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Regulation of Expression of Uropathogenic *Escherichia coli* Nonfimbrial Adhesin TosA by PapB Homolog TosR in Conjunction with H-NS and Lrp

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Urinary tract infections (UTIs) are a major burden to human health. The overwhelming majority of UTIs are caused by uropathogenic *Escherichia coli* (UPEC) strains. Unlike some pathogens, UPEC strains do not have a fixed core set of virulence and fitness factors but do have a variety of adhesins and regulatory pathways. One such UPEC adhesin is the nonfimbrial adhesin TosA, which mediates adherence to the epithelium of the upper urinary tract. The *tos* operon is AT rich, resides on pathogenicity island *aspV*, and is not expressed under laboratory conditions. Because of this, we hypothesized that *tosA* expression is silenced by H-NS. Lrp, based on its prominent function in the regulation of other adhesins, is also hypothesized to contribute to *tos* operon regulation. Using a variety of *in vitro* techniques, we mapped both the *tos* operon promoter and TosR binding sites. We have now identified TosR as a dual regulator of the *tos* operon, which could control the *tos* operon in association with H-NS and Lrp. H-NS is a negative regulator of the *tos* operon, and Lrp positively regulates the *tos* operon. Exogenous leucine also inhibits Lrp-mediated *tos* operon positive regulation. In addition, TosR binds to the *pap* operon, which encodes another important UPEC adhesin, P fimbria. Induction of TosR synthesis reduces production of P fimbria. These studies advance our knowledge of regulation of adhesin expression associated with uropathogen colonization of a host.

Urinary tract infections (UTIs), which are among the most common bacterial infections of humans (1), can occur in otherwise healthy individuals when bacteria colonizing the gastrointestinal tract gain access to the periurethral area. Most individuals with UTIs develop an infection of the bladder, referred to as cystitis (1). However, the infecting bacterium may ascend the ureters to infect the kidneys (pyelonephritis) and, in some cases, enter the bloodstream leading to bacteremia and sometimes fatal urosepsis (1–4).

A diverse group of extraintestinal pathogenic *Escherichia coli* strains, referred to as uropathogenic *E. coli* (UPEC), cause the overwhelming majority of uncomplicated UTIs (2, 5). While numerous UPEC virulence factors have been identified, including adhesins, motility systems, toxins, and iron acquisition systems, a core set of virulence factors has not been strictly defined (6–8). However, it is critical to understand specific virulence factors and how they are regulated.

Previous work identified and characterized the *E. coli* repeats-in-toxin (RTX) nonfimbrial adhesin TosA (i.e., type one secretion as the predicted secretion mechanism) (7, 9–13). In particular, it was noted that *tosA* and the other *tos* operon genes have poor *in vitro* expression (9–11). TosA, a >250-kDa surface-exposed protein, mediates UPEC adherence to epithelial cells derived from the upper urinary tract (9). This is in contrast to a number of other RTX proteins, which are fully secreted into the extracellular milieu and act as toxins (14–18). We estimated that ~32% of UPEC strains carry genes encoding TosA and its cognate type 1 secretion system, TosCBD (10). In strain CFT073, the *tos* operon resides on pathogenicity island *aspV* (PAI-*aspV*) (12). The *tos* operon, in addition to *tosA* and predicted cognate secretion system genes *tosCBD*, also contains the regulatory genes *tosR*, *tosE*, and *tosF* (9, 10). TosE and TosF together suppress motility (10), a feature also found in other adhesin operon regulators (19–21). TosR, a member of the PapB family, is a negative regulator of the *tos* operon (10).

PapB, the prototypical member of its family, is a well-characterized positive and negative transcriptional regulator of the *pap* operon (22–24) that encodes the structural and secretion machinery necessary for P-fimbria assembly (25, 26). P fimbriae are epidemiologically associated with UPEC strains (27) and have been shown to be important during experimental UTI (27–30). PapB mediates transcriptional regulation by binding within the DNA minor groove (31), which suggests that PapB might recognize structured DNA in a manner proposed for nucleoid-associated proteins (32–36). In addition, the well-known nucleoid-structuring protein Lrp also contributes to both positive and negative regulation of the *pap* operon (22, 37–39).

H-NS regulates the expression of many genes through binding structured AT-rich DNA sequences, compacting the bacterial chromosome into defined nucleoid macrodomains (40–44). PAIs are often identified by their AT richness (41, 43–45), and AT-rich genes and PAIs are often silenced by H-NS (41, 43, 44). In addition, H-NS also contributes to negative regulation of adhesin

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operons and dual regulation of motility operons (38, 46–58). Indeed, PapB was previously suggested to mediate positive regulation of the *pap* operon by anti-silencing H-NS repression (58).

Lrp and H-NS are key regulators associated with a variety of other genes, including those coding for adhesins in addition to P fimbriae (19, 22, 37-39, 43, 46-57, 59-67). In agreement with this, others have noted the possibility that Lrp and H-NS antagonize the activity of each other or could interact together to potentiate gene regulation (35, 38, 59, 68). This type of regulation resembles a regulatory switch, in which one nucleoid-structuring protein switches in predominance at key regulatory elements to perturb gene regulation. This switch may be mediated by varying the protein composition during different growth phases (i.e., Lrp levels increase during the mid-exponential phase and decrease thereafter) (40, 69). However, in the case of the *pap* operon, switch regulation may not be mediated by direct antagonism between H-NS and Lrp (38), but indirect regulation could be possible. It is unknown whether H-NS and Lrp switch regulation is responsible for *tos* operon regulation and whether H-NS and Lrp regulation of the *tos* operon is direct or indirect.

Whether TosR, like PapB, might function in the capacity of an activator in addition to repressing the *tos* operon was previously unknown. Thus, in this study, we examined the capacity of TosR to serve as both a *tos* operon activator under certain conditions and as a repressor under others. Additionally, we propose that an H-NS and Lrp regulatory switch, similar to the one described above, is responsible for *tos* operon regulation. We also examined the capacity of TosR to negatively regulate production of P fimbria. To our knowledge, TosA has become the first nonfimbrial adhesin and RTX protein to be fully integrated into the network underlying the reciprocal regulation between different adhesins and between adhesins and motility systems. This cross-regulation also suggests that hierarchical regulation of adhesins and motility is much broader than previously thought.

MATERIALS AND METHODS

Bacterial strains. A phage transductant of the original *tosR* deletion mutation (10) was unmarked using the FLP recombinase as previously described (10). The Δ*hns* Δ*lrp* CFT073 strain was engineered through phage-mediated transduction of a previous CFT073 Δ*lrp* mutation (19) into a previous lambda Red-engineered CFT073 Δ*hns* mutant unmarked as described above. Transductants were selected for on lysogeny broth (LB) agar (10 g/liter tryptone, 5 g/liter yeast extract, 0.5 g/liter NaCl, 15 g/liter agar) containing kanamycin (25 μg/ml). Deletion mutations were verified by PCR.

Engineered plasmids. Untagged *tosR* and *lrp* genes were cloned into pBAD-*myc*-HisA (Invitrogen) as previously described (10). The pBAD-*tosR*-His_6 and pRS551-P_{tos}-*lacZ* were previously engineered (10). The pBAD empty vector, pBAD-*tosR*, pBAD-*tosR*-His_6, and pBAD-*lrp* constructs were maintained in LB (10 g/liter tryptone, 5 g/liter yeast extract, 0.5 g/liter NaCl) containing 100 μg/ml ampicillin, while the pBAD-*lrp* construct was also maintained in M9 medium (12.8 g/liter $Na_2HPO_4{\cdot}7H_2O$, 3 g/liter KH_2PO_4, 0.5 g/liter NaCl, 1.0 g/liter NH_4Cl, 2 mM $MgSO_4$, 0.4% glycerol, 0.1 mM $CaCl_2$) containing ampicillin (100 μg/ml) (CFT073 Δ*tosR* strain). In addition, the pRS551-P_{tos}-*lacZ* and pRS551 empty vector constructs were maintained in LB containing ampicillin (50 μg/ml), except as noted below.

5′ RACE. Plasmid pRS551-P_{tos}-*lacZ* was transformed into CFT073 Δ*lacZ* and CFT073 Δ*tosR* Δ*lacZ* and maintained in LB containing ampicillin (100 μg/ml). The 5′ random amplification of cDNA ends (RACE) procedure was performed similar to previous methods (70). cDNA was produced using the *lacZ* cDNA primer listed in Table 1 and SuperScript II reverse transcriptase as previously described (10). Input RNAs were hydrolyzed by adding NaOH (final concentration 0.16 mM) and boiled for 10 min. This reaction was neutralized by the addition of HCl (0.16 mM). A 3′ linker, listed in Table 1, was ligated to the cDNA described above using T4 RNA ligase (New England BioLabs). After ligation, the enzyme was inactivated by incubation at 65°C for 20 min. The first-round nested PCR was performed with the forward linker primer and *lacZ* nested primer 1 listed in Table 1. The second-round nested PCR was performed with the forward linker primer and *lacZ* nested primer 2. The resulting PCR fragment from the second round of nested PCR was sequenced.

TABLE 1 Primers used in this study

Primer[a]	Sequence (5′ → 3′)
lacZ cDNA (R)	GCGGATTGACCGTAATGGGATAGGT
3′ linker	TTTAGTGAGGGTTAATAAGCGGCCGCGTC GTGACTGGGAGCGC
Linker forward (F)	GCCGCTTATTAACCCTCACTAAA
lacZ nested primer 1 (R)	GACGACGACAGTATCGGCCTCAGGAAG
lacZ nested primer 2 (R)	CATTCAGGCTGCGCAACTGTTGGGAAGG
P_{tos13} (F)	AAGTTTTGGGGTGCAGTCCAC
P_{tos13} (R)	AAAAAGTGAAATCTCAAAACAAAAAAT
P_{tos34} (F)	TAATATAGATATTATCTGCATATAA
P_{tos34} (R)	TACTAGAGATTACATCTAAAAAATT
P_{tos57} (F)	TTAGATAAAAACCCTACAGAGAAGT
P_{tos57} (R)	CTGTATATGATCTGCCATACCATTACACAT
P_{papBA} (F)	CTCACTGTAACAAAGTTTCTTCGAATA
P_{papBA} (R)	GTTTCCCCCTTCTGTCGGGCCCCTG
lacZ (F)	GCGAATACCTGTTCCGTCATAGCG
lacZ (R)	CATCGCCAATCCACATCTGTGAAAG

[a] Orientations are indicated in parentheses (F, forward; R, reverse).

EMSAs. *tosR*-His_6 was induced in wild-type CFT073 and extracted using a QIAexpressionist protocol (Qiagen) and Ni-nitrilotriacetic acid (NTA) agarose (Invitrogen) as previously described (10). Input DNAs for electrophoretic mobility shift assays (EMSAs) were generated using the P_{tos13}, P_{tos34}, P_{tos57}, P_{papBA}, and *lacZ* primers listed in Table 1. Input DNAs were terminally labeled with a digoxigenin-11-ddUTP (DIG-ddUTP) using a 2nd-generation DIG gel shift kit (Roche Applied Science) as described previously (10). DNA binding reactions and detection of shifted DNA fragments were performed using a modified Roche DIG shift kit protocol and anti-DIG–alkaline phosphatase detection antibody (Roche Applied Science) as previously described (10), with the exception that between 400 nM and 4 μM TosR-His_6 was used in each DNA binding reaction; between 2 pg/μl (P_{papBA} and *lacZ*) and 10 pg/μl (P_{tos13}, P_{tos34}, and P_{tos57}) DIG-ddUTP labeled fragments were used in the DNA binding reactions.

Western blots. To detect TosA from induced overexpression constructs, pBAD-*tosR*, pBAD-*tosR*-His_6, and pBAD-*lrp* were transformed into various CFT073 backgrounds and induced in LB containing 0, 0.06, 0.6, or 10 mM L-arabinose (pBAD-*tosR*, pBAD-*tosR*-His_6, and the pBAD empty vector) or 0, 0.6, 1.2, 2.4, or 10 mM L-arabinose (pBAD-*lrp*) for 4 h. Four hours was chosen to allow *E. coli* to transit through the exponential phase, to ensure a high titer to maximize the likelihood of observing TosA, TosR, and PapA among bacterial cells in the culture, and to avoid prolonged incubation of the cultures within the stationary phase. Prior to induction, overnight bacterial cultures were diluted 1:100 (the CFT073 wild-type strain) and 1:40 (the CFT073 Δ*lrp* and Δ*hns* strains). The pBAD-*lrp* construct transformed into CFT073 Δ*tosR* was induced for 4.5 h with 0, 0.6, 1.2, or 10 mM L-arabinose in M9 minimal medium either containing 10 mM L-leucine or no exogenous L-leucine. Prior to induction, CFT073 Δ*tosR* harboring pBAD-*lrp* was cultured overnight in LB, pelleted at 6,000 × *g*, washed in M9 medium, and diluted 1:20. Total proteins from the inductions were collected in 10 mM HEPES (pH 8.3 to 8.9), quantified with a Pierce (BCA) protein assay kit (Thermo Scientific), and assayed by Western blotting with polyclonal anti-TosA antibodies or

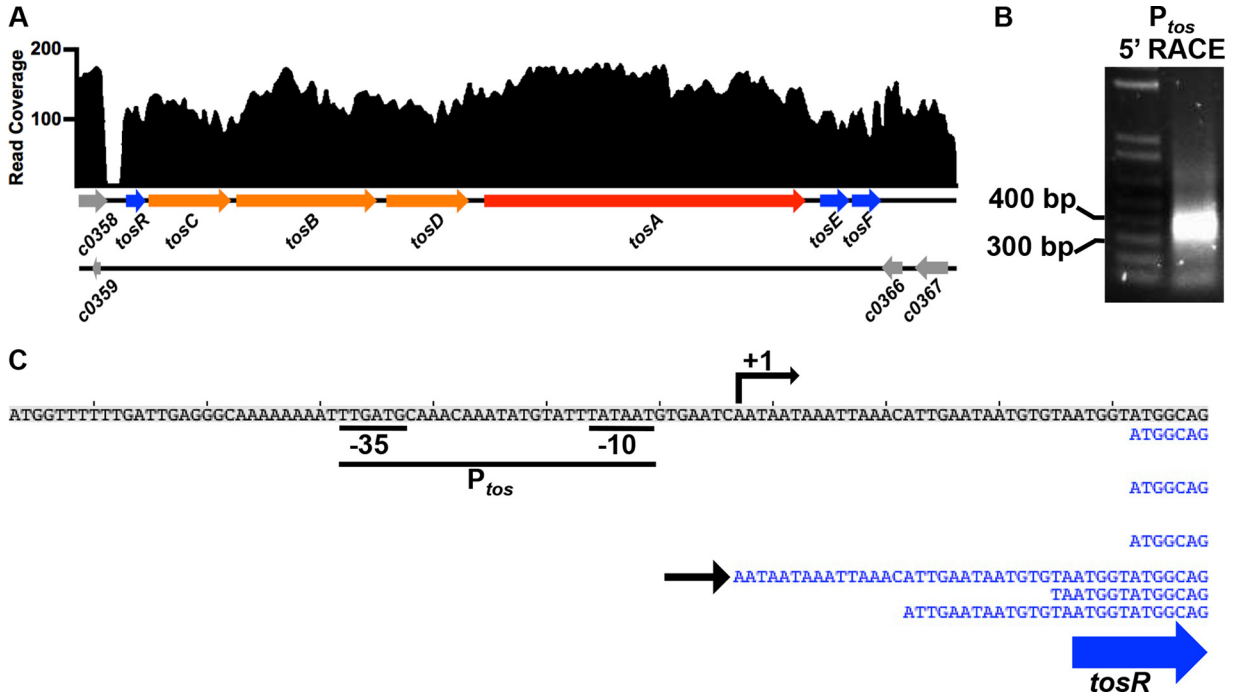

FIG 1 P_{tos} is predicted to be located upstream of *tosR*. (A) The *tos* operon is presented along with a log-transformed cDNA read plot corresponding to cDNAs obtained from the *tos* operon of UPEC strain CFT073 cultured in filter-sterilized human urine. The scale indicates mapped reads. Two parallel lines below the read plot represent the two strands of DNA, and the directions of the arrows represent the strand on which the indicated genes are carried. Only a partial sequence of *c0358* is depicted in the read plot. (B) Resolution of the 5′ RACE products obtained from transcripts expressed from the vector pRS551-P_{tos}-*lacZ* yields a product between the indicated 300- and 400-bp size markers. (C) Mapped cDNAs (in blue) are depicted below the top shaded DNA sequence; a blue arrow indicates the location of *tosR*. A black arrow to the left depicts the most upstream read obtained from the RNA-Seq experiment, which was also precisely the same sequence identified from sequencing of the PCR product obtained in panel B. An angled black arrow indicates the transcriptional start site of the *tos* operon. The predicted −35 and −10 sequences of the *tos* operon promoter, P_{tos}, are depicted.

an anti-His_6 antibody (Invitrogen) as previously described (10). To detect PapA, total proteins were assayed as described above in a CFT073 wild-type background harboring pBAD-*tosR*-His_6, induced in LB containing 0, 0.6, or 10 mM L-arabinose; the only exception was that polyclonal anti-PapA antibodies (Rockland) were used in place of anti-TosA antibodies.

To detect TosA from the CFT073 wild-type strain and CFT073 Δ*hns*, Δ*lrp*, and Δ*hns* Δ*lrp* mutants, each background construct was cultured in LB for approximately 2.5 h to the exponential phase ($A_{600} \approx 0.3$ to 0.5). Prior to being cultured to the exponential phase, CFT073 wild-type and Δ*lrp* overnight cultures were diluted 1:100 in LB, and Δ*hns* and Δ*hns* Δ*lrp* mutant overnight cultures were diluted 1:40 prior to being cultured in LB. Total proteins were collected in 10 mM HEPES (pH 8.3 to 8.9), quantified with a Pierce BCA protein assay kit (Thermo Scientific), and assayed by Western blotting with polyclonal anti-TosA antibodies as described above.

Promoter activity assay. Promoter activities from the pRS551-P_{tos}-*lacZ* or pRS551 empty construct, transformed into wild-type CFT073 or the Δ*hns*, Δ*lrp*, and Δ*hns* Δ*lrp* mutant strains, were determined using a modified Miller assay as previously described (10). The modification to the Miller assay was the use of β-methylumbelliferyl β-D-galacopyranoside (0.5 mg/ml) as the substrate instead of *o*-nitrophenyl-β-galactoside.

Growth curves. Overnight cultures of *E. coli* CFT073 harboring pRS551-P_{tos}-*lacZ* were diluted 1:100 (wild-type and Δ*lrp* mutant strains) and 1:40 (Δ*hns* and Δ*hns* Δ*lrp* mutant strains) into LB (10 g/liter tryptone, 5 g/liter NaCl) containing ampicillin (50 μg/ml). Constructs were cultured at 37°C for 24 h in a Bioscreen C automated growth curve system, with A_{600} readings recorded every 15 min.

RESULTS

The *tos* operon promoter is located upstream of the *tos* operon regulator gene, *tosR*. Our previous work localized the *tos* operon promoter (P_{tos}) to a 630-bp sequence upstream of *tosR* (10). To determine the precise location of P_{tos} and map associated promoter elements, we conducted both analysis of transcriptome sequencing (RNA-Seq [not strand specific]) and 5′ rapid amplification of cDNA ends (RACE). Mapping normalized *tos* operon cDNA reads obtained from *E. coli* CFT073, cultured in human urine (unpublished data), we predicted the *tos* operon transcriptional start site to be 23 bp upstream of *tosR*, based on the presence of a gap between *tosR* and the upstream open reading frames (ORFs) *c0358* and *c0359* (Fig. 1A). However, transcripts from genes encoded on the opposite DNA strand (*c0366* and *c0367*) at the 3′ end of the *tos* operon make it difficult to predict transcriptional termination sites, as the RNA-Seq technique employed here is not strand specific. For verification of the predicted transcriptional start site, 5′ RACE was performed on transcripts expressed from the pRS551-P_{tos}-*lacZ* transcriptional fusion, used to ensure a high concentration of transcripts containing the *tos* operon start site. Following two rounds of nested PCR on cDNAs with a 3′ linker of a known sequence ligated to this segment, we amplified a PCR product of approximately 344 bp (Fig. 1B), which was consistent with the transcriptional start site obtained from the RNA-Seq analysis.

Sequencing of the 5′ RACE PCR product identified the distal 5′ sequence (transcriptional start site) identical to that of the RNA-Seq analysis, which in turn allowed us to map a modified σ^{70} promoter upstream of the transcriptional start site (Fig. 1C). This promoter shows 67% identity (TTGAtg) to the canonical σ^{70} −35 sequence and 100% identity to the canonical σ^{70} −10 sequence

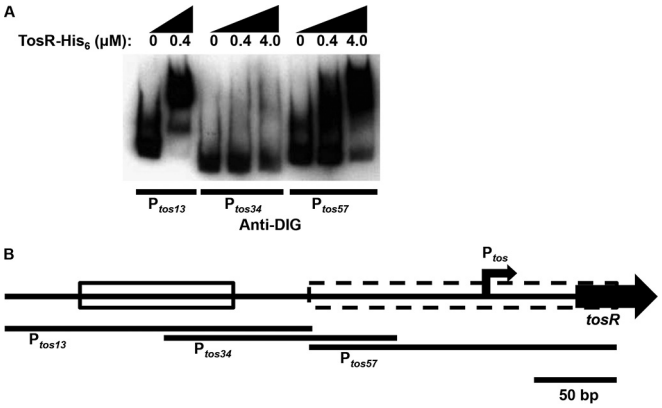

FIG 2 An EMSA indicates that TosR binds P_{tos} at promoter distal and proximal positions and with various strengths. (A) The indicated amounts of TosR-His_6 were incubated with terminally DIG-labeled P_{tos} fragments. Shifted and unshifted DNA fragments were detected with an anti-DIG antibody. The EMSA is representative of two independent experiments. (B) A schematic of P_{tos} region indicates the positions of the P_{tos} fragments used for the above EMSA, the location of the operon promoter (angled black arrow), the stronger TosR-His_6 binding site distal to the promoter (solid box), and the weaker TosR-His_6 binding site proximal to the promoter (dashed box).

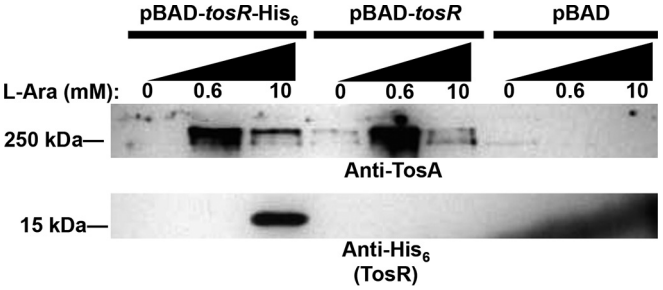

FIG 3 TosR is a dual positive and negative regulator of TosA. Western blots using polyclonal anti-TosA antibodies or an anti-His_6 antibody were performed to detect TosA (>250 kDa) or TosR (~15 kDa). Total proteins for the Western blot were obtained from UPEC strain CFT073 harboring the indicated pBAD constructs induced with the indicated concentrations of L-arabinose. Equal amounts of proteins were loaded, as determined using a Pierce BCA protein assay kit. The Western blot is representative of two independent experiments.

(TATAAT). Consistent with other σ^{70} promoters (71, 72), these −35 and −10 sequences are separated by 16 nucleotides. The transcriptional start site is 7 bp downstream from the end of last nucleotide −10 sequence, a spacing also consistent with σ^{70} promoters (72). In addition, the first base in the predicted transcript, adenosine, is also typical of many transcriptional start sites (73, 74). However, a putative ribosome-binding site upstream of the predicted TosR translational start site could not be clearly identified, which could suggest that TosR translation is inefficient.

TosR is both a positive and negative regulator of the *tos* operon. We have previously identified a repressor function for TosR (10). As the location of the previously identified TosR-binding site (10) is not near P_{tos} (160 bp upstream of the newly identified promoter), we predicted that there could be additional, weaker binding sites near the *tos* operon promoter. To test this prediction, we performed an electrophoretic mobility shift assay (EMSA) on digoxigenin (DIG)-labeled DNA fragments of P_{tos} containing the strong TosR-binding site, an intergenic region between the strong TosR binding site and P_{tos}, and a region containing P_{tos} (Fig. 2A and B). As expected, we found that the region of P_{tos} containing the strong TosR-binding site had a reduced electrophoretic mobility (i.e., was shifted) when incubated with TosR. Additionally, we found that TosR shifted the P_{tos} fragment containing the *tos* operon promoter. As the latter fragment was almost fully shifted only at the highest levels of TosR (4 μM), we also reasoned that TosR weakly binds this region, compared with the strong binding site previously identified. At least 50 bp separates the strong and weak TosR binding sites in P_{tos} (Fig. 2B). In addition, it is possible that TosR has some affinity for AT-rich sequences, as is also the case for other PapB family members (31). This is supported by the observation that TosR slightly shifts the intergenic region between the strong and weak binding sites (i.e., the intensity of the P_{tos34} unshifted fragment is lower at the highest TosR concentration). However, affinity for AT-rich sequences alone cannot explain all of the TosR binding activity, as AT-rich regions of P_{tos} failed to be effective competitors for TosR binding to the strong binding site in the vicinity of P_{tos} (10). This does not rule out the possibility that TosR recognizes a structural element, especially as another promoter, P_{papBA} (see Fig. S1A and S1B in the supplemental material), is regulated by the prototype member of the PapB family (22–24). In agreement with this, BLASTN revealed no significant sequence similarity between the weak and strong TosR binding sites.

Other PapB family members have been described as dual regulators of their cognate operons (22–24). Thus, based on the various degrees of TosR binding strengths for sites in the vicinity of P_{tos}, we speculated that TosR could also have an additional positive regulatory function on the *tos* operon. To test whether TosR could induce expression of the *tos* operon, we used a pBAD-*tosR*-His_6 construct and a pBAD-*tosR* untagged construct to assay TosA synthesis at various *tosR* induction levels. Using a Western blot of proteins from whole-cell preparations obtained from these pBAD overexpression constructs, we found that TosA levels are inversely related to induced *tosR* levels (Fig. 3). For induced levels of TosR below the detectable limit of our anti-His_6 antibody, we observed high levels of TosA synthesis; with high levels of TosR, detectable with anti-His_6 antibody, TosA levels were low. Likewise, these functions appear independent of the presence of the His_6 tag, as both tagged and untagged TosR proteins yielded similar results. Expression was also independent of the presence of arabinose alone, as an empty pBAD vector failed to regulate TosA synthesis. A similar result was also observed when *tosR* was induced in another urinary tract isolate background, ABU 83972, harboring the *tos* operon (not shown). It is important to note, however, that each of these findings is based on overexpression of TosR. Therefore, it may be the case that additional regulators supplement TosR-mediated activation and repression under native conditions. However, it is also important to note that *E. coli* CFT073 has no arabinose utilization gene mutations, and arabinose will be metabolized during these assays, which may contribute to the absence of TosR at some induction levels. Induction from the pBAD vector may also be subject to the "all-or-nothing" phenomenon (75–77). Titration from this vector may, therefore, be limited (78), especially when considering both the all-or-nothing phenomenon and arabinose utilization.

Nucleoid-structuring proteins contribute to TosR regulation of the *tos* operon. The *tos* operon is localized to the PAI-*aspV*

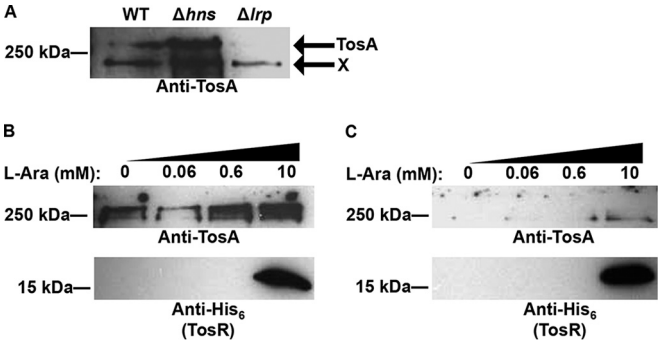

FIG 4 TosR-mediated negative and positive regulation is perturbed in the Δ*hns* and Δ*lrp* backgrounds. (A) A Western blot using polyclonal anti-TosA antibodies was performed on total proteins obtained from the indicated CFT073 backgrounds. Bands corresponding to TosA are indicated in the figure, and a nonspecific band is indicated with an X. The Western blot is representative of two independent experiments. Equal amounts of proteins were loaded, as determined using a Pierce BCA protein assay kit. Western blots were also performed as described above using polyclonal anti-TosA antibodies or an anti-His_6 antibody in the CFT073 Δ*hns* (B) or Δ*lrp* (C) background harboring pBAD-*tosR*-His_6 induced with the indicated concentrations of L-arabinose. The Western blot is representative of two independent experiments.

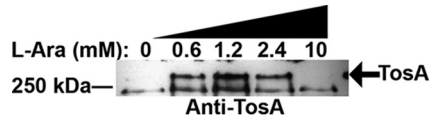

FIG 5 Lrp is a positive regulator of TosA. A Western blot using polyclonal anti-TosA antibodies was performed on total proteins obtained from CFT073 harboring pBAD-*lrp* and induced with the indicated concentrations of L-arabinose. Bands corresponding to TosA are indicated in the figure. Equal amounts of proteins were loaded, as determined using a Pierce BCA protein assay kit. The Western blot is representative of two independent experiments.

pathogenicity island in UPEC strain CFT073 (12). It is well accepted that genes on PAIs and other AT-rich sequences are often bound and regulated by nucleoid-structuring proteins, including H-NS and Lrp (41, 43, 44, 67, 79). A 400-bp region containing P_{tos} is AT rich (74%). In addition, both of these nucleoid-structuring proteins regulate the expression of many genes, including adhesin and flagellar genes (19, 22, 37–39, 43, 46–57, 59–67). To determine whether or not a 240-bp AT-rich region near P_{tos} is similarly curved to an analogous region in P_{papBA}, suggesting Lrp and H-NS-mediated nucleoid structuring, we utilized a web-based tool (http://www.lfd.uci.edu/~gohlke/dnacurve/) to predict DNA curvature. We found that both regions predicted a similar curved architecture (see Fig. S1A and S1B in the supplemental material), which suggests Lrp and H-NS could regulate the *tos* operon. To further predict whether H-NS and Lrp bind to P_{tos}, we examined this sequence for putative H-NS and Lrp binding sites (see Fig. S2 in the supplemental material). There are four clusters of putative Lrp binding sites ($GN_{2-3}TTT$), based on P_{papBA} (80), downstream and partially overlapping the predicted strong TosR binding site and upstream and partially overlapping the predicted weak TosR binding site. One of the predicted Lrp binding sites also overlaps P_{tos}. In addition, there are also two putative high-affinity H-NS binding sites with 80% (aCaATAAATT) and 70% (ataATAAATT) identity to a sequence with known high affinity for H-NS (81, 82) located upstream of the weak TosR binding site and downstream of P_{tos}, near the predicted transcriptional start site.

To determine whether H-NS and Lrp do indeed regulate the *tos* operon, we performed Western blots on proteins from whole-cell preparations obtained from CFT073 Δ*hns* and Δ*lrp* backgrounds (Fig. 4A). TosA levels were dramatically increased in the Δ*hns* background compared to wild-type CFT073, suggesting that H-NS could function as a negative regulator of the *tos* operon. However, it is important to note that H-NS perturbs the expression of a number of different genes (22, 43, 46–57); therefore, it remains unclear if additional regulators supplement H-NS-mediated negative regulation of the *tos* operon. The loss of Lrp failed to increase *tos* operon expression, as was observed for loss of H-NS.

With respect to the multitude of PapB family members, however, it is not always obvious how nucleoid structure and the cognate PapB family members integrate to govern expression of the adhesin. To determine whether H-NS and Lrp contribute to TosR regulation of the *tos* operon, we performed the same pBAD-*tosR*-His_6 overexpression experiment described above in the CFT073 Δ*hns* (Fig. 4B) and Δ*lrp* (Fig. 4C) backgrounds. As described above, loss of *hns* resulted in increased TosA synthesis, but high levels of TosR did not decrease TosA levels in the CFT073 Δ*hns* background. Conversely, we found that the TosR-mediated activation was dependent on Lrp; no change in TosA levels could be detected regardless of TosR level in the CFT073 Δ*lrp* background. Likewise, a shift to a lower antibiotic concentration for the Δ*hns* and Δ*lrp* backgrounds harboring pBAD-*tosR*-His_6 does not perturb TosR regulation itself; the lower antibiotic concentration did not perturb TosR-mediated regulation in the wild-type *E. coli* CFT073 background (data not shown). As for H-NS, Lrp is also a global regulator (19, 22, 37–39, 59–67). Therefore, it remains unclear if additional gene products also supplement Lrp- and TosR-mediated positive regulation of the *tos* operon.

Induction of *lrp* expression is sufficient to drive TosA synthesis. Observing that Lrp is required for *tos* operon expression, we tested whether exogenous expression of Lrp alone would be sufficient to induce *tos* operon expression. To determine whether Lrp acts as an activator of the *tos* operon, as is the case with the *pap* operon (22, 37, 38), we performed a pBAD-*lrp* overexpression experiment in wild-type CFT073. Western blotting of whole-cell proteins from this overexpression construct revealed that low levels of *lrp* induction increased TosA levels (Fig. 5). In turn, high levels of *lrp* induction diminished TosA levels. However, this effect was dependent on the presence of TosR (see Fig. S3 in the supplemental material). Taken together, Lrp appears to be an activator of the *tos* operon, but it can also contribute to *tos* operon repression in the presence of TosR. It is important to note, however, that the same caveats of *lrp* overexpression should also be considered, as with *tosR* overexpression described above.

Some genes regulated by Lrp are positively or negatively regulated by exogenous leucine (63–65, 67). To test whether exogenous leucine positively or negatively regulates the *tos* operon, we performed our pBAD-*lrp* overexpression assay in the CFT073 Δ*tosR* background in M9 minimal medium with and without exogenous leucine (10 mM). The CFT073 Δ*tosR* background was chosen over wild-type CFT073 due to the fact that pBAD-*lrp* was unable to induce expression of the *tos* operon in M9 minimal medium (data not shown). In the CFT073 Δ*tosR* background, induction of *lrp* expression resulted in higher TosA levels only in the absence of exogenous leucine (see Fig. S4 in the supplemental material). This demonstrates that Lrp-mediated positive regulation of the *tos* operon is subject to regulation by leucine.

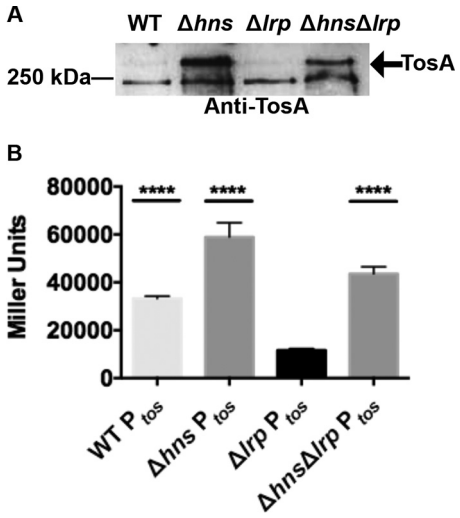

FIG 6 Lrp is not required for P_{tos} transcriptional activation in the Δ*hns* background. (A) A Western blot using polyclonal anti-TosA antibodies was performed on total proteins obtained from the indicated CFT073 backgrounds. Equal amounts of proteins were loaded, as determined using a Pierce BCA protein assay kit. (B) A Miller assay was performed using β-galactosidase translated from the *lacZ* gene of the pRS551-P_{tos}-*lacZ* vector harbored in the indicated backgrounds. Bars represent mean values of Miller units obtained from two biological replicates with two technical replicates each. Error bars represent 1 standard deviation around the mean, and **** represents *P* values of <0.0001 obtained by comparing *lacZ* expression from pRS551-P_{tos}-*lacZ* construct harbored in the respective mutant or wild-type CFT073 background with the Δ*lrp* background (determined using analysis of variance [ANOVA] followed by Tukey's multiple-comparison test).

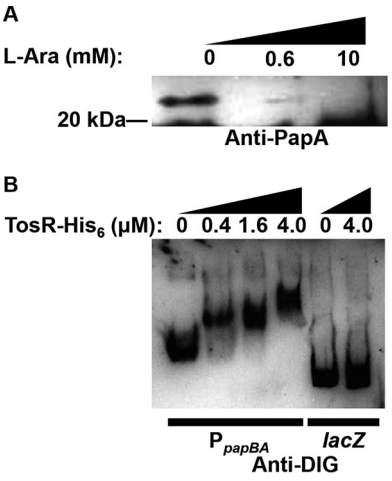

FIG 7 TosR negatively regulates P-fimbria synthesis. (A) A Western blot using polyclonal anti-PapA antibodies to detect PapA2 (~23 kDa) was performed on total proteins obtained from CFT073 harboring pBAD-*tosR*-His_6 and induced with the indicated concentrations of L-arabinose. This blot is representative of two biological replicates. Equal amounts of proteins were loaded, as determined using a Pierce BCA protein assay kit. (B) The indicated amounts of TosR-His_6 were treated along with terminally DIG-labeled P_{papBA} or *lacZ* fragments. Shifted and unshifted DNA fragments were detected using an anti-DIG antibody. The EMSA is representative of two independent experiments.

An H-NS and Lrp regulatory switch drives *tos* operon transcriptional regulation. It has been previously proposed and noted that Lrp might act to anti-silence H-NS repression (35, 38, 59, 68). We hypothesize, therefore, that an H-NS and Lrp regulation switch (i.e., predominance of either H-NS or Lrp) explains the observed regulation of the *tos* operon. In particular, if H-NS-mediated negative regulation is abolished, Lrp-mediated positive regulation of the *tos* operon would no longer be required in the Δ*hns* background. To test this hypothesis, we performed a Western blot on a CFT073 Δ*hns* Δ*lrp* mutant (Fig. 6A). Loss of Lrp in the Δ*hns* background only slightly decreased TosA levels, compared to the Δ*hns* background alone. This further strengthens the premise that Lrp is a positive regulator of the *tos* operon, as even these slightly reduced levels were much higher than the nearly undetectable levels of TosA in the CFT073 wild-type background. Coupled with the finding that TosR regulation is abolished in the Δ*lrp* background, these results suggest that an H-NS and Lrp regulation switch likely contributes to *tos* operon regulation, and TosR has a function within this regulatory switch. It is important to note, however, that both H-NS and Lrp are global regulators (19, 22, 37–39, 59–67). Therefore, it cannot be ruled out that regulation between H-NS and Lrp, at P_{tos} is indirect.

Furthermore, we tested whether the proposed H-NS and Lrp regulatory switch would function at the transcriptional level at P_{tos}. To determine whether P_{tos} is transcriptionally responsive to H-NS and Lrp, we measured the activity of our pRS551-P_{tos}-*lacZ* transcriptional fusion in both wild-type CFT073 and CFT073 nucleoid-structuring mutants using a Miller assay (Fig. 6B). As previously observed (10), the P_{tos} promoter showed high activity in wild-type CFT073. Additionally, P_{tos} showed high activity in the CFT073 Δ*hns* mutant. P_{tos} promoter activity was greatly reduced in the CFT073 Δ*lrp* background. In the CFT073 Δ*hns* Δ*lrp* background, however, P_{tos} activity was restored to slightly higher than wild-type levels. No growth differences were observed among bacterial strains harboring pRS551 (see Fig. S5 in the supplemental material). These findings suggest that native Lrp levels induce P_{tos} on the pRS551 construct by overcoming H-NS-mediated negative regulation. Thus, regulation of the *tos* operon by the H-NS and Lrp regulatory switch is at the transcriptional level. From its P_{tos} DNA binding activities and association with H-NS and Lrp regulation of the *tos* operon, we further suggest that TosR also transcriptionally regulates the *tos* operon.

TosR is a negative regulator of the *pap2* operon, another component of the H-NS and Lrp regulatory switch. Both H-NS and Lrp are global regulators that affect the expression of a variety of genes, including adhesin and flagellar genes (19, 22, 37–39, 43, 46–57, 59–67). It is, therefore, not surprising that fimbrial regulators associated with H-NS and Lrp could also participate in cross-regulation between adherence and motility genes (62, 83–86). Both PapB (P-fimbrial operon) and FocB (F1C fimbrial operon) share approximately 80% amino acid sequence identity and regulate their respective *pap* and *foc* operons. Cross-regulation between PapB and FocB is a well-characterized phenomenon (84). In contrast, TosR has only 28% amino acid sequence identity to PapB. To determine whether TosR can also regulate the *pap* operon, we performed our pBAD-*tosR*-His_6 overexpression assay and Western blotting with an anti-PapA antibody (Fig. 7A). With increased TosR levels, PapA2 levels were decreased.

Previous work characterizing PapB and FocB cross-regulation explored the abilities of both proteins to mediate this regulation through binding to P_{papBA} and P_{focBA} (84). To determine whether TosR might also mediate *pap* operon cross-regulation through binding P_{papBA}, we performed an EMSA on a DIG-labeled P_{papBA}

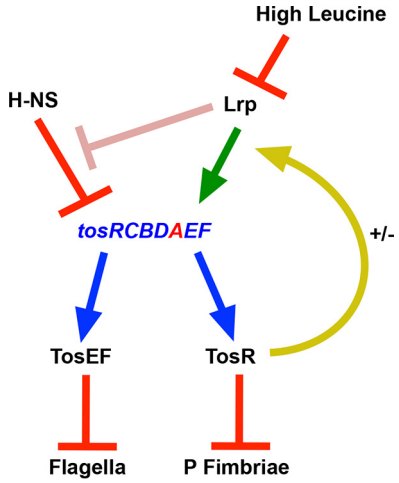

FIG 8 Model of *tos* operon regulation and its involvement in reciprocal regulation of adhesins and flagella. The *tos* operon is indicated by blue text, and *tosA* is represented by red text. A blue arrow indicates that TosR, TosE, and TosF are translated from genes transcriptionally linked to the *tos* operon. Under typical laboratory conditions, H-NS silences expression of the *tos* operon (red bar). At low and high concentrations, respectively, TosR both positively and negatively (+/−) regulates (yellow arrow) Lrp-mediated positive regulation of the *tos* operon (green arrow). Lrp may also directly or indirectly relieve H-NS negative regulation of the *tos* operon (pink bar). In turn, TosR also negatively regulates P-fimbria synthesis (red bar), and together the cognate regulators TosE and TosF negatively regulate flagellum synthesis (red bar). High levels of leucine, in addition, negatively regulate Lrp-mediated positive regulation of the *tos* operon (red bar).

fragment and, as a control, a fragment of *lacZ* (Fig. 7B). TosR shifted the P_{papBA} fragment but failed to shift the control *lacZ* fragment. Thus, we conclude that despite markedly low amino acid identity between PapB and TosR, TosR mediates negative regulation of the *pap* operon through specific binding of P_{papBA}. Intriguingly, like the weak P_{tos} binding site, BLASTN reveals no substantial sequence homology with the strong P_{tos} binding site and P_{papBA}.

DISCUSSION

Here, we present a model of *tos* operon regulation involving PapB family member TosR and two global gene regulators, H-NS and Lrp (Fig. 8). TosR is a positive and negative transcriptional regulator of the *tos* operon. This regulation is predicted to be mediated through differential binding of the chromosomal region containing P_{tos}, the *tos* operon promoter, by TosR. The global regulator H-NS transcriptionally silences expression of the *tos* operon, while another global regulator, Lrp, overcomes H-NS silencing to mediate positive regulation of the *tos* operon. When TosR levels are low, TosR promotes Lrp-mediated positive regulation of the *tos* operon. However, when TosR levels are high, TosR promotes H-NS-mediated negative regulation of the *tos* operon. We also predict that H-NS and Lrp interact either directly or indirectly to modify *tos* operon positive regulation. Additionally, TosR also negatively regulates expression of the P-fimbrial (*pap*) operon.

Using RNA-Seq and 5′ RACE, we identified the transcriptional start site of the *tos* operon 23 bp upstream of *tosR* and P_{tos} 30 bp upstream of *tosR*. The promoter sequence has only a few modifications from the canonical σ^{70}, which include two base substitutions from the canonical −35 sequence and spacing between the −35 and −10 sequences 1 bp shorter than that for the average σ^{70} promoter (71, 72). Additionally, the first base of the transcript, adenosine, is also typical of other σ^{70} promoters (73, 74), and the spacing between the promoter and the start of the transcript (7 bp) is also observed with other σ^{70} promoters (72). Thus, together these results suggest that P_{tos} could be a strong promoter, which is consistent with our previous observation of strong activity from the pRS551-P_{tos}-*lacZ* transcriptional fusion (10). This finding, however, is confounded by the weak expression of the *tos* operon previously observed (9–11), which points to negative transcriptional regulation at P_{tos} by other proteins as a possible mechanism of *tos* operon regulation.

Our group previously reported that the *tos* operon is repressed when cultured under laboratory conditions (LB broth, both aerated and static, at 37°C) (9–11). Some of this negative regulation was attributed to TosR (10). However, other PapB family members act as dual regulators (both activator and repressor) of their cognate operons (22–24). We found this was also the case for TosR, which shows a reciprocal relationship with TosA levels: if TosR levels are low, TosA levels are high, and when TosR levels are high, TosA levels are significantly reduced. Thus, TosR is a dual regulator of the *tos* operon. We predict that at least some of this differential behavior is mediated through TosR binding to two sites within P_{tos}—one site when TosR levels are low (strong binding site) and the other when TosR levels are higher (weak binding site).

Regulation of the *tos* operon not only involves TosR but also includes both H-NS and Lrp. We predict that TosR positive regulation of the *tos* operon, when TosR levels are low, may be mediated through an alleviation of negative regulation by H-NS, thereby promoting Lrp-mediated positive regulation. In terms of the predicted H-NS binding site upstream of P_{tos} (see Fig. S2 in the supplemental material), we speculate that TosR binding either displaces an H-NS filament or prevents further H-NS polymerization at this site, both known mechanisms of overcoming H-NS silencing (87). Subsequently, this activity may allow Lrp to bind to predicted binding sites in the vicinity of P_{tos} to promote positive regulation. This model is further supported by TosR-mediated positive regulation of the *tos* operon no longer being required in the Δ*hns* background. Furthermore, Lrp-mediated positive regulation is no longer necessary in the Δ*hns* background, and TosR regulation is abolished in the Δ*lrp* background. Lrp alone is also sufficient to promote expression of the *tos* operon, especially in the absence of leucine, which further supports our prediction of Lrp-mediated positive regulation of the *tos* operon. Thus, in terms of the predicted Lrp binding sites (see Fig. S2) in the vicinity of P_{tos} and previous work on Lrp by others (88–90), we speculate that Lrp binding may facilitate RNA polymerase contact with either Lrp itself or additional unknown elements near P_{tos}. Similarly, we propose that TosR-negative regulation of the *tos* operon, when TosR levels are high, may be mediated through interference of Lrp-promoted positive regulation. This effect is also predicted to be dependent on H-NS. It is speculated, in terms of the second predicted TosR and H-NS binding sites (see Fig. S2) in the vicinity of P_{tos}, that TosR binding to this site occludes Lrp binding to the predicted binding sites above and subsequent occlusion of RNA polymerase from P_{tos} at the second predicted H-NS binding site near P_{tos}. Further support for this conclusion comes from the finding that TosR is no longer a negative regulator of the *tos* operon in a Δ*hns* background. Also by integrating the two predicted H-NS

binding sites into this regulation model, it is possible that bridging at these sites could promote negative regulation (87, 91–94).

From the strong activity of P_{tos} in a Δ*hns* Δ*lrp* background, we also predict that an H-NS and Lrp regulation switch is responsible for much of the *tos* operon regulation. The switch may act through alteration of the predominance of H-NS and Lrp regulation at P_{tos}, which is consistent with modulation of nucleoid levels of associated proteins during different growth phases (40, 69). However, as H-NS and Lrp are pleiotropic regulators (19, 22, 37–39, 43, 46–57, 59–67), this switch may also be through indirect interactions. To emphasize the possibility that H-NS and Lrp indirectly interact, a pink bar is depicted in our model (Fig. 8). In agreement with this idea of a switch between the two nucleoid proteins, the strong decrease in P_{tos} activity observed with the loss of Lrp is increased when H-NS is absent. This leads us to predict that Lrp functions to overcome H-NS negative regulation of the *tos* operon, consistent with our belief that an H-NS and Lrp switch governs *tos* operon expression. It is also an intriguing possibility that the same switch is similar to a previous description of nucleoid contributions to reciprocal regulation of adherence and motility (54). However, whether this H-NS and Lrp regulation switch is mediated by direct antagonism of each component or indirect effects will need to be examined further.

We note that the estimated leucine content of pooled human urine (~0.01 mM) (95) is much lower than that of LB (~8 mM) (96). This suggests that UPEC would adjust gene expression to accommodate the low leucine levels found in human urine. Thus, growth in an environment with relatively low levels of leucine is an environmental stress encountered by UPEC during an infection. As exogenous Lrp does not positively regulate the *tos* operon in the presence of high leucine levels, we also conclude that a low leucine level is an environmental cue that upregulates the *tos* operon. We also propose that the presence of higher levels of leucine in LB at least partially accounts for poor *tos* operon expression when cultured in this medium.

As evident from the variety of genes, including those localized to adhesin operons in addition to flagellum-mediated motility genes regulated by H-NS and Lrp (19, 22, 37–39, 43, 46–57, 59–67), cross-regulation appears to be a feature of this regulatory switch (62, 83–86). We found that TosR is a negative regulator of P-fimbria production. We predict that this negative regulation is potentiated through TosR binding to P_{papBA}, the *pap* operon promoter. This is a surprising finding in that the previously well-described cross-regulation between PapB family members occurred between PapB and FocB, which shared 80% amino acid sequence identity (84). TosR and PapB share only 28% amino acid sequence identity. We believe that these results have important implications for studying adhesin expression. Further work should explore whether TosR, like PapB and FocB, also regulates FimA and FocA levels (84). Nevertheless, it is now our conclusion that such cross-regulation between PapB family members and different types of adhesins (i.e., fimbrial and nonfimbrial adhesins) is a broader phenomenon than previously thought. Thus, a more detailed exploration of adhesin cross-regulation, especially between unrelated or poorly related adhesins and adhesin regulators, should be undertaken to gain a more accurate picture of microbial adhesin regulation. These future explorations should include determination of whether reciprocal regulation between adhesins is an important fitness trait during infection. For example, P fimbria and TosA both make contributions during experimental UTI (27–30), but it is unknown whether TosR inactivation could suppress a *tosA* mutation through allowing UPEC to continue to synthesize P fimbria instead of simultaneously inhibiting P-fimbria production and attempting to produce a defective TosA adhesin.

Finally, previous work (10) has already established that TosEF, expressed when the *tos* operon is expressed, negatively regulate FliC levels. Together with the TosR findings presented above, we have also found that *tos* operon regulation participates in reciprocal regulation of adherence and motility. It is intriguing to note that a protein encoded by the terminal gene of the *pap* operon, *papX*, suppresses motility in UPEC strain CFT073 (19–21). Future work may thus also explore whether overexpression of *lrp* in *tosEF* and *papX* mutant constructs decreases motility. Taken together, these results could delineate the function of the H-NS and Lrp regulatory switch in reciprocal regulation and during infection.

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