

## QseC Mediates *Salmonella enterica* Serovar Typhimurium Virulence *In Vitro* and *In Vivo*<sup>∇†</sup>

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**The autoinducer-3 (AI-3)/epinephrine (Epi)/norepinephrine (NE) interkingdom signaling system mediates chemical communication between bacteria and their mammalian hosts. The three signals are sensed by the QseC histidine kinase (HK) sensor. *Salmonella enterica* serovar Typhimurium is a pathogen that uses HKs to sense its environment and regulate virulence. *Salmonella* serovar Typhimurium invades epithelial cells and survives within macrophages. Invasion of epithelial cells is mediated by the type III secretion system (T3SS) encoded in *Salmonella* pathogenicity island 1 (SPI-1), while macrophage survival and systemic disease are mediated by the T3SS encoded in SPI-2. Here we show that QseC plays an important role in *Salmonella* serovar Typhimurium pathogenicity. A *qseC* mutant was impaired in flagellar motility, in invasion of epithelial cells, and in survival within macrophages and was attenuated for systemic infection in 129x1/SvJ mice. QseC acts globally, regulating expression of genes within SPI-1 and SPI-2 *in vitro* and *in vivo* (during infection of mice). Additionally, dopamine  $\beta$ -hydroxylase knockout (*Dbh*<sup>-/-</sup>) mice that do not produce Epi or NE showed different susceptibility to *Salmonella* serovar Typhimurium infection than wild-type mice. These data suggest that the AI-3/Epi/NE signaling system is a key factor during *Salmonella* serovar Typhimurium pathogenesis *in vitro* and *in vivo*. Elucidation of the role of this interkingdom signaling system in *Salmonella* serovar Typhimurium should contribute to a better understanding of the complex interplay between the pathogen and the host during infection.**

Prokaryotes and eukaryotes achieve cell-to-cell signaling utilizing chemical signals. The signaling systems allow intra- and interspecies and intra- and interkingdom communication (48). The autoinducer-3 (AI-3)/epinephrine (Epi)/norepinephrine (NE) interkingdom signaling system allows communication between bacteria and their mammalian hosts. AI-3 is produced by many bacterial species (80), and Epi and NE are host stress hormones (36, 56). This system was first described as a system activating virulence traits in enterohemorrhagic *Escherichia coli* (EHEC) (55, 75). The signals are sensed by histidine sensor kinases (HKs), two of which have been identified: QseC, which senses AI-3, Epi, and NE (24), and QseE, which senses Epi, sulfate, and phosphate (67).

QseC is found in several bacteria, including *Salmonella enterica* serovar Typhimurium, which causes human gastroenteritis. However, this organism causes also systemic infection in murine models (8, 28). QseC's homolog in *Salmonella* serovar Typhimurium shares 87% sequence identity with EHEC's QseC (66), and a *Salmonella* serovar Typhimurium *qseC* mutant can be complemented with the EHEC *qseC* gene, demonstrating that the HKs of these two organisms are functionally interchangeable (59). QseC of *Salmonella* serovar Typhimurium is important for efficient colonization of swine (9). There have been contradictory reports concerning the role of Epi/NE in QseC-mediated motility in *Salmonella* serovar

Typhimurium (9, 60), and the variations can be accounted for by the labile nature of the Epi/NE signals *in vitro* (4; <http://www.sigmaaldrich.com/sigma-aldrich/home.html>). Transcriptome studies of *Salmonella* serovar Typhimurium also revealed a role for Epi and QseC in the antimicrobial peptide and oxidative stress resistance responses (50). QseC's response to its signals can be inhibited by the  $\alpha$ -adrenergic antagonist phen-tolamine (24) and the small molecule LED209. LED209 inhibits QseC in three different pathogens, EHEC, *Salmonella* serovar Typhimurium, and *Francisella tularensis*, and prevents pathogenesis *in vitro* and *in vivo* (66).

The second bacterial adrenergic HK, QseE, senses Epi, sulfate, and phosphate to activate expression of virulence genes in EHEC. Together with QseG and QseF, QseE has been implicated in pedestal formation directly related to EHEC pathogenesis (67, 68). A QseE homolog is also present in *Salmonella* serovar Typhimurium and shares 87% sequence identity with EHEC's QseE. However, the role of QseE in *Salmonella* serovar Typhimurium pathogenesis remains unknown.

*Salmonella* serovar Typhimurium pathogenesis is a complex process. The role of flagellar motility in *Salmonella* serovar Typhimurium pathogenesis is controversial. This motility was reported previously to not play a role in *Salmonella* virulence *in vivo* (53). However, flagella induce inflammation via Toll-like receptor 5 during *Salmonella* infection of the intestinal mucosa (32, 35, 77). During its evolution, *Salmonella* serovar Typhimurium acquired many pathogenicity islands (57). The main pathogenicity islands involved in *Salmonella* serovar Typhimurium infection are *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2, which encode type III secretion systems (T3SSs) essential for *Salmonella* serovar Typhimurium viru-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description or relevant genotype	Reference or source
<b>Strains</b>		
SL1344	<i>Salmonella enterica</i> serovar Typhimurium prototype	46
CGM220	<i>qseC</i> mutant $\lambda$ Red generate	66
CGM222	<i>qseC</i> complemented strain (in XhoI/HindIII pBADMycHisA)	This study
TOP10	F <sup>-</sup> <i>mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> $\Delta$ ( <i>ara-leu</i> )7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str <sup>r</sup> ) <i>endA1</i> <i>nupG</i> <i>supE44</i> $\Delta$ <i>lacU169</i> ( $\phi$ 80 <i>lacZ</i> $\Delta$ M15) <i>hsdR17</i>	Invitrogen
DH5 $\alpha$		Stratagene
<b>Plasmids</b>		
pBADMycHisA	Cloning vector	Invitrogen
pBlueScript KSII	Cloning vector	Stratagene
TOPO PCR Blunt	PCR blunt cloning vector with topoisomerase	Invitrogen
pKD3	pANTS $\gamma$ derivative containing FRT-flanked chloramphenicol resistance	26
pKD46	1 red recombinase expression plasmid	26
pKD20	TS replication and thermal induction of FLP synthesis	19

lence (38–40, 62, 71). The SPI-1-encoded T3SS is required for efficient invasion of the intestinal epithelium (39), while the SPI-2-encoded T3SS is essential for *Salmonella* serovar Typhimurium replication and survival within macrophages and systemic infection in mice (23, 43, 62). In *Salmonella enterica* serovar Typhi, the flagellar sigma factor FliA regulates SPI-1 expression and affects bacterial invasion of epithelial cells. However, this phenotype has not been observed in *Salmonella* serovar Typhimurium (29).

The SPI-1 locus encodes effectors and regulators, such as HilA, HilD, HilC, and InvF. SPI-1 contains three operons, the *prg-org*, *inv-spa*, and *sic-sip* operons. The *prg-org* and *inv-spa* operons encode the needle complex, and the *sic-sip* operon encodes the translocon that embeds itself in the host cell membrane. Additional effectors are encoded in other SPIs in the *Salmonella* serovar Typhimurium chromosome (30). HilD mediates transcriptional cross talk between SPI-1 and SPI-2, providing sequential activation of the loci. This mechanism allows *Salmonella* serovar Typhimurium to invade using the SPI-1-encoded T3SS and survive within the cellular vacuole using the SPI-2-encoded T3SS (17). InvF, encoded by the first gene of the *inv-spa* operon, is an AraC-type transcriptional activator required for SPI-1 expression.

The SPI-1 effectors SopE, SopE2, and SopB in a pathogen, as well as the Rho GTPases Cdc42, Rac1, and RhoG in a host, are required for orchestrated and efficient invasion. Together, these molecules lead to actin cytoskeletal reorganization, membrane ruffling, and bacterial internalization by macropinocytosis. The presence of SopB, together with the effectors mentioned above, leads to chloride secretion through lipid dephosphorylation (85), and SopB indirectly stimulates Cdc42 and RhoG through its phosphoinositide phosphatase activity (16, 42, 64, 85). SopB also promotes intestinal disease by increasing the intracellular concentration of D-myo-inositol 1,4,5,6-tetrakisphosphate, which stimulates cellular chloride secretion and fluid flux (85). Although SipA is not required for cell invasion, it helps initiate actin polymerization at the site of *Salmonella* serovar Typhimurium entry by decreasing the critical concentration and increasing the stability of actin filaments (44, 58, 86).

The effector SifA (encoded outside SPI-2) is secreted

through the SPI-2 T3SS and is essential for inducing tubulation of the *Salmonella* serovar Typhimurium phagosome. SifA binds to the mammalian kinesin-binding protein SKIP. SifA coexpressed with SseJ induces tubulation of mammalian cell endosomes, indicating that SifA likely mimics or activates a RhoA family of GTPases (63).

SPI-3 (containing the *mgtBC* genes), together with SPI-2, is required for intramacrophage survival, virulence in mice, and growth in magnesium-depleted medium. The *mgtC* gene is transcriptionally controlled by the PhoP-PhoQ regulatory system (15), which is responsive to Mg<sup>2+</sup>, antimicrobial peptides, and pH and is the major regulator of virulence in *Salmonella* (6, 22).

The BasRS two-component system, also known as PmrAB, is an important regulator of the *Salmonella* serovar Typhimurium virulence genes. PmrAB regulates genes that modify lipopolysaccharide, conferring resistance to cationic microbial peptides, such as polymyxin B, and subsequently aiding survival within the host (41). Expression of *pmrAB* is also indirectly regulated by the PhoPQ system, and QseC has been linked to regulation of *pmrAB* (59, 60).

Here we show that QseC plays an important role in *Salmonella* serovar Typhimurium pathogenicity *in vitro* and *in vivo*. A *qseC* mutant shows diminished flagellar motility, invasion of epithelial HeLa cells, survival within J774 macrophages, and virulence in a systemic murine infection. Additionally, *Dbh*<sup>-/-</sup> mice (which are unable to produce Epi and NE) infected with the *Salmonella* serovar Typhimurium wild type (WT) and the *qseC* mutant have different infection kinetics than WT mice. These findings suggest that the AI-3/Epi/NE signaling system plays an important role in interkingdom signaling during *Salmonella* serovar Typhimurium infection *in vivo*. QseC globally regulates transcription expression of SPI-1, the SPI-2 effector *sifA*, SPI-3, and flagellar genes *in vitro* and *in vivo*.

## MATERIALS AND METHODS

**Strains and plasmids.** All strains and plasmids used in this study are listed in Table 1. Strains were grown aerobically in LB or N-minimal medium (17, 23, 27, 54) at 37°C. Recombinant DNA and molecular biology techniques were performed as previously described (69). All oligonucleotides used in this study are listed in Table 2.

TABLE 2. Oligonucleotides used in this study

Primer	Forward sequence	Reverse sequence
<i>sipA</i>	TTTAGTAGCCGCTGCGATAA	GCCGTGGTACCGTCTACCA
<i>sopB</i>	CGGGTACCGCGTCAATTTT	TGGCGGCGAACCCCTATAAA
<i>invF</i>	CAGGACTCAGCAAACCCATTT	TTATCGATGGCGCAGGATTAG
<i>sifA</i>	GTTGTCTAATGGAACCGATAATATCG	CTACCCCTCCCTTCGACAT
<i>mgtB</i>	ACTTCATTGCGCCCATACACT	CGTCAGGGCCTACGATAGA
<i>fliA</i>	TTGGTGCGTAATCGTTTGATG	CTGGAAGTCGGCGAATCG
<i>flhDC</i>	GTCAAACCGGAAATGACAACTAA	ACCCTGCCGAGATGGT
<i>motA</i>	TTACCGCCGCGACGATA	CCAACAGTCTGGCGATGGT
<i>basS</i>	CGGTA AAAAGGCGTTTATTGTC	CGTCTCGGCGATCAATCAA
<i>qseB</i>	CCACTGGCCCTAAAACCAAAA	GGCAGCACGCGACCTTT
<i>rpoA</i>	GCGCTCATCTTCTCCGAAT	CGCGGTCTGGTTATGTG
<i>qseC-Lambda Red</i>	TGACGCAACGTCTCAGCCTGCGCGTCAGGCTG ACGCTTATTTTCTGATTGTGTAGGCTGGGA GCTGCTTC	CCAACCTACTACGGCCTCAAATCCACCTTCCCG GGCGTTGCCAAACGACACATATGAATATCCT CCTTA
<i>qseC-pBADMycHisA</i>	CTCGAGATAAATTGACGCAACGTCTCAGC	AAGCTTCGCCAACTTACTACGGCCTCAAA

**Construction of the *qseC* mutant.** An isogenic nonpolar *Salmonella* serovar Typhimurium SL1344 *qseC* mutant was constructed using  $\lambda$  red mutagenesis (26). This *qseC* mutant (CGM220) was complemented with the *qseC* gene cloned (XhoI and HindIII) in the pBADMycHisA (Invitrogen) vector, generating strain CGM222.

**Quantitative real-time RT-PCR.** Overnight cultures were grown aerobically in LB or N-minimal medium at 250 rpm to late exponential growth phase (optical density at 600 nm, 1.0) for the *in vitro* assays in the absence or presence of 50  $\mu$ M NE. The *in vivo* expression levels were measured by directly extracting the *Salmonella* serovar Typhimurium RNA from the spleens and livers of infected animals. Negative control PCRs were performed with RNA extracted from livers and spleens of uninfected animals to ensure that there was no cross-reactivity of our primers with mammalian mRNA. RNA was extracted from three biological samples using a RiboPure bacterial RNA isolation kit (Ambion) by following the manufacturer's guidelines. The primers used in the real-time assays were designed using Primer Express v1.5 (Applied Biosystems) (Table 2). Real-time reverse transcription (RT)-PCR was performed in one step using an ABI 7500 sequence detection system (Applied Biosystems). For each 20- $\mu$ l reaction mixture, 10  $\mu$ l 2 $\times$  SYBR master mixture, 0.1  $\mu$ l Multiscribe reverse transcriptase (Applied Biosystems), and 0.1  $\mu$ l RNase inhibitor (Applied Biosystems) were added. The amplification efficiency of each primer pair was verified using standard curves prepared with known RNA concentrations. The *rpoA* (RNA polymerase subunit A) gene was used as the endogenous control. Data collection was performed using the ABI Sequence Detection 1.3 software (Applied Biosystems). Data were normalized to levels of *rpoA* and analyzed using the comparative critical threshold ( $C_T$ ) method described previously (81). The levels of expression of the target genes at the different growth phases were compared using the relative quantification method (81). Real-time data are expressed below as fold changes compared to WT levels. Error bars in the figures indicate the standard deviations of the  $\Delta\Delta C_T$  values (81). Statistical significance was determined by using Student's *t* test. A *P* value of <0.05 was considered significant. All raw qRT-PCR data can be found in the supplemental tables.

**HeLa cell invasion assays.** Epithelial HeLa cells were infected with *Salmonella* serovar Typhimurium at a multiplicity of infection (MOI) of 100:1 for 90 min to examine bacterium-cell interactions at 37°C in the presence of 5% CO<sub>2</sub>, as previously described (33, 34, 65). The cells were treated with 40  $\mu$ g/ml of gentamicin for 1 h to kill extracellular bacteria and lysed with 1% Triton X-100. Bacteria were diluted and plated on LB medium plates to determine the number of CFU (33, 34, 65).

**Macrophage infection.** J774 murine macrophages were infected with opsonized *Salmonella* serovar Typhimurium with normal mouse serum at 37°C for 15 min and washed. The macrophages were infected using an MOI of 100:1 for 30 min to examine bacterium-cell interactions at 37°C in the presence of 5% CO<sub>2</sub>. The cells were treated with 40  $\mu$ g/ml of gentamicin for 1 h to kill extracellular bacteria and lysed with 1% Triton X-100. Bacteria were diluted and plated on LB medium plates to determine the number of CFU (28, 33, 65).

**Motility assays.** The motility assays were performed at 37°C using LB broth containing 0.3% agar (76). The transcriptional expression of the flagellum genes, *flhDC*, *fliA*, and *motA*, was measured by quantitative RT-PCR (qRT-PCR) using RNA extracted directly from halos cut out of the LB motility agar.

**Survival of mice with *Salmonella* serovar Typhimurium.** Mice (129x1/SvJ 7, 9 weeks old, female) were infected intraperitoneally (IP) with  $1 \times 10^8$  CFU of *E. coli* K-12 strain DH5 $\alpha$  (K-12 was used as a negative infectivity control to ensure that there were no issues with organ perforation during IP injection and that the death of mice was not due to endotoxic effects), *Salmonella* serovar Typhimurium WT strain SL1344, or the *qseC* mutant (CGM220). Ten mice were infected IP with each strain using the same IP method and  $1 \times 10^8$  CFU, and the experiments were repeated at least twice to ensure reproducibility. Mice were returned to their cages and monitored daily for signs of morbidity (anorexia, rapid shallow breathing, scruffy fur, decreased muscle tone, and lethargy) and death. At 7 days postinfection the remaining animals were euthanized by CO<sub>2</sub> asphyxiation. Systemic colonization of organs was performed as previously described (28), and after 20 h of IP infection the mice were sacrificed to remove the spleens and livers. These organs were harvested and homogenized, and the homogenates were plated on LB agar plates for bacterial cell counting to determine tissue colonization (CFU) (28, 62).

Mixed IP infection with the *Salmonella* serovar Typhimurium WT and the *qseC* mutant was performed using one-half of the WT strain inoculum usually used and one-half of the *qseC* inoculum usually used ( $1 \times 10^4$  CFU of each strain or a total of  $2 \times 10^4$  CFU). The eight mice used were sacrificed at 20 h postinfection, and organs were collected and plated to determine the bacterial loads. The ratio of  $\Delta qseC$  mutant counts to WT counts was used to determine the competitive index (12, 28, 70).

**Dbh knockout mice.** Dopamine  $\beta$ -hydroxylase knockout (*Dbh*<sup>-/-</sup>) mice, maintained with a mixed C57BL/6J and 129 SvEv background, were generated at Emory University as previously described (2), shipped to the University of Texas Southwestern Medical Center, and infected IP with the WT and the *qseC* mutant as described above. Littermate *Dbh*<sup>+/-</sup> heterozygous mice were used as controls because they had normal NE and Epi levels (78).

## RESULTS

**QseC affects flagellar motility.** The QseC system directly activates flagellar expression in EHEC by regulating transcription of the *flhDC* genes that encode the flagellar master regulators (25, 76). Moreover, Bearson and Bearson (9) have shown that NE enhances motility and flagellar gene expression via QseC in *Salmonella* serovar Typhimurium (9). Contradictory data concerning NE/Epi enhancement of motility were reported by Merighi et al. (60), suggesting that Epi did not play a role in enhancement of motility in WT *Salmonella* serovar Typhimurium. However, it is worth noting that *in vitro* NE and Epi are photosensitive and labile compounds that quickly decompose in solution (4). The labile nature of these compounds could be responsible for the variation described in the previous motility studies. Since both Bearson and Bearson and Merighi

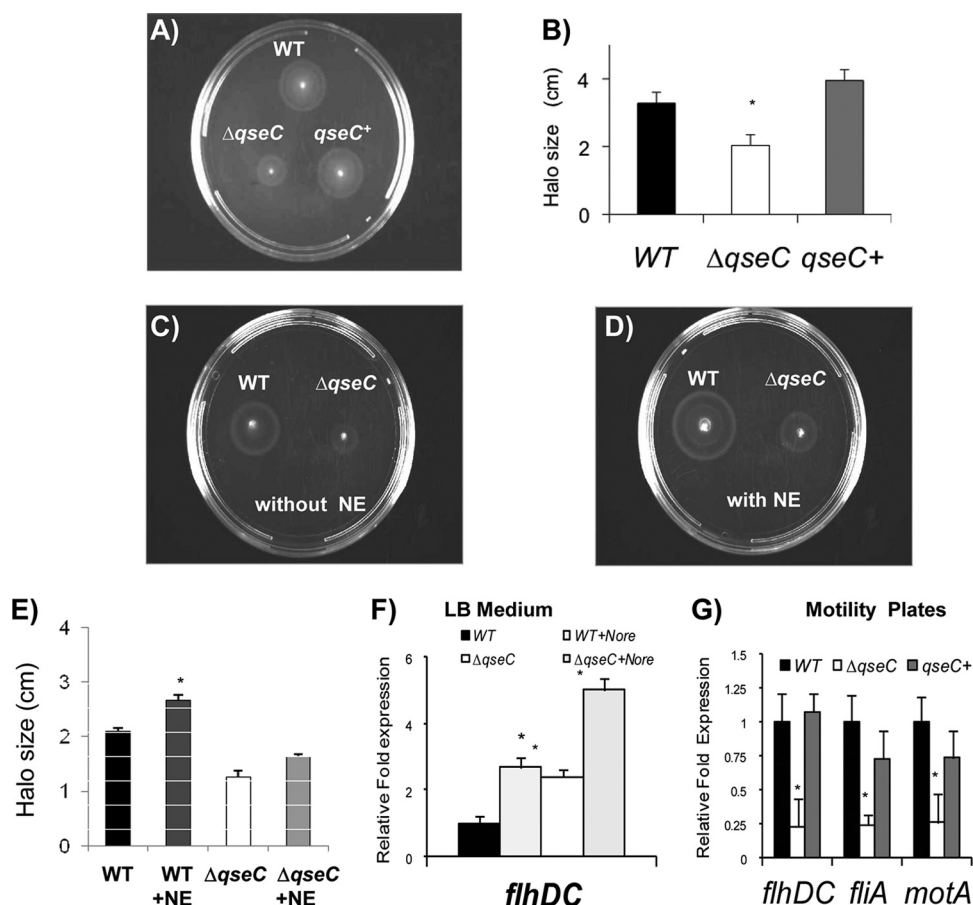


FIG. 1. QseC regulation of *Salmonella* serovar Typhimurium's flagella *in vitro*. (A) Motility assay performed in plates containing LB medium with 0.3% agar after 8 h. (B) Sizes of motility halos after 8 h. (C) Motility assay performed with the WT and  $\Delta qseC$  strains using LB medium without NE after 8 h. (D) Motility assay performed with the WT and  $\Delta qseC$  strains in LB medium with 50  $\mu$ M NE after 8 h. (E) Sizes of motility halos after norepinephrine (NE) addition at 8 h. (F) Transcription of *flhDC* in the WT strain and the  $\Delta qseC$  mutant in LB medium with and without 50  $\mu$ M norepinephrine (Nore). (G) Transcription of flagellar genes (*flhDC*, *fliA*, and *motA*) in WT,  $\Delta qseC$ , and complemented strains in motility agar. Statistical significance was determined by Student's *t* test based on comparison with the expression in the WT strain (\*,  $P < 0.001$ ).  $qseC^+$ , complemented strain.

et al. (9, 60) reported decreased motility of *qseC* mutants, we first investigated the motility phenotype of our *qseC* mutant, and congruent with both previous reports, we observed decreased motility compared to the WT and the phenotype was rescued by complementation (Fig. 1A and 1B). Although NE has been shown to increase expression of flagellum genes in WT *Salmonella* serovar Typhimurium (9), there have been no reports of QseC regulation of the flagellum regulon in this species. In agreement with the report of Bearson and Bearson (9), we observed that NE significantly increased motility of the WT strain (Fig. 1C, 1D, and 1E) but that in the presence of NE the motility of the *qseC* mutant was still decreased (Fig. 1C, 1D, and 1E). Surprisingly, in *Salmonella* serovar Typhimurium cultures grown in LB medium, transcription of *flhDC* was 2-fold greater in the *qseC* mutant than in the WT (Fig. 1F). We hypothesized that QseC-dependent flagellar gene expression may be differentially regulated in LB medium and in motility agar. To test this hypothesis, we assessed expression of the flagellar genes *flhDC*, *fliA*, and *motA* in the WT, *qseC* mutant, and complemented strains by performing qRT-PCR with RNA extracted from cultures grown in motility agar. Under these

conditions, we observed diminished transcription of all of the flagellar genes in the *qseC* mutant (transcription of *flhDC* was significantly decreased 4-fold, transcription of *fliA* was significantly decreased 4-fold, and transcription of *motA* was significantly decreased 3-fold), and this phenotype was rescued by complementation (Fig. 1G), explaining the decreased motility observed for the *qseC* mutant (Fig. 1A and B).

Consistent with previous reports (9, 24), NE increased expression of *flhDC* during growth in the WT and in the *qseC* mutant (in both strains expression was increased 2-fold) in LB medium (Fig. 1F). There are two potential explanations for the differential regulation of *flhDC* by QseC under different environmental conditions (LB medium versus motility plates). (i) Transcriptional regulation of *flhDC* is complex and can vary within a species due to the presence of insertion sequences in the regulatory region of *flhDC* (7). To add to this complexity, QseB binds to different sites according to its phosphorylated state and can act as a repressor and activator within the same promoter (47). The *flhDC* regulatory region in *Salmonella* serovar Typhimurium lacks one of the QseB binding sites present in the EHEC *flhDC* regulatory region (21), which may



account for the differential and environment-dependent regulation of these genes in *Salmonella* serovar Typhimurium compared to EHEC. (ii) *Salmonella* serovar Typhimurium NE-dependent *flhDC* expression in LB broth may also occur through a second receptor. *Salmonella* serovar Typhimurium harbors a QseE homolog, and QseE senses Epi/NE (67). Cross-signaling between the QseBC and QseEF systems has also been observed (47). Different receptors may play a role in NE modulation of *flhDC* expression under different environmental conditions, since transcription of *flhDC* in LB medium is increased in the *qseC* mutant (Fig. 1F), while it is decreased in motility agar (Fig. 1G).

Furthermore it is also worth noting that FlhDC is the master flagellar regulator and that transcriptional regulation of *flhDC* is sensitive to several environmental conditions and cell state sensors (20). The promoter of *flhDC* defines the flagellar class 1 promoter and is a crucial point at which the decision to initiate or prevent flagellar biosynthesis occurs. This promoter is controlled by a number of global regulators, such as cyclic AMP receptor protein (74, 83), heat shock proteins DnaK, DnaJ, and GrpE (72), and H-NS (11, 82), as well as by several environmental signals, such as temperature, high concentrations of inorganic salts, carbohydrates, or alcohols, growth phase, DNA supercoiling, phosphatidylethanolamine and phosphatidylglycerol synthesis, and cell cycle control (21).

**QseC plays a role in invasion of epithelial cells and intramacrophage survival.** Next we investigated whether QseC affected *Salmonella* serovar Typhimurium invasion of HeLa cells and survival within J774 macrophages. There was a significant 1-order-of-magnitude decrease in HeLa cell invasion by the *qseC* mutant compared to the WT (Fig. 2A), and there was a 5-order-of-magnitude decrease in survival of the *qseC* mutant compared to the WT in J774 macrophages (Fig. 2B). Both phenotypes were rescued by complementation (Fig. 2A and B). These data suggest that expression of both SPI-1- and SPI-2-encoded T3SSs and/or effectors is regulated by QseC.

HeLa cell invasion is an SPI-1-dependent phenotype, while SPI-2 is involved in intramacrophage survival (37–39). Expression of the SPI-1 genes *invF*, *sipA*, and *sopB* was assessed by qRT-PCR under conditions conducive to SPI-1 expression (LB medium) (17). Transcription of these genes was significantly decreased in the *qseC* mutant; expression of *invF* was decreased 2-fold, expression of *sipA* was decreased 5-fold, and expression of *sopB* was decreased 2.5-fold. Expression of all of these genes was rescued by complementation (Fig. 3A). Transcription of the gene encoding the SPI-2 T3SS effector SifA under conditions optimal for SPI-2 expression (N-minimal medium, which is a low-phosphate and low-magnesium medium [17]) was strikingly reduced in the *qseC* mutant (it was 100-fold lower than transcription in the WT) and was also rescued by complementation (Fig. 3B). These results are congruent with the decreased ability of the *qseC* mutant to invade epithelial cells and survive within macrophages (Fig. 2).

Because QseC is one of the sensors for Epi/NE (24), we investigated whether NE could activate expression of the genes mentioned above in a QseC-dependent manner. NE increased expression of *invF* (18-fold), *sopB* (17-fold), and *sifA* (6-fold) in WT *Salmonella* serovar Typhimurium (Fig. 3C). However, only NE-induced *sifA* expression was completely dependent on QseC (Fig. 3C). NE-induced expression of *invF* was signifi-

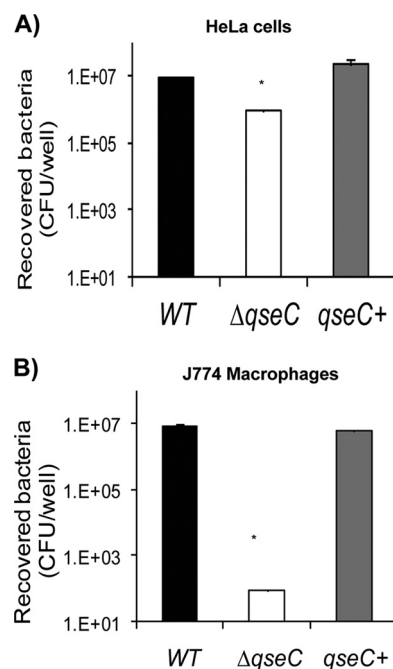


FIG. 2. Involvement of QseC in *Salmonella* serovar Typhimurium invasion of HeLa cells and intramacrophage survival. (A) Invasion of HeLa epithelial cells by WT,  $\Delta qseC$ , and complemented strains. (B) Intramacrophage survival (J774 macrophages) of WT,  $\Delta qseC$ , and complemented strains. Statistical significance was determined by Student's *t* test based on comparison with the WT strain (\*,  $P < 0.001$ ). *qseC+*, complemented strain.

cantly decreased but not eliminated in the *qseC* mutant, and there was a significant 2-fold decrease in the *qseC* mutant with NE compared to the WT with NE (Fig. 3C). Meanwhile, NE-induced expression of *sopB* was similar in the WT and the *qseC* mutant (Fig. 3C). These data suggest that *in vitro* induction of *sifA* transcription by NE is completely QseC dependent, which is congruent with our previous studies (66). However, NE-induced transcription of *invF* is only partially dependent on QseC (there was a significant 2-fold decrease in the *qseC* mutant with NE compared to the WT with NE), while transcription of *sopB* *in vitro* can be induced by NE even in the absence of QseC, suggesting that another NE receptor, maybe QseE, could play a role in this regulation.

**QseC in *Salmonella* serovar Typhimurium systemic infection of mice.** Inasmuch as QseC regulates expression of the SPI-1 and *sifA* genes (Fig. 3) and plays an important role in epithelial cell invasion and intramacrophage survival (Fig. 2), one would expect that a *qseC* mutant would be attenuated for murine infection. Indeed, we found previously that a *qseC* mutant was attenuated for systemic infection of mice by performing survival studies (66). Here we conducted further murine infection experiments to assess in more detail this attenuation for infectivity. We used the systemic (intraperitoneal [IP] infection) typhoid-like model with 129x1/SvJ mice, which are *Nramp1*<sup>+/+</sup> (79) and more resistant to *Salmonella* serovar Typhimurium infection (18). This murine strain was chosen because we obtained knockout mice that do not produce either epinephrine or norepinephrine in this background (see Fig. 7 and 8) and we needed consistency in the genetic background of the animals to

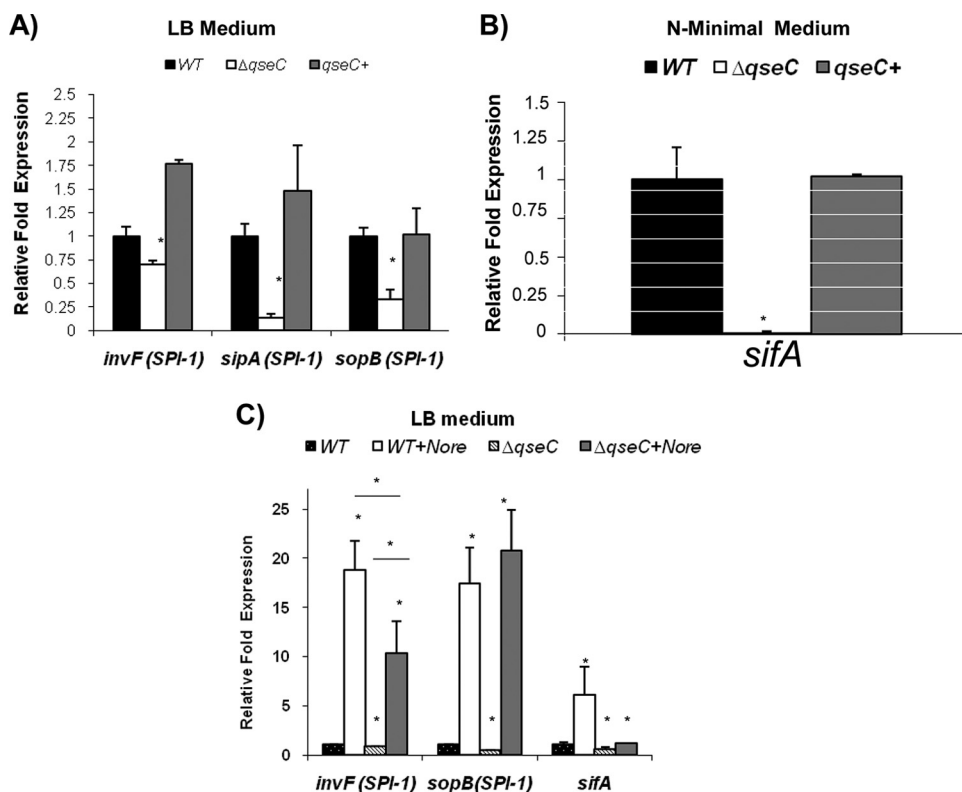


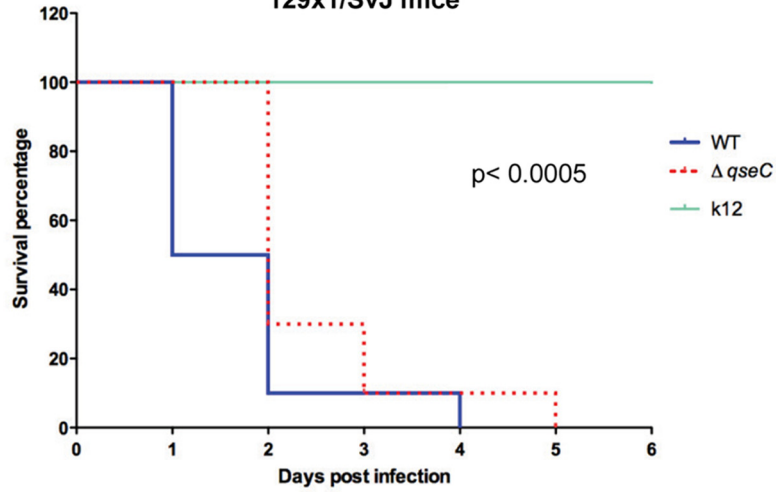
FIG. 3. QseC regulates transcriptional expression of SPI-1 and *sifA* *in vitro*. (A) Transcription of SPI-1 genes in WT,  $\Delta qseC$ , and complemented strains in LB medium. (B) Transcription of *sifA* in WT,  $\Delta qseC$ , and complemented strains in N-minimal medium. (C) Transcription of *invF* (SPI-1), *sopB* (SPI-1), and *sifA* in WT and  $\Delta qseC$  strains in LB medium in the absence and presence of 50  $\mu$ M NE. In all cases expression levels were compared to WT expression levels without NE. In addition, a second analysis of the expression of *invF* was performed by comparing the expression in the WT strain with NE and the expression in the  $\Delta qseC$  mutant with NE, as indicated. Statistical significance was determined by Student's *t* test (\*,  $P < 0.001$ ). Nore, norepinephrine;  $qseC+$ , complemented strain.

compare and contrast data. As a negative control we also performed IP infection using *E. coli* K-12 (Fig. 4A and B; see Fig. 7) to ensure that endotoxic effects were not responsible for the morbidity and mortality observed in the animals. After infection with WT *Salmonella* serovar Typhimurium, 50% of the mice were still alive at day 1 postinfection, while 100% of the *qseC* mutant-infected mice were still alive at this time (Fig. 4A). By day 2 only 10% of the WT strain-infected mice were still alive, while 30% of the *qseC* mutant-infected mice were still alive. All WT strain-infected mice were dead by day 4 postinfection, while all *qseC* mutant-infected mice were dead by day 5 (Fig. 4A). Colonization of the spleens and livers of mice (at 20 h postinfection) was significantly less (2 orders of magnitude less) for the *qseC* mutant than for the WT (Fig. 4B). We also determined a competitive index for the WT and the *qseC* mutant in mice. Competition studies were performed to avoid issues concerning individual variation between different mice when the abilities of the strains to become established in the spleens and livers of the animals were scored. As an initial control, an *in vitro* competition experiment with the WT and the *qseC* mutant was performed to ensure that the *qseC* mutant did not have growth defects (data not shown). Figure 4C shows that the WT strain outcompeted the *qseC* mutant, further indicating that the *qseC* mutant was attenuated for systemic murine infection.

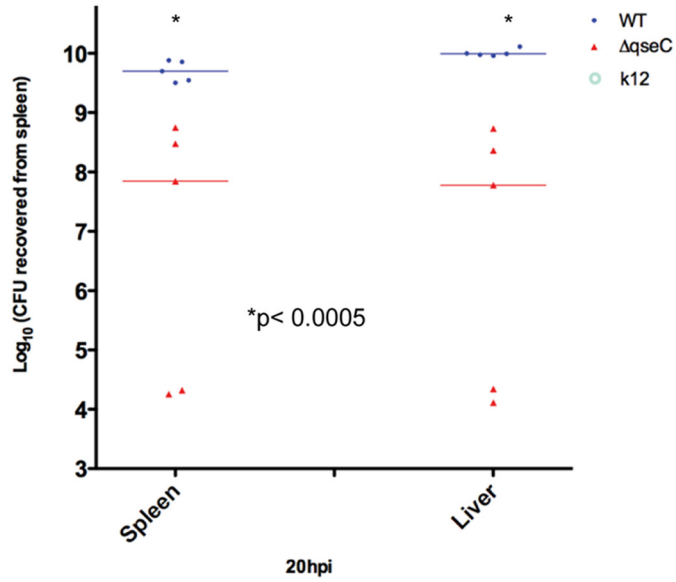
**QseC-dependent gene regulation *in vivo*.** The attenuation of the *qseC* mutant during systemic infection could have been a result of the QseC regulation of SPI-1 genes and *sifA* (Fig. 3). However, *in vitro* conditions do not exactly reflect the complexity of the *in vivo* environment. Hence, we investigated the role of QseC in the regulation of these virulence genes during infection. We harvested the spleens and livers of 129x1/SvJ mice infected with either the WT or the *qseC* mutant (three mice each) 20 h postinfection, extracted the RNA, and performed qRT-PCR using *rpoA* as an internal control. Transcription of the SPI-1 genes *sipA* (4-fold) and *sopB* (3-fold) in both spleens and livers and transcription of the *sifA* gene (4-fold in spleens and 1.4-fold in livers) were significantly decreased in the *qseC* mutant from infected animals (Fig. 5). We also examined transcription of the SPI-3 *mgfB* gene, which is known to be important for intramacrophage survival (14, 15). Transcription of the *mgfB* gene was also decreased (4-fold) in the *qseC* mutant in both spleens and livers (Fig. 5B). These data suggest that QseC activates expression of the SPI-1, *sifA*, and SPI-3 genes *in vitro* (Fig. 3) and *in vivo* (Fig. 5).

We also compared expression of these genes in the *Salmonella* serovar Typhimurium WT strain *in vitro* and *in vivo* (Fig. 6) and observed that the transcription of the SPI-1 genes *sipA* and *sopB* was significantly decreased *in vivo* compared to the transcription *in vitro*. Given that our *in vivo* assessment was

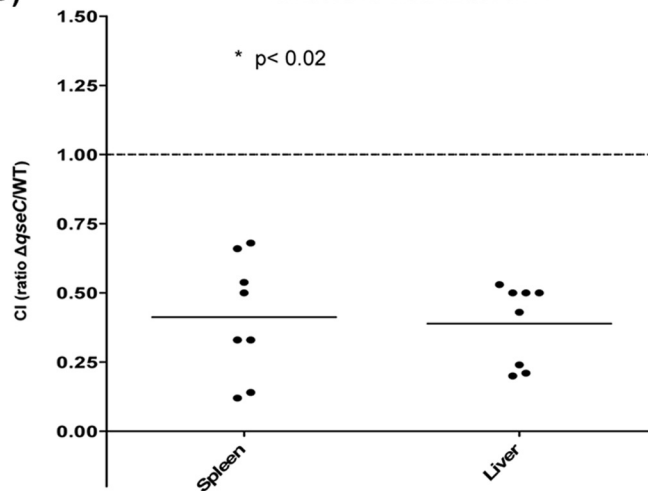
**A) Mice survival during *S. Typhimurium* SL1344 infection in 129x1/SvJ mice**



**B) Organ colonization during *S. Typhimurium* SL1344 infection in 129x1/Svj mice**



**C) Competitive Index ΔqseC/WT**



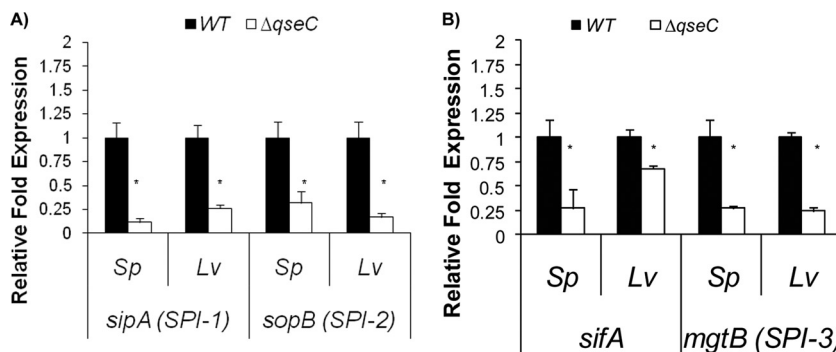


FIG. 5. QseC regulates transcriptional expression *in vivo* of SPI-1, *sipA*, and SPI-3. (A) Transcription of *sipA* and *sopB* in spleens and livers harvested from 129x1/SvJ mice 20 h after IP infection with the WT or the  $\Delta qseC$  mutant. (B) Transcription of *sipA* and *mgtB* in spleens and livers harvested from 129x1/SvJ mice 20 h after IP infection with the WT or the  $\Delta qseC$  mutant. Statistical significance was determined by Student's *t* test (\*,  $P < 0.001$ ). Sp, spleens; Lv, livers.

performed with livers and spleens and that SPI-1's major role seems to be a role in the invasion of the intestinal epithelia, these results were not unexpected. One exception is the expression of *invF*, which is an SPI-1 gene and is highly upregulated *in vivo*. InvF is a transcriptional factor, and given the cross regulation of SPI-1 and SPI-2 by the SPI-1-encoded HilD regulator (17), it is possible that *in vivo* *invF* is needed to control other genes in *Salmonella* involved in systemic disease. Expression of *sifA*, *mgtB*, and *qseB* was significantly upregulated *in vivo*, reflecting of the role of these genes in *Salmonella* serovar Typhimurium systemic infection.

**Role of NE/Epi in *Salmonella* serovar Typhimurium infection *in vivo*.** Since QseC is important for systemic infection of mice by *Salmonella* serovar Typhimurium (Fig. 4) and since QseC senses AI-3, Epi, and NE (24), we assessed the role of QseC-sensing Epi and NE during infection of mice by *Salmonella* by using *Dbh*<sup>-/-</sup> mice that lack NE and Epi (3). It is worth noting that although NE and Epi are not required for normal development of the immune system of these mice, they play an important role in the modulation of T-cell-mediated immunity to infection. Specifically, these mice have impaired Th1 T-cell responses to infection (3). Th1 T cells are important in controlling intracellular pathogens (51, 61, 73). Consequently, *Dbh*<sup>-/-</sup> mice are more susceptible to intracellular pathogens (*Listeria monocytogenes* and *Mycobacterium tuberculosis*) (3).

To assess whether the *Dbh*<sup>-/-</sup> knockout mice were more susceptible to *Salmonella* serovar Typhimurium infection, we infected *Dbh*<sup>-/-</sup> and *Dbh*<sup>+/-</sup> control mice with the WT strain. Congruent with previous reports, the *Dbh*<sup>-/-</sup> mice were more susceptible to *Salmonella* serovar Typhimurium infection than the *Dbh*<sup>+/-</sup> mice (Fig. 7A). By day 2 postinfection 80% of the *Dbh*<sup>-/-</sup> mice were dead, while only 45% of the *Dbh*<sup>+/-</sup> were dead. All of the *Dbh*<sup>-/-</sup> mice were dead by day 6, while all of

the *Dbh*<sup>+/-</sup> mice were dead by day 7 (Fig. 7A). We next infected *Dbh*<sup>-/-</sup> and *Dbh*<sup>+/-</sup> mice with the *qseC* mutant and observed that during the first 3 days, the levels of mortality due to the *qseC* mutant were similar for the two mouse strains (Fig. 7B). These results highlight an important difference compared with infection by WT *Salmonella* serovar Typhimurium, in which the defect in the Th1 immune response to infection by intracellular pathogens in the *Dbh*<sup>-/-</sup> mice impairs control of *Salmonella* serovar Typhimurium of infection (Fig. 7A). On day 3 20% of the WT-infected *Dbh*<sup>-/-</sup> mice were still alive, while 40% of the WT-infected *Dbh*<sup>+/-</sup> mice were still alive (Fig. 7A). Meanwhile, 70% of both the *Dbh*<sup>-/-</sup> and *Dbh*<sup>+/-</sup> mice infected with the *qseC* mutant were still alive on day 2 postinfection, and on day 3 60% of the *Dbh*<sup>+/-</sup> mice and 50% of the *Dbh*<sup>-/-</sup> mice were still alive (Fig. 7B). However, from day 4 to day 7, *Dbh*<sup>-/-</sup> mice were more susceptible to infection by the *qseC* mutant than *Dbh*<sup>+/-</sup> mice, and the scenario was similar to that observed with mice infected with WT *Salmonella* serovar Typhimurium (Fig. 7A and 7B). These data are congruent with data for infection of 129x1/SvJ mice (*Nramp1*<sup>+/+</sup>) (Fig. 4), where attenuation due to a *qseC* mutation was observed only until day 3 postinfection, suggesting that QseC-independent regulation is involved in *Salmonella* serovar Typhimurium pathogenesis at later time points. These results suggest that QseC, Epi, and NE play a significant role in the infection of mice by *Salmonella* serovar Typhimurium.

***In vivo* QseC-Epi/NE-dependent gene regulation.** Although *Dbh*<sup>-/-</sup> mice are more susceptible to intracellular pathogens due to an immune deficiency, these mice could yield valuable information concerning QseC-dependent Epi/NE signaling during *Salmonella* serovar Typhimurium infection. To assess QseC-Epi/NE-dependent regulation *in vivo*, we performed qRT-PCR using RNA extracted from the livers and spleens of control and *Dbh*<sup>-/-</sup> mice infected with WT and  $\Delta qseC$  mutant

FIG. 4. QseC's role in murine systemic infection. (A) Survival curves for 129x1/SvJ mice infected intraperitoneally (IP) with WT *Salmonella* serovar Typhimurium, the  $\Delta qseC$  isogenic mutant, and *E. coli* K-12 as control ( $P < 0.0005$ , as determined using Student's *t* test and comparing the *qseC* mutant to the WT at every time point on the survival curve until 5 days postinfection). (B) Organ loads of WT *Salmonella* serovar Typhimurium and the  $\Delta qseC$  isogenic mutant during systemic dissemination in 129x1/SvJ mice at 20 h postinfection (hpi) ( $P < 0.0005$ ). (C) Competitive index (CI) for the *qseC* mutant and WT strain SL1344 in 129x1/SvJ mice 20 h postinfection (ratio of  $\Delta qseC$  systemic dissemination to WT systemic dissemination). Statistical significance was determined by Student's *t* test ( $P < 0.02$ ).



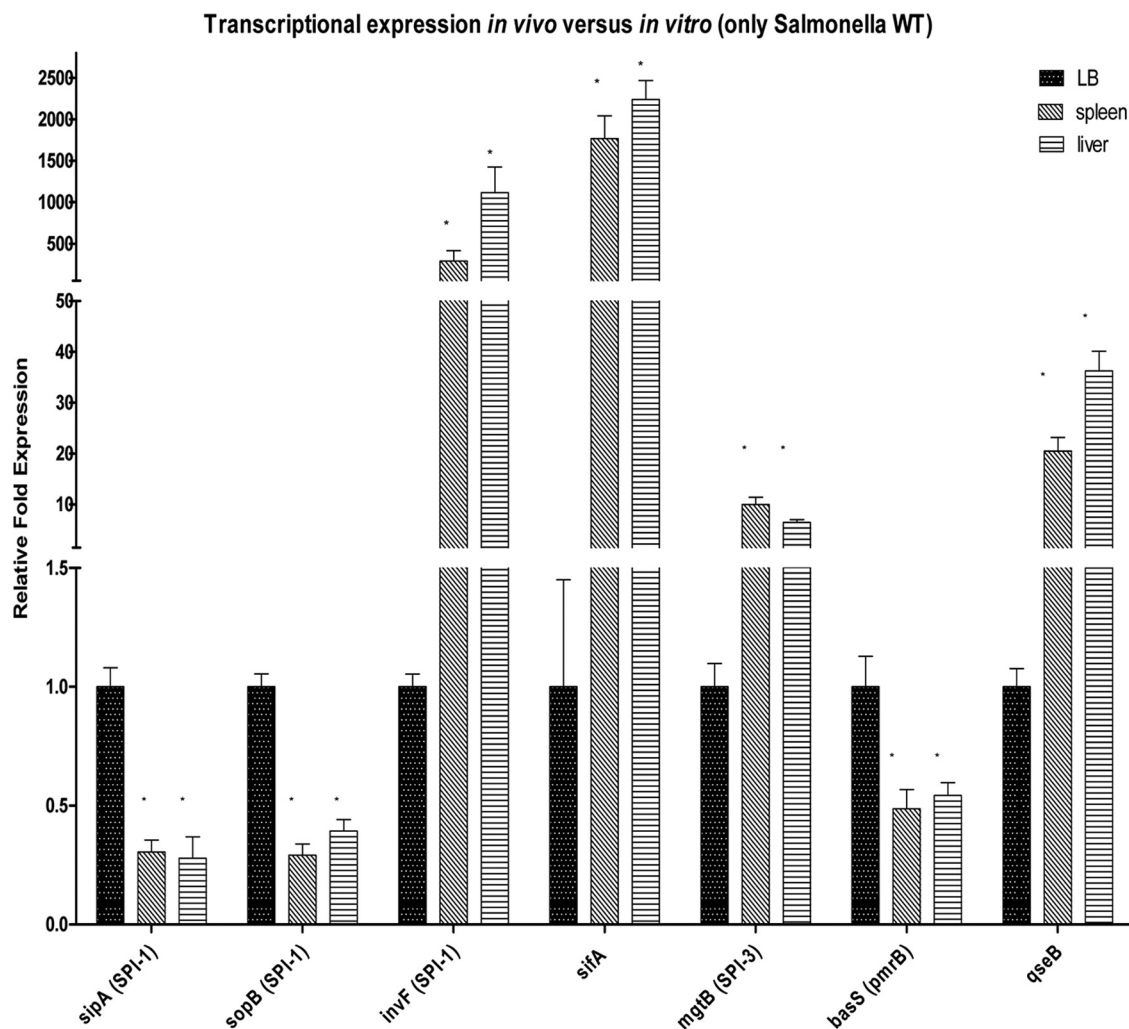


FIG. 6. Comparison of transcriptional expression in WT strain SL1344 *in vitro* (LB broth) and *in vivo* (spleens and livers). All statistical and expression analyses were performed by comparing expression levels to LB broth expression levels. Statistical significance was determined by Student's *t* test (\*,  $P < 0.001$ ).

*Salmonella* serovar Typhimurium. Expression of the SPI-1 genes *sipA* and *sopB* and the SPI-3 gene *mgtB* in the livers and spleens was significantly diminished in the absence of Epi/NE or QseC and was even further reduced in *Dbh*<sup>-/-</sup> mice infected with  $\Delta$ *qseC* *Salmonella* serovar Typhimurium (Fig. 8). These results suggest that NE/Epi facilitates expression of these genes *in vivo* and that QseC is involved in this regulation (Fig. 8). The data also show that during murine infection Epi/NE modulates virulence gene expression in *Salmonella* and that this modulation is QseC dependent.

## DISCUSSION

The complex interaction between a bacterial pathogen and its mammalian host relies on signaling through proteins, bacterial effectors, or toxins delivered to host cells, as well as chemicals (48). Here we show the intersection of the two signaling systems *in vitro* and *in vivo* in the important pathogen *Salmonella* serovar Typhimurium. *Salmonella* serovar Typhimurium is a food-borne pathogen that after ingestion invades

the gastrointestinal epithelia, reaching the mesenteric lymph nodes. It then can establish a systemic infection, replicating in the spleen and liver of the host (35, 39, 45, 49, 84). During pathogenesis, bacteria encounter several different microenvironments in the host (10), and several bacteria, including *Salmonella* serovar Typhimurium, effectively adapt by utilizing HKs (5). QseC is an HK that senses the bacterial signal AI-3 and the host hormones Epi/NE (24). The concentrations of NE are much higher in lymphoid organs than in the blood, suggesting that NE plays a physiological role in immune modulation (1, 31). NE/Epi is known to have profound systemic effects and plays important roles in energy balance, behavior, cardiovascular tone, thermoregulation, and the stress response (52). Hence, a pathogen's ability to sense these two signals may allow precise modulation of virulence gene expression, while the physiological state of the host is gauged.

Here we show that QseC is a global regulator of virulence genes of *Salmonella* serovar Typhimurium *in vitro* and *in vivo* (Fig. 1 to 8). A *qseC* mutant exhibits decreased expression of the SPI-1, *sifA*, and SPI-3 genes and a decreased ability to

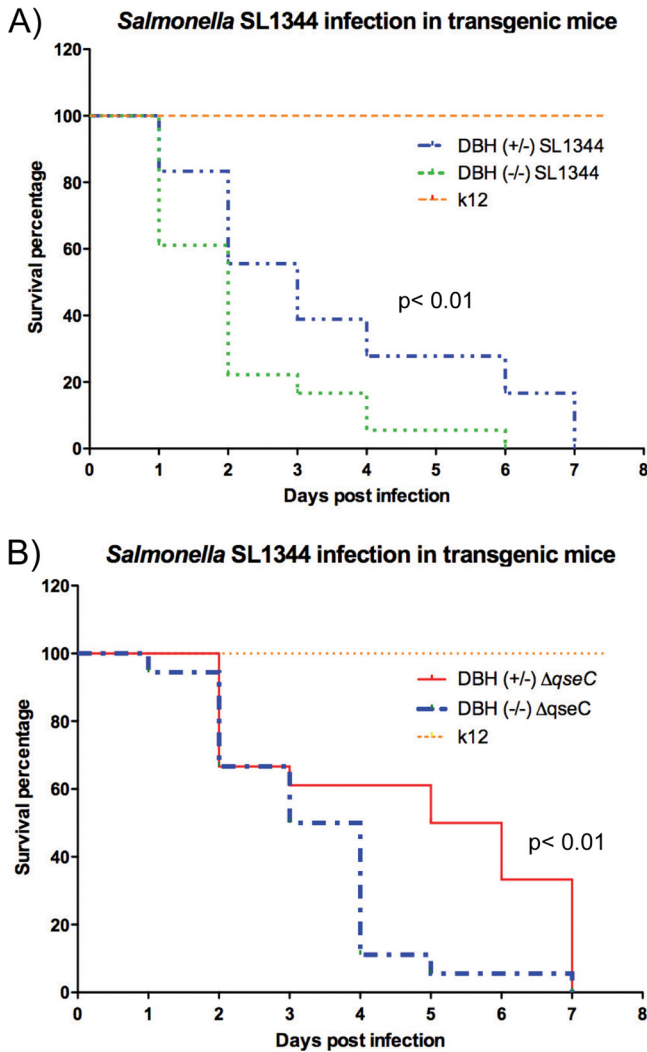


FIG. 7. *Salmonella* serovar Typhimurium infection in *Dbh*<sup>-/-</sup> knockout mice. (A) Survival plots for *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice infected IP with WT *Salmonella* serovar Typhimurium strain SL1344. (B) Survival plots for *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice infected IP with the  $\Delta$ *qseC* mutant. Statistical significance was determined by Student's *t* test.

invade epithelial cells and replicate within macrophages, and it is attenuated for systemic infection of mice (Fig. 1 to 8). In contrast to our results, Merighi et al. (60) reported that a *qseC* mutant did not exhibit attenuation for the invasion of epithelial cells. These conflicting data could be due to differences in methodology; Merighi et al. preincubated *Salmonella* serovar Typhimurium with epithelial cells at 4°C for 30 min and then at 37°C for 1 h before they added gentamicin (60), while we incubated bacteria at 37°C for 90 min before gentamicin was added, as previously described (33, 34, 65), without performing the 4°C preincubation step used in the studies of Merighi et al. However, the cell invasion defect that we observed is congruent with the downregulation of SPI-1 gene expression that we observed in the *qseC* mutant (Fig. 3).

The *qseC* mutant was attenuated for systemic infection of mice (Fig. 4) (66), congruent with the reduced expression of

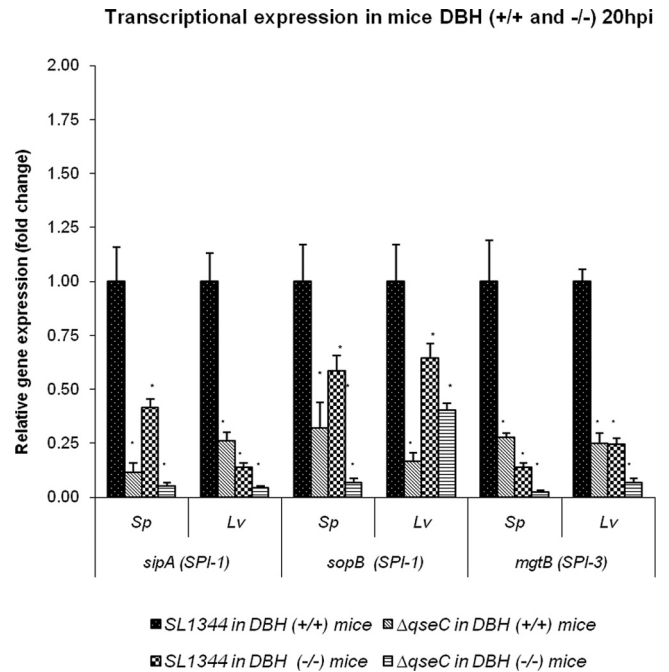


FIG. 8. Differential transcriptional expression of SPI-1 and SPI-3 in *Dbh*<sup>-/-</sup> mutant mice. Transcription of *sipA*, *sopB*, and *mgtB* in spleens and livers harvested from WT and *Dbh*<sup>-/-</sup> mice 20 h after IP infection with the WT and the  $\Delta$ *qseC* mutant. Statistical significance was based on a comparison of expression levels to expression levels in WT *Salmonella* serovar Typhimurium in *Dbh*<sup>+/+</sup> mice and was determined by Student's *t* test (\*, *P* < 0.001). Sp, spleens; Lv, livers; hpi, hours postinfection.

the *sifA* gene in the *qseC* mutant (Fig. 3B) (66) and the 5-order-of-magnitude decrease in the intramacrophage survival of the *qseC* mutant (Fig. 2B). The SifA effector is secreted by the SPI-2 TTSS and has been shown to be important for systemic infection of mice and intramacrophage survival (13). Our results also show that expression of *sifA* and *mgtB* (an SPI-3 gene also important for intramacrophage survival [14, 15]) is down-regulated in the *qseC* mutant compared to the WT in the spleens and livers of infected mice (Fig. 5).

To further address the interaction between Epi/NE and *Salmonella* serovar Typhimurium pathogenesis, we utilized *Dbh*<sup>-/-</sup> mice that do not produce the Epi/NE signals. These mice were more susceptible to *Salmonella* serovar Typhimurium infection than control mice (Fig. 7A). Although this may seem to be a conundrum given that Epi/NE enhances *Salmonella* serovar Typhimurium pathogenesis *in vitro* and a *qseC* sensor mutant is attenuated for murine infection (Fig. 4), one has to take into consideration the finding that Epi/NE is important for Th1 T-cell activation, which mediates the defense against intracellular pathogens (3). Hence, the greater susceptibility of the *Dbh*<sup>-/-</sup> mice to *Salmonella* serovar Typhimurium infection is likely due to a defective Th1 immune response. However, it is still possible to utilize these animals to gain important insights into the role of Epi/NE during systemic *Salmonella* serovar Typhimurium infection. It is noteworthy that there was not any difference in the infectivity of the *qseC* mutant in *Dbh*<sup>-/-</sup> and *Dbh*<sup>+/-</sup> mice until 3 days postinfection (Fig. 7B), suggesting that early in infection QseC sensing of

Epi/NE may play an important role in the kinetics of *Salmonella* serovar Typhimurium infection of mice. These data are further corroborated by the observations that transcription of *Salmonella*'s SPI-1 and SPI-3 genes *in vivo* is decreased in the absence of Epi/NE and that QseC is involved in this regulation (Fig. 8).

The transcription of the *sipA*, *sopB*, and *mgtB* genes in WT *Salmonella* serovar Typhimurium (which contains QseC and produces AI-3) in the spleens and livers of *Dbh*<sup>-/-</sup> mice (which do not produce Epi/NE) is significantly decreased compared to the expression of these genes in WT *Salmonella* serovar Typhimurium (which contains QseC and produces AI-3) in the spleens and livers of WT mice (which produce Epi/NE) (Fig. 8). These data suggest that during murine infection, the presence of Epi/NE in the livers and spleens of mice activates expression of *Salmonella* serovar Typhimurium virulence genes.

QseC senses AI-3, Epi, and NE (24). To address QseC's involvement in sensing Epi/NE *in vivo*, we infected WT mice (which produce Epi/NE) and *Dbh*<sup>-/-</sup> mice (which do not produce Epi/NE) with the *qseC* mutant and compared the expression of the *sipA*, *sopB*, and *mgtB* genes to the expression of these genes in WT *Salmonella* serovar Typhimurium (which contains QseC and makes AI-3) infecting WT mice. During infection of WT mice, the host signal Epi/NE is present, as is the bacterial AI-3 signal produced by the *qseC* mutant. We observed that under these conditions transcription of all three of these genes was decreased in the *qseC* mutant compared to the transcription in WT *Salmonella* serovar Typhimurium (Fig. 8), suggesting that the ability to sense the three signals is involved in the transcriptional activation of these virulence genes during murine infection. Transcription of *sipA* (in livers and spleens), *sopB* (in spleens), and *mgtB* (in livers and spleens) is decreased even further in the *qseC* mutant infecting *Dbh*<sup>-/-</sup> mice, suggesting that both QseC and Epi/NE are necessary for full activation of these genes *in vivo*. Although we show that QseC is an important sensor of these cues, it is clear that other bacterial adrenergic sensors exist. NE-induced transcription of the SPI-1 genes (Fig. 3C) is not completely dependent on QseC. We have identified a second adrenergic receptor in EHEC, QseE (67), and *Salmonella* serovar Typhimurium contains a homolog of this protein. The QseC-independent NE gene regulation could occur through this second sensor and would impart further kinetics and refinement to this signaling cascade. Further support for a role for a secondary adrenergic sensor in this regulation can be obtained if transcription of *sipA* and *mgtB* in the *qseC* mutant in the livers and spleens of WT mice is compared with transcription of these genes in the livers and spleens of *Dbh*<sup>-/-</sup> mice (Fig. 8). Transcription of these genes in the *qseC* mutant is further decreased during infection of *Dbh*<sup>-/-</sup> mice, suggesting that a second adrenergic receptor (maybe QseE) may be responsible for the residual QseC-independent Epi/NE activation of these genes in WT mice. In this study we assessed the contribution of interkingdom chemical signaling to *Salmonella* serovar Typhimurium pathogenesis by combining the powerful genetics of murine models and a genetically tractable pathogen. Further understanding of the molecular mechanisms underlying these signaling events in the bacteria and the host should allow us to examine interkingdom signaling in more depth.

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