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# QseC-mediated dephosphorylation of QseB is required for expression of genes associated with virulence in uropathogenic *Escherichia coli*

**Maria Kostakioti**[†], **Maria Hadjifrangiskou**[†], **Jerome S. Pinkner**, and **Scott J. Hultgren**[*]
Department of Molecular Microbiology and Microbial Pathogenesis, Washington University in Saint Louis School of Medicine, 660 S Euclid, St Louis, MO 63110-1010, USA

## Summary

Bacteria sense environmental cues and regulate gene expression accordingly so as to persist in diverse niches. QseC is a membrane sensor kinase shown in enterohemorrhagic *Escherichia coli* to respond to host and bacterial signals by phosphorylating the QseB response regulator at residue D51, resulting in QseB activation and presumably upregulation of virulence genes. We studied QseBC in uropathogenic *E. coli* (UPEC). UPEC establish infection by colonizing and invading bladder cells. After invasion, UPEC can escape into the cytoplasm where they can form intracellular bacterial communities. Deletion of *qseC* significantly attenuated intracellular bacterial community formation and virulence, whereas paradoxically *qseB* deletion did not impact pathogenesis. We found that QseB upregulates its own expression in the *qseC* mutant, arguing that it is activated even in the absence of QseC. However, expression of QseB, but not a QseB_D51A mutant, in the absence of QseC resulted in downregulation of type 1 pili, curli and flagella. We observed similar phenotypes with enterohemorrhagic *E. coli*, showing that this is not a UPEC-specific phenomenon. Target gene expression is restored when QseC is present. We discovered that QseC has phosphatase activity required for QseB dephosphorylation. Thus, the QseC phosphatase capacity is critical for modulating QseB activity and subsequent gene expression.

## Introduction

Bacterial pathogens often use two-component signal transduction systems as a means to respond to environmental cues, primarily by altering their gene expression patterns in a mode that is beneficial at the time (Hoch, 2000). Two-component systems are composed of a membrane-embedded sensor kinase and a cytoplasmic response regulator (Stock *et al.*, 2000). In most cases, signal recognition results in sensor kinase activation via autophosphorylation at a histidine residue (Hoch, 2000). The signal is subsequently transduced to the response regulator, which becomes phosphorylated at a conserved aspartate residue and undergoes a conformational change that results in its activation (Hoch, 2000). The activated response regulator then exerts its regulatory function usually by binding to DNA to promote or repress target gene expression (Hoch, 2000). Some sensor kinases are also responsible for dephosphorylation of their phosphorylated cognate response regulator when the signal is no longer present (Stock *et al.*, 2000). Two-component systems have been extensively implicated in pathogenesis, such as the PhoPQ (Groisman, 2001) and

[*]For correspondence. hultgren@borcim.wustl.edu; Tel. (+1) 314 362 6772; Fax (+1) 314 362 3203.
[†]These authors contributed equally to this work.

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EnvZ-OmpR systems (Bang *et al.*, 2000) in *Salmonella* spp., and the BvgAS system in *Bordetella pertussis* (Cotter and Jones, 2003), and thus the molecular mechanisms underlying their role in virulence are important to elucidate (Beier and Gross, 2006).

Studies in enterohemorrhagic *Escherichia coli* (EHEC) identified QseBC, a two-component system that responds to quorum sensing and is involved in pathogenesis (Sperandio *et al.*, 2002; Clarke *et al.*, 2006). Deletion of the QseC histidine kinase attenuates virulence of EHEC, *Francisella tularensis* and *Salmonella typhimurium* (Weiss *et al.*, 2007; Bearson and Bearson, 2008; Rasko *et al.*, 2008) and compounds that interfere with QseC signalling were shown to compromise pathogen survival *in vivo* (Rasko *et al.*, 2008). *In vitro* phosphorylation assays identified the bacterial autoinducer 3, and the host hormones epinephrine/norepinephrine, as signals that trigger QseC phosphorylation and transfer of the phosphoryl group to the QseB response regulator (Clarke and Sperandio, 2005a; Clarke *et al.*, 2006). Primer extension assays revealed that *qseC* deletion abolished the expression of *qseBC* and *flhDC* in EHEC (Clarke and Sperandio, 2005a,b). In addition, Clarke *et al.* demonstrated that phosphorylated QseB binds to the *qseBC* promoter and the promoter of the major flagella regulator *flhDC in vitro* (Clarke and Sperandio, 2005a,b). In conjunction with the motility defects and the *in vivo* attenuation of the *qseC* deletion mutant, QseB was suggested to be an activator of its target genes (Clarke and Sperandio, 2005a,b; Clarke *et al.*, 2006). Yet, the role of QseB in bacterial pathogenesis is not well understood.

To gain a better understanding of the regulatory mechanisms linking QseBC to bacterial pathogenesis, we investigated the role of QseB in uropathogenic *E. coli* (UPEC), the predominant causative agent of urinary tract infections (UTIs), which are among the most frequent bacterial infections afflicting women (Foxman, 2002). UPEC produce type 1 pili, which mediate colonization and invasion of the bladder epithelium (Mulvey *et al.*, 1998). After invasion, bladder epithelial cells are capable of expelling UPEC, presumably as part of an innate defence (Song *et al.*, 2007). However, when UPEC escape into the cytoplasm of superficial umbrella cells, they are capable of rapidly replicating into biofilm-like intracellular bacterial communities (IBCs) comprised of $10^4$–$10^5$ bacteria (Anderson *et al.*, 2003). Upon IBC maturation, bacteria detach from the IBC biomass, flux out of the host cell and spread to neighbouring cells forming next-generation IBCs (Justice *et al.*, 2004). Thus, IBC formation represents a mechanism by which invasion of a single bacterium can result in rapid expansion of UPEC numbers in the urinary tract leading to disease (Mulvey *et al.*, 2001; Anderson *et al.*, 2003). The regulatory mechanisms governing IBC development and dispersal in response to environmental cues are still unknown.

We discovered that deletion of *qseC* significantly attenuated IBC formation and UPEC virulence. Moreover, absence of this histidine kinase resulted in reduced type 1 pili expression, which most likely would explain the IBC defect and subsequent virulence attenuation. Surprisingly, double deletion of *qseBC*, or deletion of *qseB* alone, had no effect on type 1 pili expression. Similarly, deletion of *qseC* abolished expression of curli and severely decreased flagella production, but a double *qseBC* deletion or single deletion of *qseB* had no effect, suggesting that expression of *qseB* in the absence of *qseC* resulted in downregulation of these genes. We found that in the absence of QseC, the QseB response regulator remained constitutively active and positively regulated its own expression, implying an alternative pathway of QseB phosphorylation. Paradoxically, we found that presence of active QseB (but not of an inactive QseB_D51A variant) results in downregulation of type 1 pili and abolished expression of curli and flagella in the absence of QseC. We resolved this paradox by demonstrating biochemically that QseC dephosphorylates QseB. Thus, we argue that dephosphorylation of QseB by QseC, restores expression of type 1 pili, curli and flagella by preventing the negative effects that active

QseB has on these target genes. This study demonstrates that QseC is a bifunctional sensor whose dual activity is required for controlled QseB-mediated gene regulation.

## Results

### QseC but not QseB mutants are attenuated in UPEC virulence

UTI89 QseB and QseC exhibit 99% and 98% identity, respectively, to the EHEC strain EDL933 homologues. We studied the effects of single deletions in the *qseBC* gene pair on UPEC virulence using a well-established murine cystitis model (Mulvey *et al.*, 1998; Anderson *et al.*, 2003). Female C3H/HeN mice were transurethrally inoculated with $10^7$ UTI89, UTI89Δ*qseB* or UTI89Δ*qseC*. Bladder Colony-forming units (cfu) and IBC formation were monitored at 6 h post infection (h.p.i.), marking the middle of IBC development, and 16 h.p.i., marking the end of the first IBC cycle at which time bacteria from the IBC biomass disperse, filament and spread to neighbouring cells reinitiating the IBC cycle (Justice *et al.*, 2004). At 6 h.p.i. the recovered bladder cfu were comparable among all strains (Fig. 1A), yet UTI89Δ*qseC* formed significantly fewer IBCs than UTI89 and UTI89Δ*qseB* (Fig. 1C), as determined by confocal microscopy and LacZ staining following the method described by Justice *et al.* (2006). At 16 h.p.i. UTI89Δ*qseC* was significantly attenuated, having sevenfold lower cfu than UTI89, and dramatically fewer IBCs. Notably, the detected IBCs often lacked presence of filamentous UPEC, indicating a defective IBC developmental pathway. Surprisingly, UTI89Δ*qseB* produced similar levels of cfu and IBCs as wild type (wt) UTI89 (Fig. 1A and C). UTI89Δ*qseB* also produced kidney titers similar to wt, while UTI89Δ*qseC* was significantly attenuated producing 11-fold lower cfu at 16 h.p.i. (Fig. 1B). These data argue that QseC plays a role in IBC formation and maturation, and that the defect in IBC formation in the *qseC* mutant leads to attenuation of virulence. The lack of a phenotype in UTI89Δ*qseB* led us to investigate the effects of the *qse* mutants on gene regulation and the interplay between QseC and QseB.

### Deletion of *qseC* affects expression of type 1 and S pili in strain UTI89

Given the role of type 1 pili in bladder colonization and IBC formation (Wright *et al.*, 2007), we investigated the effects of the *qseB* and *qseC* deletions on type 1 pilus expression as measured by the ability of UPEC to mediate mannose-sensitive haemagglutination (HA) (Hultgren *et al.*, 1986). The FimH adhesin at the tips of type 1 pili binds to mannosylated receptors on the surface of red blood cells, typically exhibiting an HA titer of 8–9 (Hultgren *et al.*, 1986). Soluble D-mannose (2%) inhibits the HA reaction by competitively binding FimH, thereby blocking bacterial binding to the mannosylated erythrocytes (Hultgren *et al.*, 1986). Thus, UTI89 has an HA titer of 0 in the presence of 2% D-mannose. UTI89Δ*qseC* exhibited a fourfold reduction in HA titer compared with wt UTI89 in the absence of mannose (Fig. S1A), indicating a defect in the expression of type 1 pili. Whole-cell immunoblots probing for FimA, the major type 1 pilin subunit, showed a 56.2% reduction of FimA amounts in UTI89Δ*qseC* compared with wt, determined by ImageJ analyses (Fig. 2A). FimA expression and HA properties were restored in UTI89Δ*qseC* when *qseC* was introduced *in trans* under its native promoter (pQseC), confirming that deletion of *qseC* is responsible for the type 1 pili defect (Fig. 2A and Fig. S1A). Type 1 pili are regulated by ON/OFF phase variation involving an invertible DNA element that contains the *fim* promoter (*fimS*) (Abraham *et al.*, 1985). To assess whether deletion of *qseC* influences type 1 pili phase variation, we used PCR to amplify *fimS*, combined with restriction digests that yield different-size fragments depending on promoter orientation. Our results revealed that 72.5% of *fimS* was found in the OFF orientation in UTI89Δ*qseC* compared with 45% in wt UTI89 (Fig. 2B), showing that in the absence of *qseC*, an increased proportion of bacteria have *fimS* in a phase OFF orientation. Interestingly, production of type 1 pili in

UTI89Δ*qseB* was identical to that of the parent strain (Fig. 2A and B and Fig. S1A). This suggested that the *qseC*-deletion phenotype was not due to the inactivity of QseB.

In the presence of 2% D-mannose, the HA properties of wt UTI89 and UTI89Δ*qseB* were abolished (Fig. S1A), whereas UTI89Δ*qseC* retained an HA titer of 3, suggesting that a mannose-resistant adhesin, not expressed in wt or UTI89Δ*qseB* cells, is expressed in UTI89Δ*qseC*. Genomic analyses have revealed the presence of 10 chaperone/usher pilus operons in the UTI89 chromosome (Chen *et al.*, 2006). Alterations in type 1 pili expression have been shown to affect expression of S pili, which confer mannose-resistant HA (J.S. Pinkner, *et al.*, unpubl. data). Thus, we assessed the expression of *sfaA*, encoding the S pilus major subunit, in the parent strain and the *qse* mutants. Semi-quantitative RT-PCR showed that the *sfaA* transcript was significantly higher in UTI89Δ*qseC* than in wt UTI89 or UTI89Δ*qseB* (Fig. 2C). Thus, the mannose-resistant HA (titer equal to 3) observed in UTI89Δ*qseC* may be due to increased expression of S and/or some other type of pilus (Fig. S1A).

These data indicate that UTI89 QseC is part of a network controlling the expression of both type 1 and S pilus systems (directly and/or indirectly) and may have a more global function in regulating UPEC extracellular fibres. However, the QseC-mediated regulation of pili seems to occur via a mechanism in which QseB does not directly act as a positive regulator or is not essential.

## Curli formation depends on QseC but not QseB

To further understand the distinct *qseB* and *qseC* effects on gene expression, we examined how these deletions impact the production of other adhesive organelles. Curli are extracellular amyloid fibres implicated in biofilm formation. They are encoded by two divergently transcribed operons, *csgBA* and *csgDEFG* (Barnhart and Chapman, 2006). CsgA comprises the bulk of the fibre, CsgB is the nucleator and CsgEFG make up the assembly machinery. CsgD is a transcriptional activator of *csgBA* (Barnhart and Chapman, 2006). Curli formation can be assessed by growth on Yeast Extract/Casamino Acids agar supplemented with the Congo red (CR) dye, as curli bind CR giving rise to red, dry and rough colonies (Barnhart and Chapman, 2006). Deletion of CsgA abolishes this phenotype and yields smooth and white colonies (Hammar *et al.*, 1996). Deletion of *qseC,* but not *qseB*, abolished curli formation as determined by CR binding (Fig. 2D), electron microscopy (Fig. S1B) and immunoblot analyses with anti-CsgA and anti-CsgG antisera (Fig. 2E). Accordingly, UTI89Δ*qseC* was unable to form curli-dependent biofilms *in vitro,* in contrast to UTI89Δ*qseB* (data not shown). Expression of *qseC in trans* restored curli formation in UTI89Δ*qseC* (Fig. 2E). RT-PCR revealed that the *csgD* transcript levels were negligible in UTI89Δ*qseC* but remained unaffected in UTI89Δ*qseB* (Fig. S1C). Our data demonstrate that QseC but not QseB is required for curli expression.

## Absence of QseB does not influence flagella expression

Deletion of *qseC* in an EHEC strain abolished *flhDC* transcription, resulting in defective motility. Additional studies showed that QseB binds the *flhDC* promoter *in vitro* and, it was therefore thought to be a positive regulator of this promoter (Sperandio *et al.*, 2002; Clarke and Sperandio, 2005b; Gonzalez Barrios *et al.*, 2006). Consistent with previous observations, UTI89Δ*qseC* had severe motility defects, depicted by a 73 ± 1% reduction in swimming and 52 ± 7.4% reduction in swarming, and expressed significantly fewer flagella than wt UTI89 (Fig. S2A–C). Also, as reported previously in EHEC, we found that deletion of *qseC* resulted in decreased *flhDC* transcription as revealed by semi-quantitative RT-PCR (Fig. S2D). However, the effects of a *qseB* deletion on *flhDC* expression and motility have not previously been addressed. Thus, we compared the motility of UTI89Δ*qseB* and

UTI89Δ*qseC*. Surprisingly, UTI89Δ*qseB* was indistinguishable from the wt strain (Fig. S2A–C). Thus, flagella expression was dependent on QseC but not QseB (Fig. S2D), similar to type 1 pili and curli. Our results with the UTI89Δ*qseB* strain are unexpected and argue for a new paradigm for the mechanism by which QseB functions. As Δ*qseB* mutants have not previously been studied, we investigated the molecular basis of the Δ*qseB* phenotype.

## Expression of QseB in the absence of QseC results in downregulation of flagella, pili and curli genes but upregulation of *qseB*

As deletion of *qseB* did not affect the expression of type 1 pili, curli or flagella, we hypothesized that QseC may function through different response regulators in the absence of QseB. In this case, a double *qseBC* deletion should decrease or abolish the expression of type 1 pili, curli and flagella similar to what is observed in UTI89Δ*qseC*. In contrast, UTI89Δ*qseBC* behaved like UTI89Δ*qseB*, exhibiting wt expression of all of these genes (Fig. 3A and B and data not shown). Similar results were obtained with an EHEC *qseBC* deletion mutant, ruling out the possibility that this is a UPEC-specific phenomenon (data not shown). These findings argue that the *qseB* deletion phenotype is not likely due to QseC functioning via other response regulators.

We hypothesized that QseB is actively responsible for the phenotypes observed in the absence of QseC and that expression of QseB in the Δ*qseBC* background should therefore lead to downregulation of motility and curli production. To test this, *qseB* was cloned under its native promoter in vector pTrc99A (pQseB), and introduced in UTI89Δ*qseBC*. The resulting strain was non-motile and curli-negative, resembling UTI89Δ*qseC* (Fig. 3A and B). Similar phenotypes were obtained with UTI89Δ*qseC*/pQseB. Downregulation of motility in these cases was more pronounced due to the fact that QseB is expressed from a high-copy plasmid. However, when pQseB was transformed into UTI89Δ*qseB* or wt UTI89, curli and flagella expression was unaffected (Fig. 3A and B), arguing that the function of QseC is dominant under these conditions.

QseB cloned from EHEC strain EDL933 was used to complement UTI89Δ*qseBC* or UTI89Δ*qseC* and it yielded the same phenotypes as the UPEC QseB (data not shown), suggesting that the EHEC and UPEC QseB proteins are interchangeable. The EHEC QseB protein has one amino acid difference compared with UTI89 QseB (residue 86). These data support that the effects of the *qseC* deletion on curli, type 1 pili and flagella are due to the function of QseB that negatively influences the expression of these genes in a direct or indirect manner, and that presence of QseC overrides these negative effects. These observations support the hypothesis that QseB is functioning in a manner not previously expected and this is not specific for UPEC, as complementation of the EHEC *qseBC* mutant with either EHEC or UPEC QseB also abolished motility (data not shown).

We found that expression of QseB from a high-copy plasmid in UTI89Δ*qseBC* or UTI89Δ*qseC* led to more pronounced decrease of motility than that observed in the *qseC* deletion mutant, supporting the hypothesis that QseB is active in the absence of QseC. Given that QseB has been previously shown to bind its promoter and direct its own expression (Clarke and Sperandio, 2005a), we investigated the effect of *qseC* deletion on *qseB* expression. Q-RT-PCR revealed that the *qseB* transcript in UTI89Δ*qseC* was 84.9-fold higher relative to wt UTI89 (data not shown). Similar results were obtained with a transcriptional fusion of the *qseBC* promoter to a GFP reporter gene. GFP expression was undetectable in wt UTI89, UTI89Δ*qseB* and UTI89Δ*qseBC* whereas high GFP expression was observed in UTI89Δ*qseC* as determined by fluorescence microscopy and immunoblot analyses with anti-GFP antibody (Fig. 3C and Fig. S3). When pQseB was introduced, GFP expression increased in all strains with greater increases seen in those lacking QseC (Fig. 3C and Fig. S3). Thus, deletion of *qseC* results in higher *qseB* expression, which is presumably

due to autoregulation of the *qseBC* promoter by QseB, indicating that in the absence of its cognate histidine kinase, QseB remains functional.

### QseB requires QseC for dephosphorylation and deactivation

Our data argue that in the absence of QseC, QseB is active. As the D51 residue is essential for QseB phosphorylation and activation, we hypothesized that a mutation of this residue to an alanine (D51A), which would render QseB unable to accept a phosphoryl group, would result in a protein (QseB_D51A) unable to mediate the down-regulation of type 1 pili, curli and flagella. We created pQseB_D51A by site-directed mutagenesis using pQseB as a template, and assessed its effects in UTI89Δ*qseBC*, utilizing curli expression and motility as representative assays. UTI89Δ*qseBC*/pQseB_D51A remained motile and CR-positive in contrast to UTI89Δ*qseBC*/pQseB (Fig. 3A and B), demonstrating that the D51A mutation alleviated the downregulation of curli and flagella. We also mutated D51 to glutamic acid, a mutation that has been previously shown to alter the conformation of numerous DNA-binding response regulators with that resembling the active phosphorylated state (Lan and Igo, 1998; Freeman and Bassler, 1999). Complementation of UTI89Δ*qseBC* with the plasmid encoding QseB_D51E resulted in reduced motility and curli expression (Fig. 3A and B). These data argue that in the absence of QseC, QseB is found in an active form, which upregulates its own expression and negatively affects transcription of type 1 pili, curli and flagella, either directly or indirectly.

### QseC dephosphorylates QseB

QseC has been shown to phosphorylate QseB leading to its activation (Clarke *et al.*, 2006). Our studies show that QseB is active in the absence of its cognate sensor, arguing that QseC is not necessarily required for QseB activation. The mechanism of QseB activation in the absence of QseC is unknown. However, our above analysis of QseB argues that QseC is essential for alleviating the downregulation of flagella, curli and type 1 pili. Thus, we hypothesized that QseC may be required for QseB dephosphorylation in order to inactivate it and thereby relieve gene downregulation. To address this, we performed *in vitro* phosphotransfer assays using QseC-enriched membrane vesicles and purified QseB. Membrane vesicles lacking QseC were used as a negative control. We first verified that QseC is functional, by monitoring its auto-phosphorylation properties when incubated with [$\gamma$-$^{32}$P]-ATP (Fig. 4A). We then confirmed the ability of QseC to phosphorylate its cognate response regulator as previously reported (Clarke *et al.*, 2006). Interestingly, this assay revealed that QseC not only phosphorylated QseB, but that dephosphorylation of QseB also occurred in the presence of QseC (Fig. S4), suggesting a phosphatase activity.

This putative QseC phosphatase activity was directly tested in phosphatase assays. QseB was phosphorylated *in vitro* (QseB~P), incubated with QseC-containing membrane vesicles, and loss of the phosphoryl group was monitored over a 60 min time-course. QseB~P incubated in the absence of membrane vesicles was used as a control to account for spontaneous dephosphorylation. We found that rapid dephosphorylation of QseB~P occurs when QseC is present, with only 28.4% of QseB~P remaining phosphorylated by 15 min of incubation (Fig. 4B). Interestingly, dephosphorylation of QseB was coupled with QseC phosphorylation, showing that, at least *in vitro*, there is reverse phosphotransfer to QseC (Fig. 4B). Although some spontaneous QseB~P dephosphorylation occurred in the absence of membrane vesicles, 96.5% of QseB remained phosphorylated at 15 min of incubation, and 54.34% of QseB retained the phosphoryl group throughout the 60 min period (Fig. 4B). When incubated with membrane vesicles lacking QseC, QseB~P remained stably phosphorylated (75.7%) by 60 min of incubation (Fig. 4B). These data argue that QseC exhibits phosphatase activity and is required to directly dephosphorylate QseB.

## Discussion

The QseBC two-component signal transduction system has been implicated in virulence gene expression in a number of pathogenic bacteria. Previous studies focusing on understanding the role of QseC and the mechanisms of its activation have shown that *qseC* deletion attenuates EHEC, *S. typhimurium* and *F. tularensis* (Weiss *et al.*, 2007; Bearson and Bearson, 2008; Rasko *et al.*, 2008), and impairs the motility of EHEC and *S. typhimurium* (Sperandio *et al.*, 2002; Clarke and Sperandio, 2005b; Bearson and Bearson, 2008). However, the effects of a *qseB* deletion had not previously been studied. We discovered that deletion of *qseC* attenuates UPEC virulence in a cystitis mouse model but surprisingly, deletion of *qseB* had no observable effects on pathogenesis. Thus, we investigated the role of QseB and the molecular interplay with QseC in UPEC. We discovered that the function of QseB, and the mechanism by which QseC controls QseB activity, is more complex than previously thought. We found that in the absence of QseC, QseB remained active, and its activity resulted in downregulation of type 1 pili, explaining virulence attenuation. Similarly, the expression of curli and flagella was downregulated in the *qseC* mutant, due to the direct or indirect effects of an active QseB. We discovered that QseC has a phosphatase activity and thus is able to regulate the activity of QseB by acting as a bifunctional sensor kinase/phosphatase that controls the phosphorylation state of QseB, and thereby maintains optimal gene expression.

QseB is known to bind its own promoter and upregulate its own expression (Clarke and Sperandio, 2005a). Previous analyses indicated the presence of two transcriptional start sites in the *qseBC* promoter region. Deletion of QseC abolished transcript initiation from the first site and it was thus reported to be QseB-dependent (Clarke and Sperandio, 2005a). In contrast, in the same analysis, upon deletion of *qseC*, transcript initiation occurred from the second transcriptional start site. However, the second site was proposed to function as a constitutive and QseB-independent site based on the premise that QseB is inactive in the absence of its cognate sensor (Clarke and Sperandio, 2005a). However, our data showing increased *qseB* expression in the *qseC* deletion mutant support that QseB is active in the absence of QseC. Thus, the abundant transcription from the second *qseBC* transcriptional start site, observed by Clarke and Sperandio, may be the result of a QseB-dependent process. This is further corroborated by the increased *qseBC* promoter activity observed when *qseB* is expressed in strains lacking QseC using a *qseBC-GFP* reporter system. Thus, we propose that QseB is able to regulate *qseBC* expression even in the absence of its cognate sensor. It will be interesting to determine whether the presence of QseC favours QseB-mediated transcription from one of the reported *qseBC* start sites and whether its absence results in QseB predominantly directing transcription from the second promoter. Such a shift in promoter preference could possibly be related to the phosphorylation state of QseB and warrants further investigation.

Complementation of a UPEC or EHEC *qseBC* deletion mutant with either EHEC or UPEC QseB led to downregulation of flagella, arguing that QseB activity is, directly or indirectly, responsible for the repressive effects observed in both strain backgrounds. This was further corroborated by the fact that expression of a QseB_D51E variant, which mimics the phosphorylated state, as has been previously reported for some response regulators, led to downregulation of type 1 pili, flagella and curli in the *qseBC* deletion strain, in a manner similar to UTI89Δ*qseC*. Thus, QseC does not appear to be essential for QseB activation, supporting that QseB phosphorylation can occur even in the absence of QseC by an unknown mechanism. Previous studies characterizing two-component signal transduction specificity reported that a response regulator can be phosphorylated in the absence of its cognate histidine kinase via cross-talk with other two-component signal transduction systems (Siryaporn and Goulian, 2008), or orphan sensor kinases (Saini *et al.*, 2004).

However, while cross phosphorylation of a response regulator by a non-cognate sensor is reported to always be less efficient resulting in low-level target gene regulation (Siryaporn and Goulian, 2008), QseB activity in the absence of QseC results in striking phenotypes. We are currently exploring the source of QseB phosphorylation in a *qseC* deletion background.

Downregulation of target gene transcription by an active QseB could arise by direct binding of QseB at promoter sites and repression of transcription, or indirectly by positively regulating a repressor of these genes. Dual repressor/activator function has been shown for other regulators, including PmrA in *Salmonella enterica* (Kato *et al.*, 2003), which is highly homologous to QseB, and LldR in *E. coli* (Aguilera *et al.*, 2008).

The negative effects on expression of type 1 pili, curli or flagella were not detectable when QseC was present. Our phosphotransfer data support that this is due to the ability of QseC to deactivate QseB by dephosphorylation. Thus, whereas the kinase activity of QseC may be bypassed, its phosphatase activity is critical for optimal QseB-mediated target gene expression.

Collectively, our data point to a model for the QseBC regulatory mechanism where phosphorylation of QseB may occur in a QseC-dependent or -independent manner. The latter could be the result of cross-talk with the kinase of other two-component systems, or a phosho-donor molecule present in the cytosol (Fig. 5). In wt *E. coli*, the cognate sensor QseC actively removes the phosphoryl group, thereby modulating QseB activity and allowing for controlled target gene expression. In the absence of QseC, QseB activity remains un-restrained, thus upregulating its own expression and directly or indirectly mediating repression of target genes (see model, Fig. 5).

In summary, our study demonstrates that QseC is a bifunctional sensor whose kinase and phosphatase capacities regulate gene transcription by controlling the phosphorylation state of QseB. We have discovered a new paradigm explaining the mechanism by which QseBC functions to control downstream gene expression. This is the first quorum-sensing system implicated in IBC formation and uropathogenesis. Further studies will characterize the role of QseBC in responding to environmental cues and how this governs IBC formation and/or dispersal. A better understanding of the QseBC mechanism will advance our knowledge on the role of this two-component system on disease development by a number of pathogenic bacteria.

## Experimental procedures

### Strains and constructs

Primers are listed in Table S1. Deletion mutants were created using λ Red Recombinase (Murphy and Campellone, 2003). pQseC and pQseB were created in pTrc99A (Invitrogen), by cloning *qseC* (UTI89_C3451) and *qseB* (UTI89_C3450) downstream of their native promoter. pPqse was created by cloning the *qseBC* promoter in pSSH10-1 (Wright *et al.*, 2005) and subcloning P*qse*::GFP in pBAD33 (Wright *et al.*, 2005). Site-directed mutagenesis was performed on pQseB using *Pfu* Ultra DNA polymerase (Stratagene).

### Motility assays

Motility assays were performed as previously described (Wright *et al.*, 2005). Briefly, bacteria were incubated statically in LB for 18 h. To assess swarming, 5 μl of culture was spotted on 0.6% LB agar containing 0.5% glucose. Swimming was assessed in 0.25% LB agar/0.001% 2,3,5-triphenyltetrazolium chloride. Plates were incubated at 37°C for 18 and 7 h respectively. Motility was evaluated by measuring the motility diameters. Experiment was repeated five times with triplicate plates/strain.

### RNA extraction and RT-PCR

RNA was extracted using the RNeasy kit (Qiagen), DNase-treated and reverse-transcribed with Superscript II Reverse Transcriptase (Invitrogen). For semi-quantitative RT-PCR, 1 μl of the resulting cDNA pool was used with primers specific to *sfaA*, *csgD, flhDC* or *rrsH*. For Q-RT-PCR, serial dilutions of wt UTI89 and UTI89Δ*qseC* cDNA (25, 12.5, 6.25, 3.125 and 1.5625 ng $\mu l^{-1}$) were used with *qseB*- and *rrsH*-specific primers (Table S1) according to the manufacturer's instructions (Bio-Rad). Relative fold difference was determined by the ΔΔCt method (Pfaffl, 2001). Experiment was repeated five times with triplicate reactions/cDNA dilution.

### Immunoblots

For type 1 pili, cells were grown statically in LB for 18 h at 37°C. For curli, cells were harvested from Yeast Extract/Casamino Acids (1 g yeast extract, 10 g casamino acids, 20 g agar $l^{-1}$) plates incubated at 26°C for 48 h. For flagella, cells were harvested from the swarming front on motility plates. Normalized cells ($OD_{600}$ = 1) were processed for SDS-PAGE. Pili samples were treated with 1 M HCl prior to boiling and neutralized with 1 M NaOH prior to electrophoresis. Membranes were probed with type 1 pili (Pinkner *et al.*, 2006), CsgA, CsgG (Robinson *et al.*, 2006), H7 (BD-Difco) or GFP (US Biologicals) antisera (1:3000 dilution). ImageJ software was used for densitometry (http://rsbweb.nih.gov/ij/).

### Phase assays

Phase assays were performed as described by Struve and Krogfelt (1999). ImageJ was used to quantify band intensities from three independent experiments.

### HA assays

HA analyses were performed on normalized cells ($OD_{600}$ = 1) as described previously (Hultgren *et al.*, 1986).

### Mouse infections

Female C3H/HeN mice (National Cancer Institutes), 7–9 weeks old, were transurethrally infected with $10^7$ bacteria carrying the GFP-expressing plasmid pCom-GFP (Cormack *et al.*, 1996), according to Hannan *et al.* (2008). IBC enumeration was carried out by confocal microscopy (see below) and by lacZ staining (Justice *et al.*, 2006). Experiments were repeated three times.

### Microscopy

Electron and confocal microscopy samples were treated as previously described (Wright *et al.*, 2005) with the following minor modifications for confocal microscopy: bladders were bisected, splayed and fixed in 3% paraformaldehyde for 1 h. Fixed bladders were washed and counterstained for 20 min with the nuclear ToPro3 (Molecular Probes) stain, and r-WGA to outline the facet cells (1:700 and 1:500 dilution respectively). Images were obtained using Zeiss LSM 510 Meta Laser Scanning inverted confocal microscope (Thornwood). For fluorescence microscopy, bacteria were grown to $OD_{600}$ = 1, and visualized using a Zeiss fluorescence microscope (Thornwood).

### QseB purification

The *qseB* gene was cloned in pBADmyc-HisA (Invitrogen) and expressed using 0.1% arabinose. QseB was affinity-purified using a Talon column (Clontech), followed by anion exchange chromatography through a MonoQ column (GE Healthcare).

### Preparation of QseC-enriched membranes

The *qseC* gene was cloned in pBADmyc-HisA and induced with 0.02% arabinose, from UTI89Δ*qseC* at $OD_{600}$ = 0.6. Cells were broken by French Press (1000 psi). Total membranes were isolated by ultracentrifugation at 15 000 r.p.m. for 1 h, resuspended in 20 mM Tris pH 8.0/1 mM $MgCl_2$. Membranes from UTI89Δ*qseC*/pBADmyc-HisA were extracted under identical conditions.

### Autokinase and phosphotransfer assays

Membranes from UTI89Δ*qseC*/pQseCmyc-His or UTI89Δ*qseC*/pBADmyc-HisA (7 μg) were incubated in the absence or presence of purified QseB (14 μg) with 0.7 μCi [$\gamma$-$^{32}$P]-ATP, in 1× TBS/0.5 mM DTT/0.5 mM $MgCl_2$ per reaction. A 10-reaction mastermix was prepared and 10 μl aliquots were removed at different time points, mixed with SDS loading buffer and kept on ice until SDS-PAGE. Gels were dried, exposed to Phosphor Imaging plates and developed using the BAS-5000 scanner (Fujifilm).

### *In vitro* phosphorylation of QseB and phosphatase assays

Phosphorylated $PmrB_c$ beads were prepared as previously described (Kato and Groisman, 2004), and used to *in vitro* phosphorylate QseB according to Kato and Groisman (2004). QseB~P (0.2 nmol, equal to 9000 cpm) was incubated at room temperature with 7 μg of membrane vesicles in 1× TBS/0.5 mM DTT/0.5 mM $MgCl_2$. Aliquots (10 μl) were withdrawn from the mastermix reaction and treated as described above.

### Statistical analyses

Statistical analyses were performed using the Student's *t*-test (two-tailed). A *P*-value < 0.05 was considered significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

Abraham JM, Freitag CS, Clements JR, Eisenstein BI. An invertible element of DNA controls phase variation of type 1 fimbriae of *Escherichia coli*. Proc Natl Acad Sci USA. 1985; 82:5724–5727. [PubMed: 2863818]

Aguilera L, Campos E, Gimenez R, Badia J, Aguilar J, Baldoma L. Dual role of LldR in regulation of the lldPRD operon, involved in 1-lactate metabolism in *Escherichia coli*. J Bacteriol. 2008; 190:2997–3005. [PubMed: 18263722]

Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ. Intracellular bacterial biofilm-like pods in urinary tract infections. Science. 2003; 301:105–107. [PubMed: 12843396]

Bang IS, Kim BH, Foster JW, Park YK. OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar typhimurium. J Bacteriol. 2000; 182:2245–2252. [PubMed: 10735868]

Barnhart MM, Chapman MR. Curli biogenesis and function. Annu Rev Microbiol. 2006; 60:131–147. [PubMed: 16704339]

Bearson BL, Bearson SM. The role of the QseC quorum-sensing sensor kinase in colonization and norepinephrine-enhanced motility of *Salmonella enterica* serovar Typhimurium. Microb Pathog. 2008; 44:271–278. [PubMed: 17997077]

Beier D, Gross R. Regulation of bacterial virulence by two-component systems. Curr Opin Microbiol. 2006; 9:143–152. [PubMed: 16481212]

Chen SL, Hung CS, Xu J, Reigstad CS, Magrini V, Sabo A, et al. Identification of genes subject to positive selection in uropathogenic strains of *Escherichia coli*: a comparative genomics approach. Proc Natl Acad Sci USA. 2006; 103:5977–5982. [PubMed: 16585510]

Clarke MB, Sperandio V. Transcriptional auto-regulation by quorum sensing *Escherichia coli* regulators B and C (QseBC) in enterohaemorrhagic *E. coli* (EHEC). Mol Microbiol. 2005a; 58:441–455. [PubMed: 16194231]

Clarke MB, Sperandio V. Transcriptional regulation of flhDC by QseBC and sigma (FliA) in enterohaemorrhagic *Escherichia coli*. Mol Microbiol. 2005b; 57:1734–1749. [PubMed: 16135237]

Clarke MB, Hughes DT, Zhu C, Boedeker EC, Sperandio V. The QseC sensor kinase: a bacterial adrenergic receptor. Proc Natl Acad Sci USA. 2006; 103:10420–10425. [PubMed: 16803956]

Cormack BP, Valdivia RH, Falkow S. FACS-optimized mutants of the green fluorescent protein (GFP). Gene. 1996; 173:33–38. [PubMed: 8707053]

Cotter PA, Jones AM. Phosphorelay control of virulence gene expression in Bordetella. Trends Microbiol. 2003; 11:367–373. [PubMed: 12915094]

Foxman B. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Am J Med. 2002; 113 (Suppl 1A):5S–13S. [PubMed: 12113866]

Freeman JA, Bassler BL. A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. Mol Microbiol. 1999; 31:665–677. [PubMed: 10027982]

Gonzalez Barrios AF, Zuo R, Hashimoto Y, Yang L, Bentley WE, Wood TK. Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). J Bacteriol. 2006; 188:305–316. [PubMed: 16352847]

Groisman EA. The pleiotropic two-component regulatory system PhoP-PhoQ. J Bacteriol. 2001; 183:1835–1842. [PubMed: 11222580]

Hammar M, Bian Z, Normark S. Nucleator-dependent intercellular assembly of adhesive curli organelles in *Escherichia coli*. Proc Natl Acad Sci USA. 1996; 93:6562–6566. [PubMed: 8692856]

Hannan TJ, Mysorekar IU, Chen SL, Walker JN, Jones JM, Pinkner JS, et al. LeuX tRNA-dependent and – independent mechanisms of *Escherichia coli* pathogenesis in acute cystitis. Mol Microbiol. 2008; 67:116–128. [PubMed: 18036139]

Hoch JA. Two-component and phosphorelay signal transduction. Curr Opin Microbiol. 2000; 3:165–170. [PubMed: 10745001]

Hultgren SJ, Schwan WR, Schaeffer AJ, Duncan JL. Regulation of production of type 1 pili among urinary tract isolates of *Escherichia coli*. Infect Immun. 1986; 54:613–620. [PubMed: 2877947]

Justice SS, Hung C, Theriot JA, Fletcher DA, Anderson GG, Footer MJ, Hultgren SJ. Differentiation and developmental pathways of uropathogenic *Escherichia coli* in urinary tract pathogenesis. Proc Natl Acad Sci USA. 2004; 101:1333–1338. [PubMed: 14739341]

Justice SS, Lauer SR, Hultgren SJ, Hunstad DA. Maturation of intracellular *Escherichia coli* communities requires SurA. Infect Immun. 2006; 74:4793–4800. [PubMed: 16861667]

Kato A, Groisman EA. Connecting two-component regulatory systems by a protein that protects a response regulator from dephosphorylation by its cognate sensor. Genes Dev. 2004; 18:2302–2313. [PubMed: 15371344]

Kato A, Latifi T, Groisman EA. Closing the loop: the PmrA/PmrB two-component system negatively controls expression of its posttranscriptional activator PmrD. Proc Natl Acad Sci USA. 2003; 100:4706–4711. [PubMed: 12676988]

Lan CY, Igo MM. Differential expression of the OmpF and OmpC porin proteins in *Escherichia coli* K-12 depends upon the level of active OmpR. J Bacteriol. 1998; 180:171–174. [PubMed: 9422609]

Mulvey MA, Lopez-Boado YS, Wilson CL, Roth R, Parks WC, Heuser J, Hultgren SJ. Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. Science. 1998; 282:1494–1497. [PubMed: 9822381]

Mulvey MA, Schilling JD, Hultgren SJ. Establishment of a persistent *Escherichia coli* reservoir during the acute phase of a bladder infection. Infect Immun. 2001; 69:4572–4579. [PubMed: 11402001]

Murphy KC, Campellone KG. Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic *E. coli*. BMC Mol Biol. 2003; 4:11. [PubMed: 14672541]

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29:e45. [PubMed: 11328886]

Pinkner JS, Remaut H, Buelens F, Miller E, Aberg V, Pemberton N, et al. Rationally designed small compounds inhibit pilus biogenesis in uropathogenic bacteria. Proc Natl Acad Sci USA. 2006; 103:17897–17902. [PubMed: 17098869]

Rasko DA, Moreira CG, Li de R, Reading NC, Ritchie JM, Waldor MK, et al. Targeting QseC signaling and virulence for antibiotic development. Science. 2008; 321:1078–1080. [PubMed: 18719281]

Robinson LS, Ashman EM, Hultgren SJ, Chapman MR. Secretion of curli fibre subunits is mediated by the outer membrane-localized CsgG protein. Mol Microbiol. 2006; 59:870–881. [PubMed: 16420357]

Saini DK, Malhotra V, Tyagi JS. Cross talk between DevS sensor kinase homologue Rv2027c, and DevR response regulator of Mycobacterium tuberculosis. FEBS Lett. 2004; 565:75–80. [PubMed: 15135056]

Siryaporn A, Goulian M. Cross-talk suppression between the CpxA-CpxR and EnvZ-OmpR two-component systems in *E. coli*. Mol Microbiol. 2008; 70:494–506. [PubMed: 18761686]

Song J, Bishop BL, Li G, Duncan MJ, Abraham SN. TLR4-initiated and cAMP-mediated abrogation of bacterial invasion of the bladder. Cell Host Microbe. 2007; 1:287–298. [PubMed: 17710226]

Sperandio V, Torres AG, Kaper JB. Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. Mol Microbiol. 2002; 43:809–821. [PubMed: 11929534]

Stock AM, Robinson VL, Goudreau PN. Two-component signal transduction. Annu Rev Biochem. 2000; 69:183–215. [PubMed: 10966457]

Struve C, Krogfelt KA. In vivo detection of *Escherichia coli* type 1 fimbrial expression and phase variation during experimental urinary tract infection. Microbiology. 1999; 145 (Part 10):2683–2690. [PubMed: 10537190]

Weiss DS, Brotcke A, Henry T, Margolis JJ, Chan K, Monack DM. In vivo negative selection screen identifies genes required for Francisella virulence. Proc Natl Acad Sci USA. 2007; 104:6037–6042. [PubMed: 17389372]

Wright KJ, Seed PC, Hultgren SJ. Uropathogenic *Escherichia coli* flagella aid in efficient urinary tract colonization. Infect Immun. 2005; 73:7657–7668. [PubMed: 16239570]

Wright KJ, Seed PC, Hultgren SJ. Development of intracellular bacterial communities of uropathogenic *Escherichia coli* depends on type 1 pili. Cell Microbiol. 2007; 9:2230–2241. [PubMed: 17490405]

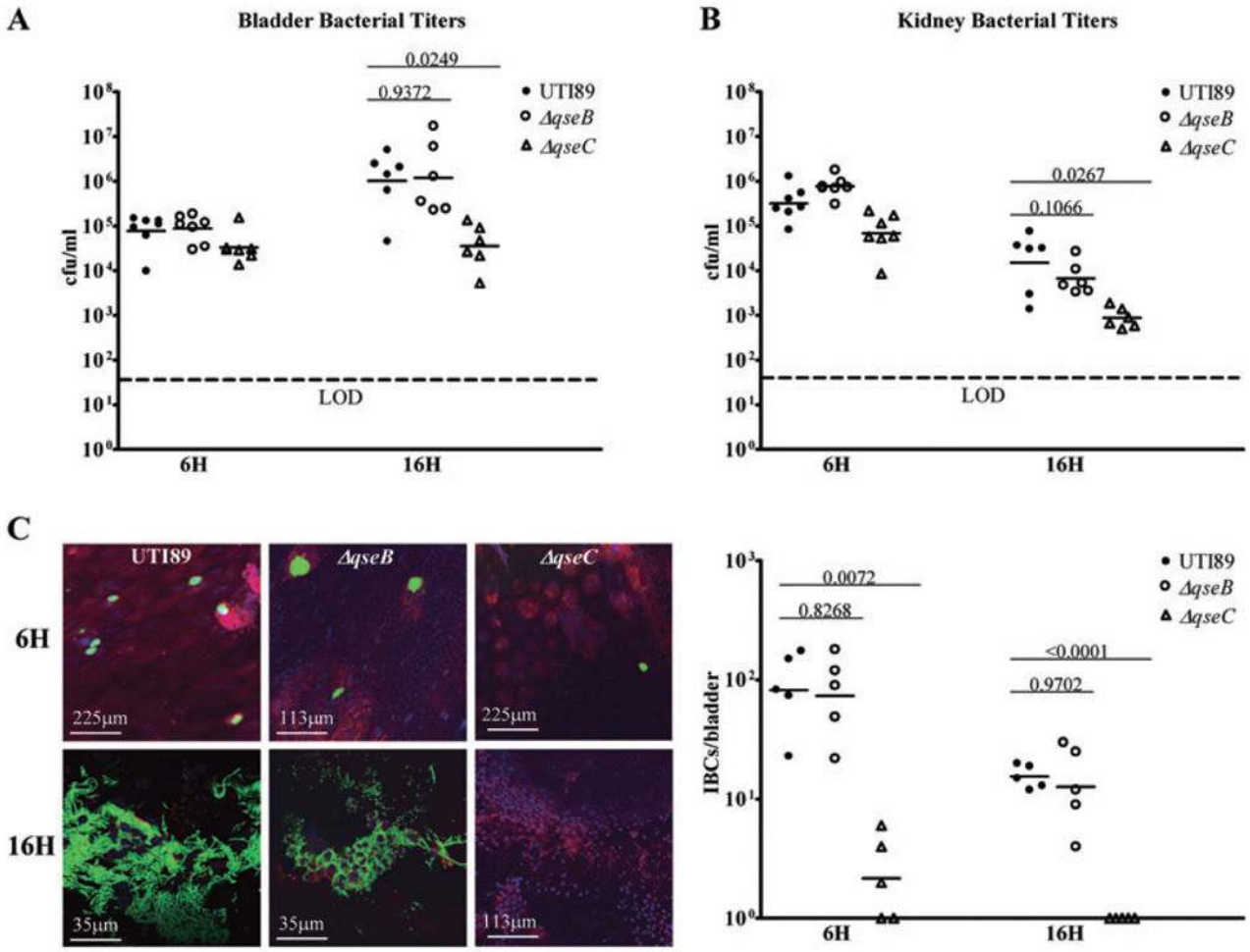


**Fig. 1.**

UTI89Δ*qseC* is attenuated *in vivo* while UTI89Δ*qseB* exhibits wt virulence.

A and B. Graphs showing the reduced bladder and kidney UTI89Δ*qseC* cfu at 16 h.p.i. compared with UTI89 ($P = 0.0249$ and $P = 0.0267$ respectively). UTI89Δ*qseB* titers are similar to UTI89 ($P > 0.1$). A representative of three independent experiments is shown. LOD, limit of detection.

C. IBC production by UTI89 and the *qse* mutants. Confocal microscopy images of whole mounted bladders depicting IBCs and bacterial filaments in green, nuclei in blue and bladder facet cells in red. The graph on the right depicts the number of IBCs scored per mouse bladder infected with wt UTI89 or each of the *qse* mutants. IBCs are significantly reduced at 6 and 16 h.p.i ($P = 0.0072$ and $P < 0.0001$ respectively) in UTI89Δ*qseC*. IBC formation is unaffected in UTI89Δ*qseB*.

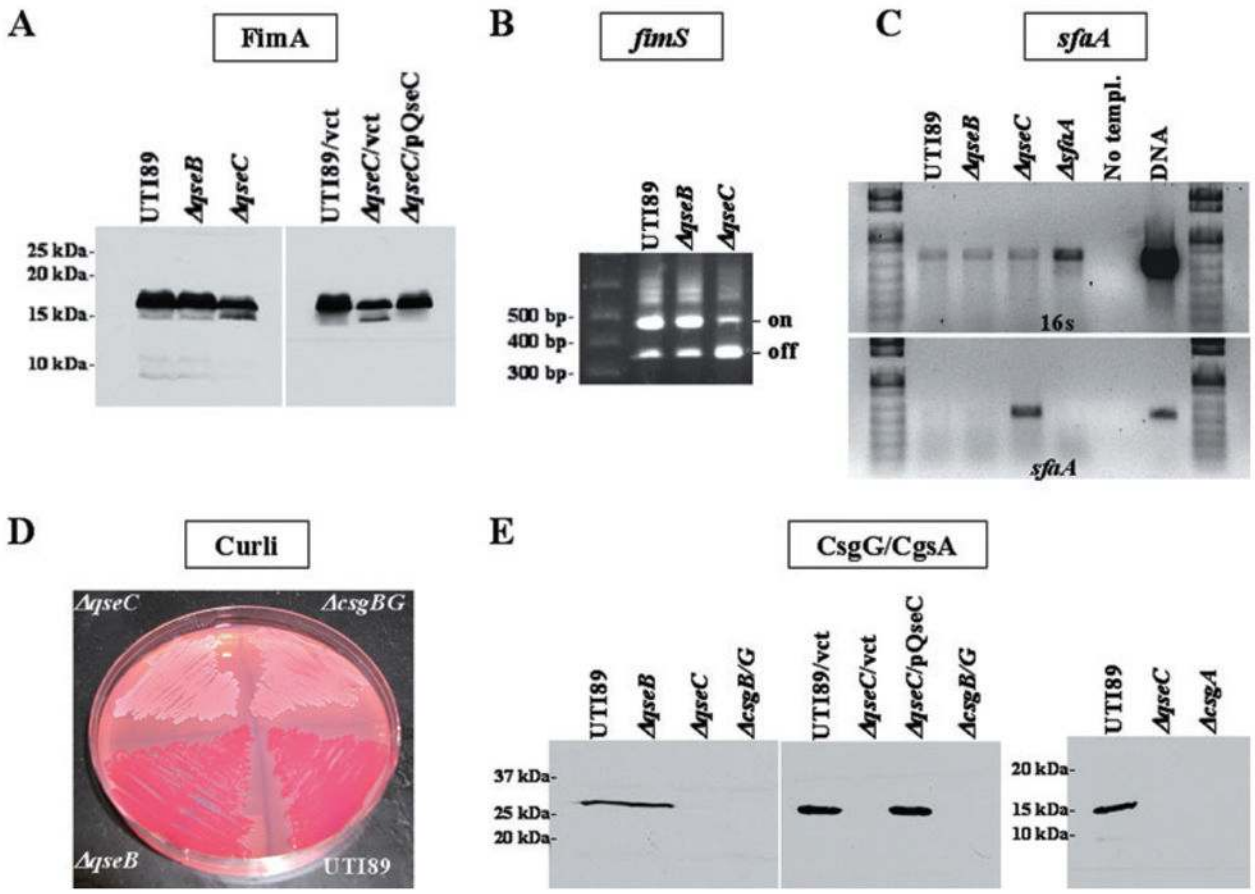


**Fig. 2.**

Deletion of *qseC* but not *qseB* affects type 1 pili, S pili and curli expression.

A. Whole-cell immunoblot showing a 56.2% reduction of FimA expression in UTI89Δ*qseC* compared with UTI89 and UTI89Δ*qseB*. FimA levels are restored in UTI89Δ*qseC*/pQseC.

B. Phase assay showing that *fimS* is primarily in the OFF orientation in the absence of QseC (OFF and ON band intensities equal to 161% and 50% of wt UTI89 respectively).

C. Semi-quantitative RT-PCR demonstrating elevated *sfaA* levels in UTI89Δ*qseC* but not in UTI89Δ*qseB*.

D. CR binding by UTI89 and the *qse* mutants, indicating that only *qseC* deletion abolishes curli production.

E. Immunoblot analyses showing the divergence of UTI89Δ*qseB* and UTI89Δ*qseC* on CsgA and CsgG production. All experiments were repeated three times. Densitometry in A and B was performed by ImageJ.

vct, empty vector control; No templ., no template control.

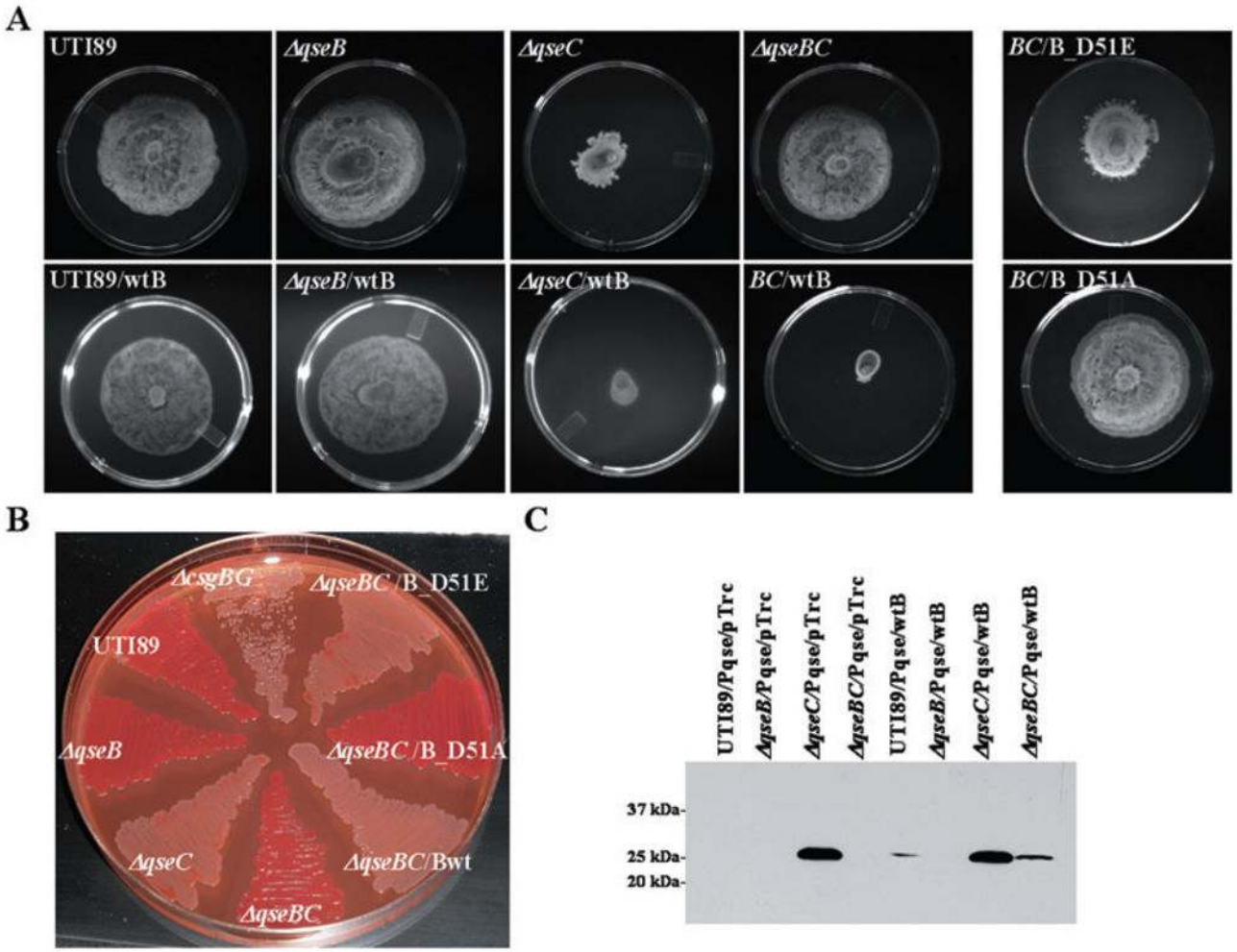


**Fig. 3.**

QseB activity is responsible for the UTI89Δ*qseC* defects.

A and B. Effects of *qseBC* deletion and QseB expression on motility and curli production. UTI89Δ*qseBC* exhibits wt phenotypes. Expression of wt QseB and QseB_D51E in the absence of QseC abolishes motility and curli production. Experiments shown are representative of three, with triplicate samples per strain.

C. Immunoblot analysis indicating *qseBC* promoter-driven GFP expression in UTI89 and the *qse* mutants in the absence or presence of wt QseB. Pqse, plasmid pPqse carrying the P*qse*::GFP transcriptional fusion; wtB, plasmid pQseB carrying *qseB* under its native promoter; pTrc, pTrc99A vector control.

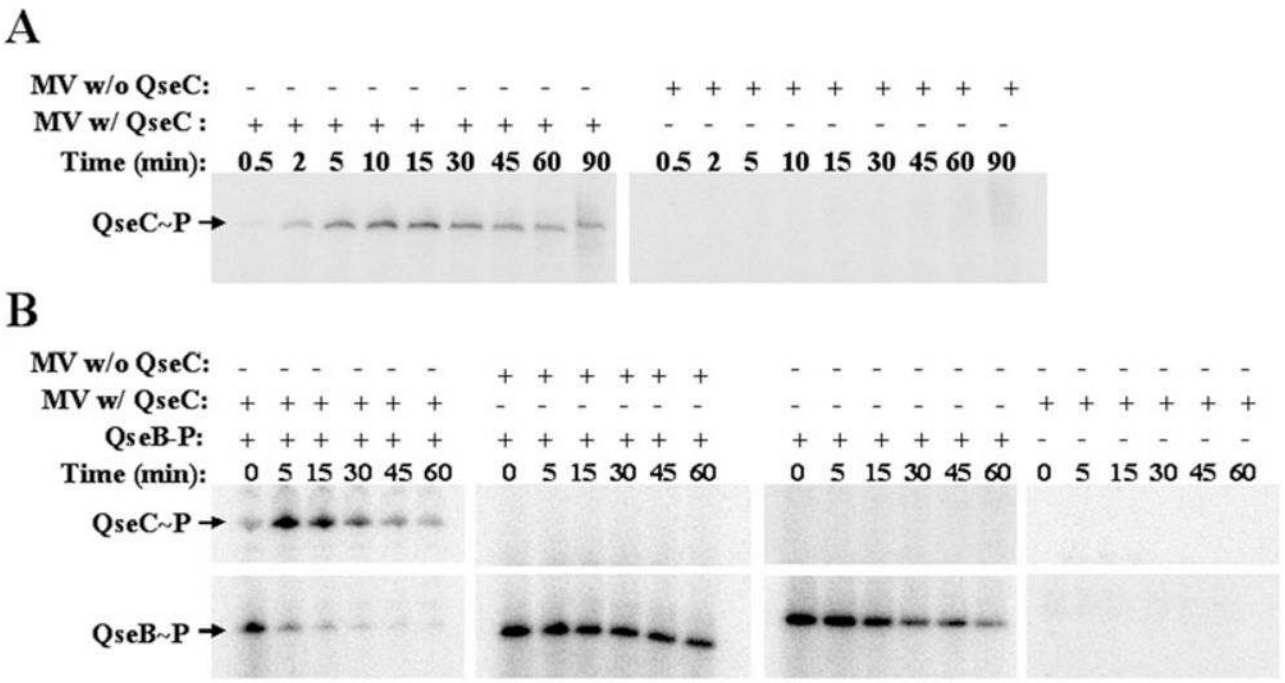


**Fig. 4.**

QseC exhibits phosphatase activity towards QseB.

A. Autokinase assay showing QseC phosphorylation in membrane vesicles (MV).

B. Phosphatase assay indicating that *in vitro* phosphorylated QseB is rapidly dephosphorylated in the presence of QseC. The last panel depicts a mock phosphatase assay using buffer treated in the same manner as QseB during *in vitro* phosphorylation to verify that the observed QseC re-phosphorylation is due to reverse phosphotransfer. Results shown are representative of three independent experiments.

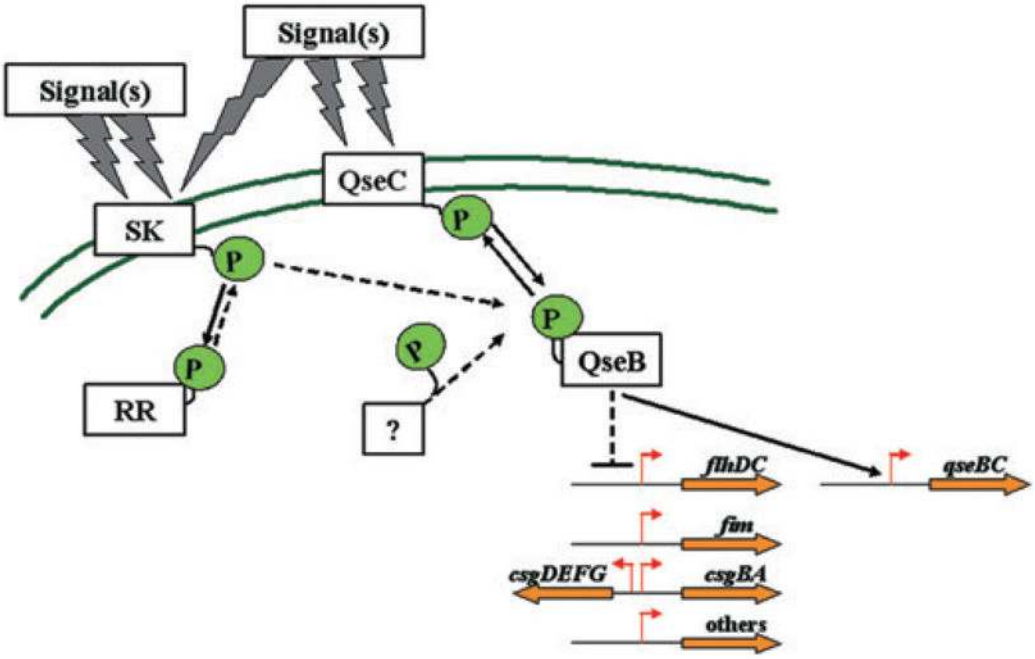


**Fig. 5.**

Model depicting the QseBC interplay and the effects on downstream gene regulation. Phosphorylation of QseB can occur in a QseC-dependent or independent manner (the latter possibly favoured upon deletion of QseC), leading to direct upregulation of *qseB* but downregulation of type 1 pili, curli and flagella genes in a direct or indirect manner. SK, sensor kinase; RR, response regulator; ?, phosphodonor molecule; P, phosphoryl group; solid lines denote direct interactions, dashed lines denote interactions that may be direct or indirect, red arrows indicate transcriptional start sites and yellow arrows indicate operons.