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YbcL of Uropathogenic *Escherichia coli* Suppresses Transepithelial Neutrophil Migration

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Uropathogenic *Escherichia coli* (UPEC) strains suppress the acute inflammatory response in the urinary tract to ensure access to the intracellular uroepithelial niche that supports the propagation of infection. Our understanding of this initial cross talk between host and pathogen is incomplete. Here we report the identification of a previously uncharacterized periplasmic protein, YbcL, encoded by UPEC that contributes to immune modulation in the urinary tract by suppressing acute neutrophil migration. In contrast to wild-type UPEC, an isogenic strain lacking ybcL expression (UTI89 ΔybcL) failed to suppress transepithelial polymorphonuclear leukocyte (PMN) migration *in vitro*, a defect complemented by expressing ybcL episomally. YbcL homologs are present in many *E. coli* genomes; expression of the YbcL variant encoded by nonpathogenic *E. coli* K-12 strain MG1655 (YbcLMG) failed to complement the UTI89 ΔybcL defect, whereas expression of the UPEC YbcL variant (YbcLUTI) in MG1655 conferred the capacity for suppressing PMN migration. This phenotypic difference was due to a single amino acid difference (V78T) between the two YbcL homologs, and a majority of clinical UPEC strains examined were found to encode the suppressive YbcL variant. Purified YbcLUTI protein suppressed PMN migration in response to live or killed MG1655, and YbcLUTI was detected in the supernatant during UPEC infection of bladder epithelial cells or PMNs. Lastly, early PMN influx to murine bladder tissue was augmented upon *in vivo* infection with UTI89 ΔybcL compared with wild-type UPEC. Our findings demonstrate a role for UPEC YbcL in suppression of the innate immune response during urinary tract infection.

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Uncomplicated UTI primarily afflict otherwise healthy women, though anatomical and urodynamics, genetic variation, and behavior can predispose individuals to infection. Despite appropriate antibiotic therapy, resolution is often short-lived, and recurrent UTI are a major problem (25% of women experience recurrent infection within 6 months of initial infection) (11). As the gastrointestinal (GI) tract serves as a reservoir for uropathogenic bacteria, recurrent infections are typically thought to arise through re inoculation of the urinary tract with fecal flora. However, recent investigations have identified a bacterial reservoir within the bladder epithelium that is refractory to antibiotic and immune clearance and may also contribute to recurrence (28, 31). The recent emergence of antibiotic-resistant isolates further complicates the effective treatment of UTI (37).

The majority of community-onset UTI are caused by a heterogeneous group of uropathogenic *Escherichia coli* (UPEC) strains that employ a variety of strategies to effectively colonize and persist within the urinary tract. This is evidenced by an array of disease manifestations, which include asymptomatic bacteriuria, acute and recurrent cystitis, and pyelonephritis. Investigations using a murine model of cystitis and UPEC isolate UTI89 have revealed a complex pathogenic cascade that begins with bacterial binding and invasion of the superficial umbrella cells of the bladder epithelium through type 1 pili-uroplakin interactions (24, 25, 38). Internalized bacteria rapidly multiply within the epithelial cell cytoplasm to form intracellular bacterial communities (IBCs) that are protected from the mounting immune response (2, 26). Expansion of the IBC and associated epithelial cell rupture release UPEC to initiate binding and invasion events with neighboring cells, leading to additional rounds of IBC formation and propagating the infection (19). The importance of bacterial amplification within the intracellular niche for UPEC pathogenesis is demonstrated by the attenuation of UPEC mutants unable to form mature IBCs (1, 29), the conservation of IBC formation among clinical UPEC isolates in multiple murine backgrounds (12), and the presence of IBCs in samples from human patients (30). Given the significance of the IBC, the events that precede bacterial invasion facilitating intracellular replication likely dictate disease outcome.

As the urinary tract is typically a sterile environment, the proliferation of UPEC within the bladder elicits a robust inflammatory response characterized by the production of cytokines and chemokines and the recruitment of leukocytes, primarily polymorphonuclear leukocytes (PMN) or neutrophils, which are essential for clearance of bacteria from the urinary tract (13). UPEC strains have acquired mechanisms to modulate the innate immune response during acute infection to access the intracellular niche (reviewed in reference 17). Recent studies have demonstrated inhibition of proinflammatory signaling pathways and attenuated cytokine production by cultured bladder epithelial cells during infection with UPEC relative to nonpathogenic *E. coli* (3, 15, 18, 20). Similarly, UPEC strains inhibit PMN functions such as production of reactive oxygen species, phagocytosis, and chemotaxis (9, 10, 23). Though bacterial effectors responsible for some of these phenotypes have been identified in some UPEC strains, the conservation of innate immune modulation (3, 15) and the considerable genome plasticity among UPEC strains (5, 6, 15, 18, 23).

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33) suggest that additional mechanisms of immune modulation exist.

In this study, we identified a previously uncharacterized bacterial protein, YbcL, that contributes to modulation of the host immune response by UPEC during acute UTI. While both nonpathogenic and uropathogenic E. coli strains encode YbcL homologs, only the uropathogenic variant, YbcLUTI, suppressed PMN migration in an in vitro model of acute inflammation, dependent upon a threonine at amino acid 78 (where the nonpathogenic allele encodes a valine). The suppressive phenotype was confirmed YbcLUTI protein to the bacterial inoculum. Furthermore, YbcLUTI was detected in the supernatant during UPEC infection of bladder epithelial cells and PMN infiltration to the bladder at early time points in a murine cystitis model. Taken together, these results describe a novel bacterial product that contributes to UPEC pathogenesis by influencing the innate immune response in the urinary tract.

### MATERIALS AND METHODS

**Bacterial strains and culture.** E. coli strains were grown statically in Luria-Bertani (LB) broth at 37°C for 18 h. Where indicated, chloramphenicol, ampicillin, or isopropyl-ß-D-1-thiogalactopyranoside (IPTG) was added at 20 µg/ml, 100 µg/ml, or 100 µM, respectively. UPEC strain UTI89 was isolated from a patient with cystitis (6), and MG1655 is a well-characterized K-12 laboratory strain that is type 1 piliated (4). Heat-killed bacterial suspensions were generated by a 30-min incubation at 55°C, and an aliquot of the suspension was plated to confirm bacterial death. UTI89 suspensions were generated by a 30-min incubation at 55°C.

**MATERIALS AND METHODS**

**TABLE 1 Primers used in this study**

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**Primer Sequence (5’ → 3’)**

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**Infection and Immunity**
inverted Transwell insert (0.33-cm² polycarbonate membranes with 3-µm diameter pores; Corning number 3472) and allowed to adhere to the membrane for 16 h. Transwells were then moved to a 24-well plate containing tissue culture medium, and additional medium was added to the upper reservoir. Fresh medium was applied every 2 days until the epithelial monolayers reached confluence, as assessed by impermeability to liquid (21).

**Human PMN isolation.** In accordance with a protocol approved by the Washington University Human Research Protection Office, PMN were isolated from venous blood of healthy adult volunteers after verbal consent was obtained. The isolation of human PMN from blood was adapted from a previously published protocol (14). In short, erythrocyte numbers were reduced by dextran sedimentation, contaminating immune cells (other than PMN) were removed using a Ficoll density gradient (Ficoll-Paque Plus; GE Healthcare), and the remaining erythrocytes were lysed hypotonically. PMN viability was >99% as assessed by trypan blue exclusion, and purity was >99% as determined by visualization of nuclear morphology after staining (Hema3; Fisher Scientific). Purified PMN were resuspended in RPMI 1640 medium (Gibco) to a concentration of 10⁶ PMN/ml and used immediately. 

**Transepithelial PMN migration assay.** Transepithelial PMN migration assays were conducted in accordance with previously published protocols (23). Briefly, Transwells with confluent 5637 monolayers were washed three times in RPMI. Bacterial cells were washed in phosphate-buffered saline (PBS) and diluted in RPMI. A bacterial inoculum of 6 × 10⁶ CFU/ml (a multiplicity of infection [MOI] of 40 CFU/cell) or an equivalent volume of RPMI was applied to the apical sides of inverted Transwells and incubated for 1 h at 37°C. The Transwells were then righted into 24-well plates (Ultra Low Attachment plates; Corning number 3472) and allowed to adhere to the membrane for 16 h. Transwells were then moved to a 24-well plate (Ultra Low Attachment plates; Corning number 3473) containing 0.6 ml RPMI, and 10⁶ PMN were added to the upper reservoir. After 1 h at 37°C, PMN in the lower reservoir were collected and enumerated using a hemacytometer, and the number of PMN recruited into the lower reservoir was normalized to input PMN. Data represent the mean and standard deviation from at least three independent experiments. Statistically significant differences were determined using an unpaired Student’s t test.

To generate conditioned medium, 5637 cells grown to confluence in 15-cm dishes were infected with the indicated strains of E. coli at an MOI of 40. After 1 h of incubation at 37°C, the supernatant was collected and filter sterilized using syringe-driven filter units (0.22-µm pore size; Millipore). The filter-sterilized supernatant (conditioned medium) was used as the inoculum and replaced 0.6 ml RPMI in the lower reservoir in the transepithelial PMN migration assay. PMN migration in response to the conditioned medium was assessed as described above.

**YbcL localization by Western blotting.** To mimic the transepithelial PMN migration assay, 5637 cells or freshly isolated PMN were infected with the indicated strains of E. coli at an MOI of 40 or 10, respectively. After 1 h of incubation at 37°C, the supernatant was collected and the eukaryotic cells were washed with PBS and lysed using 0.1% Triton X-100 containing protease inhibitors (Roche). The supernatant and cell lysate samples were filter sterilized using syringe-driven filter units (0.22-µm pore size; Millipore), and protein was precipitated using 15% trichloroacetic acid (TCA) (Sigma). Samples were separated by SDS-PAGE using 4 to 20% precast gels (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking with 2% nonfat milk plus 2% bovine serum albumin (BSA) (Sigma), blots were probed with mouse anti-FLAG antibody (1:1,000; Sigma) followed by goat anti-mouse IgG antibody (1:2,000; Sigma) and were developed using Tropix CDP-Star (Applied Biosystems).

**Sequencing of ybcL alleles in clinical isolates.** A collection of 74 UPEC isolates, including strains from women with acute cystitis, recurrent cystitis, asymptomatic bacteriuria, or pyelonephritis, was obtained from Scott Hultgren (6, 12, 30). Chromosomal DNA was isolated from each UPEC strain using the Wizard genomic DNA purification kit (Promega) according to the manufacturer’s instructions. Primers MEL231 and MEL232 were designed to bind conserved regions within the ybcL ORF identified through nucleotide alignment of ybcL alleles present in sequenced E. coli genomes. PCR was conducted using Phii DNA polymerase (Stratagene), and product formation was assessed by agarose gel electrophoresis. Amplicons of the predicted size were purified with the QIAquick PCR purification kit (Qiagen) and submitted for sequencing (SeqWright). Nucleotide alignments were performed using Vector NTI software (Invitrogen). The prevalences of specific amino acids at position 78 in YbcL proteins from various E. coli groups were compared using Fisher’s exact test.

**Purification of YbcL variants.** ybcL alleles were amplified from the constructs described above using the following primer sets for pYbcL_UTI and pYbcL_MG and pYbcL_MG217: MEL62 and MEL30 (where the reverse primer contains a sequence encoding a 6-histidine tag [6xHis] in place of the FLAG epitope). The ampiclons were cloned into pTRC99A as described above. The constructs were confirmed by direct sequencing, and expression of the YbcL variants was confirmed by SDS-PAGE. Periplasmics were prepared from E. coli Top10 carrying these plasmids and dialyzed overnight in PBS before being applied to an Ni-nitrilotriacetic acid (NTA) column (Qiagen). Protein purification was conducted according to the instructions of the manufacturer, using an elution buffer containing 200 mM imidazole. Protein concentrations were determined using a bicinchononic acid protein assay (Thermo Scientific).

**Murine cystitis and tissue MPO activity assay.** All animal procedures were approved in advance by the Animal Studies Committee at Washington University. In accordance with a well-described model of murine cystitis (16), 8-week-old female C3H/HeN mice (Harlan) were transurethrally inoculated with 50 µl of bacterial suspension (2.5 × 10⁹ CFU) or sterile PBS. At 1 h postinfection (p.i.), animals were sacrificed, bladders were harvested and homogenized in 1 ml PBS, and an aliquot of each bladder homogenate was plated on LB agar to determine tissue bacterial burden. The myeloperoxidase (MPO) content of bladder tissue was measured as described previously (23). Aliquots of undiluted bladder homogenates were transferred to a 96-well plate, and a standard curve was generated using purified MPO. Samples were incubated with the reaction buffer for 1 h (Fluoro MPO; Cell Technology) according to the manufacturer’s instructions. Enzyme activity was measured by fluorescent detection of an MPO product using a microplate reader (Synergy 2; BioTek). MPO activity in the bladder samples is reported in units/ml, and data points represent the means of triplicate measurements from individual bladders. At least 12 mice were infected for each bacterial strain tested. Differences in MPO levels were examined for significance using an unpaired Student’s t test, and bacterial loads were compared using the Mann-Whitney U test.

**RESULTS**

**YbcL encoded by UTI89 suppresses transepithelial PMN migration.** Given the ability of UPEC strain UTI89 to suppress innate immune responses by undefined mechanisms (17, 18, 23), we sought to further characterize the early host-pathogen interaction. Guided by preliminary transcriptional profiling data (23; J. Loughman, unpublished data), we identified a periplasmic protein, YbcL, with structural homology to mammalian Raf-1 kinase-inhibitory protein (RKP) (32), a modulator of eukaryotic signal transduction pathways (22, 35, 36). To investigate a role for YbcL in suppression of innate responses by UTI89, we utilized an in vitro model of acute inflammation that quantifies PMN migration across a bladder epithelial monolayer. Transwells bearing confluent 5637 uropathogenic monolayers were infected with E. coli strains or mock infected for 1 h before freshly isolated human PMN were applied to the upper reservoir, and PMN migration into the lower reservoir was enumerated using a hemacytometer. Consistent with our prior results (23), the nonpathogenic E. coli
E. coli

YbcL 

PMN Migration (×10^6)

Mock
MG1655
UTI89
ΔybcL
ybcL UTI

FIG 1 UPEC YbcL suppresses transepithelial PMN migration in vitro. 5637 bladder epithelial cell monolayers grown on Transwell inserts were infected at their apical surfaces with the indicated strains of E. coli or mock infected, and freshly isolated human PMN were added at the basolateral surface. The number of PMN recruited to the apical surface was enumerated at 1 h p.i. and is shown normalized to input PMN. Infection with MG1655 or UTI89 ΔybcL elicited significantly more PMN than that with wild-type UTI89 (*, P < 0.0001).

strain MG1655 stimulate robust PMN migration, while infection with the UPEC strain UTI89 resulted in significantly fewer PMN in the lower reservoir (Fig. 1) (P < 0.0001). The low level of PMN migration upon UPEC infection reflects active suppression of the inflammatory response by UPEC rather than failure to induce inflammatory signaling, as coinfection with MG1655 plus UTI89 yields the uropathogenic phenotype (23). In contrast to wild-type UTI89, UTI89 ΔybcL elicited significantly more PMN (P < 0.0001), and episomal expression of YbcL in the ybcL mutant restored wild-type levels of PMN migration (Fig. 1). The differential PMN migration observed was not the result of differences in either 5637 or PMN viability; both cell types survived equally well in the presence of the E. coli strains used at early time points, as assessed by lactate dehydrogenase (LDH) release (data not shown). These data suggest that YbcL encoded by UTI89 contributes to UPEC-mediated suppression of PMN migration.

Suppression of PMN migration by YbcLUTI relies on threonine 78. To investigate the properties of YbcL responsible for UPEC-specific suppression of the innate immune response, we first explored sequence conservation among YbcL homologs encoded by E. coli. The nonpathogenic strain MG1655 (4) contains a ybcL allele that is 95% identical at the nucleotide level to the UTI89 allele (6), resulting in six predicted amino acid differences. Four are contained within the mature protein, and three of these amino acid differences represent conservative or semiconservative changes (Fig. 2A and B, blue). In the single nonconservative difference, the UTI89 variant (denoted YbcLUTI) contains a threonine at position 78, while the MG1655 variant (YbcLMG) contains a valine (Fig. 2A and B, green). The crystal structure of YbcL encoded by K-12 strain W3110 (100% identical at the amino acid level to YbcLMG) has been solved (32). However, any effect that these amino acid differences may have on the tertiary structure of YbcLUTI is unclear.

Because MG1655 was unable to suppress in vitro PMN migration and YbcL contributed to this phenotype during infection with UTI89, we hypothesized that the YbcL variants encoded by these E. coli strains were functionally divergent. In accordance with this hypothesis, episomal expression of the YbcLMG variant failed to complement UTI89 ΔybcL in the transepithelial PMN migration model (Fig. 2C) (P < 0.0001 compared to UTI89). To assess the importance of the nonconservative amino acid substitution in suppression of PMN migration by YbcLUTI, we generated additional YbcL variants. Expression of YbcLUTI(T78A) (containing a threonine-to-alanine mutation at position 78) in UTI89 ΔybcL did not suppress PMN migration (Fig. 2C) (P > 0.005), demonstrating that this mutation resulted in a loss of function for the uropathogenic variant. Conversely, expression of YbcLUTI(T78V) (containing a valine-to-threonine mutation at position 78) in UTI89 ΔybcL reduced PMN levels in the lower reservoir (Fig. 2C), demonstrating a gain of function for the nonpathogenic variant. These data demonstrate the functional divergence of the YbcL variants encoded by nonpathogenic and uropathogenic E. coli strains and highlight the importance of threonine 78 in YbcL for UPEC-mediated suppression of PMN migration.

Given the functional consequence of the nonconservative amino acid difference between MG1655 and UTI89 YbcL variants, we hypothesized that threonine 78 would be conserved among UPEC. We therefore assessed the distribution of YbcL homologs among sequenced E. coli strains, focusing on the amino acid at position 78. A BLAST search using the full UTI89 YbcL amino acid sequence revealed YbcL homologs in many but not all sequenced E. coli genomes, including laboratory strains, uncharacterized fecal isolates, and human pathogens classified as either gastrointestinal E. coli (GIPEC) (including adherent-invasive E. coli [AIEC], enteropathogenic E. coli [EPEC], and enterohemorrhagic E. coli [EHEC]) or extraintestinal E. coli (ExPEC) (including neonatal meningitis E. coli [NMEC], avian-pathogenic E. coli [APEC] and UPEC). Among the sequenced strains encoding YbcL homologs, position 78 contained a threonine in 100% of ExPEC isolates, compared to 39% of uncharacterized fecal isolates, 14% of GIPEC isolates (all that contained T78 were AIEC), and 0% of laboratory strains (Fig. 2D) (P < 0.05 for ExPEC versus each group). To further examine the correlation between threonine 78 in YbcL and ExPEC, we amplified and sequenced ybcL alleles from clinical UPEC isolates associated with a range of disease manifestations (6, 12, 30). We were unable to generate an amplicon from 26 of the 74 isolates despite using multiple primer sets, suggesting that like sequenced E. coli strains, clinical isolates also vary in their genomic content. Among 48 clinical isolates from which a ybcL homolog could be amplified, 39 (81%) contained a threonine at position 78 (Fig. 2E). In total, 83% of UPEC strains (including both sequenced and clinical strains), compared to 25% of other E. coli strains, encoded a threonine at position 78 (P < 0.0001).

In addition to threonine and valine, alanine was also found at position 78 in some YbcL homologs encoded by these various E. coli strains. As with valine, episomal expression of the alanine-containing variant YbcLUTI(T78A) failed to complement UTI89 ΔybcL in the transepithelial PMN migration model (Fig. 2C) (P < 0.0001). Taken together, these data demonstrate the prevalence of threonine 78 in YbcL among UPEC strains and illustrate its importance in suppression of the innate immune response by these diverse uropathogens.

YbcLUTI confers suppressive activity on nonpathogenic E. coli MG1655. We next aimed to investigate whether other UPEC-encoded factors were required for YbcLUTI-mediated suppression of PMN migration. To define the bacterial context required for this phenotype, we assessed PMN migration in response to MG1655 episomally expressing a panel of YbcL variants. Expression of YbcLUTI or YbcLMG(V78T) in MG1655 yielded PMN migration levels similar to
those for wild-type UTI89 (Fig. 3A), demonstrating conferral of the uropathogenic phenotype upon the nonpathogenic strain. In contrast, episomal expression of YbcLMG or YbcLUTI(T78V) in MG1655 allowed significantly more PMN migration than UTI89 (Fig. 3A) \( (P < 0.05) \), consistent with the nonpathogenic phenotype.

To demonstrate that suppression of PMN migration was mediated directly by YbcL, we added purified YbcL protein to MG1655 immediately before infection of the epithelial layer. An initial concentration of 225 ng/ml was chosen to approximate the amount of YbcL present in bacterial inocula used above that contained pYbcLUTI, as determined by Western blotting (data not shown). The addition of purified YbcLUTI or YbcLMG(V78T) upon infection with MG1655 resulted in PMN levels similar to those seen upon infection with UTI89 (Fig. 3B). Conversely, MG1655 plus purified YbcLMG or YbcLUTI(T78V) stimulated significantly more PMN migration than the uropathogen UTI89 (Fig. 3B) \( (P < 0.01) \). Analogous experiments conducted using these purified YbcL variants and UTI89 \( \Delta ybcL \) as the bacterial stimulus resulted in the same trends in PMN migration (data not shown). Furthermore, YbcLUTI maintained migration-suppressing potency at concentrations as low as 150 pg/ml or 8 pM, and a decrement in effect was observed with further dilution (Fig. 3C).

To explore whether YbcL activity required live bacteria (i.e., an intact periplasm), we next used heat-killed MG1655 (HKMG) as the bacterial stimulus, which elicited robust PMN migration in contrast to UTI89 in the transepithelial PMN migration model (Fig. 3D) \( (P < 0.05) \). Infection with HKMG plus purified YbcLUTI or YbcLMG(V78T) yielded the uropathogenic phenotype, eliciting low levels of PMN migration similar to those for UTI89 (Fig. 3D), while the addition of YbcLMG or YbcLUTI(T78V) to the same bacterial stimulus had no effect on PMN migration (Fig. 3D) \( (P < 0.005) \), in agreement with data generated using live MG1655. Taken together, these data demonstrate that YbcLUTI confers the capacity to suppress PMN migration upon nonpathogenic \( E. coli \) and that this activity is independent of other pathogen-specific attributes or active bacterial processes.
YbcL is secreted from the bacterial periplasm. As purified YbcL	UTI suppressed PMN migration elicited by both live and heat-killed bacteria in the transepithelial PMN migration model, we hypothesized that YbcL	UTI was secreted from the bacterial periplasm during UPEC infection. To demonstrate a requirement for YbcL	UTI secretion in the suppression of PMN migration by UTI89, we engineered two fusion proteins composed of the lipoprotein NlpA and YbcL (36) (see Materials and Methods) to tether YbcL to the inner or outer bacterial membrane (YbcL	IM and YbcL	OM, respectively) and assessed the ability of these variants to complement UTI89/ybcL in the transepithelial PMN migration model. UTI89/ybcL episomally expressing either YbcL	IM or YbcL	OM stimulated significantly more PMN migration than wild-type UTI89 (Fig. 4A) (P < 0.05). The membrane-tethered YbcL variants failed to complement the ybcL mutation, suggesting that YbcL	UTI does not act to suppress PMN migration from within the bacterial periplasm.

To support these data, we sought to demonstrate secretion of YbcL	UTI by wild-type UTI89 during infection of bladder epithelial cells using a biochemical approach. 5637 cells in 10-cm dishes were infected with the indicated strains of E. coli at an MOI of 40 for 1 h at 37°C. The supernatant (conditioned medium) was filter sterilized and used in place of the bacterial inoculum in the transepithelial PMN migration model. Conditioned medium from infection of 5637 cells with UTI89 stimulated a low level of PMN migration (Fig. 4B). In contrast, conditioned medium generated during UTI89ΔybcL infection stimulated significantly more PMN migration (Fig. 4B) (P < 0.01). This phenotype could be reversed by expression of YbcL	UTI in UTI89ΔybcL but not by expression of either of the membrane-tethered YbcL variants, YbcL	IM or YbcL	OM (P < 0.01 compared to UTI89). These data suggest that YbcL	UTI is secreted from the bacterial periplasm and mediates suppression of PMN migration from the exterior of the bacterial cell.

To corroborate evidence from the transepithelial PMN migration model suggesting that YbcL	UTI is secreted, we assessed localization of the YbcL variants during UPEC infection of bladder epithelial cells or neutrophils. 5637 cells or PMN were infected with the indicated strains of E. coli at an MOI of 40 or 10, respectively, for 1 h at 37°C. The supernatant and eukaryotic cell lysate fractions were filter sterilized, concentrated by TCA precipitation, and resolved using SDS-PAGE. During UPEC infection of 5637...
cells or PMN, YbcL\textsubscript{UTI} and YbcL\textsubscript{OM} were clearly detected in the supernatant, in contrast to YbcL\textsubscript{IM}, which was minimally detected in that fraction (Fig. 4C). All three YbcL variants were detected in the PMN lysate. However, only YbcL\textsubscript{UTI} was detected in the 5637 cell lysate (Fig. 4C). When these cell types were infected with either MG1655 or UTI89 Δybcl episomally expressing the MG1655 YbcL variant, YbcL\textsubscript{MG} exhibited the same localization pattern as YbcL\textsubscript{UTI} (data not shown), confirming that the differential PMN migration observed in the transepithelial PMN migration model was not the result of differences in secretion of the YbcL variants. These data demonstrate that YbcL\textsubscript{UTI} is secreted from the bacterial periplasm during infection of bladder epithelial cells and PMN. Although it was detected in the supernatant, YbcL\textsubscript{MG} mutant in the transepithelial PMN migration model, suggesting that localization to the supernatant is not sufficient for suppression of PMN migration by YbcL\textsubscript{UTI}.

**YbcL\textsubscript{UTI} suppresses acute PMN migration in vivo.** We used a murine model of cystitis to assess a potential contribution by YbcL\textsubscript{UTI} to UPEC-mediated suppression of the innate response in vivo (16, 23). Female C3H/HeN mice were transurethrally inoculated with the indicated strains of *E. coli* or PBS, and myeloperoxidase (MPO) activity in bladder homogenates was determined at 1 h p.i. as a surrogate for PMN influx into bladder tissue. In accordance with our in vitro observations, MG1655 and UTI89 Δybcl elicited significantly more PMN than wild-type UTI89 (Fig. 5A) (*P* < 0.0001). Suppression of PMN migration was nearly completely restored to the ybcl mutant upon complementation with pYbcL\textsubscript{UTI} (Fig. 5A). Modestly lower bacterial titers were recovered after infection with the ybcl mutant or the complemented strain compared to wild-type UTI89 (Fig. 5B) (*P* < 0.05). It is unlikely that these two strains exhibited a defect in colonization at this early time point, as both assembled levels of type 1 pili similar to those for wild-type UTI89 as assessed by microscopy, hemagglutination titers, and in vitro binding and invasion assays using 5637 cells (data not shown). In addition, UTI89 Δybcl formed IBCs that were indistinguishable from those of wild-type UTI89 as assessed by confocal fluorescence microscopy (data not shown), and bacterial titers recovered from wild-type- or ybcl mutant-infected mice were similar at 6, 16, 24, and 48 hours p.i. and at 1 and 2 weeks p.i. (data not shown). In agreement with results obtained using the in vitro model of inflammation, these in vivo data argue that UPEC-encoded YbcL suppresses early PMN migration in a murine model of cystitis.

**DISCUSSION**

The present study identifies a novel bacterial protein encoded by UPEC that contributes to modulation of the innate immune response during UTI. UPEC-encoded YbcL suppressed early PMN migration in an in vitro model of acute inflammation and an in vivo model of murine cystitis. Examination of the YbcL homolog encoded by the nonpathogenic *E. coli* K-12 strain MG1655 revealed three conservative or semiconservative and one nonconservative amino acid difference compared to the UTI89 homolog. We demonstrated that threonine at the nonconservative position 78 is required for suppression of PMN migration by the uropathogenic variant YbcL\textsubscript{UTI}. In contrast, the nonpathogenic variant YbcL\textsubscript{MG} contains a valine at this position and has no effect on PMN migration. We hypothesize that threonine 78 is required directly or indirectly for protein-protein interactions. Future work will in-
vestigate how the identity of a single amino acid dictates YbcL functionality in this model of transepithelial PMN migration.

The presence of YbcL homologs in many but not all *E. coli* strains exemplifies both the genomic heterogeneity within the species and the variation in mechanisms of immune modulation among pathogenic strains. We were not surprised to find YbcL homologs containing threonine 78 in some uncharacterized fecal isolates and GIPEC strains, as the GI tract serves as a reservoir for *E. coli* in addition to the resident (commensal) microflora and supports a considerable amount of horizontal gene transfer. Like *E. coli* in the urinary tract, GIPEC influences local immune responses within the GI tract (7), although it is unlikely to be pathogen specific, as the localization pattern of the YbcL variants (YbcLUTI and YbcLMG) was independent of the bacterial strain, MG1655 or UTI89

Using a murine cystitis model, we demonstrated that YbcL encoded by UTI89 suppresses acute PMN migration to the bladder. Compared to wild-type UTI89, both the *ybcL* mutant and the complemented strain yielded modestly lower bacterial titers at 1 h p.i. We hypothesize that the lower *ybcL* mutant titers may be the result of increased PMN recruitment to those bladders, as evidenced by elevated MPO levels. In agreement with that hypothesis, MG1655 titers also trended lower than UTI89 titers at 1 h p.i. The slightly lower titers in the complement-infected bladders might relate to decreased bacterial fitness caused by maintenance of the plasmid or overexpression of YbcLUTI, as PMN levels were similar to those measured in wild-type infection. Examination of IBC formation and bacterial titers at subsequent time points revealed no significant differences between wild-type- and ΔybcL-infected mice, suggesting that YbcL facilitates the establishment of UTI rather than persistence. Considering the large bacterial inoculum (∼10⁷ CFU) and the capacity of IBCs to amplify and propagate infection, it is not surprising that increased PMN recruitment in the UTI89 ΔybcL-infected bladders early did not adversely affect bacterial titers at later time points. In the human urinary tract, where the inoculum is likely to be significantly lower and varying host genetics influence susceptibility to UTI, the activity of YbcLUTI may significantly favor bacterial survival prior to epithelial invasion, tipping the balance toward infection rather than clearance.

Suppression of PMN migration by YbcLUTI was conferred by episomal expression or the addition of purified protein to either live or nonviable MG1655, demonstrating that YbcLUTI functions independently of bacterial context. Using multiple approaches, we demonstrated that YbcL was secreted by UTI89 during infection of bladder epithelial cells or PMN. We were unable to detect YbcLUTI by Western blotting in filter-sterilized, TCA-precipitated conditioned medium from UTI89/pYbcLUTI ΔybcL grown in LB (M. Lau and D. Hunstad, unpublished data), suggesting that secretion of YbcLUTI is regulated. Given that the localization pattern of YbcLUTI mimicked the pattern of YbcLMG during infection of eukaryotic cells, it is unlikely that the amino acid at position 78 regulates secretion. While YbcL was detected in the supernatant, the mode of delivery from the bacterial cell remains unclear, although it is unlikely to be pathogen specific, as the localization pattern of the YbcL variants (YbcLMG and YbcLUTI) was independent of the bacterial strain, MG1655 or UTI89 ΔybcL. In light of these observations, we hypothesize that secretion of YbcLUTI, a periplasmic protein, occurs through outer membrane proteins (such as secretins) or via outer membrane vesicles (OMVs). Given its presence in the bacterial outer membrane, we hypothesize that YbcLUTI in the supernatant fraction during UPEC infection is associated with OMVs and that the membrane tether prevents that YbcL variant from suppressing PMN migration. As periplasmic proteins as well as outer membrane proteins are packaged in OMVs and precedent exists for the delivery of UPEC effectors via OMVs (e.g., cytotoxic necrotizing factor 1) (9), it is possible that these vesicles mediate YbcLUTI secretion. Future work will address these hypotheses.

In addition to localization to the supernatant during UPEC infection, similar levels of the three YbcL variants were also detected in the filtered PMN lysate. It is unlikely that the PMN-associated YbcL signal originated from internalization of supernatant YbcL, as YbcLUTI was not present in the supernatant but was detected in the PMN lysate. Rather, as PMN are professional phagocytes, we hypothesize that the PMN-associated YbcL signal was generated via bacterial lysis within the phagolysosome. In ad-
dition to the supernatant and PMN lysate, YbcL<sub>UTI</sub> also was detected in the 5637 cell lysate. As the membrane-tethered YbcL variants were not 5637 cell associated and were unable to complement the ybcL mutant in the transepithelial PMN migration model, it is possible that association with epithelial cells is required for suppression of PMN migration by YbcL<sub>UTI</sub>. Future experimentation will focus on specifying the relative contribution of YbcL activity on these cell types to the suppression of PMN migration in our models.

Elucidation of the YbcL crystal structure by Serre and colleagues revealed structural homology to the mammalian protein RKIP (32), which modulates signal transduction pathways, including the mitogen-activated protein (MAP) kinase and NF-κB pathways (35, 36). Klumpp and colleagues demonstrated that UPEC strain NU14 inhibits signaling through the MAP kinase and NF-κB pathways during <i>in vitro</i> infection of cultured bladder epithelial cells (20), although the mechanism underlying this inhibition remains unclear. Like NU14, UTI89 also inhibits signaling through these pathways, though this occurs independent of YbcL<sub>UTI</sub> and RKIP have distinct functions despite their structural homology. Furthermore, UTI89 YbcL, like wild-type UTI89, elicits minimal interleukin-6 (IL-6) and IL-8 from cultured bladder epithelial cells or human PMN relative to that elicited by MG1655 (Lau and Hunstad, unpublished data), suggesting that differences in the induction of these cytokines are not responsible for the increased PMN migration observed with ybcL deletion. Given the structural homology between YbcL and RKIP, the low concentration of YbcL<sub>UTI</sub> required to suppress PMN migration, and the presence of YbcL<sub>UTI</sub> in eukaryotic cell lysates, we hypothesize that YbcL<sub>UTI</sub> attenuates an eukaryotic signaling cascade that promotes transepithelial PMN migration. Ongoing work aims to elucidate the mechanism underlying the differential PMN migration and the role that YbcL<sub>UTI</sub> plays in mediating this phenotype, with specific attention to the importance of threonine 78.

The success of many mucosal pathogens relies on strategies to modulate host immune processes at the epithelial interface. By suppressing acute PMN recruitment, YbcL may extend the window in which UPEC can accomplish epithelial invasion and establish the protected intracellular niche required for propagating infection. YbcL represents a novel example of a bacterial exoprotein that influences early host-pathogen interactions within the urinary tract.

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