18, 28). A null mutation in *grvA* increased virulence, as measured by competitive index experiments in mouse spleens and small intestine. Interestingly, *grvA* is carried on the lambdaoid phage Gifsy-2 near *srfH*, which was shown to be activated by the SsrAB regulon (42). However, it is not known whether SsrB regulates *GrvA*. Another study determined that inactivation of the dipeptidase *pcgL* led to an accumulation of D-Ala-D-Ala, which caused an increase in bacterial numbers in mouse liver and spleen 24 h postinoculation. The occurrence of the phenotype within 24 h of inoculation suggested that the accumulation of D-Ala-D-Ala somehow compromised the innate immune system leading to faster bacterial growth in host tissues. Both of these studies demonstrate that it is possible for an inactivated gene to lead to an increase in bacterial numbers in host tissues. Increased bacterial loads in a murine host would likely lead to more rapid sepsis and toxic shock, thus increasing lethality. The increased intracellular numbers observed in an *sciS* mutant appear to be consistent with this idea, as an *sciS* mutant is hypervirulent in orally infected mice. Most studies in bacterial pathogenesis are directed toward finding genes that promote virulence in the host. *sciS* is a unique example of a horizontally acquired gene that is temporally regulated and limits *S. enterica* serovar Typhimurium virulence.

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