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Bacterial Lysis Liberates the Neutrophil Migration Suppressor YbcL from the Periplasm of Uropathogenic Escherichia coli

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Uropathogenic Escherichia coli (UPEC) modulates aspects of the innate immune response during urinary tract infection to facilitate bacterial invasion of the bladder epithelium, a requirement for the propagation of infection. For example, UPEC-encoded YbcL suppresses the traversal of bladder epithelia by neutrophils in both an in vitro model and an in vivo murine cystitis model. The suppressive activity of YbcL requires liberation from the bacterial periplasm, though the mechanism of release is undefined. Here we present findings on the site of action of YbcL and demonstrate a novel mode of secretion for a UPEC exoprotein. Suppression of neutrophil migration by purified YbcLUTI, encoded by cystitis isolate UTI89, required the presence of a uroepithelial layer; YbcLUTI did not inhibit neutrophil chemotaxis directly. YbcLUTI was released to a greater extent during UPEC infection of uroepithelial cells than during that of neutrophils. Release of YbcLUTI was maximal when UPEC and bladder epithelial cells were in close proximity. Established modes of secretion, including outer membrane vesicles, the type II secretion system, and the type IV pilus, were dispensable for YbcLUTI release from UPEC. Instead, YbcLUTI was released during bacterial death, which was augmented upon exposure to bladder epithelial cells, as confirmed by detection of bacterial cytoplasmic proteins and DNA in the supernatant and enumeration of bacteria with compromised membranes. As YbcLUTI acts on the uroepithelium to attenuate neutrophil migration, this mode of release may represent a type of altruistic cooperation within a UPEC population during colonization of the urinary tract.

Urinary tract infections (UTIs), which are among the most common bacterial infections in humans, are caused chiefly by uropathogenic Escherichia coli (UPEC) ([1]). The economic and societal burdens associated with UTIs are substantial; in the United States alone, UTIs result in approximately $4 billion in direct and indirect costs annually, including millions of physician visits and antibiotic prescriptions ([2, 3]). While antibiotics typically help to resolve acute, uncomplicated UTIs, UPEC strains are acquiring resistance to commonly used antibiotic classes at an alarming rate ([4]). Additionally, complicated and recurrent UTIs plague a notable subset of the population, despite antibiotic treatment and apparent resolution of prior infections ([5]). To address these ongoing challenges, it is imperative to understand the mechanisms by which UPEC causes disease in the urinary tract.

The establishment of a UTI represents a critical point in the UPEC infectious cycle. To facilitate colonization of the bladder, UPEC dampens the innate immune response ([6]), characterized by the production of cytokines and chemokines and the recruitment of leukocytes, primarily polymorphonuclear leukocytes (PMN) or neutrophils, from the periphery to the bladder lumen ([7, 8]). One strategy for suppressing acute inflammation relies on the activity of UPEC-encoded YbcL, a periplasmic protein that inhibits transurethral PMN migration in an in vitro model and during in vivo murine cystitis ([9]). The delayed arrival of PMN to the bladder provides an interval, free of phagocytic pressure, during which UPEC can accomplish invasion of the uroepithelium, a step that is essential for the propagation of infection. Conservation of YbcL homologs among UPEC isolates associated with various disease manifestations suggests that the suppressive activity of this protein is important for colonization of the urinary tract ([9]). Nonpathogenic E. coli strain MG1655 also encodes a YbcL variant (denoted YbcLMG), though a single amino acid substitution (T78V) in this variant abrogates its effect on PMN migration ([9]). However, the suppressive activity of UPEC YbcL can be conferred on MG1655 through episomal expression of YbcLUTI, the variant encoded by cystitis isolate UTI89, or by exogenous addition of purified YbcLUTI to the bacterial inoculum at concentrations as low as 8 μM ([9]). Lastly, liberation of YbcLUTI from the periplasm is required for suppression of transurethral PMN migration ([9]), though the mechanism by which YbcLUTI is released and the site of action of YbcLUTI are unclear.

Aside from the type V secretion systems (T5SS), few proteins secreted from the periplasm and their corresponding modes of secretion have been well characterized in UPEC. Outer membrane vesicles (OMVs) have been shown to deliver cytotoxic necrotizing factor 1 (Cnf1), a UPEC toxin, to the extracellular environment and eukaryotic cells ([10–12]). However, OMVs have not been extensively investigated in the context of UPEC infection, and no other UPEC effectors delivered by OMVs have been identified. Additionally, the type II secretion system (T2SS) and type IV pilus (T4P) encoded by UPEC appear to contribute to pathogenesis in the urinary tract ([13]), though periplasmic substrates of these systems that are important for pathogenesis have yet to be identified. Though far less common, bacterial death can also serve as a mechanism for release of intracellular proteins into the extracellular...
milio. In other E. coli pathotypes, Shiga toxin is liberated from the periplasm and colicin from the cytoplasm during bacterial-phage-mediated lysis and quasisexis, respectively (14, 15). However, liberation of UPEC effectors solely through bacterial death has not been demonstrated. As the mode of release has bearing on protein stability, delivery, and targeting, elucidating the route by which YbcLUTI leaves the periplasm may provide insight into its activity.

In this study, we investigated the site of action and the mode of release of YbcLUTI during UPEC infection. Suppression of PMN migration by YbcLUTI required the presence of bladder epithelial cells. Release of YbcLUTI from the periplasm was augmented when UPEC was exposed to cultured uroepithelial cells, compared with exposure to PMN or medium alone; this increase depended on proximity of UPEC to bladder epithelial cells. Additionally, YbcLUTI was released from UPEC in soluble form, though the T2SS and T4P encoded by UTI89 were dispensable for YbcLUTI release. However, a significant increase in bacterial death, liberating YbcLUTI, was demonstrated upon UPEC exposure to bladder epithelial cells. Taken together, these results contribute to our understanding of the site of action of YbcLUTI and describe a novel mode of delivery for a UPEC exoprotein.

MATERIALS AND METHODS

Bacterial strains and culture. E. coli was grown statically in Luria-Bertani (LB) broth for 18 h at 37°C. Ampicillin, chloramphenicol, or isopropyl β-D-thiogalactopyranoside (IPTG) was added at 100 µg/ml, 20 µg/ml, or 100 µM, respectively, unless otherwise indicated. MG1655 is a K-12 laboratory strain of E. coli (16), and UTI89 is a UPEC isolate from a patient with cystitis (17). UTI89 fimH::kan was a kind gift from S. Hultgren. UTI89/pcomGFP contains the GFPmut3 gene in pcom100, where green fluorescent protein (GFP) is constitutively expressed (18). UTI89 ΔybcL was generated by excision of the chromosomal resistance cassette from UTI89 ybcL::cat (9) through introduction of the Flp recombinase-expressing vector pCP20 (19). pYbcLUTI, containing the UTI89 ybcL allele in pTRC99A (Amp′), was created as previously described (9). UTI89 ϕE::cat was created by linear transformation of UTI89/ϕKm208 (20) with a fragment amplified from template plasmid pKD3 (19) using the primers MEL296 and MEL297 (primer sequences are given in Table S1 in the supplemental material). UTI89 hofQ::cat was created similarly using primers MEL300 and MEL301. The deletions were verified by direct sequencing.

To replace the ampicillin resistance cassette in pTRC99A with a chloramphenicol resistance cassette, pTRC99A was amplified using primers MEL245 and MEL246, and the chloramphenicol resistance cassette was amplified from template plasmid pKD3 (19) using primers MEL247 and MEL248. The resulting PCR products were digested with PciI and SpeI and then ligated.Transformed clones of E. coli Top10 (Invitrogen) were selected on chloramphenicol plates, and the accuracy of the resulting construct pMEL25, was tested by PCR and restriction enzyme digestion. To generate a translational fusion between YbcL and the β-lactamase variant TEM-1, the ybcL open reading frame (ORF) was amplified from UTI89 genomic DNA using primers MEL253 and MEL275 and digested with SacI and NotI. The DNA sequence encoding the mature form of TEM-1 (excluding the signal sequence) was amplified from plasmid pBRR32 using primers MEL278 and MEL284, with the reverse primer containing the FLAG epitope sequence, and digested with NotI and BamHI. The digested PCR products were ligated into pMEL25 (Chf′) that had been digested with SacI and BamHI. Transformed clones of E. coli Top10 were selected on chloramphenicol plates, and the accuracy of the resulting construct pYbcL::TEM-1 was confirmed by direct sequencing. Expression of the fusion protein upon IPTG induction was confirmed by Western blotting of bacterial lysates.

UTI89 surA::kan was generated as described previously (21). To control expression and monitor localization, a plasmid (denoted pSurA) encoding SurA with a C-terminal hemagglutinin (HA) tag under the control of an IPTG-inducible promoter was constructed. The surA ORF was amplified from UTI89 genomic DNA using primers JLP253 and JLP254, with the reverse primer containing the HA epitope sequence, and digested with XbaI and HindIII. The digested PCR product was ligated into pTRC99A (Amp′) that had been similarly digested. Transformed clones of Top10 were selected on ampicillin plates, and the accuracy of the resulting construct was confirmed by direct sequencing. The tetracycline resistance cassette from pCL328 skp::tet (a kind gift of T. Silhavy) was transferred by P1 phage transduction to UTI89, generating UTI89 skp::tet. As with pSurA, the skp ORF was amplified from UTI89 genomic DNA using primers MEL243 and MEL244, with the reverse primer containing the FLAG epitope sequence, and digested with BamHI and XbaI. The digested PCR product was ligated into pTRC99A (Amp′) that had been similarly digested. Transformed clones of Top10 were selected on ampicillin plates, and the accuracy of the resulting construct, pSkp, was confirmed by direct sequencing.

Human PMN isolation and migration. In accordance with a protocol approved by the Washington University Human Research Protection Office, PMN were isolated from venous blood of healthy adult volunteers according to previously established protocols (9, 22). Briefly, dextran sedimentation was used to reduce erythrocytes, leukocytes were separated using a Ficoll density gradient (Ficoll-Paque Plus; GE Healthcare), and remaining erythrocytes were lysed hypotonically. Purified PMN were resuspended in serum-free RPMI 1640 medium ( Gibco) to a concentration of 107 PMN/ml and used immediately. 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).

To evaluate PMN migration, 100 nM N-formyl-Met-Leu-Phe (fMLF) (Sigma) was added to 0.6 ml RPMI in a 24-well attachment plate (Corning). YbcL variants, YbcLUTI and YbcL::MC, expressed by UTI89 and MG1655, respectively, were purified as previously described (9) and added to the 24-well plate at a final concentration of 225 ng/ml, where indicated. Transwell inserts (0.33-cm² polyester membranes with a 3-µm pore size; Corning), either empty and uncoated or bearing confluent 3D epithelial layers prepared as described previously (9, 22), were added to each well, and 100 freshly isolated human PMN, prepared as described above, were applied to the upper reservoir. PMN migration across the Transwell insert into the lower reservoir was enumerated after 1 h using a hemacytometer and is shown normalized to 105 input PMN. The mean and standard error of the mean (SEM) from at least 3 experiments is shown.

Tissue culture and in vitro infection. Human bladder epithelial cell lines 5637 (ATCC HTB-9) and T24 (ATCC HTB-4) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma). UROtsa cells, derived from the uroepithelium of human ureter (23), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) containing 10% FBS. Human lung epithelial cell lines A549 (obtained from A. Hamilton) and HClC827 (ATCC CLR-2868) were grown in RPMI with 10% FBS. All cell lines were grown and infected at 37°C in a humidified atmosphere with 5% CO2 and then washed three times with Dulbecco’s phosphate-buffered saline (DPBS) ( Gibco) before use in experiments. All infections were performed in serum-free RPMI or DPBS as noted below.

To assess localization by Western blotting, 5637 cells or freshly isolated PMN were infected with the indicated strains of E. coli at a multiplicity of infection (MOI) of 40 bacteria/cell for 1 h unless otherwise noted. The supernatant was collected, and the epithelial cells were washed with PBS. Lysis was achieved by addition of 0.1% Triton X-100. The supernatant and cell lysate samples were processed using syringe-driven filters (0.22-µm pore size; Millipore), and protein was precipitated using 15% trichloroacetic acid (TCA) (Sigma) and then probed by Western
blotting. 1-Mannose (2%, wt/vol; Sigma) was added in selected experiments, as noted in Results.

To spatially separate the bacterial inoculum from 5637 cells grown to confluence in the bottom of a 6-well plate, UTI89/pYbcL::TEM-1 ΔybcL was applied to 1 ml DPBS in the upper reservoir of a Transwell insert (4.67-cm² polyester membranes with a 0.4-μm pore size; Corning), while 2 ml DPBS was applied to the lower reservoir. After 1 h of incubation, the supernatants from the upper and lower reservoirs and the cell lysate were sterilized, TCA precipitated, and probed by Western blotting as described above.

**Protease protection and ultracentrifugation assays.** The protease protection assay is based on previously published protocols (11, 24). To generate sterile supernatant containing YbcL, 5637 cells in RPMI in 15-cm dishes were infected with UTI89/pYbcL::TEM-1 ΔybcL at an MOI of 40. After 1 h of incubation, the supernatant was filter sterilized and separated into four aliquots. Where indicated, proteinase K (Sigma), Triton X-100, or phenylmethylsulfonyl fluoride (PMSF) (Sigma), a protease inhibitor, was added to the aliquots at 200 μg/ml, 0.1%, or 5 mM, respectively. After incubation at 37°C for 45 min, protein was precipitated from the reaction mixtures using 15% TCA and then probed by Western blotting. In parallel experiments, sterile supernatant, generated as described above, was ultracentrifuged at 245,000 × g for 1 h at 4°C. Protein was precipitated from the supernatant using 15% TCA, while the ultracentrifuged pellet was resuspended using Laemmli sample buffer. The samples were probed by Western blotting.

**Western blotting.** Proteins were separated by SDS-PAGE using 12% polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking with 2% nonfat milk plus 2% BSA, blots were probed with mouse anti-FLAG (1:1,000; Sigma), rabbit anti-ΔHA (1:4,000; Invitrogen), rabbit anti-GroEL (1:100; Sigma), or rabbit anti-GFP (1:4,000; Clontech) antibodies followed by goat anti-mouse or goat anti-rabbit IgG antibodies (1:2,000; Sigma) and visualized using a ChemiDoc MP (Bio-Rad). For experiments to detect bacterial fragments not identified by BacLight staining, bacteria were also incubated with rabbit anti-E. coli (E3500-06; US Biologicals) or anti-OmpA antiserum, followed by detection with Alexa Fluor 350-conjugated anti-rabbit IgG secondary antibody (A11046; Life Technologies).

**β-Lactamase reporter assays.** Cultured epithelial cells in 6-well plates were infected with UTI89/pYbcL::TEM-1 ΔybcL at an MOI of 40 for 1 h unless otherwise indicated. The supernatant was cleared by centrifugation at 16,000 × g for 5 min, and then aliquots were pipetted into a 96-well plate. CCF2-FA (Invitrogen), a β-lactamase substrate consisting of a cephalosporin core linking a 7-hydroxycoumarin to a fluorescein, was added at a final concentration of 500 nM. In the presence of β-lactamase activity, the cephalosporin core is cleaved, disrupting fluorescence resonance energy transfer (FRET) between the coumarin and fluorescein molecules and changing the emitted fluorescence from green (520 nm) to blue (447 nm). The reaction mixtures were incubated in the dark at 37°C for 4 h. At the end of the incubation, fluorescence was quantified using a microtiter plate reader (Synergy 2; BioTek). Fluorescence is represented as a ratio of 447 nm to 520 nm.

**RESULTS**

**Suppression of PMN migration by YbcL**

**UTI requires a bladder epithelial barrier.** Given the localization of YbcL to both bladder epithelial cells and PMN during UPEC infection (9), we sought to further characterize the site of action of YbcL by investigating the requirement for bladder epithelial cells during suppression of PMN migration. We employed an *in vitro* assay, analogous to the transuroepithelial PMN migration assay (9, 22), to quantify PMN migration across empty Transwell inserts with pores sufficiently small to prevent passive movement of PMN. The chemoattractant peptide fMLF was placed in the lower reservoir of a Transwell insert, freshly isolated PMN were applied to the upper reservoir, and the level of PMN migration across the Transwell insert into the lower reservoir was quantified after 1 h using a hemacytometer. In the presence or absence of 5637 bladder epithelial cells, the Transwell inserts, fMLF elicited significantly more PMN migration than mock treatment (Fig. 1A and 1B, respectively) (P < 0.005). The addition of purified YbcL to the lower reservoir significantly reduced the level of PMN migration when empty Transwell inserts were used (Fig. 1B), even at a concentra-
These observations demonstrate that suppression of PMN migration by YbcLUTI in this model requires the presence of a bladder epithelial barrier.

To further investigate the site of action of YbcLUTI, we compared the localization of exogenous purified YbcLUTI with that of YbcLUTI episomally expressed by UPEC. Cultured 5637 cells in 6-well plates were either infected with UTI89/pYbcLUTI ybcL::cat or incubated with 100 ng purified YbcLUTI for 1 h. The supernatant and 5637 cell lysate were filter sterilized to remove intact bacteria, protein was precipitated using 15% TCA, and the samples were probed by Western blotting. Consistent with prior results (9), YbcLUTI was detected in both the supernatant and 5637 cell lysate during UPEC infection (Fig. 1C, bacteria). However, YbcLUTI was detected only in the supernatant when purified protein was substituted for a live bacterial inoculum (Fig. 1C, protein).

Similar results were observed when the experiment was scaled up to 15-cm dishes, suggesting that the absence of purified YbcLUTI from the 5637 cell lysate did not reflect insensitivity of the Western blotting reagents. As purified YbcLUTI suppressed PMN migration in the transuroepithelial PMN migration assay (9) (Fig. 1A), these data suggest that YbcLUTI functions primarily from an extracellular location.

YbcLUTI is released into the supernatant in soluble form. While purified YbcLUTI suppressed transuroepithelial PMN migration in vitro, it was unclear whether YbcLUTI was released from the periplasm in soluble form during UPEC infection. In addition to canonical secretion systems such as the T2SS, OMVs have been implicated in secretion of bacterial effectors (25). To explore the mode of delivery from the periplasm, we examined the characteristics of extracellular YbcLUTI. Cultured 5637 cells were infected with UTI89/pYbcLUTI ybcL::cat (bacteria) or 100 ng purified YbcLUTI (protein) for 1 h, and then the supernatant and 5637 cell lysate were filter sterilized, TCA precipitated, and probed by Western blotting. Consistent with prior results (9), YbcLUTI was detected in both the supernatant and 5637 cell lysate during UPEC infection (Fig. 1C, bacteria). However, YbcLUTI was detected only in the supernatant when purified protein was substituted for a live bacterial inoculum (Fig. 1C, protein). Similar results were observed when the experiment was scaled up to 15-cm dishes, suggesting that the absence of purified YbcLUTI from the 5637 cell lysate did not reflect insensitivity of the Western blotting reagents. As purified YbcLUTI suppressed PMN migration in the transuroepithelial PMN migration assay (9) (Fig. 1A), these data suggest that YbcLUTI functions primarily from an extracellular location.

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YbcLUTI is released from the bacterial periplasm in soluble form.
The localization pattern of YbcLUTI, when episomally expressed in wild-type UTI89, UTI89 yheF::cat, or UTI89 hofQ::cat, was assessed upon infection of 5637 cells as described above. Similar levels of YbcLUTI were detected in the supernatant and 5637 cell lysate irrespective of these mutations (Fig. 3B). These data indicate that neither the T2SS nor the T4P encoded by UTI89 is responsible for delivering YbcLUTI to the extracellular milieu. Furthermore, an isogenic strain lacking expression of flgH, which encodes the secretin of the flagellar biosynthesis machinery, exhibited similar YbcLUTI localization (data not shown).

YbcL is released into the supernatant in a dose-, time-, and epithelial cell-dependent manner. As the canonical secretion systems encoded by UPEC appeared to be dispensable for delivering periplasmic YbcLUTI to the extracellular space, we further characterized the parameters of YbcLUTI release using a quantitative in vitro assay based on the enzyme activity of β-lactamase (TEM-1). With E. coli expressing a translational fusion between full-length YbcLUTI and the mature form of TEM-1 (denoted YbcL::TEM-1), the level of β-lactamase activity in the supernatant reflects the amount of YbcL::TEM-1 liberated. Of note, episomal expression of YbcL::TEM-1 complemented the phenotype of UTI89 ΔybcL in the transuroepithelial PMN migration assay (data not shown). Cultured 5637 cells in 6-well plates were infected with UTI89/pYbcL::TEM-1 ΔybcL after overnight induction with various concentrations of IPTG, the level of β-lactamase activity in the supernatant correlated with the level of IPTG induction (see Fig. S1A in the supplemental material), demonstrating a relationship between the amount of YbcL::TEM-1 in the periplasm and the amount released into the supernatant. When 5637 cells were infected with UTI89/pYbcL::TEM-1 ΔybcL for increasing durations before β-lactamase activity was measured, the level of fluorescence in the supernatant increased with longer incubation (see Fig. S1B in the supplemental material), suggesting that release of YbcL::TEM-1 from the periplasm occurs over time.

To investigate release of YbcL::TEM-1 in the presence of alternative eukaryotic cell types, UTI89/pYbcL::TEM-1 ΔybcL was incubated in RPMI alone or in the presence of cultured uroepithelial cells (5637, T24, or UROtsa) for 1 h before the level of YbcL::TEM-1 in the supernatant was measured as described above. Significantly (3- to 4-fold) higher levels of fluorescence were detected in supernatant generated in the presence of uroepithelial cells than in supernatant generated in RPMI alone (Fig. 4A) (P < 0.05). Of note, release in response to A549 or HCC827 human lung epithelial lines was similar to that with uroepithelial cells (data not shown). These results indicate that release of YbcL::TEM-1 from UTI89 is not constitutive but rather is dependent upon specific conditions.

To investigate extracellular release of YbcL::TEM-1 in the presence of professional phagocytes, 10⁵ PMN were infected with UTI89/pYbcL::TEM-1 ΔybcL for 1 h before β-lactamase activity in the cleared supernatant was quantified. With these experimental parameters, no β-lactamase activity was detected in the supernatant (data not shown). However, previous results had suggested that the level of YbcLUTI in the supernatant during infection of the outer membrane-localized secretin essential for T2SS function. As the T4P is evolutionarily related to the T2SS, we generated a separate deletion of hofQ, the gene that encodes the T4P secretin. To investigate release of YbcLUTI from UPEC and E. coli K-12. 5637 cells were infected with the indicated strains of E. coli for 1 h. The supernatant and 5637 cell lysate were filter sterilized, TCA precipitated, and probed by Western blotting. An equivalent volume of each bacterial inoculum (input) was included to demonstrate similar YbcLUTI expression across bacterial strains. (A) As seen during infection with UTI89/pYbcLUTI, YbcLUTI was detected in the supernatant and cell lysate during infection with nonpathogenic E. coli strain MG1655/pYbcLUTI. (B) The levels of YbcLUTI in the supernatant and cell lysate were unaffected by lack of YheF or HofQ, secretins T4P independent.

UTI89/pYbcLUTI ybcL::cat as described above, at a speed previously determined to pellet OMVs, separating them from the soluble fraction. By Western blotting, YbcLUTI was detected exclusively in the supernatant fraction after ultracentrifugation (Fig. 2B). Taken together, these data indicate that YbcLUTI is released from the periplasm in soluble form and is not packaged within OMVs.

**Release of YbcLUTI from UPEC and K-12 E. coli is T2SS and T4P independent.** We previously observed that YbcLUTI expressed from nonpathogenic E. coli strain MG1655 (9). As YbcLUTI was unable to influence PMN migration from within the bacterial periplasm (9), we hypothesized that the mode of release was conserved between uropathogenic and nonpathogenic E. coli strains. To test this hypothesis, 5637 cells were infected with either UTI89 or MG1655 episomally expressing YbcLUTI for 1 h, and the supernatant and 5637 cell lysate were filter sterilized, TCA precipitated, and probed by Western blotting. As with UTI89/pYbcLUTI, YbcLUTI was detected in both the supernatant and 5637 cell lysate during infection with MG1655/pYbcLUTI (Fig. 3A). These data suggest that YbcLUTI release from the periplasm is mechanistically conserved between these strains and does not require additional UPEC-specific factors.

We next focused on canonical secretion systems encoded by both UTI89 and MG1655 that would be capable of translocating periplasmic substrates. To investigate a role for the T2SS in release of YbcLUTI from the periplasm, we deleted yheF, which encodes the flagellar secretin essential for T2SS function. As the T4P is evolutionarily related to the T2SS, we generated a separate deletion of hofQ, the gene that encodes the T4P secretin. The localization pattern of YbcLUTI, when episomally expressed in wild-type UTI89, UTI89 yheF::cat, or UTI89 hofQ::cat, was assessed upon infection of 5637 cells as described above. Similar levels of YbcLUTI were detected in the supernatant and 5637 cell lysate irrespective of these mutations (Fig. 3B). These data indicate that neither the T2SS nor the T4P encoded by UTI89 is responsible for delivering YbcLUTI to the extracellular milieu. Furthermore, an isogenic strain lacking expression of flgH, which encodes the secretin of the flagellar biosynthesis machinery, exhibited similar YbcLUTI localization (data not shown).

**YbcL is released into the supernatant in a dose-, time-, and epithelial cell-dependent manner.** As the canonical secretion systems encoded by UPEC appeared to be dispensable for delivering periplasmic YbcLUTI to the extracellular space, we further characterized the parameters of YbcLUTI release using a quantitative in vitro assay based on the enzyme activity of β-lactamase (TEM-1). With E. coli expressing a translational fusion between full-length YbcLUTI and the mature form of TEM-1 (denoted YbcL::TEM-1), the level of β-lactamase activity in the supernatant reflects the amount of YbcL::TEM-1 liberated. Of note, episomal expression of YbcL::TEM-1 complemented the phenotype of UTI89 ΔybcL in the transuroepithelial PMN migration assay (data not shown). Cultured 5637 cells in 6-well plates were infected with UTI89/pYbcL::TEM-1 ΔybcL after overnight induction with various concentrations of IPTG, the level of β-lactamase activity in the supernatant correlated with the level of IPTG induction (see Fig. S1A in the supplemental material), demonstrating a relationship between the amount of YbcL::TEM-1 in the periplasm and the amount released into the supernatant. When 5637 cells were infected with UTI89/pYbcL::TEM-1 ΔybcL for increasing durations before β-lactamase activity was measured, the level of fluorescence in the supernatant increased with longer incubation (see Fig. S1B in the supplemental material), suggesting that release of YbcL::TEM-1 from the periplasm occurs over time.

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To investigate extracellular release of YbcL::TEM-1 in the presence of professional phagocytes, 10⁵ PMN were infected with UTI89/pYbcL::TEM-1 ΔybcL for 1 h before β-lactamase activity in the cleared supernatant was quantified. With these experimental parameters, no β-lactamase activity was detected in the supernatant (data not shown). However, previous results had suggested that the level of YbcLUTI in the supernatant during infection of
PMN was low (9). In a further attempt to detect Ybcl::TEM-1 in the supernatant, 10^7 5637 cells or PMN were infected with UTI89/ pYbcL::TEM-1 ΔybcL for 1 h. The supernatants were filter sterilized and concentrated 10-fold using centrifugal filtration, and β-lactamase activity was measured. Under this modified protocol, Ybcl::TEM-1 was detectable in the supernatant during infection of PMN, though the level was markedly lower than that observed during infection of 10^7 5637 cells (Fig. 4B) (P < 0.0001). In agreement with these findings, about 7-fold less YbcL was detected in the supernatant during infection of PMN than during infection of 5637 cells by Western blotting (data not shown). Of note, addition of protease inhibitors did not alter detection of YbcL (data not shown), indicating that measured extracellular levels of the target protein were not lowered by PMN-derived protease digestion. Taken together, these data demonstrate that extracellular release of YbcL from the periplasm occurs in a dose-, time-, and epithelial cell-dependent manner.

UPEC proximity to bladder epithelial cells augments Ybcl_UUTI release. We next explored the uroepithelial cell-dependent increase in YbcL::TEM-1 release during UPEC infection. We employed a UPEC mutant (UTI89 fimH::kan) defective in the production of type 1 pili, surface appendages that mediate mannose-dependent binding and invasion of bladder epithelial cells (26–28). Cultured 5637 cells in 6-well plates were infected with UTI89/pYbcL_UUTI or UTI89/pYbcL_UUTI fimH::kan for 1 h before the supernatant and 5637 cell lysate were sterilized, TCA precipitated, and probed by Western blotting. Infection with the ΔfimH strain resulted in substantially less Ybcl_UUTI detected in the cell lysate (~10% of the wild-type level) (see Fig. S2 in the supplemental material), confirming that type 1 pili are a major determinant of UPEC internalization into these cells. Of more interest, lower levels of Ybcl_UUTI were also observed in the ΔfimH supernatants (~70% of the wild-type level) (see Fig. S2 in the supplemental material). Further, supernatant fluorescence after infection of 5637 cells with UTI89/pYbcL::TEM-1 ΔybcL was diminished by 27% upon addition of mannose (data not shown). These data support the hypothesis that though contact is not strictly required, proximity of UPEC to bladder epithelial cells augments extracellular Ybcl_UUTI release.

To further test this hypothesis, Transwell inserts with 0.4-μm pores were employed to spatially separate the bacterial inoculum from the 5637 cells, while allowing passive diffusion of small molecules. UTI89/pYbcL_UUTI ybcL::cat was applied directly to the 5637 cells or to the upper reservoir of Transwell inserts. After 1 h of incubation, the supernatants from both the upper and lower reservoirs and the 5637 cell lysate were processed for Western blotting as described above. In the presence of a physical barrier separating the bacterial inoculum from the 5637 cells, very little Ybcl_UUTI was detected in the supernatant, and Ybcl_UUTI was absent from the cell lysate (Fig. 5A).

FIG 4 Release of Ybcl_UUTI is uroepithelial cell dependent. Cultured epithelial cells in 6-well plates were infected with UTI89/pYbcL::TEM-1 ΔybcL for 1 h unless otherwise indicated. The supernatant was cleared, aliquots were loaded into a 96-well plate, and the fluorescent β-lactamase substrate CCF2 was added to each well. After 1 h of incubation, fluorescence was measured and is represented as a ratio of 447 to 520 nm. Background fluorescence from medium is indicated by the dashed line. (A) Significantly more β-lactamase activity was detected in the supernatant after infection of 5637, T24, or UROtsa cells than after incubation in RPMI (*, P < 0.05). (B) Supernatant generated from infection of 5637 cells or PMN in 15-cm dishes with UTI89/pYbcL::TEM-1 ΔybcL was sterilized and concentrated 10-fold, and β-lactamase activity was determined as described above. Significantly more β-lactamase activity was detected in the supernatant after infection of 5637 cells than after infection of PMN (*, P < 0.0001).
on YbcL::TEM-1 release (data not shown). Finally, similar levels of fluorescence were detected when the conditioned medium was boiled before addition of the bacterial inoculum (Fig. 5C). These data suggest that the soluble factors associated with YbcL::TEM-1 release are host cell derived and largely heat stable.

Bacterial death occurs during infection of bladder epithelial cells. The canonical secretion systems appeared to be dispensable for YbcL::UTI release from the periplasm during UPEC infection of bladder epithelial cells. Given that a few E. coli-encoded proteins (e.g., Shiga toxin and colicin) reach the extracellular milieu exclusively via bacterial death (14, 15), we tested the hypothesis that YbcL::UTI is liberated from the UPEC periplasm in an analogous manner. First, 5637 cells were infected with UTI89/pYbcL::UTI ybcL::cat, UTI89/pSurA surA::kan, UTI89/pSkp skp::tet, UTI89, or UTI89 pcomGFP. At 0 and 60 min, supernatants were filter sterilized, TCA precipitated, and probed by Western blotting. To ensure that intracellular proteins detected in the supernatant would not represent contamination from the overnight bacterial culture, each bacterial inoculum was washed repeatedly in PBS before infection of the 5637 cells. Accordingly, very little of the periplasmic proteins YbcL, SurA, and Skp and the cytoplasmic proteins GroEL and GFP were detected in the supernatant at time zero (Fig. 6A). In contrast, these intracellular proteins were readily detected in the supernatant after infection of 5637 cells than after incubation in RPMI ($P < 0.05$).

FIG 5 Release of YbcL::UTI is augmented by proximity to bladder epithelial cells. 5637 cells were infected with UTI89/pYbcL::UTI ybcL::cat (A) or UTI89/pYbcL::TEM-1 ybcL::cat (B and C) for 1 h. (A) The supernatant and 5637 cell lysate were filter sterilized, TCA precipitated, and probed by Western blotting. (B and C) β-Lactamase activity in cleared supernatant was measured using CCF2 as described for Fig. 4. Less YbcL::UTI was detected in the supernatant when a Transwell insert (TW) physically separated the bacterial inoculum from the 5637 cells (A and B) or when conditioned medium (CM) was used (C) (*, $P < 0.05$). Boiling of the conditioned medium (CM-B) did not significantly alter YbcL::UTI release (C).

FIG 6 UPEC viability decreases in the presence of bladder epithelial cells. (A) 5637 cells were infected with UPEC strains expressing the indicated proteins. After 0 or 60 min, the supernatant was sterilized, TCA precipitated, and probed by Western blotting. A portion of the bacterial inoculum (input) is also shown. (B) 5637 cells were mock infected or infected with UTI89 for 0 or 60 min before the supernatant was sterilized, DNA was precipitated with isopropanol, and PCR was conducted using UTI89-specific primers. PCRs serving as negative (−) and positive (+) controls contained water and UTI89 genomic DNA, respectively, as the template. Bacterial periplasmic (YbcL, SurA, and Skp) and cytoplasmic (GroEL and GFP) proteins (A) and bacterial DNA (B) were detected in the supernatant after infection of 5637 cells. (C) UTI89 was incubated in the presence or absence of 5637 cells for 1 h. Bacteria were fixed to coverslips, stained for membrane integrity, and imaged and enumerated by fluorescence microscopy. Significantly more dead bacteria exhibiting severely compromised membranes were observed after infection of 5637 cells than after incubation in RPMI ($P < 0.05$).
In addition to intracellular proteins, we also probed the supernatant for bacterial DNA after infection of 5637 cells with wild-type UTI89. At selected time points, the supernatant was filter sterilized, DNA was precipitated using isopropanol, and PCR (using primers complementary to the UTI89 chromosomal ybcL allele) was conducted using an equivalent volume of each sample as the template. The rounds of amplification were minimized so that the intensity of the resulting amplicon would be proportional to the amount of template DNA. As with the protein detection described above, the bacterial inoculum was washed repeatedly in PBS before use. While a faint amplicon was generated when precipitated DNA from supernatant processed at time zero was used as the template, the amplicon generated after 60 min infection was greater in intensity, indicating a higher concentration of template DNA as the template. The presence of bacterial DNA and intracellular proteins in the supernatant under conditions shown to stimulate release of YbcLUTI is consistent with a model in which bacterial lysis mediates this release.

To quantify bacterial death directly, we employed fluorescence microscopy after bacterial staining with a viability kit to visualize and enumerate bacteria with intact membranes (live; green) versus bacteria with severely compromised membranes (dead; red). UTI89 was incubated in RPMI in the presence or absence of 5637 cells, conditions shown to stimulate significantly different levels of YbcLUTI release. After 1 h of incubation in RPMI, 2.48% (±0.57% [SEM]) of the total bacteria were dead (Fig. 6C). However, the percentage of dead bacteria isolated from the medium increased approximately 5.6-fold to 13.8% (±3.63%) after 1 h of incubation in the presence of 5637 cells (Fig. 6C) (P < 0.05). Of note, only very rare “ghosts” (bacterial remnants not containing DNA) were evident when a blue-fluorescent antibody combination against bacterial envelope components was included (data not shown). The significant increase in bacteria with severely compromised membranes upon exposure to uroepithelial cells further implicates bacterial death as the mechanism by which YbcLUTI is released into the extracellular milieu.

**DISCUSSION**

The present study furthers our understanding of the mechanism by which UPEC-encoded YbcL is liberated from the bacterial periplasm and influences the innate immune response in the bladder. Suppression of PMN migration by purified YbcLUTI required the presence of bladder epithelial cells. YbcLUTI was released from the periplasm in soluble form, though independent of the T2SS and T4P conserved in nonpathogenic and uropathogenic E. coli. Liberation of YbcLUTI from the periplasm occurred specifically in response to uroepithelial cells and was maximal when UPEC and bladder epithelial cells were in close proximity. Lastly, UPEC death upon exposure to bladder epithelial cells implicates bacterial lysis as the mechanism by which YbcLUTI is released from the periplasm.

As purified YbcLUTI had no effect on PMN migration in the absence of bladder epithelial cells, we conclude that YbcLUTI does not inhibit PMN chemotaxis directly. These data suggest that the site of action of YbcLUTI is the uropatholium, though purified YbcLUTI was detected solely in the supernatant and not in the 5637 cell lysate. Given the low concentration of YbcLUTI sufficient to suppress PMN migration (9), it is possible that the amount of purified protein associated with the 5637 cell lysate was too low to detect by Western blotting, even when larger dishes were used. When UPEC was physically separated from bladder epithelial cells, YbcLUTI was not detected in 5637 cell lysates, further demonstrating that bacterial binding or internalization is required for YbcLUTI to reach the cell interior. YbcLUTI was released by UPEC in soluble form rather than as OMV cargo, and a mechanism by which YbcLUTI could traverse the epithelial membrane in the absence of bacterial internalization is not obvious. Taken together, these data support a model in which YbcLUTI acts on uroepithelial cells from the extracellular milieu.

The specificity of YbcLUTI release during UPEC infection of uroepithelial cells also supports a model in which the bladder epithelium, rather than PMN, is the site of YbcLUTI activity. The spatial relationship between the bacterial inoculum and host cells influences the magnitude of YbcLUTI release from the periplasm. Supernatant YbcLUTI levels were lower when close association between UPEC and bladder epithelial cells was limited through mutation of type 1 pil, addition of mannose, interposition of a physical barrier, or use of conditioned medium, indicating that proximity augments YbcLUTI release. While the component(s) of the conditioned medium associated with YbcLUTI release from the periplasm. Supernatant YbcLUTI levels were lower when close association between UPEC and bladder epithelial cells was limited through mutation of type 1 pil, addition of mannose, interposition of a physical barrier, or use of conditioned medium, indicating that proximity augments YbcLUTI release. While the component(s) of the conditioned medium associated with YbcLUTI release from the periplasm appears to be largely heat stable and constitutively expressed by 5637 cells, additional work is required to identify this molecule(s). One explanation for these observations is that microenvironments at the bladder epithelial surface, where the local concentration of molecules secreted by 5637 cells would be highest, are responsible for the augmented YbcLUTI release. However, it is also possible that constituents of the bladder epithelial surface contribute to release of YbcLUTI from the periplasm.

Our interrogation of conserved secretion systems demonstrated that YbcLUTI release is independent of the T2SS and T4P. We have not generated a UPEC mutant deficient in both systems, as it is unlikely that the T2SS and T4P function redundantly to secrete YbcLUTI. Though autotransporters contribute to UPEC pathogenesis (29, 30), it is unlikely that YbcLUTI belongs to the T5SS family given the lack of homology (31). Though an as-yet-identified secretion system could be responsible for mediating YbcLUTI secretion, our subsequent experiments implicated bacterial death as the mechanism by which YbcLUTI is liberated from the bacterial cell, a novel mode of delivery for a UPEC exoprotein. First, multiple bacterial intracellular proteins and DNA were detected in supernatants overlying UPEC-infected bladder epithelial cells. While secretion systems exist to deliver cytoplasmic and periplasmic proteins as well as bacterial DNA to the extracellular space, it is unlikely that the coordinated activity of multiple such systems is responsible for the simultaneous detection of all these molecules in the supernatant. Second, bacterial death was substantially augmented upon exposure to bladder epithelial cells. Our initial efforts to detect bacterial death by plating for survival revealed an overall increase in CFU, reflecting interval bacterial growth (M. Lau and D. Hunstad, unpublished data). Using a fluorescence-based approach, significantly more bacteria with severely compromised membranes (representing a prelytic state) were observed upon UPEC exposure to bladder epithelial cells, correlating with increased release of YbcLUTI. Future work will specify whether the presence of bladder epithelial cells and derived molecules provokes this death via a bacterial mechanism (e.g., a toxin-antitoxin system or bacteriophage-mediated lysis) or via a eukaryotic mechanism (e.g., antimicrobial peptides).
Our studies contribute to a model of the early host-pathogen interactions occurring upon introduction of UPEC into the mammalian bladder. The observed proximity-dependent increase in YbcLUTI release suggests that bacteria nearest the uroepithelial surface are the minority that undergo lysis. Given the potency of YbcLUTI in suppressing PMN migration (9), lysis of very few bacteria at the epithelial surface would likely release a sufficient amount of YbcLUTI to exert a local effect on an epithelium-derived target, either secreted or present in the apical membrane. Spatially controlled release of YbcLUTI at the epithelial surface would maximize the probability of target binding and may explain how a low concentration of YbcLUTI is sufficient to suppress PMN migration despite the potential for diffusion into the bladder lumen. Though UPEC lysis would likely release bacterial products capable of activating host proinflammatory signaling, UPEC also encodes effectors (e.g., HlyA and TcpC) that block these signaling cascades (32, 33). Liberated YbcLUTI would inhibit early migration of PMN into the bladder lumen, extending a critical interval in which surviving UPEC can invade the bladder epithelium in the absence of phagocyte pressure. As shown in multiple studies, accessing the intracellular niche protects UPEC from the PMN that ultimately arrive (34, 35), facilitating the development of the intracellular bacterial community that is critical to the propagation of infection.

Release of YbcLUTI during UPEC death represents an example of bacterial altruism, a type of cooperation where some bacteria in a bacterial niche protect UPEC from the PMN that ultimately arrive (34, 35), facilitating the development of the intracellular bacterial community that is critical to the propagation of infection. Conditions that likely characterize UPEC as it colonizes the human bladder. YbcLUTI activity may therefore enable a very small (and otherwise insufficient) UPEC inoculum to establish the foothold required to initiate cystitis. A more detailed understanding of the molecular target and function of YbcLUTI and contemporaneously expressed UPEC effectors may illuminate strategies for prevention of symptomatic infection in susceptible host populations.

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