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Type VI Secretion System-Associated Gene Clusters Contribute to Pathogenesis of *Salmonella enterica* Serovar Typhimurium

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The enteropathogen *Salmonella enterica* serovar Typhimurium employs a suite of tightly regulated virulence factors within the intracellular compartment of phagocytic host cells resulting in systemic dissemination in mice. A type VI secretion system (T6SS) within *Salmonella* pathogenicity island 6 (SPI-6) has been implicated in this process; however, the regulatory inputs and the roles of noncore genes in this system are not well understood. Here we describe four clusters of noncore T6SS genes in SPI-6 based on a comparative relationship with the T6SS-3 of *Burkholderia mallei* and report that the disruption of these genes results in defects in intracellular replication and systemic dissemination in mice. In addition, we show that the expression of the SPI-6-encoded Hcp and VgrG orthologs is enhanced during late stages of macrophage infection. We identify six regions that are transcriptionally active during cell infections and that have regulatory contributions from the regulators of virulence SsrB, PhoP, and SlyA. We show that levels of protein expression are very weak under *in vitro* conditions and that expression is not enhanced upon the deletion of *ssrB*, *phoP*, *slyA*, *qseC*, *ompR*, or *hfq*, suggesting an unknown activating factor. These data suggest that the SPI-6 T6SS has been integrated into the *Salmonella* Typhimurium virulence network and customized for host-pathogen interactions through the action of noncore genes.

Protein secretion is an essential determinant of the virulence of pathogenic bacteria. Multiple systems have evolved in order to secrete proteins into the extracellular environment or across the membranes of target cells (18). In Gram-negative *Proteobacteria*, these systems include the well-characterized type III secretion system (T3SS) and T4SS that permit the translocation of protein effectors from the bacterial cytoplasm directly into target cells to modulate the host environment (8, 25). An additional type of protein secretion system involved in protein translocation, known as a T6SS, has been found to contribute to interactions between bacteria and both bacteria and eukaryotic cells, including unicellular and multicellular eukaryotes (47). This system employs an assortment of membrane-associated proteins in order to coordinate the localization and assembly of the system; however, the secretory apparatus itself is composed of proteins that are structurally analogous to that of an inverted contractile bacteriophage tail (1, 29, 39). This coordinated contractile tail is utilized to deliver effector proteins to targets including the actin cytoskeleton in eukaryotic cells and peptidoglycan in bacterial cells (41, 45).

Salmonella enterica subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) employs two well-characterized T3SSs to manipulate host cells through effector translocation in order to invade gut epithelial cells and disseminate systemically within phagocytic cells (18, 20). Most serovars of *S. enterica* subsp. *enterica* also encode a T6SS within *Salmonella* pathogenicity island 6 (SPI-6), while two other classes of T6SS have been described in *S. bongori* and *S. enterica* subsp. *arizonae* at SPI-2 and SPI-6, respectively (5, 17, 46). The *S. enterica* subsp. *enterica* T6SS was first described as part of the *S. enterica* centisome 7 genomic island (SCI) as a contributing factor in eukaryotic pathogenesis (16). Aside from the T6SS, SCI also contains the adhesin and invasin PagN, the fimbrial gene cluster *safABCD*, and the transcriptional regulator *sinR* (15, 26). Deletion of SCI resulted in an approximately 50% reduction in internalization of HEp-2 cells that could not be complemented by PagN expression; however, complementation with *safABCD* or *sinR* was not tested (16). Disruption of SPI-6 has also been found to result in a defect in systemic dissemination in orally infected BALB/c mice (19). In support, a number of SPI-6 T6SS genes have been implicated in long-term persistence in macrophages and mice through transposon mutagenesis; however, these transposon mutations have not been verified by precise deletions (9, 24, 28). Contrasting with these reports, another study described a hypervirulence phenotype in BALB/c mice upon deletion of the core structural gene *sciS* and found an increase in the number of bacteria within macrophages after 6 h, suggesting a time-dependent effect on replication (38). Further supporting a role in interactions with macrophages, genome-wide transcriptional profiling of *S.* Typhimurium during macrophage infections found that many of the SPI-6 T6SS genes were upregulated during macrophage infection although an increase over time between 4 and 12 h was not observed (21). An earlier gene expression profiling study also observed a trend toward the increased expression of some of these genes; however, statistical significance was not met (13). A mechanism leading to upregulation in this environment is unknown; however, an *in vivo* host-dependent activation mechanism has been previously described for the T6SS of *Vibrio cholerae* which requires endocytosis by phagocytic cells (32).

Distinct classes of T6SS maintain a core set of 13 genes required for localization of the system to the bacterial inner membrane and for functional secretion (7). T6SSs employ the proteins encoded by these genes to coordinate the secretion of protein substrates via

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the assembly, contraction, and disassembly of a phage-like tail (1, 43). For some T6SS-harboring organisms, the core gene encoding the tailspike protein encodes a fusion protein that allows the translocation of an effector domain (43). However, in *S.* Typhimurium and other T6SS-harboring organisms, no evolved tailspike protein is present (5). In addition to the core genes, organisms that possess a T6SS typically encode less-well-conserved, T6SS-associated genes that differ extensively in their number and location throughout the genome, and some of these genes have been reported to act as effector or regulatory proteins (2, 14, 43). Recently, a set of genes encoding T6SS-secreted toxin proteins were identified in *Pseudomonas aeruginosa* that are involved in interbacterial competition (45). While *S.* Typhimurium contains a number of noncore T6SS-associated genes, their role in the pathogenesis of the host small intestine or intracellular environment is poorly understood (5).

Noncore T6SS genes are known to play a role in the integration of T6SS into global regulatory networks. In *V. cholerae*, some strains constitutively express T6SS-associated proteins while others do not, suggesting niche adaptation (43). Regulatory control at the transcriptional level by the activator of alternative sigma factor 54, VasH, has been observed to occur in *V. cholerae* (23). In addition, posttranslational regulation by a T6SS-encoded threonine phosphorylation pathway has been described for the T6SS of *P. aeruginosa* (36). The noncore T6SS-associated gene TagR has been characterized as acting as a posttranslational repressor of this phosphorylation-dependent mechanism (50). Cues from within the host intracellular environment likely play a role in the regulation of the *S.* Typhimurium T6SS, as it has been determined that transcription of *sciS* increases approximately 16 h after the invasion of macrophages, and transcript levels are negatively correlated with the activity of the two-component response regulator of the SPI-2 T3SS, SsrB (38). Two-component signaling plays a major role in the regulation of virulence in *S.* Typhimurium, and these systems may regulate T6SS activity in concert with noncore T6SS genes, as is the case for the sensor kinases LadS and RetS in *Pseudomonas syringae* (44). A hallmark of T6SS activity has been the stable expression of phage-like proteins Hcp and VgrG, which form the contractile sheath and tailspike, respectively (with the ortholog of the latter annotated as VrgS in *S.* Typhimurium). The expression of these proteins in some organisms is absent under experimental conditions, likely due to the absence of inducing stimuli. This can be circumvented by the use of strains with constitutive T6SS activity, as in the case of a *retS* mutant of *P. syringae* or the V52 *V. cholerae* strain (43, 44). Whether or not stable expression and secretion of the Hcp and VrgS proteins occur under *in vitro* or *in vivo* conditions in *S.* Typhimurium is unknown. However, plasmid-based overexpression of the *hcp* ortholog in the SPI-6 T6SS of *S.* Typhi results in secretion *in vitro*, suggesting a functional system that can contribute to bacterial pathogenesis (53).

In this study, we determined that both the SPI-6 core T6SS and associated genes contribute to systemic dissemination during *in vivo* infections of mice and contribute to intracellular replication in macrophages. We used cell infections to determine that the Hcp and VrgS proteins are most highly expressed during late stages of infection of macrophages. We identified six transcriptionally active regions within the SPI-6 T6SS, three of which receive transcriptional input from SsrB, PhoP, and SlyA during growth *in vitro*. We then showed that expression of the Hcp and VrgS proteins *in vitro* does not change significantly upon the deletion of these and other key virulence regulatory proteins, suggesting that the induction of this system may rely on an unknown activator specific to the T6SS *in vivo*.

MATERIALS AND METHODS

Ethics statement. All experiments with animals were conducted according to guidelines set by the Canadian Council on Animal Care. The Animal Review Ethics Board at McMaster University approved all of the protocols developed for this work.

Informatics. Genome sequences were retrieved from NCBI GenBank (*S. enterica* subsp. *enterica* serovar Typhimurium strain LT2 [accession number AE006468.1], *Burkholderia mallei* ATCC 23344 [accession number NC_006349.2], and *Burkholderia pseudomallei* K96243 [accession number NC_006351.1]) and the Wellcome Trust Sanger Institute (*S. enterica* subsp. *enterica* serovar Typhimurium strain SL1344). BLASTP searches were performed online using the NCBI BLAST server against the nonredundant protein sequence database, and protein sequences were obtained from the *S.* Typhimurium strain LT2 genome file NC_003197.faa. The noncore gene search analysis employed an expect threshold of 1, and the number of target sequences was limited to 1,000. A custom Python script was used to parse the BLAST results for organisms with at least two of the proteins queried that did not belong to the genus *Salmonella*.

Strains and growth conditions. All of the *S. enterica* strains used in this study for experimental work were *S. enterica* subsp. *enterica* serovar Typhimurium strain SL1344 derivatives. Constructs were designed using the *S.* Typhimurium LT2 genome and tested in *S.* Typhimurium SL1344. These genomes differ within the SPI-6 T6SS region by a silent mutation in *sciG*, an M1175T mutation in *sciS*, an in-frame 5-amino-acid mutation (A50 to L54) in *sciI*, and an in-frame 166-amino-acid mutation (V620 to Q786) in *rhs1*. None of these mutations are expected to have affected the results of experiments. The media employed included LB broth (1% [wt/vol] tryptone, 1% [wt/vol] sodium chloride, 0.5% [wt/vol] yeast extract); acidic, low-phosphate, low-magnesium (LPM) minimal medium [5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 80 mM morpholinepropanesulfonic acid, 0.3% glycerol, 0.1% Casamino Acids, 24 μM $MgCl_2$, 337 μM PO_4^{3-}]; M9 minimal medium (5 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.6 mM NH_4Cl, 11.1 mM glucose, 2 mM $MgSO_4$, 100 μM $CaCl_2$, 0.1% Casamino Acids, pH 7.4); and Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies). Bacteria were grown at 37°C with shaking at 225 rpm.

Construct inserts were amplified from *S.* Typhimurium SL1344 genomic DNA using platinum *Taq* high-fidelity DNA polymerase (Life Technologies), purified on PCR purification spin columns (Qiagen), digested using restriction endonucleases (NEB), and ligated using T4 DNA ligase (NEB). Constructs were propagated through *Escherichia coli* DH5α and electroporated into *S.* Typhimurium SL1344 competent cells. Integrated chromosomal transcriptional fusion reporters were generated by cloning the intergenic regions of interest into integrative plasmid pIVET5n and conjugating these into *S.* Typhimurium SL1344 through *E. coli* SM10 lambda *pir* (10). Deletion mutants were generated using the lambda red mutagenesis method (12). Hemagglutinin (HA) tags were introduced into the chromosome using a modified lambda red mutagenesis method (52). Lambda red cassettes were amplified using platinum *Taq* DNA polymerase (Life Technologies) from template plasmid pKD3 (chloramphenicol [Cm] replacement), pKD4 (kanamycin [Kn] replacement), pSUB314 (Cm-marked HA fusion), or pSUB315 (Kn-marked HA fusion), purified on PCR purification spin columns (Qiagen), and electroporated into water- and glycerol-washed *S.* Typhimurium SL1344 competent cells containing the helper plasmid pKD46 (12, 52). Plasmid-based luciferase transcriptional fusion reporters were generated by cloning the intergenic regions of interest into the pGEN-luxCDABE plasmid (27). Flag epitope expression constructs were generated by cloning the indicated genes into the pFLAG-CTC expression vector (Sigma) and in-

duced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and secretion assays were performed with LPM minimal medium as previously described (11). Briefly, after 6 h of growth in LPM minimal medium, bacteria were pelleted at 10,000 × *g*, medium was removed and filtered through a 0.2-μm acrodisc syringe filter (PALL), trichloroacetic acid was added to 10%, and the mixture was incubated on ice at 4°C overnight. Samples were pelleted at 18,000 × *g* for 30 min at 4°C, washed with 1 ml acetone, and pelleted again at 18,000 × *g* for 30 min, and the pellet was allowed to air dry. The pellet was then resuspended in an optical density at 600 nm (OD_{600})-normalized volume of 2× SDS loading dye. For *sciG* complementation, *sciG* and its upstream region were cloned into low-copy-number pWSK29 plasmid (54). The following antibiotics were added to the medium when necessary unless otherwise specified: ampicillin (100 μg/ml), Cm (34 μg/ml), Kn (50 μg/ml), streptomycin (50 μg/ml), and gentamicin (10 or 100 μg/ml). All of the strains and plasmids employed here are listed in Table S1 in the supplemental material. All of the primers used to generate constructs are listed in Table S2 in the supplemental material.

Mouse infections. Bacteria from stationary-phase LB cultures were washed and diluted in inoculation buffer (0.1 M HEPES buffer, 0.9% NaCl), and 10- to 15-week-old female C57BL/6 mice were infected orally with 100 μl of 2 × 10^7 CFU/ml in groups of three to five. Endpoint analyses and competitive infections (CIs) were performed as previously described (11). For endpoint experiments, mice were monitored for endpoint and sacrificed when they had lost 20% of their initial body weight. For CI experiments, mixed inoculums of wild-type (pseudogene *ushA*::Cm marked) and mutant strains were used. At 72 h postinfection, mice were sacrificed and their spleens and livers were harvested, homogenized in phosphate-buffered saline (PBS), and plated to determine bacterial numbers, which were compared to initial inoculum plating numbers as CFU ratios ([mutant output/wild-type output]/[mutant input/wild-type input]). For *sciG* complementation, the wild-type reference strain also possessed the empty pWSK29 plasmid. Statistical analyses were performed in Prism using the nonparametric Wilcoxon signed-rank test to determine whether mutant CI values differed from 1 ($P < 0.05$ at a 95% confidence interval).

Cell infections. RAW 264.7 murine macrophage-like cells were seeded at 2 × 10^5 CFU per well in 24-well plates and grown at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS (Life Technologies). Bacteria from overnight LB cultures were pelleted, resuspended in DMEM–10% FBS, and opsonized in human serum, and macrophages were infected at a multiplicity of infection (MOI) of 50 for 30 min. This was followed by two PBS rinses, a 1.5-h incubation in DMEM–10% FBS plus 100 μg/ml gentamicin, two PBS rinses, and incubation to the given lysis time point in DMEM–10% FBS plus 10 μg/ml (11). At the indicated time points, infected cells were washed with PBS, lysed in cell lysis buffer (1 ml PBS, 1% Triton X-100, 0.1% SDS), and plated to establish bacterial counts. For epithelial cell coculture experiments, HeLa and HEp-2 cells were seeded at 1 × 10^5 CFU per well in 24-well plates and cocultured at an MOI of 10 from pelleted and DMEM–10% FBS-washed 3-h exponential-phase LB cultures. For epithelial cell infections, HeLa or HEp-2 cells were seeded at 1 × 10^5 CFU per well in 24-well plates and infected at an MOI of 50 from pelleted and DMEM–10% FBS-washed 3-h exponential-phase LB cultures for 15 min, washed twice with PBS, and allowed to complete invasion in fresh DMEM–10% FBS for 20 min. Cells were then washed twice with and incubated in DMEM–10% FBS plus 100 μg/ml gentamicin for 1.5 h, washed twice with PBS, and lysed in cell lysis buffer. Lysates and infection inoculum were plated and counted to calculate invasion efficiency. For *sciG* complementation, the wild-type reference strain also possessed the empty pWSK29 plasmid. Statistical analyses were performed in Prism using a one sample *t* test.

Transcriptional reporter assays. For beta-galactosidase assays, strains were grown overnight in M9 medium, subcultured at 1:50 in LPM minimal medium, and then grown at 37°C with shaking. At defined time intervals, OD_{600} was read using a spectrophotometer and 200-μl culture samples were pelleted and frozen for analysis. For analysis, pellets were resuspended in 200 μl PBS and lysed by the addition of 50 μl chloroform. A 2-μl volume of the lysate was combined with 100 μl of Tropix Galacto-Star detection reagent (Applied Biosystems), and luminescence in relative light units (RLU) was measured using an Envision plate reader (Perkin-Elmer) (10). For intracellular luminescence assays, macrophages in four sets of wells were identically infected as previously described. At each time point, one set of wells was washed with PBS and luminescence was measured using an Envision plate reader. Cells were then lysed in cell lysis buffer and plated to determine bacterial counts. Statistical analyses were performed in Prism using a one-sample *t* test.

Immunoblotting. Strains were grown overnight in LB medium at 37°C. Cultures were pelleted, washed, and diluted in DMEM containing 10% FBS. Inoculum was added to 12-well tissue culture plates with or without eukaryotic cells for coincubation protocol or used for infection as previously described. Plates were incubated at 37°C under 5% CO_2. At defined time points, the contents of the well were collected, pelleted, and washed in PBS. Pellets were lysed in 200 μl 2× SDS sample buffer (100 mM Tris-HCl [pH 6.8], 20%% [vol/vol] glycerol, 4% [wt/vol] SDS, 0.002% [wt/vol] bromophenol blue, 4 M urea, 0.2 M dithiothreitol). Samples were boiled for 10 min at 100°C, centrifuged for 1 min at 17,900 × *g*, and subjected to 15% SDS-PAGE. Protein was transferred to polyvinylidene difluoride membrane and incubated overnight with 1:10,000 mouse anti-DnaK (Stressgen), 1:1,000 mouse anti-HA (Covance), or 1:5,000 mouse anti-FLAG (Sigma) antibodies. Blots were then washed in Tris-buffered saline–Tween 20 (TBST), incubated with 1:5,000 goat anti-mouse–horseradish peroxidase antibodies (Sigma), washed in TBST, and detected using enhanced chemiluminescence (Western Lightning; Perkin-Elmer). Low-signal Western blot assays of samples collected from regulator mutant bacteria were performed with SuperSignal West Femto (Thermo Scientific).

RESULTS

The SPI-6 T6SS noncore genes include T6SS-associated gene pairs. The *S.* Typhimurium SPI-6 T6SS contains 13 core T6SS genes in addition to other uncharacterized genes (5). Many T6SSs have undergone preliminary characterization in other sequenced bacteria, and we hoped to gain insight into these uncharacterized genes by identifying a similar characterized system. Using the SPI-6 T6SS core protein sequences, we performed a BLASTP search of the NCBI BLAST nonredundant protein sequence database to identify an organism that encodes a similar T6SS. We observed that the T6SS-3 (Tss3) of *Burkholderia mallei* and *B. pseudomallei* is highly similar (49) (Fig. 1; see Table S3 in the supplemental material). These systems have been previously reported to be closely related through phylogenetic analyses (4, 5, 7). The *Burkholderia* sp. system has 15 genes in common with that of *S.* Typhimurium, representing 13 core genes, and they share synteny in core T6SS genes aside from their VgrG ortholog, which is positioned at opposite sides of the island. The two systems differ in the number of additional nonconserved genes: four genes in a single cluster in the *Burkholderia* variants (BMAA cluster) and 10 genes within three clusters in the *S.* Typhimurium T6SS (STM clusters 1 to 3). These clusters contain genes *sciJ* to *sciL* (*STM0274A* to *STM0278*), *sciQ* and *sciR* (*STM0283* and *STM0284*), and *sciT* to *sciV* (*STM0286* to *STM0288*), respectively. A fourth cluster of genes (STM cluster 4), *sciW* to *sciY* (*STM0290* to *STM0298*), located between the T6SS and *safABCD* operon contains recombination hot spot (Rhs) elements, poorly conserved genes, and transposases.

In order to better understand the noncore genes that are not conserved between the *S.* Typhimurium SPI-6 T6SS and the *Burkholderia* Tss3, we searched for organisms in which orthologs of at least two of these genes exist (see Table S4 in the supplemental

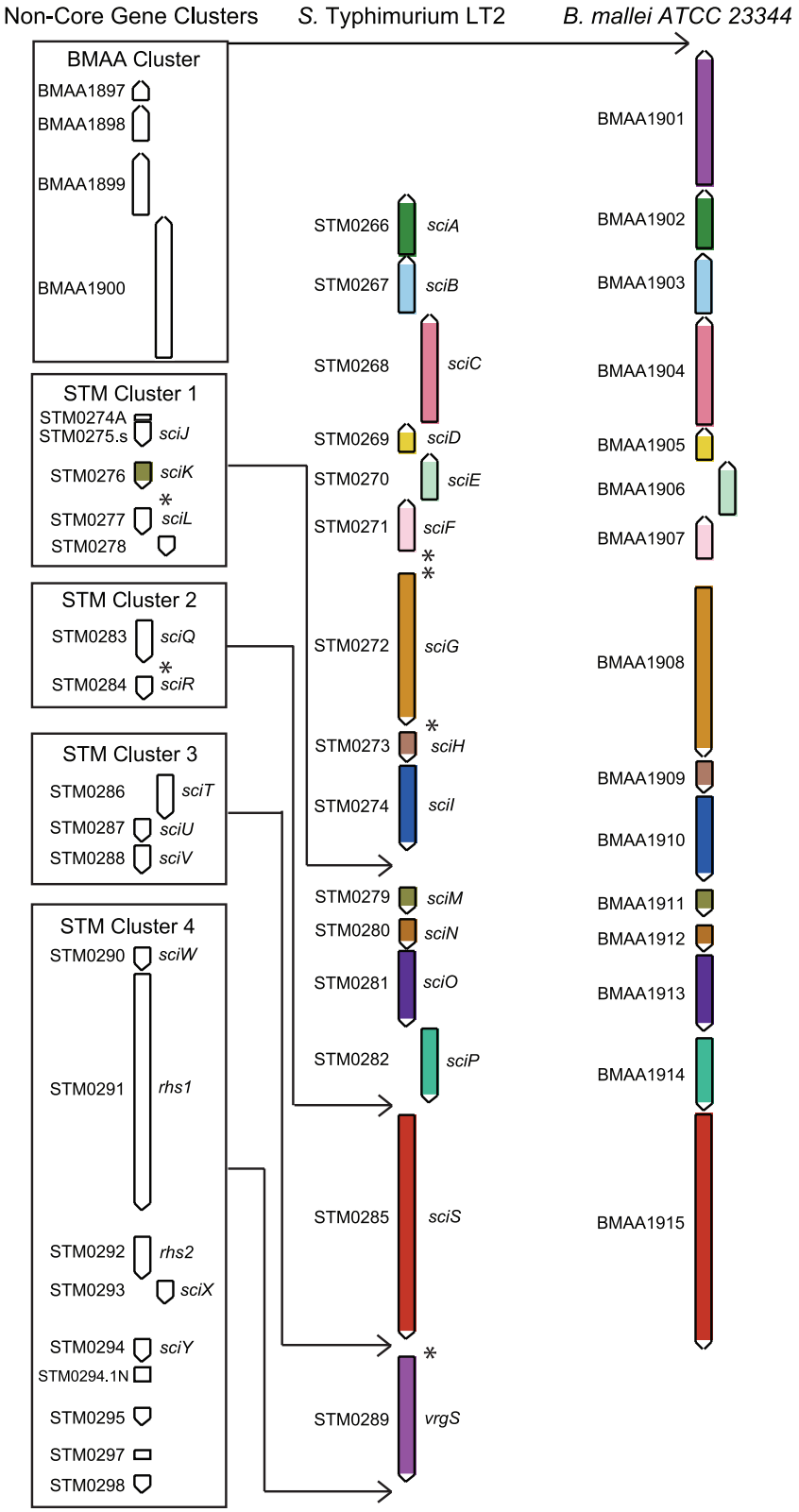

FIG 1 Diagram showing the 15 T6SS genes that the *S.* Typhimurium LT2 SPI-6 T6SS and the *B. mallei* ATCC 23344 Tss3 T6SS have in common. Orthologs are indicated by shading with the same color. Nonconserved genes in *B. mallei* (BMAA cluster) and *S.* Typhimurium (STM clusters) are displayed in boxes on the left, and their positions relative to the core genes are indicated by black lines. Observed transcriptionally active regions are denoted by asterisks.

material). We did not include *sciK*, a paralog of *sciM*, in cluster 1, given its extensive conservation as an Hcp-encoding gene even though it is absent from the *B. mallei* Tss3. In cluster 1, *STM0274A* and *sciJ* were not well conserved outside *Salmonella* and were found to exist in only *Escherichia* sp. strain TW09308. In contrast, orthologs of *sciL* and *STM0278* were found to co-occur in 14 other genomes, including those of species of *Escherichia*, *Enterobacter*, *Erwinia*, and *Frateuria*, in close proximity to an *sciS* ortholog (suggestive of a T6SS), and in those of species of *Advenella*, *Pantoea*, and *Pseudomonas*, not in close proximity to an *sciS* ortholog. In cluster 2, orthologs of *sciQ* and *sciR* were found to co-occur only in *Agrobacterium tumefaciens* in close proximity to an *sciS* ortholog. In cluster 3, orthologs of *sciU* and *sciV* were found to co-occur in four genomes, including those of species of *Bordetella*, in close proximity to an *sciS* ortholog, and in those of species of *Delftia* and *Vibrio*, not in close proximity to an *sciS* ortholog. While not always in close proximity, all of the organisms indicated here that possessed these gene pairs did contain at least one *sciS* ortholog. While many genomes were found to contain orthologs of genes within cluster 4, only *Enterobacter hormanechei* ATCC 49162, *Cronobacter sakazakii* ATCC BAA-894, *Cronobacter turicensis* z3032, *E. coli* TA280, and *Pantoea* sp. strain SL1_M5 orthologs of these genes were found near orthologs of genes from clusters 1 to 3.

Our search also recovered the previously identified orphan Hcp-encoding gene *STM3131*, which is not linked with the SPI-6 T6SS and likely has an origin distinct from that of the *S. enterica* subsp. *enterica* and *arizonae* SPI-6 T6SS (5). Recognizing the possibility that *STM3131* could contain a gene product functionally relevant to the *Salmonella* SPI-6 T6SS, we included this gene in downstream experiments.

The SPI-6 T6SS contributes to pathogenesis in a mouse model of typhoid. We generated mutants of both *sciG* (the *clpV* ortholog), and *sciS* (the *icmF* ortholog), genes which have been established as essential for T6SS assembly and functional secretion in other systems (6, 42). When mice were infected with wild-type and mutant strains of *S.* Typhimurium, mice infected with the mutant strains reached an endpoint approximately 1 to 2 days later than mice infected with wild-type *S.* Typhimurium (Fig. 2A). The major processes leading to death in this model are systemic dissemination and replication of the bacteria in organs including the spleen and liver. To determine whether the T6SS contributes to these processes, we performed CI experiments with the *sciG* and *sciS* mutants against the wild-type strain (Fig. 2B). We obtained combined (liver and spleen) CI values for these mutants of 0.39 and 0.43, respectively (see Table S5 in the supplemental material), that were in agreement with previously reported data for deletion of the entire SPI-6 locus (19). This defect in systemic dissemination was successfully complemented by plasmid-based expression of *sciG* under the control of its native promoter.

The SPI-6 noncore gene clusters contribute to pathogenesis in mice. Since we were able to measure a virulence effect for the T6SS core genes by oral infection of mice, we employed this model to test the contribution of the SPI-6 noncore gene clusters to systemic dissemination. We generated cluster 1 through 4 deletion mutants. When competed against the wild type in oral infections, cluster 2 and 4 deletion mutants yielded combined organ CI values of 0.47 and 0.37 (see Table S5 in the supplemental material), values similar to that obtained by deletion of the core genes *sciG* and *sciS* (Fig. 2C). The combined CI values of cluster 1 and 3 mutants did not differ significantly from that of the wild type, although we observed clustering of values for the cluster 3 deletion near the CI value of the core mutant deletion (average = 0.82, median = 0.49) and therefore included this cluster for further analysis. To identify specific genes contributing to this fitness defect, we generated additional single gene deletion strains for clusters 2, 3, and 4. When competed against the wild type, the individual cluster 2 mutants both had a combined CI value of 0.69 that was not as low as the cluster 2 mutant combined CI value of 0.47 (Fig. 2D). CI experiments with individual cluster 3 mutants showed that the *sciT* and *sciV* mutants had combined CI values below that of the wild type (0.55 and 0.67) while the *sciU* deletion mutant was found to trend toward increased systemic dissemination with a combined CI value of 1.39, albeit not significant (Fig. 2E). CI experiments with selected individual cluster 4 mutants revealed a low combined CI value of 0.32, indicating a strong systemic dissemination defect in the *rhs2* mutant (Fig. 2F).

The SPI-6 T6SS contributes to intracellular replication in macrophages. Systemic dissemination is dependent on survival within phagocytic cells. To determine whether the SPI-6 T6SS contributes to intracellular replication, we assessed the intracellular survival/replication rates of T6SS core gene and noncore gene mutants 24 h postinfection in the murine macrophage-like cell line RAW 264.7 (Fig. 3A). Compared to the wild-type *S.* Typhimurium parent strain, the *sciG* and *sciS* core T6SS gene mutants were found to have an intracellular replication ratio of approximately 0.5, compared to the intracellular replication ratio of 0.13 of an *sseC* SPI-2 T3SS translocon mutant that is defective in intracellular persistence. Plasmid-based expression of *sciG* was able to complement the *sciG* mutant to near-wild-type levels of intracellular replication. We observed a significant decrease in replication over the wild-type strain for clusters 1 and 3. Individual deletions within cluster 1 revealed a significant contribution for *STM0278*, while no individual gene in cluster 3 was found to make a significant contribution (Fig. 3B). While this is not significant, we again observed a trend toward increased replication in the cluster 3 *sciU* single gene mutant.

To determine whether this replication phenotype is specific to macrophages, we performed invasion assays with HeLa and HEp-2 epithelial cells. While we observed a clear defect in invasion by our *hfq* mutant control, which is defective in invasion, we saw no significant effect on invasion in both cell types for any of our core and noncore SPI-6 T6SS mutants (see Fig. S1 in the supplemental material).

Hcp and VrgS protein expression is enhanced during infection of macrophages. Secretion of Hcp and VgrG orthologs is the hallmark of a functional T6SS. Hcp-1, Hcp-3, and VrgS were cloned and overexpressed as C-terminal FLAG fusion proteins and assayed for expression and secretion following IPTG induction in wild-type and *sciG* mutant strains (see Fig. S2 in the supplemental material). While all of the proteins were observed in the cytoplasmic fraction, only Hcp-3 was found to be secreted. In addition, it was determined that Hcp-3 secretion into the medium was not dependent on the T6SS ATPase SciG. The Hcp proteins were observed at their predicted sizes of approximately 17 kDa, while the VrgS protein was observed to migrate at approximately 70 kDa rather than the predicted 80 kDa based on the *S.* Typhimurium LT2 annotation, suggesting a possible misannotated start codon. BLASTP searches indicate low-complexity sequences within the first 100 amino acids, and this region falls outside the conserved Vgr protein domains TIGR03361, COG3501, and

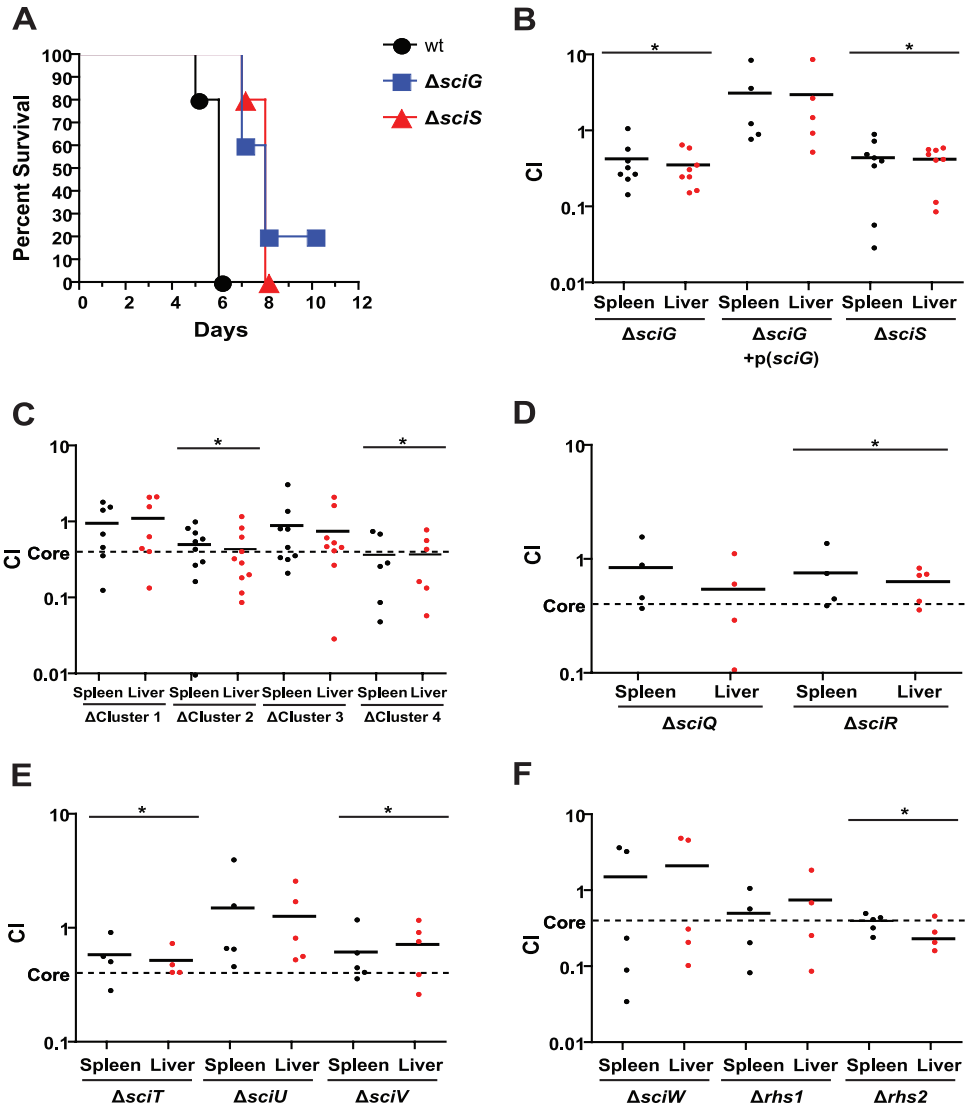

FIG 2 Contribution of the SPI-6 T6SS to *S.* Typhimurium pathogenesis in mice. (A) Survival curve of SPI-6 T6SS mutant bacteria in C57BL/6 mice. Mice infected with Δ*sciG* (ATPase) and Δ*sciS* (*icmF*) mutant bacteria reached an endpoint 2 days later than those infected with wild-type (wt) bacteria. (B to F) CI experiments with wild-type and SPI-6 T6SS mutant bacteria and C57BL/6 mice orally infected with 10^6 CFU. (B) Δ*sciG* and Δ*sciS* mutants were less fit in the liver and spleen than wild-type bacteria, a defect that could be resolved by plasmid-based complementation of *sciG* (*, $P < 0.05$). (C) Deletion of noncore gene clusters 2 and 4 resulted in CI values similar to those produced by the deletion of essential T6SS genes (core), while deletion of cluster 3 resulted in an intermediate CI value. (D) Deletion of individual genes within cluster 2 could not recapitulate the defect caused by deletion of the entire cluster. (E) Deletion of individual genes within cluster 3 with a nonsignificant trend toward increased replication in the *sciU* mutant. (F) Deletion of individual genes within cluster 4 showed a strong defect in the Δ*rhs2* mutant.

TIGR01646 identified by the BLAST integrated conserved domain database (35). Interestingly, trace levels of DnaK were observed only in the secreted fraction of VrgS, suggesting that overexpression of VrgS may be slightly toxic.

In order to characterize the stable expression of SPI-6 T6SS proteins, we constructed chromosomal HA epitope fusion proteins with the Hcp paralogs SciK (Hcp-1), SciM (Hcp-2), and STM3131 (Hcp-3) and with the VgrG ortholog VrgS such that their expression was controlled by their native chromosomal promoters. Under *in vitro* growth conditions, only Hcp-3 was detectable after 24 h when bacteria were grown in rich (DMEM–10% FBS) or minimal (LPM) medium (Fig. 4A).

Some T6SSs have been shown to require target cell contact for activation (32). To determine whether the absence of chromosomal expression of the Hcp and VrgS proteins *in vitro* was due to the lack of induction by host cell signals, strains harboring chromosomally tagged genes were either used to infect RAW 264.7 murine macrophage-like cells or grown in tissue culture medium in the absence of macrophages (Fig. 4B). We observed only weak expression of Hcp-1, Hcp-2, and VrgS in the absence of macrophages, whereas the abundance of these proteins was increased following macrophage infection. In contrast, Hcp-3 appears to be constitutively expressed under all of the conditions tested. To determine whether this induction is specific to phagocytic cells or the intracellular environment, HeLa cells were grown in the presence of wild-type or invasion-deficient *invA* mutant strains. No induc-

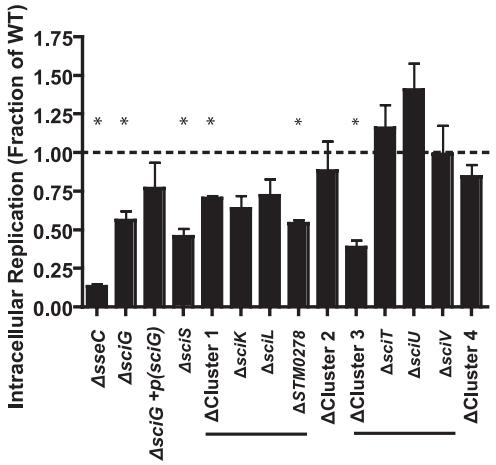

FIG 3 Assessment of SPI-6 T6SS contribution to replication in macrophage cell culture. Intracellular replication of SPI-6 T6SS mutants in RAW 264.7 murine macrophage-like cells expressed as a fraction of that of the wild type (WT) at 24 h postinfection compared with that of a Δ*sseC* SPI-2 T3SS translocon mutant (*, $P < 0.05$). Decreased intracellular replication of core gene Δ*sciG* and Δ*sciS* mutants was observed, with near-wild-type levels for the *sciG* complemented strain. Replication defects were also observed in noncore gene cluster 1 and 3 mutants. Decreased intracellular replication for the *STM0278* mutant was observed when single gene mutants were tested, while a nonsignificant increase in that of individual noncore gene cluster 3 mutants was observed.

tion of Hcp-1, Hcp-2, or VrgS expression was observed after 24 h (see Fig. S3 in the supplemental material). Hcp-3 was expressed in both backgrounds. It was noted that Hcp-1 appears to be more strongly expressed than Hcp-2 and VrgS under cell culture conditions both in the absence of macrophages and by wild-type cells in the presence of HeLa cells.

A previous report had shown that transcript levels of the *icmF* ortholog *sciS* remain low until late time points (>16 h) in macrophage infections (38). To determine whether Hcp and VrgS expression follows this pattern, we collected the contents of individual wells of infected RAW 264.7 cells at 4, 8, 16, and 24 h postinfection. Hcp-3 expression was evident at early time points, while VrgS, Hcp-1, and Hcp-2 expression was weak. By 16 h, expression of both Hcp-2 and VrgS was observed (Fig. 4C).

The SPI-6 T6SS is not induced by deletion of regulators of intracellular virulence. A previous report suggested that the SPI-6 T6SS is induced in response to host cell signaling as a result of regulatory derepression, possibly through the transcription factor SsrB (38). In order to further investigate regulatory contributions, we identified transcriptionally active regions by cloning intergenic regions of greater than 40 bp in the SPI-6 T6SS as transcriptional fusions to luciferase (see Table S6 in the supplemental material). Measurement of luminescence normalized to the number of recovered bacterial cells was performed during RAW 264.7 macrophage-like cell infections to assess transcriptional activity (Fig. 5A). At 24 h postinfection, active transcription from regions upstream of the six genes *sciF*, *sciG*, *sciH*, *sciL*, *sciR*, and *vrgS* was observed. Transcription from regions upstream of *sciF*, *sciL*, and *sciR* was much lower than that from regions upstream of *sciG*, *sciH*, and *vrgS* during exponential growth *in vitro* (see Fig. S4 in the supplemental material).

To determine if these transcriptionally active regions are targets of regulatory input from SsrB, *lacZ* transcriptional reporters were chromosomally integrated downstream of these regions to generate merodiploid reporter strains. The transcriptional activity of these reporters was measured *in vitro* in defined LPM minimal medium, which provides an environment in which SsrB and upstream regulators of virulence genes, PhoPQ and SlyA, are active (10). We assessed the transcriptional activities of our reporters in backgrounds deficient in these proteins involved in intracellular pathogenesis (56). We observed altered profiles of transcriptionally active regions upstream of *sciF*, *sciG*, and *vrgS* (Fig. 5B). In an *ssrAB* mutant background, the transcriptional activity of the regions upstream of *sciF* and *vrgS* was increased slightly (less than 2-fold). The transcriptional profiles in the *phoP* and *slyA* mutant backgrounds were identical and revealed an increase in transcription in the region upstream of *sciF* by approximately 2.5-fold and a 2-fold decrease in the regions upstream of *sciG* and *vrgS*.

To determine whether the expression of the SPI-6 T6SS is repressed by the activity of regulators of intracellular virulence, we assessed the levels of chromosomal fusion protein VrgS::HA expressed under the control of its native promoter in backgrounds deficient in regulators of intracellular pathogenesis (56). These included the two-component systems PhoPQ, OmpR/EnvZ, QseBC, PmrAB, and SsrAB and the RNA binding protein Hfq, regulators important for intracellular pathogenesis (Fig. 5C). Expression of VrgS in these mutant backgrounds in LB medium after 24 h remained barely detectable by an enhanced-sensitivity chemiluminescence system, although expression appeared slightly higher in an *hfq* mutant background. When expression of Hcp-1, Hcp-2, and VrgS was assessed after 24 h of growth in DMEM under cell culture conditions, we failed to see an increase in expression in either *ssrB* or *hfq* mutant background strains over that by the wild type (Fig. 5D). Expression of these proteins was weaker during growth in LPM minimal medium and also failed to show increased expression over that by the wild type (data not shown).

A PmrA box motif was previously identified upstream of the orphan Hcp-3 gene *STM3131*, although the regulatory input was not confirmed (34). In order to determine whether a regulatory interaction with PmrA occurs at this site, we generated a *lacZ* transcriptional fusion on the chromosome downstream from *STM3131*. We also generated *lacZ* transcriptional fusions on the chromosome for the PmrA-activated promoter of *pmrC* and the PmrA-repressed promoter of *pmrD*. Assessment of transcriptional activity indicated that transcription increased 2-fold in a *pmrA* mutant background, similar to that of the PmrA-repressed promoter of *pmrD*, suggesting that PmrA acts as a transcriptional repressor of this gene (see Fig. S4 in the supplemental material).

DISCUSSION

T6SS variants have been acquired at least three times within the *Salmonella* lineage, and little is known about their function and mechanism, although most reports have implicated roles for the *S.* Typhimurium SPI-6 T6SS in the pathogenesis of mice and infection of macrophages (5, 9, 17, 19, 24, 28, 30, 38). We found that the disruption of noncore T6SS clusters 2 and 4 caused significant defects in systemic dissemination in mice and that disruption of noncore gene clusters 1 and 3 resulted in a significant intracellular replication defect in macrophages. Further supporting this role in intracellular pathogenesis, we showed an increase in Hcp and VrgS protein expression in association with macrophages. Finally, we showed that deletion of a previously identified negative regu-

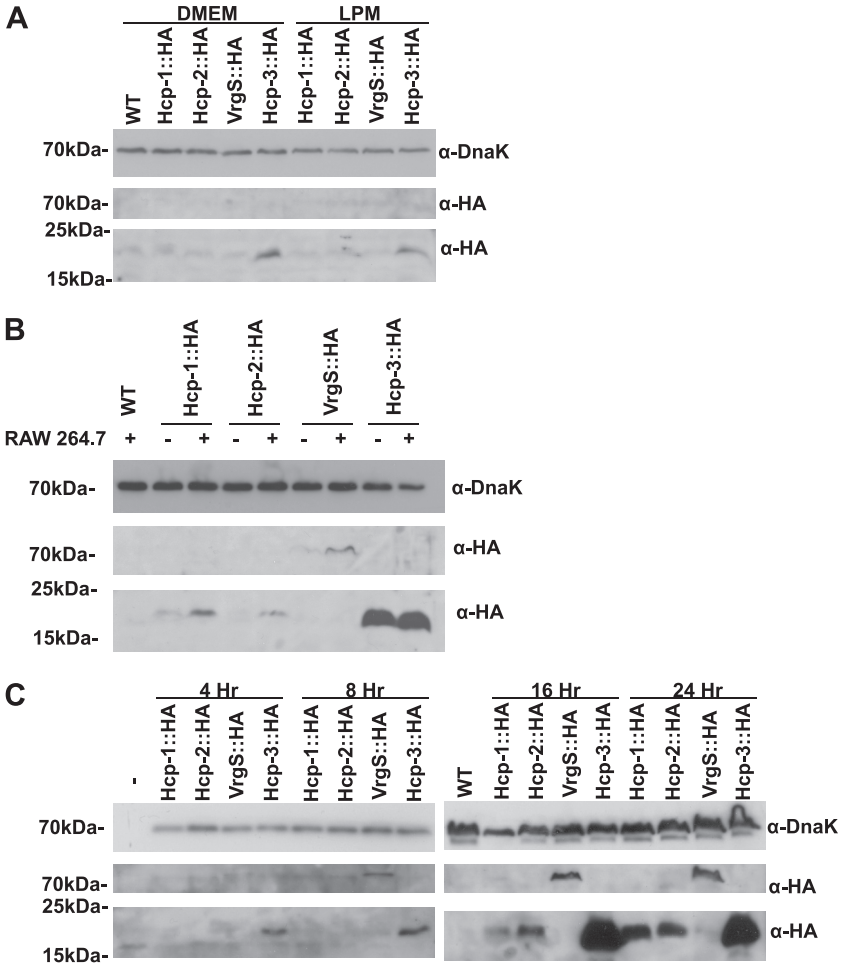

FIG 4 Expression of Hcp and VrgS C-terminal chromosomal HA epitope fusion proteins assessed by Western blot assay. (A) Following 24 h of growth in DMEM or LPM minimal medium, expression of only Hcp-3::HA was observed. (B) At 24 h postinfection of RAW 264.7 macrophage-like cells with opsonized bacteria, Hcp-1::HA, Hcp-2::HA, and VrgS::HA expression was enhanced compared to that in bacteria grown under identical cell culture conditions in the absence of eukaryotic cells. Expression of Hcp-3::HA was not enhanced. (C) Expression of Hcp-2::HA and VrgS::HA was detected by 16 h postinfection of RAW 264.7 macrophage-like cells with opsonized bacteria. WT, wild type.

lator of this system enhances levels of transcription of this system from defined transcriptionally active regions but does not lead to increased levels of Hcp and VrgS protein expression upon deletion.

Distinct T6SSs are encoded by *S. bongori*, *S. enterica* subsp. *arizonae*, and serovars of *S. enterica* subsp. *enterica* as the most proximal element to the tRNA genes within SPI-6 or SPI-2, suggesting relatively recent acquisition events (5, 17). In spite of the number of T6SS in *Salmonella*, an evolved VgrG effector gene has been identified only in *S. enterica* subsp. *arizonae*, suggesting that if the *S.* Typhimurium SPI-6 T6SS functions to deliver effector proteins, they are not encoded as evolved VgrG proteins (5). The SPI-6 T6SS closely resembles the *Burkholderia* Tss3 (49) present in *B. mallei* and *B. pseudomallei* but lacks a reported role in pathogenesis and is absent from *B. thailandensis* (48). In order to understand the role of the SPI-6 T6SS, we focused on noncore T6SS genes. Noncore T6SS gene transposon mutants of *S.* Typhimurium have been found to be defective in long-term persistence in mice and macrophages; these include *sciR* in cluster 2 (9), *sciU* in cluster 3 (9), and *sciW*, *rhs1*, and *STM0298* in cluster 4, suggesting that these genes may encode proteins important for T6SS activity (9, 24, 28). By BLAST-based analysis, we found that some of these genes are restricted to *Salmonella* while others exist as gene pairs in other T6SS-encoding organisms. The *sciL* and *STM0278* genes in cluster 1 are extensively conserved and are particularly interesting candidates for further investigation. Cluster 2 was found to be poorly conserved and carries a conserved gene pair found in only one other non-*Salmonella* organism. The genes *sciU* and *sciV* also exist as a conserved pair in a limited number of organisms, although the association with T6SS is not as strong as that of *sciL* and *STM0278*. Many orthologs of genes within cluster 4 were identified, including the *sciW* and *rhs1* genes; however, the extensive conservation of *rhs*-associated genes and their close similarity made drawing conclusions about these genes difficult. Evidence of conservation of gene pairs within each of these clusters is particularly interesting given the presence of toxin-antitoxin gene pairs found in other T6SS-encoding organisms (22).

In order to quantify the contribution of the SPI-6 T6SS in host pathogenesis, we assessed the systemic dissemination of core and noncore mutants in a murine mouse model of typhoid and found

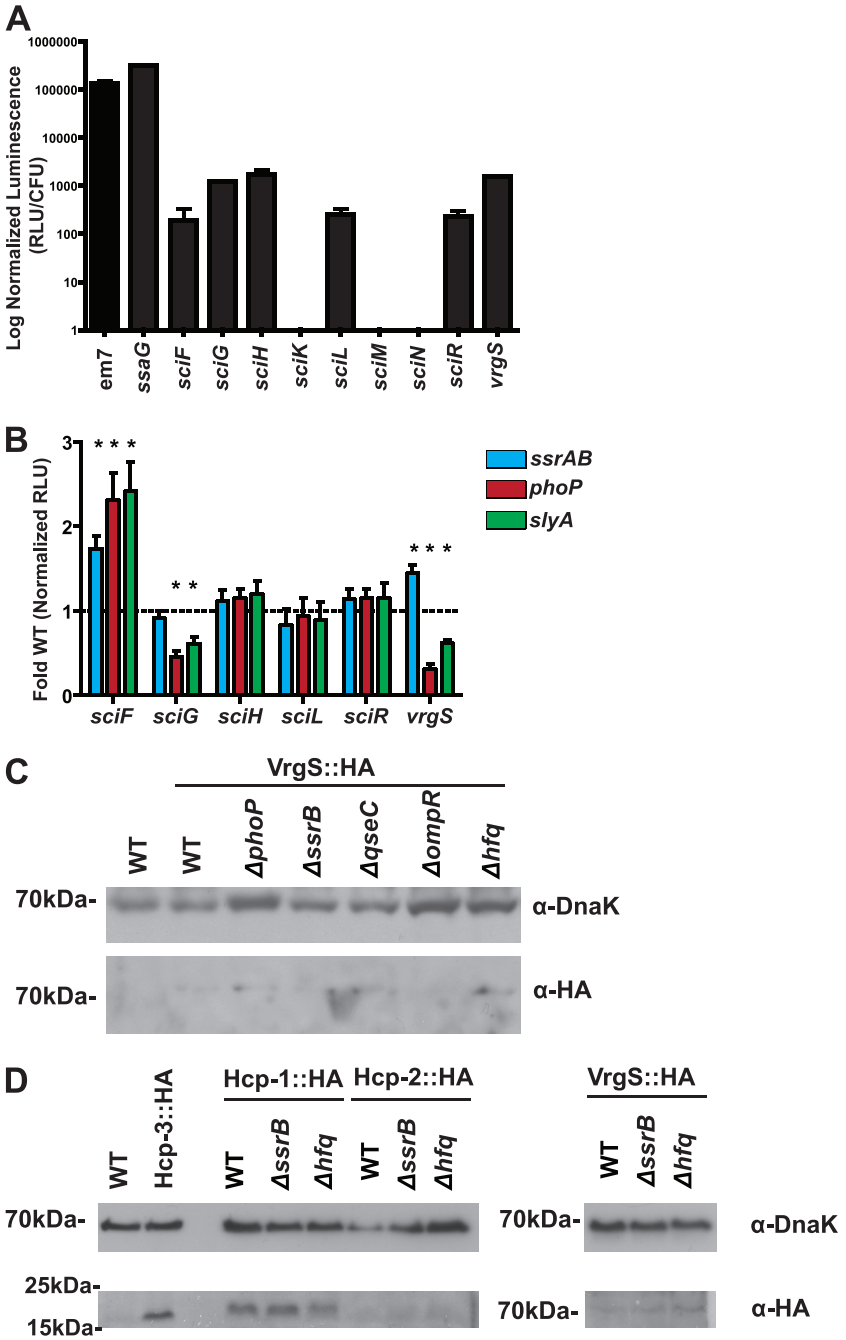

FIG 5 Effect of regulator mutations on SPI-6 T6SS gene expression. (A) Activities of nine SPI-6 T6SS intergenic regions directly upstream of the indicated genes that were cloned as transcriptional fusions with the *luxCDABE* genes. Luminescence data were collected as RLU and normalized to CFU. At 24 h postinfection of RAW 264.7 macrophage-like cells, six of the nine intergenic regions were transcriptionally active. The constitutive synthetic promoter *em7* and the SsrB-dependent promoter of *ssaG* are also shown as controls. (B) Activities of the six transcriptionally active intergenic regions when cloned as *lacZ* chromosomal transcriptional fusion reporters in regulator mutant backgrounds and measured during log-phase growth in LPM minimal medium (*, $P < 0.05$). Luminescence data were collected as RLU and normalized to OD_{600}. The transcriptional activity of the region upstream of *sciF* is increased in the *phoP*, *slyA*, and *ssrAB* mutant backgrounds, and that of *vrgS* is increased only in an *ssrAB* mutant background. Those of *sciG* and VrgS are decreased in the *phoP* and *slyA* mutant backgrounds. (C) Expression of VrgS::HA protein assessed by Western blot assay. Trace levels of the VrgS::HA fusion protein expressed under native promoter control on the chromosome can be detected after 24 h of growth in LB medium, and expression is not strongly upregulated in the *phoP*, *ssrAB*, *qseC*, *ompR*, and *hfq* mutant backgrounds. (D) Expression of native-promoter-expressed Hcp-1::HA, Hcp-2::HA, and VrgS::HA chromosomal fusion proteins assessed by Western blot assay in the *ssrAB* and *hfq* mutant backgrounds after 24 h of growth in DMEM–10% FBS under cell culture conditions. No significant difference in expression was observed. WT, wild type.

significant contributions for noncore gene clusters 2 and 4. Deletion of individual genes within noncore gene cluster 2 did not decrease systemic dissemination to the same extent as deletion of the entire cluster, suggesting that the transcriptionally active region upstream of *sciR* may be a contributing element in pathogenesis. Interestingly, the orthologous *sciQ* and *sciR* genes in *A. tumefaciens* are in a different orientation and lack this intergenic region. In the deletions within cluster 3, individual *sciT* and *sciV* mutants revealed decreased fitness and a trend toward increased fitness of the *sciU* mutant. Deletion of all three genes may result in either increased or decreased fitness *in vivo*, depending on uncontrolled host factors. The *sciU* and *sciV* genes may function as a gene pair, given that these two genes exist as an orthologous gene pair in the absence of *sciT* in four other *Proteobacteria* member genomes. Deletion of cluster 4 had the greatest effect on systemic dissemination of all of the clusters tested, indicating that it may contain an element essential for T6SS-associated pathogenesis *in vivo*. This cluster contains Rhs family genes and Rhs-associated genes that are commonly linked with T6SSs and which are extensively distributed in Gram-negative proteobacteria (55). We disrupted *sciW*, *rhs1*, and *rhs2* and found that disruption of *rhs2* produced the same defect in systemic dissemination in mice as disruption of core T6SS genes and the entire SPI-6 locus (19). This Rhs element is located with *sciX* in a bicistronic operon and may also function as a toxin/immunity gene pair participating in contact-dependent growth inhibition (40).

Survival and replication within host cells permits systemic dissemination *in vivo*, and disruption of the SPI-6 T6SS resulted in reduced intracellular replication in macrophages. In contrast to systemic dissemination, we found that only noncore gene cluster 1 and 3 mutations had significant effects on intracellular replication in cell culture. Deletion of individual genes within cluster 1 revealed a significant replication defect for *STM0278*. Like *rhs2-sciX*, this gene is also part of a bicistronic gene pair with *sciL* that may also function as a toxin/immunity gene pair. We found that orthologs of these genes are present in close association with T6SS loci in a number of other members of the family *Enterobacteriaceae*. Given that this gene pair is the most strongly conserved of all of the noncore T6SS-associated genes in clusters 1 to 3, we are further pursuing their characterization. Deletion of individual genes within cluster 3 did not significantly attenuate intracellular replication and in fact produced a nonsignificant increase in intracellular replication. Interestingly, deletion of cluster 3 gene *sciU* produced a trend toward increased intracellular replication and systemic dissemination. These genes may have redundant functions, or attenuation in the noncore gene cluster 3 mutant may be due to polar effects of the disruption. The difference in mutant fitness between *in vivo* systemic dissemination and cell culture intracellular replication experiments may be explained by differences in the length of experiment and cell types encountered as assessment of intracellular replication in cell culture is a simplified model of one aspect of systemic dissemination. Oral infection of mice with *S.* Typhimurium involves passage through the gut environment before systemic dissemination, and it is possible that genes in clusters 2 and 4 are involved in interbacterial competition in this milieu. These multiple gene sets may act to repurpose the general type 6 secretory apparatus for delivery of effector proteins to different cell targets. While other T6SSs, such as that of *P. aeruginosa* and *V. cholerae*, have been found to have antibacterial properties, we were unable to observe this phenomenon in the *S. enterica* T6SS in solid LB agar-based competition assays against *E. coli* (data not shown) (22, 33). In addition, a previous report found that deletion of the entire SCI genomic island resulted in a 50% reduction of HEp-2 epithelial cells but we were unable to observe an effect on the invasion of both HeLa and HEp-2 epithelial cells by our *sciG* and *sciS* mutants, suggesting that gut epithelial cells may not be the target of this system and that this reduction in invasion may be due to a different SCI-encoded factor (16). Interestingly, *sciL* in cluster 1 and *rhs2* and *sciX* in cluster 4 were found to be upregulated in macrophages and not in HeLa epithelial cells (21). *sciL* is located in a bicistronic operon with *STM0278* in cluster 1, which had the most notable contribution to intracellular replication in macrophages. *rhs2* and *sciX* are located as a bicistronic operon in cluster 4, and *rhs2* made the most notable contribution to systemic dissemination in mice. Additionally, the *sciL*, *STM0278*, *rhs1*, and *rhs2* genes have the largest number of orthologs of all of the genes in clusters 1 to 4.

Given the number of environments encountered by *S.* Typhimurium, virulence systems require regulatory control to achieve appropriate situational expression. We observed only weak chromosomal expression and did not observe plasmid-based secretion of Hcp-1, Hcp-2, or VrgS following growth under *in vitro* conditions after 24 h, which argues against a general role for the SPI-6 T6SS. In contrast, Hcp-3 appears to be constitutively expressed, negatively regulated by PmrA, and secreted under these conditions; however, the secretion of Hcp-3 is, curiously, not dependent on *sciG*. The ClpV ATPase is encoded by a core T6SS gene, and its abrogation results in the inability of the T6SS to assemble and secrete Hcp in *V. cholerae* (6). The Hcp-3 coding sequence differs significantly from those of Hcp-1 and Hcp-2, more closely resembling those of the Hcp proteins of *S. bongori* and *Pseudomonas*, and may have evolved to perform alternative functions, as is the case for the fourth *S. enterica* Hcp-encoding gene paralog *hilE* (16). Hcp-3 may therefore have an unrelated function and may be accessing the extracellular environment through an alternate secretory pathway. *sciG* may be dispensable for functional secretion in *S.* Typhimurium; however, the equivalent defects observed in the *sciG* and *sciS* mutants in both systemic dissemination in mice and intracellular replication in macrophages make this unlikely. The expression of Hcp-1 and that of Hcp-2 are not identical, as we observed expression of Hcp-1 in the absence of macrophages in DMEM, in contrast to that of Hcp-2 and VrgS, which was barely detectable. Hcp-1 is encoded by *sciK* within noncore gene cluster 1, and its expression is perhaps a reflection of promoter changes during its acquisition or duplication from *sciM*.

The absence of expression and secretion of the Hcp-2 and VrgS proteins suggests a system under tight regulatory control. The expression of *S.* Typhimurium SPI-2 T3SS virulence genes important for replication and survival in macrophages is upregulated upon exposure to the intracellular compartment (31). In a similar manner, we found that the genes for Hcp and VrgS are differentially expressed in the presence or absence of macrophages and between *in vitro* and tissue culture conditions. Hcp-1 is encoded within noncore gene cluster 1, and like that of Hcp-2 and VrgS, its expression was clearly enhanced during late stages of macrophage infection. The difference in the expression of these proteins between *in vitro* and cell culture conditions suggests an activating signal specific to growth in the presence of macrophages but may also be in response to general cell stress. Expression of the SPI-6 T6SS may rely on regulatory inputs from the host environment in

a manner similar to that of the T6SS of *V. cholerae*, which requires internalization of bacteria (32). With regard to *S.* Typhimurium, expression of the T6SS does not appear to occur until after 8 h of infection and has been previously hypothesized to be a result of the loss of repression through an SsrB-dependent regulatory pathway. It was hypothesized that induction may be relevant to host cell death and bacterial escape (38). Another group reported the upregulation of some SPI-6 T6SS genes during macrophage infection but did not observe an increase over time (21). It is possible that this induction relies on the onset of host cell death, which can be affected by differing MOIs and experimental conditions. Indeed, when MOIs were much reduced, we observed that Hcp and VrgS expression was lower (data not shown). An important consideration is that expression levels of this system, even in the context of macrophage infection, remain low and we have not yet been able to observe translocation of tagged proteins to the host cells and therefore further work is necessary to determine whether this system is activated within the intracellular compartment or activated simply in the presence of macrophages.

Induction of the SPI-6 T6SS could be similar to that of the HSI-I T6SS in *Pseudomonas* spp., where deletion of the sensor kinase RetS leads to enhanced secretory activity of effector proteins (22). We pursued this hypothesis by identifying transcriptionally active regions within the SPI-6 T6SS. We found six transcriptionally active regions, including one within noncore gene cluster 2, active *in vitro* and in association with host macrophages. A previous report observed increased numbers of transcripts of *sciS* in *ssrB* null mutants during late stages of host cell infection (38). We also found increased levels of transcription of the T6SS in an *ssrB* mutant background, with increased transcriptional activity from regions upstream of *sciF* and *vrgS*. The regulatory proteins PhoP and SlyA are involved in the activation of SsrB (3, 37), and we found that transcriptional activity upstream of *sciF* also increases in this mutant background; however, *sciG* and *vrgS* transcriptional activity decreased, suggesting additional regulatory inputs to this system between PhoP and SsrB. The transcriptional profiles of all six reporters in both the *phoP* and *slyA* mutant backgrounds were identical, suggesting that they act through a common regulatory input. We previously reported the genome-wide interaction map of SsrB using chromatin immunoprecipitation and found only two interaction sites within the SPI-6 T6SS, upstream of *sciF* and *sciR* (51). Given our observation of increased transcriptional activity upstream of *sciF* and the previously reported increase in *sciS* transcripts in a *ssrB* mutant background, we asked whether the loss of these regulatory inputs has a significant effect on VrgS protein expression through regulatory deregulation. Other regulatory proteins aside from PhoP-PhoQ, SsrA-SsrB, and SlyA are necessary for intracellular survival during host infection (56). VrgS levels were not affected in the *phoP*, *ssrB*, *qseC*, *ompR*, or *hfq* mutant background, although the disruption of these proteins results in extensive changes in *S.* Typhimurium gene expression and leads to abrogation of pathogenesis (56). While these results are not extensive, we believe that they argue for the presence of an activating factor that controls T6SS upregulation in response to inducing signals. An unbiased transposon-screening approach is likely to be necessary to identify such a regulator.

In summary, these findings further support a role for the SPI-6 T6SS during host infection, likely mediated through interactions with macrophages and enabled by the SPI-6 T6SS noncore genes *STM0278* and *rhs2*. Two transcriptionally active regions of this system receive negative regulatory input through the SsrB regulatory network; however, the expression of this system appears to be controlled by an as-yet-unknown regulatory factor.

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