The QseG lipoprotein impacts the virulence of enterohemorrhagic *E. coli* and *Citrobacter rodentium* and regulates flagellar phase variation in *Salmonella enterica* serovar Typhimurium

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The QseEF histidine kinase/response regulator system modulates expression of enterohemorrhagic *E. coli* (EHEC) and *Salmonella enterica* serovar Typhimurium (Stm) virulence genes in response to the host neurotransmitters epinephrine and norepinephrine. *QseG*, which encodes an outer membrane lipoprotein, is co-transcribed with *qseEF* in these enteric pathogens, but there is little knowledge of its role in virulence. Here, we found that in EHEC QseG interacts with the type three secretion system (T3SS) gate protein SepL, and modulates the kinetics of attaching and effacing (AE) lesion formation on tissue-cultured cells. Moreover, an EHEC Δ*qseG* mutant had reduced intestinal colonization in the infant rabbit model. Additionally, in *Citrobacter rodentium*, an AE lesion-forming pathogen like EHEC, QseG is required for full virulence in a mouse model. In Stm, we found that QseG regulates the phase switch between the two flagellin types, FliC and FljB. In an Stm Δ*qseG* mutant, the phase variable promoter for *fljB* is preferentially switched into the ‘on’ position, leading to an overproduction of this ‘phase two’ flagellin. In infection of tissue-cultured cells, the Δ*qseG* Stm mutant provokes increased inflammatory cytokine production vs wild-type; in vivo, in a murine infection model, the Δ*qseG* strain caused a more severe inflammatory response, and was attenuated vs the wild-type strain. Collectively, our findings demonstrate that QseG is important for full virulence in several enteric pathogens and controls flagellar phase variation in Stm, and highlight both the complexity and conservation of the regulatory networks that control the virulence of enteric pathogens.
INTRODUCTION

The gastrointestinal tract is a diverse ecosystem with many physiologically distinct niches. To mount a productive infection, bacterial pathogens that infect the gut must be able to sense these niches and deploy their virulence regime in the appropriate environment. Aberrant deployment of virulence factors may lead to wasted energy or detection by the host immune system. To accomplish this task many bacterial pathogens have evolved highly complex regulatory networks to control expression of their virulence factors. Understanding these complex regulatory systems will lead to a greater understanding of disease progression (1).

Two component systems are a common strategy used by bacterial pathogens to regulate virulence genes in response to environmental cues (2). Both enterohemorrhagic *E. coli* (EHEC) and *Salmonella enterica* serovar Typhimurium (Stm) use the QseEF two-component system to sense host hormones and regulate expression of virulence genes. The sensor kinase QseE senses epinephrine, norepinephrine, phosphate and sulfate and phosphorylates its cognate regulator, QseF. In EHEC, QseF induces transcription of *espFu* (3, 4), a type III secretion system (T3SS) effector that interacts with host proteins to activate actin polymerization and initiate formation of attaching and effacing (AE) lesions (5-7). In Stm, QseEF signaling enhances expression of the SPI-1 pathogenicity island (8), which encodes a T3SS and is necessary for host cell invasion (9). In a mouse model of systemic infection a Stm strain lacking *qseE* is significantly attenuated compared to WT (8). Although conserved across these two species, the QseEF system has undergone species specific specialization to regulate virulence factors unique to each pathogen.

The *qseG* gene is located between *qseE* and *qseF* on the chromosome, and co-transcribed with this two-component system. Homologs of *qseG* are present in many of the *Enterobacteriaceae*, however, all previous work investigating QseG function has been carried
out in EHEC. QseG is an outer membrane lipoprotein that is transported through the general secretory pathway (Sec) or the twin arginine translocation (Tat) system (known to transport folded proteins (10, 11)) (4). QseG is predicted to contain alpha-helices but lacks significant homology to other proteins in the database. In an EHEC \textit{qseG} deletion mutant expression of \textit{qseE} is increased, suggesting that QseG modulates expression of the two-component system. QseG is not required for secretion of T3SS components or effectors, but impacts the translocation of the translocated intimin receptor (Tir) (4). Tir is the first effector translocated into the host cell via the T3SS (12). It interacts with intimin on the bacterial membrane to promote tight adherence, and interacts with host proteins to promote actin polymerization and A/E lesion formation (13). Because QseG impacts translocation of Tir, and potentially other effectors, a strain lacking \textit{qseG} does not form A/E lesions efficiently (4). These data suggest that \textit{qseG} is important for EHEC virulence; moreover, since \textit{qseG} along with \textit{qseEF} is conserved among many of the pathogenic \textit{enterobacteriaceae}, it may play an important role in regulating virulence across many pathogens.

Here we show that \textit{qseG} is required for full virulence in animal models of EHEC, \textit{Citrobacter rodentium}, a related AE pathogen, and Stm infection. We show that although QseG is important for full virulence in animal models of both EHEC and Stm infection, its mechanism of action has diverged. In Stm QseG plays a role in modulating flagellar phase variation—a system that is missing from EHEC. This phase variation regulation leads to decreased inflammation at systemic sites during Stm infection. The \textit{qseEFG} operon is found in the genomes of many \textit{Enterobacteriaceae} and it is likely that QseG plays an important role in other species as well. Our work highlights both the complexity of the regulatory networks that
bacterial pathogens employ, as well as that proteins common to different pathogens can adapt to
serve species specific roles.

RESULTS

QseG faces the periplasm and interacts with SepL. QseG contains a predicted lipid
attachment site and previous work using cell fractionation demonstrated that QseG is located in
the EHEC outer membrane (4). To determine whether QseG is exposed to the outside of the cell,
or instead faces the periplasm, a proteinase K protection assay was performed (Fig. 1A). Cells
were treated with the membrane impermeable protease proteinase K, and the effect on QseG and
OmpA, as a control, was assessed by western blot. Levels of OmpA, which is exposed to the
outside of the cell, were diminished by proteinase K treatment. In contrast, levels of QseG were
unchanged by proteinase K treatment, suggesting it is not exposed to the outside of the cell and
therefore likely faces the periplasm.

QseG is not involved in the expression of the LEE or espFu genes, or in the assembly of
the TTSS, but impacts the translocation of Tir into host cells (4). We hypothesized that QseG,
because of its cellular location, may physically interact with components of the T3SS apparatus
and thereby have a role in modulating the T3SS. Using a yeast two-hybrid assay, QseG was
tested for its ability to interact with various components of the T3SS thought to be present in the
periplasm. Of the proteins screened, only SepL was identified as a potential binding partner for
QseG (Fig 1B). SepL, along with SepD, are required for secretion of EspA, EspB and EspD (14,
15), the structural components of the T3SS known as the translocon. Furthermore they are
responsible for regulating the switch from secretion of the translocon to secretion of effector
proteins (14, 16). SepL directly binds to Tir to prevent its early secretion (17), but the signal that
initiates the switch to begin secretion of effectors is not fully defined. Co-immunoprecipitation was employed to confirm the interaction of QseG with SepL. An HA-tagged allele of sepL was expressed in a ΔsepL or ΔsepLΔqseG strain of EHEC and a pull-down performed using an anti-HA antibody. When both SepL-HA and QseG were present, QseG was detected via western blot in the precipitated sample using an anti-QseG antibody (Fig. 1C), confirming the SepL-QseG interaction. The direct interaction of QseG with SepL in EHEC suggests that QseG may play a role in regulation of the T3SS, and may play a role in the switch from translocon to effector secretion. Because Tir translocation is a key event in AE lesion formation we investigated AE lesion formation by the ΔqseG using live cell imaging. The ΔqseG mutant exhibited delayed and was almost abrogated in its ability to form AE lesion on epithelial cells compared to WT. The ΔqseG mutant does not have any growth defects compared to WT (18), and previous AE lesion assays are congruent with the live cell image, showing that ΔqseG is defective for AE lesion formation and that this defect can be complemented (4). Taken together, these data suggest that differences in Tir translocation impact the kinetics of pedestal formation (Fig. 2, Movies S1 and S2).

QseG is required for robust colonization and full virulence in in vivo models of EHEC infection. QseG impacts Tir translocation and is required for efficient pedestal formation (4). To test if this defect in T3SS activity translates to attenuated colonization and virulence in vivo, we used two different small animal models of EHEC infection. We infected infant rabbits with wild-type (WT) or a ΔqseG mutant of the 86-24 EHEC strain and measured bacterial load in the colon 5 days post infection. Significantly fewer CFUs were recovered from rabbits infected with ΔqseG compared to WT (Fig. 3A), demonstrating that QseG contributes to efficient colonization in this model. We also tested the role of QseG in vivo using Citrobacter rodentium,
a natural pathogen of mice that is closely related to EHEC. The *C. rodentium* genome also harbors the LEE (19) and many of the regulatory pathways that control its expression are conserved between *C. rodentium* and EHEC. Importantly, where EHEC’s virulence in mice can be solely attributed to Shiga-toxin (20), *C. rodentium* infection in mice exhibits many of the hallmarks of EHEC disease in humans including AE lesion formation, colonic hyperplasia and diarrhea (21, 22), making it a useful model for studying LEE regulation *in vivo*. Mice infected with a ΔqseG mutant survived significantly longer than those infected with WT *C. rodentium* (Fig. 3B). ΔqseG infected mice also displayed attenuated disease symptoms compared to WT. The ΔqseG infected mice had decreased colon weights (consistent with decreased colonic hyperplasia (16, 23, 24)) (Fig. 3C), and their stool was formed in normal pellets where WT infected mice displayed unformed, liquid diarrheal feces. (Fig. 3D). The increased survival and attenuation of disease symptoms where qseG is absent indicates that this gene is required for full virulence *in vivo*.

**Absence of qseG leads to overproduction of the Stm type II flagellin.** The QseEF two-component system regulates type III secretion in both EHEC and Stm and is required for full virulence in both pathogens (4, 8, 25, 26). We hypothesized that qseG modulates virulence in *Salmonella* as well, and studying its role in this pathogen could help uncover its mechanism of action. A sequence alignment of the *qseEFG* operon of EHEC and Stm revealed that the sequence similarity of qseG was lower at both the nucleotide and protein level (59/88% respectively) than the surrounding *qseE* (71/94%) and *qseF* (81/98%) genes (Fig. 4A). This suggests that QseG has undergone greater adaptation than the QseEF two-component system with which it is co-transcribed. We know that QseEF regulon differs between EHEC and Stm,
controlling virulence factors specific to each species, therefore we hypothesized that the role of QseG may differ between these two pathogens as well.

We created a qseG deletion mutant in the Stm SL1344 strain and confirmed that, as with the corresponding EHEC mutant (18), it displayed identical growth kinetics to WT Stm in vitro (data not shown). We then analyzed the secreted proteome of WT vs ΔqseG Stm by SDS-PAGE and Coomaisie staining. The supernatant of an ΔinvAΔspiB mutant, which is deficient in both the Salmonella T3SSs encoded in pathogenicity islands SPI-1 and SPI-2, was run as a control to identify proteins secreted through T3SS. Compared to WT the ΔqseG mutant did not appear to have any change in the amounts of T3SS secreted proteins. However, a large band appeared in the ΔqseG supernatant that was not observed in the WT supernatant (Fig. 4B). The unknown band was extracted and identified by mass spectrometry as FljB (Table 1), the Stm phase II flagellin. Unlike E. coli, many strains of Salmonella possess two antigenically distinct alleles of the flagellin protein: fliC and fljB (27). fljB, the “phase II” flagellin, is under the control of an invertible promoter that in the on position leads to transcription of fljB and the repressor of fliC, fljA, leading to reciprocal control of the two flagellin types (28-33). A relative quantification of the total secreted proteome was performed by mass spectrometry (Table 1), confirming that ΔqseG mutant supernatants contained nearly 50-fold higher FljB protein levels compared to WT supernatants (Fig. 4B). A corresponding decrease in FliC levels was observed as expected, but no other significant changes in flagella-associated proteins were observed. The secreted proteome analysis also confirmed that no significant, consistent change in SPI-1 secreted proteins was observed (Table 1).

QseG decreases fljB transcription by modulating its invertible promoter to favor the off position. To test whether increased FljB secretion in the ΔqseG mutant was modulated at the
transcriptional level, we measured \(fljB\) RNA levels using qPCR. \(fljB\) transcript was increased over 100-fold in \(\Delta qseG\) Stm compared to WT and a corresponding, but more modest, decrease in \(fliC\) transcript was also observed. Importantly, complementation of \(qseG\) in trans restored \(fljB\) and \(fliC\) transcript to WT levels (Fig. 4C). As \(fljB\) is controlled by an invertible promoter, we tested whether the observed increase in \(fljB\) transcription was due to an increase in the proportion of \(fljB\) promoters that were in the “on” position. A qPCR based assay was designed where one primer is located within the invertible region and one outside, such that the molar ratio of the orientations can be calculated. In the WT strain the “off” orientation was favored by a ratio of over 100:1. In the absence of \(qseG\) the ratio was altered to closer to 1:1, with the “on” position being slightly favored (Fig. 4D). \(qseG\) complementation restored the on/off ratio to WT levels. These observations reveal that QseG modulates the orientation of the \(fljB\) promoter to repress \(fljB\) expression.

**Absence of \(qseG\) does not affect Stm motility, cell invasion or intracellular replication.** Because QseG was involved in regulation of flagellin transcription, we hypothesized that the mutant may have an altered motility phenotype. Motility was assessed using a soft agar swimming assay but no significant alteration in motility was observed (Fig. 5A). This was not entirely unexpected, as we have not demonstrated that QseG represses overall flagella expression, but rather modulates which flagellin allele is favored. In a HeLa cell-based invasion assay the \(\Delta qseG\) mutant did not display any defects in cell invasion *in vitro* (Fig. 5B). Similarly, no defects in intracellular replication were observed in the \(\Delta qseG\) mutant either in human (Fig. 5C) or mouse (Fig. 5C) macrophages. Defects in cell invasion and intracellular replication are typically phenotypes associated with defects in the T3SS1 or T3SS2 respectively,
and since we did not observe any differences in the amount of secreted proteins associated with either of these two systems, a lack of phenotype is not unexpected.

**QseG is required for full Stm virulence in mice.** To assess the importance of QseG in vivo we utilized a murine model of systemic disease where mice are infected intraperitoneally with Stm. At days two and three post infection, groups of five mice were sacrificed and spleens and livers were collected to enumerate bacterial loads. Data shown is combined from two independent experiments. Mice infected with the $\Delta$qseG mutant survived significantly longer than those infected with WT Stm (Fig. 6A), demonstrating that qseG is required for full virulence in this model of Stm infection. The $\Delta$qseG mutant infected mice also appeared to lose slightly less weight compared to WT infected mice (Fig. 6B), although this was not statistically significant. Interestingly, no significant differences in bacterial loads were observed at either day two or three in the liver or spleen of infected animals (Fig. 6C). Since QseG contributes to Stm virulence, but is not required for efficient colonization or proliferation in this model, it is possible that it augments virulence by modulating the host response to infection.

**QseG limits inflammation during Stm infection.** QseG modulates expression of the flagellin proteins, which are “pathogen associated molecular patterns” and potent activators of the immune system. Flagellins are recognized by Toll-like receptor-5 (34) leading to cytokine and nitric oxide production (35) and by the NOD-like receptors NLRC4 and NAIP5, leading to activation of the inflammasome (36). To test whether QseG has a role in modulating the inflammatory response during Stm infection we exposed T84 colonic epithelial cells to supernatants from different strains of Stm and monitored the induction of IL-8 transcription by qPCR (Fig. 7A). Cells exposed to supernatants from WT Stm showed approximately 50-fold induction of IL-8 transcript compared to uninfected cells. Supernatants from $\Delta$fliC$\Delta$fljB Stm
caused no IL-8 induction, suggesting that any IL-8 induction observed in this assay can be attributed to the flagellin response. Supernatants from ΔqseG Stm significantly increased IL-8 transcription compared to WT supernatants, resulting in nearly 100-fold induction over uninfected cells. Supernatants from a ΔqseGΔfljB double mutant resulted in a slightly dampened IL-8 response compared to ΔqseG Stm however, this was not statistically significant and IL-8 levels were not reduced to those measured with WT Stm supernatants. We hypothesize that because there is significant crosstalk between fliB and fliC expression and that deletion of fljB could lead to increased fliC expression compensating for the of FljB. Additionally, it is possible that the ΔqseG Stm increases inflammatory cytokine production through another mechanism other than modulating fljB expression.

To confirm the role of QseG in modulating the fljB promoter in vivo, DNA from infected livers and spleens was collected and the orientation of the fljB promoter was assessed via the qPCR-based assay described above. A higher proportion of the fljB promoter was in the on position in livers and spleens from ΔqseG Stm infected mice compared to WT infected mice (Fig. 7B), confirming that the modulation of the fljB promoter by QseG is occurring in vivo during infection. To test the effect of QseG on the inflammatory response we collected RNA from infected livers and spleens and assayed for host expression of the inducible nitric oxide synthase, NOS2. A significant increase in NOS2 expression was observed in livers infected with ΔqseG Stm compared to WT (Fig. 7C), suggesting that QseG does indeed dampen the inflammatory response to Stm, perhaps through its modulation of fljB expression.

DISCUSSION
To establish a productive infection, intestinal pathogens must tightly regulate their virulence systems, deploying them at the appropriate sites and times during infection. Likewise, pathogens need to dampen expression of genes that provoke inflammatory responses when they are not needed to escape detection by the host immune system.

The signaling networks that control pathogen gene expression during infection are extremely complex, and often integrate many different environmental signals. Here, we investigated the function of QseG, a protein conserved in many pathogenic Enterobacteriaceae, but which lacks significant homologues in the database. qseG is co-transcribed with qseEF, a two-component system known to control critical virulence genes in both EHEC and Stm. Notably, we found that QseG is required for full virulence in EHEC, C. rodentium and Stm. However, we found that the sequence similarity of qseG between EHEC and Stm was lower than for the surrounding genes, suggesting that its function may have diverged in these two species. Our data indeed supports that although qseG is required for full virulence by both EHEC and Stm, its mechanism of action differs between these two pathogens.

In EHEC, we found that QseG interacts with a component of the T3SS, SepL, but the nature of this interaction, and its significance, remains undefined. Interestingly, SepL and its Stm homolog SsaL share only 35% similarity (37) and sepL cannot fully complement an ssaL mutant (38). Therefore we do not expect that SepL and SsaL will play equivalent roles in regards to their interactions with QseG.

Previous studies have shown that QseG impacts the translocation of Tir but not its secretion. This, along with its interaction with SepL, suggests that QseG is involved in regulation of T3SS function. QseG’s location in the inner leaflet of the outer membrane, and its lack of any predicted signal transduction motifs, suggest that its regulatory role may be carried
out through protein-protein interactions, with SepL and potentially other components of the
T3SS apparatus. The QseEF two-component system controls transcription of the T3SS effector,
*espFU*; thus, the *qseEGF* operon may control the activity of EHEC’s T3SS at several levels.

In *Stm*, we found that QseG modulates the invertible promoter controlling expression of
the flagellin gene *fljB*, leading to repression of this flagellin allele. However, QseG does not alter
motility in *Stm*. This flagellin switch does not exist in EHEC, and in EHEC QseG also does not
play any role in motility or flagella expression (data not shown). QseG also has a role in
controlling the inflammatory response to *Stm* that we hypothesize is mediated through its role in
controlling *fljB* expression. Previous studies have shown that strains that are locked into
expressing *fljB* are attenuated in mouse models of infection compared to both WT and a “locked
off” strain that expresses *fliC*, suggesting a disadvantage to expressing *fljB* in a mammalian host
(39). Interestingly, purified recombinant FliC and FljB activate NF-κB to comparable levels *in
vitro* (40), suggesting that FljB is not inherently more inflammatory than FliC.

We propose that while flagella is important during the intestinal phase of infection,
expression of flagellin, and particularly FljB, is detrimental for *Stm* at systemic sites (Fig. 8).
During the systemic phase of infection, QseG is important for modulating the *fljB* promoter to
favor the off position—leading to diminished FljB secretion. This leads to decreased NOS2 and
cytokine induction and decreased overall inflammation at systemic sites.

Several studies have demonstrated extensive cross-talk between regulation of SPI-1 and
flagella in *Stm* (41-49). We have previously shown that QseEF activates expression of SPI-1 and
now we have demonstrated that QseG regulates expression of flagellin, suggesting that the
*qseEGF* operon is another player in the crosstalk between these two critical systems. However,
further studies are needed to define whether there are interactions between QseEF and QseG and
their respective regulons.

While we have demonstrated that QseG modulates the $fljB$ promoter in Stm, we have also
shown previously that it is localized to outer membrane in EHEC (4). The localization of QseG
poses an interesting question on how it excerts its effect on the orientation of the $fljB$ DNA
promoter. One possibility is that it interacts with other proteins that relay the message from the
periplasm to the cytoplasm and exert effects on the promoter. We have found that QseG has at
least one direct binding partner in EHEC, SepL, but we have yet to identify any proteins that
interact with QseG in Stm. The focus of future studies will be to determine proteins that QseG
may interact with to carry out its function modulating the $fljB$ promoter. Another potential,
albeit less likely, mechanism is that QseG itself interacts with DNA or cytoplasmic DNA binding
proteins before it is lipidated and secreted to the outer membrane. As there are no putative DNA
binding domains we do not expect QseG to bind DNA itself but future studies will seek to
identify protein binding partners.

QseG modulates flagellar phase variation in Stm but paradoxically, QseG is present
EHEC and other *Enterobacteriaceae* whose genomes encode only a single flagellin, *fliC*.
Clearly, there has been some functional divergence of QseG across species, which is reflected by
the 62% sequence identify between the Stm and EHEC proteins. This specialization is also seen
in the QseEF two-component system that is co-transcribed with *qseG*. In both EHEC and Stm,
QseEF controls expression of critical virulence factors, however the particular genes it controls
are species specific. However, as the presence of the *qseEGF* operon has been conserved across
many pathogenic *Enterobacteriaceae*, there may still be a common, undefined function.
The work presented here highlights the idea that conserved systems across different species can evolve and be tuned to perform species-specific functions. Thus, while QseG is required for full virulence in both EHEC and Stm, this outer membrane protein performs distinct functions in these two species. Furthermore, this work highlights the complexity of the regulatory networks that bacterial pathogens use to modulate expression of their virulence genes. Many systems incorporate several layers of regulation as well as cross-talk between different virulence-associated systems, such as a T3SS and flagellins, which we still do not fully understand. Uncovering the mechanistic basis for virulence gene regulation will allow us to more fully understand how pathogens cause disease and may help in developing more effective therapeutics.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are listed in Table 2. EHEC and Stm deletion mutants were created using λ red mutagenesis (50). Stm QseG complementation plasmid was created via restriction digest/ligation into the pBAD/Myc-His A backbone. The complementation plasmid was induced with 0.2% arabinose. Primers used in this study are listed in Table 3.

Proteinase K protection assay. Proteinase K protection assays were performed as previously described (51).

Yeast two-hybrid screen. A yeast two hybrid was used as previously described (52) to test for possible protein-protein interaction between QseG and QseG, EscC, EspA, SepD, SepL and Tir. Briefly, the yeast report strain L40 was transformed (53) with all combinations of the proteins in both the bait (pLEX-ADE) and the library (pVP16) vector. Dual transformants were
selected on yeast minimal media lacking leucine, and tryptophan and then assayed for protein-protein interactions using the integrated \textit{LacZ} and \textit{HIS3} reporters. Association of the two protein fusions was determined by growth on yeast minimal media lacking histadine.

**Co-immunoprecipitation.** Co-immunoprecipitation of SepL and QseG was performed using SepL-HA (14) and anti-HA antiserum, and an anti-QseG antiserum (4) from EHEC expressing SepL-HA on a plasmid, as previously described (54).

**Live cell imaging.** Live cell imaging was performed as previously described (55). The Lifeact::GFP expressing cell line was created using the Flip-In System (Invitrogen). HeLa cells were transfected with pLacZ::Zeocin using Fugene 6 (Promega) to create FRT sites in the genome. Cells were then selected with 100µg/ml zeocin. Resistant foci were grown and assayed by Southern blot against \textit{lacZ} for single insertions, and then β-galactosidase assays were performed to measure expression of the inserted locus. High expressing single insertions were then transfected with the flippase helper plasmid pOD44 and Lifeact::GFP cloned into pFRT plasmid. These transfected cells were then selected in 100µg/ml hygromycin, and resistant foci were visualized under fluorescent microscopy to measure levels of Lifeact::GFP expression. These cells were then maintained in 50µg/ml hygromycin. When they are split before infection hygromycin is not added. EHEC were transformed with the mCherry expressing plasmid pDP151 and grown ON statically in LB. The Lifeact::GFP cell’s media was replaced with low glucose DMEM supplemented with 10% FBS and infected with a 1:100 dilution of the ON. The infection was allowed to continue for 2hrs at 37°C and 5% CO₂ and then the cells are washed 3 times with DMEM and then visualized by live cell imaging with a Zeiss scope. Images are taken every 2 minutes for 2 hours.
Infant rabbit model of EHEC infection. To prepare the inoculum, bacteria were grown overnight in LB broth at 37°C with appropriate antibiotics, harvested by centrifugation and resuspended in sterile PBS (pH 7.2) and adjusted to a cell density of ~ $10^9$ CFU ml$^{-1}$. Infant rabbit experiments were carried out as described previously (56). Briefly, 3-day-old New Zealand White rabbits were intragastrically inoculated with $\sim 5 \times 10^8$ CFU of WT EHEC or the qseG mutant using a size 5 French catheter. Rabbits were monitored twice daily for signs of illness or diarrhea. Diarrhea was described as i) none – normal pellets are dark green, hard and formed, ii) mild – diarrhea consisting of a mix of soft yellow-green unformed and formed pellets resulting in light staining of the hind legs, or iii) severe – diarrhea consisting of unformed or liquid feces, resulting in significant staining of the hind legs. Rabbits were euthanized at 2 and 5 days post-infection. At necropsy, the intestinal tract from the duodenum to the anus was removed and samples obtained for microbiologic analyses. To limit any litter-specific effects, at least two different litters were used to test each bacterial strain.

Citrobacter rodentium murine infections. For mouse survival experiment, ten 3.5 week old female C3H/HeJ or 129x1/SvJ mice per group were infected by oral gavage with multiple infectious doses of wild type, or $\Delta$qseG C. rodentium strains by oral gavage with 100 µl of PBS. Mouse survival in each group (10 animals per group) was accessed over the course of 14 to 26 days. Kaplan meyer test was used to determine statistical significance. For colon weight measurement, five 3.5 week old female C3H/HeJ mice per group (5 animals per group) were infected with $1 \times 10^9$ cells of wild type wild type or $\Delta$qseG C. rodentium. The infected mice were sacrificed on day 6 post infection, and their colons were taken, washed and weighed.

Salmonella typhimurium. 9-12 week old female Balb/c mice were infected intraperitoneally with $1 \times 10^4$ CFU SL1344 in PBS or sterile PBS as a control. Two independent
experiments were performed, each with 15 mice per group (10 for survival, 5 for organ collection). Survival was monitored and weight measured daily. On day 2 (1st experiment) or day 3 (2nd experiment) 5 randomly selected mice were sacrificed and livers and spleens homogenized and plated on LB agar + streptomycin to enumerate CFUs. Weight and survival data from the two independent experiments were combined. A student’s t-test was used to compare weight loss between the different groups on each day and the survival curves were analyzed using the Mantel-Cox test.

To measure fljB promoter orientation and NOS2 expression in infected tissues, portions of liver and spleen were collected into Trizol and total DNA and RNA was extracted according to manufacturer’s instructions. The fljB promoter orientation was measured in the extracted DNA using the qPCR standard curve method outlined below. NOS2 levels were assayed from extracted RNA via qPCR as described below. NOS2 readings were normalized to GAPDH levels and reported as a fold-change over levels from uninfected (PBS treated) mice. Average and standard deviations across the five mice are reported and statistical significance was calculated using a student’s unpaired t-test.

**Secreted proteome analysis.** SL1344 strains of Stm were grown to late log phase in LB. Cells were pelleted and supernatants filtered through 0.2um filter and concentrated using Amicon 10kDA MWCO concentrators (Millipore). Samples from concentrated supernatants were run under denaturing conditions on 12% SDS-PAGE gels and stained using Bio-safe Coomaisie (Biorad). The unknown band that appeared in the ΔqseG sample was extracted from the gel and submitted for identification by LC-MS/MS by the UT-Southwestern proteomics core facility. For total protein identification and quantification concentrated supernatants from WT and ΔqseG Stm were loaded onto SDS-PAGE gel and run just until samples entered the
resolving gel. The sample was extracted from the resolving gel and submitted for LC-MS/MS analysis to the UT-Southwestern proteomics core.

**Quantitative real-time PCR.** For *in vitro* transcription analysis Stm was grown to OD$_{600}$ 1.0 in LB. RNA was extracted and DNase treated as described in (57). Quantification of RNA transcript levels was performed as described previously(58). Primer pairs were validated for amplification efficiency and specificity using a standard curve of diluted RNA. Sample RNA was mixed with SYBR master mix, validated primers, RNase inhibitor and reverse transcriptase. A one-step reaction was run using ABI 7500 system and data was analyzed using the ABI sequence detection 1.2 software. Values were normalized to endogenous *rpoA* and analyzed by the comparative critical threshold method. Values are presented as fold-change over WT levels and the average and standard deviation from three independent replicates is represented.

**fljB promoter orientation assay.** DNA was extracted from strains grown as described above using Sigma bacterial genomic DNA extraction kit. A “universal” primer was designed within the invertible element and “on” and “off” primers were designed outside the invertible element such that a product would only be amplified when the promoter was in the designated position. Amplicons for each orientation were synthesized via conventional PCR, purified and diluted to make a standard curve of known quantities. Standard curves were used to determine the quantity of each orientation in unknown samples, which were converted to mol and the molar ratio of on to off was calculated.

**Swimming motility assay.** Motility assays were performed as described previously (59). Briefly, strains were grown in LB broth + 0.2% arabinose to induce complementation plasmid shaking at 37°C until they reached OD 1.0. Cells were stabbed into LB plates containing 0.3%
agar and incubated at 37°C. Motility halos were measured at indicated time points. Average and standard deviation between three replicates is displayed.

**Cell invasion assay.** HeLa epithelial cells were routinely cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum in 5% CO₂. Invasion assay was performed as described previously (60). Briefly, HeLa cells were seeded at 1x10⁵ per well in 24-well plates 24 hours prior to infection. Overnight Stm cultures were diluted into fresh LB and grown to OD 1.0, washed in sterile PBS and resuspended to 1X in pre-warmed DMEM media. Cells were washed x3 with pre-warmed PBS and infected at an MOI of 10. Plate was spun at 1000xg for 5 minutes and incubated at 37°C in 5% CO₂ for 90 minutes. Media was removed, cells washed and DMEM + gentamycin (30ug/mL) was added to kill extracellular bacteria. Cells were incubated for an additional 60 minutes before removing media and washing with PBS x3. Cells were lysed by incubating in 1mL 1% Triton X-100 in PBS at room temperature for 5 minutes. Cell lysates and the bacterial inoculum were diluted in PBS and plated on LB agar with streptomycin to enumerate CFUs.

**Intracellular replication assay.** J774 mouse macrophages or THP-1 human macrophages were seeded in 24-well plates and grown to confluency over 2 days. To prepare inoculum bacteria were diluted from an overnight culture 1:10 into fresh LB medium and grown to OD 1.0 at 37°C shaking. Bacteria were washed with PBS x2 then resuspended in cell culture media. Cells were infected at an MOI of 5 and plates were spun at 1000xg for 5min to maximize bacteria-cell contact. Infected cells were incubated for 30 minutes at 37°C, 5%CO₂. Media was removed and cells were washed x3 with warm PBS. Cell culture media + 30µg/mL gentamycin was added to kill extracellular bacteria. After 3 hours samples were collected by removing media, washing wells x3 with PBS then adding 1mL 0.1% Triton X-100 to each well. After
incubating for 5 minutes cell lysates were collected. For 24 hour samples, media was removed at
3 hours and replaced with media containing 10µg/mL gentamycin. At 24 hours post infection
wells were washed and cells lysed as described above. Serial dilutions of lysates were plated on
LB agar + streptomycin to enumerate CFUs. Averages and standard deviations for three
independent experiments are shown.

**IL-8 expression by T84 cells.** The T84 (ATCC CCL-248) human colorectal carcinoma
cell line was routinely cultured in DMEM-F12 media (Gibco) supplemented with 10% heat
inactivated fetal bovine serum (Gibco). Cells were seeded in 6-well plates at a density of 1x10^5
cells per well, grown for 48 hours. 24 hours prior to infection cell culture media was removed
and replaced with serum-free media. SL1344 strains were grown to OD 1.0 shaking in LB media.
Cells were pelleted and supernatants were filtered and concentrated ~60X with 10kDa MWCO
Amicon concentrators (Millipore). Concentrated supernatants or purified flagellin (InvivoGen)
were added to T84 cells in triplicate. One hour post-addition, media was removed and cells were
washed x3 with pre-warmed sterile PBS. 1mL Trizol was added to each well and cells were
collected into Eppendorf tubes. RNA was extracted via manufacturer’s instructions. DNA was
degraded using Turbo DNase (Ambion) and cDNA was synthesized using Superscript II reverse
transcriptase (Invitrogen) according to manufacturer’s instructions. 50ng of cDNA was used per
reaction with SYBR master mix and validated primers using ABI 7500 system. To ensure no
gDNA remained, control reactions were run with RNA as the template. Data was analyzed using
the ABI sequence detection 1.2 software. Values were normalized to endogenous GAPDH and
analyzed by the comparative critical threshold method. Values are presented as fold-change over
untreated cells. Average and standard deviation were calculated across triplicate wells and
statistically significant differences were determined via student’s t-test. One representative
experiment is shown but three independent experiments were performed to ensure reproducibility.

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL FILE 1, live cell movie of WT EHEC on HeLa cells
SUPPLEMENTAL FILE 2, live cell movie of ΔqseG EHEC on HeLa cells

ACKNOWLEDGEMENTS

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REFERENCES


Table 1: Mass spec analysis of secreted proteins in WT vs *qseG*- SL1344

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**Figure legends**

**FIG 1** QseG interacts with SepL. (A) Proteinase K protection assays of OmpA and QseG. WT EHEC cells were treated with proteinase K and then proteins were analyzed by western blot. Known outer-membrane protein OmpA is included as a control (B) Yeast two hybrid assays to probe possible protein-protein interaction between QseG and QseG, EscC, EspA, SepD, SepL and Tir. Briefly, the yeast report strain L40 was transformed with all combinations of the proteins in both the bait (pLEX-ADE) and the library (pVP16) vector. Dual transformants were selected on yeast minimal media lacking leucine, and tryptophan and then assayed for protein-protein interactions using the integrated *LacZ* and *HIS3* reporters. Association of the two protein fusions was determined by growth on yeast minimal media lacking histidine. (C) Pull down assays with SepL-HA and QseG. A plasmid expressing an HA-tagged *sepL* allele was transformed into ∆*sepL* and ∆*sepLΔqseG* EHEC strains. SepL was captured using HA antisera and the precipitate was probed for QseG by western blot using QseG antisera.

**FIG 2** AE lesion timing and dynamics. Time lapse microscopy of Lifeact::GFP expressing HeLa cells being infected with mCherry expressing WT and ∆*qseG* EHEC. The minutes elapsed after imaging was begun are listed above each panel. EHEC pedestal formation can be observed as yellow areas—where actin polymerization (green puncta) co-localizes with attached EHEC bacteria (red).

**FIG 3** QseG is necessary for EHEC and C. rodentium virulence. (A) EHEC infection in infant rabbits. 3-day old rabbits were inoculated intragastrically with ~5x10^8 CFU or WT or ∆*qseG*
EHEC. At 5 days post infection colonic contents was plated on LB containing streptomycin to
determine EHEC CFUs **p<0.01. *Citrobacter rodentium* murine infections. 3.5 week old
C3H/HeJ mice were gavaged with 10⁹ CFU of WT or ΔqseG *C. rodentium* or PBS. (B) survival
was monitored daily (n=10). Statistical significance was determined via Kaplan Meyer test (C)
Colon weight. Mice were infected as described in B (n=5). At day 6 post infection mice were
sacrificed, and their colons collected, washed and weighed. (D) Colons collected as described in
C were examined for gross pathology differences.

**FIG 4** QseG controls flagellin phase variation in *Salmonella enterica* Typhimurium (Stm). (A)
Nucleotide and protein sequence similarities of *qseE*, *qseF* and *qseG* between EHEC and Stm are
displayed (B) Stm strains were grown statically in LB broth to late log phase. A standard amount
of BSA was spiked into cultures just prior to collection to serve as a concentrating/loading
control. Cells were pelleted and proteins in filtered, concentrated supernatants were separated via
SDS-PAGE and stained using Coomaisie blue. Presumed identifications of selected proteins
based on molecular weight are indicated and the band of interest that is overexpressed in the
*qseG* mutant supernatant is indicated with an asterisk. Relative quantification of FljB and FliC
levels from the pictured supernatants was performed via LC-MS/MS and is listed below. (C)
WT, ΔqseG and the complemented strain were grown to OD 1.0 in LB broth + 0.2% arabinose to
induce the complementation plasmid. RNA extracted from these cells was probed via qPCR for
expression levels of *fliC* and *fljB*. Levels of genes of interest were normalized to *rpoA* levels and
expressed as a fold change over WT levels. The average and standard deviation of three
replicates is shown. (D) Using a qPCR-based assay, the orientation of the *fljB* promoter was
assessed in Stm grown as described in 3B. The data is displayed as the molar on:off ratio of the
*fljB* promoter. The average and standard deviation of three replicates is shown and a student’s t-test was used to determine statistical significance.

**FIG 5** QseG does not contribute to motility, cell invasion or intracellular replication in Stm. (A) Indicated Stm strains were grown to OD 1.0 in LB broth + 0.2% arabinose (to induce complementation plasmid) then stabbed into 0.3% agar LB plates. At 6,8 and 24 hours the diameter of the motility halos were measured. Averages and standard deviations from three replicates are reported and representative plates at 24 hours are pictured. (B) HeLa cells were infected with the indicated Stm strains to assess cell invasion efficiency. The ∆*invA∆spiB* mutant represents an invasion deficient strain. Averages and standard deviations from three replicates are shown. (C) J774 (mouse) or THP1 (human) macrophages were infected with the indicated Stm strains to assess intracellular replication ability. The ∆*invA∆spiB* mutant represents a intracellular replication deficient strain. The averages and standard deviation of three replicates are shown.

**FIG 6** QseG impacts Stm murine infection. Balb/c mice were mice were infected intraperitoneally with 1x10⁴ CFU of the indicated Stm strain or sterile PBS. Survival (A) and weight (B) were monitored over time. Data from two independent experiments, each using 10 mice per group, are displayed in A and B. Significant differences between survival curves were determined using the Mantel-Cox method. (C) On day 2 (top) or 3 (bottom), 5 mice were randomly selected, sacrificed and serial dilutions of organ homogenates plated for CFU counts.
FIG 7 Altered inflammation during murine infection by the qseG Stm mutant. (A) Concentrated supernatants from the indicated Stm strains were applied to T84 intestinal epithelial cells. Induction of IL-8 transcription was assessed via qPCR. IL-8 levels were normalized to GAPDH levels and values are reported as a fold change over uninfected cells. The averages and standard deviations from three replicates are displayed and statistical significance was determined using a student’s t-test. (B) From the murine infection experiment described in Figure 6, total DNA was extracted from infected spleens and livers on Day 3 post-infection. The orientation of the fljB promoter was assayed via the qPCR-based method described in Fig. 3C (n=5). Significant differences were determined using a student’s t-test. (C) From the infection experiment described in Figure 6, total RNA was extracted from infected spleens and livers on Day 3 post-infection. Host NOS2 expression levels were assayed via qPCR, normalized to GAPDH levels and reported as a fold change over levels from PBS treated mice. (n=5) Significant differences were determined using a student’s t-test.

FIG 8 Model of the role of QseG in Stm. Flagellin expression is advantageous during the intestinal portion of the life cycle and the intestinal Stm population is mixed: with some expressing FliC and others FljB. Once the bacteria cross the epithelial barrier and go systemic, flagellin expression is no longer advantageous. Flagellin secretion at systemic sites, particularly FljB, can lead to an increased inflammatory response and an attenuated infection. QseG is involved in limiting the inflammatory response to flagellin, and modulates the fljB promoter such that the off orientation is favored. This leads to less FljB secretion, lowered NOS2 induction by the host and a more virulent phenotype in a mouse model of systemic infection.
Fig. 1

A

Proteinase K

Proteinase K

OmpA

OmpA

QseG

LPS

B

Tir

SepL

QseG

EspA

SepD

SepL-HA

QseG

Input

IP

C

SepL-HA

QseG

- + + - + + -

- + + - + + -

QseG
Colon weights of infected C3H/HeJ mice

**Fig. 3**

Colon Day 5

A

Log$_{10}$ CFU/g

WT  ▲

ΔqseG  ▲

B

% survival

WT ▲

ΔqseG ▲

P<0.01

days post infection

C

Colon weights of infected C3H/HeJ mice

PBS  ▲

WT  ▲

ΔqseG ▲

D

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ΔqseG vs WT Stm

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**Fig. 4**
Fig. 5

A

B

C

Fig. 5
Fig. 6

**p = 0.004**

A. Survival rates over days post-infection.

B. Percentage change in weight over days post-infection.

C. CFU/g levels in spleen and liver on Day 2 and Day 3.

Legend:
- WT
- ΔqseG
- PBS
A

![Graph showing IL-8 mRNA fold change vs uninfected](image)

- **p < 0.01**
- **p < 0.001**
- ns

B

![Graph showing NFκB ON/OFF ratio](image)

- **p = 0.003**
- p = 0.024

C

![Graph showing fold change vs uninfected](image)

- **p < 0.02**

Fig. 7
Fig. 8

Invading S. Tm

Intestinal lumen

FljB

FliC

QseG

Inflammation

Liver

Spleen

NOS2
SUPPLEMENTAL MATERIAL

SUPPLEMENTAL FILE 1, live cell movie of WT EHEC on HeLa cells

SUPPLEMENTAL FILE 2, live cell movie of ΔqseG EHEC on HeLa cells