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Interplay between the QseC and QseE Bacterial Adrenergic Sensor Kinases in *Salmonella enterica* Serovar Typhimurium Pathogenesis

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The bacterial adrenergic sensor kinases QseC and QseE respond to epinephrine and/or norepinephrine to initiate a complex phosphorelay regulatory cascade that modulates virulence gene expression in several pathogens. We have previously shown that QseC activates virulence gene expression in *Salmonella enterica* serovar Typhimurium. Here we report the role of QseE in *S.* Typhimurium pathogenesis as well as the interplay between these two histidine sensor kinases in gene regulation. An *S.* Typhimurium *qseE* mutant is hampered in the invasion of epithelial cells and intramacrophage replication. The Δ*qseC* strain is highly attenuated for intramacrophage survival but has only a minor defect in invasion. However, the Δ*qseEC* strain has only a slight attenuation in invasion, mirroring the Δ*qseC* strain, and has an intermediary intramacrophage replication defect in comparison to the Δ*qseE* and Δ*qseC* strains. The expressions of the *sipA* and *sopB* genes, involved in the invasion of epithelial cells, are activated by epinephrine via QseE. The expression levels of these genes are still decreased in the Δ*qseEC* double mutant, albeit to a lesser extent, congruent with the invasion phenotype of this mutant. The expression level of the *sifA* gene, important for intramacrophage replication, is decreased in the *qseE* mutant and the Δ*qseEC* double mutant grown *in vitro*. However, as previously reported by us, the epinephrine-dependent activation of this gene occurs via QseC. In the systemic model of *S.* Typhimurium infection of BALB/c mice, the *qseC* and *qseE* mutants are highly attenuated, while the double mutant has an intermediary phenotype. Altogether, these data suggest that both adrenergic sensors play an important role in modulating several aspects of *S.* Typhimurium pathogenesis.

The QseC and QseE adrenergic histidine sensor kinases sense the host hormones epinephrine (Epi) and/or norepinephrine (NE). Both of these sensors were first described as regulating virulence in enterohemorrhagic *Escherichia coli* (EHEC) (11, 48, 54). However, Epi and NE and/or QseC regulation of virulence gene expression is not exclusive to EHEC, since these hormones were also reported to play a role in the pathogenesis of other bacterial species, such as enteropathogenic *Escherichia coli* (EPEC) (52), *Francisella tularensis* (46), *Vibrio parahaemolyticus* (39), as well as *Salmonella enterica* serovar Typhimurium (4, 46, 54). Moreover, QseC has been reported to be a central virulence factor in other pathogens, such as uropathogenic *Escherichia coli* (UPEC) (24, 34). In addition to sensing Epi/NE, QseC also senses bacterial autoinducer-3 (AI-3) (11), and QseE also senses phosphate and sulfate sources (48).

The QseC and QseE sensor kinases are part of a two-component system, where their respective cognate response regulators are QseB and QseF. QseE phosphorylates QseF only, while QseC, in addition to phosphorylating QseB, also phosphorylates QseF and KdpE (31, 61). Next, these response regulators bind to their target genes, promoting changes in gene expression.

The role of QseC in *S.* Typhimurium pathogenesis has been under investigation (4, 37, 38, 45, 46); however, a more complete understanding of this regulatory cascade in this gastrointestinal pathogen is still missing. We have shown previously that QseC plays an important role in *S.* Typhimurium systemic infection in mice (38, 46). It was also reported previously that NE induced motility and augmented swine infection via QseC in *S.* Typhimurium (4). However, QseC and QseE do not seem to play a role in *S.* Typhimurium colonization in a bovine ligated ileal loop model (45). Using recessive homozygous Dbh (dopamine beta hydroxylase) mice, which lack Epi/NE, we have previously shown that the kinetics of infection of *S.* Typhimurium in these animals differed from those of infection in wild-type (WT) (Epi/NE-producing) animals and that QseC played an important role in the recognition of these two hormones *in vivo*. Moreover, the *qseC* mutant presented decreased motility, had a mild defect in the invasion of epithelial HeLa cells, and had a striking decrease in survival within J774 macrophages. QseC regulates the transcription of *Salmonella* pathogenicity island 1 (SPI-1) genes, the SPI-2 effector *sifA*, and flagellar genes during pathogenesis *in vivo* and *in vitro* (38).

S. Typhimurium pathogenesis is a truly complex and orchestrated process. Classically, the main islands involved in *S.* Typhimurium infection are SPI-1 and SPI-2, both of which encode type III secretion systems (T3SSs) essential for *S.* Typhimurium virulence (20, 21, 23, 41, 51). The SPI-1-encoded T3SS is required for the efficient invasion of the intestinal epithelium (21), while the SPI-2-encoded T3SS is essential for *S.* Typhimurium replication and survival within macrophages and systemic infection in mice (10, 28, 41).

The SPI-1 locus also contains genes that encode effectors and regulators. Among these effectors, SopE, SopE2, and SopB, on the pathogen side, as well as the Rho GTPases Cdc42, Rac1, and RhoG, on the host side, are required for a coordinated and efficient invasion. Together, they lead to actin cytoskeletal reorganization, membrane ruffling, and bacterial internalization by macro-

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
SL1344	*Salmonella enterica* serovar Typhimurium prototype	30
CGM220	SL1344 *qseC* mutant	46
CGM224	SL1344 *qseC*-complemented strain	This study
CGM225	SL1344 *qseE* mutant	This study
CGM226	SL1344 *qseE*-complemented strain	This study
CGM227	SL1344 *qseE* and *qseC* double mutant	This study
CGM228	SL1344 *qseC* (pBADMycHisA) and *qseE* (pACYC184) complemented	This study
TOP10	*E. coli* F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
DH5α	*E. coli supE44* Δ*lacU169* (80d*lacZ*ΔM15) *hsdR17*	Stratagene
Plasmids		
pBADMycHisA	Cloning vector and C-terminal Myc-His expression vector	Invitrogen
pACYC184	Cloning vector	NEB
TOPO PCR Blunt	PCR blunt cloning vector with topoisomerase	Invitrogen
pKD3	λ Red template plasmid	12
pKD46	λ Red helper plasmid (recombinase)	12
pKD20	λ Red resolvase plasmid	9

pinocytosis. SopB, together with the above-mentioned effectors, leads to chloride secretion through lipid dephosphorylation (62) and indirectly stimulates Cdc42 and RhoG through its phosphoinositide phosphatase activity (19, 27, 43, 62). SopB also promotes intestinal disease by increasing the intracellular concentration of D-*myo*-inositol 1,4,5,6-tetrakisphosphate, which stimulates cellular chloride secretion (62). Although SipA is not exclusively required for cell invasion, it helps to initiate the actin polymerization at the site of *S.* Typhimurium entry by decreasing the critical concentration and increasing the stability of actin filaments (29, 36, 64).

Encoded outside SPI-2, the effector SifA is secreted through the SPI-2 T3SS and is crucial for inducing the tubulation of the *S.* Typhimurium phagosome. SifA binds to the mammalian kinesin-binding protein SKIP. SifA coexpressed with SseJ induces the tubulation of mammalian cell endosomes, indicating that SifA likely mimics or activates the RhoA family of GTPases (42).

Here we describe the contribution of QseE and its interplay with QseC to *S.* Typhimurium pathogenesis. Our data suggest that QseE is also important for *S.* Typhimurium virulence both *in vitro* and *in vivo* and that QseC and QseE have a complex relationship in virulence gene regulation in *S.* Typhimurium. These results further highlight the multifaceted role of virulence chemical cues and their cascades in bacterial pathogenesis.

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 at pH 4.5 or pH 7.0, where indicated (7, 10, 13, 35), at 37°C. Recombinant DNA and molecular biology techniques were performed as previously described (50). Most of the oligonucleotides used in this study were previously reported (38). Otherwise, they are listed in Table 2.

Construction of the *qseE* and *qseEC* mutants. The construction of the isogenic nonpolar *S.* Typhimurium SL1344 *qseE* mutant and *qseEC* double knockout mutant were achieved by using λ Red mutagenesis (12). The *qseC* mutant (CGM220) (46) was complemented with the *qseC* gene cloned into the pBADMycHisA (KpnI and EcoRI) vector (Invitrogen), generating strain CGM224. Similarly, the *qseE* mutant (CGM225) was complemented with the *qseE* gene cloned into the pBADMycHisA (KpnI and EcoRI) vector (Invitrogen), generating strain CGM226. The *qseEC* double mutant (CGM227) was also complemented with *qseC* cloned into pBADMycHisA (Invitrogen) and *qseE* cloned into the pACYC184 vector (EcoRI site into the Cm^r cassette), generating strain CGM228.

Quantitative real-time RT-PCR. Cultures were grown aerobically overnight in LB or N-minimal medium at 250 rpm to the late-exponential growth phase (optical density at 600 nm [OD_{600}] of 1.0) for the *in vitro* assays, in the absence or presence of 50 μM Epi. RNA from three biological samples was extracted by using the RiboPure-Bacteria RNA isolation kit (Ambion), according to the manufacturer's instructions. The primers used for the real-time assays were designed by using Primer Express v1.5 (Applied Biosystems) (Table 2). Real-time reverse transcription (RT)-PCR was performed in a one-step reaction using an ABI 7500 sequence detection system (Applied Biosystems). For each 20-μl reaction mixture, 10 μl 2× SYBR master mix, 0.1 μl Multi-Scribe reverse transcriptase (Applied Biosystems), and 0.1 μl RNase inhibitor (Applied Biosystems) were added. The amplification efficiency of each primer pair was verified by using standard curves of known RNA concentrations. The *rpoA* (RNA polymerase subunit A) gene was used as the endogenous control. Data collection was performed by using ABI Sequence Detection 1.3 software (Applied Biosystems). Data were normalized to levels of *rpoA* and analyzed by using the comparative critical threshold (C_T) method, as previously described (60). The expression levels of the target genes under different growth conditions were compared by using the relative

TABLE 2 Oligonucleotides used in this study

Primer	Sequence
qseC-F	GGTACCAAATTGACGCAACGTCTCAG
qseC-R	GAATTCGCCCAACTTACTACGGCCTC
qseE-F	GGTACCAGCGACACGTTGAAGCGC
qseE-R	GAATTCGCGTGTTTGTCAGATGCAGG
qseE-F Lambda Red	TTGTCAGATGCAGGCAATGGCAGCGA GATGCGAAAACAAACTTCCTGCGC GTGTAGGCTGGGAGCTGCTTC
qseE-R Lambda Red	GACACGTTGAAGCGCTGGTCTGTTTT CCCCCGTTCTTTACGACAATTGGT CATATGAATATCCTCCTTA

quantification method (60). Real-time data are presented as fold changes compared to WT levels. Error bars represent the standard deviations of the $\Delta\Delta C_T$ values (60). Statistical significances were determined by one-way analysis of variance (ANOVA) and posttested with the Bonferroni correction. An ANOVA P value of <0.0001 and a posttest P value of <0.01 were considered highly significant.

HeLa cell invasion and adhesion assays. Epithelial HeLa cells were infected with *S.* Typhimurium at a multiplicity of infection (MOI) of 100:1 for 90 min at 37°C in 5% CO_2, as previously described (17, 18, 44). To ensure the uniform inoculation of all strains in these assays, the inocula were normalized to the same OD_{600} value and plated onto LB, and CFU were enumerated through serial dilutions. These cells were treated with 40 μg/ml of gentamicin for 1 h to kill extracellular bacteria and lysed with 1% Triton X. Bacteria were diluted and plated onto LB plates for CFU determinations (17, 18, 44). All the assays in the presence of Epi-bitartrate (50 μM) were performed by preconditioning the cells with 10% dialyzed fetal bovine serum (FBS) to avoid Epi/NE traces from bovine sera and light protection to avoid Epi degradation during the assay. HeLa cell adhesion control assays were performed by employing cytochalasin D, as previously reported (15). Statistical significances were determined by one-way ANOVA compared to WT CFU. P values of <0.05 were considered significant.

Macrophage infection. J774 murine macrophages were infected with opsonized *S.* Typhimurium cells with normal mouse serum at 37°C for 15 min and washed. These macrophages were infected at an MOI of 100:1 for 30 min of bacterium/cell interactions at 37°C in 5% CO_2. To ensure the uniform inoculation of all strains in these assays, the inocula were normalized to the same OD_{600} and plated onto LB, and CFU were enumerated through serial dilutions. These cells were treated with 40 μg/ml of gentamicin for 1 h to kill extracellular bacteria and lysed with 1% Triton X. Bacteria were diluted and plated onto LB plates for CFU determinations (14, 17, 44). All the assays in the presence of Epi-bitartrate (50 μM) were performed by preconditioning the cells with 10% dialyzed FBS to avoid Epi/NE traces from bovine sera and light protection to avoid Epi degradation during the assay. Statistical significances were determined by one-way ANOVA compared to WT CFU. P values of <0.05 were considered significant.

Mouse survival experiments with *Salmonella* Typhimurium. Mice (BALB/c, 7 to 9 weeks old, and female) were infected i.p. (intraperitoneally) with a predetermined lethal dose of 1×10^6 CFU of *S.* Typhimurium WT strain SL1344 or the same CFU of the innocuous *E. coli* K-12 strain DH5α (the K-12 strain was used as a negative infectivity control to ensure that there were no issues with organ perforations during i.p. injection and that the death of mice was not due to endotoxic effects) or specific *S.* Typhimurium mutants, as indicated. We employed 10 mice per strain using the same i.p. infection with 1×10^6 CFU, and these 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. After 9 days postinfection, the remaining animals were euthanized by CO_2 asphyxiation. Systemic infection (i.p. route) for replication within tissues was performed as previously described (14). For CFU enumeration in spleens and livers after 20 h of i.p. infection, the mice were sacrificed to remove the spleens and livers. Those organs were harvested, homogenized, and plated onto LB agar plates for bacterial cell counting to determine tissue colonization (CFU) (14, 41).

RESULTS

QseE plays a role in invasion of epithelial cells. Invasion is the first step in infection, since the gastrointestinal epithelium is the first barrier to be surpassed (22–24). Here we investigated the role of the sensor kinases QseC and QseE in invasion. Previously, we reported that QseC has a minor, although significant, role in the invasion of epithelial cells (38). Here we performed gentamicin protection assays to measure epithelial cell invasion by the WT and the *qseC*, *qseE*, and *qseEC* mutants as well as their respective complemented strains. As most commercially available fetal bovine serum (FBS) formulations used to supplement HeLa epithelial cell culture media contain traces of Epi/NE, we used dialyzed FBS (Gibco, Invitrogen), which has all molecules with a molecular mass of less than 10,000 Da removed. In the absence of Epi/NE, the *qseC* mutant had a significant ($P = 0.034$) 1-order-of-magnitude decrease in HeLa cell invasion compared to the WT, and a similar reduction was observed for the *qseEC* double mutant ($P = 0.024$) (Fig. 1A). In contrast, the *qseE* mutant had a striking decrease in invasion of approximately 5 orders of magnitude compared to the WT ($P = 0.018$) (Fig. 1A). All of these phenotypes were restored upon complementation in *trans*. These results suggested that although QseC plays a minor role in the invasion of epithelial cells, QseE has an important role in this process, and the phenotype of the double kinase mutant mirrors the Δ*qseC* phenotype (Fig. 1A).

The initial assays were performed in the absence of Epi, whereas QseC would have only the *S.* Typhimurium self-produced AI-3 signal and QseE SO_4 and PO_4 sources present in the media to sense. Because both QseC and QseE sense Epi, we also performed these assays in the presence of Epi. Epi increased the invasiveness of WT *S.* Typhimurium by 1.5 orders of magnitude compared to invasion in the absence of Epi ($P = 0.04$). Epi also increased the invasiveness of the *qseE* mutant by 2 orders of magnitude, although it never reached the levels of invasiveness of the WT strain either in the presence or in the absence of Epi ($P = 0.018$). These data suggest that the *qseE* mutant can still respond to Epi, probably because QseC is still intact in this strain, and that other factors sensed or regulated exclusively through QseE also play an important role in invasion. The complementation of the *qseE* mutant restored its ability to sense Epi ($P = 0.014$). The *qseC* mutant did not increase its invasiveness in the presence of Epi, and the complementation of *qseC* restored its ability to increase its invasiveness in the presence of this hormone ($P = 0.015$). These results may reflect the fact that QseC acts upstream of QseE, with the transcription of *qseE* being activated by QseC (49). The phenotype of the *qseEC* mutant again mirrored the *qseC* phenotype (Fig. 1A). The differences in the invasive abilities among these strains cannot be attributed to either differences in growth or differential adhesion to HeLa cells, given that the growth rates of all strains were similar to that of the WT (Fig. 1B) and cytochalasin D adhesion assays with HeLa cells (15) showed no significant differences in adhesion among these strains (Fig. 1C).

These differences in the invasion assays prompted us to investigate whether the expression levels of SPI-1 genes were influenced by QseC and QseE, given that the SPI-1 T3SS is responsible for the invasion of epithelial cells (22–24). To test this hypothesis, we performed quantitative RT-PCR (qRT-PCR) to measure the expression levels of SPI-1 genes under in *vitro* conditions conducive to SPI-1 expression (LB). We previously reported that the expression levels of *sipA* and *sopB* were significantly decreased, by 5- and 2.5-fold, respectively, in a *qseC* mutant (38). Here we show that the expression levels of both *sipA* and *sopB* were also significantly decreased in the *qseE* mutant and the *qseEC* double mutant albeit to a lesser extent in the double mutant than in the single mutant (Fig. 2). The *qseE* mutant had a 4-fold decrease in *sopB* and a 10-fold decrease in *sipA* expression levels, while the *qseEC* double mutant had a 2-fold decrease in the expression levels of both *sopB* and *sipA* compared to the WT. These decreases in expression lev-

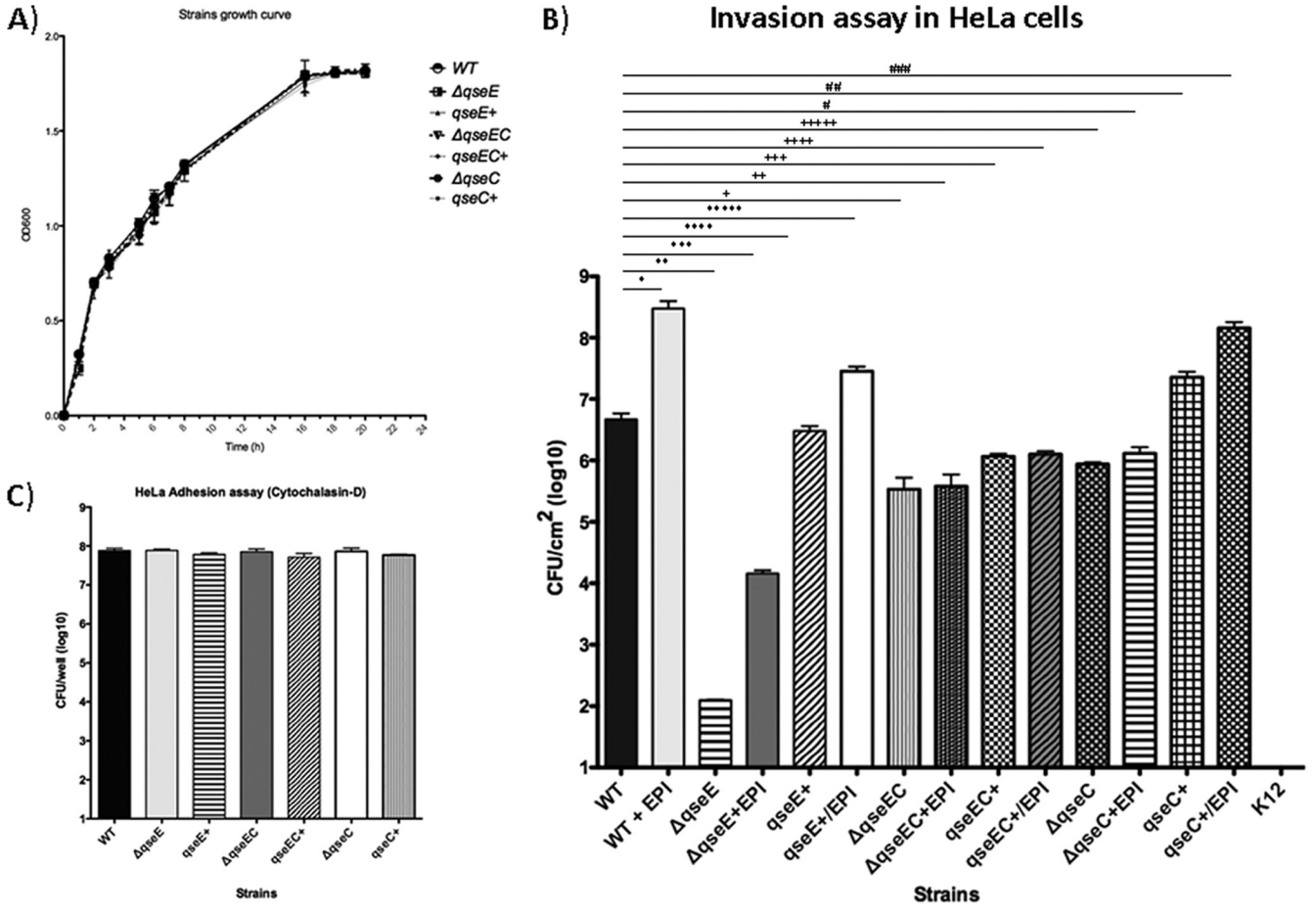

FIG 1 (A) Invasion assay of HeLa epithelial cells by the WT; *qseE*, *qseC*, and *qseEC* mutant; and complemented strains in the absence and presence of 50 μM Epi. Statistical differences were considered significant compared to WT levels at a *P* value of <0.05, determined by ANOVA. *, $P = 0.041$; **, $P = 0.018$; ***, $P = 0.018$; ****, not significant ($P = 0.293$); *****, $P = 0.014$; +, $P = 0.024$; ++, $P = 0.025$; +++, $P = 0.044$; ++++, $P = 0.048$; +++++, $P = 0.034$; #, not significant ($P = 0.055$); ##, $P = 0.029$; ###, $P = 0.015$. (B) Growth curves of the WT, mutants, and the respective complemented strains. No statistically significant differences were observed, according to one-way ANOVA ($P = 1.00$). (C) HeLa cell adhesion control assay with cytochalasin D (Sigma).

els were rescued upon complementation (Fig. 2). These qRT-PCR data are congruent with the invasion phenotypes observed for the *qseE* and *qseEC* mutants. Therefore, the interplay between QseE and QseC has a significant role in SPI-1-mediated epithelial cell invasion.

Role of QseE and QseC in *S.* Typhimurium survival within macrophages. *S.* Typhimurium intracellular survival and replication are critical steps for the progression of *S.* Typhimurium infection, and they are SPI-2-mediated processes (42). Next, we investigated whether QseE and QseC influence *S.* Typhimurium survival within murine J774 macrophages. Here again, we used dialyzed FBS (Gibco, Invitrogen), which has all molecules with a molecular mass of less than 10,000 Da removed to avoid traces of Epi/NE present in normal undialyzed FBS. We previously reported that QseC plays an important role in intramacrophage survival (38), with a decrease of over 4 orders of magnitude, as further confirmed in Fig. 3. The other two mutants, the *qseE* and *qseEC* mutants, also presented significant decreases in survival within macrophages. The *qseE* mutant presented a decreased survival of approximately 4 orders of magnitude within macrophages compared to the WT ($P = 0.046$), while the *qseC* mutant had a reduction of 6 orders of magnitude ($P = 0.0001$). The *qseEC* double mutant showed an intermediate phenotype, with a reduction of survival of 5 orders of magnitude within macrophages compared to the WT ($P = 0.0001$). These differences were rescued upon their respective complementations in *trans* (Fig. 3). Epi was also added to this assay mixture, similarly to the HeLa cell invasion assay described above. The addition of Epi significantly increased macrophage replication for the WT and *qseE* mutant strains ($P = 0.046$ and 0.001, respectively) but did not change the ability of either the *qseC* or the *qseEC* mutant to replicate within these cells, suggesting again that QseC may be the primary, most upstream sensor of Epi in this signaling cascade. Epi could still be sensed by the *qseE* mutant but not by the *qseC* and double mutants in this assay (Fig. 3). The complemented strains' ability to replicate within macrophages was not changed, supposedly because the overexpression of these sensors, albeit in low-copy-number vectors, may be sufficient to bypass the need for the sensing of these signals in certain assays.

The SPI-2 T3SS effector SifA is critical for intramacrophage survival and replication. The transcription of the *sifA* gene is optimal under conditions of SPI-2 expression in N-minimal medium, which has low concentrations of phosphate and magnesium (7). *S.* Typhimurium also encounters an acidic pH within the

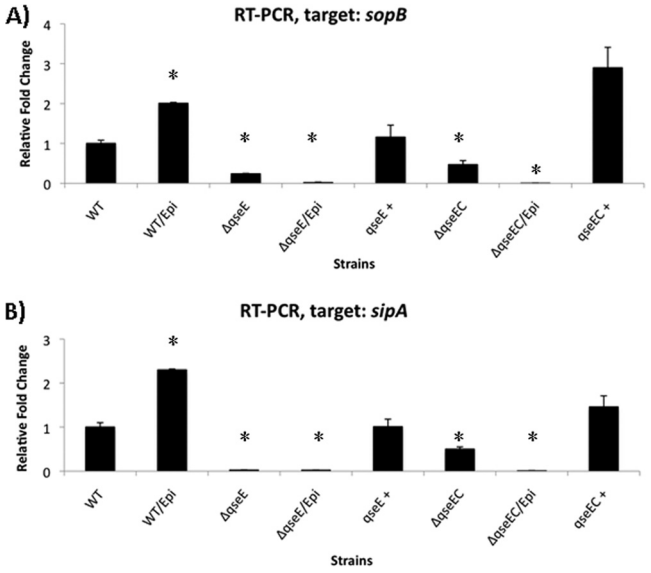

FIG 2 QseE and QseEC regulate the transcriptional expression of SPI-1 genes in the absence and presence of 50 μM Epi. (A) Transcriptional expression of *sopB* in LB in the WT (wild type), the mutants, and the respective complemented strains. (B) Transcriptional expression of *sipA* in LB in the WT, the mutant, and the respective complemented strains. ∗, statistical differences are considered highly significant compared to WT expression levels ($P < 0.0001$ by ANOVA and $P < 0.01$ by posttest).

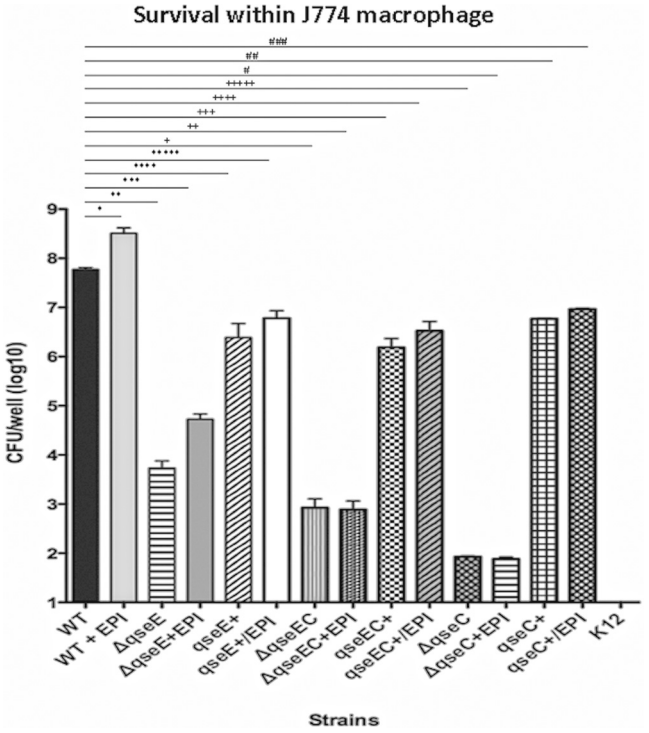

FIG 3 Intramacrophage survival assays with J774 cells infected with the WT; *qseE*, *qseC*, and *qseEC* mutant; and complemented strains in the absence and presence of 50 μM Epi. Statistical differences were considered significant compared to WT levels at a *P* value of <0.05, determined by ANOVA. ∗, $P = 0.046$; ∗∗, $P = 0.0001$; ∗∗∗, $P = 0.0001$; ∗∗∗∗, $P = 0.001$; ∗∗∗∗∗, $P = 0.001$; +, $P = 0.0001$; ++, $P = 0.0001$; +++, $P = 0.0001$; ++++, $P = 0.001$; +++++, $P = 0.0001$; #, $P = 0.0001$; ##, $P = 0.001$; ###, $P = 0.001$.

vacuole (5). To assess whether pH influences *sifA* expression, we assessed *sifA* transcription under conditions of acidic and neutral pHs. The expression level of *sifA* was increased 10-fold at an acidic pH (4.5) compared to a neutral pH (7.0) (Fig. 4A). Consequently, from here on, we assessed *sifA* expression levels only under acidic conditions (pH 4.5). As previously observed for a *qseC* mutant (38), the transcription of *sifA* was decreased approximately 100-fold in both the *qseE* and *qseEC* mutants compared to the WT, and these differences were rescued upon complementation (Fig. 4B). The expression level of *sifA* was also measured, via qRT-PCR, within J774 macrophages by employing the same conditions as those used for the intramacrophage survival assays (Fig. 3). The expression level of *sifA* within macrophages was increased 100-fold compared to its expression level under *in vitro* conditions (minimal medium at pH 4.5) in WT *S.* Typhimurium (Fig. 4C), and the *sifA* expression level was decreased over 10-fold in the *qseC* and *qseEC* mutants but not in the *qseE* mutant (Fig. 4C). These data suggest that QseC plays an important role in *sifA* expression both during growth *in vitro* and during intramacrophage replication and that although QseE is important for the expression of this gene *in vitro*, it is dispensable within macrophages. This pattern of *sifA* expression level decreases is congruent with the diminished intramacrophage survival of these mutants (Fig. 3), indicating that the QseC and QseE sensor kinases play a pleiotropic role in *S.* Typhimurium pathogenesis.

Epinephrine increases *S.* Typhimurium virulence gene expression. Since Epi is a common cue that can be sensed by both QseC and QseE (11, 48, 54), we assessed gene expression in the presence of Epi. Epi increased the expression levels of *sopB*, *sipA*, and *sifA* (Fig. 2 and 4) (38). We previously reported that the Epi/NE-dependent increase in the *sopB* expression level is not dependent on QseC, leading us to propose that the sensor of Epi toward the activation of the expression of this gene was QseE. In agreement with our initial hypothesis, the Epi activation of *sopB* expression is dependent on QseE (Fig. 2B), given that a *qseE* mutant does not respond to Epi and cannot activate *sopB* expression. Interestingly, the *qseCE* double mutant presented a decrease in the *sopB* expression level in the presence of Epi, suggesting that in the absence of both of these sensors, another Epi-dependent signaling pathway represses *sopB* expression (Fig. 2B). In support of these data, a third adrenergic receptor in *S.* Typhimurium was previously proposed (53). The expression of *sipA* follows the same trend as that of *sopB*, with the Epi-dependent regulation of this gene also occurring through QseE (Fig. 2B).

Next, we assessed Epi-dependent *sifA* expression (Fig. 4B). We previously reported that the *qseC* mutant could not respond to Epi/NE to activate *sifA* expression, leading us to suggest that the Epi activation of *sifA* expression occurred through QseC (38). Here again, congruent with our previously reported hypothesis, the *qseE* mutant still responds to Epi to activate *sifA* expression (Fig. 4B), given that QseC is present in this strain and the double kinase mutant is irresponsive. These data suggest that during *in vitro* growth, the Epi activation of SPI-1 effectors occurs primarily through QseE, while the expression of the *sifA* SPI-2 effector occurs primarily through QseC.

Role of QseE and QseC *in vivo*. Using 129x1/SvJ mice, which are more resistant to *S.* Typhimurium infection, we previously showed that a *qseC* mutant was attenuated for infection. We used this resistant mouse strain in the past, because dopamine hydrox-

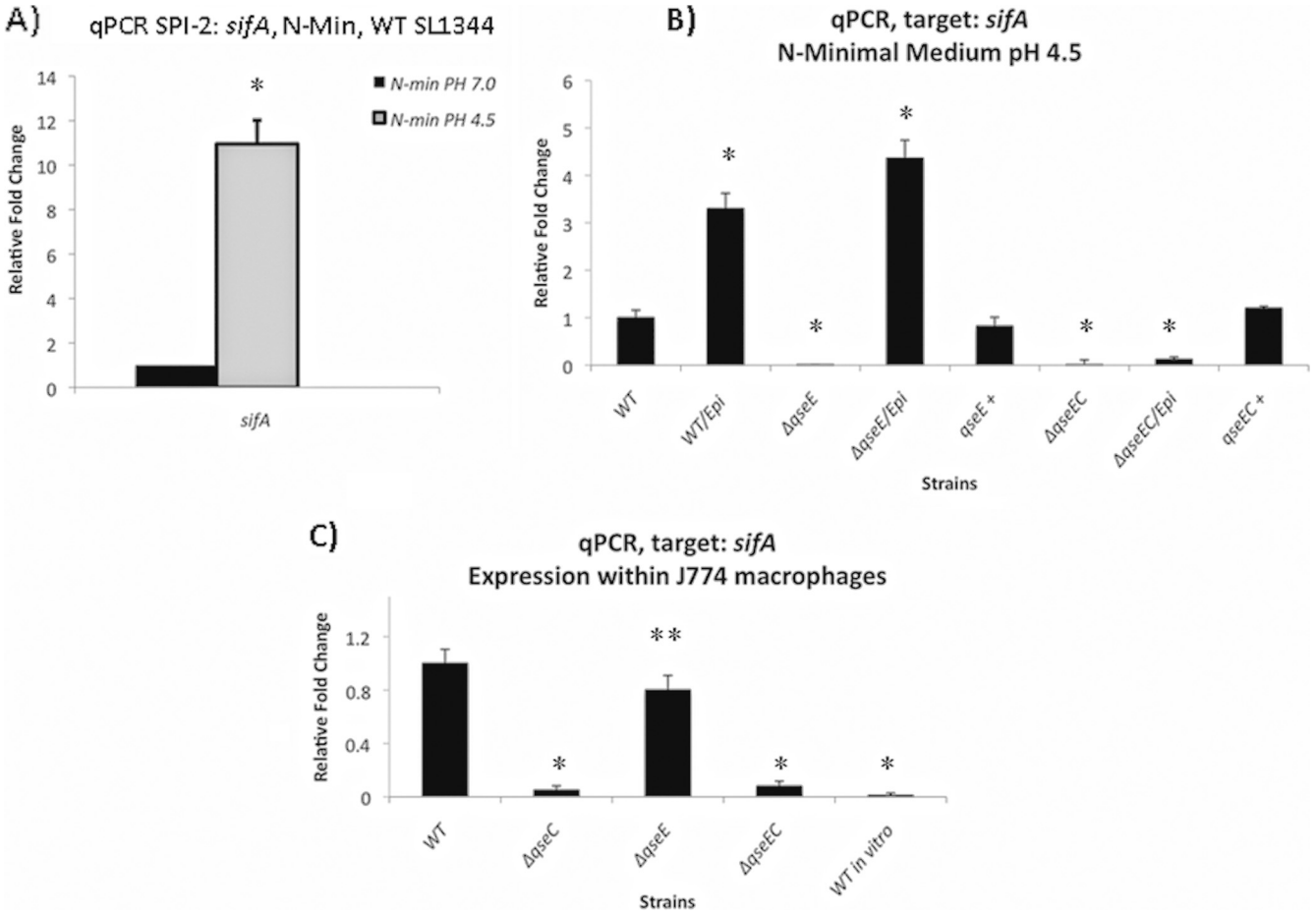

FIG 4 QseE and QseEC regulate the transcriptional expression of *sifA* (SPI-2) in the absence and presence of 50 μM Epi. (A) Transcriptional expression of *sifA* in N-minimal medium (N-Min) in acidic and neutral milieus in the WT (wild type), the mutants, and the respective complemented strains. (B) Transcriptional expression of *sifA* in N-minimal medium in an acidic milieu in the WT, the mutants, and the respective complemented strains. (C) Assay of intramacrophage transcriptional expression of *sifA* within J774 cells of the WT and mutants, also compared to *in vitro* conditions (N-minimal medium). *, statistical differences considered highly significant compared to N-minimal medium at pH 7.0 (A) and WT expression levels (B and C) ($P < 0.0001$ by ANOVA and $P < 0.01$ by posttest); **, not statistically significantly changed ($P = 0.085$ by ANOVA and posttest [Bonferroni correction]).

ylase knockout mice (these mice do not produce either Epi or NE) had this genetic background, and in these initial studies, we also employed these animals to show that QseC was involved in the sensing of Epi/NE during murine infection. In the animal studies performed here, we employed systemic (intraperitoneal [i.p.]) infection to reproduce the typhoid-like model using BALB/c mice, which are Nramp1$^{-/-}$ or Nramp1 and are more susceptible to *S.* Typhimurium infection (8). i.p. infections were also performed by using *E. coli* K-12 as a negative control, to ensure that endotoxic effects were not responsible for the death of these animals. After 1 day postinfection with WT *S.* Typhimurium, 85% of the mice were alive, while 100% of the *qseC* and *qseE* mutant-infected mice were also alive at this time (Fig. 5A). On day 2, 100% of the WT strain-infected mice were dead, while 100% of *qseC*, 80% of *qseE*, and 30% of *qseEC* mutant-infected mice were alive. By day 3, the survival rate of the *qseC* mutant-infected mice dropped slightly, to approximately 85%. Mice infected with the *qseE* mutant succumbed to death only at day 7. The survival rate of *qseEC* mutant-infected mice also dropped by day 4, and none survived by day 5 postinfection. Meanwhile, the survival rate of the *qseC* mutant-infected mice was constant at 85% throughout the experiment, and the K-12-infected negative controls had no deaths, as expected (Fig. 5A).

Next, to better understand the role of QseE during systemic infection, we harvested spleens and livers of mice infected with either the WT or the *qseE* mutant (20 h postinfection), similarly to experiments that we previously performed using mice infected with the *qseC* mutant (38). *S.* Typhimurium replication in the spleens and livers of mice during early stages (at 20 h postinfection) was significantly reduced for the *qseE* mutant, with an approximate decrease of 5 orders of magnitude in spleens and livers compared to WT-infected animals (Fig. 5B). These data indicate that QseE also has an important role during *in vivo* colonization within spleen and liver replication in a systemic *S.* Typhimurium infection model.

DISCUSSION

Bacterial cell-to-cell chemical communication is a complex process, which can aid pathogens to sense their surroundings in order to successfully colonize specific niches, survive host defenses, and outcompete indigenous microbiota (3). Many histidine sensor kinases were implicated in this regulation to fine-tune the expres-

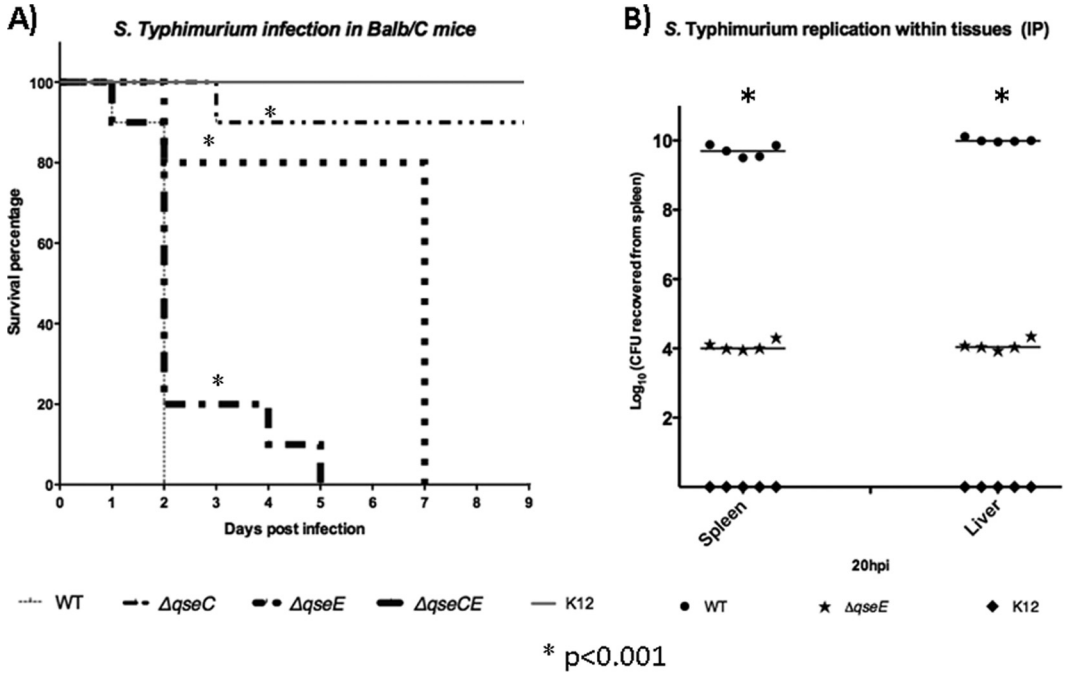

FIG 5 QseE is important for murine infection. (A) Survival plots of BALB/c mice infected (i.p.) with the WT and mutants, as indicated, using lethal doses of *S.* Typhimurium. (B) Quantification of replication within tissues as loads in spleens and livers harvested from BALB/c mice after 20 h postinfection. The infection route was oral gavage with lethal doses of the WT or the *qseE* mutant. *, $P < 0.001$.

sion of virulence factors (54). Therefore, cell communication in *S.* Typhimurium has been the subject of various studies (33, 55–58), although only recently has a role for QseC and QseE been investigated (4, 37, 38, 45, 46). QseC has been shown to increase virulence by the upregulation of SPI-1 gene expression; however, the most striking QseC-dependent gene regulation pertains to the expression of SifA (38), which is an SPI-2 T3SS effector essential for *Salmonella*-induced filament formation, i.e., intracellular survival and replication (6, 46). Prolonged survival within macrophages and increased motility are QseC-mediated phenotypes (38). However, the role of QseE as well as the interplay between these two kinases in *S.* Typhimurium pathogenesis remained undefined. Considering that QseE plays a role in EHEC virulence and interfaces with QseC in this regulatory cascade, (11, 48, 54), our initial hypothesis was that QseE might also have a role in the regulation of *S.* Typhimurium virulence.

The invasion of epithelial cells is one of the initial steps in *S.* Typhimurium pathogenesis. Here we showed that the *qseE* mutant has a striking reduction in HeLa cell invasion (Fig. 1). This is in contrast to the *qseC* mutant, where a mild 1-log reduction was observed. These results were consistent with the transcription profiles of these mutants: while QseC mildly regulates the expression levels of SPI-1 genes and effectors (38) primarily involved in invasion, QseE has a more pronounced effect (Fig. 2). Even the Epi-dependent activation of these genes (*sipA* and *sopB*) seems to occur primarily through QseE rather than QseC (Fig. 2) (38). SipA facilitates actin stability, while SopB is directly involved in the stimulation of cellular chloride secretion (26, 62). Although both are not required for invasion, their presence is essential for efficient invasion, an essential initial step in *S.* Typhimurium pathogenesis (16, 22, 25, 29, 59, 63, 64). The invasion difference in the *qseE* mutant was supported by the reductions in the expression levels of *sopB* and *sipA*. Recently, SopB was also described to be important to vacuole maturation; i.e., it helps *S.* Typhimurium to evade lysosomal fusion and host defenses (2). The phenotype of the *qseEC* double mutant mirrored the *qseC* mutant phenotype (Fig. 1), and congruent with the invasion phenotype, the levels of transcription of SPI-1 genes were also decreased less in the double mutant than in the *qseE* single mutant (Fig. 2). These data suggest a novel role for QseE during *S.* Typhimurium pathogenesis, with emphasis on epithelial cell invasion, while QseC seems to play a more important role in systemic disease and intramacrophage replication. Furthermore, there seems to be an intricate interplay between these two sensor kinases in the timing of the extensive repertoire of *S.* Typhimurium virulence gene expression.

Following invasion, intramacrophage survival and replication are an essential second step in *S.* Typhimurium pathogenesis (6, 19). Hence, we investigated survival within J774 macrophages to assess the role of QseC and QseE and their interplay during intracellular survival. We observed that the *qseE* mutant presented a significant decrease in survival, similarly to the double knockout in intramacrophage survival/replication, while the *qseC* mutant presented a striking reduction (Fig. 3) (38). These results are again congruent with the transcription of virulence factors, where *sifA* transcriptional regulation is dependent mostly on QseC, especially within macrophages (Fig. 4) (38).

Given that both kinases regulated many aspects of *S.* Typhimurium virulence *in vitro*, we next assessed the role of these sensors during murine infection. To examine the role of QseC and QseE *in vivo*, we assessed mouse survival using lethal systemic infection with *S.* Typhimurium. Previously, we reported that QseC was important during systemic murine infection, using a resistant murine strain (38, 46). Here, using an *S.* Typhimurium-susceptible murine strain (BALB/c), we observed that the contribution of

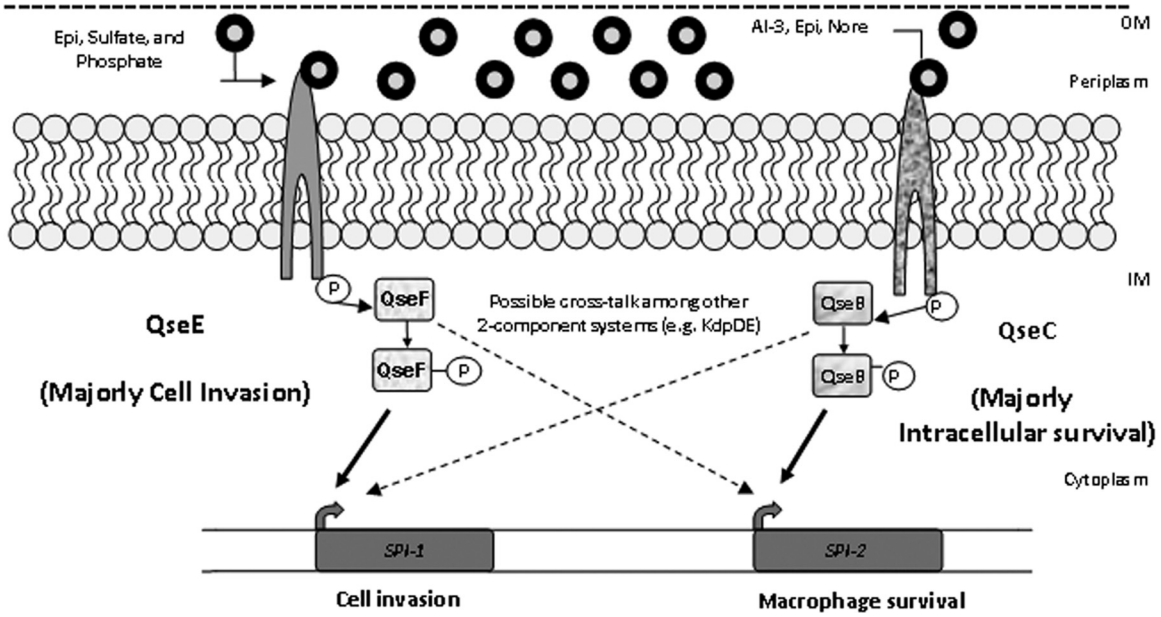

FIG 6 Model for AI-3/epinephrine/norepinephrine (Nore) regulation in *Salmonella enterica* serovar Typhimurium via QseEF and QseBC cross talk during pathogenesis. QseC senses AI-3, Epi, and NE to augment its autophosphorylation, while QseE senses PO_4, SO_4, Epi, and NE. QseE exclusively phosphorylates QseF, while QseC phosphorylates QseB, QseF, and KdpE, whose cognate sensor kinase, KdpD, senses potassium. Additionally, QseF is phosphorylated by UhpB, BaeS, EnvZ, and RstB. An additional level of cross talk involves the QseEF repression of *rcsB* transcription. Besides phosphorylating QseB, QseC also dephosphorylates this response regulator to exert its control in gene expression. Finally, the concerted and integrated action of QseBC and QseEF with several two-component systems, forming a network, leads to the optimal activation of SPI-1 and *sifA* expression and the enhancement of *S.* Typhimurium pathogenesis. However, which of the response regulators is directly activating the transcription of SPI-1 and *sifA* remains to be determined. OM, outer membrane; IM, inner membrane.

QseC to systemic infection is even more profound in this model (Fig. 5A). Additionally, we also report that QseE plays a significant role in systemic infection (Fig. 5) albeit to a lesser degree than QseC. It is, however, puzzling that each individual mutant has a more profound effect during murine infection than the double mutant, which, although significantly attenuated compared to the WT, is much less attenuated than the single mutants (Fig. 5).

There is extensive interplay at the genetic and biochemical levels between the QseBC and the QseEF systems as well as with other two-component systems. QseBC activates the transcription of *qseEF*, and this activation is enhanced in the presence of Epi, suggesting a hierarchal relationship between these two systems, with QseBC acting upstream of QseEF (49). There is also cross talk between these systems as well as with other two-component systems at the biochemical level, with QseC phosphorylating not only its cognate response regulator, QseB, but also the noncognate response regulators QseF and KdpE (31) (Fig. 6). The cognate sensor kinase for KdpE is KdpD, which senses potassium, and the KdpD sensor kinase is known to be important for *S.* Typhimurium pathogenesis in *Caenorhabditis elegans* (1). Additional layers of complexity also exist, with the QseEF system repressing the expression of *rcsB* (40, 47) and with QseF, in addition to being phosphorylated by QseE and QseC, also being phosphorylated by UhpB, BaeS, EnvZ, and RstB (61). Finally, one also has to take into consideration that QseC and QseE, in addition to being kinases, are also phosphatases, and response regulators such as QseB can bind to different sites depending on their phosphorylation state (31). It was also reported previously that QseC acts through QseB not only by phosphorylating this response regulator but also by dephosphorylating it (34). Combinatorial sensing by several two-component systems was proposed previously to enable the integration of multiple stimuli and the amplification of signals. This is especially relevant given that bacteria are exposed to different gradients of environmental signals and/or cues during host infection (32).

These data further highlight the importance of the cross talk among different two-component systems in bacterial pathogens. The data also support the concept that histidine sensor kinases and response regulators are network components rather than stand-alone systems. They are organized in intricate networks that confer plasticity and rapid responses to change gene expression toward adaptation to complex and dynamic environments (32). A more complete understanding of two-component system regulation in bacterial pathogenesis is essential for the future development of novel antibacterial therapies.

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