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Poultry Body Temperature Contributes to Invasion Control through Reduced Expression of *Salmonella* Pathogenicity Island 1 Genes in *Salmonella enterica* Serovars Typhimurium and Enteritidis

Bryan Troxell,[a] Nicholas Petri,[a] Caitlyn Daron,[a] Rafaela Pereira,[b] Mary Mendoza,[a] Hosni M. Hassan,[a] Matthew D. Koci[a]

Prestage Department of Poultry Science, North Carolina State University, Raleigh, North Carolina, USA[a]; Department of Animal Science, University of Sao Paulo, Piracicaba, SP, Brazil[b]

Salmonella enterica **serovars Typhimurium (*S.* Typhimurium) and Enteritidis (*S.* Enteritidis) are foodborne pathogens, and outbreaks are often associated with poultry products. Chickens are typically asymptomatic when colonized by these serovars; however, the factors contributing to this observation are uncharacterized. Whereas symptomatic mammals have a body temperature between 37°C and 39°C, chickens have a body temperature of 41°C to 42°C. Here, *in vivo* experiments using chicks demonstrated that numbers of viable *S.* Typhimurium or *S.* Enteritidis bacteria within the liver and spleen organ sites were ≥4 orders of magnitude lower than those within the ceca. When similar doses of *S.* Typhimurium or *S.* Enteritidis were given to C3H/HeN mice, the ratio of the intestinal concentration to the liver/spleen concentration was 1:1. In the avian host, this suggested poor survival within these tissues or a reduced capacity to traverse the host epithelial layer and reach liver/spleen sites or both. *Salmonella* pathogenicity island 1 (SPI-1) promotes localization to liver/spleen tissues through invasion of the epithelial cell layer. Following *in vitro* growth at 42°C, SPI-1 genes *sipC*, *invF*, and *hilA* and the SPI-1 *rtsA* activator were downregulated compared to expression at 37°C. Overexpression of the *hilA* activators *fur*, *fliZ*, and *hilD* was capable of inducing *hilA-lacZ* at 37°C but not at 42°C despite the presence of similar levels of protein at the two temperatures. In contrast, overexpression of either *hilC* or *rtsA* was capable of inducing *hilA* and *sipC* at 42°C. These data indicate that physiological parameters of the poultry host, such as body temperature, have a role in modulating expression of virulence.**

Salmonella enterica serovars Typhimurium (*S.* Typhimurium) and Enteritidis (*S.* Enteritidis) are major causes of foodborne diseases worldwide. In the United States, *S.* Typhimurium and *S.* Enteritidis accounted for the majority of confirmed cases of *Salmonella* outbreaks between 1970 and 2011 (1). These two are nontyphoid *Salmonella* (NTS) serovars that are capable of causing disease signs in a variety of animals, which contrasts with typhoid fever serovars that exclusively infect humans. Numerous food products have been associated with *Salmonella* outbreaks and illnesses in humans; however, poultry products are frequently implicated in outbreaks associated with NTS (www.cdc.gov/Salmonella/outbreaks.html). In 2010, a major poultry-related outbreak occurred that involved *S.* Enteritidis infections across 11 states and resulted in the recall of 380 million eggs (2).

S. Typhimurium invades the host epithelial cell layer and migrates to liver and spleen tissue sites through the action of a type 3 secretion system (T3SS), encoded by *Salmonella* pathogenicity island 1 (SPI-1). SPI-1 is a DNA segment that is approximately 40 kb in size and encodes the structural components of the secretion system, secreted and chaperone proteins, and transcription factors that activate expression of the SPI-1 genes (3–5). Three regulators that are carried within SPI-1, *hilA*, *hilC*, and *hilD*, and *rtsA*, which is carried outside SPI-1, are critical activators of the island. Collectively, the protein products encoded by genes within SPI-1 promote uptake of the pathogen by nonphagocytic cells of the host's epithelial cell layer (6–8). Oral infection with *S.* Typhimurium mutants lacking the entire pathogenicity island or with Δ*spi1* or Δ*hilA* mutants results in severely limited localization of the mutant to the spleen compared to wild-type strain results, but this difference is not observed when mice are inoculated through the intraperitoneal route (9). This supports the concept that SPI-1 plays an important role in traversing the intestinal epithelial layer.

Invasion of the host epithelial cell layer is a critical aspect of *S.* Typhimurium virulence (10). RtsA forms a complex regulatory network with HilC and HilD that ultimately activates expression of *hilA*, which activates components of SPI-1 (9, 11). Recently, several works have contributed to our understanding of the complex regulation of SPI-1 (12–14). In addition, the DNA binding sites of the HilD protein have been mapped and include several genes that are coregulated by HilC and RtsA (15), suggesting that reduced activation by one of the three activators may influence the activation of coregulated genes.

Although poultry are associated with *Salmonella* outbreaks, poultry are largely asymptomatic. In general, oral administration of *S.* Typhimurium or *S.* Enteritidis to poultry results in poor localization and colonization of systemic tissues such as the liver and spleen compared to bacterial concentration within the ceca

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Address correspondence to Bryan Troxell, bryan_troxell@ncsu.edu.

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(16–20). Although the reasons for these results are likely multifactorial, the 3 to 5°C difference in the body temperature of poultry (41°C to 42°C) compared to susceptible mammals (37°C to 39°C) may be a factor contributing to the lack of systemic localization by these serovars in poultry. Since both serovars are capable of reaching systemic tissues in other animals, we hypothesized that the body temperature of chickens exerts a regulatory effect that limits expression of SPI-1 and localization to systemic tissues.

Here, we show that *S.* Typhimurium or *S.* Enteritidis colonized the ceca of a commercial breed of chicks but localized poorly to the liver and spleen, suggesting a low level of invasion. These results contrasted with data from the murine host, which exhibited similar concentrations of *S.* Typhimurium or *S.* Enteritidis within the intestines, spleen, and liver. Therefore, the effect of temperature on the regulation of SPI-1 was evaluated in cells grown at 37°C versus 42°C. Following growth at 42°C, there was reduced expression of SPI-1 genes. Gene expression studies conducted at 42°C demonstrated reduced activation of the *rtsA* gene, which is directly activated by HilD (15), suggesting a reduction in the level of either HilD or HilD protein activity in response to growth at 42°C. To gain insight into the mechanism resulting in the inability to activate SPI-1 at 42°C, we utilized an inducible system to test the roles of Fur, FliZ, HilC, RtsA (STM14_5188), and HilD. Inducible expression of either HilC or RtsA protein, but not of Fur, FliZ, or HilD, was sufficient to activate *hilA* following growth at 42°C. As previously shown, the Lon protease inhibited activation of SPI-1; however, the lack of *lon* still resulted in the inability to activate SPI-1 following growth at 42°C. Our results supported the hypothesis that the body temperature of poultry caused a regulatory change in the expression of SPI-1 genes that likely contributed to the poor localization to the liver and spleen. Thus, the body temperature of poultry is a significant barrier to activation of SPI-1 and may contribute to diminished invasion of *S.* Typhimurium or *S.* Enteritidis *in vivo*.

MATERIALS AND METHODS

Bacterial strains, plasmids constructions, and reagents. The bacterial strains used throughout this study and their construction are described and listed in Table S1 in the supplemental material.

***S.* Typhimurium and *S.* Enteritidis challenge of 1-day-old chicks.** A total of 50 1-day-old layer-type chicks (W-36; Hy-Line North America, West Des Moines, IA) were randomly assigned to two HEPA-filtered 934-WP animal isolators (L. H. Leathers, Inc., Athens, GA) (25 birds each). Each isolator is equipped with wire mesh racks located above plastic trays to collect waste. Chicks were provided water and feed (All Grain Start-N-Grow; Southern States, Richmond, VA) *ad libitum*. Isolators were temperature controlled and were maintained between 29°C and 31°C for the duration of the experiment. The Institutional Animal Care and Use Committee (IACUC) approved the animal study protocol (protocol 15-065-A).

S. Typhimurium strain NC1040 was cultivated overnight under standing conditions in Luria-Bertani broth (LB) medium (per liter, 10 g tryptone, 5 g yeast extract, and 10 g NaCl) at 37°C, concentrated by centrifugation, and washed with phosphate-buffered saline (PBS). The cell pellet was resuspended in PBS to a concentration of $\sim 5 \times 10^9$ CFU/ml (optical density at 600 nm [OD_{600}] of ~10 for *S.* Typhimurium and ~20 for *S.* Enteritidis). Chicks were individually inoculated by oral gavage with 100 μl of the cell suspension ($\sim 5 \times 10^8$ CFU per bird). The inoculum of strain NC1040 was quantified by serial dilution and plating to ensure the accuracy of the dose given to animals. Control birds were housed separately and given equal volumes of PBS. At the indicated days postinoculation (dpi), 5 birds from each treatment group were euthanized and the cecal contents, liver, and spleen were aseptically removed and placed in PBS–1 mM $MgCl_2$. The weights of cecal content, liver, and spleen samples were recorded. Cecal contents were serially diluted and plated on XLT4 agar plates with 100 mM MOPS (morpholinepropanesulfonic acid) (pH 7.4) without Tergitol (BD Difco, Franklin Lakes, NJ). Liver and spleen samples were homogenized (Kimble Chase Kontes tissue grinder; VWR International, Radnor, PA), serially diluted, and plated on XLT4 agar plates. XLT4 agar plates contained kanamycin sulfate to select for strain NC1040 and 100 μg/ml rifampin to select for strain BTNC0025, and H_2S-positive (black) colonies were counted. CFU counts were normalized to the weight of cecal contents or tissue.

The detection limit for this procedure was determined by spiking liver samples from control birds with strain BTNC0025. Livers were homogenized and plated on XLT4-MOPS with rifampin to determine mean levels of CFU per gram. Three separate experiments were used to determine the means ± standard deviations (SD) corresponding to the detection limit ($\log_{10}$ CFU per gram, 2.3 ± 1.7). When 50% or less of the tissue samples were culture negative, the mean detection limit was substituted for the zero value to determine the mean value for the samples for that population and time point (simple replacement approach).

Measurement of cloacal temperatures within chicks. The body temperatures of a W-36 line of chicks, reared at North Carolina State University, were determined by measuring the cloacal temperature over time. Seventy-one birds were housed in two separate isolators as mentioned above, and birds were euthanized over time to maintain the appropriate space requirements for the animals within the isolators. A digital thermometer (Easy-Read Flex-Tip digital thermometer; Walgreens, Deerfield, IL) was used to measure cloacal temperatures at 1 ($n = 71$), 2 ($n = 71$), 4 ($n = 61$), 6 ($n = 43$), 8 ($n = 32$), 13 ($n = 19$), and 22 ($n = 12$) days posthatch. A body temperature reading was obtained following insertion of the temperature probe approximately 1 to 2 in. within the cloacal orifice. A smooth, sterile probe cover was used during measurements to prevent tissue damage.

***S.* Typhimurium and *S.* Enteritidis challenge of mice.** Sixteen female C3H/HeN mice (Ity^r, *Salmonella* resistant) were purchased from Harlan Laboratories (Indianapolis, IN) and subjected to gavage with *S.* Typhimurium strain NC1040 or *S.* Enteritidis strain BTNC0025 as described above. Mice were housed in disposable cages (4 mice per cage), and each mouse was given $\sim 5 \times 10^8$ CFU of strain NC1040. In accordance with previous work (21), disease symptoms were monitored and mice were given a body condition score (BCS). At the indicated dpi, mice were euthanized and the concentration of strain NC1040 within the intestines, liver, and spleen was determined as described above. The IACUC approved the animal study protocol (protocol 15-035-B).

β-Galactosidase assays, SDS-PAGE, and immunoblotting. For all experiments, frozen stocks (−80°C) of bacteria were inoculated into LB (Fisher) medium and incubated overnight at 37°C. Samples were then diluted at either 1:50 or 1:500 into LB medium containing 1 mM glucose and 100 mM MOPS buffered to pH 7.4 with NaOH or were left unbuffered (pH 6.0) and then were split into separate 15-ml conical tubes (Fisher) and cultured overnight at 37°C or 42°C. To induce expression of IPTG (isopropyl-β-D-thiogalactopyranoside)-controlled promoters, 0.1 or 0.01 mM IPTG was added to the growth medium. To induce expression of the *hilD* gene, tetracycline-HCl was added to reach a final concentration of 2.5 μg/ml. Transcriptional fusions to the *lacZ* gene were assayed for β-galactosidase as described previously (22). β-Galactosidase activity measured in experiments performed with the pSP417 multicopy plasmid and derivatives was determined by the use of 10 μl of sample to measure activity, whereas all other fusions used 100 μl of sample. β-Galactosidase activity was measured in stationary-phase cells following overnight growth (approximately 16 to 18 h) under the specified conditions, and, when appropriate, cells were pelleted from a portion of the sample and prepared for SDS-PAGE as described below. When expression studies used derivatives of rifampin-resistant *S.* Enteritidis strain BTNC0025, to

avoid SPI-1 repression through metabolism of the solvent dimethyl sulfoxide (DMSO), rifampin was not added (14, 23).

Expression of FLAG-tagged proteins was determined by Western blotting, where the cell pellets were suspended in Laemmli sample buffer and samples were reduced and denatured by boiling. Samples were separated by size on 15% acrylamide gels (SDS-PAGE) and transferred to 0.2 μM nitrocellulose membranes (Bio-Rad, Hercules, CA). Immunoblotting was performed as described previously (24, 25). Briefly, membranes were stained with Ponceau S (0.1% Ponceau S [wt/vol], 1% acetic acid) to ensure equivalent loading of samples. For immunoblotting, membranes were blocked in a blocking buffer (i.e., PBS containing 0.05% Tween 20 and 1% powered nonfat milk, pH 7.4) and probed with primary antibody (monoclonal anti-FLAG M2; Sigma-Aldrich) at 1:2,000 for 2 to 3 h. Membranes were washed 2 to 3 times with the blocking buffer and probed with secondary antibody (peroxidase-conjugated goat anti-mouse antibody; Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:10,000 for ~1 h. Membranes were washed 2 to 3 times with Tris-NaCl (50 mM Tris, 200 mM NaCl, pH 7.6), and detection of horseradish peroxidase activity was determined in Tris-NaCl using 4-chloro-1-naphthol (4CN; dissolved in methanol) and H_2O_2 (Thermo Fisher Scientific, Waltham, MA).

qRT-PCR. In response to growth at 42°C, changes in gene expression of target genes *hilC*, *rtsA*, and *hilD* and the *rrsA* reference gene were determined by quantitative reverse transcriptase PCR (qRT-PCR). Bacteria were grown overnight in LB medium and diluted 100-fold in 15-ml conical tubes containing 10 ml of LB-MOPS (pH 7.4) with 1 mM glucose. Cultures were incubated at 37°C or 42°C until an OD_{600} of ~1 was reached (~5.5 h after dilution). When expression studies used derivatives of the rifampin-resistant *S.* Enteritidis BTNC0025 strain, to avoid SPI-1 repression through metabolism of the solvent DMSO, rifampin was not added (14, 23). A 20-ml volume of cold RNA*later* solution (Life Technologies, Grand Island, NY) was added to stabilize RNA. Samples were centrifuged at ~10,000 × *g*, and cell pellets were resuspended in 1 ml of TRIzol reagent (Life Technologies). RNA was extracted according to manufacturer's specifications and treated with DNase I (New England BioLabs) for 1.5 h at 37°C. Then, the DNase-treated RNA was purified using the RNA cleanup protocol with an RNeasy miniprep kit (Qiagen). cDNA was synthesized as described previously (24). Briefly, 1 μl of a 10 mM deoxynucleoside triphosphate (dNTP) mixture (2.5 mM [each] dNTP), 0.5 μl of 100 μM gene-specific primer, total RNA, and H_2O were added to reach a final volume of 13 μl. The sample was heated at 65°C for 5 min and then placed on ice for 1 min. Then, 5 μl of 5× first-strand synthesis buffer (Invitrogen), 1 μl of 0.1 M dithiothreitol (DTT; Invitrogen), 1 μl of RNase OUT (Invitrogen), and 1 μl of Superscript reverse transcriptase III (Invitrogen) were added. The sample was incubated at room temperature for 5 min and then at 50°C for 60 min and at 70°C for 15 min. Following cDNA synthesis, 20 or 30 μl of double-distilled water (ddH_2O) was added to the sample. A control receiving no reverse transcriptase was included for each RNA sample.

qRT-PCR was performed, using RT^2 SYBR green ROX quantitative PCR (qPCR) Mastermix, as follows: 10 μl of qPCR Mastermix, 0.75 μM (each) primers, 2 μl of cDNA, and ddH_2O (used to adjust the reaction mixture to a 20-μl final volume) were added to a 96-well plate. An iCycler (Bio-Rad, Hercules, CA) machine was used with the following PCR parameters: 95°C for 15 min with 40 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 30 s. The data were analyzed using Bio-Rad Optical System software, version 3.1, according to manufacturer specifications. Melting-curve analysis confirmed the presence of a single PCR product for each sample. A standard curve of *rrsA* DNA (10^2 to 10^8 copies per reaction mixture) was used to quantify transcripts, and expression data were normalized per the numbers of copies of *rrsA*, which is the R001 gene in *S.* Enteritidis. qPCR performed with no reverse transcriptase control, targeting *rrsA*, confirmed the reduction of ≥5 orders in magnitude of copy numbers in the samples, indicating that genomic DNA contamination was near the background level.

Primers are listed in Table S2 in the supplemental material and were purchased from IDT DNA Technologies (Coralville, IA) and were designed to target the *hilC* (*STM14_3465*), *rtsA* (*STM14_5188*), *hilD* (*STM14_3474*), and *rrsA* (*STM14_4794*) genes in *S.* Typhimurium; these genes correspond to *SEN_2709*, *SEN_4086*, *SEN_2717*, and the R001 gene in *S.* Enteritidis, respectively.

Statistical analysis. Figures and statistical analysis were accomplished using GraphPad Prism v4.0. Throughout the study, Student's *t* test, with Bonferroni's correction for multiple comparisons when appropriate, was used to determine significance. In addition, an unpaired Student *t* test with Welch's correction was used to determine significant differences in *Salmonella* concentrations within livers and spleens of infected chicks compared to C3H/HeN mice. With strains that harbor derivatives of pSP417, β-galactosidase activities of some samples had a broad range (~3 orders of magnitude). To address this, the reporter activities were $\log_{10}$ transformed prior to analysis.

TABLE 1 Colonization of 1-day-old chickens following challenge with *S.* Typhimurium[a]

No. of days postinoculation with NC1040	NC1040 CFU/g (no. of culture-positive birds/total no. of birds)		
	Cecum	Liver	Spleen
3	8.2 ± 0.3 (5/5)	2.8 ± 0.3 (5/5)	2.6 ± 0.3 (2/5)
6	7.8 ± 0.2 (5/5)	2.5 (1/5)	2.8 ± 0.4 (4/5)
10	8.0 ± 0.2 (5/5)	<2.3 (0/5)	3.3 ± 0.9 (3/5)
15	7.5 ± 1.4 (5/5)	<2.3 (0/5)	2.7 ± 0.6 (2/5)
21	7 ± 0.4 (5/5)	<2.3 (0/5)	<2.3 (0/5)

[a] W-36 chicks (1 day old) were inoculated with ~5×10^8 CFU of the kanamycin-resistant (*fnr′*::*ha*) "wild-type" strain (NC1040). Data shown are the mean $\log_{10}$ CFU values per gram of cecal content or tissue homogenate from the indicated number of culture-positive birds among those sampled (shown in parentheses), with a detection limit of 2.3 $\log_{10}$ CFU/g. Results below the detection limit are shown as <2.3 (0/5).

RESULTS

_S._ Typhimurium and _S._ Enteritidis exhibit temporal localization to the liver and spleen in chicks compared the murine host. To determine the tissue burden of *S.* Typhimurium *in vivo*, 25 1-day-old chicks of the W-36 line were subjected to gavage with ~5×10^8 CFU/bird of the kanamycin-resistant "wild-type" strain (NC1040). At the indicated days postinoculation (dpi), 5 birds per group were euthanized and the concentration of NC1040 was enumerated from the cecal contents and tissue homogenates of the liver and spleen. At day 3, all livers were positive for NC1040 but at a much lower level than the concentrations in the ceca (Table 1). In addition, after 3 dpi, NC1040 was detected in the liver of only 1 bird (Table 1). Eleven of 25 birds were positive for NC1040 in the spleen at any time point during the study, with decreasing levels of NC1040 from 10 dpi until undetectable levels were seen at 21 dpi. All the liver-positive birds were identified before 10 days postinoculation (Table 1). In contrast, 25 of 25 birds had *S.* Typhimurium in the cecal content, with a mean concentration of $>10^7$ CFU/g throughout the course of the study (Table 1). Taken together, these results demonstrated that *S.* Typhimurium colonization of liver and spleen tissues was temporal among the birds and that, when detected, the concentrations were low ($<10^4$ CFU/g tissue), whereas ceca concentrations were $>10^7$ CFU/g and were detectable for the duration of the study.

To test whether these results were specific to *S.* Typhimurium, the experiment described above was repeated using strain BTNC0025, which is a rifampin-resistant isolate of *S.* Enteritidis ATCC 31194. W-36 1-day-old chicks were inoculated with ~5×10^8 CFU of BTNC0025, and the bacterial burdens in the ceca,

TABLE 2 Colonization of 1-day-old chickens following challenge with *S.* Enteritidis[a]

No. of days postinoculation with BTNC0025	BTNC0025 CFU/g (no. of culture-positive birds/total no. of birds)		
	Cecum	Liver	Spleen
1	7.7 ± 0.6 (5/5)	<2.3 (0/5)	<2.3 (0/5)
3	6.4 ± 1.1 (5/5)	<2.3 (0/5)	<2.3 (0/5)
6	6.9 ± 0.4 (5/5)	<2.3 (0/5)	<2.3 (0/5)
10	5.1 ± 0.8 (5/5)	<2.3 (0/5)	<2.3 (0/5)
15	5.0 ± 2.0 (4/5)	<2.3 (0/5)	<2.3 (0/5)
21	<2.3 (0/5)	<2.3 (0/5)	<2.3 (0/5)

[a] W-36 chicks (1 day old) were inoculated with ~5×10^8 CFU of the rifampin-resistant "wild-type" strain (BTNC0025). Data shown are the mean $\log_{10}$ CFU values per gram of intestinal content or tissue homogenate from the indicated number of culture-positive birds among those sampled (shown in parentheses), with a detection limit of <2.3 $\log_{10}$ CFU/g.

TABLE 4 Colonization of murine tissues following challenge with *S.* Enteritidis[a]

No. of days postinoculation with BTNC0025	BTNC0025 CFU/g (no. of culture-positive mice/total no. of mice)		
	Intestine	Liver	Spleen
1	5.3 ± 1.1 (4/4)	2.4 ± 0.1 (3/4)	2.5 ± 0.3 (2/4)
3	3.6 ± 0.8 (4/4)	5.2 ± 1.5 (4/4)	5.5 ± 1.5 (4/4)
6	4.1 ± 0.9 (4/4)	5.6 ± 1.1 (4/4)	6.9 ± 1.0 (4/4)
8[b]	5.1 (1/1)	4.9 (1/1)	5.9 (1/1)

[a] C3H/HeN female mice (4 to 6 weeks old) were inoculated with ~5×10^8 CFU of the rifampin-resistant "wild-type" strain (BTNC0025). Data shown are the mean $\log_{10}$ CFU values per gram of intestinal content or tissue homogenate from the indicated number of culture-positive mice among those sampled (shown in parentheses), with a detection limit of <2.3 $\log_{10}$ CFU/g.
[b] Three mice in this group were found dead on the day of sampling.

spleens, and livers were determined. Similarly to data obtained with *S.* Typhimurium strain NC1040, BTNC0025 did not localize well to these tissue sites. Throughout the study, concentrations of BTNC0025 within the spleen were undetectable (Table 2). Collectively, these data indicated that BTNC0025 exhibited poor localization to these tissue sites, which supported results obtained with *S.* Typhimurium. Although BTNC0025 was poorly detected within these tissue sites, the ceca was colonized to a level of >10^5 CFU/g within all birds until 10 dpi (Table 2). In contrast to the data obtained with NC1040, the cecal concentrations of the BTNC0025 strain declined after 10 dpi to undetectable levels at 21 dpi (Table 2), which was in agreement with earlier data (26). The results indicated that the Enteritidis strain exhibited poor localization to the livers and spleens.

S. Typhimurium causes typhoid-like disease signs in susceptible strains of mice; in resistant strains of mice, however, there are gastroenteritis-like symptoms, accompanied by an acute systemic infection, which can be resolved in approximately 20% to 60% of infected mice (27, 28). These resistant strains of mice develop disease signs more similar to what occurs during human infections with invasive NTS. Therefore, we used C3H/HeN mice (NRAMP$^{+/+}$ TLR4$^{+/+}$) to determine the phenotype of NC1040 or BTNC0025 in this host background. Oral gavage of mice with strain NC1040 (~5×10^8 CFU) gave positive results in the intestines, livers, and spleens of all mice at 3 dpi (Table 3). From 3 to 8 dpi, the concentrations of strain NC1040 within the intestines were similar to those measured in liver and spleen tissues, i.e., an approximately 1:1 ratio, which contrasts with data from the avian host, which exhibited a difference of ~4 orders of magnitude (Table 1). Moreover, this experiment was repeated with *S.* Enteritidis strain BTNC0025 and demonstrated results similar to those obtained with the *S.* Typhimurium strain. From 3 to 8 dpi, the concentrations of BTNC0025 within the intestines and livers/spleens were similar (Table 4).

TABLE 3 Colonization of murine tissues following challenge with *S.* Typhimurium[a]

No. of days postinoculation with NC1040	NC1040 CFU/g (no. of culture-positive mice/total no. of mice)		
	Intestine	Liver	Spleen
1	5.7 ± 1.3 (4/4)	2.8 (1/4)	2.7 ± 0.6 (2/4)
3[b]	5.0 ± 1.2 (3/3)	3.8 ± 1.3 (3/3)	4.3 ± 1.0 (3/3)
4[b]	5.1 ± 1.7 (3/3)	4.4 ± 1.4 (3/3)	4.4 ± 1.3 (3/3)
8	5.5 ± 1.5 (4/4)	4.4 ± 1.4 (4/4)	5.1 ± 0.6 (4/4)

[a] C3H/HeN female mice (4 to 6 weeks old) were inoculated with ~5×10^8 CFU of the kanamycin-resistant (*fnr′::ha*) "wild-type" strain (NC1040). Data shown are the mean $\log_{10}$ CFU values per gram of intestinal content or tissue homogenate from the indicated number of culture-positive mice among those sampled (shown in parentheses), with a detection limit of <2.3 $\log_{10}$ CFU/g.
[b] A mouse in this group was found dead on the day of sampling.

Expression of SPI-1 is decreased at 42°C. The body temperature of poultry is known to increase during development. To determine the body temperatures of chickens under our conditions, we measured the internal body temperatures in a W-36 line of birds. As demonstrated previously (29–31), there was a distinct developmental increase in the body temperature of birds (Fig. 1A). At 1, 2, 4, 6, 8, 13, and 22 days posthatch, the birds had mean body temperatures of 40.3 ± 0.4°C, 40.7 ± 0.4°C, 41.3 ± 0.2°C, 41.6 ± 0.3°C, 41.5 ± 0.3°C, 41.5 ± 0.3°C, and 41.7 ± 0.3°C, respectively (Fig. 1A). At day 8, the body temperature was not significantly different from that measured at day 6 ($P < 0.05$). This

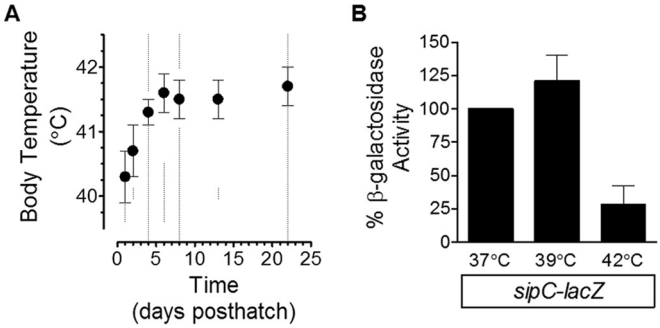

FIG 1 Expression of the SPI-1 gene *sipC* is repressed following growth at 42°C. (A) The body temperatures of a W-36 line of chickens were measured 1, 2, 4, 6, 8, 13, and 22 days posthatch (indicated by dashed lines). By day 8, the body temperature was not significantly different than at day 6 ($P < 0.05$ [Student's *t* test and Bonferroni adjustment for multiple comparisons]). (B) A *sipC-lacZ* fusion strain (RM5385) was used to test the impact of different temperatures on the regulation of SPI-1. Bacteria were grown overnight in LB medium under standing conditions at 37°C and diluted 50-fold in LB medium containing MOPS (pH 7.4, 100 mM) and glucose (1 mM) and incubated at different temperatures under standing conditions for approximately 16 to 18 h. β-Galactosidase activity was determined from experiments performed on three separate occasions. Data shown are the means ± SD, with reporter activity under the 37°C conditions set to 100%.

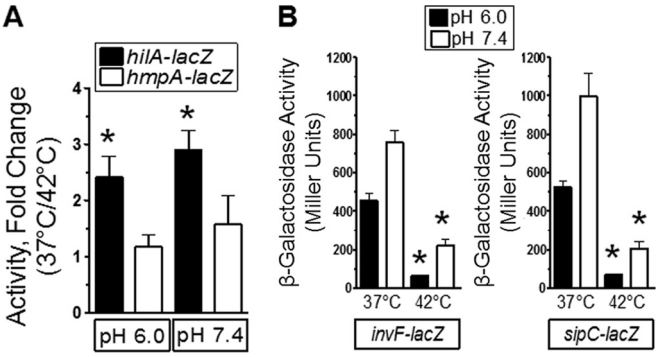

FIG 2 Growth at 42°C repressed SPI-1 genes independently of pH. (A) Bacteria were grown as described in the Fig. 1B legend. The pH of the medium was left unchanged or buffered to 7.4 with NaOH. Bacteria were grown overnight, and β-galactosidase activity was determined. The *hmpA-lacZ* strain was used as a control. Data are from 4 separate experiments, and an asterisk indicates a significant difference in the fold change measured for the *hilA-lacZ* strain compared to the control, the *hmpA-lacZ* strain. Strains used were RM5948 and AV0305. (B) Bacteria were treated as described for panel A, and the reporter activity of strains was measured. Data are from 3 separate experiments, and an asterisk indicates a significant difference in reporter activity at 42°C compared to 37°C under the same pH conditions. Strains used were CA701 and RM5385.

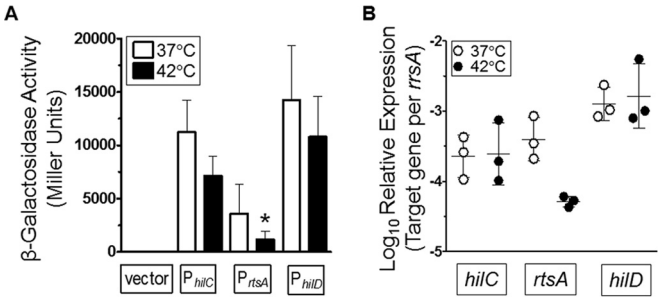

FIG 3 The promoters of the SPI-1 activators, *hilC* and *rtsA*, are differentially regulated by growth at 42°C. (A) The promoters of *hilC*, *rtsA*, and *hilD* were cloned into the pSP417 multicopy shuttle vector (empty vector) upstream of a promoterless *lacZ* gene. Bacteria were grown as described for Fig. 1B and diluted 1:50 into LB-MOPS medium with 1 mM glucose at pH 7.4 at 37°C or 42°C. β-Galactosidase activity was measured after overnight growth. Data are from 4 separate experiments, and a statistically significant result compared to activity at 37°C was determined. The strains used were BTNC0002 to BTNC0005. (B) Bacteria were grown in LB-MOPS medium with 1 mM glucose at pH 7.4 at 37°C or 42°C. Total RNA was extracted at an OD_{600} of ~1. cDNA was generated using gene-specific primers, and expression data were normalized to the *rrsA* 16S rRNA gene.

indicated that the body temperature had stabilized between days 6 and 8 posthatch. Based on these data and earlier work regarding heat shock regulation within *S.* Typhimurium (32), gene regulation experiments were conducted at 42°C.

SPI-1 is an important factor that promotes invasion of the intestinal epithelium and, ultimately, systemic infection. Because infected chicks had significantly lower concentrations of NC1040 within the livers and spleens while simultaneously having significantly higher concentrations within the ceca than C3H/HeN mice, the results suggested that there may be reduced expression of SPI-1 within the avian host. Although this could have been due to a variety of host factors, the body temperature of chickens is higher than that of some other animals. Therefore, the expression of the SPI-1 genes, *sipC-lacZ*, was tested in response to different temperatures. Compared to expression following growth at 37°C, there was no change in expression of *sipC-lacZ* at 39°C, which contrasts with the data determined following growth at 42°C that resulted in reporter activity that was ~25% of that seen at 37°C (Fig. 1B). This suggested that an environmental temperature of 42°C is a crucial factor in the regulation of SPI-1 genes.

To further test the role of 42°C in the regulation of SPI-1, expression of a *hilA-lacZ* fusion was monitored following growth at 37°C and 42°C. In addition, the expression of a *hmpA-lacZ* fusion was monitored to determine the specificity of temperature for SPI-1 gene regulation within *S.* Typhimurium. The influence of pH 6.0 was also tested, since this is a known environmental condition that represses expression of *hilA-lacZ* (33). The data showed that expression of *hilA-lacZ* was repressed at 42°C independently of pH, whereas that of *hmpA-lacZ* was not influenced by temperature (Fig. 2A). Moreover, expression of the HilA-activated *invF-lacZ* and *sipC-lacZ* reporter genes was also repressed at 42°C independently of pH (Fig. 2B). These data demonstrated that the expression of the SPI-1 activator, *hilA*, as well as of the HilA-activated genes *invF* and *sipC*, was transcriptionally repressed by growth at 42°C.

Effect of 42°C on transcriptional activators of SPI-1. To understand how growth at 42°C repressed SPI-1 genes, the promoters of the three AraC/XylS-type activators, *hilC*, *rtsA*, and *hilD*, were cloned into the multicopy, promoterless pSP417 *lacZ* shuttle vector (34). These constructs were transformed into strains NC1040 and BTNC0025, and expression of each reporter fusion was determined following growth at 37°C or 42°C. Importantly, these plasmid constructs do not disrupt the chromosomal copies of *hilC*, *rtsA*, or *hilD*, which avoids complications in the interpretation of the data when one of these activators is mutated (9, 35). The activity seen with the empty vector, P_{hilC}, or the P_{hilD} fusion was not significantly altered by growth at 42°C. However, the expression of the HilD-activated P_{rtsA}-*lacZ* fusion was significantly reduced in both serovars (Fig. 3A; see also Fig. S1A in the supplemental material). Thus, growth at 42°C reduced transcriptional control of *sipC*, *invF*, *hilA*, and *rtsA* and suggested that the activation of SPI-1 was diminished at 42°C.

Expression data from *lacZ* fusions were confirmed in strains NC1040 and BTNC0025 by measuring mRNA levels of *hilC*, *rtsA*, and *hilD*. Bacteria were grown to an OD_{600} of ~1 at 37°C or 42°C, and RNA was extracted for qRT-PCR. As with the *lacZ* data, expression of *hilD* was not influenced by growth at 42°C; however, expression of *rtsA* was reduced ~9-fold (Fig. 3B). Although β-galactosidase levels from the pP_{hilC}-*lacZ* construct approached statistical significance under conditions of growth at 42°C, the expression of *hilC* was not reduced (Fig. 3B). With strain BTNC0025, expression of *rtsA* was reduced >5-fold following growth at 42°C (see Fig. S1 in the supplemental material). Considering the results from both serovars, the data supported the conclusion that growth at 42°C reduced expression of the *rtsA* gene.

Previous work from our laboratory and others demonstrated that the Fur transcription factor is required for activation of SPI-1 genes (35–38). Therefore, we determined the effect of Fur on the expression of the P_{hilC}-*lacZ*, P_{rtsA}-*lacZ*, and P_{hilD}-*lacZ* fusions following growth at 37°C. Because growth at 42°C repressed expression of the P_{rtsA}-*lacZ* fusion, identifying that Fur also regulates SPI-1 in this manner would suggest that temperature control of SPI-1 may act through Fur. However, activation of the P_{rtsA}-*lacZ*

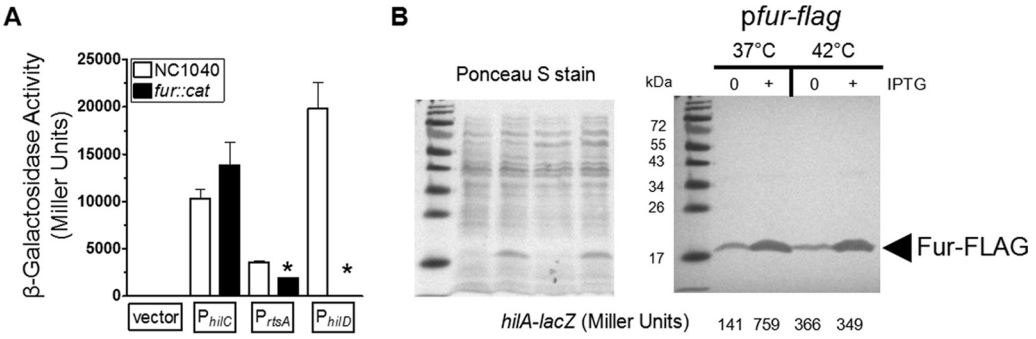

FIG 4 Growth at 42°C repressed expression of SPI-1 genes independently of the activator Fur. (A) The promoters of *hilC*, *rtsA*, and *hilD* were cloned into the pSP417 multicopy shuttle vector (empty vector) upstream of a promoterless *lacZ* gene. Bacteria were grown as described for Fig. 1B, and the promoter activities were determined for the NC1040 and *fur*::*cat* strains following overnight growth at 37°C. Data are from 3 separate experiments. The strains used were BTNC0002 to BTNC0005 and BTNC0006 to BTNC0009. (B) Expression of a *hilA-lacZ* fusion at the chromosomal *att* site was determined following overnight growth with or without induction of the Fur-FLAG protein. Bacteria were grown as described for Fig. 1B, except that samples were diluted 500-fold and cultivated at either 37°C or 42°C. A portion of each sample was removed to measure β-galactosidase activity, and the remainder was treated for SDS-PAGE and Western blotting to detect the FLAG epitope. Following transfer of proteins to the nitrocellulose membrane, the membrane was stained with Ponceau S to ensure that equivalent levels of protein were loaded for samples and that ~2×10^8 cells were loaded per lane (left panel). IPTG was added to the growth medium to reach a concentration of 0.1 mM to induce Fur-FLAG. Western blotting with the anti-FLAG antibody revealed cross-reactivity to the Fur protein of the expected size, indicated by the arrowhead with the appropriate label (right panel). The β-galactosidase activity for each sample is listed below each lane (right panel). Samples shown are representative of the results of 3 separate experiments. The complete β-galactosidase activity data are listed here. For BTNC0017 (p*fur-flag*), the values measured at 37°C were 156 ± 33 under uninduced conditions and 657 ± 89 under induced conditions and the values measured at 42°C were 316 ± 70 under uninduced conditions and 345 ± 4 under induced conditions.

and P_{hilD}-*lacZ* fusions was inhibited by deletion of the *fur* gene (Fig. 4A). The reduction in P_{rtsA} and P_{hilD} activation was consistent with earlier data, which showed that Fur regulated SPI-1 by controlling *hilD* expression when the *hilD* gene was present (35, 37). Following growth at 37°C and consistent with earlier findings (35, 36), the overexpression of Fur increased expression of *hilA-lacZ* by ~4-fold (Fig. 4B, right panel). Despite the enhanced expression of the *hilA-lacZ* fusion following induction of the Fur protein with IPTG at 37°C, induction at 42°C did not increase expression of *hilA-lacZ* even though the levels of the Fur protein under the two sets of conditions were strikingly similar (Fig. 4B, right panel). These data indicated that the inability to activate SPI-1 following growth at 42°C was not related to Fur.

Lon and ClpPX differentially control regulation of SPI-1 independently of growth at 42°C. Earlier work showed that the heat shock response influences regulation of SPI-1 genes (32, 39). Because the heat shock response involves activation of the two major proteases, Lon and Clp, regulation of SPI-1 genes at 42°C was studied in the presence and absence of these two factors. Consistent with earlier results, deletion of the *lon* gene enhanced expression of *sipC-lacZ* (Fig. 5A). However, the temperature-dependent expression of *sipC* was reduced ~3-fold to 5-fold following growth at 42°C for both the NC1040 and *lon*::*cat* strains (Fig. 5A). The role of the Clp protease in the regulation of SPI-1 appeared to be operating at the level of activation of the pathway instead of repression. Expression of the *sipC-lacZ* fusion was reduced ~3-fold upon deletion of the *clpP* and *clpX* (*clpPX*) genes (Fig. 5A). Furthermore, deletion of *clpPX* resulted in a level of inactivation similar to that seen with strain NC1040 following growth at 42°C (Fig. 5A). Thus, the *clpPX* heat shock genes do not appear to be the cause for reduced expression of SPI-1 at 42°C.

Additional experiments were performed to further test the contribution of *lon* to the temperature-dependent regulation of SPI-1. Even though the basal expression of a *hilA-lacZ* fusion was ~4-fold higher in the absence of *lon*, a similar level of inactivation still occurred following growth at 42°C (Fig. 5B). Thus, it is apparent that Lon does influence the basal expression of SPI-1 genes, but Lon does not appear to be responsible for inactivation of SPI-1 following growth at 42°C.

The inability to activate SPI-1 expression by growth at 42°C is overcome by overexpression of RtsA or HilC but not by overexpression of FliZ or HilD. To determine how the overexpression of SPI-1 activators influences the regulation of *hilA-lacZ* following growth at 42°C, three plasmid constructs carrying *fliZ*, *hilC*, and *rtsA* whose protein production is under the control of the allactose analog IPTG were generated. Each activator was tagged with the FLAG epitope at the 3′ end, cloned into the pUHE21-2*lacI*^{q}

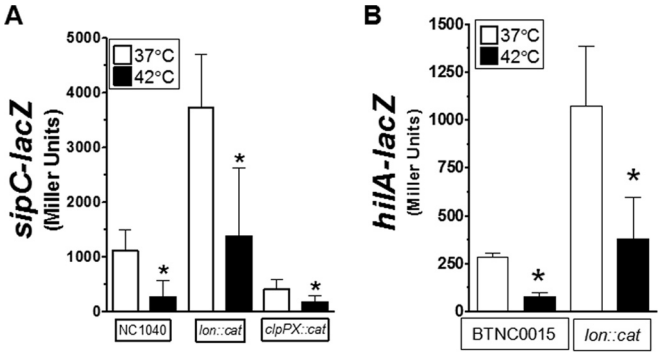

FIG 5 The Lon heat shock protease contributes to the basal level of SPI-1 repression but does not perturb inactivation following growth at 42°C. (A) The *sipC-lacZ* fusion was used to determine the contribution of *lon* or *clpPX* to the regulation of SPI-1 following growth at 42°C. Bacteria were grown as described for Fig. 1B, and reporter activity was measured after overnight growth. The strains used were BTNC0010 to BTNC0012. (B) The *hilA-lacZ* fusion integrated into the *att* within the chromosome was used to determine the influence of *lon* on the reduced expression following growth at 42°C. Samples were cultivated as described for Fig. 1B after overnight growth. Data are from 4 separate experiments. The strains used were BTNC0015 and BTNC0016.

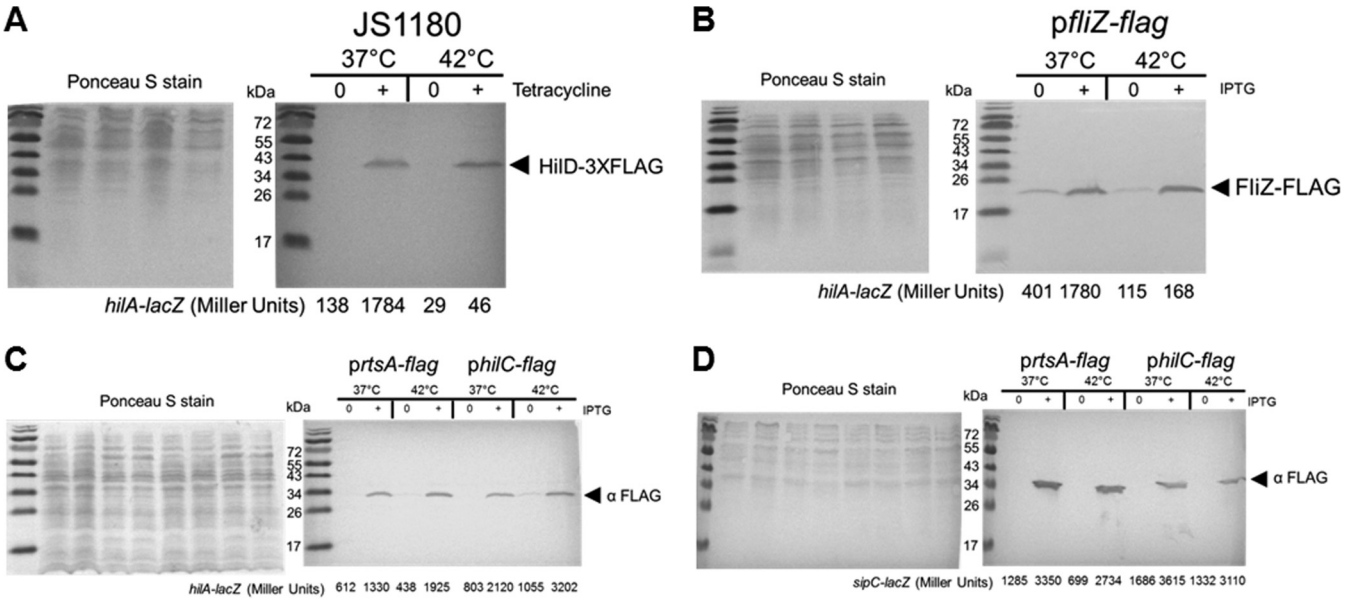

FIG 6 Overexpression of either the HilD or the FliZ SPI-1 activator does not activate SPI-1 at 42°C, but overexpression of either HilC or RtsA does. (A) Expression of a *hilA-lacZ* fusion at the chromosomal *att* site was determined following overnight growth with or without induction of the HilD-3×FLAG protein. Bacteria were grown as described for Fig. 1B, except that samples were diluted 500-fold and cultivated at either 37°C or 42°C. A portion of each sample was removed to measure β-galactosidase activity, and the remainder was treated for SDS-PAGE and Western blotting to detect the FLAG epitope. Following transfer of proteins to the nitrocellulose membrane, the membrane was stained with Ponceau S to ensure the equivalent levels of protein were loaded for samples and that ~2×10^8 cells were loaded per lane (left panel). Tetracycline was added to reach a concentration of 2.5 μg/ml to induce HilD-3×FLAG. Western blotting with the anti-FLAG antibody revealed cross-reactivity to the HilD protein of the expected size, indicated by the arrowhead with the appropriate label (right panel). The β-galactosidase activity for each sample is listed below each lane (right panel). Samples shown are representative of the results of 3 separate experiments. The complete β-galactosidase activity data are listed here. For JS1180, the values measured at 37°C were 133 ± 13 under uninduced conditions and 1,716 ± 146 under induced conditions and the values measured at 42°C were 28 ± 1 under uninduced conditions and 62 ± 34 under induced conditions. (B) Expression of a *hilA-lacZ* fusion at the chromosomal *att* site was determined following overnight growth with or without induction of FliZ-FLAG. Samples were prepared as described for panel A for the Ponceau S staining (left panel), and the Western blot analysis was performed with anti-FLAG (right panel). IPTG was used at 0.1 mM to induce FliZ-FLAG. Sample data shown are representative of the results of 3 separate experiments. The complete β-galactosidase activity data for BTNC0018 (p*fliZ-flag*) are listed here; the values measured at 37°C were 527 ± 133 under uninduced conditions and 1,539 ± 243 under induced conditions, and the values measured at 42°C were 89 ± 36 under uninduced conditions and 177 ± 92 under induced conditions. (C) Samples were treated as described for panel A for the RtsA-FLAG or HilC-FLAG strains containing the *hilA-lacZ* fusion and induced with 0.1 mM IPTG. Samples data are representative, and the β-galactosidase activity for each sample is shown below each lane. The complete β-galactosidase activity data are listed here. For BTNC0019 (p*rtsA-flag*), the values measured at 37°C were 580 ± 30 under uninduced conditions and 1,490 ± 317 under induced conditions, and the values measured at 42°C were 367 ± 66 under uninduced conditions and 2,177 ± 504 under induced conditions. For BTNC0020 (p*hilC-flag*), the values measured at 37°C were 811 ± 9 under uninduced conditions and 1,936 ± 319 under induced conditions, and the values measured at 42°C were 965 ± 113 under uninduced conditions and 3,142 ± 649 under induced conditions. (D) Strain RM5385 was transformed with p*rtsA-flag* or p*hilC-flag* and reporter activity was determined at different temperatures. IPTG was used at 0.01 mM to induce RtsA-FLAG and HilC-FLAG. The Ponceau S (left panel) and Western blotting (right panel) are shown. The complete β-galactosidase activity data are listed here. For BTNC0023 (p*rtsA-flag*), the values measured at 37°C were 1,966 ± 781 under uninduced conditions and 3,409 ± 103 under induced conditions, and the values measured at 42°C were 712 ± 469 under uninduced conditions and 2,666 ± 171 under induced conditions. For BTNC0024 (p*hilC-flag*), the values measured at 37°C were 2,801 ± 769 under uninduced conditions and 3,727 ± 906 under induced conditions, and the values measured at 42°C were 1,774 ± 521 under uninduced conditions and 3,122 ± 790 under induced conditions.

plasmid (40), and transformed into the *hilA-lacZ att* site strain (BTNC0015). To study the influence of *hilD*, we utilized published strain JS1180, which carries a tetracycline-inducible chromosomal copy of *hilD-3×flag* and the *hilA-lacZ* fusion integrated into the chromosomal *att* site (13). Along with measurement of the β-galactosidase levels in each sample, a portion was treated for SDS-PAGE and immunoblot analysis to detect expression of the FLAG-tagged proteins.

Induction of the HilD-3×FLAG protein induced expression of the *hilA-lacZ* fusion by ~13-fold following growth at 37°C; however, when cells were cultivated at 42°C, induction of HilD-3×FLAG resulted in modest (only ~2-fold) activation of the *hilA-lacZ* fusion (Fig. 6A). This was surprising, since similar levels of the HilD-3×FLAG protein were produced at 37°C and 42°C (Fig. 6A). This suggested that the activity of the HilD protein was regulated by growth at 42°C and not by the level of the protein.

The FliZ regulator is known to enhance activity of the HilD protein (41) through an unknown posttranslational step; therefore, the influence of overexpression of FliZ on *hilA-lacZ* in response to temperature was tested. Similarly to the results seen with Fur and HilD, overexpression of FliZ increased expression of *hilA-lacZ* ~4-fold at 37°C but not at 42°C even though the protein levels of FliZ were similar at the two temperatures (Fig. 6B). Collectively, these data indicated that expression of SPI-1 genes was reduced at 42°C through decreased function of the HilD protein, which resulted in reduced expression of *rtsA* and *hilA*. In addition, this implied that expression of SPI-1 genes at 42°C could be restored through HilD-independent expression of HilC or RtsA.

To test this, HilC and RtsA were placed under IPTG control to determine if expression of *hilA-lacZ* could be activated by either regulator at 42°C. In contrast to results seen with Fur, HilD, and FliZ, IPTG induction of either RtsA or HilC following growth at

37°C or 42°C was sufficient to enhance expression of the *hilA-lacZ* fusion to a similar level of activity (Fig. 6C). Moreover, overexpression of either HilC or RtsA was sufficient to activate expression of *sipC-lacZ* (Fig. 6D), supporting the conclusion that HilD-independent expression of HilC or RtsA is capable of restoring expression of SPI-1 genes at 42°C.

DISCUSSION

In pathogenic bacteria, temperature is a known environmental cue that alters expression of virulence factors. In the Lyme disease agent *Borrelia burgdorferi*, growth at different temperatures is used to model gene expression within the tick vector or the mammalian host (42–44). In addition, the infectivity of *Yersinia pestis* (formerly *Pasteurella pestis*) decreases 100-fold when cultivated at temperatures below the host body temperature (45). More recently, Elhadad et al. demonstrated that *Salmonella* bacteria causing typhoid fever alter gene regulation and invasion in response to temperatures consistent with a febrile response (46). After ~6 days posthatch, the body temperature of chickens stabilized near 42°C (Fig. 1A), a temperature that negatively influences the regulation of SPI-1 genes. The data indicated that the activity of the HilD protein was inhibited during growth at 42°C. This effect was specific for HilD since overexpression of either RtsA or HilC, the other AraC/XylS type activators of *hilA*, was sufficient to activate SPI-1 expression at 42°C (Fig. 6C). Lon and ClpPX do not appear involved in the lack of activation of SPI-1 by growth at 42°C. Instead, the function of the HilD protein is diminished following growth at the body temperature of poultry (42°C). It is becoming evident that activation of the HilD protein through an unknown posttranslational mechanism(s) is a critical aspect in the regulation of SPI-1 (13, 14, 41). Future work is required to determine how growth at 42°C integrates into the existing understanding of the regulation of SPI-1. Regardless, it is clear from this work that activators of SPI-1 do not activate SPI-1 at 42°C, suggesting that the body temperature of the avian host appears to have adapted to limiting invasion of the liver and spleen by these two serovars.

A difference in body temperature may explain the marginal requirement of SPI-1 for *S.* Typhimurium and *S.* Enteritidis within the avian host. Numerous studies have tested the contribution of SPI-1 genes to the cecal and systemic colonization of *S.* Typhimurium and *S.* Enteritidis in birds. Despite differences in experimental designs, *Salmonella* strains, and chickens, the collective data indicate a marginal contribution of SPI-1 to invasion of the liver and spleen during infection. When 1-day-old chicks were orally infected with a strain containing deletion of the SPI-1 gene *spaS*, Jones et al. (47) observed a delay in colonization of the liver of infected birds without a reduction in the cecal burden compared to the wild type. However, even the livers of birds infected with the wild-type strain exhibit low concentrations of *S.* Typhimurium by 14 days postinoculation (47). Sivula et al. (48) observed a similar phenotype when White Leghorn chicks were infected with *S.* Typhimurium. Moreover, mutation of the *invA* SPI-1 gene did not influence invasion of the cecal epithelium of birds (48). However, other work did detect a reduction in the concentration of a Δ*spi1* strain in the spleens of infected chickens compared to the wild-type strain (49). Conclusions from this finding are complicated by the observation that the Δ*spi1* strain also had a defect in cecal colonization compared to the wild-type strain (49). Thus, the reduction in localization of the Δ*spi1* strain to the spleen may be due to a fitness defect within the ceca. Indeed, gene expression data corresponding to colonization of the avian ceca by *S.* Typhimurium *in vivo* demonstrate that expression of *hilC* and other activators of SPI-1 (e.g., *tdcA*) is significantly downregulated (50).

Experiments performed with *S.* Enteritidis in chickens exhibit a trend similar to that seen with data from *S.* Typhimurium experiments. Rychlik et al. (51) tested the contribution of SPI-1, SPI-2, SPI-3, SPI-4, and SPI-5 to cecal and systemic colonization in chickens. Deletion of *spi1* resulted in a significant reduction in liver and spleen colonization at an early time point (5 dpi) but not at a later time point (51). In addition, earlier work showed that SPI-1 marginally contributed to systemic localization (26). Moreover, deletion of the *hilA* SPI-1 activator reduced localization to systemic organs early during infection but not at a later time point (52). These results, in combination with our gene regulation data, suggest that SPI-1 may be important early in infection before the body temperature of birds has plateaued at ~42°C. Although our data support the idea of a reduced ability of these two serovars to reach the livers and spleens of chicks, a thorough screening performed with additional isolates from these serovars may reveal more-invasive phenotypes. Recent work shows that *S.* Typhimurium isolates exhibit broad ranges of invasive phenotypes (46).

In contrast to data from the murine host, our results indicated that both *S.* Typhimurium and *S.* Enteritidis poorly localized to the spleen and liver sites within chickens despite colonization of the ceca at high concentrations. Expression of SPI-1 genes is decreased when the isolates are cultivated at the body temperature of poultry, which may explain the reduced ability of each serovar to reach the livers and spleens in chickens. In addition to contributing to invasion, expression of SPI-1 is linked to inflammation and tissue damage within the digestive tract of the animal host (53). For these two serovars, the reduced expression of SPI-1 genes at the body temperature of poultry (41°C to 42°C) may be a factor contributing to the overall lack of disease signs (i.e., liver abscesses and splenomegaly) in chickens.

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