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Recommended Citation
Kostakioti, Maria; Hadjifrangiskou, Maria; Cusumano, Corinne K.; Hannan, Thomas J.; Janetka, James W.; and Hultgren, Scott J., "Distinguishing the contribution of type 1 pili from that of other QseB-misregulated factors when QseC is absent during urinary tract infection." Infection and Immunity.80,8. 2826-2834. (2012).
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Distinguishing the Contribution of Type 1 Pili from That of Other QseB-Misregulated Factors when QseC Is Absent during Urinary Tract Infection

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Urinary tract infections (UTI), primarily caused by uropathogenic Escherichia coli (UPEC), are one of the leading bacterial infections due to their high frequency and rate of recurrence. Both type 1 pilus adhesive organelles (fim) and the QseC sensor kinase have been implicated in UPEC virulence during UTI and have been individually reported to be promising drug targets. Deletion of qseC leads to pleiotropic effects due to unregulated activation of the cognate response regulator QseB, influencing conserved metabolic processes and diminishing expression of virulence genes, including type 1 pili. Here, we discern the type 1 pilus-dependent and -independent effects that contribute to the virulence attenuation of a UPEC qseC deletion mutant in a murine model of experimental UTI. We show that although a ΔqseC mutant restored for type 1 pilus expression regains the ability to colonize the host and initiate acute infection up to 16 h postinfection, it is rapidly outcompeted during acute infection when coinoculated with a wild-type strain. As a result, this strain has a diminished capacity to establish chronic infection. A prophylactic oral dose of a FimH small-molecular-weight antagonist (ZFH-02056) further reduced the ability of the qseC mutant to establish chronic infection. Thus, loss of QseC significantly enhances the efficacy of ZFH-02056. Collectively, our work indicates that type 1 pili and QseC become critical in different infection stages, and that dual targeting of these factors has an additive effect on abating UPEC virulence.
The IBC pathogenic cascade, which has been characterized in a murine model of infection (2, 27, 29) and has been extensively documented in samples from human clinical studies (17, 42), is FimH dependent (52). It has been shown that the fimH gene is under positive selection in human clinical isolates of UPEC (6), further supporting its role in human disease. Thus, it has been reported that type 1 pilin fulfill the molecular Koch’s postulates of microbial pathogenesis (44). Type 1 pilin are encoded by the fim gene cluster, and their expression is directed by a phase-variable promoter (fimS), which facilitates a switch between piliated and nonpiliated bacterial states (30). UPEC populations are typically heterogeneous in terms of pilus expression, consisting of bacteria that are observed by transmission electron microscopy to be bald or lightly, moderately, or highly piliated (40). The ratio of each piliated fraction shifts depending on the environmental niche. Studies investigating the expression of type 1 pilin revealed that UPEC associated with epithelial cells are highly piliated, consistent with the critical role of type 1 pilin in colonization of the urothelium (26, 33, 54, 55). In contrast, the fimS promoter in bacteria recovered from urine samples of patients has been shown to be predominantly in the OFF phase (32). Regulation of fimS phase variation is controlled by the FimB, FimE, and FimX recombinases, the expression of which is influenced by numerous regulatory factors (16, 20, 30). We recently identified the QseBC two-component system as one of the factors implicated in the regulation of several CUPs in UPEC, including type 1 pilin (19, 31). In a UPEC mutant lacking the sensor kinase QseC, the fim promoter is found primarily in the OFF orientation, resulting in reduced type 1 pilus expression, an effect that stems from uncontrolled activation of the QseB response regulator in this mutant (31). In addition to influencing type 1 pilus expression in the absence of qseC, overactive QseB (31) dysregulates conserved cellular pathways and impacts several other virulence-associated genes, resulting in UPEC attenuation (19, 31).

While the effects of disruption of type 1 pilus production on UPEC pathogenesis are fairly well understood (6, 8, 24, 52) and likely explain at least some of the attenuation of the ΔqseC mutant, the contributions of QseB-regulated factors in the attenuation of ΔqseC, independent of type 1 pilin, are unknown. Therefore, in this study we disengaged type 1 pilin from QseBC control to determine the role of other QseB-regulated factors in acute and chronic stages of bladder infection. By locking the invertible fim promoter element in the ON orientation, we restored type 1 pilus expression in the ΔqseC mutant. The resulting strain, UTI89ΔqseC_LON (LON indicates locked in the ON orientation), was rescued in its ability to cause acute UTI but was rapidly outcompeted by wild-type (wt) UTI89 during acute competitive infection studies and was less efficient at causing chronic cystitis. Thus, despite expression of type 1 pilin, the QseB-regulated processes in the ΔqseC mutant decrease bacterial fitness for acute infection and reduce the ability of UPEC to develop chronic cystitis. We then used small-molecular-weight FimH inhibitors, also known as mannosides, as molecular scalps to show that transiently blocking FimH function during infection, by oral prophylactic administration, further impeded the ability of ΔqseC to cause chronic cystitis compared to ΔqseC alone. Collectively, our studies extend our understanding of the basis of ΔqseC attenuation, distinguishing the effect of type 1 pilin from that of other QseB-regulated factors during the course of infection, and support that simultaneously targeting QseC and type 1 pilin represents a promising avenue for potentiating therapeutics against UPEC.

**MATERIALS AND METHODS**

**Strains, constructs, and growth conditions.** UTI89 is a human cystitis isolate that is highly virulent in a mouse model of UTI (36). UTI89_LON and UTI89ΔqseC_LON were created using a Red recombination and primers LON_L (TAAAAAGGAAGAAGGTTATGATTATTAATTTGATAT AAGTTAAAAAGGTTAGCGGCTGTCGTTCTC) and LON_R (CGATGGTTCTCTCTGATGAGTCAAAAGAGATCTAATTGTCTTGT ATTATTTGGATATATATCTCCCTTATG) so as to mutate out of 9 nucleotides in the left invertible repeat (LIR) of the fim promoter in the chromosome (37) (the wt LIR sequence 5’-TGGCCCAAAA-3’ was mutated to 5’-GAGATCTAA-3’ and is underlined in the sequences above). Chromosomally marked LON strains were constructed as shown in the Fig. 2A schematic but without inducing excision of the Kan cassette (the last step of the schematic). Similarly, chromosomally marked UTI89 and UTI89ΔqseC strains were constructed with the Kan resistance cassette in the α-attor qseC locus, respectively. All strains have previously been tested and behave identically to their nonmarked counterparts. Bacteria were incubated in Luria-Bertani (LB) medium at 37°C for 4 h under shaking conditions, subcultured (1:1000) in fresh LB medium, and incubated statically at 37°C for 18 h.

**Mouse infections.** All procedures involving mice were performed in compliance with current federal guidelines and institutional policies at Washington University in Saint Louis and ensured the proper and humane treatment of animals. Female C3H/HeN mice (Harlan), 7 to 9 weeks old, were used for all studies described below, and in each case mice were infected with 10⁷ bacteria.

**Short-term infections.** Mice were transurethrally infected with bacteria carrying the green fluorescent protein (GFP)-expressing plasmid pANT4 (9) as previously described (31), and they were sacrificed at 1 and 3 h postinfection (hpi). Bladders were aseptically removed, homogenized, and plated for total bacterial enumeration or gentamicin treated to determine intracellular bacterial titers. The experiment was repeated 3 times, and statistical analysis was performed using two-tailed Mann-Whitney tests (P < 0.05 was considered significant).

**Ex vivo gentamicin assay.** Bladders were bisected and washed 3 times in 500 μl phosphate-buffered saline (PBS). The washes were collected and plated for CFU enumeration to determine luminal bacteria. Washed bladders were incubated for 90 min at 37°C with 100 μg/ml gentamicin to kill adherent extracellular bacteria. Following washes (3×) with PBS, bladders were homogenized in 1 ml PBS and plated to determine intracellular bacterial titers. The two-tailed Mann-Whitney test (P < 0.05) was used for statistical analysis.

**Acute infection studies.** Mice were transurethrally infected with 10⁷ bacteria carrying the plasmid pANT4 as previously described (31). Experiments were repeated three times and statistically analyzed using the two-tailed Mann-Whitney test (P < 0.05 was considered significant).

**Long-term infection studies.** Mice were infected with chromosomally marked strains of wt UTI89, UTI89_LON, UTI89ΔqseC, or UTI89ΔqseC_LON. Mice were sacrificed at 2 weeks postinfection (wpi), and organs were processed for CFU enumeration on LB-kanamycin agar plates. The experiment was repeated 2 times. Cumulative data from all experiments are presented.

**Competition assays.** Cohorts of 10 mice were used per time point. Bacterial inocula were prepared such that they comprised 50% UTI89_LON and 50% UTI89ΔqseC_LON (Kan⁺) in a total of 3×10⁶ to 4×10⁷ bacteria per 50 μl, and the inocula were transurethrally introduced in each mouse. Mice were sacrificed at 6, 16, 30, 48, and 72 hpi, and organs were processed for CFU enumeration on LB and LB-Kan agar plates. To account for possible variation due to antibiotic resistance, experiments were repeated using UTI89_LON (Kan⁺) and UTI89ΔqseC_LON. The experiment was repeated 3 times. Cumulative
data from all experiments are presented. Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test.

Mannoside studies. One hundred µl of a 10 mg/ml (50 mg/kg of body weight) solution of ZFH-02056 [IUPAC name: N1,N3-dimethyl-5-[4-[(2R,3S,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]oxyphenyl]benzene-1,3-dicarboxamide] was administered to each mouse by oral gavage 30 min prior to bacterial inoculation. Control mice were gavaged with 100 µl of PBS 30 min prior to bacterial inoculation. Mice were euthanized at 2 and 4 wpi, and organs were processed for CFU enumeration.

IBC enumeration by confocal microscopy. For IBC enumeration, mice were infected with fluorescent bacteria carrying the GFP-plasmid pANT4 as described above for acute infections. Bladders excised from infected animals were treated for confocal microscopy as previously described (53). Briefly, bladders were bisected, splayed, and fixed in 4% paraformaldehyde overnight at 4°C. Fixed bladders were washed and counterstained for 20 min with the nuclear ToPro3 (Molecular Probes) stain and rhodamine-conjugated wheat germ agglutinin to outline the facet cells (1:700 dilution for each). For IBC enumeration, the entire bladder was scanned using a Zeiss LSM 510 Meta laser scanning inverted confocal microscope (Thornwood).

Immunoblotting, HA, and phase assays. Bacteria were grown statically in LB for 18 h at 37°C. Immunoblotting (using anti-type 1 pilus antibody), hemagglutination (HA), and phase assays were performed on normalized cells (optical density at 600 nm [OD₆₀₀] of 1) as previously described (25, 31).

qPCR analyses. RNA extraction, DNase treatment, and reverse transcription were performed using reagents and methods as reported by Kostakioti et al. (31). Relative transcript abundance was determined by quantitative PCR (qPCR) as previously described (31) using aceB-specific (19) or qseB-specific (31) primers.

RESULTS

Deletion of QseC impairs the ability of UPEC to colonize and invade the bladder. We have previously shown that deletion of qseC in UTI89 leads to reduced bladder bacterial titers during the acute stages of infection (6 and 16 h postinfection) in a cystitis murine model (31). Given that type 1 pili are critical for bladder adherence and invasion (33, 35) and are significantly reduced in the absence of QseC, we rationalized that the in vivo attenuation of UTI89ΔqseC could be, at least partly, attributed to reduced type 1 pilus-mediated colonization and invasion of the bladder. Thus, we assessed the ability of UTI89ΔqseC to adhere to and invade the bladder epithelium compared to the parent strain. Female C3H/HeN mice were transurethrally inoculated with 10⁷ wild-type (wt) UTI89 or UTI89ΔqseC, and bacterial colonization and invasion were assessed at 1 and 3 h postinfection (hpi), time points previously shown to be suitable for studying the invasion events in vivo (29, 33, 35). Enumeration of CFU recovered from infected bladders indicated that compared to wt UTI89, UTI89ΔqseC had significantly lower overall bacterial titers (which include both intracellular and luminal populations) at both time points (Fig. 1A).

Treatment of infected bladders with gentamicin to eradicate the extracellular, adherent bacteria (luminal population) while leaving the intracellular population unharmed revealed decreased intracellular numbers for UTI89ΔqseC (Fig. 1B). Despite the observed reduced internalization of UTI89ΔqseC, the number of luminal UTI89ΔqseC bacteria was not higher but rather was comparable to wt UTI89 luminal populations (Fig. 1B); given the reduced expression of type 1 pili by UTI89ΔqseC, these data suggest that reduced adherence of UTI89ΔqseC leads to faster clearance by micturition. Thus, our data indicate that deletion of qseC affects the ability of UTI89 to initiate infection in part by compromising its ability to colonize and invade the host bladder.

Expression of type 1 pili rescues the adherence properties of UTI89ΔqseC in vitro but does not influence other ΔqseC-related defects. Our findings demonstrate that attenuation of UTI89ΔqseC is linked to reduced bacterial internalization due to decreased type 1 pilus production. We therefore investigated whether restoration of type 1 pilus expression alone would be sufficient to overcome the in vivo defects of UTI89ΔqseC. We have previously reported that in the absence of QseC, the phase-variable fim promoter driving type 1 pilus expression, fimS, is primarily switched to the OFF orientation (31). We thus locked fimS in the ON orientation in the chromosome of wt UTI89 and UTI89ΔqseC to attain expression of type 1 pili independently of QseC (Fig. 2A). UTI89_LON and UTI89ΔqseC_LON exhibited comparable mannose-sensitive hemagglutination (HA) properties, which were higher than that of the wt UTI89 because the inversion of the promoter to the OFF state can no longer occur (Fig. 2B). Consistent with this, Western blot analyses probing for FimA, the major type 1 pilus subunit, verified that the level of type 1 pilus production in UTI89ΔqseC_LON is comparable to that of UTI89_LON, but again it is higher than that of wt UTI89 (Fig. 2C). Electron microscopy revealed that the resulting strains, UTI89_LON and UTI89ΔqseC_LON, were not hyperpililated,
FIG 2 Restoring production of type 1 pili in UTI89ΔqseC does not influence other ΔqseC-mediated defects. (A) Schematic showing the strategy used to lock the phase-variable fim promoter in the ON orientation. LIR, left invertible repeat within the fim promoter; RIR, right invertible repeat within the fim promoter; LIR*, mutated left invertible repeat of the fim promoter. (B and C) Hemagglutination assays and Western blot analyses depicting restoration of type 1 pilus expression in UTI89ΔqseC locked-ON strain (ΔqseC_LON). (D) Locking the fim promoter ON does not interfere with the QseB-mediated dysregulation in the qseC deletion mutant. Relative fold change of qseB and aceB in UTI89_LON and UTI89ΔqseC_LON compared to UTI89 and UTI89ΔqseC, measured by qRT-PCR. Values are normalized to the 16S rrsH gene. (E) Assessment of curli production on YESCA-CR agar verifies that, similar to UTI89ΔqseC, UTI89ΔqseC_LON remains defective for curli expression, exhibiting a white and smooth morphotype. Graphs depict averages of three independent experiments; error bars denote standard errors of the means. Statistical analysis was performed by unpaired two-tailed Student’s t test (*, P < 0.05; **, P < 0.0099; ***, P < 0.0001).
rather a larger population of piliated versus nonpiliated bacteria was observed compared to wt UTI89 (data not shown).

In addition to defects in type 1 pilus expression, deletion of qseC results in perturbation of central cellular processes due to aberrant upregulation and activation of the QseB response regulator (19, 31). This prompted us to verify that restoration of type 1 pilus expression does not affect the QseB-mediated dysregulation. We first performed quantitative reverse transcription-PCR (qRT-PCR) to probe the expression of qseB in the fim-locked-ON (LON) strains compared to wt UTI89 and UTI89ΔqseC. We found that the LON strains behaved similarly to their non-LON counterparts, with UTI89ΔqseC and UTI89ΔqseC_LON exhibiting increased qseB levels compared to both wt UTI89 and UTI89_LON (Fig. 2D). These data suggest that the massive pleiotropy imparted by QseB-mediated misregulation in the absence of QseC remains unaffected upon restoration of type 1 pilus expression. To test this, we examined the expression of AceB, a metabolic factor, and curli, a bacterial amyloid, both known to be misregulated in the absence of QseC (19). We confirmed by qRT-PCR that aceB, which is upregulated in UTI89ΔqseC (19), displays elevated transcript levels in UTI89ΔqseC_LON compared to both UTI89 and UTI89_LON (Fig. 2D), while curli expression was abolished, which is the previously reported defect of UTI89ΔqseC (Fig. 2E). Curli formation can be assessed by growth on yeast extract/Casamino Acids agar supplemented with the Congo red dye (YESCA-CR), since curli bind CR, giving rise to red, dry, and rough colonies (3). When grown under curli-inducing conditions, UTI89ΔqseC_LON had a white and smooth morphotype on YESCA-CR medium similar to UTI89ΔqseC and in contrast to the red, dry, and rough curli-positive phenotype of UTI89 and UTI89_LON (Fig. 2E). As expected, based on these results, production of the CsgG outer membrane pore, critical for secretion of the curli components, was diminished in UTI89ΔqseC_LON but remained unaffected in UTI89_LON (data not shown). These findings show that locking the fim promoter in the ON orientation does not interfere with qseB overexpression and the associated downstream defects imparted by the qseC deletion.

UTI89ΔqseC_LON is restored for virulence in the acute infection stages. We investigated whether restoration of type 1 pilus expression would rescue the qseC phenotype during the acute stages of infection. Our previous studies demonstrated that deletion of qseC results in reduced bladder titers and diminished IBC formation at 6 and 16 hpi (31). Since type 1 pili are essential for attachment, invasion, and IBC formation (52), we tested the ability of UTI89ΔqseC_LON to survive within the urinary tract and form IBCs during these times. Mice were infected with wt UTI89, UTI89ΔqseC, UTI89_LON, or UTI89ΔqseC_LON, and bacterial invasion was evaluated at 1 hpi by gentamicin protection assays. UTI89_LON and UTI89ΔqseC_LON had comparable amounts of internalized bacteria, in contrast to what was observed for UTI89ΔqseC (Fig. 3A). In contrast to UTI89ΔqseC, the bacterial titers recovered from UTI89ΔqseC_LON-infected mice were comparable to those recovered for wt UTI89 and UTI89_LON at both 6 and 16 hpi (Fig. 3B). Similarly, UTI89ΔqseC_LON formed IBCs as efficiently as wt UTI89 and UTI89_LON (Fig. 3C). Thus, restoring expression of type 1 pili in the absence of QseC is sufficient for rescuing IBC formation and survival within the bladder at the acute infection stages.

UTI89ΔqseC_LON is outcompeted by UTI89_LON within the host. To investigate whether QseB-mediated misregulation in the UTI89ΔqseC_LON strain imparted a fitness disadvantage independent of the conferred type 1 piliation defect in UPEC acute pathogenesis, we monitored the infection outcome in mice coinfected with equal bacterial loads of differentially marked
UTI89\textsubscript{LON} and UTI89\textsubscript{ΔqseC_LON} strains during a 72-h period, marking the acute and subacute stages. Our data revealed that UTI89\textsubscript{ΔqseC_LON} began exhibiting a fitness disadvantage as early as 6 hpi in the presence of UTI89\textsubscript{LON}, as indicated by a median competitive index (CI) of 0.1860 (CIs ranged from 0.0496 to 6.951) (Fig. 4A). The fitness disadvantage was more prominent at the later stages of infection, with the CI median dropping 16-fold, from 0.1860 to a value of 0.0115 at 16 hpi, with a range of 0.000074 to 0.2368 (Fig. 4A). These data suggest that restoration of type 1 pili does not suffice for complete restoration of \textit{in vivo} fitness.

\textbf{UTI89\textsubscript{ΔqseC_LON} is less efficient in establishing chronic cystitis.} We used enumeration of bladder titers at 2 weeks postinfection (wpi) as a surrogate marker of chronic cystitis to determine whether QseB-mediated misregulation reduces the ability of UPEC to establish chronic cystitis, even in the presence of type 1 pilus expression. Cohorts of female C3H/HeN mice were inoculated with wt UTI89, UTI89\textsubscript{ΔqseC}, UTI89\textsubscript{LON}, or UTI89\textsubscript{ΔqseC_LON}, and CFU were enumerated at 2 wpi. Our findings demonstrated that, compared to wt UTI89, mice infected with UTI89\textsubscript{ΔqseC} had significantly lower titers by 2 wpi, with only 2 out of 20 mice (10\%) displaying high bladder titers (>10\textsuperscript{5}) indicative of chronic cystitis (Fig. 4B). These results are consistent with the lower bacterial burden observed for UTI89\textsubscript{ΔqseC} at the acute infection stages (Fig. 3). The LON strains were significantly more competent in establishing chronic cystitis, due to their inability to revert \textit{fimS} in the OFF orientation, than their non-LON counterparts. UTI89\textsubscript{ΔqseC_LON} was as efficient as wt UTI89 in developing chronic cystitis. However, significantly fewer mice infected with UTI89\textsubscript{ΔqseC_LON} had high bacterial titers indicative of chronic cystitis (51.7\%) compared to the mice infected with UTI89\textsubscript{LON} (70.0\% developed chronic infection) (Fig. 4B). These findings argue that QseB-mediated misregulation in the absence of QseC imparts a fitness disadvantage independent of the conferred type 1 pilus regulatory defects.

\textbf{The effect of FimH inhibitors that blunt type 1 pilus function is potentiated by deletion of qseC.} Deletion of qseC reduces but does not abolish expression of type 1 pili (31). We rationalized that if we temporarily blunt type 1 pilus function in the \textsubscript{ΔqseC} mutant during the acute infection stage, we will exacerbate the inability of this mutant to establish chronic infection. Compounds ZFH-02056 and ZFH-04269 (mannosides) bind with high affinity to the mannose-binding pocket of the FimH adhesin and thus are potent inhibitors of type 1 pilus function (11). Specifically, oral administration of ZFH-02056 and ZFH-04269 significantly reduces bacterial titers in chronically infected mice within 6 h (11). We used compound ZFH-02056 as a molecular scalpel to temporarily blunt FimH function in wt UTI89 and UTI89\textsubscript{ΔqseC} early in infection and assess long-term infection outcome. Mice were pretreated with one dose of 50 mg/kg ZFH-02056 and infected 30 min after treatment with wt UTI89 or UTI89\textsubscript{ΔqseC}. Compound ZFH-02056 becomes bioavailable in the bladder within 30 min of oral delivery and at this dosage is sustained for 6 h above the MIC (11). Mice treated with PBS prior to infection were included as controls. Bladder bacterial titers were enumerated at 2 wpi to estimate the propensity for chronic infection. Even though ZFH-02056 is bioavailable at the dose delivered in the bladder for only 6 h (11), we observed that this single dosing significantly affected the course of infection 2 wpi. Compared to nontreated mice, at 2 wpi there was a 2-log reduction in CFU in mannoside-treated mice infected with wt UTI89 (Fig. 5, UTI89 versus UTI89\textsubscript{MAN}). Thus, this single-dosemannoside treatment of wt UTI89 resulted in the same degree of attenuation observed with UTI89\textsubscript{ΔqseC}; both had a similar reduction in CFU (Fig. 5, UTI89\textsubscript{MAN} versus UTI89\textsubscript{ΔqseC}). Notably, in mannoside-treated animals infected with UTI89\textsubscript{ΔqseC}, bacterial reduction was further enhanced, as indicated by an additional 1.5-log drop in CFU, compared to mannoside-treated animals infected with wt UTI89 (Fig. 5, UTI89\textsubscript{MAN} versus UTI89\textsubscript{ΔqseC_MAN}). Our findings strongly indicate that dual targeting of QseC and
Type 1 pili and QseC have been extensively implicated in UPEC virulence. Type 1 pili physically participate in the interactions that lead to host colonization and establishment of infection, while the QseC sensor kinase influences numerous cellular processes that in turn affect virulence-associated factors, including type 1 pili (19, 31, 33, 35, 51, 52). In this study, we show that restoring type 1 pilus expression in the absence of qseC rescues the adherence and invasion defects of this mutant and restores its ability to properly form IBCs inside the host during the acute infection stages. Thus, attenuation of UTI89ΔqseC is largely linked to reduced expression of type 1 pili, which decreases the population of internalized bacteria. Of note, locking the fim promoter in the ON orientation shifted the overall population toward piliated bacteria without yielding more pili per cell, which indicates that the levels of pili on the bacterial surface are not exclusively a function of phase variation but also are a function of transcriptional and/or posttranscriptional regulation. Our studies show that by shifting the population toward piliated bacteria, the LON strains are more efficient at colonizing and invading the host than their non-LON counterparts, leading to more bacteria being internalized, which is likely perpetuated with every IBC cycle. This explains the higher levels of colonization in the LON bacteria at 2 wpi compared to the non-LON counterparts, as a previous study demonstrated an association between the number of IBCs formed during acute infection and the development of chronic cystitis (21, 43). Interestingly, although UTI89ΔqseC_LON is enhanced in its ability to cause chronic infection compared to UTI89ΔqseC, it is still significantly less fit for establishing chronic cystitis than UTI89_LON. We have found that the establishment of chronic cystitis is dependent on host-pathogen interactions within the first 24 h of acute infection (21, 43). Indeed, although UTI89ΔqseC_LON alone does not have a defect during acute infection in single-infection experiments, it is rapidly outcompeted by UTI89_LON in the bladder when coinoculated with this strain. Thus, QseB-mediated misregulation in the absence of QseC results in a significant defect in sustaining infection and establishing chronic cystitis, even when type 1 pilation is restored. These data suggest that simultaneous inactivation of these factors have an additive effect on interfering with the onset of chronic UTI.

Research aimed at the development of therapeutics for UTIs has shown that compound ZFH-02056, an inhibitor of type 1 pilus function, significantly reduces UPEC titers in the host bladder when used as a treatment for chronic cystitis (11). The qseC deletion leads to reduced but not abolished expression of type 1 pili (31). We showed that pretreatment with one oral dose of ZFH-02056 significantly reduced the ability of UTI89ΔqseC to establish chronic cystitis. Thus, drugs targeting QseC may potentiate mannosides in the treatment of UTI. Previous studies identified LED209 as an inhibitor of the kinase activity of QseC (41). However, our studies have established that the defects of a qseC deletion mutant are associated with the phosphatase function of QseC, the absence of which results in an overactive QseB response regulator that cannot be dephosphorylated and deactivated (31). In vitro analyses demonstrated that LED209 had no inhibitory effect on the phosphatase activity of UPEC QseC and did not impact virulence-associated gene expression in UPEC (see Fig. S1 in the supplemental material). These data further indicate that it is the phosphatase activity of QseC that needs to be targeted to attenuate virulence, an area that we are currently exploring.

**FIG 5** Coinhibition of type 1 pili and QseC as a prophylactic measure for UTIs. Chart depicting the bladder CFU obtained at 2 wpi from mice pretreated with mannoside and subsequently infected with either wt UTI89 or UTI89ΔqseC (used as a proxy for a QseC inhibitor). Significantly fewer CFU (1.5-log reduction) were obtained from pretreated mice infected with UTI89ΔqseC. The averages from 3 independent experiments are shown (**, P < 0.01; ***, P < 0.001; each by two-tailed Mann-Whitney). UTI89_MAN, mice pretreated with mannoside and challenged with wt UTI89; ΔqseC_MAN, mice pretreated with mannoside and challenged with ΔqseC; UTI89, mice pretreated with PBS and challenged with wt UTI89; and ΔqseC, mice pretreated with PBS and challenged with ΔqseC.
Collectively, our work extends our understanding of the molecular basis of attenuation of UPEC QseC and shows that drugs targeting QseC hold promise to further potentiate mannose efficacy for the treatment and prevention of UTIs. Patients who suffer from chronic or recurrent UTIs currently require suppressive antibiotics which disrupt the normal flora, causing unwanted side effects (14). The development of antivirulence agents would provide an alternative prevention means with the potential to eliminate UPEC colonization by effectively targeting both bacterial attachment and invasion, but most importantly persistence within the host, thereby greatly reducing the possibility of chronic and nonretractable infections.

ACKNOWLEDGMENTS

We are grateful to Zhenfu Han for synthesizing compound ZFH-02056.

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